Molecular survey of tick microbiome and tick-borne pathogens in ixodid ticks and rodents collected in Sarawak, Malaysian Borneo

（マレーシアボルネオ島サラワク州におけるマダニ微生物叢ならびにマダニおよびネズミ類でのマダニ媒介性病原体の分子調査）

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Abbreviations

12S	12 small subunit
16S	16 small subunit
18S	18 small subunit
23S	23 small subunit
AF	Adult female
AM	Adult male
ANCOM	Analysis of compositions of microbiomes
Bbsl	*Borrelia burgdorferi* sensu lato
Bbss	*Borrelia burgdorferi* sensu sricto
CO1	Cytochrome c oxidase subunit 1
DA	*Dermacentor atrosignatus*
DC	*Dermacentor compactus*
DNA	Deoxyribonucleic acid
dnaK	Chaperone protein gene
DS	*Dermacentor steini*
flaB	Flagellin B gene
ftsZ	Cell division protein gene
fopA	Outer membrane protein
GGNP	Gunung Gading National Park
gltA	Citrate synthase gene
groEL	Chaperone protein gene
HH	*Haemaphysalis hystricis*
HS	*Haemaphysalis shimoga*
htrA	17-kDa common antigen gene
ID	Identification
IG	*Ixodes granulatus*
KNP	Kubah National Park
L	Larva
LD	Lyme disease
LEfSe	Linear discriminant analysis Effect Size
MLSA	Multilocus sequence analysis

mt-rrs	Mitochondrial 16S ribosomal deoxyribonucleic acid
N	Nymph
n	Number
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
No.	Number
OP	Oil palm
ompA	Outer membrane protein A
ompB	Outer membrane protein B
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCoA	Principal Coordinates Analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
rpoB	Beta subunit of bacterial ribonucleic acid polymerase gene
rDNA	Ribosomal deoxyribonucleic acid
RF	Relapsing fever
RNA	Ribonucleic acid
sca4	Surface cell antigen-4
ssrA	Transfer-messenger ribonucleic acid
TBD	Tick-borne disease
TBP	Tick-borne pathogen
tul4	T-cell epitope
Qiime2	Quantitative Insights Into Microbial Ecology 2

Preface

Malaysia is geographically located in the Southeast Asian region and is divided into Peninsular Malaysia and Malaysian Borneo (Figure 1). Malaysian Borneo consists of Sarawak and Sabah states and is located in Borneo Island with two other countries: Brunei and Indonesia. Sarawak is the largest state in Malaysia and has tropical geography and an equatorial climate. These relatively high daily average temperature and all-year-round humidity climatic conditions are ideal for tick survival [194]. Furthermore, Sarawak has undergone massive forest degradation and fragmentation, mainly because of logging activities and oil palm plantations [37, 78], and land conversion has been significantly related to increasing emerging or re-emerging zoonotic diseases, including vector-borne diseases [284, 296].

Ticks are obligate hematophagous arthropods, feeding on a wide range of animal hosts, including humans. It is established that ticks have high public health importance by harboring various pathogenic agents and play a crucial role as a vector in disease transmission to humans and animals. Tick-borne diseases (TBDs), which afflict humans and animals, are caused by infectious agents transmitted by tick bites. These infectious agents transmitted by ticks are tick-borne pathogens (TBPs), which include bacteria, viruses, and protozoa. For example, ticks are known to harbor medically-important bacterial species from a wide range of genera, including:

Anaplasma and Ehrlichia

Anaplasmosis and ehrlichiosis are TBDs caused by obligate intracellular bacteria of the genera *Anaplasma* and *Ehrlichia*, respectively. Among six *Anaplasma* spp., *A. phagocytophilum* is the causative agent of human and animal granulocytic anaplasmosis. Human granulocytic infections were first reported in 1994 [64]. Other *Anaplasma* spp. have been reported as the causative agents of anaplasmosis in animals. For example, *A. platys* causes infectious canine cyclic thrombocytopenia. *A. marginale*, *A. centrale*, and *A. ovis* are the causative agents for bovine anaplasmosis, whereas *A. bovis* infects a wide range of mammal species [65]. Among *Ehrlichia* spp., *E. chaffeensis* and *E. ewingii* are associated with human infections. The first human monocytic ehrlichiosis caused by *E. chaffeensis* was documented in 1991 [7, 55], followed by in 1999, when *E. ewingii* was first identified as an agent of human disease [39]. Clinical manifestations of human anaplasmosis and ehrlichiosis are similar and nonspecific, mimicking other acute febrile illnesses.

Seroprevalence study of farmworkers and indigenous people residing in Peninsular Malaysia revealed 29.9% and 34.3% of IgG antibodies against *E. chaffeensis* detected from farmworkers and indigenous people, respectively. In addition, 6.9% of the indigenous people were seropositive to *A. phagocytophilum* [138]. Similarly, there was seropositive evidence of *E. chaffeensis* being documented from neighboring countries in Indonesia and Thailand [28, 97, 236]. In conjunction with the serological survey, Koh et al. [138] also conducted tick surveillance from farm animals, wildlife, peri-domestic

animals, and vegetation adjacent to the survey areas. They reported the presence of several species of *Anaplasma* spp., including *A. marginale, A. bovis, A. platys, A. phagocytophilum,* and *Anaplasma* spp. closely related to *Candidatus* Cryptoplasma californiense in ticks. They did not detect the DNA of *E. chaffeensis,* instead they found *Ehrlichia* sp. strain EBm52, *E. mineirensis* and *Candidatus* Ehrlichia shimanensis. Molecular detection of *E. canis* in dogs [201] and *A. platys* in cattle were also recently documented [3].

Bartonella

Members of the genus *Bartonella* are fastidious, gram-negative, short rod intracellular bacteria that infect a diverse array of mammalian hosts via the blood-sucking arthropods, including sand fly, human body louse, cat fleas, and ticks [8, 187]. At least 20 species are known to cause host-specific intraerythrocytic infections in their specific mammalian reservoir hosts. Moreover, at least 13 species of *Bartonella* have been identified as pathogenic to humans, with the most common causative agents being *B. bacilliformis, B. quintana,* and *B. henselae* [35, 117]. *Bartonella bacilliformis* is the first *Bartonella* species identified as a human pathogen in the early 1900s, causing Carrion's disease or Oroya fever in the acute phase with high mortality, verruga peruana or Peruvian wart in the chronic phase. *Bartonella bacilliformis* is transmitted by the sand fly *(Lutzomyia verrucarum*), and the disease is geographically specific, reported in South American countries. *Bartonella quintana* causes trench fever and is transmitted by the human body louse (*Pediculus humanus humanus*). Endocarditis, generalized lymphadenopathy, and bacillary angiomatosis are the common symptoms reported in immunocompromised people infected by *B. quintana*. *Bartonella henselae* causing cat-scratch disease is the most common symptomatic *Bartonella* infection reported worldwide. Overall, *Bartonella* spp. infections are often chronic or asymptomatic in their reservoir hosts [12, 111]. Recently, hallucinations, weight loss, muscle fatigue, and neurological manifestations such as partial paralysis and so on have been related to *Bartonella* infection [33, 34]. Case studies have also reported *Bartonella* in tumors such as those of vasoproliferative and mammary tissue [180, 181, 222]. Therefore, bartonellosis has public health importance and warrants investigation.

Although *Bartonella* DNA was detected in several tick species in multiple studies [35, 45, 98, 114, 232], the transmission of *Bartonella* spp. by ticks to humans and animals is still controversially discussed as the vector competence of naturally infected ticks have not been confirmed. Nevertheless, there have been experimental studies that demonstrated vector competence for *B. henselae* [50] and *B. birtlesii* infection in ticks [234]. Rodents are considered important reservoir hosts for tick-borne pathogens as they are the preferred hosts of tick larvae and nymphs. Several *Bartonella* spp. have been detected in rodents which further supports the possibility that ticks may play a role in *Bartonella* transmission [44, 92, 134, 171]. Recently, Blasdell et al. [30] reported a high prevalence of *Bartonella* spp. in rodents from urban areas of a growing city in Sarawak, Borneo.

Borrelia

Members of the *Borrelia burgdorferi* sensu lato (Bbsl) complex are etiological agents of Lyme disease (LD), which currently, more than 20 genospecies have been reported worldwide [179]. The natural transmission cycles of Bbsl are maintained between their vertebrate reservoir hosts and ixodid (hard) ticks (Acari: *Ixodidae*) as the vector [96, 220]. LD is endemic in east Asian countries as there have been reports from China, Japan, Taiwan, South Korea, and Russia [119, 185]. Relapsing fever *Borrelia* (RFB) is categorized into three genetic groups: New World RFB, Old World RFB, and RFB harbored by ixodid ticks. RFB harbored by ixodid ticks, including *B. miyamotoi*, *B. lonestari*, and *B. theileri*, are mainly transmitted by the genus *Ixodes*, *Amblyomma*, and *Rhipicephalus* ticks, respectively [17, 76, 252]. Among these three, *B. miyamotoi* is pathogenic to humans [221], and *B. theileri* is responsible for bovine borreliosis [253]. *Borrelia miyamotoi* infection in humans was first reported in Russia in 2011, and, since then, cases have been documented in the United States, Europe, Japan, and northeastern China [105, 115, 143, 221, 245]. Only a few studies on *Borrelia* spp. were conducted in Peninsular Malaysia, in which most of the prior studies conducted focused on tick surveillance, and little is known about the occurrence of these etiological agents in most parts of Malaysia.

Coxiella

Q fever is a worldwide zoonotic disease caused by the obligate intracellular bacterium *Coxiella burnetii* [286]. The primary mode of infection is through inhalation of contaminated aerosols, and livestock like ruminants are the primary source of human infections [11, 188]. Although ruminants are the main reservoir of *C. burnetii*, the bacterium has also been detected from various vertebrates and invertebrates, including birds, dogs, horses, small mammals, and ticks [40, 239, 286]. Ruminants infected with *C. burnetii* show signs of abortion and decreased milk production [279]. In humans, infection with *C. burnetii* is often asymptomatic and self-limiting, but an acute phase of Q fever causes high fever and malaise. Furthermore, the chronic phase is often debilitating even though with treatment. It is also reported that *C. burnetii* DNA could still be detected in the bone marrow of patients with Q fever previously, as long as up to 12 years [279].

The majority of Q fever studies have been conducted on humans and livestock, while only a fraction involves other mammalian hosts. Among them, wild terrestrial small mammals such as rodents are thought to be maintenance hosts of *C. burnetii*, particularly the genus *Rattus* is considered as a true reservoir because all studies conducted in Europe revealed that it is consistently positive to *C. burnetii* DNA [110, 191, 235]. Further support that small mammals as reservoirs was the detection of specific *C. burnetii* antibodies by Meredith et al. [192]. In addition, the detection of *C. burnetii* DNA in small mammal spleen samples and the rat feces suggested that replication and shedding of *C. burnetii* did occur in rodents [1, 84].

Francisella

Genus *Francisella* is a gram-negative, facultative intracellular bacterium that is comprised of five species, *F. tularensis*, *F. philomiragia*, *F. hispaniensis*, *F. noatunensis*, and *F. novicida*. The existence of *Francisella* in the environment is divided into the terrestrial cycle and aquatic cycle [90]. *Francisella tularensis* is a highly infectious tick-borne bacterium causing tularemia in humans and animals worldwide. Four subspecies of *F. tularensis* are recognized, *F. tularensis* subp. *tularensis* (type A), *F. tularensis* subp. *holarctica* (type B), *F. tularensis* subp. *mediasiatica*, and *F. tularensis* subp. *novicida* [92]. All the reported subspecies are pathogenic, with the most virulent being *F. tularensis* ssp. *tularensis*, while other subspecies can cause disease in immunocompromised individuals. *Francisella tularensis* is primarily a pathogen of the orders Lagomorpha and Rodentia but has been reported in hundreds of other species, including birds, amphibians, and fish [92]. Other species of *Francisella*, such as *F. philomiragia*, *F. novicida*, and *F. hispaniensis* are generally less virulent, but diseases have been reported in immunocompromised patients [100, 106]. *F. tularensis* was first detected in ticks of the species *Dermacentor andersoni* [214], and later on, ticks have been reported as having a substantial role both in maintaining *F. tularensis* in nature and disease transmission. Furthermore, *F. tularensis* infections occur via ingestion, inhalation, as well as tick bites [4].

Rickettsia

Rickettsia are gram-negative, obligate intracellular bacteria transmitted by a variety of hematophagous arthropods, including ticks, lice, mites, and fleas. Rickettsioses are emerging infectious diseases that are often neglected in tropical regions. The genus is comprised of four groups, a basal ancestral group (AG), the spotted fever group (SFG), the typhus group (TG), and the transitional group. All but the ancestral group contain established human pathogens [59, 82, 216].

The SFG rickettsiae are tick-transmitted rickettsioses. Ticks act as vectors and reservoirs for most SFG rickettsiae by transmitting the bacteria transtandially and transovarially [255]. There are 25 validated SFG rickettsiae, and among *R. rickettsia* is considered the most pathogenic rickettsial species causing Rocky Mountain Spotted Fever mainly in the United States and in part of Mexico [6, 59, 216]. In Asia, SFG rickettsiae that have been reported include *Rickettsia conorii*, *R. sibirica*, *R. japonica*, *R. honei*, *R. heilongjiangensis*, *R. tamurae*, and *R. raoultii* [216]. Typhus group consists of louse-borne typhus, caused by *R. prowazekii* transmitted by body louse (*Pediculous humanus humanus*) [29]. Murine typhus is flea-borne (*Xenopsylla cheopis*) typhus caused by *R. typhi*, which occurs worldwide at endemic levels, especially in tropical and subtropical regions [29]. *Rickettsia akari*, *R. australis*, and *R. felis* are pathogenic rickettsial species in the transitional group. Disease attributed to *R. felis* is transmitted by cat fleas (*Ctenocephalides felis*) and has a worldwide distribution [36].

In Malaysia, serological surveillances revealed high antibody prevalence for SFG rickettsiae involving rural febrile patients (42.5% against *R. honei* TT118 strain), rubber estate workers (~50%),

indigenous people (50% against *R. conorii* and 22.5% against *R. felis*), farm workers (13.8% against *R. conorii* and 16.1% against *R. felis*), blood donor and so on in Peninsular Malaysia [126, 266, 269, 271,]. Case reports on Spotted Fever Rickettsiosis and murine typhus have also been diagnosed in Peninsula and Sabah, Malaysia [125, 243].

Babesia spp. are tick-borne protozoan parasites of the phylum Apicomplexa that infect erythrocytes of mammals and birds [246]. Over 100 *Babesia* spp. have been reported worldwide from a vast range of vertebrate hosts including: Bovidae, Canidae, Cervidae, Felidae, Mustelidae, Rodentia, Marsupialia, and humans [104, 113]. Despite its significant public health and veterinary implications, the tick-borne protozoal diseases caused by *Babesia* spp. have remained understudied in Malaysia. Currently, reported studies in Malaysia have been limited to bovine and canine babesiosis, with the detection of *B. bigemina* and *B. bovis* in cattle, and *B. gibsoni* and *B. vogeli* in dogs, as well as from the ticks infesting these animals [142, 210, 223].

In Malaysia, TBDs are regarded to be a potential emerging threat to public health. Thus far, tick bite cases and TBDs were rarely being reported in Malaysia, which could be expected due to a general lack of awareness of TBDs. Nevertheless, there was a report of a human bite case by *A. testudinarium*, which caused erythema at the biting sites [288]. In addition, based on a survey conducted in 2013 on some farms in Peninsular Malaysia, it was revealed that a large number of farmworkers, including administrative workers, had experienced tick bites [80]. With that remark, the risk of exposure to tick bites may generally be underestimated in Malaysia.

Likewise, studies eliciting tick microbiome are limited in Malaysia and not reported from Sarawak, Malaysia Borneo [128, 157]. The tick microbiome consists of largely non-pathogenic microorganisms essential for tick survival, development, and reproduction. Ticks acquire symbiotic microorganisms via the environment, blood meal hosts, and parents by transtandial and transovarial routes. The presence of a high abundance of non-pathogenic organisms in ticks suggests a constant interaction between the tick, pathogen, and endosymbionts. Therefore, understanding the microbial structure in ticks may be crucial to unravel the dynamics of pathogen colonization and transmission.

Taken together, the status of TBPs in Malaysia warrants further investigation, especially in Sarawak state that has undergone massive landscape alteration in past decades due to the logging and oil palm plantations. In addition, precise identification of tick species is crucial for epidemiological investigation, prevention, and control of TBDs in general. Obtaining baseline information and insights on tick microbiome and TBPs from Sarawak, Malaysian Borneo, is pivotal for the direction and design of upcoming research from this region, for example, by pinpointing the TBPs that required immediate attention. Therefore, this study aimed to conduct a molecular survey on different tick and rodent species collected from primary forests and an oil palm plantation in Sarawak, Malaysian Borneo.

The present thesis consists of five chapters. In chapter I, tick species collected from flagging and rodent hosts were identified with morphological and molecular methods. In chapter II, rodents and *Ixodes* ticks were screened for *Borrelia* spp., followed by species characterization using multilocus sequence analysis. In chapter III, high-throughput screening using Next-generation sequencing (NGS) for tick microbiome and tick-borne bacterial pathogens was conducted, followed by the multi-species comparative analysis of tick microbiome. In chapter IV, a molecular survey of tick-borne bacterial

pathogens was conducted in rodents. Finally, in chapter V, the tick-borne protozoal screening on *Babesia* was carried out on rodent and tick samples.

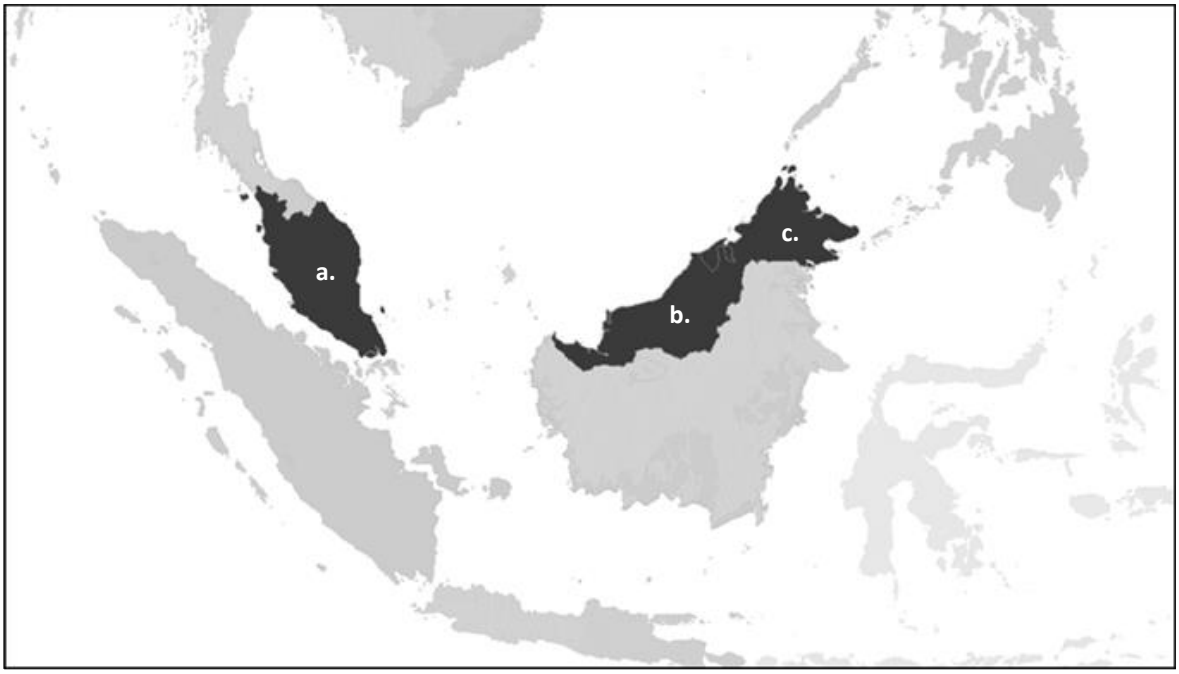

Figure 1. Malaysia is divided into a. Peninsular Malaysia and b. & c. Malaysian Borneo. Malaysian Borneo consists of b. Sarawak and c. Sabah states and is located in Borneo Island.

Chapter I

Tick species identified from primary forests and an oil palm plantation in Sarawak, Borneo

Introduction

In the tropics such as Malaysia, ticks are the second most important vector after mosquitoes causing human vector-borne diseases [56]. To date, at least 45 tick species have been documented in Malaysia [219]; however, their identification and systematics remain mostly unresolved. Moreover, tick morphological identification relies on dichotomous keys availability and requires intensive training. Nevertheless, the advance of molecular characterization using DNA markers, such as cytochrome c oxidase subunit I (*COI*) [95] and 16S rDNA (*mt-rrs*) [27], have enabled corroboration of several tick species in Malaysia based upon phenotypic and genotypic traits.

To date, tick species belonging to the genera of *Ixodes*, *Haemaphysalis*, *Dermacentor*, and *Amblyomma* have been previously described and characterized in Malaysia [46, 70, 71, 263]. These tick genera have been frequently documented from the vegetation and wildlife of primary, fringe, and secondary forest habitats or livestock farms in Malaysia [199]. Amongst, *Ixodes* is considered the most common and abundant tick species infesting mammals, especially rodents, in a study undertaking multiple sites in Peninsular Malaysia [170]. In addition, *Ixodes* is the only reported tick species where adult individuals can be found on rodents in Malaysia [199]. *Haemaphysalis hystricis, H. wellingtoni, and H. bispinosa* are the commonly reported species for the genus *Haemaphysalis* [70, 128]. Meanwhile, *D. steini*, *D. compactus*, *D. auratus*, and *D. atrosignatus* are the commonly reported *Dermacentor* ticks in Malaysia [71, 199]. *Amblyomma testudinarium* and *A. helvolum* parasitizing on large mammals and reptiles and amphibians, respectively, have been documented. Additionally, *A. cordiferum* was recently described from a reticulated python [263]. A list of literature that includes the recent morphological or molecular phylogenic identification of tick species in Malaysia is presented in Table 1.

Precise identification of tick species is essential for TBP investigation and effective TBD control and prevention. However, despite the importance, tick species distributed in Sarawak, Malaysian Borneo remains understudied. Thus, the aim of this study was to identify the tick species collected from the Sarawak state with morphological and molecular methods.

Table 1. A list of literature on morphological and molecular phylogenic identification on Malaysian tick species until the year 2020.

No	Title	Year	Reference
1	Ticks (Ixodoidea) of Borneo and Malaya	1957	[140]
2	Description of new *Dermacentor* (Acari: Ixodidae) species from Malaysia and Vietnam	2015	[15]
3	Precise identification of different stages of a tick, *Ixodes granulatus* Supino, 1897 (Acari: Ixodidae)	2016	[46]
4	Description of two new species of *Dermacentor* Koch, 1844 (Acari: Ixodidae) from Oriental Asia	2016	[16]
5	Phenotypic and genotypic identification of hard ticks of the genus *Haemaphysalis* (Acari: Ixodidae) in Peninsular Malaysia	2017	[70]
6	*Amblyomma cordiferum* Neumann, 1899 (Acari: Ixodidae) parasitizing reticulated pythons, *Malayopython reticulatus* (Schneider, 1801) (Reptilia: Pythonidae) in Peninsular Malaysia	2019	[263]
7	A checklist and key to the tick fauna (Acari: Ixodidae, Argasidae) of Pulau Tioman, Malaysia	2020	[151]
8	Description and characterization of questing hard tick, *Dermacentor steini* (Acari: Ixodidae) in Malaysia based on phenotypic and genotypic traits.	2020	[71]

Materials and Methods

Ethics approvals

The collecting of rodents and ticks was approved by the Forest Department Sarawak, Malaysia (Permit No. (91) JHS/NCCD/600-7/2/107 and Park Permit No. WL47/2018; Permit No. (11) JHS/NCCD/600-7/2/107(Jld2) and Park Permit No.WL5/2019). The sampling methods were approved by the Animal Care and Use Committee of Hokkaido University, Japan (Approval No. 18-0081). The samples were exported with the permission of the Forest Department Sarawak (No.18650).

Survey sites and sample collection

Two protected primary forests, Gunung Gading National Park (GGNP hereafter) (1.69°N, 109.85°E) and Kubah National Park (KNP hereafter) (1.61°N, 110.20°E), and an oil palm (OP hereafter) plantation (3.36°N, 113.69°E) in Sarawak were selected as the study sites. The rodents and ticks were collected from GGNP and KNP during the wet season in November 2018 and from the OP plantation during the dry season in March 2019 (Figure 2). The sampling period for each site ranged from 5–10 days. The rodents were captured using collapsible cage traps; their tentative species, sex, breeding status, and body measurements were recorded. The captured rodents were individually anesthetized using isoflurane, and the ticks attached to each rodent were removed. Questing ticks were also collected by dragging white flannel cloths over the forest floor at the rodent trapping sites. All ticks collected from rodent hosts and flagging were kept separately in 70% ethanol and stored at −20℃ until sample processing and DNA extraction.

DNA preparation and morphological identification of ticks

The tick genera or species were morphologically identified based on the taxonomic keys [46, 71, 140, 265] before the DNA extraction, followed by removing one leg of the ticks for DNA extraction. DNA was extracted by using the hot alkaline extraction method previously described by Mtambo et al. [197], with some modifications. Briefly, 10 µL of 100 nM of sodium hydroxide was added and incubated at 95℃ for 10 min, followed by adding 2 µL of tris-hydrochloride buffer (pH 7.0).

PCR amplification and sequencing

Tick mitochondrial 16S ribosomal DNA

For molecular identification and confirmation, a fragment of tick mitochondrial 16S ribosomal DNA (16S rDNA) was amplified with the primer pair mt-rrs1 (5′-CTGCTCAATGATTTTTTAAATTGCTGTGG-3′) and mt-rrs2 (5′-CCGGTCTGAACTCAGATCAAGTA-3′), which amplified about 400-bp fragment [277]. The PCR was

conducted using Tks Gflex DNA Polymerase (Takara Bio, Shiga, Japan), with the following conditions: initial denaturation at 94℃ for 1 min, followed by 40 cycles of 98℃ for 10 sec, 55 ℃ for 15 sec, and 68℃ for 24 sec, and a final extension at 68℃ for 5 min. The amplification products were electrophoresed on a 1.2% agarose gel with Midori Green Direct DNA stain (Nippon Genetics, Tokyo, Japan) and visualized with a BLooK LED transilluminator (GeneDireX, Las Vegas, NV, USA). The Sanger sequencing was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were analyzed on an ABI Prism 3130 x genetic analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Cytochrome c oxidase subunit 1 and TA cloning

Further characterization using PCR was conducted on 28 tick samples (Table 4) with the primer pair LC01490 (5′- GGTCAACAAATCATAAAGATATTGG-3′) and HC02198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), which amplified a 710-bp fragment of Cytochrome c oxidase subunit 1 (*CO1*) gene. The PCR was conducted using KOD FX Neo, with the following conditions: initial denaturation at 95℃ for 1 min, followed by 40 cycles of 98℃ for 15 sec, 50℃ for 15 sec, and 72℃ for 30 sec, and a final extension at 72℃ for 2 min. The amplification products were electrophoresed on a 1% agarose gel with ethidium bromide dye and visualized for the targeted bp size. After that, 17 amplified PCR products were purified with AxyPrep Magnetic Bead Purification Kit, and sequencing was performed by Genewiz (Azenta Life Sciences, Tokyo, Japan).

Five tick samples, including three samples of the genus *Amblyomma* (Sample IDs: Q023, Q015, and Q005) and two samples of the genus *Haemaphysalis* (Sample IDs: Q011 and Q013), were included for TA cloning. For the TA cloning, 1 µL of 10X A attachment mix (Toyobo, Osaka, Japan) was added to 9 µL of the PCR product from KOD FX Neo (Toyobo, Osaka, Japan), and the mixture was incubated at 60℃ for 10 min. Ligation was conducted using 2X rapid ligation buffer following the quick protocol for the Promega pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) and incubated at 12℃ for 3 hrs. The transformation was performed using Competent Quick DH5α (Toyobo, Osaka, Japan), followed by overnight incubation at 37℃. Colonies were cultivated in SOB (Super Optimal Broth) medium overnight before conducting the PCR using the *Ex Taq* Hot Start Version (Takara Bio, Shiga, Japan). Finally, PCR products were verified by electrophoresis and purified for Sanger sequencing.

12S ribosomal DNA

Seven tick samples of the genera *Amblyomma* (Sample IDs: Q023, Q015, and Q005), *Dermacentor* (Sample IDs: Q007 and Q017), and *Haemaphysalis* (Sample IDs: Q011 and Q013) were further selected for 12S ribosomal DNA (12S rDNA) amplification and characterization. The

amplification was performed with primer pair Tick-12S+1 (5′- TACTATGTTACGACTTA-3′) and Tick-12S-1 (5′-AAACTAGGATTAGATACCC-3′), which yielded about 400-bp fragment [205]. The PCR reaction mixture was prepared using KOD FX Neo (Toyobo, Osaka, Japan), and the PCR conditions were as follow: initial denaturation at 95℃ for 1 min, followed by 40 cycles of 95℃ for 15 sec, 50℃ for 15 sec, and 72℃ for 30 sec, and a final extension at 72℃ for 5 min. The PCR products were visualized on a 1% agarose gel with ethidium bromide dye. Purification of PCR products was done using AxyPrep Magnetic Bead Purification Kit (Axygen, Tewksbury, MA, USA), and sequencing was performed by Eurofins Genomics.

Phylogenetic analysis

Sequences obtained were compared with public databases using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (*https://blast.ncbi.nlm.nih.gov/Blast.cgi)*. For phylogenetic analysis, 28 representative tick samples were included for 16S rDNA analysis and phylogenetic tree construction. Phylogenetic tree for *CO1* gene was also constructed to elucidate the genetic relatedness of three *Dermacentor* spp. in this study. All phylogenetic trees were constructed in MEGA version X [147] using the Maximum Likelihood or Neighbour-Joining method and Kimura 2-parameter model with a bootstrap of 1000 replications.

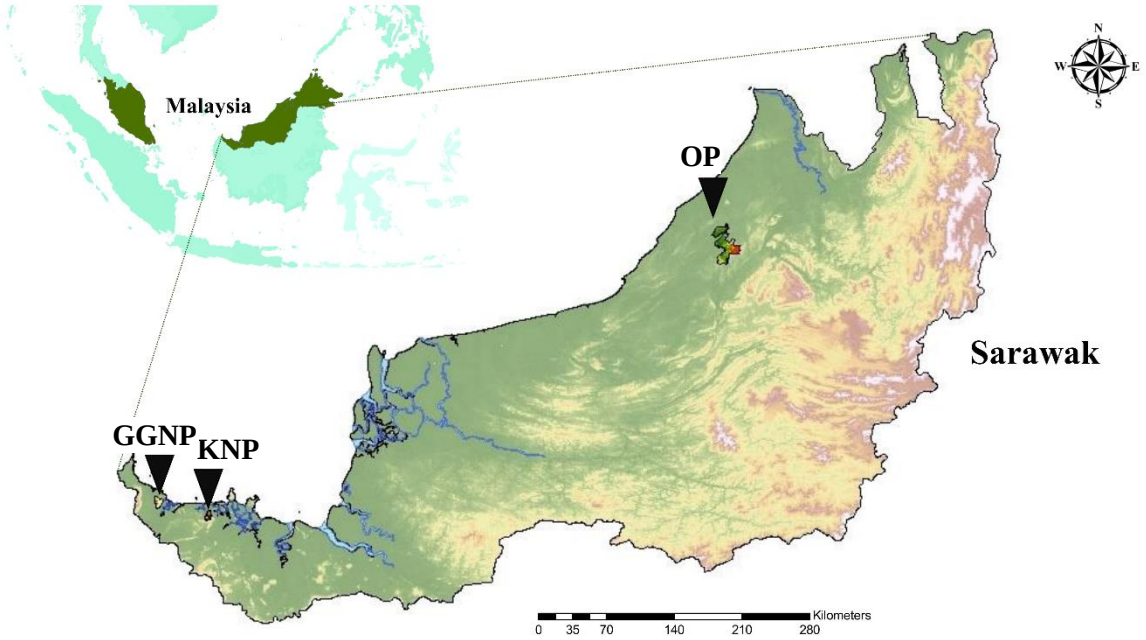

Figure 2. Map of the sampling sites. GGNP: Gunung Gading National Park; KNP: Kubah National Park; and OP: Oil palm plantation.

Results

Overall, 238 ticks from nine species of four genera were collected from GGNP, KNP, and OP plantation. Most of the ticks (n = 191) were collected from the OP plantation, while 47 ticks were from the primary forests, in which 83 ticks were collected from the rodents and 155 ticks were questing ticks. In general, a total of eight different tick species of *Ixodes*, *Haemaphysalis*, *Dermacentor*, *Amblyomma* genera were collected from both primary forests, GGNP and KNP. In comparison, four different tick species of three genera, including one *Ixodes*, two *Haemaphysalis*, and one *Dermacentor* species, were collected from the OP plantation. The tick species with the highest number collected was *H. shimoga* (n = 115) from the OP plantation, with all the developmental stages collected. This species was not found in primary forests during the sampling. Out of the four genera, three different species from the genera of *Haemaphysalis* and *Dermacentor*, two different species belong to the *Amblyomma* genus, and one *Ixodes* species were successfully being identified based on the morphological and molecular identification. The number, developmental stage, feeding status, and collection site of each tick species are shown in Table 2.

By using the mitochondrial 16S rDNA, most of the ticks in this study were able to be identified up to their species level, except for one *Dermacentor* sp. (Sample IDs: Q007 and Q017) and one *Haemaphysalis* sp. (Sample IDs: Q011 and Q013) (Table 3, Figure 3). The unknown *Haemaphysalis* sp. and *Dermacentor* sp. were moderately related to *H. mageshimaensis* (AB819211) and *D. taiwanensis* (AB819169), respectively, with 92% identity (Table 3). Two individuals of *Amblyomma* ticks (Sample IDs: Q015 and Q005) were closely related to *Amblyomma* sp. UG (AB602349), a larva sample obtained from an adult man in Peninsular Malaysia, with 99% identity (398/402 bp) (Table 3, Figure 3). The samples were found to cluster together with *A. testudinarium* identified from Thailand, Taiwan, and Japan in the phylogenetic tree (Figure 3). Another *Amblyomma* tick in this study (Sample ID: Q023) showed a close relation with *A. geoemydae* (KT382864) collected from a compressed tortoise in Thailand with 95.6% identity (389/407 bp) (Table 3). This sample also appeared to cluster together with other *A. geoemydae* in the phylogenetic tree (Figure 3). Phylogenetic inference based on mitochondrial 16S rDNA of the tick species collected in this study is depicted in Figure 3.

Further characterization with tick *CO1* gene has successfully identified *Dermacentor* sp. (Sample IDs: Q007 and Q017) as *D. atrosignatus*. The *CO1* sequence obtained showed high identity with *D. atrosignatus* collected in Malaysia (MW766912), with 98.9% identity (635/643 bp) (Table 4, Figure 4). The molecular identification of *D. atrosignatus* was also compatible with the morphological observation. In general, sequence analysis results for the *CO1* gene for most of the tick species were consistent with the findings of 16S rDNA. For instance, *D. compactus* (Sample IDs: Q006 and Q021) and *H. hystricis* (Sample IDs: Q016 and F018) revealed over 99% identity with their respective reference sequence from

the database (Table 4). Meanwhile, *I. granulatus* from this study (Sample IDs: F028 and F059) showed 94.1% (619/658 bp) and 93.6% identity (615/657 bp) with the *I. granulatus* from China (MG721046) (Table 4). In addition, one *Amblyomma* tick (Sample ID: Q005) showed 97.4% identity (602/618 bp) with the *Amblyomma* sp. FLMNH 42105 (MF983562) collected in Myanmar, while another sample (Sample ID: Q015) showed 89.7% identity (590/658 bp) with *A. testudinarium* AT19B37 (LC553841) collected in Japan (Table 4). For *Amblyomma* tick sample ID: Q023, the *CO1* sequence showed 92.5% identity (608/657 bp) with the *A. geoemydae* (MK814531) collected in China (Table 4).

Sequence analysis of tick 12S rDNA for *Amblyomma* ticks with the sample IDs: Q015 and Q005 showed 93.3% identity (350/375 bp) with *A. testudinarium* AT19B37 (LC553841) collected in Japan. In comparison, sample ID: Q023 showed 93.9% identity (367/391 bp) with *A. geoemydae* (MK814531) collected in China (Table 4). These findings were consistent with the sequence analysis for the *CO1* gene. Furthermore, morphological observation based on the taxonomic keys for *Amblyomma* ticks (Sample IDs: Q015 and Q005) was consistent with their molecular identification of *A. testudinarium*. However, for sample ID: Q023, the morphological observation did not fully support the molecular identification of *A. geoemydae* and is listed as *A. geoemydae*-like hereafter. Thus far, sequence analysis of all three genes for *Haemaphysalis* sp. (Sample IDs: Q011 and Q013) could not resolve its taxonomic assignment up to the species level (Tables 3 and 4).

Table 2. Total number of ticks and their developmental stage and status identified from Gunung Gading National Park (GGNP), Kubah National Park (KNP), and an oil palm (OP) plantation.

		Sampling period				Feeding status	
		November 2018		March 2019			
No	**Tick species**	**GGNP**	**KNP**	**OP**	**Total**	**Feeding**	**Questing**
1	***Ixodes granulatus***						
	Adult male (AM)	1	N/A	N/A	1	N/A	1
	Adult female (AF)	3	10	11	24	24	N/A
	Nymph (N)	N/A	2	4	6	6	N/A
	Larva (L)	N/A	N/A	6	6	6	N/A
	Total	4	12	21	37	36	1
2	***Haemaphysalis hystricis***						
	Adult female (AF)	1	N/A	1	2	N/A	2
	Nymph (N)	1	N/A	N/A	1	1	N/A
	Larva (L)	1	N/A	37	38	4	34
	Total	3	N/A	38	41	5	36
3	***Haemaphysalis shimoga***						
	Adult male (AM)	N/A	N/A	23	23	N/A	23
	Adult female (AF)	N/A	N/A	29	29	N/A	29
	Nymph (N)	N/A	N/A	22	22	18	4
	Larva (L)	N/A	N/A	41	41	18	23
	Total	N/A	N/A	115	115	36	79
4	***Haemaphysalis* sp.**						
	Adult male (AM)	1	N/A	N/A	1	N/A	1
	Adult female (AF)	1	N/A	N/A	1	N/A	1
	Total	2	N/A	N/A	2	N/A	2
5	***Dermacentor compactus***						
	Adult male (AM)	2	1	N/A	3	N/A	3
	Adult female (AF)	1	2	N/A	3	N/A	3
	Total	3	3	N/A	6	N/A	6
6	***Dermacentor steini***						
	Adult female (AF)	5	3	N/A	8	3	5
	Nymph (N)	1	N/A	N/A	1	1	N/A
	Larva (L)	2	N/A	17	19	2	17
	Total	8	3	17	28	6	22
7	***Dermacentor atrosignatus***						
	Adult male (AM)	3	N/A	N/A	3	N/A	3
	Adult female (AF)	2	1	N/A	3	N/A	3
	Total	5	1	N/A	6	N/A	6
8	***Amblyomma testudinarium***						
	Adult male (AM)	1	N/A	N/A	1	N/A	1
	Nymph (N)	1	N/A	N/A	1	N/A	1
	Total	2	N/A	N/A	2	N/A	2

9	***Amblyomma geoemydae*-like**						
	Nymph (N)	N/A	1	N/A	1	N/A	1
	Total	N/A	1	N/A	1	N/A	1
	Total	**27**	**20**	**191**	**238**	**83**	**155**

Table 3. The sequence analysis results for mitochondrial 16S ribosomal DNA for 28 tick samples of eight species and four genera from this study.

Sample ID	**Developmental stage**	**BLASTn**	**Identity**	**Accession no.**
Q015	AM	*Amblyomma* sp. UG (Malaysia)	99.0% (398/402 bp)	AB602349
Q005	N	*Amblyomma* sp. UG (Malaysia)	99.0% (398/402 bp)	AB602349
Q023	N	*Amblyomma geoemydae* isolate AGMI15 (Thailand)	95.6 % (389/407 bp)	KT382864
F007	AF	*Ixodes granulatus* (Malaysia)	98.5% (405/411 bp)	U95885
F055	AF	*Ixodes granulatus* (Malaysia)	99.3% (407/410 bp)	U95885
F028	AM	*Ixodes granulatus* (Malaysia)	98.5% (405/411 bp)	U95885
F059	N	*Ixodes granulatus* (Malaysia)	99.3% (407/410 bp)	U95885
F085	L	*Ixodes granulatus* (Malaysia)	98.8% (405/410 bp)	U95885
Q006	AM	*Dermacentor compactus* (Malaysia)	99.3% (402/405 bp)	MK316361
Q021	AF	*Dermacentor compactus* (Malaysia)	99.0% (401/405 bp)	MK316361
Q007	AM	*Dermacentor taiwanensis* (Tokushima, Japan)	92.1% (373/405 bp)	AB819169
Q017	AF	*Dermacentor taiwanensis* (Tokushima, Japan)	92.1% (373/405 bp)	AB819169
Q020	AF	*Dermacentor steini* (Malaysia)	99.5% (399/401 bp)	MK296407
F019	N	*Dermacentor steini* (Malaysia)	99.5% (399/401 bp)	MK296407
Q054	L	*Dermacentor steini* (Malaysia)	99.3% (398/401 bp)	MK296407
F022	L	*Dermacentor steini* (Malaysia)	99.8% (400/401 bp)	MK296407
Q016	AF	*Haemaphysalis hystricis* from *Sus scrofa* (China)	100% (401/401 bp)	MT294298
F018	N	*Haemaphysalis hystricis* from *Sus scrofa* (China)	100% (401/401 bp)	MT294298
Q134	AF	*Haemaphysalis hystricis* from *Rattus tiomanicus* (Malaysia)	98.0% (394/402 bp)	MT912970
Q105	L	*Haemaphysalis hystricis* from *Rattus tiomanicus* (Malaysia)	98.0% (394/402 bp)	MT912970
Q043	L	*Haemaphysalis hystricis* from *Rattus tiomanicus* (Malaysia)	98.0% (394/402 bp)	MT912970
F042	AF	*Haemaphysalis shimoga* (Thailand)	99.8% (403/404 bp)	KC170730
Q133	AF	*Haemaphysalis shimoga* (Thailand)	99.8% (403/404 bp)	KC170730
Q120	AM	*Haemaphysalis shimoga* (Thailand)	99.8% (403/404 bp)	KC170730
Q135	AM	*Haemaphysalis shimoga* (Thailand)	99.8% (403/404 bp)	KC170730
Q117	N	*Haemaphysalis shimoga* (Thailand)	99.8% (403/404 bp)	KC170730
Q011	AM	*Haemaphysalis mageshimaensis* (Kagoshima, Japan)	91.9% (373/406 bp)	AB819211
Q013	AF	*Haemaphysalis mageshimaensis* (Kagoshima, Japan)	91.9% (373/406 bp)	AB819211

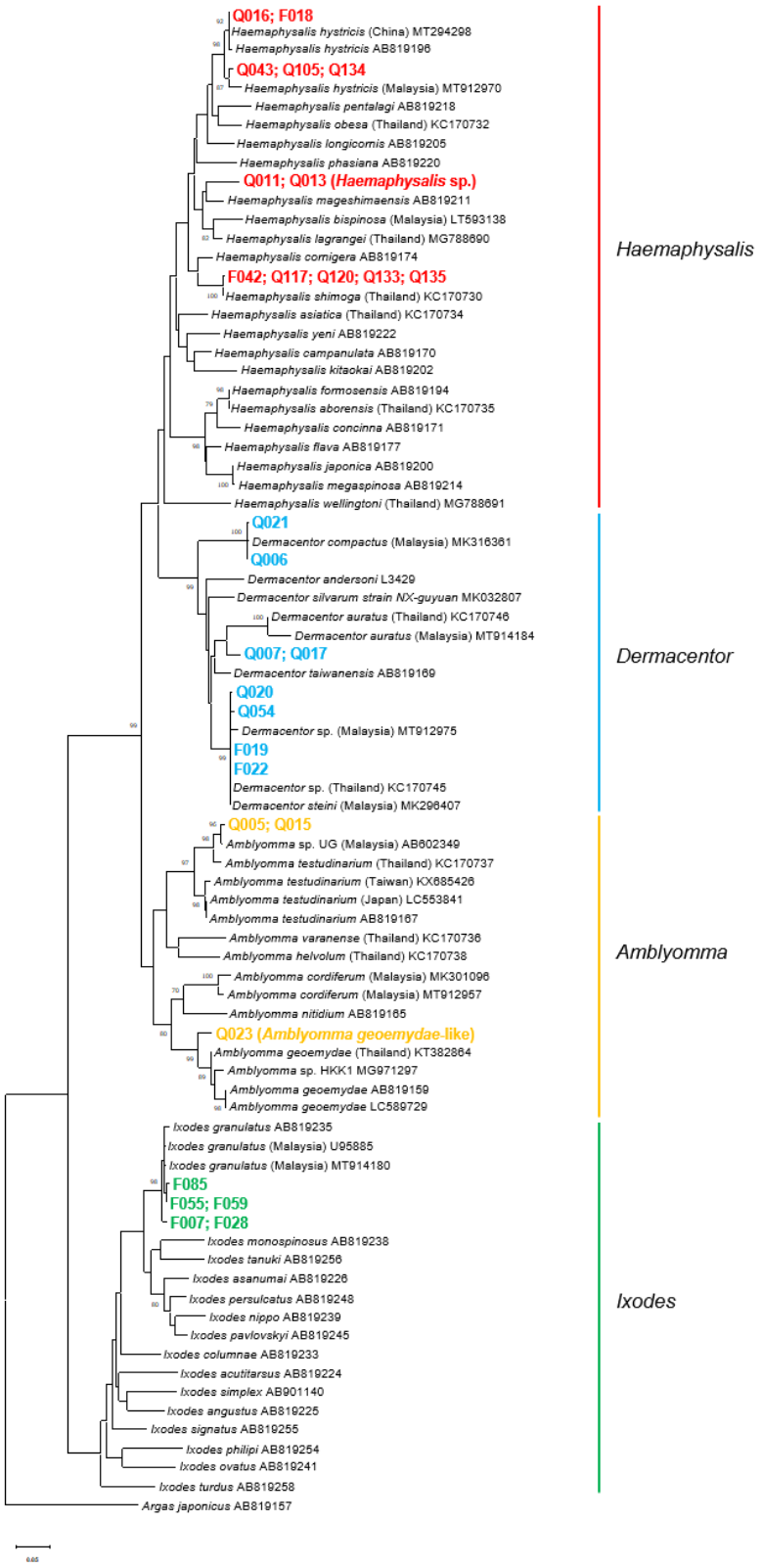
Q016; F018
Haemaphysalis hystricis (China) MT294298
Haemaphysalis hystricis AB819196
Q043; Q105; Q134
Haemaphysalis hystricis (Malaysia) MT912970
Haemaphysalis pentalagi AB819218
Haemaphysalis obesa (Thailand) KC170732
Haemaphysalis longicornis AB819205
Haemaphysalis phasiana AB819220
Q011; Q013 (Haemaphysalis sp.)
Haemaphysalis mageshimaensis AB819211
Haemaphysalis bispinosa (Malaysia) LT593138
Haemaphysalis lagrangei (Thailand) MG788690
Haemaphysalis cornigera AB819174
F042; Q117; Q120; Q133; Q135
Haemaphysalis shimoga (Thailand) KC170730
Haemaphysalis asiatica (Thailand) KC170734
Haemaphysalis yeni AB819222
Haemaphysalis campanulata AB819170
Haemaphysalis kitaokai AB819202
Haemaphysalis formosensis AB819194
Haemaphysalis aborensis (Thailand) KC170735
Haemaphysalis concinna AB819171
Haemaphysalis flava AB819177
Haemaphysalis japonica AB819200
Haemaphysalis megaspinosa AB819214
Haemaphysalis wellingtoni (Thailand) MG788691
Haemaphysalis
Q021
Dermacentor compactus (Malaysia) MK316361
Q006
Dermacentor andersoni L3429
Dermacentor silvarum strain NX-guyuan MK032807
Dermacentor auratus (Thailand) KC170746
Dermacentor auratus (Malaysia) MT914184
Q007; Q017
Dermacentor taiwanensis AB819169
Q020
Q054
Dermacentor sp. (Malaysia) MT912975
F019
F022
Dermacentor sp. (Thailand) KC170745
Dermacentor steini (Malaysia) MK296407
Dermacentor
Q005; Q015
Amblyomma sp. UG (Malaysia) AB602349
Amblyomma testudinarium (Thailand) KC170737
Amblyomma testudinarium (Taiwan) KX685426
Amblyomma testudinarium (Japan) LC553841
Amblyomma testudinarium AB819167
Amblyomma varanense (Thailand) KC170736
Amblyomma helvolum (Thailand) KC170738
Amblyomma cordiferum (Malaysia) MK301096
Amblyomma cordiferum (Malaysia) MT912957
Amblyomma nitidium AB819165
Q023 (Amblyomma geoemydae-like)
Amblyomma geoemydae (Thailand) KT382864
Amblyomma sp. HKK1 MG971297
Amblyomma geoemydae AB819159
Amblyomma geoemydae LC589729
Amblyomma
Ixodes granulatus AB819235
Ixodes granulatus (Malaysia) U95885
Ixodes granulatus (Malaysia) MT914180
F085
F055; F059
F007; F028
Ixodes monospinosus AB819238
Ixodes tanuki AB819256
Ixodes asanumai AB819226
Ixodes persulcatus AB819248
Ixodes nippo AB819239
Ixodes pavlovskyi AB819245
Ixodes columnae AB819233
Ixodes acutitarsus AB819224
Ixodes simplex AB901140
Ixodes angustus AB819225
Ixodes signatus AB819255
Ixodes philipi AB819254
Ixodes ovatus AB819241
Ixodes turdus AB819258
Argas japonicus AB819157
Ixodes
0.05

Figure 3. A maximum-likelihood phylogenetic tree based on mitochondrial 16S ribosomal DNA was constructed using 28 sequence samples from this study, the sequences from Takano et al. [260], and other published sequences from Malaysia and Thailand. Samples from this study are highlighted in bold and colors. Tick species not resolved are labeled as *Haemaphysalis* sp. and *Amblyomma geoemydae*-like in parentheses after their sample ID. Samples with identical sequences are listed in parallel with semicolons.

Table 4. The sequence analysis results for Cytochrome c oxidase subunit 1 (*CO1*) and 12S ribosomal DNA (rDNA) for selected tick samples. Samples with identical sequences are listed in parallel.

Gene	Sample ID	BLASTn	Identity	Accession no.
CO1	Q015	*Amblyomma testudinarium* AT19B37 (Japan)	89.7% (590/658 bp)	LC553841
	Q005	*Amblyomm*a sp. FLMNH 42105 (Myanmar)	97.4% (602/618 bp)	MF983562
	Q023	*Amblyomma geoemydae* (China)	92.5% (608/657 bp)	MK814531
	F028	*Ixodes granulatus* (China)	94.1% (619/658 bp)	MG721046
	F059	*Ixodes granulatus* (China)	93.6% (615/657 bp)	MG721046
	Q006	*Dermacentor compactus* (Malaysia)	99.4% (635/639 bp)	MW766910
	Q021	*Dermacentor compactus* (Malaysia)	99.5% (636/639 bp)	MW766910
	F019	*Dermacentor everestianus*	87.4% (574/657 bp)	NC_042764
	Q020	*Dermacentor everestianus*	87.2% (573/657 bp)	NC_042764
	Q007	*Dermacentor atrosignatus* (Malaysia)	98.9% (636/643 bp)	MW766912
	Q017	*Dermacentor atrosignatus* (Malaysia)	98.8% (635/643 bp)	MW766912
	Q016; F018	*Haemaphysalis hystricis* (China)	99.1% (652/658 bp)	MT013253
	F042	*Haemaphysalis bancrofti*	88.7% (583/657 bp)	NC_041076
	Q133	*Haemaphysalis bancrofti*	88.7% (583/657 bp)	NC_041076
	Q011; Q013	*Haemaphysalis verticalis*	88.3% (581/658 bp)	KR108850
12S rDNA	Q005; Q015	*Amblyomma testudinarium* AT19B37 (Japan)	93.3% (350/375 bp)	LC553841
	Q023	*Amblyomma geoemydae* (China)	93.9 % (367/391 bp)	MK814531
	Q007; Q017	*Dermacentor auratus* (Singapore)	92.5% (343/371 bp)	MW034677
	Q011; Q013	*Haemaphysalis longicornis* (China)	91.2% (333/365 bp)	MK450606

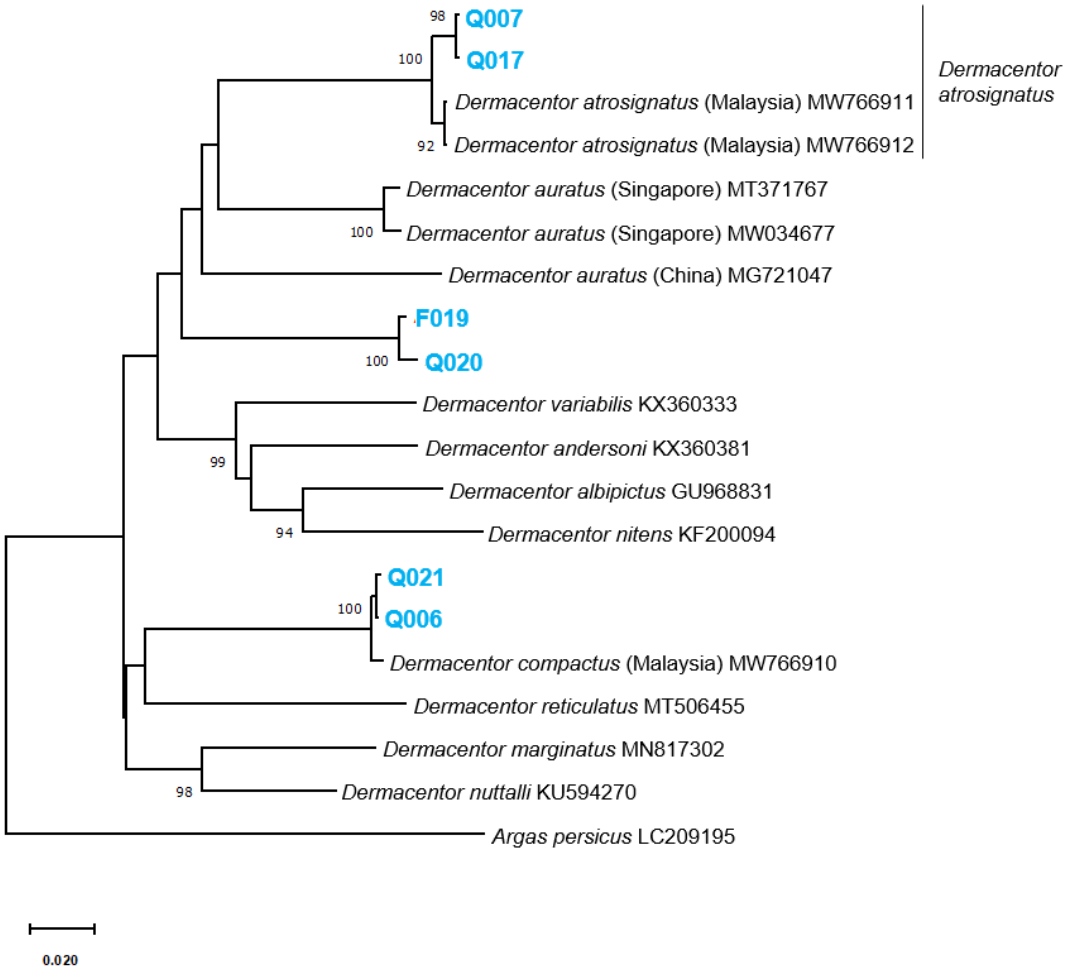

Figure 4. A neighbor-joining phylogenetic tree for *Dermacentor* spp. was constructed based on the Cytochrome c oxidase subunit 1 (*CO1*) gene. Samples from this study are highlighted in bold and blue colors. *Dermacentor atrosignatus*, which could not be identified with mitochondrial 16S ribosomal DNA, was identified based on the *CO1* gene.

Discussion

In this study, ticks collected from primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak, Borneo, were identified. A total of nine species of four genera were collected from these locations, in which seven species: *I. granulatus*, *H. hystricis*, *H. shimoga*, *D. compactus*, *D. steini*, *D. atrosignatus*, and *A. testudinarium* were successfully identified up to species level based on both taxonomic keys and molecular methods. However, although multiple genes (16S rDNA, *CO1*, and 12S rDNA) of ticks were used to investigate the tick species, one species of the *Haemaphysalis* genus could not be resolved to its species level. Furthermore, one *Amblyomma* sp., which was revealed as closely related to *A. geoemydae* by molecular phylogeny, did not fully match the morphological description for the species. Nevertheless, this study provided the first molecular evidence of multiple tick species in Sarawak, with the first report of *H. shimoga* in Malaysia.

Petney et al. [218] presented the historical perspective of Southeast Asian ticks and pointed out that the current status in most countries of the region has stagnated, except for a few regional reports and important collections. The only two major studies on regional ticks came from Tanskul et al. [264] for Thailand, and Kolonin [141] for Vietnam. The enormous efforts by Harry Hoogstraal and colleagues from the 1960s to the 1980s had contributed to our knowledge of ticks in this region. Nevertheless, most of the knowledge is scattered throughout the specialist literature, and much earlier works are no longer published [218]. The lack of information and synopsis of tick species in the region has added to the difficulties of ticks identification based on morphological phylogenies.

Precise identification of tick species is essential to control TBDs, and traditionally, ticks identification relies mainly on the published morphological keys. However, species identification based on morphological data is laborious and requires well-trained personnel. Furthermore, it can be difficult when the specimens are physically damaged, engorged, or in sub-adult stages (larvae or nymphs). Molecular identification provides accurate and fast identification based on several genes [168]; however, it can be costly to perform in some Southeast Asian countries when resources are limited. Another limitation is the availability of reference sequence in the database; as for the *Haemaphysalis* sp. in this study, it could not be resolved to species level even though several genes were examined.

Haemaphysalis shimoga was first described by Tapido and Hoogstraal [275] as *H. cornigera shimoga* subspecies from Southern India, and its distribution has been recorded from Thailand, Cambodia, Myanmar, Vietnam, and China [141, 156, 264]. To the best of our knowledge, *H. shimoga* has not been documented in Malaysia, or at least the species may not be identified in the available published literature from Malaysia. However, there have been records of the *H. cornigera*, both male and female individuals reported from Peninsula and Borneo Island [140], collected from sambar deer (*Cervus unicolor*), wild boar, and humans. This study presented *H. shimoga* from all developmental stages collected from an oil

palm plantation but not from the primary forests. More investigation on distribution will be needed to better understand this tick species in the future. For *A. geoemydae*-like species in this study, only a single specimen (nymph) was available for morphological and molecular phylogeny comparisons. Therefore, more specimens of different developmental stages will be helpful for better phylogenic elucidations and confirmation.

Overall, morphological and molecular data contribution for ticks is crucial, especially for Southeast Asian countries like Malaysia, which will be beneficial for accurate identification of tick species in the future. Furthermore, the most basic data on tick distribution, prevalence, and interaction with hosts are still lacking, let alone information on TBDs surveillance. Therefore, more works are essential to elucidate the tick species from this region.

Summary

Precise identification of tick species is essential for TBP investigation and effective TBD control and prevention. However, tick species distributed in Sarawak, Borneo remains understudied. Thus, this study aimed to identify the tick species collected from two primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak with morphological and molecular methods. A total of 238 ticks collected from flagging and rodent hosts were first morphologically identified up to their genus or species level based on the available taxonomic keys. Molecular identification of ticks was conducted based on three genes, 16S rDNA, cytochrome c oxidase subunit 1 (*CO1*), and 12S rDNA. Overall, nine species of four genera were collected from GGNP, KNP, and OP plantation, in which seven species, *Ixodes granulatus*, *Haemaphysalis hystricis*, *H. shimoga*, *Dermacentor compactus*, *D. steini*, *D. atrosignatus*, and, *Amblyomma testudinarium*, were successfully identified up to species level. However, one *Haemaphysalis* sp. was not be resolved to its species level. Furthermore, one *Amblyomma* sp., which revealed as closely related to *A. geoemydae* by molecular phylogeny, did not fully match the morphological description for the species. Nevertheless, this study provides the molecular evidence of multiple tick species in Sarawak, with the first report of *H. shimoga* in Malaysia. Overall, tick morphological and molecular data is crucial, especially for Southeast Asian countries like Malaysia, which will be beneficial for accurate identification of tick species in the future.

Chapter II

Detection of *Borrelia burgdorferi* sensu lato and Relapsing fever *borrelia* in feeding *Ixodes* ticks and rodents in Sarawak, Malaysia: New geographical records of *Borrelia yangtzensis* and *Borrelia miyamotoi*

Introduction

Members of the *Borrelia burgdorferi* sensu lato (Bbsl) complex are etiological agents of Lyme disease (LD), which currently, more than 20 genospecies have been reported worldwide [179]. High incidences of LD and high occurrences of its causative bacterium have been continuously reported since the discovery of LD spirochetes in 1982 in North America and later in 1983 in Europe [220]. In North America, *B. burgdorferi* sensu stricto (Bbss), *B. afzelii*, *B. garinii*, *B. californiensis*, *B. bissettiae*, *B. kurtenbachii*, *B. mayonii*, and *B. spielmanii* are known to be responsible for LD [74, 174, 176, 225, 237]. While in Europe, Bbss, *B. garinii*, *B. afzelii*, and *B. bavariensis* are the predominant causative bacterium for LD cases [257]. The natural transmission cycles of Bbsl are maintained between their vertebrate reservoir hosts and ixodid (hard) ticks (Acari: *Ixodidae*) as the vector [96, 220]. LD remains one of the most important infectious diseases in these two continents. Moreover, this disease is also endemic in east Asian countries as there have been reports from China, Japan, Taiwan, South Korea, and Russia [119, 185].

Relapsing fever *Borrelia* (RFB) is categorized into three genetic groups: New World RFB, Old World RFB, and RFB harbored by ixodid ticks. Among them, New World and Old World RFB are transmitted by soft ticks and louse [21] and are endemic to the African countries, Middle East, Central Asia, southern Europe, and North America [21, 274]. On the other hand, RFB harbored by ixodid ticks, including *B. miyamotoi*, *B. lonestari*, and *B. theileri*, are mainly transmitted by the genus *Ixodes*, *Amblyomma*, and *Rhipicephalus* ticks, respectively [17, 76, 252]. Among these three, *B. miyamotoi* is pathogenic to humans [221], and *B. theileri* is responsible for bovine borreliosis [253]. *Borrelia miyamotoi* infection in humans was first reported in Russia in 2011, and, since then, cases have been documented in the United States, Europe, Japan, and northeastern China [105, 115, 143, 221, 245].

To date, the number of studies on TBPs, such as *Borrelia*, has been limited and largely overlooked in Malaysia. Only a few studies on *Borrelia* spp. were conducted in Peninsular Malaysia, in which most of the prior studies conducted focused on tick surveillance, and little is known about the occurrence of these etiological agents in most parts of Malaysia. For instance, serological evidence for *Borrelia* was provided by Tay et al. [267] by screening the blood donors and patients; also recently, by Khor et al. [132]

from the survey of indigenous people of Peninsular Malaysia. Detection of *Borrelia* species has been reported from *I. granulatus* collected from rodents in Peninsular Malaysia [131], in which the *Borrelia* species closely related to *B. yangtzensis*, a member of Bbsl complex was reported. *Borrelia* sp. closely related to relapsing fever *Borrelia* was also detected from *H. hystricis* [130]. Nevertheless, no study on *Borrelia* has been conducted in Malaysian Borneo or Sarawak, so its status is unknown.

Most of the prior studies conducted in Peninsular Malaysia focused on tick surveillance, and little is known about the occurrence of these etiological agents in most parts of Malaysia, including Sarawak. Hence, this study investigated the presence of *Borrelia* spp. in rodents and *Ixodes* ticks collected in Sarawak, Malaysian Borneo.

Materials and Methods

Sample collection

Rodent and *Ixodes* tick samples used for this study were collected as mentioned in the Materials and Methods of Chapter I. All *Ixodes granulatus* ticks used in this study were partially or fully engorged individuals removed from rodent hosts.

DNA preparation and species identification of rodents

For the *Borrelia* spp. screening in rodents, the selected rodents were euthanized following the method described by Taylor et al. [270] for internal organs collection. The collected organs were kept in 70% ethanol and subsequently stored at −20℃ after being transferred back to the facility; the spleen samples of the rodents were used in this study. DNA was extracted from the rodent spleens at the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer's instructions. The DNA samples and ticks were sent to Hokkaido University, Japan, where the subsequent screenings and analyses of the samples were conducted.

For the molecular identification of the rodent species, a fragment of the cytochrome c oxidase subunit 1 (*CO1*) was amplified by PCR using the primer pairs BatL5310 and R6036R (Table 5) [238]. The PCRs were conducted in a 20 µL reaction mixture using the *Ex Taq* Hot Start version (Takara Bio, Shiga, Japan) with the following conditions: 30 cycles of denaturation at 94℃ for 30 sec, annealing at 48℃ for 30 sec, and extension at 72℃ for 60 sec. The sequences obtained from Sanger sequencing and analysis were compared with public databases using the Nucleotide Basic Local Alignment Search Tool (BLASTn). In addition, the Barcode of Life Data System (BOLD; *http://www.barcodinglife.org*) was also used for the identification of rodent species [231].

DNA preparation of *Ixodes* ticks

After identifying the tick species, *Ixodes* ticks were selected for *Borrelia* investigation. Ticks were washed with sterile phosphate-buffered saline (pH 7.4) and individually crushed with Micro Smash MS-100R (TOMY, Tokyo, Japan) for 30 sec at 2500 rpm. Next, the DNA was extracted using the Wizard® Genomic DNA Purification Kit, as specified in the manufacturers' protocol for animal tissue.

Screening of *Borrelia* spp.

DNA from the rodent spleens and *Ixodes* ticks were subjected to the screening of Bbsl and RFB using a nested PCR targeting the *flaB*, which produces a 345 bp amplicon [259]. The PCR conditions were

as follows: 25 and 30 cycles of denaturation at 94℃ for 30 sec, 55℃ and 50℃ of annealing for 30 sec, and extension at 72℃ for 1 min in the first and nested PCR, respectively. The positive samples of the *flaB*-PCR were further characterized by additional PCRs, targeting 16S rDNA, which is approximately a 1,370 bp amplicon. For the *Ixodes* ticks, a single PCR with BF1 and BR1 primers was performed [240]. While, for the rodent samples, universal primers targeting bacterial 16S rDNA were added for the first PCR [284], BF and BR primers were added for the nested PCR. The PCR conditions for the single and nested PCRs were identical, i.e., 35 cycles of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, followed by extension at 72℃ for 90 sec. The PCR was conducted using the *Ex Taq* Hot Start version with a reaction mixture of 20 μL. DNA from "*Candidatus* Borrelia fainii" strain Qtaro [228] and molecular-grade water were used for the positive and negative controls, respectively. Finally, the electrophoresis, PCR product purification, and Sanger sequencing were performed. All primers used are listed in Table 5.

Multilocus sequence analysis of the *Borrelia* spp.

Four rodent and six tick samples were randomly selected from the *flaB* and 16S rDNA PCR positive samples and were used for multilocus sequence analysis (MLSA) based on the sequences of eight housekeeping genes (*clpA, clpX, nifS, pepX, pyrG, recG, rplB,* and *uvrA*). In order to obtain the sequences from these genes, previously described methods were employed with slight modification [173]. Briefly, nested PCRs using *Ex Taq* Hot Start version (Takara Bio, Shiga, Japan) were performed without the touchdown step initially. Then, for the samples that failed to amplify, PCRs were repeated with the touchdown step. Finally, the PCR products were observed with gel electrophoresis and purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), followed by Sanger sequencing.

Sequencing and phylogenetic analyses

The sequences were assembled and trimmed using the ATGC software version 6.0.4 (GENETYX, Tokyo, Japan) and compared with the sequences available in public databases using BLASTn. The phylogenetic trees were constructed using MEGA version X [147] with Neighbor-Joining or maximum likelihood models and the Kimura 2-parameter model with pairwise deletion and 1000 bootstrap replications. The phylogenetic relationships of the *Borrelia* spp. were also analyzed using the concatenated sequences of the eight genes and those of closely related *Borrelia* spp. downloaded from the PubMLST database (*https://pubmlst.org/*).

All the sequences obtained in this study are available in the GenBank database with following accession numbers: *flaB:* LC572294–LC572312; 16S rDNA: LC572071–LC572081; *clpA*: LC572082–LC572087; *clpX*: LC572088–LC572095; *nifS*: LC572096–LC572101; *pepX*: LC572102–LC572111*;*

pyrG: LC572112–LC572119; *recG*: LC572120–LC572128; *rplB*: LC572129–LC572136; and *uvrA*: LC572137–LC572145.

Table 5. Primers used in this study.

Primer name	Sequence (5' to 3')	Target gene (PCR type)	Annealing temperature (ºC)	Amplicon size (bp)	Reference
BatL5310	CCTACTCRGCCATTTTACCTATG	Cytochrome oxidase subunit 1 of rodents (Single PCR)	48	750	[238]
R6036R	ACTTCTGGGTGTCCAAAGAATCA				
BflaPAD	GATCARGCWCAAYATAACCAWATGCA	*flaB* of *Borrelia* (1st PCR)	55	800	[259]
BflaPDU	AGATTCAAGTCTGTTTTGGAAAGC				
BflaPBU	GCTGAAGAGCTTGGAATGCAACC	*flaB* of *Borrelia* (2nd PCR)	50	345	[259]
BflaPCR	TGATCAGTTATCATTCTAATAGCA				
fD1	AGAGTTTGATCCTGGCTCAG	Universal primer for 16S rDNA of bacteria	55	1,400	[284]
rp2	ACGGCTACCTTGTTACGACTT	(1st PCR for rodent samples)			
BF1	GCTGGCAGTGCGTCTTAAGC	16S rDNA of *Borrelia* (Single PCR for tick and 2nd PCR for rodent samples)	55	1,371	[240]
BR1	GCTTCGGGTATCCTCAACTC				
*BF3_seq	AGATACCCTGGTAGTCTACGCT	16S rDNA of *Borrelia*	N/A	N/A	This study
*BR3_seq	GCTGCTGGCACGTAATTAGC				

*Primers used for sequencing.

Results

Identification of rodent species

Overall, 97 rodents were trapped from two primary forests, Gunung Gading National Park (GGNP) and Kubah National Park (KNP), and an oil palm (OP) plantation, out of which 56 rodents were selected and used for this study based on the research permissions. Sample identity was shown as sampling site abbreviation (KNP-, GGNP-, and OP-), followed by a numerical assignment. The morphological and molecular identification of the rodents revealed 4 *Leopodamys sabanus*, 2 *Maxomys rajah*, 2 *M. whiteheadi*, 45 *Rattus* spp., and 3 *Sundamys muelleri* (Table 5). The rodents that were morphologically assigned to the *Rattus* spp., *R. tiomanicus*, and *R. tanezumi* were collectively grouped as one *Rattus* spp., as they were not molecularly identified as a single species.

Detection of the *Borrelia* spp.

Out of the 56 rodent samples, four *Rattus* spp. (Sample IDs: OP-007, -014, -018, and -033) from the OP plantation and one *S. muelleri* (Sample ID: GGNP-04) from the GGNP were positive for the borrelial flagellin gene (*flaB*) in a PCR (Table 6). The prevalence of *Borrelia* spp. in rodents from GGNP, KNP, and the OP plantation were 16.7% (1/6), 0% (0/7), and 8.9% (4/45), respectively (Table 6). Subsequent analysis revealed that sequences from these five samples were different from each other. The sequences from OP-007 and OP-014 showed 100% (300/300 bp) and 95% identity (285/300 bp), respectively, with *B. yangtzensis* in *I. granulatus* extracted from rodent *Niviventer fulvescens* in China (EU135602). The sequence from OP-018 showed 100% identity (300/300 bp) with *B. valasiana*-related genospecies from rodent *Suncus murinus* in Japan (AB091710). The sequence from OP-033 showed 99.3% identity (298/300 bp) with *B. valasiana*-related genospecies from rodent *Apodemus agrarius* in China (AB022136). Finally, the sequence from GGNP-04 showed 100% identity (294/294 bp) with *B. miyamotoi* from *I. nipponensis* in South Korea (MH102393). For further characterization of the *Borrelia* spp., subsequent nested PCRs targeting the borrelial 16S rDNA were carried out and successful in four of the samples (OP-014, -018, -033, and GGNP-04). The sequence from OP-014 showed 99.6% identity (1,348/1,354 bp) with *B. yangtzensis* from *H. longicornis* in China (EU135595). The sequence from OP-018 showed 99.7% identity (1,350/1,354 bp) with *B. valasiana*-related genospecies from rodent *A. agrarius* in China (AB022141). The sequence from OP-033 showed 99.9% identity with *B. valasiana*-related genospecies from South Korea (U44938). Finally, the sequence from GGNP-04 showed 99.8% identity (1,352/1,355 bp) with *B. miyamotoi* from a febrile patient in Russia (CP037471).

Out of 32 *I. granulatus* samples, 14 samples (43.8%) were positive for *flaB*-PCR. The 14 samples included 1 female from KNP and 10 females, 1 nymph, and 2 larvae from the OP plantation. None of the *I. granulatus* samples from GGNP were positive for *flaB*-PCR. In addition, only one positive *I. granulatus* sample (Sample ID: IG-218) was collected from the Bbsl positive rodent, OP-033. Among the three sampling sites, the OP plantation recorded the highest number of tested samples and the highest prevalence of positive samples (72.2%; 13/18; Table 6). The details for the sequencing results of both *flaB* and 16S rDNA are shown in Table 6. From the sequence analysis, six *I. granulatus* (Sample IDs: IG-204, -206, -208, -213, -217, and -218) had an identical sequence of *flaB*, i.e., 100% identity (300/300 bp) with *B. yangtzensis* (EU135602). This sequence was also identical to that from the rodent sample, OP-007. Additionally, sequences from sample IDs: IG-214, -216, -220, -221, -222, and -228) were identical with Bbsl sequences from *I. granulatus* in Taiwan (HM853004), China (MG717513 and MG717514), and Malaysia (LT969779) (Table 7). Finally, the sequences from sample IDs: IG-215 and -219 showed 99.3% (298/300 bp) and 100% identity (300/300 bp), respectively, with *B. valaisiana*-related genospecies (AB091710), and the sequence from IG-215 was identical to that from the rodent sample, OP-018. Additionally, 7 out of the 14 *I. granulatus* samples with positive for *flaB*-PCR were successfully sequenced for 16S rDNA. The sequences showed high similarity with *B. yangtzensis* from China (EU135595 and EU135598) and South Korea (L39080) and with *B. valaisiana*-related genospecies from China (AB022140 and AB022141) (Table 7). Similarly, the same sequences were also detected in both tick and rodent samples. The sequence from IG-219 had an identical sequence to the rodent sample, OP-018.

Phylogenetic analysis

Collectively, 19 samples (5 rodents and 14 *I. granulatus*) were positive for *flaB*-PCR and were included in the phylogenetic tree construction. Out of 19 samples, 18 were assigned to the clade of Bbsl and clustered together with *B. yangtzensis* or *B. valaisiana*-related genospecies (Figure 5). The remaining rodent sample (GGNP-04) was assigned to the RFB and clustered together with *B. miyamotoi* (Figure 5). In addition, the phylogenetic tree based on the 16S rDNA sequences revealed consistent clustering, as observed in *flaB* (Figure 6).

Multilocus sequence analysis of the *Borrelia* spp.

All *I. granulatus* samples included for the MLSA were successfully amplified for the eight housekeeping genes. In the phylogenetic tree based on the concatenated MLSA genes, the *Borrelia* spp. from *I. granulatus* were located in the clade of *B. yangtzensis* (Figure 7). This trend was also confirmed

in the other phylogenetic trees based on *flaB* and 16S rDNA (Figures 5 and 6). For the four rodent samples included in MLSA, a minimum of two (1/4), four (2/4), and six (1/4) housekeeping genes were successfully amplified. Therefore, the phylogenetic inferences of the *Borrelia* species in both rodent and tick samples were made on a per gene basis. The phylogenetic analysis based upon each housekeeping gene showed that the rodent samples (OP-007, -014, -018, and -033) were located in the clade of *B. yangtzensis* with *I. granulatus* from this study.

Table 6. The number of rodent species and *Ixodes granulatus* used for the screening of *Borrelia* spp.

	Sampling period			
	November 2018		March 2019	
Rodent species	**GGNP**	**KNP**	**OP**	**Total**
Leopodamys sabanus	0/2	0/2	N/A	**0/4**
Maxomys rajah	N/A	0/2	N/A	**0/2**
Maxomys whiteheadi	0/2	N/A	N/A	**0/2**
Rattus spp.	N/A	0/3	4/42	**4/45**
Sundamys muelleri	1/2	N/A	0/1	**1/3**
Total	**1/6**	**0/7**	**4/43**	**5/56**
Ixodes granulatus	**GGNP**	**KNP**	**OP**	**Total**
Female	0/3	1/9	10/10	**11/22**
Nymph	N/A	0/2	1/3	**1/5**
Larva	N/A	N/A	2/5	**2/5**
Total	**0/3**	**1/11**	**13/18**	**14/32**

No. of positive/No. of tested. Gunung Gading National Park (GGNP), Kubah National Park (KNP), oil palm (OP).

Table 7. The sequence analysis results for the borrelial flagellin gene (*flaB*) and 16S ribosomal DNA (rDNA) of *Ixodes* ticks.

Gene	Sample ID	BLASTn	Similarity (bp)	Accession no.
flaB	IG-204	*Borrelia yangtzensis*	300/300	EU135602
	IG-206			
	IG-208			
	IG-213			
	IG-217			
	IG-218			
	IG-215	*Borrelia valaisiana*-related genospecies	298/300	AB091710
	IG-219		300/300	
	IG-214	*Borrelia* sp. TKM-30 from *Ixodes granulatus (*Taiwan)	298/300	HM853004
	IG-216			
	IG-220	Uncultured *Borrelia* sp. clone Borr65 from *Ixodes granulatus* (China)	300/300	MG717514
	IG-221	Uncultured *Borrelia* sp. clone BorrIg from *Ixodes granulatus* (China)	297/300	MG717513
	IG-222	Uncultured *Borrelia* sp. from *Ixodes granulatus* (Malaysia)	299/300	LT969779
	IG-228		300/300	
16S rDNA	IG-213	*Borrelia yangtzensis* strain QX-S13 (China)	1347/1354	EU135598
	IG-216	*Borrelia* sp. 9MT (South Korea)	1351/1354	L39080
	IG-217	*Borrelia valaisiana*-related genospecies from rodent *Apodemus agrarius* (China)	1348/1354	AB022140
	IG-220		1346/1354	
	IG-218	*Borrelia yangtzensis*	1351/1354	EU135595
	IG-219	*Borrelia valaisiana*-related genospecies	1350/1354	AB022141
	IG-221		1349/1354	

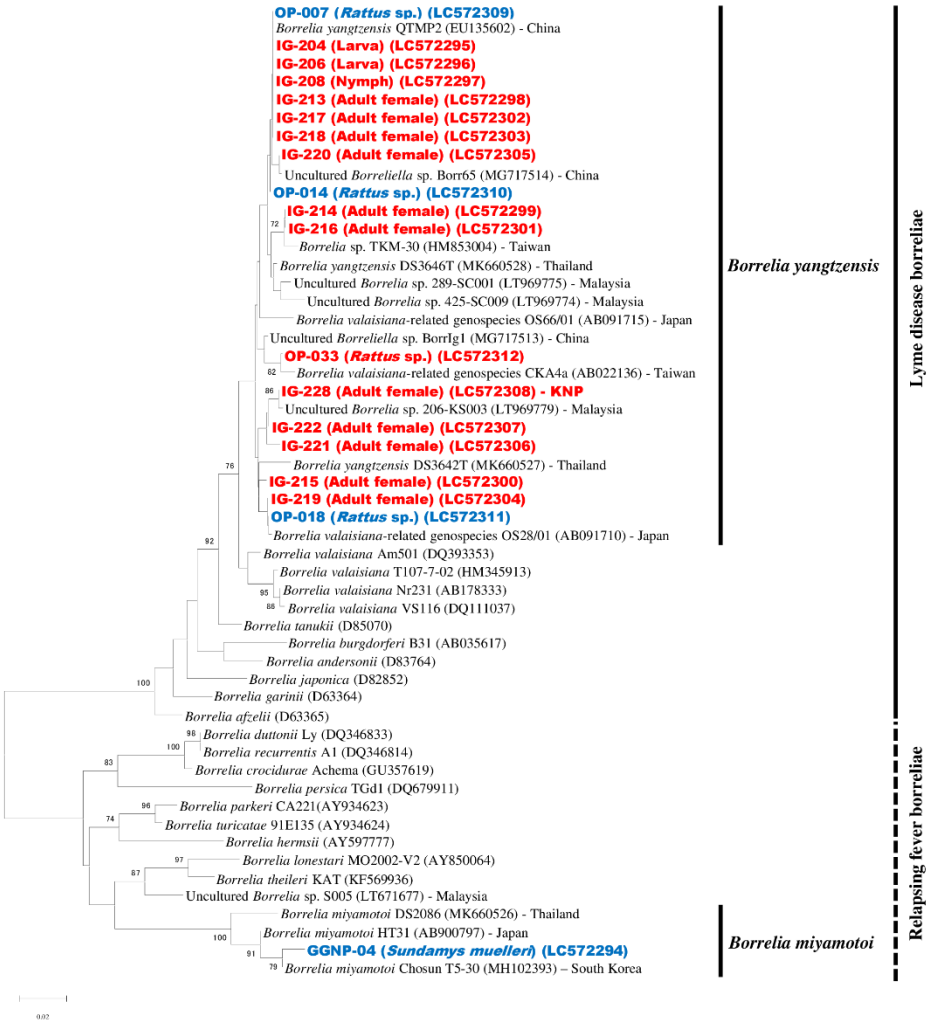

Figure 5. Phylogenetic tree based on *flaB* sequences of the *Borrelia* species. The phylogenetic tree was constructed in MEGA version X [147] by the Neighbor-Joining model with Kimura-2 parameter and 1000 bootstrap replications. The sequences from ticks and rodents obtained in this study are shown in red and blue, respectively. All positive tick samples were collected from an oil palm (OP) plantation; the location is not indicated except for one sample from Kubah National Park (KNP).

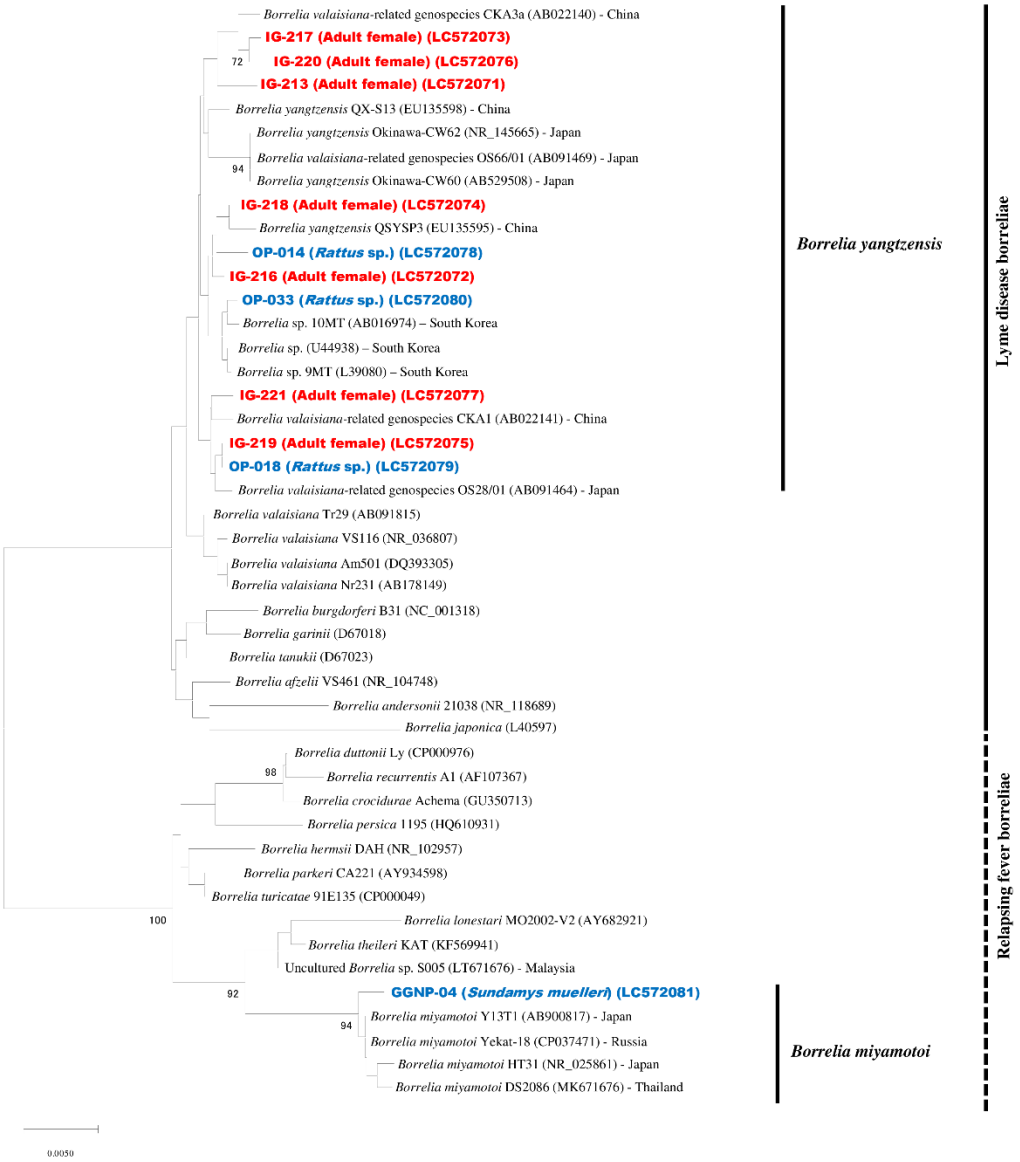

Figure 6. Phylogenetic tree based on 16S rDNA sequences of the *Borrelia* species. The phylogenetic was constructed in MEGA version X [147] by the Neighbor-Joining model with Kimura-2 parameter and 1000 bootstrap replications. The sequences from ticks and rodents obtained in the present study are shown in red and blue, respectively. All samples were collected from an oil palm (OP) plantation, except one sample, which was from Gunung Gading National Park (GGNP).

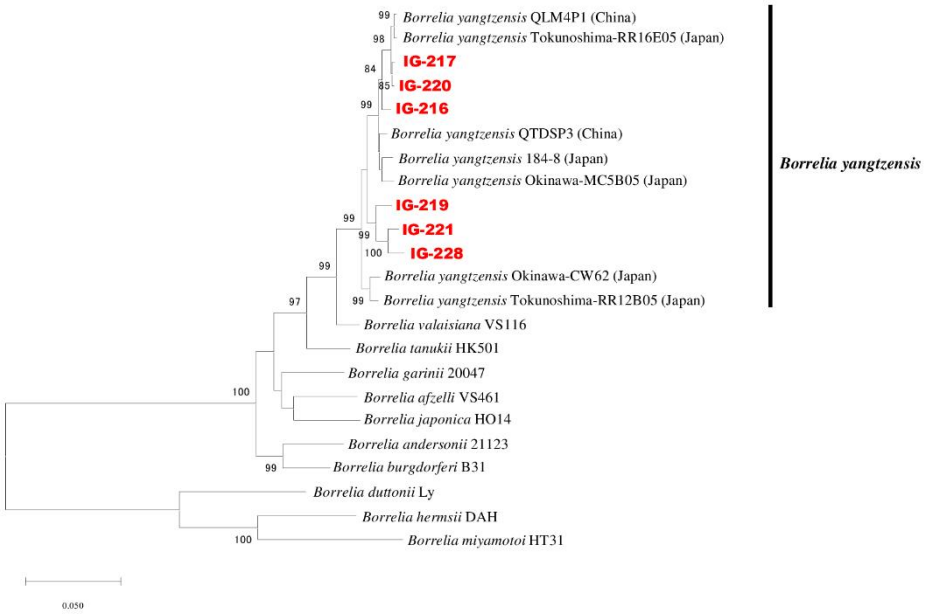

Figure 7. Phylogenetic inference of the *Borrelia burgdorferi* sensu lato. The samples are shown in red. The sequences obtained from eight housekeeping genes were trimmed and concatenated in the order of *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*, according to the *Borrelia* PubMLST database. The maximum likelihood model was used with 1000 bootstrap replications for the phylogenetic construction in MEGA version X [147].

Discussion

This study investigated *Borrelia* spp. in *I. granulatus* and rodents, *L. sabanus*, *M. rajah*, *M. whiteheadi*, *Rattus* spp., and *S. muelleri* from primary forests (GGNP and KNP) and an OP plantation in Sarawak, Malaysia. *Borrelia yangtzensis* from *I. granulatus* and *Rattus* spp. and *B. miyamotoi* from *S. muelleri* were identified. This study is the first evidence of *B. miyamotoi* in Malaysia and *B. yangtzensis* in Sarawak, Malaysian Borneo.

Borrelia yangtzensis detected from *I. granulatus* in this study was formerly known as *B. valaisiana*-related genospecies since phylogenetic inferences showed a close relation but a clear distinction to *B. valaisiana* (a member of the Bbsl complex in Europe) [175]. However, unlike *B. valaisiana* that utilizes birds as the reservoir host, and *Ixodes* ticks as the vector [175, 281], *B. yangtzensis* is maintained and transmitted through a natural infection cycle between rodents and *Ixodes* ticks [120]. Isolations of *B. yangtzensis* from different rodent species were recorded from *Rattus* spp., *S. murinus*, *Mus* spp., and *A. agrarius* in Japan, China, and Taiwan [120, 184, 186], as well as from different *Ixodes* tick species, such as *I. nipponensis* in South Korea and *I. granulatus* in Japan and China [48, 120, 183]. Recently, a *Borrelia* sp. closely related to *B. yangtzensis* was detected in Peninsular Malaysia from *I. granulatus* collected from different rodent species [131]. Takhampunya et al. [261] also identified *B. yangtzensis* from one rodent and two tick pools of *Ixodes* spp. collected from rodents in northern Thailand. This is similar to this study, as *B. yangtzensis* was detected from *Rattus* spp. and *I. granulatus* for the first time in Sarawak. These results suggest that *B. yangtzensis* is circulated, and *Rattus* spp. and *I. granulatus* play the roles of the natural reservoir and vector, respectively, in Sarawak. However, there is a limitation in this study; as all of the positive *Ixodes* ticks were engorged, whether borrelial DNA detected from the tick samples was from the blood meal host could not be ruled out. Thus, further investigations of *B. yangtzensis* in unfed *I. granulatus* are required to confirm the vector species of this bacterium in Sarawak. In addition, *I. granulatus* has been documented from migratory birds in Taiwan by Kuo et al. [146]. Migratory birds are known to play an important role in the dispersal of Bbsl, with previous reports involving *Ixodes* ticks from Japan, South Korea, and Russia [116, 193, 196]. Moreover, studies in Canada by Scott et al. [247, 248] showed that migratory birds disperse Bbsl-infected ticks over a long distance and across geographical barriers. Thus, investigation of ticks collected from migratory birds in Malaysia might help expand knowledge of Bbsl, including *B. yangtzensis*.

Multilocus sequence analysis was first introduced by Margos et al. [173] for depicting the evolutionary processes of *B. burgdorferi*. By targeting multi loci, eight housekeeping genes were developed for the Bbsl complex [173] and have been subsequently used in other studies to characterize

the complexity of the Bbsl genospecies [120, 175]. In recent years, this method has also proven to be useful in comparing the intraspecific diversity, elucidating population genetic structure, and other ecological aspects that may contribute to the transmission dynamics of the Bbsl genospecies [198, 207]. Further, MLSA has been used to confirm *B. yangtzensis* from the isolates of ticks and rodents from China and Japan [175]. Based on MLSA, the phylogeny inference revealed that the isolates formed two sister clusters, with each cluster consisting of isolates from both China and Japan. Concordantly, in this study, the concatenated sequences of *B. yangtzensis* from *I. granulatus* were also located in two sister clades.

On the human pathogenic aspect, *B. valaisiana* had been regarded as the causative agent of LD in humans but was recently proven otherwise [177]. Two human LD cases caused by *B. valaisiana*-related genospecies were reported from Japan and China [202, 242], but Margos et al. [175, 177] later ratified them be *B. yangtzensis* and suggested *B. valaisiana* as a non-human pathogenic. Although *B. yangtzensis* may potentially be a human pathogenic, there is currently no study providing further evidence. Even though the significance of *B. yangtzensis* in humans and animals is not yet fully understood, the findings of *B. yangtzensis* in this study imply the likelihood that the bacterium circulates within the *Ixodes* ticks and rodents in primary forests and OP plantation in Sarawak. *Borrelia* spp. closely related to *B. yangtzensis* were also detected from *I. granulatus* in Peninsular Malaysia [131]. Of note, *I. granulatus* is a rare parasite of humans with few related reports. Yun et al. [290] identified only one female of *I. granulatus* from 261 ticks they collected from humans in South Korea. A checklist of ticks from Thailand, dated back to 1983, documented that humans could be hosts for this tick species [264]. However, it was not clear in these studies whether the *I. granulatus* collected were biting humans. Thus, more investigations are required to evaluate the pathogenicity to humans and understand the transmission cycle of *B. yangtzensis* in Malaysia.

Borrelia miyamotoi, the causative agent of RF, was first isolated from *I. persulcatus* ticks in Japan [76]. To date, several *Ixodes* tick species are considered as a vector of *B. miyamotoi*. For instance, *I. scapularis* and *I. pacificus* are the vectors reported in the United States and Canada, *I. ricinus* in Europe, and *I. persulcatus* in Europe and Asia [58, 76, 115, 144, 226]. So far, the reservoir hosts, based on the geographical distribution, for *B. miyamotoi* are still not well understood, but rodents and birds have been considered as the reservoir hosts in some regions [162, 270]. In this study, *B. miyamotoi* was detected from *S. muelleri* in GGNP; this is the first report of *B. miyamotoi* in Malaysia. However, none of the *I. granulatus* examined in this study were positive for *B. miyamotoi*. Furthermore, the infection rate of *B. miyamotoi* was much lower than that of *B. yangtzensis* in this study. Generally, the infection rate of *B. miyamotoi* in rodents and ticks appears to be lower than that of Bbsl species as per previous studies in Japan and Russia [226, 270]. Furthermore, another study on the prevalence of *B. miyamotoi* infection in *I. scapularis* conducted in Canada was low (<1%) [58]. Moreover, the reported prevalence of *B. miyamotoi*

in questing *Ixodes* ticks ranged from 1.3% in *I. ricinus* to 3.6% in *I. persulcatus* [281]. For future studies, the sample size of the rodents and ticks should be increased to find the vector tick species and to describe the diversity and distribution of *B. miyamotoi* in Sarawak. In addition, *B. miyamotoi* has been recently reported from *H. concinna* in Northeastern China [48]. In Europe, migratory birds have been reported as the reservoir host of *B. miyamotoi* or play a role in the dispersal of tick vectors [162]. Thus, the investigations of other tick species and birds may provide more in-depth insights into *B. miyamotoi* in Sarawak.

The sampling in this study was conducted only once for the primary forests (GGNP and KNP) and the OP plantation in different seasons, which yielded a small sample size, especially in the sampling during the wet season. Small sample size and lack of sampling repetition may have contributed to the low number of positive samples in this study, as *B. miyamotoi* was only positive in one rodent, and *B. yangtzensis* was not detected in the rodents from GGNP and KNP. In addition, only rodent spleens were used in this study for Bbsl and RFB screening. Future works to estimate the prevalence should include ear biopsies and other internal organs, as Bbsl and *B. miyamotoi* may not have the same strategies for the maintenance and dissemination in the same reservoir host [22]; therefore, different tissue may yield different detection rates [291]. Despite the incomparable rodent numbers, the number of *Ixodes* ticks from the primary forests and OP plantation were fairly similar (14 and 18, respectively). In the OP plantation, 13 ticks were positive for *B. yangtzensis*; in contrast, in the primary forests, only one tick from KNP was positive. Land conversion with a human-dominated ecosystem could have a potent effect on reservoirs and the zoonotic risk because of the alterations of host diversity and composition [81, 161]. The difference observed in this study might be reflected by the variation between the primary forest and OP plantation. A follow-up study to evaluate this hypothesis should encompass a larger sampling size with repetition.

In conclusion, this study examined *Borrelia* spp. in rodents and ticks from primary forests and an OP plantation. This study showed the presence of *B. miyamotoi* for the first time in Malaysia and also reported the first detection of *B. yangtzensis*, which was characterized by using MLSA in both rodents and *I. granulatus* in Sarawak. These findings of *Borrelia* spp. in Sarawak provide evidence of a new geographical record. This study warrants the need for further investigations as it is important to determine how the *Borrelia* spp. may impact public health in Malaysia.

Summary

Members of the *Borrelia* burgdorferi sensu lato (Bbsl) complex are etiological agents of Lyme disease (LD), and *Borrelia miyamotoi* is one of the relapsing fever *Borrelia* (RFB). Despite the serological evidence of LD in Malaysia, there has been no report from Sarawak, Malaysian Borneo. Thus, this study aimed to detect and characterize *Borrelia* species in rodents and *Ixodes* ticks from primary forests and an oil palm (OP) plantation in Sarawak. *Borrelia yangtzensis* (a member of the Bbsl complex) was detected in 43.8% (14/32) of *Ixodes granulatus*, and most of the positive ticks were from the OP plantation (13/14). Out of 56 rodents, *B. yangtzensis* was detected in four *Rattus* spp. from the OP plantation, and *B. miyamotoi* was detected in one rodent, *Sundamys muelleri*, from the primary forest. Furthermore, the positive samples of *B. yangtzensis* were randomly selected for multilocus sequence analysis (MLSA). The MLSA results of successfully amplified tick samples revealed a clustering with the sequences isolated from Japan and China. This study is the first evidence of *B. miyamotoi*, a known human pathogen in Malaysia, and *B. yangtzensis*, which is circulating in ticks and rodents in Sarawak, Malaysian Borneo, and presenting a new geographical record of the *Borrelia* spp.

Chapter III

Insights on the microbiome and tick-borne pathogens of ixodid ticks in Sarawak, Malaysian Borneo

Introduction

Ticks harbor a high abundance of symbiotic and commensal microorganisms that can be obligate or facultative presence. The obligate endosymbionts are maternally inherited microorganisms essential for tick survival and development. For instance, *Coxiella*-like endosymbiont and the *Francisella*-like endosymbiont (*Coxiella*-LE and *Francisella*-LE hereafter) are two well-established non-pathogenic microorganisms, which are essential for tick survival and development [67, 68]. Both *Coxiella*-LE and *Francisella*-LE act in providing vitamin B and co-factor, which are deficient in the tick-specific hematophagous diet but crucial for growth and development. It was evident that eliminating *Francisella*-LE impaired tick development [68]. Similar to *Francisella*-LE, vitamin B biosynthesis pathways were also discovered in the genome of *Coxiella*, evident in its functionality as nutritional bacteria [85]. Facultative endosymbionts, on the contrary, although not fundamental in the tick life cycle, these microorganisms may be involved in the manipulation of the tick immune system and reproduction. Some known facultative endosymbionts like arsenophonos, rickettsia, spiroplasma, and wolbachia are commonly present in arthropods and have a role in manipulating the reproduction [69], have also been described in ticks [10, 49, 203, 276].

Ticks can acquire microorganisms via the environment and blood meal hosts, and these factors can add to the bacterial complexity [150, 293]. The different ontogeny stages and sex of ticks have been reported in previous studies in shaping the bacterial community [208, 298]. Furthermore, the interaction between non-pathogenic microorganisms and pathogens in ticks has gained substantial interest as it may be fundamental to the disease control measurements. For instance, Abraham et al. [2] revealed the capability of *A. phagocytophilum* in modulating the gut microbiota of *Ixodes scapularis* ticks to favor its colonization. Narasimhan et al. [200] also demonstrated the link between gut microbiota and *Borrelia burgdorferi* colonization in *Ixodes scapularis* ticks. Besides, shifting between pathogenic and non-pathogenic forms may occur for some bacterial genera as an evolutionary process due to ecological and epidemiological implications [31, 66]. The roles of non-pathogenic microorganisms in ticks have continuously been corroborated in different contexts such as tick development and pathogen transmission, which can be resolved with the advent of high throughput methods such as NGS. Hence, revealing the tick microbiome on the top of pathogen detection has become the new paradigm as glancing into the

microbiome structure of different tick species under different circumstances may be pivotal to unravel the complexity and subsequently provide probable solutions to control the disease transmission.

Studies employing NGS elucidating tick endosymbionts have appeared to be limited in Malaysia. The bacterial community in three *Haemaphysalis* tick species (*H. hystricis*, *H. wellingtoni*, and *H. bispinosa*) collected from domestic animals from the indigenous people settlements was reported by Khoo et al. [128]. In addition, another study presented bacterial communities for *Haemaphysalis*, *Dermacentor*, and *Amblyomma* collected from wild boars in the peninsula [157]. Overall, the information for tick microbiome in large parts of Malaysia is still unknown.

TBDs afflicting humans and animals can be caused by a vast range of bacterial genera, such as *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Rickettsia*, and so on. In Malaysia, *C. burnetii*, the causative agent for Q fever, has been detected from two tick samples: *D. steini* and *H. hystricis* collected from wildlife and livestock [129]. In addition, a study in Peninsular Malaysia detected *Rickettsia* spp. closely related to *R. raoultii*, *R. tamurae*, *R. heilongjiangensis*, and *R. asiatica* from ticks, pointing out the diversity of rickettsial species and potential tick vectors in this part of Malaysia [127]. Several genotypes of *Bartonella bovis* have been detected from cattle and ticks infesting this animal, *H. bispinosa* [124]. Recently, *Bartonella* sp. closely related to *B. phoceensis* was also reported from *Dermacentor* sp. and rodent host [18]. However, it is noteworthy that the transmission of *Bartonella* spp. by ticks to humans and animals is still controversially discussed. Nevertheless, studies on TBDs are generally lacking in Malaysia and have remained under-investigated in Sarawak, Borneo.

In this study, tick samples collected from the primary forests and an oil palm plantation in Sarawak, Malaysian Borneo (Figure 1) were used for the screening. We targeted the microbiome in the collected tick species using NGS and bioinformatics. We further identified the main endosymbionts and characterized the potential pathogens detected for each tick species. We also explored the factors that may contribute to the difference of microbial composition and richness in ticks. Altogether, this was the first initiative we took to outline the tick microbiome and TBPs from Sarawak state, which provided us with important insights that will become the direction of upcoming research on TBP prevalence study and control strategy.

Materials and Methods

Bacterial 16S rDNA amplification

A total of 210 ticks consisting of different developmental stages and feeding statuses as identified in Chapter I were included in this study. Overall, the samples consisted of six tick species: *I. granulatus* (n = 32), *H. hystricis* (n = 36*), H. shimoga* (n = 110), *D. compactus* (n = 4), *D. steini* (n = 24), and *D. atrosignatus* (n = 4). Amplification targeted the 16S rDNA V3-V4 hypervariable regions, and sample preparation was performed following the procedure in the Illumina 16S Metagenomic Sequencing library preparation manual (Illumina, Inc., San Diego, CA, USA). The targeted region was amplified by PCR with primer set (338F and 806R) as in Klindworth et al. [135]. A total volume of 25 µL PCR reaction mixture was prepared with 12.5 µL of 2 x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 1 µM of each forward and reverse primers, and 2 µL of the extracted DNA. Negative control PCR reactions were prepared using molecular-grade water in place of DNA samples, and mock DNA extractions were also subjected for PCR. Amplification was run using the following thermal cycling conditions: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, followed by a final extension for 5 min at 72°C. The amplicon PCR products were electrophoresed on a 1.2% agarose gel with Midori Green Direct DNA stain and visualized with a BLooK LED transilluminator for the expected size of 460 base pair.

Library preparation and sequencing

Amplified PCR products were purified via Agencourt AMPure XP beads. Nextera XT Index Kit (Illumina, Inc., San Diego, CA, USA) was used to provide unique dual indices for each purified sample. The purification step was repeated, and the size integrity of the amplicons was verified on a Bioanalyzer. Finally, all samples were quantified and pooled in equimolar concentrations, and paired-end sequencing was conducted on an Illumina MiSeq platform using a MiSeq v3 reagent kit (600-cycle, paired-end) (Illumina, Inc., San Diego, CA, USA). The combined library included negative controls, which were later on used as a standard to identify and remove suspected contaminants during the data analysis. Library preparation, samples purifications, and high throughput sequencing were conducted at the National Institute for Environmental Studies (NIES, Ibaraki, Japan).

Data analysis

Microbiome data sequences were analyzed in Quantitative Insights in Microbial Ecology 2 (QIIME2 2019.10) [32]. Illumina Fastq sequence data were demultiplexed and quality-filtered using the

q2-demux plugin followed by denoising with DADA2 [41], then assigned to amplicon sequence variants (ASVs). Potential contaminants were then identified using the Decontam package [53] in R (version 4.0.2, Core R Team, 2020) by the frequency method with a threshold of 0.4 and checked manually in reference to the negative controls before filtering out using the QIIME2 sequence identifiers. Next, taxonomy was assigned using Greengenes 13_8 99% reference sequences [189]. Unidentified ASVs and those identified as chloroplast and in negative controls were removed as well as bacterial sequences not assigned to phylum level. Paired-end reads were aligned with mafft [118] and used to construct a rooted phylogenetic tree with fasttree2 [224].

Diversity analysis was performed on decontam-filtered feature table at the species level in QIIME2 after samples were rarefied for sufficient sequencing depth for the observed number of OTUs from all samples and removed samples with low sequence read counts. Four alpha-diversity metrics: Shannon's diversity, observed OTUs, Faith's Phylogenetic Diversity (Faith' PD) [73], and Pielou evenness and four beta-diversity metrics: weighted UniFrac [166], unweighted UniFrac [165], Jaccard distance, and Bray-Curtis dissimilarity were quantified using QIIME2. The statistical significances for both alpha- and beta- diversity metrics were then configured using the vegan package in R software [209]. Furthermore, the significance of beta-diversity was tested by Permutational multivariate analysis of variance (PERMANOVA) [9] using 999 permutations. Principal coordinate analysis (PCoA) was plotted based on the four distance matrices using the R package phyloseq [190] to visualize the differences.

In addition, the differential abundance of the taxonomic groups was visualized using taxa_heatmap function in the qiime2 R package in R (version 2.13.0). Furthermore, Analysis of Composition of Microbiome (ANCOM) [172] was conducted to determine the dissimilarity among different tick species and the effect of life stage and feeding status for *H. shimoga*. Linear discriminant analysis effect size (LEfSe) was done using the Huttenhower lab Galaxy pipeline [249] to test these dissimilarities in the context of relative abundances. Additionally, for some indicated tick species, the pairwise analysis was performed to test the difference between the ticks that harbored the bacteria species and those that were negative (e.g., *Borrelia*- positive vs. *Borrelia*-negative *Ixodes granulatus*).

PCR amplification and sequencing of bacteria

PCR was performed to characterize the species of each bacteria detected in tick samples in NGS screening. The following bacteria genera: *Anaplasma, Ehrlichia, Bartonella, Coxiella, Francisella,* and *Rickettsia,* were targeted in the PCR amplification and sequencing. The details of all primers used in the bacteria species identification are described in Table 8.

DNA amplification for *Bartonella*, *Francisella*, and *Rickettsia* was conducted using *Ex Taq* Hot Start Version in a reaction mixture of 20 µL. The conditions used in the PCR assays were as follows: 35 or 40 cycles of denaturation at 94℃ for 30 sec, annealing temperature according to each respective primer set for 30 sec, and extension at 72℃ for 30 sec, 60 sec or 90 sec depending on the targeted amplicon size. Single PCRs were performed in all the DNA amplification, except semi-nested PCR was conducted for the *gltA* gene of *Bartonella*.

For *Anaplasma*, *Ehrlichia*, and *Coxiella* identification, Tks Gflex DNA Polymerase was used for DNA amplification with a reaction mixture of 25 µL. Nested PCR was conducted for *Anaplasma* and *Ehrlichia* with the following conditions: initial denaturation at 95℃ for 3 min, followed by 40 cycles of denaturation step at 95℃ for 30 sec, 48℃ and 54℃ of annealing for 30 sec, and extension at 68℃ for 90 sec, with a final extension at 68℃ for 5 min in the first and second PCR, respectively. DNA of *Coxiella* was amplified with nested or semi-nested PCRs. The conditions were as follows: initial denaturation at 94℃ for 1 min, followed by 40 cycles of denaturation step at 98℃ for 10 sec, 54℃ or 56℃ of annealing for 15 sec, and extension at 68℃ for 1 min, with a final extension at 68℃ for 5 min.

Finally, all the PCR products were verified with electrophoresis, followed by the Sanger sequencing. The resulting sequences were assembled and trimmed using the ATGC software version 9.0.0 and compared with the sequences available in the public databases using the BLASTn.

All the sequences obtained in this study are available in the GenBank database with following accession numbers: *Anaplasma* (LC602250); *Ehrlichia* (LC602251); *Francisella* (16S rDNA: LC602252–LC602256; *tul4*: LC602776–LC602781); *Rickettsia* (16S rDNA: LC602357–LC602360; *ompA*: LC602733–LC602736; *ompB*: LC602737–LC602740; *gltA*: LC602741–LC602744; *Sca4*: LC602745–LC602748; *htrA*: LC602770–LC602773); *Bartonella* (*ftsZ*: LC602774; *gltA*: LC602775); *Coxiella* (23S rDNA: LC602368–LC602388; 16S rDNA: LC602389–LC602400; *dnaK*: LC602703–LC602712; *rpoB*: LC602713–LC602732; *groEL*: LC602749–LC602769).

Table 8. Primers used in this study.

Organism	Primer name	Sequence (5' to 3')	Target gene (PCR type)	Annealing temperature (ºC)	Amplicon size (bp)	Reference
Bacteria	338F	ACTCCTACGGGAGGCAGCAG	16S ribosomal DNA V3 and V4 hypervariable regions (Single PCR)	55	460	[135]
	806R	GGACTACHVGGGTWTCTAAT				
Anaplamatacea	EC9	TACCTTGTTACGACTT	16S ribosomal DNA (1st PCR)	48	1,400	[7]
	EC12A	TGATCCTGGCTCAGAACGAACG				[215]
	A17a	GCGGCAAGCCTCCCACAT	(Nested PCR)	54	1,300	[121]
	IS58-1345R	CACCAGCTTCGAGTTAAACC				
	*Ana16_seq1	TCCTTAGTTGCCAGCGGGTT		N/A	N/A	This study
	*EA16_seq2	GGCATAGCTGGATCAGGCTT	*Anaplasma*: Ana16_seq1 and EA16_seq2			
	*Ehr16_seq1	TCGATGCTACGCGAAAAACC	*Ehrlichia*: Ehr16_seq1 and EA16_seq2			
Bartonella	CS443f	GCTATGTCTGCATTCTATCA	citrate synthase gene, *gltA*			[26]
	CS1210r	GATCYTCAATCATTTCTTTCCA	CS443f and CS1210r (1st PCR)	48	767	[25]
	BhCS1137.n	AATGCAAAAAGAACAGTAAACA	CS443f and BhCS1137.n (Semi-nested PCR)	48	694	[206]
	Bfp1	ATTAATCTGCAYCGGCCAGA	cell division protein gene, *fts*Z (Single PCR)	55	900	[292]
	Bfp2	ACVGADACACGAATAACACC				
	*ftz_seq3	TCTGGTGTTGCTTCCATTACAGA		N/A	N/A	This study
Coxiella	CoxdnaKF3	GGTACKTTYGATATTTCCATC	chaperone protein DNAK, *dna*K (1st PCR)	54	636	[66]
	CoxdnaKR	CGTCATGAYKCCGCCYAAGG				
	CoxdnaKF2	GAAGTGGATGGCGARCAYCAATT	(Nested PCR)	54	512	
	CoxdnaKR3	CTTGAATAGCYGCACCAATAGC				
	CoxGrF1	TTTGAAAAYATGGGCGCKCAAATGGT	chaperone protein GROEL, *gro*EL (1st PCR)	56	655	
	CoxGrR2	CGRTCRCCAAARCCAGGTGC				
	CoxGrF2	GAAGTGGCTTCGCRTACWTCAGACG	(Nested PCR)	56	619	
	CoxGrR1	CCAAARCCAGGTGCTTTYAC				
	CoxrpoBF2	GGGCGNCAYGGWAAYAAAGGSGT	β subunit of bacterial RNA polymerase gene, *rpo*B (1st PCR)	56	607-610	
	CoxrpoBR1	CACCRAAHCGTTGACCRCCAAATTG				
	CoxrpoBF3	TCGAAGAYATGCCYTATTTAGAAG	(Nested PCR)	56	539-542	
	CoxrpoBR3	AGCTTTMCCACCSARGGGTTGCTG				

	Cox16SF1	CGTAGGAATCTACCTTRTAGWGG	16S ribosomal DNA	52-56		
	Cox16S_07F	AGAGTTTGATYMTGGCTCAG	Cox16SF1 and Cox16SR2 (1st PCR)		1,321-1,429	
	Cox16SR2	GCCTACCCGCTTCTGGTACAATT	Cox16S_07F and Cox16SR2 (1st PCR)		1,434-1,542	
	Cox16SR1	ACTYYCCAACAACAGCTAGTTCTCA	Cox16S_07F and Cox16SR1 (Semi-nested PCR)		832-939	
	Cox23SF1	GCCTGCGAWAAGCTTCGGGGAG	Large ribosomal subunit (1st PCR)	56	694-1188	
	Cox23SR2	CTCCTAKCCACASCTCATCCCC				
	Cox23SF2	GATCCGGAGATWTCYGAATGGGG	Large ribosomal subunit (Nested PCR)	56	583-867	
	Cox23SR1	TCGYTCGGTTTCGGGTCKACTC				
Francisella	FT393	ATGGCGAGTGATACTGCTTG	membrane protein/T-cell epitope, TUL4 (Single PCR)	53	248	[163]
	FT642	GCATCATCAGAGCCACCTAA				
	MS1	CAGCTACTACACAAAGCAGTGG	Outer membrane protein, *fop*A (Single PCR)	58	707	[99]
	MAI	CACCATTTACTGTATAGCACGC				
	F11	TACCAGTTGGAAACGACTGT	16S ribosomal DNA (Single PCR)	51	1,000	[75]
	F5	CCTTTTTGAGTTTCGCTCC				
	*Fran16F_seq1	TCGTCAGCTCGTGTTGTGAA	16S ribosomal DNA	N/A	N/A	This study
	*Fran16R_seq2	GCATTTCACCGCTACACCAG				
Rickettsia	gltA_Fc	CGAACTTACCGCTATTAGAATG	citrate synthase gene, *glt*A (Single PCR)	55	580	[77]
	gltA_Rc	CTTTAAGAGCGATAGCTTCAAG				
	Rr.190.70p	ATGGCGAATATTTCTCCAAAA	outer membrane A gene, *omp*A (Single PCR)	48	542	[233]
	Rr.190.602n	AGTGCAGCATTCGCTCCCCCT				
	120_2788	AAACAATAATCAAGGTACTGT	outer membrane B gene, *omp*B (Single PCR)	48	816	[241]
	120_3599	TACTTCCGGTTACAGCAAAGT				
	17K_5	GCTTTACAAAATTCTAAAAACCATATA	17-kDa common antigen gene, *htr*A (Single PCR)	52	550	[152]
	17K_3	TGTCTATCAATTCACAACTTGCC				
	Rick_16S_F3	ATCAGTACGGAATAACTTTTA	16S ribosomal DNA (Single PCR)	52	1,328	[14]
	Rick_16S_F4	TGCCTCTTGCGTTAGCTCAC				
	*SeqRick16SF	CAGCTCGTGTCGTGAGATGT		N/A	N/A	This study
	*SeqRick16SR	TACGCCAGTAATTCCGAAC				
	D1f	ATGAGTAAAGACGGTAACCT	surface cell antigen-4, *sca*4 (Single PCR)	50	928	[250]
	D928r	AAGCTATTGCGTCATCTCCG				

Results

A total of 6,110,903 raw paired-end reads were obtained from the Illumina MiSeq sequencer. Sequences obtained were demultiplexed and quality filtered, resulting in 509,467 high-quality reads assigned to 9287 features retained after the DADA2 quality control analysis. Among 210 ticks from six species of three genera, four samples were excluded from the analysis due to quality reasons. Tick samples were categorized into three groups for microbial analysis. The first group included all ticks from their available life stages. The second group consisted of only the adult stage ticks because not all tick species collected had all life stages represented (*H. hystricis* excluded). Finally, since *H. shimoga* had the most comprehensive sample structure, we examined the effect of different life stage and feeding status for this species.

Tick species microbial variations

Microbial diversity analysis revealed significant differences among tick species, regardless of tick life stage, when all ticks or only adult ticks were included in the analysis. Amongst, genus *Dermacentor* showed the highest microbial diversity in Faith's PD ($p < 0.001$), in observed OTUs ($p < 0.05$ and $p < 0.001$), and Shannon diversity ($p < 0.05$) (Figure 8). Next, *I. granulatus* had significantly higher microbial diversity than *H. shimoga* in Faith's PD ($p < 0.05$) (Figure 8). When comparing the microbial diversity between the three species of *Dermacentor*, *D. steini* was significantly less diverse than *D. compactus* in observed OTUs ($p < 0.05$) and in Faith's PD ($p < 0.001$) and *D. atrosignatus* in Faith's PD ($p < 0.001$) (Figure 8). However, it was not significant when only adult stage *Dermacentor* was included in the analysis. Pairwise LEfSe analyses also supported these findings as indicated that the genus *Dermacentor* displayed a greater number of taxonomy groups that were significantly more abundant than other tick species, consistent with the alpha diversity results that the genus had the highest microbial diversity. Finally, species evenness was relatively high for all species examined, and a significant difference was observed between *H. hystricis* and *H. shimoga* ($p < 0.001$) (Figure 8).

Next, the pairwise PERMANOVA comparisons for the beta diversity analyses revealed that microbial composition was significantly different among the tick species, except between the *D. compactus*, *D. steini*, and *D. atrosignatus* were not significantly different (Table 9). Consistently, results with only adult ticks showed significant difference between tick species, except for the three *Dermacentor* spp. Of all tick species, *H. shimoga* had greater pseudo-F values estimated in pairwise comparisons with other tick species for all four beta diversity metrics that were significant ($p = 0.001$) (Table 9). Adult *H. shimoga* ticks also formed a distinct cluster from *I. granulatus* and *Dermacentor* species in the Bray-

Curtis dissimilarity and Jaccard distance plots (Figure 9). Overall, results revealed a highly significant microbial composition difference in *H. shimoga*. Meanwhile, *H. hystricis* also showed greater difference in unweighted and weighted UniFrac distances with *D. compactus* (pseudo-F = 27.76 and 18.95; $p = 0.001$) and *D. atrosignatus* (pseudo-F = 26.79 and 18.2; $p = 0.001$). No significant difference was observed between three *Dermacentor* species ($p > 0.05$) and they clustered together as shown in unweighted and weighted UniFrac distance plots of adult ticks (Figure 9).

Microbial composition and main endosymbionts for each tick species

Overall, the most abundant phylum identified in ticks was Proteobacteria (76.77%). The rest of the phyla accounted for less than 10%, and they were Actinobacteria (6.69%), Chlamydiae (6.67%), Spirochaetes (3.26%), Firmicutes (1.44%), Bacteroidetes (1.43%), and Planctomycetes (1.12%). Notably, Spirochaetes were only present in *I. granulatus* and accounted for 18.9% of its total relative abundance, which was characterized as *B. yangtzensis*, a member of Bbsl complex, in Chapter II for *Borrelia* screening. Heatmap (Figure 10) showed the taxonomy groups that were more abundantly represented in each tick species. For instance, Rickettsiales could be found in most tick species such as *I. granulatus*, *H. hystricis*, *H. shimoga*, and *D. steini*, while order Legionellales was mainly distributed in *H. shimoga*. Differentially abundant taxonomy groups for each tick species were identified by pairwise LEfSe analyses. Taxonomy groups always in high abundance including Planctomycetes, Actinobacteria, Bacteroidetes, and Rhizobiales for *D. compactus* and *D. atrosignatus*, while Gammaproteobacteria, Pseudomonadales, Legionellales, and *Francisellaceae* for *D. steini*. LEfSe results also showed that *H. shimoga* harbored more taxonomy groups which were more abundant than *H. hystricis*, including *Coxiellaceae*, Actinomycetales, and *Mycobacteriaceae*. Additionally, while *Coxiellaceae* was highlighted to be the more abundant group found in *H. shimoga*, *Borreliaceae* was evident for *I. granulatus*, and *Francisellaceae* was noted for *D. steini*. Pseudomonadales and Enterobacteriales were among the taxonomy groups that were found more abundant in *H. hystricis* when compared with other tick species.

Main endosymbionts were identified in some tick species, except for *D. compactus* and *D. atrosignatus* (Table 10). For *I. granulatus*, Rickettsiales (25.63%) was the dominant bacteria, and for *H. shimoga*, *Coxiella* accounted for 50% of its relative abundance. Some tick species had more than one endosymbionts dominating, for instance, *Acinetobacter* and Rickettsiales were the bacteria that found dominating in *H. hystricis*. In *D. steini*, *Acinetobacter* (21.96%), Rickettsiales (17.90%), and *Francisella* (17.87%) were most abundant. Meanwhile, in *D. compactus* and *D. atrosignatus*, most taxonomy groups were present with a relative abundance of less than 10%. Furthermore, *Francisella* was not detected in *D. compactus* and *D. atrosignatus*, but we detected *Francisella* (2.25%) in *I. granulatus*.

Ontogenic and sex on microbial variations of *Haemaphysalis shimoga*

Alpha diversity analysis of different developmental stages of *H. shimoga* ticks revealed that adult ticks showed significantly higher microbial diversity than nymphs and larvae by observed OTUs ($p < 0.05$ and $p < 0.001$, respectively) and Shannon diversity ($p < 0.05$) (Figure 11). However, microbial diversity was not significantly different between adult male and female and between the nymph and larva of *H. shimoga* ticks. The Pielou's evenness analysis showed that the microbiota was evenly distributed in all *H. shimoga* developmental stages (Figure 11). Furthermore, the microbial composition of the adult females was significantly different ($p = 0.001$) from nymphs and larvae, in which beta diversity analysis results showed distinct clusters between adult females and larvae were observed in weighted UniFrac distance (pseudo-F = 34.29), Bray-Curtis dissimilarity (pseudo-F = 24.62), and Jaccard distance (pseudo-F = 20.87) plots of *H. shimoga* (Table 11, Figure 12). Similarly, adult male and larva *H. shimoga* ticks were significantly different ($p = 0.001$) in microbial composition in unweighted (pseudo-F = 5.84) and weighted (pseudo-F = 10.50) UniFrac distance, Bray-Curtis dissimilarity (pseudo-F = 8.57), and Jaccard distance (pseudo-F = 8.37) analyses. Adult male *H. shimoga* ticks were also significantly different from *H. shimoga* nymphs with unweighted (pseudo-F = 3.52; $p = 0.001$) and weighted (pseudo-F = 4.79; $p = 0.004$) UniFrac distance, Bray-Curtis dissimilarity (pseudo-F = 3.53; $p = 0.002$), and Jaccard distance (pseudo-F = 4.32; $p = 0.003$). There was a significant difference detected between the microbial composition of adult male and female *H. shimoga* ticks in unweighted (pseudo-F = 1.91; $p = 0.03$) and weighted (pseudo-F = 3.57; $p = 0.006$) UniFrac distance, Bray-Curtis dissimilarity (pseudo-F = 2.65; $p = 0.004$), and Jaccard distance (pseudo-F = 2.63; $p = 0.003$) analyses. Additionally, nymph and larva of *H. shimoga* ticks were not greatly different in microbial composition, with unweighted (pseudo-F =1.65; $p = 0.126$) and weighted (pseudo-F = 1.85; $p = 0.11$) UniFrac distance showed not significantly different, while Bray-Curtis dissimilarity (pseudo-F =2.13; $p = 0.006$), and Jaccard distance (pseudo-F =2.10; $p = 0.001$) revealed significance (Table 11).

Furthermore, pairwise LEfSe analyses revealed the differential abundance for adult ticks was indicated by *Coxiellaceae* and *Mycobacteriaceae*. Rickettsiales, Burkholderiales, Xanthomonadales, and Caulobacterales were significantly more abundant in nymph than adult female ticks, whereas only Rickettsiales and Comamonadacea family were significantly more abundant than adult male ticks. One phylum, three classes, six orders and five families were found significantly more abundant in larva when compared with adult female ticks, including orders Chlamydiales and Burkholderiales, which also significantly more abundant than adult male ticks. These microbial difference observed was supported by the presence of main endosymbionts in different life stage and sex. As shown in the Table 12 and Heatmap

(Figure 13), genus *Coxiella* was found to dominate the adult ticks and dual symbiosis was observed for nymph and larva. For instance, the presence of Rickettsiales and *Coxiella* in nymphs and Rickettsiales and *Candidatus* Rhabdochlamydia in larvae. Contrary to nymph and larva, order Rickettsiales was found in low relative abundance in adult ticks.

The effect of *Borrelia* infection on *Ixodes granulatus* microbiome

Alpha and beta diversity analyses were conducted on 13 *Borrelia*-positive and 15 *Borrelia*-negative *I. granulatus* ticks. Based on PERMANOVA pairwise comparison, the microbial composition between the positive and negative ticks was significantly different (p = 0.001) for unweighted and weighted UniFrac distance, Jaccard distance, and Bray-Curtis dissimilarity analyses (Table 13). For microbial diversity analyses, we detected significantly different values for faith's PD (p = 0.006). Additionally, pairwise LEfSe analysis revealed taxonomy groups with significant differential abundance for the positive and negative ticks. Additionally, pairwise LEfSe analysis revealed taxonomy groups with significant differential abundance for the positive and negative ticks (Figure 14). For instance, *Borrelia*-negative ticks had more abundant Rickettsiales, *Acinetobacter*, Moraxellaceae, and Pseudomonadales (Figure 14).

Bacteria species characterization

Bacteria identified in NGS were verified and characterized using conventional PCRs. The number of NGS- and PCR- positive ticks are shown in Table 14. Table 15 listed the sequence analysis from the successfully amplified bacteria from their respective tick species and the targeted genes that amplified in PCRs. Overall, bacteria species from six genera: *Coxiella*, *Francisella*, *Rickettsia*, *Anaplasma*, *Ehrlichia*, and *Bartonella* were identified from the ticks.

Amongst, *Coxiella* sp. closely related to *Coxiella*-LE was identified in *H. shimoga* and *H. hystricis* ticks with 86.9-99.8% identity. *Francisella* sp. closely related to *Francisella*-LE strain FLE011 from *Hyalomma marginatum marginatum* in Bulgaria (HQ705174) was identified from *D. steini* and *I. granulatus* ticks with 94.9% identity (203/214 bp) based on *tul4* gene. In addition, *Francisella* sp. closely related to *Francisella* endosymbiont of *D. atrosignatus* isolate DASSD4 in Thailand (KC170748) was identified in *D. steini* with 99.9% identity (1,100/1,101 bp) based on 16S rDNA. Furthermore, *R. heilongjiangensis*, the causative agent for spotted fever rickettsiosis in humans, was identified from *H. shimoga* ticks. Phylogenetic trees constructed with multiple target genes also showed the clustering with *R. heilongjiangensis* (Figure 15). *Anaplasma* sp. closely related to *A. platys* isolated in deer in China (KJ659044), with 98.9% identity (1,313/1,327 bp) was identified from *D. atrosignatus*. *Ehrlichia* sp.

identified from *H. shimoga* in this study had 98.9% identity (1,315/1,330 bp) with the *Ehrlichia* sp. EBm52 of *Rhipicephalus microplus* reported in Thailand (AF497581). Phylogenetic tree based on 16S rDNA for *Anaplasma* and *Ehrlichi*a inferred that the *Anaplasma* sp. and *Ehrlichia* sp. in this study clustered with *A. platys* and *E. ewingii*, respectively (Figure 16). *Anaplasma platys* is known to cause canine cyclic thrombocytopenia, while *E. ewingii* caused human monocytic ehrlichiosis in humans. In addition, two *Bartonella* spp. were identified from *D. steini* and *I. granulatus* ticks. *Bartonella* sp. closed related to *B. rattimassiliensis* isolated from European *Rattus norvegicus* in France (AY515124), with 97.8% identity (703/719 bp) was identified in *D. steini* based on the *gltA* gene. *Bartonella* sp. closely related to *B. tribocorum* strain MVT04 detected in human blood in France (HG969192), with 97.1% identity (868/894 bp) was identified in *I. granulatus* tick based on the *ftsZ* gene.

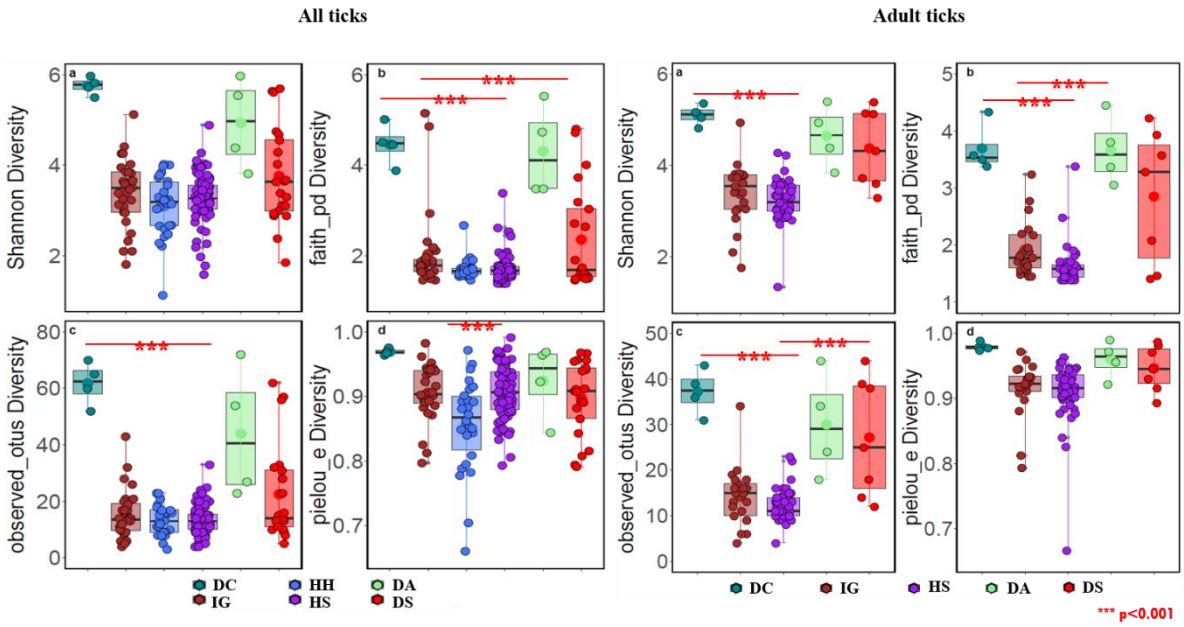

Figure 8. Alpha-diversity analysis of All ticks and Adult ticks with a) Shannon, b) Faith's Phylogenetic Diversity, c) observed Operational Taxonomic Units, and d) Pielou's evenness. Tick species are abbreviated as follows: IG (*Ixodes granulatus*), HH (*Haemaphysalis hystricis*), HS (*H. shimoga*), DC (*Dermacentor compactus*), DS (*D. steini*), and DA (*D. atrosignatus*). Genus *Dermacentor* (DC, DA, and DS) was significantly ($p < 0.001$) higher in microbial diversity than other tick species regardless of the developmental stage.

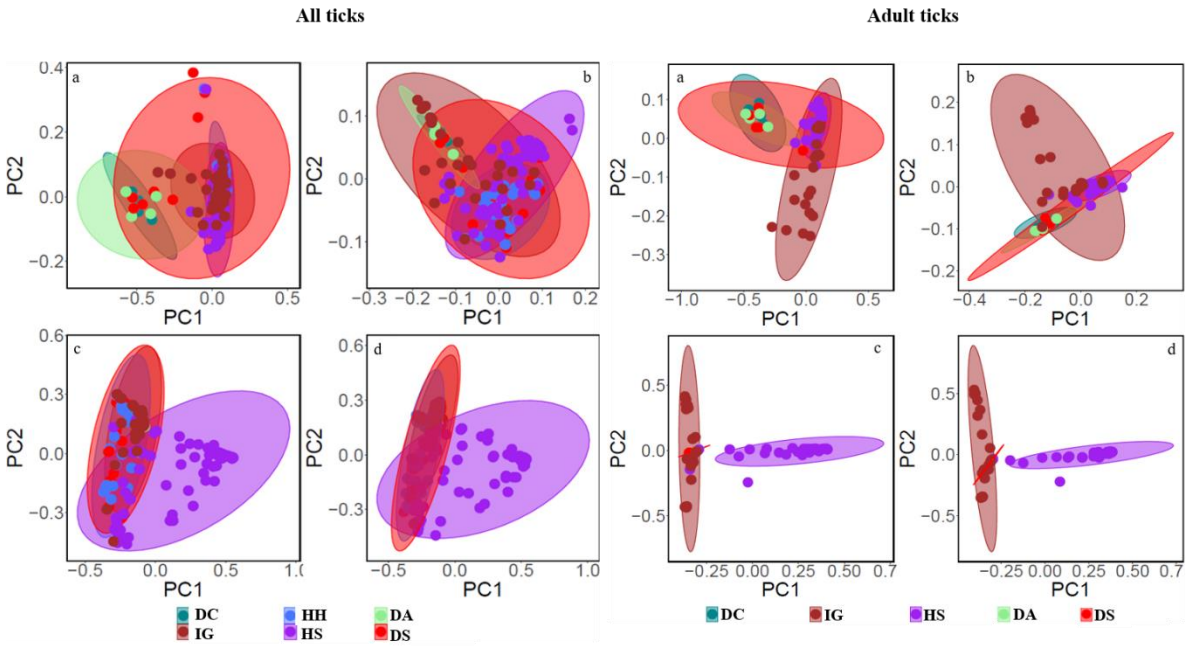

Figure 9. Principal Coordinate Analysis plots of All ticks and Adult ticks with a) unweighted UniFrac distance, b) weighted UniFrac distance, c) Jaccard distance, and d) Bray-Curtis dissimilarity showed significant dissimilarity in microbial composition between tick species, especially for *Haemaphysalis shimoga* (HS) (p = 0.001) in c) Jaccard distance and d) Bray-Curtis dissimilarity plots of Adult ticks.

Table 9. Pairwise PERMANOVA (Permutational multivariate analysis of variance) results for All ticks and Adult ticks. Significance of unweighted and weighted UniFrac distance, Bray-Curtis dissimilarity, and Jaccard distance was tested with 999 permutations. Significance *p*-values are italicized in the table. Tick species are abbreviated as follows: IG (*Ixodes granulatus*), HH (*Haemaphysalis hystricis*), HS (*H. shimoga*), DC (*Dermacentor compactus*), DS (*D. steini*), and DA (*D. atrosignatus*).

			unweighted UniFrac distance			**weighted UniFrac distance**			**Bray-Curtis dissimilarity**			**Jaccard distance**		
Group 1	**Group 2**	**Sample size**	**pseudo-F**	***p*-value**	**q-value**	**pseudo-F**	***p*-value**	**q-value**	**pseudo-F**	***p*-value**	**q-value**	**pseudo-F**	***p*-value**	**q-value**
All ticks														
HS	**DC**	83	26.35	*0.001*	0.002	14.03	*0.001*	0.002	3.92	*0.002*	0.004	3.43	*0.003*	0.005
	IG	107	9.13	*0.001*	0.002	19.68	*0.001*	0.002	14.00	*0.001*	0.003	12.04	*0.001*	0.002
	HH	105	9.71	*0.001*	0.002	10.70	*0.001*	0.002	17.20	*0.001*	0.003	14.20	*0.001*	0.002
	DA	83	25.39	*0.001*	0.002	14.57	*0.001*	0.002	3.91	*0.003*	0.005	3.42	*0.002*	0.004
	DS	100	8.37	*0.001*	0.002	5.59	*0.001*	0.002	12.34	*0.001*	0.003	11.09	*0.001*	0.002
DC	**IG**	32	10.84	*0.001*	0.002	4.05	*0.003*	0.004	1.64	*0.015*	0.017	1.58	*0.016*	0.020
	HH	30	27.76	*0.001*	0.002	18.95	*0.001*	0.002	2.91	*0.001*	0.003	2.52	*0.001*	0.002
	DA	8	0.93	0.645	0.645	1.01	0.437	0.437	0.96	0.972	0.972	0.97	0.91	0.910
	DS	25	5.69	*0.002*	0.003	6.55	*0.002*	0.003	1.90	*0.012*	0.015	1.89	*0.01*	0.015
IG	**HH**	54	5.12	*0.001*	0.002	10.02	*0.001*	0.002	4.26	*0.001*	0.003	3.41	*0.001*	0.002
	DA	32	10.28	*0.001*	0.002	4.25	*0.004*	0.005	1.63	*0.032*	0.034	1.57	*0.018*	0.021
	DS	49	3.05	*0.004*	0.004	6.02	*0.002*	0.003	2.76	*0.002*	0.004	2.54	*0.001*	0.002
HH	**DA**	30	26.79	*0.001*	0.002	18.27	*0.001*	0.002	2.90	*0.001*	0.003	2.51	*0.001*	0.002
	DS	47	3.62	*0.003*	0.003	2.11	0.052	0.056	1.88	*0.01*	0.015	1.42	0.083	0.089
DA	**DS**	25	5.19	*0.002*	0.003	6.55	*0.002*	0.003	1.90	*0.012*	0.015	1.89	*0.012*	0.016
Adult ticks														
HS	**DC**	50	27.19	*0.001*	0.001	26.16	*0.001*	0.003	9.07	*0.001*	0.003	7.91	*0.001*	0.003

	IG	67	10.68	*0.001*	0.001	28.50	*0.001*	0.003	23.79	*0.001*	0.003	20.69	*0.001*	0.003
	DA	50	29.33	*0.001*	0.001	28.44	*0.001*	0.003	9.01	*0.001*	0.003	7.87	*0.001*	0.003
	DS	53	13.78	*0.001*	0.001	11.86	*0.001*	0.003	14.26	*0.001*	0.003	12.42	*0.001*	0.003
DC	**IG**	25	8.81	*0.001*	0.001	3.44	*0.009*	0.015	1.62	*0.028*	0.047	1.50	*0.02*	0.033
	DA	8	1.06	0.39	0.390	0.77	0.726	0.726	0.97	0.948	0.948	0.98	0.888	0.888
	DS	11	1.33	0.187	0.229	1.72	0.18	0.200	1.42	0.11	0.137	1.39	0.1	0.125
IG	**DA**	25	9.13	*0.001*	0.001	4.00	*0.003*	0.006	1.62	*0.036*	0.051	1.50	*0.026*	0.037
	DS	28	4.37	*0.001*	0.001	3.08	*0.017*	0.024	2.44	*0.004*	0.008	2.21	*0.002*	0.004
DA	**DS**	11	1.35	0.206	0.229	1.90	0.127	0.159	1.38	0.124	0.138	1.36	0.126	0.140

Table 10. Main endosymbionts and their relative abundance identified in each tick species, except for *Dermacentor compactus* and *D. atrosignatus*.

Tick species	Bacterial taxa	Relative abundance (%)
Ixodes granulatus	Rickettsiales	25.63
	Borrelia	18.9
	Acinetobacter	11.75
	Candidatus Rhabdochlamydia	6.36
Haemaphysalis hystricis	*Acinetobacter*	30.06
	Rickettsiales	25.3
	Stenotrophomonas	10.41
	Candidatus Rhabdochlamydia	10.02
Haemaphysalis shimoga	*Coxiella*	50
	Rickettsiales	13.69
	Candidatus Rhabdochlamydia	7.32
	Stenotrophomonas	5.2
Dermacentor steini	*Acinetobacter*	21.96
	Rickettsiales	17.9
	Francisella	17.87
	Burkholderiales	7.96

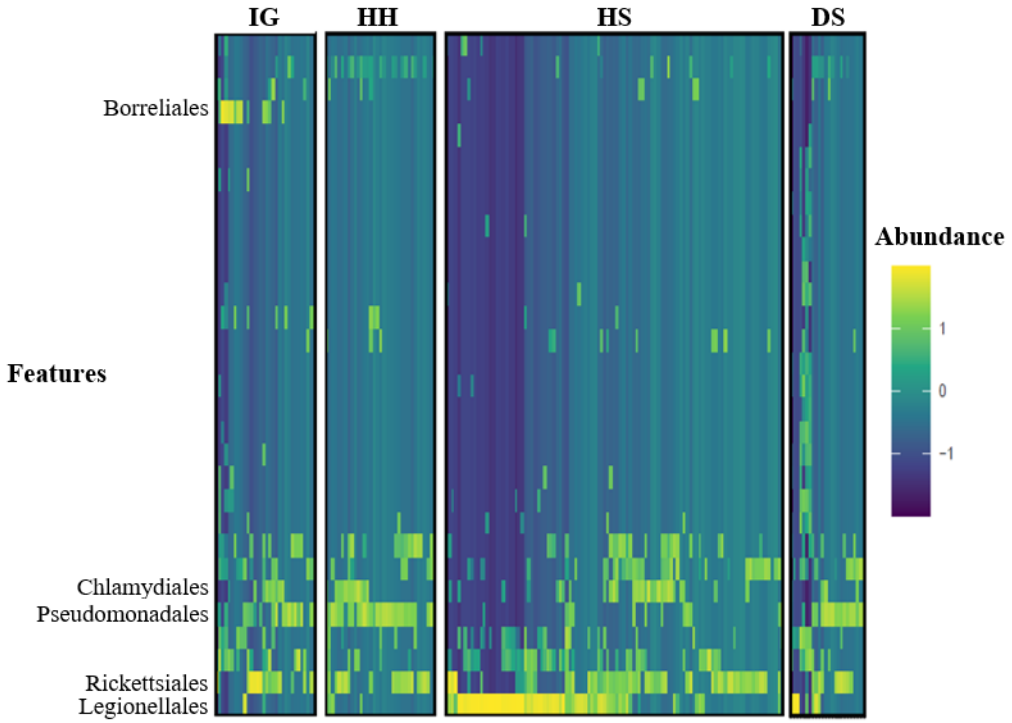

Figure 10. Heatmap for *Ixodes granulatus* (IG), *Haempahysalis hystricis* (HH), *H. shimoga* (HS), and *Dermacentor steini* (DS) at the taxonomy Order level.

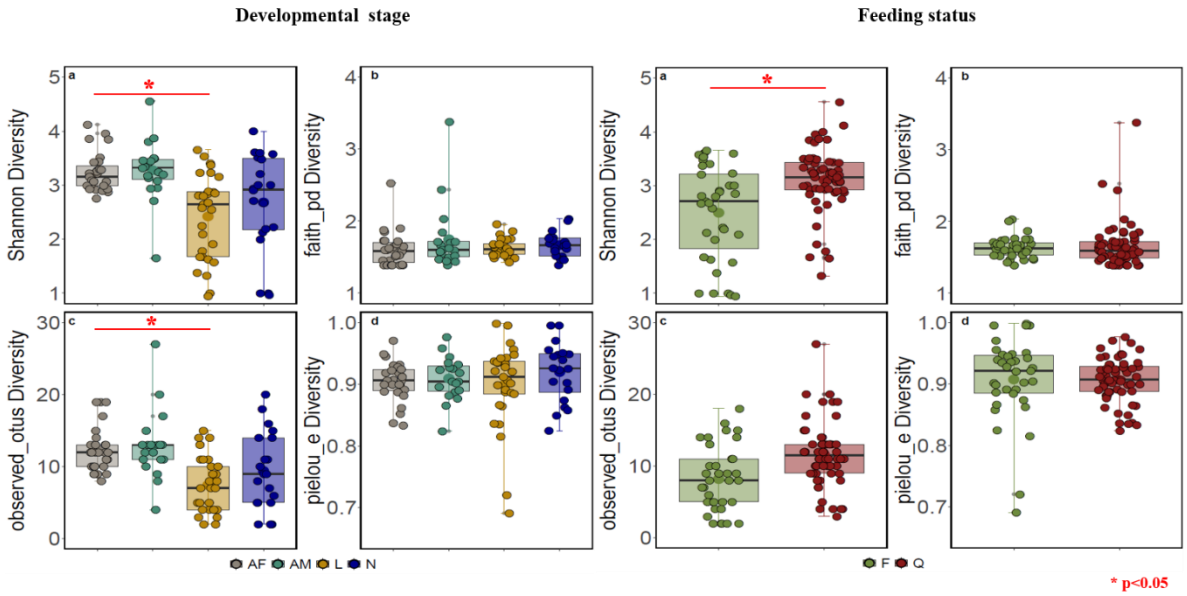

Figure 11. Alpha-diversity analysis of different developmental stages and feeding status of *Haemaphysalis shimoga* in a) Shannon, b) Faith's Phylogenetic Diversity, c) observed Operational Taxonomic Units, and d) Pielou's evenness. The developmental stage and feeding status of the ticks are abbreviated as follows: AF (Adult female), AM (Adult male), N (Nymph), L (Larva), F (Feeding), and Q (Questing). A significant difference in microbial diversity was observed between adult ticks (AF and AM) and larvas (L) with $p < 0.05$.

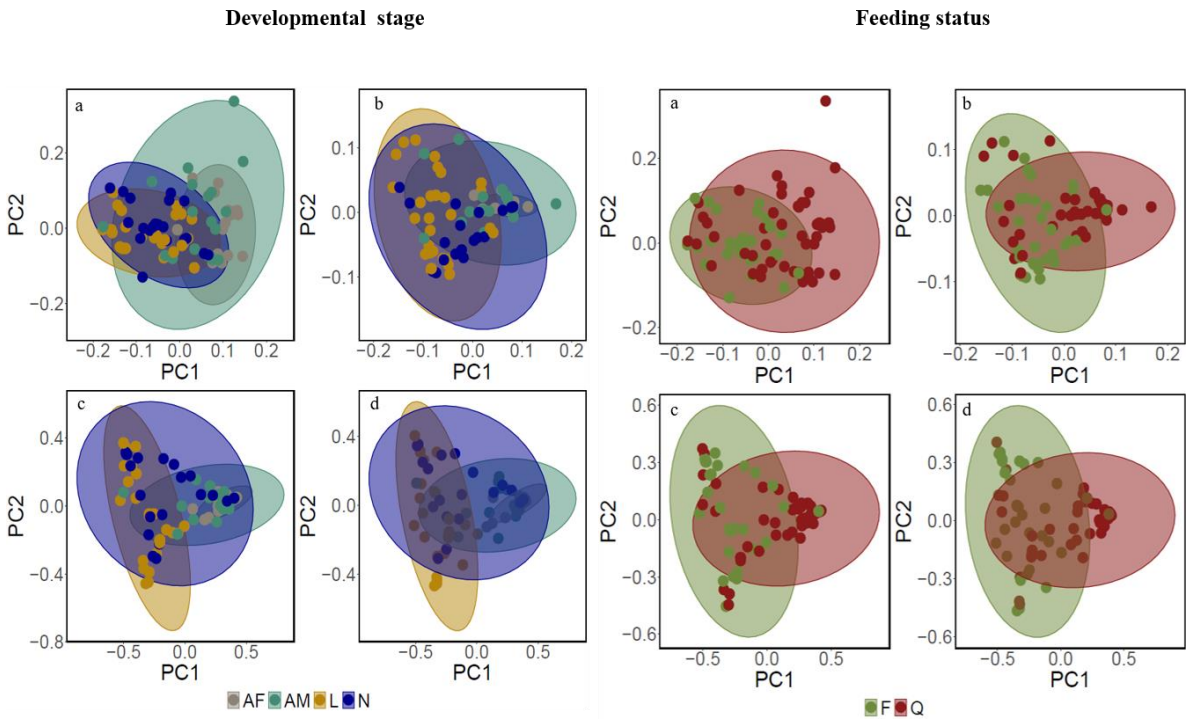

Figure 12. Principal Coordinate Analysis plots of different developmental stages and feeding status of *Haemaphysalis shimoga* in a) unweighted UniFrac distance, b) weighted UniFrac distance, c) Jaccard distance, and d) Bray-Curtis dissimilarity showed significant dissimilarity in microbial composition between different developmental stages with $p < 0.05$.

Table 11. Pairwise PERMANOVA (Permutational multivariate analysis of variance) results between different developmental stages and feeding status of *Haemaphysalis shimoga*. Significance of unweighted and weighted UniFrac distance, Bray-Curtis dissimilarity, and Jaccard distance was tested with 999 permutations. Significance *p*-values are italicized in the table. The developmental stage and feeding status of the ticks are abbreviated as follows: AF (Adult female), AM (Adult male), N (Nymph), L (Larva), F (Feeding), and Q (Questing).

			unweighted UniFrac distance			**weighted UniFrac distance**			**Bray-Curtis dissimilarity**			**Jaccard distance**		
Group 1	**Group 2**	**Sample size**	**pseudo-F**	***p*-value**	**q-value**	**pseudo-F**	***p*-value**	**q-value**	**pseudo-F**	***p*-value**	**q-value**	**pseudo-F**	***p*-value**	**q-value**
Developmental stage														
AF	**AM**	46	1.91	*0.03*	0.036	3.57	*0.006*	0.007	2.65	*0.004*	0.005	2.63	*0.003*	0.003
	L	56	13.81	*0.001*	0.002	34.29	*0.001*	0.002	24.62	*0.001*	0.002	20.87	*0.001*	0.002
	N	47	8.73	*0.001*	0.002	20.91	*0.001*	0.002	13.24	*0.001*	0.002	12.82	*0.001*	0.002
AM	**L**	48	5.84	*0.001*	0.002	10.50	*0.001*	0.002	8.57	*0.001*	0.002	8.37	*0.001*	0.002
	N	39	3.52	*0.001*	0.002	4.79	*0.004*	0.006	3.53	*0.002*	0.003	4.32	*0.003*	0.003
L	**N**	49	1.65	0.126	0.126	1.85	0.11	0.110	2.13	*0.006*	0.006	2.10	*0.001*	0.002
Feeding status														
F	**Q**	95	6.90	*0.001*	0.001	15.33	*0.001*	0.001	12.37	*0.001*	0.001	11.65	*0.001*	0.001

Table 12. Bacterial taxa present in high relative abundance in each developmental stage of *Haemaphysalis shimoga*.

Developmental stage	Bacterial taxa	Relative abundance (%)
Adult male (AM)	*Coxiella*	62.04
	Rickettsiales	8.8
	Mycobacterium	7.01
	Rickettsia	5.12
Adult female (AF)	*Coxiella*	80.25
	Rickettsia	7.18
	Rickettsiales	2.42
	Mycobacterium	1.83
Nymph (N)	Rickettsiales	29.91
	Coxiella	25.8
	Burkholderiales	13.11
	Stenotrophomonas	9.6
Larva (L)	Rickettsiales	24.45
	Candidatus Rhabdochlamydia	21.92
	Stenotrophomonas	11.51
	Coxiella	8.09

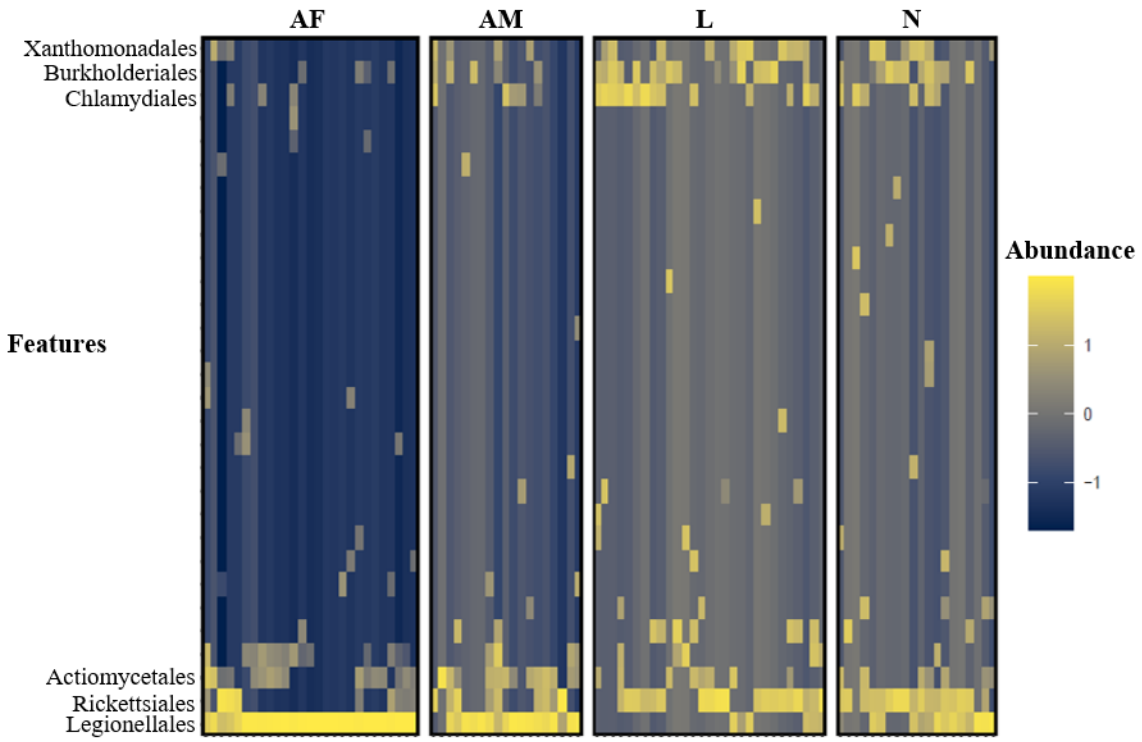

Figure 13. Heatmap for different developmental stages of *Haemaphysalis shimoga*, Adult female (AF), Adult male (AM), Nymph (N), and Larva (L) at the taxonomy Order level.

Table 13. Pairwise PERMANOVA (Permutational multivariate analysis of variance) results between *Borrelia*-positive and -negative *Ixodes granulatus*. Significance of unweighted and weighted UniFrac distance, Bray-Curtis dissimilarity, and Jaccard distance was tested with 999 permutations. Significance p-values are italicized in the table.

unweighted UniFrac distance	**pseudo-F**	4.97
	***p*-value**	*0.001*
	q-value	0.001
weighted UniFrac distance	**pseudo-F**	10.40
	***p*-value**	*0.001*
	q-value	0.001
Bray-Curtis dissimilarity	**pseudo-F**	3.93
	***p*-value**	*0.001*
	q-value	0.001
Jaccard distance	**pseudo-F**	3.46
	***p*-value**	*0.001*
	q-value	0.001

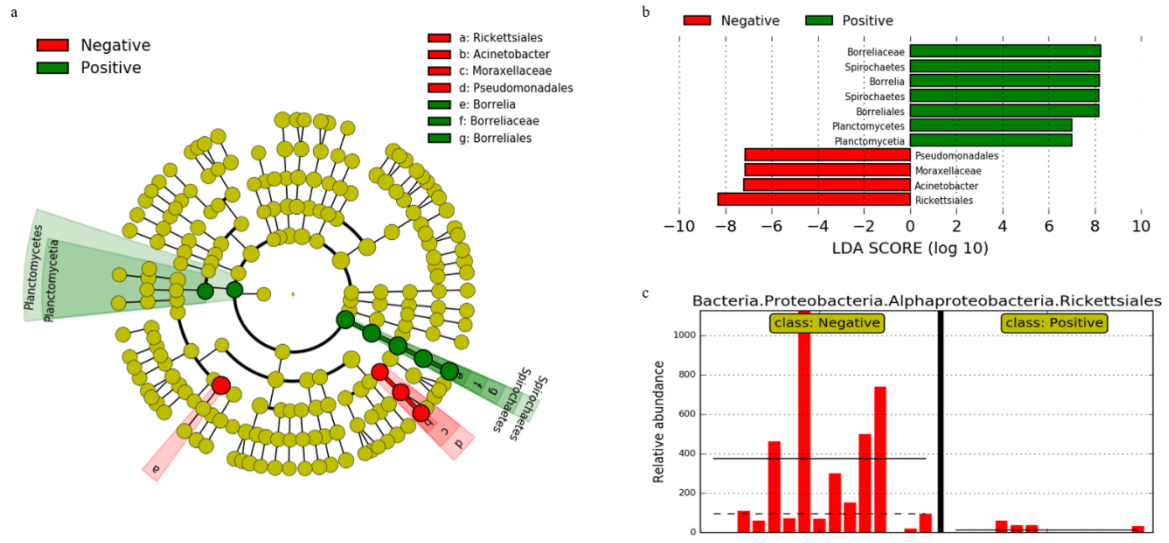

Figure 14. LEfSe (Linear discriminant analysis Effect Size) a. cladogram, b. barplots, and c. relative abundance for Rickettsiales for *Borrelia*-positive and –negative *Ixodes granulatus*. LEfSe analysis revealed that bacterial taxa such as Rickettsiales, *Acinetobacter*, Moraxellacea, and Pseudomonadales showed significant differential abundance.

Table 14. The number of tick samples included in Polymerase chain reactions (PCRs) for bacteria characterization after Next-generation sequencing (NGS).

	No. of positive samples in PCR /No. of positive samples in NGS					
Tick species	***Anaplasma***	***Ehrlichia***	***Bartonella***	***Coxiella***	***Francisella***	***Rickettsia***
Ixodes granulatus	0/1	0/1	1/3	N/A	1/1	N/A
Haemaphysalis hystricis	N/A	N/A	N/A	1/2	N/A	N/A
Haemaphysalis shimoga	N/A	1/3	N/A	**20/66*	N/A	4/5
Dermacentor compactus	N/A	N/A	N/A	N/A	N/A	0/2
Dermacentor steini	N/A	N/A	1/2	0/2	5/5	N/A
Dermacentor atrosignatus	1/1	N/A	N/A	N/A	N/A	N/A

* 20 NGS-positive samples were randomly selected for PCRs, as all 66 samples had the same feature.

Table 15. The sequence analysis results for the bacterial genera of *Coxiella*, *Francisella*, *Rickettsia*, *Anaplasma*, *Ehrlichia*, and *Bartonella* in different tick species based on each successfully amplified gene.

Bacteria	Genes	Tick species	BLASTn	Identity	Accession No.
Coxiella	16S rRNA	*Haemaphysalis hystricis*	Uncultured *Coxiella* sp. isolate S006 from *Haemaphysalis hystricis* (Malaysia)	99.8% (1,273/1,275 bp)	LT009433
		Haemaphysalis shimoga	Bacterium symbiont of *Haemaphysalis shimoga* clone HSKY-3 (Thailand)	99.8% (1,275/1,277 bp)	HQ287535
	23S rRNA	*Haemaphysalis hystricis*	*Coxiella burnetii*strain RSA439 from *Dermacentor andersoni* (USA)	92.0% (496/539 bp)	CP040059
		Haemaphysalis shimoga	*Coxiella burnetii*strain RSA439 from *Dermacentor andersoni* (USA)	91.9% (500/544 bp)	CP040059
	GroEL	*Haemaphysalis hystricis*	Uncultured *Coxiella* sp. clone PK179-183 from chicken tick (Thailand)	93.7% (479/511 bp)	MG874468
		Haemaphysalis shimoga	Uncultured *Coxiella* sp. clone T3115 from *Ixodes uriae* (Canada)	86.7% (494/570 bp)	KJ459059
	rpoB	*Haemaphysalis hystricis*	Uncultured *Coxiella* sp. isolate S002 from *Haemaphysalis hystricis* (Malaysia)	99.8% (490/491 bp)	LT174612
		Haemaphysalis shimoga	Uncultured *Coxiella* sp. isolate S002 from *Haemaphysalis hystricis* (Malaysia)	89.9% (438/487 bp)	LT174612
	dnaK	*Haemaphysalis shimoga*	*Coxiella* endosymbiont of *Ixodes* sp. isolate Isp1iso2 (Cote d'Ivoire)	86.9-87.1% (391-392/450 bp)	KP985406
Francisella	*tul4*	*Ixodes granulatus*	*Francisella*-like endosymbiont strain FLE011from *Hyalomma marginatum marginatum* (Bulgaria)	94.9% (203/214 bp)	HQ705174
		Dermacentor steini	*Francisella*-like endosymbiont strain FLE011from *Hyalomma marginatum marginatum* (Bulgaria)	94.9% (203/214 bp)	HQ705174
	16S rRNA	*Dermacentor steini*	*Francisella* endosymbiont of *Dermacentor atrosignatus* isolate DASSD4 (Thailand)	99.9% (1,100/1,101 bp)	KC170748
Rickettsia	*gltA*	*Haemaphysalis shimoga*	*Rickettsia heilongjiangensis* Sendai-58 from *Haemaphysalis concinna* (Japan)	100% (537/537 bp)	AP019865
	ompA		*Rickettsia japonica* strain PMK (Thailand)	100% (491/491 bp)	DQ909072

	ompB		*Rickettsia heilongjiangensis* Sendai-58 from *Haemaphysalis concinna* (Japan)	99.9% (769/770 bp)	AP019865
	htrA		*Rickettsia heilongjiangensis* Sendai-58 from *Haemaphysalis concinna* (Japan)	100% (495/495 bp)	AP019865
	sca4		*Rickettsia heilongjiangensis* Sendai-58 from *Haemaphysalis concinna* (Japan)	100% (888/888 bp)	AP019865
	16S rRNA		*Rickettsia heilongjiangensis* Sendai-58 from *Haemaphysalis concinna* (Japan)	100% (1,243/1,243 bp)	AP019865
Anaplasma	16S rRNA	*Dermacentor atrosignatus*	*Anaplasma platys* isolate 2ax1 from sika deer (China)	98.9% (1,313/1,327 bp)	KJ659044
Ehrlichia	16S rRNA	*Haemaphysalis shimoga*	*Ehrlichia* sp. EBm52 of *Rhipicephalus microplus* (Thailand)	98.9% (1,315/1,330 bp)	AF497581
Bartonella	*gltA*	*Dermacentor steini*	*Bartonella rattimassiliensis* sp. nov. from European *Rattus norvegicus* (France)	97.8% (703/719 bp)	AY515124
	ftsZ	*Ixodes granulatus*	*Bartonella tribocorum* strain MVT04 from human blood (France)	97.1% (868/894 bp)	HG969192

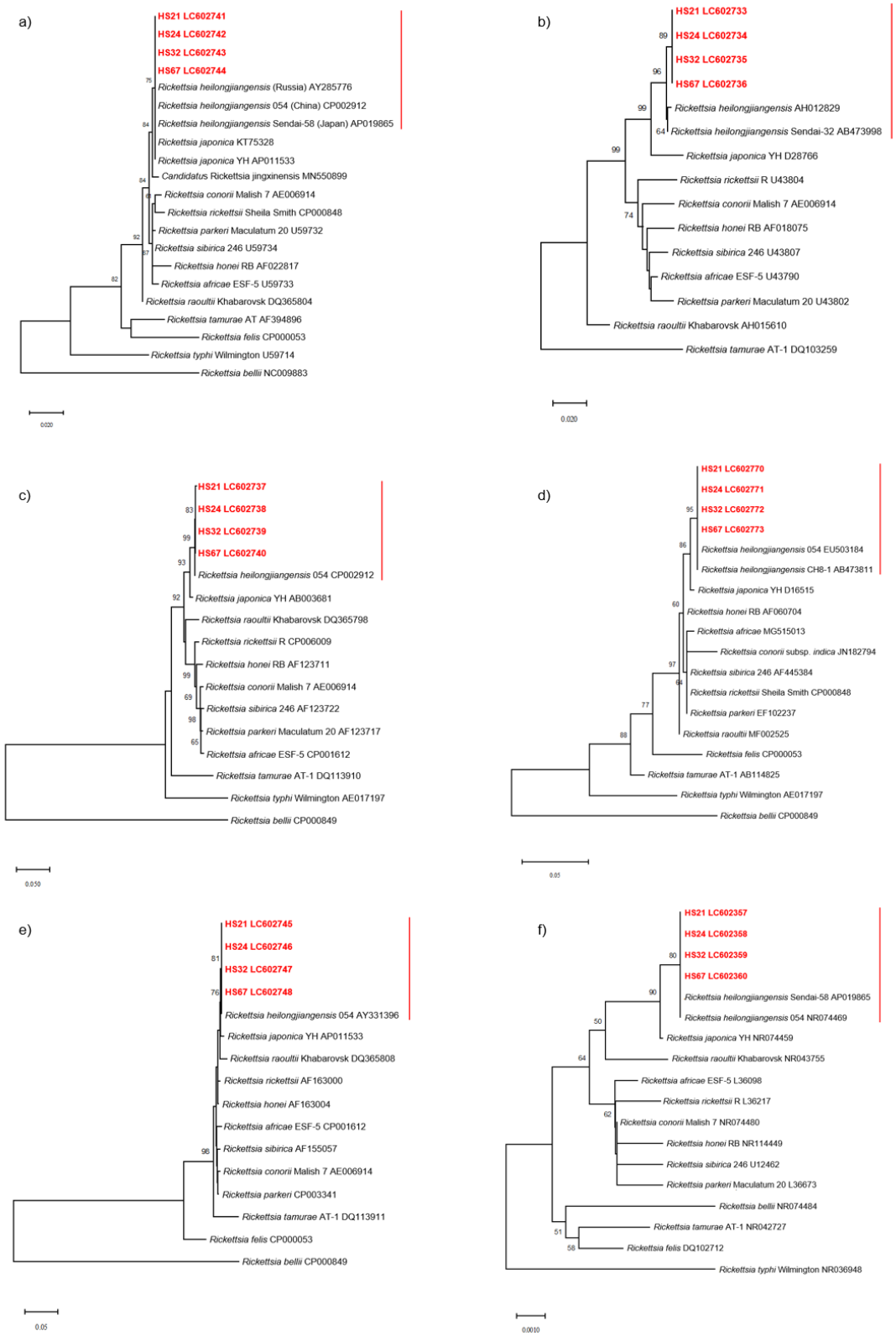
a)
HS21 LC602741
HS24 LC602742
HS32 LC602743
HS67 LC602744
Rickettsia heilongjiangensis (Russia) AY285776
Rickettsia heilongjiangensis 054 (China) CP002912
Rickettsia heilongjiangensis Sendai-58 (Japan) AP019865
Rickettsia japonica KT75328
Rickettsia japonica YH AP011533
Candidatus Rickettsia jingxinensis MN550899
Rickettsia conorii Malish 7 AE006914
Rickettsia rickettsii Sheila Smith CP000848
Rickettsia parkeri Maculatum 20 U59732
Rickettsia sibirica 246 U59734
Rickettsia honei RB AF022817
Rickettsia africae ESF-5 U59733
Rickettsia raoultii Khabarovsk DQ365804
Rickettsia tamurae AT AF394896
Rickettsia felis CP000053
Rickettsia typhi Wilmington U59714
Rickettsia bellii NC009883
0.020
b)
HS21 LC602733
HS24 LC602734
HS32 LC602735
HS67 LC602736
Rickettsia heilongjiangensis AH012829
Rickettsia heilongjiangensis Sendai-32 AB473998
Rickettsia japonica YH D28766
Rickettsia rickettsii R U43804
Rickettsia conorii Malish 7 AE006914
Rickettsia honei RB AF018075
Rickettsia sibirica 246 U43807
Rickettsia africae ESF-5 U43790
Rickettsia parkeri Maculatum 20 U43802
Rickettsia raoultii Khabarovsk AH015610
Rickettsia tamurae AT-1 DQ103259
0.020
c)
HS21 LC602737
HS24 LC602738
HS32 LC602739
HS67 LC602740
Rickettsia heilongjiangensis 054 CP002912
Rickettsia japonica YH AB003681
Rickettsia raoultii Khabarovsk DQ365798
Rickettsia rickettsii R CP006009
Rickettsia honei RB AF123711
Rickettsia conorii Malish 7 AE006914
Rickettsia sibirica 246 AF123722
Rickettsia parkeri Maculatum 20 AF123717
Rickettsia africae ESF-5 CP001612
Rickettsia tamurae AT-1 DQ113910
Rickettsia typhi Wilmington AE017197
Rickettsia bellii CP000849
0.050
d)
HS21 LC602770
HS24 LC602771
HS32 LC602772
HS67 LC602773
Rickettsia heilongjiangensis 054 EU503184
Rickettsia heilongjiangensis CH8-1 AB473811
Rickettsia japonica YH D16515
Rickettsia honei RB AF060704
Rickettsia africae MG515013
Rickettsia conorii subsp. indica JN182794
Rickettsia sibirica 246 AF445384
Rickettsia rickettsii Sheila Smith CP000848
Rickettsia parkeri EF102237
Rickettsia raoultii MF002525
Rickettsia felis CP000053
Rickettsia tamurae AT-1 AB114825
Rickettsia typhi Wilmington AE017197
Rickettsia bellii CP000849
0.05
e)
HS21 LC602745
HS24 LC602746
HS32 LC602747
HS67 LC602748
Rickettsia heilongjiangensis 054 AY331396
Rickettsia japonica YH AP011533
Rickettsia raoultii Khabarovsk DQ365808
Rickettsia rickettsii AF163000
Rickettsia honei AF163004
Rickettsia africae ESF-5 CP001612
Rickettsia sibirica AF155057
Rickettsia conorii Malish 7 AE006914
Rickettsia parkeri CP003341
Rickettsia tamurae AT-1 DQ113911
Rickettsia felis CP000053
Rickettsia bellii CP000849
0.05
f)
HS21 LC602357
HS24 LC602358
HS32 LC602359
HS67 LC602360
Rickettsia heilongjiangensis Sendai-58 AP019865
Rickettsia heilongjiangensis 054 NR074469
Rickettsia japonica YH NR074459
Rickettsia raoultii Khabarovsk NR043755
Rickettsia africae ESF-5 L36098
Rickettsia rickettsii R L36217
Rickettsia conorii Malish 7 NR074480
Rickettsia honei RB NR114449
Rickettsia sibirica 246 U12462
Rickettsia parkeri Maculatum 20 L36673
Rickettsia bellii NR074484
Rickettsia tamurae AT-1 NR042727
Rickettsia felis DQ102712
Rickettsia typhi Wilmington NR036948
0.0010

Figure 15. Phylogenetic trees for *Rickettsia heilongjiangensis* based on a) citrate synthase gene, *gltA*; b) outer membrane protein A gene, *ompA*; c) outer membrane protein B gene, *ompB*; d) 17-kDa common antigen gene, *htrA*; e) surface cell antigen 4, *sca4*; f) 16S ribosomal DNA. The phylogenetic analyses were done using neighbor-joining or maximum likelihood methods with the Kimura 2-parameter model and 1000 bootstrap replications. Samples from this study are highlighted in bold and red with accession numbers.

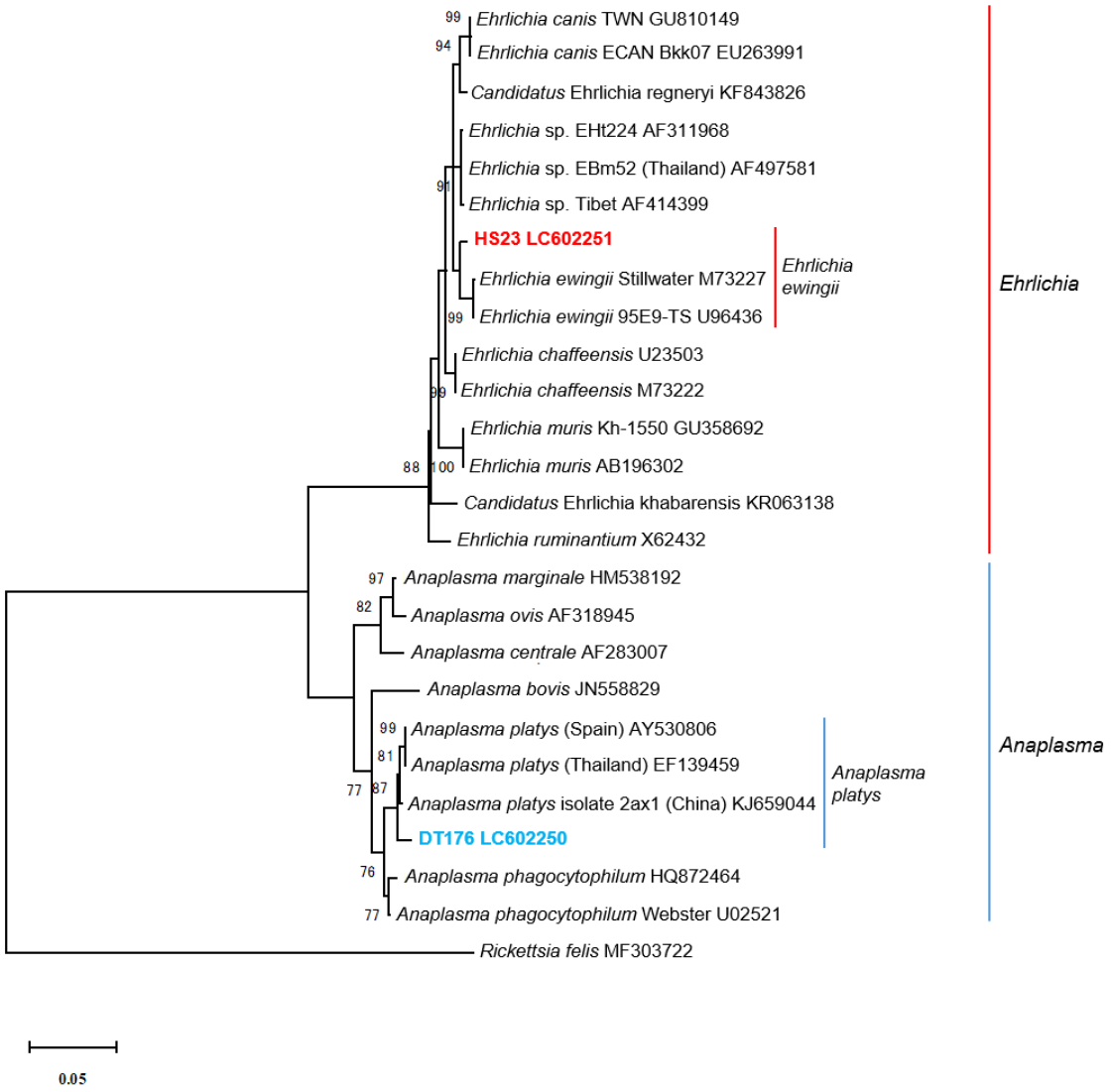

Figure 16. A maximum-likelihood phylogenetic tree based on 16S ribosomal DNA of *Anaplasma* and *Ehrlichia* was constructed with the Kimura 2-parameter model with 1000 bootstrap replications. Samples from this study are highlighted in bold and color with accession numbers.

Discussion

Microbiome investigation in ticks has become a new paradigm in the past decade due to the significance of tick-associated microorganisms for tick biological processes, and the interaction between the microorganisms (pathogenic and non-pathogenic) has been positively or negatively associated with the mechanisms of colonization and transmission of pathogens. In recent years, the tick microbiome study has been considered as a potential approach in controlling ticks and tick-borne diseases. Despite the importance, there has been no study reported from Sarawak, Malaysian Borneo up to now. Furthermore, studies on tick species and tick-borne diseases have also been very limited. This study compared microbial variations from six tick species of three genera: *Ixodes*, *Haemaphysalis*, and *Dermacentor* collected from primary forests and an oil palm plantation in Sarawak, Malaysian Borneo, and analyzed the ontogeny and sex variation from one tick species, *H. shimoga*. This study also managed to characterize human and animal pathogens from different tick species, and further, the changes in microbiota from the *Borrelia*-infected and non-infected tick *Ixodes* ticks were examined. This study is the first initiative to outline different tick microbiome profiles in addition to the pathogens screening from this region and included tick species that have never been studied previously.

In this study, multispecies comparison of ticks revealed a significant difference in microbial diversity and composition. From the alpha and beta diversity analyses, genus *Dermacentor* had higher microbial diversity, while *H. shimoga* had greater microbial composition differences than other tick species. A previous study conducted in Peninsular Malaysia did not find microbial diversity difference between *Dermacentor* (same *Dermacentor* species as in this study), *Amblyomma*, and *Haemaphysalis* (*H. hystricis*) species collected from wild boar [157]. The difference with this study could be related to blood meal feeding, as most of the *Dermacentor* ticks in this study were collected from vegetation. Some studies have reported the association between blood meal host and tick microbiome [94, 154, 293] and implied that host blood is generally low in bacterial richness [94]. While the blood meal speculation may be true for most tick species, including *I. granulatus*, in this study, it did not explain the lower microbial diversity in *H. shimoga* (Figure 8). The result was also consistent when only adult ticks were included in the analysis, which consisted of only *H. shimoga* collected from vegetation (Figure 8). However, a high relative abundance of endosymbiont has been found to obscure detection of DNA sequences of the rare bacterial community in *Ixodes* ticks [150], which could explain the lower microbial diversity of *H. shimoga* in this study. Downstream analysis revealed that *Coxiella* dominated in *H. shimoga* with as high as 80% relative abundance in adult female ticks. Furthermore, the microbial composition of *H. shimoga* showed the

greatest difference, probably due to the high relative abundant endosymbiont they harbored in the dominant.

Although there were variations in tick samples such as blood meal status, developmental stage, and sex, this study did not find a greater difference in alpha and beta diversity analyses. One of the reasons could be the majority of ticks were collected from vegetation, except for *I. granulatus* ticks were all engorged, and some tick species did not or had few nymph and larva samples. To overcome the variation that could be resulted from ontogenic difference, analysis was conducted with all ticks and only adult ticks. For instance, no significant difference was observed among adult *Dermacentor* species in microbial richness, whereas analysis with all tick samples showed a difference. In addition, adult *H. shimoga* ticks formed distinct clusters from other tick species in PCoA plots (Figure 9), showing that the adult *H. shimoga* ticks had significantly different microbial compositions. These findings supported that ontogenic variation could contribute to the difference in the bacterial community in ticks. Ontogenic variation in the tick microbiome has been reported in previous studies [47, 298].

Main endosymbionts were observed in some tick species, with multiple symbioses existing in *H. hystricis* and *D. steini*. Main endosymbionts have frequently been reported in ticks, and some of their functional roles have been determined. In this study, the order Rickettsiales were detected from all of the tick species. Primarily, Rickettsiales were found dominating in *I. granulatus*, co-symbiont with *Acinetobacter* in *H. hystricis*, and co-symbiont with *Acinetobacter* and *Francisella* in *D. steini* (Table 10). Rickettsiales group consists largely of pathogenic agents under genera *Anaplasma, Ehrlichia, Rickettsia*, and *Wolbachia* [244]. And this study also characterized *Anaplasma* sp., *Ehrlichia* sp., and *Rickettsia heilongjiangensis*. However, the rest of Rickettsiales strains remained unidentified. *Rickettsia* endosymbiont is commonly reported in arthropods and has also been reported in other tick species [149, 203]. In other arthropod species, they functioned as reproductive manipulators [69] and defensive mechanisms in other insects [167], but its functional role in ticks is currently unknown. With the high proportion of Rickettsiales detected in this study, it could be speculated that *Rickettsia* endosymbiont might be dominating in this tick species, which required further clarification. In addition, *Acinetobacter* was found in high proportion in *H. hystricis* and *D. steini*, which the bacteria has also been detected in other tick genera of *Ixodes*, *Amblyomma*, and *Dermacentor* [38, 148]. *Acinetobacter*, however, was not reported from *Haemaphysalis* species, including *H. hystricis*, in another study conducted in Malaysia [128]. The potential bacteria pathogen *Candidatus* Rhabdochlamydia was detected in high relative abundance in most of the tick species, including 2.57% in *D. steini* (Table 10). Although the species for *Candidatus* Rhabdochlamydia were not identified, it is important to know that most of the tick species in this study

were capable of harboring the bacteria. It warrants future investigation as tick-transmitted human cases have been previously reported [72].

Francisella has been reported from several tick species, and recently Duron et al. [68] demonstrated that this bacteria was maternally inherited and essential in hematophagous ticks for vitamin B synthesis. In this study, *Francisella*-LE was characterized from *D. steini* and *I. granulatus* but not from *D. compactus* and *D. atrosignatus*. Furthermore, there were no primary endosymbionts identified from *D. compactus* and *D. atrosignatus*. Although the sample size for these two species was small, most of the bacterial taxa detected had less than 1% relative abundance, with a few taxa exceptional, which also below 10%. The adult of these three *Dermacentor* species feed chiefly on wild boars (*Suidae*), and a wide range of mammalian hosts, including humans, as well as reptiles, have also been reported [102, 103, 282]. It is interesting that despite the similarity of the feeding hosts, the microbial composition was different with *D. steini*. A previous study suggested that ecological and physiological factors could play a role in shaping the tick microbiome and allow the tick to not require harboring a dominant endosymbiont to survive and develop [47]. The findings for *Dermacentor* also differed from the previous study in Malaysia [157] with their samples collected from wild boar. Therefore, investigating environmental and host association factors in these species would be of particular interest.

The main endosymbionts identified for *H. shimoga* showed ontogenic and sex variations, with *Coxiella* sp. dominating the adult ticks. A previous study has demonstrated that *Coxiella*-LE is essential for *Amblyomma* tick survival and reproduction [295]. The organism is also commonly reported from many other tick species [66, 67, 153]. In this study, *Coxiella*-LE (characterized by PCR) was the obligate endosymbiont for *H. shimoga*, with an overall relative abundance of 50% and was highest in female ticks 80% and 62% in male ticks. *Coxiella*-LE was reported to provide Vitamin B and co-factor that was essential for hematophagous arthropods like ticks [85, 254], so its presence in adult ticks and nymphs (25.8%) may be essential for feeding and reproduction for female ticks. However, the presence of *Coxiella*-LE was only 8.09% in larva samples, contrary to previous reports as a maternally inherited organism in *Amblyomma*, *Rhipicephalus*, and *Ornithodoros* [5, 89, 136, 169]. A recent study by Ben-Yosef et al. [24] has demonstrated that *Coxiella*-LE is essential for ontogeny development, fitness, fertility, and fecundity in *Rhipicephalus sanguineus*, but also pointed out that it may not be mandatory for oocyte development and hatching. It is yet to determine for *H. shimoga* if the other bacteria taxa have replaced the *Coxiella*-LE functional role in the larva. However, Rickettsiales and *Candidatus* Rhabdochlamydia were the main endosymbionts reported from the larva samples. In addition, *H. shimoga* ticks could also obtain *Coxiella*-LE through feeding, as previous studies have detected it from the salivary gland of some tick species [136, 169, 227]. Furthermore, tick-transmitted *Coxiella*-LE was responsible for mild

infectious cases in humans [13], which means the organism could be transmitted via tick bites. It is noteworthy that human was documented host for *H. shimoga* ticks, and in this study, this species was collected exclusively from oil palm plantation. Finally, while *Coxiella*-LE was the single primary endosymbiont in adult male and female ticks, *Coxiella*-LE and Rickettsiales were dual symbioses in nymph samples. Dual symbiosis also occurred in larva samples. Overall, it was evident that ontogeny affected the microbial structures in *H. shimoga*, and different bacterial taxonomies were seen to dominate in different life stages, with *Coxiella*-LE present mainly in adult ticks.

In this study, a high proportion of *Borrelia* was detected in *I. granulatus*, and the presence of *Borrelia* has a significant effect on ticking microbial composition. A high proportion of *Borrelia* was reported in *I. scapularis*, in which the researcher found microbial composition differences between *Borrelia*-positive males and females collected from two regions [272]. In their study, the geography factor was significantly related to pathogen detection. Consistently, in this study, the majority of the *Borrelia*-positive *I. granulatus* ticks were collected from the oil palm plantation (Chapter II). While environmental factors could have contributed to the high relative abundance of *Borrelia* in the ticks, other factors such as sex and blood-feeding could not be examined in this study, as all *I. granulatus* samples were engorged females. Other than *Borrelia*, several other bacterial species such as *Anaplasma* sp., *Ehrlichia* sp., *Bartonella* spp., *Rickettsia heilongjiangensis*, and *Coxiella*- and *Francisella*-LE were also identified from different tick species (Tables 14 and 15). However, due to the low number of samples and detection rate, correlation analysis was not conducted. Nevertheless, given that there has been human serological evidence for *Anaplasma*, *Ehrlichia*, and rickettsial reported previously from Malaysia [126, 138], the identification of pathogenic and potentially pathogenic bacteria species in this study warrants more investigation. Furthermore, the positive tick species such as *H. shimoga* and *Dermacentor* species have a reported wide range of feeding hosts, including humans. It is therefore important to understand the actual prevalence and transmission mechanism. Besides, *Coxiella*-LE and *Francisella*-LE were closely related to their pathogenic type, and shifting between pathogenic and non-pathogenic forms could occur [31, 66].

All in all, this is the first multispecies microbial comparisons in ticks collected from Sarawak, Malaysian Borneo, with the identification of human and animal pathogens. Microbial variations were found to be significant between tick species. Factors contributing to the variations included the development stage, and potentially blood meal feeding may have played a role in shaping the microbiome profile. Further investigation with *H. shimoga* revealed the ontogenic and sex variations affecting microbial composition, with some bacterial taxa found more represented in one developmental stage than another. However, comparing the feeding status of *H. shimoga* did not show conclusive results, probably because questing ticks consisted mainly of the adult ticks, and there were only nymph and larva samples

in the feeding category. Most tick species in this study harbored one or multiple endosymbionts, except for *D. compactus* and *D. atrosignatus*. It could be speculated that other factors such as ecological variation such as habitat and host, could probably have a greater effect on *D. compactus* and *D. atrosignatus*, enabling them to survive and develop without main endosymbiont, which requires further study. Finally, it was certain that microbial structure could be affected by the presence of specific bacteria taxa in high abundance, evidenced by *Borrelia*-positive and negative *I. granulatus*. The findings of current study provide important insights into the tick microbiome difference and the presence of pathogenic and potentially pathogenic bacteria circulating in ticks from primary forests and an oil palm plantation in Sarawak. More studies are required to unravel the factors associated with the variations we observed in this study.

Summary

Ticks harbor a high abundance of symbiotic and commensal microorganisms that can be obligate or facultative presence. The obligate endosymbionts are maternally inherited microorganisms essential for tick survival and development. Facultative endosymbionts, on the contrary, may be involved in the manipulation of the tick immune system and reproduction. Furthermore, understanding the interaction between non-pathogenic microorganisms and pathogens in ticks may be fundamental for the control measures. There has been few studies on ticks and tick-borne pathogens, and no tick microbiome study published up-to-date from Sarawak, Borneo. Thus, this study aimed to perform the microbiome and pathogen screening of each tick species collected from two primary forests and an oil palm plantation by employing Next-generation sequencing (NGS). A total of 210 feeding and questing ticks consisting of different life stage and status of *Ixodes granulatus* (n = 32), *Haemaphysalis hystricis* (n = 36*), H. shimoga* (n = 110), *Dermacentor compactus* (n = 4), *D. steini* (n = 24), and *D. atrosignatus* (n = 4) were included for NGS. The 16S rRNA gene V3-V4 hypervariable regions were targeted, and sequencing was conducted on an Illumina MiSeq platform. The following bacteria: *Anaplasma, Ehrlichia, Bartonella, Coxiella, Francisella,* and *Rickettsia,* were verified with conventional PCR and sequencing. Data analyses were performed in Quantitative Insights in Microbial Ecology 2 (Qiime 2), Decontam package and vegan package in R software, and Huttenhower lab Galaxy pipeline. Tick samples were categorized into three groups for microbial analysis. The first group included all ticks from their available life stages. The second group consisted of only the adult stage ticks because not all tick species collected had all life stages represented (*H. hystricis* excluded). Finally, since *H. shimoga* had the most comprehensive samples, the effect of different developmental stages and feeding statuses for this species were examined. The study findings revealed that microbial variations were significant between tick species. From the multispecies comparison, genus *Dermacentor* had the highest microbial diversity, while *H. shimoga* had greater microbial composition differences than other tick species. Factors contributing to the variations included the development stage, and potentially blood meal feeding may have played a role in shaping the microbiome profile. Further investigation with *H. shimoga* revealed the ontogenic and sex variations affecting microbial composition, with some bacterial taxa found more represented in one developmental stage than another. Most tick species in this study harbored one or multiple endosymbionts, except for *D. compactus* and *D. atrosignatus*. Finally, the microbial structure could be affected by the presence of specific bacteria taxa in high abundance, evidenced by *Borrelia*-positive and negative *I. granulatus*. This study also managed to characterize human and animal pathogens from genera *Coxiella*, *Francisella*, *Rickettsia*, *Anaplasma*, *Ehrlichia*, and *Bartonella*. Amongst, *Coxiella*-like endosymbiont from *H. shimoga*

and *Francisella*-like endosymbionts from *D. steini* and *I. granulatus* were characterized. Furthermore, human pathogens such as *Rickettsia heilongjiangensis* and *Ehrlichia* sp. closely related to *E. ewingii* were also identified. This study is the first initiative to outline different tick microbiome profiles in addition to the pathogens screening from this region and included tick species that have never been studied previously. More works are required to unravel the factors associated with the variations we observed in this study.

Chapter IV

Molecular survey of tick-borne bacterial pathogens in rodents collected in Sarawak

Introduction

Worldwide, TBDs are a persistent growing problem at the One Health interface. The surveillance and control of TBDs are also challenging issues, as well as for the detection and diagnosis of many tick-borne infections in humans and animals due to multi-tiered approaches needed to confirm pathogens. A wide range of TBDs caused by the bacteria are constantly being reported worldwide. Some of them cause the most prevalent diseases, such as Lyme disease (e.g. *Borrelia afzelii*), human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), tularemia (*Francisella tularensis*), and spotted fever (e.g. *Rickettsia ricketsii*). Thus far, the role of rodents has been highlighted as important reservoir hosts in maintaining and circulating pathogens.

Furthermore, rodents are the typical blood meal feeding hosts for tick larvae and nymphs, and some adult ticks of some species. Importantly, pathogens can be acquired and transmitted during blood meal feeding. Therefore, investigating TBPs in rodents is essential to understanding the transmission dynamics of the pathogen of interest. In Malaysia, the seroprevalence rickettsiosis, anaplasmosis, and ehrlichiosis in humans have been reported [126, 132, 138, 266–268]. Potential vectors of these bacteria have also been investigated, which involved collecting feeding and questing ticks at and adjacent to study areas. Nonetheless, most of these studies have been limited to specific populations and regions that only included indigenous communities and farm workers residing in Peninsular Malaysia. Moreover, the actual bacterial strains causing the infections were mostly unknown, as well as the vector and reservoir of the pathogens were not well elucidated in these studies.

In this study, the target was to screen the rodents collected from primary forests and an oil palm plantation in Sarawak, Borneo, for the tick-borne bacterial pathogens from the following genera: *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Francisella*, and *Rickettsia*.

Materials and Methods

Rodents were captured from three sampling sites (GGNP, KNP, and OP plantation) (Figure 2), and DNA was extracted from the spleen samples with the method as described in the Materials and Methods of Chapter II. Polymerase chain reactions (PCRs) were conducted for the detection of *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Francisella*, and *Rickettsia*. The details of primers and PCR types used for each bacteria identification are described in Table 16.

Real-time PCR for *Bartonella* spp. screening

Real-time PCR was conducted for the screening of *Bartonella* infection by using the THUNDERBIRD® Probe qPCR Mix (TOYOBO, Osaka, Japan) and primers (*ssrA*-F, 5′-GCTATGGTAATAAATGGACAATGAAATAA-3′; *ssrA*-R, 5′-GCTTCTGTTGCCAGGTG-3′; *ssrA*-P, 5′-6FAM-ACCCCGCTTAAACCTGCGACG-TAMRA-3′), which were designed to amplify a 300-bp fragment of the transfer messenger RNA, *ssrA* gene of *Bartonella* [57]. Real-time PCR was performed using a LightCycler 96 (Roche Diagnostics GmbH, Mannheim, Germany). The DNA of *Candidatus* bartonella rousetti isolated by Qiu et al. [229] and molecular grade water were used as positive and negative controls, respectively.

Conventional PCR amplification, sequencing, and phylogenetic analysis

All PCRs were conducted using *Ex Taq* Hot Start Version in a reaction mixture of 20 µL. The conditions used in the PCR assays were as follows: 40 cycles of denaturation at 94°C for 30 sec, annealing temperature according to each respective primer set for 30 sec, and extension at 72°C for 30 sec, 60 sec or 90 sec depending on the targeted amplicon size. Single PCR was performed for most of the target genes (Table 16). For *Coxiella*, nested and semi-nested PCRs were conducted for the amplification of the chaperone protein (*groEL*) and β subunit of bacterial RNA polymerase gene (*rpoB*) and 16S rDNA, respectively. Nested PCRs were also conducted for Anaplasmatacea for the *groEL* gene and 16S rDNA. Similarly, the *Bartonella*-positive samples from real-time PCR were included in conventional PCRs targeting both *ssrA* and citrate synthase gene (*gltA*) for species characterization.

Finally, all the PCR products were verified with electrophoresis, followed by the Sanger sequencing. The resulting sequences were assembled and trimmed using the ATGC software version 9.0.0 and compared with the sequences available in the public databases using the BLASTn. Phylogenetic analyses for each identified bacteria were constructed using MEGA version X [147].

Table 16. Primers used in this study.

Organism	Primer name	Sequence (5' to 3')	Target gene (PCR type)	Annealing temperature (ºC)	Amplicon size (bp)	Reference
Anaplasmatacea	EHRCS-131F	CAGGATTTATGTCTACTGCTGCTTG	citrate synthase gene, *gltA*	50	748	[160]
	EHRCS-879R	TIGCKCCACCATGAGCTG	EHRCS-131F and EHRCS-879R (Single PCR 1)			
	EHRCS-754F	ATGCTGATCATGARCAAAATG	EHRCS-754F and EHRCS-1226R (Single PCR 2)	50	473	
	EHRCS-1226R	CCAGTATATAAYTGACGWGGACG				
	EHR16SD	GGTACCYACAGAAGAAGTCC	16S ribosomal DNA (Single PCR)	55	345	[215]
	EHR16SR	TAGCACTCATCGTTTACAGC				
	EC9	TACCTTGTTACGACTT	16S ribosomal DNA (1st PCR)	48	1,400	[7]
	EC12A	TGATCCTGGCTCAGAACGAACG				[211]
	A17a	GCGGCAAGCCTCCCACAT	(Nested PCR)	54	1,300	[121]
	IS58-1345R	CACCAGCTTCGAGTTAAACC				
	HS1a	AITGGGCTGGTAITGAAAT	chaperone protein GROEL, *gro*EL (1st PCR)	54		[258]
	HS6a	CCICCIGGIACIAIACCTTC				
Anaplasma	HS3-F	ATAGTYATGAAGGAGAGTGAT	(nested PCR)	50	1,256	[159]
	HSV-R	TCAACAGCAGCTCTAGTWG				
Ehrlichia	groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	(nested PCR)	50	1,100	[83]
	groeEL_rev2	GCCGACTTTTAGTACAGCAA				
Bartonella	CS443f	GCTATGTCTGCATTCTATCA	citrate synthase gene, *gltA*			[26]
	CS1210r	GATCYTCAATCATTTCTTTCCA	CS443f and CS1210r (1st PCR)	48	767	[25]
	BhCS1137.n	AATGCAAAAAGAACAGTAAACA	CS443f and BhCS1137.n (Semi-nested PCR)	48	694	[206]
	ssrA-F	GCTATGGTAATAAATGGACAATGAAATAA	tmRNA *ssr*A (Real-time PCR & Single PCR)	60	300	[57]
	ssrA-R	GCTTCTGTTGCCAGGTG				
	ssrA-P	6FAM-ACCCCGCTTAAACCTGCGACG-TAMRA	Real-time PCR			
Coxiella	CoxGrF1	TTTGAAAAYATGGGCGCKCAAATGGT	chaperone protein GROEL, *gro*EL (1st PCR)	56	655	
	CoxGrR2	CGRTCRCCAAARCCAGGTGC				
	CoxGrF2	GAAGTGGCTTCGCRTACWTCAGACG	(Nested PCR)	56	619	
	CoxGrR1	CCAAARCCAGGTGCTTTYAC				

	CoxrpoBF2	GGGCGNCAYGGWAAYAAAGGSGT	β subunit of bacterial RNA polymerase gene, *rpo*B (1st PCR)	56	607-610	
	CoxrpoBR1	CACCRAAHCGTTGACCRCCAAATTG				
	CoxrpoBF3	TCGAAGAYATGCCYTATTTAGAAG	(Nested PCR)	56	539-542	
	CoxrpoBR3	AGCTTTMCCACCSARGGGTTGCTG				
	Cox16SF1	CGTAGGAATCTACCTTRTAGWGG	16S ribosomal DNA	52-56		
	Cox16S_07F	AGAGTTTGATYMTGGCTCAG	Cox16SF1 and Cox16SR2 (1st PCR)		1,321-1,429	
	Cox16SR2	GCCTACCCGCTTCTGGTACAATT	Cox16S_07F and Cox16SR2 (1st PCR)		1,434-1,542	
	Cox16SR1	ACTYYCCAACAACAGCTAGTTCTCA	Cox16S_07F and Cox16SR1 (Semi-nested PCR)		832-939	
Francisella	FT393	ATGGCGAGTGATACTGCTTG	membrane protein/T-cell epitope, TUL4 (Single PCR)	53	248	[163]
	FT642	GCATCATCAGAGCCACCTAA				
	MS1	CAGCTACTACACAAAGCAGTGG	Outer membrane protein, *fop*A (Single PCR)	58	707	[99]
	MAI	CACCATTTACTGTATAGCACGC				
Rickettsia	gltA_Fc	CGAACTTACCGCTATTAGAATG	citrate synthase gene, *glt*A (Single PCR)	55	580	[77]
	gltA_Rc	CTTTAAGAGCGATAGCTTCAAG				

Results

Overall, 55 rodent spleens were included in the screening, and among the targeted bacteria, samples were positive for four bacteria genera, *Anaplasma*, *Ehrlichia*, *Bartonella*, and *Coxiella*. None of the rodent samples were found positive for *Francisella* and *Rickettsia* spp. despite the attempts with different primer pairs. For example, T-cell epitope, *tul4* and outer membrane protein, *fopA*, both genes were used for *Francisella* screening (Table 16). In addition, coinfections were detected in two rodent samples. *Anaplasma bovis* and *Bartonella rattimassiliensis* were detected in Sample ID: WM002, and *B. phoceensis* and *Coxiella burnetii* were detected in Sample ID: WM003.

Anaplasma and *Ehrlichia*

Screening of all rodent samples was done with 16S rDNA that amplified 345-bp fragment, revealing four *Anaplasma*-positive (Sample IDs: WM002, WM006, WM065, and WM081) and one *Ehrlichia*-positive (Sample ID: WM082) samples from OP plantation, all of which belonged to *Rattus* spp. Sequences of all *Anaplasma*-positive samples were identical and blasted to *A. bovis* detected in *Amblyomma triguttatum* in Australia (KY425420), with 100% identity (305/305 bp) (Table 17). *Ehrlichia* sp. detected in this study was 100% (305/305 bp) identical with *E. chafeensis* strain Arkansas (NR_074500) (Table 17). Positive samples from the screening were included for the amplification of a longer fragment (1,300-bp) using different primers pairs of 16S rDNA, in which one *Anaplasma*- (Sample ID: WM002) and the *Ehrlichia*-positive (Sample ID: WM082) samples were amplified. Sequence analysis for *Anaplasma* showed 99.7% identity (1,323/1,327 bp) with uncultured *Anaplasma* sp. detected from the liver and spleen samples of rodent, *Niviventer confucianus* in China (JN862824) (Table 17). Additionally, the phylogenetic inference revealed that the detected *Anaplasma* sp. in this study clustered with sequences of *A. bovis* (Figure 17). *Ehrlichia* sp. in this study showed high identity, 99.8% (1,329/1,331 bp) with *Ehrlichia* sp. NS101 detected in deer in Japan (AB454074) (Table 17) and clustered with sequences of *E. chaffeensis* in the phylogenetic tree (Figure 17). Unfortunately, PCRs targeting both *gltA* and *groEL* genes that would be helpful for species characterization were not amplified, probably due to low DNA templates or the suitability of primers.

Bartonella

Real-time PCR with the *ssrA* gene revealed 24 positive samples, including one sample from primary forest, GGNP, and 23 samples from OP plantation. All positive samples belonged to *Rattus* spp., except for two *Sundamys muelleri* from GGNP (Sample ID: GGNP01) and OP plantation (Sample ID:

WM026). For species characterization, all positive samples from real-time PCR were included in conventional PCRs with *ssrA* and *gltA* genes. As a result, eight samples (Sample IDs: WM002, WM003, WM004, WM012, WM026, WM028, WM033, and WM049) from the OP plantation were successfully amplified and sequenced for the *ssrA* gene. No samples were amplified by the *gltA* gene. With further sequence analysis, five samples (Sample IDs: WM002, WM004, WM012, WM028, and WM049) were identified as *Bartonella rattimassiliensis*, with three samples (Sample IDs: WM002, WM004, and WM049) having identical sequences and showed 100% identity (253/253 bp) with *B. rattimassiliensis* strain R1023 detected in rodent in Thailand (KT355804). Meanwhile, another two samples, sample IDs: WM012 and WM028, showed 99.2% (251/253 bp) and 98.4% identity (249/253 bp), respectively. One sample (Sample ID: WM003) had 100% identity (251/251 bp) with *B. phoceensis* strain GDHL62 identified in rodent in China (MF765679). Finally, two samples (Sample IDs: WM026 and WM081) showed 100% identity (252/252 bp) with *Bartonella* sp. strain GDLD09 detected from a rodent in China (MF765616). The results of sequence analysis for *Bartonella* are shown in Table 17. Inferring the genetic relatedness of the *Bartonella* spp. from this study with the phylogenetic tree revealed the identification of three species of *Bartonella*, *B. rattimassiliensis*, *B. phoceensis*, and lastly, *B. coopersplainsensis* (Figure 18), supported the findings in sequence analysis.

Coxiella

Two samples (Sample IDs: WM003 and WM090) were detected for *Coxiella* by using the *groEL* gene. Both positive samples were from *Rattus* spp. from OP plantation. Further attempts for species characterization with the *rpoB* gene and 16S rDNA were not successful. Based on the sequence analysis of the *groEL* gene, the *Coxiella* sp. detected in this study had 99.8% identity (559/560 bp) with *C. burnetii* (CP014563) (Table 17). From the phylogenetic tree, samples in this study were clustered with the other reported *C. burnetii* samples and *Coxiella* sp. of soft ticks in the Clade A (Figure 19).

Table 17. Sequence analysis for successfully sequenced genes of Anaplasmatacea, *Bartonella*, and *Coxiella*. Samples with identical sequences are listed together.

Organism	**Gene**	**Sample ID**	**BLASTn**	**Identity**	**Accession no.**
Anaplasmatacea	**16S rDNA**	WM002; WM006; WM065; WM081	*Anaplasma bovis* isolate Y11 from *Amblyomma triguttatum* (Australia)	100% (305/305 bp)	KY425420
		WM082	*Ehrlichia chaffeensis* strain Arkansas	100% (305/305 bp)	NR_074500
		WM002	Uncultured *Anaplasma* sp. clone ZJ05/2009 from *Niviventer confucianus* (China)	99.7% (1,323/1,327 bp)	JN862824
		WM082	*Ehrlichia* sp. NS101 in deer (Japan)	99.8% (1,329/1,331 bp)	AB454074
Bartonella	***ssrA***	WM002; WM004; WM049	*Bartonella rattimassiliensis* strain R1023 from rodent (Thailand)	100% (253/253 bp)	KT355804
		WM012		99.2% (251/253 bp)	
		WM028		98.4% (249/253 bp)	
		WM003	*Bartonella phoceensis* strain GDHL62 from rodent (China)	100% (251/251 bp)	MF765679
		WM026; WM033	*Bartonella* sp. strain GDLD09 from rodent (China)	100% (252/252 bp)	MF765616
Coxiella	***groEL***	WM003; WM090	*Coxiella burnetii* strain Schperling	99.8% (559/560 bp)	CP014563

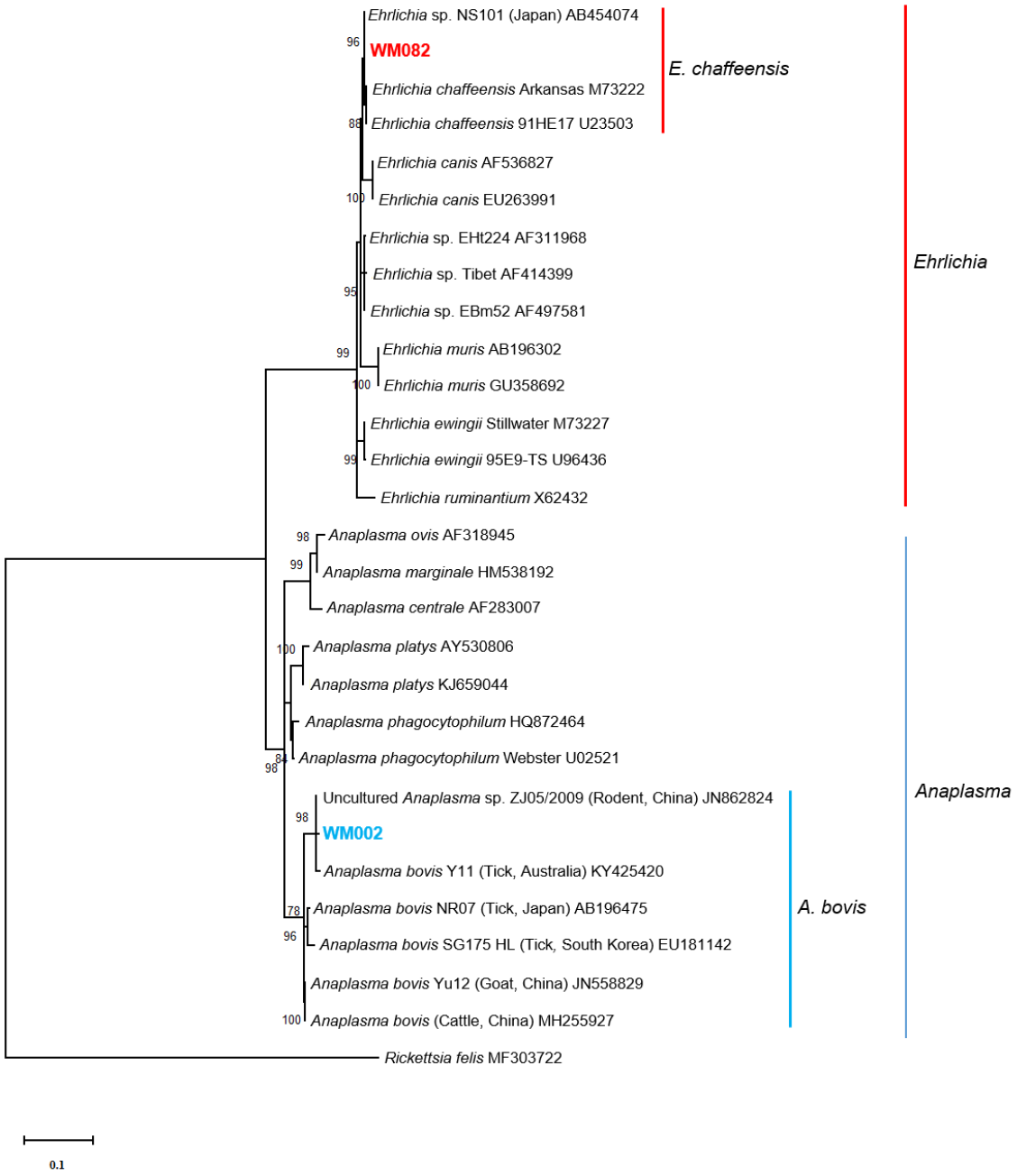

Figure 17. A maximum-likelihood phylogenetic tree based on 1300-bp of 16S ribosomal DNA of *Anaplasma* and *Ehrlichia* was constructed using the Hasegawa-Kishino-Yano model with 1000 bootstrap replications. Samples from this study were highlighted in bold and color.

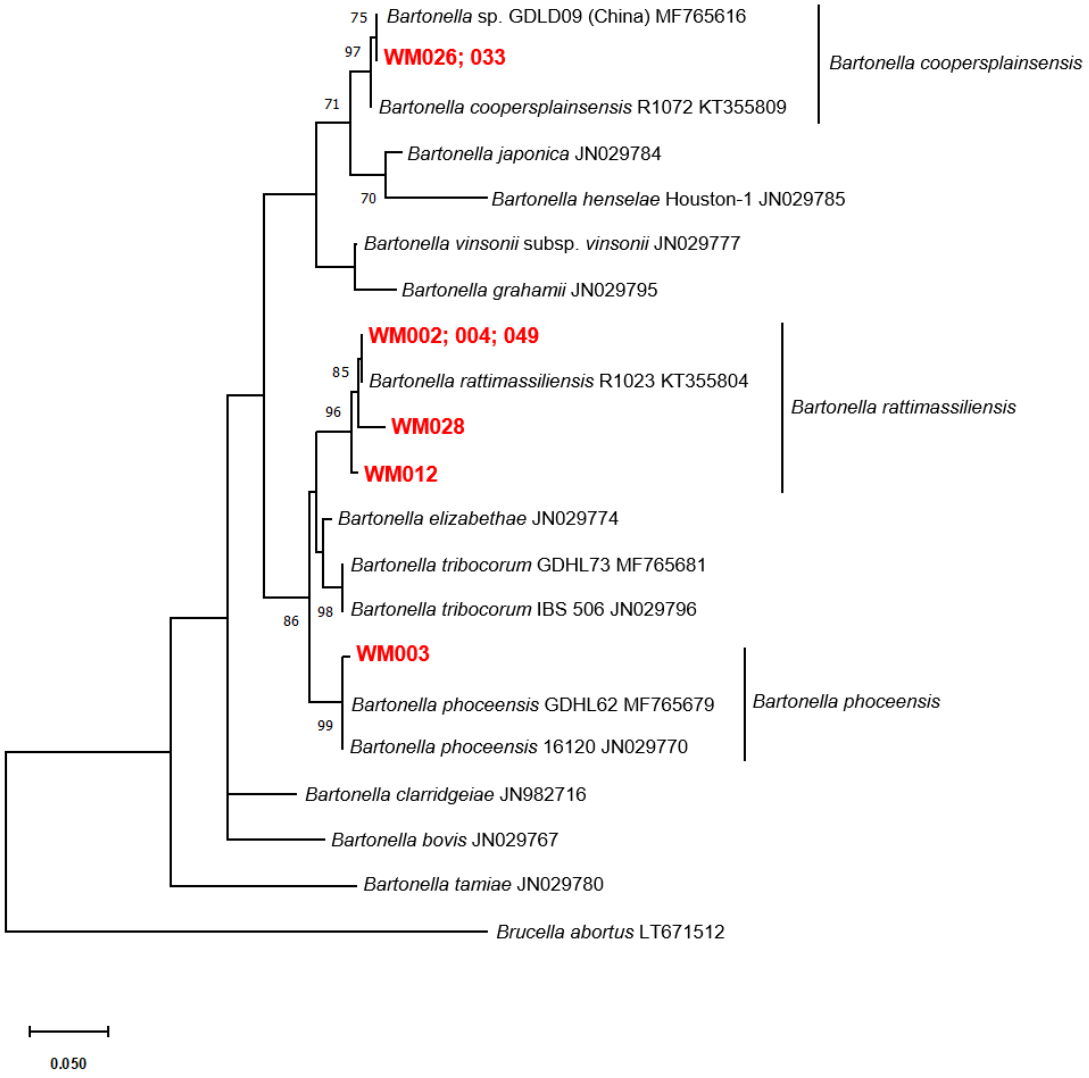

Figure 18. Phylogenetic tree based on transfer messenger RNA, *ssrA* gene of *Bartonella* was constructed with maximum likelihood method and Kimura 2-parameter model with 1000 bootstrap replications. Samples from this study were highlighted in red and bold. Samples with identical sequences are listed in parallel with semicolons.

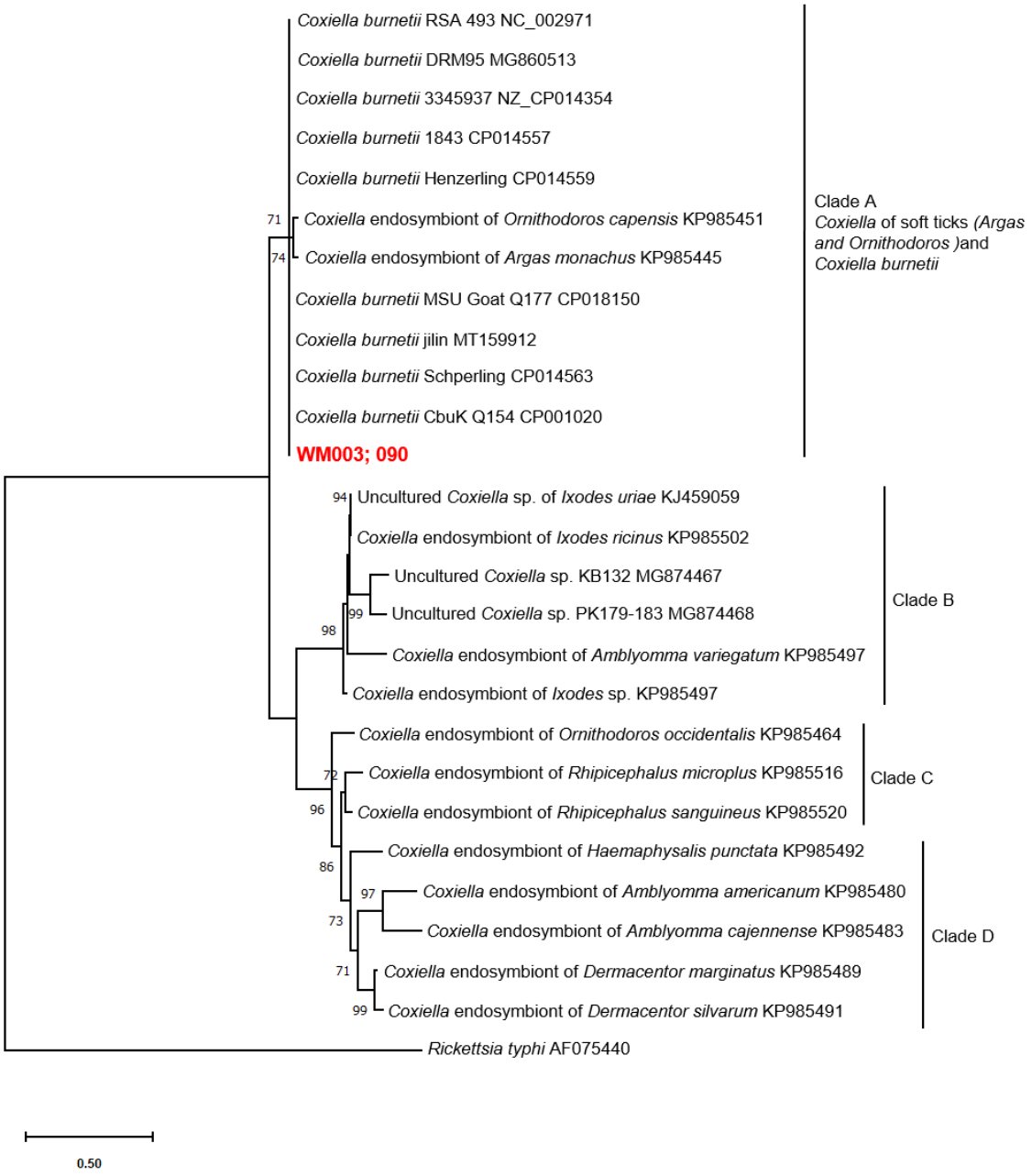

Figure 19. Phylogenetic tree based on the chaperone protein, *groEL* gene of *Coxiella* was constructed with maximum likelihood method and Tamura 3-parameter model and 1000 bootstrap replications. Clade designation is based on the study by Duron et al. [63]. Samples in this study, highlighted in red and bold, are clustered in Clade A with *Coxiella* sp. detected in soft ticks and *Coxiella burnetii*. Both samples with identical sequences and are listed in parallel with semicolons.

Discussion

Rodents are well adapted to living in a wide variety of habitats, especially in close proximity with humans, and thus, they play an important role in public health. Additionally, rodents constantly interact with hematophagous arthropods like ticks, flea, mites, and lice as their blood meal host, and as a result, rodents act as reservoirs for numerous vector-borne pathogens. In this study, 55 rodent spleen samples from two primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak were screened for tick-borne bacteria from six genera of *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Francisella*, and *Rickettsia*. From this molecular survey, *Anaplasma bovis* and *Ehrlichia chaffeensis* were identified based on 16S rDNA, three *Bartonella* spp., namely *B. rattimassiliensis*, *B. phoceensis*, and *B. coopersplainsensis* were detected with *ssrA* gene, and *Coxiella sp.* located in the same clade with *C. burnetii* and *Coxiella* spp. of soft ticks was detected by using the *groEL* gene. None of the samples were positive for *Francisella* and *Rickettsia*. This study provides the first evidence of *E. chaffeensis*, a causative agent of human monocytic ehrlichiosis, and potentially *C. burnetii*, a causative agent for Q fever in humans in Malaysia. Furthermore, all positive samples belonged to rodent species collected from the oil palm plantation, *Rattus* spp., except for one *Bartonella*-positive sample from *Sundamys muelleri* (Sample ID: WM026).

Ehrlichia chaffeensis is the first *Ehrlichia* sp. reported to be zoonotic [7] and has been largely investigated in ticks worldwide [42, 133, 211, 273], and mammal hosts such as deer, human, dog, coyote, and fox [7, 52, 122, 137], but has rarely been reported from rodents [63]. In this study, *Ehrlichia* sp. detected from *Rattus* sp. that was closely related to *Ehrlichia* sp. NS01 detected in the deer in Japan, with 99.8% identity (1,329/1,331 bp), which was an *E. chaffeensis* [122]. Furthermore, *A. bovis* was identified in four *Rattus* spp. from the oil palm plantation. *A. bovis* was first described in cattle in 1936 by Donatien and Lestoquard [62]. Since then, it has been reported from diverse host species worldwide, including ruminants, dogs, raccoons, and cats, has also been documented [19]. Infection in ruminants is typically characterized by fever, drop in milk production, weakness, weight loss, and so on, and death can occur in stressed or naïve animals [164, 256]. *A. bovis* has been detected in goats, deer, buffaloes, and monitor lizard in a study conducted in Malaysia [139]. Serological detection of *E. chaffeensis* and *A. phagocytophilum* in humans has been reported previously in Peninsular Malaysia; however, the actual causative agents were not determined [138]. Although the overall sample size was small and attempts to characterize the species with other target genes were not successful in this study, it is still noteworthy that the pathogens, *E. chaffeensis* and *A. bovis* are potentially circulating in rodents in Borneo Island. These findings will require further investigation to determine the potential role of rodents in causing the diseases.

Rodents are the prominent host for *Bartonella* spp., and in this study, three species were successfully characterized using the *ssrA* gene. *Bartonella rattimassiliensis*, *B. phoceensis*, and *B. coopersplainsensis* are among the *Bartonella* spp. that have been reported from *Rattus* spp., and the same species have also been reported by Tay et al. [269] in the Peninsula and Blasdell, Perera, and Firth [30] in Sarawak, Malaysia. *Bartonella* spp. detected in this study are not the zoonotic species, but Blasdell, Perera, and Firth [30] had documented *B. elizabethae* in their study, in which human is the accidental host [35] for the infection.

In Malaysia, the presence of *C. burnetii* has been reported from two tick samples of *D. steini* and *H. hystricis* collected from wildlife and livestock [129]. However, the disease prevalence in hosts, including small mammals, has not been investigated. The role of small mammals, particularly rodents of the *Rattus* genus, has recently been elucidated as important reservoirs for *C. burnetii* [1, 84, 158, 192, 235]. For instance, the Norway rats (*R. norvegicus*) and black rats (*R. rattus*) were implicated in the reintroduction of *C. burnetii* into endemic areas of the Netherlands [235]. In this study, *C. burnetii* was detected in two *Rattus* spp. (2/55) 3.6% from the oil palm plantation. A previous study reported the prevalence of *C. burnetii* from Norway rats and black rats was 3.6% (2/55) and 15% (3/20), respectively, by using the fecal samples [1]. Other studies reported a total prevalence of *C. burnetii* ranging from 9.7% using the spleen sample [84] to as high as 17.3% in the seroprevalence study [192], with observed variations in different rodent species. Thus, it is crucial to investigate the rodents in order to determine their role and what species could be relevant hosts in *C. burnetii* maintenance in Sarawak.

Little is known about the occurrence of tick-borne bacterial pathogens and the role of rodents for the described pathogens in this study. This study had a small sample size, which might have resulted in negative detection of *Francisella* and *Rickettsia* spp. Nevertheless, this study provides significant evidence that the rodent species harbored multiple tick-borne bacteria, in which *E. chaffeensis* and potentially *C. burnetii* pose zoonotic threats to public health. Future investigation with a larger sample size will be required to elucidate the rodent role and prevalence of the detected pathogens.

Summary

Worldwide, tick-borne diseases are a persistent growing problem at the One Health interface. The role of rodents has been highlighted as important reservoir hosts in maintaining and circulating tick-borne pathogens. In this study, a molecular survey was conducted on the rodents collected from two primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak, Borneo, for the tick-borne bacteria from the following genera, *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Francisella*, and *Rickettsia*. Overall, 55 rodent spleens were screened with PCRs. As a result, *Anaplasma* sp. closely related to *A. bovis* and *Ehrlichia chaffeensis* were detected based on 16S ribosomal DNA, three *Bartonella* spp., namely *B. rattimassiliensis*, *B. phoceensis*, and *B. coopersplainsensis*, were confirmed with transfer messenger RNA, *ssrA* gene, and *Coxiella sp.* closely related to *C. burnetii* was detected by using the chaperone protein, *groEL* gene. None of the rodent samples were positive for *Francisella* and *Rickettsia* spp. Nevertheless, this provides the first evidence of *E. chaffeensis*, a causative agent of human monocytic ehrlichiosis in Malaysia. *Coxiella* sp. located in the clade of *Coxiella burnetii*, a causative agent for Q fever in humans, was also identified in two rodent samples. Furthermore, all positive samples belonged to rodent species collected from the oil palm plantation, *Rattus* spp., except for one *Bartonella*-positive sample identified in *Sundamys muelleri*. Despite the small sample size limitation, this is significant evidence that the rodent species in Sarawak harbored multiple causative agents, in which *E. chaffeensis* and *Coxiella* sp. closely related to *C. burnetii* pose zoonotic threats to public health. These findings warrant more investigation to determine the prevalence and infection risk in the next step.

Chapter V

Detection of a *Babesia* sp. genotype closely related to marsupial-associated *Babesia* spp. in *Haemaphysalis shimoga* from Sarawak, Borneo

Introduction

Babesia spp. are tick-borne protozoan parasites of the phylum Apicomplexa that infect erythrocytes of mammals and birds [246]. Since the discovery of *Babesia* species by Victor Babes in 1888 [20], over 100 *Babesia* spp. have been reported worldwide from a vast range of vertebrate hosts including: Bovidae, Canidae, Cervidae, Felidae, Mustelidae, Rodentia, Marsupialia, and humans [104, 112]. Recently updated phylogeny-based classification for piroplasmids designated *Babesia* spp. into *Babesia* sensu stricto (Clade X/(VI)), and *Babesia microti*-like (Clade I), Western clade (Clade III/(II), and Peircei group (Clade V) collectively as *Babesia* sensu lato [113, 246]. Furthermore, human babesiosis has been detected from all the clades, except from the Peircei group to date.

The main etiological agents for human babesiosis are *Babesia microti*, *Babesia divergens*, *Babesia duncani*, and *Babesia venatorum* [145, 278]. Two human cases inflicted by *B. microti* have been reported at China-Myanmar border [296]. In addition, previous study in Thailand reported a *Babesia* sp. detected in *Haemaphysalis lagrangei*, which was phylogenetically related to *Babesia* sp. KO1 from a human case in Korea [283]. Dantrakool et al. [51] reported a *Babesia* sp. from *Bandicota indica* rats in Thailand, which was morphologically resembled *B. microti*, but closely related to *B. canis* in phylogenetic analysis. Research into *Babesia* spp. from countries in Southeast Asian region has largely focused on bovine and canine babesiosis. For example, *B. bovis* and *B. bigemina*, the causative agents for bovine babesiosis have been reported from Malaysia, Indonesia, Thailand, and Vietnam [43, 91, 156, 210, 230, 251]. Both *Babesia gibsoni* and *B. vogeli* have been detected in dogs from Malaysia and Thailand [60, 142, 223], while *B. vogeli* has been reported from the dogs in Cambodia [107, 109]. Additionally, *B. gibsoni* and *B. vogeli* were detected in *Rhipicephalus sanguineus* parasiting dogs in Peninsular Malaysia [223].

Over the years, *Babesia* spp. are continuously being detected from a vast range of hosts, including bears, badgers, kangaroos and many more [23, 108, 213]. It can be speculated that all vertebrates could potentially be susceptible to *Babesia* infection, as long as they are compatible hosts for the vector ticks [246]. Furthermore, vector ticks are obligated in the life cycle of *Babesia* spp. and during the blood meal feeding ticks can acquire and transmit the agent [112, 287]. In addition, transovarial transmission can occur for *Babesia* spp. of *Babesia* sensu stricto clade (Clade X/(VI)) in ticks, identification of the *Babesia*

species is therefore crucial to determine the transmission mechanism in vectors. Moreover, identification of *Babesia* species in its vector tick is necessary to understand the host-*Babesia*-vector interactions.

Babesia spp. and other piroplasms are understudied in Malaysia and limited to studies of dogs, cattle, and the ticks infesting these animals [142, 210, 223, 230]. Furthermore, previous studies undertaking in Sarawak state involved molecular and serological survey in dogs [142] and cattle [230]. There has been no research into *Babesia* spp. in ticks and rodents from this state of Malaysia. In this study, we aimed to conduct molecular survey in different tick and rodent species collected from primary forests and an oil palm plantation in Sarawak, Malaysian Borneo.

Materials and Methods

Screening of *Babesia* spp.

DNA from the rodent spleens and ticks were used for the *Babesia* screening and characterization using conventional PCR targeting the 18S rDNA. All PCRs were conducted using KOD One PCR Master Mix (Toyobo, Osaka, Japan) with a reaction mixture of 20 µL. First, screening of all samples was conducted using RLBF and RLBR primers, which amplified to 400-500 bp fragment [88]. The PCR condition was as follows: 40 cycles of denaturation at 98°C for 10 sec, 60°C of annealing for 5 sec, and extension at 68°C for 1 sec. The initial PCRs with ticks showed no amplification for larvae in electrophoresis, and therefore, the remaining larvae were excluded in subsequent PCR screenings. After that, the positive samples were further characterized to obtain longer sequence by nested PCR using BTH18S 1st F and BTH18S 1st R primers in the first PCR and BTH18S 2nd F and BTH18S 2nd R primers in the nested PCR, which yielded an approximately 1.4 – 1.6 kb fragment [182]. The PCR conditions for the first and nested PCRs were identical, i.e., 40 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 5 sec, followed by extension at 68°C for 2 sec. For rodents, nested PCR using BTH18S primer sets were also used to screen all spleen samples under the same PCR conditions. The sample details used in *Babesia* screening are documented in Table 18. Finally, the amplification products were observed with gel electrophoresis and purified using a FastGene Gel/PCR Extraction Kit, followed by Sanger sequencing as mentioned above.

Phylogenetic analysis

Forward and reverse sequences were assembled and trimmed using the ATGC software version 9.0.0 to obtain the consensus. Consensus sequences were then compared with those in public databases using BLASTn. For phylogenetic analysis, sequences were aligned in MEGA X [147] using ClustalW, then trimmed to the length of the sample sequences in this study. Maximum-likelihood phylogenetic tree was constructed with Tamura-Nei model, which was selected based on the lowest Bayesian information criterion (BIC) scores for nucleotide substitution. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G, parameter = 0.1613) and phylogeny was tested with 1,000 bootstrap replications. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The sequences obtained in this study are available in GenBank with following accessions: LC655684 and LC655685.

Table 18. Tick and rodent samples used in *Babesia* screening.

Tick species	**Number**
Ixodes granulatus	32
Haemaphysalis hystricis	38
Haemaphysalis shimoga	69
Dermacentor compactus	4
Dermacentor steini	13
Dermacentor atrosignatus	4
Total	**160**

Rodent species	**Number**
Leopodamys sabanus	**3**
Maxomys rajah	**2**
Maxomys whiteheadi	**2**
***Rattus* spp.**	**45**
Sundamys muelleri	**3**
Total	**55**

Results

Babesia sequences were obtained from two tick samples (Sample IDs: HS55 and HS57) by the PCR screening with RLBF and RLBR primers. The *Babesia*-positive samples were identified as male *Haemaphysalis shimoga* (2.9%, 2/69) collected from the oil palm plantation via flagging. In further characterization, both samples were successfully amplified and sequenced with BTH18S primer pairs. Sequence analysis for BTH18S sequences revealed that both *H. shimoga* samples had identical sequences. Furthermore, the sequences showed 98.6% (1,433/1,451 bp) identity with *Babesia macropus* detected from eastern grey kangaroos (*Macropus giganteus*) in Australia (JQ437265). Consistently, the phylogenetic tree based on the 18S rDNA sequences inferred that both our samples clustered in *Babesia* sensu stricto clade (Clade X/(VI)) and were closely related to the marsupial-associated *Babesia* spp. (Figure 20). None of the rodent samples were positive for *Babesia* spp. in the screening with both 18S rDNA primer sets.

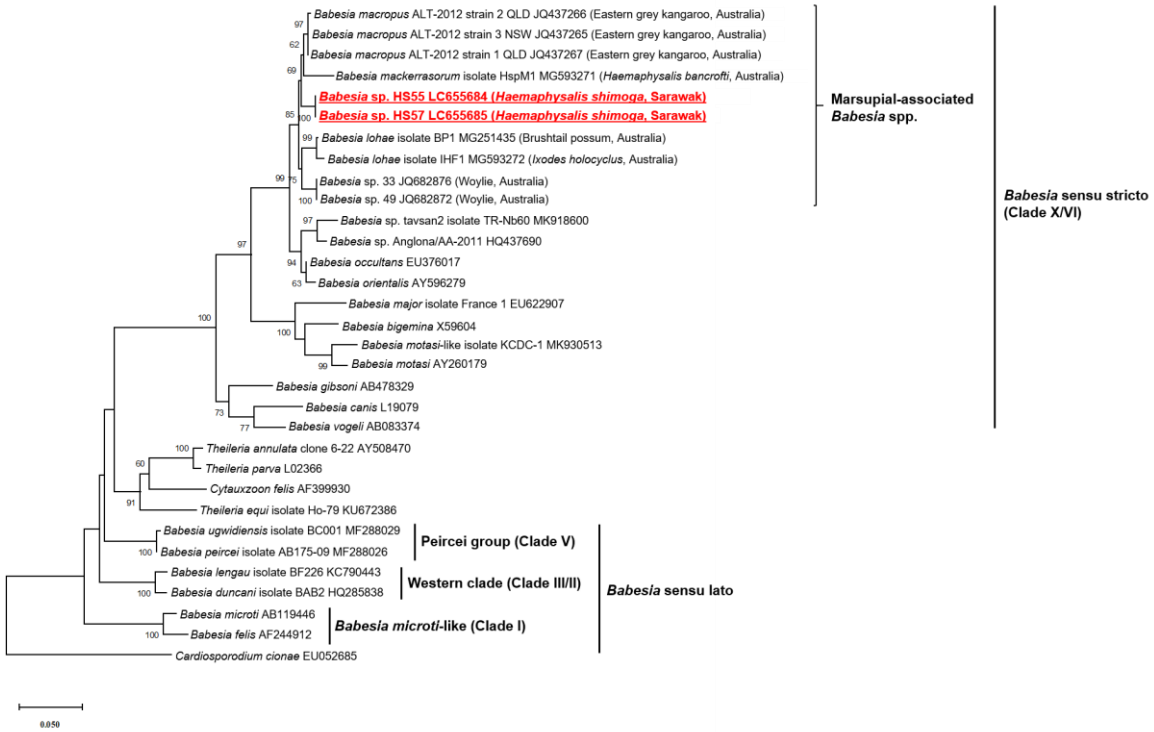

Figure 20. A maximum likelihood phylogenetic tree of 18S rDNA sequences (~ 1.5 kbp). The sequences from the present study are underlined and labelled in bold. Only bootstrap values more than 60% are shown. Clade designation and numbers are based on Schnittger et al. [246] and Jalovecka et al. [112].

Discussion

This study investigated *Babesia* spp. in six different tick species, *I. granulatus*, *H. hystricis*, *H. shimoga*, *D. compactus*, *D. steini*, and *D. atrosignatus* and rodents collected in Sarawak, Borneo. *Babesia* sp. closely related to *B. macropus*, described from eastern grey kangaroos in Australia [54] was identified from *H. shimoga* ticks collected from the oil palm plantation. This is the first insight into *Babesia* infection status using ticks and rodents in Sarawak, Borneo.

To our knowledge, there have been no reports of the vectors for *B. macropus* to-date in the study locations. However, *Ixodes* and *Haemaphysalis* ticks were speculated to be the tick vector of this agent [54, 61]. The two positive samples in this study were from adult *H. shimoga* ticks collected from an oil palm plantation in Sarawak, Malaysian Borneo. *Haemaphysalis shimoga* was first described from Southern India [275], and its distribution included Cambodia, China, Myanmar, Thailand, and Vietnam [141, 156, 264]. Nevertheless, its role as the competent vector for the detected *Babesia* sp. still requires further investigation.

Babesia macropus has only been reported from marsupials, including Agile wallabies (*Macropus agilis*), Swamp wallaby (*Wallabia bicolor*), Red-necked wallaby (*M. rufogriseus*), and Eastern bettong (*Bettongia gaimardi*) in Australia [61]. On the other hand, the adult *H. shimoga* ticks are known to feed on sambar deer (*Cervus unicolor*), but other hosts such as cattle, sheep, goats, and humans have also been recorded [79, 264]. The phylogenetic positions of the *Babesia* sp. in this study and marsupial-associated *Babesia* spp., showed a close relationship to *Babesia* spp. reported in the domestic pig, water buffalo, and sable antelope suggested a common ancestor (Figure 20). Moreover, these *Babesia* spp. are positioned in the clade of *Babesia* sensu stricto (Clade X/(VI)) that consists of a broad range of mammalian and avian species. It is important to note that transovarial transmission is exclusive to *Babesia* sensu stricto. In contrast, transtandial transmission is a feature in *Babesia* sensu stricto and *Babesia* sensu lato (*Babesia microti*-like, Western clade, Peircei group) [86, 113, 195]. And this feature of *Babesia* sensu stricto could have facilitated host switches by increasing the parasite-tick capacity to switch to unrelated vertebrate host families [113], and resulted in *Babesia* spp. being reported from a vast range of hosts in this clade. Future investigation into the vector capacity and potential hosts of the *Babesia* sp. detected in this study will help understand the transmission and risk.

Although *Ixodes* are the principal vector for many reported *Babesia* spp., including the agents of human babesiosis [145], *Babesia* sp. was not detected from *I. granulatus* ticks in this study. It is noteworthy that *I. granulatus* used in this study included the individuals infected with *Borrelia yangtzensis*, a Lyme disease group borreliae (Chapter II), and coinfection between *Borrelia* and *Babesia* are common

[289]. Likewise, *Babesia* was not detected from rodent samples. Future investigation should encompass a more extensive sampling size to confirm the presence of other *Babesia* spp. in ticks and rodents.

Detection of *Babesia* spp. is imperative due to its significant impacts, and *Babesia* spp. of *Babesia* sensu stricto are pontentially of economic importance in the livestock industry. In this study, a *Babesia* sp. genotype closely related to marsupial-associated *Babesia* spp. belonging to the *Babesia* sensu stricto clade was detected for the first time from *H. shimoga* collected from the oil palm plantation. Clinical cases of *Babesia* infection are mainly associated with stress-induced immunosuppressed animals, in which young animals are more prone to severe clinical manifestation [61, 217]. Marsupial-associated *Babesia* spp., such as *B. macropus* is of veterinary importance with clinical symptoms such as severe anemia, emaciation, lethargy, neurologic signs, and death have been reported in macropods [54, 61]. The potential of the *Babesia* sp. identified in this study in causing disease should also be investigated. Future *Babesia* surveillance in Malaysia should cover more tick and host species.

Summary

Babesia spp. are tick-borne Apicomplexan parasites that infect erythrocytes of mammals and birds. Since their discovery, over 100 *Babesia* species have been reported worldwide from a vast range of vertebrate hosts. However, research into *Babesia* spp. in Malaysia has been limited to dogs and livestock and the ticks collected from these animals. Thus, we investigated *Babesia* spp. in different rodent and tick species collected from primary forests and an oil palm plantation in Sarawak Borneo. A total of 55 rodents and 160 questing and engorged ticks were included for *Babesia* spp. detection by PCR targeting the 18S ribosomal DNA. The presence of *Babesia* spp. DNA were detected in two questing *Haemaphysalis shimoga* collected from the oil palm plantation. Sequence analysis revealed that both sequences obtained were identical and showed high identity (98.6%) with *Babesia macropus* detected from eastern grey kangaroos (*Macropus giganteus*) in Australia. Phylogenetic tree showed that the *Babesia* sp. in this study and marsupial-associated *Babesia* spp. clustered together and were positioned in the *Babesia* sensu stricto clade. To date, no vector has been reported for *B. macropus*, while adult *H. shimoga* ticks are known to feed primarily on sambar deer (*Cervus unicolor*). Future investigation is warranted to understand the vector-host relationship and risks of the *Babesia* sp. detected in this study.

Conclusion

Ticks are obligate hematophagous arthropods, feeding on a wide range of animal hosts, including humans. Additionally, ticks have high public health importance by harboring various pathogenic agents and play a crucial role as a vector in disease transmission to humans and animals. Tick-borne diseases (TBDs), which afflict humans and animals, are caused by infectious agents transmitted by tick bites. There are a variety of tick-borne pathogens (TBPs), for example, ticks are known to harbor bacterial species that can cause the most prevalent diseases, including Lyme disease (*Borrelia*), human granulocytic anaplasmosis (*Anaplasma*), tularemia (*Francisella*), and spotted fever (*Rickettsia*). In addition, tick-borne protozoa such as *Babesia* has significant health implications for humans and animals worldwide. In Malaysia, TBDs are regarded to be a potential emerging threat to public health. The tick microbiome consists of largely non-pathogenic microorganisms essential for tick survival, development, and reproduction. Understanding the microbial structure in ticks is believed to be crucial to unravel the dynamics of pathogen colonization and transmission. Taken together, tick microbiome and the status of TBPs in Malaysia warrants further investigation, especially in Sarawak state, as the status is largely unknown. In addition, precise identification of tick species is crucial for epidemiological investigation, prevention, and control of TBDs in general. With that study objectives, tick and rodent samples were collected from two primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak.

In chapter I, identification of tick species based on the available taxonomic keys and molecular methods were done with ticks collected from flagging and rodent hosts. As a result, nine species of ticks from four genera were collected from GGNP, KNP, and OP plantation, in which seven species, *Ixodes granulatus*, *Haemaphysalis hystricis*, *H. shimoga*, *Dermacentor compactus*, *D. steini*, *D. atrosignatus*, and, *Amblyomma testudinarium*, were successfully identified up to species level. This study provides the first molecular evidence of multiple tick species in Sarawak, with the first report of *H. shimoga* in Malaysia.

In chapter II, screenings of rodents and *Ixodes* ticks for *Borrelia* spp. were conducted, and two *Borrelia* spp. were identified in this study. Furthermore, *Borrelia yangtzensis*, a member of *Borrelia burgdorferi* sensu lato, was characterized using the multilocus sequence analysis. Importantly, the Relapsing Fever *Borrelia*, *B. miyamotoi*, was also identified from one rodent sample collected in the primary forest, GGNP. This was the first evidence of *B. miyamotoi* in Malaysia. Overall, this study has provided evidence of a new geographical record for *B. yangtzensis* and *B. miyamotoi*.

Chapter III elucidated the tick microbiome from six species of three genera and used Next-generation sequencing (NGS). From the multi-species comparative analysis of tick microbiome, microbial diversity and structure were significant among tick species, in which genus *Dermacentor* has the highest

microbial diversity and *H. shimoga* had the greatest microbial structure difference. Additionally, main endosymbionts were identified in different tick species and the developmental stage of *H. shimoga*. Furthermore, the presence of *Borrelia* in *I. granulatus* showed a significant difference in microbial structure compared with *Borrelia*-negative *Ixodes* ticks. Finally, the bacteria species detected by NGS was characterized with conventional PCRs. Among the detected bacteria, *Ehrlichia* sp. closely related *E. ewingii* and *Rickettsia heilongjiangensis* are zoonotic agents causing ehrlichiosis and spotted fever, respectively. *Anaplasma* sp. closely related to *A. platys*, a causative agent for anaplasmosis in dogs, was also detected. Besides, two *Bartonella* spp. closely related to *B. tribocorum* and *B. rattimassiliensis* were also identified in ticks. This study provides important insights into the tick microbiome difference and the presence of pathogens circulating in ticks from primary forests and an oil palm plantation in Sarawak.

In chapter IV, molecular screening targeting the tick-borne bacterial pathogens was conducted with the rodent samples. As a result, *E. chaffeensis*, a causative agent for human monocytic ehrlichiosis was detected in one *Rattus* sp. collected from the oil palm plantation. *Coxiella* sp. located in the clade of *Coxiella burnetii*, a causative agent for Q fever in humans, was also identified in two rodent samples. *Anaplasma* sp. closely related to *A. bovis*, a causative agent for monocytic anaplasmosis in cattle and potentially other species was also identified. Furthermore, three *Bartonella* spp., *B. coopersplainsensis*, *B. rattimassiliensis*, and *B. phoceensis* were also identified. The finding of *E. chaffeensis* was the first evidence of the presence of this zoonotic species in Malaysia, as well as the detection of *C. burnetii* in rodent species. Due to the limited sample size, future investigation with more samples is required to confirm the prevalence and infection risk of these pathogens.

Finally, in chapter V, rodent and tick samples were screened for tick-borne protozoa, *Babesia*. *Babesia* sp. closely related to *B. macropus* identified from eastern grey kangaroos in Australia were detected in two *H. shimoga* ticks collected in the oil palm plantation. From the phylogenetic inference, *Babesia* sp. in this study clustered with marsupial babesias. This study suggests that marsupial babesias may have a broader host and vector range, as well as geographical distribution. Furthermore, *Haemaphysalis* ticks could be the potential vector.

Overall, this thesis contributed to the imperative information and insights on tick species, tick microbiome, and tick-borne pathogens in Sarawak, Malaysian Borneo. This data is fundamental and valuable for the upcoming research planning from this region.

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