MOLECULAR DETECTION OF BACTERIAL MICROBOME OF TICKS PARASITIZING WILD BOARS (*SUS SCROFA*) IN AN ORANG ASLI COMMUNITY

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Molecular Detection of Bacterial Microbiome of Ticks Parasitizing Wild Boars (*Sus scrofa*) in an Orang Asli Community

ABSTRACT

Ticks are competent hematophagous vectors of arthropod-borne diseases globally**.** In Malaysia, the extent of tick-borne diseases is still rarely studied and neglected. There are still many tick species from different animal hosts here in Asia region which the associated bacterial communities are unexplored. Next generation sequencing (NGS) has been used effectively recently in many studies to dissect the microbiomes of tick vectors, to reveal novel bacterial pathogen. Here, we performed a survey of the bacterial communities associated with ticks recovered from wildlife host, wild boar (n=3) trapped near forested area surrounding the Orang Asli Community. Ticks (n=72) were morphologically identified as *Haemaphysalis hystricis* (n=32), *Dermacentor compactus* (n=15), *Amblyomma testudinarium* (n=13), *Dermacentor steini* (n=10) and *Dermacentor atrosignatus* (n=2). Taxonomic summary of these ticks shown that there are 16 dominant bacterial taxa (relative abundance >1%), including known bacteria associated with ticks (*Rickettsia, Coxiella* and *Francisella*) and possibly environmental or skin bacteria from the sampled host (*Acinetobacter, Staphylococcus*). From the bacterial community analysis, it was shown that the abundance of *Coxiella*, *Rickettsia* and *Francisella* appeared to be associated with *H. hystricis*, *D. compactus* and *D. steini* tick species respectively, regardless of the hosts. Specific gene amplification was performed on selected sample to identify the *Coxiella*, *Rickettsia* and *Borrelia*. Sequences of spotted fever group (SFG) rickettsia were identified from 4 tick samples, with one shared high similarity to the pathogenic *Rickettsia raoultii* strain. A relapsing fever (RF) group *Borrelia* was identified from one sample, a first discovery in Malaysia. *Coxiella burnetii* and potential *Coxiella* endosymbionts were identified from ten individual samples. The

zoonotic potential of the newly found *Borrelia* sp., *Rickettsia* sp. and *Coxiella* sp. merits further investigation. This study provides the baseline knowledge of the microbiome of *Haemaphysalis*, *Dermacentor* and *Amblyomma* ticks commonly found in parasitizing wild boar in an Orang Asli community. Further studies are required to verify if the findings here are representative of the common bacterial community of ticks in Malaysia.

Keywords: next-generation sequencing, microbiome, ticks, tick-borne diseases

Pengesanan Molekular Mikrobiota Bakteria dalam Sengkenit Babi Hutan (*Sus scrofa*) di kawasan Orang Asli

ABSTRAK

Sengkenit (ticks) adalah vektor penyakit bawaan artropod di seluruh dunia. Di Malaysia, pengesahan penyakit bawaan sengkenit masih jarang dikaji dan tidak dihargai. Penjujukan generasi hadapan telah digunakan dalam banyak kajian untuk kaji komuniti bakteria yang dikaitkan dengan spesies sengkenit. Terdapat banyak spesies sengkenit dari haiwan yang berbeza di Asia yang mana komuniti bakteria yang dikaitkan masih belum dijelajahi. Tinjauan terhadap komuniti bakteria yang dikaitkan dengan sengkenit yang dikumpul dari hidupan liar, iaitu 3 babi hutan liar telah dilakukan. Sengkenit (n = 72) yang dikumpul dikenali sebagai *Haemaphysalis hystricis* (n=32), *Dermacentor compactus* (n=15), *Amblyomma testudinarium* (n=13), *Dermacentor steini* (n=10) dan *Dermacentor atrosignatus* (n=2) secara morfologi dan molekul. Taksonomi komuniti bakteria sengkenit ini menunjukkan terdapat 16 jenis bakteria yang dominan (kelimpahan relatif> 1%), termasuk endosymbiont yang berkaitan dengan sengkenit (*Rickettsia*, *Coxiella* dan *Francisella*) dan bakteria yang biasa dijumpai dari alam sekitar atau atas kulit binating (*Acinetobacter, Staphylococcus*). Menerusi analisis komuniti, berdasarkan segi spesies, ia ditunjukkan bahawa *Coxiella, Rickettsia* dan *Francisella* dikaitkan dengan spesies sengkenit *H. hystricis, D. compactus* dan *D. steini* masing-masing. PCR dilakukan pada sampel terpilih untuk mengenal pasti *Coxiella, Rickettsia* dan *Borrelia*. *Rickettsia* kumpulan demam berbintik (SFG) dikenal pasti dari 4 sampel, dengan satu persamaan tinggi yang dikongsi dengan *Rickettsia raoultii*. Kumpulan demam berulang (RF) *Borrelia* telah dijumpai dari satu sampel, dan merupakan kali pertama dijumpai di Malaysia. *Coxiella burnetii* dan Coxiella *endosymbiont* telah dikenal pasti daripada 10 sampel. Potensi zoonotik terhadap bakteria yang baru dijumpai patut dikaji dengan

selanjutnya. Kajian ini menyumbang pengetahuan asas sengkenit microbiome *Haemaphysalis, Dermacentor* dan *Amblyomma* yang biasa dijumpai pada babi hutan. Kajian lanjut diperlukan untuk mengesahkan sama ada penemuan di sini mewakili komuniti bakteria sengkenit yang boleh dijumpai di Malaysia.

Kata kunci: penjujukan generasi hadapan, mikrobiota, sengkenit, penyakit bawaan sengkenit

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LIST OF SYMBOLS AND ABBREVIATIONS

CDC	:	Centre of Disease Control and Prevention
PGM	:	Personal Genome Machine
RMSF	:	Rocky Moutain spotted fever
TIBOLA	:	Tick-borne lymphadenopathy
TBEV	:	Tick-borne encephalitis virus
CCHF	:	Crimean-Congo hemorrhagic fever
SFTSV	:	Severe Fever with Thrombocytopenia Syndrome virus
IZI	:	Interfacial zones of inhabitants (IZI)
PCR	:	Polymerase Chain Reaction
NGS	:	Next generation sequencing
DNA	:	Deoxyribonucleic acid
rRNA	:	Ribosomal ribonucleic acid
PBS	:	Phosphate buffer saline
QIIME	:	Qualitative Insight Into Microbial Ecology
OTU	:	Operational Taxonomic Unit
NMDS	:	Non-metric multidimensional scaling
NCBI	:	National Center for Biotechnology Information
SFG	:	Spotted Fever group
RFL	:	*Rickettsia felis*-like group
TG	:	Typhus group
RF	:	Relapsing fever
LD	:	Lyme Disease
CLB	:	*Coxiella*-like bacteria

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Ticks are obligate hematophagous ectoparasites. They belong to the Arachnida class and there are two major tick families, the Ixodidae, more commonly known as the 'hard ticks' due to the presence of the dorsal shield (scutum) and Argasidae, the 'soft ticks', which lacked the dorsal shield. Ticks are among the primary source of arthropod-borne diseases with global distribution across different host species including humans, livestock and companion animals (Jongejan & Uilenberg, 2005). When feeding for blood on the animal or human hosts, ticks are able to transmit pathogenic microorganisms to the hosts causing diseases. Tick-borne diseases are generally caused by microorganisms such as bacteria, viruses and piroplasms. There has been a significant increase in the incidence of tick-borne diseases worldwide. According to the United States (US) Centre of Disease Control and Prevention (CDC), from the year of 2000 to 2010, over 25000 cases of Lyme disease caused by *Borrelia burgdoferi* transmitted by ticks were reported in the US. Other notable tick-borne diseases described by US CDC include rickettsioses that affect both humans and animals, as well as a number of tick-borne viral fevers that cause high mortalities in humans. On top of that, other tick-borne viral illness such as tick-borne encephalitis virus (TBEV) (Gils et al., 2018) and the recent discovered severe fever with thrombocytopenia syndrome virus (SFTSV) (Cai et al., 2019) were constantly being reported in Europe and Asia, respectively. As ticks are commonly found in forested areas, human populations living or working in or near to forested areas are at risk of exposure to tick bites, as well as the transmission of zoonotic agents from the animal reservoirs in the forested areas. Tick-borne bacterial infections are also of economic importance as it may serve as a threat to livestock industries. Tick-borne diseases such as theileriosis and anaplasmosis affect cattle and goats, causing poor growth, reducing herd fertility and dairy yields.

The Orang Asli people are the indigenous people of Malaysia, with many of them still live in deep forest and near forest fringes in rural areas. Past seroprevalence studies on the Orang Asli communities suggested the presence of rickettsial and borrelial infections among the population. Over 45% seroprevalence of rickettsiosis was reported, with 20.6% against spotted fever group rickettsiae (SFGR) and 27.9% against typhus group rickettsiae (TG) among 544 healthy Orang Asli people in Pennisular Malaysia (Tappe et al., 2018). On the other hand, 9.6% and 8.1% seroprevalence against *C. burnetti* and Lyme disease borreliae (LD) were reported among 900 Orang Asli people across Pennisular Malaysia, respectively (Khor et al., 2018; Khor et al., 2019). The exact etiological agents of these infections, as well as the arthropod vectors and the animal reservoirs are still unclear.

Ticks recovered from wild boar have been reported to carry various pathogenic tick-borne pathogens, including *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Rickettsia slovoca* and *Borrelia* sensu lato (s.l.) in Germany and Spain (Silaghi et al., 2014; de la Fuente et al., 2004). Orang Asli communities in Malaysia practice the subsistence way of life, hunting and foraging in the forests routinely. Their lifestyle involves regular close contact with wildlife present in the forests, which include wild boar. It is possible that wild boar and its associated ticks may contribute to the transmission of tick-borne pathogens to the Orang Asli population.

1.1 Study Objectives

The objective of the study is to establish the baseline information of the common bacterial community of ticks infesting wild boar from an Orang Asli community living in the forest fringe area. Two specific aims are designed to achieve the overall objective of this study:

1) To investigate the bacterial microbiome of ticks collected from wild boar from an Orang Asli community living in a forest fringe area in Malaysia using NGS approach.
2) To confirm the presence and assess the phylogenetic positions of tick-associated bacteria, including *Borrelia*, *Rickettsia* and *Coxiella* identified from the bacterial community analyses.

Fortunately, during the studies, several publications on tick-borne diseases in Malaysia relevant to this dissertation appeared in literature (2015 to 2018). These publications will be considered in the Discussion section (Chapter 5) of the dissertation.

CHAPTER 2: LITERATURE REVIEW

2.1 The life cycle of ticks

Generally, ticks go through four different life stages (Figure 2.1), the egg, six-legged larva, eight-legged nymph and lastly the adult stage, and feeding on a host during each stage of the life cycle (Anderson & Magnarelli, 2008). After fully engorged with the host blood, a fed tick may fall off from the hosts to moult in sheltered areas. After moulting, the newly emerged tick at the subsequent life stage will then proceed to feed on a different host, and may spend an extended period of time before encountering another suitable blood feeding host. Commonly, tick species of medical importance feed on two or three different hosts in their lives (Estrada-Pena & de la Fuente, 2014). As a result, ticks have the possibility of acquiring and cross-transmitting potential haemoparasitic pathogens, including protozoans, bacteria and viruses, responsible for blood-borne diseases to susceptible hosts, including humans. More than often, the blood-feeding hosts of juvenile ticks, including larvae and nymphs, are limited to smaller animals including rodents and birds, while adult ticks feed on larger animals. Humans are often accidental hosts to ticks, especially the juveniles (Jongejan & Uilenberg, 2005). However, some tick species, namely cattle ticks of *Rhipicephalus* genus, are able to spend most of its life cycle on a single host, feeding and moulting on the same, usually larger animal host such as cattle (Maruyama et al., 2017).

2.2 Transmission of tick-borne pathogens

Ticks are able to acquire pathogens, either during feeding on infected hosts, or by transstadial or transovarial transmissions. Certain infectious agents may be transmitted via all the methods above. According to Jongejan and Uilenberg (2005), a tick species can only be considered as a potential vector only if it fits the following criteria: (1) the

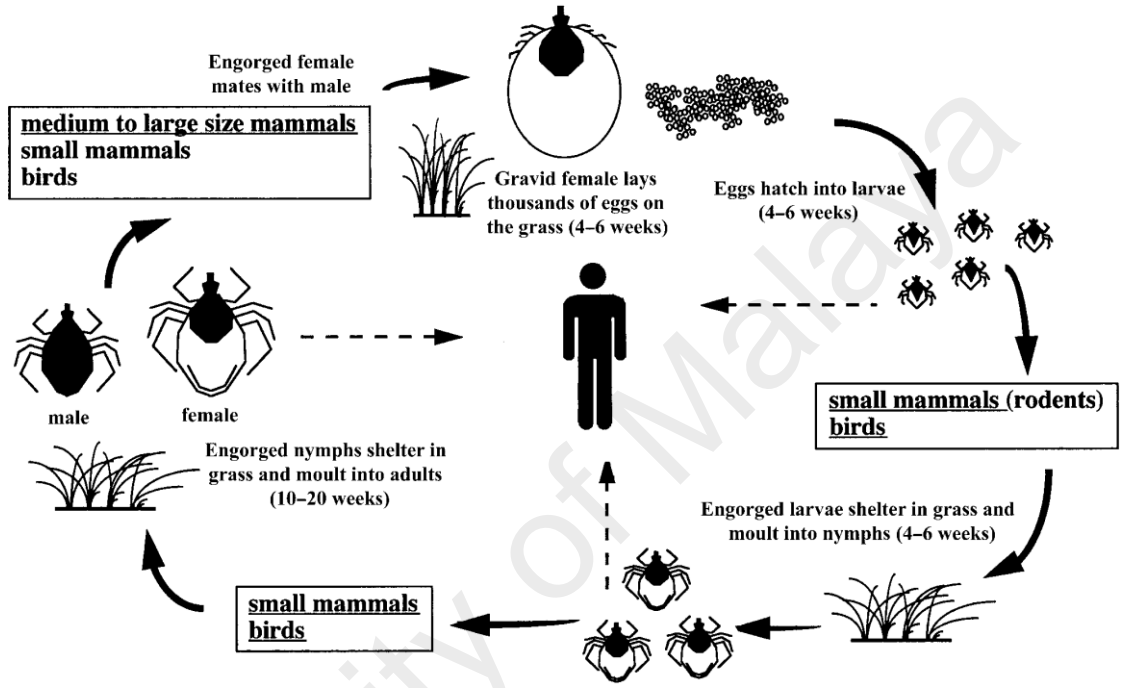

Figure 2.1: Life cycle of ticks (Parola & Raoult, 2001)

tick will feed on an infected host, (2) the tick is able to acquire the infectious agent via blood feeding process, (3) the acquired agent is maintained in tick life cycle during different life stages, and lastly (4) the tick is able to pass on the agent to the next host during blood feeding. *Rickettsia rickettsii* is an example of a disease agent vectored by ticks. Studies conducted by Niebylski et al. (1999) and Ponnusamy et al. (2014) demonstrated that *R. rickettsii* possess the ability to reproduce in almost all organs and fluid of ticks, including the salivary gland and ovaries, hence, enabling it to be both transmitted to the blood meal host and the ticks' offspring. On the other hand, microorganisms that could not colonize or reproduce in organs important during blood feeding, such as the salivary glands, may not be transmitted to the animal hosts during blood feeding and therefore are not pathogenic (Wang et al., 2018). Symbiotic microorganisms in ticks are known to colonize reproductive organs but not organs important for blood feeding, which may explain their maintenance within the tick population via vertical transmission but are not transmitted to the blood feeding animal hosts (Wang et al., 2018).

2.3 An overview of tick-borne diseases and the causative agents

2.3.1 Tick-borne bacterial diseases

Ticks are able to transmit a number of pathogenic bacteria, including the causative agents of rickettisal and Lyme diseases. In humans, spotted fever and tick typhus are caused by the genetically diverse *Rickettsia* bacterial species transmitted by ticks, with world-wide distribution (Parola et al., 2013). Many of these rickettsial bacteria exist in close association with its tick vector, in which the vertical transmission of the rickettsiae in ticks helps to maintain the infection in nature. Some rickettsial bacteria are known to be highly pathogenic, including *Rickettsia ricketsii*, the causative agent for the Rocky Mountain spotted fever (RMSF) (Dantas-Torres, 2007). On the other hand, some

rickettsial species, including *Rickettsia raoultii*, the novel and emerging rickettsial agent causing tick-borne lymphadenopathy (TIBOLA) in Europe, appears to be mildly pathogenic (Parola et al., 2009). Similarly, the severity of rickettsial diseases in humans may range from life-threatening, such as for the case of RMSF, to fairly mild for TIBOLA (Parola et al., 2009).

Other causative agents of tick-borne rickettsial diseases include *Ehrlichia* and *Anaplasma* bacteria. *Ehrlichia chafeensis* and *A. phagocytophilum* are most commonly implicated in human infections, in which both cause potentially acute, life-threatening infections in humans (Thomas et al., 2009). *Ehrlichia* and *Anaplasma* infections are also present as significant veterinary health threats. *Ehrlichia ruminantium* transmitted by cattle ticks is known to cause a fatal infection known as heartwater disease among domestic ruminants, which severely impacts the livestock industries in many African countries (Bell-Sakyi et al., 2000). Bovine anaplasmosis is caused by *Anaplasma marginale*, which can cause persistent infection in both cattle and tick hosts, in which infected animals are difficult to treat (Kocan et al., 2004).

Lyme disease, a disease caused by the spirochete, *Borrelia burgdorferi*, is another tick-borne disease with increasing global concern. *B. burgdorferi* and a number of genetically related *Borrelia* species are primarily transmitted by ticks of the *Ixodes* genera, including *Ixodes ricinus* in Europe (Mannelli et al., 2012) and *Ixodes scapularis* in the US (Qiu et al., 2002). Numerous animal species, including small mammals and birds, have been implicated as the reservoirs for *Borrelia* spirochetes, with ticks playing the key role in maintaining the transmission cycle of the spirochete between animal hosts in nature and nymphal ticks contributing to diseases in humans via summer tick bites (Mannelli et al., 2012). Lyme disease caused by *Borrelia* is usually acute and highly treatable with antibiotics. However, a proportion of patients could develop persistent symptoms, including fatigue, general muscoskeletal pain and cognitive impairment, long after

antibiotic treatments, which may have a negative impact on the quality of living for these patients (Wills et al., 2016).

As the use of molecular techniques expanded in disease surveillance efforts in recent years, novel rickettsial or borrelial genospecies closely related to the existing etiological agents to rickettsioses (Kho et al., 2015; Wijnveld et al., 2016) or borreliosis (Pritt et al., 2016) are continuously being discovered and reported from humans, ticks or the animal hosts. Although not all of these newly describe genospecies can cause diseases in humans or animals, these findings suggest that the true diversity of tick-borne bacteria remains largely unexplored.

2.3.2 Tick-borne viral diseases

Tick-borne encephalitis is endemic in Europe, as is also reported in Asian countries including China, Japan, South Korea (Süss, 2011). It is caused by tick-borne encephalitis virus (TBEV), a flavivirus transmitted by *Ixodes* ticks. Crimean-Congo hemorrhagic fever (CCHF), a disease caused by a tick-borne nairovirus commonly transmitted by *Hyalomma* ticks, was documented in Southeast Europe, Middle East and Asia (Xia et al., 2011). Recently, outbreaks involving a novel tick-borne phlebovirus, the Severe Fever with Thrombocytopaenia Syndrome virus (SFTSV), were reported in China, Japan and Korea (Lei et al., 2015). Most tick-borne viral diseases exhibit high mortalities in humans, currently with no known vaccines or treatment measures (Lei et al., 2015; Mansfield et al., 2009). The potential spread of SFTSV globally has been of great concern recently as its primary vector, the *Haemaphysalis longicornis* tick, is increasingly being reported in greater geographical regions, including the US and Europe, beyond its natural home range in Eastern Asia (Beard et al., 2018).

2.3.3 Tick-borne piroplasmic diseases

Theileria and *Babesia* species are tick transmitted intracellular protozoa belonging to the phylum Apicomplexa, family Theileridae. They infect wide range of both domestic and wild animals, and have caused great economic losses in livestock production worldwide (Mehlhorn, 1985). Two infectious *Theileria* species, *Theileria annulata* and *Theileria prava*, occur worldwide in tropical regions of the world. *T. annulata* causes tropical theileriosis in Southern Europe, Northern Africa, Western, Southern and Eastern Asia (Bishop et al., 2004). *T. parva* is responsible for East Coast fever with limited distribution across Africa (Gachohi et al., 2012). Similar to the virulent *Theileria* spp., *Babesia bovis, Babesia bigemia* and *Babesia divergens* are described as the parasitic species potentially involved in several clinical babesiosis worldwide (Bock 2et al., 2004). *Theileria* and Babesia species are known to be transmitted by Ixodidae ticks of the *Rhipicephalus, Amblyomma, Hyalomma, Ixodid* and *Haemaphysalis* genera (Bishop et al., 2004; Bishop et al., 2008; Erwanas et al., 2014; Mans et al., 2015).

2.4 Tick-borne diseases in Malaysia

At the inception of this study at 2015, there was still a lacking of information on the status of tick-borne diseases in Malaysia. Most studies conducted were serological surveys, which indicated the presence of tick-borne infections, for instance babesiosis (Rahman et al., 2010a), theleiriosis (Haron et al., 2015; Tay et al., 2000), ehrlichiosis (Rahman et al., 2010b) and rickettsial infections (Tay et al., 1999) in domestic animals and humans. There was also limited serological evidence of the exposure to tick-borne encephalitis virus among farm workers that have experienced tick bite in Malaysia (Mohd Shukri et al., 2015). Langat virus, a flavivirus genetically related to TBEV, was isolated from a pool of *Ixodes granulatus* ticks in Malaysia, however, it is not known to cause diseases in humans (Smith, 1956).

Serological surveys suggest that individuals involved in the agricultural sector (ie. rubber plantations), primarily in semi-forested areas and farmlands, are most affected by rickettsial infections (Tay et al., 2000). These areas, also known as the interfacial zones of inhabitants (IZI), allow for frequent cross contact of ticks and human, thus, contributing to higher exposure risk of tick bites and disease transmission to individual occupying these areas. Examples of IZI areas include the Orang Asli settlement and livestock farms. However, the exact etiological agents for these diseases affecting the communities at IZI are still under-appreciated hence, under-studied and. By 2015, limited number of published studies provided molecular evidence to the presence of potentially novel rickettsial species in ticks collected from wildlife such as snakes and macaques (Kho et al., 2015; Tay et al., 2015).

Furthermore, the exact tick vector for the tick-borne diseases identified in the serological surveys are largely unknown. Studies in the taxonomic identification and distribution of various tick species present in Malaysia were largely conducted during the pre-independence days (Kohls, 1957). It is unclear at present which tick species are human-biting and contributes to the transmission cycle of tick-borne pathogens. The epidemiology of ticks in Malaysia remains to be a great concern across our nation since about two thirds of Malaysia is still covered with tropical forests which serve as a natural habitat for ticks and their animal hosts (Hock, 2007). The indigenous people of Malaysia, locally known as the Orang Asli, are communities living the forest fringes in rural areas in Malaysia. Many of these communities still practice the subsistence way of living, often hunting and foraging in the forests. Wildlife such as rodents and wild boar are often hunted by Orang Asli for food or to be sold for supplementary income.

Various tick-borne pathogens have been detected in wild boar globally, suggesting its role as the reservoir of these agents. DNA of *Rickettsia tamurae*, a spotted fever group rickettsia strain was identified from the skin biopsy and ticks collected from the wild boar

in Japan (Motoi et al., 2012). In Germany, *A. phagocytophilum* was detected from the both the blood of the wild boar and the ticks found parasitizing the wild boar (Silaghi et al., 2014). DNA of *Rickettsia helvetica* was detected from the whole blood of wild boar in Netherland (Sprong et al., 2009). Little is known about the tick-borne pathogens present in the wild boar in Malaysia. As wild boar are natural hosts for ticks, Orang Asli could be at risk of tick bites and tick-borne diseases due to frequent contact and handling of the wild boar during their regular hunting activities.

2.5 Methods for detecting bacteria in ticks

Microorganisms transmitted by ticks are obligate intracellular microorganisms. To be able to detect these intracellular bacteria via the conventional bacterial cultivating method, it will be necessary to inoculate the tick homogenate onto appropriate cell cultures, allowing these bacteria to infect and propagate in the cells. This can be rather time consuming and requires specifically trained personnel to handle cell cultures. In addition, over 99% of active microorganisms are not detectable by cultivating method, hence, information retrieved from this method is limited (Hugenholtz et al., 1998). Furthermore, most tick-borne pathogens are classified as risk group-3 organisms that require handling in biosafety containment level-3 facilities, which may not be available or are expensive and difficult to operate in resource-poor settings.

2.5.1 DNA sequence amplification

Molecular biology techniques such as PCR provide an alternative approach independent of cultivation to detect tick-borne pathogens (Aktas, 2014; Ioannou et al., 2011; Widmer et al., 2011). This technique has been widely used for the screening of tick-borne bacteria. By targeting highly conserved nucleic acid sequences specific to the pathogenic bacterial genus or species (Han, 2006; Sumrandee et al., 2014), primers designed from these conserved regions can be used in PCR to amplify the conserved

microbial sequence from DNA materials extracted from whole tick or tick tissues. This molecular approach allows for targeted detection, identification and phylogenetic characterization of known bacterial pathogens. For examples, Parola et al. (2003) and Kernif et al. (2012) described the use of PCR protocols targeting the *Rickettsia*-specific *gltA* gene sequence in identifying potentially novel *Rickettsia* from tick DNA samples and studying the phylogenetic positions in relation to known rickettsial species. Furthermore, the relative abundance of specific microbes can be determined by the quantitative-PCR (qPCR) approach (Jasinskas et al., 2007).

To be able to detect co-existing bacteria, or the whole bacteria community within a tick, PCR targeting the hypervariable region bacterial 16s rRNA sequences could be performed to amplify non-specific bacteria DNA materials within the tick samples. The resulting PCR amplicons can then be cloned and transformed into a bacterial host (ie. *Escherichia coli*), and sequenced individually for each clone. Identification of the individual microorganisms can then be deduced by comparison with existing gene sequence databases.

2.5.2 Next-generation sequencing (NGS) in studying ticks microbiome

Sequencing hundreds of clones individually may be time consuming and costly. Recently, NGS technology has been applied widely in characterizing the bacterial community, also termed the microbiome, in ticks in a number of studies (Bonnet et al., 2014; Carpi et al., 2011; Vayssier-Taussat et al., 2013). The application of this high-throughput DNA sequencing technique allows for the in-depth exploration of the microbial diversity associated with ticks, which surmounts the limitation of low throughput molecular techniques. Unlike the conventional cloning-based techniques, with the ability to retrieve thousands to millions of sequences in a single run, NGS is able to detect a diverse range of bacterial species using minute amount of tick DNA. Most of

the current NGS procedures utilize the PCR amplified DNA fragments of the hypervariable regions of 16s rRNA gene in characterizing the existing microbial communities (Neelakanta & Sultana, 2013). Since this approach does not target any specific bacterial species, it has resulted in the detection and identification of bacteria previously not known to be associated with ticks, or a particular geographical locality in which the ticks were sampled from (Vayssier-Taussat et al., 2013). More importantly, this approach also revealed the existence of wide range of non-pathogenic bacterial species possibly existing as endosymbionts or commensals in ticks (Bonnet et al., 2014). *Coxiella* (Smith et al., 2015) and *Rickettsia* (Kurtti et al., 2005) are examples of known tick-borne bacteria acting as endosymbionts with potential roles in tick physiology. These microbiome studies are important in providing the fundamental knowledge to identify potential pathogens from tick and to determine the prevalence and transmission of infectious agents from ticks. This information will be crucial in formulating strategies for the prevention and managing of disease transmission. Therefore, this study proposes for the application of NGS technology in addition to conventional PCR methods in characterizing the bacterial communities associated with ticks parasitizing wild boar found in an Orang Asli community areas.

CHAPTER 3: MATERIAL AND METHODS

3.1 Collection of ticks

Feeding ticks were sampled from 3 different wild boar carcasses during regular hunting activities in an Orang Asli settlement in Selangor, Malaysia. The Orang Asli community was located near the town of Ulu Langat (3.1131° N, 101.8157° E) and surrounded by secondary forest. Tick collections were conducted from January 2014 to August 2014 with approval from the Department of Orang Asli Development, Malaysia (JAKOA). Ticks were transported back in zip-lock bags to be stored in -80^{o}C freezer.

3.2 Ticks identification

Non-engorged adult ticks from each wild boar host were included in the study to minimize the presence of bacteria originated from the host blood in identifying the bacterial communities of ticks. Each tick was microscopically identified down to genus, or species level whenever possible as described, according to published taxonomic keys for *Dermacentor*, *Amblyomma* and *Haemaphysalis* ticks (Hoogstraal et al., 1965; Volcit & Keirans, 2003; Wassef & Hoogstraal, 1983, 1984a, 1984b; Wassef & Hoogstraal, 1988).

3.3 DNA extraction from tick samples

Prior to DNA extraction, tick samples were washed twice in 70% ethanol followed by sterile distilled water to remove traces of environmental contaminants (Carpi et al., 2011). Each tick was submerged in liquid nitrogen and crushed with a pre-chilled sterile mortar and pestle. Each set of mortar and pestle was soaked in 10% sodium hypochlorite solution, rinsed with deionized water and baked at 160^{o}C to eliminate contaminants before use. After grinding, the fine powder was resuspended in 500 µL of 1 X sterile phosphate buffered saline (PBS). DNA extraction of tick sample was performed using QIAamp DNA Mini Kit (Qiagen, Hilden Germany) with 200 µL of tick homogenate according to

manufacturer's protocol. DNA was eluted in 60 µL of Ultrapure DNA/RNA-free distilled water (Invitrogen Life Technologies, MA, USA). The extracted DNA was kept in -80°C until further use. To ensure the integrity and quality of the extracted tick genomic DNA, partial tick 16s rRNA gene was amplified using previous published primer (16S+1 and 16S-1) to serve as internal control (Black & Piesman, 1994). The PCR mixture reaction was prepared in a final volume of 50 µL containing 2.5 units of Dreamtaq DNA polymerase (Thermo Scientific, MA, USA), 0.2 µM of dNTPs (Promega, WI, USA) and 0.2 µM of forward and reverse primers, respectively. Amplification was performed in a programmable thermal cycler (Applied Biosystems, MA, USA) with initial denaturation step of 5 minutes at 95°C, two three-steps cycling programs as follow; (1) 1 minute at 92°C, 1 minute at 48°C and 1 minute and 30 seconds at 72°C for 10 cycles followed by (2) 1 minute at 92°C, 35 seconds at 54°C and 1 minute and 30 seconds at 72°C for 32 cycles. The PCR reaction ended with a final extension step of 72°C for seven minutes. The amplicons were then separated and visualized under a 1.5% agarose gel electrophoresis stained with 10000X SYBR® Safe nucleic acid stain (Invitrogen Life Technologies, MA), Amplicons with the correct band size were excised, gel purified using NucleoSpin® Gel and PCR Clean-up Kit (MACHERY-NAGEL, Düren, Germany) according to manufacturer's protocol. In parallel, a mock extraction control was performed using PBS added to the sterile morta and pestle without any ticks. The elution from the mock extraction were subjected to the same bacterial gene amplification protocol below.

3.4 Amplification of V6 hypervariable region of 16s bacterial rRNA gene

Bacterial 16s rRNA gene were amplified by polymerase chain reaction (PCR) from DNA sample using set of barcoded tagged V6 oligonucleotide primers (nucleotide 872-1052bp of the complete 16s rRNA of *Escherichia* coli, NCBI Accession: AJ605115) as described previously (Carpi et al., 2011; Khoo et al., 2016a) (Figure 3.1). Specifically,

each forward primer was linked with Ion adapter "A" sequence (5' CCATCTCATCCCTGCGTGTCTCCGACTCAG 3') (in red), Ion Xpress barcode sequence (Ion Xpress ^{TM} Barcode Adapters 60-96) (in green), a linker sequence (GAT) (in orange) and lastly the primer (in blue) (Carpi et al., 2011). Each of the sample was assigned to one of 36 barcode sequences used for sample identification in latter stage. Reverse primer (in blue) was fused with Ion adapter "p1" sequence (5' CCTCTCTATGGGCAGTCGGTGAT 3') (in pink) and a linker sequence (CC) (in yellow). V6 hypervariable region has the highest sensitivity and suggested to represent the optimal sub-regions for phylogenetic studies of bacteria (Yang et al., 2016). The barcodes will allow for multiplexing of 36 samples per NGS run,

Using tick genomic DNA as template, the reaction was performed in a final volume of 50 µL PCR mixture containing 2.5 units of Dreamtaq DNA polymerase (Thermo Scientific, MA, USA), 0.2 µM of dNTPs (Promega, WI, USA) and 0.2 µM of forward and reverse primers, respectively. PCR condition included an initial denaturation at 94^{o}C for 3 minutes, 32 cycles of denaturing step at 94^{o}C for one-minute, annealing step at 56^{o}C for one minute and extension step at 72^{o}C for two minutes. The PCR reaction ended with a final extension step of 72^{o}C for two minutes. The amplicons were then separated and visualized under a 1.5% agarose gel stained with 10000X SYBR® Safe nucleic acid stain (Invitrogen Life Technologies, MA, USA). Amplicons with expected band size were gel purified using NucleoSpin® Gel and PCR Clean-up (MACHERY-NAGEL, Düren, Germany) according to manufacturer's protocol. The elutions from the mock extractions did not yield any visible PCR amplicons on the electrophoreses gels.

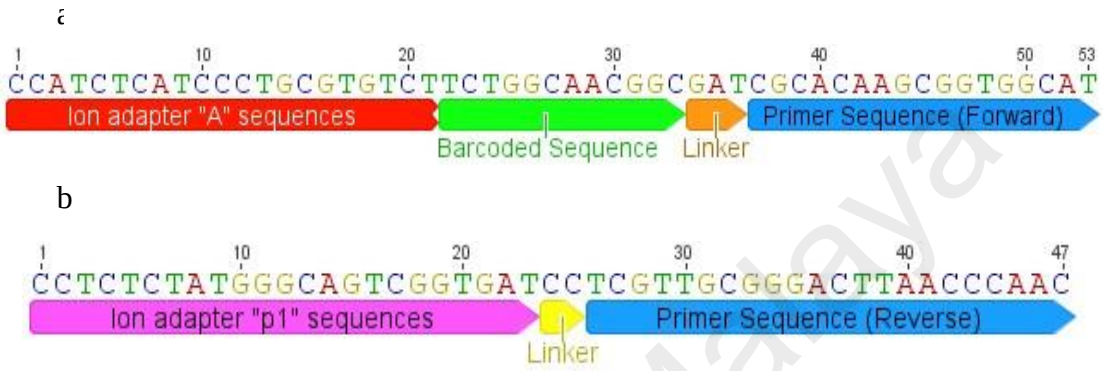

Figure 3.1: Primers used for amplification of V6 hypervariable region of the bacterial 16s rRNA gene. (a) Forward primer and (b) reverse primer.

3.5 Preparation of barcoded V6 16s rRNA amplicon libraries

After gel purification, each amplicon was quantified using Qubit dsDNA HS assay kit (Life Technologies, MA, USA) to determine their respective concentration according to manufacturer's protocol. Amplicons were diluted to 1 ng/µL respectively prior to pooling of sample for library preparation. Each amplicon library accommodates 36 individual samples with their individual barcode sequences. Two libraries were generated, each with 36 samples, to accommodate the total number of 72 tick samples used in this study. The amplicon libraries were then adjusted to the final concentration of 13 pM. Emulsion PCR was carried out with Ion PGM™ Hi-Q™ OT2 kit (Life Technologies, MA, USA) in the Ion One Touch™ 2 Instrument according to manufacturer's protocol (Catalog No: A27739, Publication No: MAN0010902). The template-positive Ion sphere particles (ISPs) were recovered and enriched using Dynabeads® MyOne™ Streptavidin C1 Beads (Life Technologies, MA, USA) in the Ion One Touch™ ES (Life Technologies, MA, USA). Each amplicon library was loaded into a single 316 chip and sequencing was performed using Ion PGM™ Hi-Q™ Sequencing Kit (Life Technologies, MA, USA) according to manufacturer's protocol on the Ion Personal Genome Machine™ System (Life Technologies, MA, USA) (Catalog No: A25592, Publication No: MAN0009816).

3.6 Sequence analysis

Low quality and polyclonal sequences were removed from the preliminary filter of sequence data using the Ion PGM software. The resulting data were exported as individual FastQ files for each tick sample. Downstream sequence analysis was performed using Quantitative Insights Into Microbial Ecology (QIIME) (Version 1.9.0) in Oracle VM Virtual Box (Version 5.0.12) following protocol as previously described (Caporaso et al., 2010). The resultant FastQ files were split into constituent fasta (sequence) and qual (quality) files using Seqtk version 1.2 (https://github.com/lh3/seqtk.git). Individual fasta files were then concatenate as a single fasta file containing the data for all 72 tick samples

to be used for analysis on the QIIME platform. Briefly, the following QIIME scripts and default parameters were applied unless otherwise noted: 1) 'add_qiime_labels.py' matched each sample with their respective variable (species, gender and host) with a single mapping file; 2) 'split_libraries.py -l 125 -L 220' removed poor quality sequences with length less than 125 and longer than 220 nucleotides, together with adaptors and primers from each sample; 3) 'pick_de_novo_otus.py' allowed de novo Operational Taxanomic Unit (OTU) picking on quality-filtered sequences using the UCLUST method at 97% similarity. Subsequently, taxonomic assignment was performed using RDP Classifier (Version 2.2) based on Greengenes reference database (Version 13_8); 4) 'identify_chimeric_seqs.py' and 'filter_otus_from_otu_table.py' were chained to removed chimeric sequence from OTU Table using ChimeraSlayer (Haas et al., 2011).

3.7 Rarefaction curve

Sequence alignment was performed using PyNAST (1.2.2) and FastTree (Price et al., 2010) was used to build a phylogenetic tree from the aligned sequences. Rarefaction plots were generated based on OTU abundance information, with random subsampling performed at incremental steps of 5000 reads and 10 iterations at each step

3.8 Community structure analysis

Quality filtered sequences for each sample were rarefied to 20000 reads prior to community structure analysis to avoid bias in bacterial community analysis due to uneven PCR amplification or sequencing efficiency (Ponnusamy et al., 2014). Community structure analysis was performed using the VEGAN package (version 2.2-1) implemented in R studio (Oksanen et al., 2010). Permutational Multivariate Analysis of Variance (PERMANOVA) as implemented in the VEGAN function *adonis* was performed to assess the tick species and the sampling host factor on their respective bacterial taxonomic profile based on Bray-Curtis distance matrix (permutation=1000). The Bray-Curtis distance matrix take into account of the presence and the abundance of each bacterial

OTUs to compare the communities (Jones et al., 2015; Kurilshikov et al., 2015; Williams-Newkirk et al., 2014). Beta dispersion test (beta-disper function) was performed to assess the multivariate dispersion for each group (Oksanen et al., 2013). Non-metric multidimensional scaling (NMDS) ordination was performed based on calculated Bray-Curtis distance matrix to visualize data sets on a two-dimensional ordination space (Minchin, 1987).

3.9 Molecular verification of *Coxiella, Rickettsia* and *Borrelia*

The presence of bacteria genera known to be associated with ticks as endosymbionts or pathogens, specifically *Rickettsia, Coxiella* and *Borrelia*, were verified by PCR. All PCR amplifications were performed in a programmable thermal cycler (Applied Biosystems, MA, USA). Unless otherwise stated, all PCR reactions consisted Dreamtaq DNA polymerase (Thermo Scientific, MA, USA) at the indicated concentrations, 0.2 µM of dNTPs (Promega, WI, USA) and 0.2 µM of forward and reverse primers.

3.9.1 Coxiella

Nested-PCR was performed to detect *Coxiella* sp. by amplifying the 16s rRNA partial gene using primers (Table 3.1) and protocol as described previously (Duron et al., 2014). The first reaction was performed in a final volume of 50 µL containing 0.2 µM of external forward and reverse primers. Post PCR, 2 µL of PCR product was added into the second round of amplification to serve as template. The second reaction was performed in 2 tubes of 25 µL PCR mixtures, each with 0.2 µM of internal forward and reverse primers (Cox16sF1 and Cox16sR1, Cox16sF2 and Cox16sR2), respectively. PCR amplification was performed with the following condition: initial denaturation step of 3 minutes at 95^{o}C, 30 cycles of denaturation at 93^{o}C for 30 seconds, annealing at 56^{o}C for 30 seconds, extension at 72^{o}C for 2 minutes and a final extension of 5 minutes at 72^{o}C.

Table 3.1: PCR primers for amplification of bacteria specific genes

Target bacteria	Target gene		Fragment size	References
Coxiella				
	16s rRNA	Cox16SF1 Cox16SR2 Cox16SF2 Cox16s R1	Cox16sF1/ Cox16sR2: **1321-1461bp*** Cox16sF1/ Cox16sR1: **719-813bp**** Cox16sF2/ Cox16sR2: **624-625bp****	Duron et al. (2014)
Rickettsia				
	gltA	CS1d CS890r	CS1d/ Cs890r: **850bp**	Roux et al. (1997)
Borrelia				
	flaB	132f 905r 220f 823r	132f/ 905r: **774bp** 220f/ 823r: **604bp**	Wodecka et al. (2010)

3.9.2 Rickettsia

Partial amplification of *gltA* gene was performed to detect *Rickettsia* sp. using previously published primers (Table 3.1) and protocol (Roux et al., 1997). Specifically, the reaction was performed in a final volume of 25 µL PCR mixture containing 1.25 units of Dreamtaq DNA polymerase, 0.4 µM of dNTPs and 0.4 µM of forward and reverse primers, respectively. The amplification protocol included an initial denaturation at 94^{o}C for 30 seconds, 40 cycles of denaturing step at 94^{o}C for 30 seconds, annealing step at 45^{o}C for 30 seconds and extension step at 65^{o}C for 55 seconds. The PCR reaction ended with a final extension step of 72^{o}C for three minutes.

3.9.3 Borrelia

Nested-PCR was performed to detect *Borrelia* sp. by amplifying the *flaB* gene using primers (Table 3.1) and protocol as described previously (Wodecka et al., 2010). Specifically, the first reaction was performed in a final volume of 25 µL PCR mixture containing 1.25 units of Dreamtaq DNA polymerase, 0.4 µM of dNTPs and 0.4 µM of external forward and reverse primers, respectively. Post PCR, 2 µL of PCR product was then added into the second round of amplification to serve as template. The second reaction was performed in 25 µL PCR mixtures, each with 0.2 µM of internal forward and reverse primers respectively, 1.25 units of Dreamtaq DNA polymerase, 0.4 µM of dNTPs. The amplification protocol included an initial denaturation at 94^{o}C for three minutes, 40 cycles of denaturing step at 94^{o}C for one-minute, annealing step at 50^{o}C for 45 seconds for first reaction (external primer) and 54^{o}C for 45 seconds for second reaction (internal primer), respectively and extension step at 72^{o}C for one minute. The PCR reaction ended with a final extension step of 72^{o}C for seven minutes.

3.10 Gel purification, sequencing and phylogenetic analysis

Upon completion of the PCR reaction, the amplicons were separated and visualized under a 1.5% agarose gel stained with 10000X SYBR® Safe nucleic acid stain (Invitrogen Life Technologies, MA). Amplicons from representative positive sample with the correct band size for respective bacteria (*Rickettsia, Borrelia, Coxiella*) were excised, gel purified using NucleoSpin® Gel and PCR Clean-up (MACHERY-NAGEL, Düren, Germany) according to manufacturer's protocol. Purified amplicons were subjected to sequencing by a third-party service provider (First Base Laboratories, Malaysia).

3.11 Sequence analysis and phylogenetic analysis

Sequences obtained were primer trimmed and subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for homologous sequence in the NCBI GenBank database. Sequences of bacteria (*Borrelia, Coxiella* and *Rickettsia*) from this study were aligned with reference sequence for respective bacteria (Appendix B, C and D) retrieved from NCBI database using CLUSTALW, as implemented in MEGA 6 (Tamura et al., 2013). Poorly aligned sequence and divergent regions of the alignment were removed using GBLOCK (Talavera & Castresana, 2007). Phylogenetic placement of bacterial sequences obtained from this study was inferred using the Bayesian Markov chain Monte Carlo method implemented in BEAST version 1.8.3 (Drummond & Rambaut, 2007).

CHAPTER 4: RESULTS

4.1 Tick sample and amplification of V6 hypervariable region

This study presents the bacterial microbiome of ticks parasitizing wild boar at an Orang Asli community in Malaysia. A total of 72 ticks were used in this study, sampled from 3 separate wild boar carcasses. Ticks were morphologically identified as *Haemaphysalis hystricis* (n=32), *Dermacentor steini* (n=10), *Dermacentor compactus* (n=15), *Dermacentor atrosignatus* (n=2) and *Amblyomma testudinarium* (n=13) from three separate wild boar hosts (Host 1 to 3) (Table 4.1). *Haemephysalis* ticks were commonly identified from the presence of the short palp in the gnathostome, with the 2nd segment of the palp extending laterally (Figure 4.1A and B, arrow). *Dermacentor* ticks were identified from the presence of generally enlarged coxa IV (Figure 4.1D, F, H, arrow). *Amblyomma* ticks were identified from the presence of long and palps (Figure 4.1I). Individual species were identified based on previously published species description: *H. hytricis* (Hoogstral et al., 1965), *D. steini* (Wassef & Hoogstral, 1988), *D. compactus* (Hoogstral & Wassef, 1984), *D. atrosignatus* (Hoogstral & Wassef, 1985) and *A. testudinarium* (Volcit & Keirans, 2003).

4.2 Ion Torrent PGM sequencing and taxonomic assignment

Sequencing of the 72 tick samples generated a total of 6343982 raw reads, ranging from 36995 to 162124 reads per sample (Appendix A). After the initial quality filtering step in the QIIME workflow, a total of 5027324 quality reads, ranging between 28522 to 118201 reads per sample were retained. A total of 910 bacterial taxa were identified after taxonomic assignment. Out of the 910 bacterial taxa, 450 of the bacterial taxa were assigned to the genus level. Each tick species exhibited different richness and variation of bacterial community. There were 597 and 478 taxa detected for *H. hystricis* ticks from Host 1 (n=22) and Host 2 (n=10) respectively. For *D. compactus*, taxonomic assignment revealed a total of 318, 397 and 624 taxa for ticks from Host 1 (n=1), Host 2 (n=3) and

Host 3 (n=11) respectively. *D. steini* from Host 1 (n=2), Host 2 (n=6) and Host 3 (n=2) each exhibited 278, 554 and 187 bacterial taxa respectively. Two individuals of *D. atrosignatus* from Host 1 and 3 separately exhibited 43 and 138 taxa. *A. testudinarium* ticks (n=13) collected solely from Host 3 displayed 518 taxa. All the sequences were deposited into the European Nucleotide Archive (ENA, Study Accession: PRJEB31681)

4.3 Rarefaction curves

Tick samples were divided into eleven groups according to their respective species and hosts for generating rarefaction curves. The rarefaction curves for each group of samples showed increasing number of observed OTUs with increasing number of reads (Figure 4.2). To assess if the sequencing depth is enough to cover the most prevalent bacterial taxa in these samples, the Good's coverage estimator was calculated. Good's coverage estimates the probability of subsequent reads belonging to an existing OTU in the sample (Good, 1953). In this study, Good's coverage value ranged from 97% to 99% for each group of samples, indicating the majority of bacterial diversity was captured with the sequencing depth.

Table 4.1: Tick species identified with their respective wild boar host

	Number of individual ticks on each host (n)		
Wild boar	Host 1	Host 2	Host 3
H. hystricis	22	10	-
D. steini	2	6	2
D. compactus	1	3	11
D. atrosignatus	1	-	1
A. testudinarium	-	--	13

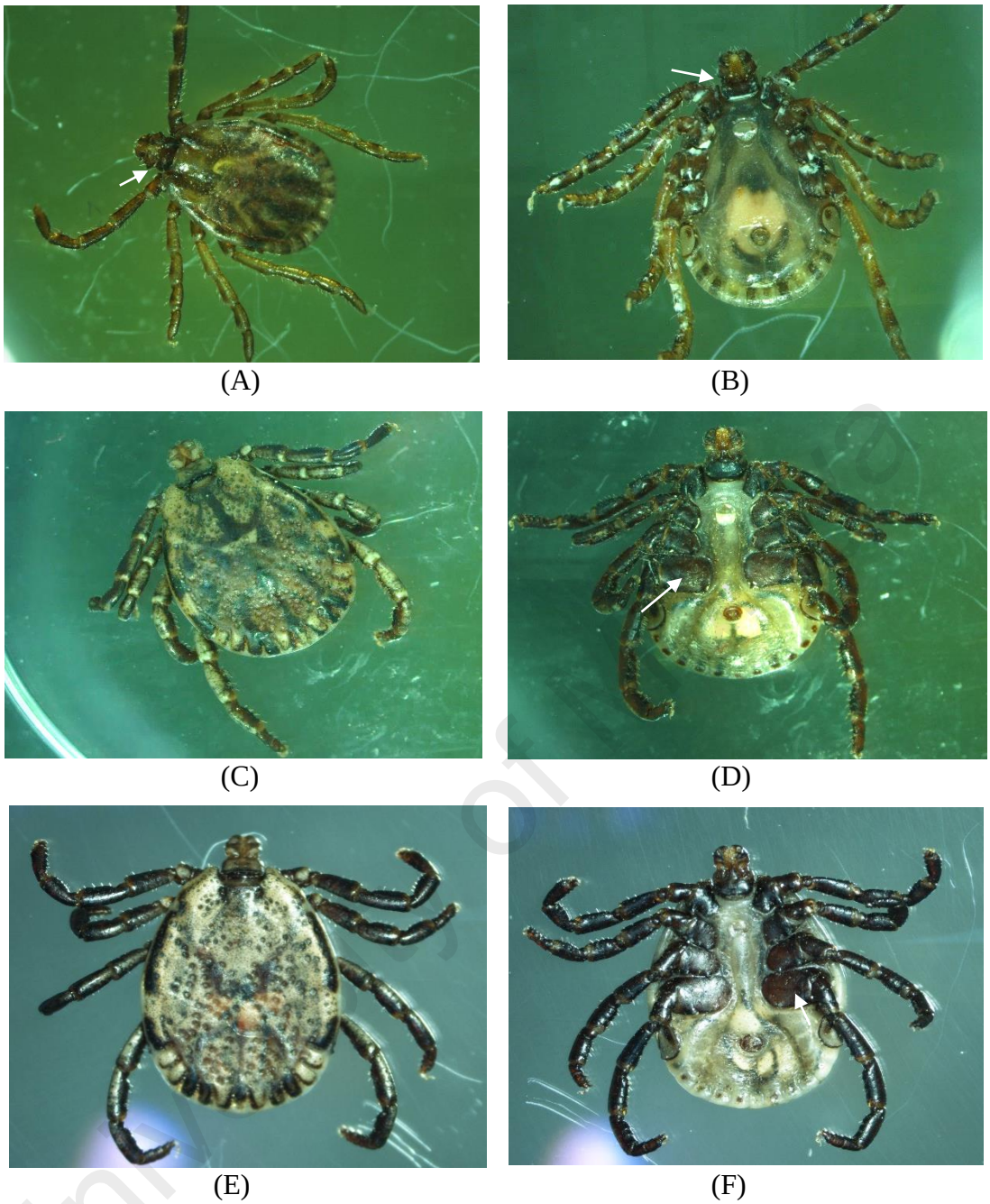

Figure 4.1: Representative images from each tick species. *Haemaphysalis hytricis*, (A) dorsal and (B) ventral view. *Dermacentor steini*, (C) dorsal and (D) ventral view. *Dermacentor compactus*, (E) dorsal and (F) ventral view. *Dermacentor atrosignatus*, (G), dorsal and (H) ventral view. *Amblyomma testudinarium*, (I) dorsal and (J) ventral view.

(G)

(H)

(I)

(J)

Figure 4.1, continued

4.4 Bacterial 16s rRNA diversity and richness

Combining the bacterial taxa identified in all 72 samples, 16 bacterial taxa were identified with greater than 1% relative abundance, representing the most dominant bacterial taxa found in these samples. These include known tick-associated bacteria, such as *Coxiella* (16.6%), *Rickettsia* (16.6%) and *Francisella* (5.45%). Other abundant bacterial taxa include *Staphylococcus* (14.7%), *Acinetobacter* (4.4%), *Erwinia* (4.1%), *Corynebacterium* (2.1%), *Stenotrophomonas* (2.1%), *Pseudomonas* (1.7%), *Arthrobacter* 1.7%) and *Brevibacterium* (1.2%), which are commonly found in the environment or on animal skin (Table 4.2). Among the 16 abundant bacterial taxa, there were several bacterial taxa that were not assigned to genus level due to limited taxonomy resolution caused by the short partial 16s *rRNA* gene sequences, including *Staphylococcaceae* (10.9%), *Bacillales* (4.2%), *Gammaproteobacteria* (3.6%), *Enterobacteriaceae* (2.9%) and *Actinomycetales* (1.7%). In general, 120 out of the 910 bacterial taxa identified were found shared between all five tick species.

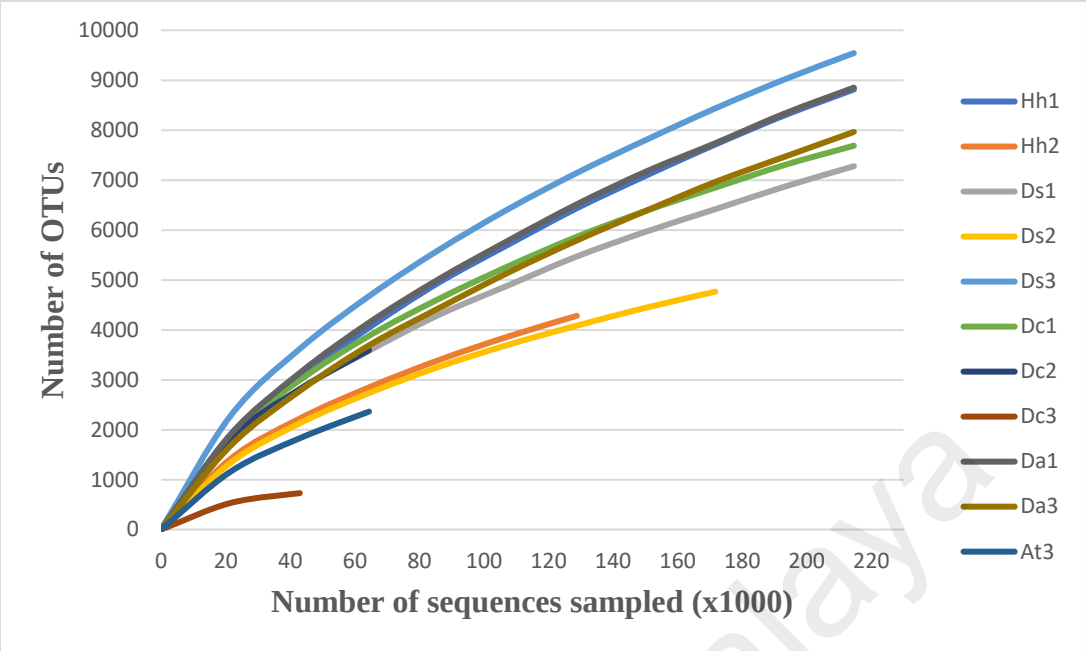

Figure 4.2: Rarefaction curves of operational taxonomic units (OTUs) defined by 97% sequence similarity for different group of samples. Hh1, *H. hystricis* from Host 1: Hh2, *H. hystricis* from Host 2: Ds1, *D. steini* from Host 1: Ds2, *D. steini* from Host 2: Ds3, *D. steini* from Host 3: Dc1, *D. compactus* from Host 1: Dc2, *D. compactus* from Host 2: Dc3, *D. compactus* from Host 3: Da1, *D. atrosignatus* from Host 1: Da3, *D. atrosignatus* from Host 3: At3, *A. testudinarium* from Host 3. Host here refer to individual wild boar in which the ticks were sampled from.

Table 4.2: Assigned taxa with relative abundance > 1% of total bacterial population in tick samples as identified by QIIME

Assigned taxa	Abundance (%)
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__Coxiella	16.6
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus	14.7
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;Other	10.9
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae;g__Rickettsia	9.1
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Francisellaceae;g__Francisella	5.4
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	4.4
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other;Other	4.2
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Erwinia	4.1
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other;Other;Other	3.6
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	2.9
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas	2.1
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	2.1
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Arthrobacter	1.7
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other;Other	1.7
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	1.7
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Brevibacteriaceae;g__Brevibacterium	1.2

k: kingdom; p: phylum; c: class; o: order; f: family; g: genus

Other: OTU was unable to be matched with known reference sequences

The relative abundance of some of these most represented bacteria taxa were presented in Table 4.3 and Figure 4.3. Looking at the known tick-associated bacteria, when broken down into individual tick species, *Coxiella* was present in all tick species, ranging from 0.002% to 95.2%. The relative abundance of *Coxiella* identified in *H. hystricis* appeared to be higher, at 23.1% from Host 1 (n=22) and 66.8% from Host 2 (n=10), compared to other tick species (Figure 4.3). While *Rickettsia* was present in the majority of ticks here (64/72), it was more abundant in *D. compactus*, at 12.6% from Host 1 (n=1), 40.2% from Host 2 (n=3), and 23.0% from Host 3 (n=11). On the contrary, *Fransciella* was found to be more prevalent in *D. steini*, at 20% from Host 1 (n=2), 31.2% from Host 2 (n=6) and 61.9% from Host 3 (n=2). Given these observations, it appeared that the presence of *Coxiella, Rickettsia* and *Francisella* are associated specifically with *H. hystricis*, *D. compactus* and *D. steini* respectively regardless of which host the sample was collected from. In addition, *Coxiella* was found only to be highly abundant in *A. testudinarium* ticks from Host 3 at 8.2% (n=13) and *D. steini* from Host 2 at 2.4% (n=6). A single *D. astrosignatus* from Host 1 was found with exceptionally high relative abundance of *Rickettsia* (99.8%). High occurrence of *Rickettsia* was also found in *H. hystricis* (n=22) from Host 1 (1.9%), *D. steini* (n=6) from Host 2 (2.2%) and *A. testudinarium* (n=13) from Host 3 (5.9%).

On the other hand, several bacteria taxa commonly found in the environment or animal skin appeared to be associated with specific wild boar host. Ticks sampled from Host 1 appeared to have high occurrence of bacteria belonging to the genus *Acinetobacter* with relative abundance of 10.5% in *H. hystricis* (n=22), 27.0% in *D. steini* (n=2) and 16.6% in *D. \compactus* (n=1) (Table 4.3, Figure 4.3). *Arthrobacter* was also highly abundant in *H. hystricis* (3.2%), *D. steini* (8.3%) and *D. compactus* (11.7%) from Host 1.

Table 4.3: Relative abundance (5) of the most dominant bacterial taxa based on tick species and hosts

Bacterial taxa	*Haemaphysalis hystricis*		*Dermacentor steini*			*Dermacentor compactus*			*Dermacentor atrosignatus*		*Amblyomma testudinarium*
	Host 1 (n = 22)	Host 2 (n = 10)	Host 1 (n = 2)	Host 2 (n = 6)	Host 3 (n = 2)	Host 1 (n = 1)	Host 2 (n = 3)	Host 3 (n = 11)	Host 1 (n = 1)	Host 3 (n = 1)	Host 3 (n = 13)
Coxiella	23.1	68.6	0	2.4	0	0.1	0.9	0.8	0	0	8.2
Rickettsia	1.9	0.5	0	2.2	0	12.6	40.2	23.0	99.8	0	5.9
Franciscella	0.1	0	20.0	31.2	61.9	0	0.7	0.1	0	0	0.3
Acinetobacter	10.5	0.7	27.0	1.3	0	16.6	0.5	0.5	0	0	0.2
Staphylococcus	3.5	9.6	2.1	19.0	4.3	1.2	23.5	25.1	0	85.2	21.7
*Staphylococcaceae**	1.6	3.4	0.1	10.4	24.6	0.1	9.1	14.6	0	7.2	28.8
Erwinia	5.2	0.4	0.4	0.4	0	1.1	1.7	8.1	0	0	5.2
*Enterobacteriaceae**	8.5	0.4	0.1	1.9	0	0.3	0.8	0.6	0	0	0.3
*Bacillales**	0.5	1.3	0.4	3.6	5.6	0.6	4.8	8.9	0	4.4	8.3
*Gammaproteobacteria**	4.0	2.9	1.5	2.4	0.3	2.9	1.0	7.8	0	0	2.1
Stenotrophomonas	7.1	0.1	0.6	0.1	0.1	0.5	0	0	0	0	0.1
Pseudomonas	4.9	0.5	1.9	0.5	0	1.8	0.3	0.1	0	0.2	0.1
Corynebacterium	0.5	0.4	1.1	1.1	0.2	0.1	0.1	0.8	0	0.3	8.8
Arthrobacter	3.2	0.2	8.3	0.7	0.1	11.7	0.1	0.4	0	0.1	1.0
Brevibacterium	0.4	0.4	0.3	2.5	0.6	0.2	0.2	2.2	0	0.4	2.0

* Bacterial taxa not assigned to genus level

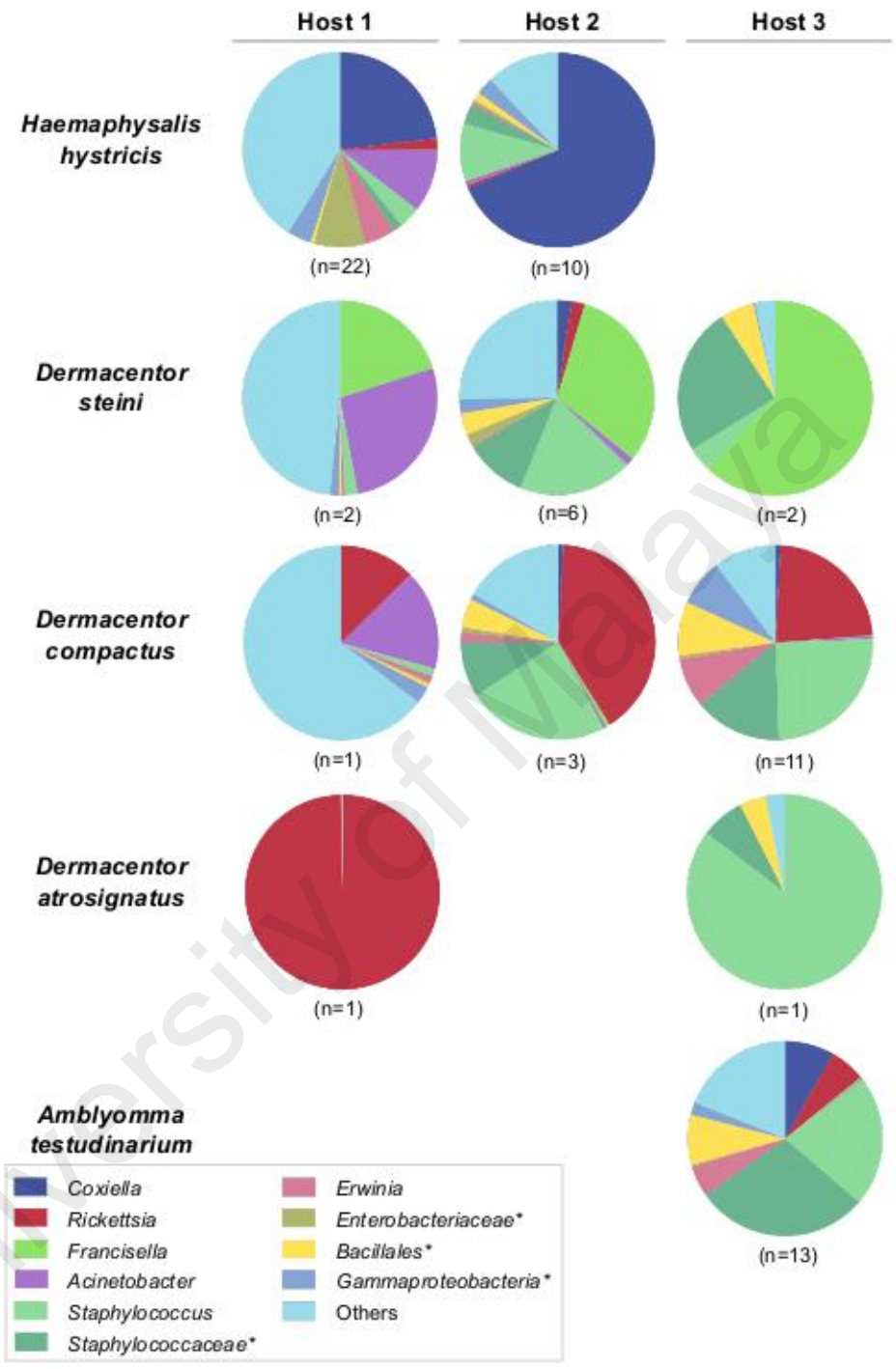

Figure 4.3: Relative abundance (%) of top ten most represented bacterial taxa according to tick species and hosts.

Pseudomonas was observed with the high relative abundance in *H. hystricis* (4.9%), *D. Steini* (1.9%) and *D. compactus* (1.8%) from Host 1 compared to ticks from other hosts. In contrast, members of family *Staphylococcaceae*, order *Bacillales* and genus *Staphylococcus* were found to be more abundant in all species of ticks collected from Host 2 and 3 compared to Host 1.

For the remaining dominant bacterial taxa, they were not correlated with any specific host or tick species. *Erwinia* was more abundant in *H. hystricis* from Host 1 at 5.2%, as well as *D. compactus* (8.1%) and *A. testudinarium* (5.2%) from Host 3. Highest occurrence of bacteria belonging to the family of *Enterobacteriaceae* and class of *Gammaproteobacteria* were observed in *H. hystricis* from Host 1 at 8.5% and *D. compactus* from Host 3 at 7.8% respectively. High relative abundance (8.8%) of *Corynebacterium* was observed in *A. testudinarium* from Host 3. *Brevibacterium* appeared to be more abundant in *D. steini* from Host 2 at 2.5% and *D. compactus* from Host 3 at 2.2%

For other known tick-associated bacteria that may cause diseases, high relative abundance of *Borrelia* was observed in a single *H. hyrticis* from Host 1 at 20.3%. It was detected at low abundance (>1% relative abundance) in another 22 tick samples. Another tick-borne bacteria, *Ehrlichia* was found in three *H. hystricis* from Host 1, two *H. hystricis* in Host 2, all at low abundance (>1%). *Anaplasma* was detected at <0.01% abundance in two *H. hystricis* ticks from Host 1.

4.5 Beta diversity and bacterial community structure

Beta diversity compared the diversity in microbial community between different tick species (Lagkouvardos et al., 2017). Due to the small sample size, *D. steini* from both Host 1 and Host 3, *D. compactus* from Host 1, as well as *D. atrosignatus* from both Host 1 and Host 3 were removed from subsequent analysis. For the remaining samples, multivariate analysis using PERMANOVA based on Bray-Curtis distance matrix revealed that tick species ($F=6.30$, $p<0.001$) and host ($F=9.05$, $p<0.001$) factor were significant predictors of the variations observed for the bacterial communities, without significant differences in beta-dispersion (Table 4.4). From the two-dimensional NMDS ordination generated, clustering of samples along the lines of tick species and animal host was observed (Figure 4.4). Two different clusters of *H. hystricis* sampled from Host 1 and Host 2 respectively were observed. The same tick species could have distinct bacterial community structures due to host factor. In addition, separation of community structures were observed for *H. hystricis*, *D. steini* and *D. compactus*, suggesting distinct bacterial community based on tick species, and the separation is most likely due to the association with *Coxiella*, *Rickettsia*, *Fransciella*. There was also more overlap of bacterial communities for ticks sampled from Host 2 and Host 3, most likely due to the sharing of higher abundance for skin or environment-associated bacteria, such as *Staphylococcaceae*, *Bacillales* and *Staphylococcus*. The bacterial community structure in ticks sampled from Host 1 appeared to be contributed by *Acinetobacter*, *Pseudomonas*, *Arthrobacter*, *Erwinia*, *Stenotrophomonas* and the unassigned *Enterobacteriaceae.*

Table 4.4: PERMANOVA and beta-dispersion of bacterial community composition with tick species and host as factors

	Pseudo-*F*	df	R2	PERMANOVA (p value)	Beta-dispersion (p value)
Factor					
Tick species	6.3026	4	0.2734	< 0.001	0.290
Host	9.0545	2	0.20789	< 0.001	0.148

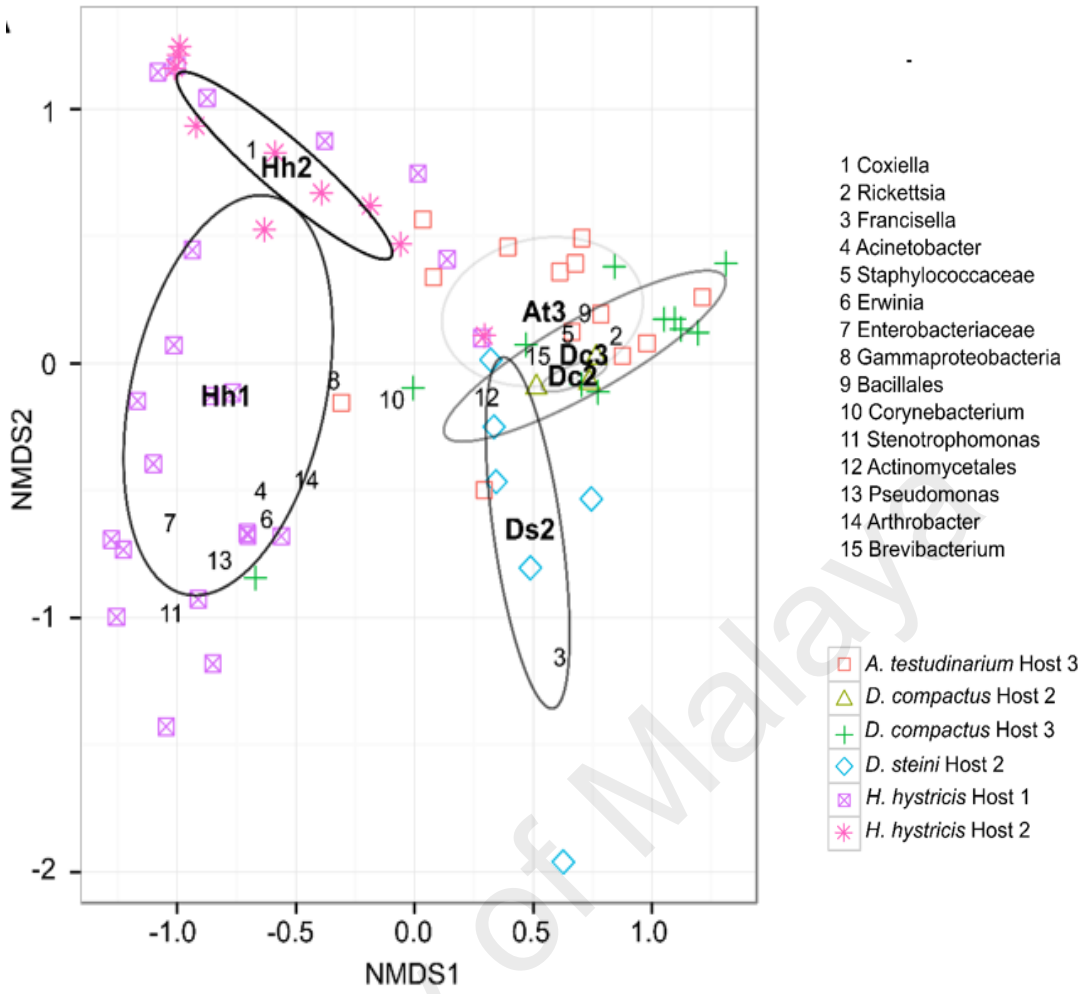

Figure 4.4: NMDS plot of the bacterial community structure between tick species and sampling hosts based on Bray-Curtin distance metric. Hh1: *H. hystricis* from Host 1, Hh2: *H. hystricis* from Host 2, Ds1: *D. steini* from Host 1, Ds2: *D. steini* from Host 2, Ds3: *D. steini* from Host 3, Dc1: *D. compactus* from Host 1, Dc2: *D. compactus* from Host 2, Dc3: *D. compactus* from Host 3, At3: *A. testudinarium* from Host 3. Host here refer to individual wild boar in which the ticks were sampled from. The centroid, representing the weighted mean for the individual sample scores, is indicated in the plot by the labels Hh1, Hh2, S11, Ds2, Ds3, Dc1, Dc2, Dc3, At3 respectively for each species-host group. The ellipse show the dispersion (1 standard deviation) for the individual sample scores

4.6 Molecular Detection and Phylogenetic analysis of *Coxiella*, *Rickettsia* and *Borrelia*

The short sequence length of bacterial V6 hypervariable region used in the high throughput sequencing only allows for the classification of each bacterial taxa up to family or genus level. To verify the presence and provide species level characterization of tick-associated bacteria, *Coxiella*, *Rickettsia* and *Borrelia*-specific genes were amplified from positive samples identified in the high-throughput sequencing

4.6.1 Coxiella

4.6.1.1 Sequencing results

Partial *Coxiella* 16s rRNA gene was successfully amplified from 43 out of the 72 positive tick samples. Ten representative samples based on host and tick species were selected for sequencing (S002, S006, S012, S014, S026, S038, S043, S058, S073 and S076). When compared to existing sequences in NCBI Genbank, *Coxiella* detected from two *H. hystricis* (S014, S038) and one *D. steini* (S026) displayed similarities of 97.5-99.6% to *Coxiella burnetti* reference strains (NCBI Accession No: CP014563.1). *Coxiella* detected from three *H. hystricis* (S002, S006 and S043) demonstrated closest match (96.7-98.3%) to *Coxiella* sp. detected from *H. obesa* from Thailand (NCBI Accession No: KC170759.1). *Coxiella* detected from two *A. testudinarium* (S058 and S073) and a single *D. compactus* (S076) displayed similarities of 94.2-97.6% to uncultured *Coxiella* sp. clone T3115 from *I. uriae* from Canada (NCBI Accession No: KJ459074.1). *Coxiella* detected from another *D. compactus* (S012) showed closest match (93.4%) to *Coxiella* endosymbiont of a soft tick, *Ornithodoros sonrai* from Senegal, Africa.

4.6.1.2 Phylogenetic analyses

Phylogenetic relationship of the of *Coxiella* partial 16s *rRNA* gene sequences from this study and other published *Coxiella* sequences from NCBI Genbank was inferred using Bayesian likelihood method (Drummond & Rambaut, 2007). The published sequences of

Coxiella (Appendix B) were selected based on previously reported *Coxiella* strains from various tick hosts. Several *Coxiella* sequences detected from *Haemaphysalis bispinosa* collected from goats in Malaysia in a separate study were also included in the analysis (Khoo et al., 2016b).

Coxiella from a *H. hystricis* (S014) and *D. steini* (S026), both taken from the same wild boar host, were clustered in Clade A, alongside with various *C. burnetti* strains (Figure 4.5). *Coxiella* from *A. testudinarium* ticks (S073 and S058) clustered together with *Coxiella* sp. detected from *A. variegatum* and *I. uriae*. *Coxiella* from three *H. hystricis* (S043, S002 and S006) formed a clade distinct from the *Coxiella* detected in other *Haemaphysalis* ticks, including *H. bispinosa* detected from goats in Malaysia (Khoo et al., 2016b), *Haemaphysalis longicornis* and *Haemaphysalis shimoga* found in other parts of Asia. *Coxiella* from another *H. hystricis* (S038) appeared to be distinct from all the other *Coxiella* detected in *Haemaphysalis* ticks included in this analysis. These *Coxiella* detected from *Haemaphysalis* ticks were also separated from the *Coxiella* endosymbiont of *Haemaphysalis punctata*, which is commonly found in Africa. *Coxiella* from *D. compactus* (S076 and S012) formed a separate clade from the other *Coxiella* strains detected in this study.

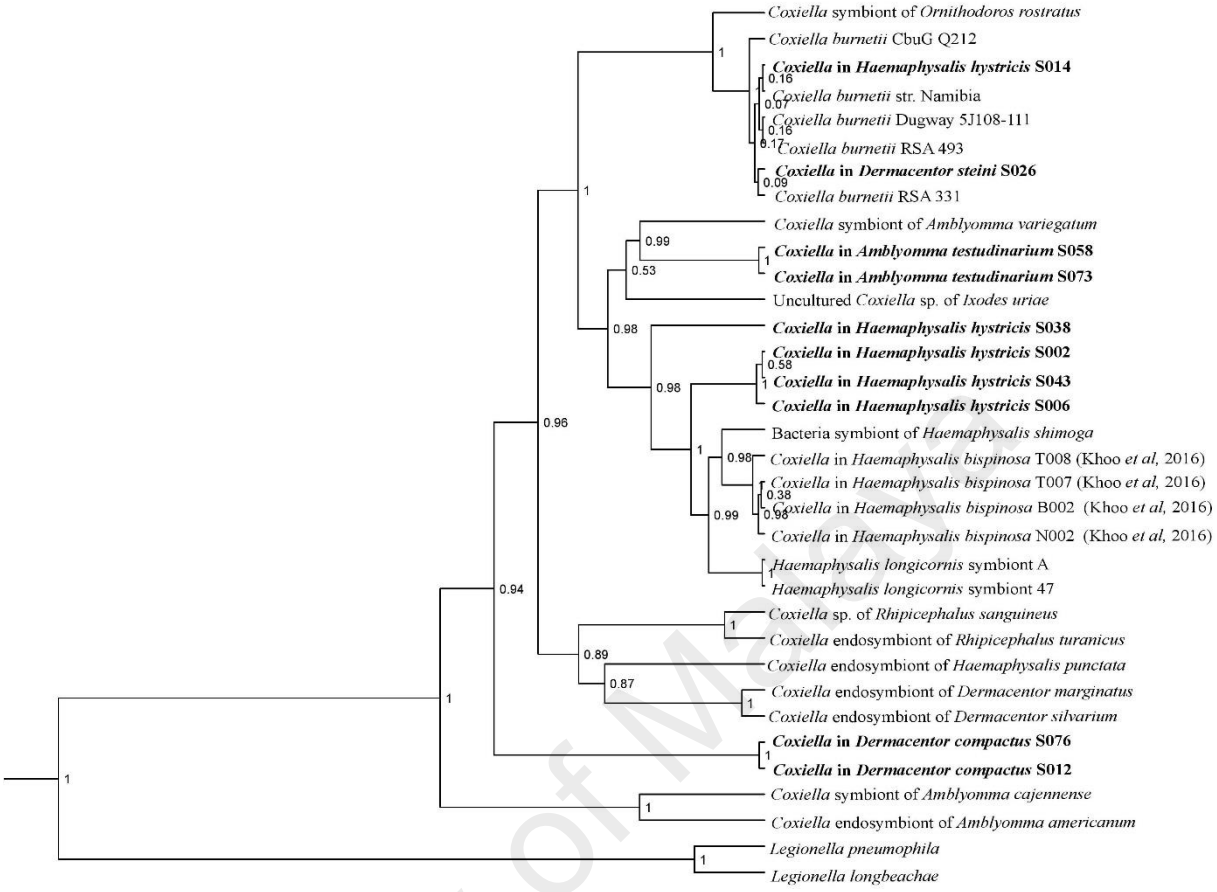

Figure 4.5: Bayesian-inferred phylogenetic tree of *Coxiella* sp. based on partial 16s rRNA sequences (1165 aligned nucleotides). Posterior probabilities are displayed adjacent to the nodes. Samples from this present study were highlighted in bold. Each clade is labelled based on clustering of various *Coxiella* strains previously described by Duron et al. (2015). The 16s sequences of *Legionella pneumophila* and *Legionella longbeachae* serve as outgroup in the tree.

4.6.2 Rickettsia

4.6.2.1 Sequencing results

Partial *Rickettsia gltA* gene was successfully amplified in 5 out of 63 positive tick samples and subjected to nucleic acid sequencing, even though the amplification was weak for sample S002. Despite multiple attempts, sequencing of the *gltA* gene from sample S002 failed to generate satisfactory results. Among the four samples with clean sequencing results, *Rickettsia* detected from *D. atrosignatus* (S036) and a *D. compactus* (S059) demonstrated closest match (99.2 to 99.4%) to *R. raoultii* strain IM16 (NCBI Accession No: KY474576.1), a strain causing infection among human population in China. *Rickettsia* sp. from another *D. compactus* (S069) showed closest match (96%) to *Rickettsia monacensis* strain Crimea-3 detected from *H. punctata* (NCBI Accession No: KU961539.1). *Rickettsia* sp. of *H. hystricis* (S005) showed closest match (98.6%) to *Rickettsia* sp. strain MC16 (NCBI Accession No: U59722.1).

4.6.2.2 Phylogenetic analyses

Phylogenetic relationship of *Rickettsia* partial *gltA* gene sequences from this study and published *Rickettsia* sequences in NCBI Genbank was inferred using Bayesian likelihood method (Drummond & Rambaut, 2007). Representative *Rickettsia* strains of different groups (Appendix C), namely Spotted Fever -Group (SFG) rickettsiae, *Rickettsia felis* and *R. felis*-like group, and Typhus Group (TG) rickettsiae were included in the phylogenetic analysis. All *Rickettsia* sp. detected in this study were clustered together with the SFG rickettsiae (Figure 4.6), forming a large clade with a number of pathogenic SFG rickettsiae, including *R. raoultii, R. helvetica, R. africae, R. aeschimannii, R. sibrica, R. parkeri,* as well as *R. tamurae*, *R. japonica* and *R. montana*. Separation of clades within SFG rickettsiae was only partially supported, with posterior probabilities varying between 0.09 to 0.98.

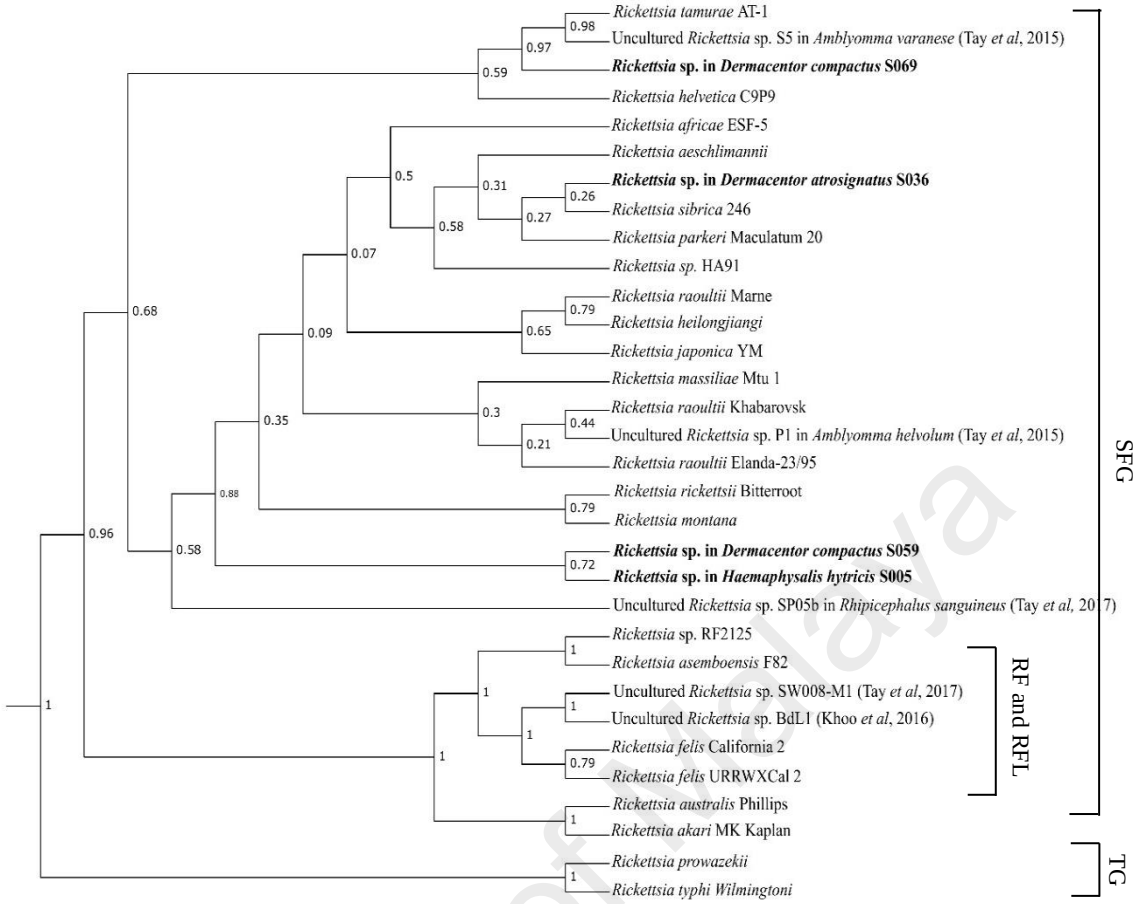

Figure 4.6: Bayesian-inferred phylogenetic tree of *Rickettsia* sp. based on partial *gltA* sequences (352 aligned nucleotides). Values displayed adjacent to nodes represent the posterior probabilities. Samples detected from this study are highlighted in bold. SFG: Spotted fever group, RF and RFL: *Rickettsia felis and Rickettisa felis*-like. TG, typhus group.

4.6.3 Borrelia

4.6.3.1 Sequencing results

Partial *Borrelia flaB* gene was successfully amplified from a single *H. hystricis* (S005) displaying high relative abundance of *Borrelia* (20.33%) in the high-throughput sequencing. Sequence from the sample demonstrated closest match (99.9%) to Relapsing fever group *Borrelia* sp. strain tHM16w of *Haemaphysalis megaspinosa* described in Japan (NCBI Accession No: LC170035).

4.6.3.2 Phylogenetic analyses

Phylogenetic tree of *Borrelia* partial *flaB* gene sequence from this study and published *Borrelia* sequences in NCBI Genbank was inferred using Bayesian likelihood method (Drummond & Rambaut, 2007). *Borrelia* strains of different groups (Appendix D), namely Relapsing fever (RF) group borreliae and Lyme Disease (LD) group borreliae were included in the phylogenetic analysis. Two different distinct clades of *Borrelia* clustering can be seen (Figure 4.7), with *Borrelia* sp. of *H. hystricis* (S005) clustered with *B. theileri, B. miyamotoi, B. lonestari* and *Borrelia* strain from hard ticks including *H. punctata, H. japonica* and *R. sanguineus* in the RF group borreliae.

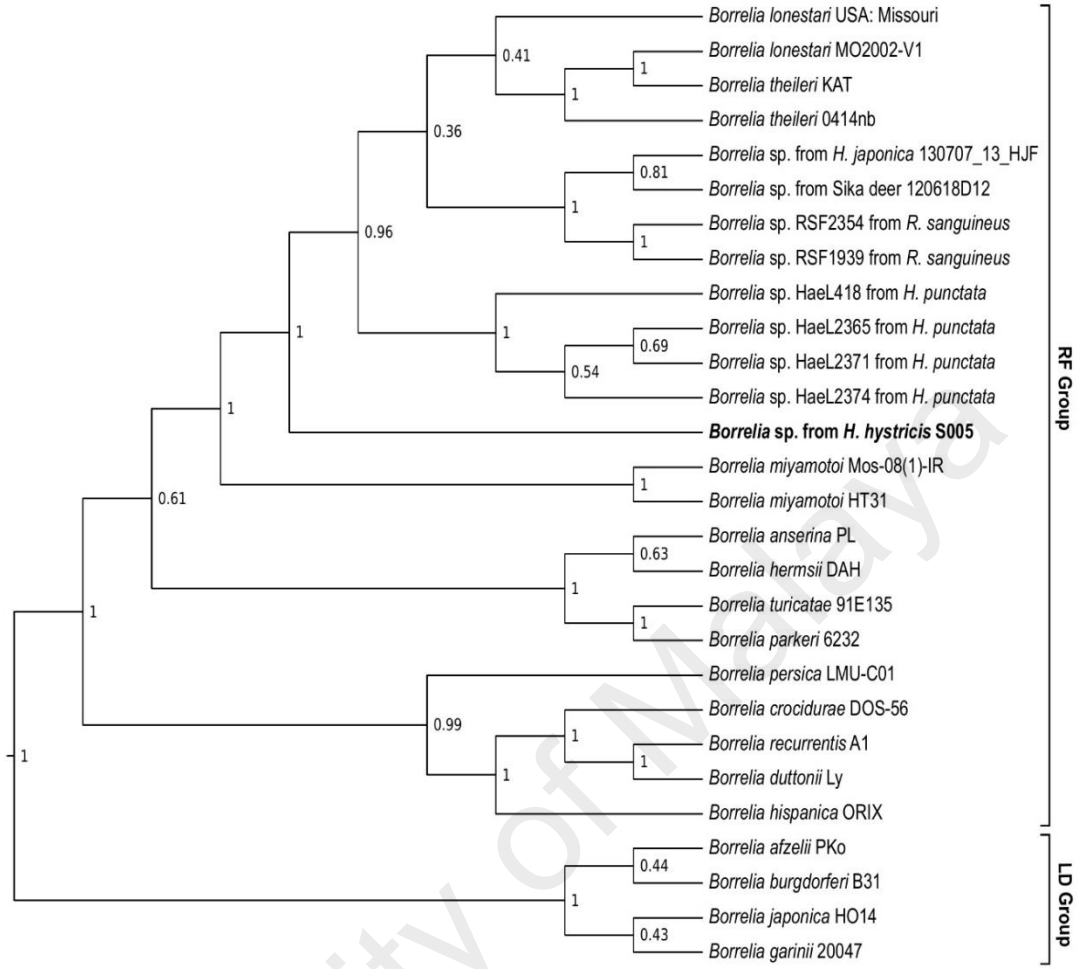

Figure 4.7: Bayesian-inferred phylogenetic tree of *Borrelia* sp. based on partial *flaB* sequences (1165 aligned nucleotides). Values displayed adjacent to the nodes represented the posterior probabilities. Samples detected from this study are highlighted in bold. RF, relapsing fever: LD, Lyme disease.

CHAPTER 5: DISCUSSION

The present study described the use of high throughput 16s amplicons sequencing in establishing the common bacterial community of ticks parasitizing wild boar from an Orang Asli community living in a forest fringe area of Malaysia. As a wild mammal of worldwide distribution, wild boar is known to be reservoir for many human pathogens (Meng et al., 2009), and these include a number of important of tick-borne pathogens. For example, *Anaplasma phagocytophilum* have been detected in both the wild boar host and the ticks feeding on it, suggesting potential role of wild boar in the epidemiological cycle of anaplasmosis in Netherlands, Belgium (Jahfari et al., 2014) and Germany (Silaghi et al., 2014). Furthermore, Faria et al. (2015) described the first evidence of *Borrelia burgdorferi* sensu lato DNA, primary causative agent for Lyme disease in wild boar in Portugal, asserting its potential role as reservoir for numerous zoonotic pathogens. In Malaysia, previous studies into zoonotic agents from wild board are only limited to the surveillance for *Toxoplasma gondii* (Puvanesuaran et al., 2013). Since wild boar hunting and the consumption of wild boar meat are still common practise among the Orang Asli and Chinese communities here respectively, there is a real risk of zoonotic agent transmission from wild boar and their ectoparasites to humans. At the time of writing, this thesis represents the first investigation into the potential zoonotic agents of ticks parasitizing wild boar in Malaysia.

Ticks sampled from this study include *H. hystricis*, *D. steini*, *D. atrosignatus*, *D. compactus* and *A. testudinarium*. The ticks species list are consistent with previous studies of the occurrences of these *Dermacentor* (Hoogstraal & Wassef, 1984, 1985; Mariana et al., 2011; Wassef & Hoogstraal, 1988), *Haemaphysalis* (Khoo et al., 2016a) and *Amblyomma* (Mariana et al., 2008) tick species in South East Asia. *H. hystricis* are three-host ticks known to parasitize a wide variety of animal hosts, including wild boar, goats, dogs and cats (Hoogstraal et al., 1965). In Japan, H. *hystricis* has been linked to

the transmission of Japanese spotted fever to humans, suggesting its role as a vector for the zoonotic diseases (Seki et al., 2006). As for A. *testudinarium*, apart from infesting a wide range of wildlife, previous report shown that *A. testudinarium* is able to feed on humans in Malaysia (Yamauchi et al., 2012) and Korea (Kim et al., 2010). A number of medically important infectious agents were detected in this tick species, including *R. tamurae* (Fournier et al., 2006), spotted fever group *Rickettsia* (Fournier et al., 2002) and SFTS virus (Imaoka et al., 2011; Suh et al., 2016). *D. steini*, *D. compactus* and *D. atrosignatus* have been known to feed on humans and wild life (Hoogstraal et al., 1965; Hoogstraal & Wassef, 1984; Vongphayloth et al., 2016; Yamaguti et al., 1971), even though their status as a carrier for diseases is still unclear. As the lifestyle of Orang Asli involves routine hunting, especially the omnipresence wild boar, thus they are at higher risk of acquiring accidental tick bites and tick-borne illnesses.

High-throughput sequencing of tick samples from the study revealed the presence a vast diversity of bacteria. The bacteria community analyses suggested that the bacterial communities in *H. hystricis*, *D. steini* and *D. compactus* ticks are primarily characterized by the presence and abundance of *Coxiella*, *Francisella* and *Rickettsia*, respectively. It is possible that these bacteria were specifically associated to the three tick species. The analyses suggested that the bacterial communities in these ticks could also be influenced by the bacterial species most likely found on the animal host skin or the environment. The PERMANOVA tests suggested that the composition of the bacterial communities was distinct in each of the individual tick species, and the composition of the bacterial communities were more similar in ticks collected from the same animal host. The beta-dispersion tests indicated that the bacterial communities from each of the tick species and host group were similarly heterogenous in composition.

Bacteria such as *Acinetobacter*, *Staphylococcus*, *Pseudomonas*, *Corynebacterium* and *Methylobacterium* have been reported in a handful of studies investigating the

microbiome of ticks globally (Carpi et al., 2011; Kurilshikov et al., 2015; Nakao et al., 2013). The distinct bacterial communities based on the animal hosts in which the ticks were sampled from, such as the separation of the *H. hystricis* ticks from Host 1 and Host 2 in the NMDS plots, may be explained by the differences of the bacteria carried on the skin of the animal hosts that have been transferred onto the exoskeleton of the ticks. Host 1 appeared to exhibit higher abundance of *Acinetobacteria* while Host 2 appeared to have greater proportion of *Staphylococcus* and the unidentified *Staphylococcaceae*.

These findings were in slight disagreement from previous reports in that the bacterial communities of ticks are most likely influenced by the tick species itself and not the environment (Hawlena et al., 2013). It is important however, to note that the sampling strategies in the two studieswere different. In this study, ticks were only collected from animal hosts, while questing ticks were used in the previous study (Hawlena et al., 2013). In addition, it has been shown that host blood meal could also influence bacterial diversity in ticks (Swei & Kwan, 2017). Although only ticks that were visibly non-engorged were selected for this study, the presence of host meal blood could not be completed excluded. Therefore, the differences in the bacterial communities could also reflect the presence of host blood meal and this could not be ascertained in the present study. Overall, the findings here suggest that bacterial communities in ticks are influenced by both the tick species and the animal hosts, which may be include to the skin microflora or from earlier blood meal.

The findings from high-throughput nucleic acid sequencing performed here highlighted the high abundance of bacteria belonging to genus *Coxiella*, *Francisella* and *Rickettsia* associated with *H. hystricis*, *D. steini* and *D. compactus*, respectively. *Coxiella*, *Francisella* and *Rickettsia* have been shown to be potential endosymbionts for various species of ticks (Bonnet et al., 2017).

Coxiella-like bacteria (CLB) have been studied extensively in two species of ticks, namely *Amblyomma americanum* tick (lone star tick) (Jasinskas et al., 2007) and *Rhipicephalus sanguineus* tick (brown dog tick) (Tsementzi et al., 2018). CLB found in these two tick species were shown to be endosymbionts with essential roles in ticks' fitness, with evidence of involvement in vitamin B biosynthesis pathway and cofactors in ticks (Jasinskas et al., 2007; Smith et al., 2015; Tsementzi et al., 2018; Zhong et al., 2007). Furthermore, CLB have been shown to be prevalent in many of the tick species globally (Duron et al., 2017; Duron et al., 2014; Duron et al., 2015) and was found to be infecting the salivary glands as a maternally inherited bacteria (Klyachko et al., 2007). In this study, *Coxiella* bacteria were found to be present in all tick samples and species, but at varying relative abundance. The presence of *Coxiella* in a sub-sample of the ticks here was confirmed by sequence analyses of the longer 16s rRNA fragment, in which the results suggested the presence of several *Coxiella* species genetically distinct from *C. burnetii* (published in (Khoo et al., 2016b)). Phylogenetic analyses also revealed that the *Coxiella* species from the same tick species or genus clustered into the same clade, suggesting close association of the bacteria with the respective tick host. Findings from the present study is consistent with the earlier findings of the presence of CLB in multiple tick species found in this region. These fndings adding more evidence supporting the hypothesis of CLB as a universal tick-associated bacteria. The analyses for bacteria community structure revealed that high abundance of *Coxiella* was detected in *H. hystricis* ticks. The potential endosymbiotic role of *Coxiella* in this tick species, and the zoonotic potential of this bacteria, nonetheless merits further investigation. Although previously thought to be non-pathogenic, there has been a report of a tick-associated CLB, *Candidatus Coxiella massilensis*, causing mild infection in human in France (Angelakis et al., 2016). Additionally, CLB infection in pet birds have been occasionally reported in North

America although the role of ticks in transmitting CLB to birds remain unclear (Shivaprasad et al., 2008; Vapniarsky et al., 2012).

Phylogenetic analyses performed here also suggested the presence of *C. burnetii* in two ticks, *D. steini* and *H. hystricis*. These two ticks were collected from the same wild boar host (Host 1). Although only non-engorged ticks were included in this study, the possibility of the presence of host blood in these ticks cannot be excluded. The *C. burnetii* detected may be have originated from the tick itself, or from the wild boar host. This is the first evidence describing the presence of *C. burnetii* in ticks from wildlife in Malaysia. *C. burnetii* was previously identified from *R. sanguineus* ticks collected from dogs seen in a veterinary hospital in Malaysia (Watanabe et al., 2015), although the role of ticks in transmitting *C. burnetii* to humans or animals is unknown. *C. burnetii* is the causative agent of Q fever, a zoonotic disease that can lead to death in humans and livestock animals (Bina Rai et al., 2011). The last reported Q fever outbreak in Malaysia occurred in 2009 among a herd of dairy goats (Bina Rai et al., 2011). Recent studies also provided serological evidence to the past exposure of *C. burnetii* among the Orang Asli people in Malaysia, although the source of the exposure is still not known (Khor et al., 2018). The role of ticks and wild boar in the transmission cycle of *C. burnetii* at the Orang Asli communities warrants further investigation.

Bacteria community studies performed here revealed potential association of *Francisella* and *Rickettsia* with *D. steini* and *D. compactus*, respectively, suggesting the possible presence of tick-specific endosymbionts. Rickettsial endosymbionts have been reported in a number of tick species in the US, including *Dermacentor andersoni* (Hunter et al., 2015) and *Ixodes pacificus* (Felsheim et al., 2009). Similar to *Coxiella* endosymbionts, rickettsial symbionts may play an important role in the biosynthesis of vitamins in the tick host (Hunter et al., 2015).

A recent study also emphasized the role of *Francisella*-like endosymbionts in *Amblyomma maculatum* in amino acid (cysteine) synthesis which is lacking in the tick's usual hematophagy diet (Gerhart et al., 2016). Further investigation, however, is necessary to confirm the presence and significance of the endosymbiotic relationship between the *Francisella* or *Rickettsia* and the tick hosts in this study.

Phylogenetic analyses performed in the present study suggested that the *Rickettsia* detected in this study belonged to the SFG group. It is important to note that the separation of clades within the SFG rickettsiae was only partially supported, suggesting that partial *gltA* sequence alone may not be able to provide sufficient phylogenetic resolution to distinguish among the SFG rickettsiae. Since the gene sequence used for the analysis did not allow for the full resolution of the rickettsial species, the actual identity of these *Rickettsia* could not be determined. It is possible that some of the *Rickettsia* identified here were endosymbionts, while others may be pathogenic rickettsiae or represent novel species. During the course of the present study, a handful of publications emerged, reporting the presence of multiple known and novel *Rickettsia* in fleas and ticks in Malaysia (Kho et al., 2015; Kho et al., 2018; Kho & Tay, 2018; Tay et al., 2014). The epidemiological cycle of the identified rickettsiae nonetheless, has yet to be investigated. Seroprevalence of TG and SFG rickettsiae among the Orang Asli communities were also previously reported, although the etiological agent of rickettsioses in Malaysia is yet to be determined (Kho et al., 2017; Kho et al., 2016; Tappe et al., 2018). Notably, a recent study reported the presence of potentially novel *Rickettsia* sp. closely related to *R. raoultii* in an ill-patient in Malaysia (Kho et al., 2016), suggesting that rickettsiosis in Malaysia may be caused by this novel rickettsiae.

This present study presents another important finding, that is the detection of *Borrelia* sp. in *H. hystricis* [published in (Khoo et al., 2017)] The *Borrelia* sp. detected in the study, together with *Borrelia* sp. detected from *H. punctata*, *R. sanguineus*, *H. japonica* and the

Japanese Sika deer (Lee et al., 2014; Nunes et al., 2016), were genetically related to the relapsing fever group borreliae, *B. lonestari, B. miyatomi* and *B. theileri*. The zoonotic potential of these novel borreliae are still unknown. At the inception of the present study, there was only one previous study documenting exposure of humans to borreliae infections in Malaysia, but the epidemiology of these infections was not known (Tay et al., 2002). More recent serological surveys conducted in Malaysia (Khor et al., 2019) revealed the possible past *Borrelia* infections among the Orang Asli. This current study provides the first evidence of the presence of *Borrelia* bacteria in ticks in Malaysia, which may account for the human infection and exposures. The presence of another *Borrelia* sp. closely related to the LD group borreliae, *Borrelia yangtzensis*, from *Ixodes granulatus* ticks in Malaysia (Khoo et al., 2018). Further investigation including isolation, propagation and infection studies are warranted to determine the medical and veterinary significance of these newly identified borreliae noted in ticks in Malaysia.

CHAPTER 6: CONCLUSION

Findings in the present study provide an insight to the microbiome of *Amblyomma testudinarium, Haemaphysalis hystricis*, *Dermacentor steini*, *Dermacentor atrosignatus* and *Dermacentor compactus* sampled from wild boar present in the Orang Asli community in Malaysia. Our results suggest the presence of diverse bacterial communities in each tick species. Beta diversity analysis suggests that tick species and animal hosts influenced the differences observed within the bacterial communities in ticks recovered from the wildboars. Molecular verification of *Coxiella, Rickettsia* and *Borrelia* in these tick samples revealed the presence potential agents of tick-borne illness, including *C. burnetii* and relapsing fever group *Borrelia* and spotted fever group *Rickettsia*. These findings are consistent with the earlier findings describing the seropositivity among the Orang Asli populations of Peninsular Malaysia. Some of the bacteria identified may also serve as endosymbionts since there appeared to be a close association of the bacteria with the tick host species in the diversity and phylogenetic analysis. Further investigation including isolation, propagation and full genome sequencing, and infections are required to verify the phylogenetic position in relation to other known species, and to examine the potential implications of these organisms to both human and animal health.

These findings presented in the present study provide the foundation in which further research could be performed to dissect the relationships between the tick bacterial communities and the vector capacity of the tick species studied. Studies such as to investigate bacterial communities in respective tick's organs are crucial in the future to investigate the impact of these bacterial on the specific tick species and the potential transmission of zoonotic agents to the animal hosts, and possibly leading to diseases in humans and animals.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications arising from this thesis

Jing-Jing, Khoo, **Fang-Shiang, Lim**, Fezshin, Chen, Wai-Hong, Phoon, Chee-Sieng, Khor, Li-Yen, Chang, Sazaly AbuBakar (2016) *Coxiella* detection in ticks from Malaysia. *Vector-Borne and Zoonotic Diseases*. DOI: 10.1089/vbz.2016.1959. (ISI-Cited Publication, IF: 1.956)

Jing-Jing, Khoo, **Fang-Shiang, Lim**, Kim-Kee, Tan, Fezshin, Chen, Wai-Hong, Phoon, Chee-Sieng, Khor, Brian L, Pike, Li-Yen, Chang, Sazaly AbuBakar. Detection in Malaysia of a *Borrelia* sp. detected *Haemaphysalis hystricis*. Journal of Medical Entomology. DOI: https://doi.org/10.1093/jme/tjx131 Published 13 July 2017 (ISI-Cited Publication, IF: 1.712)

Other publications

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