Taxonomic study of trypanosomes from small mammals: bats, rodents and soricids in the Oriental region

(東洋区のコウモリ、げっ歯類、トガリネズミ類など小型哺乳類に寄生する

トリパノソーマの系統分類学的研究)

DISSERTATION

Presented for partial fulfillment of the requirement of Doctor of Philosophy in the United Graduate School of Veterinary Science, Yamaguchi University.

United Graduate School of Veterinary Science, Yamaguchi University

Eliakunda Mafie

March 2019.

Acknowledgement

The current study was conducted in the Laboratory of Parasitology, The United Graduate School of Veterinary Science, Yamaguchi University from 2015 – 2019. I thank my Almighty God, Jesus Christ for health and his protection during this study.

Firstly and foremost I would like to thank my main supervisor Prof. Dr. Hiroshi SATO, for accepting me to do my PhD in his laboratory under his supervision and for his guidance and support of my study until graduation. I am grateful for his tireless advices and patience during the whole study period.

I am grateful to Yamaguchi University Management, for the support provided to me in terms of Tuition fees exemption and Otsuka Toshimi foundation and JEES for their financial support during my study.

I am forwarding my sincere thanks to co-supervisor Assoc. Prof. Dr. Tetsuya YANAGIDA for his tireless guidance and advices during my whole study period and for his critical review of my graduation thesis.

My sincere thanks are extended to all members of the Laboratory of Parasitology, United Graduate School, Yamaguchi University for their cooperation and support during my study. I am indebted to Mr. Eigo KATO for being my Japanese Tutor during this study. I am also grateful to Dr. Zhang for reviewing my Graduation thesis.

I also thank Yamaguchi City for providing the daycare for my children and all the daycare teachers who have been taking care of my children. My sincere thanks are extended to teachers in Yamaguchi Daini, Yuda and Nyuji Aijien daycares for good care of my children.

Lastly but not least I would like to thank my family, first my dear husband Isaac Makundi for his love, care, spiritual and physical support during my study. I thank my two children Jotham and Joanna for being my motivation during my study. I am indebted to Joanna for letting me continue with my study after only one month old. I also thank my dear parents: my dear father who passed away during this study and my mother for raising me and allowing me to be away from them and study in Japan.

Table of contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRIVIATIONS

apoL-I = apolipoprotein L-I

BSK = Barbour-Stoenner-Kelly

BW = maximum trypanosome width without undulating membrane

CO_2 = carbon dioxide

FF = length of the free flagellum

gGAPDH = glycosomal glyceraldehyde-3-phosphate dehydrogenase

ITS1 = internal transcribed spacer 1

KI = kinetoplast index

KL = length of the kinetoplast

KN = distance between the middle of the kinetoplast and the middle of the nucleus

KW = width of the kinetoplast

LSU = large subunit

ML = maximum likelihood

MW = maximum trypanosome width with undulating membrane

NA = distance between the middle of the nucleus and the anterior end

NHS = normal human serum

NI = nuclear index

NL = length of the nucleus

NW = width of the nucleus

PCR = Polymerase chain reaction

PK = distance between the posterior end and the middle of the kinetoplast

rDNA = ribosomal deoxyribonucleic acid.

SSU = small sununit

TL = total length

ABSTRACT

Taxonomy of the genus *Trypanosoma* Gruby, 1843 (Kinetoplastea: Trypanosomatida: Trypanosomatidae), is at challenging yet, particularly that for trypanosomes classified in the section Stercoraria. Representative are so-called rodent trypanosomes of the subgenus *Herpetosoma*, i.e. *Trypanosoma lewisi* (Kent, 1880) and its relatives, and trypanosomes of the subgenus *Schizotrypanum*, such as *Trypanosoma cruzi* Chagas, 1909 and its relatives. For a long time, morphological differentiation of stercorarian trypanosomes depended considerably upon our assumption of their rigid host-specificity, but molecular approaches introduced widely in the taxonomical study in the last two decades have demonstrated that this taxonomy is subjective enough. In the present study, I have approached taxonomically trypanosomes in small mammals such as rodents, soricids and bats, clarifying localization of taxonomic problems related to them.

In Chapter I, I outlined briefly records of atypical human trypanosomiasis caused by *T. lewisi* and *T. evansi* as an opportunistic infection, and updated the current status of molecular approaches to *T. lewisi* or *T. lewisi*-like trypanosomes in rodents based mainly on the ribosomal RNA gene (rDNA) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene. A limited number of deposited nucleotide sequences of nominal rodent trypanosome species, eight out of ca. 50 species, at the databases made reliable diagnosis and specific taxonomy of *T. lewisi*-like trypanosomes difficult. In addition, some nominal species such as *T. lewisi*, *T. musculi* and T. *blanchardi* have an almost identical rDNA sequences with few nucleotide substitutions, while *T. grosi* showes substantial intraspecific genetic variations even more than interspecific genetic variations of the aforementioned *Herpetosoma* trypanosomes.

In Chapter II, I collected blood samples of 89 murid rodents of 15 species and 11 soricids of four species in Indonesia, Philippines, Vietnam, Taiwan, and mainland China

with help of colleagues, and characterized trypanosomes based on morphology and nucleotide sequences of the rDNA and gGAPDH gene. *T. lewisi* and *T. lewisi*-like trypanosomes were found in the blood smears of 10 murids, which included *Bandicota indica* (two rats), *Rattus argentiventer* (one rat), and *Rattus tiomanicus* (two rats) in Indonesia; *Rattus rattus* (one rat) in the Philippines; and *Niviventer confucianus* (four rats) in mainland China. Furthermore, large- or medium-sized non-*T. lewisi*-like trypanosomes were detected in two soricids, *Crocidura dracula* in Vietnam and *Anourosorex yamashinai* in Taiwan, respectively. At last, I erected three new species, i.e. *T. niviventerae* n. sp., *T. sapaensis* n. sp., and *T. anourosoricis* n. sp. for isolates from *N. confucianus* in mainland China, *C. dracula* in Vietnam and *A. yamashinai* in Taiwan, respectively.

In chapter III, I characterized trypanosomes in the blood samples of bats (*Miniopterus fuliginosus*) from Japan, and reported for the first time the distribution of *T. dionisii* Bettencourt et França, 1905 of the *T. cruzi* clade in Asia. Our genetic characterization of the Asian isolate had biogeographical significance in discussion of the speciation and dispersion of bat-infecting trypanosomes and zoonotic *T. cruzi* endemic in the Latin America.

We are still a long way from understanding the real biodiversity of trypanosomes in the Stercoraria section. I believe, however, our effort to collect and characterise more isolates not only by morphology but also genetically of trypanosomes in small mammals such as rodents, soricids and bats may unveil their real ‘species’ in science.

GENERAL INTRODUCTION

Background information.

Trypanosomes are unicellular eukaryotes and obligate haemoflagellates of the genus *Trypanosoma* Gruby, 1843 (Kinetoplastea: Trypanosomatida: Trypanosomatidae). They infect almost all classes of vertebrates, such as fish, amphibians, birds and mammals, and are transmitted by vectors such as haematophagous arthropods (Insecta: Diptera and Hemiptera) and leeches (Clitellata: Hirudinea) (Hoare 1972). The history of genus *Trypanosoma* goes back to 1843 when D. Gruby named the parasites he found in frog's blood as *Trypanosoma sanguinis*. The genus name was derived from a Greek word "*trypano*" meaning screw-like and "*soma*" means body (Hoare 1972), to describe its corkscrew swimming motion. Trypanosomes range from non-pathogenic species to those that are highly pathogenic causing important diseases in animals as well as in human beings.

Once the goal of taxonomic understanding of trypanosomes seemed to be near to us based on morphological criterion (Hoare 1972), it is our current view that we are still a long way from understanding the real biodiversity of trypanosomes, particularly those in the Stercorarian section. Except for well-known zoonotic *T. cruzi*, less attention has been directed towards non-pathogenic stercorarian trypanosomes, while human and animal pathogenic trypanosomes of the salivarian section are given more and intensive attention. Some trypanosome species that are normally considered non-pathogenic, however, can cause detrimental effects especially when the infected host is exposed to the increased level of stress or is immunologically naïve (Botero et al. 2013). Stress, gestation, poor nutrition and concurrent infections have been reported to cause non-pathogenic *T. theileri* to become potentially pathogenic in cattle (Hussain et al. 1985; Doherty et al. 1993; Botero 2013). Botero et al. (2013) also speculated that trypanosome infection caused the decline

of a formerly abundant marsupial in Australia. In addition, rodent trypanosomes (*T. lewisi* and *T. lewisi*-like) transmitted by rat fleas, are emerging causes of atypical human trypanosomiasis in Southeast Asia and Africa (Howie et al. 2006; Sarataphan et al. 2007; Doke and Kar 2011; Shah et al. 2011; Tang et al. 2012; Pumhom et al. 2015). According to Lun et al. (2015), *T. lewisi* is a potentially underestimated, neglected human pathogen and it can resist normal human serum (NHS) like other pathogenic human trypanosomes, e.g. *T. brucei gambiense* and *T. brucei rhodesiense*. In addition, *Trypanosoma (Schizotrypanum) dionisii* which is a bat trypanosome of the *T. cruzi* clade, considered to be non-pathogenic, has recently been identified from the heart tissue of fatal human case, in mixed infection with *T. cruzi* (Dario et al. 2016).

Apart from zoonotic potential of trypanosomes, questions about evolution, taxonomy and biogeographical speciation of trypanosomes have not clearly been answered (Vickerman 1994; Stevens et al. 1999a, 1999b, 2001; Hamilton et al. 2012a, 2012b; Lukes et al. 2014; Votypka et al. 2015), needing more collection, genetic characterisation and phylogenetic analysis of trypanosomes isolated from different parts of the world.

Morphology of mammalian trypanosomes

Bloodstream trypomastigotes are lanceolate in shape and in transverse section their body is elliptical or oval (Hoare 1972). They have tapered ends with the anterior end more pointed compared to the posterior end, which is broader tapering more abruptly or terminating in a blunt or rounded tip (Hoare 1972). The outer covering of trypanosomes is known as pellicle or periplast which is an envelope surrounding the trypanosome body and give its ability to maintain constant shape (Hoare 1972).

The main organellae of trypanosomes, which can easily be identified by light microscope are flagellum, kinetoplast and nucleus (Fig. 1A). The nucleus lies near the

center or in the anterior half of trypomastigote form and is spherical or ellipsoid in shape, surrounded by thin membranous envelope. The flagellum starts within the cytoplasm behind the nucleus in the posterior part of the body and is seen as a delicate filament with an even thickness throughout its whole length, running forward along the outer margin of undulating membrane (Hoare 1972). The appearance of trypomastigotes depends on the form assumed when they were killed, either C-shaped, S-shaped or convoluted in different ways with flagellum and undulating membrane following the bending of the body, but always running along the convex side of the curve (Hoare 1972). The kinetoplast is always situated near the base of flagellum and is disc-shaped or round (Hoare 1972; also see Fig. 1 in the current study). It stains ruby-red or purple-red in Giemsa stain. Depending on trypanosome species, the kinetoplast can either be terminal or sub-terminal in the posterior end, for example in most salivarian trypanosomes or it can be situated away from the posterior end (close to the nucleus), for *Megatrypanum* trypanosomes (Hoare 1972; Lizundia et al. 2011). The trypomastigote forms can either be thin slender or broad stumpy.

In addition, amastigote and epimatigote forms can also be found in mammalian host, especially during multiplication stage of some trypanosome species (Hoare 1972; Pinto et al. 1999; Tyler et al. 2003). Amastigote forms are round or elongated and has no external flagellum (see Fig. 1B). On the other hand, epimastigote forms are elongated with kinetoplast anterior to nucleus, and the flagellum arise close to the kinetoplast and emerge from the side of the body to run along its surface or along a short undulating membrane (see Fig. 1B).

Trypanosome morphotypes are defined basing on cell morphology, arrangement of intracellular organellae and the presence or absence of free flagellum (Hoare 1972; Votypka et al. 2015). Morphometric features commonly used are total trypanosome length including free flagellum (TL), trypanosome width (W), distances from the posterior end to

the kinetoplast (PK), posterior end to nucleus (PN), kinetoplats to nucleus (KN), nucleus to anterior end (NA) and the length of free flagellum (FF) (Hoare 1972; also see Fig. 2).

Haore (1972) defined a species as an assemblage of genetically identical populations possessing common structural characters that do not intergrade with those of allied species. However, some of trypanosome subgenera have indistinguishable morphology and therefore the structural characters intergrade. This makes their identification basing on morphology difficult, especially in *Trypanozoon, Herpetosoma* and *Schizotrypanum* subgenera (Hoare 1972).

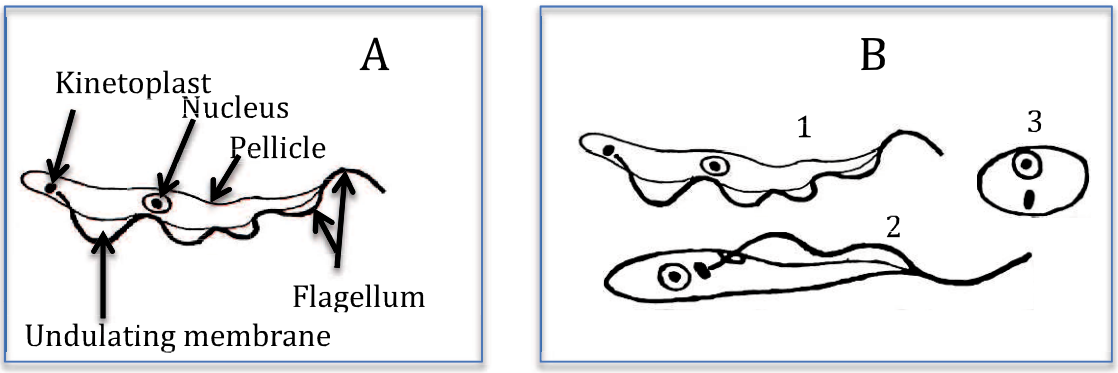

Fig. 1. Trypanosome sketches indicating trypanosome structure (A) and different forms of trypanosomes (B). 1 is trypomastigote form, 2 is epimastigote form and 3 is amastigote form. (Origainal drawing with reference to Hoare (1972)).

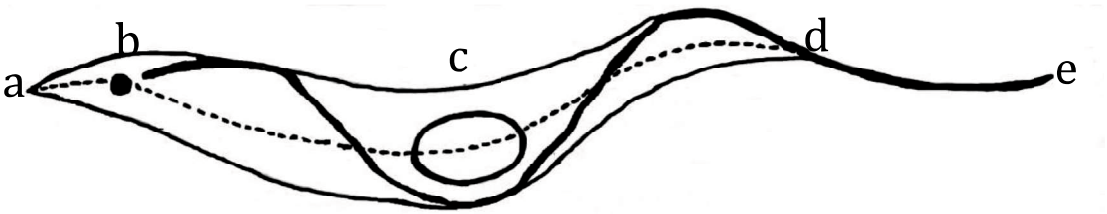

Fig. 2. Sketch of trypomastigote form of trypanosome to indicate morphometric features. Distances a-b is from posterior end to the kinetoplast (PK), a-c is from posterior end to middle of nucleus (PN), b-c is from kinetoplast to middle of nucleus (KN), c-d is from middle of nucleus to anterior end (NA), d-e is free flagellum and a-e is total length including free flagellum (TL). All the distances are measured through the trypanosome midline indicated by the dotted line. (Origainal drawing with reference to Hoare 1972)

Classification and taxonomy of mammalian trypanosomes

Haore (1972) divided mammalian trypanosomes into two major sections: Salivaria and Stercoraria, based on mode of transmission to their mammalian host and development in the vector. Developments of salivarian trypanosomes terminate in the anterior station of the vector and are transmitted through saliva. Section Salivaria contains most pathogenic trypanosomes such as *T. congolense*, *T. vivax* and *T. brucei* which cause African animal trypanosomiasis (nagana), and *T. brucei gambiense* and *T. brucei rhodensiense* which cause human African trypanosomiasis (sleeping sickness). Salivarian trypanosomes belong to four subgenera, which are *Trypanozoon* (e.g. *T. brucei*), *Duttonella* (e.g. *T. vivax*), *Pycnomonas* (e.g. *T. suis*), and *Nannomonus* (e.g. *T. congolense*).

On the other hand, the section Stercoraria contains trypanosomes whose developments terminate in the posterior station of the vector and are transmitted through contamination, either by ingestion of vector or vector feces. Trypanosomes of the Stercoraria section are less pathogenic (with exception of *T. cruzi*) and they belong to three subgenera, which are *Megatrypanum*, *Herpetosoma* and *Schizotrypanum* (Hoare, 1972). *Trypanosoma rangeli* which was initially placed in the subgenus *Herpetosoma*, as its morphology conforms to those of *T. lewisi*-like trypanosomes (Hoare 1972), was latter grouped in subgenus *Tejeraia* (Añex 1982).

The subgenus *Megatrypanum* is a heterogeneous group of large trypanosomes and is phylogenetically considered as the most primitive representative of genus *Trypanosoma* in mammals (Haore 1972). The type species of subgenus *Megatrypanum* is *T. theileri* and majority of species in this subgenus, conform in structure with the type species although some differ in certain morphological features (Hoare 1972). *Megatrypanum* trypanosomes infect a wide range of animal orders, but are commonly found in Chiroptera (bats) and Artiodactyla (Hoare 1972). Many trypanosome species in this subgenus appear to be

restricted to their individual mammalian host, but very little is known about their mode of reproduction in mammalian host, vectors and their life cycle (Hoare 1972; Naiff and Barrett 2013).

The subgenus *Herpetosoma* is a homogeneous group of trypanosomes and the type species of this subgenus is *T. lewisi*. The adult bloodstream forms of *Herpetosoma* trypanosomes are morphologically indistinguishable from that of *T. lewisi*. They have rigid host–restriction, a characteristic used to name the species in this subgenus in the past, and infections in heterologous animals have only been achieved experimentally, with or without host manipulation (Hoare 1972; Noyes et al. 2002; Sato et al. 2003). As described later in the present study (Chapter I), this idea has been changed recently. According to Hoare (1972), groups of trypanosomes with different morphology are separated as different species, provided no overlap in the characters distinguishing them, and therefore there is a gap between the species. When the characters of various populations intergrade, they are regarded as subspecies of the same species (Hoare 1972). If no morphological differences, biological characters distinguishing them, such as host restriction and geographical distribution is used. Hoare (1972) stated that *Herpetosoma* trypanosome groups, with identical morphology but different mode of reproduction in the mammalian host, are regarded as different species. The systematic position of *Herpetosoma* trypanosomes differing only on host restriction is uncertain. However, Hoare (1972) proposed to retain distinctive names for *T. lewisi*–like trypanosomes, which can be distinguished by some biological features as it is easier to re-group the named parasites when the knowledge advances than to separate anonymous ones from a common pool. A majority of species of the subgenus *Herpetosoma* are found in rodents, lagomorphs and soricids with few reports in monkey and bats (Hoare 1972; Maia da Silva et al. 2010).

The subgenus *Schizotrypanum* is also a homogeneous group of trypanosomes with an indistinguishable morphology among the species of the same group (Hoare 1972; Baker et al. 1978). The type species of this subgenus is *T. cruzi*, which infects a wide range of mammals and is mainly found in New World, widely distributed in Latin and Central America. In human beings, *T. cruzi* causes a devastating disease known as Chagas disease which affect people of all age. Other *Schizotrypanum* trypanosomes are considered non-pathogenic and are restricted to Chiropteran (bats) hosts, mostly found in New World and a few species have been reported in Europe and Africa (Hoare 1972; Baker et al. 1972; Hamilton et al. 2012b; Lima et al. 2012; Marcili et al. 2013; Cottontail et al. 2014; Hodo et al. 2016). According to Hoare (1972), classification of trypanosomes in the *Schizotrypanum* is done based on biological distinctions especially host-parasite relationship as morphological characters do not provide reliable criteria. Haore (1972) stated that the subgenus *Schizotrypanum* is a complex of *T. cruzi*-like trypanosomes, and its taxonomic status and mutual affinities need further investigation. In Chapter III of the present study, I will explain the current status of trypanosomes classified in this subgenus.

Life cycle of Mammalian trypanosomes

Trypanosomes are digenetic parasites. Their life cycle involves alteration of vertebrates which are the definitive vertebrate host and haematophagous invertebrate host referred as vectors (Hoare 1972; Lizundia et al. 2011; Peacock et al. 2012; Carrea and Diambra 2016). In the mammalian host the trypanosomes are introduced as the metacyclic trypomastigotes (metatrypanosomes), either through contamination for stercorarian trypanosomes or through a bite during blood feeding for salivarian trypanosomes (Hoare 1972; Pinto et al. 1999; Tyler and Engman 2003; Lizundia et al. 2011; Peacock et al. 2012; Mathew et al. 2004; Mathew 2005).

For salivarian trypanosomes, when metacyclic trypomastigotes inoculated into the mammalian host by the vector, they first develop locally in the site of inoculation and transform into slender trypomastigote forms (Hoare 1972; Mathew et al. 2004). The slender trypomastigote forms enter the circulation and continue its proliferation to initiate first peak of parasitaemia (Hoare 1972; Mathew et al. 2004; Mathew 2005). During the ascending phase of parasitaemia the parasite population is slender forms and at the peak of parasitaemia the population is mainly of stumpy form which is non-dividing form (Mathew et al. 2004; Mathew 2005).

The vector of salivarian trypanosomes, tsetse fly (*Glossina* spp.) picks the stumpy form of trypomastigotes during blood feeding. In the midgut of tsetse fly they undergo their differentiation to the procyclic trypomastigote form (Mathew et al. 2004; Peacock et al. 2012), which proliferate in the midgut and then migrate anteriorly to the mouthparts (Peacock et al. 2012). The parasites then differentiate into epimastigote forms which will either invade the salivary gland or proboscis depending on the species. In the salivary gland/proboscis they will differentiate to the metacyclic trypomastigotes which are the infective stage to mammalian host (Peacock et al. 2012).

For the stercorarian trypanosomes, the life cycle has been best studied in type species for each subgenus; namely, *T. cruzi* (Hoare 1972; Pinto et al. 1999; Tyler and Engman 2003), *T. theileri* (Hoare 1972; Dirie et al. 1990; Bose and Heister 1993) and *T. lewisi* (Hoare 1972). For *T. cruzi*, the vector is a reduviid bugs (*Triatoma* spp.). Metacyclic trypomastigotes present in the vector faeces are introduced to the mammalian host through the contamination of insect bite wound. The metacyclic trypomastigotes enters the nucleated phagocytic and non-phagocytic cells, where they undergo morphological transformation to form amastigote forms. The parasite proliferates in the cell as amastigote forms until the cell is full. Then the parasites become elongated acquiring its long

flagellum; the stage referred as intermediate epimastigote. Intermediate epimastigotes differentiate to slender trypomastigotes, which escape the cell and can invade the adjacent cells to start a new cycle or enter the bloodstream. In the bloodstream, extracellular trypomastigotes differentiate to broad trypomastigotes or extracellular amastigotes. The three forms can all be found in the mammalian blood circulation and can be picked by the insect during blood feeding (Tyler et al. 2003; Pinto et al. 1999).

For *T. theileri* and *T. lewisi,* multiplication in mammalian hosts mainly occurs extracellularly in the blood stream where the parasites divide by the binary fission of

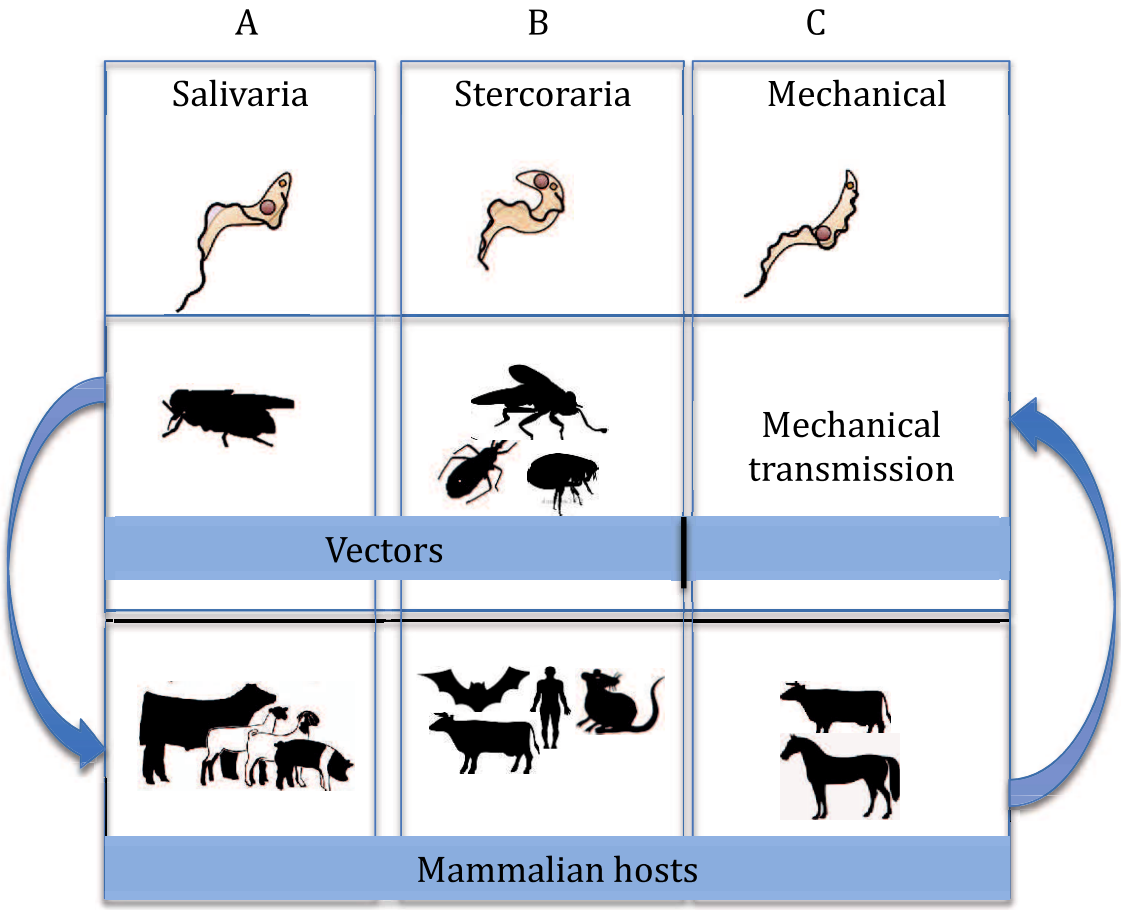

Fig. 3. Schematic life cycle of trypanosomes. Panel **A** represents lifecycles of salivarian trypanosomes transmitted by tstse fly, **B** stercorarian trypanosomes transmitted by tabanid flies, rat fleas and triatomine bugs, and **C** represents mechanically transmitted trypanosomes such as *T. evansi* and *T. equiperdum*

epimastigote stage or multiple fission of plasmodial stage (Hoare 1972). The epimastigotes then differentiate to slender and broad trypomastigotes forms which are picked by the intermediate host; Tabanidae flies in case of *T. theileri* (Hoare 1972; Dirie et al. 1990; Bose and Heister 1993) or rat fleas in the case of *T. lewisi* (Hoare 1972). In the vector midgut, the parasites multiply as epimatigotes. Development and differentiation continues until the parasites migrate from midgut to the hindgut. Then parasites differentiate from epimastigotes to amastigotes and then metacyclic trypomastigotes which are the infective form to the mammalian host (Hoare 1972; Dirie et al. 1990; Bose and Heister 1993).

For many other trypanosomes of the stercorarian section, their vectors and life cycles remain unknown. Reduviid bugs (*Triatoma* spp.) have been reported as the vector of most *Schizotrypanum* species in the New world while bat bugs (Cimicidae) been reported as the vector of *T. dionisii* in Old world.

Evolution of trypanosomes.

The origin of genus *Trypanosoma* is controversial due to lack of paleontological evidence (Vickerman 1994). According to Hoare (1972), speculations about the origin of trypanosomes were centered on the close resemblance of some trypanosome developmental stages, in the insect vector, to the monogenic trypanosomatid specific to insects, especially *Blastocrithidia* spp. The first hypothesis suggested the ancestors of trypanosomes to be monogenic parasites of invertebrates that were transferred to vertebrates through blood sucking and adapted to the life in vertebrate host (Hoare 1972; Vickerman 1994; Maslov and Simpson 1995). Another hypothesis suggested that the ancestors of trypanosomes were gut parasites of vertebrate hosts disseminated through cyst in faeces, and at some point, those primitive parasites penetrated the gut of vertebrates, to the blood where they were picked by blood-sucking insect and adapted to the life in the gut

of invertebrates (Hoare 1972; Vickerman 1994; Maslov and Simpson 1995). Both hypotheses have been criticized by several researches; some supporting the latter (Vickerman 1994) while others supporting the former (Hoare 1972). Those supported the former, they based their argument on the fact that there is no trypanosmatid parasites of vertebrate gut known today, and also some trypanosomes (e.g. *T. melophagium*) are not yet fully adapted to blood medium but are still essentially insect-parasites (Hoare 1972). On the other hand, those supported the latter, their arguments centered to the fact that, no successful experimental infection of vertebrate hosts by invertebrate trypanosomatid, has been accomplished, and it is highly possible to accidentally transfer a parasite from the blood of large animal to blood-sucking insect than vise versa (Vickerman 1994). Vickerman (1994) speculated the ancestors of trypanosomes to be a parasite of aquatic vertebrates transmitted by leech vectors as it is for today's fish and amphibian's trypanosomes.

The development of molecular technology, which enable use of molecular sequencing in phylogenetic studies, has not clearly resolved the problem of trypanosome evolution. The phylogenetic trees constructed using nucleotide sequences of the small subunit (SSU) rDNA, had different topologies depending on the number of taxa included and the outgroup (Vickerman 1994; Maslov and Simpson 1995; Haag et al. 1998; Stevens et al. 1999b, 2001). Early constructed phylogenetic trees, indicated trypanosomes are paraphyletic (Vickerman 1994; Maslov and Simpson 1995) with *T. brucei* and *T. cruzi* branching out earlier than monogenetic trypanosomatids of insects. Paraphyletic trees suggested that parasitism and digenetic lifecycle in trypanosome lineages had arisen more than once (Stevens et al. 2001). However, reconstruction of phylogenetic trees by addition of more trypanosome taxa in the phylogenetic analyses revealed that trypanosomes are monophyletic and they evolved from monogenetic trypanosomatids of insects (Haag et al

1998; Stevens et al. 1999b, 2001; Simpson et al. 2006, Hamilton et al. 2007; Lukes et al. 2014).

Molecular methods and current taxonomy

Several molecular methods have been employed in the identification of trypanosomes. DNA probes of non-coding satellite repeats, were among the first molecular methods used to identify trypanosomes both in mammalian host and in the vectors (Kukla et al. 1987; Adams and Hamilton 2008; Hutchinson and Stevens 2017). DNA probes were overtaken by polymerase chain reaction (PCR), which is more sensitive and can identify trypanosomes to the species or subspecies level, when species-specific primers are used (Adams and Hamilton 2008; Hutchinson and Stevens 2017). Species-specific PCR method can detect trypanosomes in samples with a very low number of parasites, hence frequently used for detection and identification of trypanosomes (Adams and Hamilton 2008). However, its use is limited to the known species whose species-specific primers are available. To solve this problem generic primers targeting a conserved region of the genome are used (Adams and Hamilton 2008). Species identification is made by comparing the size of amplified fragments, or by restriction length polymorphism-PCR (RFLP-PCR) method (Adams and Hamilton 2008; Hutchinson and Stevens 2017).

Development of DNA sequencing made possible to identify the species by comparing the unknown DNA sequences with that of known species from the sequence database (Adams and Hamilton 2008). The growth of Next-Generation sequencing decreased the cost of sequencing (Hutchinson and Stevens 2017) and hence molecular sequencing rapidly became indispensable (Votypka et al. 2015). Several target genes have been used as molecular markers for trypanosome characterization. These include SSU rDNA, internal transcribed spacers (ITS) 1 and 2, glycosomal glyceraldehyde 3-phosphate dehydrogenase

(gGAPDH), spliced leader RNA (SL RNA) genes, etc. (Haag et al. 1998, Hamilton et al. 2004, Adams and Hamilton 2008; Votypka et al. 2015; Hutchinson and Stevens 2017). Among these markers SSU rDNA and gGAPDH gene, are commonly used for trypanosome identification and phylogenetic analysis. SSU rDNA is a highly expressed multicopy gene, which contain both conserved region suitable for universal primers and variable region suitable for providing taxonomic information (Hutchinson and Stevens 2017). As DNA sequencing increased its popularity, SSU rDNA has become the gene of choice for nearly all trypanosome evolution analysis and has formed the basis of all modern trypanosome taxonomic frameworks (Vickerman 1994; Maslov and Simpson 1995; Haag et al. 1998; Stevens et al. 1999b, 2001; Sato et al. 2005, 2008, 2009; Hutchinson and Stevens 2017). The gGAPDH gene is used as an additional marker in phylogenies, especially when there are inadequate signals at certain depth of phylogenetic trees, constructed using SSU rDNA (Hamilton et al. 2004; Hutchinson and Stevens 2017). Targetted nucleotide sequences of gGAPDH are shorter than that of SSU rDNA and relatively conserved, but provides a complementary depth of phylogenetic information (Hamilton et al. 2004; Hutchinson and Stevens 2017).

Taxonomy of trypanosomes based on molecular phylogeny using SSU rDNA and gGAPDH genes, grouped mammalian trypanosomes into four clades, which are *T. brucei*, *T. cruzi*, *T. lewisi* and *T. theileri* clades (Hamilton et al. 2007). *T. brucei* clade contain tsetse transmitted African trypanosomes (*T. brucei, T. congolense*, *T. vivax*, and *T. simiae*) together with *T. evansi* and *T. equiperdum* transmitted mechanically by tabanid flies and by coitus, respectively (Stevens et al. 2001; Hamilton et al. 2007). *T. cruzi* clade contain *T. cruzi cruzi*, *T. cruzi marinkellei, T. rangeli, T. dionisii*, *T. vespertilionis*, *T. conorhini*, and *T. minasense* (Stevens et al. 2001; Hamilton et al. 2007; Sato et al. 2008). *T. lewisi* clade contain mainly rodent trypanosomes (*T. lewisi*, *T. musculi*, *T. microti*, *T. nabiasi, T. grosi,*

T. otospermophili, and T. kuseli) and *T. theileri* clade contain *T. theileri* and *T. cyclops* (Hamilton et al. 2007).

Gibson (2003) defined the species as a group of species with a genetically similarity. However, the challenges arised on how to determine what level of similarity define a species and how long ago the lineages separated from each other (Gibson 2003). Molecular taxonomy also group *T. rangeli*, initially grouped in subgenus *Herpetosoma* (Hoare 1972), into *T. cruzi* clade, which mainly contain trypanosomes initially grouped into subgenus *Schizotrypanum* (Stevens et al. 1999a; Hamilton et al. 2007). Likewise, *T. conorhini* and *T. minasense* were initially grouped into subgenus *Megatrypanum*, but according to molecular taxonomy, they cluster together with *T. cruzi* clade (Stevens et al. 2001; Hamilton et al. 2007), making *Megatrypanum* trypanosomes paraphyletic. This has raised different opinions among the researchers; some arguing the validity of using subgenera in taxonomy (Stevens et al. 1999a, 2001), while others still insisting the use of subgenera in taxonomy of trypanosomatids (Votypka et al. 2015). Votypka et al. (2015) stated that "the traditional taxonomy has not been designed for protists, but nor has modern molecular phylogenetics". In my study, traditional taxonomy using morphology was combined with molecular phylogenetics to characterize trypanosomes from bats, rodents and soricids to clarify their taxonomic status as well as molecular phylogenetic relationship.

Bat trypanosomes

Bats mostly harbour trypanosomes of the *T. cruzi* clade, and hence bat trypanosomes have been associated with the evolutionary history of *T. cruzi* (Hamilton et al 2012a). Extensive studies of bat trypanosomes have been conducted in the North and South American continents where *T. cruzi* is endemic. *T. cruzi* is grouped into six discrete typing units (DTUs) named *T. cruzi* I – *T. cruzi* VI (TcI-TcVI) (Zingales et al. 2009, 2012). TcI

predominantly infect human in endemic areas, but also commonly infect bats in the sylvatic cycle in South and North America, together with 'Tcbat' (a lineage of *T. cruzi* commonly found in bats) (García et al. 2012; Hodo et al. 2016). Other bat trypanosomes are *T. cruzi marinkellei*, *T. wauwau T. erneyi*, *T. livingstonei* and *T. dionisii. T. cruzi marinkellei* is widely distributed in South America (Maia da Silva 2009 ; Pinto et al. 2015) while *T. wauwau* have only been reported in Brazil (Lima et al. 2015). *T. dionisii* is widely distributed in the American continents and in Europe. Initially *T. dionisii* was considered to be restricted to bats, but recently it has also been reported from a human patient (Dario et al. 2016). *T. erneyi* and *T. livingstonei* are both found in African continent (Lima et al. 2012, 2013).

The studies of bat trypanosomes have indicated that bats can play a great role in parasite dispersion due to its ability to fly a long distance. The study of Hamilton et al. (2012b) reported that the *T. dionisii* genotypes isolated from bats in Europe were closely related to those found in South America. These imposed a question on the route of movement of the parasites and their host. However, the information about bat trypanosomes in Asia, which could contribute to unveil the parasite movement from Old World to New World or vise versa, is so scarce. Combination of morphology, molecular genetics, natural hosts and geographical distribution of bat trypanosomes is important to understand the speciation of *T. cruzi* in Latin America.

Rodent trypanosomes

Rodents generally harbour trypanosomes of the subgenus *Herpetosoma*, but also some *Megatrypanum* trypanosomes such as *T. conorhini* and *T. zeledoni* are found in rodents (Hoare 1972). As mentioned earlier, *Herpetosoma* trypanosomes are morphologically indistinguishable and therefore they are referred as *T. lewisi*-like trypanosomes. Among the

trypanosomes found in rodents are *T. lewisi, T. musculi, T. grosi, T. otospermophili, T. rabinowitschae, T. microti* and *T. evotomys*. Most rodent trypanosomes are specific to their vertebrate hosts and therefore the species of rodent trypanosomes are named based on their host-restriction and development into their mammalian host. *T. lewisi* is specific to rodents of genus *Rattus*. It commonly infects black and brown rats (*Rattus rattus* and *Rattus norvegicus*) throughout the world, but it can also infect other species in the same genus (Hoare 1972). Under natural conditions *T. lewisi* is not pathogenic to rats and upon infection, rats can recover spontaneously and become immune to the reinfection. Several cases of *T. lewisi* and *T. lewisi*-like infections in human patients have been reported in Asia and Africa some of them culminating into death.

T. musculi is the *T. lewisi*-like trypanosomes of house-mouse (*Mus musculus*). They are mainly distributed in warm countries of Mediterranean basin and the west coast of Africa (Hoare 1972). *T. grosi* is another *T. lewisi*-like trypanosomes which mainly infect wild mice of the genus *Apodemus* with high level of host restriction, but can also experimentally infect Mongolian jirds (*Meriones unguiculatus*) (Sato et al. 2003). Distribution of *T. grosi* is the same as that of its host and is widely distributed in Europe and Asia (Hoare 1972).

All other *T. lewisi*-like trypanosomes are specific to their vertebrate host to some extent, generally infecting only one rodent genus. *T. otospermophili* infect ground squirrels of genus *Citellus* while *T. rabinowitschae, T. microti* and *T. evotomys* infect rodents of the family Cricetidae and genera *Cricetus, Microtus* and *Clethrionomys* respectively (Hoare 1972). Many other *T. lewisi*-like trypanosomes are found in rodents but majority of them were only characterized basing on their morphology and host restriction. The emergence of human infection caused by *T. lewisi*-like trypanosomes, is prompting molecularly

characterizing the so-called *T. lewisi*-like trypanosomes to validate their taxonomy, which is the basis for the diagnosis, treatment and control of their caused zoonosis.

Context of the study

Small mammals such as rodents, bats and soricids have worldwide distribution and commonly found in close association with human settlements (Datiko et al. 2007; Bantihun and Bekele 2015; Habtamu and Beleke 2008; Kunz and Reynolds 2003), which pose the great risks of disease transmission from small mammals to human residents as disease vectors are closely associated with their host and are not strictly host specific. Sometimes they can feed on humans as an opportunistic host. As stercorarian trypanosomes are transmitted through contamination of vector faeces or ingestion of the infected vectors, it is commonly a problem of unhygienic environment of rural poor people. Most rural families especially in Sub-saharah Africa are living in areas where there is no access to modern health centers and many human fatal cases; the cause of death is unknown. However, many poor families either share their shelters with small mammals or the small mammals are found in their surrounding. Howie et al. (2006) who reported the case of *T. lewisi*-like trypanosomiasis in two-months old girl in Gambia, stated that the patient's twin brother and their mother died with unknown cause. The visit to the patient's house, revealed the rodent's droppings in patient's bed and even a dead rodent on the porch, indicating the house was infested with rodents. In addition, almost all *T. lewisi*-like infection cases in Southeast Asia have been reported in families which shared their dwellings or surrounding with small mammals (Doke and Kar 2011; Verma et al. 2011). Nevertheless Hamilton et al. (2012a) suggested that the ancestors of trypanosomes of the *T. cruzi* clade were bat trypanosomes that switched the host and acquired the ability to infect other mammals including human (bat seeding hypothesis). So, it is important to understand the diversity,

taxonomy and phylogeny of trypanosomes infecting small mammals of wider geographical distribution.

Many trypanosome studies have focused on the pathogenic trypanosomes that mainly affect large mammals such as cattle (*T. vivax*, *T. brucei*, *T. congolense*, *T. evansi*), human (*T. b. gambiense*, *T. b. rhodensiense*, *T. cruzi*), and horses (*T. equiperdum*), but little attention has been directed to small mammal trypanosomes.

In my PhD study, I firstly reviewed atypical human trypanosomiasis of *T. lewisi* and *T. lewisi*-like and summarized the reported cases, the background of their incidences and discussed the molecular diagnostic approaches. Then I employed morphological features and two genetic markers (SSU rDNA and gGAPDH), to characterize trypanosomes from rodents and soricids collected from Indonesia, China, Philippines, Taiwan and Vietnam, and from bats collected in Japan, and clarify their taxonomy, molecular characteristics and their phylogenetic relationships.

Study Objectives.

The main objective of my study was to employ morphological and molecular characteristics to clarify taxonomy, diversity and phylogenetic relationships of small mammals' trypanosomes. The specific objectives were (i) To determine the prevalent species, natural hosts and their geographical distribution in the selected parts of Asia, (ii) To determine their genotypic diversity, (iii) To determine their phylogenetic relationships. Two molecular markers: SSU rDNA and gGAPDH were used to deduce genotype diversity and phylogenetic relationship.

CHAPTER I

Brief review on atypical human trypanosomiasis of *Trypanosoma lewisi*

Information described in this chapter has been published as follows;

Eliakunda Mafie, Fatema Hashem Rupa, Aogu Setsuda, Atsuko Saito-Ito, Hiroshi Sato (2016). Brief review on atypical human trypanosomiasis of *Trypanosoma lewisi*. Japanese Journal of Veterinary Parasilogy 15:24-33.

Abstract

Trypanosomes (Kinetoplastea: Trypanosomatida: Trypanosomatidae), particularly salivarian trypanosome species as well as stercorarian *Trypanosoma cruzi*, are important parasites of humans and other animals that cause often fatal diseases. Stercorarian *T. lewisi* is known as a rat-specific species of the subgenus *Herpetosoma*. Rodent trypanosomes cause latent infections, and *T. lewisi* infection beyond the genus border, e.g. infection in mice, is considered to be virtually impossible. Nevertheless, nine human cases of *T. lewisi* infection have been reported in recent decades, with an increased incidence (five cases) in the last two decades. In the present review, I have summarized the records of atypical human trypanosomiasis ascribed to *T. lewisi* infection and provide information on the background of disease incidences and possible PCR-based diagnostic approaches.

Introduction

Haemoflagellates of the genus *Trypanosoma* (Kinetoplastea: Trypanosomatida: Trypanosomatidae) have a wide range of hosts (fish, amphibians, reptiles, birds, and mammals) and are transmitted by blood feeding invertebrates. Members of the genus are conveniently divided into two major groups, i.e. Salivaria and Stercoraria, based on their mode of transmission (Hoare 1972). Salivarian trypanosomes are transmitted to the recipient via the saliva of vectors, whereas stercorarian trypanosomes are transmitted via vector faeces or vectors themselves. The former group of trypanosomes (e.g. *Trypanosoma brucei*, *T. vivax*, and *T. congolense*), transmitted by tsetse flies, is of medical and/or veterinary importance. The diseases caused by the latter group (stercorarians), with the exception of *T. cruzi*, are latent.

Trypanosoma lewisi with a cosmopolitan distribution is the type species of the

subgenus *Herpetosoma*, which includes a variety of trypanosome species of rodents (ca. 50 nominal species) (Hoare 1972). Members of *Herpetosoma* exhibit an almost identical morphology of bloodstream trypomastigote forms. However, they usually have a strict host specificity, so no infection occurs beyond the families, subfamilies, or genera of host rodents (Hoare 1972). By use of special rodent hosts such as Mongolian jirds (*Meriones unguiculatus*) treated with immunosuppressants, the partial host barrier to *Herpetosoma* infection can be overcome. This was demonstrated by the successful experimental infection of *T. grosi* and *T. lewisi*, which take *Apodemus* spp. or *Rattus* spp. as natural hosts, respectively, in immunosuppressed Mongolian jirds (Sato et al. 2003, 2004, 2005).

Human cases of *T. lewisi* infection

Human cases of *T. lewisi* infection have been reported in different parts of the world. The first noted infection was in 1933 in a four-month-old infant living in Malaysia (Table 1) (Johnson 1933; Truc et al. 2013; Sousa 2014). More than 40 years later, two other cases of *T. lewisi* infection were reported – a married couple living in a village in Madhya Pradesh, India (Shrivastava and Shrivastava 1974). In these three cases, the trypanosomes in blood films were identified morphologically, and all the cases were spontaneously cured. After another extensive time period, six more human cases of *T. lewisi* infection were reported (Gambia, one case in 2003; Thailand, one case in 2003; and India, four cases in 2006, 2007, 2010, and 2014) (Howie et al. 2006; Kaur et al. 2007; Sarataphan et al. 2007; Doke and Kar 2011; Shah et al. 2011; Verma et al. 2011; Warpe and More 2014). Major symptoms of these *T. lewisi*-infected patients included fever, coughing, anorexia, and depression (Joshi et al. 2005; Sarataphan et al. 2007; Shah et al. 2011; Verma et al. 2011). In the most recent cases, anti-trypanosome drugs were effectively used with a good prognosis (Truc et al. 2013; Warpe and More 2014).

In addition to these nine human cases of *T. lewisi* infection, atypical human trypanosomiasis has been caused by *T. brucei brucei* (four cases in 1930, 1947, 1987, and 2003), *T. congolense* (one case in 1998), and *T. evansi* (five cases in 1977, 1999, 2004, 2005, and 2010) (Joshi 2013; Truc et al. 2013). The five human cases of *T. evansi* infection were reported in India (three cases), Sri Lanka (one case), and Egypt (one case), and other human cases of salivarian animal trypanosomes have been reported in the Sub-Saharan region (Truc et al. 2013). Patients infected with salivarian animal trypanosomes had a fluctuating trypanosome parasitemia associated with febrile episodes for a long time period, e.g. five months (Joshi et al. 2005). Due to an increasing number of reports of patients with atypical trypanosomiasis in the last two decades, *T. evansi* and *T. lewisi* are now regarded as emerging protozoan pathogens in humans. Truc et al. (2013) proposed that the actual prevalence is probably underestimated based on the following study. From a seroepidemiological survey conducted in a village where a patient with *T. evansi* infection was identified, 22.7% of the residents (410/1,806) were found to be antibody-positive by a card agglutination test for *T. evansi* (CATT/*T. evansi*; Institute of Tropical Medicine, Antwerp, Belgium) with a 1:4 dilution of whole blood. Positive residents were subsequently subjected to serosurvey using serum samples and a card agglutination test for trypanosomiasis (CATT; Institute of Tropical Medicine). The prevalence of confirmed sero-positive residents was reduced to 4.5% (81/1,806) and no trypanosomes were microscopically detected in them (Shegokar et al. 2006), suggesting substantial exposure occasions of human residents to *T. evansi* in the surveyed area. Considering the recent geographical expansion of *T. evansi* in North Africa, Middle East, southern Eurasia, South Asia, and South America (Luckins 1988; Desquesnes et al. 2013), it is highly possible that human exposure occasions to the trypanosomes might be increased in the near future, as is the case with domestic and wild animals at present.

In India, epidemiological surveys of *T. lewisi* in rats, conducted in areas surrounding patients' houses, showed prevalences of around 20% (Doke and Kar 2011; Verma et al. 2011). These values are similar to those from other field studies in other areas in different countries such as Brazil, Ecuador, Cambodia, Lao PDR, Thailand, and China (Linardi and Botelho 2002; Pinto et al. 2006; Jittapalapong et al. 2008; Tang et al. 2012; Pumhom et al. 2014). Maia da Silva et al. (2010) reported *T. lewisi* infection in Brazilian monkeys in captivity at a rate of 3/160 (two *Callithrix jacchus* and one *Alouatta fusca*).

Factor(s) responsible for host specificity of trypanosomes

Trypanosoma lewisi and other rodent trypanosomes, often referred to as *T. lewisi*-like due to their similar morphology, are transmitted by fleas whereby rats are infected by either licking flea feces on their fur or ingesting infected fleas (Albright and Albright 1991; Hoare 1972). Human infection requires an infestation of fleas or flea feces, with most human cases being associated with patients living in non-hygienic houses with potential exposure to rat fleas and/or their feces. Once infected, human innate immunity against atypical trypanosomes must work to eliminate the haemoflagellates.

Typical human trypanosomiasis is caused by *T. brucei gambiense* which causes chronic sleeping sickness in West Africa, *T. brucei rhodesiense* which causes acute sleeping sickness in East Africa, and *T. cruzi* which causes Chagas disease in South America. These human trypanosomes are capable of infecting humans because they are resistant to normal human serum (NHS), i.e. trypanolysis induced by human serum protein apolipoprotein L-I (apoL-I), while animal trypanosomes are susceptible to this protein and cannot survive in human blood (Vanhamme et al. 2003; Pérez-Morga et al. 2005). A lack of apoL-I was demonstrated in patients with clinical manifestation of atypical trypanosomiasis due to *T. evansi* (Vanhollebeke et al. 2006).

The apoL-I protein contains an ionic pore-forming domain and an adjacent pH-sensitive membrane-addressing domain (Pérez-Morga et al. 2005). After endocytosis, the protein triggers the formation of anion selective pores in the lysosomal membrane, inducing osmotic swelling of the lysosomes and subsequent trypanolysis (Vanhamme et al. 2003; Pérez-Morga et al. 2005; Vanwalleghem et al. 2015). Human trypanosomes such as *T. brucei rhodesiense* inhibit apoL-I activity by expressing a truncated form of the variant surface glycoprotein, termed serum resistance-associated protein, which is a lysosomal protein (Vanhamme et al. 2003).

Lun et al. (2015) recently reported that *T. lewisi* is resistant to NHS containing trypanolytic apoL-I. This perception is based on their findings that *T. lew*isi could resist up to 90% NHS or 30μg/ml recombinant human apoL-I (rhapoL-I) *in vitro*. In contrast, *T. brucei brucei* could not survive in 0.0001% NHS or 0.1 μg/ml rhapoL-I. Furthermore, their *in vivo* experiments using *T. lewisi*- and *T. brucei brucei*- infected rats and NHS injections supported their *in vitro* results, revealing that *T. lewisi* might be fully resistant to NHS (Lun et al. 2015). Apart from this finding, it is thought that the inability of *T. lewisi* to infect a range of mammals is associated with the trypanolytic activity of both the complement and granulocytes (Ferrante 1985; Albright and Albright 1991). Consequently, immature or depressed immune systems allow the host to be infected with rat trypanosomes as an opportunistic parasite. Therefore, considering that five out of the nine aforementioned human cases of *T. lewisi* infection were reported in unweaned babies between 1.5 and 4 months of age (Table 1), it appears that atypical human trypanosomiasis requires not only exposure chances to infected fleas but also a defective innate immunity in hosts.

Molecular diagnosis of atypical trypanosomiasis

The levels of parasitaemia in atypical trypanosomiasis are usually too low to provide sufficient numbers of bloodstream trypomastigotes for a specific diagnosis based on morphology. In addition, as mentioned above, rodent trypanosomes of the subgenus *Herpetosoma* show an almost identical morphology, making specific identification difficult. Hence, molecular diagnosis of atypical trypanosomiasis is essentially necessary for all cases (Truc et al. 2013). Cases of atypical trypanosomiasis caused by *T. lewisi* or *T. lewisi*-like trypanosomes in human patients and non-human primates have been diagnosed by molecular technologies using PCR amplification and/or nucleotide sequencing of the ribosomal RNA gene (rDNA) (Sarataphan et al. 2007; Maia da Silva et al., 2010). As illustrated in Figures 3 and 5 of Sato et al. (2008), almost the full length of the 18S rDNA has been sequenced and deposited for only a portion of the rodent trypanosomes. Out of approximately 50 nominal species of the subgenus *Herpetosoma* (Hoare 1972), at present just eight of them are contained in the database provided by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm. nih.gov/taxonomy). However, a few more sequences of other nominal rodent trypanosomes (such as *T. nabiasi* and *T. evotomys*) and multiple anominal species have been deposited. Previous studies on the genetic characterization of the SSU rDNA and/or the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene were conducted to clarify the interspecific relationships of mammalian trypanosomes. To improve the resolution of species identification, the internal transcribed spacer 1 (ITS1) region of rDNA has recently been used to discriminate *T. lewisi* and its isolates from atypical trypanosomiasis in humans and non-human primates (Desquesnes et al. 2002; Sarataphan et al. 2007; Maia da Silva et al. 2010; Desquesnes et al. 2011).

As shown in Fig. 4 and Table 2, as far as is currently known, multiple nominal species such as *T. lewisi, T. musculi*, and *T. blanchardi* have an almost identical SSU rDNA nucleotide sequence with few nucleotide substitutions. Furthermore, Brazilian *T. lewisi* isolates consistently display a nucleotide substitution in comparison to ones from other places (USA, UK, and Asia) (Table 2). Contrastingly, *T. grosi* isolates from *Apodemu*s spp. in the Far East show substantial intraspecific nucleotide variations, comparable to or even more than the interspecific variations among the aforementioned *Herpetosoma* species. The ITS1 region of *T. lewisi* and *T. lewisi*-like trypanosomes is characterized by multiple repeats of single, double, or longer nucleotide units, such as 'TA', 'TG', and 'AC'. The numbers of repeats of such few nucleotide units often vary even in a single parasite individual or an isolate of some helminths (Makouloutou et al. 2013; Setsuda et al. 2016); however, little is known in the case of trypanosomes. Tang et al. (2012) reported that 16 *T. lewisi* isolates from Guangzhou (Southern China) had the same ITS1 sequence, which was slightly different from other isolates from Jilin (Northern China) and Thailand. Table 3 summarizes the nucleotide differences in the ITS1 region among *T. lewisi* and *T. lewisi*-like trypanosomes (only those closest to *T. lewisi*, i.e. *T. blanchardi* and *T. rabinowitschae*). Although these sequences are only representative at present, it would appear that ITS1 nucleotide sequences can successfully discriminate *T. lewisi* from closely related *T. lewisi*-like species such as *T. blanchardi* and *T. rabinowitschae*. When eliminating nucleotide unit repeats, 11 nucleotide positions (16, 69, 94, 101, 149, 167, 193, 212, 230, 236, and 352 in Table 3) are relatively significant to discriminate *T. lewisi* from these two closely related species, and five nucleotide positions (24, 33, 89, 93, and 364 in Table 3) are important to discriminate major genotypes of *T. lewisi*. The almost identical ITS1 nucleotide sequences of *T. blanchardi* and *T. rabinowitschae* (see Table 3) are in contrast to the more divergent 18S rDNA nucleotide sequences of these two species (see

Table 2). Thus, the collection of more ITS1 nucleotide sequences of *Herpetosoma* trypanosomes is required to clarify the significance of this region in inter- and intraspecific discrimination. Fig. 5 illustrates the relationships among *T. lewisi* isolates collected to date based on their ITS1 nucleotide sequences.

Finally, I would like to emphasize that phylogenetic trees using the ITS region of closely related *Herpetosoma* trypanosome species may only be valid if they are constructed extremely carefully. Usually it is not appropriate to use ITS-based phylogenetic trees to determine the relationships among trypanosomes in general or even in limited categories. I also need to surmount the difficulties of sequencing the ITS1 region with its multiple repeats of certain nucleotide units.

Conclusive remarks

Records of atypical human trypanosomiasis caused by *T. evansi* and *T. lewisi* appear to have increased in number in the last two decades. Considering the geographical expansion in distribution and increased prevalence of *T. eva*nsi in the Middle East, India, Asia, and South America, and the wide spectrum of vectors transmitting the trypanosomes (including the genera *Tabanus*, *Stomoxys, Haematopota*, *Chrysops*, and *Lyperosia*) (Luckins 1988; Desquesnes et al. 2013), the chance of human exposure to infective *T. evansi* is likely to be increased at the present time and in the future regardless of the hygienic status of human living. In contrast, as vectors of *T. lewisi* are rat fleas and transmission of *T. lewisi* occurs by human ingestion of flea faeces or fleas themselves, the hygienic status of human living is closely related to the occurrence of atypical human trypanosomiasis regardless of geographical location because the host (rats) and parasite (*T. lewisi*) are cosmopolitan in distribution. Little is known in relation to how *T. lewisi* is able to infect humans. However, it is apparent that some humans and nonhuman primates,

particularly lactating babies, are more susceptible to the infection and/or the disease can manifest itself more easily (Maia da Silva et al. 2010; Truc et al. 2013). Although microscopic discrimination of *T. lewisi* infection in atypical hosts is extremely difficult, molecular approaches using the rDNA sequence are emerging. However, due to a low social interest in non-pathogenic trypanosomes, the current number of basic studies of rodent trypanosomes of the subgenus *Herpetosoma* (Hoare 1972), particularly their genetic characterization, is not sufficient, and only a limited number of nucleotide sequences is available for a limited number of rodent trypanosome species. Presently, specific discrimination of the subgenus *Herpetosoma* largely relies on the host category since the morphology of trypomastigotes in the bloodstream is almost identical (hence the term "*T. lewisi*-like") and little is known about the biology of each species. As shown in Table 2, the intraspecific nucleotide variations of *T. grosi* exceed the interspecific nucleotide variations between, for example, *T. blanchardi* and *T. lewisi* or *T. musculi.* In order to provide the basis for an accurate specific discrimination in atypical human trypanosomiasis, greater effort in both the field and laboratory needs to be directed towards the collection of more rodent trypanosome isolates for genetic and biological characterization.

Table 1. Human cases of *Trypanosoma lewisi* or *T. lewisi*-like trypanosome infection *

Case No.	Country	Age and sex of patient	Trypanosome Species	Year of notice	Method of species identification	Symptoms	Prognosis	Reference
1	Malaysia	4-month-old infant	*T. lewisi*	1933	Morphology	Fever	Self cure	Johnson (1933)
2	India (Madhya Pradesh)	One of a married couple	*T. lewisi*	1974	Morphology	Fever	Self cure	Shrivastava and Shrivastava (1974)
3	India (Madhya Pradesh)	One of a married couple	*T. lewisi*	1974	Morphology	Fever	Self cure	Shrivastava and Shrivastava (1974)
4	Gambia	a baby	*T. lewisi*-like	2003	Genetic (ITS1)	Fever	Cure	Howei et al., (2006)
5	Thailand	45-day-old infant	*T. lewisi*-like	2003	Genetic	Fever	Cure	Sarataphan et al., (2007)
6	India (Mumbai)	1.5-month-old girl	*T. lewisi*	2006	Morphology	Fever	Self cure	Kaur et al. (2007; Shah et al. 2011)
7	India (Pune)	57-year-old man	*T. lewisi*	2007	Genetic	Fever	Death	Doke and Kar, (2011)
8	India (Bagpat)	37-day-old infant	*T. lewisi*	2010	Genetic	Fever	Cure	Verma et al. (2011)
9	India (Madhya Pradesh)	40-year-old man **	*T. lewisi*	2014	Morphology	Fever	cure	Warpe et al. (2014)

* Modified after Truc et al. (2013).

** Immunocompromised person with HIV infection and intermittent febrile episodes for 12 months due to chronic malaria.

Table 2. Nucleotide variations in the 18S rDNA of *T. lewisi* and closely related *T. lewisi*-like trypanosomes.

Species	Isolate	Host	Locality	Accession no.	Sequence length	18S rDNA*																										
						40	135	186	272	274	277	289	345	501	559	620	706	764	814	1023	1085	1323	1411	1477	1503	1540	1769	1965	2016	2127	2132
T. lewisi	ATCC 30085	*Rattus norvegicus*	USA	AJ223566	2187 bp	A	T	C	T	C	C	T	T	T	—	—	T	T	T	A	G	T	T	G	A	G	T	A	T	T	T
T. lewisi	Molteno B3	*Rattus sp.*	UK	AJ009156	2155 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryRrPHL201	*Rattus rattus*	Philippines	AB242273	2147 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryRaIDN202	*Rattus argentiventer*	Indonesia	AB242273	2147 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryBiIDN203	*Bandicota indica*	Indonesia	AB242273	2147 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryRoIDN204	*Rattus omanicus*	Indonesia	AB242273	2147 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 34	*Rattus rattus*	Brazil	GU252210	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 35	*Rattus rattus*	Brazil	GU252211	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 44	*Rattus rattus*	Brazil	GU252213	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 1148	*Rattus norvegicus*	Brazil	GU252215	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	Af	*Alouatta fusca*	Brazil	GU252209	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 43	*Aotus sp.*	Brazil	GU252212	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 124	*Callithrix jacchus*	Brazil	GU252214	2168 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
T. musculi	LUM 343	*Mus musculus*	UK	AJ223568	2187 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
T. blanchardi		*Eliomys quercinus*	France	AY491764	2155 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·
Trypanosoma sp.	TryNcCHN503	*Niviventer confucianus*	China	AB242274	2147 bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	C	·	·	·	·
T. rabinowitschae		*Cricetus cricetus*	France	AY491765	2155 bp	—	·	·	·	·	·	·	·	·	C	T	·	·	·	·	·	C	·	A	—	A	·	·	·	·	·
T. grosi	Cha1	*Apodemus agrarius*	China	FJ694763	2159 bp		C	A	C	·	·	C	·	A	·	·	C	C	C	A	A	·	C	·	·	·	·	C	A	C	C
T. grosi	SESUJI	*Apodemus agrarius*	Russia	AB175622	2219 bp	·	C	A	C	T	·	C	C	A	·	·	C	C	C	A	·	·	C	·	·	·	·	·	·	·	C
T. grosi	AKHA	*Apodemus speciosus*	Japan	AB175624	2219 bp	·	C	A	C	·	·	C	·	A	·	·	C	C	C	A	·	·	C	·	·	·	·	·	·	·	C
T. grosi	HANTO	*Apodemus peninsulae*	Russia	AB175623	2219 bp	·	·	·	C	·	A	C	·	A	·	·	·	C	C	A	·	·	C	·	·	·	·	·	·	·	C

∗ Twenty-one 18S rDNA nucleotide sequences of *T. lewisi* and *T. lewisi*-like trypanosomes shown above were aligned by Clustal W multiple alignment program, with subsequent manual adjustment. By this processing, nucleotide positions were settled, and nucleotide position is expressed relative to the 5'-terminus of *T. grosi* SESUJI (DDBJ/EMBL/GenBank accession no. AB175622). Dots denote an identical nucleotide to that of *T. lewisi* (AJ223566), and gaps are indicated by '—'. Nucleotide substitutions which separate *T. lewisi* in Brazil from *T. lewisi* in other places are encased in a rectangle.

Table 3. Nucleotide variations in the ITS1 region of *T. lewisi* and closely related *T. lewisi*-like trypanosomes（*T. blanchardi* and *T. rabinowitschae*)

Species	Isolate	Host	Locality	Accession no.	ITS1																		
					16	24	33	35–44	47–49	69	83–84	89	93	94	101	104	121	124	143	147	149	162	167
T. lewisi	TryCC 44	*Rattus rattus*	Brazil	GU252220	T	A	C	(TA)×3	——A	T	——	G	T	A	A	A	T	A	C	—	T	C	A
T. lewisi	TryCC 1148	*Rattus norvegicus*	Brazil	GU252222	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi		*Rattus norvegicus*	China	EU599639	·	·	·	·	·	·	TG	·	·	·	·	·	·	·	T	·	·	·	·
T. lewisi	TryCC 35	*Rattus rattus*	Brazil	GU252218	·	·	·	·	·	·	TG	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi		*Rattus norvegicus*	China	FJ011094	·	G	T	(TA)×5	—CA	·	·	A	G	·	·	·	·	·	·	·	·	·	·
T. lewisi		*Rattus norvegicus*	China	EU861192	·	G	T	(TA)×5	—CC	·	·	A	G	·	·	·	·	·	·	·	·	·	·
T. lewisi	Af	*Alouatta fusca*	Brazil	GU252216	·	G	T	(TA)×5	·	·	·	A	G	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 124	*Callithrix jacchus*	Brazil	GU252221	·	G	T	(TA)×5	·	·	·	A	G	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 34	*Rattus rattus*	Brazil	GU252217	·	G	T	(TA)×5	·	·	·	A	G	·	·	·	·	·	·	·	·	·	·
T. lewisi	TryCC 43	*Aotus* sp.	Brazil	GU252219	·	G	T	(TA)×4	—CA	·	·	A	G	·	·	·	·	·	·	·	·	·	·
T. lewisi	Rn02	*Rattus tanezumi*	Thailand	HQ437158	·	G	T	(TA)×4	—CA	·	·	A	G	·	·	·	·	G	·	·	·	·	·
T. lewisi		*Rattus norvegicus*	China	FJ011095	·	G	T	(TA)×4	CAC	·	·	A	G	·	·	·	·	·	·	C	·	·	·
T. lewisi		*Homo sapiens*	Thailand	DQ345394	·	G	T	·	—CA	·	·	A	G	·	·	G	·	·	·	·	·	—	·
T. blanchardi		*Eliomys quercinus*	France	GU252223	A	·	T	(TA)×1	·	C	·	·	G	G	G	·	·	·	·	·	C	·	G
T. rabinowitschae		*Cricetus cricetus*	France	GU252224	A	·	T	(TA)×1	·	C	·	·	G	G	G	·	—	·	·	·	C	·	G

Table 3.（continued）

Species	Accession no.	ITS1																						
		169	170	189	191	193	212	218	219	223	230	236	241–254	255–257	260–264	352	364	370–373	374–375	376–383	387	412	414	428	437
T. lewisi	GU252220	C	A	G	A	T	G	T	—	—	A	A	(TA)×2	CAT	ATATA	A	A	(AT)×2	——	(AT)×1	A	A	G	T	G
T. lewisi	GU252222	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
T. lewisi	EU599639	·	·	·	·	·	·	·	·	·	·	·	(TA)×5 +T	———	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	GU252218	·	·	·	·	·	·	·	·	·	·	·	(TA)×7	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	FJ011094	·	·	·	·	·	·	·	A	·	·	·	(TA)×4 +T	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	EU861192	·	·	·	·	·	·	·	·	·	·	·	(TA)×5	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	GU252216	·	·	·	·	·	·	·	·	·	·	·	(TA)×6	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	GU252221	·	·	·	·	·	·	·	·	·	·	·	(TA)×6	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	GU252217	·	·	·	·	·	·	·	·	·	·	·	(TA)×6	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	GU252219	·	·	·	·	·	·	·	·	·	·	·	(TA)×7	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	HQ437158	·	·	·	·	·	·	C	·	·	·	·	(TA)×6	·	·	·	G	(AT)×0	·	(AT)×0	·	·	·	·	·
T. lewisi	FJ011095	·	C	T	·	·	·	·	G	T	·	·	(TA)×4 +T	·	·	·	G	(AT)×0	·	(AT)×0	G	·	·	—	—
T. lewisi	DQ345394	—	·	·	G	·	·	·	·	·	·	·	(TA)×6	·	·	·	G	(AT)×0	·	(AT)×0	·	T	T	·	·
T. blanchardi	GU252223	·	·	·	·	C	A	·	·	·	C	G	(TA)×3	———	—————	G	·	·	GT	(AT)×4	·	·	·	·	·
T. rabinowitschae	GU252224	·	·	·	·	C	A	·	·	·	C	G	(TA)×3	———	—————	G	·	·	GT	(AT)×4	·	·	·	·	·

＊Fifteen ITS1 nucleotide sequences of either *T. lewisi*, *T. blanchardi* or *T. rabinowitschae* shown above were aligned by Clustal W multiple alignment program, with subsequent manual adjustment. By this processing, 444 nucleotide positions were settled, and nucleotide position is expressed relative to the 5'-terminus of *T. lewisi* (DDBJ/EMBL/GenBank accession no. GU252220). Dots denote an identical nucleotide to that of *T. lewisi* (GU252220) and gaps are indicated by '—'.

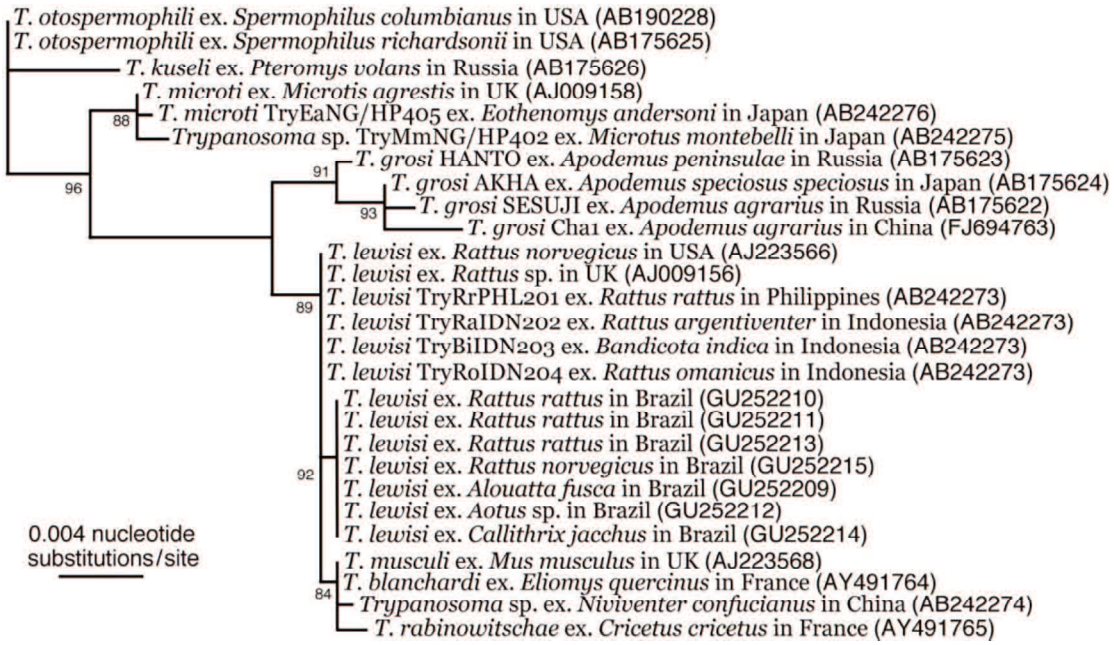

Fig. 4. Maximum likelihood (ML) phylogenetic tree based on the 18S rDNA sequences.
The nucleotide sequences of rodent trypanosomes （the subgenus *Herpetosoma*） were retrieved from the DDBJ/EMBL/GenBank databases, and ML analysis was performed as described in (Setsuda, et al., 2016). Regions judged to be poorly aligned and characters with a gap in any sequences were excluded from subsequent analyses; 2,109 characters, of which 49 were variable, remained for subsequent analysis. The probability of inferred branch was assessed by the approximate likelihood-ratio test as previously described (Setsuda et al., 2016). Two trypanosome species of Sciuridae（ two isolates of *T. otospermophili* and one isolate of *T. kuseli*） were used as an outgroup for the construction of the ML phylogenetic tree.

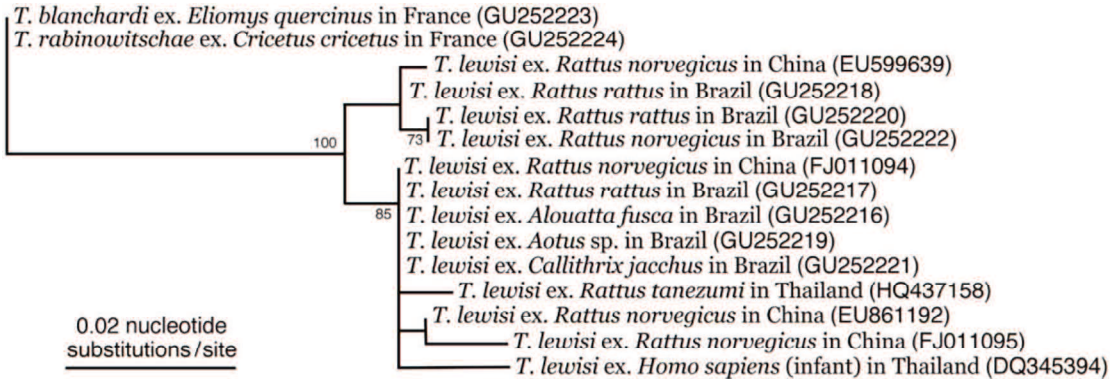

Fig. 5. Maximum likelihood (ML) phylogenetic tree of *T. lewisi* isolates based on the ITS1 region of rDNA sequences. The nucleotide sequences of *T. lewisi* and its closely related *T. lewisi*-like species (*T. blanchardi* and *T. rabinowitschae*) were retrieved from the DDBJ/EMBL/GenBank databases, and ML analysis was performed as stated in the legend for Fig. 4. Regions judged to be poorly aligned and characters with a gap in any sequences were excluded from subsequent analyses; 392 characters, of which 27 were variable, remained for subsequent analysis. Two trypanosome species (*T. blanchardi* and *T. rabinowitschae*) were used as an outgroup for the construction of the ML phylogenetic tree.

CHAPTER II

Integrative taxonomic approach of trypanosomes in the blood of rodents and soricids in Asian countries, with the description of three new species

Information described in this chapter has been published as follows;

Eliakunda Mafie, Atsuko Saito-Ito, Masatoshi Kasai, Mochammad Hatta, Pilarita T. Rivera, Xiao-Hang Ma, Eng-Rin Chen, Hiroshi Sato, Nobuhiro Takada (2018). Integrative taxonomic approach of trypanosomes in the blood of rodents and soricids in Asian countries, with the description of three new species. Parasitology Research (in press) Published online: 23 October 2018 [https://doi.org/10.1007/s00436-018-6120-3]

Abstract

Trypanosoma lewisi (Kinetoplastea: Trypanosomatida: Trypanosomatidae) with a cosmopolitan distribution is the type species of the subgenus *Herpetosoma,* which includes ca. 50 nominal species isolated mainly from rodents. Since members of *Herpetosoma* in different host species have an almost identical morphology of bloodstream forms, these trypanosomes are referred to as '*T. lewisi*-like', and the molecular genetic characterization of each species is necessary to verify their taxonomy. In the present study, 89 blood samples were collected from murid rodents of 15 species and 11 soricids of four species in Indonesia, Philippines, Vietnam, Taiwan, and mainland China for the detection of haemoprotozoan infection. *T. lewisi* and *T. lewisi*-like trypanosomes were found in the blood smears of 10 murid animals, which included *Bandicota indica* (two rats), *Rattus argentiventer* (one rat), and *Rattus tiomanicus* (two rats) in Indonesia; *Rattus rattus* (one rat) in the Philippines; and *Niviventer confucianus* (four rats) in mainland China. Furthermore, large- or medium sized non-*T. lewisi*-like trypanosomes were detected in two soricids, *Crocidura dracula* in Vietnam and *Anourosorex yamashinai* in Taiwan, respectively. Molecular genetic characterization of the small subunit (SSU) ribosomal RNA gene (rDNA) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene indicated that the trypanosomes from all the murid hosts had identical SSU rDNA or gGAPDH gene nucleotide sequences except for those in *N. confucianus* in mainland China. These *N. confucianus*-infecting trypanosomes also showed several unique morphological features such as smaller bodies, anteriorly positioned nuclei, and larger rod-shaped kinetoplasts when compared with *T. lewisi* trypomastigotes. *Trypanosoma* (*Herpetosoma*) *niviventerae* n. sp. is erected for this new species. Similarly, based on morphological and molecular genetic characterization, *Trypanosoma sapaensis* n. sp. and *Trypanosoma anourosoricis* n. sp. are proposed for the trypanosomes in *C. dracula* in

Vietnam and *A. yamashinai* in Taiwan, respectively. More effort directed toward the morphological and molecular genetic characterization of the trypanosomes of rodents and soricids is required to fully understand the real biodiversity of their haemoflagellates.

Introduction

Haemoflagellates of the genus *Trypanosoma* (Kinetoplastea: Trypanosomatida: Trypanosomatidae) take a wide spectrum of hosts such as fish, amphibians, reptiles, birds, and mammals and are transmitted by blood-feeding invertebrates (Hoare 1972; Bardsley and Harmsen 1973; Simpson et al. 2006; Cooper et al. 2017). The subgenus *Herpetosoma* with the type species *Trypanosoma lewisi* in rats (*Rattus* spp.) is given mainly to rodent trypanosomes with *T. lewisi*-like morphology (Hoare 1972) and includes ca. 50 nominal species. Members of the subgenus, *T. lewisi* and '*T. lewisi*-like' species, were believed to have a rigid host specificity irrespective of their almost identical morphology of bloodstream forms (Hoare 1972). Over the last two decades, however, several sporadic cases of human infection with *T. lewisi* have been recorded in Gambia, Thailand, and India under special circumstances where close contact was found between human residents and rat fleas with metacyclic *T. lewisi* (reviewed by Truc et al. 2013). Lun et al. (2015) demonstrated that *T. lewisi* is resistant to normal human serum, i.e., trypanolysis induced by human serum protein apolipoprotein L-1, while other animal trypanosomes are susceptible to this protein and cannot survive in human blood (Vanhamme et al. 2003; Pérez-Morga et al. 2005). Due to this recently recognized zoonotic potential as a cause of atypical human trypanosomiasis, the taxonomy and biological characters of trypanosomes of *Herpetosoma* are now a major priority for the specific diagnosis of the disease and also for the basic taxonomic concerns relating to morphologically similar trypomastigotes in diverse rodent species.

Prior to the stimulus of the recent recognition of zoonotic infection with *T. lewisi*, the molecular genetic characterization of *Herpetosoma* trypanosomes has been attempted over the last two decades using the ribosomal RNA gene (rDNA) and protein-coding genes such as the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene (Haag et al. 1998; Stevens et al. 1998; Noyes et al. 1999, 2002; Sato et al. 2003, 2005, 2007; Hamilton et al. 2004, 2005, 2007). These basic data contributed to the specific diagnosis of *T. lewisi* infection in humans and nonhuman primates, as well as molecular epidemiological surveys of the species in wild rodents (Maia da Silva et al. 2010; Pumhom et al. 2014). An additional polymerase chain reaction (PCR)-based specific differentiation of *T. lewisi* and its related species using a different genetic marker has recently been attempted (Hong et al. 2017; Ortiz et al. 2018). At present, the available nucleotide sequences of *Herpetosoma* species are limited, making the taxonomic discussion on special DNA sequences with a few or several nucleotide substitutions compared with those of *T. lewisi* rather difficult. For example, *T. lewisi* in rats and *Trypanosoma musculi* in the house mouse (*Mus musculus*), showing distinct biological characters in their vertebrate hosts and unsuccessful cross infection between these two murid hosts, have only one nucleotide substitution in the small subunit (SSU) rDNA over a 2187-bp length (e.g., DDBJ/EMBL/GenBank accession nos. AJ223566 and AJ223568), whereas *Trypanosoma blanchardi* in the garden dormouse (*Eliomys quercinus*) has an identical SSU rDNA nucleotide sequence (AY491764) to that of *T. musculi*. On the contrary, different isolates of *Trypanosoma grosi* in field mice (*Apodemus* spp.) show occasional nucleotide substitutions ranging from two to eight over a 2159-bp length (AB175622–AB175624 and FJ694763; Sato et al. 2005; Guan et al. 2011). The collection of more isolates for nucleotide sequencing and biological characterization is necessary in order to clarify the taxonomic significance of such molecular genetic uniqueness of different *Herpetosoma*

isolates.

In the present study, 89 blood samples collected from murid rodents and 11 soricids in Indonesia, the Philippines, Vietnam, Taiwan, and mainland China were examined for the detection of haemoprotozoan infection. Trypanosomes isolated from 10 murids and two soricids were morphologically and molecular-genetically characterized, thus providing additional data for the taxonomic discussion of trypanosomes of rodents and soricids.

Materials and methods

Parasitological survey

Between 2002 and 2005, cooperative surveys of zoonotic hemoprotozoan agents such as *Babesia microti* and related species in murid rodents were conducted at three localities in Indonesia, one locality in the Philippines, one locality in Vietnam, four localities in Taiwan, and two localities in mainland China (Table 4). In total, 89 murids of 15 species and 11 soricids of four species were trapped alive using Sherman traps. Species identification of the collected murids and soricids in Indonesia and the Philippines was conducted by mammalogists in the Faculty of Medicine, Hasanuddin University, Indonesia, and the College of Veterinary Science and Medicine, Central Luzon State University, Philippines, respectively. The species identification of trapped animals in Vietnam, Taiwan, and mainland China was carried out following the principles of Ma et al. (1987) and Zhang et al. (1997). Blood was collected by cardiac puncture from each animal under diethyl ether anesthesia and immediately mixed in plastic tubes with a drop of heparin sodium (Mochida Pharmaceutical, Tokyo, Japan). Thin blood films were prepared individually, fixed in absolute methanol, then stained in Giemsa's solution. Blood films were rigorously examined at x200 magnification under a light microscope. The ethics of animal trapping and sample collection adhered to in this study followed the guidelines

outlined by each survey participant's university, but at that time, no individual permission numbers of sample collection were provided from these organizations.

Morphological examination

Trypanosome-positive blood films were further examined under oil immersion at x1000 magnification. Undistorted, well-stained trypanosomes were photographed at x1000 magnification, transformed into photographs with Adobe® Photoshop® v. 5.0, then printed at a higher magnification. Measurements were conducted on printed photographs, and a digital curvimeter type S (Uchida Yoko, Tokyo, Japan) was used when necessary. The measurements (expressed in μm) included total length (TL), maximum width with undulating membrane (MW), maximum width without undulating membrane (BW), distance between the posterior end and the middle of the kinetoplast (PK), distance between the middle of the kinetoplast and the middle of the nucleus (KN), distance between the middle of the nucleus and the anterior end (NA), length of the free flagellum (FF), and lengths and widths of the kinetoplast (KL and KW) and nucleus (NL and NW). The nuclear index (NI) and kinetoplast index (KI) were calculated as follows: NI = (PK + KN)/NA and KI = (PK + KN)/KN, following Hoare (1972). Blood films on glass slides examined in this study were deposited in the Meguro Parasitological Museum, Tokyo, Japan, under the collection numbers 21386–21394.

DNA extraction, PCR, and nucleotide sequencing

Parasite DNA was extracted from 0.2 ml of each blood sample from 12 trypanosome-positive animals using a nucleic acid purification kit, MagExtractor™-Genome- (Toyobo, Osaka, Japan), according to the instructions of the manufacturer.

PCR amplification of three overlapping fragments of the SSU rDNA was conducted

using the primer pairs shown in Table 5 with the following protocol: 5 min at 95 °C followed by 40 cycles of 20 s at 94 °C, 40 s at 55 °C, and 60 s at 72 °C, then a final extension at 72 °C for 5 min. The gGAPDH gene was amplified using degenerate primers G3 (5′-TTY GCC GYA TYG GYC GCA TGG-3′) and G5 (5′-ACM AGR TCC ACC ACR CGG TG-3′) with the following protocol: 3 min at 95 °C followed by 35 cycles of 60 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C, then a final extension at 72 °C for 7 min, according to Hamilton et al. (2004). Next, a nested PCR using a primer pair of G1 (5′-CGC GGA TCC ASG GYC TYM TCG GBA MKG AGA T-3′) and G4a (5′-GTT YTG CAG SGT CGC CTT GG-3′), or that of G1 and G4b (5′-CCAMGASACVAYCTTGAAGAA-3′) in the second round, was conducted according to Hamilton et al. (2004). The PCR protocol for the second round was similar to that of the first-round PCR detailed above. As sequencing primers, G6 (5′-GYG GTK TCS VTS AAG GAC TG-3′) and G7 (5′ -CSC CTG TBG TGC TBG GTA TG-3′) were used (Hamilton et al. 2004).

Phylogenetic analysis

For phylogenetic analyses, nucleotide sequences of the SSU rDNA and gGAPDH gene were used. The newly obtained SSU rDNA sequences of trypanosomes isolated in the present study (AB242273, AB242274, AB242822, and AB242823) and those of related trypanosome species retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson et al. 1994), with subsequent manual adjustment. Trypanosomes of the '*Trypanosoma cruzi* clade' (such as *T. cruzi*, *Trypanosoma dionisii*, and *Trypanosoma erneyi*) were used as an outgroup due to their close phylogenetic relationships with trypanosomes of the *Herpetosoma*/soricid trypanosome (*Trypanosoma talpae*) clade (Sato et al. 2008; Paparini et al. 2011; Chapter III of the present study). The accession numbers of the sequences analysed in the present

study are given in the figures showing phylogenetic trees. Regions judged to be poorly aligned and characters with a gap in any sequence were excluded from subsequent analyses; 1710 characters, of which 125 were variable, remained for subsequent analyses. Similarly, the newly obtained nucleotide sequences of the gGAPDH gene of trypanosomes isolated in the present study (LC369597 and LC369598) and those of related trypanosome species retrieved from the aforementioned databases were aligned and processed as described above; 804 characters, of which 340 were variable, remained for subsequent analyses. As an outgroup, trypanosomes of the *T. cruzi* clade and marsupial mammals were again used due to their close phylogenetic relationships. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the 'phylogeny.fr' website (http://www.phylogeny.fr/). The probability of inferred branches was assessed by the approximate likelihood ratio test (aLRT), an alternative to the nonparametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

Results

Incidence of trypanosomes in the blood films

As shown in Table 4, trypanosomes were found in 12 blood films prepared from the cardiac blood of 10 murids (*Bandicota indica* (two animals), *Rattus argentiventer* (one animal), and *Rattus tiomanicus* (two animals) in Indonesia; *Rattus rattus* (one animal) in the Philippines; and *Niviventer confucianus* (four animals) in mainland China) and two soricids (*Crocidura dracula* (one animal) in Vietnam and *Anourosorex yamashinai* (one animal) in Taiwan. The levels of parasitaemia were variable but generally low in all blood films, particularly those prepared from soricids in which only a few trypomastigotes were detected.

Morphological and molecular genetic characterization of trypanosomes in the blood

The trypomastigotes in the blood of *B. indica* (two animals) and the three *Rattus* spp. (four animals) collected in Indonesia and the Philippines were typical *T. lewisi* bloodstream forms (Figs. 6a–c and 7a and Table 6). Molecular genetic characterisation using the SSU rDNA nucleotide sequences supported this specific identification of *T. lewisi*; the nucleotide sequences of all isolates of trypanosomes from these animals (IN6, IN8, IN26, IN28, IN29, and Ph7), 2147 bp in length, were identical to each other (AB242273) and to SSU rDNA reference sequences of *T. lewisi*, i.e., Molteno B3 and ATCC 30085 isolates (AJ009156 and AJ223566). The trypomastigotes in the blood of *N. confucianus* exhibited a somewhat unique *T. lewisi*-like appearance and were consistently smaller in size (Figs. 6d–f and 7b and Table 6) in all blood smears prepared from different host individuals. The SSU rDNA nucleotide sequences of all successfully characterized isolates (Ch II-24, Ch II-27, and Ch II-32) collected at Hangzhou, Zhejiang Province, 2147 bp in length, were identical to each other (AB242274) and showed the highest identity with those of *T. blanchardi* and *T. musculi* (AY491764 and AJ223568) with a single nucleotide substitution site over the 2147-bp length, followed by the aforementioned *T. lewisi* sequences with two nucleotide substitution sites over a similar length. The gGAPDH nucleotide sequences of trypanosomes from *N. confucianus*, 842 bp in length, were identical to each other (LC369598) and showed the highest identity with those of *T. lewisi* isolates collected from *Rattus* spp. in Indonesia and the Philippines (IN28, IN29, and Ph7; LC369597) with two nucleotide substitution sites over the 842-bp length or that of a *T. lewisi* isolate collected from *R. rattus* in the UK (AJ620272) with three nucleotide substitution sites, followed by those of *T. grosi* isolates (AB362557 andAB362558) with seven or eight nucleotide substitution sites over the same length.

The trypanosomes from *C. dracula* in Vietnam and those from *A. yamashinai* in

Taiwan showed a rigid appearance of bodies with granular structures in the central cytoplasm and the kinetoplast localized rather near the nucleus, features distinct from *T. lewisi* and other species in the subgenus *Herpetosoma* (Figs. 6g–i and 7c–d and Table 7). The SSU rDNA nucleotide sequences of these two isolates from different soricid species in Vietnam (Vietnam5) and Taiwan (Tw II-1) were successfully obtained: 2158-bp long (AB242822) and 2166-bp long (AB242823), respectively. These sequences showed the highest affinities with the SSU rDNA sequence of *T. talpae* from the European mole (*Talpa europaea*) in the UK (1828-bp long; AJ620545), the only deposited SSU rDNA sequence of soricid trypanosomes. The sequence of trypanosomes from *C. dracula* had the higher nucleotide identity (99.56%; 1813/1821) with that of *T. talpae* but contained 19 insertion/deletion sites (indels) and that of trypanosomes from *A. yamashinai* had 98.93% (2132/2155) identity with 14 indels. The *T. talpae* and Tw II-1 isolates showed 99.39% (1810/1821) identity with 28 indels. Based on the morphological uniqueness of trypomastigotes in the blood as well as their molecular genetic characters, three new species, *Trypanosoma* (*Herpetosoma*) *niviventerae* n. sp., *Trypanosoma sapaensis* n. sp., and *Trypanosoma anourosoricis* n. sp., are erected for the three morphotypes in *N. confucianus*, *C. dracula*, and *A. yamashinai*, respectively.

Description

***Trypanosoma* (*Herpetosoma*) *niviventerae* n. sp. (Kinetoplastea: Trypanosomitida: Trypanosomatidae) (Figs. 6d–f and 7b and Table 6)**

The body of the trypomastigotes in the cardiac blood (n = 6), which measured 19.7–30.0 (24.0) x 1.6–2.4 (2.0), had a curved anterior end and a spindle-shaped posterior end, with the free flagellum measuring 3.1–5.6 (3.9) in length. The undulating membrane was apparently narrow, adhering closely to the body. The oval nucleus, which measured 2.4–

3.4 (2.7) x 1.0–2.1 (1.4), was situated near the 1/3 point of the body from the anterior end, with NI 1.2–2.2 (1.9). The nucleus occupied the complete width of the body. The rod-shaped, relatively large kinetoplast, which measured 0.9–1.3 (1.1) x 0.5–0.7 (0.6), was situated at the posterior 1/3 point between the nucleus and posterior end, with KI 1.3–1.5 (1.4). Additional measurements of this new species in *N. confucianus* (Ch II-27) are given in Table 6, along with the measurements of *T. lewisi* in *B. indica* (IN6) and *R. argentiventer* (IN8). The SSU rDNA and gGAPDH nucleotide sequences of this new species are deposited in and available from the DDBJ/EMBL/GenBank databases under the accession numbers AB242274 and LC369598, respectively.

Taxonomic summary

Host: *Niviventer confucianus* (Milne-Edwards, 1871), Chinese white-bellied rat (Mammalia: Rodentia: Muridae).

Locality: Hangzhou, Zhejiang, China.

Materials deposited: Hapantotype no. 21393 (Ch II-27), Meguro Parasitological Museum, Tokyo, Japan. Paratype nos. 21394b (Ch II-24, Ch II-31, and Ch II-32) in the same museum.

Prevalence: Four of seven rats at Hangzhou, Zhejiang. Relatively high intensity as the level of parasitemia.

Etymology: The species is named after the name of the host genus.

Remarks

This new *T. lewisi*-like species was found in four rats at different levels of parasitaemia. The observed trypomastigotes were consistently smaller in size when compared with *T. lewisi* (Table 6). The cytoplasm often assumed a granular or mottled

appearance due to heterogeneous basophilic deposition. As discussed above, the molecular genetic characterization based on the SSU rDNA and gGAPDH gene supports the morphological differentiation of *T. niviventerae* n. sp. from *T. lewisi* or *T. lewisi*-like trypanosomes such as *T. musculi*.

Description

***Trypanosoma sapaensis* n. sp. (Kinetoplastea: Trypanosomitida: Trypanosomatidae) (Figs. 6g–h and 7c and Table 7)**

The body of the trypomastigotes in the blood (n = 3), which measured 36.8–38.8 (37.9) x 3.7–5.4 (4.8), had a unique morphological appearance: broad body with posteriorly situated kinetoplasts near the nucleus rather than the posterior end, granular or mottled cytoplasm, and elongated sharpened ends. The free flagellum measured 5.8–7.8 (6.7) in length. The undulating membrane was distinct, measuring 1.0 maximal width. The oval nucleus, which measured 3.1–3.7 (3.3) x 1.7–2.0 (1.8), was situated almost at the midpoint between both ends, with NI 0.8–1.1 (1.0). The nucleus was located close to the side of the undulating membrane and the kinetoplast. The small oval kinetoplast, which measured 0.7–0.9 (0.8) x 0.6–0.7 (0.6), was situated rather near the nucleus, with KI 3.2–3.8 (3.4). Additional measurements of this new species in *C. dracula* (Vietnam5) are given in Table 7, along with the measurements of *T. talpae* and other *Trypanosoma* spp. recorded from the Soricidae. The SSU rDNA nucleotide sequence of this new species is deposited in and available from the DDBJ/EMBL/GenBank databases under the accession number AB242822.

Taxonomic summary

Host: *Crocidura dracula* (Thomas, 1912), large white-toothed shrew (Mammalia:

Eulipotyphla: Soricidae), based on Abramov et al. (2012).

Locality: Sapa, Lào Cai Province, Vietnam.

Materials deposited: Hapantotype no. 21391 (Vietnam5), Meguro Parasitological Museum, Tokyo, Japan.

Prevalence: One of one shrew examined. Extremely low intensity as the level of parasitemia.

Etymology: The species is named after the name of the location where the host was trapped.

Remarks

Hoare (1972) listed two trypanosome species with non-*T. lewisi*-like morphology from soricides, i.e., *T. talpae* in European moles and *Trypanosoma hoarei* in North American shrews of the *Sorex* genus (*Sorex trowbridgii*, *Sorex vagrans*, *Sorex ornatus*, and *Sorex palustris*). The present new species is differentiated from *T. talpae* in having distinct undulating membranes and a different location of the nucleus (near the convex side vs. close to the side opposite to the undulating membrane and kinetoplast). Similarly, the new species is differentiated from *T. hoarei* in having a different location of the nucleus (approximately at the midpoint of the body vs. posterior localization, reflected in different average NI values, 1.0 vs. 0.6), a long tapering posterior end, and the presence of a distinct free flagellum (5.8–7.8 vs. 0–3). As discussed above, the molecular genetic characterization based on the SSU rDNA supports the morphological differentiation of *T. sapaensis* n. sp. from *T. talpae*. No gGAPDH nucleotide sequence has been obtained for this isolate probably due to an extremely low parasitaemia.

Description

***Trypanosoma anourosoricis* n. sp. (Kinetoplastea: Trypanosomitida: Trypanosomatidae) (Figs. 6i and 7d and Table 7)**

The body of the trypomastigotes in the blood (n = 6), which measured 22.9–38.3 (28.7) x 2.4–3.6 (2.9), had granular or mottled cytoplasm and elongated sharpened ends. The free flagellum measured 3.6–5.4 (4.7) in length. The undulating membrane was indistinct, measuring 0–0.7 (0.3) in width. The ovoid nucleus, which measured 2.6–3.0 (2.8) x 1.4–1.9 (1.5), was situated almost at the midpoint between both ends, with NI 0.8–1.1 (1.0). The tiny indistinct kinetoplast, which measured 0.4–0.9 (0.6) x 0.3–0.6 (0.5), was situated midway between the nucleus and posterior end, with KI 1.8–2.7 (2.2). Additional measurements of this new species in *A. yamashinai* (Tw II-1) are given in Table 7, along with the measurements of other trypanosomes recorded from the family Soricidae. The SSU rDNA nucleotide sequence of this species is deposited in and available from the DDBJ/EMBL/GenBank databases under the accession number AB242823.

Taxonomic summary

Host: *Anourosorex yamashinai* (Kuroda, 1935), Taiwanese mole shrew (Mammalia: Eulipotyphla: Soricidae).

Locality: Renai, Nantou County, Taiwan.

Materials deposited: Hapantotype no. 21392 (Tw II-1), Meguro Parasitological Museum, Tokyo, Japan.

Prevalence: One of two mole shrews examined. Extremely low intensity as the level of parasitemia.

Etymology: The species is named after the genus name of the host from which this species was detected.

Remarks

The present species, *T. anourosoricis* n. sp., showed a rigid, narrow but elongated spindle-shaped body with an indistinguishable undulating membrane. These morphological characters are distinct from *T. sapaensis* n. sp., *T. talpae*, and *T. hoarei*, all of which have undulatory broad bodies. As discussed above, the molecular genetic characterization based on the SSU rDNA supports the morphological differentiation of the present specimens from *T. sapaensis* n. sp. and *T. talpae*, although these species have close phylogenetic relationships with the present specimens (discussed below). Karbowiak et al. (2005) described a new species, *Trypanosoma ornata*, from the Eurasian water shrew, *Neomys fodiens* (Pennant, 1771), in Poland solely based on the morphology of trypomastigotes. Since morphological differentiation between the present specimens and *T. ornata* is difficult, the molecular genetic characterization of *T. ornata* is important. The collection and intensive morphological examination of additional trypomastigote of *T. anourosoricis* n. sp. is also necessary. At the same time, genetic characterization of the gGAPDH nucleotide sequence of the species is important, since it has not been obtained in this study probably due to an extremely low parasitemia.

Phylogenetic relationships of newly isolated trypanosomes with known species

Trypanosomes of the subgenus *Herptetosoma* formed a well-supported clade in the ML phylogenetic tree based on the SSU rDNA (Fig.8), which consisted of four major subgroups: (1) *Trypanosoma microti* and related species, (2) sciurid trypanosomes such as *Trypanosoma otospermophili* and *Trypanosoma kuseli*, (3) *T. grosi* isolates, and (4) *T. lewisi* and related species. The available SSU rDNA nucleotide sequences of *T. lewisi* were divided into two groups: a group of European and Asian isolates from *Rattus* spp. and *B. indica* (AJ009156, AJ223566, and AB242273) and another of Brazilian isolates from

various hosts such as *Rattus* spp. and nonhuman primates (GU252209–GU252215). This division was based on a single nucleotide substitution at the 1013th base relative to the 5′-end of the deposited reference sequence of *T. lewisi* (ATCC 30085 isolate; AJ223566). In the same way, *T. niviventerae* n. sp., *T. musculi, T. blanchardi*, and *T. rabinowitschae* formed a clade having consistently and minimally a single nucleotide substitution at the 1313th base. The SSU rDNA nucleotide sequences of trypanosomes with morphology of 'the subgenus *Megatrypanum*' (Hoare 1972) from soricids, *T. talpae, T. sapaensis* n. sp., and *T. anourosoricis* n. sp., formed a distinct clade from that for *Herpetosoma* trypanosomes, but a close relationship clearly existed (Fig. 8). In an earlier study from our laboratory, *Megatrypanum* trypanosomes of ruminants such as *Trypanosoma theileri* and those of primates such as *Trypanosoma minasense* were found to locate distantly from a clade of these soricid trypanosome species (see Fig. 3 of Sato et al. 2008). The available gGAPDH nucleotide sequences of *Herpetosoma* trypanosomes were limited to eight taxa of six species (Fig. 9). The phylogenetic topology of *Herpetosoma* species to other groups of trypanosomes was almost identical to the relationships observed using the SSU rDNA sequences. There were no available gGAPDH nucleotide sequences of soricid trypanosomes. Indeed, we were unable to obtain gGAPDH nucleotide sequences from the two trypanosome isolates from soricids in this study.

Discussion

Hoare (1972) devoted the subgenus *Herpetosoma* to *T. lewisi* and its related species recorded from the blood of rodents, ca. 50 in number, which included unnamed species. Although the recent occurrence of sporadic human cases of *T. lewisi* infection in Thailand and India (reviewed by Truc et al. 2013) as well as *T. lewisi* infection in Brazilian monkeys in captivity (Maia da Silva et al. 2010) has promoted the molecular genetic

characterization of rodent trypanosomes, there are still only a limited number of deposited nucleotide sequences, thus making a proper discussion on the taxonomy of *Herpetosoma* trypanosomes difficult. My previous study described in Chapter I, reported the latest status of research on the molecular genetic characterization of *Herpetosoma* trypanosomes. Briefly, *T. lewisi* with a cosmopolitan distribution was found to have a fixed SSU rDNA nucleotide sequence, which is represented by the deposited reference sequences AJ009156 (Molteno B3 isolate) and AJ223566 (ATCC 30085 isolate) in the DDBJ/EMBL/GenBank databases. Brazilian isolates of *T. lewisi* from *R. rattus, R. norvegicus, Alouatta fusca, Aotus* sp., and *Callithrix jacchus* were discovered to be the exception to this finding, consistently having a single nucleotide substitution at base position 1013 relative to the 5′-terminus of AJ223566 (aforementioned deposited reference sequence of *T. lewisi*). Contrary to *T. lewisi* isolates, *T. musculi, T. blanchardi,* and *T. rabinowitschae*, as well as *T. niviventerae* n. sp. in the present study, had at least a single nucleotide substitution at base position 1313, forming a separate subclade (Fig. 8). Zhang et al. (2015) reported a partial SSU rDNA nucleotide sequence (KP098535), 682 bp in length, of *Herpetosoma* trypanosomes in *Rattus losea*, in addition to a partial but typical *T. lewisi* SSU rDNA sequence (KP098536) of trypanosomes from *Rattus flavipectus*. The former SSU rDNA sequence (KP098535) is identical to that of *T. niviventerae* n. sp. rather than that of *T. lewisi*. Furthermore, the SSU rDNA of *T. niviventerae* n. sp. has an additional nucleotide substitution at base position 1759 compared with *T. lewisi*, *T. musculi*, *T. blanchardi*, and *T. rabinowitschae* (see Table 2); however, no information is available on the trypanosomes in the blood of *R. losea* mentioned above. In order to clarify the taxonomic status of the *Herpetosoma* trypanosomes in *R. losea* reported by Zhang et al. (2015), i.e., identical to or distinct from *T. niviventerae* n. sp. reported here, a longer SSU rDNA sequence including parts close to both the 5′- and 3′- termini of the gene needs to be obtained. Although

molecular genetic differences are few in the SSU rDNA sequences among *Herpetosoma* trypanosomes of murid hosts, the morphological uniqueness of *T. niviventerae* n. sp., different from *T. lewisi*, justifies our erection of the new species for trypanosomes isolated from *N. confucianus* in China. Due to a dearth of morphological differences among *T. musculi*, *T. blanchardi*, *T. rabinowitschae*, and *T. lewisi* (Hoare 1972), the new species is also differentiated from the three former species. Surprisingly, the two genetic groups of *T. lewisi* based on a single nucleotide substitution in the SSU rDNA sequence are not consistent with two genetic groups based on different profiles of nucleotide sequences of the internal transcribed spacer (ITS) 1 region (see Table 3 and Fig. 5). Therefore, it is vital to collect more data on not only the SSU rDNA but also other regions of the gene in order to fully discern the nucleotide changes critical for specific differentiation.

In the present study, two trypanosome isolates of the so-called subgenus *Megatrypanum*, not of the subgenus *Herpetosoma*, were obtained from soricids (*C. dracula* in Vietnam and *A. yamashinai* in Taiwan). The level of parasitaemia was extremely low in both hosts, a similar finding to the majority of previous studies investigating the so-called *Megatrypanum* trypanosomes in the peripheral blood of hosts (Hoare 1972). Long SSU rDNA nucleotide sequences were successfully obtained for these two soricid trypanosome isolates, making them the second and third deposited nucleotide sequences of soricid '*Megatrypanum*' trypanosomes after that of *T. talpae* from the European mole (AJ620545). Intriguingly, these three nucleotide sequences were found to have frequent indels, 14, 19, or 28 in number against each other, with eight or 23 nucleotide substitutions over a 1821- or 2155-bp length, respectively. Hoare (1972) considered members of the subgenus *Megatrypanum* to be primitive forms of trypanosomes, although species of this subgenus from different mammalian hosts are dispersed in the phylogenetic tree and do not form a special subgenus clade. Indeed,

Megatrypanum trypanosomes from soricids form a well-supported clade comprised of only themselves, which has a sister relationship with a clade of *Herpetosoma* trypanosomes (Fig. 8), but is distant from *T. theileri*, the type species of the *Megatrypanum* subgenus.

The taxonomic relationship of soricid trypanosomes in *A. yamashinai* from Taiwan with *T. ornata* from the Eurasian water shrew (*N. fodiens*) in Poland is uncertain, although they appear to be morphologically closer to each other. Molecular genetic characterization of *T. ornata* could determine the precise taxonomic relationship between these two species. It is well recognized that the morphological differentiation of trypanosomes in the blood of rodents and soricids is difficult due to very similar morphotypes, i.e., *T. lewisi*-like and soricid '*Megatrypanum*' morphology, respectively. Indeed, sometimes only biological characters such as host specificity and reproductive manner enable species to be separated, as in the case of *T. lewisi* and *T. musculi*.

Which target genes should be considered when undertaking molecular taxonomy to differentiate *Herpetosoma* or soricid '*Megatrypanum*' trypanosomes? As detailed here and in Chapter I there is only a single or just a few nucleotide substitution(s) among 2140-bp long SSU rDNA sequences of apparently distinct species (e.g., *T. lewisi* vs. *T. musculi*). Furthermore, a Brazilian population of *T. lewisi* isolates showed a single nucleotide substitution when compared with European and Asian isolates of *T. lewisi*. Contrarily, *T. grosi* showed more greater intraspecific variation of the SSU rDNA sequence, two to four nucleotide substitutions over a 2150-bp length. All the current data suggest a low resolution of molecular analyses using the SSU rDNA sequences of *Herpetosoma* species. In contrast, based on just three available sequences at present, interspecific nucleotide variation is high in the SSU rDNA sequences of soricid trypanosome species. Similar to the SSU rDNA nucleotide sequences, those of the gGAPDH gene of *Herpetosoma* trypanosomes are also well conserved with only a few nucleotide substitutions between

different species. Regarding the ITS region, direct sequencing may be hampered by the presence of repeats of nucleotide units, making sequencing after gene cloning likely to be necessary, which increases the amount of labor and cost. Additionally, genetic groups based on the SSU rDNA and ITS nucleotide sequences do not tend to show consistency.

We are still a long way from establishing an ideal target gene(s) for the molecular taxonomic purpose of separating a wide range of *Herpetosoma* trypanosomes. At present, the molecular genetic characterization of almost the entire length of SSU rDNA sequences of isolated trypanosomes from various sources must be a basic minimum requirement to support the morphological characterization of trypanosomes.

Table 4. Blood smears from rodents and soricids and detected trypanosomes

Locality and collected hosts	No. of animals	Date of collection	No. of positive animals	Trypanosomes detected	SSU rDNA[b]	gGAPDH[b]
Indonesia (Sulawesi): Toraja and Makassar	(28 in total)	March 3, 2003				
Bandicota indica	15		2 (IN6; IN26)	*T. lewisi*	AB242273	-
Rattus argentiventer	4		1 (IN8)	*T. lewisi*	AB242273	-
Rattus exulans	3		0			
Rattus tiomanicus	5		2 (IN28; IN29)	*T. lewisi*	AB242273	LC369597
Mus caroli	1		0			
Indonesia (Sulawesi): Palopo	(6 in total)	Mar 7, 2003				
Bandicota indica	1		0			
Rattus exulans	2		0			
Rattus tiomanicus	1		0			
Rattus argentiventer	2		0			
Philippines (Luzon): Nueva Ecija	(7 in total)	Mar 19-21, 2003				
Rattus rattus	7		1 (Ph7)	*T. lewisi*	AB242273	LC369597
Vietnam (Lào Cai Province): Sapa	(6 in total)	Dec 18 and 19, 2003				
Rattus flavipectus	2		0			
Apodemus draco	2		0			
Crocidura dracula	1		1 (Vietnam5)	*T. sapaensis* n. sp	AB242822	-
Crocidura horsfieldii	1		0			
Taiwan: Taipei	(3 in total)	Sep 6, 2004				
Rattus losea	2		0			
Apodemus agrarius	1		0			
Taiwan (Nantou): Renai, Guoshing, and Puli	(23 in total)	Dec 22, 2002 – Dec 23, 2004				
Mus caroli	2		0			
Apodemus semotus	15		0			
Anourosorex yamashinai	2		1 (Tw II-1)	*T. anourosoricis* n. sp.	AB242823	-
Crocidura horsfieldii	2		0			
Suncus murinus	2		0			
Taiwan (Chiayi): Minshiung	(3 in total)	Dec 23, 2002				
Suncus murinus	3		0			
Taiwan (Kaohsigung): Touyuan and Linyuan	(8 in total)	Dec 23, 2002 - Dec 23, 2004				
Bandicota indica	7		0			
Rattus coxingi	1		0			
China (Shaanxi Province): Qinling Mountains	(8 in total)	Oct 31 – Nov 3, 2004				
Apodemus chevrieri	2		0			
Apodemus peninsulae	6		0			
China (Zhejiang Province): Hangzhou	(8 in total)	Feb 11, 2005				
Niviventer confucianus	7		4 (Ch II-24; Ch II-27; Ch II-31; Ch II-32)	*T. niviventerae* n. sp.	AB242274	LC369598
Apodemus agrarius	1		0			

[a]Number of positive animals is followed by the name of isolates in parentheses
[b]DDBJ/EMBL/GenBank accession number

Table 5. Primers used for amplification of SSU rDNA nucleotide fragments in the present study.

Segment No.	Primer for PCR amplification	Primer for sequencing	Direction	Sequence	Position of 5'-end[b]	Reference
1	SSU/U1m		F	5′-TTGATTCTGCCAGTAGTCATA-3′	− 21	Haag et al. (1998), modified
	TRY2R		R	5′-AACCAACAAAAGCCGAAACG-3′	929	Present study
		TRY4R	R	5′-CTATTTCTTTTCGCTGCCTC-3′	505	Present study
2	TRY1F		F	5′-CTACCACTTCTACGGAGGG-3′	427	Present study
	TRY927mR		R	5′-CTACTGGGCAGCTTGGATC-3′	1688	Noyes et al. (1999), modified
		SSU561mF	F	5′-TGGGATAACAAAGGAGCAT-3′	879	Noyes et al. (1999), modified
		SSU561mR	R	5′-TGAGACTGTAACCTCAAAGC-3′	1446	Noyes et al. (1999), modified
3	TRY3F		F	5′-ATGACACCCATGAATTGGGG-3′	1261	Present study
	SSU/L1		R	5′-CTACAGCTACCTTGTTACGA-3′	2167	Haag et al. (1998), modified
		TRY5F	F	5′-TCGGTGGAGTGATTTGTTTG-3	1629	Present study

Table 6. Morphometric comparison of *Herpetosoma* trypanosomes from murid hosts

Species	*T. lewis*			*T. musculi*	*T. niviventerae* n. sp.
Isolate	IN6	IN8	Lab T3	lab LGT	CHN II-27
Host	*Bandicota indica*	*Rattus argentiventer*	*Rattus argentiventer*	*Mus musculi*	*Niviventer confucianus*
Reference	Present study	Present study	Davis (1952)	Davis (1952)	Present study
	n = 15	n = 41	n = 50	n =50	n = 6
TL	34.3 ± 0.8 (33.2–35.9)	34.7 ± 1.1 (32.2–37.0)	29.4 ± 1.1	29.2 ± 0.8	24.0 ± 4.6 (19.7–30.0)
PK	4.2 ± 0.5 (3.6–5.3)	5.3 ± 0.5 (3.7–6.6)	2.6 ± 0.4	5.3 ± 0.5	3.5 ± 0.8 (2.7–4.9)
KN	10.8 ± 0.5 (11.2–12.9)	11.5 ± 0.6 (10.2–12.5)	10.9 ± 0.7	8.5 ± 0.4	9.4 ± 1.4 (8.1–11.5)
NA	10.8 ± 0.8 (8.8–12.5)	11.1 ± 1.0 (8.8–14.4)	8.6 ± 0.9	9.7 ± 1.0	7.2 ± 2.3 (5.1–11.2)
FF	7.4 ± 0.8 (6.6–9.3)	6.8 ± 0.9 (4.8–9.7)	7.2 ± 1.0	5.8 ± 0.9	3.9 ± 0.9 (3.1–5.6)
BW	2.2 ± 0.3 (1.5–2.7)	1.8 ± 0.4 (1.2–2.7)	1.8	1.8	2.0 ± 0.3 (1.6–2.4)
MW	2.6 ± 0.4 (2.0–3.4)	2.3 ± 0.3 (1.7–2.9)	–[a]	–[a]	2.0 ± 0.3 (1.6–2.4)
NL	3.0 ± 0.3 (2.4–3.6)	2.5 ± 0.2 (1.9–2.8)	2.3	3.2	2.7 ± 0.4 (2.4–3.4)
NW	1.2 ± 0.2 (0.8–1.4)	1.2 ± 0.2 (0.9–1.5)	1.2	1.3	1.4 ± 0.4 (1.0–2.1)
KL	0.8 ± 0.1 (0.7–1.0)	0.8 ± 0.1 (0.6–1.0)	1.0	1.1	1.1 ± 0.2 (0.9–1.3)
KW	0.6 ± 0.1 (0.5–0.7)	0.5 ± 0.1 (0.4–0.7)	0.6	0.5	0.6 ± 0.1 (0.5–0.7)
NI	1.5 ± 0.2 (1.3–1.9)	1.5 ± 0.2 (1.2–1.9)	1.6	1.4	1.9 ± 0.4 (1.2–2.2)
KI	1.4 ± 0.1 (1.3–1.5)	1.5 ± 0.1 (1.3–1.6)	1.2	1.6	1.4 ± 0.1 (1.3–1.5)

All measurements are in micrometers and expressed as mean ± standard deviation with range in parentheses

BW, maximum body width without undulating membrane; FF, free flagellum; KI, kinetoplast index; KL, length of kinetoplast; KN, distance between kinetoplast and themiddle of nucleus;KW, width of kinetoplast;MW,maximumwidth including undulatingmembrane; NA, distance between themiddle of nucleus and anterior end; NI, nuclear index; NL, length of nucleus;NW, width of nucleus; PK, distance between posterior end and kinetoplast; TL, total length

[a]No available data

Table 7. Morphometric comparison of non-T. lewisi-like trypanosomes from soricid hosts

Species	*T. sapaensis* n. sp.	*T. talpae*	*T. hoarei*	*T. anourosoricis* n. sp.	*T. ornata*
Isolate	Vietnam5			Tw II-1	
Host	*Crocidura Dracula*	*Talpa* spp.	*Sorex* spp.	*Anourosorex yamashinai*	*Neomys fodiens*
Reference	Present study	Hoare (1972)	Davis (1969)	Present study	Karbowiak et al. (2005)
	n = 3			n = 6	n = 100
TL	37.9 ± 1.0 (36.8–38.8)	ca. 21–38	34 (24.7–44.3)	28.7 ± 6.2 (22.9–38.3)	29.4 ± 4.1 (20.5–40.9)
PK	10.7 ± 1.4 (9.5–12.2)	6–20	–[a]	6.2 ± 1.2 (4.8–8.1)	4.3 ± 1.5 (2.6–10.7)
KN	4.5 ± 0.2 (4.4–4.8)	3–4.7	–	5.6 ± 2.3 (3.9–8.6)	6.5 ± 1.4 (2.5–14.4)
NA	15.9 ± 1.9 (13.7–17.5)	–[a]	–	12.3 ± 3.4 (9.2–18.1)	15.9 ± 2.3 (11.0–21.0)
FF	6.7 ± 1.0 (5.8–7.8)	3.5–6.3	Max. ca. 3	4.7 ± 0.8 (3.6–5.4)	3.1 ± 1.3 (1.0–9.2)
BW	4.1 ± 1.0 (3.1–4.9)	–	–	2.6 ± 0.2 (2.4–2.9)	–[a]
MW	4.8 ± 0.9 (3.7–5.4)	3.5–6.5	5.8 (2.8–10.3)	2.9 ± 0.4 (2.4–3.6)	3.1 ± 0.4 (1.9–4.1)
NL	3.3 ± 0.4 (3.1–3.7)	–	–	2.8 ± 0.1 (2.6–3.0)	3.8 ± 0.9 (1.2–6.9)
NW	1.8 ± 0.2 (1.7–2.0)	–	–	1.5 ± 0.2 (1.4–1.9)	1.2 ± 0.4 (0.7–3.0)
KL	0.8 ± 0.1 (0.7–0.9)	–	–	0.6 ± 0.2 (0.4–0.9)	1.2
KW	0.6 ± 0.1 (0.6–0.7)	–	–	0.5 ± 0.1 (0.3–0.6)	0.6
NI	1.0 ± 0.2 (0.8–1.1)	–	ca. 0.6	1.0 ± 0.1 (0.8–1.1)	0.7 ± 0.2 (0.3–1.2)
KI	3.4 ± 0.3 (3.2–3.8)	ca. 3	ca. 2	2.2 ± 0.3 (1.8–2.7)	1.7 ± 0.2 (1.3–2.5)

Abbreviations similar to Table 6

[a]No available data

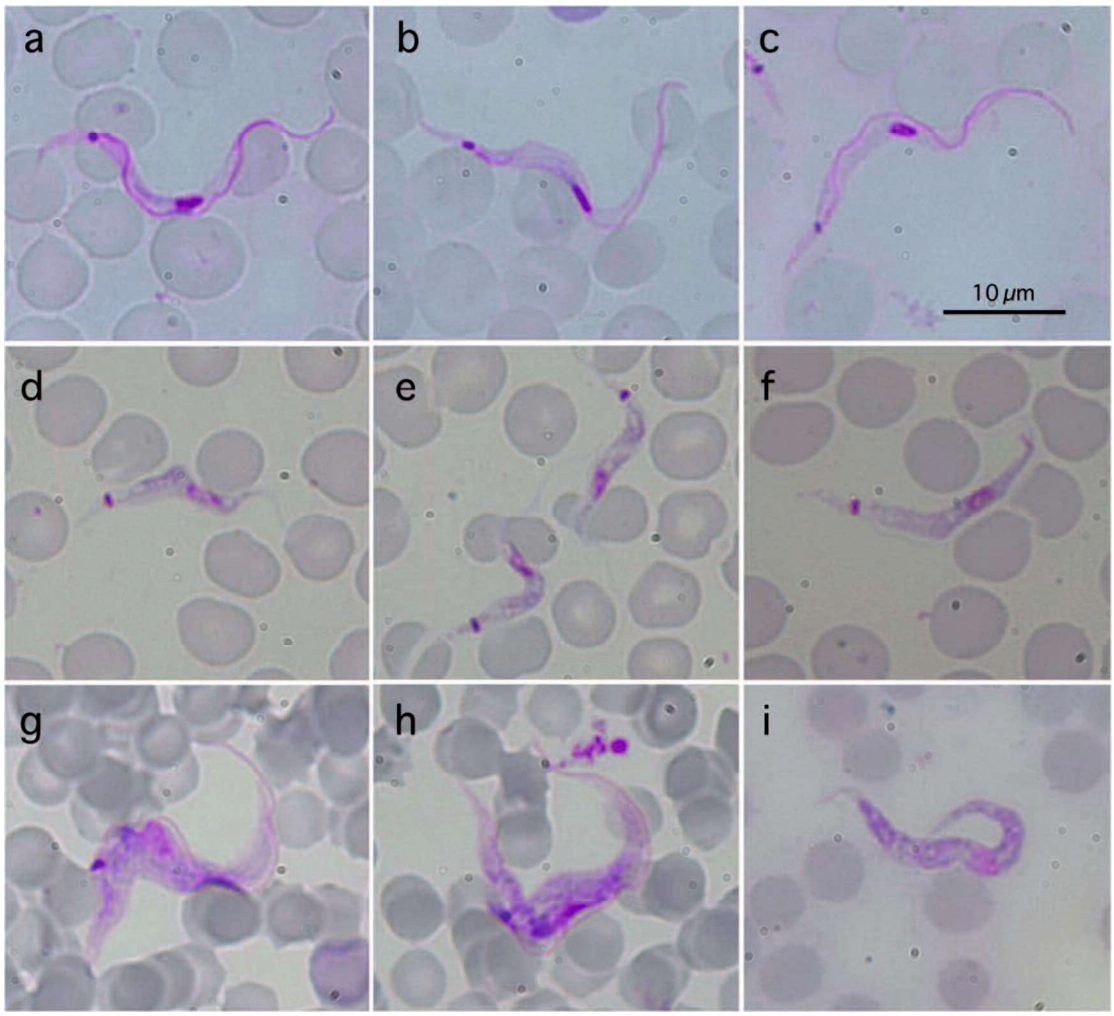

Fig. 6 Photographs of trypomastigotes of *Trypanosoma* (*Herpetosoma*) l*ewisi* in the blood of *Rattus tiomanicus* in Indonesia (a–c); *Trypanosoma* (*Herpetosoma*) *niviventerae* n. sp. in the blood of *Niviventer confucianus* in China (d–f); *Trypanosoma sapaensis* n. sp. in the blood of *Crocidura dracula* in Vietnam (g, h); and *Trypanosoma anourosoricis* n. sp. in the blood of *Anourosorex yamashinai* in Taiwan (i). All photographs are at the same magnification and the scale bar is shown in c

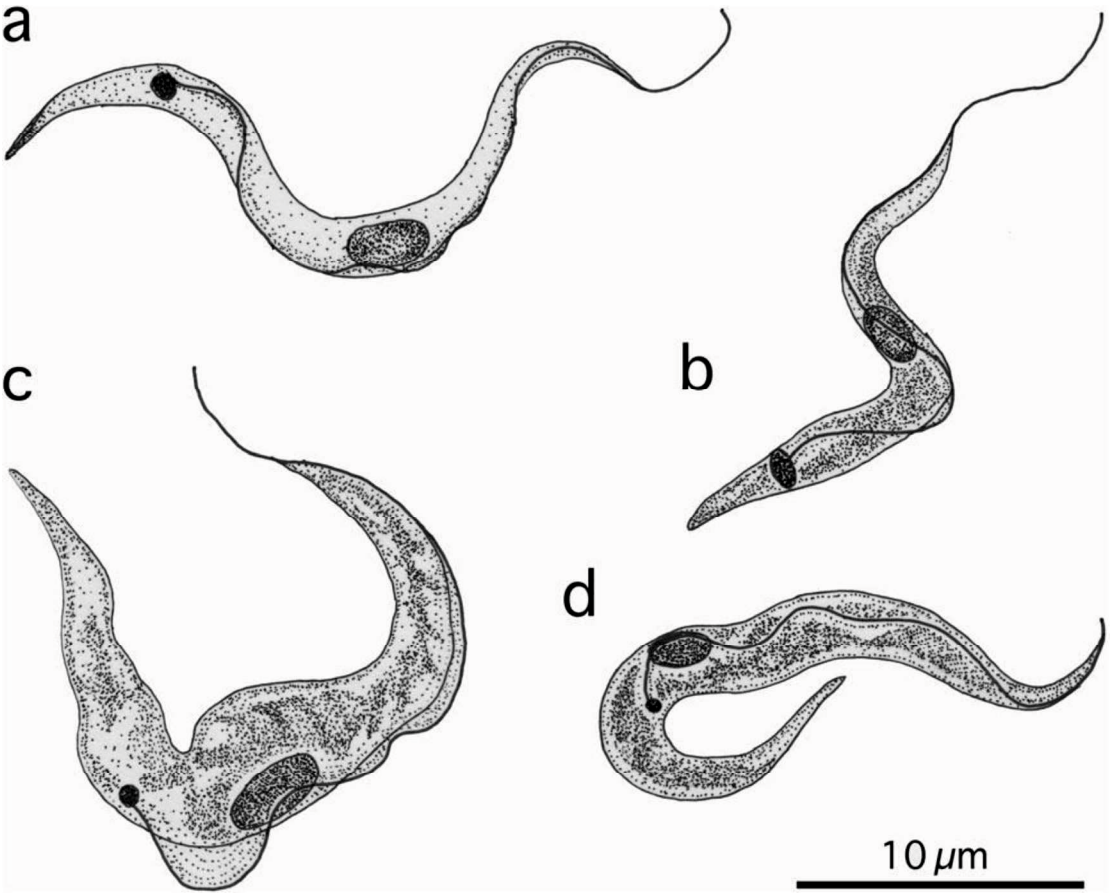

Fig. 7. Stylized drawings of the four *Trypanosoma* spp. shown in Fig.3*: T.* (*H.*) *lewisi* (a), *T.* (*H.*) *niviventerae* n. sp. (b), *T. sapaensis* n. sp. (c), and *T. anourosoricis* n. sp. (d). All drawings are at the same magnification

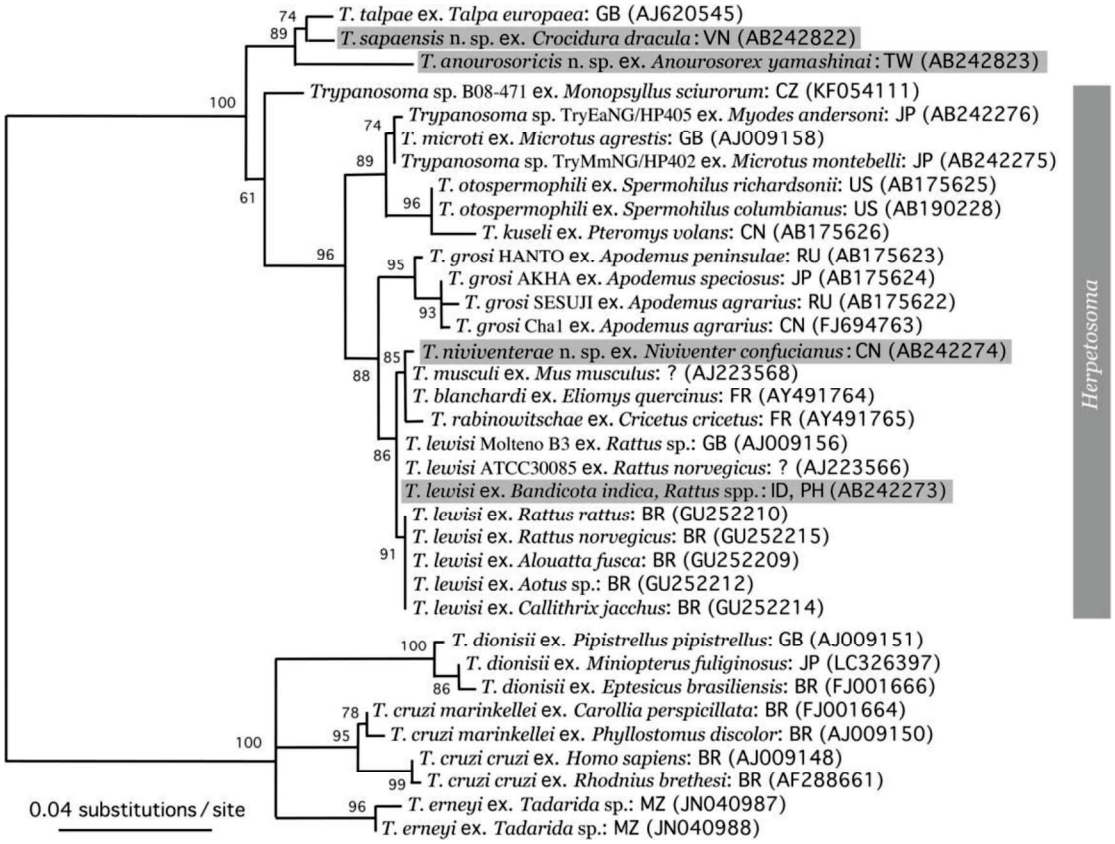

Fig. 8. ML phylogenetic tree based on the SSU rDNA nucleotide sequence. Species names are followed by host names and localities (DDBJ/EMBL/GenBank accession numbers in parentheses). The newly sequenced isolates are indicated with a gray background. Brazil (BR), China (CN), Czech Republic (CZ), France (FR), United Kingdom (GB), Indonesia (ID), Japan (JP), Mozambique (MZ), Philippines (PH), Russian Federation (RU), Taiwan (TW), United States of America (US), and Vietnam (VN)

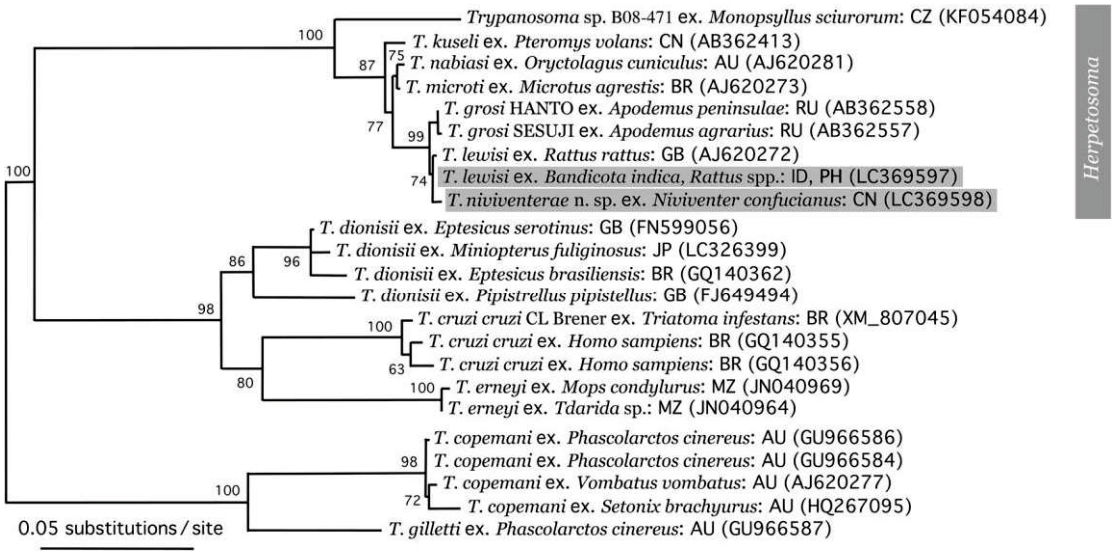

Fig.9. ML phylogenetic tree based on the gGAPDH nucleotide sequence. See Fig. 8 legend for details. Australia (AU)

CHAPTER III

First record of *Trypanosoma dionisii* of the *T. cruzi* clade from the Eastern bent-winged bat (*Miniopterus fuliginosus*) in the Far East

Information described in this chapter has been published as follows;

Eliakunda Mafie, Fatema Hashem Rupa, Ai Takano, Kazuo Suzuki, Ken Maeda, Hiroshi Sato (2018). First record of *Trypanosoma dionisii* of the *T. cruzi* clade from the Eastern bent-winged bat (Miniopterus fuliginosus) in the Far East. Parasitology Research 117:673–680

Abstract

Chiropteran mammals worldwide harbour trypanosomes (Euglenozoa: Kinetoplastea: Trypanosomatida) of the subgenus '*Schizotrypanum*' in the classical sense. Latterly, these trypanosomes have been referred to as members of the '*Trypanosoma cruzi* clade' as their phylogenetic relationships, structure and life cycle conform to *T. cruzi*, parasitising various terrestrial mammals as well as humans in Latin America. Little is known, however, about the trypanosome species in Asian bats. During a survey on *Borrelia* spp. in the Eastern bent-winged bat (*Miniopterus fuliginosus*) living in a cave in Wakayama Prefecture, Japan, incidental proliferation of trypanosomes was detected in two of 94 haemocultures. Squat or slender trypanosomes that proliferated in the cultures were 7.5–20.5 μm in length between both body ends and 1.0–3.8 μm in width with/without free flagella up to 14.5 μm (n = 29). The nucleotide sequences of the small subunit ribosomal RNA gene (SSU rDNA; 2176 bp), large subunit ribosomal RNA gene (1365 bp) and glycosomal glyceraldehyde-3-phosphate dehydrogenase gene (gGAPDH; 843 bp) of the present isolates were characterized to clarify their molecular phylogenetic position in *T. cruzi*-like trypanosomes. The newly obtained SSU rDNA and gGAPDH nucleotide sequences showed the highest identities with Brazilian and European isolates of *Trypanosoma dionisii* of the *T. cruzi* clade, ranging between 99.4 and 99.7% or between 95.6 and 99.3% identities, respectively. Although multiple *T. dionisii* isolates from the North and South American continents showed the closest molecular genetic relatedness to the present Far East isolates, only short SSU rDNA segments of the former isolates were deposited. Therefore, a definitive conclusion cannot be made until full nucleotide sequencing of at least the American isolates' SSU rDNA is available. This is the first confirmation of a Far East distribution of *T. dionisii*, demonstrating a wide geographical distribution of the species in the Eurasian and American continents with a limited nucleotide variation.

Introduction

Hoare (1972) divided mammalian *Trypanosoma* spp. (Euglenozoa: Kinetoplastea: Trypanosomatida) into seven subgenera (three for stercorarians and four for salivarians), and chiropteran mammals, or bats, are hosts for more than 30 stercorarian trypanosome species classified in either *Megatrypanum*, *Herpetosoma* or *Schizotrypanum*. Most species of the *Schizotrypanum* subgenus are currently referred to as members of the *T. cruzi* clade. *Trypanosoma cruzi*, which is well established as the cause of Chagas disease in Latin American residents, infects a wide spectrum of terrestrial mammals (Bern et al. 2011; Kirchhoff 2011). Other members of this group are exclusively found in bats distributed widely throughout the world (North and South America, Europe, Africa and Australia) (Hoare 1972). Like *T. cruzi*, the trypanosomes enter cells, transform into amastigotes then multiply to form amastigote aggregates (Baker et al. 1971; Oliveira et al. 2009). Hamilton et al. (2012a) proposed the 'bat seeding hypothesis', which considers the ancestor trypanosomes of the *T. cruzi* clade in bats as the origin of terrestrial mammalian trypanosome species (such as *T. cruzi*, *T. rangeli* and *T. conorhini*) and marsupial trypanosome species at different occasions and/or places through host-switching. Intensive research on bat trypanosomes in Europe, Africa, South America and Australia has disclosed intriguing new biogeographical findings on *T. cruzi*-like trypanosomes; however, little is known about the species in Asian bats (Hamilton et al. 2012b). During a survey on *Borrelia* spp. in the Eastern bent-winged bat (*Miniopterus fuliginosus*) in a cave in western Japan, incidental proliferation of trypanosomes was detected in two of the 94 haemocultures. This provided me with a rare opportunity to characterize the molecular genetic properties of Asian bat trypanosomes.

Materials and methods

Parasite isolation

A survey of *Borrelia* spp. in Eastern bent-winged bats living in a cave in Asso, Kamitonda-cho, Wakayama Prefecture, Japan (N 33° 42′, E 135° 26′), was conducted on 27 June and 30 July, 2016. Haemocultures were cultivated using the modified Barbour-Stoenner-Kelly (BSK) medium at 30 °C as described previously (Sato et al. 2009; Takano et al. 2011). The collection of 56 and 38 bats (86 males and 8 females; body weight 5.6–20.1 g [mean ± standard deviation 12.6 ± 2.2 g]) in each month for this survey was permitted by the Wakayama Prefectural Office. Weekly checking of haemocultures for the 94 individuals revealed two trypanosome-positive samples (no. 42 male bat with 11.6 g body weight, caught on 27 June, and no. 82 male bat with 5.7 g body weight, caught on 30 July) at 25 or 41 days of culture.

DNA Extraction, PCR and Sequencing

Trypanosome DNA was extracted from centrifuged sediments of two culture samples (KTD42 and KTD82) using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the instructions of the manufacturer. Polymerase chain reaction (PCR) amplification of overlapping fragments of the ribosomal RNA gene (rDNA) was performed in a 20-μl volume containing a DNA polymerase, Blend Taq -Plus- (TOYOBO, Dojima Hama, Osaka, Japan), and multiple combinations of primers (Sato et al. 2005). PCR products for sequencing were purified using a FastGene Gel/PCR extraction kit (NIPPON Genetics Co., Tokyo, Japan). Following the direct sequencing of PCR amplicons, sequences were assembled manually with the aid of the CLUSTAL W multiple alignment program (Thompson et al. 1994). The glycosomal glyceraldehyde-3-phosphate dehydrogenase gene (gGAPDH) was amplified as described

previously (Hamilton et al. 2004; Sato et al. 2008). The nucleotide sequences reported in the present study are available in the DDBJ/EMBL/GenBank databases under the accession nos. LC326397–LC326399.

Phylogenetic Analysis

For phylogenetic analysis, the newly obtained small subunit (SSU) rDNA and gGAPDH nucleotide sequences and those of related trypanosomes, retrieved from the DDBJ/EMBL/GenBank databases, were aligned using the CLUSTAL W multiple alignment program (Thompson et al. 1994), with subsequent manual adjustment. The accession numbers of the sequences analysed in the present study are given in the figures showing phylogenetic trees. Regions judged to be poorly aligned and characters with a gap in any sequence were excluded from subsequent analyses; 2034 characters, of which 281 were variable, and 755 characters, of which 283 were variable, remained for subsequent analysis of the SSU rDNA and gGAPDH nucleotide sequences, respectively. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the 'phylogeny.fr' website (http://www.phylogeny.fr/). The probability of inferred branches was assessed by the approximate likelihood ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

Results

In two culture samples, numerous trypanosomes swam free in the culture medium and did not form clusters. Microscopic examination of Giemsa-stained smears of the culture medium revealed slender trypomastigote forms and stumpy epimastigote forms of variable sizes, sometimes showing a process of binary fission as described by Baker et al. (1972),

elongated oval nuclei near the middle of the body, prominent kinetoplasts near the posterior end, undeveloped or developed free flagella and no undulating membranes (Fig. 10). These trypanosomes measured 7.5–20.5 μm in length between the anterior and posterior ends, and 1.0–3.8 μm in width with/without free flagella up to 14.5 μm in length when present (n = 29). Smears of the cultured trypanosomes examined in the present study (KTD isolate) were deposited in the Meguro Parasitological Museum, Tokyo, Japan, under collection no. 21385.

The newly obtained SSU and large subunit (LSU) rDNA nucleotide sequences of the two isolates KTD42 and KTD82 were absolutely identical to each other (2176-bp and 1365-bp long sequences, respectively). This was also the case for their 843-bp long gGAPDH nucleotide sequences. The SSU rDNA and gGAPDH nucleotide sequences of the present bat trypanosome isolates were highly similar to deposited sequences of Brazilian and European isolates of *T. dionisii*, with 99.4–99.7% identities or 95.6–99.4% identities, respectively (Figs. 11 and 12). Since no LSU rDNA nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank databases, direct comparison of nucleotide identity with any hitherto recorded *T. dionisii* isolates could not be conducted.

With regard to long *T. dionisii* SSU rDNA nucleotide sequences (i.e. more than 2000 bp long; Table 8), the nucleotide sequence of the present KTD isolate was closest to *T. dionisii* from *Carollia perspicillata* from Brazil (DDBJ/EMBL/GenBank accession no. FJ001667) with six nucleotide substitutions and one nucleotide insertion/deletion (indel), followed by the species from *Eptesicus brasiliensis* from Brazil (FJ001666) with five nucleotide substitutions and six indels, or the species from *Pipistrellus pipistrellus* from the UK (AJ009151 and AJ009152) with eight nucleotide substitutions and four indels. When short SSU rDNA sequences of *T. dionisii* from various bats from the UK, Brazil and the USA (Texas), notably 457–757 bp in length and containing a hypervariable region of

nucleotides, were included for sequence comparison, deposited sequences from all three countries (Brazil: e.g. FJ001652, FJ001653, FJ001655–FJ001657, FJ001663 and EU867810; the UK: FN599057; and the USA: KX227600 and KX227601) were identical to the corresponding regional sequence of the aforementioned Brazilian isolate from *Carollia perspicillata* (FJ001667). Likewise, multiple short SSU rDNA sequences of *T. dionisii* from bats from Brazil (e.g. KJ817043, KJ817044, EU867811, FJ001654 and FJ001660) and the USA (KX227505–KX227509) were identical or almost identical to the corresponding regional sequence of the aforementioned Brazilian isolate from *Eptesicus brasiliensis* (FJ001666).

When long gGAPDH nucleotide sequences ranging between 793 and 902 bp were compared, the closest ones to that of the present KTD isolate were sequences of Brazilian and the UK isolates of *T. dionisii* (GQ140363, FN599055 and FN599056) with 99.3% (837/843) to 99.4% (788/793) nucleotide identities, followed by those of a Brazilian isolate (GQ140362) with 98.5% (830/843) and the UK isolates (FN599054 and FJ649494) with 95.6% (789/825 or 806/843) nucleotide identities (Table 9). These nucleotide substitutions caused one to four amino acid substitutions as shown in Table 9. In these cases, the nucleotide substitutions occurred at the first or second nucleotide of the amino acid codons. In other words, the majority of nucleotide substitutions in the gGAPDH sequences occurred at the third nucleotide of codons.

Discussion

Amongst members of the *T. cruzi* clade, *T. cruzi marinkellei* in South American bats, *T. erneyi* in African bats and *T. dionisii* in European and American bats show the closest molecular phylogenetic relatedness to zoonotic *T. cruzi cruzi* in a variety of Latin American mammals including humans (Stevens et al. 1998; Hamilton et al. 2004, 2012a,

2012b; Maia da Silva et al. 2009; Cavazzana et al. 2010; Lima et al. 2012; Marcili et al. 2013; Botero et al. 2016; Hodo et al. 2016). Furthermore, Dario et al. (2016) recently recorded a human infection with *T. dionisii* in Brazil through demonstration of its specific DNA in cardiac tissue. As mentioned above, Hamilton et al. (2012a) recently proposed their compelling 'bat seeding hypothesis' to explain the biogeographical speciation of *T. cruzi* in the South American continent. Although subsequent research has supported this hypothesis (Lima et al. 2012, 2013, 2015; Hodo et al. 2016), additional and more detailed molecular data on chiropteran *T. cruzi*-like trypanosomes are needed, particularly from areas where there is little information, such as Asia. In this regard, the present molecular genetic characterization of *T. dionisii* from *Miniopterus fuliginosus* in Japan is very important for the elucidation of the precise route of geographical dispersion of *T. cruzi*-related ancestor trypanosomes. Hamilton et al. (2012b) have suggested a possible route of parasite movement from the Old World to the New World, namely from Siberia to Alaska, across the Bering Land Bridge during a period of low sea level. To attest this speculation, molecular genetic data of *T. dionisii* isolated in the Far East and North America are critical.

Hamilton et al. (2012b) divided *T. dionisii* into two major genotypes: type A only in bats from the UK and type B in bats from Brazil and the UK. As depicted in this study, nucleotide sequences of the *T. dionisii* SSU rDNA are further divided into two subgenotypes, referred to here as *T. dionisii* B-I and B-II, represented by DDBJ/EMBL/GenBank accession nos. FJ001667 and FJ001666, respectively (see Table 8). Similarly, nucleotide sequences of the *T. dionisii* gGAPDH are divided into *T. dionisii* A, B-I and B-II, represented by FN599054, FN599056 and GQ140362, respectively (see Table 9). Clearly, the Japanese isolate of *T. dionisii* reported in the present study is a lineage of the subgenotype B-I. Analysing a 457–459-bp long hypervariable region of the SSU rDNA, Hodo et al. (2016) also reported the *T. dionisii* B-I subgenotype from *Antrozous pallidus*

and *Parastrellus hesperus* as well as the B-II subgenotype from *Tadarida brasiliensis* in Texas, USA. These molecular genetic documentations indicate the worldwide distribution, including the Far East, of *T. dionisii* subtypes and in the future, may aid the elucidation of the precise route of parasite movement by inferring biogeographical relationships amongst local isolates in the world. Due to the important intraspecific nucleotide substitutions localized near the 5′- and 3′-termini of the SSU rDNA sequences or dispersed widely in the gGAPDH sequences (see Tables 8 and 9), longer SSU rDNA and gGAPDH nucleotide sequences are necessary to infer their phylogenetic relationships.

In the present study, the incidental proliferation of trypanosomes in blood cultures from *M. fuliginosus* and subsequent phylogenetic analyses enabled me to demonstrate the distribution of *T. dionisii* B-I subtype in Japan. Japan has 37 species of chiropteran mammals: Pteropodidae (three species), Rhinolophidae (four species), Hipposideridae (one species), Vespertilionidae (27 species) and Molossidae (two species) (Ohdachi et al. 2010). Since *T. dionisii* has been recorded from a variety of bats having insectivorous, frugivorous and haematophagous feeding (Cavazzana et al. 2010; Hamiltonet al. 2012b), and is transmitted by common cimicid bugs (Hamilton et al. 2007), it is likely that more host chiropteran species for *T. dionisii* and other *Trypanosoma* spp. will be recorded in Japan. Considering that *T. dionisii* proliferation occurs principally in host cells through amastigote formation (Baker et al. 1971, 1972), blood cultures using the BSK medium are not suitable for epidemiological studies designed to clarify the precise prevalence of trypanosomes and/or isolate a spectrum of trypanosome genotypes. However, as the transportation of wild mammals like bats from field to laboratory is procedurally difficult, field samplings of trypanosomes using the BSK medium, in which blood culture is possible at 30 °C without a specialized CO_2 incubator, are advantageous in some limited circumstances. Additionally, cultures can also be maintained in the Novy-MacNeal-Nicolle

medium with a liver infusion tryptose overlay at 27 °C without CO_2 incubation (Ziccardi et al. 1996). To fully understand the biogeography or evolutionary history, i.e. geographical dispersion and individual speciation of the *T. cruzi* clade, phylogenetic analyses should be extended to more trypanosome isolates collected at different localities in the world. Such studies, particularly in Asia and North America, may help us to elucidate more precisely the parasite dispersion and speciation from Africa to Latin America, or the movement between different continents.

Table 8. Nucleotide substitutions observed in partial SSU rDNA sequences of *Trypanosoma dionisii* of different origins.

Genotype	Isolate	Host	Locality	DDBJ/EMBL/GenBank Accession no[a].	Position of nucleotide changes[b]																							Number of nucleotide Substitutions[c]
					182	186	187	240	698	753	782	805	806	967	977	995	1003/1004	1045	1325	1336	1390	1393-1395	1397	1430	1913	1970	2124	
B-I	KTD	*Miniopterus fuliginosus*	Japan	LC326397 (2174 bp)	G	C	A	A	A	G	T	G	G	C	A	T	-	C	T	A	T	TTT	T	C	C	T	C	-
A	P3	*Pipistrellus pipistrellus*	UK	AJ009151 (2178 bp)	A	.	.	.	.	A	C	.	.	.	.	C	.	.	C	-	C	-	C	.	.	.	T	8 (4 indels)
A	PJ	*Pipistrellus pipistrellus*	UK	AJ009152 (2178 bp)	A	.	.	.	.	A	C	.	.	.	.	C	.	.	C	-	C	-	C	.	.	.	T	8 (4 indels)
B-I	TryCC 495	*Carollia perspicillata*	Brazil	FJ001667 (2242 bp)	.	T	G	G	.	.	.	.	.	.	.	.	.	T	.	.	.	TT-	.	.	T	C	.	6 (1 indel)
B-I	GNASH	*Eptesicus serotinus*	UK	FN599057 (526 bp)										.	.	.	.	.	.	.	.	TT-	.	A				
B-I	R12-302	*Antrozous pallidus*	USA	KX227600 (457 bp)										.	.	.	.	T	.	.	.	TT-	.					
B-II	TryCC 211	*Eptesicus brasiliensis*	Brazil	FJ001666	.	T	G	.	.	.	.	-	-	.	G	.	TT	T	.	.	.	T--	.	.	T	.	.	5 (6 indels)
B-II	TryCC 633	*Sturnira lilium*	Brazil	EU867812 (756 bp)					G	.	.	.	.	.	G	.	TT	T	.	.	.	T--	.					
B-II	A14-6383	*Tadarida brasiliensis*	Brazil	KX227595 (458 bp)										T	G	.	TT	T	.	.	.	T--	.					

[a] Length of deposited sequence in number of base pairs is given in parentheses
[b] Nucleotide position relative to the 5'-terminus of the SSU rDNA sequence of Japanese isolate (KTD) of *T. dionisii* (LC326397). Dots denote an identical base of the uppermost nucleotide sequence of *T. dionisii* (KTD isolate), and blanks indicate no available data
[c] Number of base positions where nucleotide insertion/deletion (indel) occurs is given in parentheses

Table 9. Nucleotide substitutions observed in partial gGAPDH sequences of *Trypanosoma dionisii* of different origin

Genotype	Isolate	Host	Locality	DDBJ/EMBL/GenBank accession no.[a]	Position of nucleotide substitutions																	
					5	20	44	62	77	80	105	107	118	126	128	134	143	158	188	242	245	248
B-I	KTD	*Miniopterus fuliginosus*	JPN	LC326399 (843 bp)	C	C	C	T	T	T	A	G	T	G	G	T	T	T	T	A	C	C
A	Z3126	*Pipistrellus pygmaeus*	U.K	FN599054 (825 bp)			T	C	C	C	G	.	C	A	T	C	C	C	C	G	T	G
A	P3	*Pipistrellus pipistrellus*	U.K	FJ649494 (902 bp)	T	.	T	C	C	C	G	.	C	A	T	C	C	C	C	G	T	G
B-I	TryCC 495	*Carollia perspicillata*	Brazil	GQ140363 (793 bp)					.	.	.	T	.	.	.	.	.	.	.	.	.	G
B-I	Gnash	*Eptesicus serotinus*	U.K	FN599056 (853 bp)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
B-I	x842	*Nyctalus noctula*	U.K	FN599055 (853 bp)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
B-II	TryCC 211	*Eptesicus brasiliensis*	Brazil	GQ140362 (853 bp)		T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G

(Continued)

Genotype	Isolate	Host	Locality	DDBJ/EMBL/GenBank accession no.[a]	Position of nucleotide substitutions																	
					254	261	262	279	284	314	350	386	392	404	440	509	521	524	557	565	575	578
B-I	KTD	*Miniopterus fuliginosus*	JPN	LC326399 (843 bp)	T	G	C	T	C	C	T	T	G	T	C	G	T	T	G	T	T	G
A	Z3126	*Pipistrellus pygmaeus*	U.K	FN599054 (825 bp)	.	.	T	C	T	G	C	C	C	A	.	A	C	C	.	.	.	A
A	P3	*Pipistrellus pipistrellus*	U.K	FJ649494 (902 bp)	.	.	T	C	T	G	C	C	C	A	.	A	C	C	.	.	.	A
B-I	TryCC 495	*Carollia perspicillata*	Brazil	GQ140363 (793 bp)	.	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.
B-I	Gnash	*Eptesicus serotinus*	U.K	FN599056 (853 bp)	.	A	.	C	.	.	C	.	.	G	T	.	.	.	.	.	.	.
B-I	x842	*Nyctalus noctula*	U.K	FN599055 (853 bp)	.	A	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.
B-II	TryCC 211	*Eptesicus brasiliensis*	Brazil	GQ140362 (853 bp)	C	A	.	.	.	.	C	.	.	G	.	.	.	.	A	.	C	.

(Continued)

Genotype	Isolate	Host	Locality	DDBJ/EMBL/GenBank accession no.[a]	Position of nucleotide substitutions														Number of nucleotide substitutions
					602	614	665	671	710	728	731	734	737	767	779	803	806	821	
B-I	KTD	*Miniopterus fuliginosus*	JPN	LC326399 (843 bp)	G	G	C	C	C	C	C	T	A	G	C	C	A	T	-
A	Z3126	*Pipistrellus pygmaeus*	U.K	FN599054 (825 bp)	A	T	.	.	T	T	.	C	G	.	.	T	G	C	36
A	P3	*Pipistrellus pipistrellus*	U.K	FJ649494 (902 bp)	A	T	.	.	T	T	.	C	G	.	.	T	G	C	37
B-I	TryCC 495	*Carollia perspicillata*	Brazil	GQ140363 (793 bp)	.	.	.	.	.	.	.	.	.	A	.	.	.	.	5
B-I	Gnash	*Eptesicus serotinus*	U.K	FN599056 (853 bp)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6
B-I	x842	*Nyctalus noctula*	U.K	FN599055 (853 bp)	.	.	.	.	.	.	.	.	.	.	T	.	.	.	6
B-II	TryCC 211	*Eptesicus brasiliensis*	Brazil	GQ140362 (853 bp)	.	.	T	T	.	.	T	.	G	.	.	.	.	.	13

[a] Length of deposited sequence in number of base pairs is given in parentheses

[b] Nucleotide position relative to the 5′-terminus of the gGAPDH sequence of Japanese isolate (KTD) of *T. dionisii* (LC326399) is shown. Dots denote an identical base to the uppermost nucleotide sequence of *T. dionisii* (KTD isolate), and blanks indicate no available data. Nucleotide substitutions causing amino acid substitutions are encased in a box

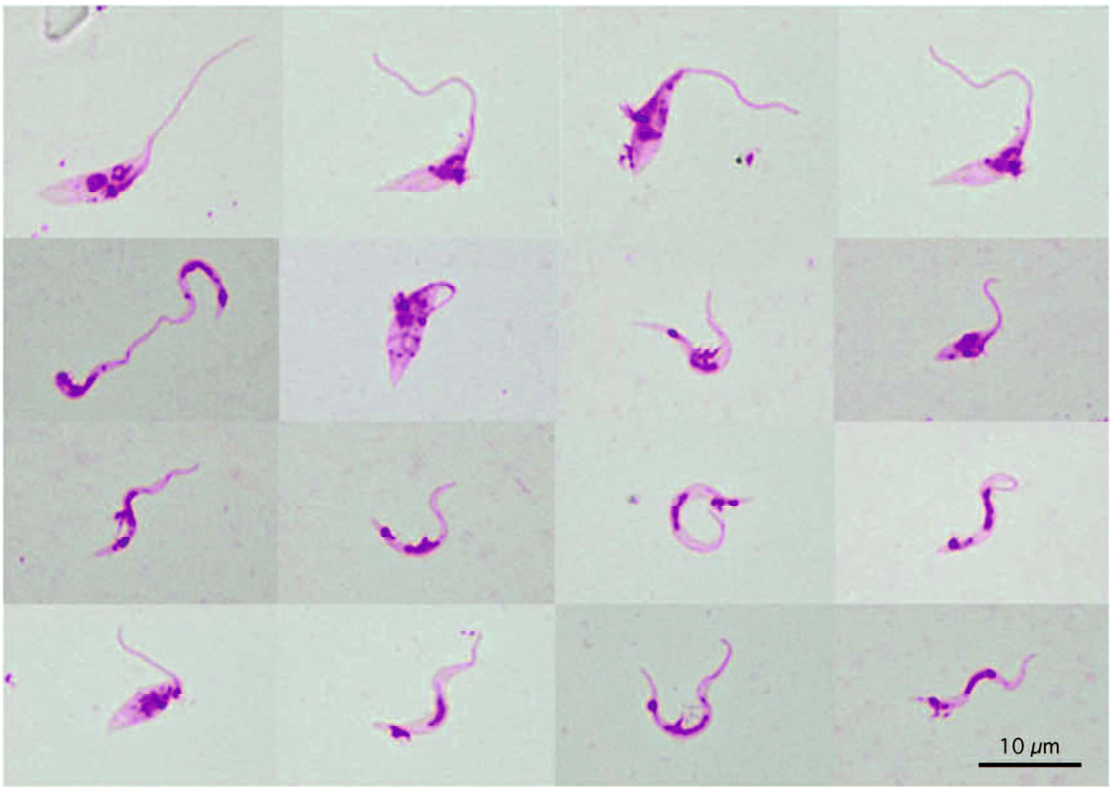

Fig. 10. Cultured forms of *Trypanosoma dionisii* from the blood of the Eastern bent-winged bat in Japan. Representative Giemsa-stained forms after haemoculture for 25 days in the BSK medium are shown at the same magnification.

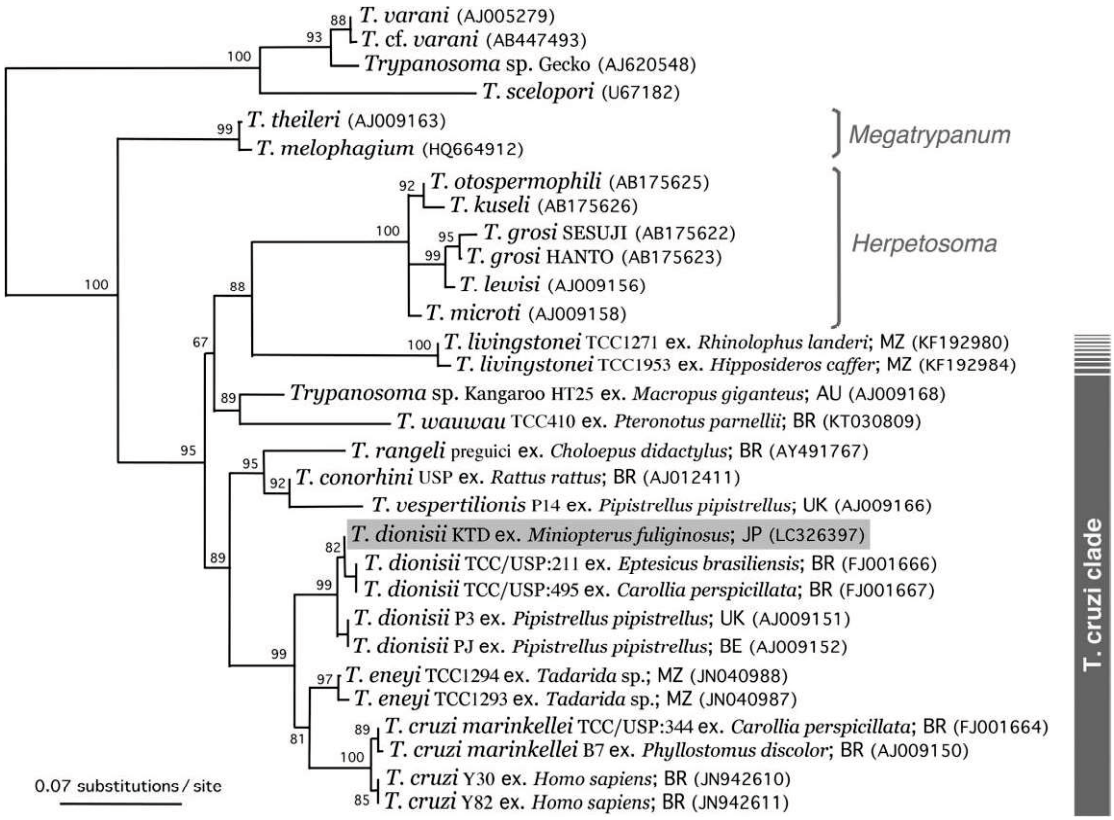

Fig.11. ML phylogenetic tree based on the SSU rDNA sequence. Reptile trypanosomes are placed as an outgroup. For trypanosome species in the *T. cruzi* clade, species names are followed by isolate names, host names and country names (DDBJ/EMBL/GenBank accession numbers are in parentheses). The newly sequenced isolate is indicated with a grey background. Australia (AU), Belgium (BE), Brazil (BR), Japan (JP), Mozambique (MZ) and the United Kingdom (UK)

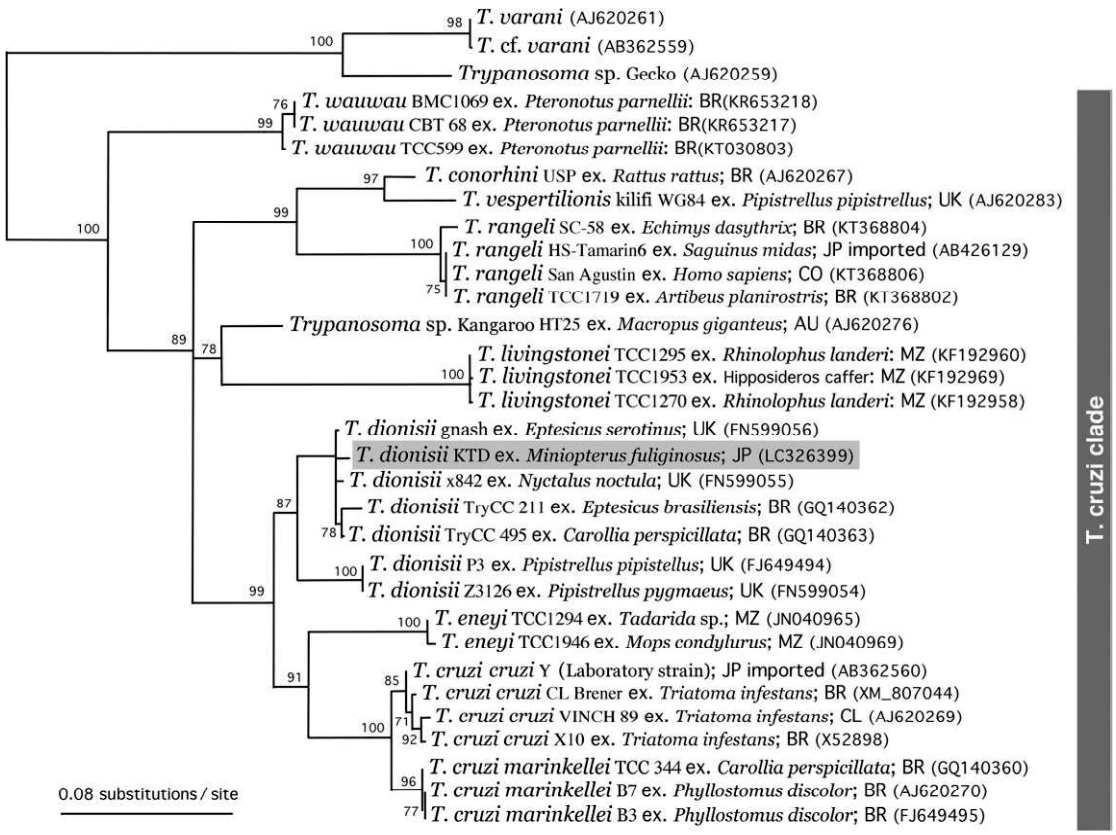

Fig. 12. ML phylogenetic tree based on the gGAPDH sequence. See Fig. 11 legend for details. Chile (CL) and Colombia (CO).

GENERAL DISCUSSION AND CONCLUSION

The knowledge on specific taxonomy and biogeographical distributions of trypanosomes is important not only for basic researches but also maintenance of the community health and economy. Extensive number of researches has focused on large animal trypanosomes of veterinary importance and human trypanosomes of medical importance, whereas very little attention has been directed to less/non-pathogenic stercorarian trypanosomes of small mammals. However, it is not uncommon for small mammals such as rodents and bats to share their dwellings with human residents. Sharing of niches by animals of different species has great risks of parasite switches from their natural host to the new host which share the habitat. Hamilton et al. (2012a) reported that various trypanosomes of the *Trypanosoma cruzi* clade have independently switched from bats into terrestrial mammals, and one of such host switches gave rise to *T. cruzi cruzi*. Habitats sharing between bats, terrestrial mammals and invertebrate vectors which feed on both bats and terrestrial mammals was pointed out as the factor facilitating and may continue to facilitate the host switch (Hamilton et al. 2012a). This might have been the case for *T. lewisi* which naturally infect rats, but due to niches sharing between rats, human and rat fleas, it can accidentally infect human and it is now regarded as an emerging human pathogen (Lun et al. 2015).

In Chapter I, I reviewed human cases of *T. lewisi* and molecular diagnosis of atypical human trypanosomiasis. Lun et al. (2015) reported that *T. lewisi* is resistant to NHS containing trypanolytic apoL-I, a characteristic of all human trypanosomes and the details of *T. lewisi* pathogenesis in human is still to be unveiled. Identification of *Herpetosoma* trypanosomes using morphology is difficult due to indistinguishable morphology and therefore it is necessary for all human cases of *T. lewisi* or *T. lewisi*-like trypanosomes to be confirmed by molecular methods. PCR amplification and/or nucleotide sequencing of

SSU rDNA and ITS1 have been used to diagnose *T. lewisi* and *T. lewisi*-like trypanosomes in human and non-human primates. However, yet a few full-length SSU rDNA sequences have been deposited in the gene database. More research efforts should be directed towards collection and genetic characterization of more rodent trypanosomes for accurate diagnosis of atypical human trypanosomiasis and for basic taxonomy of morphologically indistinguishable trypomastigotes in different rodent species.

In Chapter II, I characterized rodent and soricid trypanosomes in blood samples collected from Indonesia, Philippines, Vietnam, Taiwan, and mainland China by uses of morphology and nucleotide sequences of SSU rDNA and gGAPDH gene. Integrated, namely morphological and molecular characterization revealed that the *Herpetosoma* trypanosomes from Indonesia and Philippines were typical *T. lewisi* but those from *Niviventer confucianus* in mainland China were different based on both morphological and molecular genetic criteria; a new species, *Trypanosoma* (*Herpetosoma*) *niviventerae* n. sp. was erected for this isolate. In the phylogenetic tree constructed based on the SSU rDNA sequences, *T. niviventerae* n. sp. clustered together with *T. musculi*, *T. blanchardi* and *T. rabinowitschae* forming an independent cluster from that of *T. lewisi* but within the same *Herpetosoma* clade. Gibson et al. (2003) stated that there are challenges on how to determine what level of similarity define a species and how long ago the lineages separated from each other. Indeed *T. lewisi* is grouped into two genetic groups by a single nucleotide substitution in the SSU rDNA sequences, i.e. Brazilian isolates and those from other parts of the world, but these genetic groups are not consistent with groups based on nucleotide sequences of the ITS 1 region. It is therefore important to collect more data on not only the SSU rDNA but also other regions of the gene or other nuclear genes to fully identify the nucleotide changes critical for specific differentiation. Trypanosomes from soricids in Vietnam and Taiwan, were described as new species, *T. sapaensis* n. sp. and *T.*

anourosoricis n. sp. were proposed for them, respectively. Soricid trypanosomes (i.e. two new species in the current study and *T. talpae*) formed a well-supported clade comprised of only themselves, showing a sister relationship with *Herpetosoma* clade.

In Chapter III, I characterized trypanosomes from bats (*Miniopterus fuliginosus*) samples collected from Wakayama Prefecture in Japan. Bats harbour trypanosomes of subgenus *Schizotrypanum* which is a complex of *T. cruzi*-like trypanosomes. The current study employed morphological and molecular genetic characteristics to clarify the molecular phylogenetic position of an Asian bat trypanosome isolate. This study is the first report of *T. dionisii* in Asia. Characterisation of *T. dionisii* in Far East has critical importance to understand parasite dispersal between the continents.

Description of three new species and one new geographical distribution of small mammalian trypanosomes depicted in this study is an indication that the biodiversity and geographical distribution of them is still to be fully explored. More research efforts should be directed to small mammalian trypanosomes of the stercorarian section to unveil their real 'species' in science.

REFERENCES

Abramov A.V., Bannikova A.A., Rozhnov V.V. (2012). White-toothed shrews (Mammalia, Soricomorpha, Crocidura) of coastal islands of Vietnam. ZooKeys 2007:37–47.

Adams E.R., Hamilton P.B. (2008). New molecular tools for the identification of trypanosome species. Future Microbiology 3:167–176.

Albright J.W., Albright J.F. (1991). Rodent trypanosomes: their conflict with the immune system of the host. Parasitology Today 7:137–140.

Añex N. (1982). Studies on *Trypanosoma rangeli* Tejera, 1920 IV. A reconsideration of its systematic position. Memórias do Instituto Oswaldo Cruz 77:405–415.

Anisimova M., Gascuel O. (2006). Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Systematic Biology 55:539–552.

Baker J.R., Chaloner L.A., Green S.M. (1971). Intracellular development in vitro of *Trypanosoma dionisii* of bats. Royal Society of Tropical Medicine and Hygiene 65:427.

Baker J.R., Green S.M., Chaloner L.A., Gaborak M. (1972). *Trypanosoma* (*Schizotrypanum*) *dionisii* of *Pipistrellus pipistrellus* (Chiroptera): intra- and extracellular development in vitro. Parasitology 65:251–263.

Baker J.R., Miles M.A., Godfey D.G., Barrett T.V. (1978). Biochemical characterization of some species of *Trypanosoma* (*Schizotrypanum*) from bats (Microchiroptera). American Society of Tropical Medicine and Hygiene 27:483–491.

Bantihun G., Bekele A. (2015). Diversity and habitat association of small mammals in Aridtsy forest, Awi Zone, Ethiopia. Zoological Research 36:88–94.

Bardsley J.E., Harmsen R. (1973). The trypanosomes of anura. Advances in Parasitology 11:1–73.

Bern C., Kjos S., Yabsley M.J., Montgomery S.P. (2011). *Trypanosoma cruzi* and Chagas' disease in the United States. Clinical Microbiology Reviews 24:655–681.

Bose R., Heister N.C. (1993). Development of *Trypanosoma* (*M.*) *theileri* in tabanids. Journal of Eukaryotic Microbiology 40:788-792.

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

Botero A., Thompson C.K., Peacock C.S., Clode P.L., Nicholls P.K., Wayne A.F., Lymbery A.J., Thompson R. (2013). Trypanosomes genetic diversity, polyparasitism and the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*). International Journal for Parasitology: Parasites and Wildlife 2:77–89.

Carrea A., Diambra L. (2016). Systems biology approach to model the life cycle of *Trypanosoma cruzi*. PLoS One 11:e0146947.

Cavazzana M. Jr., Marcili A., Lima L., Maia da Silva F., Junqueira Â.C.V., Veludo H.H., Viola L.B., Campaner M., Nunes V.L.B., Paiva F., Coura J.R., Camargo E.P., Teixeira M.M.G. (2010) Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats. International Journal for Parasitology 40:345–355.

Cooper C., Clode P.L., Peacock C., Thompson R.C.A. (2017). Host–parasite relationships and life histories of trypanosomes in Australia. Advances in Parasitology 97:47–109.

Cottontail V.M., Kalko E.K.V., Cottontail I., Wellinghausen N., Tschapka M., et al. (2014). High local diversity of *Trypanosoma* in a common bat species, and

implications for the biogeography and taxonomy of the *T. cruzi* clade. PLoS One 9:e108603.

Dario M.A., Rodrigues M.S., Barros J.H., Xavier S.C., D'Andrea P.S., Roque A.L., Jansen A.M. (2016). Ecological scenario and *Trypanosoma cruzi* DTU characterization of a fatal acute Chagas disease case transmitted orally (Espírito Santo state, Brazil). Parasites & Vectors 9:477.

Datiko D., Bekele A., Belay G. (2007). Species composition, distribution and habitat association of rodents from Arbaminch forest and farmlands, Ethiopia. African Journal of Ecology 45:651–657.

Davis B.S. (1952). Studies on the trypanosomes of some California mammals. University of California Publications in Zoology 57:145–249.

Davis B.S. (1969) *Trypanosoma* (*Megatrypanum*) *hoarei* sp. nov. (Prozoa: Trypanosomatidae) from shrews of the genus *Sorex* in California. Parasitology 59:469–491.

Dereeper A., Guignon V., Blanc G., Audic S., Buffet S., Chevenet F., Dufayard J.F., Guindon S., Lefort V., Lescot M., Claverie J-M., Gascuel O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research 36:465–469.

Desquesnes M., Ravel S. Cuny G. (2002). PCR identification of *Trypanosoma lewisi,* a common parasite of laboratory rats. Kinetoplastid Biology and Disease 1:2

Desquesnes M., Kamyingkird K., Yangtara S., Milocco C., Ravel S., Wang M.-H., Lun Z.-R., Morand S. and Jittapalapong S. (2011). Specific primers for PCR amplification of the ITS1 (ribosomal DNA) of *Trypanosoma lewisi*. Infection, Genetics and Evolution 11:1361–1367.

Desquesnes M., Holzmuller P., Lai, D.-H., Dargantes A., Lun Z.-R. and Jittaplapong S.

(2013). *Trypanosoma evansi* and surra: a review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. BioMed Research International 2013:194176.

Dirie M.F., Bornstein S., Wallbanks K.R., Stiles J.K., Molyneux D.H. (1990). Zymogram and life-history studies on trypanosomes of the subgenus *Megatrypanum*. Parasitology Research 76:669–674.

Doherty M.L., Windle H., Voorheis H.P., Larkin H., Casey M., Clery D. (1993). Clinical disease associated with *Trypanosoma theileri* infection in a calf in Ireland. Veterinary Record 132:653–656.

Doke P. P. and Kar A. (2011). A fatal case of *Trypanosoma lewesi* in Maharashtra, India. Annals of Tropical Medicine and Public health 4:91–95.

Ferrante A. (1985). Trypanolytic activity, agglutinins, and opsonins in sera from animals refractory to *Trypanosoma lewisi*. Infection and Immunity 49:378–382.

García E.S., Castro D.P., Figueiredo M.B., Azambuja P. (2012). Parasite-mediated interactions within the insect vector: *Trypanosoma rangeli* strategies. Parasites & Vectors 5:105.

Gibson W. (2003). Species concepts for trypanosomes: from morphological to molecular definitions? Kinetoplastid Biology and Disease 2:10

Guan G., Niu Q., Yang J., Li Y., Gao J., Luo J., Yin H. (2011). *Trypanosoma* (*Herpetosoma*) *grosi*: first isolation from Chinese striped field mouse (*Apodemus agrarius*). Parasitology International 60:101–104.

Guindon S., Gascuel O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52:696–704.

Haag J., O'hUigin C., Overath P. (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. Molecular and Biochemical

Parasitology 91: 37–49.

Habtamu T., Bekele A. (2008). Habitat association of insectivores and rodents of Alatish National Park, northwestern Ethiopia. Tropical Ecology 49:1-11.

Hamilton P.B., Stevens J.R., Gaunt M.W., Gidley J., Gibson W.C. (2004). Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. International Journal for Parasitology 34:1393–1404.

Hamilton P.B., Stevens J.R., Holz P., Boag B., Cooke B., GibsonW.C. (2005). The inadvertent introduction into Australia of *Trypanosoma nabiasi*, the trypanosome of the European rabbit (*Oryctolagus cuniculus*), and its potential for biocontrol. Molecular Ecology 14:3167–3175.

Hamilton P.B., Gibson W.C., Stevens J.R. (2007). Patterns of co-evolution between trypanosomes and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. Molecular Phylogenettics and Evolution 43:15–25.

Hamilton P. B., Marta M.G. Teixeira M. M. G. Jamie R. Stevens J. R. (2012a). The evolution of *Trypanosoma cruzi*: the 'bat seeding' hypothesis. Trends in Parasitology 28:136–141.

Hamilton P.B., Cruickshank C., Stevens J.R., Teixeira M.M.G., Mathews F. (2012b). Parasites reveal movement of bats between the New and Old Worlds. Molecular Phylogenettics and Evolution 63:521–526.

Hoare C.A. (1972). The trypanosomes of mammals: a zoological monograph. Blackwell Scientific Publications, Oxford, U.K., 749 p.

Hodo C. L., Goodwin C. C., Mayes B.C., Mariscal J. A., Waldrup K. A., Hamer S. A. (2016). Trypanosome species, including *Trypanosoma cruzi*, in sylvatic and peridomestic bats of Texas, USA. Acta Tropica 164:259–266.

Hong X.K., Zhang X., Fusco O.A., Lan Y.G., Lun Z.R. (2017). PCR-based identification of *Trypanosoma lewisi* and *Trypanosoma musculi* using maxicircle kinetoplast DNA. Acta Tropica 171:207–212.

Howie, S., Guy, M., Fleming, L., Bailey, W., Noyes. H., Faye, J. A., Pepin, J., Greenwood, B., Whittle, H., Molyneux, D. and Corrah, T. (2006). A Gambian infant with fever and an unexpected blood film. PLoS Medicine 3 e355.

Hussain K., Brodie B., Ott R.S., Montealegre F. (1985). Prevalence of *Trypanosoma theileri* in cows and fetuses at slaughter. American Journal of Veterinary Research 46:1256–1258.

Hutchinson R., Stevens J.R. (2017). Barcoding in trypanosomes. Parasitology 145:563–573.

Jittapalapong S., Inpankaew T., Sarataphan N., Herbreteau V., Hugot J.P., Morand S. Stich R.W. (2008). Molecular detection of divergent trypanosomes among rodents of Thailand. Infection, Genetics and Evolution 8:445–449.

Johnson P.D. (1933). A case of infection by *Trypanosoma lewisi* in a child. Transactions of The Royal Society of Tropical Medicine and Hygiene 26:467–468.

Joshi P.P., Shegokar V.R., Powar R.W., Herder S., Katti R., Salkar H.R., Dani V.S., Bhargava A., Jannin J., Truc P. (2005). Human trypanosomiasis caused by *Trypanosoma evansi* in India: the first case report. American Journal of Tropical Medicine and Hygiene 73: 491–495.

Joshi P.P. (2013). Human trypanosomiasis in India: Is it an emerging new zoonosis? p10-13. [www.apiindia.or/medicine-update-2013/chaper04.pdf]

Karbowiak G., Wita I., Rychlik L. (2005). *Trypanosoma* (*Megatrypanum*) *ornata* sp. n., a parasite of the Eurasian water shrew *Neomys fodiens* (Pennant, 1771). Acta Protozoologica 44:363–367.

Kaur R., Gupta V.K., Dhariwal A.C., Jain D.C., Shiv L. (2007). A rare case of trypanosomiasis in a two month old infant in Mumbai, India. Journal of Communicable Diseases 39:71–74.

Kirchhoff L.V. (2011) Epidemiology of American trypanosomiasis (Chagas disease). Advances in Parasitology 75:1–18.

Kukla BA, Majiwa PAO, Young JR, Moloo SK and Ole-Moiyoi OK (1987). Use of species specific DNA probes for detection and identification of trypanosome infection in tsetse flies. Parasitology 95:1–16.

Kunz T.H., Reynolds D.S. (2003). Bat colonies in buildings. Information and Technology Report–2003–003:91–102.

Lima L., Maia da Silva F., Neves L., Attias M., Takata C.S.Z., Campaner M., de Souza W., Hamilton P.B., Teixeira M.M.G. (2012). Evolutionary insights from bat trypanosomes: morphological, developmental and phylogenetic evidence of a new species, *Trypanosoma* (*Schizotrypanum*) *erneyi* sp. nov., in African bats closely related to *Trypanosoma* (*Schizotrypanum*) *cruzi* and allied species. Protist 163:856–872.

Lima L., Espinosa-Álvarez O., Hamilton P.B., Neves L., Takata C.S.A., Campaner M., Attias M., de Souza W., Camargo E.P., Teixeira M.M.G. (2013). *Trypanosoma livingstonei*: a new species from African bats supports the bat seeding hypothesis for the *Trypanosoma cruzi* clade. Parasites & Vectors 6:221

Lima L., Espinosa-Álvarez O., Pinto M., Cavazzana M Jr., Pavan A.C., Carranza J.C., Lim B.K., Campaner M., Takata C.S.A., Camargo E.P., Hamilton P.B., Teixeira M.M.G. (2015). New insights into the evolution of the *Trypanosoma cruzi* clade provided by a new trypanosome species tightly linked to Neotropical *Pteronotus* bats and related to an Australian lineage of trypanosomes. Parasites & Vectors 8:657.

Linardi P.M. Botelho J. R. (2002). Prevalence of *Trypanosoma lewisi* in *Rattus norvegicus* from Belo Horizonte, State of Minas Gerais, Brazil. Memórias do Instituto Oswaldo Cruz 97:411–414.

Lizundia R., Newman C., Buesching C.D., Ngugi D., Blake D., Sin Y.W., Macdonald D.W., Wilson A., McKeever, D. (2011). Evidence for a role of the host-specific flea (*Paraceras melis*) in the transmission of *Trypanosoma* (*Megatrypanum*) *pestanai* to the European badger. PLoS One 6:e16977.

Luckins, A. G. (1988). *Trypanosoma evansi* in Asia. Parasitology Today 4:137–142.

Lukes J., Skalicky T., Tyc J., Votypka J., Yurchenko V. (2014). Evolution of parasitism in kinetoplastid flagellates. Molecular and Biochemical Parasitology 195:115–122.

Lun, Z. R., Wen, Y. Z., Uzureau, P., Lecordier, L., Lai, D. H., Lan, Y. G., Desquesnes, M., Geng, G. Q., Yang, T. B., Zhou, W. L., Jannin, J. G., Simarro, P. P., Truc, P., Vincendeau, P. and Pays, E. (2015). Resistance to normal human serum reveals *Trypanosoma lewisi* as an underestimated human pathogen. Molecular and Biochemical Parasitology 199:58–61.

Ma Y., Wang F., Jin S., Li S. (1987). Glires (rodents and lagomorphs) of northern Xinjiang and their zoogeographical distribution. Science Press, Beijing, p 274 (in Chinese).

Maia da Silva F., Marcilli A., Lima L., Cvazzana M.Jr., Ortiz P.A., Campaner M., Takeda G.F., Paiva F., Nunes V.L.B., Camargo E.P., Teizeira M.M.G. (2009). *Trypanosoma rangeli* isolates of bats from Central Brazil: genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences. Acta Tropica 109:199–207.

Maia da Silva F., Marcili A., Ortiz P.A., Epiphanio S., Campaner M., Catão-Dias J.L., Shaw J.J., Camargo E.P., Teixeira M.M.G (2010). Phylogenetic, morphological and behavioural analyses support host switching of *Trypanosoma* (*Herpetosoma*) *lewisi*

from domestic rats to primates. Infection, Genetics and Evolution 10:522–529.

Makouloutou, P., Setsuda, A., Yokoyama, M., Tsuji, T., Saita, E., Torii, H., Kaneshiro, Y., Sasaki, M., Maeda, K., Une, Y., Hasegawa, H. and Sato, H. (2013). Genetic variation of *Gongylonema pulchrum* from wild animals and cattle in Japan based on ribosomal RNA and mitochondrial cytochrome *c* oxidase subunit I genes. Journal of Helminthology 87: 326–335.

Marcili A., da Costa A.P., Soares H.S., Acosta I.C.L., Lima J.T.R., Minervino A.H.H., Melo A.T.L., Agular D.M., Pacheco R.C., Gennari S.M. (2013) Isolation and phylogenetic relationships of bat trypanosomes from different biomes in Mato Grosso, Brazil. Journal of Parasitology 99:1071–1076.

Maslov D.A., Simpson L. (1995). Evolution of parasitism in kinetoplastid protozoa. Parasitology Today 11:30–32.

Matthews K.R. (2005). The developmental cell biology of *Trypanosoma brucei*. Journal of Cell Science 118:283–290.

Matthews K.R., Ellis J.R., Paterou A. (2004). Molecular regulation of the life cycle of African trypanosomes. Trends in Parasitology 20:40–47.

Naiff R.D., Barrett T.V. (2013). *Trypanosoma* (*Megatrypanum*) *lainsoni* n. sp. from *Mesomys hispidus* (Rodentia: Echimyidae) in Brazil: trypomastigotes described from experimentally infected laboratory mice. Parasite 20:51.

Noyes H.A., Stevens J.R., Teixeira M., Phelan J., Holz P. (1999). A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. International Journal for Parasitology 29:331–339 (Corrigendum: International Journal for Parasitology 30:228 [2000]).

Noyes, H.A., Ambrose, P., Barker, F., Begon, M., Bennet, M., Bown, K.J., Kemp, S.J., (2002). Host specificity of Trypanosoma (*Herpetosoma*) species: evidence that bank

voles (*Clethrionomys glareolus*) carry only one *T*. (*H*.) *evotomys* 18S rRNA genotype but wood mice (*Apodemus sylvaticus*) carry at least two polyphyletic parasites. Parasitology 124:185–190.

Ohdachi S.D., Ishibashi Y., Iwasa M.A., Saitoh T. (2010). The wild mammals of Japan. Shoukadoh book Sellers, Kyoto, Japan, p544.

Oliveira M.P.C., Cortex M., Maeda F.Y., Fernandes M.C., Haapalainen E.F., Yoshida N. (2009). Unique behavior of *Trypanosoma dionisii* interacting with mammalian cells: invasion, intracellular growth, and nuclear localization. Acta Tropica 110:65–74.

Ortiz P.A., Garcia H.A., Lima L., Maia da Silva F.M., Campaner M., Pereira C.L., Jittapalapong S., Neves L., Desquesnes M., Camargo E.P., Teixeira M.M.G. (2018). Diagnosis and genetic analysis of the worldwide distributed *Rattus*-borne *Trypanosoma* (*Herpetosoma*) *lewisi* and its allied species in blood and fleas of rodents. Infection, Genetics and Evolution 63:380–390.

Paparini A., Irwin P.J., Warren K., McInnes L.M., de Tores P., Ryan U.M. (2011). Identification of novel trypanosome genotypes in native Australian marsupials. Veterinary Parasitology 183:21–30.

Peacock L., Cook S., Ferris V., Bailey M., Gibson W. (2012). The life cycle of *Trypanosoma* (*Nannomonas*) *congolense* in the tsetse fly. Parasites & Vectors 5:109.

Pérez-Morga, D., Vanhollebeke, B., Paturiaux-Hanocq, F., Derek P. Nolan, D.P., Lins, L., Homblé, F., Vanhamme, L., Tebabi, P., Pays, A., Poelvoorde, P., Jacquet, A., Brasseur, R. and Pays, E. (2005). Apolipoprotein L-I promotes trypanosome lysis by forming pores in lysosomal membranes. Science 309:469–472.

Pinto P.L.S., Takami R., Nunes E.V., Guilherme C.S., Oliveira Jr. O.C., Gama-Rodrigues J., Okumura M. (1999). Life Cycle of *Trypanosoma cruzi* (Y Strain) in mice. Rev.

Hosp. Revista do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo 54:141–146.

Pinto, C.M., Ocaña-Mayorga, S., Lascano, M.S., Grijalva, M.J. (2006). Infection by trypanosomes in marsupials and rodents associated with human dwellings in Ecuador. Journal of Parasitology 92:1251–1255.

Pumhom, P., Pognon, D., Yangtara, S., Thaprathorn, N., Milocco, C., Douangboupha, B., Herder, S., Chaval, Y., Morand, S., Jittapalapong, S. and Desquesnes, M. (2014). Molecular prevalence of *Trypanosoma* spp. in wild rodents of Southeast Asia: influence of human settlement habitat. Epidemiology and Infection 142:1221–1230.

Pumhom P., Morand S., Tran A., Jittapalapong S., Desquesnes M. (2015). *Trypanosoma* from rodents as potential source of infection in human-shaped landscapes of South-East Asia. Veterinary Parasitology 208:174–180.

Sarataphan N., Vongpakorn M., Nuansrichay B., Autarkool N., Keowkarnkah T., Rodtian P., Stich R.W., Jittapalapong S. (2007). Diagnosis of a *Trypanosoma lewisi*-like (*Herpetosoma*) infection in a sick infant from Thailand. Journal of Medical Microbiology 56:1118–1121.

Sato H., Ishita K., Matsuo K., Inaba T., Kamiya H., Ito M. (2003). Persistent infection of Mongolian jirds with a non-pathogenic trypanosome, *Trypanosoma (Herpetosoma) grosi.* Parasitology 127:357-363.

Sato, H., Ishita, K., Osanai, A., Yagisawa, M., Kamiya, H. and Ito, M. (2004). T cell-dependent elimination of dividing *Trypanosoma grosi* from the bloodstream of Mongolian jirds. Parasitology 128:295-304.

Sato H., Osanai A., Kamiya H., Obara Y., Jiang W., Zhen Q., Chai J., Une Y., Ito M. (2005). Characterization of SSU and LSU rRNAgenes of three *Trypanosoma* (*Herpetosoma*) grosi isolates maintained in Mongolian jirds. Parasitology 130:157–

167.

Sato H., Al-Adhami B.H., Une Y., Kamiya H. (2007). *Trypanosoma* (*Herpetosoma*) *kuseli* sp. n. (Protozoa: Kinetoplastida) in Siberian flying squirrels (*Pteromys Volans*). Parasitology Research 101:453–461.

Sato H., Leo N., Katakai Y., Takano J., Akari H., Nakamura S., Une Y. (2008). Prevalence and molecular phylogenetic characterization of *Trypanosoma* (*Megatrypanum*) *minasense* in the peripheral blood of small Neotropical primates after a quarantine period. Journal of Parasitology 94:1128–1138.

Sato H., Takano A., Kawabata H., Une Y., Watanabe H., Mukhtar M. (2009). *Trypanosoma* cf. *varani* in an imported ball python (*Python reginus*) from Ghana. Journal of Parasitology 95:1029–1033.

Setsuda, A., Da, N., Hasegawa, H., Behnke, J.M., Rana, H.B., Dhakal, I.P., Sato, H. (2016). Intraspecific and interspecific genetic variation of *Gongylonema pulchrum* and two rodent *Gongylonema* spp. (*G. aegypti* and *G. neoplasticum*) with the proposal of *G. nepalensis* n. sp. for the isolate in water buffaloes from Nepal. Parasitology Research 115:787–795.

Shah, I., Ali, U.S., Andankar, P., Joshi, R.R. (2011). Trypanosomiasis in an infant from India. Journal of Vector Borne Diseases 48:122–123.

Shegokar, V.R., Powar, R.M., Joshi, P.P., Bhargava, A., Dani, V.S., Katti, R., Zare, V.R., Khanande, V.D., Jannin J., Truc, P. (2006). Short report: Human trypanosomiasis caused by *Trypanosoma evansi* in a village in India: preliminary serologic survey of the local population. American Journal of Tropical Medicine and Hygiene 75:869–870.

Shrivastava, K.K., Shrivastava, G.P. (1974). Two cases of *Trypanosoma* (*Herpetosoma*) species infection of man in India. Transactions of the Royal Society of Tropical

Medicine and Hygiene 68:143-144.

Simpson A.G.B., Stevens J.R., Lukes J. (2006). The evolution and diversity of kinetoplastid flagellates. Trends in Parasitology 22:168-174.

Sousa, M.A. 2014. On opportunist infections by *Trypanosoma lewisi* in humans and its differential diagnosis from *T. cruzi* and *T. rangeli*. Parasitology Research 113:4471-4475.

Stevens J., Noyes H., Gibson W. (1998). The evolution of trypanosomes infecting humans and primates. Memórias do Instituto Oswaldo Cruz 93:669–676.

Stevens J.R., Teixeira M.M.G., Bingle L.E.H., Gibson W.C. (1999a). The taxonomic position and evolutionary relationships of *Trypanosoma rangeli*. International Journal for Parasitology 29, 749–757.

Stevens J.R., Noyes H.A., Dover G.A., Gibson W.C. (1999b). The ancient and divergent origins of the human pathogenic trypanosomes, *Trypanosoma brucei* and *T. cruzi*. Parasitology 118:107–116.

Stevens J.R., Noyes H.A., Schofield C.J., Gibson W. (2001). The molecular evolution of Trypanosomatidae. Advances in Parasitology 48:1-56.

Takano A., Nakao M., Masuzawa T., Takada N., Yano Y., Ishiguro F., Fujita H., Ito T., Ma X., Oikawa Y., Kawamori F., Kumagai K., Mikami T., Hanaoka N., Ando S., Honda N., Taylor K., Tsubota T., Konnai S., Watanabe H., Ohnishi M., Kawabata H. (2011). Multilocus sequence typing implicates rodents as the main reservoir host of humanpathogenic *Borrelia garinii* in Japan. Journal of Clinical Microbiology 49: 2035–2039.

Tang, H., Lan Y., Wen Y., Zhang, X., Desquesnes, M., Yang, T., Hide, G. and Lun, Z. (2012). Detection of *Trypanosoma lewisi* from wild rats in Southern China and its genetic diversity based on the ITS1 and ITS2 sequences. Infection, Genetics and

Evolution 12:1046–1051.

Thompson J.D., Higgins D.G., Gibson T.J. (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673–4680.

Truc, P., Büscher, P., Cuny, G., Gonzatti, M. I., Jannin, J., Joshi, P., Juyal, P., Lun, Z.-R., Mattioli, R., Pays, E., Simarro, P. P., Teixeira, M. M. G., Touatier, L., Vincendeau, P. and Desquesnes, M. (2013). Atypical human infections by animal trypanosomes. PLoS Neglected Tropical Diseases 7:e2256.

Tyler K.M., Engman D.M. (2003). The life-cycle of *Trypanosoma cruzi*. *In:* Tyler K.M., Miles M.A. (Eds.) American trypanosomiasis. World class parasites. Vol 7. Kluwer Academic Publishers, Boston, MA; pp.1–11.

Vanhamme, L., Paturiaux-Hanocq, F., Poelvoorde, P., Nolan, D. P., Lins, L., Van Den Abbeele, J., Pays, A., Tebabi, P., Van Xong, H., Jacquet, A., Moguilevsky, N., Dieu, M., Kane, J. P., De Baetseller, P., Brasseur, R., Pays, E. (2003). Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature 422:83–87.

Vanhollebeke, B., Truc, P., Poelvoorde, P., Pays, A., Joshi, P. P., Katti, R., Jannin J. G. and Pays, E. (2006). Human *Trypanosoma evansi* infection linked to a lack of apolipoprotein L-1. The New England Journal of Medicine 355: 2752–2756.

Vanwalleghem, G., Fontaine, F., Lecordier, L., Tebabi, P., Klewe, K., Nolan, D. P., Yamaryo-Botte, Y., Botte, C., Kremer, A., Burkard, G. S., Rassow, J., Roditi, I., Perez-Morga, D., Pays, E. (2015). Coupling of lysosomal and mitochondrial membrane permeabilization in trypanolysis by APOL1. Nature Communications 6:8078.

Verma A., Manchanda S., Kumar, N., Sharma A., Goel, M., Banerjee P. S., Garg R., Singh B.P., Balharbi F., Lejon V., Deborggraeve S., Singh Rana U.V., Pullyel J. (2011). *Trypanosoma lewisi* or *T. lewisi*-like infection in a 37-day-old Indian infant. American Journal of Tropical Medicine and Hygiene 85:221–224.

Vickerman K. (1994). The evolutionary expansion of the trypanosomatid flagellates. Internarional Journal for Parasitology 24:1317–1331.

Votýpka J., d'Avila-Levy C.M., Grellier P., Maslov D.A., Lukeš J., Yurchenko V. (2015). New approaches to systematics of Trypanosomatidae: criteria for taxonomic (Re)description. Trends in Parasitology 31:460–469.

Warpe, B.M., More, S.V. (2014). A rare Indian case of human trypanosomiasis caused by *Trypanosoma lewisi*-like parasites. Indian Journal of Medical Microbiology 5:564–567.

Zhang L.L., Ni Q.X., Yao L.N., Zhang X.H., Chen H.L., Feng Y., Zhang X., Yu K.G., Ruan W. (2015) [Identification of *Trypanosoma* species in *Rattus flavipectus* and *R. losea* using microscopy and PCR method]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 33:277–280.

Zhang Y., Jin S., Quan G., Li S., Ye Z., Wang F., Zhang M. (1997). Distribution of mammalian species in China. China Forestry Publishing House, Beijing, pp.280.

Ziccardi M., Lourenço-de-Oliveira R., Nogueira R. (1996). The haemoculture of *Trypanosoma minasense* Chagas, 1908. Memórias do Instituto Oswaldo Cruz 91:501–505.

Zingales B., Andrade S.G., Briones M.R.S., Campbell D.A., Chiari E., Fernandes O., Guhl F., Lages-Silva E., Macedo A.M., Machado C.R., Miles M.A., Romanha A.J., Sturm N.R., Tibayrenc M., Schijiman A.G. (2009). A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI.

Memórias do Instituto Oswaldo Cruz 104:1051–1054.

Zingales B., Miles M.A., Campbell D.A., Tibayrenc M., Macedo A.M., Teixeira M.M., Schijman A.G., Llewellyn M.S., Lages-Silva E., Machado C.R., Andrade S.G., Sturm N.R. (2012). The revised *Trypanosoma cruzi* subspecific nomenclature: rationale, epidemiological relevance and research applications. Infection, Genetics and Evolution 12:240–253.