Phylogenetic study of hemoparasites in domestic and wildlife animals with special reference to avian and chiropteran hemosporidians and ruminant trypanosomes

家畜および野生動物に寄生する住血原虫類の分子系統学的研究

―特に家禽とコウモリに寄生する住血胞子虫類および

反芻動物トリパノソーマに注目してー

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LIST OF ABBREVIATIONS

aLRT = approximate likelihood ratio test

BL = body length

BLAST = basic local alignment search tool

BSK = barbour-stoenner-kelly

BW = body width

CatL-like = the cathepsin L-like cysteine protease

Clpc = apicoplast caseinolytic protease C

Cox-1 = cytochrome *c* oxidase subunit I

Cytb = cytochrome *b*

DNA = deoxyribonucleic acid

DDBJ = DNA data bank of Japan

EDTA = ethylenediaminetetraacetic acid

EF2 = nuclear elongation factor 2

EMBL = european molecular biology laboratory

FF = free flagellum

gGAPDH = the glycosomal glyceraldehyde-3-phosphate dehydrogenase

IACUC = institutional animal care and use committee

ITS = the internal transcribed spacer

ITS1 = internal transcribed spacer 1

K = kinetoplast

KI = kinetoplast index

KN = distance between the kinetoplast and nucleus

Mal-Avi = a database for avian parasites

MCH = mean corpuscular hemoglobin

MCHC = mean corpuscular hemoglobin concentration

MCV = mean corpuscular volume

ML = maximum likelihood

NA = distance of the nucleus from the anterior end

NCBI = national center for biotechnology information

NI = nuclear index

NL = nucleus length

Nos = number of sequences

NW = nucleus width

PCR = polymerase chain reaction

PCV = packed cell volume

PK = distance of the kinetoplast from the posterior end

PN = distance of the nucleus from the posterior end

rDNA = ribosomal RNA

SL = the spliced leader RNA

SSU = small subunit

TthI = *Trypanosoma theileri* lineage I

TthII = *Trypanosoma theileri* lineage II

UM = undulating membrane

ABSTRACT

Hemoparasites live in the bloodstreams of mammals, including humans, and depending on the species or strains of the pathogen, they can cause an acute illness with a wide range of symptoms. Several hemoparasitic illnesses and associated vectors continue to pose the greatest risks to the health of domestic and wild animals, which result in significant population and financial losses. The accurate identification of a disease-causing pathogen is crucial for comprehending disease transmission in the nature. Hemoparasite identification of intra-erythrocytic protozoa (Apicomplexa: Haemosporidia) in avian and chiropteran and flagellate protozoa (Kinetoplastea: Trypanosomatidae) in ruminants is still solely based on morphological characterization. Recently, molecular techniques have identified more precisely or finely the species, cryptic species, and additional genotypes or lineages for hemoparasites transmission in nature. In the present study, I have endeavored to understand the diversity of avian and chiropteran hemosporidians and ruminant trypanosomes in animals with natural infection.

In Chapter I, I studied an avian hemosporidian of the genus *Haemoproteus* (Apicomplexa: Haemosporida: Haemoproteidae). Members of the genus are found worldwide in domestic and wild birds from various families with almost identical features. It is also generally suggested that this genus have limited pathogenicity and no veterinary relevance. To investigate this problem, I characterized morphologically and genetically pigeon haemoproteosis in farmed domestic pigeons (*Columba livia* f. *domestica* Gmelin, 1789) from Indonesia, with special references to hemogram analysis. In this study, we demonstrated for the first time a high prevalence of *Haemoproteus* infection with high degrees of coexistence of immature and mature gametocytes, indicating a chronic infection phase with repeated relapse and/or

reinfection, in farmed domestic pigeons from Yogyakarta, Central Java. The morphology and measurements of parasite were identical to *Haemoproteus columbae* Kruse, 1890, distributed worldwide. The cytochrome *b* gene (*cytb*) of our isolates exhibited four lineages that are prevalent in *H. columbae* lineages recorded worldwide. Our findings also demonstrated negative impact of subclinical *H. columbae* infection on the hemogram health of pigeons.

In Chapter II, I studied morphology and genetic diversity of bat hemosporidian parasites of the genus *Polychromophilus* Dionisi, 1899 (Haemosporidia: Haemoproteidae) in four bat species from Yamaguchi Prefecture, Japan. This study is the first to report the isolation and molecular characterization of two hemosporidian species of the genus *Polychromophilus* in bats distributed in Asian temperate region. *Polychromophilus melanipherus* from Japan is restricted to bats of the family Miniopteridae family in which gametocytes were dominant, and *Polychromophilus murinus* is limited to the family Vespertilionidae in which trophozoites were dominant. Host specificities of these two species correspond to previous studies on bats from Gabon, Madagascar, Europe, and Australia. The 15 *Polychromophilus cytb* sequences from 11 miniopterid and 4 vespertilionid bats were categorized into six haplotypes (three for each species), with no region-specific variation in the Old World. Multiple haplotypes (seven for cytochrome *c* oxidase subunit I (*cox-1*) and nine for apicoplast caseinolytic protease *c* (*clpc*)) and genotypes (three for nuclear elongation factor 2 (EF2)) were defined for Japanese *Polychromophilus* isolates, and the findings were compatible with hemosporidian *cytb* analyses. Our finding also identified bat flies of the genus *Nycteribia* as a potential invertebrate vector of *P. melanipherus.*

In Chapter III, I studied morphologically and genetically the cervid *Trypanosoma theileri* (Kinetoplastida: Trypanosomatidae) in Honshu sika deer (*Cervus nippon*) from

Japan. Seven genotypes of ruminant Megatrypanum-type trypanosomes were characterized based on the putative secondary structure of the hair-pin loop of the 18S ribosomal RNA gene (rDNA) hypervariable region. Of *T. theileri* and ruminant Megatrypanum-type trypanosomes, internal transcribed spacer 1 (ITS1) sequences were grouped into 10 major groups. The phylogenetic relationships of glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), cathepsin L-like cysteine protease (CatL-like) and spliced leader RNA (SL) gene is almost similar to those observed based on the 18S rDNA. Our findings demonstrated the distribution of genetic lineages of cervid Megatrypanum-type trypanosomes in Japan, where deer trypanosomes in western part are genetically closer to European cervid trypanosomes and the bovine *T. theileri* TthII lineage, while deer trypanosomes in northern part are genetically closer to North American cervid trypanosomes and the bovine *T. theileri* TthI lineage.

Through morphological and molecular characterization using genetic markers, I have resolved some complex biodiversities of avian and chiropteran hemosporidians and ruminant trypanosomes. By appropriately selecting hemoparasite genetic markers, we may be able to predict the potential risk of epizootic disease to humans and domestic animals that may be in contact with wildlife animals.

GENERAL INTRODUCTION

Bloodstream pathogens, also known as hemoparasites, such as bacteria, mycoplasma and protozoa, can cause an acute illness with a variety of clinical signs depending on the species or strains of the pathogen involved. Similarly, infection are often caused by some species, leading to wasting conditions (Springer *et al.*, 2015; Stuen, 2020). Hemoparasites occur on all continents, although their distribution is continually changing due to animal and vector mobility and transit, as well as the increased globalization of both live animals and their products (Ghai *et al.*, 2016; Stuen, 2020). Short- and long-distance vector and host migrations are either directly or indirectly influenced by climate change (Shope, 1991). These alterations will have a profound influence on the propagation and establishment of diseases and vectors (Sándor *et al.*, 2021; Fetene *et al.*, 2021). In the tropical region, several hemoparasitic illnesses and associated vectors continue to pose the greatest risks to the health of domestic animals, which result in significant financial losses for the animal sector (Melendez, 2006).

Hemosporidians (Sporozoa: Haemosporida) are a peculiar and phylogenetically isolated group of obligate heteroxenous protists, that become the focus of avian and chiropteran hemoparasites, and use blood-sucking dipteran insects (Insecta: Diptera) as vectors (Valkiūnas, 2005). Hemosporidians are one of the best-known and most-studied parasitic protist families because they contain the agents of malaria, a widespread human illness in warm-climate settings with imported cases reported globally (Valkiūnas, 2005; Fecchio *et al.*, 2020). However, most research focuses on human malaria agents and a few Plasmodiidae species that are used as model organisms, while other families such as Haemoproteidae, Leucocytozoidae, and Garniidae are limited

(Valkiūnas, 2005; Schaer *et al.*, 2013; Springer *et al.*, 2015; Marroquin-Flores *et al.*, 2017; Valkiūnas and Iezhova, 2018; Lotta *et al.*, 2019).

The unicellular parasitic flagellate protozoa of the genus *Trypanosoma* (Euglenozoa; Kinetoplastea; *Trypanosoma*tida), the most significant and pervasive hemoparasite in mammals, face the same issues. Human and animal pathogenic trypanosomes have received greater focus and research than less/nonpathogenic trypanosomes (Gautret *et al.*, 2009; Gómez-Junyent *et al.*, 2017; Sato and Mafie, 2022). However, certain trypanosome species (e.g. *T. theileri*) that are often regarded as non-pathogenic may induce harmful consequences, particularly when the infected host is subjected to a high amount of stress or is immunologicall depressed (Doherty *et al.*, 1993; Stuen, 2020; Suganuma *et al.*, 2022).

In addition, there are still unclear problems regarding the evolution, taxonomy, and biogeographical speciation of hemosporidians in avian and chiropteran hosts, as well as trypanosomes in mammals (Stevens and Gibson, 1999; Hellgren *et al.*, 2004; Hamilton *et al.*, 2012a; Schaer *et al.*, 2013; Votýpka *et al.*, 2015; Fecchio *et al.*, 2020). In the current study, hemosporidians isolated from domestic birds and wild bats, and trypanosomes isolated from ruminants were morphologically and molecular genetically characterized to provide additional data for discussions on evolution and taxonomy as well as geographical distribution.

Avian hemosporidians

The avian malaria parasite of the genus *Plasmodium* and related hemosporidians (genera *Haemoproteus, Leucocytozoon,* and *Fallisia*) are members of a worldwide group of protozoan parasites that have been isolated from all bird clades and zoogeographical areas with the exception in the polar region (Bensch *et al.*, 2009;

Fecchio *et al.*, 2020). Avian hemosporidians can be separated into four families: Plasmodiidae, Haemoproteiidae, Leucocytozooiidae, and Garnidae, each containing one genus: *Plasmodium, Haemoproteus, Leucocytozoon,* and *Fallisia*, respectively (Valkiūnas, 2005).

Light microscopy has traditionally been used to investigate the life cycle, morphology, and categorization of avian hemosporidians. There are around 200 nominal hemosporidian species and hundreds of possibly undescribed cryptic species that appear to be genetically separate yet commonly display convergent morphology with other known taxa (Merino *et al.*, 1997; Jones *et al.*, 2002; Quillfeldt *et al.*, 2010; Valkiūnas, 2011). These parasites can cause either acute disease or chronic infection with wasting (Atkinson *et al.*, 2001; Donovan *et al.*, 2008; Valkiūnas and Iezhova, 2018). The recent use of molecular methods to identify infections (e.g. PCR) has resulted in an expanded study, bringing fresh insights into parasite genetic diversity and host specificity (Hellgren *et al.*, 2009; Ventim *et al.*, 2012).

Life cycle and morphology of avian hemosporidians

Active transmission of these three hemosporidian genera (*Plasmodium*, *Haemoproteus,* and *Leucocytozoon*) in birds necessitates the presence of three factors: a parasite, a competent dipteran vector, and a competent bird (Figure 1). The parasite is transmitted to each bird by the dipteran vector through infective sporozoites located in the salivary glands at the time of blood collection. Sporozoites travel through the bloodstream of the bird until they infect the proper tissue cells (e.g. liver, spleen). Once inside the host body, the sporozoites reproduce asexually to create schizonts (which include numerous merozoites), a process known as schizogony or merogony. When tissue cells break, merozoites are discharged. Merozoites can infect blood cells and

develop into micro- or macro- gametocytes, the sexual forms of male and female parasites, or they can continue the cycle of infecting tissue cells to generate additional schizonts and merozoites. Gametogony refers to the process of gametocyte production. *Plasmodium* is the only genus that reproduces asexually in the bloodstream. Some *Plasmodium* merozoites continue to infect red blood cells during this phase, producing new schizonts and merozoites (Valkiūnas, 2005; Valkiūnas and Iezhova, 2018; Cepeda *et al.*, 2019).

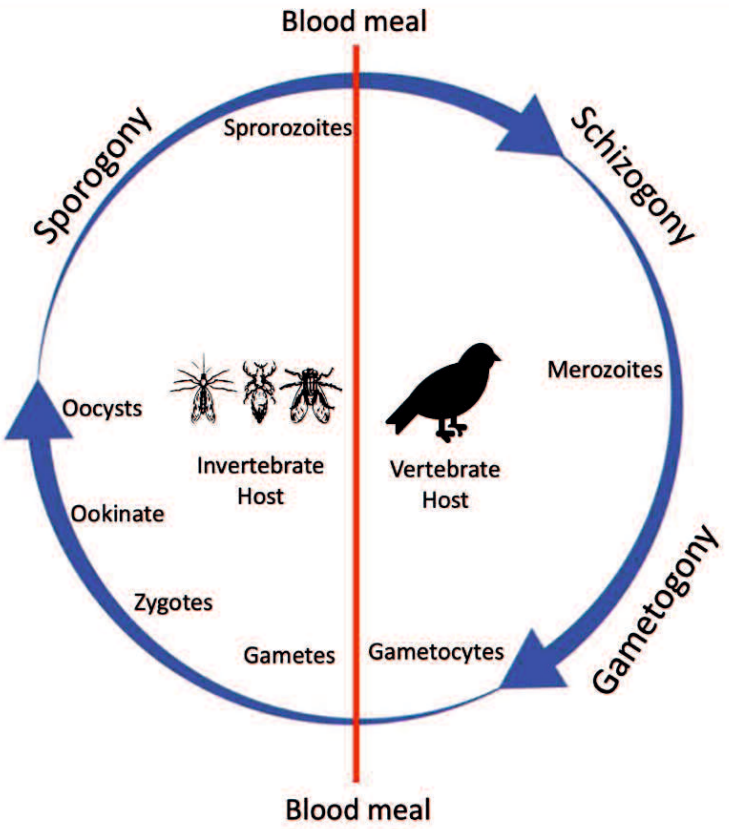

Figure 1. General schematic of the avian hemosporidian life cycle.

During feedings, dipterans consume gametocytes as well as other blood components from infective birds. Each macrogametocyte detaches from the blood cell it is in while in the dipteran's gut (creating macrogametes). Simultaneously, each microgametocyte undergoes fast asexual reproduction, producing eight flagella-like microgametes that exit from the blood cell, a process known as exflagellation.

Microgametes fertilize macrogametes, resulting in the formation of a diploid zygote, an elongated structure known as an ookinete. The ookinete enters the gut and adheres to the exterior of the intestinal wall, forming a ball known as an oocyst. Asexual reproduction allows oocysts to develop and expand by creating a large number of haploid sporozoites. The oocyst eventually ruptures, releasing the sporozoites to go to the salivary glands. Sporogony refers to the process of creating sporozoites. When the infective dipteran feeds on another bird host, the parasite will be ready to continue the life cycle (Valkiūnas, 2005; Valkiūnas and Iezhova, 2018; Cepeda *et al.*, 2019).

The three hemosporidian genera become detectable in avian blood at different times (i.e. they have different prepatent periods). *Haemoproteus* becomes visible in the blood after around 11-21 days, with mature gametocytes that are sausage-shaped with blunt ends and are seen to shift the nucleus of host erythrocytes laterally (Valkiūnas, 2005). *Plasmodium* becomes detectable in blood stages two days to several months after sporozoite infection with malarial pigment (hemozoin) (Valkiūnas, 2005). Fully grown erythrocytic meronts and gametocytes occupy more than half of the cytoplasmic space in infected erythrocytes but do not occupy all available cytoplasmic space in erythrocytes. *Leucocytozoon* has a five-day prepatent phase (Merino and Potti, 1995), during which gametocytes develop in roundish and/or fusiform host cells. The nucleus of fusiform host cells is more or less dumbbell-shaped, with obvious thickenings at both ends, and is tightly appressed to the gametocytes. During persistent parasitemia and relapses, gametocytes in fusiform host cells are frequent in peripheral circulation.

Molecular methods and current taxonomy of avian hemosporidians

PCR has evolved into an essential molecular method for screening of hemosporidian infection in birds, and it is frequently used in concert with microscopy.

Use of both microscopy and molecular data can aid in determining genus and/or species, mixed infection, and revealing fascinating insights into the parasites' genetic diversity and host-specificity (Hellgren *et al.*, 2009; Ventim *et al.*, 2012).

Bensch et al. (2000) helped to revolutionize avian malaria screening by designing a primer set to amplify a 478-nucleotide portion of the mitochondrial cytochrome *b* gene from avian *Haemoproteus* and *Plasmodium* which was refined by Hellgren et al. (2004) to detect all three parasites simultaneously a nested PCR. Other molecular identification methods included restriction fragment length polymorphism (RFLP) (Beadell and Fleischer, 2005), serological testing (Atkinson *et al.*, 1995, 2001), quantitative PCR, real-time PCR, DNA amplification via PCR from a single cell selected using a laser (Palinauskas *et al.*, 2010) or post-PCR cloning manipulation (e.g., TA cloning) to separate mixed infections in the same host. Furthermore, genetic screening of hemosporidians in wild-caught insect vectors was also conducted even not common, such as in mosquitoes (Gager *et al.*, 2008; Ishtiaq *et al.*, 2008; Kim *et al.*, 2009; Hughes *et al.*, 2010; Kim and Tsuda, 2010; Kimura *et al.*, 2010; Njabo *et al.*, 2011; Inci *et al.*, 2012; Kim and Tsuda, 2012; Ferraguti *et al.*, 2013a; Tanigawa *et al.*, 2013; Lalubin *et al.*, 2013), black flies (Hellgren *et al.*, 2008; Sato *et al.*, 2009), or biting midges (Martínez-de la Puente *et al.*, 2011; Santiago-Alarcon *et al.*, 2012; Ferraguti, *et al.*, 2013b; Synek *et al.*, 2013) but the evidence does not support the vector's potential to transmit parasites into vertebrate hosts (Valkiūnas, 2011; Valkiūnas *et al.*, 2013).

Systematic taxonomy of avian hemosporidians based on morphological and life cycle characteristics revealed ten subgenera: *Parahaemoproteus* and *Haemoproteus* grouped as *Haemoproteus* genera; *Haemamoeba*, *Giovannolaia, Novyella, Bennettinia,* and *Huffa* grouped as *Plasmodium* genera; *Plasmodiodes* grouped as *Fallisi* genera, and *Leucocytozoon* and *Akiba* grouped in *Leucoytozoon* genera. However, molecular

phylogeny using mitochondrial *cytb* genes identified more than 250 morphospecies and over 3900 hemosporidian lineages prompting the creation of a coordinated database (Mal-Avi, http://mbio-serv2.mbioekol.lu.se/Malavi) to record the distributions of lineages and facilitate the investigation of global patterns (Bensch *et al.*, 2009; Nourani *et al.*, 2020). Lineages in avian hemoparasites are used to identify novel hosts, study host-parasite interactions, and understand the geographical distribution patterns of parasites by considering at least one mutation in comparison with other known *cytb* sequences deposited in GenBank and Mal-Avi database (Nourani *et al.*, 2020).

Pigeon hemosporidians

According to Valkūinas (2005), 19 species of avian hemosporidians have been reported infecting birds of the Columbiformes order. *Haemoproteus columbae* (Columbidae), *H. sacharovi* (Columbidae), *H. turtur* (Columbidae), *H. palumbis* (Columbidae), *H. krylovi* (Pteroclididae), and *H. pteroclis* (Pteroclididae) are all members of the *Haemoproteus* genera. In genera *Plasmodium* there are *Plasmodium relictum* (Passeridae), *P. cathemerium* (Passeridae), *P. circumflexum* (Turdidae), *P. polare* (Hirundinidae), *P. lophurae* (Phasianidae), *P. gabaldoni* (Columbidae), *P. vaughani* (Turdidae), *P. columbae* (Columbidae), *P. nucleophilum* (Mimidae), *P. dissanaikei* (Psittacidae), and *P. elongatum* (Passeridae**).** Other parasites are *Fallisia neotropicalis* (Columbidae) and *Leucocytozoon marchouxi* (Columbidae) which belongs to the genera *Fallisia* and *Leucocytozoon*, respectively.

Haemoproteus columbae is the most common blood parasite that is widely found especially in domestic pigeons (*Columba livia* f. *domestica*), i.e. rock doves, wild pigeons i.e. mourning doves (*Zenaidura macroura*), turtle doves (*Streptopelia turtur*) and other wild columbid species (Valkiūnas, 2005; Samani *et al.*,

2013). *Haemoproteus columbae* has not yet been found in a non-columbid host and might be specific to pigeon and dove hosts (Valkiūnas, 2005). Most infections of these parasites produce subclinical infections with over 50% of erythrocytes frequently parasitized at the peak of parasitemia and anemia, anorexia, and a marked enlargement of the liver and spleen even death were recorded (Valkiūnas, 2005; Samani *et al.*, 2013, 2016; Hussein and Abdelrahim, 2016).

The life cycle of this parasite involves two hosts, with merogony stages and gametogony stage in rock doves (*Columba livia*) and sporogony the stage in pigeon louse flies (*Pseudolynchia canariensis*) (Figure 2) (Valkiūnas, 2005; Borji *et al.*, 2011; Hussein and Abdelrahim, 2016; Samani *et al.*, 2016; Maharana and Kumar, 2017). This parasite characterized by fully grown gametocytes markedly displaces the nucleus of infected erythrocytes laterally, slightly encloses the nucleus with their ends but does not encircle it completely. Pigment granules that tend to aggregate into large compact masses which frequently exceed 1 µm in diameter in microgametocytes. The nucleus in macrogametocytes is median or submedian in position (Valkiūnas, 2005).

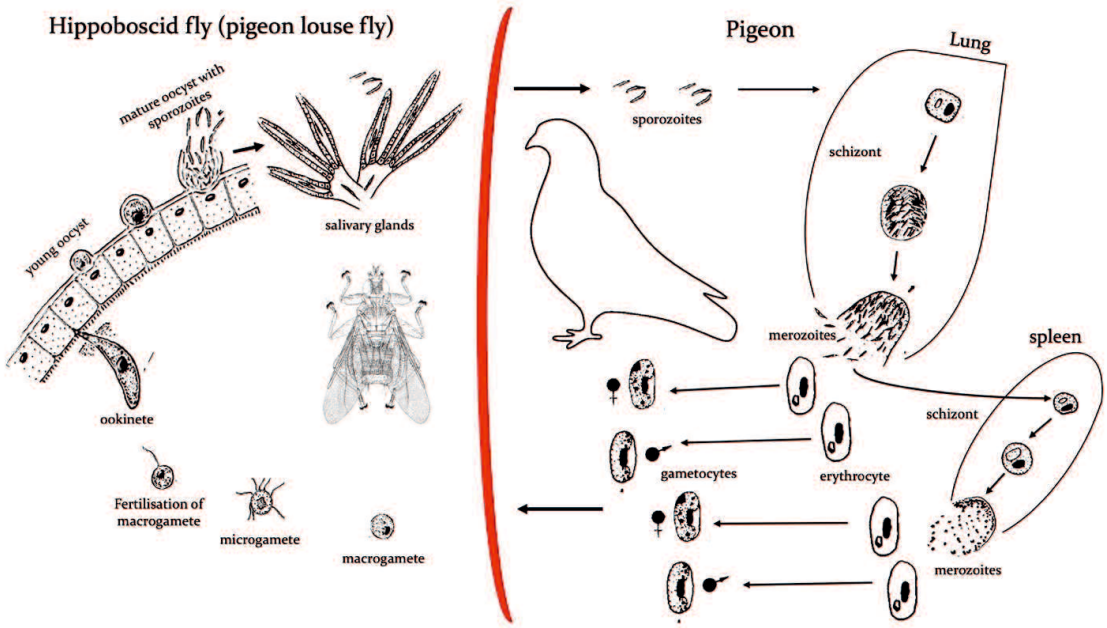

Figure 2. Diagrammatic representation of the life cycle of *Haemoproteus columbae* in pigeon and hippoboscid fly (pigeon louse fly) according to Valkūinas (2005) .

Despite the lack of sequencing data on avian hemosporidian in pigeons, four frequent *cytb* lineages of *H. columbae* in pigeons have been identified: HAECOL1, COQUI05, COLIV07, and COLIV03 (Nebel *et al.*, 2020). Those lineages have been isolated from feral pigeons *Columba livia* in Peru and Brazil (Pacheco *et al.*, 2018a; Nebel *et al.*, 2020), European common wood pigeon *Columba palumbus* (Dunn *et al.*, 2017), African olive pigeon *Columba arquatrix* in Malawi (Lutz *et al.*, 2015), Amboyna cuckoo-dove *Macropygia amboinensis* in Papua New Guinea (Beadell *et al.*, 2004), band-tailed pigeon *Patagioenas fasciata* in Peru (JQ988683, JQ988729; published on GenBank only), and two mosquitoes (Culicidae, Diptera) in Cameroon (Njabo *et al.*, 2011).

Bat hemosporidians

Bats are classified under the order Chiroptera which is one of the mammalian orders that has the clearest definition (Taylor and Tuttle, 2019). It is also the second biggest, with more than 1,300 species around the planet and accounting for about 20 percent of the world's living mammals (Schaer *et al.*, 2013; Taylor and Tuttle, 2019; Sato and Mafie, 2022). Bats are second only to rodents (of which there are well over 2,000 species) in terms of the variety of species. The order Chiroptera is often subdivided into two suborders: Microchiroptera, which consists of the smaller, mostly insectivorous "microbats," and Megachiroptera, which consists of the primarily bigger fruit bats and flying foxes—the "megabats," and only contains one family, Pteropodidae (Schaer *et al.*, 2013; Taylor and D.Tuttle, 2019; Arnuphapprasert *et al.*, 2020).

A wide range of pathogens, including viruses (Plowright *et al.*, 2014), bacteria (Mühldorfer, 2013), and protozoa (Molyneux and Bafort, 1971; Cabral *et al.*, 2013; Hornok *et al.*, 2015), that cause developing infectious illnesses are either hypothesized

to exist in bats. Studying the hemosporidian of bats (Garnham, 1953) can also provide one with an in-depth understanding of the evolutionary links between vertebrates and malarial parasites (Duval *et al.*, 2007; Witsenburg *et al.*, 2012; Schaer *et al.*, 2013).

All around the world, bats are infected by eight different Plasmodiidae genera: *Biguetiella, Dionisia, Hepatocystis, Johnsprentia, Nycteria, Plasmodium, Polychromophilus,* and *Sprattiella* (Perkins and Schaer, 2016). Of the 8 genera, 6 of them are exclusively found in bats except for *Plasmodium* and *Hepatocytis* (Schaer *et al.*, 2013, 2015; Arnuphapprasert *et al.*, 2020).

Life cycle and morphology of bat hemosporidians

The life cycle of *Polychromophilus* is fairly typical of hemosporidians life cycle (Figure 3), with gametocytes and gametes being confined to the circulation of the host and meronts infecting organs, particularly the lungs and the liver (Gardner and Molyneux, 1988a). Sporozoites of parasites reside in the salivary glands of their insect vectors until a blood meal is consumed. The sporozoites enter the primary host's bloodstream and infect erythrocytes, where the parasite develops into merozoites stages and matures becoming gametocytes. Sporozoites are elongated, straight or mildly curved, and not particularly motile (Gardner and Molyneux, 1988; Valkiūnas and Iezhova, 2018; Arnuphapprasert *et al.*, 2020).

The gametocytes then proceed to sexually reproduce via gametogony, involving the exflagellation of the parasite forming numerous microgametes and macrogametes (Gardner and Molyneux, 1988). Malaria parasites (*Plasmodium* genus) are the only hemosporidian that can grow asexually in red blood cells, making them highly pathogenic (Venugopal *et al.*, 2020). The microgametes and macrogametes remain within the blood until they are taken in by an insect vector during another blood

feeding. The gametocytes are known to doubly infect the same erythrocyte, a feature considered characteristic of some species (Gardner and Molyneux, 1988; Duval *et al.*, 2012; Witsenburg *et al.*, 2014; Arnuphapprasert *et al.*, 2020).

The gametes combine in pairs inside the insect's gut, and the fertilized macrogamete transforms into an ookinete. The parasite develops into an oocyst between the midgut's epithelial cells and basement membrane (Fayer, 1986; Gardner and Molyneux, 1988). The oocyst generally contains multiple cristate mitochondria, and mature oocysts are packed with sporozoites (Gardner and Molyneux, 1988). When mature, the oocyst explodes, releasing the sporozoites into the insect carrier, and moving to the salivary gland to restart the cycle (Gardner and Molyneux, 1988).

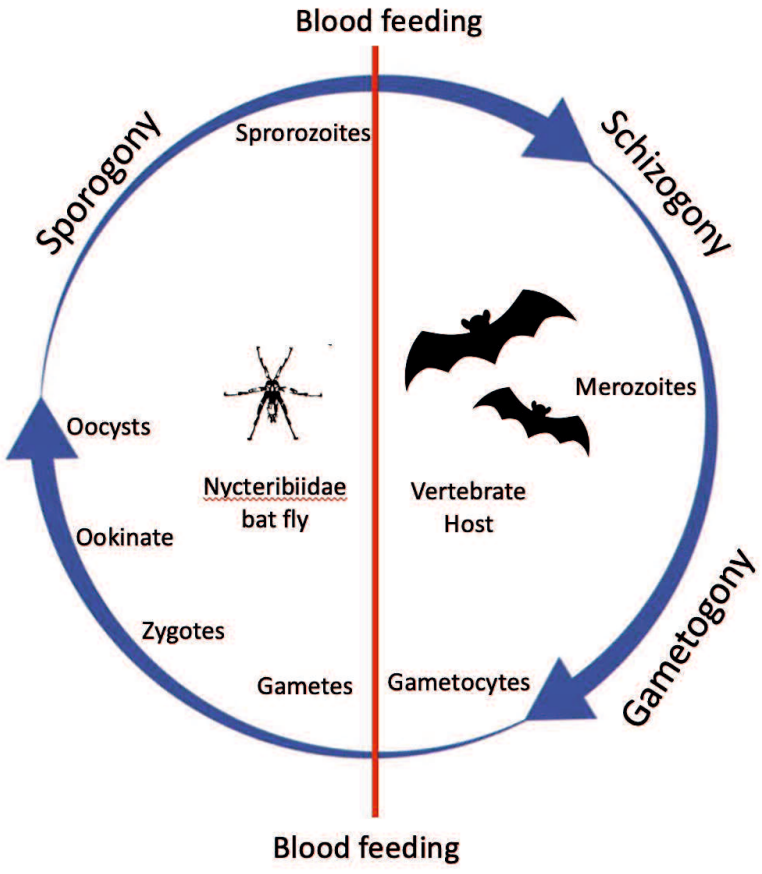

Figure 3. General schematic of the bat hemosporidianlife cycle with special reference to *Polychromophilus* spp.

Molecular methods and current taxonomy of avian hemosporidians

Our understanding of the evolutional relationships and species/genetic lineage differentiation of intra-erythrocytic protozoans has improved over the last two decades as a result of molecular approaches to studying malarial parasites (diverse *Plasmodium* parasites with different host specificities, infection courses, and pathogenicity) and other hemosporidian parasites (Martinsen *et al.*, 2008; Schaer *et al.*, 2013; 2015; Boundenga *et al.*, 2018; Galen *et al.*, 2018).

Four signature genes representing the three parasite genomes, mitochondrial: cytochrome *b*, cytochrome *c* oxidase subunit I; plastid: caseinolytic protease *c;* nuclear: elongation factor 2A, are used to characterize phylogenetically bat hemosporidians (Duval *et al.*, 2007; Martinsen *et al.*, 2008; Schaer *et al.*, 2013; Arnuphapprasert *et al.*, 2020). The most popular is *cytb*, which uses nested PCR and has a sequence length of around 741 base pairs (Duval *et al.*, 2007).

The avian and squamate *Plasmodium* species are placed as polyphyletic to the mammalian *Plasmodium* species, and bat hemosporidians form a single clade rooted with *Leucocytozoon* species, suggesting that bats were likely the first mammalian hosts of *Plasmodium* before the parasites started to use rodents and primates, including humans, as vertebrate hosts (Outlaw and Ricklefs, 2011; Schaer *et al.*, 2013). The phylogeny also implies *Plasmodium* parasites never shifted to nonmammalian hosts after invading mammals (Duval *et al.*, 2007; Martinsen *et al.*, 2008).

***Polychromophilus* spp. in bats**

The hemosporidian genus *Polychromophilus* has only been associated with bats and is transmitted by ectoparasitic, highly specialized nycteribiid flies (Corradetti *et al.*, 1961; Garnham, 1973a; Witsenburg *et al.*, 2012; Sándor *et al.*, 2021). The only

hemosporidian taxon that infects mammalian hosts in both tropical and temperate climatic zones is *Polychromophilus*. According to many studies (Schaer *et al.*, 2013; Ramasindrazana *et al.*, 2018; Arnuphapprasert *et al.*, 2020; Sándor *et al.*, 2021), these parasites are prevalent in bats throughout Europe as well as the tropical parts of Africa, Asia, and South America. Despite being frequent, only three legitimate species of *Polychromophilus* parasites have been properly characterized up to this point. Most cases of *Polychromophilus murinus* and *Polychromophilus melanipherus* have been found in bats of the families Vespertilionidae and Miniopteridae, respectively (Duval *et al.*, 2007; Arnuphapprasert *et al.*, 2020). The African *Miniopterus* parasitic species that gave rise to the species *Polychromophilus corradetti* and *Polychromophilus adami* were described (Landau *et al.*, 1980) but were subsequently mistakenly thought to be a junior synonym of *Polychromophilus melanipherus* recently based on molecular analyses (Duval *et al.*, 2012; Rosskopf *et al.*, 2019).

The description of *Polychromophilus deanei* from *Myotis nigricans* (Vespertilionidae) in Brazil, as well as three additional records of *Polychromophilus* from bats in Brazil and the southeastern United States, gave evidence of chiropteran hemosporidian parasites in the New World (Wood, 1952; Garnham *et al.*, 1971; Foster, 1979).

Phylogenetic investigations have verified that *P. murinus* and *P. melanipherus* are separate species (Megali *et al.*, 2011; Witsenburg *et al.*, 2012). *Polychromophilus* of *M. nigricans* from Panama, which may be *P. deanei*, groups closely with *P. murinus* parasite sequences in molecular phylogenies (Borner *et al.*, 2016). However, *Polychromophilus* sequences from *P. corradetti* and *P. adami* from African miniopterid hosts clustered with *P. melanipherus* and are considered synonymous species as mentioned above (Duval *et al.*, 2012; Rosskopf *et al.*, 2019).

The morphological and molecular examinations of *Polychromophilus* parasites in Asia, particularly in the temperate zone, have only been the subject of a very small number of research. Studies regarding *Polychromophilus* in Asian bats are limited in Cambodia (Chumnandee *et al.*, 2021), Malaysia (Eyles *et al.*, 1962) and Thailand (Landau *et al.*, 1984; Duval *et al.*, 2007; Arnuphapprasert *et al.*, 2020) while in Asian temperate region is scarce. In this study, we were able to provide significant new information on the phylogeny of these understudied parasites, which included the isolation and genetic analysis of *Polychromophilus* infections in Japanese bats.

Ruminant trypanosomes

Trypanosomes are the most concerning hemoparasites in ruminants due to their zoonotic potential. All vertebrate hosts are susceptible to *Trypanosoma* Gruby, 1843 (Euglenozoa: Kinetoplastea: Trypanosomatidae), but most research is focused on species that cause sickness in humans and animals as well as significant economic losses (Shope, 1991). Trypanosome members of the subgenus *Megatrypanum* (*T. theileri, T. cervi, T. melophagium, T. stefanskii,* and *T. theodori*, as well as other species described in the past based on morphology) are generally considered as ruminant trypanosomes (Magri *et al.*, 2021). *T. theileri* is well-known as a large ruminant trypanosome. This parasite infects ruminants and is transmitted by a variety of vectors including tabanids and triatomine bugs (Thompson *et al.*, 2014; Brotánková *et al.*, 2022).

T. theileri, in terms of phylogenetic analysis, it is grouped within the "*Trypanosoma* with unspecified subgenus". *T. theileri* is considered a mildly pathogenic species that typically infects wild and domestic ruminants (Magri *et al.*, 2021). Different tabanid species are common vectors of *T. theileri*, transmitting the

pathogen by laying infected feces on the skin of the mammalian host or by ingestion of infected insects (Böse *et al.*, 1993); however, during a study concerning *Leishmania infantum* in the Emilia-Romagna region (Italy), the presence of *T. theileri*-like trypanosomes has been recently reported in sandflies (*Phlebotomus* spp.) (Ganyukova *et al.*, 2018; Kostygov *et al.*, 2022), although their role as vectors has not been established. Exploiting abraded skin or mucosae, *T. theileri* invades the bloodstream of the mammalian host; the prepatent period ranges from 4 to 20 days and parasitemia decreases after 2–4 weeks (Mansfield, 1977).

Arnold Theiler, a veterinary bacteriologist, discovered this species while immunizing cattle against rinderpest in Africa. He noticed *Trypanosoma*-like structures in blood smears and described the parasite as *T. theileri* (Hoare, 1972; Magri *et al.*, 2021). In the following years, numerous reports on the discovery of *Trypanosoma* were published; however, they were often have been erroneous due to the large morphological variation, which led to the creation of several new species (e.g., *Trypanosoma frank* from cattle in Germany, *Trypanosoma wrublewskii* from the European bison *Bison bonasu* in Poland, *Trypanosoma americanum* and *Trypanosoma rutherfordi* from cattle in North America (Hoare, 1972), which were later recognized as *T. theileri* by Herbert (1964). Recent reports of the presence of *T. theileri* in ruminants ranged from Australia (Turner and Murnane, 1930), the United Kingdom (Wells *et al.*, 1965; Hoyte, 1972), the USA and Canada (Woo *et al.*, 1970; Matthews *et al.*, 1979), Brazil (Melo *et al.*, 2011; Pacheco *et al.*, 2018b), Europe (Magri *et al.*, 2021), and Asia (Suganuma *et al.*, 2022).

Although these infections are typically asymptomatic, clinical symptoms have occasionally been described (Garcia, *et al.*, 2011; Magri *et al.*, 2021). The disease primarily affects immunocompromised animals, characterized by mild leukocytosis

and spleen enlargement, anemia, weight loss, and a substantial drop in milk production, especially when the infection is mixed with the infection by bovine leukemia virus (Amato *et al.*, 2019; Magri *et al.*, 2021).

Life cycle and morphology of ruminant trypanosomes

Trypanosomes are obligatory parasites of the Protozoa subkingdom, Euglenozoa phylum, Kinetoplastea class, and *Trypanosomatida* order (Votýpka *et al.*, 2015; Kaufer *et al.*, 2017; Borges *et al.*, 2020). Typically, the genus *Trypanosoma* belongs to the subfamily Blechomonadinae, which mostly harbors dixenous parasites (Votýpka *et al.*, 2013), and is traditionally divided into two groups based on the replication site inside the invertebrate host: Salivaria and Stercoraria.

In mammalian hosts, trypanosome of the Salivaria multiply at the trypomastigote stage, which retains the kinetoplast in a terminal or subterminal location and has a blunt posterior end. Salivaria consists of four subgenera: *Duttonella, Nannomonas, Pycnomonas,* and *Trypanozoon*, for tsetse flies are the primary vectors (Gibson, 2015; Radwanska *et al.*, 2018).

Trypanosomes belonging to the subgenus *Megatrypanum*, *T. theileri*, are stercorarian. Stercoraria is protozoans that reproduce as epimastigote/amastigote forms in mammalian hosts and exhibit nonreproductive trypomastigote forms in the blood. Typically, Stercoraria is not pathogenic (except for *T. cruzi*). Other subgenera that comprise this category include: *Schizotrypanum* contains *Trypanosoma cruzi* (Sutherland *et al.*, 2015); *Herpetomonas*, which Molyneux (1969) classifies as a subgenus, has *Trypanosoma lewisi* (Desquesnes *et al.*, 2013; Magri *et al.*, 2021).

Trypanosoma theileri reproduces almost exclusively extracellularly in mammalian hosts, namely in circulation, either by the binary fission of epimastigotes

or the multiple fission of plasmodia (Hoare, 1972). Trypomastigotes develop into wide and thin forms after differentiating from epimastigotes. These forms are taken up by the vector hosts, which are *Tabanidae* flies (Hoare, 1972; Böse *et al.*, 1993). The parasites replicate in the form of epimastigotes inside the vector's midgut. To proceed with their growth and differentiation, parasites move from the midgut to the hindgut of their hosts. The infective form for mammalian hosts is the metacyclic trypomastigote, which develops from the epimastigote stage (Hoare, 1972; Dirie *et al.*, 1990; Böse *et al.*, 1993).

Molecular methods and current taxonomy of ruminant trypanosomes

The species-specific PCR approach may identify trypanosomes in samples with a very low parasite level, and as a result, it is often employed for the detection and identification of trypanosomes (Hamilton *et al.*, 2009). Several target genes have been used as molecular markers to characterize ruminant trypanosomes (classically *Megatrypanum* trypanosomes). A few examples of these include the ribosomal RNA gene (rDNA), the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene, the internal transcribed spacer (ITS) gene, the spliced leader RNA (SL) gene, and the cathepsin L-like cysteine protease (CatL-like) genes (Rodrigues *et al.*, 2006, 2010a, 2010b; Garcia, *et al.*, 2011a, 2011b, 2020; Martinković *et al.*, 2012; Yokoyama *et al.*, 2015; Pacheco *et al.*, 2018). In the process of identifying trypanosomes and doing phylogenetic research, the SSU rDNA gene and the gGAPDH gene are often used. Highly expressed multicopy SSU rDNA genes have both a conserved section that is appropriate for universal primers and a variable region that is appropriate for supplying taxonomic information (Vickerman, 1994; Votýpka *et al.*, 2015; Hutchinson and Stevens, 2018; Mafie *et al.*, 2019; Sato and Mafie, 2022). gGAPDH is employed as a

supplementary genetic marker (Hamilton *et al.*, 2004; Hutchinson and Stevens, 2018; Sato and Mafie, 2022).

In phylogenies constructed by utilizing SSU rDNA and gGAPDH genes, *T. theileri* from cattle clustered along with *T. theileri*-like trypanosomes from water buffalo in Brazil, deer in Europe and Asia, and antelope in Africa. This formed the *T. theileri* clade, TthI, and TthII. This clade was located at a location that was very distinct from species that infected orders other than Artiodactyla (Stevens *et al.*, 1999; Rodrigues *et al.*, 2006; Hamilton *et al.*, 2007, 2009; Hatama *et al.*, 2007).

Phylogenies of ruminant *T. theileri*-like trypanosomes based on SSU and ITS1 rDNA sequences showed 5 lineages: (A) Brazilian water buffalo genotypes; (B) and (C) two divergent Brazilian cattle genotypes; and (D) and (E) European cattle and deer genotypes (Rodrigues *et al.*, 2006). Recently, African *T. theileri* trypanosomes from "antelopes" were compared using V7-V8 rDNA, and one isolate from Sitatunga was given a new lineage designation of F, whilst isolates from Duikers were given lineage designations of (C) or (E) despite also having distinct genotypes (Hamilton *et al.*, 2009). Host restriction experimentally demonstrated for trypanosomes infecting cattle, sheep, and goats, served as the basis for the description of *T. theileri*-related species (Hoare, 1972; Rodrigues, *et al.*, 2010).

***Trypanosoma theileri*-like in cervids**

There have also been reports of *T. theileri*-like species in wild ruminants including roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), red deer (*Cervus elaphus)*, and sika deer (*Cervus nippon*) which are morphologically closed to *T. theileri* (Böse *et al.*, 1987a; Dirie *et al.*, 1990; Hatama *et al.*, 2007; Magri *et al.*, 2021).

Additionally, *T. theileri*-like strains were found by molecular biology in vectors (i.e., tabanid flies) in Russia (Ganyukova *et al.*, 2018) and Poland (Werszko *et al.*, 2020b).

T. theileri-like trypanosomes from cattle, water buffalo, antelopes (duiker and sitatunga), and deer represent a monophyletic group of trypanosomes specific to ruminant hosts over a wide geographical range in Europe, South America, Asia, and Africa, according to current phylogenies (Rodrigues *et al.*, 2006; Hatama *et al.*, 2007). However, limited sample sizes and the fact that the majority of available data are restricted to SSU rDNA sequences, which have proven to be too conserved to distinguish closely related species of trypansonomes, have hampered the evaluation of their true genetic diversity and host-associated genotypes (Rodrigues *et al.*, 2006; Hatama *et al.*, 2007; Hamilton *et al.*, 2009). In this study, we used various gene markers to identify genetic diversity of *T. theileri* present in Japanese cervids.

Context of the study

Hemoparasites of the order Haemosporida and Trypanosomatidae are responsible for major animal diseases (for example, malaria infection in animals can result in a decline in poultry populations). Certain species also play an important role in regulating wildlife populations. The impact of hemoparasites is considered important in the extinction of several animal species. Recently, the avian malaria parasite was suspected to be the cause of the widespread decline in domestic and wild animals. This avian hemosporidians are phylogenetically closely related to bat hemosporidians. Meanwhile, in ruminants, the main focus of hemoparasitic disease is *Trypanosoma*. The existence of a close relationship between *Trypanosoma* in domestic and wild ruminant animals causes the possibility of a pattern of disease spread from wild to domestic animals.

The evolution, taxonomy, and biogeographical speciation of avian and bat hemosporidian and ruminant trypanosomes are currently unknown (Vickerman, 1994; Stevens *et al.*, 1999; Stevens *et al.*, 2001; Hamilton, *et al.*, 2012a; Hamilton, *et al.*, 2012b; Votýpka *et al.*, 2015), necessitating more collection of samples from around the world for genetic characterization and phylogenetic analysis. In this study, an avian hemosporidian isolated from domestic poultry, a bat hemosporidian isolated from a temperate region in Asia, and trypanosomes from domestic and wild ruminants were morphologically and molecular-genetically characterized to contribute to the discussion of hemoparasites evolution, taxonomy, diagnosis, and host effects.

Study Objectives

The main objective of my study was to employ morphological and molecular characteristics to clarify the taxonomy, diversity, and phylogenetic relationships of hemosporidians (Apicomplexa: Haemosporidia) in chiropteran and avian host especially the impact on the health status in domestic Avian, and trypanosomes (Kinetoplastea: Trypanosomatidae) in ruminant hosts. The specific objectives were (i) to determine the prevalent species, natural hosts, and their geographical distribution, (ii) to determine their genotypic diversity, (iii) to determine their phylogenetic relationships (iv) to describe their impact on farmed domestic animal health.

CHAPTER I

Impact of subclinical *Haemoproteus columbae* infection on farmed domestic pigeons from Central Java (Yogyakarta), Indonesia, with special reference to changes in the hemogram

Information described in this chapter has been published as follows:

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Abstract

Pigeon haemoproteosis caused by *Haemoproteus columbae* (Apicomplexa: Haemosporida: Haemoproteidae) is globally prevalent in rock doves (*Columba livia*), although little is known regarding this disease in pigeons and doves in Indonesia. Blood samples of 35 farmed domestic pigeons (*C. livia* f. *domestica*) from four localities in Yogyakarta Special Region, Central Java, Indonesia, were collected from March to June, 2016, subjected to a hemogram, and analyzed for the presence of hemoprotozoan infections. Microscopic examination of blood smears revealed a prevalence of 62.5–100% of *H. columbae* at the four localities (n = 8–10 for each locality), and geometric means of 3.0–5.6% of erythrocytes were parasitized by young and mature gametocytes, suggesting that all infected pigeons were in the chronic phase of infection with repeated recurrences and/or reinfections. Nucleotide sequencing of mitochondrial cytochrome *b* gene (*cytb*) for hemosporidian species demonstrated the distribution of four major *cytb* lineages of *H. columbae* (mainly HAECOL1, accompanied by COLIV03, COQUI05, and CXNEA02 according to the Mal-Avi database). Hemogram analysis, involving the estimation of packed cell volume, erythrocyte counts, mean corpuscular volume, mean corpuscular hemoglobin concentration, and plasma protein and fibrinogen levels of 20 parasitized pigeons and five non-infected pigeons, demonstrated significant macrocytic hypochromic anemia with hypoproteinemia and hyperfibrinogenemia in the infected pigeons. This study shows the profound impact of long-lasting subclinical pigeon haemoproteosis caused by *H. columbae* on the health of farmed domestic pigeons.

Introduction

Avian hemosporidian infection is caused mainly by the genera *Plasmodium* Marchiafava et Celli, 1885, *Haemoproteus* Kruse, 1890, or *Leucocytozoon* Sambon,

1908 (Apicomplexa: Haemosporidia). There are more than 200 nominal hemosporidian species and presumably thousands of undescribed cryptic species, which are differentiated genetically but often exhibit convergent morphology with other known taxa (Bensch *et al.*, 2004, 2009; Valkiūnas, 2005; Martinsen *et al.*, 2008) . In rock doves (*Columba livia* Gmelin, 1789), including domestic pigeons (*C. livia* f. *domestica* Gmelin, 1789), *Haemoproteus columbae* Kruse, 1890 is globally the representative cause of the hemosporidian infection (Bennett and Peirce, 1990; Valkiūnas, 2005; Atkinson, 2009). In addition to this species, at least five other *Haemoproteus* spp. infect pigeons and doves (Columbiformes: Columbidae), are transmitted by hippoboscid flies, and are classified in the subgenus *Haemoproteus* Kruse, 1890. In contrast, a large variety of birds from other families are infected by more than 126 different *Haemoproteus* spp. that are transmitted by biting midges (Ceratopogonidae) and are classified in the subgenus *Parahaemoproteus* Bennett, Garnham and Fallis, 1965 (Bennett *et al.*, 1965; Valkiūnas, 2005). *Haemoproteus* spp. of these two subgenera are transmitted by different families of vectors, are phylogenetically distinct, and form separate clades (Valkiūnas, 2005; Martinsen *et al.*, 2008; Levin *et al.*, 2011; Križanauskienė *et al.*, 2013; Dimitrov *et al.*, 2014; González *et al.*, 2015; Nourani *et al.*, 2018; Harl *et al.*, 2019).

Pigeon haemoproteosis caused by *H.* (*Haemoproteus*) *columbae* is transmitted by the bite of the pigeon louse fly *Pseudolynchia canariensis* (Macquart, 1839). It has a prepatent period of 22–38 days (ca. 30 days in average), wherein repeated merogony occurs in the lungs (Aragão, 1955; Ahmed and Mohammed, 1977, 1978; Valkiūnas, 2005). Merozoites released from the meront invade erythrocytes and develop into gametocytes. Pathogenicity of natural *H. columbae* infections is conventionally believed to be virtually minimal and of no veterinary importance (Atkinson and Van

Riper, 1991; Valkiūnas, 2005; Atkinson, 2009). However, in the accidental non-columbid hosts, such as captive naive birds translocated from non-endemic areas to zoos and aviaries in the endemic areas, clinical and moribund *Haemoproteus* (*Parahaemoproteus*) infections have been often reported (Ahmed and Mohammed, 1977; Valkiūnas, 2005; Atkinson, 2009). In cases of accidental infection, severe haemoproteosis and death of infected birds have been found to be closely related to the pre-erythrocytic stage of parasites that occurs before the development of parasitemia. This illness is caused by the rupture of megalomeronts in the muscle and liver, and subsequent pathogenic changes (Atkinson and Van Riper, 1991; Ferrell *et al.*, 2007; Donovan *et al.*, 2008; Olias *et al.*, 2011; Valkiūnas, 2011; Cannell *et al.*, 2013). In other words, virulence of *Haemoproteus* infection is seen in "abortive" infections. Thence, pathogenic mechanisms underlying accidental fatal or moribund cases caused by members of the subgenus *Parahaemoproteus* can be extended to explain the potential pathogenicity of *H.* (*Haemoproteus*) *columbae* infection in the natural host, although the latter species does not have megalomeronts in its life-cycle (Ilgūnas *et al.*, 2019). Recently, however, Nebel et al. (2020) demonstrated a negative relationship between the level of *H. columbae* parasitemia and body mass in feral domestic pigeons in urban areas of Cape Town; a 1% increase in the number of infected erythrocytes was correlated with a 5.42 g reduction in body mass.

In the present study, we examined the prevalence and levels of parasitemia of hemosporidian infection in farmed domestic pigeons that are bred for racing and meat, generally called "squab" (Bolla, 2007; Darwati *et al.*, 2010), at four localities in Yogyakarta Special Region, Central Java, Indonesia. Hemosporidians detected in the blood were genetically characterized based on mitochondrial DNA sequences of partial cytochrome *b* gene (*cytb*), as recommended by Bensch et al. (2009). To assess the

impact of natural hemosporidian infection on the health of the pigeons, analyses of several hemogram parameters were performed.

Materials and methods

Blood collection and microscopic examination

Thirty-five healthy-looking domestic pigeons were purchased between March and June 2016 from local breeders at four localities in Yogyakarta Special Region, namely, Mlati (n = 10), Ngemplak (n = 9), Kalasan (n = 8), and Sedayu (n = 8), with at least 11– 18 km distance between different localities. Pigeons were 6 to 18 months old; however, information such as sex and body weight were not recorded. Approximately 0.5–1.0 mL of blood was individually collected from the brachial vein using clean syringes with fine needles, and immediately transferred to 3-mL BD VacutainerTM K3EDTA glass tubes (Fisher Scientific, Arendalsvägen, Göteborg, Sweden). Two to three thin blood films were prepared on clean glass slides for each blood sample, air-dried, and fixed in absolute methanol for 10 min. Blood films were stained with Giemsa's solution (Sigma-Aldrich, St. Louis, MS, USA). Several drops of blood from 14 arbitrarily selected pigeons (Mlati (n = 4), Ngemplak (n = 5), Kalasan (n = 3), and Sedayu (n = 2)) were placed on a circle of WhatmanTM FTA^{TM} Classic Card (GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK). The residual amount of blood was used for hematological analyses, which are mentioned below. After blood collection, all pigeons were released. All animal experiments were performed according to the Guidelines on Animal Experimentation as set out by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Gadjah Mada University.

Microscopic observation of blood films was first conducted at ×400 magnification, and then at ×1000 with oil immersion using an Olympus BX60 light microscope equipped with a DP72 digital camera (Olympus, Nishi-Shinjuku, Tokyo, Japan). The level of parasitemia was determined by counting the number of parasitized cells per 1000 erythrocytes, according to Godfrey et al. (Godfrey *et al.*, 1987). Measurements of parasites and their identification were conducted according to Valkiūnas (2005). Representative blood films collected in the present work were deposited in the National Museum of Nature and Science, Tokyo, Japan.

Hematological analyses

Hematological analyses were manually conducted in the Department of Clinical Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, referring to Benjamin (1985) and Coles (1986) with slight modifications. Parameters examined included number of erythrocytes, number of leukocytes, PCV, MCV, MCH, MCHC, plasma protein, and fibrinogen per mm^3.

Three microhematocrit tubes, coated by heparin, were filled with pigeon blood in the BD VacutainerTM K3EDTA glass tubes (Fisher Scientific, Arendalsvägen, Göteborg, Sweden), and centrifuged at 3500 rpm for 10 min. PCV value were determined using a microhematocrit tube reader. Plasma fraction of one tube was individually dispensed onto the prism of the Goldberg TS Meter refractometer (Reichert Technologies, Depew, NY, USA) to obtain total plasma protein concentration. Fibrinogen concentration was determined by the heat precipitation method. Briefly, the second tube was incubated in the 58 °C water bath for 3 min to precipitate the fibrinogen as a white ring around the bottom of plasma fraction. These microhematocrit tubes were recentrifuged, and plasma fraction was measured by the method described above to

obtain plasma protein concentration without fibrinogen. Fibrinogen concentration was calculated as the difference of plasma protein concentrations of the first and second microhematocrit tubes. Numbers of erythrocytes and leucocytes were determined by the hemocytometer method using phosphate-buffered saline and Turk's solution. Hemoglobin concentration was determined by the colorimetric method using Drabkin's reagent (Sigma-Aldrich, St. Louis, MS, USA) following manufacturer' instruction. MCV, MCH, and MCHC were calculated using the standard formulae.

DNA extraction, amplification and sequencing

As mentioned above, blood from 14 arbitrarily selected pigeons (Mlati (n = 4), Ngemplak (n = 5), Kalasan (n = 3), and Sedayu (n = 2)) were used for DNA extraction. Using a 2-mm Harris Uni-Core puncher (Whatman®; GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK), four punches from each sample circle of Whatman™ FTA™ Classic Card were placed in an Eppendorf tube. The DNA of each sample was extracted from these four punches using an IllustraTM tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK) according to the instructions of the manufacturer. PCR amplification of mitochondrial *cytb* DNA fragments was performed in a 20-μL PCR solution containing DNA polymerase packed in Blend Taq-Plus- (TOYOBO, Dojima Hama, Osaka, Japan), a primer pair of the forward HaemNF1 (5′-CAT ATA TTA AGA GAA NTA TGG AG-3′) and the reverse HaemNR3 (5′-ATA GAA AGA TAA GAA ATA CCA TTC-3′) for hemosporidians of genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Hellgren *et al.*, 2004), and one μL of template DNA. Another primer pair of the forward HaemF (5′-ATG GTG CTT TCG ATA TAT GCA TG-3′) and the reverse HaemR2 (5′-GCA TTA TCT GGA TGT GAT AAT GGT-3′), usually used as a second-round primer pair

of nested PCR using products of the aforementioned PCR (Hellgren *et al.*, 2004), was independently performed (Bensch *et al.*, 2000). The following PCR cycling protocol was used: 3 min at 94 ˚C followed by 35 cycles of 30 s at 94 ˚C, 30 s at 50 ˚C, and 60 s at 72 ˚C; this was followed by a final extension at 72 ˚C for 7 min. The PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequenced directly from both ends with the original primers. When direct sequencing was not satisfactory, the purified PCR products were cloned into the plasmid vector pTA2 (TArget CloneTM; TOYOBO, Dojima Hama, Osaka, Japan) and transformed into *Escherichia coli* JM109 cells (TOYOBO, Dojima Hama, Osaka, Japan) according to the instructions of the manufacturer. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co., Ltd., Bunkyoku, Tokyo, Japan) and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers according to the instructions of the manufacturer (TOYOBO, Dojima Hama, Osaka, Japan). The nucleotide sequences obtained in the present study are available from the DDBJ/EMBL/GenBank databases under the accession nos. LC605998–LC606013.

Phylogenetic analysis

Fragments of the newly obtained *cytb* sequences (571-bp or 478-bp) were analyzed to identify highly similar nucleotide sequences using the BLAST of the National Center for Biotechnology Information (NCBI) website. The lineage names were identified by perform ing BLAST searches in the Mal-Avi database (http://130.235.244.92/Malav i/; accessed on 26 January 2021) (Bensch *et al.*, 2009). For phylogenetic analysis, the newly obtained *cytb* sequences and closely related sequences of *Haemoproteus* (*Haemoproteus*) spp. retrieved from the

DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson *et al.*, 1994), with subsequent manual adjustments. Trimmed sequences of 465 characters, of which 99 were variable, were subjected to subsequent analysis. Maximum likelihood 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/; accessed on 26 January 2021). The probability of inferred branching was assessed by the approximate likelihood-ratio test, an alternative to the nonparametric bootstrap estimation of branch support (Anisimova and Gascuel, 2006).

Statistical analysis

Hemogram data obtained in the present study are expressed as a range with its mean or mean ± standard deviation in parentheses. Hemogram comparison between uninfected (n = 5) and infected pigeons (n = 20) were statistically analyzed using one-way analysis of variance, ANOVA (StatView ver. 5; Abacus Concepts Inc., Berkeley, CA, USA). Correlations between values of infection intensities and hemogram parameters were analyzed via regression analysis using the same statistical package. A p-value less than 0.05 was considered statistically significant.

Results

Prevalence and morphology of hemosporidian species

Upon microscopic examination, 85.7% (30/35) of blood samples from domestic pigeons exhibited the presence of *Haemoproteus* sp. The geometric mean of infection intensity (parasitemia levels) was higher at Mlati (3.9% (n = 10)) and Ngemplak (5.6% (n = 9), wherein all pigeons were infected with the hemosporidian species, and

comparatively lower at Kalasan (3.9% (n = 6)) and Sedayu (3.0% (n = 5)), wherein two and three pigeons were uninfected, respectively (Table 1). We identified the species as *H. columbae* (Table 2 and Figure 4), based on the morphological features of the gametocytes (Valkiūnas, 2005), and we did not detect any other hemosporidian species. Mature gametocytes, which were sausage-shaped with blunt ends, were observed to displace the nucleus of host erythrocytes laterally. Parasitized erythrocytes were enlarged (Table 2), but not evidently deformed (Figure 4). Dimensions of non-parasitized erythrocytes in pigeons infected by *H. columbae* were significantly larger than those of uninfected pigeons (Table 2). Macrogametocytes had a darker cytoplasm with randomly dispersed pigment granules than microgametocytes which had pigment granules with a polarized distribution. Nuclei were located in the central one-third area of gametocytes. In most cases, immature gametocytes were more frequently observed than mature gametocytes (Table 1). Usually, a single erythrocyte contained one gametocyte, but occasionally they also had two gametocytes.

Molecular characterization of *cytb* sequences, and phylogenetic analyses

Sixteen *cytb* nucleotide sequences from 14 pigeons were successfully obtained—seven 571 bp-long sequences and nine 478 bp-long sequences with six polymorphic sites. Basic Local Alignment Search Tool (BLAST) search confirmed that all sequences were partial *cytb* sequences of *H. columbae*. Consequently, four lineages (HAECOL1, COLIV03, COQUI05, and CXNEA02 according to Mal-Avi database (Bensch *et al.*, 2009) were differentiated (Table 3), wherein there was one dominant lineage (HAECOL1; 11 sequences), and three other lineages (one or two sequences/lineage). All these lineages were translated into an identical amino acid sequence. Mixed infection of *H. columbae* of two lineages was found in the blood

samples of two pigeons. A phylogenetic tree of *Haemoproteus* (*Haemoproteus*) spp. that infect pigeons and doves of Columbidae was constructed based on the *cytb* sequences (Figure 6).

Hematological analyses

Twenty-five blood samples from 20 *H. columbae*-infected pigeons and five uninfected pigeons were subjected to a hemogram test (Table 4). Upon comparison of the infected and uninfected groups, statistically significant reductions were found in erythrocyte numbers, packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin concentration, and plasma protein concentration/mm^3, whereas statistically significant increases were found in leukocyte numbers, mean corpuscular volume (MCV), and fibrinogen concentration/mm^3. Increases in the leukocyte numbers were largely ascribed to increased numbers of monocytes. This hemogram profile suggests that *H. columbae* infection causes a statistically significant degree of macrocytic hypochromic anemia with hypoproteinemia and inflammation.

Relationships between parasitemia level and degree of anemia (number of erythrocytes/mm^3 and MCHC) are shown in Figure 5. There was a statistically significant linear negative relationship between parasitemia level and number of erythrocytes (1% increase in number of infected erythrocytes led to a 6×10^4 decrease in erythrocytes/mm^3; $R^2 = 0.297$) or MCHC (1% increase in number of infected erythrocytes lead to a 0.49 g/dL decrease in MCHC; $R^2 = 0.285$).

Discussion

In this study, we demonstrated a high prevalence (85.7%) of *H. columbae* infection in farmed domestic pigeons from Yogyakarta, Central Java; this is comparable to previous studies that have reported a *H. columbae* prevalence of 72.7% (24/33) or 96.9% (186/192) in feral domestic pigeons in Cape Town, South Africa (Earle and Little, 1993; Nebel *et al.*, 2020), a prevalence of 82% (41/50) in the island of Tenerife, Canary Archipelago, Spain (Foronda *et al.*, 2004), a prevalence of 57.3% (59/103) in Qena, Egypt (Hussein and Abdelrahim, 2016), and a prevalence of 100% (20/20) in São Paulo, Brazil (Chagas *et al.*, 2016).

Based on the high levels of prevalence and mean levels of parasitemia ranging from 3.0% to 5.6% of erythrocytes in the examined pigeons in this study and the temporal changes seen in parasitemia in domestic pigeons experimentally infected with *H. columbae* (Ahmed and Mohammed, 1978; Cepeda *et al.*, 2019), it is likely that all or a majority of infected pigeons examined in our study might be in the long-lasting chronic phase of *H. columbae* parasitemia with evident recurrences or continued reinfections, which were demonstrated by erythrocytes actively parasitized not only with mature gametocytes, but also young gametocytes, as observed in the blood films (Table 1; Figure 4). In immunocompetent natural vertebrate hosts for *H. columbae*, i.e., *C. livia*, pre-erythrocytic growth or merogony occurs mainly in the lungs (the prepatent period ranging between 22 and 37 days), followed by gametogony in the erythrocytes after the cell invasion of merozoites (the acute phase ranging between nine and 20 days), and is ended by crisis (Aragão, 1955; Cepeda *et al.*, 2019). Primary as well as recurrent infection induces a partial immunity to *H. columbae* reinfection, i.e., incomplete premunition which allows for superinfection (Coatney, 1933; Ahmed and Mohammed, 1978; Valkiūnas, 2005; Cepeda *et al.*, 2019), resulting in the long-lasting

parasitemia with immature and mature gametocytes, as seen in this study. Here, the parasitemia levels (1.1–25.7% (geometric mean, 6.1%)) with high degrees of coexistence between immature and mature gametocytes (0.47–15.0 (geometric mean, 2.4):1, respectively) in the blood films of all 30 infected farmed pigeons suggested the existence of growing exoerythrocytic forms, such as merozoites, that might be continuously released from meronts in the lungs of pigeons bitten by pigeon louse flies infected with *H. columbae*.

As described above, in the natural avian hosts for *Haemoproteus* spp., the pathogenicity of the hemosporidians is conventionally believed to be virtually minimal, especially when compared with the pathogenicity of the genera *Plasmodium* and *Leucocytozoon* in avian hosts (Atkinson and Van Riper, 1991; Valkiūnas, 2005; Atkinson, 2009; Cepeda *et al.*, 2019). In accidental hosts, such as captive birds in zoos and aviaries; however, clinical and fatal *Haemoproteus* infections have been reported (Atkinson *et al.*, 1988; Ferrell *et al.*, 2007; Atkinson, 2009; Olias *et al.*, 2011; Valkiūnas, 2011; Cannell *et al.*, 2013; Chagas *et al.*, 2016; Lee-Cruz *et al.*, 2016). In such accidental hosts, severe haemoproteosis and death in infected birds have been reported mostly in the prepatent period due to the rupture of megalomeronts in muscle and liver and subsequent pathogenic changes (Atkinson, 2009).

Some evidence has shown that natural haemoproteosis can exert an important selective pressure on the survival, reproductive success, behavior, and community structure of the hosts, as evidenced by studies that involve the experimental interruption of natural *Haemoproteus* infection and analysis of its consequences (Sorci and Møller, 1997; Merino *et al.*, 2000; Marzal *et al.*, 2005; Valkiūnas *et al.*, 2006; Møller and Nielsen, 2007). Recently, Nebel *et al.* (2020) demonstrated a negative relationship between *H. columbae* parasitemia level and body mass in feral domestic pigeons in

urban areas of Cape Town. In this study, we demonstrated subclinical but substantial symptoms such as macrocytic hypochromic anemia and hypoproteinemia in *H. columbae*-infected pigeons, and we speculate that this could cause body mass reduction and other negative health conditions. The demonstration of these substantial negative effects on the health of domestic pigeons triggered by the latent infection by *H. columbae* is important as domestic pigeons are used as racing birds or for meat production. Cooked meats of farmed pigeons, such as "squab", is a delicious, high-quality product that is consumed across many cultures or countries, such as European countries, U.S.A., Egypt, and Asian countries, including Indonesia (Jeffrey *et al.*, 2001; Bolla, 2007; Darwati *et al.*, 2010; Hussein and Abdelrahim, 2016). Although negative correlation between parasitemia level and development of anemia was found to be statistically significant, the values of the coefficient determination was fairly low, less than 0.3 (Figure 5). As speculated above, the pigeons sampled in this study were chronically infected ones with reinfections. Therefore, a point parameter, i.e., levels of parasitemia, might not be the best in reflecting the actual state of infection. We suppose that an experimental observation on pigeons regularly exposed to *H. columbae*-carrying *P. canariensis* would clearly show the negative impact of natural *H. columbae* infection in pigeons and doves, in contrast to experimental *H. columbae* infections, wherein a single exposure to infected pigeon louse flies was used (Garvin *et al.*, 2003).

The Mal-Avi database is a unified taxonomic database for avian hemosporidian genera of *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* (Bensch *et al.*, 2009), and the epidemiological or ecological significance of genetic lineages of isolated hemosporidian species can be understood with ease. The core sequence lengths of *cytb*, 478-bp for *Plasmodium* and *Haemoproteus* spp. and 479 bp-long for *Leucocytozoon* spp., cover almost all taxonomically important variations. For example, mitochondrial

cytb lineages of parasites differing by as few as one nucleotide frequently indicate distinctly different areas of transmission and range of host species (Waldenström *et al.*, 2002; Beadell *et al.*, 2004; Reullier *et al.*, 2006), although whether hemosporidians showing such closely similar *cytb* lineages represent independent species require detailed analyses of multiple genetic markers as well as other biological characteristics (Bensch *et al.*, 2009). In the present study, we detected four *cytb* lineages (HAECOL1, COLIV03, COQUI05, and CXNEA02 according to the Mal-Avi database for *cytb* lineages) that are prevalent *H. columbae* lineages recorded worldwide, such as in Botswana, Cameroon, Nigeria, South Africa, Brazil, Colombia, and Italy (Waldenström *et al.*, 2002; Karamba K., 2012; González *et al.*, 2015; Scaglione *et al.*, 2015; Chagas *et al.*, 2016; Nebel *et al.*, 2020).

The ecology and biting behavior of hippoboscid flies is substantially different from those of biting midges and might explain the highest host specificity of *H. columbae* to pigeons and doves. Adult stages of pigeon louse flies are virtually flightless and crawl on the host body surface (Bennett *et al.*, 1965). Detecting only *H. columbae* in farmed domestic pigeons in Yogyakarta, Central Java, suggests feasible control of the infection by vector control and better housing. This preventative measure has advantages when considering the findings of the present study that *H. columbae* infection causes subclinical but substantial hypochromic anemia and hypoproteinemia.

Conclusions

Pigeon haemoproteosis caused by *H.* (*H.*) *columbae* is prevalent mostly in the tropics and subtropics, and the pathogenicity of natural *H. columbae* infection is conventionally believed to be virtually minimal and of no veterinary importance. However, we observed that farmed domestic pigeons in Yogyakarta, Central Java,

Indonesia has an 85.7% (30/35) prevalence and a high level of parasitemia, wherein 3.0–5.6% erythrocytes were parasitized with immature and mature gametocytes, indicating a long-lasting subclinical infection with incomplete premunition allowing reinfection. Careful hemogram examination of pigeons with/without *H. columbae* parasitemia suggested that *H. columbae* infection might cause a statistically significant degree of macrocytic hypochromic anemia with hypoproteinemia and inflammation.

Table 1. Farmed domestic pigeons examined with detected hemosporidians.

Locality	Number of pigeons examined	Prevalence of hemosporidian	Detected hemosporidian	Parasitemia (%) [1]	Ratio of immature gametocytes: mature gametocytes
Mlati, Yogyakarta (7˚43′53″S, 110˚19′52″E)	10	100% (10/10)	*Haemoproteus columbae*	1.1–18.9 (3.9)	0.67–4.39 (1.61): 1
Ngemplak, Yogyakarta (7˚41′57″S, 110˚26′42″E)	9	100% (9/9)	*Haemoproteus columbae*	1.6–25.7 (5.6)	0.80–15.00 (3.35): 1
Kalasan, Yogyakarta (7˚45′18″S, 110˚29′06″)	8	75.0% (6/8)	*Haemoproteus columbae*	2.5–6.7 (3.9)	2.15–11.75 (4.10): 1
Sedayu, Yogyakarta (7˚48′49″S, 110˚16′17″)	8	62.5% (5/8)	*Haemoproteus columbae*	2.1–7.1 (3.0)	0.47–15.00 (1.47): 1

[1] Percentage of parasitized cells by counting 1000 erythrocytes. Values are expressed by range with geometric mean in parentheses

Table 2. Morphometric values of erythrocytes and *Haemoproteus columbae* gametocytes in infected pigeons (expressed in μm).

Feature	The present study (*n* = 30)	Valkiūnas (2005) [1] (*n* = 31)
Uninfected erythrocyte [2]		
Length	12.3–14.9 (13.4)	12.8–14.7 (13.7)
Width	6.6–7.7 (7.0)	6.4–7.7 (7.0)
Length of nucleus	6.1–7.7 (6.7)	6.2–7.7 (6.7)
Width of nucleus	2.3–3.4 (2.8)	2.1–2.9 (2.4)
Infected erythrocyte with a mature microgametocyte		
Length	12.2–16.6 (14.1)	12.9–15.9 (14.4)
Width	6.1–7.8 (7.0)	5.3–7.8 (6.9)
Length of nucleus	5.5–7.4 (6.4)	5.5–7.4 (6.5)
Width of nucleus	2.3–3.5 (2.7)	2.1–2.6 (2.3)
Infected erythrocyte with a mature macrogametocyte		
Length	13.0–15.9 (14.5)	13.8–16.0 (15.0)
Width	6.2–7.9 (7.2)	6.0–7.9 (7.1)
Length of nucleus	4.9–7.5 (6.3)	6.0–7.4 (6.5)
Width of nucleus	2.2–2.9 (2.6)	1.7–2.7 (2.3)
Mature microgametocyte		
Length	10.5–16.6 (13.7)	11.6–15.5 (13.3)
Width	2.7–5.0 (3.6)	2.6–4.3 (3.6)
Length of nucleus	— [3]	—
Width of nucleus	2.6–4.5 (3.1)	2.6–4.3 (3.6)
Mature macrogametocyte		
Length	12.4–16.4 (14.6)	13.4–16.7 (14.8)
Width	2.5–4.2 (3.5)	3.0–4.2 (3.4)
Length of nucleus	2.1–3.2 (2.6)	2.1–3.6 (2.9)
Width of nucleus	1.5–3.2 (2.3)	1.5–3.4 (2.3)

[1] According to Valkiūnas and Iezhova (Valkiūnas and Iezhova, 1990). [2] Measurements of 10 erythrocytes each from five uninfected pigeons were as follows ($n = 50$ in total): Length, 9.5–12.2 (11.4); width, 4.6–7.8 (6.2); length of nucleus, 4.13–6.7 (5.6); and width of nucleus, 1.9–3.7 (2.7). Differences in all parameters of dimensions of uninfected erythrocytes, except for width of nucleus, between uninfected and *H. columbae*-infected pigeons were statistically significant ($p < 0.05$). [3] Difficult to measure due to unclear borders.

Table 3. Detected *cytb* lineages of *Haemoproteus columbae* in farmed pigeons in Central Java.

Locality	Number of pigeons examined molecular–genetically	Detected *cytb* lineages of *H. columbae* (Number of pigeons)	DDBJ/EMBL/GenBank Accession No.
Mlati, Yogyakarta	4	HAECOL1 (3) COLIV03 (1)	LC605998–LC606001
Ngemplak, Yogyakarta	5	HAECOL1 (5) CXNEA02 (2) [1]	LC606002–LC606008
Kalasan, Yogyakarta	3	HAECOL1 (2) COLIV03 (1)	LC606009–LC606011
Sedayu, Yogyakarta	2	HAECOL1 (1) COQUI05 (1)	LC606012–LC606013

[1] Two pigeons were co-infected with two lineages (HAECOL1 and CXNEA02) of *H. columbae*.

Table 4. Hemograms of pigeons infected and uninfected with *Haemoproteus columbae* gametocytes.

Parameter	Unit	Infected pigeons (*n* = 20)	Uninfected pigeons (*n* = 5)	Normal range reported by Ihedioha et al. (2016) [1]	Change [2]	Statistical significance
Number of erythrocytes	10^6/mm^3	2.10–3.15 (2.49 ± 0.29)	3.25–4.60 (3.93 ± 0.55)	2.12–3.95 (3.34 ± 0.38)	↓	$p < 0.001$
Number of leukocytes	10^3/mm^3	11.95–12.85 (12.44 ± 0.34)	8.15–11.20 (9.61 ± 1.22)	12.50–35.50 (23.36 ± 7.06)	↑	$p < 0.007$
PCV	%	34–50 (39.8 ± 4.6)	45–48 (46.2 ± 1.6)	32–55 (44.5 ± 4.7)	↓	$p < 0.001$
MCV	fl	134.10–218.60 (161.04 ± 18.44)	97.83–138.46 (119.24 ± 15.34)	109.82–169.09 (133.86 ± 19.37)	↑	$p < 0.001$
Hemoglobin	g/dl	4.5–10.9 (6.30 ± 1.28)	11.5–17.2 (13.54 ± 2.26)	7.76–16.00 (12.89 ± 1.55)	↓	$p < 0.001$
MCH	pg	18.60–35.16 (25.39 ± 4.11)	26.30–41.45 (34.79 ± 5.95)	n.d.[3]	↓	$p < 0.001$
MCHC	g/dl	10.00–21.80 (15.90 ± 2.72)	25.56–35.83 (29.22 ± 3.97)	23.57–33.75 (28.97 ± 2.59)	↓	$p < 0.001$
Plasma protein	g/dl	1.0–4.0 (2.4 ± 1.0)	4.1–5.2 (4.7 ± 0.5)	n.d.[3]	↓	$p < 0.001$
Fibrinogen	g/dl	0.2–1.0 (0.8 ± 0.3)	0.1–0.4 (0.2 ± 0.1)	n.d.[3]	↑	$p < 0.001$

[1] Values were calculated based on 64 adult domestic pigeons of both sexes. [2] Status of infected pigeons, compared with that of uninfected pigeons. [3] No data.

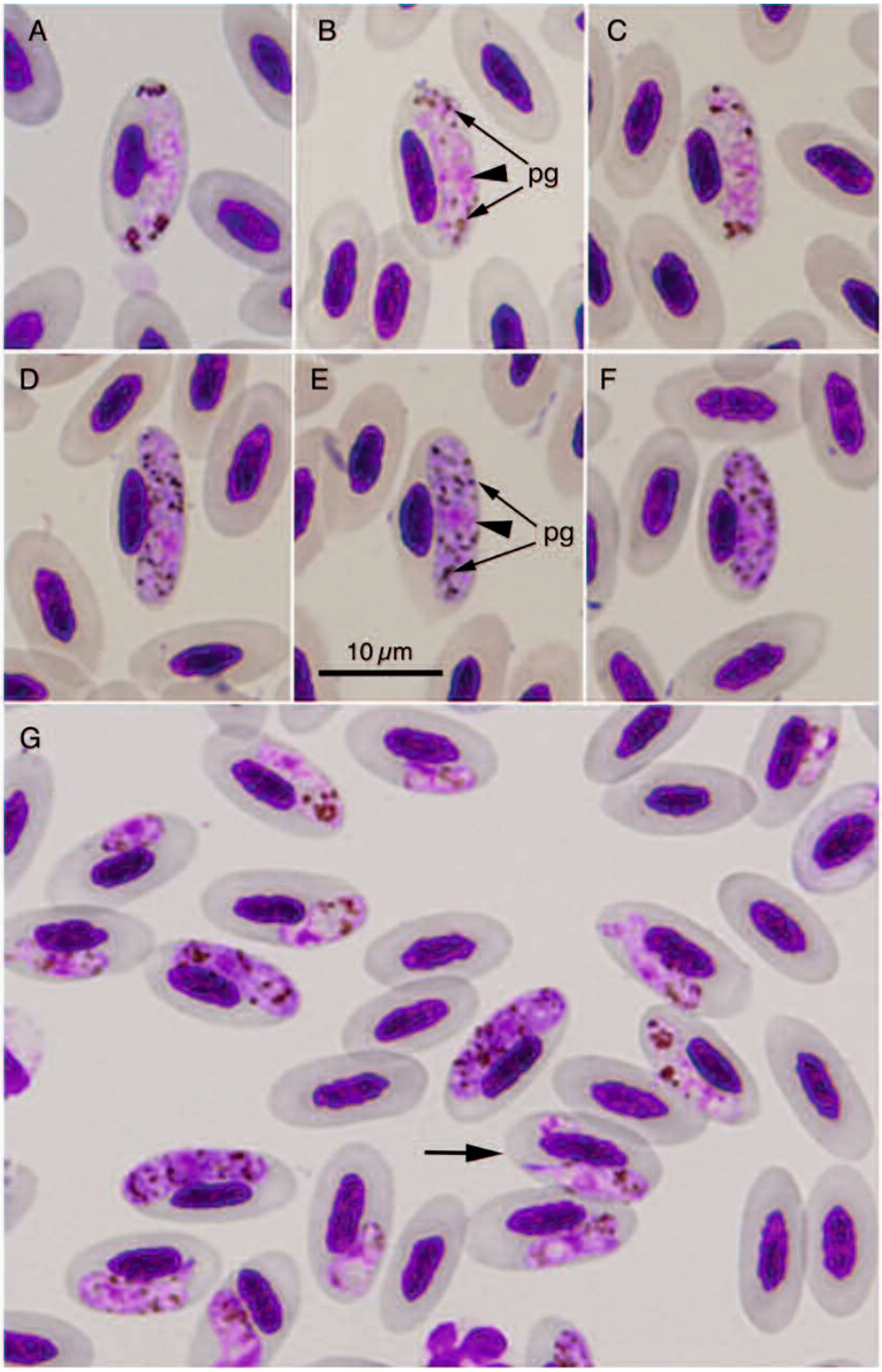

Figure 4. *Haemoproteus columbae* gametocytes in the blood of farmed domestic pigeons (*Columba livia* f. *domestica*) from Yogyakarta Special Region, Indonesia. Mature microgametocytes (A–C), mature macrogametocytes (D–F), and various developmental stages of gametocytes (**G**) in erythrocytes of a pigeon from Ngemplak, Yogyakarta. All photographs are at the same magnification, and the scale bar (10 μm) is shown in the photograph (**E**). Nuclei (arrowheads) and pigment granules (pg) of *H. columbae* are shown in the photographs (**B**, **E**). Arrow in the photograph (**G**) indicates an erythrocyte with two gametocytes.

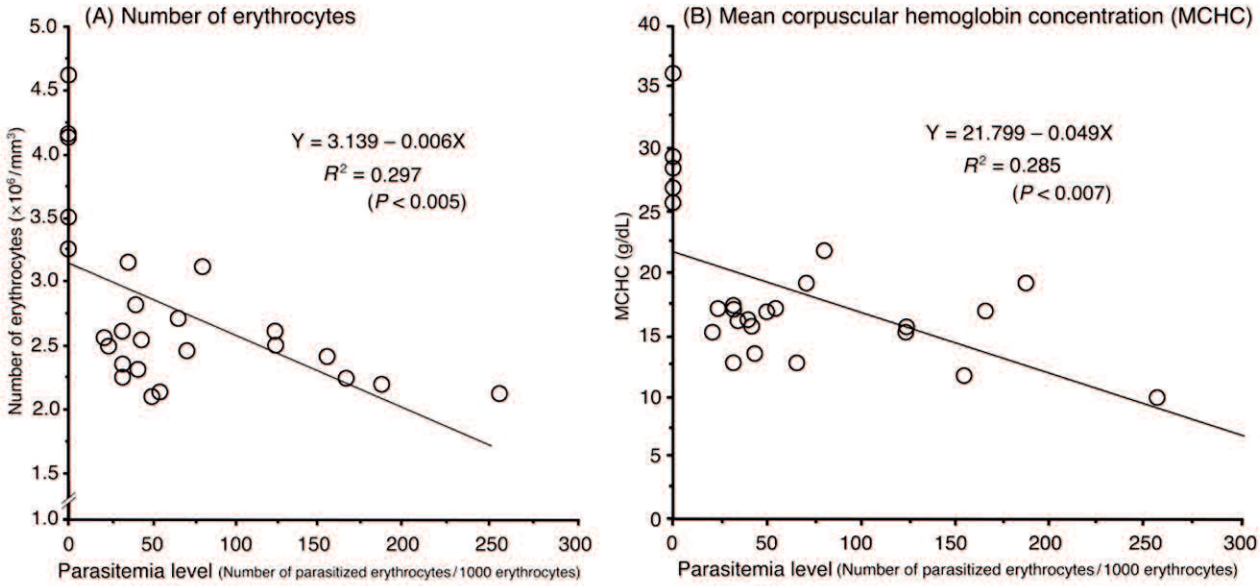

Figure 5. Relationship between *Haemoproteus columbae* parasitemia level (number of parasitized erythrocytes/1000 erythrocytes) and number of erythrocytes ($\times 10^6/mm^3$) (A). or mean corpuscular hemoglobin concentration (MCHC; g/dL) (B). Each open circle represents one individual farmed pigeon. Linear regressions shown in A and B are statistically significant with $R^2 = 0.297$ and 0.285, respectively.

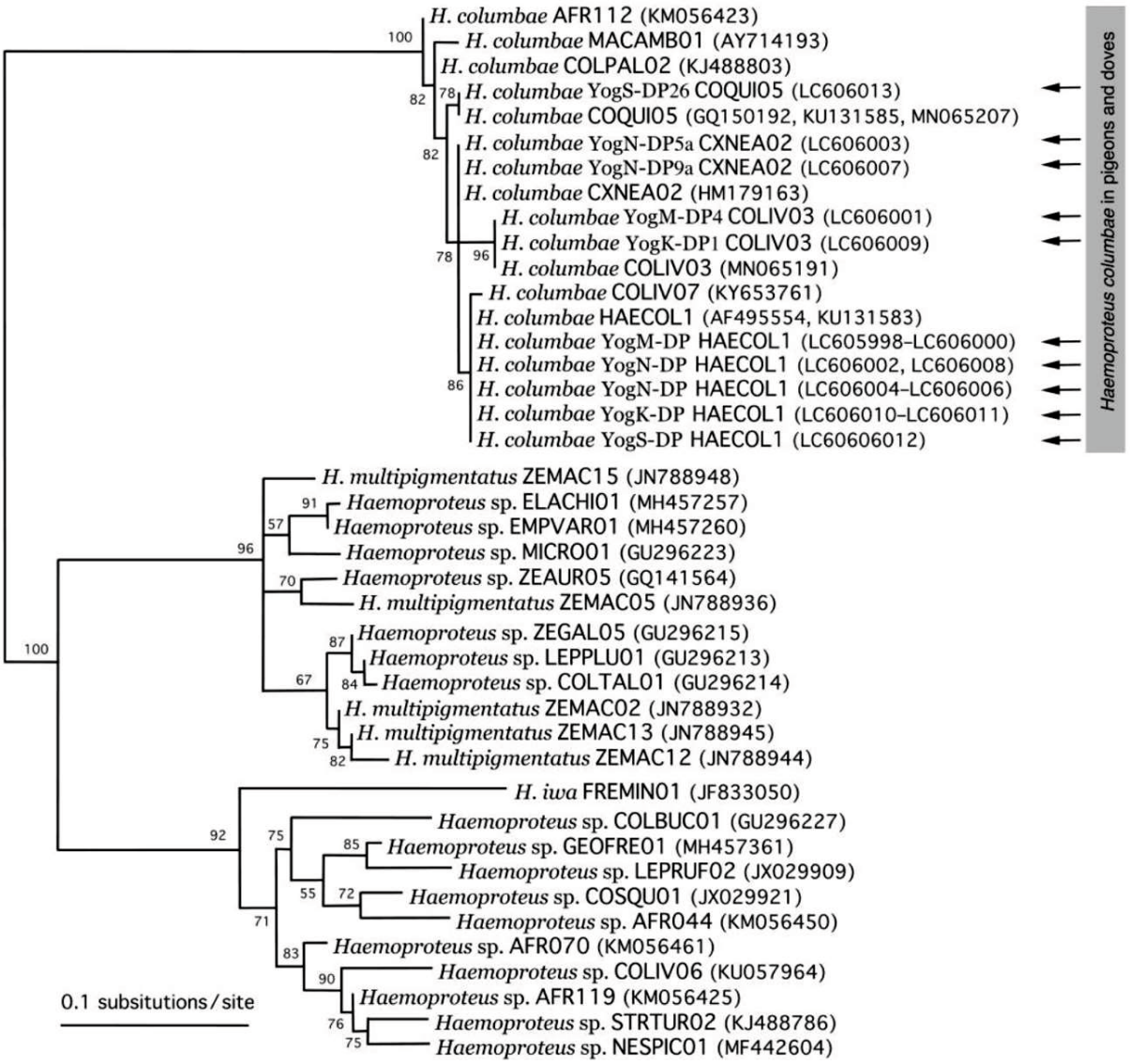

Figure 6. Maximum likelihood phylogenetic trees of *Haemoproteus* spp. of the subgenus *Haemoproteus* based on the *cytb* sequences (465 characters). The species name (for the new isolates in this study with their isolate names; arrows) is followed by the Mal-Avi lineage tag, and DDBJ/EMBL/GenBank accession number in parentheses.

CHAPTER II

Isolation and molecular characterization of *Polychromophilus* spp. (Haemosporida: Plasmodiidae) from the Asian long-fingered bat (*Miniopterus fuliginosus*) and Japanese large-footed bat (*Myotis macrodactylus*) in Japan

Information described in this chapter has been published as follows;

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Abstract

Bats (order, Chiroptera) account for more than one-fifth of all mammalian species in the world and are infected by various intra-erythrocytic parasites of the family Plasmodiidae (Apicomplexa: Haemosporida), including *Polychromophilus* Dionisi, 1899. Recent advance in the molecular characterization of hemosporidian isolates has enabled their accurate identification, particularly in the last decade. Studies are actively conducted in tropical regions, Europe, and Australia; however, data on hemosporidian infection in bats in Asian temperate areas, including Japan, remain limited. In this study, 75 bats of 4 species (*Miniopterus fuliginosus*, *Myotis macrodactylus*, *Rhinolophus nippon*, and *Rhinolophus cornutus*) were captured at three sites in western Japan (Yamaguchi Prefecture), and hemosporidian parasites were screened microscopically and molecularly via nested polymerase chain reaction (PCR) targeting the cytochrome *b* (*cytb*), cytochrome *c* oxidase subunit I (*cox-1*), apicoplast caseinolytic protease C (*clpc*), and nuclear elongation factor 2 (EF2) genes. The survey detected *Polychromophilus melanipherus* in 15 (40.5%) miniopterid bats (*M. fuliginosus*) and *Polychromophilus murinus* in 6 (46.2%) vespertilionid bats (*M. macrodactylus*), whereas none of the 25 rhinolophid bats (*R. nippon* and *R. cornutus*) was infected, indicating the robust host specificity for miniopterid (*P. melanipherus*) and vespertilionid (*P. murinus*) bats regardless of orthotopic nesting. The 15 *Polychromophilus cytb* sequences obtained from 11 miniopterid and 4 vespertilionid bats were classified into six *cytb* haplotypes (three for each species), showing no region-specific variation in a phylogenetic tree of *Polychromophilus* isolates in the Old World. Similarly, multiple haplotypes (seven for *cox-1* and nine for *clpc*) and genotypes (three for EF2) were characterized for the Japanese isolates of *Polychromophilus*, and the results were consistent with those based on a hemosporidian *cytb* analysis. Bat flies (*Nycteribia allotopa* and another undetermined *Nycteribia* sp.) collected from the body surface of bats harbored *Polychromophilus* oocysts on the external surface of the midgut. This is the first study to report

the isolation and molecular characterization of *Polychromophilus* spp. in miniopterid and vespertilionid bats in the temperate area of Asia (western Japan). Future studies should evaluate the global prevalence of hemosporidian infections in bats.

Introduction

Bats represent a major mammalian group (order, Chiroptera) in the world, accounting for more than 20% of extant mammalian species (Teeling *et al.*, 2005). They serve as hosts to a variety of hemosporidian parasites of the family Plasmodiidae (Apicomplexa: Haemosporida), including protozoans of the genera *Plasmodium*, *Hepatocystis*, *Nycteria*, and *Polychromophilus*. A number of parasites are found exclusively in bats, including six out of the nine genera of the family Plasmodiidae recorded in mammalian hosts (Schaer *et al.*, 2013; Schaer *et al.*, 2015; Perkins and Schaer, 2016). Malarial parasites (members of the genus *Plasmodium*) undergo an intra-erythrocytic replication phase, called schizogony, whereas hemosporidian parasites of the other eight genera lack the ability to reproduce asexually in erythrocytes, which is reflected in lower pathogenicity in hosts than that of symptomatic malaria (Holz et al., 2019; Witsenburg et al., 2014).

Parasites of the genus *Polychromophilus* Dionisi, 1899 have been isolated from insectivorous bats in Europe, Middle East, Australia, and the tropical regions of Africa, Southeast Asia, and Central and South Americas (Mackerras, 1959; Goedbloed *et al.*, 1964; Dew and McMillan, 1970; Garnham *et al.*, 1971; Garnham, 1973b; a; Foster, 1979; Landau *et al.*, 1980; Gardner *et al.*, 1987; Duval *et al.*, 2007; Megali *et al.*, 2011; Duval *et al.*, 2012; Witsenburg *et al.*, 2015; Holz *et al.*, 2019; Arnuphapprasert *et al.*, 2020; Rasoanoro *et al.*, 2021; Sándor *et al.*, 2021). Although *Polychromophilus* parasites are widespread and highly prevalent worldwide, only five morphospecies have been described to date. *Polychromophilus melanipherus* Dionisi, 1899 has been reported mainly in bats of the family Miniopteridae, and

Polychromophilus murinus Dionisi, 1899 has been identified in bats of the family Vespertilionidae (Garnham, 1973b; Landau *et al.*, 1980; Gardner and Molyneux, 1988; Megali *et al.*, 2011; Duval *et al.*, 2012; Ramasindrazana *et al.*, 2018). *Polychromophilus corradetti* and *Polychromophilus adami* were isolated and described by Landau et al. (1980) from *Miniopterus* spp. of the family Miniopteridae in Central Africa (Gabon and Congo). Garnham et al. (1971) isolated and described *Polychromophilus deanei* from *Myotis nigricans* (Vespertilionidae) in the Amazon region of Brazil; additionally, *P. deanei*-like species have been isolated from *Glossophaga soricina* (Phyllostomidae) in the Amazon region, *Eptesicus fuscus* (Vespertilionidae) in central Colombia, and *Myotis austroriparius* in North Central Florida, USA (Deane and Deane, 1961; Foster, 1979; Marinkelle, 1995). It is difficult to differentiate *Polychromophilus* spp. solely based on the microscopic analysis of gametocytes in the erythrocytes due to minor morphological differences (Duval *et al.*, 2012; Ramasindrazana *et al.*, 2018; Chumnandee *et al.*, 2021; Rasoanoro *et al.*, 2021).

In the last two decades, molecular approaches to studying malarial parasites (diverse *Plasmodium* parasites with different host specificities, infection courses, and pathogenicities) and other hemosporidian parasites have increased our understanding of the phylogenetic relationships and species/genetic lineage differentiation of intra-erythrocytic protozoans (Martinsen *et al.*, 2008; Schaer *et al.*, 2015, 2017, 2018; Borner *et al.*, 2016; Boundenga *et al.*, 2018; Galen *et al.*, 2018). Regarding *Polychromophilus* parasites, phylogenetic studies based on mitochondrial cytochrome *b* gene (*cytb*) sequences have confirmed the occurrence of two species, *P. melanipherus* and *P. murinus*, mainly in bats of the families Miniopteridae and Vespertilionidae, respectively (Megali *et al.*, 2011; Duval *et al.*, 2012; Witsenburg *et al.*, 2012). Furthermore, *Polychromophilus* sp. isolated from the blood of *Myotis nigricans* in Panama as well as isolates from *Myotis riparius*, *Myotis ruber*, and *Eptesicus diminutus* (all belonging to the family Vespertilionidae) in southern Brazil, which may represent *P. deanei*, have been

shown to be phylogenetically close to *P. murinus*; however, they form a distinct clade (Borner *et al.*, 2016; Minozzo *et al.*, 2021). In contrast, when *Polychromophilus* isolates obtained from *Miniopterus inflatus* in Gabon, which represent the type host and locality for *P. corradetti*, and other African isolates from miniopterid bats in Guinea and Madagascar were phylogenetically analyzed based on *cytb* nucleotide sequences, all isolates have been found to be grouped in the clade of *P. melanipherus*; this suggested that *P. corradetti* and *P. adami* may represent junior synonyms of *P. melanipherus* (Duval *et al.*, 2012; Schaer *et al.*, 2013; Rosskopf *et al.*, 2019). Apart from the three aforementioned *Polychromophilus* clades including *P. melanipherus*, *P. murinus*, and putative *P. deanei*, three more genetic lineages of *Polychromophilus* isolated from vespertilionid bats in Cambodia, Thailand, Madagascar, and Guinea have been described in latest phylogenetic studies (Duval *et al.*, 2007; Schaer *et al.*, 2013; Chumnandee *et al.*, 2021; Rasoanoro *et al.*, 2021).

Morphological and molecular analyses of *Polychromophilus* parasites in Asia are limited in the tropical region such as Thailand, Malaysia, and Cambodia (Eyles *et al.*, 1962; Landau *et al.*, 1984; Duval *et al.*, 2007; Arnuphapprasert *et al.*, 2020; Chumnandee *et al.*, 2021) and data on hemosporidian infections in bats in the Asian temperate region, including Japan, are scarce. Therefore, the present study performed microscopic and molecular analyses of hemosporidian infections in four chiropteran species of three families (Miniopteridae, Vespertilionidae, and Rhinolophidae) in the western part of Japan (temperate region) for the first time.

Materials and methods

Bats were captured in February and September 2021 from two tunnels in Iwakuni (34° 18′ 39″ N, 131° 59′ 02″ E, and 34° 20′ 57″ N, 131° 00′ 05″ E) and one cave in Mine (34° 13′ 42.3″ N, 131° 18′ 10.5″ E), Yamaguchi Prefecture, Japan (Prefectural Government Approval

No. 429-1), using a sweep net with an extended rod (Table 5). A total of 75 bats were captured, and four chiropteran species of three families were identified (Ohdachi *et al.*, 2010). Bats were transported to the laboratory in Yamaguchi University, and blood samples were collected. Animal experiments were performed according to the Declaration of Helsinki, and the Guidelines on Animal Experimentation as set out by the Institutional Animal Care and Use Committee, Yamaguchi University. Blood samples (50–250 μl per bat) were collected via cardiac puncture and transferred to sterile heparinized Eppendorf tubes. One drop of blood from each bat was used to prepare a thin blood smear, and the remaining few blood drops were used for DNA extraction. The remaining blood samples and carcasses were used to isolate viral, microbial, and helminth pathogens. Bat flies of the family Nycteribiidae crawling on the skin surface of each bat were collected in a labeled plastic bag and frozen at − 80 °C until use.

Thin blood smears on clean slides were dried, submerged in 100% methanol for 2 min, and stained with 5% Giemsa's solution (Sigma-Aldrich, St. Louis, MS, USA) in phosphate buffer (pH 7.2) for 20 min. Blood smears were washed with clean water, examined under an Olympus BX60 light microscope equipped with a DP72 digital camera (Olympus, Nishi-Shinjuku, Tokyo, Japan) at × 400 magnification, and further observed at × 1000 magnification using oil immersion for at least 20–25 min (20–71 fields) for each blood smear. Levels of parasitemia were calculated as the proportion (%) of parasite-infected erythrocytes in total erythrocytes as described by Godfrey et al. (1987). For this calculation, more than 1000 erythrocytes were counted individually. Blood smear slides with hemosporidian parasites were deposited in the Meguro Parasitological Museum, Tokyo, Japan (MPM collection nos. 21808–21810).

Genomic DNA was extracted from blood samples in Eppendorf tubes using an illustraTM tissue and cell genomicPrep Mini Spin Kit (GE Healthcare UK, Ltd., Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. Nested polymerase chain

reaction (PCR) of mitochondrial DNA (*cytb* and cytochrome *c* oxidase subunit I gene (*cox-1*)) and apicoplast DNA (caseinolytic protease C gene (*clpc*)) fragments and one-step PCR of nuclear DNA (elongation factor 2 (EF2) gene) fragments were performed in a 20 μl PCR solution containing DNA polymerase packed in Blend Taq-Plus-(Toyobo, Dojima Hama, Osaka, Japan), a pair of the forward primer and the reverse primer (see Table 6), and 1 μl of template DNA. The following PCR cycling protocol was used: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at a different annealing temperature according to the target DNA fragments (see Table 6), and 60 s at 72 °C, and then a final extension at 72 °C for 7 min. The PCR products were purified using a FastGene Gel/ PCR Extraction Kit (Nippon Genetics Co., Tokyo, Japan) and sequenced directly from both ends using the primers used for PCR amplification, and additional ones for nucleotide sequencing (see Table 6). Sequences were manually assembled using the CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994). The nucleotide sequences obtained in the present study are available from the DDBJ/ EMBL/GenBank databases under the accession nos. LC668428–LC668433 (*cytb*), LC715187–LC715196 (*cox-1*), LC715197–LC715205 (*clpc*), and LC715206–LC715208 (EF2 gene).

The newly obtained nucleotide sequences excluding the primer-annealing parts (*cytb*, 741 bp; *cox-1*, 981 bp; EF2 gene, 565 bp; and *clpc*, 537 bp) were analyzed to identify highly similar nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information website (https://www.ncbi.nlm. nih.gov/). For phylogenetic analysis, the newly obtained DNA sequences and those of related hemosporidian parasites retrieved from the DDBJ/EMBL/GenBank databases were aligned using MEGA7 software (Kumar *et al.*, 2016), with manual adjustments. The accession numbers of the sequences analyzed in the present study are specified in the figure showing a phylogenetic tree. Poorly aligned regions and characters with a gap in any sequence were excluded from

subsequent analyses. Ultimately, 464 characters (*cytb*) of which 239 were variable, 768 characters (*cox-1*) of which 168 were variable, 485 characters (*clpc*) of which 193 were variable, or 466 characters (EF2 gene) of which 144 were variable were selected for subsequent analyses. Maximum likelihood analysis was performed using the PhyML tool (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 via the approximate likelihood ratio test (aLRT), an alternative to the nonparametric bootstrap estimation of branch support (Anisimova and Gascuel, 2006).

Bat flies stored at −80 °C were thawed and then dissected under a dissection microscope. The head with salivary glands was cut from the body using a clean blade, the salivary glands were separated using forceps and stamped on a clean glass slide, and the remaining part was immersed in DNA extraction buffer. The tissue stamps on glass slides were stained with 5% Giemsa's solution as described above. The midgut and hindgut were carefully observed under a dissection microscope and a conventional light microscope. To identify the bat fly species, partial insect *cox-1* fragments were amplified via one-step PCR using salivary gland DNA extracts and the primer pair LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HC02198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) according to Folmer et al. (1994). The PCR cycling protocol was similar to that mentioned previously, although it excluded the annealing temperature at 48 °C. To screen hemosporidian parasites in the salivary gland, nested PCR amplification of parasite *cytb* fragments was performed as described previously. The nucleotide sequences obtained in the present study are available from the DDBJ/EMBL/GenBank databases under the accession nos. LC715183–LC715185 (bat fly *cox-1*) and LC715186 (hemosporidian *cytb*).

Results

Microscopic analysis of hemosporidian parasites in blood smears identified 18 positive samples, and nested PCR screening for hemosporidian *cytb* fragments identified 21 positive samples. All infected bats comprised the Asian long-fingered bat *Miniopterus fuliginosus* (Hodgson, 1835) of the Miniopteridae family and the Japanese large-footed bat *Myotis macrodactylus* (Temminck, 1840) of the Vespertilionidae family (Table 5). Twenty-five bats of the Rhinolophidae family, including the greater Japanese horseshoe bat *Rhinolophus nippon* Temminck, 1835 and the little Japanese horseshoe bat *Rhinolophus cornutus* Temminck, 1835, were negative for hemosporidian infection based on both methods. The average parasite intensity in these infected bats was 0.19% (range, 0.07–0.63%), excluding one bat (#Nbat65), in which trophozoites were frequently observed with an intensity of 7.25%.

A majority of hemosporidian parasites in the blood of miniopterid bats were gametocytes exhibiting coarse hemozoin pigment grains scattered in the cytoplasm (Figure 7a–e). The cytoplasms of microgametocytes and macrogametocytes were stained light pink and purple-blue, respectively, and contained eccentrically or centrally located nuclei of irregular shapes, and mature gametocytes accounted for all of the slightly enlarged erythrocyte cytoplasm (Table 7). In contrast, a majority of hemosporidian parasites in the blood of vespertilionid bats were trophozoites (Figure 7f), and rarely gametocytes were seen.

Fifteen hemosporidian *cytb* sequences (741 bp) were successfully sequenced and assigned to six haplotypes (Table 8). Phylogenetic analysis demonstrated that three *cytb* haplotypes of miniopterid *Polychromophilus* parasites were clustered in a clade of *P. melanipherus*, whereas three *cytb* haplotypes of vespertilionid parasites were clustered in a clade of *P. murinus* (Figure 8). These *Polychromophilus* clades, including *P. deanei* from New World bats and several additional isolates from vespertilionid bats in Southeast Asia

(Cambodia and Thailand), Madagascar, and Central Africa (Guinea), showed a sister relationship to avian and saurian *Plasmodium* parasites in a phylogenetic tree based on the *cytb* sequences.

Three *cytb* haplotypes of Japanese *P. melanipherus* isolates showed 98.92% (733/741) to 99.87% (740/741) similarity with each other, and three *cytb* haplotypes of Japanese *P. murinus* isolates showed 98.38% (729/741) to 99.33% (736/741) similarity with each other. The highest similarity of nucleotide sequences between Japanese *P. melanipherus* and *P. murinus cytb* haplotype groups was 97.84% (725/741). BLAST analysis of the six new *cytb* sequences determined in this study could not find the same *cytb* haplotypes in other regions; however, *cytb* sequences showing the same degree of intraspecific nucleotide variation as observed among Japanese isolates were easily found as follows: *P. melanipherus* isolates from miniopterid bats in Gabon, Madagascar, Europe, and Australia with 99.01% (702/709) to 99.57% (699/702) similarity and *P. murinus* isolates from vespertilionid bats in Europe with 99.01% (699/706) to 99.58% (703/706) similarity. Therefore, *P. melanipherus* and *P. murinus* isolated from chiropteran hosts could not be classified into any geographical lineages based on the *cytb* sequences.

Thirteen hemosporidian *cox-1* sequences (981–1226 bp) were successfully sequenced and assigned to eight haplotypes (Table 8). Phylogenetic analysis demonstrated that six *cox-1* haplotypes of miniopterid *Polychromophilus* parasites were clustered in a clade of *P. melanipherus* and two *cox-1* haplotypes of vespertilionid parasites were clustered in a clade of *P. murinus* (Figure 9). Six *cox-1* haplotypes of Japanese *P. melanipherus* isolates showed more than 99.10% similarity (1215/1226), and two *cox-1* haplotypes of Japanese *P. murinus* isolates showed 99.18% similarity (1216/1226). The highest similarity among nucleotide sequences between the Japanese *P. melanipherus* and *P. murinus cytb* haplotype groups was 96.90% (1188/1226).

Twelve apicoplast *clpc* nucleotide sequences (537 bp) were successfully sequenced and assigned to nine haplotypes (Table 8). Phylogenetic analysis demonstrated that six *clpc* haplotypes of miniopterid *Polychromophilus* parasites were clustered in a clade of *P. melanipherus* and three *clpc* haplotypes of vespertilionid parasites were clustered in a clade of *P. murinus* (Figure 10). Six *clpc* haplotypes of Japanese *P. melanipherus* isolates showed more than 96.64% similarity (519/537), and three *clpc* haplotypes of Japanese *P. murinus* isolates showed 97.20% similarity (522/537). The highest similarity among nucleotide sequences between the Japanese *P. melanipherus* and *P. murinus clpc* haplotype groups was 94.60% (508/537). Only four *P. melanipherus* EF2 DNA sequences (565 bp) were successfully sequenced, and they were assigned to three genotypes (Table 8). These Japanese *P. melanipherus* genotypes showed more than 97.34% similarity (550/565) (Figure 11). The highest similarity of nucleotide sequences between the EF2 genotypes of Japanese *P. melanipherus* obtained in this study and a deposited genotype of a tropical *Polychromophilus* sp. (accession no. KF159742) was 92.04% (474/515).

Eight bat flies from three miniopterid bats that exhibited *P. melanipherus* gametocytes in their blood were molecularly identified as either *Nycteribia allotopa* Speiser, 1901 or undetermined *Nycteribia* sp., as previously described by Nabeshima et al. (2020) (Figure 7). One or two *Polychromophilus* oocysts were microscopically observed on the external surface of midguts in two bat flies (Figure 12). Screening of *Polychromophilus* sporozoites in the salivary glands identified two miniopterid bats with positive reactions based on nested PCR targeting of a hemosporidian *cytb* fragment. Sequencing of one of two amplicons was successful, and it corresponded to the *P. melanipherus* haplotype JMin*Cytb*-**H3** (Table 8). Tissue stamps of the salivary glands of one bat fly demonstrated a single sporozoite.

Discussion

This study is the first to report the isolation and molecular characterization of two hemosporidian species of the genus *Polychromophilus* in bats distributed in Japan: *P. melanipherus* in *Miniopterus fuliginosus* and *P. murinus* in *Myotis macrodactylus*. Chiropteran hosts of *P. melanipherus* and *P. murinus* species mainly represent miniopterid and vespertilionid bats, respectively (Garnham, 1973b; Landau *et al.*, 1980; Gardner and Molyneux, 1988; Megali *et al.*, 2011; Duval *et al.*, 2012; Schaer *et al.*, 2013; Ramasindrazana *et al.*, 2018), and regardless of their orthotopic nesting in an identical tunnel, this host specificity of *Polychromophilus* parasites was observed in the present study as well. This host specificity has been suggested to be attributed to the natural immunity of hosts against different *Polychromophilus* spp. in chiropteran hosts, not attributable to rigorous host specificity of vectors, or ecological relationships between hosts and vectors (Ramasindrazana *et al.*, 2018; Rasoanoro *et al.*, 2021; Sándor *et al.*, 2021). Ramasindrazana et al. (2017) have demonstrated that nycteribiid flies (bat flies) parasitizing insectivorous bats, which are known to transmit *Polychromophilus* spp. as a vector (Mer and Goldblum, 1947; Adam and Landau, 1973; Gardner and Molyneux, 1988b; Duval *et al.*, 2012; Witsenburg *et al.*, 2015; Obame-Nkoghe *et al.*, 2016; Szentiványi *et al.*, 2020), show little host preference (polyxenous); bats of both genera *Miniopterus* (Miniopteridae) and *Myotis* (Vespertilionidae) are parasitized by *Penicillidia leptothrinax*, *Penicillidia* cf. *fulvida*, and *Nycteribia stylidiopsis* in Madagascar. Japanese bats in the genera *Miniopterus* (Miniopteridae) and *Myotis* (Vespertilionidae) are infested with a variety of nycteribiid bat flies, such as *Nycteribia allotopa*, *Nycteribia pleuralis*, *Nycteribia pygmaea*, *Penicillidia jenynsii*, *Penicillidia monoceros*, *Phthiridium hindlei*, *Basilia rybini*, and *Basilia truncate* (Maa, 1967; Nikoh *et al.*, 2011; Nabeshima *et al.*, 2020). To explore the potential vectors of *Polychromophilus* spp. in Japan, this study identified *P.*

melanipherus oocysts and sporozoites on the external surface of the midguts and the salivary gland, respectively, in the examined bat flies of the genus *Nycteribia*.

A notable intraspecific genetic diversity of *cytb*, *cox-1*, *clpc*, and EF2 gene sequences was found in Japanese isolates of both *P. melanipherus* and *P. murinus* which have been reported in Central Africa, Europe, Southeast Asia, and Australia (Witsenburg *et al.*, 2015; Obame-Nkoghe *et al.*, 2016; Ramasindrazana *et al.*, 2018; Holz *et al.*, 2019; Rasoanoro *et al.*, 2021; Sándor *et al.*, 2021) however, these isolates did not cluster by origin, such as region or host species. However, as mentioned above, the putative *cytb* and *cox-1* sequences of *P. deanei*, a *Polychromophilus* morphospecies isolated from New World bats of the family Vespertilionidae (Garnham *et al.*, 1971), have been shown to be phylogenetically closest to *P. murinus*, although they cluster in a separate subclade (Borner *et al.*, 2016; Minozzo *et al.*, 2021; see Figs. 2 and 3 in this study). This contradicts the taxonomical classification of the other two *Polychromophilus* morphospecies: *P. corradetti* from *Miniopterus schreibersii* in Gabon and *P. adami* from *Miniopterus minor* in Congo (Landau *et al.*, 1980), which may represent junior synonyms of *P. melanipherus* (Duval *et al.*, 2012; Rosskopf *et al.*, 2019). Based on latest phylogenetic studies (Duval *et al.*, 2007; Schaer *et al.*, 2013; Arnuphapprasert *et al.*, 2020; Chumnandee *et al.*, 2021; Rasoanoro *et al.*, 2021) the distribution of at least three unrecorded *Polychromophilus* spp. has been suggested in vespertilionid bats (*Kerivoula hardwickii*, *Scotophilus kuhlii*, *Scotophilus robustus*, *Laephotis capensis*, and *Pipistrellus* aff. *grandidieri*) in Cambodia, Thailand, Madagascar, and Guinea (cf. Figs. 2 and 3). To elucidate the biogeography of these genetic lineages and clarify their taxonomic status, further morphological and molecular investigations of hemosporidian parasites in bats are necessary globally, particularly in regions where few studies have been conducted and little is known about hemosporidian parasites in bats, such as Japan.

Molecular characterization of hemosporidian parasites is a feasible approach to study morphologically indistinguishable species. In addition to miniopterid bats, several *P. melanipherus* infections have been recorded in bats of the families Emballonuridae (*Coleura afra* in Gabon and *Taphozous melanopogon* in Thailand), Hipposideridae (*Hipposideros caffer* in Gabon), Pteropodidae (*Rousettus aegyptiacus* in Gabon), and Rhinolophidae (*Paratriaenops furculus* in Madagascar) (Obame-Nkoghe *et al.*, 2016; Ramasindrazana *et al.*, 2018; Arnuphapprasert *et al.*, 2020; Sándor *et al.*, 2021). Additionally, *P. murinus* infections have been reported in bats of the family Rhinonycteridae (*Rhinolophus* spp. and *Triaenops persicus* in Italy and Africa) (Garnham, 1973a; Sándor *et al.*, 2021). In such exceptional hosts, hemosporidian species can be reliably identified by molecular approaches.

Bats are parasitized by hemosporidian species of eight out of the nine genera of the family Plasmodiidae recorded in mammalian hosts (*Plasmodium*, *Hepatocystis*, *Nycteria*, *Polychromophilus*, *Sprattiella*, *Johnsprentia*, *Dionisia*, and *Biguetiella*), and except for the two genera *Plasmodium* and *Hepatocystis*, species of six genera are isolated exclusively from bats (Adam and Landau, 1973; Landau and Chabaud, 1978; Duval *et al.*, 2007; Landau *et al.*, 2012; Schaer *et al.*, 2013, 2015; Lutz *et al.*, 2016; Perkins and Schaer, 2016; Boundenga *et al.*, 2018). When a phylogenetic tree was constructed based on the *cytb* and *cox-1* sequences, the genus *Polychromophilus* exhibited a sister relationship to saurian and avian *Plasmodium* spp. (Figs. 2 and 3). However, when multiple genes are incorporated for the construction of a phylogenetic tree, the topologies of different genera may change; *Polychromophilus* parasites are speculated to be the most basal group of mammalian hemosporidian parasites (Schaer *et al.*, 2013, 2015). Regardless of its exact evolutionary origin, Duval et al. (2012) speculated that *Polychromophilus* ancestors may have originated before the diversification of chiropteran Miniopteridae and Vespertilionidae (approximately 45 million years ago; Miller-Butterworth *et al.*, 2007), and they might have coevolved independently of the host groups to *P.*

melanipherus and *P. murinus*. This coevolution may be partly supported by the vectors of the family Nycteribiidae, which comprise flattened flies without eyes and wings that live on the host body surface during their entire lifecycle (Dick and Patterson, 2006), in addition to a possible natural immunity of the host group to different *Polychromophilus* spp. as mentioned above.

In Japan, 35 chiropteran species are distributed among its subtropical region (Okinawa) to subarctic region (Hokkaido) and classified as Vespertilionidae (25 species), Miniopteridae (2 species), Pteropodidae (2 species), Rhinolophidae (3 species), Hipposideridae (1 species), and Molossidae (2 species) (Ohdachi *et al.*, 2010; Preble *et al.*, 2021). In addition to two *Polychromophilus* spp. in two chiropteran species in Japan, it is highly possible that additional host species for these hemosporidian species may exist along with different genetic lineages/species of the genus *Polychromophilus* or other genera as well.

Table 5. Bats examined in this study and prevalence of hemosporidian infections.

Host species	Locality	Date of collection	Sex (M, male; F, female)	Body weight[a] (g)	Number of bats examined	Number of bats with haemosporidian infection (%)
Miniopteridae Dobson, 1875						
Miniopterus fuliginosus (Hodgson, 1835)	Tunnel Y, Nishiki, Yamaguchi Prefecture	Feb. 15, 2021	14M, 14 F	9.6–13.0 (11.0 ± 0.8)	28	8 (28.6)
		Sep. 27, 2021	6M, 3F	10.0–14.5 (12.8 ± 1.2)	9	7 (77.8)
Vespertilionidae Gray, 1821						
Myotis macrodactylus (Temminck, 1840)	Tunnel Y, Nishiki, Yamaguchi Prefecture	Sep. 27, 2021	11M, 2F	6.0–10.7 (7.5 ± 1.5)	13	6 (46.2)
Rhinolophidae Gray, 1825						
Rhinolophus nippon Temminck, 1835	Tunnel M, Nishiki, Yamaguchi Prefecture	Feb. 15, 2021	5M, 7F	16.9–22.1 (18.8 ± 1.8)	12	0
		Sep. 27, 2021	2M, 1F	21.8–29.9 (24.9 ± 4.3)	3	0
	Cave A, Mine, Yamaguchi Prefecture	Feb. 25, 2021	2M, 2F	18.0–21.5 (19.9 ± 1.4)	4	0
Rhinolophus cornutus Temminck, 1834	Cave A, Mine, Yamaguchi Prefecture	Feb. 25, 2021	4M, 2F	4.7–5.4 (5.1 ± 0.2)	6	0

[a]Range (mean ± standard deviation)

Table 6. Primers used to amplify and sequence *Polychromophilus* genes.

Gene	Primer action	Primer name	Sequence (5`-3`)	Annealing temperature in PCR (°C)	Relative position of primer`s 5`-terminus nucleotide in the first-round amplicon[a]	Reference
cytb	1st round–forward	PLAS1	5`-GAG AAT TAT GGA GTG GAT GGTG-3′	50	1	Duval et al. (2007)
	1st round–reverse	PLAS2	5`-GTG GTA ATT GAC ATC CWA TCC-3′		816	Duval et al. (2007)
	2nd round–forward	PLAS3	5`-GGT GTT TYA GAT AYA TGC AYGC-3′	50	19	Duval et al. (2007)
	2nd round–reverse	PLAS4	5`-CAT CCW ATC CAT ART AWA GCA TAG -3′		805	Duval et al. (2007)
cox-1	1st round–forward	coI/outerF	5`-CTA TTT ATG GTT TTC ATT TTT ATT TGGTA-3′	60	1	Martinsen et al. (2008)
	1st round–reverse	coI/outerR	5`-AGG AAT ACG TCT AGG CAT TAC ATT AAA TCC -3′		1288	Martinsen et al. (2008)
	2nd round–forward	coI/innerF	5`-ATG ATA TTT ACA RTT CAY GGW ATT ATT ATG -3′	52	122	Martinsen et al. (2008)
	2nd round–reverse	coI/innerR	5`-GTA TTT TCT CGT AAT GTT TTA CCA AAGAA-3′		1188	Martinsen et al. (2008)
	sequencing–forward	coI/midF	5`-TTA TTC TGG TTT TTT GGT CAT CCA G-3′		662	Martinsen et al. (2008)
	sequencing–reverse	coI/midR	5`-CTG GAT GAC CAA AAA ACC AGA ATA A-3′		686	Martinsen et al. (2008)
clpc	1st round–forward	Clpc/outerF	5`-AAA CTG AAT TAG CAA AAA TATTA-3′	50	1	Martinsen et al. (2008)
	1st round–reverse	Clpc/outerR	5`-CGW GCW CCA TAT AAA GGA T-3′		640	Martinsen et al. (2008)
	2nd round–forward	Clpc/innerF	5`-GAT TTG ATA TGA GTG AAT ATA TGG -3′	50	61	Martinsen et al. (2008)
	2nd round–reverse	Clpc/innerR	5`-CCA TAT AAA GGA TTA TAW G-3′		634	Martinsen et al. (2008)
EF2 gene	Forward	EF2F	5`-GTT CGT GAG ATC ATG AAC AAAAC-3′	55	1	Schaer et al. (2013)
	Reverse	EF2R	5`-CCT TGT AAA CCA GAA CCA AA-3′		608	Schaer et al. (2013)

cytb cytochrome b gene, *cox-1* cytochrome c oxidase subunit I gene, *clpc* caseinolytic protease C gene, EF2 elongation factor 2

[a]For each gene, relative positions of the 5`-terminus nucleotide of primers are determined with reference to deposited sequences (DDBJ/EMBL/GenBank accession nos. MK098848 (*cytb*), KU726004 (*cox-1*), LT594503 (*clpc*), and KF159731 (EF2 gene))

Table 7. Measurements of erythrocytes with/without *Polychromophilus* gametocytes.

	Miniopterus fuliginosus (P. melanipherus)	*Myotis macrodactylus (P. murinus)*
Uninfected erythrocyte	*n* = 12	*n* = 5
Length	5.5 ± 0.2 (5.1-5.8)	5.4 ± 0.1 (5.3-5.6)
Width	5.0 ± 0.3 (4.5-5.6)	5.1 ± 0.1 (4.9-5.2)
Microgametocyte	*n* = 31	*n* = 4
Length	6.4 ± 0.6 (5.1-7.6)	6.9 ± 0.9 (5.8-7.8)
Width	5.2 ± 0.5 (4.8-6.4)	4.4 ± 1.4 (3.2-5.9)
Macrogametocyte		
Length	6.2 ± 0.6 (5.1-7.3)	6.4 ± 1.2 (5.1-7.5)
Width	5.1 ± 0.2 (4.8-6.0)	3.6 ± 0.4 (3.3-4.1)

Table 8. Haplotypes/genotypes of *Polychromophilus* DNA sequences obtained in this study.

Isolate	Host	*cytb*	*cox-1*	*clpc*	EF2 gene
P. melanipherus					
Nbat16	*Miniopterus fuliginosus*	JMinCytb-**H1** (LC668428)	FA	FA	FA
Nbat22	*Miniopterus fuliginosus*	JMinCytb-**H1** (LC668428)	JMinCox1-**H1** (LC715187)	JMinClpc-**H1** (LC715197)	FA
Nbat31	*Miniopterus fuliginosus*	JMinCytb-**H1** (LC668428)	JMinCox1-**H1** (LC715187)	JMinClpc-**H2** (LC715198)	JMinEF2-**G1** (LC715206)
Nbat33	*Miniopterus fuliginosus*	JMinCytb-**H1** (LC668428)	JMinCox1-**H2** (LC715188)	JMinClpc-**H3** (LC715199)	FA
Nbat36	*Miniopterus fuliginosus*	JMinCytb-**H1** (LC668428)	JMinCox1-**H3** (LC715189)	JMinClpc-**H4** (LC715200)	FA
Nbat39	*Miniopterus fuliginosus*	JMinCytb-**H1** (LC668428)	FA	FA	FA
Nbat53	*Miniopterus fuliginosus*	JMinCytb-**H2** (LC668429)	JMinCox1-**H4** (LC715190)	JMinClpc-**H5** (LC715201)	FA
Nbat59	*Miniopterus fuliginosus*	JMinCytb-**H2** (LC668429)	JMinCox1-**H4** (LC715190)	JMinClpc-**H5** (LC715201)	JMinEF2-**G2** (LC715207)
Nbat60	*Miniopterus fuliginosus*	JMinCytb-**H2** (LC668429)	JMinCox1-**H5** (LC715192)	JMinClpc-**H5** (LC715201)	JMinEF2-**G3** (LC715208)
Nbat62	*Miniopterus fuliginosus*	JMinCytb-**H2** (LC668429)	JMinCox1-**H4** (LC715193)	JMinClpc-**H5** (LC715201)	FA
Nbat64	*Miniopterus fuliginosus*	JMinCytb-**H3** (LC668430)	JMinCox1-**H6** (LC715191)	JMinClpc-**H6** (LC715202)	JMinEF2-**G2** (LC715207)
Batfly36-9	*Nycteribia* sp. (bat fly)	JMinCytb-**H3** (LC715186)	FA	FA	FA
P. murinus					
Nbat51	*Myotis macrodactylus*	JMyoCytb-**H1** (LC668431)	JMyoCox1-**H1** (LC715194)	FA	FA
Nbat65	*Myotis macrodactylus*	JMyoCytb-**H2** (LC668432)	JMyoCox1-**H1** (LC715195)	JMyoClpc-**H1** (LC715203)	FA
Nbat69	*Myotis macrodactylus*	JMyoCytb-**H1** (LC668431)	JMyoCox1-**H1** (LC715195)	JMyoClpc-**H2** (LC715204)	FA
Nbat71	*Myotis macrodactylus*	JMyoCytb-**H3** (LC668433)	JMyoCox1-**H2** (LC715196)	JMyoClpc-**H3** (LC715205)	FA

Haplotype/genotype name with DDBJ/EMBL/GenBank accession number is presented in parentheses. “FA” denotes no obtained sequence due to failure of successful PCR amplification of the gene segment.

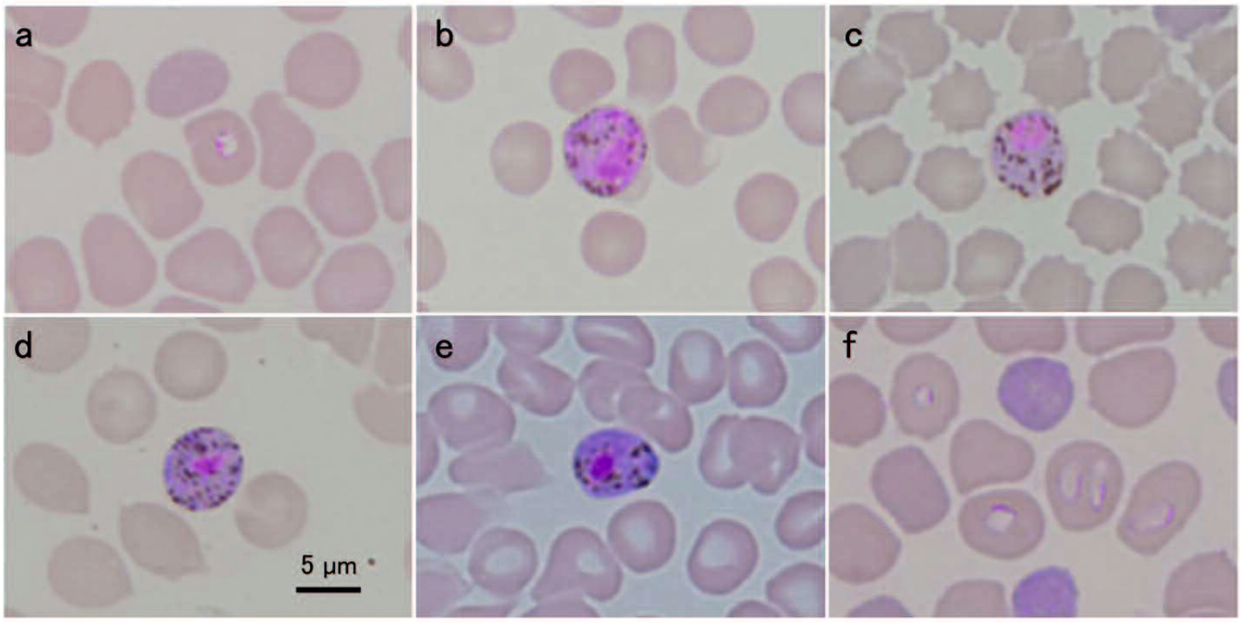

Figure 7. Hemosporidian parasites detected in the erythrocytes of **a–e** *Miniopterus fuliginosus* and **f** *Myotis macrodactylus* in Yamaguchi Prefecture, Japan. **a** *Polychromophilus melanipherus* trophozoite, **b, c** microgametocyte, **d,** e macrogametocyte, and **f.** *Polychromophilus murinus* trophozoites. All photographs are shown at the same magnification.

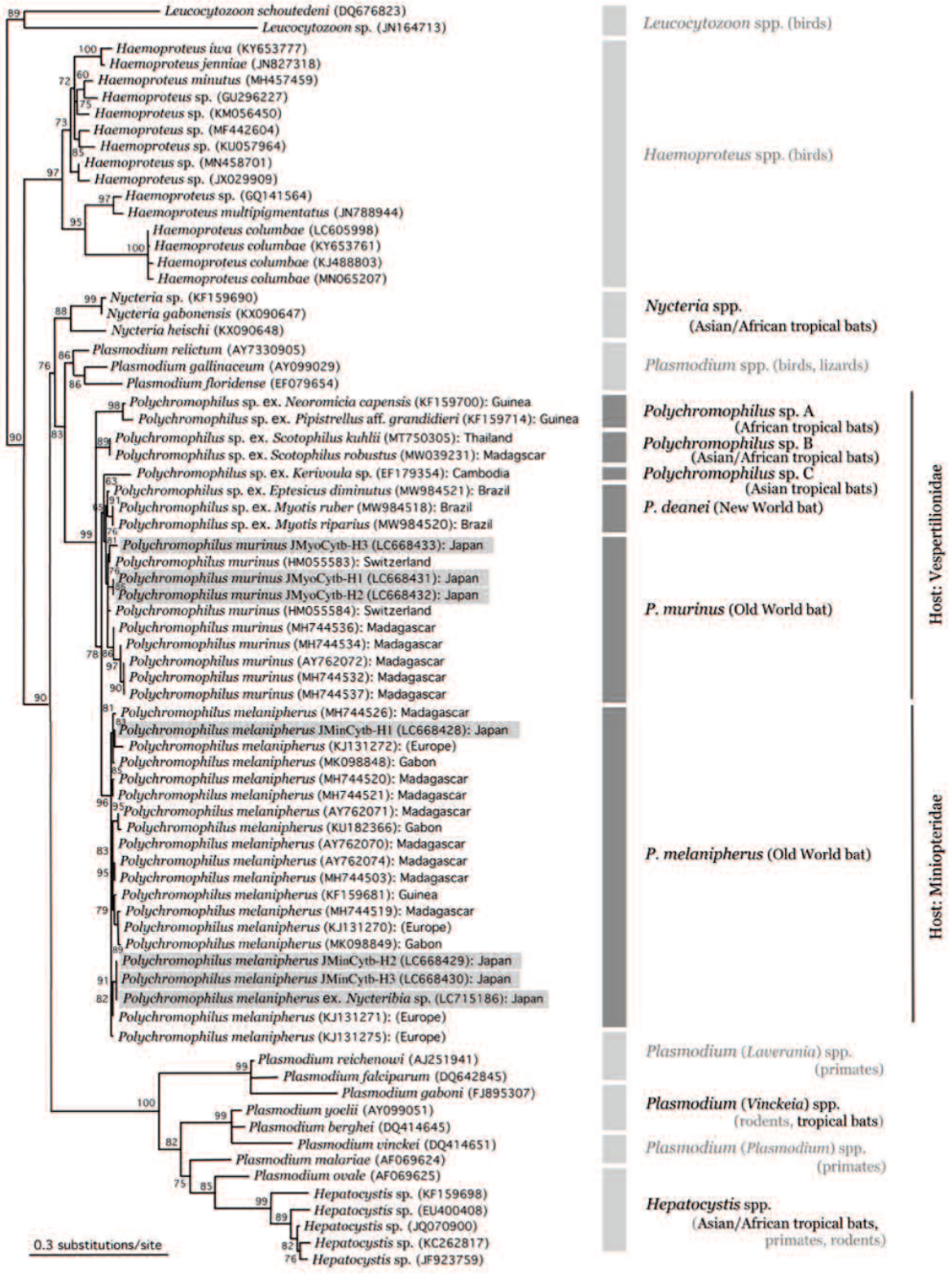

Figure 8. Maximum likelihood phylogenetic tree based on the *cytb* nucleotide sequence. *Leucocytozoon* spp. are placed as an outgroup. For *Polychromophilus* spp., the species names are followed by DDBJ/ EMBL/GenBank accession numbers in parentheses and geographical origins are omitted. The newly sequenced isolate (representative *cytb* haplotypes, which are shown after species names) is indicated with a gray background.

Figure 9. Maximum likelihood phylogenetic tree based on the *cox-1* nucleotide sequence. Primate *Plasmodium* spp. and *Hepatocystis* spp. are placed as an outgroup. Sequences are labeled as described in the legend for Figure 8.

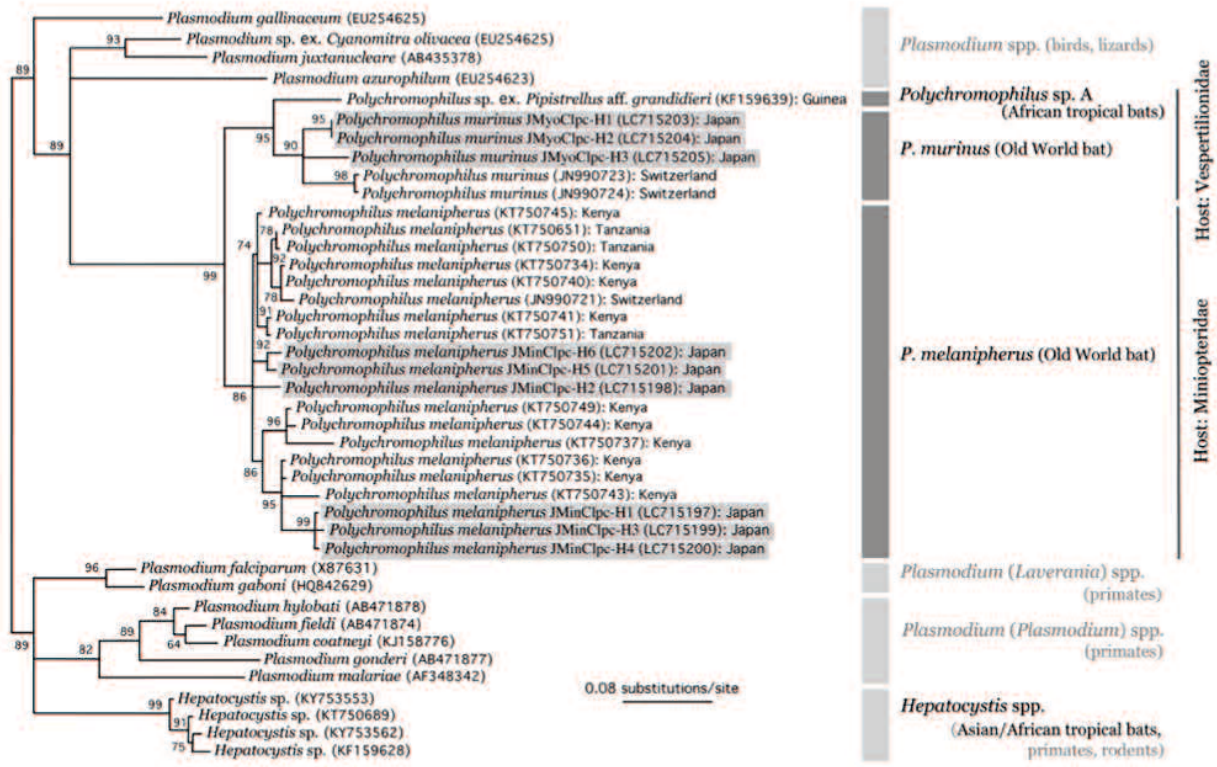

Figure 10. Maximum likelihood phylogenetic tree based on the *clpc* nucleotide sequence. Primate *Plasmodium* spp. and *Hepatocystis* spp. are placed as an outgroup. Sequences are labeled as described in the legend for Figure 8.

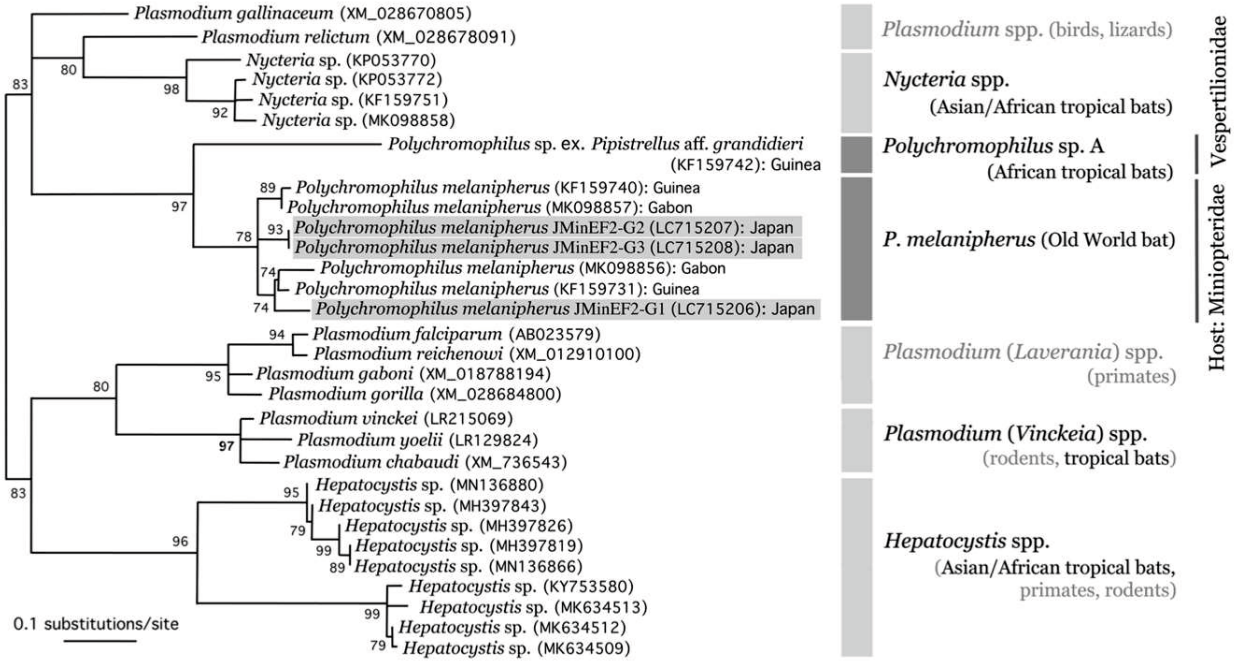

Figure 11. Maximum likelihood phylogenetic tree based on the EF2 nucleotide sequence. Mammalian *Plasmodium* spp. and *Hepatocystis* spp. are placed as an outgroup. Sequences are labeled as described in the legend for Figure 8.

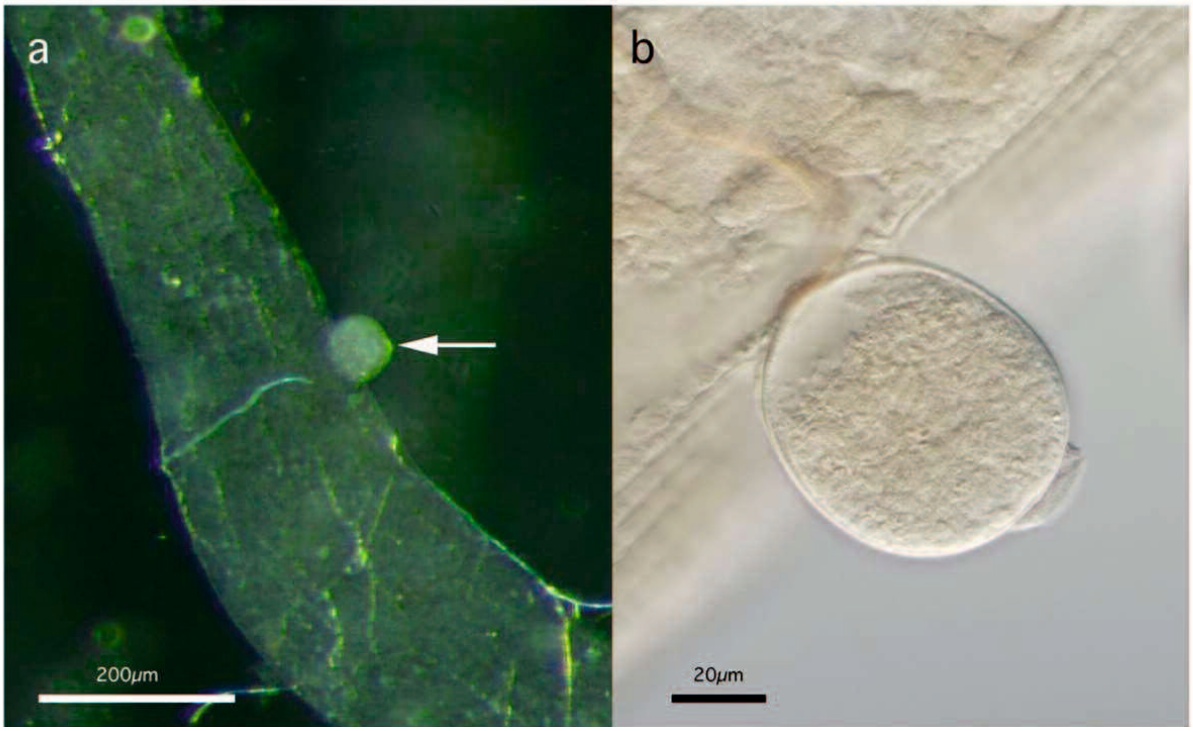

Figure 12. *Polychromophilus melanipherus* oocyst on the external surface of the midgut of a *nycteribiid* bat fly, **a** under a dissection microscope (arrow) and **b** under a conventional light microscope.

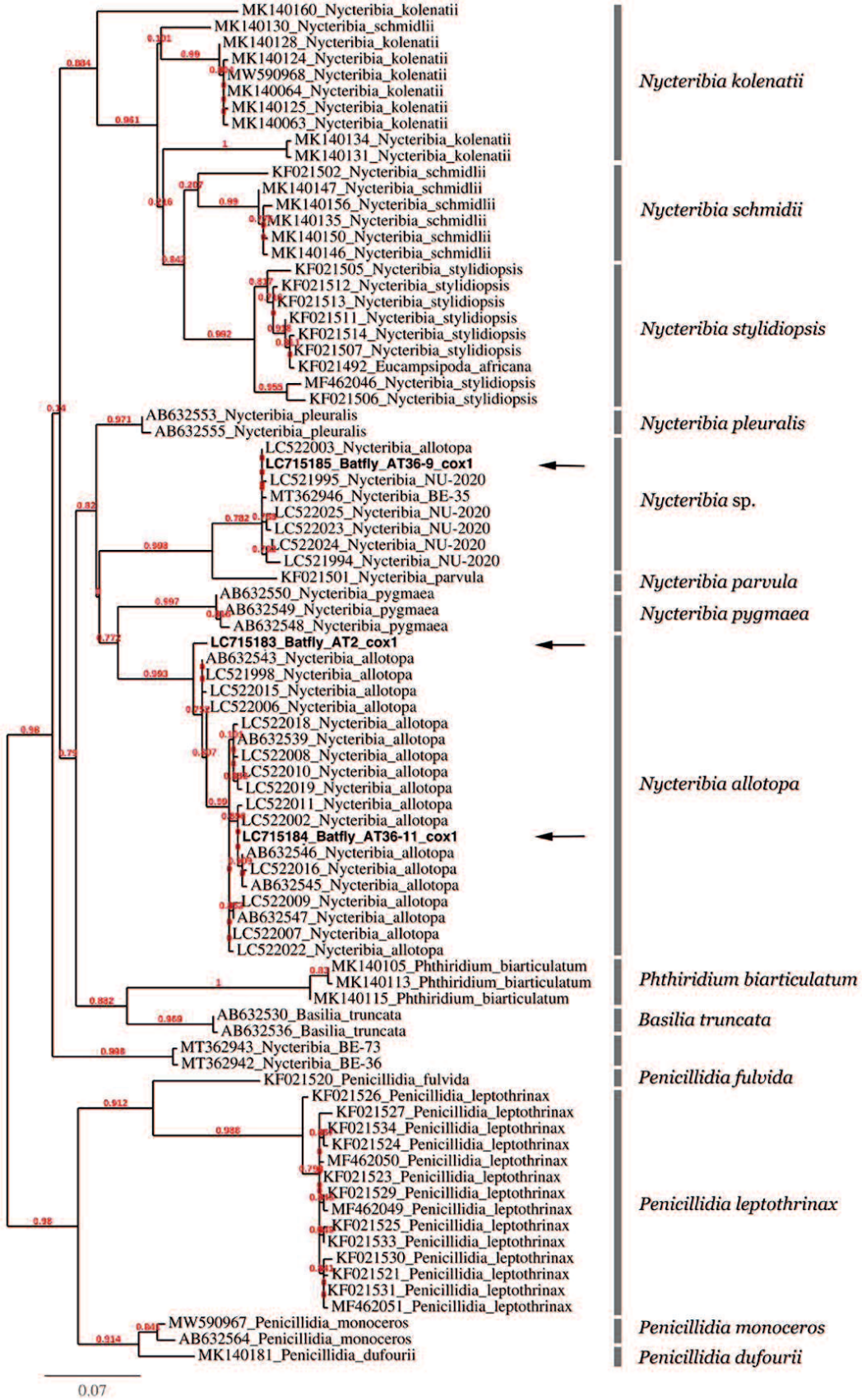

Figure 13. Maximum likelihood phylogenetic tree based on the *cox-1* nucleotide sequence (658-bp) for bat fly identification.

CHAPTER III

Genetic diversity of cervid *Trypanosoma theileri* in Honshu sika deer (*Cervus nippon*) in Japan

Information described in this chapter has been published as follows:

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Abstract

The taxonomy of ruminant *Trypanosoma theileri* and its relatives (Kinetoplastida: Trypanosomatidae) is controversial, with recent phylogenetic studies segregating *T. theileri* in cattle and other ruminants worldwide into two major genetic lineages (the TthI and TthII clades) based on genetic markers. In the present study, *T. theileri*-like trypanosomes isolated from Honshu sika deer (*Cervus nippon*) in the western Japan (YMG isolate) were genetically characterized using a number of genetic markers. Sika deer trypanosomes of the YMG isolate were genetically different from the *Trypanosoma* sp. TSD1 isolate previously recorded from Hokkaido sika deer in northern Japan, with the former trypanosome isolate being genetically closer to European cervid trypanosomes and the bovine *T. theileri* TthII lineage. In contrast, the latter isolate exhibited greater relatedness to North American cervid trypanosomes and the bovine *T. theileri* TthI lineage, although a clear genetic distinction between these was apparent. Furthermore, trypanosomes in Honshu sika deer from the central part of Japan harboured additional genetic diversity and were closer to either TSD1 or YMG isolates, while distinct from known *T. theileri*-related genotypes. Importantly, cervids and wild ruminants worldwide might harbour divergent descendants of a *T. theileri* ancestor, which exhibit rigid host specificity to either bovines or cervid species.

Introduction

Trypanosoma theileri Laveran, 1902 (Euglenozoa: Kinetoplastida: Trypanosomatidae) is a cosmopolitan trypanosome of bovines, including domestic cattle, Zebu cattle, water buffaloes, European bison *Bison bonasus (L.)* and various African antelopes. It is transmitted *via* horse-flies, members of *Tabanidae* (Touré, 1968; Hoare, 1972; Schlafer, 1979; Kingston *et al*., 1986; Böse *et al.*, 1987a; Böse *et al.,* 1987b; D'Alessandro and Behr, 1991). Although appreciable numbers of *T. theileri* are occasionally found in the blood of cattle and other

bovines, the infection is latent without any specific symptoms in healthy-looking ruminants (Hoare, 1972; D'Alessandro and Behr, 1991), detectable only *via* haemoculture (Touré, 1968; Schlafer, 1979) prior to the genetic era. Extremely low levels of parasitaemia persist, however, often for more than one year (Hoare, 1972). Classically, large-sized stercorarian trypanosomes such as *T. theireli* in mammals are classified in the subgenus *Megatrypanum* Hoare, 1964 (Section Stercoraria). The hosts of *Megatrypanum* trypanosomes include ruminants (Table 9), monotremes, marsupials, insectivores, bats, edentates and monkeys, among others (Hoare, 1972; Kingston and Morton, 1975; Kingston *et al.*, 1992; Wita and Kingston, 1999; Hamilton *et al.*, 2004, 2005; Thompson *et al*., 2014). Recent phylogenetic studies have demonstrated that this subgenus is solely based on morphological criteria and is clearly polyphyletic, lacking evolutionary as well as taxonomic relevance, thus considered *nomen nudum* in the latest classification (Stevens *et al.*, 1999; Hamilton *et al.*, 2005; Hamilton *et al*., 2007). In the present study, however, the term 'Megatrypanum-type' is used solely for convenience in order to specify trypanosomes of unique *T. theileri*-like morphology without taxonomical implications.

Since most of Megatrypanum-type species from non-bovines (see Table 9) were identified based on morphological characterization using trypanosomes occasionally found on blood smears, it was difficult to assume their precise taxonomic positions or relationships with *T. theileri* in the past. However, strict host specificity has been demonstrated *via* cross-infection experiments of *T. theileri* in sheep, *T. melophagium* in cattle (Hoare, 1972; D'Alessandro and Behr, 1991) and *T. theileri* in deer (Böse *et al.*, 1987a; Fisher *et al.*, 2013), suggesting their taxonomic relationships as independent species. A successful experimental infection of American bison *Bison bison (L.)* with *T. theileri* of cattle origin has been previously reported, indicating that cattle and bison share trypanosome species (Kingston *et al.*, 1986). With regard to the Megatrypanum-type trypanosomes in wild ruminants, including deer, cross-infection experiments are a challenge, with the oral application of tabanids containing cervid

trypanosomes being successful only in fallow deer and not in cattle calves (Böse *et al.*, 1987a), indicative of the parasites' rigid host specificity. Due to the taxonomic uncertainty with regard to deer trypanosomes, in the present study, '*T. cf. cervi*' and '*T. cf. stefanskii*' are used for convenience to describe Megatrypanum-type trypanosomes prevalent in North American and European cervids, respectively.

Modern molecular technology utilizing various genetic markers has demonstrated a close evolutional relationship between *T. theileri* and Megatrypanum-type trypanosomes in wild ruminants (Rodrigues *et al.*, 2003, 2006, 2010; Hamilton *et al.*, 2005, 2009; Adriana C. Rodrigues *et al.*, 2010; Gibson *et al.*, 2010; Garcia, *et al.*, 2011a, 2011b, 2020; Martinković *et al.*, 2012; Fisher *et al.*, 2013). In parallel, these studies have highlighted the genetic divergence of *T. theileri* and related Megatrypanum-type trypanosomes of different host and geographical origins based on multiple genetic markers such as the ribosomal RNA gene (rDNA), the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene, the spliced leader RNA (SL) gene and cathepsin L-like cysteine protease (CatL-like) genes (Rodrigues *et al.*, 2006, 2010a, 2010b; Garcia, *et al.*, 2011a, 2011b, 2020; Martinković *et al.*, 2012; Yokoyama *et al.*, 2015; Pacheco *et al.*, 2018).

In the present study, trypanosomes isolated from Honshu sika deer *Cervus nippon aplodontus* (Heude, 1884) in Yamaguchi Prefecture, the westernmost part of Honshu Island, Japan, were genetically characterized based on the multiple genetic markers mentioned above. The Megatrypanum-type trypanosome isolate, referred to as YMG, was distinct from the TSD1 isolate from a Hokkaido sika deer *C. nippon yesoensis* (Heude, 1884) on Hokkaido Island, the northernmost part of Japan, recorded previously (Hatama *et al.*, 2007). In other words, the present study demonstrated that two distinct genetic lineages of cervid Megatrypanum-type trypanosomes, fairly closer to '*T. cf. stefanskii'* and '*T. cf. cervi*', are distributed across Japan, having Honshu sika deer (at least *C. n. aplodontus*) and Hokkaido sika deer (*C. n. yesoensis*)

as natural hosts, respectively. Furthermore, the analyses of additional cervid trypanosomes in the Kii Peninsula around the central part of Honshu suggest the more complicated genetic diversity of Megatrypanum-type sika deer trypanosomes.

Materials and methods

Sample collection and trypanosome detection

To control the population size of sika deer (*C. n. aplodontus*) and wild boars *Sus scrofa leucomystax* Temminck in rural areas of Shimonoseki City, Yamaguchi Prefecture, Japan, a maximum of 1500 animals for each species are shot throughout the year based on the annual plan introduced by the municipal office. Wild mammals shot as part of this program were analyzed in the present study. Cardiac blood samples from 75 male and 40 female deer were collected monthly throughout a period between 9 November 2013 and 2 August 2014. In the same period, cardiac blood samples were also collected from 65 wild boars (21 males, 39 females and five animals of unknown sex). The blood was transferred from clean syringes to sterile vacuum blood collection tubes with EDTA-2Na (TERUMO Co., Shibuya-ku, Tokyo, Japan), kept at 4 °C, and transported to the laboratory of Yamaguchi University within 5 h after collection. Thin blood smears for each sample were taken for microscopic examination after Giemsa staining. For a survey of *Borrelia* infection in deer, 0.2 mL of blood from individual samples was added to 4.0 mL of modified Barbour-Stoenner-Kelly (BSK) medium, using MEM-alpha (BioWest, Essen, Germany) as a substrate for CMRL-1066, and incubated at 32 °C. Bacterial growth checks were performed on day 7 of culture and at arbitrary intervals thereafter. When free or aggregated trypanosomes were detected in the culture supernatant, these were collected in Eppendorf tubes, washed repeatedly with cold MEM medium and preserved at −20 °C until use. A few drops of the supernatant with trypanosomes were placed on a clean glass slide, air dried, fixed in 100% methanol and processed for Giemsa staining.

Stained specimens were examined with a ×100 oil immersion objective lens, photographed at a magnification of ×1000 and edited with Adobe® Photoshop® ver. 11.0 (Adobe Systems, San Jose, California, USA). Photographs were then printed at a high magnification. Printed photographs were analyzed as described previously (Sato *et al.*, 2008).

Similarly, one and 10 cardiac blood samples were obtained from deer hunted in Tanabe City, Wakayama Prefecture, on 20 November 2008, and in Kushimoto Town, Wakayama Prefecture, on 14 October 2009, respectively. Hunting was conducted under the municipal annual plan to control the population size of sika deer. The blood samples were collected in sterile vacuum blood collection tubes with EDTA-2Na kept at 4 °C and were transported to the laboratory of Hyogo University of Health Sciences within 24 h after collection. Thin blood smears for each sample were taken for microscopic examination after Giemsa staining. These 11 deer blood samples were not used for culture, but for DNA extraction.

From slaughtered cattle at the Nanko Division of Osaka Municipal Central Market, 175 blood samples were collected on four separate days during the period between 10 and 31 August 2015. These cattle were bred in 19 prefectures throughout Japan and included 93 Japanese black breed, seven Holstein-Friesian breed, and 75 F1 between the Japanese black and Holstein- Friesian breeds. Blood samples were collected in sterile vacuum blood collection tubes with EDTA-2Na kept at 4 °C and were transported to the laboratory of Yamaguchi University within 32 h of collection. Thin blood smears for each sample were taken for microscopic examination after Giemsa staining. Simultaneously, approximately 0.2mL of each blood sample were added to two culture wells of 24-well culture plates (MS-80240; Sumitomo Bakelite Co., Shinagawa-ku, Tokyo, Japan), containing 1.5 mL well^{-1} of RPMI1640 medium (Nissui Pharmaceutical Co., Sugamo, Tokyo, Japan) supplemented with 0.3% L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B and 10% heat-inactivated fetal bovine serum, in addition to feeder cells placed according to (Sato *et al.*, 2003).

Feeder cells used in the present study were derived from the primary cell culture of kidney cells from a deer fetus. Culture was maintained under sterile conditions of 5% CO2 at 37 °C and checked weekly under an inverted microscope. Blood and culture smear slides were deposited in the Meguro Parasitological Museum, Tokyo, Japan.

DNA extraction, polymerase chain reaction (PCR) and nucleotide sequencing

Parasite DNA was extracted from the primary trypanosome-positive haemocultures of deer collected in Yamaguchi Prefecture and cattle using an Illustra^{TM} tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the instructions of the manufacturer. Blood DNA of Wakayama Prefecture deer was extracted from 0.2 mL of each blood sample using a nucleic acid purification kit, MagExtractor^{TM}-Genome (TOYOBO, Dojima Hama, Osaka, Japan), according to the instructions of the manufacturer. PCR amplification of partially overlapping rDNA fragments was performed in a 20 μL solution containing a DNA polymerase, Blend Taq-Plus (TOYOBO), and primers, as previously described (Sato *et al*., 2005). The PCR cycling protocol for rDNA fragments included 3 min at 94 °C, 40 cycles of 45 s at 94 °C, 1 min at 64, 62 or 60 °C (according to primer pairs), and 1 min at 72 °C, followed by a final extension step at 72 °C for 7 min. The hypervariable region (V7/V8) of the 18S rDNA was amplified *via* nested PCR using the TRY927F and TRY927R primer pair in the first round and SSU561F with SSU561R in the second round (see Sato et al., 2005). The gGAPDH gene was amplified *via* nested PCR with degenerate primers G3 and G5 in the first round, followed by G1 and G4a or G1 and G4b in the second round, as previously described (Hamilton *et al.*, 2004; Sato *et al.*, 2008). PCR amplification of the catalytic domain-encoding region of CatL-like genes was performed using a combination of primers DTO154 (5′-ACA GAA TTC CAG GGC CAA TGC GGC TCG TGC TGG-3′) and DTO155 (5′ -TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA-3′) according to Cortez *et al*. (2009).

The PCR cycling protocol for CatL-like genes was 3 min at 94°C, 35 cycles of 1min at 94°C, 1 min at 56°C and 1 min at 72 °C, followed by a final extension step at 72 °C for 10 min. The PCR amplification of SL genes was performed using a combination of primers LSL1 (5′-TTC TGT ACT TCA TGG TAT G-3′) and LSL2 (5′ -CCA ATG AAG TAC AGA AAC TG-3′) as per Rodrigues *et al*. (2010a). The PCR cycling protocol for SL genes was 3 min at 94°C, 35 cycles of 1 min at 94°C, 2 min at 50 °C and 2 min at 72 °C, followed by a final extension at 72 °C for 10 min. When direct sequencing was not satisfactory, the purified PCR products were cloned into a plasmid vector, pTA2 (TArget Clone™; TOYOBO), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.), and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

Alignment and phylogenetic analysis

The rDNA, gGAPDH gene, CatL-like gene and SL gene sequences of trypanosome isolates obtained in the present study (DDBJ/ EMBL/GenBank accession nos. LC618030–LC618052) and related sequences 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 Mfold web server (Zuker, 2003) was employed for predicting the secondary structure of partial 18S rDNA molecules (V7/V8 hypervariable region) using the energy minimization approach for each sequence. The accession numbers of the sequences analysed for each gene fragment are given in Table 10 and figures showing phylogenetic trees. Regions judged to be poorly aligned and characters with a gap in any sequences were excluded from subsequent analyses: 623 characters, of which 198 were variable, remained for subsequent gGAPDH gene analysis; 450 characters, of which 90 were

variable, remained for subsequent CatL-like gene analysis; and 658 characters, of which 119 were variable, remained for subsequent SL gene analysis. Additional CatL-like gene analysis including *T. trinaperronei* sequences (accession nos. MN747149–MN747155) was conducted using 256 characters, of which 60 were variable. *Trypanosoma trinaperronei* Teixeira, Camargo et García, 2020 is a recently proposed Megatrypanum-type trypanosome species from the white-tailed deer in Venezuela (Garcia *et al.*, 2020). Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel, 2003; Dereeper et al., 2008) available on the 'phylogeny.fr' website (http://www.phylogeny.fr/). The probability of inferred branches was assessed by the approximate likelihood-ratio test, an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel, 2006). Relationships of different 18S rDNA V7/V8 genotypes were visualized using an automated haplotype network layout and visualization software 'HapStar', downloaded at http://fo.am/hapstar (Teacher and Griffiths, 2011). As variable lengths of the hypervariable 18S rDNA arising due to sites of nucleotide insertion/deletion (indel) were applied to this software in the present study, the position of a deleted nucleotide was treated as 'X' character, and the numbers of nucleotide differences between sequences were counted for the analysis (Tabel 11).

Results

Incidence of trypanosomes in deer and cattle

Blood samples were collected monthly from two to 28 deer in Yamaguchi Prefecture, and blood culture analysis detected trypanosome infection in eight (10.7%) male and five (12.5%) female deer (Figure 14A). Microscopic detection of trypanosomes on the blood smear was thoroughly attempted but unsuccessful except for in one blood sample with a few trypomastigotes (Figure 14B and C). Their measurements (n = 4) are summarized in Table 13 for comparison with other ruminant Megatrypanum-type *Trypanosoma* spp. Wild boar blood

culture detected no trypanosome infection. Six out of 11 (54.5%) deer from Wakayama Prefecture were positive for trypanosomes, as determined *via* nested PCR screening targeting the hypervariable region of 18S rDNA. Microscopic examination of blood smears revealed only one bloodstream form in a deer from Wakayama Prefecture (Figure 14D). Haemoculture of 175 cattle blood samples identified six (3.43%) positive animals bred and reared in the western part of Japan (see Table 10). Microscopic examination of blood smears revealed no trypanosomes in the peripheral blood of slaughtered cattle. Metacyclic trypomastigotes of *T. theileri* in haemoculture are shown in Figure 15.

Characterization of the rDNA of sika deer trypanosomes and cattle *T. theileri* in the western part of Japan

Three haemoculture samples of deer from Yamaguchi Prefecture (YMG-11, 14 and 15) exhibited no genetic variation in the rDNA sequence over 6777-bp length (DDBJ/EMBL/GenBank accession no. LC618030). Similarly, all six cultured trypanosome samples of cattle blood (OSK-16, 120, 148, 150, 155 and 172) showed no genetic variation in the rDNA sequence over 6132-bp length (DDBJ/EMBL/GenBank accession no. LC618031).

The hypervariable region of 18S rDNA is frequently utilized for detecting stercorarian trypanosomes in terrestrial vertebrates. The sequence was compared with those of *T. theileri* from cattle and bisons, *T. melophagium* from sheep, and other Megatrypanum-type trypanosomes from a variety of wild ruminants such as deer and antelopes (Table 11.). Compared sequences were divided into seven groups based on the hair-pin tip structure of the hypervariable region, corresponding to the 82nd to 92nd nucleotide of the amplicon as determined *via* nested PCR using the SSU561F and SSU561R primer pair for YMG isolate trypanosomes (LC618030), or to 82nd to 94th nucleotides of the *T. theileri* KM strain (TthI lineage) amplicon recorded in cattle from Japan (AB007814), as shown in Figure 16. The

relationships between ruminant Megatrypanum-type trypanosomes of various origins retrieved from the DDBJ/EMBL/GenBank databases are shown in Figure 17. Genotype 1 (loop-tip sequence UUUUCCCAUCUUU) and Genotype 4 (AUUCCCC--UUUC) represented *T. theileri* from domestic ruminants, corresponding to the 'TthI' and 'TthII' genotypes of *T. theileri* described by Rodrigues *et al*. (2006, 2010a, 2010b) and Garcia *et al*. (2011a, 2011b).

A majority of North American deer trypanosomes ('*T. cf. cervi*') represented Genotypes 2 (UUUUCCCAUUUUU) and 3 (UUUUCCCA–UUUU), having loop-tip nucleotide sequences closer to Genotype 1 (*T. theileri* TthI lineage). The TSD1 isolate of Megatrypanum-type trypanosomes, previously reported from a Hokkaido sika deer in the northernmost part of Japan, had a loop-tip nucleotide sequence identical to Genotype 1 and close loop-tip nucleotide sequences identical to Genotypes 2 and 3 ('*T. cf. cervi*'). In contrast, a majority of European deer trypanosomes ('*T. cf. stefanskii*') were of Genotype 6 (AUUCCCC— UUCU), having a loop-tip nucleotide sequence closer to Genotype 4 (*T. theileri* TthII lineage; AUUCCCC—UUUC). YMG isolates of Honshu sika deer trypanosomes and *T. trinaperronei* from the white-tailed deer in Venezuela and North America shared the same loop-tip nucleotide sequence of Genotype 5 (AUUCCCC--UCUU), which was closer to Genotype 4 (*T. theileri* TthII lineage) and Genotype 6 ('*T. cf. stefanskii*').

Trypanosomes from the sika deer in Tanabe and Kushimoto, Wakayama Pref. (TNB872, KSM941, 944, 946, 948 and 949 isolates) had several diverse genotypes, distinct from the YMG isolate (Genotype 5), representing Genotype 1 and additional non-specified genotypes (AUUCCCC--AUUC, AUUCCCC--ACUC or AUUCCCC--GUUU) (DDBJ/EMBL/GenBank accession no. LC618032–LC618036). Multiple genotypes were often found in a single host deer (TNB872, KSM946, KSM944 and KSM948). Megatrypanum-type trypanosomes from various antelopes in Africa and other isolates exhibited further additional genotypes (Figure 17).

Characterization of the ITS1 sequence of sika deer trypanosomes and cattle *T. theileri* in the western part of Japan

The ITS1 nucleotide sequence of the YMG isolate obtained in the present study was 282-bp long. Of *T. theileri* and ruminant Megatrypanum-type trypanosomes, 149 ITS1 sequences retrieved from the DDBJ/EMBL/GenBank databases (see Table 10), 186–272-bp in length, were aligned and grouped into 10 major groups. Alignment revealed a great amount of indels, including some repeated units of a few nucleotides. Of *T. theileri* TthI, the lowest nucleotide identity recorded among 27 sequences was 90.8%, excluding indels. Similarly, of *T. theileri* TthII from cattle in Brazil, Croatia and Japan, the lowest nucleotide identity among 25 retrieved sequences was 80.6%. OSK isolates of *T. theileri* TthII (LC618031) had identical ITS1 sequences with the species isolated from cattle in Mongolia (LC440408), Italy (MK163554), Austria (KY412803), USA (JX178186), the Philippines (LC546921) and Brazil (HQ664817). Between 52 nucleotide sequences of TthI and TthII *T. theileri* lineages, the lowest identity was 45.1%.

Trypanosoma trinaperronei from white-tailed deer from Venezuela and Texas (MN752208, MN752209 and JX178172) had highly similar ITS1 sequences (99.57–100% with one indel over 232/233-bp of length), but exhibited lower similarity to the YMG isolate (<90.52% with a great number of indels), although their 18S rDNA was of Genotype 5 (Figure 17). Venezuelan and North American isolates of *T. trinaperronei* and '*T. cf. stefanskii*' D30 (HQ664845 or AY773714) had ITS sequences with a similarity of 88.79% and frequent indels. ITS1 sequences of the YMG isolate and '*T. cf. stefanskii*' D30 exhibited 83.86% similarity with several indels. Between the YMG isolate of Genotype 5 and TSD isolate of Genotype 1 (AB569248), ITS regions showed 76.70% nucleotide identity with a great amount of indels.

Characterization of gGAPDH sequences of sika deer trypanosomes

Two gGAPDH nucleotide sequences of sika deer trypanosomes YMG-11 and YMG-14 isolates were identical to each other (DDBJ/EMBL/GenBank accession no. LC618037). An ML phylogenetic tree constructed with sequences available at the DDBJ/ EMBL/GenBank databases is shown in Figure 18, and the phylogenetic relationships of different taxa were almost similar to those observed based on the 18S rDNA. Intra-lineage identities of *T. theileri* nucleotide sequences were fairly high [the lowest identities in the TthI and TthII lineages were 98.94% (838/847) and 98.11% (831/847), respectively, and inter-lineage identities of nucleotide sequences varied between 91.97% (779/847) and 93.27% (790/847).

The gGAPDH nucleotide sequence of the YMG isolate exhibited higher identity to those of *T. theileri* TthII lineage [95.28% (807/847) −95.75% (811/847)] than those of *T. theileri* TthI lineages [highest 92.21% (781/847)]. The same YMG isolate nucleotide sequence showed 95.97% (785/818) identity with that of the '*T. cf. stefanskii*' D30 isolate from a fallow deer in Germany (HQ664806) and 96.58% (818/847) identity with that of *T. trinaperronei* WTD2268 in a white-tailed deer from Venezuela (MN756794). Between the *T. trinaperronei* WTD2268 (MN756794) and '*T. cf. stefanskii*' D30 isolate, a gGAPDH nucleotide identity of 97.76% (828/847) was observed. Two gGAPDH nucleotide sequences of the KSM isolate harboured 96.08% (616/638) similarity between each other, as well as 91.36% (772/845) and 91.69% (585/638) similarity with the YMG isolate sequence.

Characterization of CatL-like and SL gene nucleotide sequences of sika deer trypanosomes

CatL-like gene sequences, 450-bp in length, were obtained with one each for the YMG-11 (LC 618041), YMG-14 (LC618042) and YMG-15 (LC618043) isolates with identities ranging between 96.00% (432/450) and 99.78% (449/450). In the ML phylogenetic tree, deer

Megatrypanum-type trypanosomes formed an independent clade from the TthI and TthiII lineages of *T. theileri* and other African ruminant Megatrypanum-type trypanosomes (Figure 19). Nucleotide identities between the D30 isolate from *Cervus dama* in Germany ('*T. cf. stefanskii*') and the YMG isolate ranged between 94.44% (425/450) and 97.78% (440/450). Nucleotide identities of *T. trinaperronei* and the YMG isolate ranged between 92.97% (238/256) and 98.05% (251/256), while those of *T. trinaperronei* and the '*T. cf. stefanskii*' D30 ranged between 97.27% (249/256) and 98.44% (252/256).

As for the SL gene of Honshu sika deer trypanosomes, three YMG-11 clones and six YMG-14 clones were sequenced, and all of them differed in length, ranging between 891 and 922-bp due to some repeated units of a few nucleotides (LC618044– LC618052). To construct an ML phylogenetic tree based on SL sequences, these short-repeated units were omitted from the analysis. YMG-11 and YMG-14 sequences formed an independent branch from *T. melophagium* as well as *T. theileri* TthI and TthII lineages (Figure 20).

Discussion

In the current work, molecular genetic analyses of cultured trypanosomes isolated from the blood of Honshu sika deer in Japan (YMG isolate) revealed a unique divergent of cervid trypanosomes, distinct from either the TthI or TthII lineages of *T. theileri*, *T. melophagium* in domestic ruminants or cervid Megatrypanum-type trypanosomes in Europe ('*T. cf. stefanskii*') as well as those in the Americas ('*T. cf. cervi*' and *T. trinaperronei*). Further, the YMG isolate was also distinct from a previously known Japanese isolate of sika deer trypanosomes (TSD1 isolate) collected from a Hokkaido sika deer in the northern part of Japan (Hatama *et al.*, 2007), which was genetically closer to '*T. cf. cervi*' or bovine *T. theileri* TthI (Figure 17). It is likely that these two lineages of sika deer trypanosomes represent different phylogenetic populations of the parasite maintained by the southern and northern populations of sika deer in Japan, which

were recently identified to harbour differences in their mitochondrial genes (Tamate et al., 1998; Nagata et al., 1999; Ohdachi et al., 2010). These two host groups arrived in the eastern part of the Eurasian continent prior to their dispersal to the Japanese Archipelago from (1) the Korean Peninsula to Kyushu and Honshu Islands (southern group) and (2) the northern part of the continent (through Sakhalin Island) to Hokkaido Island (northern group). Phylogenetic segregation of Japanese sika dear into two genetically distinct populations, i.e. southern and northern groups, divides the Honshu sika deer (a subspecies of sika deer *C. nippon* Temminck, 1838) based on morphological aspects. The Kii Peninsula (Wakayama Prefecture), where additional sika deer trypanosomes were collected (TNB/KSM isolate), is located near the border of distribution between the southern and northern Honshu sika deer groups. Accordingly, it was hypothesized that TNB/KSM isolates might resemble either YMG, TSD1 or a mix of both isolates.

TNB/KSM isolates exhibited multiple genotypes, closer to either YMG or TSD1 isolates from Japanese sika deer, but identical to neither. The nucleotide variation observed in TNB/KSM trypanosome isolates was exceptional when considering the homogeneous nucleotide sequences of *T. theileri* from all six OSK culture isolates. Similarly, the 18S rDNA of all three YMG cultured trypanosome isolates were identical. Other researchers have also reported unexpected variations of *T. theileri* sequences using field-collected blood samples without culture (Rodrigues et al., 2010b; Garcia et al., 2011a). It is uncertain whether this phenomenon can be ascribed to the source of PCR templates (trypanosomes in the original blood or grown in the culture), or endogenous features of the target genes analysed. Due to the use of field-collected blood, only two additional gGAPDH sequences were obtained (Figure 18). The two gGAPDH sequences of TNB/KSM isolates showed a close relationship to the TthI lineage of *T. theileri*, and the same isolates exhibited an identical hair-pin loop-tip sequence (CCCAUCUUU) with the TthI lineage of bovine *T. theileri* (Figure 17). Although

sister phylogenetic relationships of *Trypanosoma cyclops* Weinman, 1972 as well as trypanosomes from Australian marsupials and terrestrial leeches to the *T. theileri* TthII clade are shown in Figure 18 as in previous studies (Hamilton *et al.*, 2005, 2007, 2009), the latest study by Ellis *et al*. (2021) has demonstrated that the *T. cyclops* clade containing newly collected leech trypanosomes forms a sister group to the *T. theileri* clade (including both TthI and TthII lineages, cervid *T. theileri*, and *T. melophagium*), indicating the monophyletic nature of ruminant Megatrypanum-type trypanosomes.

Using morphological criteria (e.g. pronounced undulating membrane, distinctly shorter free flagella and the greater body sizes of '*T. cervi'* compared to '*T. theileri*') and host specificity, '*T. cervi*' in North American cervids and *'T. stefanskii'* from European cervids were described as independent species (Kingston and Morton, 1975; Kingston et al., 1985, 1992). Morphological variations in the bloodstream form of deer trypanosomes were reported to hamper precise species identification (Kingston *et al.*, 1985, 1992). Based on isoenzyme profile differences, Böse *et al*. (1993) suggested that there are probably at least two different species of cervid Megatrypanum-type trypanosomes in Europe, one parasitizing roe deer and the other infecting red deer and fallow deer. Using the same biochemical technique, Dirie *et al*. (1990) described the uniqueness of Megatrypanum-type trypanosomes in Swedish reindeer (*Rangifer tarandus*) compared to those found in American cervids and Swedish moose (*Alces alces*). The mosaic distribution of at least two Megatrypanum-type cervid trypanosomes has not yet been demonstrated *via* rDNA and gGAPDH sequencing, although Fisher *et al*. (2013) identified two genetic lineages of Megatrypanum-type trypanosomes from the white-tailed deer in Texas based on the rDNA sequences. Recently, Garcia *et al*. (2020) proposed a new species *T. trinaperronei* for one cervid trypanosome lineage found in the white-tailed deer in Venezuela, and they postulated its evolution from the ancestor originating from Eurasia after transcontinental movement through the Bering Strait *via* the Bering Land Bridge and the

Panama Isthmus. According to this *T. trinaperronei* evolutionary scenario in Venezuela, the discovery of the YMG isolate in Japanese sika deer is essential for connecting the European cervid trypanosome D30 isolate from fallow deer (Germany) to the newly proposed species. The distribution of the TSD1 isolate, closely related to '*T. cf. cervi*', might be explained by the reverse transcontinental movement of some deer from North America to Siberia through the Bering Land Bridge during the glacial period. At the same time, it can be postulated that limited research on cervid Megatrypanum-type trypanosomes and the use of haemoculture might constrain the field discovery of existing trypanosome lineages, as suggested in this study (TNB/KSM isolates; see Figure 17). Garcia *et al*. (2011b) suggested that new genotypes (genetic lineages) are yet to be discovered in unexplored regions after they identified unknown local genotypes of *T. theileri* in cattle from Thailand.

Molecular research on *T. theileri* in cattle using the above-described genetic markers has suggested that phylogenetic relationships among trypanosome sequences are congruent with phylogeny based on other gene sequences (Cortez *et al.*, 2009; Garcia *et al.*, 2020), although the resolution degree of lineages or population diversity differs based on the gene markers assessed in relation to the inherent nucleotide conservation/divergence as well as sequencing span. Consistently, molecular studies on *T. theileri* indicated that the species of bovine '*T. theileri*' include at least two major lineages (TthI and TthII) distributed through-out the world, probably *via* the anthropogenic dispersal of their hosts, i.e. domestic ruminants (Rodrigues *et al.*, 2006, 2010a; b), even though the distribution of other *T. theileri* genotypes has been suggested in cattle from Thailand, Philippines or Sri Lanka as well as antelopes in Africa (Garcia *et al.*, 2011a; Auty *et al.*, 2012; Yokoyama *et al.*, 2015). Based on the nucleotide sequences of the rDNA hypervariable region, Megatrypanum-type trypanosomes in North American and European cervids are divided into at least two major lineages, both distinct yet closer to TthI and TthII lineages of *T. theileri* in cattle, which might correspond to species

classically grouped as '*T. cf. cervi*' and '*T. cf. stefanskii*', respectively (Figure 17). Prior to this study, the characterization of *T. trinaperronei* as *T. theileri*-like trypanosomes in white-tailed deer across the North and Central Americans (Garcia *et al.*, 2020) seemed reasonable, since trypanosomes of the TthII-related lineage of cervid trypanosomes ('*T. cf. stefanskii*', typically observed in Europe) represent a special case in the North American continent where TthI-related lineage of cervid trypanosomes might be prevalent ('*T. cf. cervi*', typically observed in North America). The current study partially confirms their speculation, as the YMG isolate, which has closer phylogenetic relationships with '*T. cf. stefanskii*' and *T. trinaperronei*, is distributed in Japan, which is located in the Far East, as well as near the continental border between the Old World and the New World. Currently, the new YMG isolate seems to occupy an intermediate phylogenetic position between the aforementioned two species, raising the question of where to draw the geographical boundary between species. Furthermore, another lineage of sika deer trypanosomes closer to either '*T. cf. stefanskii*' or '*T. cf. cervi*' (TNB/ KSM isolates) were isolated in the Japanese mainland (Honshu Island), in addition to the previously reported sika deer TSD1 trypanosomes isolate, which is phylogenetically closer to '*T. cf. cervi*', but still genetically distinct from it. It is highly probable that multiple diverse lineages of Megatrypanum-type trypanosomes can be obtained from cervids in the Far East and other continents. Thus, in order to avoid taxonomic confusion, we propose that *T. theileri* and *T. theileri*-like trypanosomes in ruminants are classified solely as '*T. theileri*', and are differentiated by lineage tags as has been recently done by various researchers (Rodrigues *et al.*, 2006, 2010a; b; Garcia *et al.*, 2011a; b, 2020; Ybañez *et al.*, 2013; Weerasooriya *et al.*, 2016). As far as retaining bovine '*T. theileri*' for both TthI and TthII lineages since this nomenclature is widely accepted at present, we suggest that '*T. melophagium'*, '*T. cervi'*, '*T. stefanskii'* and '*T. trinaperronei*' should be synonymous to *T. theileri* regardless of host specificity. The taxonomic classification of three other species, i.e. *T. ingens*, *T. mazamarum*

and *T. threodori*, remains uncertain due to a lack of genetic information. When considering that multiple lineages of cervid *T. theileri* (e.g. Japanese sika deer trypanosome isolates, genetically closer to either bovine *T. theileri* TthI or TthII, but identical to neither) could be isolated from an individual host or a local population, the use of different species names for each lineage is not feasible. Furthermore, as observed for the YMG isolate, it is difficult to define a clear border between the YMG isolate and *T. trinaperronei* or '*T. cf. stefanskii*', even if they use different vectors, as emphasized by Votýpka *et al*. (2015) with regard to species classification. Further collection of samples and genetic data of *T. theileri* and *T. theileri*-like trypanosomes (i.e., Megatrypanum-type) from wild ruminants in different regions and continents is of great relevance for understanding their biodiversity and biogeography or, in other words, the clonal structure of *T. theileri*.

Table 9. Nominal ruminant *Trypanosoma* spp. of Megatrypanum-type morphology[1].

Species	**Representative hosts recorded**
T. theileri Laveran, 1902	cattle, water buffaloe, bison, various African antelopes, etc.
T. ingens Bruce, Bruce, Hamerton, Bateman et Mackie, 1909	Cattle in Africa, African antelopes (Uganda, Congo, etc.), African and Asian chevrotains
T. mazamarum Mazza, Romaña et Fiora, 1932	Brocket deer (*Mazama* spp.) in South America
T. melophagium (Flu, 1908) Nöller, 1917	Sheep
T. threodori Hoare, 1931	Goat
T. cervi Kingston et Morton, 1975	North American cervids (elk, Alaskan moose, white-tailed deer, mule deer, reindeer) red deer in Poland
T. stefanskii Kingston, Bobek et Perzanowski, 1992	European cervids (roe deer, red deer, fallow deer)
T. trinaperronei Teixeira, Camargo et García, 2020	White-tailed deer in Venezuela and North America

[1] References: Hoare, 1972; Kingston and Morton, 1975; Kingston *et al.* 1992; Wita and Kingston, 1999; García *et al.* 2020

Table 10. DNA sequences used for phylogenetic analyses in this study.

GenBank Accession no.	Species name	Sample ID (Isolate name_ Clone name)	Host species	Locality
1. 18S rDNA sequences				
1.1. Newly obtained in the present study				
LC618030	*T. theileri*	YMG-11	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
		YMG-14	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
		YMG-15	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618031	*T. theileri*	OSK-16	*Bos taurus*	Japan: Miyazaki
		OSK-120	*Bos taurus*	Japan: Shimane-Hiroshima
		OSK-148	*Bos taurus*	Japan: Miyazaki-Saga
		OSK-150	*Bos taurus*	Japan: Nagasaki-Saga
		OSK-155	*Bos taurus*	Japan: Miyazaki-Nagano
		OSK-172	*Bos taurus*	Japan: Kagoshima-Kumamoto
LC618032	*T. theileri*	KSM-941_c7, c9, c10	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		KSM-946_c2, c4, c5	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
LC618033	*T. theileri*	TNB-872_c5, c7, c8	*Cervus nippon aplodontus*	Japan: Tanabe, Wakayama
		KSM-944_c1, c4	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
LC618034	*T. theileri*	TNB-872_c1, c2	*Cervus nippon aplodontus*	Japan: Tanabe, Wakayama
LC618035	*T. theileri*	KSM-944_c1, c2, c3	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		TNB-872_c4, c6	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		KSM-946_c1	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		KSM-948_c1, c2, c3	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		KSM-949_c2, c3	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
LC618036	*T. theileri*	KSM-948_c2	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		KSM-950	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
1.2. Retrieved from the GenBank				
AB007814	*T. theileri*	KM	*Bos taurus*	Japan: Toyama
AB569248	*Trypanosoma* sp.	TSD1	*Cervus nippon yesoensis*	Japan: Hokkaido
AB569249	*T. theileri*	Esashi_9	*Bos taurus*	Japan: Hokkaido
AB569250	*T. theileri*	Esashi_12	*Bos taurus*	Japan: Hokkaido
AJ009163	*T. theileri*	TREU_124	*Bos taurus*	Scotland
AJ009164	*T. theileri*	K127	*Bos taurus*	Germany
AJ009165	*Trypanosoma* sp.	D30	*Cervus dama*	Germany
AY773689	*T. theileri*	Tthc5	*Bos taurus*	Brazil: Dourados, Mato Grosso do Sul
AY773692	*T. theileri*	Tthc14	*Bos taurus*	Brazil: Miranda, Mato Grosso do Sul
FM202489	*T. theileri*	–	*Tragelaphus_spekei*	Cameroon
FM202490	*T. theileri*	1	*Cephalophus monticola*	Cameroon
FM202491	*T. theileri*	*2*	*Cephalophus monticola*	Cameroon
FN666409	*T. melophagium*	St Kilda	*Melophagus ovinus*	UK: St Kilda
GQ176155	*T. theileri*	Tthc28	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ176157	*T. theileri*	Tthc30	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664909	*T. theileri*	TthcV2	*Bos taurus*	Venezuela: El Baúl, Cojedes
HQ664912	*T. melophagium*	TmHR1	*Melophagus ovinus*	Croatia: Goljak, Zagreb
JN798594	*Trypanosoma* sp.	ACR-2011_TC2	*Cervus elaphus*	Croatia
JX178162	*T. theileri*	Cow-2095_Cl-8	*Bos taurus*	USA: Webb, Texas
JX178163	*T. theileri*	Cow-2095_Cl-9	*Bos taurus*	USA: Webb, Texas
JX178164	*T. theileri*	Cow-2_Cl-4	*Bos taurus*	USA: Webb, Texas
JX178165	*T. theileri*	Cow-2_Cl-7	*Bos taurus*	USA: Webb, Texas
JX178166	*T. theileri*	Cow-2_Cl-10	*Bos taurus*	USA: Webb, Texas
JX178167	*T. theileri*	Cow-3535_Cl-3	*Bos taurus*	USA: Webb, Texas
JX178168	*T. theileri*	Cow-3535_Cl-6	*Bos taurus*	USA: Webb, Texas
JX178169	*T.* cf. *cervi*	WTD-NL15_Cl-1	*Odocoileus virginianus*	USA: Zapata, Texas
JX178170	*T.* cf. *cervi*	WTD-NL15_Cl-6	*Odocoileus virginianus*	USA: Zapata, Texas
JX178171	*T.* cf. *cervi*	WTD-NL15_Cl-9	*Odocoileus virginianus*	USA: Zapata, Texas
JX178172	*Trypanosoma* sp.	WTD-A3_Cl-9.1	*Odocoileus virginianus*	USA: Zapata, Texas
JX178174	*T.* cf. *cervi*	WTD-A21_Cl-4	*Odocoileus virginianus*	USA: Zapata, Texas
JX178175	*T.* cf. *cervi*	WTD-A21_Cl-5	*Odocoileus virginianus*	USA: Zapata, Texas
JX178176	*T.* cf. *cervi*	WTD-A21_Cl-6	*Odocoileus virginianus*	USA: Zapata, Texas
JX178177	*Trypanosoma* sp.	Elk-142_Cl-9	*Cervus canadensis*	USA: Minnesota
JX178178	*Trypanosoma* sp.	Elk-142_Cl-10	*Cervus canadensis*	USA: Minnesota
JX178179	*T.* cf. *cervi*	Elk-416_Cl-8	*Cervus canadensis*	USA: Minnesota
JX178180	*T.* cf. *cervi*	Elk-328_Cl-3	*Cervus canadensis*	USA: Minnesota
JX178181	*T. theileri*	Cow-2073_Cl-7	*Bos taurus*	USA: Starr, Texas
JX178183	*T. theileri*	Cow-139_Cl-9	*Bos taurus*	USA: Starr, Texas
JX178184	*T. theileri*	Cow-139_Cl-10	*Bos taurus*	USA: Starr, Texas
JX178185	*T. theileri*	Cow-139_Cl-11	*Bos taurus*	USA: Starr, Texas
JX178186	*T. theileri*	Cow-133_Cl-1	*Bos taurus*	USA: Starr, Texas
JX178187	*T. theileri*	Cow-104_Cl-1	*Bos taurus*	USA: Starr, Texas
JX178188	*T. theileri*	Cow-104_Cl-2	*Bos taurus*	USA: Starr, Texas
JX178192	*T.* cf. *cervi*	WTD-A1_Cl-1	*Odocoileus virginianus*	USA: Zapata, Texas
JX178193	*T.* cf. *cervi*	WTD-A1_Cl-4	*Odocoileus virginianus*	USA: Zapata, Texas
JX178194	*T.* cf. *cervi*	WTD-A21_Cl-4	*Odocoileus virginianus*	USA: Zapata, Texas
JX178195	*T.* cf. *cervi*	WTD-A21_Cl-6	*Odocoileus virginianus*	USA: Zapata, Texas
JX178196	*T.* cf. *cervi*	WTD-A5_Cl-1	*Odocoileus virginianus*	USA: Zapata, Texas
JX178197	*T.* cf. *cervi*	WTD148_Cl-4	*Odocoileus virginianus*	USA: Zapata, Texas
JX853182	*T.* cf. *cervi*	WTD-A1_6	*Odocoileus virginianus*	USA

JX853184	*Trypanosoma* sp.	Elk-421_2	*Cervus canadensis*	USA
JX853185	*T. theileri*	Cow-2095_4	*Bos taurus*	USA
KF765799	*T. theileri*	Bb813	*Bison bonasus bonasus*	Poland
KF765800	*T. theileri*	Bb845	*Bison bonasus bonasus*	Poland
KF765801	*T. theileri*	Bb756	*Bison bonasus bonasus*	Poland
KF765802	*T. theileri*	Bb816	*Bison bonasus bonasus*	Poland
KF924254	*T. theileri*	Bb812	*Bison bonasus bonasus*	Poland
KF924255	*T. theileri*	Bb821	*Bison bonasus bonasus*	Poland
KF924256	*T. theileri*	Bt7201Hka	*Bos taurus*	Poland
KF924257	*T. theileri*	Bt14ZA	*Bos taurus*	Poland
KJ195879	*T. theileri*	Cn1	*Cervus nippon holturolum*	Poland
KJ195884	*T. cervi*	DdP287	*Dama dama*	Poland
KJ195885	*Trypanosoma* sp.	DdP18	*Dama dama*	Poland
KJ397590	*Trypanosoma* sp.	Ce134	*Cervus elaphus*	Poland
KJ397591	*T. theileri*	Bb823	*Bison bonasus*	Poland
KJ397592	*T. theileri*	Bb871	*Bison bonasus*	Poland
KR024688	*T. theileri*	G24	*Glossina fuscipes fuscipes*	Central African Republic
KU587631	*T. theileri*	CBT-38	*Bos taurus*	Brazil: São Paulo
KU587641	*T. theileri*	CBT-115	*Bos taurus*	Brazil: Ji-Paraná, Rondônia
KU587643	*T. theileri*	CBT-120	*Bos taurus*	Brazil: Ji-Paraná, Rondônia
KU587646	*T. theileri*	CBT-123	*Bos taurus*	Brazil: Ji-Paraná, Rondônia
KY681802	*Trypanosoma* sp.	2016/SF5	*Phlebotomus perfiliewi*	Italy: Emilia-Romagna, Bologna
MN752212	*T. trinaperronei*	WTD-2268	*Odocoileus virginianus*	Venezuela: Simón Bolíar, Anzoátegui
2. ITS1 sequences				
2.1. Newly obtained in the present study				
LC618030	*T. theileri*	YMG-11	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
		YMG-14	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
		YMG-15	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618031	*T. theileri*	OSK-16	*Bos taurus*	Japan: Miyazaki
		OSK-120	*Bos taurus*	Japan: Shimane-Hiroshima
		OSK-148	*Bos taurus*	Japan: Miyazaki-Saga
		OSK-150	*Bos taurus*	Japan: Nagasaki-Saga
		OSK-155	*Bos taurus*	Japan: Miyazaki-Nagano
		OSK-172	*Bos taurus*	Japan: Kagoshima-Kumamoto
LC618032	*T. theileri*	KSM-941_c7, c9, c10	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
		KSM-946_c2, c4, c5	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
AB007814	*T. theileri*	KM	*Bos taurus*	Japan: Toyama
AB569248	*Trypanosoma* sp.	TSD1	*Cervus nippon yesoensis*	Japan: Hokkaido
AB569249	*T. theileri*	Esashi_9	*Bos taurus*	Japan: Hokkaido
AB569250	*T. theileri*	Esashi_12	*Bos taurus*	Japan: Hokkaido
AY773698	*T. theileri*	Tthc3	*Bos taurus*	Brazil: Eldorado, São Paulo
AY773699	*T. theileri*	Tthb6 (?)	*Bos taurus* (?)	Brazil: Eldorado, São Paulo (?)
AY773700	*T. theileri*	ATCC-30017_TthATCC	*Bos taurus*	USA: Pennsylvania
AY773701	*T. theileri*	Tthb4	*Bubalus bubalis*	Brazil: Registro, São Paulo
AY773702	*T. theileri*	Tthb12	*Bubalus bubalis*	Brazil: Dourados, Mato Grosso do Sul
AY773703	*T. theileri*	Tthb13	*Bubalus bubalis*	Brazil: Dourados, Mato Grosso do Sul
AY773704	*T. theileri*	Tthc8	*Bos taurus*	Brazil: Porto Alegre, Rio Grande do Sul
AY773705	*T. theileri*	Tthc9	*Bos taurus*	Brazil: Londrina, Paraná
AY773706	*T. theileri*	Tthc10	*Bos taurus*	Brazil: Paraná
AY773707	*T. theileri*	Tthc2	*Bos taurus*	Brazil: Jacupiranga, São Paulo
AY773708	*T. theileri*	Tthc16	*Bos taurus*	Brazil: Miranda, Mato Grosso do Sul
AY773714	*Trypanosoma* sp.	D30	*Cervus dama*	Germany
GQ176146	*T. theileri*	Tthc30_3	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ176147	*T. theileri*	Tthc32_6	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ176148	*T. theileri*	Tthc37_2	*Bos taurus*	Brazil: Bujaru, Pará
GQ176149	*T. theileri*	Tthc38_2	*Bos taurus*	Brazil: Bujaru, Pará
GQ176150	*T. theileri*	Tthc39_2	*Bos taurus*	Brazil: Santarém, Pará
GQ176151	*T. theileri*	Tthc41_1	*Bos taurus*	Brazil: Bujaru, Pará
HQ664808	*T. theileri*	Tthb10	*Bubalus bubalis*	Brazil: Eldorado, São Paulo
HQ664809	*T. theileri*	Tthb14	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664810	*T. theileri*	Tthb17	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664811	*T. theileri*	Tthb19	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664812	*T. theileri*	Tthb20	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664813	*T. theileri*	Tthb22	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664814	*T. theileri*	TthbV9	*Bubalus bubalis*	Venezuela: San Felipe, Yaracuy
HQ664815	*T. theileri*	TthbV13	*Bubalus bubalis*	Venezuela: El Baul, Cojedes
HQ664816	*T. theileri*	TthbV15	*Bubalus bubalis*	Venezuela: El Baul, Cojedes
HQ664817	*T. theileri*	Tthc1	*Bos taurus*	Brazil: Jacupiranga, São Paulo
HQ664818	*T. theileri*	Tthc29_1	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664819	*T. theileri*	Tthc30_4	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664820	*T. theileri*	Tthc32_4	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664821	*T. theileri*	Tthc32_5	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664822	*T. theileri*	Tthc37_5	*Bos taurus*	Brazil: Bujaru, Pará
HQ664823	*T. theileri*	Tthc39_4	*Bos taurus*	Brazil: Santarém, Pará
HQ664824	*T. theileri*	Tthc39_3	*Bos taurus*	Brazil: Santarém, Pará
HQ664825	*T. theileri*	Tthc40_2	*Bos taurus*	Brazil: Santarém, Pará
HQ664826	*T. theileri*	Tthc40_3	*Bos taurus*	Brazil: Santarém, Pará
HQ664827	*T. theileri*	Tthc41_6	*Bos taurus*	Brazil: Santarém, Pará
HQ664828	*T. theileri*	Tthc41_3	*Bos taurus*	Brazil: Santarém, Pará
HQ664829	*T. theileri*	TthcV2_7	*Bos taurus*	Venezuela: El Baul, Cojedes
HQ664830	*T. theileri*	TthcV4_10	*Bos taurus*	Venezuela: San Felipe, Yaracuy

HQ664831	*T. theileri*	TthcV6	*Bos taurus*	Venezuela: Merida, Merida
HQ664832	*T. theileri*	CepCamp4_1	*Cephalophus monticola*	Cameroon
HQ664833	*T. theileri*	CepCamp4_2	*Cephalophus monticola*	Cameroon
HQ664834	*T. theileri*	CepCamp4_3	*Cephalophus monticola*	Cameroon
HQ664835	*T. theileri*	CepCamp4_4	*Cephalophus monticola*	Cameroon
HQ664842	*T. theileri*	SitaBip1_1	*Tragelaphus spekii*	Cameroon
HQ664843	*T. theileri*	SitaBip1_2	*Tragelaphus spekii*	Cameroon
HQ664844	*T. theileri*	SitaBip1_4	*Tragelaphus spekii*	Cameroon
HQ664845	*Trypanosoma* sp.	D30_2	*Cervus dama*	Germany
HQ664846	*Trypanosoma* sp.	D30_3	*Cervus dama*	Germany
HQ664847	*T. theileri*	Treu124_2	*Bos taurus*	Germany
HQ664848	*T. theileri*	Treu124_3	*Bos taurus*	Germany
HQ664849	*T. theileri*	Treu124_5	*Bos taurus*	Germany
HQ664850	*T. theileri*	TmHR1_1	*Melophagus ovinus*	Croatia: Goljak, Zagreb
HQ664851	*T. theileri*	TmHR1_4	*Melophagus ovinus*	Croatia: Goljak, Zagreb
HQ664852	*T. theileri*	TmHR1_3	*Melophagus ovinus*	Croatia: Goljak, Zagreb
HQ664853	*T. theileri*	TthHR1_1	*Bos taurus*	Croatia: Pula
HQ664854	*T. theileri*	TthHR1_2	*Bos taurus*	Croatia: Pula
HQ664855	*T. theileri*	TthHR1_4	*Bos taurus*	Croatia: Pula
JN673395	*T. theileri*	Z18106	*Kobus vardonii*	Zambia: Musalangu Game Management Area
JN673396	*T. theileri*	ZPU2807	*Kobus vardonii*	Zambia: North Luangwa National Park
JN673397	*T. theileri*	ZPU2707	*Kobus vardonii*	Zambia: North Luangwa National Park
JN798601	*Trypanosoma* sp.	ACR-2011_TC2	–	–
JN798602	*Trypanosoma* sp.	ACR-2011_TC3	*Cervus elaphus*	Croatia
JN798603	*Trypanosoma* sp.	ACR-2011_TC4	–	–
JN798604	*Trypanosoma* sp.	ACR-2011_TC9	–	–
JN798605	*Trypanosoma* sp.	ACR-2011_TC10	–	–
JN798606	*Trypanosoma* sp.	ACR-2011_TC603	–	–
JN798607	*Trypanosoma* sp.	ACR-2011_TC603	–	–
JN798608	*T. theileri*	TthHR1	–	–
JX178162	*T. theileri*	Cow-2095_Cl-8	*Bos taurus*	USA: Webb, Texas
JX178163	*T. theileri*	Cow-2095_Cl-9	*Bos taurus*	USA: Webb, Texas
JX178164	*T. theileri*	Cow-2_Cl-4	*Bos taurus*	USA: Webb, Texas
JX178165	*T. theileri*	Cow-2_Cl-7	*Bos taurus*	USA: Webb, Texas
JX178166	*T. theileri*	Cow-2_Cl-10	*Bos taurus*	USA: Webb, Texas
JX178167	*T. theileri*	Cow-3535_Cl-3	*Bos taurus*	USA: Webb, Texas
JX178168	*T. theileri*	Cow-3535_Cl-6	*Bos taurus*	USA: Webb, Texas
JX178169	*T.* cf. *cervi*	WTD-NL15_Cl-1	*Odocoileus virginianus*	USA: Zapata, Texas
JX178170	*T.* cf. *cervi*	WTD-NL15_Cl-6	*Odocoileus virginianus*	USA: Zapata, Texas
JX178171	*T.* cf. *cervi*	WTD-NL15_Cl-9	*Odocoileus virginianus*	USA: Zapata, Texas
JX178172	*Trypanosoma* sp.	WTD-A3_Cl-9.1	*Odocoileus virginianus*	USA: Zapata, Texas
JX178173	*Trypanosoma* sp.	WTD-A3_Cl-9.4	*Odocoileus virginianus*	USA: Zapata, Texas
JX178174	*T.* cf. *cervi*	WTD-A21_Cl-4	*Odocoileus virginianus*	USA: Zapata, Texas
JX178175	*T.* cf. *cervi*	WTD-A21_Cl-5	*Odocoileus virginianus*	USA: Zapata, Texas
JX178176	*T.* cf. *cervi*	WTD-A21_Cl-6	*Odocoileus virginianus*	USA: Zapata, Texas
JX178177	*Trypanosoma* sp.	Elk-142_Cl-9	*Cervus canadensis*	USA: Minnesota
JX178178	*Trypanosoma* sp.	Elk-142_Cl-10	*Cervus canadensis*	USA: Minnesota
JX178179	*T.* cf. *cervi*	Elk-416_Cl-8	*Cervus canadensis*	USA: Minnesota
JX178180	*T.* cf. *cervi*	Elk-328_Cl-3	*Cervus canadensis*	USA: Minnesota
JX178183	*T. theileri*	Cow-139_Cl-9	*Bos taurus*	USA: Starr, Texas
JX178184	*T. theileri*	Cow-139_Cl-10	*Bos taurus*	USA: Starr, Texas
JX178185	*T. theileri*	Cow-139_Cl-11	*Bos taurus*	USA: Starr, Texas
JX178186	*T. theileri*	Cow-133_Cl-1	*Bos taurus*	USA: Starr, Texas
JX178187	*T. theileri*	Cow-104_Cl-1	*Bos taurus*	USA: Starr, Texas
JX178188	*T. theileri*	Cow-104_Cl-2	*Bos taurus*	USA: Starr, Texas
JX178189	*T. theileri*	Cow-104_Cl-3	*Bos taurus*	USA: Starr, Texas
JX853182	*T.* cf. *cervi*	WTD-A1_6	*Odocoileus virginianus*	USA
JX853183	*T.* cf. *cervi*	WTD-A1_7	*Odocoileus virginianus*	USA
JX853184	*Trypanosoma* sp.	Elk-421_2	*Cervus canadensis*	USA
JX853185	*T. theileri*	Cow-2095_4	*Bos taurus*	USA
3. gGAPDH gene sequences				
3.1. Newly obtained in the present study				
LC618037	*T. theileri*	YMG-11	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
		YMG-14	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618038	*T. theileri*	OSK-16	*Bos taurus*	Japan: Miyazaki
		OSK-120	*Bos taurus*	Japan: Shimane-Hiroshima
		OSK-148	*Bos taurus*	Japan: Miyazaki-Saga
		OSK-150	*Bos taurus*	Japan: Nagasaki-Saga
		OSK-155	*Bos taurus*	Japan: Miyazaki-Nagano
		OSK-172	*Bos taurus*	Japan: Kagoshima-Kumamoto
LC618039	*T. theileri*	TNB-872	*Cervus nippon aplodontus*	Japan: Tanabe, Wakayama
LC618040	*T. theileri*	KSM-941	*Cervus nippon aplodontus*	Japan: Kushimoto, Wakayama
3.2. Retrieved from the GenBank				
AB362413	*T. kuseli*	–	*Pteromys volans*	Japan (imported from Russia)
AB362557	*T. grosi*	SESUJI	*Apodemus agrarius*	Russia: Vladivostok
AB362558	*T. grosi*	HANTO	*Apodemus peninsulae*	Russia: Vladivostok
AJ620273	*T. microti*	TRL132	*Microtus agrestis*	England
AJ620276	*Trypanosoma* sp.	kangaroo H25	*Macropus giganteus*	Australia
AJ620278	*Trypanosoma* sp.	wallaby ABF	*Wallabia bicolor*	Australia
AJ620280	*Trypanosoma* sp.	TL.AQ.22	*Philaemon* sp.	Australia
AJ620282	*T. theileri*	K127	*Bos taurus*	Germany
FJ649493	*T. cyclops*	–	–	–

FM164792	*T. theileri*	–	*Tragelaphus_spekei*	Cameroon
HF545653	*T. theileri*	uganda12_61_89	*Bos taurus*	Uganda
HF545654	*T. theileri*	uganda29	*Bos taurus*	Uganda
HQ664784	*T. theileri*	Tthb10	*Bubalus bubalis*	Brazil: Eldorado, São Paulo
HQ664785	*T. theileri*	Tthb14	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664786	*T. theileri*	Tthb16	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664787	*T. theileri*	Tthb17	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664788	*T. theileri*	Tthb19	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664789	*T. theileri*	Tthb20	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664790	*T. theileri*	Tthb22	*Bubalus bubalis*	Brazil: Santarém, Pará
HQ664791	*T. theileri*	TthbV15	*Bubalus bubalis*	Venezuela: El Baul, Cojedes
HQ664792	*T. theileri*	Tthc1	*Bos taurus*	Brazil: Jacupiranga, São Paulo
HQ664793	*T. theileri*	Tthc3	*Bos taurus*	Brazil: Eldorado, São Paulo
HQ664794	*T. theileri*	Tthc39	*Bos taurus*	Brazil: Santarém, Pará
HQ664795	*T. theileri*	Tthc40	*Bos taurus*	Brazil: Santarém, Pará
HQ664796	*T. theileri*	Tthc41	*Bos taurus*	Brazil: Santarém, Pará
HQ664797	*T. theileri*	TthcV4	*Bos taurus*	Venezuela: San Felipe, Yaracuy
HQ664798	*T. theileri*	TthcV5	*Bos taurus*	Venezuela: San Fernando, Apure
HQ664799	*T. theileri*	Tthc29	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664801	*T. theileri*	Tthc30	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664802	*T. theileri*	Tthc32	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
HQ664803	*T. theileri*	Tthc37	*Bos taurus*	Brazil: Bujaru, Pará
HQ664804	*T. theileri*	CepCamp4	*Cephalophus monticola*	Cameroon
HQ664805	*T. theileri*	CepCamp5	*Cephalophus monticola*	Cameroon
HQ664806	*T. theileri*	D30	*Cervus dama*	Germany
HQ664807	*T. melophagium*	TmHR1	*Melophagus ovinus*	Croatia: Goljak, Zagreb
MN756794	*T. trinaperronei*	WTD2268	*Odocoileus virginianus*	Venezuela: Anzoátegui, Simón Bolivar
4. CatL-like gene sequences				
4.1. Newly obtained in the present study				
LC618041	*T. theileri*	YMG-11	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618042	*T. theileri*	YMG-14	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618043	*T. theileri*	YMG-15	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
4.2. Retrieved from the GenBank				
GU299350	*T. theileri*	Tthc32_1	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299351	*T. theileri*	Tthc32_2	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299352	*T. theileri*	Tthc32_3	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299354	*T. theileri*	Tthc30_2	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299355	*T. theileri*	Tthc30_5	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299356	*T. theileri*	Tthc38_1	*Bos taurus*	Brazil: Castanhal, Pará
GU299357	*T. theileri*	Tthc38_2	*Bos taurus*	Brazil: Castanhal, Pará
GU299359	*T. theileri*	Tthc28_1	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299361	*T. theileri*	Tthc28_4	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299363	*T. theileri*	Tthc29_3	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GU299366	*T. theileri*	Tthc12_6	*Bos taurus*	Brazil: Miranda, Mato Grosso do Sul
GU299369	*T. theileri*	Tthc14_4	*Bos taurus*	Brazil: Miranda, Mato Grosso do Sul
GU299372	*T. theileri*	Tthc19_2	*Bos taurus*	Brazil: Monte Negro, Rondônia
GU299375	*T. theileri*	Tthb2_3	*Bubalus bubalis*	Brazil: Pariquera-Açu, São Paulo
GU299376	*T. theileri*	Tthb2_4	*Bubalus bubalis*	Brazil: Pariquera-Açu, São Paulo
GU299380	*T. theileri*	Tthb4_3	*Bubalus bubalis*	Brazil: Registro, São Paulo
GU299381	*T. theileri*	Tthb10_2	*Bubalus bubalis*	Brazil: Eldorado, São Paulo
GU299383	*T. theileri*	Tthb9_2	*Bubalus bubalis*	Brazil: Jacupiranga, São Paulo
GU299385	*T. theileri*	Tthb9_4	*Bubalus bubalis*	Brazil: Jacupiranga, São Paulo
GU299386	*T. theileri*	Tthb3_2	*Bubalus bubalis*	Brazil: Pariquera-Açu, São Paulo
GU299387	*T. theileri*	Tthb3_3	*Bubalus bubalis*	Brazil: Pariquera-Açu, São Paulo
GU299390	*T. theileri*	Tthb6_1	*Bubalus bubalis*	Brazil: Jacupiranga, São Paulo
GU299391	*T. theileri*	TthATCC_1	*Bos taurus*	USA: Pennsylvania
GU299392	*T. theileri*	TthATCC_2	*Bos taurus*	USA: Pennsylvania
GU299393	*T. theileri*	TthATCC_3	*Bos taurus*	USA: Pennsylvania
GU299395	*T. theileri*	Tthc16_2	*Bos taurus*	Brazil: Miranda, Mato Grosso do Sul
GU299398	*T. theileri*	Tthc2_4	*Bos taurus*	Brazil: Jacupiranga, São Paulo
GU299399	*T. theileri*	Tthc2_5	*Bos taurus*	Brazil: Jacupiranga, São Paulo
GU299400	*T. theileri*	Tthc1_2	*Bos taurus*	Brazil: Jacupiranga, São Paulo
GU299402	*T. theileri*	Tthc9_4	*Bos taurus*	Brazil: Londrina, Paraná
GU299403	*T. theileri*	Tthc10_1	*Bos taurus*	Brazil: Londrina, Paraná
GU299405	*T. theileri*	Tthc8_1	*Bos taurus*	Brazil: Porto Alegre, Rio Grande do Sul
GU299410	*T. theileri*	Tab12_1	*Tabanus* sp.	Brazil: Miranda, Mato Grosso do Sul
GU299411	*T. theileri*	Tab12_6	*Tabanus* sp.	Brazil: Miranda, Mato Grosso do Sul
GU299412	*T. theileri*	Tab12_2	*Tabanus* sp.	Brazil: Miranda, Mato Grosso do Sul
GU299413	*T. theileri*	Tab7VR_1	*Tabanus* sp.	Brazil: Registro, São Paulo
GU299414	*T. theileri*	Tab7VR_4	*Tabanus* sp.	Brazil: Registro, São Paulo
GU299415	*Trypanosoma* sp.	D30_1	*Cervus dama*	Germany
GU299416	*Trypanosoma* sp.	D30_2	*Cervus dama*	Germany
GU299417	*Trypanosoma* sp.	D30_4	*Cervus dama*	Germany
HQ664736	*T. theileri*	TthbV9_2	*Bubalus bubalis*	Venezuela: San Felipe, Yaracuy
HQ664737	*T. theileri*	TthbV13_3	*Bubalus bubalis*	Venezuela: El Baul, Cojedes
HQ664738	*T. theileri*	TthbV15_1	*Bubalus bubalis*	Venezuela: El Baul, Cojedes
HQ664743	*T. theileri*	TthcV2_1	*Bos taurus*	Venezuela: El Baul, Cojedes
HQ664744	*T. theileri*	TthcV4_3	*Bos taurus*	Venezuela: San Felipe, Yaracuy
HQ664745	*T. theileri*	SitaBip1_1	*Tragelaphus spekii*	Cameroon
HQ664746	*T. theileri*	SitaBip1_2	*Tragelaphus spekii*	Cameroon
HQ664747	*T. theileri*	SitaBip1_4	*Tragelaphus spekii*	Cameroon
HQ664748	*T. theileri*	CepCamp4_1	*Cephalophus monticola*	Cameroon
HQ664749	*T. theileri*	CepCamp5_1	*Cephalophus monticola*	Cameroon
HQ664750	*T. theileri*	CepCamp5_2	*Cephalophus monticola*	Cameroon
HQ664751	*T. theileri*	CepCamp5_4	*Cephalophus monticola*	Cameroon

MN747149	*T. trinaperronei*	WTD2268_c1	*Odocoileus virginianus*	Venezuela: Anzoátegui, Simón Bolivar
MN747150	*T. trinaperronei*	WTD2268_c2	*Odocoileus virginianus*	Venezuela: Anzoátegui, Simón Bolivar
MN747151	*T. trinaperronei*	WTD2268_c3	*Odocoileus virginianus*	Venezuela: Anzoátegui, Simón Bolivar
MN747153	*T. trinaperronei*	WTD2268_c8	*Odocoileus virginianus*	Venezuela: Anzoátegui, Simón Bolivar
MN747154	*T. trinaperronei*	Tt.LM1	*Lipoptena mazamae*	Venezuela: Anzoátegui, Simón Bolivar
MN747155	*T. trinaperronei*	Tt.LM2	*Lipoptena mazamae*	Venezuela: Anzoátegui, Simón Bolivar
5. Spliced leader (SL) gene sequnces				
5.1. Newly obtained in the present study				
LC618044	*T. theileri*	YMG-11_c1	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618045	*T. theileri*	YMG-11_c6	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618046	*T. theileri*	YMG-11_c10	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618047	*T. theileri*	YMG-14_c2	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618048	*T. theileri*	YMG-14_c3	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618049	*T. theileri*	YMG-14_c1	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618050	*T. theileri*	YMG-14_c6	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618051	*T. theileri*	YMG-14_c7	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
LC618052	*T. theileri*	YMG-14_c8	*Cervus nippon aplodontus*	Japan: Shimonoseki, Yamaguchi
5.2. Retrieved from the GenBank				
GQ162134	*T. theileri*	Tthc30_4	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ162135	*T. theileri*	Tthc32_3	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ162136	*T. theileri*	Tthc32_7	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ162137	*T. theileri*	Tthc32_10	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ162138	*T. theileri*	Tthc37_1	*Bos taurus*	Brazil: Bujaru, Pará
GQ162139	*T. theileri*	Tthc37_2	*Bos taurus*	Brazil: Bujaru, Pará
GQ162140	*T. theileri*	Tthc37_10	*Bos taurus*	Brazil: Bujaru, Pará
GQ162149	*T. theileri*	Tthc23_3	*Bos taurus*	Brazil: Sao Mamede, Paraíba
GQ162150	*T. theileri*	Tthc23_5	*Bos taurus*	Brazil: Sao Mamede, Paraíba
GQ162151	*T. theileri*	Tthc23_1	*Bos taurus*	Brazil: Sao Mamede, Paraíba
GQ162152	*T. theileri*	Tthc24_1	*Bos taurus*	Brazil: Sao Mamede, Paraíba
GQ162153	*T. theileri*	Tthc24_3	*Bos taurus*	Brazil: Sao Mamede, Paraíba
GQ162154	*T. theileri*	Tthc25_2	*Bos taurus*	Brazil: Patos, Paraíba
GQ162156	*T. theileri*	Tthc29_3	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ162157	*T. theileri*	Tthc29_7	*Bos taurus*	Brazil: Mossoró, Rio Grande do Norte
GQ162158	*T. theileri*	Tthc27_2	*Bos taurus*	Brazil: Catolé do Rocha, Paraíba
GQ162159	*T. theileri*	Tthc27_3	*Bos taurus*	Brazil: Catolé do Rocha, Paraíba
GQ162160	*T. theileri*	Tthc39_1	*Bos taurus*	Brazil: Santarém, Pará
GQ162161	*T. theileri*	Tthc39_3	*Bos taurus*	Brazil: Santarém, Pará
GQ162162	*T. theileri*	Tthc39_5	*Bos taurus*	Brazil: Santarém, Pará
GQ162163	*T. theileri*	Tthc40_1	*Bos taurus*	Brazil: Santarém, Pará
GQ162164	*T. theileri*	Tthc40_5	*Bos taurus*	Brazil: Santarém, Pará
GQ162166	*T. theileri*	Tthc41_5	*Bos taurus*	Brazil: Santarém, Pará
HQ664890	*T. melophagium*	TmHR1_2	*Melophagus ovinus*	Croatia: Goljak
HQ664891	*T. melophagium*	TmHR1_5	*Melophagus ovinus*	Croatia: Goljak
HQ664892	*T. melophagium*	TmHR1_4	*Melophagus ovinus*	Croatia: Goljak

Table 11. Nucleotide substitutions observed in the hypervariable region (V7/V8) of the 18S rDNA of ruminant Megatrypanum-type trypanosomes.

Sequence ID	Sequence Group ID	GenBank Accession no.	Species name_Isolate name_Clone name	Host species	Country of Isolation	Nucleotide position in the 18S rDNAb hypervariable region (V7/V8)																																									
						13	19	52	63	74	81	82	83	84	85	86	87	88	89	90	91	92	93	94	100	114	116	121	131	149	154	157	167	186	210/211	222	223	230	295	319	324	334	336	387	391	393	399	405	407	409	411	450	457
1	1	AB007814	*T. theileri*_KM	*Bos taurus*	Japan	C	C	T	T	T	G	T	T	T	T	C	C	C	A	T	C	T	T	T	A	C	A	T	T	C	C	C	T	G	--	G	C	G	T	A	A	A	A	T	C	T	A	G	T	C	T	C	T
2		JX178162	*T. theileri*_Cow-2095	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3		JX178166	*T. theileri*_Cow-2_Cl-10	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4		JX178184	*T. theileri*_Cow-139_Cl-10	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5		JX178188	*T. theileri*_Cow-104_Cl-2	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6		JX853185	*T. theileri*_Cow-2095_4	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7		KF765799	*T. theileri*_Bb813	*Bison bonasus bonasus*	Poland	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8		KF765801	*T. theileri*_Bb756	*Bison bonasus bonasus*	Poland	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9		KF765802	*T. theileri*_Bb816	*Bison bonasus bonasus*	Poland	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10		KF924257	*T. theileri*_Bt14ZA	*Bos taurus*	Poland	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11		KJ397592	*T. theileri*_Bb871	*Bison bonasus*	Poland	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12		JX178185	*T. theileri*_Cow-139_Cl-11	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13—18	2	LC618031	*T. theileri*_OSK-16,120,148,150,155,172	*Bos taurus*	Japan	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
19	3	JX178164	*T. theileri*_Cow-2_Cl-4	*Bos taurus*	USA	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
20	4	JX178183	*T. theileri*_Cow-139_Cl-9	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
21	5	JX178167	*T. theileri*_Cow-3535_Cl-3	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
22	6	JX178163	*T. theileri*_Cow-2095_Cl-9	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
23	7	JX178187	*T. theileri*_Cow-104_Cl-1	*Bos taurus*	USA	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
24	8	JX178165	*T. theileri*_Cow-2_Cl-7	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
25	9	JX178186	*T. theileri*_Cow-133_Cl-1	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.
26	10	JX178168	*T. theileri*_Cow-3535_Cl-6	*Bos taurus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.

Sequence ID	Sequence Group ID	GenBank Accession no.	Species name_Isolate name_Clone name	Host species	Country of Isolation	13	19	52	63	74	81	82	83	84	85	86	87	88	89	90	91	92	93	94	100	114	116	121	131	149	154	157	167	186	210/211	222	223	230	295	319	324	334	336	387	391	393	399	405	407	409	411	450	457
						Nucleotide position in the 18S rDNAb hypervariable region (V7/V8)																																															
27	**11**	JX178193	*T.* cf. *cervi*_PJH-2013_WTD-A1	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
28	**12**	KR024688	*T. theileri*_G24	*Glossina fuscipes fuscipes*	C.A.R.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
29	**13**	AB569248	*Trypanosoma* sp._TSD1	*Cervus nippon yesoensis*	Japan	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
30	**14**	JX178180	*T.*cf. *cervi*_PJH-2013_Elk-328	*Cervus elaphus canadensis*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
31	**15**	JX853184	*Trypanosoma* sp._PJH-2013b_Elk-421-2	*Cervus canadensis*	USA	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
32	**16**	JX178178	Trypanosoma sp._PJH-2013b_Elk-142_Cl-10	*Cervus canadensis*	USA	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
33	**17**	JX178175	*T.*cf. *cervi*_PJH-2013_WTD-A21_Cl-5	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
34	**18**	JX178179	*T.*cf. *cervi*_PJH-2013_Elk-416_Cl-8	*Cervus canadensis*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
35	**19**	JX178177	*Trypanosoma* sp._PJH-2013b_Elk-142_Cl-9	*Cervus canadensis*	USA	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.
36	**20**	JX178197	*T.* cf. *cervi*_PJH-2013_WTD148_Cl-4	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
37	**21**	JX178169	*T.*cf. *cervi*_PJH-2013_WTD-NL15_Cl-1	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
38		JX178170	*T.* cf. *cervi*_PJH-2013_WTD-NL15_Cl-6	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
39		JX178171	*T.* cf. *cervi*_PJH-2013_WTD-NL15_Cl-9	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
40		JX178192	*T.* cf. *cervi*_PJH-2013_WTD-A1_Cl-1	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
41		JX853182	*T.* cf. *cervi*_PJH-2013_WTD-A1_6	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
42		JX178196	*T.* cf. *cervi*_PJH-2013_WTD-A5_Cl-1	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
43		KJ195884	*T. cervi*_DdP287	*Dama dama*	Poland	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
44	**22**	JX178195	*T.* cf. *cervi*_PJH-2013_WTD-A21_Cl-6	*Odocoileus virginianus*	USA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	G	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.

Sequence ID	Sequence Group ID	GenBank Accession no.	Species name_Isolate name_Clone name	Host species	Country of Isolation	Nucleotide position in the 18S rDNAb hypervariable region (V7/V8)																																															
						13	19	52	63	74	81	82	83	84	85	86	87	88	89	90	91	92	93	94	100	114	116	121	131	149	154	157	167	186	210/211	222	223	230	295	319	324	334	336	387	391	393	399	405	407	409	411	450	457
45		JX178176	*T.* cf. *cervi*_PJH-2013_WTD-A21_Cl-6	*Odocoileus virginianus*	USA	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	G	·	C	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·
46	**23**	JX178194	*T.*cf. *cervi*_PJH-2013_WTD-A21_Cl-4	*Odocoileus virginianus*	USA	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	G	·	·	·	·	·	C	·	GG	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
47		JX178174	*T.* cf. *cervi*_PJH-2013_WTD-A21_Cl-4	*Odocoileus virginianus*	USA	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	G	·	·	·	·	·	C	·	GG	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
48	**24**	MN752212	*T. trinaperronei*_WTD-2268	*Odocoileus virginianus*	Venezuela	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	C	·	·	G	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	G	A	·	·	·	·	·
49	**25**	JX178172	*Trypanosoma* sp._PJH-2013a_WTD-A3_Cl-9.1	*Odocoileus virginianus*	USA	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	C	·	·	G	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	G	A	·	·	·	·	·
50	**26**	FM202491	*T. theileri*_2	*Cephalophus monticola*	Cameroon	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	·	·	T	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	G	A	·	·	·	·	·
51	**27**	FM202490	*T. theileri*_1	*Cephalophus monticola*	Cameroon	·	·	·	·	G	·	A	·	·	C	·	·	·	–	–	T	·	·	·	G	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
52	**28**	HQ664912	*T. melophagium*_TmHR1	*Melophagus ovinus*	Croatia	·	·	·	·	G	·	A	C	·	C	·	·	·	·	–	T	·	·	·	G	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
53		FN666409	*T. melophagium*_St Kilda	*Melophagus ovinus*	UK	·	·	·	·	G	·	A	C	·	C	·	·	·	·	–	T	·	·	·	G	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
54—56	**29**	LC618030	T. theileri_YMG-11,14,15	*Cervus nippon aplodontus*	Japan	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	C	·	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
57	**30**	AJ009165	*Trypanosoma* sp._D30	*Cervus dama*	Germany	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	C	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
58		JN798594	*Trypanosoma* sp._ACR-2011_TC2	*Cervus elaphus*	Croatia	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	C	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
59		KJ195879	*Trypanosoma* sp._JW-2014b_Cn1	*Cervus nippon holturolum*	Poland	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	C	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
60		KJ195885	*Trypanosoma* sp._JW-2014b_DdP18	Dama dama	Poland	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	C	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
61		KJ397590	*Trypanosoma* sp._JW-2014_Cel34	*Cervus elaphus*	Poland	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	C	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
62		KY681802	*Trypanosoma* sp._2016/SF5	*Phlebotomus perfiliewi*	Italy	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	C	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
63	**31**	AJ009163	*T. theileri*_TREU124	*Bos taurus*	Scotland	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
64		AJ009164	*T. theileri*_K127	*Bos taurus*	Germany	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
65		GQ176155	*T. theileri*_Tthc28	*Bos taurus*	Brazil	·	·	·	·	·	·	A	·	·	C	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·

Sequence ID	Sequence Group ID	GenBank Accession no.	Species name_Isolate name_Clone name	Host species	Country of Isolation	13	19	52	63	74	81	82	83	84	85	86	87	88	89	90	91	92	93	94	100	114	116	121	131	149	154	157	167	186	210/211	222	223	230	295	319	324	334	336	387	391	393	399	405	407	409	411	450	457
66		HQ664909	*T. theileri*_TthcV2	*Bos taurus*	Venezuela	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
67		JX178181	*T. theileri*_Cow-2073_Cl-7	*Bos taurus*	USA	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
68		KJ397591	*T. theileri*_Bb823	*Bison bonasus*	Poland	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
69		KF765800	*T. theileri*_Bb845	*Bison bonasus*	Poland	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
70		KF924254	*T. theileri*_Bb812	*Bison bonasus*	Poland	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
71		KF924255	*T. theileri*_Bb821	*Bison bonasus*	Poland	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
72		KF924256	*T. theileri*_Bt7201Hka	*Bos taurus*	Poland	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
73		KU587641	*T. theileri*_CBT-115	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
74	32	AB569249	*T. theileri*_Esashi-9	*Bos taurus*	Japan	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
75		AB569250	*T. theileri*_Esashi-12	*Bos taurus*	Japan	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	·	·	G	·	·	·	·	·	C	·	·	T	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
76	33	GQ176157	*T. theileri*_Tthc30	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	C	·	C	·	·	G	·	·	A	T	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
77	34	FM202489	*T. theileri*	*Tragelaphus_spekei*	Cameroon	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	·	–	T	·	·	·	·	·	G	·	·	T	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
78	35	AY773692	*T. theileri*_Tthc14	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	T	·	G	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	C	G	A	·	·	·	·	·
79	36	AY773689	*T. theileri*_Tthc5	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	T	·	G	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	C	G	A	·	·	·	·	·
80		KU587631	*T. theileri*_CBT-38	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	T	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
81	37	KU587643	*T. theileri*_CBT-120	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	T	·	G	·	·	·	·	G	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
82		KU587646	*T. theileri*_CBT-123	*Bos taurus*	Brazil	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	T	·	·	C	C	·	G	·	·	·	·	G	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
83	38	LC618032	*T. theileri*_KSM941_c7, c9, c10	*Cervus nippon aplodontus*	Japan	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
84	39	LC618033	*T. theileri*_TNB872_c5, c7, c8	*Cervus nippon aplodontus*	Japan	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·
85	40	LC618034	*T. theileri*_TNB872_c1	*Cervus nippon aplodontus*	Japan	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	A	C	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
86	41	LC61803	*T.*	*Cervus nippon*	Japan	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	A	·	·	C	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	A	·	·	·
87	42	LC618036	*T. theileri*_KSM948_c2	*Cervus nippon aplodontus*	Japan	·	·	·	·	·	·	·	A	·	·	C	·	·	·	·	–	–	G	·	·	·	·	·	G	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	A	·	·	·	·	·

Table 12. Number of nucleotide changes observed between isolates in the hypervariable region (V7/V8) of the 18S rDNA of ruminant Megatrypanum-type trypanosomes.

Sequence	Sequence	Sequence Group ID																																								
ID	Group ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
1—12	1	0																																									
13—18	2	0	0																																								
19	3	1	1	0																																							
20	4	1	1	2	0																																						
21	5	1	1	2	2	0																																					
22	6	1	1	2	2	2	0																																				
23	7	2	2	3	3	3	3	0																																			
24	8	2	2	3	3	3	3	4	0																																		
25	9	2	2	3	3	3	3	4	4	0																																	
26	10	2	2	3	3	3	3	4	4	4	0																																
27	11	2	2	3	3	3	3	4	4	4	4	0																															
28	12	2	2	3	3	3	3	4	4	4	4	0	0																														
29	13	3	3	4	4	4	4	5	5	5	5	1	1	0																													
30	14	3	3	4	4	4	4	5	5	5	5	1	1	2	0																												
31	15	4	4	5	5	5	5	6	6	6	6	2	2	3	1	0																											
32	16	4	4	5	5	5	5	6	6	6	6	2	2	3	1	2	0																										
33	17	4	4	5	5	5	5	6	6	6	6	2	2	3	1	2	2	0																									
34	18	4	4	5	5	5	5	6	6	6	6	2	2	3	1	2	2	2	0																								
35	19	5	5	6	6	6	6	7	7	7	7	3	3	4	2	3	3	3	3	0																							
36	20	5	5	6	6	6	6	7	7	7	7	3	3	4	2	3	3	3	3	4	0																						
37—43	21	3	3	4	4	4	4	5	5	5	5	1	1	2	1	2	2	2	2	3	3	0																					
44, 45	22	5	5	6	6	6	6	7	7	7	7	3	3	4	3	4	4	4	4	5	5	2	0																				
46, 47	23	3	4	4	7	7	7	8	8	5	5	2	2	3	2	3	3	3	3	4	4	1	3	0																			
48	24	11	11	12	12	12	12	13	13	13	13	9	9	10	8	9	9	9	9	10	10	9	11	1	0																		
49	25	11	11	12	12	12	12	13	13	13	13	9	9	10	8	9	9	9	9	10	10	9	11	10	0	0																	
50	26	10	10	11	11	11	11	12	12	12	12	8	8	9	7	8	8	8	8	9	9	8	10	9	2	2	0																
51	27	12	12	13	13	13	13	14	14	14	14	10	10	11	9	10	10	10	10	11	11	10	12	11	3	3	3	0															
52, 53	28	12	12	13	13	13	13	14	14	14	14	10	10	11	9	10	10	10	10	11	11	10	12	11	5	5	5	2	0														
54—56	29	11	11	12	12	12	12	13	13	13	13	9	9	10	8	9	9	9	9	10	10	7	11	10	2	2	3	3	5	0													
57—62	30	11	11	12	12	12	12	13	13	13	13	9	9	10	8	9	9	9	9	10	10	9	11	10	4	4	3	3	5	2	0												
63—73	31	11	11	12	12	12	12	13	13	13	13	9	9	10	8	9	9	9	9	10	10	9	11	10	4	4	3	3	5	2	2	0											
74, 75	32	12	12	13	13	13	13	14	14	14	14	10	10	9	9	10	10	10	10	11	11	10	12	11	5	5	4	4	6	3	3	1	0										
76	33	14	14	15	15	15	15	16	16	16	16	12	12	13	11	12	12	12	12	13	13	12	14	13	5	5	6	6	8	3	5	3	4	0									
77	34	10	10	11	11	11	11	12	12	12	12	8	8	9	7	8	8	8	8	9	9	8	10	9	5	5	4	4	4	3	3	3	4	5	0								
78	35	13	13	14	14	14	14	15	15	15	15	11	11	12	10	11	11	11	11	12	12	11	13	12	5	5	3	4	6	4	4	2	3	5	5	0							
79, 80	36	13	13	14	14	14	14	15	15	15	14	11	10	12	9	11	11	11	11	12	12	11	13	12	5	5	3	4	6	4	4	2	3	5	5	0	0						
81, 82	37	13	13	14	14	14	14	15	15	15	15	11	11	12	10	11	11	11	11	12	12	11	13	12	5	5	3	4	6	4	4	2	3	5	5	2	2	0					
83	38	2	2	3	3	3	3	4	4	4	4	0	0	1	1	2	2	2	2	3	3	1	3	2	9	9	8	10	10	9	9	9	10	12	8	11	11	11	0				
84	39	5	5	6	6	6	6	7	7	7	7	3	3	4	4	5	5	5	5	6	6	4	6	5	8	8	7	7	7	6	6	6	7	9	5	8	8	8	3	0			
85	40	9	9	10	10	10	10	11	11	11	11	7	7	8	7	7	8	8	8	9	9	7	9	8	5	5	6	8	10	5	7	5	6	6	8	7	7	7	7	10	0		
86	41	12	12	13	13	13	13	14	14	4	14	10	10	11	10	10	11	11	11	12	12	10	12	10	6	6	5	5	7	4	4	2	3	5	3	4	4	4	10	6	5	0	
87	42	10	10	11	11	11	11	12	12	12	12	8	8	9	8	9	9	9	9	10	10	8	10	9	4	4	3	3	5	2	2	2	3	5	3	4	4	4	9	8	6	3	0

Table 13. Morphometric comparison of Megatrypanum-type *Trypanosoma* spp. recorded from ruminants (cervids and bovids).

Species	Host	Locality	Reference	*n*	L	BL	BW	UM	PK	KN	PN	NA	FF	NL	NW	K	NI	KI
Sika deer	*Cervus nippon*	Japan	Present study	4	41—47	36—43	2.6—8.6	1.0—2.8	7.2—8.9	5.8—9.4	13.6—16.7	20.8—29.2	3.6—5.6	1.9—3.6	1.7—1.9	0.7—1.0	0.47—0.76	1.76—2.33
T. theileri					(44)	(40)	(4.8)	(1.6)	(7.9)	(7.1)	(15.0)	(24.4)	(4.5)	(2.7)	(1.9)	(0.8)	(0.63)	(2.16)
T. stefanskii	*Capreolus capreolus*	Poland	Kingston *et al.* (1992)	72	37—75	26—68	2—13	Well developed	2—24	3—15	11-30	11-42	4-17				0.41-2.27	1-6
					(55)	(47)	(6.5)		(13.5)	(6.3)	(19.6)	(27.8)	(7.7)				(0.73)	(3.3)
T. stefanskii	*Capreolus capreolus, Cervus elaphus, Cervus dama*	Germany	Hoffmann *et al.* (1984)	286	(47)	(37)			(9.6)	(6.5)	(16.0)	(20.5)	(9.7)				(0.78)	(2.58)
T. cervi	*Cervus canadensis, Odocoileus spp. Rangifer tarandus, Alces alces*	North America	Kingston *et al.* (1975)	14	40—61	32—56			5—20	4—9	11-32	20-30	3-11				0.5-1.3	2.8-3.6
					(52)	(45.4)			(12.2)	(7)	(19.4)	(24.8)	(6.6)				(0.78)	(2.77)
T. theileri	*Bos taurus*	Japan	Saisawa *et al.* (1933)	?	67—109	61—96		2.3—6.6	17—43	4—13	21—56	30—53	7—14				0.7—1.1	4.4—5
					(84.4)	(75.5)		(3.8)	(29.8)	(6.4)	(36.2)	(39.7)	(8.9)				(0.91)	(5.68)
T. theileri	*Bos taurus*	Canada	Woo *et al.* (1970)	65	31.2—64.9	31.2—64.9	1.4—5.0	2.3—6.6	17.0—21.0	13.8—22.3	—	49.2—68.9	3.6—26.5	4.6—8.5	3.9—4.6	1.6—2.3	0.47—0.76	1.76—2.33
					(47.6)	(47.6)	(11.9)	(3.8)	(18.7)	(16.7)		(57.5)	(10.7)	(6.4)	(4.4)	(1.9)	(0.63)	(2.16)
T. theileri	*Bos taurus*	North America	Kingston *et al.* (1992)	304	16—90	13—59	1—13		0—17	2—20	5—33	7—36	1—37				0.43—1.67	1—4
					(50.5)	(36.4)	(3.3)		(7.4)	(8.9)	(16.2)	(20.2)	(14.2)				(0.88)	(1.86)
T. ingens	*Bos taurus,* various antelops, African and Asian chevrotains	Africa & Asia	Hoare (1972)	?	72—130	72—130	5—10						7—24					2

T. mazamarum	*Mazama* spp.	South America	Hoare (1972)	?	36—85	36—85	6.5—7.0			7—12			7—15					
T. melophagium	*Ovis aries*	Germany	Büscher and Friedhoff (1984)	111	45±4.1	—	2.1—4.6	—	14.7±2.9	5.1±1.1	19.8±3.5	19.5±1.9	6.0±1.6	2.2—3.7	1.3—1.7	0.9—1.3	0.9—1.2	3.3—4.9
							(3.1)							(2.6)	(1.7)	(1.1)	(1.1)	(3.8)

Abbreviation: Body length (BL), excluding length of the free flagellum (FF); body width (BW), excluding the width of the undulating membrane; width of the undulating membratne (UM); distance of the kinetoplast from the posteior end (PK), distance between the kinetoplast and nucleus (KN); distance of the nucleus from the anterior end (NA); length; Length and width of the nucleus (NL and NW, respectively); diameter of the kinetoplast (K). The nuclear (NI) and kinetoplast (KI) indexes are calculated as follows: NI = PN/NA and KI = PN/KN.

Reference:

Büscher, G and Friedhoff, KT (1984) The morphology of ovine *Trypanosoma melophagium* (Zoomastigophorea: Kinetoplastida). Journal of Protozoology 31, 98–101. doi: 10.1111/j.1550-7408.1984.tb04297.x.

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Saisawa, K, Taise, K and Kaneko, K (1933) Study on *Trypanosoma theileri*. Japanese Journal of Experimental Medicine 11, 101–105.

Woo, P, Soltys, MA and Gillick, AC (1970) Trypanosomes in cattle in Southern Ontario. Canadian Journal of Comparative Medicine 34, 142–147.

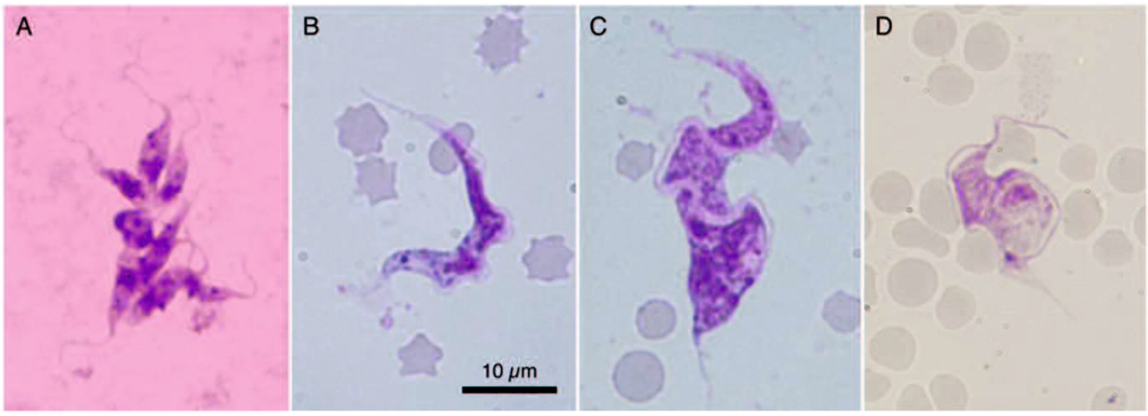

Figure 14. *Trypanosoma theileri* detected in the peripheral blood of sika deer in Japan. Cluster of epimastigotes in the culture supernatant (A); YMG isolate trypomastigotes in the blood smears (B and C); and a trypomastigote of the TNB isolate (D). The specimens have been stained with Giemsa. All photographs are shown at the same magnification with the scale bar in photograph B.

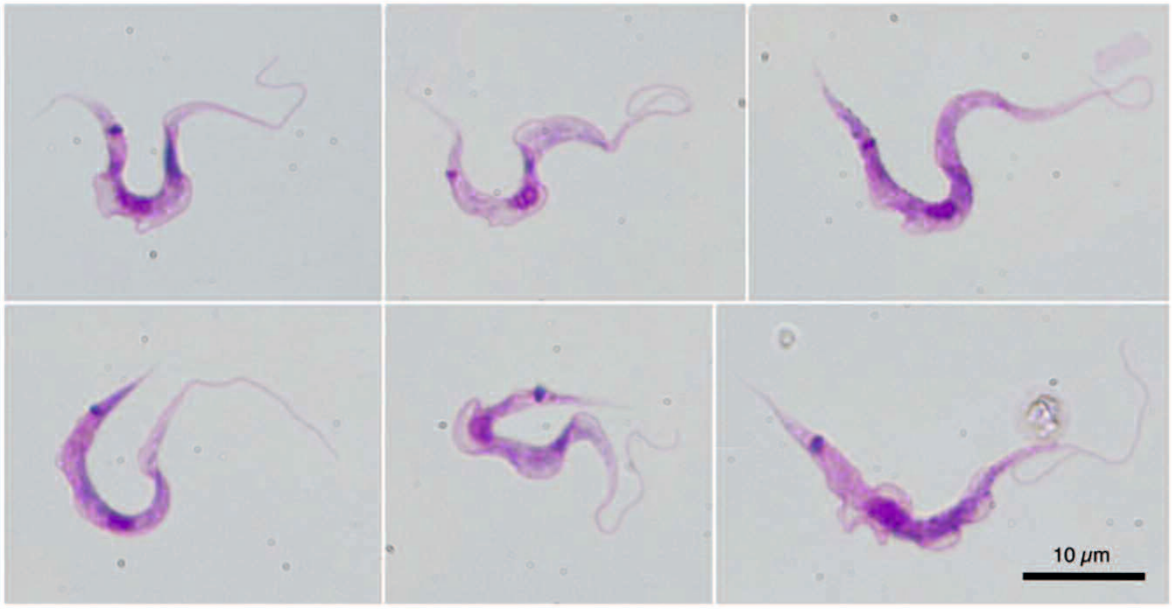

Figure 15. Metacyclic trypomastigotes of cultured *Trypanosoma theileri* from OSK isolates, originating from the peripheral blood of cattle in Japan. The specimens have been stained with Giemsa. All photographs are shown at the same magnification with the scale bar at the lower right.

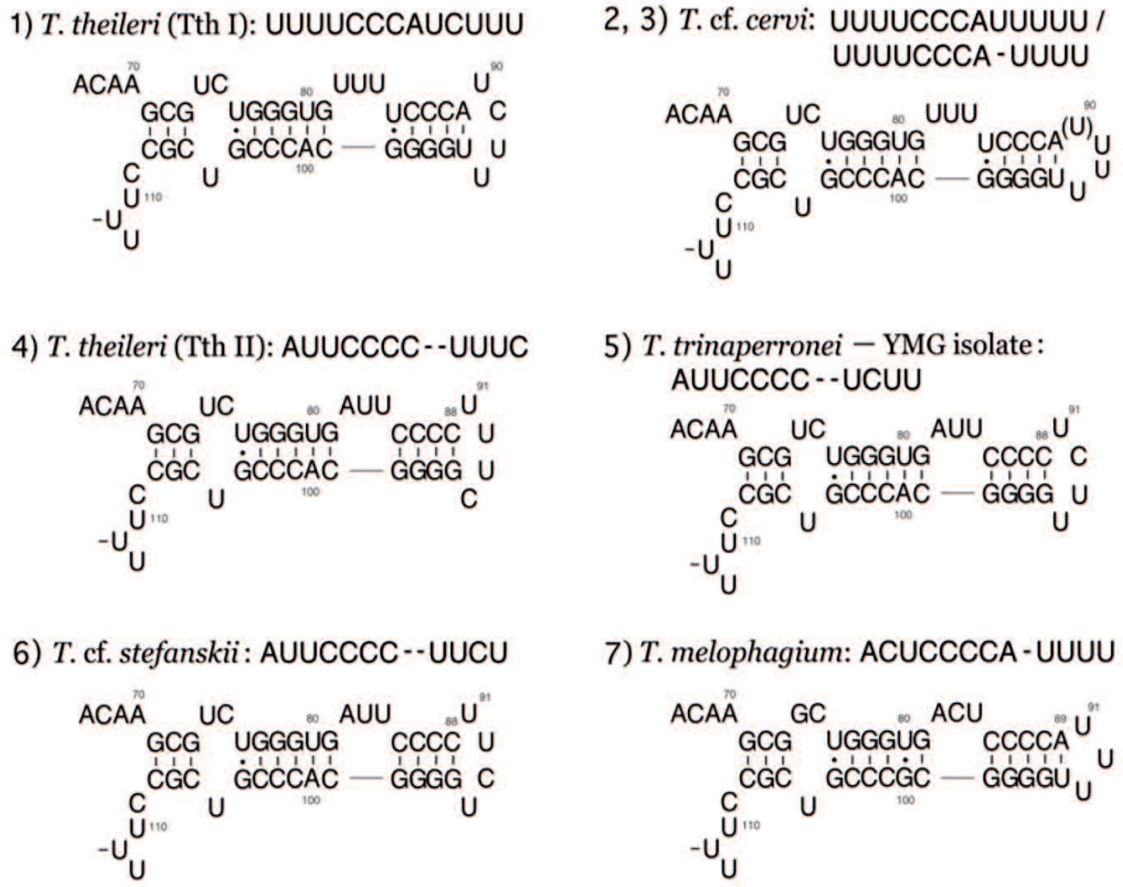

Figure 16. Seven genotypes of ruminant Megatrypanum-type trypanosomes based on the putative secondary structure of the hair-pin loop of the 18S rDNA hypervariable region.

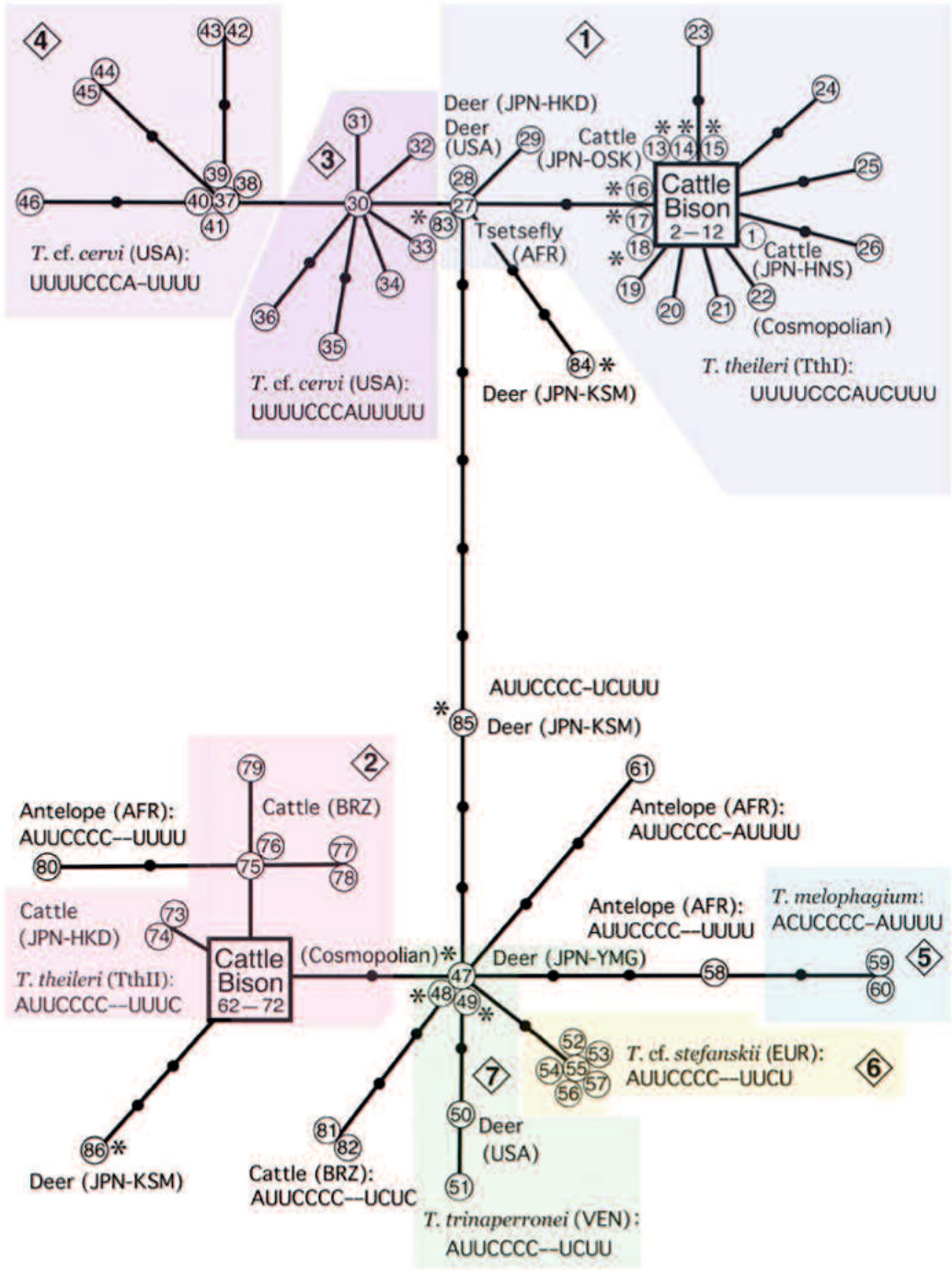

Figure 17. Relationships of ruminant Megatrypanum-type trypanosome isolates based on nucleotide sequence differences in the hypervariable region of 18S rDNA, illustrated via HapStar network analysis. Detailed data about the nucleotide sequences of analysed isolate are shown in Table 10, and numbers in the circles or squares of this figure correspond to material numbers shown in Table 11 and 8. Numbers 1–7 in the rhombus indicate genotypes shown in Figure 18.

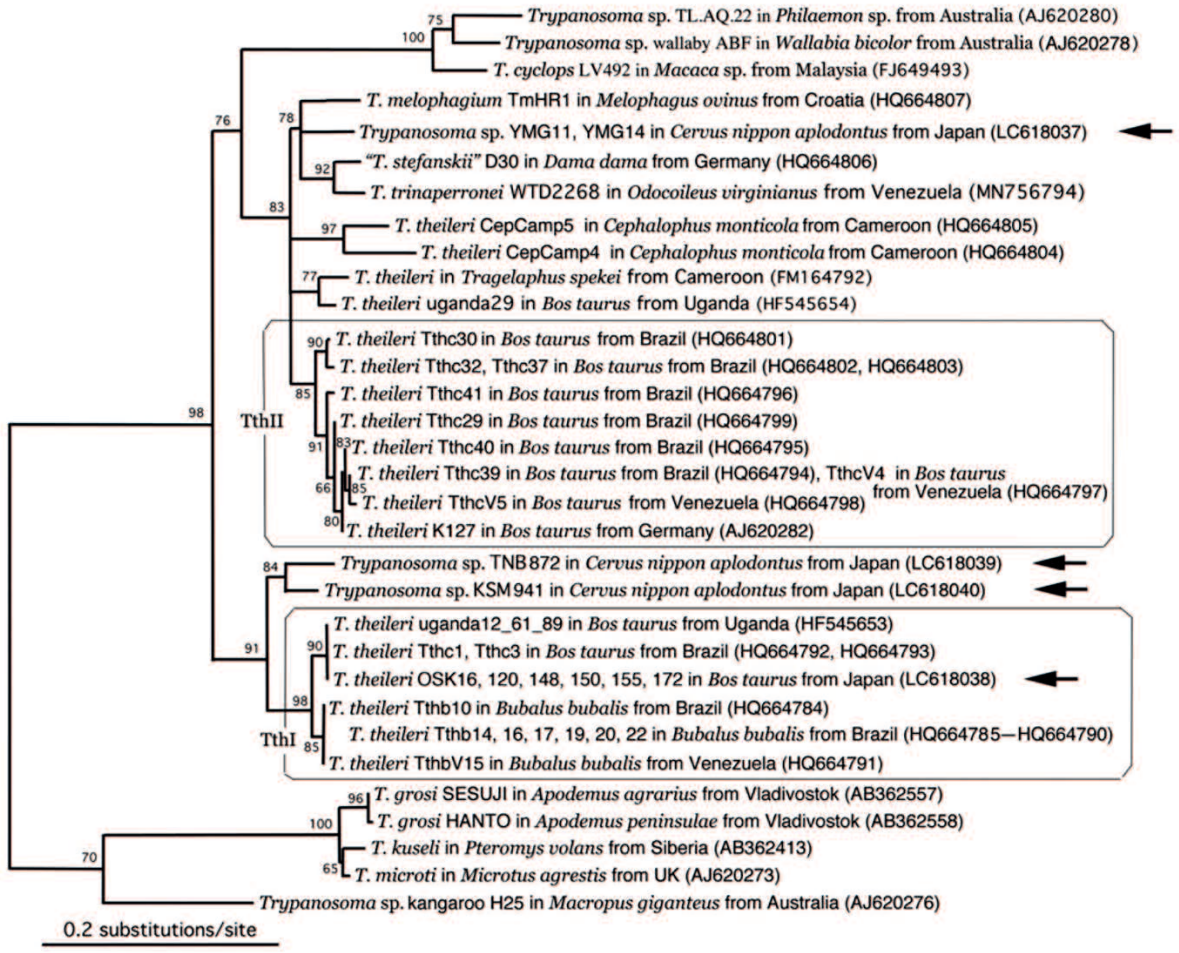

Figure 18. ML phylogenetic tree of ruminant Megatrypanum-type trypanosomes based on the 623 characters of gGAPDH gene nucleotide sequences. The species name of isolates is followed by the host species, country of collection and the DDBJ/EMBL/GenBank accession number in parentheses. Newly obtained sequences are indicated by arrows. Representative rodent trypanosomes and a Megatrypanum-type *Trypanosoma sp*. from a kangaroo were used as an outgroup due to their close phylogenetic relationships with the *T. theileri* clade (Hamilton et al., 2004).

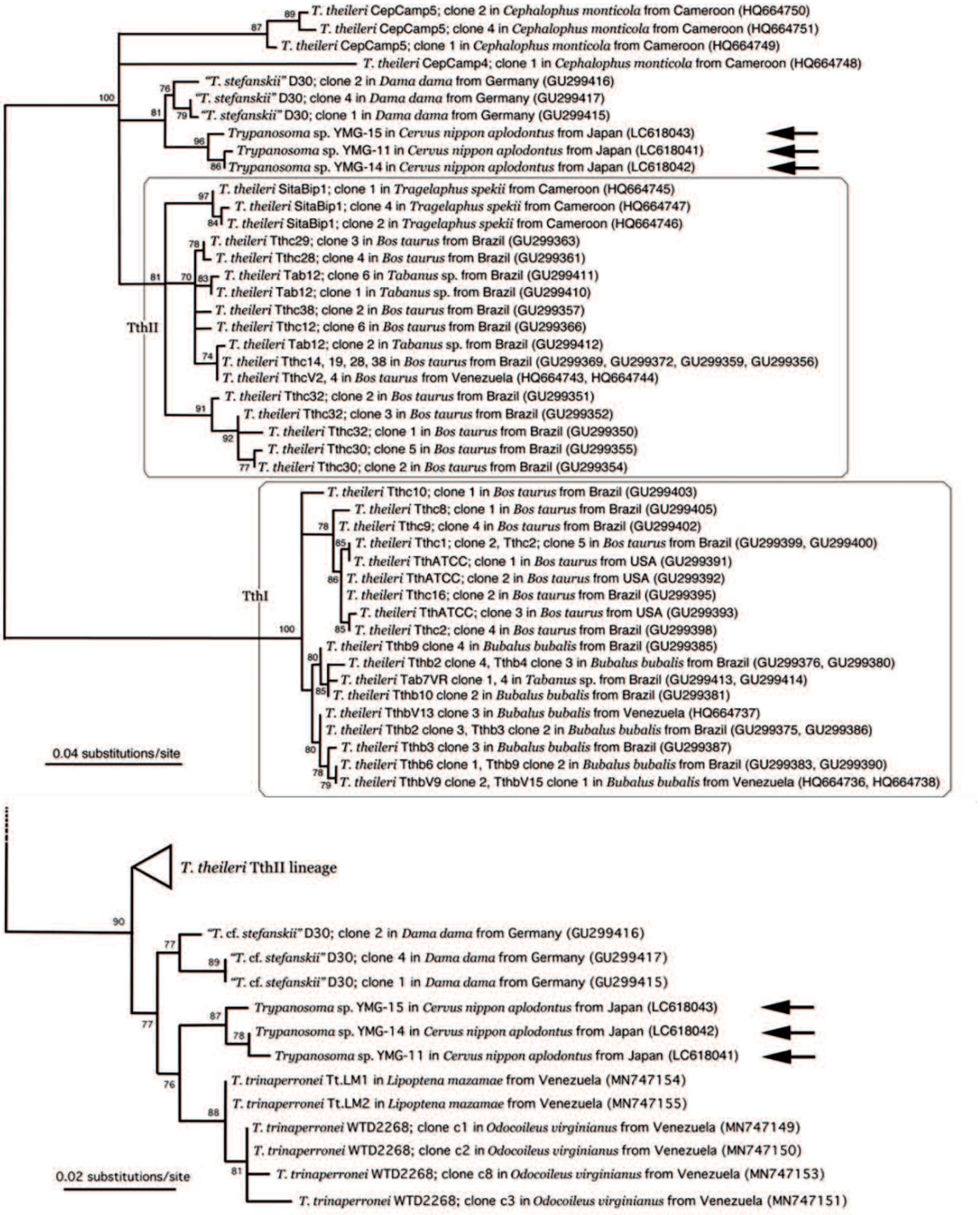

Figure 19. Unrooted ML phylogenetic tree of ruminant Megatrypanum-type trypanosomes based on the 450 characters (upper) and 256 characters of CatL-like gene nucleotide sequences (lower). The species name of isolates is followed by the host species, country of collection and the DDBJ/EMBL/GenBank accession number in parentheses. Newly obtained sequences are indicated by arrows.

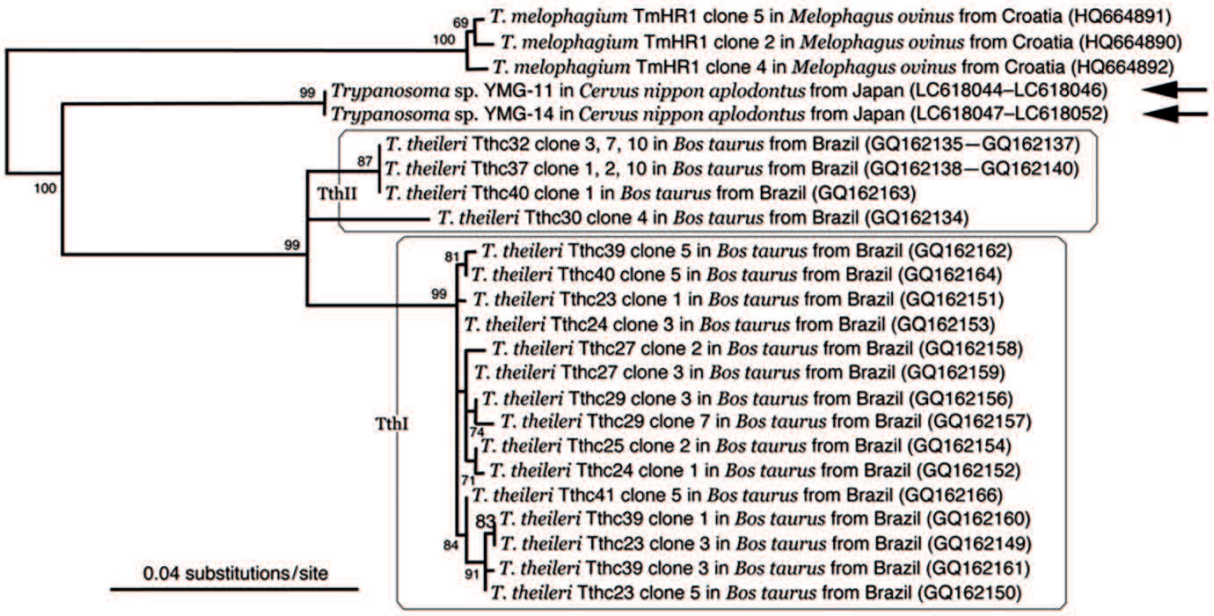

Figure 20. Unrooted ML phylogenetic tree of ruminant Megatrypanum-type trypanosomes based on the 658 characters of SL gene nucleotide sequences. The species name of isolates is followed by the host species, country of collection and the DDBJ/EMBL/GenBank accession number in parentheses. Newly obtained sequences are indicated via arrows.

GENERAL DISCUSSION AND CONCLUSION

Hemoparasites, such as trypanosomes, filarial worms, and hemosporidian protozoa (such as *Plasmodium* spp.), are significant human and domestic animal pathogens that cause diseases including malaria, sleeping sickness, lymphatic filariasis, and canine heartworm disease. The health and fitness of animals may also be impacted by endemic and invasive hemoparasites (Custer and Pence, 1981; Atkinson *et al.*, 2000; Garvin *et al.*, 2003; Donahoe *et al.*, 2015). Particularly in light of ecological instability brought on by human and domestic animal encroachment into wildlife habitats and vice versa, understanding of particular taxonomy and biogeographical ranges of hemoparasites has the potential to disclose emergent disease threats (Daszak *et al.*, 2001; Keesing *et al.*, 2010). Very little study has been done on non-zoonotic hemoparasites that are less pathogenic or of less medical or veterinary interest, such as the genera *Haemoproteus* in pigeons, *Polychromophilus* in bats, or *T. theileri*-like trypanosomes in cervids.

In the study described in Chapter I, I challenged the aforementioned speculation based on morphometric and genetic analysis of pigeon haemoproteosis in farmed domestic pigeons (*Columba livia* f. *domestica* Gmelin, 1789) from Indonesia. Finally, I could demonstrate for the first time a high prevalence of *Haemoproteus columbae* Kruse, 1890 infection in Indonesia, comparable to previous studies that have been reported in Cape Town, South Africa (Earle and Little, 1993; Nebel *et al.*, 2020), Spain (Foronda *et al.*, 2004), Egypt (Hussein and Abdelrahim, 2016), and Brazil (Chagas *et al.*, 2016). The majority of those studied are likely in the long-lasting chronic phase instead of initial infections, which are often characterized by greater infection intensities (Asghar *et al.*, 2012) with evident erythrocytes actively parasitized not only with mature gametocytes but also with immature gametocytes, as revealed in the blood

films, indicative of recurrences or ongoing reinfections. Extreme infection intensities during initial infections increase the risk of predation-related death in individuals (Temple, 1987; Møller and Nielsen, 2007) or through the effects of the parasite itself such as anemia (Earle *et al.*, 1993).

Molecular characterization of the cytochrome *b* (*cytb*) gene identified four *H. columbae* lineages (HAECOL1, COLIV03, COQUI05, and CXNEA02) that are prevalent lineages recorded worldwide. The four *cytb* lineages identified in this research seem to be widespread in feral pigeons and are morphologically related to *H. columbae* (Chagas *et al.*, 2016; Nebel *et al.*, 2020). The similarity in parasite lineage composition should be viewed in a historical context, before the Columbian Exchange, the natural resident range of rock doves was confined to western and southern Europe, North Africa, and then to Southeast Asia, where it was subsequently expanded by humans (Johnston and Janiga, 1995), probably with their louse fly (Hippoboscidae) vectors. Single and repeated infections with various blood parasite lineages may have diverse effects on hosts and have been related to detrimental consequences on their physical health, lowered hematocrit values, and lower host survival (Marzal *et al.*, 2008; Palinauskas *et al.*, 2011; Pigeault *et al.*, 2018).

In addition, our data revealed that asymptomatic *H. columbae* infection negatively affected the hemogram health of pigeons. A hemogram examination of 20 infected pigeons demonstrated that *H. columbae* infection may induce statistically significant macrocytic hypochromic anemia with hypoproteinemia and inflammation. High infection intensity of *H. columbae* can be associated with negative physiological (Earle *et al.*, 1993) and behavioral (Markus and Oosthuizen, 1972) changes. In extreme situations, infections may even be fatal, particularly in young individuals who have not yet fully matured their immune systems (Sol *et al.*, 2003).

Blood stages of various *Haemoproteus* species are highly obvious in blood smears (Valkiūnas, 2005), but species identification is challenging owing to the scarcity of distinctive morphological characteristics. In recent years, DNA sequencing utilizing PCR has been used to distinguish blood parasite lineages (Fallon *et al.*, 2003) and has also been used as a more precise approach for determining the prevalence of blood parasites, particularly at low infection intensities (Fallon and Ricklefs, 2008; Garamszegi, 2010). Additionally, genetic sequencing makes it possible to distinguish between blood parasite lineages that are closely related but that cannot be distinguished by microscopic analysis. Furthermore, sequencing makes it possible to identify coinfections of several hemosporidian lineages, which often occur (Poulin and Morand, 2000; Silva-Iturriza *et al.*, 2012; Alizon *et al.*, 2013). Mitochondrial cytochrome *b* (*cytb*) is frequently used as a genetic marker because it contains the so-called "DNA barcode" region for s. This region is used not only to distinguish species or lineages in avian hemosporidians (Bensch *et al.*, 2009; Dimitrov *et al.*, 2014), but also to distinguish between species and lineages in chiropteran hemosporidians as described in Chapter II.

Over a century ago, chiropteran parasites were first discovered (e.g. Dionisi, 1899; Garnham, 1953). *Plasmodium, Hepatocystis, Nycteria,* and *Polychromophilus* are the four different genera of Haemosporidia that have been documented to infect bats (Garnham, 1953, 1966; Witsenburg *et al.*, 2012). Parasites belonging to the genus *Polychromophilus*, which are transmitted to insectivorous bats by certain species of nycteribiid flies, seem to be prevalent among insectivorous bats in both temperate and tropical climates (Dionisi, 1898; Garnham, 1973a,b). I reported for the first time the isolation and molecular characterization of two species of the genus *Polychromophilus* in bats distributed in Japan: *P. melanipherus* in *Miniopterus fuliginosus* and *P. murinus*

in *Myotis macrodactylus* using nested PCR targeting not only *cytb* but also another genetic marker such as *cox-1*, *clpc*, and EF2 gene (Duval *et al.*, 2007; Witsenburg *et al.*, 2012). This study provides support for the conclusions that have been published in prior research carried out in Central Africa, Europe, Southeast Asia, and Australia. (Garnham, 1973b; Landau *et al.*, 1980; Gardner and Molyneux, 1988; Megali *et al.*, 2011; Duval *et al.*, 2012; Schaer *et al.*, 2013; Ramasindrazana *et al.*, 2018). According to findings from studies conducted worldwide, the level of intraspecific genetic variation found in this research among the *cytb, cox-1, clpc*, and EF2 gene sequences was very high (Witsenburg *et al.*, 2015; Obame-Nkoghe *et al.*, 2016; Ramasindrazana *et al.*, 2018; Holz *et al.*, 2019; Rasoanoro *et al.*, 2021; Sándor *et al.*, 2021)

The bat flies collected in this study were subjected to a molecular screening to identify probable candidate vectors. The findings of the screening using whole vector DNA should be taken with care, since the presence of *Polychromophilus* DNA in a nycteribiid may simply be the consequence of a recent blood meal (Ramasindrazana *et al.*, 2018). To circumvent this bias, I employed the salivary glands of *Nycteribia* bat flies to morphologically and molecularly identify the sporozoite stage, whereas the midgut was used to identify the oocyst stage of parasites. As a result, *P. melanipherus* was morphologically discovered in *Nycteribia* bat flies from a *P. melanipherus*-positive host by PCR screening. In this research, *Miniopterus fuliginosus* and *Myotis macrodactylus* share the same environment in the tunnel, yet there is no cross-contamination. This may be connected to *Miniopterus* and *Myotis* ecology. *Miniopterus* spp. and *Myotis* spp. colonies are often seen in syntropy (physical contact). Members of these two genera seem to have ectoparasites with a permissive preference for hosts (Tortosa *et al.*, 2013; Ramasindrazana *et al.*, 2017). Nevertheless, due to their near physical proximity, it may promote parasite co-infection and/or host swapping but the

absence of *P. murinus*/*P. melanipherus* co-infection in bat genera and associated nycteribiid ectoparasites (Duval *et al.*, 2012; Schaer *et al.*, 2013; Obame-Nkoghe *et al.*, 2016) suggests host-specificity.

My confirmation of avian and chiropteran hemoparasites has far-reaching ramifications for our knowledge of host-switching within the Haemosporida and the development of mammalian malaria parasites (Figure 8). The evolutionary framework presented here supports a single genesis of mammalian malaria leading to the development of *Polychromophilus*, *Nycteria, Hepatocystis,* and *Plasmodium* from an ancestor infecting sauropsids. *Plasmodium* parasites that infect birds and squamates had a secondary flip back to sauropsid hosts after they adapted to infecting mammals. Furthermore, the evolutionary concept presented here underlines the importance of bats (order Chiroptera) as significant agents of malaria diversification (Schaer *et al.*, 2013; Lutz *et al.*, 2016). Malaria parasites appear to have experienced a complex history of divergence after switching to or from bats; our preferred phylogenetic hypothesis suggests either initial wide radiation of malaria parasites in bats followed by at least four transitions to other host groups (ungulates, primates, rodents, and sauropsids) or at least four separate colonizations of bats as hosts (in *Polychromophilus, Nycteria, Hepatocystis* and mammal *Plasmodium*). The significance of bats in the evolution of malarian parasites further highlights the significance of Asia and Africa as cradles of malaria evolution, since bat hemosporidians are most varied and frequent in these locations (Perkins and Schaer, 2016).

My results also refute the hypothesis proposed by Carreno *et al.* (1997) that *Polychromophilus* is most closely related to *Haemoproteus* which also disproved the hypothesis that the nycteribid-transmitted *Polychromophilus* and the hippoboscid-transmitted *H. (Haemoproteus)* have a common ancestry (Witsenburg *et al.*, 2012). A

co-evolutionary event between these Haemosporida and their dipteran hosts may thus be ruled out. As with other present *Plasmodium* species, the progenitor of *Polychromophilus* must have been vectored by a member of the Culicidae. Culicidae is one of the most ancient families of Diptera, having a greater rate of species diversification than all terrestrial animals combined (Wiegmann *et al.*, 2011). The evolutionary divergence between Culicidae and Nycteribiidae is one of the greatest within the order, however, it seems that the adaptations necessary for this new vector were gained simultaneously with those required for the new mammalian host (Wiegmann *et al.*, 2011; Witsenburg *et al.*, 2012).

In a study described in Chapter III, I characterize *T. theileri*-like trypanosomes in cervids that are morphologically indistinguishable from other domestic and wild ruminants, including cattle, buffalo, sheep, goats, and antelopes (Hoare, 1972; Rodrigues *et al.*, 2006). Unlike avian and chiropteran , to characterize *T. theileri*-like trypanosomes (classically trypanosomes of the subgenus *T. Megatrypanum*) several target genes are used such as rDNA, gGAPDH, ITS, SL, and CatL-like genes (Rodrigues *et al.*, 2006, 2010a, 2010b; Garcia, *et al.*, 2011a, 2011b, 2020; Martinković *et al.*, 2012; Yokoyama *et al.*, 2015; Pacheco *et al.*, 2018). Our findings in morphological and molecular genetic analyses of cultured trypanosomes isolated from Honshu sika deer in Japan (YMG isolate) revealed a unique divergent of cervid trypanosomes, distinct from TthI or TthII lineages of *T. theileri, T. melophagium* in domestic ruminants, or cervid Megatrypanum-type trypanosomes in Europe (‘*T. cf. stefanskii)* as well as those in the Americas (‘*T. cf. cervi*’ and *T. trinaperronei*).

Additionally, my results of phylogenetic analyses using the sequences from the SSU rRNA, gGAPDH, spliced leader, and cathepsin L-like genes strongly supported earlier findings that the subgenus *Megatrypanum* as a taxon comprised only

trypanosomes from artiodactyls that clustered together to form the clade *T. theileri* (Rodrigues *et al.*, 2003, 2006). In my study, this clade formed by *T. theileri* trypanosomes comprises two phylogenetic lineages (TthI and TthII) and seven genotypes of ruminant Megatrypanum-type trypanosomes based on the putative secondary structure of the hair-pin loop of the SSU rDNA hypervariable region. Other genotypes (TthIA–C and TthIIA–B) were also defined by internal transcribed spacer (ITS1) rDNA and spliced leader (SL) genes, which are associated with geographic and host origin, suggesting a spatial populational structure with host-switching among closely related host species (Rodrigues *et al.*, 2010b). However, the determinant factors of genotype segregation still need to be clarified (Rodrigues *et al.*, 2006, 2010a).

There is no evidence to date that trypanosome infections may be passed between cervids and bovids, either from experiments or through molecular epidemiology (Kingston *et al.*, 1982; Böse *et al.*, 1987a; Garcia *et al.*, 2011b; Fisher *et al.*, 2013). *Trypanosoma* sp. TSD1 was discovered in Japanese sika deer, while sympatric cattle were infected with *T. theileri* of both TthI and TthII lineages (Suganuma *et al.*, 2019). Similar findings were made in Venezuela, where sympatric cattle and water buffalo were discovered to have *T. theileri* and *T. theileri*-like infections, respectively. In Croatia, host-specific trypanosomes have reportedly been found in sheep, cattle, and deer (Martinković *et al.*, 2012). These data together provide compelling evidence that Megatrypanum-type trypanosomes have a limited range of hosts, in other words strict. Although one host species may harbor trypanosomes of more than one species or genotype, each trypanosome species or genotype was only ever discovered in a single host species or closely related hosts in terms of phylogeny (Garcia *et al.*, 2011b; Fisher *et al.*, 2013; Suganuma *et al.*, 2019). In my study, I showed the genetic lineages of cervid Megatrypanum-type trypanosomes in Japan, where deer trypanosomes in the

west are genetically closer to European cervid trypanosomes and the bovine *T. theileri* TthII lineage, while deer trypanosomes in the north are closer to North American cervid trypanosomes and the bovine *T. theileri* TthI lineage. These two sika deer trypanosome lineages indicate different phylogenetic populations of the parasite maintained by the southern and northern sika deer populations in Japan, which contain variances in their mitochondrial DNA (Tamate et al., 1998; Nagata et al., 1999; Ohdachi et al., 2010).

Deer flies (tabanids) and deer keds (hippoboscids), which have a strong association with their cervid hosts, may potentially play a role in trypanosome host limitation. Deer flies, which have been experimentally shown to transmit deer trypanosomes, as well as deer keds have been identified as cervid trypanosomes vectors (Hoare, 1972; Böse and Petersen, 1991; Garcia *et al.*, 2020; Werszko *et al.*, 2020a; b). Recent research has shown deer trypanosome DNA in the guts of sand flies and culicids (Calzolari *et al.*, 2018; Schoener *et al.*, 2018; Kostygov *et al.*, 2022), but further research is needed to determine how these insects act as vectors, particularly in maintaining the genetic lineages of cervid Megatrypanum-type trypanosomes in Japan.

In the study of zoonotic epidemics, phylogenies are a useful tool for determining the origin of a virus or parasite and predicting future outbreaks by finding evolutionary lineages with high host-switching potential. Through above study, I have reported the value of selected genetic marker sequencing such as mitochondrial *cytb* sequencing to allow the species detection and phylogenies of cryptic species in avian (Bensch *et al.*, 2000; Hellgren *et al.*, 2004) and chiropteran (Witsenburg *et al.*, 2012; Schaer *et al.*, 2013; Arnuphapprasert *et al.*, 2020). However, the identification and phylogenies of hemoparasites in ruminant trypanosomes requires more attention, where usually natural infections have a low infection rate, so needs blood culture to detect the parasite (Rodrigues *et al.*, 2006) and requires multiple genetic markers (Rodrigues *et al.*, 2006,

2010a, 2010b; Garcia, *et al.*, 2011a, 2011b, 2020; Martinković *et al.*, 2012; Yokoyama *et al.*, 2015; Pacheco *et al.*, 2018). In addition, my studies demonstrate clearly that by combining morphological identification and appropriately selecting hemoparasite genetic markers, we may be able to predict the potential risk of epizootic disease to humans and domestic animals that may be in contact with wildlife animals.

FUTURE RESEARCH PLAN

Research on avian and chiropteran hemosporidian and ruminant trypanosomes diversity and evolution has benefited by veterinarians ongoing efforts to investigate the potential threat of epizootic disease to humans and domestic animals that may be connected to wildlife animals. When figuring out evolutionary links, taxon sampling is vital to take into account. We should be able to learn more about the development of parasites in the future with the help of sampling of hemosporidians and ruminant trypanosomes from new host taxa and geographical regions (such as Asian birds and mammals, Old and New World bats, ungulates, etc.). The marriage of morphologically described parasite samples with new genetic data should always be maintained in order to allow taxonomic revisions that correspond to previously characterized species. Mixed infection is a common occurrence in natural populations when genetically related parasite lineages coexist. As a result, another candidate for a conserved genetic marker that can both detect single infections and mixed infections needs to be characterized in the future. Last but not least, many genomic and transcriptome datasets from avian and chiropteran hemosporidians and ruminant trypanosomes have recently been described, although it is still challenging to ascertain how one species fits into the phylogeny of other species. Whole-genome sequencing (WGS) will be crucial in the phylogenetic and taxonomic

analyses of hemoparasites in the future. The use of WGS may anticipate finding a novel locus for molecularly identifying these species in the future that was more robust and selective than other regularly used markers for inferring the phylogeny of ruminant trypanosomes, avian and chiropteran hemosporidians, and other species.

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