MOLECULAR DETECTION OF *BORRELIA, BARTONELLA* AND *ORIENTIA* IN SMALL MAMMALS FROM OIL PALM PLANTATIONS IN MALAYSIA

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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Name of Candidate: **Siti Nurul Izzah Binti Mohd Azami**

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Name of Degree: **Master of Philosophy**

Title of Dissertation ("this Work"): **Molecular detection of *Borrelia*, *Bartonella* and *Orientia* in small mammals from oil palm plantations in Malaysia**

Field of Study: **Health (Molecular Screening of Pathogen)**

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MOLECULAR DETECTION OF *BORRELIA*, *BARTONELLA* AND *ORIENTIA* IN SMALL MAMMALS FROM OIL PALM PLANTATIONS IN MALAYSIA

ABSTRACT

Many human clinical cases due to vector-borne infections are underreported in Malaysia, especially in rural localities where healthcare infrastructures are lacking. Land clearing activities and encroachment into the forest, bring the human-animal interface closer to each other. Rodents and other small mammals in the wild also carry diseases, which are relatively different from those carried by urban rodents, increasing the risk of transmission to humans when forests are cleared. In this study, the aim was to detect the presence of vector-borne pathogens such as *Orientia* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. in the spleen of small mammals trapped on site. 217 small mammals were selected for this study, of which 100 samples were from UM Plantation Sdn. Bhd., Johor (oil palm plantation) and 117 samples from Kampung Tumbuh Hangat, Perak (oil palm plantation in the vicinity of an Orang Asli village). From the morphological identification and DNA barcoding assay performed using the mitochondrial gene, cytochrome c oxidase (COI), the individuals were identified as *Rattus rattus diardii*, *Rattus tiomanicus*, *Rattus argentiventer*, *Rattus exulans*, *Rattus tanezumi* and *Tupaia glis*. 203 spleens were collected and screened via conventional Polymerase Chain Reaction (PCR) assay. The molecular examination was performed for the detection of *Orientia* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. from the harvested spleens. From the PCR assays, there was a prevalence of each pathogen such as 12.3% (25/203) for *Orientia* sp., 5.9% (12/203) for *Borrelia* spp., 4.9% (10/203) for *Bartonella* sp. and none for *Rickettsia* spp. Almost all of the animals were found to be infested with chiggers and several were infested with ticks. Fleas were not found on the individuals. There were also lice and mites infesting the rodents. The infestation of a substantial number of chiggers

could explain the exposure of *Orientia tsutsugamushi* in the rodents and tree shrews. This speculation must be investigated further in order to confirm the vector for this pathogen. As for the *Borrelia* sp., all the infected individuals were not infested by any tick which is known to be the vector for Lyme disease. Similarly, *Bartonella phoceensis* was detected in individuals with no infestation of the tick vector. No flea was found on the trapped animals which explained the negative detection for *Rickettsia* spp. Based on the phylogenetic analysis, some of the detected *Borrelia* sp. were clustered together with the Lyme disease group and some were grouped together with the relapsing fever group. Interestingly, one positive *Borrelia* sp. sample was neither close nor similar to any of the Lyme disease and relapsing fever groups *Borreliae* in GenBank. From the result, there was a sign of current infection of *Borrelia* spp., *O. tsutsugamushi* and *B. phoceensis* in *Rattus r. diardii, R. exulans, R. argentiventer, R. tiomanicus* and *T. glis.* To the best of the current literature, this is the first report of the infection of *Borrelia* sp. and *O. tsutsugamushi* in *T. glis* in Malaysia. Further investigations are warranted to elucidate the relationships between the ectoparasites, the host and the respective pathogens.

Keywords: *Bartonella* sp., *Borrelia* sp., infectious disease, *Orientia* sp., *Rickettsia* sp., *Rattus* sp.

PENGESANAN MOLEKULAR UNTUK *BORRELIA*, *BARTONELLA* DAN *ORIENTIA* DALAM MAMALIA KECIL DARI LADANG KELAPA SAWIT DI MALAYSIA

ABSTRAK

Kebanyakan kes klinikal manusia akibat daripada jangkitan bawaan vektor adalah kurang dilaporkan di Malaysia, terutamanya di kawasan luar bandar yang serba kekurangan dari aspek infrastruktur penjagaan kesihatan. Pembangunan kawasan secara berterusan telah mengakibatkan aktiviti pembersihan tanah dan pencerobohan dalam hutan, seterusnya merapatkan jurang pertembungan antara manusia dan haiwan. Tikus dan mamalia kecil liar lain juga berisiko untuk membawa penyakit, yang mana agak berbeza daripada yang dibawa oleh spesis tikus bandar. Hal ini bakal meningkatkan risiko penularan kepada manusia apabila hutan ditebang. Dalam kajian ini, tujuannya adalah untuk mengesan kehadiran patogen bawaan vektor seperti *Orientia* spp., *Borrelia* spp., *Bartonella* spp. dan *Rickettsia* spp. di dalam limpa mamalia kecil yang diperangkap dari kajian lapangan. 217 sampel mamalia kecil telah dipilih untuk kajian ini di mana 100 sampel daripadanya adalah dari UM Plantation Sdn. Bhd., Johor (ladang kelapa sawit) dan 117 sampel adalah dari Kampung Tumbuh Hangat, Perak (kampung Orang Asli). Daripada pengenalpastian morfologi dan ujian barkod DNA yang dilakukan menggunakan protein mitokondria, iaitu penanda genetik cytochrome c oxidase (*COI*), beberapa spesis telah dikenal pasti sebagai *Rattus rattus diardii, Rattus tiomanicus, Rattus argentiventer, Rattus exulans, Rattus tanezumi* dan *Tupaia glis*. 203 limpa telah dikumpulkan dan disaring melalui ujian Tindak Balas Berantai Polimeras (*PCR*) konvensional. Pengesanan secara molekul dilakukan untuk mengesan *Bartonella* sp., *Borrelia* sp., *Orientia* sp. dan *Rickettsia* sp. daripada limpa yang dikumpulkan. Daripada ujian *PCR*, terdapat kadar jangkitan yang rendah bagi setiap patogen seperti 25/203

(12.3%) untuk *Orientia* sp., 12/203 (5.9%) untuk *Borrelia* spp., 10/203 (4.9%) untuk *Bartonella* sp., dan tiada untuk *Rickettsia* spp. Hampir kesemua haiwan itu digigit oleh kutu hama dan beberapa daripadanya dijangkiti sengkenit. Pepinjal tidak ditemui pada individu. Terdapat juga kutu *lice* dan kutu *mite* yang menggigit spesis tikus. Sejumlah besar kutu hama dijumpai boleh menjelaskan jangkitan *Orientia tsutsugamushi* pada tikus dan tupai muncung besar. Spekulasi ini perlu dikaji lebih lanjut untuk mengesahkan vektor bagi patogen ini. Bagi *Borrelia* sp., semua individu yang dijangkiti tidak digigit oleh sebarang sengkenit yang diketahui sebagai vektor penyebaran penyakit Lyme. Hal yang sama berlaku untuk *Bartonella phoceensis* yang dikesan pada individu tanpa vektor. Ini mungkin disebabkan oleh perumah itu sendiri sesuai untuk membawa patogen itu tanpa vektor. Ketiadaan pepinjal ditemui pada individu yang diperangkap menjelaskan ketiadaan jangkitan *Rickettsia* sp.. Berdasarkan pokok filogenetik, sebahagian jangkitan *Borrelia* sp. didapati tergolong dalam kumpulan penyakit Lyme dan ada yang tergolong bersama dengan kumpulan demam berulang. Menariknya, salah satu jujukan yang positif untuk *Borrelia* sp. didapati tidak hampir sama mahupun serupa dengan mana-mana kumpulan sama ada kumpulan penyakit Lyme atau kumpulan demam berulang *Borreliae* di GenBank. Daripada dapatan hasil, terdapat tanda jangkitan semasa *Borrelia* sp., *O. tsutsugamushi* dan *B. phoceensis* pada *Rattus r. diardii*, *R. exulans, R. argentiventer, R. tiomanicus* dan *T. glis*. Sepengetahuan saya, ini adalah laporan pertama jangkitan *Borrelia* spp. dan *O. tsutsugamushi* pada *T. glis* di Malaysia. Siasatan lanjut diperlukan untuk menjelaskan hubungan antara ektoparasit, perumah dan patogen masing-masing.

Kata kunci: *Bartonella* sp., *Borrelia* sp., penyakit berjangkit, *Orientia* sp., *Rickettsia* sp., *Rattus* sp.

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%	:	Percentage
°C	:	Degree Celcius
µℓ	:	Microlitre
µM	:	Micromolar
iTOL	:	Interactive Tree of Life
Bbsl	:	*Borrelia burgdorferi sensu lato*
BIC	:	Bayesian Information Criterion
BLAST	:	Basic Local Alignment Search Tool
BOLD	:	Barcode of Life Data Systems
bp	:	Base pair
CDC	:	Centers for Disease Control and Prevention
CERoPath	:	Community Ecology of Rodents and their Pathogens
clpA	:	ATP-dependent Clp protease ATP-binding subunit gene
COI	:	Cytochrome c oxidase I gene
COII	:	Cytochrome c oxidase II gene
CSD	:	Cat-scratched disease
DNA	:	Deoxyribonucleic Acid
EDTA	:	Ethylenediamine tetraacetic acid
ELISA	:	Enzyme-linked immunoassay
F	:	Female
flaB	:	Flagellin B gene
ftsZ	:	Cell division protein gene
g	:	Relative centrifugal force
gltA	:	Citrate synthase gene

HKY	:	Hasegawa–Kishono–Yano
IFA	:	Immunofluorescence assay
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
ITS	:	Intergenic spacer gene
kDa	:	Kilodalton
km	:	Kilometer
LD	:	Lyme disease
m	:	Metre
mg	:	Milligram
mg kg^{-1}	:	Milligram per kilogram
mℓ	:	Millilitre
M	:	Male
MCC	:	Maximum clade credibility
MCMC	:	Bayesian Markov Chain Monte Carlo
MEGAX	:	Molecular Evolutionary Genetics Analysis X
MEGA 11	:	Molecular Evolutionary Genetics Analysis 11
MLST	:	Multi-locus sequence typing
NCBI	:	National Center for Biotechnology Information
NFW	:	Nuclease free water
NJ	:	Neighbour-joining method
nuoG	:	NADH-quinone oxidoreductase subunit G gene
ompA	:	Outer membrane protein A gene
ompB	:	Outer membrane protein B gene
ORF	:	Open reading frame
pap31	:	31kDa major protein gene

PCR	:	Polymerase chain reaction
PP	:	Posterior probability
ppdK	:	Pyruvate, phosphate dikinase precursor gene
RF	:	Relapsing fever
rpoB	:	RNA polymerase beta-subunit gene
RrC	:	*Rattus rattus* Complex
rRNA	:	Ribosomal ribonucleic acid
rrs	:	16S rRNA protein gene
S	:	Sedimentation coefficient
s. l.	:	*sensu lato*
s. s.	:	*sensu stricto*
Sdn. Bhd.	:	Sendirian Berhad
SFG	:	Spotted fever group
ssrA	:	Transfer-messenger RNA gene
sucD	:	Succinyl-CoA synthetase gene
TAE	:	Tris base, acetic acid and EDTA
TCB	:	Tick Cell Biobank
TIDREC	:	Tropical Infectious Disease Research and Education Centre
TSA47	:	47 kDa type specific antigen gene
TSA56	:	56 kDa type specific antigen gene
UM	:	Universiti Malaya
UMH	:	UM Holdings
USA	:	United State
WHO	:	World Health Organization
V	:	Volt

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CHAPTER 1: INTRODUCTION

1.1 Background studies

Vector-borne diseases are a group of diseases transmitted via intermediate hosts such as mosquitoes, ticks, fleas, mites and lice. The diseases associated with these vectors would usually have atypical symptoms such as fever, rashes and chills. Factors such as urbanisation, rapid land surface changes and agricultural practices may influence the outbreak of these diseases (Gubler, 1998). Scrub typhus (Chakraborty & Sarma, 2017), murine typhus (Brown *et al.*, 1977; Walter *et al.*, 2012), Lyme diseases (Hamšíková *et al.*, 2017) and spotted fever group ricketsioses (Aung *et al.*, 2014) have been reported to be endemic globally, not only restricted to developed countries but also the developing countries such as Malaysia. Several vector-borne pathogens have become a concern in southeast Asia such as *Orientia* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. since they are increasingly reported to be detected in the vectors, peri-domestic animals and even humans (reviewed by Meerburg *et al.* (2009)). To date, there are increasing reports on vector-borne diseases being reported in humans by seroprevalence studies. These diseases have been detected in the aborigines locally known as the Orang Asli. Their settlements have been known to be situated near to the forest fringes, making them be the highly vulnerable for exposure towards vector-borne pathogens (Lai, 2011).

The vector-borne pathogens are residing inside the vectors which are then harboured by the animal reservoirs. The potential reservoirs are the peri-domestic animals which can be easily found in the forested areas and near to human habitation (Muul *et al.*, 1977) such as the rodents (i.e. *Rattus* spp., *Maxomys* spp. and *Bandicota* spp.) and common tree shrews (i.e. *Tupaia glis*). They have been reported to be infested by vectors such as the *Ixodes* spp. ticks (Khoo et al., 2018), *Xenopsylla* spp. fleas (Kernif *et al.*, 2012) and *Leptotrombidium* spp. chigger mites (Huang *et al.*, 2017). Several vector-borne pathogens have become a concern in southeast Asia such as *Orientia* spp., *Borrelia* spp., *Bartonella*

spp. and *Rickettsia* spp. since they are increasingly reported to be detected in the vectors, peri-domestic animals and even human infections (Meerburg *et al.,* 2009; Bai *et al.,* 2012).

Orientia spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. have been known to be the causative agents for scrub typhus (Luce-Fedrow *et al.,* 2018), Lyme disease (Pun *et al.,* 2018), trench fever (Brouqui, 2011; Boutellis *et al.*, 2012), and typhus and spotted fever group (Bermúdez & Troyo, 2018) respectively. These pathogens can be detected molecularly by using the polymerase chain reaction (PCR) assay aside from serological tests and inoculation method that are available since decades ago (Jackson *et al.*, 1957; Walker *et al.*, 1973). Since there is limited information regarding the reservoirs, vectors, ecology and distribution of these vector-borne pathogens in Malaysia, more studies are needed to understand the epidemiology of these pathogens and allow the planning for the prevention and treatment.

Rodents and common tree shrews served as the reservoirs of pathogen-carrying vectors that can potentially infect humans especially the oil palm plantation workers. The oil palm plantation was chosen as the target of this research because they are located near the forests in which rodents are mostly found as pests (Sinniah *et al.*, 1978). Rodents are known as pests that are damaging the fruits thus affecting the end product of palm oil (Puan *et al.*, 2011). Based on the study, many species of the rodents can be found abundantly in the oil palm plantation. Some of the common species of rodents that can be found in oil palm plantation are *Rattus rattus diardii* Jentink, followed by *Rattus argentiventer* and *Rattus tiomanicus* Miller. Not limited to *Rattus* spp. mentioned, other species also could be found in the oil palm plantation in Malaysia such as *Maxomys baeodon, Maxomys rajah, Maxomys whiteheadi, Mus musculus, Rattus exulans, Sundamys muelleri* and *Rattus rattus* (Mohd-Azlan *et al.*, 2019). The role of rodents in

the transmission of vector-borne pathogens to the oil palm plantation workers need to be further investigated to understand the transmission dynamics of these diseases.

1.2 Problem Statement

Most vector-borne diseases are known to cause undifferentiated and general clinical symptoms in human such as fever and body aches, and they can only be distinguished using specific laboratory tests. Several seroprevalence studies have showed that there were human exposure to *Orientia* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. in Malaysia and neighbour countries. However, there are limited study that focused on the reservoir or vector for those pathogens. Moreover, not much is known about the rodent population diversity in oil palm plantations located in rural areas of Malaysia.

1.3 Significant of the study

Many vector-borne disease is endemic in Malaysia especially when these diseases are carried by mosquitoes, ticks, fleas and mites. Since majority of previous studies have researched the prevalence of pathogens in the arthropods vectors, the presence of these pathogens in the animal reservoirs are generally overlooked. In Malaysia and Thailand, previous studies have reported the detection of *Orientia* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. in humans. These pathogens were believed to be carried by pests such as rodents and tree shrews, commonly found in the palm plantation areas. Therefore, the oil palm plantation workers have a very high likelihood of exposure to these pathogens carried by reservoirs (rodents and tree shrews).

1.4 Objective

The main objective of this research is to determine the presence of vector-borne pathogens in wild rodents and tree shrews sampled from two oil palm plantations in Malaysia. Two specific aims are designed to achieve the goal of this study as follows:

a) To identify the major rodent and tree shrew species available at the oil palm plantations by employing both morphological and molecular identification.

b) To detect the presence of vector-borne pathogens genetically, specific members of the *Orientia, Borrelia, Bartonella* and *Rickettsia* genera, in the rodents and common tree shrews found in the oil palm plantations.

CHAPTER 2: LITERATURE REVIEW

2.1 Vector-borne diseases and their pathogens

Vector-borne diseases are diseases transmitted by infected arthropods, including mosquitoes, ticks, fleas and mites. In Malaysia, one of the most prevalent (endemic) vector-borne diseases is dengue and this virus is carried by mosquito vectors. However, there has been an increase in the reports of other vector-borne diseases such as scrub typhus (caused by *Orientia tsutsugamushi*) (Muul *et al.*, 1977; Tay *et al.*, 2000; Mohamed *et al.*, 2016), Lyme disease (caused by *Borrelia burgdorferi sensu lato,* the Bbsl group) (Khor *et al.,* 2019), cat-scratch disease and trench fever (caused by *Bartonella* spp.) (Hou *et al.*, 2022) and rickettsiosis (caused by *Rickettsia* spp.) (Brown *et al.*, 1977; Tay *et al.*, 1999; Mokhtar & Tay, 2011; Tay *et al.*, 2015; Kho *et al.*, 2017). These vector-borne diseases are often transmitted to humans by arthropods, including chiggers, ticks and fleas. Previously, several seroprevalence studies have been carried out to determine the exposure of humans to these vector-borne diseases. The studies focused on the serological testing on local aborigines, also known as the Orang Asli, to detect past exposure to vector-borne diseases caused by pathogens such as *Orientia* spp. (Tay *et al.,* 2014a), *Borrelia* spp. (Khor *et al.,* 2019) and *Rickettsia* spp. (Kho *et al.,* 2017). Their nomadic lifestyle and dependence on forest produce and wildlife for sustenance have increased their risk of exposure to arthropod bites. Thus, they have a high risk of infection from these vector-borne pathogens. There is still limited information on the actual vector species (i.e., tick, chigger or flea species) as well as the animal reservoirs for these vector-borne pathogens.

Many studies have been conducted to detect the presence of vector-borne pathogens in vectors such as the *Orientia* spp. (Sanprick *et al.*, 2019), *Borrelia* spp. (Bao-Gui *et al.*, 2021), *Bartonella* spp. (Nguyen *et al.*, 2020) and *Rickettsia* spp. (Mokhtar & Tay, 2011; Pramestuti *et al.*, 2018) from different countries in Southeast Asia. These detections were

performed in the vectors such as chiggers, lice, ticks and fleas. Therefore, not much is known about the presence of vector-borne pathogens in the animal reservoir.

2.1.1 Lyme disease and relapsing fever caused by *Borrelia* sp.

Borrelia spp. is a spirochete known to be pathogenic to humans. The genus was eventually called *Borrelia* after Amédée Borrel, who studied the soft tick chicken spirochaete *Spirochaetagallinarum* (known as *Borrelia gallinarum*). This spirochete is transmitted mainly from the ticks tohumansn or peridomestic animals. It can be classified into two infectious groups namely the Lyme disease group and the relapsing fever group. Surprisingly, there was a recent study that reported the potential of a third borreliae group occurrence based on phylogenetic analyses (Binetruy *et al.*, 2020). This third borreliae group was reported to be isolated from the avian. However, more investigation must be performed to elucidate the third group of borreliae.

The name "relapsing fever" was developed to characterise the clinical condition following an outbreak of infection in Edinburgh between the years 1843 – 1848. Nevertheless, the aetiology of this infection remained unknown until Otto Obermeier's rigorous research in 1873 while working at the Berlin Charité Hospital (reviewed in (Cutler, 2010)). The name ‘Lyme disease’ originated from a clinical case diagnosing a skin lesion from a patient, came from Lyme in Connecticut in 1970s (reviewed by Elbaum-Garfinkle (2011)). Lyme disease is endemic in Europe and it is caused by Bbsl carried by ticks (Saint Girons *et al.*, 1998). Lyme disease borreliosis research has since increasing, but relapsing fever has largely been ignored, briefly viewed as a neglected tropical sickness.

There are many previous studies being reported on the infection of Lyme disease-related (LD) borreliae in human worldwide. Bbsl (also known as the Lyme disease pathogen) is commonly identified in human infections and this group contained several

close phylogenetic sister taxa such as *Borrelia burgdorferi sensu stricto* (*s. s.*), *Borrelia afzelii* and *Borrelia garinii* (Tay *et al.*, 2002a; Strle *et al.*, 2006; Khor *et al.*, 2019). A species known as *Borrelia valaisiana* closely related to the *B. burgdorferi s. s.* was reported as non-pathogenic to humans as there was not enough evidence to make a definite conclusion on its pathogenicity (Margos *et al.*, 2017). Meanwhile, *B. afzelii* and *B. garinii* are known to cause the neuroborreliosis (Strle *et al.*, 2006). The study also discovered that the symptoms caused by *B. garinii* is distinguishable compared to the *B. afzelii* (Strle *et al.*, 2006).

On the other hand, *Borrelia miyamotoi* and its closely related species such as the *Borrelia yangtzensis*, *Borrelia parkeri, Borrelia johnsonii, Borrelia recurrentis, Borrelia crocidurae, Borrelia anserina, Borrelia kalaharica, Borrrelia lonestari, Borrelia theileri* and *Borrelia turicatae*, are known as relapsing fever group pathogens. Among them, *B. yangtzensis* are still unknown for its transmission and pathogenicity. To date, there has been no relapsing fever clinical cases reported in Malaysia. In contrast, there were studies reported the infection of Lyme disease in Malaysia. For example, Tay *et al.* (2002a) reported the prevalence of *B. afzelii* in the blood donors and patients with different clinical diagnosis upon admission such as leptospirosis, tick typhus, rickettsial infection and melioidosis. There was 16.3% of Lyme disease past exposure (IgM antibodies) detected from the patients and only one out of thirty random blood donors have been exposed (Tay *et al.*, 2002a). This finding indicated that co-infection and mixed infection can possibly happen. Orang Asli is one of the communities in Malaysia, who stays near to the forest fringe and agricultural areas. A recent seroprevalence study has been reporting the occurrence of Lyme disease in Orang Asli villages. That seroprevalence was performed in 16 villages, revealed that 8.1% of the total Orang Asli individuals was seropositive for *B. burgdorferi* IgG antibodies (Khor *et al.*, 2019). There was a seroprevalence study in Russia that reported the detection of *B. miyamotoi, B. garinii* and Bbsl in patients who

were suspected to acquire tick-borne infection (Platonov *et al.*, 2011b). An infection rate of 36.1% 17%, and 7% and for Bbsl, *B. miyamotoi* and *B. garinii,* respectively, was reported in the patients diagnosed. The symptoms that occurred to the patients were fever, headache, chills, fatigue, vomiting, and myalgia. All of the infected patients were bitten by ticks prior to hospital admission. From the observation taken, the tick bite to symptom onset took 15 days for *B. miyamotoi* and shorter for *B. garinii*, 10 days. The weakness of the serological assays were the backdated assay data obtained and not being validated with new genospecies especially from the Bbsl complex. This will cause a limited information obtained. Therefore, the use of molecular detection has become popular in detecting the borreliae since it is more specific.

To date, there were many studies focusing on the reservoir or the host and vectors of the borreliae. Many seroprevalence and molecular detection were performed to collect data as many as possible, as the borreliae is also one of the vector-borne pathogens that are having genetic divergence (reviewed in (Oppler *et al.*, 2021)). Sarawak has seen major forest degradation and fragmentation, mostly as a result of logging operations and oil palm plantations (Bryan *et al.*, 2013; Gaveau *et al.*, 2014), and land conversion has been linked to an increase in developing or re-emerging zoonotic illnesses, particularly vector-borne diseases (Zohdy *et al.*, 2019). Recently, there was a report on the discovery of the relapsing fever borreliae, *B. yangtzensis* and *B. miyamotoi* in rodents using molecular method (Lau *et al.*, 2020), in which warranted further investigation as their pathogenicity on human and animal hosts is still unknown. To date, none of the clinical cases related to *B. miyamotoi* or *B. yangtzensis* was reported in Malaysia. The study focused on the protected forestry area and oil palm plantation. The prevalence rate of borreliae in Gunung Gading National Park (GGNP) was 16.7% while in oil palm plantation was 8.9%. Borreliae were detected in the spleens of forest rodent, *Sundamys muelleri* and synanthropic rodents, the *Rattus* spp. (Lau *et al.*, 2020).

A few years ago, a study reported both groups of pathogen, the LD and RF borreliae in wild rodents captured in Japan including the *B. miyamotoi, B. afzelii* and *B. garinii* (Taylor *et al.*, 2013). There was 15.8% of urinary bladder and 7.3% of blood of the rodents were *Borrelia*-positive molecularly. The hosts were identified as *Apodemys speciosus, Mus rufocanus, Apodemys argenteus* and *Mus rutilus*. None of the *Rattus* spp. was trapped in the study. The adult rodents were found as significantly risked to the *B. garinii* and *B. afzelii*. The study suggested that *B. miyamotoi* might persist in other organs instead of urinary bladder and blood, providing an example of *Borrelia duttoni* that remain in the brain of mice in a previous study (Larsson *et al.*, 2006). Prior to the study, another study in Japan as well has concluded that rodent is the main reservoir for the *B. garinii* (Takano *et al.*, 2011). The study explained that *B. garinii* isolates ST group B was the predominant strains in rodents and clinical samples, based on several reports in Japan and China. In southeast China, the closely related *B. garinii* and *B. valaisiana* have been detected in wild rodents (Chu *et al.*, 2008). The study was conducted in an area with forested hills in 2004. A total of four rodent species (8% of the total individuals) (i.e., *Niniventer confucianus*, *Niniventer coxingi*, *Apodemus sylvaticus* and *Rattus losea*) were found to be positive for both species belong to Bbsl complex (Chu *et al.*, 2008). During the time, both species were unknown to cause human disease yet. Previously, *B. burgdorferi* was detected in *Tupaia belangeri* in Yunnan Province (Liu *et al.*, 2009). Nonetheless, to date, there is no published data on the detection of *Borrelia* spp. in *T. glis*.

Lau *et al.* (2020) reported that a relatively high prevalence of borreliae, approximately 43.8% of the feeding *Ixodes* ticks collected in Sarawak, Malaysia were positive for *flaB*-PCR. In Japan, a number of 240 *Ixodes persulcatus* ticks collected by flagging and were included in a study (Taylor *et al.*, 2013). Out of total number collected, 163 were adult ticks and 77 were nymphs. PCR and sequencing analysis indicated that 49 (30.1%) adults and 6 (7.8%) nymphs were positive for the borrelial *flaB* gene. Among the adult samples,

44 (27.0%) were positive for *B. garinii*, 5 (3.1%) were positive for *B. afzelii,* and 3 (1.8%) were positive for *B. miyamotoi.* Of the nymphs, 6 (7.8%) were positive for *B. garinii,* and 1 (1.3%) sample was positive for *B. afzelii* (Taylor *et al.*, 2013). *B. garinii* comes from ticks as vector of rodents (Takano *et al.*, 2011). *Haemaphysalis longicornis* ticks may be typical in Japan and China, the subtropical countries and has been reported carrying the relapsing fever-related (RF) borreliae (Chu *et al.*, 2008; Furuno *et al.*, 2017; Nakayama *et al.*, 2019). A study has reported the detection of RF borreliae in the same genus of ticks, *Haemaphysalis hystricis* in Malaysia (Khoo *et al.*, 2017). The strain was closely related to borreliae detected in *Haemaphysalis japonica* and Sika deer from Japan. In contrast, a study in the USA suggested that *H. longicornis* has low vector competence in spreading LD borreliae (Breuner *et al.*, 2020). The vector competence of *Haemaphysalis* ticks in spreading RF borreliae remain unknown. In Malaysia, *Ixodes granulatus* ticks collected from rodents (i.e., *Sundamys muelleri*, *Maxomys whiteheadi*, *Leopoldamys sabanus*, and *R. rattus*) were carrying *B. yangtzensis* closely related (Khoo *et al.*, 2018). *I. granulatus* is predominantly found on small mammals in southeast Asia (Ishak *et al.*, 2018b; Vongphayloth *et al.*, 2018). To date, not many surveillance studies were found in Southeast Asia that detect the borreliae in ticks or other vectors.

2.1.2 Bartonellosis caused by *Bartonella* sp.

There are many infectious diseases caused by *Bartonella* spp. such as trench fever (Leulmi *et al.*, 2015), CSD (Karski *et al.*, 2018), Carrion's disease and Oroya fever (Garcia-Quintanilla *et al.*, 2019). The species known to be pathogenic to human include *Bartonella clarridgeiae*, *Bartonella tamiae, Bartonella henselae, Bartonella bacilliformis, Bartonella quintana, Bartonella alsatica, Bartonella koehlerae, Bartonella mayotimonensis, Bartonella vinsonii* and *Bartonella elizabethae* (Kordick *et al.*, 1997; Kosoy *et al.*, 2008; Ordaya & Maguiña, 2020). *Bartonella* spp. are known to infect a number of cell types, including endothelial, erythrocyte, epithelial cells, and more

recently in stem cells (Regier *et al.*, 2016). Eventually, prolonged infection will cause complication in different organs, including endocarditis (Noopetch *et al.*, 2018), bacillary angiomatosis (Brzewski *et al.*, 2020), peliosis hepatis (Ahsan *et al.*, 1998), neuroretinis (George *et al.*, 2006), bacteremia (Kordick *et al.*, 1997), and encephalopathy (Samarkos *et al.*, 2018). Non-febrile patients were reported to have symptoms such as muscle pain, rash, anemia, eye pain or conjunctivitis (Bhengsri *et al.*, 2011). Louse-borne bartonellosis, *B. quintana*, was widely detected in person experiencing homelessness in western countries (Leibler *et al.*, 2016) and can be found to be co-infected with COVID19 (McCormick *et al.*, 2021). *B. henselae* was initially discovered in 1992 from an HIV-positive patient who had a persistent fever (Regnery *et al.*, 1992). Serological test might be not accurate for surveillance because the patient can be seroreactive in several years even after being treated (Okaro *et al.*, 2017). A rapid diagnose and sensitive test such as PCR must be applied in order to arrange treatments that are suitable for the patient immediately (Bai *et al.*, 2012; Noopetch *et al.*, 2018).

Bartonelloses in southeast Asia were primarily associated with exposure to cats. In Thailand, a patient was diagnosed with *B. henselae* endocarditis (Noopetch *et al.*, 2018) and the infection was suspected to originate from the exposure to his pet cats. The patient was a 51-year-old, having symptoms for about 3 months including a mass-forming lytic lesion was discovered using computed tomography scanning of the chest and abdomen. He has been treated (i. e. ceftriaxone and azithromycin), however, the patient was not recovering. A PCR assay was conducted resulting in 99% homology with the *B. henselae* 31kDa major protein (*pap31*) gene. The patient then only recovered after being treated with levofloxacin, azithromycin, doxycycline, and gentamicin. This was not the first case being reported in Thailand (Fournier *et al.*, 2010; Habib *et al.*, 2015). There were also reports of bartonelloses associated with exposure to rodents. A study from Thailand reported the discovery of *Bartonella vinsonii arupensis* infections in patients (Bai *et al.*,

2012). These four patients exhibited similar symptoms and reported were exposed to wild rodents at least 2 weeks before the diagnosis. The PCR screening targeted three genes such as transfer-messenger RNA (*ssrA*) gene, the citrate synthase (*gltA*) gene, and the 16S–23S rRNA internal transcribed spacer, resulted in sequences that were closely related to *B. vinsonii* subsp. *arupensis*. Not surprising, the strain has been reported in human in many clinical cases previously and also discovered from dog. In fact, the 3 of 4 patients kept dogs within their houses (Bai *et al.*, 2012). In addition, bartonelloses were also reported in three patients who were involved in trapping and killing rodents in their houses (Kosoy *et al.*, 2008). Laboratory analyses found that the strains isolated from the patients were genetically similar to each other and were representatives of a novel (at that time), *B. tamiae*. The study has implemented bacteria culture using the blood of patients and confirmed the identity via PCR detection targeting the citrate synthase (*gltA*), the cell division protein (*ftsZ*), the RNA polymerase beta-subunit (*rpoB*), the heat shock protein (*groEL*), and 16S ribosomal RNA (*rRNA*) genes, as well as the 16S-to-23S rRNA intergenic spacer (*ITS*) gene. Most reports of bartonelloses are recorded in Thailand vicinity. In another seroprevalence study, there was past exposure of *B. henselae* observed in 5.5% of healthy persons (mainly blood donors) in Thailand (Maruyama *et al.*, 2000). The prevalence of *Bartonella* spp. in febrile patients was reported in a study and 71% of them were exposed to rodents prior to diagnosis (Kosoy *et al.*, 2010). 7.7% of the blood clots culture was detected as *Bartonella*-positive via *gltA* targeted PCR. The strains detected were closely related to closely related to *B. elizabethae, B. rattimassiliensis, B. tribocorum, B. henselae, B. vinsonii* and *B. tamiae*. The study reveal that the homologous strains were usually reported in *Bandicota* spp. and *Rattus* spp. rodents. In another study, in rural Thailand, 11.7% of febrile and non-febrile patients tested had past exposure to *B. elizabethae, B. quintana, B. henselae* and *B. vinsonii* (Bhengsri *et al.*, 2011). The rate of infection was slightly higher than the previous study. However, none of the patients were

reported to be exposed to rodents in the past two weeks. The study mentioned that the seropositivity was frequent in non-febrile patient compared to febrile patients, in which could be caused by several factors (i. e. the seroconversion period during sample collection). It was the first report of *B. henselae* infection in Thailand. From this preliminary data presented, more studies have been extensively conducted in Thailand in the later years. From the previously reported studies, most of the pathogens found were *B. henselae*, *B. quintana* or other closely related species in human patients. Notably, seroprevalence has shortcoming in which the occurrence of cross-reactivity among the genus.

There are many reports regarding the potential reservoir for *Bartonella* spp. other than rodents such as bats (Veikkolainen *et al.*, 2014), pets (Chomel *et al.*, 2006), ground squirrel (Kosoy *et al.*, 2003) and others. However, nothing is confirmed yet regarding the main animal reservoir for the pathogen or interaction between reservoirs and accidental hosts except for cats, known as the animal reservoir for *B. henselae*. Currently, human is considered as the reservoir and accidental host for *Bartonella* spp. (Breitschwerdt, 2017). Multiple *Bartonella* spp. have been reported in rodents in various southeast Asian countries mainly in Thailand. Thailand has started to detect bartonellae in rodents since 2004 using standard culture techniques (Castle *et al.*, 2004). The study reported the prevalence of strains closely related to *Bartonella grahamii* and *B. elizabethae* (both caused human illness). The host were identified as *B. indica*, *R. losea* and *R. rattus*. The study also suggested that there might be new genotype during that time based on the clustering showed by the strains isolated from *B. indica* and *R. rattus*. However, there was not enough reference strains that were included in the phylogenetic analyses to indicate new genotype. A similar methodology applied in a recent study reported three new strains isolated from rodents (*Rattus* spp., *M. musculus* and *B. indica*) and shrews (*Suncus murinus*) in Thailand (Pangjai *et al.*, 2022). An increase of detection (11.5%) was

recorded since there were more species detected in rodents including the *B. tribocorum, B. rattimassiliensis, B. queenslandensis, B. elizabethae, B. henselae, B. coopersplainsensis, Bartonella chanthaburi* spp. nov., *Bartonella satun* spp. nov., and *Bartonella ranong* spp. nov. based on the *rpoB* and *gltA* genes analyses. Prior to the study by Pangjai *et al.* (2022), several studies have been done extensively. A separate study that was conducted in 17 provinces in Thailand uncovered 23 unique variants of *Bartonella* spp. from the captured rodents, clustered with the *B. tribocorum, B. elizabethae, B. rattimassiliensis, Bartonella phoceensis,* and *B. coopersplainensis* (Bai *et al.*, 2009). A relatively high prevalence was reported, 41.5% of the rodents (i. e. *Berylmys berdmorei, B. indica, Bandicota savilei, Mus cervicolor, R. argentiventer, R. exulans, Rattus nitidus, Rattus norvegicus, R. rattus* and *Rattus remotus*) were *Bartonella*-positive. The study suggested that the *R. norvegicus* and *R. rattus* could serve as reservoir based on the rate of infection obtained and other findings that had been discussed. The study also found that different species of rodents also could harbour same variants despite the discrete geographical area. Several years later, Pangjai *et al.* (2014) found *B. queenslandensis* in Thailand, adding on to the previous study. There was 15.5% of the small mammals (i.e., *Rattus bukit bukit, R. exulans, R. muelleri, R. nitidus, R. tanezumi, R. norvegicus, R. rattus, B. indica* and *S. murinus*) trapped from nine provinces have been infected altogether (Pangjai *et al.*, 2014). Eight of the total provinces are rural areas. *B. tribocorum* was found to has the highest prevalence in the study. Interestingly, the study utilised the frozen blood samples instead of fresh blood for culture and still successfully isolated the bartonellae colonies. *Bartonella* spp. was detected in 35% of rodents and five flea pools via nested PCR (Panthawong *et al.*, 2020). The detected species showed 98-100% identity to *B. queenslandensis.* In 2009, Saisongkorh *et al.* (2009) reported a new variant, *Candidatus* Bartonella thailandensis in *Rattus surifer*. However, there is currently no further information on the prevalence of this new variant. The phylogenetic analysis only

showed the clustering of this strain with *B. phoceensis* with 62% confidence of bootstrapping. Further genetic and phenotypic characterisation are necessary to confirm its status as a novel species. A prevalence study reported that there was 12.5% exposure in the provinces on the border of Cambodia, 3.7% in the provinces on the border of Myanmar and 3.4% in the provinces on the border of Lao PDR. Rodents infected were *B. savilei*, *M. cervicolor*, *R. berdmorei*, *R. exulans*, *R. rattus* and *R. surifer*. The strains were found to be closely related to *B. coopersplainsensis*, *B. phoceensis*, *B. queenslandensis*, *B. rochalimae* and *Bartonella* sp. RN24BJ. Anh *et al.* (2021) reported a relatively high molecular prevalence of *Bartonella* spp. infection (31.6%) in rodents captured in Vietnam. However, full information regarding the *Bartonella* strains obtained in the study was not provided. In Malaysia, the first evidence of *B. phoceensis* was reported in synanthropic rodents with the prevalence rate of 3.73% (Low *et al.*, 2020a). Beforehand, a short report showed 13.7% of rodents trapped in Kuala Lumpur and Penang, Malaysia were positive for *Bartonella* spp. (except the *B. phoceensis*) in rodents trapped in Kuala Lumpur and Penang, Malaysia (Tay *et al.*, 2014b). A few species, such as *B. rattimassiliensis, B. tribocorum,* and *B. elizabethae,* were detected in *R. norvegicus* and *R. diardii* (Tay *et al.*, 2014b). All of the bartonellae species detected are the concern of public health. The neighbouring country, Singapore, has conducted a study to determine the prevalence of *Bartonella* spp. in small mammals, reported that 20.8% of them positive using molecular methods (Neves *et al.*, 2018). The highest prevalence found was in *R. norvegicus* (75%). However, it was vague to conclude *R. norvegicus* as the main reservoir in that country since only 4 individuals were tested. Aside from *Rattus* sp., *S. muelleri* could also serve as the reservoir since Blasdell *et al.* (2019b) reported 87% of *Bartonella* spp. prevalence in that species in Sarawak, Malaysia. Indonesia (Winoto *et al.*, 2005) has started the molecular investigation upon the animal reservoir for *Bartonella* spp. followed by Laos (Angelakis *et al.*, 2009), Cambodia (Jiyipong *et al.*,

2012) and Malaysia (Asyikha *et al.*, 2020). Approximately 10.7% of rodents and shrews trapped along the Mekong River were infected with *Bartonella* spp. (Jiyipong *et al.*, 2012). Amongst the rodents, *Bartonella* spp. was found the most in *Rattus* sp. compared to other animals (Jiyipong *et al.*, 2012). Bartonellae detected were closely related to *B. queenslandensis*, *B. rattimassiliensis*, *B. tribocorum*, *B. elizabethae*, *B. coopersplainsensis* and *B. phoceensis.* Here, there were mix of known human pathogenic bartonellae and unknown status of pathogenicity bartonellae in which detected in small mammals. Angelakis *et al.* (2009) reported the presence of *Bartonella* spp. in rodents from four provinces in Laos with a prevalence rate of 25.5%. Two new variants were reported and assigned as Lao/Nh1 and Lao/Nh2 (Angelakis *et al.*, 2009). Altogether these studies showed that rodents especially the *Rattus* sp. were competent to harbor a wide spectrum of *Bartonella* spp. Most of the current studies reported the prevalence of *Bartonella* spp. in rodents and shrews. A previous study reported the detection of *Bartonella* sp. in one *T. glis* trapped in Singapore (Neves *et al.*, 2018). The study amplified the *rpoB*, NADH-quinone oxidoreductase subunit G (*nuoG*) and 16S *rRNA* genes for *Bartonella* spp., however, the DNA sequences did not match to any species in the database. In general, there is still lack of information on *Bartonella* spp. infection in *T. glis*. Overall, most of the prevalence reported in Thailand were from agricultural sites such as rice paddies, forests and plantations in which exposing risks of vector-borne disease transmission to the farmers (Saisongkorh *et al.*, 2009; Panthawong *et al.*, 2020). Another study by Blasdell *et al.* (2019b) reported that the prevalence of *Bartonella* spp. was higher in urban and developing areas compared to rural area. However, more data must be collected to evaluate the situation. Several other settings has been reported such as city (Tay *et al.*, 2014b) and a forest habitat (Neves *et al.*, 2018). More comprehensive research must be performed to elucidate the current status of synanthropic small mammals

as reservoir considering several factors including the ecological factors and richness of ectoparasites.

Based on the study by Frank *et al.* (2018), there are seven species associated to rodents identified to cause spillover to human, including to *Bartonella washoensis, B. vinsonii, B. grahamii, B. elizabethae* and three unknown species. Interestingly, *Bartonella* sp. was detected in some of the ectoparasites such as lice, fleas, chigger mites and ticks. Based on current knowledge, the role of the ectoparasites in the enzootic transmission of *Bartonella* cannot be determined. Less information was recorded regarding the role of oriental rat fleas, *Xenopsylla* spp. in transmitting *Bartonella* spp. among the rodents except for a study discussed by (Panthawong *et al.*, 2020). Recently, strains that were closely related to *B. queenslandensis* were found in both rodents and rodent-associated *Xenopsylla cheopis* fleas collected (Panthawong *et al.*, 2020). The pathogen species was also previously reported in rodent (Pangjai *et al.*, 2014), however, no ectoparasites associated was recorded to carry the pathogen species. Further investigation must be conducted to confirm the role of fleas from rodents as the vector of *Bartonella* spp.

2.1.3 Rickettsial agents

There is an increasing body of knowledge on the prevalence of rickettsial infections or rickettsioses, including scrub typhus, murine typhus and spotted fever, in southeast Asia (reviewed by Low *et al.* (2020b)). Findings from serological studies have attributed rickettsial infections as the cause of many undifferentiated febrile illnesses in southeast Asian countries such as Thailand and Indonesia (Tay *et al.*, 2000; Wangrangsimakul *et al.*, 2018; Luvira *et al.*, 2019; Lokida *et al.*, 2020). A random study also had been done to evaluate the prevalence of scrub typhus in Indonesia (Richards *et al.*, 2003). *Orientia tsutsugamushi*, the causative agent for scrub typhus (discovered in Japan), and the various *Rickettsia* spp. are transmitted via the bite of arthropods such as chiggers, fleas and ticks.

The role of rodents in the ecology of rickettsial diseases are well-established for scrub typhus and murine typhus, as rodents are the primary animal hosts for the arthropods vector. However, the role of rodents in the transmission for spotted fever is less apparent.

2.1.3.1 Scrub typhus caused by *Orientia tsutsugamushi*

Previously, scrub typhus was thought to be restricted to the Tsutsugamushi Triangle, an area encompassing Asia (up to China, Korea and Japan), towards the west in India, and to the south near the northern regions of Australia. However, recently the disease and the etiological agent were found to exist in a wider geographical area, including Chile, Africa and the United Arab Emirates as reviewed by Xu *et al.* (2017) and (Richards & Jiang, 2020). This finding has caused a concern worldwide as the scrub typhus must be treated as soon as possible despite the typical symptoms reported in patients, in which causing difficulties in diagnosis stage. Some of the examples were headache, lymphadenopathy and the presence of eschar (Tilak *et al.*, 2011). *O. tsutsugamushi* is has been known as he causative agent since decades ago until recently, a newly reported species, namely *Orientia chuto*, has caused scrub typhus in United Arab Emirates (reviewed in (Richards & Jiang, 2020)). This new species was detected and described in 2010, from a patient harboring this unrecognised pathogen (during that time) from Dubai, showing the scrub typhus symptoms (Izzard *et al.*, 2010). This prevalence proved that scrub typhus is no longer circulating within the Tsutsugamushi Triangle. Blood from the patient had been collected and was pursued for cell culture, subsequently, extracted for genomic DNA. Then, the DNA was subjected to conventional PCRs and real-time PCR to further characterised the new *Orientia* strain. The new strain was diverged from 16S rRNA protein (*rrs*) gene reference sequences at 2%, positioned outside of the *O. tsutsugamushi* clade (have less than 1% divergence). In 56 kDa type specific antigen (*TSA56*) gene analysis showed 47 to 58% of divergence while 47 kDa type specific antigen (*TSA47*) showed 17.7 to 18.2% of divergence of *O. chuto* strain Dubai. It was

mentioned that the divergence is greater than the *O. tsutsugamushi* strain Shimokoshi. These finding merit further investigation to identify the factors that causing the occurence of new genetic identity of *Orientia*.

The role of rodents and the chigger vectors in the ecology of scrub typhus have been widely investigated since the disease was first described in Japan in the 1800s (reviewed in (Elliott *et al.*, 2019; Richards & Jiang, 2020)). From the review studies, this scrub typhus has been circulating since centuries ago without any proper detection and containment measures. Epidemiological investigations of recent outbreaks still associate the presence of rodents and chiggers with the scrub typhus outbreaks (Tilak *et al.*, 2011; Rodkvamtook *et al.*, 2018). For example, in a recent outbreak of scrub typhus among soldiers attending a training in Chonburi, Thailand, epidemiological follow-up studies revealed high prevalence of *O. tsutsugamushi* infections in rodents and chiggers within 10 km radius of the training site (Rodkvamtook *et al.*, 2018). In addition to *Leptotrombidium deliense*, a well-known vector of scrub typhus, a study by Tilak *et al.* (2011) found that *Schoengastilla ligula* were also the vector in an outbreak of scrub typhus in India.

Rodents are known to be the primary hosts for chiggers, hence contribute to the dispersal of the scrub typhus vector. Rodents themselves may also be infected with *O. tsutsugamushi*, and could possibly serve as a reservoir for that pathogen (Rodkvamtook *et al.* (2018). In a rodent survey across rural areas Thailand, approximately 42% of rodents, comprising of *R. rattus, R. exulans, R. losea, R. norvegicus* and *B. indica* species, were found positive for scrub typhus by immunofluorescence assay (IFA) and PCR (Lerdthusnee *et al.*, 2008). Other southeast Asian countries also reported the detection of *O. tsutsugamushi* in rodents across the years, such as Indonesia (Richards *et al.*, 1997), Malaysia (Hanifah, 2013) and the Philippines (Van Peenen *et al.*, 1977). Both Richards

et al. (1997) and Van Peenen *et al.* (1977) utilised the serological tests, in such indirect route like enzyme-linked immunoassay (ELISA) nowadays, because the organ of the rodents captured were crushed first and injected into the laboratory rodent intraperitoneally. This methodology might expose the specimens to cross reactions. However, scrub typhus was not being investigated extensively in recent years in some of the countries mentioned. Rodents of the Muridae family, including *Apodemus agrarius*, *R. rattus*, *R. norvegicus*, *R. tiomanicus*, and *B. indica* were commonly found to be infected *O. tsutsugamushi* as reviewed by Elliott *et al.* (2019). A study reported by Coleman *et al.* (2003) detected the presence of *O. tsutsugamushi* in one *T. glis* trapped in Thailand, suggesting that *T. glis* might be the accidental host and competent to harbor the pathogen. Generally, less information on *T. glis* carrying the *O. tsutsugamushi* was recorded in southeast Asia.

Lerdthusnee *et al.* (2008) proposed that the dry season is associated with higher risks of scrub typhus due to the increase in rodent population and chigger densities. The presence of *O. tsutsugamushi*-infected rodents were also more likely to be found in habitats with forested covering (Chaisiri *et al.*, 2017b). In a more recent study, ecological analyses presented evidence of the positive correlation of chigger species richness and the latitude with the incidence of scrub typhus in Thailand (Chaisiri *et al.*, 2019). The study also suggests that there is still much to learn on the ecology of scrub typhus. Since most of the recent ecological information of scrub typhus are based on studies from Thailand, studies in other Southeast Asian countries are necessary since the rural and agricultural landscape may be differ from one country to the other. Notably, changes in landscape and climate also could contribute to the spread of scrub typhus (Roberts *et al.*, 2021). An outbreak was reported in periurban and rural areas, predominantly the tea plantation in India (Tilak *et al.*, 2011). The study sites also reported to have poor hygiene near to the residential area.

2.1.3.2 Murine typhus caused by *Rickettsia typhi*

Members of the typhus group *Rickettsia* spp. include *Rickettsia typhi* and *Rickettsia prowazekii,* are responsible for murine typhus (flea-borne rickettsioses, or endemic typhus) and louse-borne typhus (also known as epidemic typhus) respectively (Rauch *et al.*, 2018). However, only murine typhus is endemic in southeast Asia (Barbara *et al.*, 2010; Vallée *et al.*, 2010). 20.6% of the people in a study conducted within the city of Laos, had been exposed to the murine typhus (Vallée *et al.*, 2010). The study employed an ELISA assay in which specific to anti-*R. typhi* antibody detection only. Therefore, no other species of rickettsia could be detected. Murine typhus is a mild illness but in rare cases; infections may lead to fatality if not detected and treated early (Osterloh *et al.*, 2016). In southeast Asia, murine typhus contributes to cases of undifferentiated fevers, especially in the urban areas (reviewed in (Low *et al.*, 2020b)). In Thailand, approximately 3.5% and 5% of the patients' sera from studies in Chiangrai and Bangkok respectively were positive for murine typhus (Wangrangsimakul *et al.*, 2018; Luvira *et al.*, 2019). From 2004 until 2017, there was a past exposure of 17% among the patients (Roberts *et al.*, 2021). The study reported that most of the patients were exposed to rodents, fleas, also visited rice fields and forests before being diagnosed as murine typhus. However, statistically, the rice fields and forests were not associated to the exposure but home address in Vientiane Capital showed positive association. Higher prevalence of immunoglobulin G (IgG) in human serum was reported in the central zone of Laos compared to its periphery (Vallée *et al.*, 2010). The study also highlighted that poor household and lack of hygiene may contribute to the spread of murine typhus. Diagnosis is difficult as the symptoms closely resemble other endemic diseases; some of the patients were prescribed with ineffective and inappropriate antibiotics. Multiple seroprevalence studies for murine typhus further provide evidence of the risk of exposure in both rural and urban areas in this region (Strickman *et al.*, 1994; Vallée *et al.*, 2010; Trung *et al.*,

2017; Tappe *et al.*, 2018). In Laos, a recent study has successfully isolated *R. typhi* from patients whole EDTA-anticoagulated blood or EDTA buffy coat fraction, using the mammalian cell culture (Ming *et al.*, 2020). The finding has enabled new strains to be discovered in future.

Rodent species commonly found in rural or urban areas in southeast Asia were shown to be exposed to *R. typhi* in serological studies too. These species, including *R. tanezumi* (Widjaja *et al.*, 2016; Pramestuti *et al.*, 2018), *R. rattus* (Ibrahim *et al.*, 1999), *R. exulans*, *R. norvegicus* (Ibrahim *et al.*, 1999) and *M. musculus* (Chareonviriyaphap, Leepitakrat et al. 2014), are likely to serve as the reservoir for murine typhus in this region. In an Indonesian study, seropositivity in *R. norvegicus* was found to be highly significant compared to other rodent species studied, and most of the rodents with positive antibody response were captured from Jakarta, the capital city of Indonesia (Ibrahim *et al.*, 1999). Similar findings were reported by other studies in Indonesia (Richards *et al.*, 2002) and Thailand (Siritantikorn *et al.*, 2003; Chareonviriyaphap *et al.*, 2014), in which higher prevalence of *R. typhi* was observed for *R. norvegicus* and in urban setting, adding to the evidence that murine typhus is a disease associated with urban areas. Many studies have reported the prevalence of *R. typhi* in rodents in southeast Asia, however, Malaysia still lack of concrete data despite the clinical cases and seroprevalence study reported (Kho *et al.*, 2016; Tappe *et al.*, 2018; Yuhana *et al.*, 2019).

2.1.3.3 Spotted fever group *Rickettsia*

Spotted fever group (SFG) *Rickettsia* consists of at least 30 different species of *Rickettsia* bacteria globally and 21 of them are classified as pathogenic (reviewed by Satjanadumrong *et al.* (2019)). Similar to scrub typhus and murine typhus, the seroprevalence of SFG *Rickettsia* was reported in most southeast Asian countries (reviewed in (Low *et al.*, 2020b)). However, since most of the serological assays used for

screening were developed using antigens from SFG rickettsiae not endemic to this region, the actual agent causing infections in humans in southeast Asia is largely unknown. The common serological tests can only identify a targeted species such as reported by Richards *et al.* (2003), the *Rickettsia coronii* and *Rickettsia rickettsii* for SFG *Rickettsia* seroprevalence. A handful of reports based on molecular assays identified *Rickettsia felis*, *Rickettsia* sp. RF2125 or *Rickettsia asembonensis*, *Rickettsia raoultii*, *Rickettsia honei* TT-118, and *Rickettsia japonica* in human infections (Jiang *et al.*, 2005; Gaywee *et al.*, 2007; Kho *et al.*, 2016). Richards *et al.* (2003) has reported in a seroprevalence study that there was past exposure in 20.4% of a group screened of residents without having diagnosed as SFG patient. This is very interesting as the finding was based on malaria and filariasis cases. The unknown causes of fever could be from the SFG *Rickettsia* infection as well as reported by Yang *et al.* (2021) in Taiwan. The study found that the patients infected by SFG *Rickettsia* were previously negative for murine typhus, Q fever and scrub typhus. Instead, 19% of the patients were detected as positive for *R. felis* and some of them were having seroconversion. None of the Rickettsia-positive patients were known to be infested by the fleas or exposed to animals. Serological assays must consider the recommended time frame in order to isolate the *Rickettsia* strain successfully. An exposure to ticks also could be associated to *R. raoultii* infection (Li *et al.*, 2017; Yin *et al.*, 2018). The studies have listed several symptoms reported on the positive patients such as fever, malaise, myalgia, lymphadenopathy, nausea, rash, arthralgia and eschar. Some laboratory testing also showed abnormalities. Same symptoms were reported in patients in China who were infected with *R. japonica*, in which known to be transmitted by ticks (Lu *et al.*, 2018). Another symptom reported was anemia in an infected patient (Moonga *et al.*, 2021). The study attempted in utilising the molecular detection in a rickettsia surveillance study in human resulted in low detection of *Rickettsia asembonensis* (0.39%). In southeast Asia such as Thailand, has reported 6.9% of residents near the

agricultural landscape have been exposed to SFG *Rickettsia* (Chaisiri *et al.*, 2022). None of the landscape type and socio-demographic parameters was found to be associated to the exposure of the SFG *Rickettsia* significantly. However, the number of domestic animals associated to the residents influenced the high levels of seropositivity in the SFG *Rickettsia* indeed. Similar study has been reported in Sao Tome Island, *Rickettsia africae* and *Rickettsia conorii* infecting 8.3% of the residents (Hsi *et al.*, 2020). Furthermore, 81.7% of the ticks collected from the domestic animals nearby also infected by *R. africae*. Based on the reports above, there were potentials of zoonotic and vector-borne disease transmission here. To date, the SFG *Rickettsia* has been endemic globally not limited to certain area (Faccini-Martínez *et al.*, 2018; Farovitch *et al.*, 2019).

Rickettsia was commonly reported from fleas associated to the dogs (Mongkol *et al.*, 2018) and cats (Brown *et al.*, 2022). Recently, five *Rickettsia* sp. that were closely related to *R. conorii* Brumpt, *Rickettsia felis* URRWXCal2, *Rickettsia japonica* Uchida, *Rickettsia raoultii* Mediannikov and *R. rickettsii* Brumpt; were detected in fleas collected from rodents and shrew (Wang *et al.*, 2020). Not limited to fleas as the vector, ticks are also known as the vector for SFG *Rickettsia* disease (Hsi *et al.*, 2020). A study has reported the prevalence of *R. parkerii* and *R. felis* closely related, in *I. granulatus* ticks in Taiwan (Shih *et al.*, 2021). Both species are belonged to the SFG *Rickettsia*. The ticks were collected from rodents trapped in vegetation areas. However, no information on the species of rodents was reported in that study. There is a vector known as *Pulex irritans*, the human fleas detected as positive for *Rickettsia belii* (Ghavami *et al.*, 2018). However, there is still lack of information on the host preference and their role in the transmission of pathogens (Miarinjara *et al.*, 2021). New species has been reported from ticks collected from housing areas of the infected patients, *Candidatus* R. xinyangensis (Li *et al.*, 2020). This finding indicated that a continuous surveillance must be performed in order to identify any evolution or divergence in *Rickettsia* species. In the Republic of

Korea, *Ixodes nipponensis*, *Amblyomma testudinarium*, and *Haemaphysalis longicornis* ticks species have collected from patients suspected having SFG *Rickettsia* disease (Tariq *et al.*, 2021). However, only *H. longicornis* has been reported to carry the *Rickettsia roultii*. This finding, the prevalence of *R. roultii* from the ticks infesting the patients, was the first reported in the Republic of Korea. In China, Yin *et al.* (2018) has reported the detection of SFG *Rickettsia* in 51.5% of the ticks collected from the animals. *R. aeschlimannii* and *R. raoultii* closely related were detected in *Haemaphysalis asiaticum*, *Dermacentor nuttalli* and *Haemaphysalis marginatum*. Recently, another SFG *Rickettsia* species such as *Rickettsia asiatica*, *Rickettsia helvetica,* and *Rickettsia monacensis* were detected in *Haemaphysalis flava, H. longicornis, Ixodes monospinosus, Ixodes nipponensis*, and *Ixodes ovatus* ticks collected in recreational areas such as parks, forests with hiking courses, and camping sites. A study revealed the prevalence of *Rickettsia* in mountainous area with high biodiversity and rural farming (Pascucci *et al.*, 2022). A total *Rickettsia* detection of 7.58% of the ticks collected. Environment, animals and humans showed that these three factors played an important role in the SFG *Rickettsia* transmission. Overall, this SFG *Rickettsia* has a broad geographical distribution worldwide and could impose risk to the public (Chisu *et al.*, 2018; Guo *et al.*, 2019).

Apart from the vectors, studies have also shown that rodents are exposed to the infection by SFG *Rickettsia*. In Zambia, rodent *Mastomys* sp. has been reported carrying *Rickettsia felis* in which commonly detected in cat or dog fleas (Moonga *et al.*, 2019). However, the study did not emphasize any infestation of fleas on the positive rodents. The role of rodents in this case could raise the research gap in the study of epidemiology of SFG *Rickettsia*. In Indonesia, serological study showed that *R. tanezumi, R. norvegicus, R. exulans, R. tiomanicus* and *Maxomys* sp. were exposed to SFG *Rickettsia* (Widjaja *et al.*, 2016). SFG *Rickettsia* such as *Rickettsia honei* TT-118 and *R. japonica* were detected in *B. indica* and *R. argentiventer* (Okabayashi *et al.*, 1996). In 2000, 12.2% of the rodents

captured in selected areas in the Philippines were reported to have past exposure to SFG *Rickettsia* based on IFA using antigens from *R. japonica* (Camer *et al.*, 2000). However, the actual rodents species were not reported. Ibrahim *et al.* (1999) reported up to 40% of rodents captured in Indonesia from both port and inland areas had antibodies to *R. honei* TT-118 and *R. conorii* respectively. However, these serological studies failed to determine the specific SFG *Rickettsia* species circulating in rodents. In Malaysia, SFG *Rickettsia* closely related to *R. honei, R. conorii, R. raoultii* and *Rickettsia* sp. TCM1 were identified by PCR in wild rodents caught from markets in Kuala Lumpur and Penang (Tay *et al.*, 2014a). More molecular studies are necessary to identify the circulating SFG *Rickettsia* species in rodents in this region and to determine the role of rodents in contributing to the transmission of SFG *Rickettsia* to humans or the maintenance of the bacteria among the arthropod vectors. Thailand has also reported the prevalence of SFG *Rickettsia* in small mammals such as rodents (Rungrojn *et al.*, 2021).

2.2 Rodents, common tree shrews and their associated arthropod vectors

Peri-domestic animals such as rodents and tree shrews are common wildlife usually found in forest (Dalmagro & Vieira, 2005; Ishak *et al.*, 2018b) and rural areas (Kernif *et al.,* 2012; Pumhom *et al.,* 2013).

Rodents (Order: Rodentia) have been known to be reservoirs for zoonotic agents that causes diseases in humans. Since many rodent species are considered as synanthropic animals, in which they co-habitat with humans in both rural and urban settings, they are often the cause of zoonotic diseases to humans (Minter *et al.*, 2020; Ramalho-Ortigao & Gubler, 2020; Wang *et al.*, 2020). In southeast Asia, rodents can be easily found in agricultural lands such as oil palm plantations (Samad & Sabrina, 2016; Phua *et al.*, 2018) and paddy fields (Jäkel *et al.*, 2017), cities (Castillo *et al.*, 2003), and forests (Rozental *et*

al., 2017) especially near to water source such as river stream. The rapid reproduction cycle of synanthropic rodents, that associated with zoonotic diseases contributes to their success as the reservoir of the zoonotic agent (Han *et al.*, 2015). The study highlighted those biogeographical parameters, such as range size, and intrinsic host characteristics connected to lifetime reproductive output are significant predictors of zoonotic reservoirs. From their model study, 66 zoonoses are contained in 217 species of rodents as reservoirs (Han *et al.*, 2015). Various wild rat species were captured in Carey Island, Klang which was surrounded by plantation and villages in a past study (Nursyazana *et al.*, 2013). *R. tiomanicus* was the dominant rat species (45.7%) followed by *Rattus rattus diardii* (25.9%), *R. argentiventer* (16.1%) and *R. norvegicus* (12.3%). Some rodent species such as *Myodes glareolus, R. norvegicus* and *Peromyscus leucopus* are potential reservoirs for diseases such as Lyme disease, scrub typhus, and Rocky Mountain spotted fever respectively (Meerburg *et al.*, 2009).

The prevalence of vector ectoparasites found on the rodent hosts could be influenced by the rodent host species (Moravvej *et al.,* 2015). In northern Thailand, tick (*Hemaphysalis bandicota*), mites (*Laelaps nuttali* and *Laelaps echidninus*), and flea (*X. cheopis*) were found on *B. indica, B. savilei, R. losea, R. rattus, R. exulans, R. norvegicus, Menetes berdmorei* and *Tamiops mcclellandii*. In Malaysia, based on the study by Mohd Zain *et al.* (2015), ectoparasites that were commonly found on rodents were the chiggers (*Walchiella oudemansi, Leptotrombidium deliense* and *Gahrliepia fletcheri*), mesostigmatid mites (*L. echidninus, L. nutalli, Laelaps sedlaceki, Laelaps turkestanicus, Laelaps sanguisugus* and *Longolaelaps whartoni*), fleas (*Xenopsylla cheopis*) and ticks (*Amblyomma spp., Haemphysalis spp., Dermacentor spp.,* and *Ixodes granulatus*).

The common tree shrew, *T. glis* (Scandentia: Tupaiidae) was discovered by Diard in 1820. *Tupaia glis* is native to Thailand, Malaysia, and Indonesia. It belongs to the tree

shrew family *Tupaiidae.* In Thailand, *T. glis* has been recognized as one of the potential reservoirs that contributes to the maintenance of scrub typhus (Coleman *et al.,* 2003; Paramasvaran *et al.*, 2009; Thanee *et al.*, 2009). Ectoparasites such as mite (*Laelaps* sp.), tick (*Ixodes* sp.), flea (*X. cheopsis*) and pseudoscorpion (*Chelifer cancroides*) infestation were also reported. There was a previous report on ectoparasite infestations of tree shrews in Terengganu (Sulaiman *et al.*, 2016). Out of 23 hosts sampled, 87% of *T. glis* in the Lingai agricultural area were infested by ticks (*Ixodes* sp.) and mites (*L. echidninus*). *T. glis* also was spotted in the wildlife reserve forest in Johor (Mariana *et al.*, 2008), Selangor (Adrus *et al.*, 2011) and Kedah (Mariana *et al.*, 2008) but the ectoparasite that abundantly infested the tree shrew (usually at ear lobe, eyes and limb) were chiggers. The chiggers were reported to be a potential vector for *O. tsutsugamushi* (previously named as *Rickettsia tsutsugamushi*) which can cause scrub typhus (Kundin & Jones, 1972). Aside from chiggers, sucking louse (*Sathrax durus*) was reported to be found on the head, flanks, and dorsal body of tree shrews in West Malaysia while ova were recorded mainly from the anterior flank (Durden & DeBruyn, 1984). To date, there is limited information on *T. glis* as a reservoir for vector-borne diseases in Malaysia and other tropical countries. These small mammals are able to adapt to the anthropized areas hence causing them to be commonly found in oil palm plantations. Thus, due to a huge population size, they can act as the reservoir for vector-borne pathogens and can potentially cause zoonotic disease spill over to humans living in the same area.

2.3 Vector-borne pathogens and diseases associated with ectoparasites infesting rodents and tree shrews

Vector-borne diseases are transmitted indirectly involving the transmission of pathogens via intermediate host such as sanguinivorous arthropods and livestocks.

Rodents and tree shrews may also serve as the amplifying animal reservoir for these pathogens, contributing to the maintenance of the transmission cycle due to their vast population and wide distribution (Aplin *et al.*, 2003b; Aplin *et al.*, 2011; Pages *et al.*, 2013).

Ticks, fleas and mites are vectors that can be found infesting animal hosts such as the rodents, cats and dogs. These animals can act as the reservoir for vector-borne pathogens. Majority of the studies available were fragmented and more investigations must be warranted to connect the dots between the animal host, ectoparasites and vector-borne pathogens, thus, recognising the host reservoir of vector-borne diseases. The following Table 2.1 summarised the literature review within Southeast Asia of peridomestic animals as hosts, their associated ectoparsites and vector-borne pathogens that were previously reported in certain ectoparasites species.

Table 2.1: The summary of peridomestic animals with their associated ectoparasites and the vector-borne pathogens that were reported in ectoparasites species in Southeast Asia.

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Rattus rattus	Chiggers	*Leptotrombidium delicense;* *Ascoschoengastia* spp.; *Ascoschoengastia indica;* *Leptotrombidium chiangraiensis*	*Orientia tsutsugamushi*	Rodkvamtook et al. (2013); Ruang-Areerate et al. (2011); Elliott et al. (2019)
	Ticks	*Ixodes granulatus;* *Haemaphysalis bandicota*	*Borrelia* sp. (LD); *Rickettsia honeii*; *Bartonella* sp.	Khoo et al. (2018); Ishak et al. (2018a); Kollars et al. (2001); Klangthong et al. (2015); Panthawong et al. (2020)
	Fleas	*Xenopsylla cheopis*	*Bartonella* sp.; *Bartonella grahamii;* *Bartonella rattimassiliensis;* *Bartonella rochalimae;* *Bartonella tribocorum*	Klangthong et al. (2015); Billeter et al. (2013); Parola et al. (2003)
	Mites	-	*Bartonella* sp.	Klangthong et al. (2015)
	Lice	-	*Bartonella* sp.	Klangthong et al. (2015)

LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Rattus sp.	Ticks	*Ixodes granulatus*	*Borrelia* sp. (LD)	Lau et al. (2020); Tay et al. (2014a)
	Mite	*Laelaps* spp.	-	
Rattus tanezumi	Chiggers	*Leptotrombidium* sp.	*Orientia tsutsugamushi*	Elliott et al. (2019)
	Fleas	*Xenopsylla cheopis*	*Rickettsia typhi;* *Rickettsia felis*	Barbara et al. (2010)
Rattus argentiventer	Chiggers	*Leptotrombidium delicense;* *Leptotrombidium chiangraiensis* *Leptotrombidium arenicola* *Leptotrombidium sp.* *Walchia lewthwaitei* *Walchiella oudemansi* *Ascoschoengastia indica*	*Orientia tsutsugamushi*	Elliott et al. (2019); Alkathiry et al. (2022)
Rattus argentiventer	Ticks	*Haemaphysalis bandicota*	-	Panthawong et al. (2020)
	Fleas	*Xenopsylla cheopis*	-	Parola et al. (2003)
Rattus exulans	Fleas	*Xenopsylla cheopis*	*Bartonella* sp.; *Bartonella elizabethae;* *Bartonella grahamii;* *Bartonella rochalimae;* *Bartonella tribocorum*	Klangthong et al. (2015); Billeter et al. (2013); Panthawong et al. (2020); Barbara et al. (2010)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Rattus exulans	Chiggers	*Leptotrombidium delicense; Gahrliepia (Walchia) rustica; Ascoschoengastia indica; Walchia lewthwaitei; Walchiella oudemansi; Ascoschoengastia indica; Gahrliepia fletcheri; Walchia disparunguis pingue; Leptotrombidium arenicola*	*Orientia tsutsugamushi*	Rodkvamtook et al. (2013); Elliott et al. (2019); Alkathiry et al. (2022)
Rattus surifer	Fleas	*Xenopsylla cheopis; Nosopsyllus fasciatus*	*Bartonella* sp.	Parola et al. (2003)
Rattus cremoriventer	Fleas	*Xenopsylla cheopis*	-	Parola et al. (2003)
Rattus berdmorei	Chiggers	*Leptotrombidium deliense Ascoschoengastia* spp.	*Orientia tsutsugamushi*	Elliott et al. (2019)
Rattus losea	Chiggers	*Ascoschoengastia* sp. *Leptotrombidium chiangraiensis*	*Orientia tsutsugamushi*	Ruang-Areerate et al. (2011); Elliott et al. (2019)
	Fleas	*Xenopsylla cheopis*	*Bartonella* sp.	Panthawong et al. (2020)
Rattus muelleri	Chiggers	*Leptotrombidium deliense Ascoschoengastia indica*	*Orientia tsutsugamushi*	Elliott et al. (2019)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Rattus rattus diardii	Chiggers	*Leptotrombidium arenicola* *Leptotrombidium akamushi*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Rattus norvegicus	Fleas	*Xenopsylla cheopis*	*Bartonella* sp.*;* *Bartonella elizabethae;* *Bartonella grahamii;* *Bartonella rochalimae;* *Bartonella tribocorum;* *Rickettsia typhi*	Klangthong et al. (2015); Billeter et al. (2013); Barbara et al. (2010)
	Lice	-	*Bartonella* sp.	Klangthong et al. (2015)
Rattus tiomanicus	Chiggers	*Leptotrombidium arenicola* *Leptotrombidium deliense* *Walchia lewthwaitei* *Walchia ewingi ewingi* *Ascoschoengastia indica* *Walchia disparunguis pingue* *Eutrombicula wichmanni*	*Orientia tsutsugamushi*	Elliott et al. (2019)
	Ticks	*Ixodes granulatus* *Dermacentor auratus* *Haemaphysalis hystricis*	*Bartonella* sp.	Ishak et al. (2018a); Asyikha et al. (2020)
Rattus rattus septicus	Chiggers	*Ascoschoengastia indica*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Rattus tanezumi sensu stricto	Chiggers	*Ascoschoengastia indica*	-	Alkathiry et al. (2022)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Rattus tanezumi R3 mitotype	Chiggers	*Walchia kritochaeta; Leptotrombidium delicense; Eutrombicula wichmanni; Leptotrombidium arenicola; Walchia disparunguis pingue; Gahrliepia fletcheri; Ascoschoengastia indica; Walchiella oudemansi; Walchia ewingi ewingi; Walchia lewthwaitei*	-	Alkathiry et al. (2022)
Menetes berdmorei	Chiggers	*Leptotrombidium deliense*	*Orientia tsutsugamushi*	Ruang-Areerate et al. (2011); Elliott et al. (2019)
	Fleas	*Nosopsyllus fasciatus*	-	Parola et al. (2003)
Bandicota indica	Chiggers	*Leptotrombidium deliense; Ascoschoengastia* spp. *Leptotrombidium* sp. *Walchia disparunguis pingue Leptotrombidium chiangraiensis*	*Orientia tsutsugamushi*	Rodkvamtook et al. (2013); Ruang-Areerate et al. (2011); Elliott et al. (2019)
	Lice	-	*Bartonella* sp.	Klangthong et al. (2015)
	Ticks	*Haemaphysalis bandicota*	*Bartonella* sp.	Klangthong et al. (2015); Panthawong et al. (2020)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Bandicota savilei	Chiggers	*Leptotrombidium deliense* *Ascoschoengastia* spp.	*Orientia tsutsugamushi*	Rodkvamtook et al. (2013); Elliott et al. (2019)
	Lice	-	*Bartonella* sp.	Klangthong et al. (2015)
	Ticks	*Haemaphysalis bandicota*	-	Panthawong et al. (2020)
Bandicota bengalensis	Chiggers	*Leptotrombidium deliense*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Berylmys berdmorei	Chiggers	*Ascoschoengastia* spp.	*Orientia tsutsugamushi*	Elliott et al. (2019)
Berylmys bowersi	Fleas	*Nosopsyllus fasciatus*	-	Parola et al. (2003)
Sundamys muelleri	Ticks	*Ixodes granulatus* *Dermacentor* sp.	*Borrelia* sp. (LD)	Khoo et al. (2018); Ishak et al. (2018a)
Maxomys whiteheadi	Chiggers	*Leptotrombidium deliense*	*Orientia tsutsugamushi*	Elliott et al. (2019)
	Ticks	*Ixodes granulatus* *Dermacentor* sp.	*Borrelia* sp. (LD)	Khoo et al. (2018); Ishak et al. (2018a)
Maxomys rajah	Ticks	*Ixodes granulatus* *Dermacentor* sp. *Dermacentor atrosignatus* *Haemaphysalis* sp. *Haemaphysalis bispinosa*	*Rickettsia* strain RF2125; *Rickettsia raoultii/Rickettsia heilongjiangensis*	Ishak et al. (2018a); Kho et al. (2019)
Mus sp.	Chiggers	*Leptotrombidium deliense*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Mus musculus	Fleas	*Xenopsylla cheopis*	-	Parola et al. (2003)
Niniventer cremoriventer	Chiggers	*Leptotrombidium deliense*	*Orientia tsutsugamushi*	Elliott et al. (2019)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Leopoldamys edwardsi	Chiggers	*Ascoschoengastia indica*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Leopoldamys sabanus	Ticks	*Ixodes granulatus* *Dermacentor atrosignatus* *sDermacentor* sp.	*Borrelia* sp. (LD); *Rickettsia asiatica*	Khoo et al. (2018); Ishak et al. (2018a); Kho et al. (2019)
Tupaia glis	Chiggers	*Leptotrombidium delicense;* *Ascoschoengastia indica;* *Walchia lewthwaitei;* *Walchiella oudemansi;* *Gahrliepia fletcheri;* *Leptotrombidium arenicola;* *Eutrombicula wichmanni;* *Gahrliepia rutile;* *Trombiculindus paniculatum;* *Walchia rustica;*	*Orientia tsutsugamushi*	Rodkvamtook et al. (2013); Elliott et al. (2019); Alkathiry et al. (2022)
	Ticks	*Amblyomma* sp. *Ixodes granulatus* *Amblyomma* sp.	-	Ishak et al. (2018a)
Tupaia belangeri	Chiggers	*Acariscus leachi* *Leptotrombidium deliense*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Collasciurus nigrovittatus	Chiggers	*Ascoschoengastia indica*	*Orientia tsutsugamushi*	Elliott et al. (2019)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

Host Species	Ectoparasites associated to the host	Ectoparasites species associated to the host	Pathogens previously detected in ectoparasites species associated	References
Dogs and Cats	Fleas	*Ctenocephalide felis;* *Ctenocephalides orientis;* *Pulex irritans;* *Xenopsylla cheopis;* *Ctenocephalide canis;*	*Candidatus* Rickettsia senegalensis PU01-02; *Rickettsia sp. Rf31;* *Rickettsia felis;* *Rickettsia spp.;* *Bartonella henselae;* *Bartonella clarridgeiae*	Khoo et al. (2021); Mokhtar & Tay, 2011; Kernif et al. (2012); Parola et al. (2003)
	Ticks	*Haemaphysalis hystricis;* *Rhipicephalus sanguineus;* *Rhipicephalus* spp.; *Haemaphysalis bispinosa;* *Boophilus spp.*	*Borrelia* sp. (RF); *Rickettsia* spp.	Kernif et al. (2012); Khoo et al. (2021); Tay et al. (2019)
Sundasciurus lowii	Chiggers	*Leptotrombidium sp.*	*Orientia tsutsugamushi*	Elliott et al. (2019)
Sundasciurus tenuis	Ticks	*Dermacentor sp.*	-	Ishak et al. (2018a)
Suncus murinus	Fleas	*Xenopsylla cheopis*	*Rickettsia typhi;* *Rickettsia felis*	Barbara et al. (2010)
Herpestes javanicus	Chiggers	*Leptotrombidium deliense*	*Orientia tsutsugamushi*	Rodkvamtook et al. (2013)
Eutropis multifasciata	Ticks	*Amblyomma helvolum*	*Rickettsia roultii*	Kho et al. (2019)
Pig	Ticks	*Dermacentor* spp.	-	Kernif et al. (2012)
	Lice	*Haematopinus suis*	-	Kernif et al. (2012)
Ferret-badger	Fleas	*Ctenocephalide felis*	-	Parola et al. (2003)

Table 2.1, continued. LD = Lyme disease group; RF = relapsing fever group

2.4 Oil palm plantation as the targeted area

Oil palm plantation consists different types of flora and fauna (Mathews *et al.*, 2007), that could contribute to the circulation of zoonotic and vector-borne diseases. The oil palm plantation industry in Malaysia started its development Malaysia since the 1960s and over time, this has led to lands previously designated for rice plots and rubber plantations have been converted for oil palm cultivation. This environmental change has impacted the ecology of these areas. For instance, the common tree shrew that was once confined to the forest and forest-fringe habitats are now well-adapted to the oil palm plantations (Lim, 2016; Appanan *et al.*, 2021). Some of the synanthropic small mammals were originated from a spillover from the tropical forest (Chapman *et al.*, 2019). The forest studied was home to all 12 species of small mammals; native murids, squirrels, and tree shrews. Among the listed species, there were two invasive murids (Black Rat, *R. rattus* and Polynesian Rat, *R. exulans*). While in the plantation, only these two invasive species and the native Whitehead's Rat (*Maxomys whiteheadi*) were captured. As a result, the study did not capture any species that were restricted to the plantation, but nine of them were restricted to the forest. None of the native small mammal species to the forest were caught inside the plantation at any distance, with the exception of *M. whiteheadi*. Aside from rodents, the common tree shrew, known to be a semi-arboreal, daytime small mammal can also be found abundantly in oil palm plantations. Unspecific to any one environment, it may be found in lowland to sub-montane woods up to 1100 m (Lim, 2016). The species is currently present in both urban and suburban regions as well as bush and woodland areas as a result of deforestation since 1950. Typically seen alone, although occasionally seen in groups of two to three, roaming around gardens and making brief forays inside homes. Oil palm plantations also have been reported to be infested with rodents continuously (Sinniah *et al.*, 1978; Buckle *et al.*, 1997). Burrows, palm crowns, ground vegetation, and inter-row frond piles were the habitat features whose occupancy

was recorded at the time of a fix in order to study habitat utilisation (Buckle *et al.*, 1997). The frond piles were the site of 49.6% of all rat radio fixes. This portion of the oil palm ecosystem was presumably used as a location for building nests as well as a somewhat predator-protected area for movement. Other rodent-borne diseases such as *Leptospira* sp. is common in the oil palm plantation as the pathogen was found in 33.3% of the rodents (mostly *R. tiomanicus*), soil and water samples in an oil palm plantation in Miri, Sarawak (Lesley et al., 2018). This has become a concern since Malaysia is the second-largest palm oil supplier world after Indonesia (Colchester, 2011). Some rodent- and vector-borne diseases have also been reported near oil palm plantations such as rickettsioses (Sagin *et al.*, 2000), malaria and dengue (Chang *et al.*, 1997; Tanga *et al.*, 2011). Rural areas also associated to borreliae, rickettsiae and hantavirus since they were reported had past exposure to rodents and tick bites (Stukolova et al., 2022).

As a result of massive land clearing for the development of oil palm plantations, many Orang Asli settlements were brought closer to the plantations and the Orang Asli were hired as workers in the oil palm plantations (Norhayati *et al.*, 1997; Lai, 2011). The development of oil palm plantations usually encroached into the forest-fringe areas, impacting the Orang Asli's source of income, causing them to seek employment in the oil palm plantations and assimilate to local customs (Kari *et al.*, 2016). Moreover, the Orang Asli has a high risk of exposure to infectious diseases such as parasitic infections (Al-Delaimy *et al.,* 2014; Sinniah *et al.*, 2012), leptospirosis (Loong *et al.*, 2018), SFG *Rickettsia* (Kho *et al.*, 2017), Lyme disease (Khor *et al.*, 2019) and scrub typhus (Tay *et al.*, 2000).

CHAPTER 3: METHODOLOGY

3.1 Facilities utilised for the research

3.1.1 UM Plantations Sdn Bhd Laboratory

Small mammals sample processing were performed at UM Plantations Sdn Bhd, Johore, Malaysia (N2.02916, E103.87076). It is an oil palm plantation located in the southern state of Johore. This plantation is operated under the management of UM Holdings Sdn. Bhd (UMH). The UMH Group was founded in 2001 as Universiti Malaya's (UM) business entity, with the goal of leveraging UM's assets and resources and thereby supporting UM's long-term financial stability. The Group's plantation company started with the founding of UM Plantations Sdn. Bhd. and the creation of its oil palm plantation property in Kota Tinggi, Johor. The oil palm plantation was in the mature stage (10-15 planting years). Two different trips were completed h in June 2019 and November 2019, respectively. Each trip was conducted for approximately one week. Two staff who are responsible for the safety and security of researchers, were assigned to assist during each of the trip. Their tasks include guiding the researchers using established routes to the selected study sites in the plantation.

After completing the small mammal trapping activities in the selected study site, all of those cages were brought back to the field laboratory. An on-site field laboratory was set up prior to the sample collection and it was situated outdoors, near the administrative building of the plantation to facilitate the processing of the trapped small mammals. Every corner was sanitised properly, following the standard operation procedures (SOP) based on risk assessments performed prior to the commencement of the project. All steel cages were cleaned and stored in a storage room nearby for the next sampling trip.

3.1.2 *Makmal Infodesa* Kampung Tumbuh Hangat (Community Hall)

All of the targeted tissues from the small mammals trapped at Kampung Tumbuh Hangat were processed at Makmal Infodesa Kampung Tumbuh Hangat, Bota, Perak, Malaysia (N4.313903, E100.929009). Kampung Tumbuh Hangat is a village in central Perak that is surrounded by oil palm plantations and paddy fields. The oil palm plantation was in the mature stage, similar to UM Plantation. In addition, there is a community, known as the Orang Asli, and they are very close to the plantation area, approximately 100m. Most of the Orang Asli work in the oil palm plantation while the paddy fields are being cultivated by the Malay villagers from neighbouring villages. Three different trips were conducted in December 2018, March 2019 and July 2019, respectively. Each trip was conducted for approximately one week.

After collecting the trapped small mammals in the selected study sites, all of the occupied cages were brought back to a temporary on-field processing site. Every corner was sanitised properly, following the SOPs based on risk assessments performed prior to the commencement of the project. All steel cages were cleaned and stored in a storage room nearby for the next sampling trip.

3.1.3 Tick Cell Biobank – Asia Outpost, TIDREC, UM

Molecular screening for the detection of pathogens was performed at the Tick Cell Biobank – Asia Outpost. The entire workflow from genomic DNA extraction to the purification of PCR products was performed conveniently here. Tick Cell Biobank Asia Outpost (located at the Tropical Infectious Disease Research & Education Centre (TIDREC), Universiti Malaya, Malaysia) is part of a worldwide network of laboratories that can provide tick cell lines for academic research, with the main biobank based at the University of Liverpool.

Apart from that, the Tick Cell Biobank Asia Outpost has facilitated the molecular laboratory works, particularly the genomic DNA extractions and vector-borne pathogen

screenings from all the samples collected in this study. All of the tissues harvested from the small mammals were archived at Tick Cell Biobank Asia Outpost for future research purposes.

3.2 Ethics approval for conducting the research

This study received animal ethics approval from the Universiti Malaya Institutional Animal Care and Use Committee, IACUC (G8/01082018/24052018-01/R). Permission was also obtained from the Department of Orang Asli Development (JAKOA) (JAKOA/PP.30.052Jld13 (32)) for conducting this study at Kampung Tumbuh Hangat, Perak. Approval for small-mammal trapping was also received from the University of Liverpool's Animal Welfare and Ethics Review Body with reference no. AWC0127.

3.3 Small mammal trapping and the associated ectoparasites collection

Several baits were used to lure small mammals such as bananas, dried fish, corn and palm fruit (Mohd-Taib & Ishak, 2021b). On the day of arrival, the baits were freshly prepared in the afternoon and were cut into several pieces to be placed into each steel trap. There were 150 traps prepared for each round of trapping. Each trap was properly labelled to indicate that research was being conducted and to recognise the traps easily. All of the traps were loaded onto four-wheeled drive vehicles (due to the uneven pavement and water puddles) and then placed randomly at each study site. Each trap was placed about 50 m away from each other. All of the traps were left overnight since the rodents are nocturnal. The rodents are the main target since they are known to be carriers of various pathogens. The traps were collected the next early morning. Some new traps and fresh baits were brought to the sites. All of the traps were checked for any small mammals,

damages or missing baits. Any damaged traps, it was immediately replaced with new steel trap. Missing baits were replaced with the same fresh baits. For instance, a missing banana was replaced with a fresh banana. Aside from that, if there was no trapped small mammal, the old baits were replaced with a fresh ones on the third day. If there was a trapped small mammal, ecological data was collected on the spot and the trap was then transferred into a big container with proper ventilation for transportation purposes. This is important to avoid the traps to be shaky during transportation back to the field laboratory and to reduce stress for the small mammals. Each collected trap was replaced with a new trap to ensure a consistent number of traps each day. All of the trapped small mammals were then brought back to the laboratory for processing.

Figure 3.1: A rat was trapped in the steel cage.

3.4 Identification of small mammals and DNA barcoding analysis of rodents

All captured rats were euthanised by overdosing on Zoletil®, via injection of the intramuscular area (>50 mg kg^{-1}) (Mohd-Taib & Ishak, 2021b). After injection, the small

mammals were left aside for a few minutes. Within 1.5 min, no physical movement and weak heartbeat were observed in the small mammals. During the processing period, all morphological criteria such as the measurement of feet, tail, skull, ears, body length and weight of each individual in grams were recorded. The age and sex of each individual were determined. By referring to morphological features and measurements, the small mammals were classified into the respective species accordingly, following the keys published by Francis (2019) and Ruedas (2008). Any infestation of ectoparasites or uncommon conditions observed on the individuals or their tissues were recorded. A stainless-steel tray was set up and each individual was combed carefully to check for mites. From the process, a few lice and mites would be dropped onto the tray. A fine-end paintbrush has been used to collect the dropped ectoparasites by dipping the tip of a paintbrush into the 70% ethanol solution and tapping onto each ectoparasite. As for ticks, an observation was done to check for engorged individuals. Each tick found was collected using a sharp-end forceps. All chiggers clump (orange in colour) spotted at ear lobes and skin, were collected by cutting each organ respectively. All ectoparasites collected were preserved in a screw-capped vial of 80% ethanol solution. Two sets of scissors and forceps were utilised, one was for the outer layer (skin) of the individual and one was for the cutting of internal tissues. Scissors and forceps were thoroughly washed consecutively in bleach (5% sodium hypochlorite), sterile water and 70% ethanol prior to harvesting tissues to prevent cross-contamination. An aseptic cardiac puncture was performed to collect the whole blood sample (approximately five mℓ), kept in a sterile EDTA tube and subsequently euthanised the individual. The EDTA tubes were brought back to the laboratory and kept at -20°C. Small pieces (< 50 mg) of lung, liver, kidney and spleen were aseptically collected in the same order, and placed into respective pre-labelled cryotubes. The tissues were then stored in a nitrogen tank in the field and subsequently transferred to a −80°C freezer at the Tick Cell Biobank Asia Outpost laboratory. All these

steps were performed as fast as possible to avoid the individuals from dying before organ harvesting. This is crucial in order to preserve the integrity of the bacteria residing inside the organs.

Based on the morphological species identification record, a conventional PCR assay was performed to confirm their species by targeting the cytochrome c oxidase I (*COI*) and the cytochrome b (*cytb*) genes. Primers used in the present study are listed in Table 1. The PCR assay was performed in an Applied Biosystems™ Veriti™ 96-Well Fast Thermal Cycler (California, USA). For the PCR reaction; 1X MyTaq™ Mix (Bioline Reagents Ltd, United Kingdom), 0.4 µM of forward primer (BatL5310), 0.4 µM of reverse primer (R6036R), 2.0 µℓ of DNA template and nuclease-free water (NFW) were added to make up the 25 µℓ reaction volume. Both field identification and molecular assay were referred to a protocol established in the Community Ecology of Rodents and their Pathogens (CERoPath) website (Herbreteau *et al.*, 2011). 5 µℓ of the amplified product was run in 1.0% Gene Xpress LE grade agarose gel (Gene Xpress PLT, Malaysia) under a 0.5X tris-acetate EDTA (TAE) buffer (Gene Xpress PLT, Malaysia) for 1 hour at 80 V, and the Invitrogen™ SYBR™ Safe DNA gel stain (Eugene, Oregon, United States) was used for DNA staining. DNA visualization was performed using a blue light illuminator, B-BOX™ epi-illuminator (SMOBIO Technology, Inc., Taiwan) and a 100 bp DNA ladder, GeneRuler 100 bp DNA Ladder was used as the DNA size marker (Thermofisher). The ~700 bp band was cut and purified using a DNA purification kit (NucleoSpin Gel and PCR Clean-up, Macharey-Nagel, Germany) following the manufacturer's instructions. 15 µℓ of the eluted purified DNA fragments were sent to a third-party commercial company (Apical Scientific Sdn. Bhd., Malaysia) for sequencing, utilising Sanger's method. Upon receiving the chromatograms, the DNA sequences obtained were compared to those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5 DNA extraction for pathogen detection

The spleens of the small mammals harvested were subjected to DNA extraction. The genomic DNA extraction procedure followed the protocols provided by the NucleoSpin® Tissue Extraction Kit (Macherey-Nagel, Germany). The spleens were processed in Biosafety Cabinet (BSC) Level II since they were considered infectious materials. A portion of the spleen (~ 10mg) was cut and placed into 180 µℓ of T1 buffer in a 1.5 mℓ Eppendorf tube. A set of pellet pestles with a cordless motor (Sigma-Aldrich) was used to grind the spleen tissue in the T1 buffer. The sample was homogenised for about 8 to 10 seconds. 20 µℓ of Proteinase K was pipetted into the T1 buffer and mixed with the specimen. The mixture was incubated for 3 hours at 56°C using the dry block. Following that, 200 µℓ of B3 buffer was added into the incubated Eppendorf tube and the tube was further incubated for 10 minutes at 70°C. Then, 210 µℓ of absolute ethanol was added into the incubated tube to prepare for DNA binding to the thin membrane in the column. The specimen was briefly vortexed to promote DNA aggregation. Everything in the tube was aliquoted into the column and spun for 1 minute at 11 000 g. The supernatant in the collection tube was discarded together with the collection tube. The old collection tube was replaced with a new one. Next, the column was washed with BW buffer (500 µℓ) and B5 buffer (600 µℓ) to remove all the unnecessary components leaving the DNA pellets bound to the membrane. The DNA pellets bound to the membrane were eluted with 100 µℓ nuclease-free water. Finally, all of the extracted genomic DNA was kept at -80°C until further experiment was conducted.

3.6 Pathogen detection from the spleens

The extracted genomic DNA from the spleens of rodents and tree shrews were utilized for the amplification of genes specific for the *Orientia* sp., *Borrelia* sp., *Bartonella* sp. and *Rickettsia* sp. The type surface antigen 47kDa gene, *TSA47* specific to the *Orientia tsutsugamushi* (Masakhwe *et al.*, 2018) and the flagellin gene, *flaB* specific to the *Borrelia* spp. (Lau *et al.*, 2020) were amplified according to previously published protocols. The detection of *Bartonella* spp. and *Rickettsia* spp. followed two different PCR protocols that target the same citrate synthase gene, *gltA* (Roux *et al.*, 1997; Labruna *et al.*, 2004; Inoue *et al.*, 2008). Primers used in the present study are listed in Table 1.

5 µℓ of the amplified PCR product was run in 1.0% agarose gel under a 0.5X tris-acetate EDTA (Tris base, acetic acid and EDTA, TAE) buffer (Gene Xpress PLT, Malaysia) for 1 hour at 80 V, and the Invitrogen™ SYBR™ Safe DNA gel stain (Eugene, Oregon, USA) was used for DNA staining. DNA visualization was performed using a blue light illuminator, B-BOX™ epi-illuminator (SMOBIO Technology, Inc., Taiwan) and a 100 bp DNA ladder, GeneRuler 100 bp DNA Ladder was used as the DNA size marker (Thermofisher). The ~800 bp for *Orientia* spp., and *Rickettsia* spp.; and ~400 bp band for *Borrelia* spp. and *Bartonella* spp. respectively was observed. The remaining amplified PCR product (approximately 20 µℓ) was then sealed and sent to the third-party commercial company, (Apical Scientific Sdn. Bhd., Malaysia). Upon receiving the chromatograms, the DNA sequences obtained were compared to those available in the GenBank database using the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The PCR-positive DNA samples for *Borrelia* spp. and *Orientia* spp. were further subjected to multi-locus sequence typing (MLST) according to protocols for *Borrelia* spp. (Margos *et al.*, 2008) and *O. tsutsugamushi* (Sonthayanon *et al.*, 2010). These protocols are available at the PubMed MLST website (https://pubmlst.org/organisms/borrelia-spp

and https://pubmlst.org/organisms/orientia-tsutsugamushi). All obtained amplicons were purified and subsequently sequenced in both directions. DNA sequences obtained were compared to those available in GenBank using the BLAST tool.

3.6.1 *Borrelia* spp.

A nested PCR assay was performed for the detection of *Borrelia* spp., referring to Lau *et al.* (2020) with some modifications. For a 25 µℓ reaction volume; 1X of MyTaq Redmix, 0.4 uM of primary forward primer (132f), 0.4 uM of primary reverse primer (905r), 2.0 µℓ of DNA template and NFW were added to a PCR tube and mixed. The same reaction composition was applied to the secondary PCR reactions containing the secondary forward primer (220f) and secondary reverse primer (823r). The PCR reactions were inserted into the thermal cycler with the following PCR condition:

Table 3.1: Primary reaction of PCR condition for targeting *flaB* (774 bp).

PCR Stage	Temperature (℃)	Duration	Cycle
Initial denaturation	94	10 min	1
Denaturation	94	30 sec	40
Anneal	50	45 sec	
Extend	72	1 min	
Final Extension	72	7 min	1
	4	10 min	
	10	∞	

Table 3.2: Secondary reaction of PCR condition for targeting *flaB* (604 bp).

PCR Stage	Temperature (℃)	Duration	Cycle
Initial denaturation	94	10 min	1
Denaturation	94	30 sec	40
Anneal	54	45 sec	
Extend	72	1 min	
Final Extension	72	7 min	1
	4	10 min	
	10	∞	

3.6.2 *Bartonella* spp.

A singleplex PCR assay was performed for the detection of *Bartonella* spp., referring to Inoue *et al.* (2008) with some modifications. For a 25 µℓ reaction volume; 1X of MyTaq Redmix, 0.4 uM of forward primer (BhCS.781p), 0.4 uM of reverse primer (BhCS.1137n), 2.0 µℓ of DNA template and NFW were added to a PCR tube and mixed. The PCR reactions were inserted into the thermal cycler with the following PCR condition:

Table 3.3: PCR condition for targeting gltA (379 bp).

PCR Stage	Temperature (℃)	Duration	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	20 sec	35
Anneal	56	30 sec	
Extend	72	2 min	
Final Extension	72	7 min	1
	4	10 min	
	10	∞	

3.6.3 *Orientia tsutsugamushi*

A nested PCR assay was performed for the detection of *O. tsutsugamushi*, referring to Masakhwe *et al.* (2018) with some modifications. For a 25 µℓ PCR reaction volume; 1X of MyTaq Redmix, 0.4 µM of primary forward primer (Ot-145F), 0.4 µM of primary reverse primer (Ot-1780R), 2.0 µℓ of DNA template and NFW were added to a PCR tube and mixed. The same reaction composition was applied to the secondary PCR reactions containing the secondary forward primer (Ot-263F) and secondary reverse primer (Ot-1133R). Both primary and secondary reactions followed the same PCR conditions. Both PCR reactions were inserted in the thermal cycler with the following PCR condition:

Table 3.4: PCR condition for targeting *TSA47* (821 bp).

PCR Stage	Temperature (℃)	Duration	Cycle
Initial denaturation	95	2 min	1
Denaturation	94	30 sec	40
Anneal	54	30 sec	
Extend	68	2 min	
	72	7 min	
Final Extension	4	10 min	1
	10	∞	

3.6.4 *Rickettsia* spp.

A singleplex PCR assay was performed for detecting *Rickettsia* spp., referring to Roux *et al.* (1997) and then repeated using another primer by Labruna *et al.* (2004) protocol with some modification. For a 25 µℓ reaction volume; 1X of MyTaq Redmix, 0.4 uM of forward primer (CS1d and CS-239), 0.4 uM of reverse primer (CS890r and CS-1069), 2.0 µℓ of DNA template and NFW were added to a PCR tube and mixed. The PCR reactions were inserted into the thermal cycler with the following PCR condition:

Table 3.5: PCR condition for targeting *gltA* (889 bp) (Roux *et al.*, 1997).

PCR Stage	Temperature (℃)	Duration	Cycle
Initial Denaturation	95	3 min	1
Denature	95	20 sec	35
Anneal	48	30 sec	
Extend	60	2 min	
Final Extension	72	7 min	1
	4	10 min	
	10	∞	

Table 3.6: PCR condition for targeting *gltA* (830 bp) (Labruna *et al.*, 2004).

PCR Stage	Temperature (℃)	Duration	Cycle
Initial Denaturation	95	3 min	1
Denature	95	15 sec	40
Anneal	48	30 sec	
Extend	72	30 sec	
Final Extension	72	7 min	1
	4	∞	

Table 3.7: List of primers utilised in this study.

Species	Target	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
Rodents	*COI*	BatL5310[a,c]	ACTTCTGGGTGTCCAAAGAATCA	726	(Herbreteau *et al.*, 2011)
		R6036R[b,c]	CCTACTCRGCCATTTTACCTATG		
O. tsutsugamushi	*TSA47*	Ot-145F[a]	ACAGGCCAAGATATTGGAAG	871	(Masakhwe *et al.*, 2018)
		Ot-1780R[b]	AATCGCCTTTAAACTAGATTTACTTATTA		
		Ot-263F[a,c]	GTGCTAAGAAARGATGATACTTC	821	
		Ot-1133R[b,c]	ACATTTAACATACCACGACGAAT		
Bartonella spp.	*gltA*	BhCS.781p[a,c]	GGGGACCAGCTCATGGTGG	379	(Inoue *et al.*, 2008)
		BhCS.1137n[b,c]	AATGCAAAAAGAACAGTAAACA		
Borrelia spp.	*flaB*	BflaPAD[a]	GATCARGCWCAAYATAACCAWATGCA	800	(Takano *et al.*, 2010; Lau *et al.*, 2020)
		BflaPDU[b]	AGATTCAAGTCTGTTTTGGAAAGC		
		BflaPBU[a,c]	GCTGAAGAGCTTGGAATGCAACC	345	
		BflaPCR[b,c]	TGATCAGTTATCATTCTAATAGCA		
Rickettsia spp.	*gltA*	CS1d[a,c]	ATGACTAATGGCAATAATAA	889	(Roux *et al.*, 1997; Labruna *et al.*, 2004)
		CS890r[b,c]	GCTTTIAGCTACATATTTAGG		
		CS-239[a,c]	GCTCTTCTCATCCTATGGCTATTAT	830	
		CS-1069[b,c]	CAGGGTCTTCGTGCATTTCTT		

a: Forward primer; b: reverse primer; c: sequencing primer

3.7 The DNA sequence analysis

The DNA sequences obtained from the sequencing were trimmed using the Geneious version 7.1 software (https://www.geneious.com) employing the default low-quality end trimming conditions (minimum 75% of good quality bases and excluding the primer templates). The trimmed DNA chromatograms were edited using the same software. The targeted gene sequences obtained were compared to existing sequences in the National Center for Biotechnology Information (NCBI) GenBank using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer-trimmed sequences of *COI* and *cytb* obtained were aligned using CLUSTALW program, as implemented in Molecular Evolutionary Genetics Analysis, MEGAX (Kumar *et al.*, 2018). Phylogenetic relationships of the rodents in this study were presented in phylogenetic trees using the Neighbour-joining (NJ) method with 1000 bootstrapping phylogeny tests by MEGAX to confirm the genetic identity.

Primer-trimmed sequences of *TSA47, flaB* and *gltA* obtained were aligned using CLUSTALW program, as implemented in MEGAX (Kumar *et al.*, 2018). All positions containing gaps and missing data were eliminated (complete deletion option). Phylogenetic relationships of the pathogens in this study were presented in phylogenetic trees using the Bayesian Markov Chain Monte Carlo (MCMC) approach, as implemented in BEAST 1.10.4 (Drummond *et al.*, 2012). The Hasegawa–Kishono–Yano (HKY) model with the Gamma site (HKY + G) was selected for all genes studied using the Bayesian Information Criterion (BIC) as implemented in MEGA11 (Tamura *et al.*, 2021). The analysis was performed under a strict molecular clock model with an MCMC chain length of 5 million samplings every 1000 generations. The resulting MCMC trace file was analyzed and visualized using Tracer Version 1.7.1 (Rambaut *et al.*, 2018). The maximum clade credibility (MCC) tree was produced using TreeAnnotator 1.10.4 and visualized using Interactive Tree of Life (iTOL) (https://itol.embl.de/itol.cgi).

CHAPTER 4: RESULTS

4.1 Ectoparasites associated with the small mammals

The table below (Table 4.3) showed the types of ectoparasites found in the small mammals in this study. Each species of small mammal was observed to be infested by ectoparasites. Amongst the observed ectoparasites, the chiggers were the predominant group, followed by lice, mites, laelaps and ticks. A separate study was conducted to count the total number of chiggers collected from this study, resulting in 40 736 individuals altogether (Alkathiry *et al.*, 2022). A total of 14 species were identified morphologically under the microscope. Several genera were identified in the study such as *Ascoschoengastia indica, Eutrombicula wichmanni, Gahrliepia fletcheri, Gahrliepia rutila, Leptotrombidium arenicola, Leptotrombidium deliense, Trombiculindus paniculatum* (a new record for Malaysia), *Walchia disparunguis pingue, Walchia ewingi ewingi, Walchia krito chaeta* (a new record for Malaysia), *Walchia lewthwaitei, Walchia rustica, Walchiella oudemansi* and an undetermined species. The other ectoparasites collected, however, were reserved for another study in the future.

Table 4.1: The ectoparasites collected from each host species.

Species of small mammals trapped	Ectoparasites associated
Rattus tanezumi R3 mitotype	Chiggers, Ticks, Laelaps, Mesostigmatid mites, Lice, Sarcoptidae mites, other mites
Rattus tanezumi sensu stricto	Ticks
Rattus argentiventer	Chiggers, Ticks, Laelaps, other mites
Rattus exulans	Chiggers, Ticks, Laelaps, other mites
Rattus tiomanicus	Chiggers, Ticks, Laelaps, Lice, Sarcoptidae mites, other mites
Tupaia glis	Chiggers, Ticks, Lice, other mites

4.2 Distribution of small mammal species

Morphological identification conducted on the captured small mammals resulted in the identification of rat and tree shrew species. This was further strengthened by the use of DNA barcoding, revealing the identification of six small mammal species (rat, n=5 and tree shrew, n=1) (Table 4.1). Analyses of the *COI* sequences identified five rat species. These species were *Rattus tanezumi sensu stricto, Rattus tiomanicus, Rattus tanezumi* R3 mitotype, *Rattus exulans*, and *Rattus argentiventer*. The *COI* sequences were deposited in the Barcode of Life Data System (BOLD) database using the following process IDs: UMNPA004-20 - UMNPA056-20, and UMNPA058-20 - UMNPA068-20 for rodents captured from Johor, and UMNPA069-20, UMNPA071-20 – UMNPA076-20, UMNPA078-20 – UMNPA080-20, UMNPA082-20 – UMNPA083-20, UMNPA085-20, UMNPA087-20 – UMNPA091-20, UMNPA093-20 – UMNPA102-20, UMNPA161-20 – UMNPA194-20, UMNPA196-20 – UMNPA216-20, and UMNPA218-20 - UMNPA223-20 for rodents captured from Perak. The Johor study site consists of one habitat (oil palm plantation) while the study site at Perak contains three different habitats (paddy field, oil palm plantation and human residential area). *Rattus tanezumi* R3 mitotype (n=113, 52.1%) predominated both sites followed by *T. glis* (n=40, 18.4%), *R. argentiventer* (n=24, 11.1%), *Rattus tiomanicus* (n=22, 10.1%), *Rattus exulans* (n=17, 7.8%) and *Rattus tanezumi sensu stricto* (*s. s.*) (n=1, 0.5%). Both sites have similar number of small mammals trapped. In Johor, *T. glis* (n=33) outnumbered *R. tiomanicus* (n=13) and *R. exulans* (n=3), while and *R. tanezumi s. s.* and *R. argentiventer* were not found. In Perak, *R. tanezumi s. s.* was solely found in the paddy field, while *R. tiomanicus* and *T. glis* were absent. Additionally, *R. argentiventer* was absent in the residential areas and *R. tanezumi* R3 mitotype can be found in all habitats but was mostly found at the oil palm plantations. Out of the 217 captured animals, 105 of them were females and 112 of them were males. A majority of the captured animals

were mature adults (n=148, 68.2%) and subadults (n=41, 18.9%), followed by juveniles (n=25, 11.5%) and the age of the remaining three individuals could not be identified.

Table 4.2: The identification of small mammals trapped in Perak and Johor based on morphological and molecular identification.

No.	Species	Trapping Site				Total of individuals
		Perak (n)			Johor (n)	
		Residential areas	Paddy field	Oil palm plantation	Oil palm plantation	
1.	*Rattus tanezumi* R3 mitotype	14	2	45	52	113
2.	*Rattus tiomanicus*	2	0	7	13	22
3.	*Rattus exulans*	3	2	9	3	17
4.	*Rattus tanezumi sensu stricto*	0	1	0	0	1
5.	*Rattus argentiventer*	0	21	3	0	24
6.	*Tupaia glis*	3	0	4	33	40
Total number of individuals			116		101	217

4.3 DNA barcoding of the rodent species

4.3.1 Cytochrome c oxidase I gene marker

A number of rodents' *COI* partial sequences were successfully amplified with fragments size of 580bp to 727bp. From the sequences obtained, an alignment with 706 sites was generated. The *COI* phylogenetic analyses (Figure 4.1) revealed that there were five species of rodents available in both oil palm plantations.

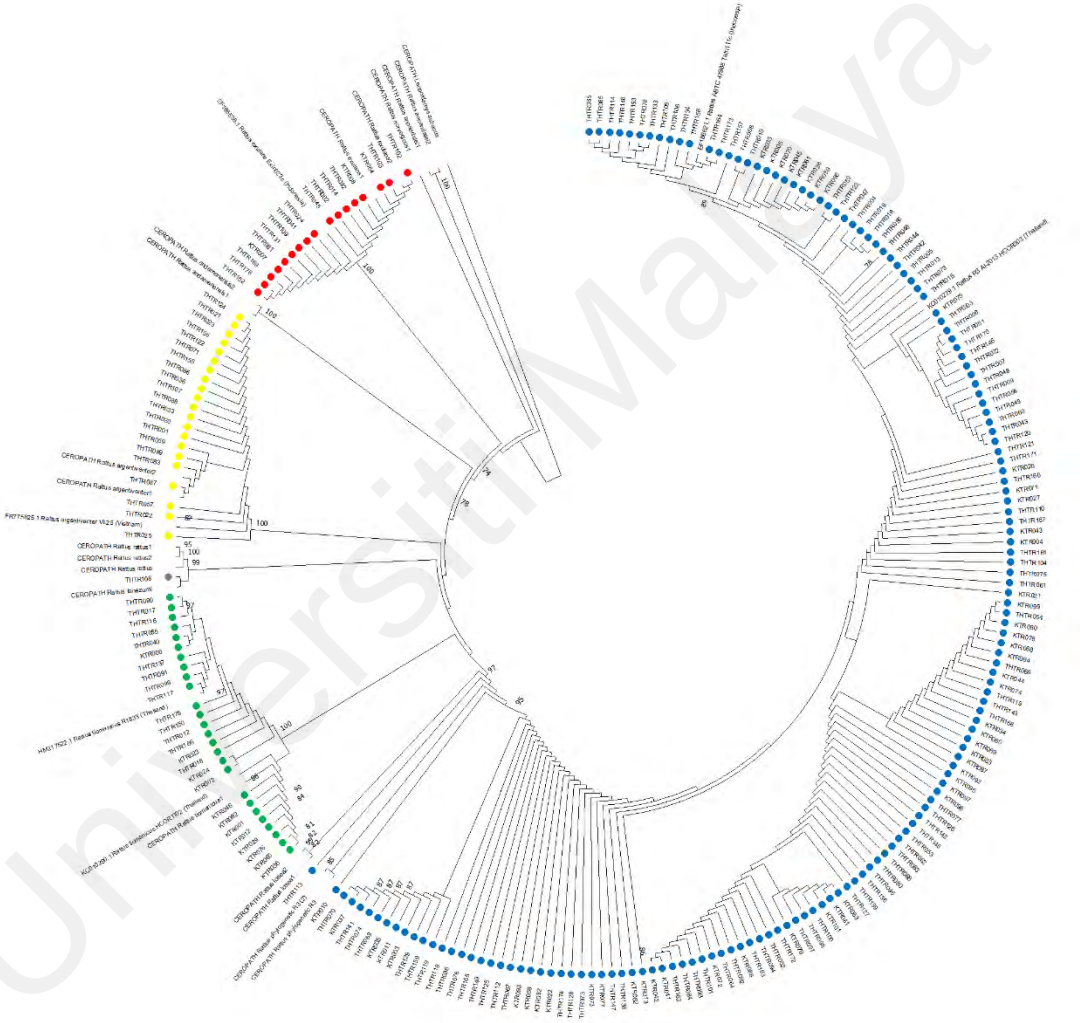

Figure 4.1: The segregation of the rodent species using the *COI* gene marker and analysed using the Neighbor-Joining method with 1000 bootstrapping. Each rodent species was presented in the phylogenetic tree with different coloured dots; blue dot for *R. tanezumi* R3 mitotype; green dot for *R. tiomanicus*; yellow dot for *R. argentiventer*; red dot for *R. exulans*; and grey dot for *R. tanezumi s.s*.

The analyses showed that *R. tanezumi* R3 mitotype clustered together with the R3 phylogenetic group from Thailand (retrieved from database http://www.ceropath.org/barcoding_tool/rodentsea). The same *COI* partial sequence data has been published and further analysed (Nasir *et al.*, 2022a; Nasir *et al.*, 2022b). Both studies supported that *R. tanezumi* R3 mitotype (referred as *Rattus diardii* by Nasir *et al.* (2022a)) in this study was closely related to the *Rattus* sp. R3 (Pagès *et al.*, 2010) and *Rattus rattus* Complex (RrC) Lineage IV (Aplin *et al.*, 2011). This species was found to be 99-100% similar to several partial sequences of *COI* deposited in GenBank such as the *Rattus* sp. R3 AL-2013, *Rattus* sp. ABTC and *Rattus* R3 MP-2010. All the successful *COI* sequences of rats were deposited to Barcode of Life Data Systems (BOLD) (http://boldsystems.org) under project code UMNPA as described by previous study.

The topology of the Old-World *R. tanezumi* group has diverged into two groups, the *R. tanezumi s.s.* and *R. tanezumi* R3 mitotype. This was supported by morphological identification as different morphology has been observed in *R. tanezumi s.s.* and *R. tanezumi* R3 mitotype. The obvious observation was the *R. tanezumi s.s.* has a full greyish-black body and fur compared to *R. tanezumi* R3 mitotype which has reddish-brown fur with pinkish feet (Figure 4.2 and 4.3).

Figure 4.2: The *Rattus tanezumi sensu stricto*.

Figure 4.3: The *Rattus tanezumi* R3 mitotype.

4.3.2 Cytochrome b gene marker

A number of rodents' *cytb* partial sequences were successfully amplified with fragments size 588bp to 1161bp. From the sequences obtained, an alignment with 1144 sites was generated. The *cyt b* phylogenetic analyses (Figure 4.4) revealed that there were five species of rodents available at the oil palm plantation.

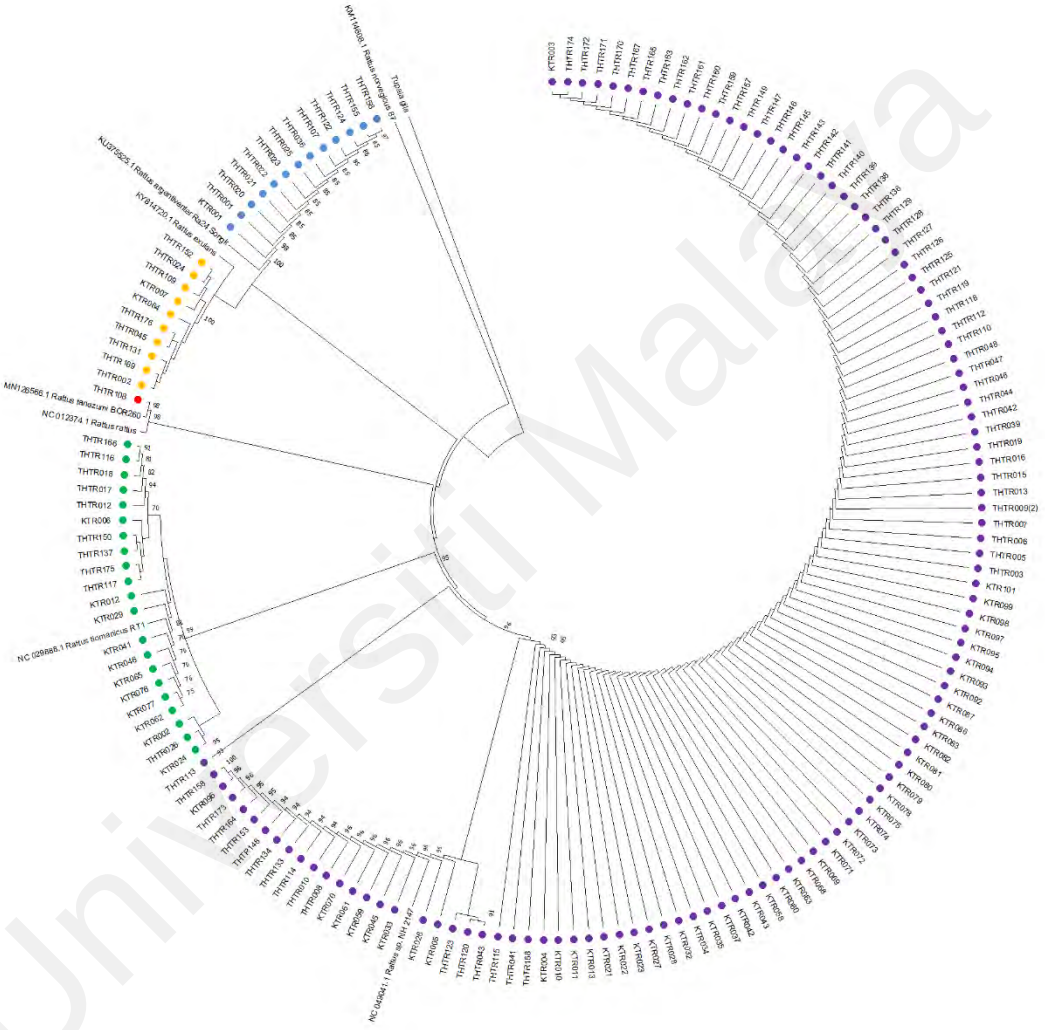

Figure 4.4: The segregation of the rodent species using the *cyt b* gene marker and analysed using the Neighbor-Joining method with 1000 bootstrapping. Each rodent species was presented in the phylogenetic tree with different coloured dots; purple dot for *R. tanezumi* R3 mitotype; green dot for *R. tiomanicus*; blue dot for *R. argentiventer*; yellow dot for *R. exulans*; and red dot for *R. tanezumi s.s*.

Notably, the topology of the phylogenetic tree was similar to the *COI* sequence analyses. Apparently, the *R. tanezumi* R3 mitotype formed a clade that was unique from the *R. rattus* and *R. tanezumi* groups, which was clustered together with the *R. tanezumi*

s.s. in this study. The other species (i. e. *R. tiomanicus, R. exulans,* and *R. argentiventer*) were found to be segregated from the cryptic species, *R. tanezumi* R3 mitotype.

Based on the BLAST analyses, the cryptic species *cytb* partial sequences were found to be closely related to several strains from RrC lineage IV from Phillipines such as EAR1655 (JQ823535.1), FMNH193812 (JQ823506.1), and LRH3530 (JQ823514.1). Some were identical to *R. rattus* strains from Malaysia such as J12 (MT037741.1), M10 (MT037729.1), NS7 (MT037714.1), PHG8 (MT037679.1) and ZOKL8 (MH818026.1). Several other individuals were closely related to *Rattus* sp. R3 MP-2010 (HM217399.1) from Thailand which similar to the *COI* analyses in this study. The percentage of identities obtained were ranging from 99% to 100% identity when compared to GenBank database, suggesting the three groups from Southeast Asia were from the same genospecies. However, more information must be included such as the morphological resemblance and comprehensive mitogenome analyses. Notably, the *R. tanezumi s.s.* identified in this study was similar to several strains from China (HM031689.1), Vietnam (JQ823463.1) and Japan (AB211040.1) with 99.9% identity. This species also was closely related to RrC lineage II from Myanmar (LC510810.1) with 99.38% identity. Both *R. tanezumi s.s.* and *Rattus tanezumi* R3 mitotype have diverged from the Old-World *R. rattus*. Meanwhile, many sequences of *R. tiomanicus* were deposited from Johor, Malaysia resulting in a clade of Malaysia origin, which is also closely related to *Rattus baluensis* recorded in Sabah, Malaysia (KY611367.1) with 98% identity. *R. argentiventer* was closely related to species captured from Vietnam (FR775875.1) and Thailand (KU375523.1) with 98.4% to 99.4% identity. Lastly, *R. exulans* has various origins of similar sequences such as Australia (OM908891.1), Germany (KJ530564.1), Myanmar (LC510797.1), and Vietnam (FR775885.1) ranging from 96.9% to 100% identity.

4.4 The screening of pathogens in small mammals captured

DNA extracted from spleens, of the captured small mammals (n=203) were examined for the presence of *O. tsutsugamushi, Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. (Table 4.2). The pathogen screening assays targeted the *TSA47* gene for *O. tsutsugamushi*; the *flaB* gene for *Borrelia* spp.; and the *gltA* gene for *Bartonella* spp. and *Rickettsia* spp., each with their respective protocols. Overall, 12.3% (25/203) of the small mammals were positive for *O. tsutsugamushi* followed by *Borrelia* spp. 5.9%, (12/203) and *B. phoceensis* 4.9% (10/203). *Rickettsia* spp. however, was not detected in all spleen specimens. The *COI* gene was successfully amplified for all the extracted DNA specimens, indicating that negative pathogen amplification was not due to the low quality of DNA samples. Moreover, two different sets of primers were utilised for *Rickettsia* spp. detection (Roux *et al.* (1997) and Labruna *et al.* (2004)) and both returned negative. The Table 4.2 below shows the location, host species, pathogens detected and number of positive individuals for specific hosts.

The pathogen detection rate is relatively higher in Perak (15.8%) as compared to Johore (7.4%). Based on Table 4.2, PCR amplification for the respective pathogens showed that *O. tsutsugamushi* was detected in five small mammal species, except for *R. tanezumi s. s.*; *Borrelia* spp. was detected in four species except *R. tanezumi s. s.* and *R. argentiventer*; and *B. phoceensis* was detected in *R. tanezumi* R3 mitotype and *R. argentiventer*. *O. tsutsugamushi* was detected the most in *R. tanezumi* R3 mitotype at both study sites (Perak, n=11; Johor, n=7). *B. phoceensis* and LD borreliae were the second most detected pathogens in Perak (n=9) and Johore (n=4), respectively (Table 4.3). Furthermore, there were four individuals (i. e. *R. tanezumi* R3 mitotype) found to have been co-infected with *B. phoceensis* and *O. tsutsugamushi*.

Table 4.3: The vector-borne bacteria detected from the spleens of rodents and tree shrews.

Location	Host Species	Bacteria detected	Number of positive individuals (n)
Kampung Tumbuh Hangat, Bota Kanan, Perak	*R. tanezumi* R3 mitotype	*Bartonella phoceensis*	8
		Orientia tsutsugamushi	11
		Borrelia sp. (LD)	1
		Borrelia sp. (RF)	4
	R. exulans	*Borrelia sp.* (undetermined)	1
		Orientia tsutsugamushi	2
	R. argentiventer	*Bartonella phoceensis*	1
		Orientia tsutsugamushi	2
	R. tiomanicus	*Borrelia sp.* (RF)	1
	T. glis	*Borrelia sp.* (RF)	1
UM Plantation Sdn. Bhd., Kota Tinggi, Johor	*R. tanezumi* R3 mitotype	*Bartonella phoceensis*	1
		Orientia tsutsugamushi	7
		Borrelia sp. (LD)	3
	R. tiomanicus	*Orientia tsutsugamushi*	1
	T. glis	*Orientia tsutsugamushi*	2
		Borrelia sp. (RF)	1

LD=Lyme disease group; RF=relapsing fever group

4.5 Sequence analyses of the detected pathogens

4.5.1 *Borrelia* spp.

The borrelial *flaB* sequences generated from this study were segregated into two clusters (Figure 4.5), one with members of the LD borreliae and the other with members of the RF borreliae, consistent with previous reports (Khoo *et al.*, 2017; Khoo *et al.*, 2018; Binetruy *et al.*, 2020).

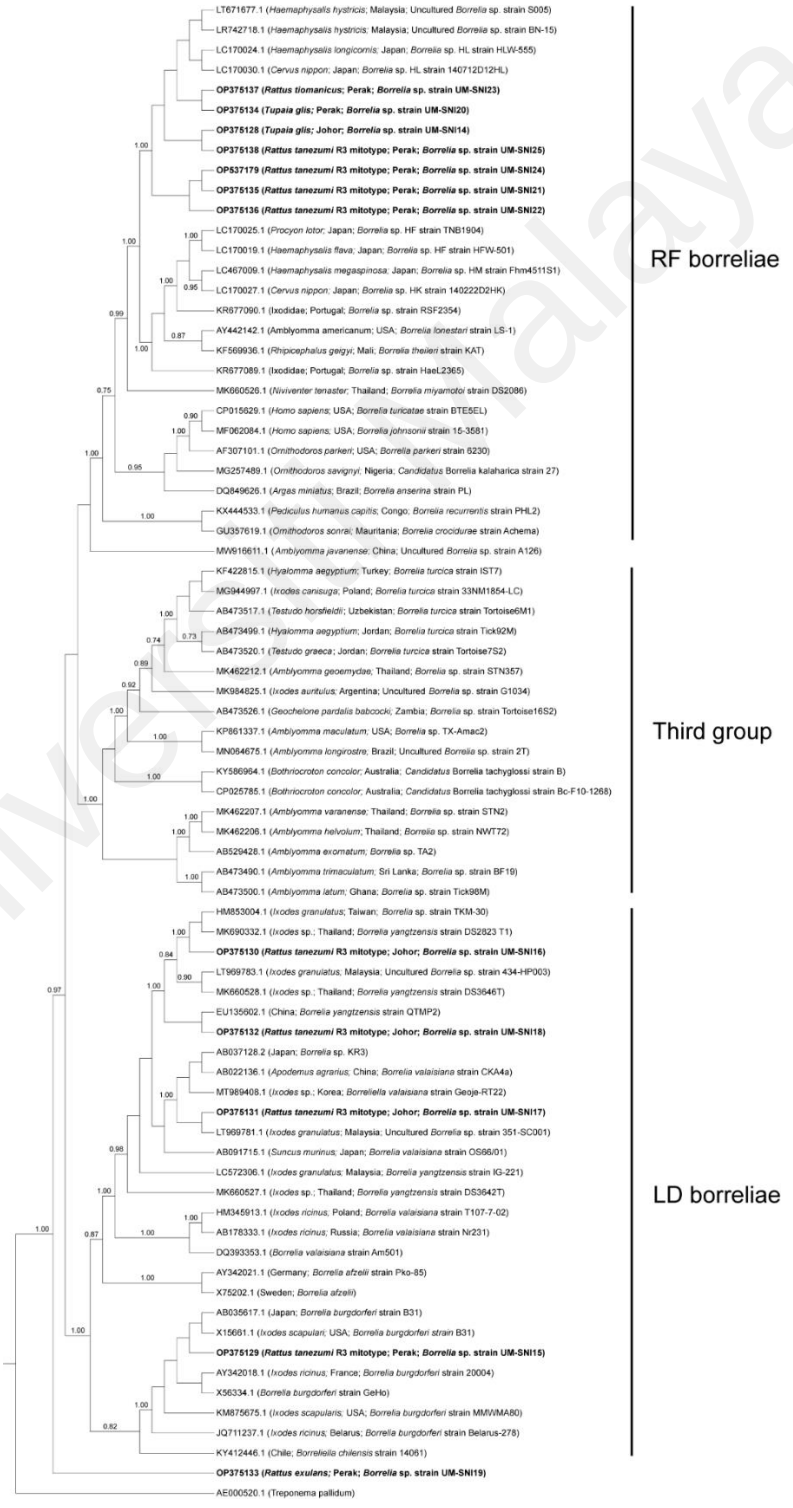

Figure 4.5, continued: Bayesian inference phylogenetic tree of *Borrelia* spp. based on the partial sequences (270 - 300 bp) of the *flaB* gene. Posterior probability (PP) is shown on the branches. Only PP of >0.7 are shown. Newly generated sequences are labelled in bold text, with their accession numbers followed by the animal host species, location and strain name in parentheses. The reference sequences are labelled with their accession numbers followed by the *Borrelia* spp. strain with the ectoparasite/animal host species and location in parentheses. LD = Lyme disease-related, RF = relapsing fever-related.

A third cluster whose members did not belong to the former two groups was also included in the analysis but none of our specimens clustered with this group. Both LD and RF borreliae were detected in specimens collected from both study sites (Figure 2). LD borreliae were only detected in *R. tanezumi* R3 mitotype. On contrary, the RF borreliae were detected in *R. tanezumi* R3 mitotype, *R. exulans*, *R. tiomanicus* and *T. glis*.

A closer observation of the RF borreliae from this study indicated that they form a sister clade independent from the other RF borreliae strains. This clade includes the uncultured *Borrelia* sp. detected in Malaysia and Japan (Figure 2). Conversely, the LD borreliae topology showed that most of our specimens clustered with *B. yangtzensis* and *B. valaisiana* genospecies group members reported from other Asian countries. One specimen (UM-SNI19) clustered with various strains of *B. burgdorferi*, including the *Borrelia burgdorferi sensu stricto* (*s. s.*) B31. Only the ATP-dependent Clp protease ATP-binding subunit (*clpA*) gene from the *Borrelia* spp. MLST scheme was successfully amplified in one specimen, *R. tanezumi* R3 mitotype from Johor. It was found to be phylogenetically related to *B. yangtzensis* (Accession no. LC572085.1) detected in Malaysia at 98.63% homology. An enquiry was made in the PubMLST database and found that the *clpA* gene has no match with existing alleles. The closest sequence type to our sequence was isolated from *M. caroli* and *I. granulatus* (Kawabata *et al.*, 2013).

Notably, specimen UM-SNI19 was ambiguous to any of the groups. UM-SNI19 forms a sister clade to other LD borreliae members with less than 0.7 PP. BLAST analysis showed that UM-SNI19 was less than 90% identity to both LD and RF borreliae. The

BLAST match with the highest percentage identity was the LD borreliae, *B. afzelii* at 88.3% identity with a low 47% query cover. This was followed by members of RF borreliae, including *B. anserina* Es isolate UTHSCSA (85.88% identity with 62% query coverage). On the other hand, the BLAST matches with the highest query cover include the uncultured *Borrelia* sp. clone T207 (79.65% identity with 98% query cover (Ehlers *et al.*, 2020), uncultured *Borrelia* sp. clone IR-1 (84.09% identity with 95% query cover, (Ghasemi *et al.*, 2021)) and *Borrelia microti* strain Abyek (84.09% identity with 95% query cover, NCBI Accession: JF708951, unpublished), which are all RF borreliae strains.

4.5.2 *Bartonella phoceensis*

BLAST analyses of the amplified sequences specific to *gltA* of *Bartonella* spp. revealed that all the specimens were positive for *B. phoceensis* with 99-100% identity. As such, the phylogenetic analyses clustered all the amplified specimens together with *B. phoceensis* representatives from Thailand and France at 1.00 and 0.81 PP. The phylogenetic tree (Figure 4.6) displayed the relationship of the *Bartonella*-positive samples clustered into one clade known as *B. phoceensis*.

Figure 4.6: Bayesian inference phylogenetic tree of *B. phoceensis* based on the partial sequences (338 bp) of the *gltA* gene. Posterior probability (PP) is shown on the branches. Only PP of >0.7 are shown. Newly generated sequences are labelled in bold text, with their accession numbers followed by the *Bartonella* spp. strain with the ectoparasite/animal host species and location in parentheses. The reference sequences are labelled with their accession numbers followed by the *Bartonella* spp. strain and location in parentheses.

From the phylogenetic tree, all the samples were clustered together with *B. phoceensis* from Taiwan, Thailand and France. All of the samples were homogenous to the GU056197 and AY515126 (France), and grouped under a sister taxa to the KT324563 (Thailand) with 100% homogeneity. Interestingly, the predominant host from this study was the *Rattus* phylogenetic R3 in which abundantly found in both sites. After enquiring the sequences to BLAST, KTR043 and THTR008 were 100% closely related to *B. phoceensis* strain L1181 (Accession no. KT324563). UM-SNI07 and UM-SNI09 have 99.63% identical to the uncultured *B. phoceensis* sequences reported by Blasdell *et al.* (2019b). The remaining sequences have 100% identical to the same uncultured *B. phoceensis*.

4.5.3 *Orientia tsutsugamushi*

Phylogenetic analyses of the 825bp sequences from *O. tsutsugamushi TSA47*-positive specimens grouped all those specimens together with two strains from Thailand (UT176 and TA763) at 0.95 posterior probability (PP) (Figure 4.7).

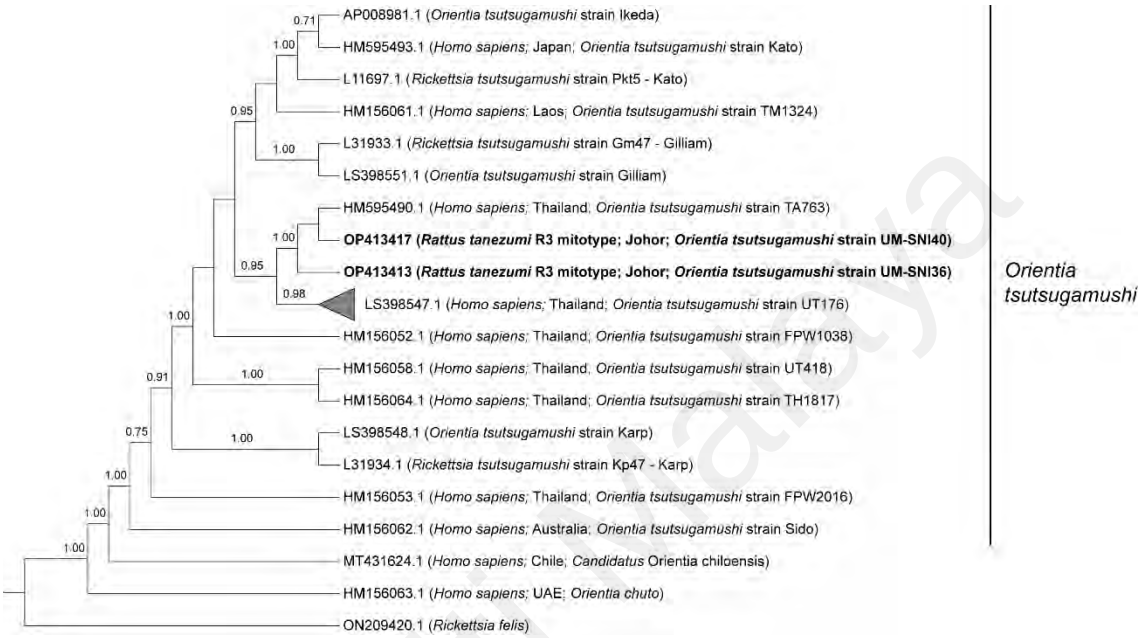

Figure 4.7: Bayesian inference phylogenetic tree of *O. tsutsugamushi* based on the partial sequences (825 bp) of the *TSA47* gene. Posterior probability (PP) is shown on the branches. Only PP of >0.7 are shown. Newly generated sequences are labelled in bold text, with their accession numbers followed by the animal host species, location and strain name in parentheses. The reference sequences are labelled with their accession numbers followed by *O. tsutsugamushi* strain with the ectoparasite/animal host species and location in parentheses. The collapsed branch consisted of the 23 new sequences from this study, which clustered together with strain UT176 (Accession no. LS398547.1) at 0.98 PP.

In contrast to our phylogenetic tree, the UT176 strain has been reported as Karp sub-genotype while TA763 was a separate genotype based on the *TSA56* genotyping (Blacksell *et al.*, 2008). Subsequently, Batty *et al.* (2018) reported that the UT176 strain was closely related to Kato and Ikeda strains. In Thailand, there were eight clades known to be circulated such as Karp, Kato, Gilliam, TA678, TA686, TA716, TA763 and TH1817 since 1960s (Elisberg *et al.*, 1968; Shirai *et al.*, 1981; Wongprompitak *et al.*, 2013). In addition, an epidemiology study reveal that at least 5 isolates were circulating in Cambodia while 3 isolates in Vietnam (Duong *et al.*, 2013). UM-SNI36 and UM-SNI40

clustered with *O. tsutsugamushi* strain TA763 (1.00 PP). The other 23 specimens clustered with *O. tsutsugamushi* strain UT176 (0.98 PP). A pairwise distance was generated resulting in a range of 0% to 1.61%. During manual inspection of the sequencing chromatogram for the *TSA47* sequences, we noticed double peaks (ie. 2 different bases) at some nucleotide positions. Out of all the genes from the MLST scheme, we only managed to amplify succinyl-CoA synthetase (*sucD*) and pyruvate, phosphate dikinase precursor (*ppdK*) genes from one specimen, *R. tanezumi* R3 mitotype. Subsequent BLAST analyses revealed that the amplified *ppdK* and *sucD* genes were identical to *O. tsutsugamushi* isolate Karp (Accession no. LS398548.1) at 100% and *O. tsutsugamushi* strain Wuj/2014 (Accession no. CP044031.1) at 98.9% identities, respectively. This result was supported by an inquiry made in PubMLST that showed *ppdK* gene were closer to the Karp serotype detected in human from Papua New Guinea (Jolley *et al.*, 2018). The *ppdK* gene sequence also was close to sequence type reported in human from Laos and Thailand (Jolley *et al.*, 2018). Additionally, the *sucD* gene revealed that the strain was close to the allele in sequence type found in human from Thailand as well (Jolley *et al.*, 2018). However, both gene sequences were not able to be submitted to the PubMLST database as their chromatograms contained mixed signals (Sonthayanon *et al.*, 2010).

CHAPTER 5: DISCUSSION

5.1 Small mammals identified in the present study

The current study employed a combination of morphological and genotyping analyses to identify the small mammals captured from the oil palm plantation study sites. Six species of small mammals were trapped in oil palm plantations in Johor and Perak, respectively. The lateral and dorsal features of the rodent species in this study were published in a separate report (Nasir *et al.*, 2022b). Based on the morphological analyses, five rodent and one tree shrew species were identified. To further support morphological identification of the rodents, DNA barcoding assays successfully identified five rodent species; *R. tanezumi* R3 mitotype, *R. tiomanicus*, *R. exulans*, *R. argentiventer* and *R. tanezumi s. s*.

R. tanezumi R3 mitotype was found to be the predominant small mammal species in oil palm plantation area followed by *T. glis*. This was congruent to a study conducted by Andru *et al.* (2013) who found *R. tanezumi* R3 mitotype (also known as the R3 group) as the predominant species found at oil palm plantation in Riau and Bangka respectively. The findings by Nasir *et al.* (2022b), also supported *R. tiomanicus* as one of the common species found in oil palm plantations. This suggested that both *R. tanezumi* R3 mitotype and *R. tiomanicus* have adapted to living in oil palm plantation habitats. *T. glis* and *R. tanezumi* R3 mitotype are species of small mammals that were found abundantly at oil palm plantation areas based on data collected from the current study. A similar number of individuals were trapped for both species, suggesting the competition of food sources. Interestingly, (Nasir *et al.*, 2022b) reported that there were no *R. tanezumi* R3 mitotype found in oil palm plantations with young trees. Therefore, more investigation must be performed to determine the influence of food sources on species diversity.

Interestingly, the *R. tanezumi* R3 mitotype belonged to a group of indistinguishable species that has been speculated as one of the lineages from the Black Rat group (*Muridae*). *R. tanezumi* R3 mitotype was considered a cryptic species and indistinguishable via morphological methods alone (Robins *et al.*, 2007). The lineage sorting in this group is still considered incomplete, making them difficult to be distinguished from each other especially the *R. tanezumi* and *R. rattus* (Bazin *et al.*, 2012; Pages *et al.*, 2013). The delineation of the species of the rodents using the partial mitochondrial *COI* gene in this study has been discussed by (Nasir *et al.*, 2022a; Nasir *et al.*, 2022b). According to the study, the *R. tanezumi* R3 mitotype was very similar to the morphological description of *R. r. diardii* group described by Aplin *et al.* (2003a) and Paramasvaran *et al.* (2013). Genetically, based on *COI* DNA barcoding analyses, *R. tanezumi* R3 mitotype was homologous to the *Rattus* sp. R3 (Pagès *et al.*, 2010) and the RrC Lineage IV (Aplin *et al.*, 2011) that have been previously described. These indistinguishable groups were close to each other based on the pairwise genetic distance (0.41 to 0.52%). However, a new species name was not suggested since there were not enough data analyses to confirm the new species and also due to the multiple terminologies utilised by researchers (Nasir *et al.*, 2022a). Therefore, this genetic diversity must be further investigated such as conducting a comprehensive mitochondrial and nuclear gene barcoding study (Lecompte *et al.*, 2008). Based on *cytb* partial sequences of the rodents deposited in the GenBank for comparison, similar species identity was obtained revealing the *Rattus* sp., *R. argentiventer*, *R. exulans*, *R. tanezumi* s. s. and *R. tiomanicus.* A study has been established using the *cytb* gene marker to detect host species in a tick's blood meal resulting in rodents, common tree shrews and mammals as hosts (Che Lah *et al.*, 2015). This study indicated that *cytb* was a reliable gene marker in narrowing the species, however, not accurate enough to determine the species delineation, especially the *R. tanezumi* R3 mitotype.

Nuclear gene barcoding has also been suggested as one of the techniques to sort the species lineage of rats (Pagès *et al.*, 2010). There were studies from Europe (Martin *et al.*, 2000), Africa (Lecompte *et al.*, 2008), Japan (Motokawa *et al.*, 2022) and China (Zhang *et al.*, 2013), describing the identification of rat species using nuclear genes. Based on the *COI* phylogenetic analysis in the present study, there were four confirmed rat species and one cryptic species, the *R. tanezumi* R3 mitotype. When we performed DNA barcoding using the *cytb* gene, the sequencing results and subsequent phylogenetic analyses revealed findings that were largely congruent to the *COI* phylogenetic analysis. All the rat species were separated from each other and the *R. tanezumi* R3 group formed its own cluster. Limited information was extracted from the *cytb* analysis because of the limited entries in the GenBank as compared to the *COI* gene. A recent study reported the identification of RrC Lineage IV in Japan (Motokawa *et al.*, 2022). This rat lineage is commonly found in Southeast Asia (Aplin *et al.*, 2011), suggesting that it has a broader geographical spread than was previously thought. The study also reported that there was no significant difference in morphological characteristics between the RrC Lineage II (*R. tanezumi*) and the RrC Lineage IV. However, there was a slight difference in molar size between them (Motokawa *et al.*, 2022). The Lineage II recorded in Africa (Lecompte *et al.*, 2008) was congruent to the Southeast Asian *Rattus* reported by Watts and Baverstock (1995), Verneau *et al.* (1998) and Steppan *et al.* (2005). Therefore, further investigation on the delineation of rodents based on genetic features must be performed in Malaysia in the nearest future, especially on the indistinguishable *R. tanezumi* R3 mitotype group, RrC Lineage IV (Aplin *et al.*, 2011) and *Rattus* sp. R3 (Pagès *et al.*, 2010). Since both *COI* and *cytb* barcoding analyses were consistent with the morphological features, the rodents identified as *R. tanezumi* R3 mitotype in this study are accepted as it is and part of the identification data have already gone through peer review and published.

Among the numerous species within the genus *Rattus*, only three species; the Norway rat (*R. norvegicus*), the black or roof rat (*R. rattus*), and the Asian black rat (*R. tanezumi*) have synanthropically roamed in the urban ecosystems globally for a historically long period of time. The fourth invasive species, *R. exulans*, is limited to tropical Asia–Pacific areas (Kosoy *et al.*, 2015).

5.2 Ectoparasites associated with small mammals in the present study

Ectoparasites commonly found on small mammals are fleas, chiggers, mites and ticks (Raharjo; Mariana *et al.*, 2008; Ishak *et al.*, 2018a). The present study has identified all of them except for fleas from rodents and common tree shrews. Studies investigating the presence of ectoparasites on small mammals have been reported in the USA (Nieto *et al.*, 2007), Argentina (Nava *et al.*, 2003) and several other countries (Martinů *et al.*, 2018). A more recent of similar study was conducted in South Africa as well (Stevens *et al.*, 2022). However, very limited information can be found on the comprehensive study of the abundance of ectoparasites on small mammals in the Southeast Asia region. A study from Thailand reported the infestation of mites (*L. echidinus*), ticks (*Ixodes* spp.), fleas (*X. cheopis*) and pseudoscorpions (*Chelifer cancroides*) in rodents and common tree shrews (Thanee *et al.*, 2009). A recent attempt of using molecular identification (using the *COI* and cytochrome c oxidase II (*COII*) genes) reported the presence of *Ctenocephalides felis orientis* fleas and *H. hystricis* ticks on rodents and domestic mammals (Chaorattanakawee *et al.*, 2021). Both species were important as they potentially carry the *Rickettsia* spp. that causes murine typhus. The chigger species richness associated with small mammals was discussed by Chaisiri *et al.* (2019b) and the authors identified 38 species collected from 11 provinces in Thailand. The majority of the chigger species were found on *R. tanezumi* (Asian house rat) and *B. indica* (greater bandicoot rat). The richness of chigger

species was reported the highest in forest followed by dry land, rain-fed land and settlements. The chiggers also have a higher number of individuals collected during the dry season as compared to the wet season. A similar study in Peninsular Malaysia found that a higher number of chiggers during the dry season (Alkathiry *et al.*, 2022), concurring with the previous Thai report. Low chigger abundance was found in human dwellings and paddy fields with no significant effect of temperature throughout the sampling (Alkathiry *et al.*, 2022).

In Malaysia, a number of ticks (i.e. *Amblyomma cordiferum*, *I. granulatus*, *H. hystricis*, *Dermacentor auratus* and *Dermacentor atrosignatus*) and mite species (i.e. *L. echidninus*) have been found infesting the *R. tiomanicus* captured from the mangrove forests (Mohd-Taib *et al.*, 2021a). Several tick species (i.e. *Amblyomma*, *Dermacentor*, *Haemaphysalis* or *Ixodes*), mesostigmatid mites (i.e. *Laelaps* spp. and *Longolaelaps longulus*) and chiggers (i.e. *Eutrombicula* sp., *Leptotrombidium* spp. and Garliephia spp.) were found on small mammals such as *R. tiomanicus, Leopoldamys sabanus*, *Maxomys rajah*, *Maxomys surifer*, *Maxomys whiteheadi*, *Niniventer cremoriventer* and *Sundamys mueller* at Gunung Stong, Kelantan, Malaysia (Mariana *et al.*, 2005). A separate study also found hard ticks (*I. granulatus, Haemaphysalis* sp. 1, *Haemaphysalis* sp. 2), mesostigmatid mite (*L. echidninus, Laelaps sedlaceki* and *Laelaps nuttalli*), trombiculid mite (chigger species), and louse species (*Hoplopleura* sp.) from rodents trapped in Sarawak, Malaysia (Ng *et al.*, 2017), a finding similar to our present study. These studies suggest that small mammals harbour various ectoparasites regardless of the location where the animals are found.

5.3 Rodent and tree shrew as a reservoir for the detected pathogens

The spleen is an organ that functions to fight invading pathogens and act as a filter for the blood. Therefore, pathogens that circulate in the bloodstream can be found in the spleen, making it the most studied tissue by researchers trying to detect pathogens in rats (Winoto *et al.*, 2005; Gajda *et al.*, 2017; Lau *et al.*, 2020). This however, depends on the specific characteristics of the pathogens as some can be easier detected in certain tissues such as blood or lungs (Hanifah *et al.*, 2013; Chaisiri *et al.*, 2017b). In this study, four vector-borne bacteria (*O. tsutsugamushi*, *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp.) were screened from the spleens harvested from small mammals trapped in two oil palm plantations. These bacterial pathogens have previously been detected in the spleens. Additionally, we employed established molecular detection methods with proper positive and negative controls in place for the detection of *O. tsutsugamushi*, *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp. (Roux *et al.*, 1997; Labruna *et al.*, 2004; Inoue *et al.*, 2008; Masakhwe *et al.*, 2018; Lau *et al.*, 2020).

There were current infections of *O. tsutsugamushi, Borrelia* spp. and *B. phoceensis* in all host species except for *R. tanezumi s.s.* The data, however, was insufficient to conclude the *R. tanezumi s.s.* is excluded from potential host species for the detected pathogens. This was because only a single individual was collected from this study as compared to other studies (Hanifah *et al.*, 2013; Chaisiri *et al.*, 2017b). The *R. tanezumi* R3 mitotype was abundantly found in the oil palm plantation making them predominant hosts for all detected bacteria. The species also has been reported to carry the *Leptospira interrogans* and *Leptospira borgpetersinii* in Sarawak, Malaysia (Blasdell *et al.*, 2019a). Helminth parasites have been reported in RrC rodents emphasizing the role of *R. tanezumi* in carrying parasites as well (Chaisiri *et al.*, 2017a; Chaisiri & Morand, 2021). The helminths are known to cause several diseases such as onchocerciasis, lymphatic filariasis, soil-transmitted helminthiases, schistosomiasis, food-borne trematodiases and

taeniasis/cysticercosis. A unique flavivirus species has been reported in *Sundamys muelleri* rodent, that are commonly found in forest areas (Blasdell *et al.*, 2021). Several members of this genus are known as dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese-encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV). All of the viruses have been reported to cause fatality in humans. These findings indicate that rodent is a competent host to carry several types of pathogens that could risk the human in their vicinity. There was a study that reported the effectiveness of using chemical control and intensive coumatetralyl on rodents in oil palm plantations in Indonesia (Andru *et al.*, 2013). The study showed that rodents in Riau were more susceptible to coumatetralyl compared to Bangka. More investigation of species identification is needed in order to recognise the potential of using rodenticides as pest control. Anthropogenic factors other than agriculture, such as urbanisation also can contribute towards the zoonotic disease risk (Blasdell *et al.*, 2022). Therefore, more investigation must be conducted to identify each species and its pathogenic traits in order to understand the epidemiology of vector-borne diseases in Southeast Asia.

Tupaia glis captured in this study has been recorded to carry *O. tsutsugamushi* and *Borrelia* spp. Although no *Bartonella* sp. has been detected in this study, another study by Neves *et al.* (2018) has successfully detected *Bartonella* sp. from *T. glis* captured in Singapore. The study recorded four genotypes of *Bartonella* sp. that were circulated in that country based on the phylogenetic analyses. Less information is reported by other researchers regarding the vector-borne pathogens in *T. glis*. However, recent studies reported the parasite known as filarial in *T. glis* captured in Peninsular Malaysia (Uni *et al.*, 2017; Mat Udin *et al.*, 2020).

5.4 Detection of *Borrelia* spp.

Borrelia spp. closely related to *B. valaisiana, B. burgdorferi, B. theileri* and *B. yangtzensis* were detected in rodents and tree shrews trapped in the current study. Based on Figure 4.6 the phylogenetic analyses suggested there was exposure of infectious Bbsl group within the oil palm plantations. However, no associated vector in which tick, was found on the hosts throughout the sampling process. In contrast, some specimens were clustered with Thailand variants which were isolated from *I. granulatus* ticks collected off rodents, strain DS2823 T1 (Takhampunya *et al.*, 2021) and from the rodent host itself (i.e. *N. tenaster*) DS3646T (Takhampunya *et al.*, 2019) (Figure 4.6). *B. burgdorferi* is known to be pathogenic as it causes Lyme disease (Johnson *et al.*, 1984). A strain of *B. burgdorferi s. s.* was detected from a *R. tanezumi* mitotype R3 trapped at the oil palm plantation area in Kampung Tumbuh Hangat, Perak. Strain UM-SNI15 was revealed as *B. burgdorferi s. s.* as the sequence also was unique to other Bbsl strains detected. This is the first evidence of *Rattus* sp. being the potential host for *B. burgdorferi*. Other Bbsl strains listed were found to be closely related to *B. yangtzensis* and *B. valaisiana*.

In Southeast Asia, *B. yangtzensis*-related strains were first reported in *I. granulatus* tick collected from *S. muelleri* from a recreational forest in Malaysia (Khoo *et al.*, 2018). A separate study reported the second finding of *B. yangtzensis* in rodent-associated ectoparasites (chiggers and ticks) collected in northern Thailand (Takhampunya *et al.*, 2019). The study reported the detection of *B. yangtzensis* in *I. granulatus* and *Haemaphysalis bandicota* ticks. Schutzer *et al.* (2012) and Margos *et al.* (2015) reported the clustering of *B. yangtzensis* sp. with strains from China and Japan, but they were distant from *B. valaisiana* VS116 (detected in Switzerland), which was congruent with observations from this study as *B. yangtzensis* and *B. valaisiana*-related strains are distinctly clustered. While *B. yangtzensis* was previously detected in rodents in Sarawak, Malaysia, the researchers only identified the rodents using morphological keys and

rodents of the *Rattus* genera were all grouped as a single, *Rattus* spp (Lau et al., 2020). Hence, our present study is the first to show the detection of *B. yangtzensis*-related strains in *R. tanezumi* R3 mitotype in Southeast Asia.

Furthermore, Lau *et al.* (2020) also emphasized a higher *B. yangtzensis* detection rate in oil palm plantations in agreement with this study. *Borrelia yangtzensis*-related strains were also detected by Khoo *et al.* (2018) in *I. granulatus* ticks collected from forest and residential areas in Selangor, with a prevalence of almost 50%. This strongly suggests that *I. granulatus* could serve as the vector and rodents as the host for borreliae. It has been reported the presence of tick-borne relapsing fever borreliae, *Borrelia hermsi* in rodents and the pathogen was carried by *Ornithodoros hermsi* ticks in the USA (Schwan *et al.*, 2009; Nieto & Teglas, 2014). *Borrelia miyamotoi* has been reported in rodent-associated ticks, *Haemaphysalis inermis* and *I. ricinus* in Slovakia (Heglasová *et al.*, 2020). The first reports on rodents infected with borreliae in Chile and Poland have been described, however, there was no information on the vectors associated with the infected rodents (Sánchez *et al.*, 2020; Gryczyńska *et al.*, 2021). Interestingly, RF borreliae not only can be carried via ticks, but by louse as well (Warrell, 2019). More studies are required to evaluate the role of these small mammals in the ecology of the identified RF borreliae.

The RF borreliae from the present study were found to be closely related to the *Borrelia* sp. detected from Japanese Sika deers (*Cervus nippon*) and the associated tick ectoparasite, *Haemaphysalis longicornis* (Furuno *et al.*, 2017; Kumagai *et al.*, 2018; Nakayama *et al.*, 2019), a tick species not previously reported in Malaysia. Several strains from the present study were also clustered with RF borreliae that were previously detected in *H. hystricis* collected from a wild boar (Khoo *et al.*, 2017) and a dog (Khoo *et al.*, 2021). These findings suggest that both *H. longicornis* and *H. hystricis* might

harbour closely related borrelial strains. Our study presented evidence of the detection of RF borreliae strains in *Rattus* spp. rodents and *T. glis*. In Thailand, a previous study reported the detection of RF borreliae in rodents (*Rattus* spp.*, B. indica, Niviventer* spp., *L. sabanus, Crocidura fuliginosa, M. caroli* and *M. cookii*) and ticks (*H. bandicota, Rhipicephalus sanguineus, I. granulatus and Dermacentor* spp.), however, they were more closely related to *B. theileri*, *B. lonestari* and *B. miyamotoi* (Takhampunya *et al.*, 2019; Takhampunya *et al.*, 2021). *B. crocidurae*, the causative agent for tick-borne relapsing fever in West Africa, was commonly detected in small mammals, suggesting their importance in disease epidemiology (Schwan *et al.*, 2012; Ndiaye *et al.*, 2021). Small mammals were also known as reservoirs for *B. miyamotoi*, another RF borreliae, in different geographical regions including Malaysia (Taylor *et al.*, 2013; Siński *et al.*, 2016; Lau *et al.*, 2020). The findings from our study add to the evidence of the potential role of small mammals, especially *Rattus* spp. rodents and *T. glis*, in the ecology of the identified RF borreliae in the studied areas. *B. miyamotoi* was previously assumed to be non-pathogenic until the first human infection was reported in Russia (Platonov *et al.*, 2011a). Although the currently identified RF borreliae strains have yet to be associated with human infections, increased surveillance is important as small mammal infestation is widespread in oil palm plantations, which may lead to pathogen transmission to humans residing or working within the plantations. In Malaysia, *B. yangtzensis*-related strains were first reported in *I. granulatus* tick collected from *S. muelleri* from a recreational forest (Khoo *et al.*, 2018; Loong *et al.*, 2018). The MLST finding revealed that our *clpA* allele (allele 310) was closest to ST360 reported in Japan (Figure 5.1 and Figure 5.2) (Kawabata *et al.*, 2013). The ST360 strains were isolated from *M. caroli* rodent and *I. granulatus* tick.

Allele	% Identity	Mismatches	Gaps	Alignment	Compare
clpA: 82	99.136	5	0	579/579	
clpA: 81	99.136	5	0	579/579	
clpA: 76	98.964	6	0	579/579	
clpA: 100	98.791	7	0	579/579	
clpA: 79	98.618	8	0	579/579	
clpA: 78	98.618	8	0	579/579	
clpA: 83	98.273	10	0	579/579	
clpA: 89	98.100	11	0	579/579	
clpA: 84	98.100	11	0	579/579	
clpA: 75	98.100	11	0	579/579	
clpA: 80	97.582	14	0	579/579	
clpA: 50	96.718	19	0	579/579	
clpA: 257	96.718	19	0	579/579	
clpA: 49	96.546	20	0	579/579	
clpA: 110	96.546	20	0	579/579	
clpA: 96	96.373	21	0	579/579	
clpA: 77	96.200	22	0	579/579	
clpA: 297	94.646	31	0	579/579	
clpA: 179	91.883	47	0	579/579	
clpA: 32	91.537	49	0	579/579	
clpA: 299	91.537	49	0	579/579	
clpA: 234	91.537	49	0	579/579	
clpA: 74	91.379	48	2	580/579	
clpA: 74	91.379	48	2	580/579	
clpA: 28	91.364	50	0	579/579	
clpA: 26	91.364	50	0	579/579	

Figure 5.1: Similarities of *clpA* allele 310 (*Borrelia yangtzensis*) with closely related *clpA* alleles in the PubMLST database.

Isolate fields							MLST								
id	isolate	aliases	country	species	year	source	clpA	clpX	nifS	pepX	pyrG	recG	rplB	uvrA	ST
1262	Okinawa-MC8B05		Japan	Borrelia yangtzensis		animal host	81	66	59	70	74	62	58	58	360
1272	066-5		Japan	Borrelia yangtzensis		tick	81	66	59	70	74	62	58	58	360

Figure 5.2: *Borrelia yangtzensis* ST360 strains deposited in the PubMLST database.

This suggests that small mammals in the present study were competent to harbour borreliae strains accustomed to multiple host species. A previous study reported the detection of *B. yangtzensis* in rodents and the respective *Ixodes* tick and chigger ectoparasites in northern Thailand (Takhampunya *et al.*, 2019). *B. yangtzensis* was also

detected in rodents and the ticks that were found (*I. granulatus* and *H. longicornis*) in the rodents in China and Japan (Margos *et al.*, 2015). This indicates that *B. yangtzensis*-related strains are widespread in East and Southeast Asia. Additionally, findings from our study included *R. tanezumi* R3 mitotype as another potential host for the pathogen. Since *B. yangtzensis* is pathogenic to humans (Kim *et al.*, 2021), our findings suggest that *B. yangtzensis* could impose risks of infection on the residents in the oil palm plantations.

One unique strain, UM-SNI19 was separated from the remaining of RF borreliae based on the phylogenetic analysis in Figure 4.6. Currently, we were unable to ascertain the phylogenetic placement of strain UM-SNI19 in this study. BLAST analyses suggest that strain UM-SNI19 may be more closely related to the RF borreliae as the highest query cover and similarity scores matched with members of the RF borreliae strains, even though a portion of the sequences also exhibited high similarity to a single member of the LD borreliae, *B. afzelii*. Moreover, this strain revealed a unique gap compared to other strains in the multiple sequence alignment provided in Figure 5.3 below.

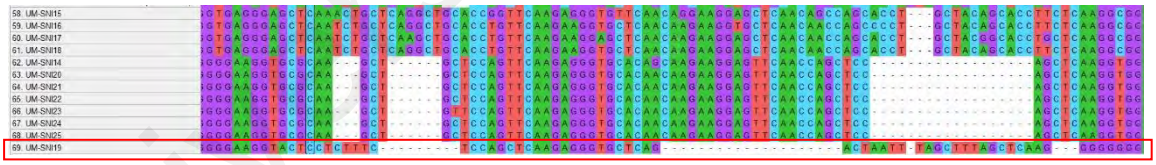

Figure 5.3: The unique gap in the *flaB* sequences of strain UM-SNI19 (red box), compared to other borreliae strains.

These findings suggest that strain UM-SNI19 *Borrelia* sp. may be a distinctive genotype based on the *flaB* sequences, however, investigation into more genes and more specimens will be necessary to confirm this observation. Overall, there were nine out of twelve individuals *Borrelia*-positive hosts found in oil palm plantation areas and three of them were trapped in residential areas, indicating the potential of spillover to the villagers and double the risk of exposure to the villagers who work at oil palm plantations.

5.5 Detection of *Bartonella phoceensis*

Bartonellae is common in cats as the cat scratch disease is caused by *B. henselae*. In the present study, *B. phoceensis* was detected in *R. argentiventer* and *R. tanezumi* R3 mitotype that were found in Johor and Perak (Figure 4.7). The phylogenetic tree (Figure 4.3) displayed the relationship of the *Bartonella*-positive samples clustered into one clade known as *B. phoceensis*. Based on Table 4.2, the prevalence of *B. phoceensis* in small mammals in the current study was relatively low (4.9%) as compared to the study reported by Blasdell *et al.* (2019b), 57.3%. The authors reported the prevalence of *B. phoceensis* was low in rural areas, similar to the localities of the present study. This suggests that rural areas may have a lower rodent population density, discouraging intraspecies interaction and causing lower *Bartonella* spp. transmission. Blasdell *et al.* (2019b) also hypothesized that hosts with mites and lice had higher chances of pathogen infection. This was in contrast to our findings whereby there were a large number of rodents infested with mites and lice, however, only a handful of hosts were infected with *Bartonella* spp. To date, the vector for *B. phoceensis* has yet to be found. Apart from *B. phoceensis*, there are other *Bartonella* spp. associated with rodents, such as *B. rattimassiliensis, B. coopersplainsensis* and *B. tribocorum*. In Thailand, Saengsawang et al. (2021) detected *B. tribocorum, B. phoceensis, B. grahamii*, and *B. rattimassiliensis* in the blood samples of *R. exulans* (Pacific rat) and *R. tanezumi* (Asian house rat). Anh *et al.* (2021) reported a high detection rate for *Bartonella* spp. in rodents trapped in Vietnam. They found 42 out of 133 rodents (31.6%) (*R. tanezumi, Niniventer fulvescens, R. rattus*, and *B. savilei*) positive for the presence of *Bartonella* spp.

To the best of our knowledge, there were only a few studies reporting the prevalence of *Bartonella* spp. in rodents in Malaysia. A recent study reported a relatively low prevalence of *B. phoceensis* in rodents captured in urban areas (Low *et al.*, 2020a) when compared to the present study. The authors found 3.73% prevalence of *B. phoceensis* in

synanthropic rodents trapped in Kuala Lumpur. Another study found various bartonellae group members such as *B. tribocorum*, *B. rattimassiliensis*, *B. coopersplainsensis*, *B. elizabethae*, and *B. queenslandensis* from rodents captured in Kuala Lumpur and Penang, at 13.7% prevalence (Tay *et al.*, 2014b). These data suggested that synanthropic rodents especially, the *Rattus* spp. could serve as a potential reservoir for bartonellae. Although the pathogenicity of *B. phoceensis* is yet to be proven, we should not overlook the potential of this microorganism in causing disease.

5.6 Detection of *Orientia tsutsugamushi*

Detection of *O. tsutsugamushi* in small mammals in Malaysia was first recorded as early as 1973 (Walker *et al.*, 1973). In the present study, small mammals (rodents and tree shrews) trapped in two locations, Johor and Perak, were studied for the presence of selected vector-borne pathogens (*Orientia* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp.) by PCR amplification of bacteria-specific genes. *O. tsutsugamushi* has been detected in various small mammal species across Southeast Asia (Elliott *et al.*, 2019), and conventional detection was based on bacteria isolation or serology (Tay *et al.*, 1998; Frances *et al.*, 1999; Frances *et al.*, 2001; Rodkvamtook *et al.*, 2011; Chareonviriyaphap *et al.*, 2014; Linsuwanon *et al.*, 2018; Rodkvamtook *et al.*, 2018; Elders *et al.*, 2021). However, more recent efforts focused on PCR assays targeting the *TSA47* gene for *Orientia* detection (Blacksell *et al.*, 2008; Hanifah, 2013; Wongprompitak *et al.*, 2013; Elliott *et al.*, 2021). In the present study, *O. tsutsugamushi* was detected in 12.3% of the small mammals. The data revealed an equal number of individuals being infected from both study sites in Perak and Johor. This rate of detection was in contrast to the low prevalence reported in a previous study detecting *O. tsutsugamushi,* in only 1 out of 88 small mammals (i.e. *L. sabanus*) (~1%) captured from eight different states in

Peninsular Malaysia (Hanifah, 2013). Another study employing the PCR detection of *O. tsutsugamushi* in rodents captured near the Selangau Health Center, Sarawak, Malaysia, however, did not yield any positive results (Tay *et al.*, 2002b). The highest prevalence according to the literature searches, was the 20% *O. tsutsugamushi*–positive detection in the liver and spleen of rodents sampled in Si Racha, Chonburi province, Thailand (Rodkvamtook *et al.*, 2018). Other than that, most studies resulted in very low *O. tsutsugamushi* prevalences ranging from 0.7 to 2.3%, as compared to our study. These studies also employed the PCR method, but some of them were detecting the pathogen in different tissues, such as the lungs (Chaisiri *et al.*, 2017b; Linsuwanon *et al.*, 2018; Takhampunya *et al.*, 2018; Takhampunya *et al.*, 2019). The relatively low prevalence of *O. tsutsugamushi* could also be caused by insensitive primers used to amplify the sequence of the *TSA47* gene from the specimens. Chiggers were also found in less than half of the obtained small mammals, possibly explaining the low prevalence of *O. tsutsugamushi*. Nevertheless, the current study presented evidence of the molecular detection of *O. tsutsugamushi* in small mammals captured from oil palm plantations in Malaysia, adding on to the knowledge of *O. tsutsugamushi* prevalence in Southeast Asia.

In the current study, there was an increase in *O. tsutsugamushi* detection in November and December, coinciding with the wet season in Malaysia (Nasir *et al.*, 2022a). This was in contrast to the data presented by Linsuwanon *et al.* (2021) in terms of the season despite the same month of collection, December. The study found the highest prevalence of *O. tsutsugamushi* in rodents during December in which considered as dry-cool season in Thailand. However, the other data presented in the same study led to rats as dead-end hosts for the transmission. Alkathiry *et al.* (2022) reported that there was an association of habitat type (highest in forest border), state (highest in Perak), and season (highest in dry) to the abundance and species richness of chiggers. This data suggests that there is a risk of transmission of *O. tsutsugamushi* in the forest border and dry season.

More analysis must be performed in order to correlate the climate with the abundance of small mammals and pathogen transmission.

The majority of *O. tsutsugamushi* surveys in small mammals in Southeast Asia were carried out in Thailand. The Thai researchers have discovered the presence of *O. tsutsugamushi* in *R. bukit*, *R. rattus*, *R. argentiventer*, *R. berdmorei*, *R. losea*, *B. indica*, *Rattus koratensis*, *B. savilei*, *R. exulans* and *T. glis* (Coleman *et al.*, 2003). Thailand has consistently reported the detection of *O. tsutsugamushi* in small mammals using molecular method (i.e. *R. rattus* complex, *B. indica*, *T. glis*, *Rattus tanezumi*, *R. andamanensis, R. exulans, B. indica, Mus cookie, R. nitidus, B. berdmorei, B. savilei, Berylmys bowersi, Leopoldamys edwardsi, Rattus sp.* phylogenetic clade 3 and *M. berdmorei)* as well as in chiggers associated with the small mammals (Frances *et al.*, 1999; Chaisiri *et al.*, 2017; Linsuwanon *et al.*, 2018; Rodkvamtook *et al.*, 2018; Takhampunya *et al.*, 2018; Takhampunya *et al.*, 2019; Elliott *et al.*, 2021; Linsuwanon *et al.*, 2021). A study from Vietnam reported the detection of *O. tsutsugamushi* in *Rattus flavipectus* (Lan Anh *et al.*, 2020) and *R. norvegicus* (Hotta *et al.*, 2016). All data obtained from the literature supported the observation that rodents and tree shrews are competent hosts for *O. tsutsugamushi,* similar to the present study.

A recent study detected *O. tsutsugamushi* in chiggers parasitizing *R. rattus* and *Tupaia* sp. in Malaysia, albeit from a different state; Kelantan (Ernieenor *et al.*, 2021). The animals were trapped in areas near the house of a scrub typhus patient and it was surrounded by mixed ecologies such as shrubs, coconut, fruit and sugar cane orchards. That study also reported two out of 16 pools of *L. deliense* mites (12.5%) tested positive for *O. tsutsugamushi* (Ernieenor *et al.*, 2021). Chaisiri *et al.* (2017b) reported that *O. tsutsugamushi*-infected rodents in Thailand were also trapped in similar ecotypes such as forested and reforestation areas, fallows, cassava plantations, and rice fields. Although

the main ecotype covered in our study was the oil palm plantation, there were rice fields and also residential areas nearby the Perak study site. *O. tsutsugamushi* was detected in all small mammal species collected from this study except for *R. tanezumi s. s.*, and this can be explained as *R. tanezumi s. s.* has been shown to be an incidental rodent species in oil palm plantations in Malaysia (Nasir *et al.*, 2022b). The two synanthropic species; *R. exulans* and *R. tanezumi* R3 mitotype, live in close association with humans (Bordes *et al.*, 2015; Kosoy *et al.*, 2015; Morand *et al.*, 2015). From the findings of the current study, both species have been found to carry *O. tsutsugamushi*. A separate project that studied the ectoparasites infesting the five small mammal species captured in the present study found close to 41 000 chiggers in those animals (Alkathiry *et al.*, 2022). A majority of them were identified as *A. indica* and *L. deliense* (both are vectors for scrub typhus) (Alkathiry *et al.*, 2022). The large number of chiggers infesting the animals could explain the detection of *O. tsutsugamushi* in rodents and tree shrews in the current study. Similarly, Thailand has also reported the prevalence of *O. tsutsugamushi* in chiggers associated with rodents (Takhampunya *et al.*, 2019). The detection of *O. tsutsugamushi* in rodents however, was lower (3%) (Takhampunya *et al.*, 2019) as compared to our study, which detected *O. tsutsugamushi* in 12.3% of the total small mammals analysed in Perak and Johor.

The current study also detected *Orientia TSA47* sequences similar to the *O. tsutsugamushi* isolated from scrub typhus patients (Blacksell *et al.*, 2008; Paris *et al.*, 2009; Jiang *et al.*, 2013). This was congruent to studies reporting the infection of febrile patients, healthy villagers and rubber estate workers from rural areas with *O. tsutsugamushi* (Tay *et al.*, 1999; Sagin *et al.*, 2000; Tay *et al.*, 2000). A serological study of the Orang Asli, the aborigines of the Malay Peninsula, showed that as many as 73% of persons over the age of 20 years living in the deep forest had antibodies to *Rickettsia tsutsugamushi* (currently known as *O. tsutsugamushi*), while the figures for those living

in the fringe jungle and on smallholdings were 48% and 8% respectively. For persons aged under 20 years, the percentages were 56%, 18% and 0%. Similar figures were obtained from habitats widely separated in Malaysia (Cadigan Jr *et al.*, 1972). Residents who live near the forest fringe are considered as the high-risk group to be infected with vector-borne pathogens. These data suggested that small mammals potentially increase the risk of transmitting *O. tsutsugamushi* to humans working and living in the oil palm plantations since they all share the same habitat.

In the present study, we employed MLST on the *TSA47*-PCR positive specimens to strengthen and complement *O. tsutsugamushi* identification. We, however, could only amplify two different alleles from two specimens. The amplified *ppdK* and *sucD* alleles contained mixed sequences at several positions (Figures 5.4 and 5.5), hence allele numbers could not be assigned to them by the PubMLST curators.

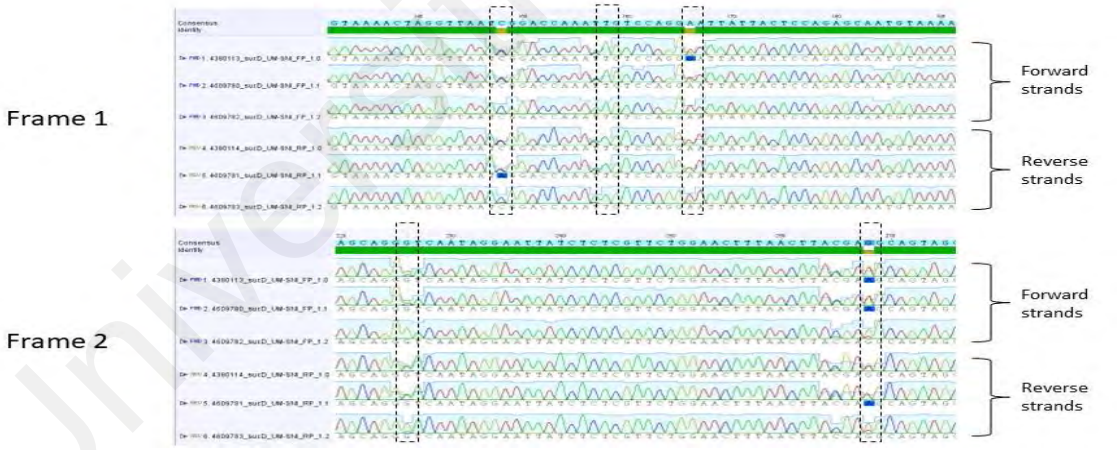

Figure 5.4: Positions with heterozygous double peaks in the *sucD* allele of *Orientia tsutsugamushi*.

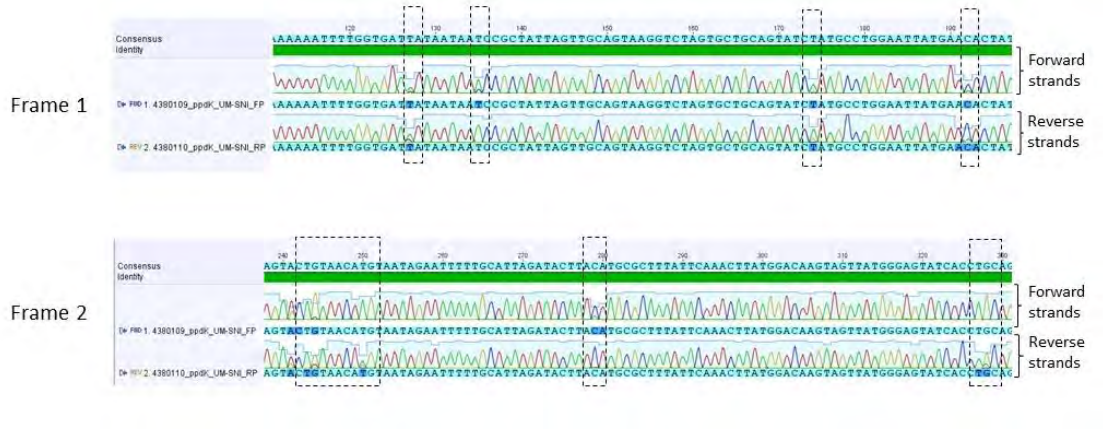

Figure 5.5: Positions with heterozygous double peaks in the *ppdK* allele of *Orientia tsutsugamushi*.

The presence of mixed sequences very likely indicates the existence of more than one *O. tsutsugamushi* strain in the specimen (Sonthayanon et al., 2010). Additionally, a recent publication also reported the detection of mixed *O. tsutsugamushi* strains in humans (Tang *et al.*, 2022), indicating that this phenomenon is not uncommon. Similar to our study, Ernieenor *et al.* (2021) reported the presence of multiple bases at some nucleotide positions in the sequences of the amplified PCR product. This was possible as Sonthayanon *et al.* (2010) explained that different *O. tsutsugamushi* strains may often co-exist in the same host at the same time, allowing for genetic exchange and diversity to occur. Additionally, *O. tsutsugamushi* genes are known to have a high rate of pseudogenisation and gene degradation (Batty *et al.*, 2018), supporting our MLST findings that only yielded partial amplification. These characteristics could influence sequencing efficiency and also the underestimation of the *O. tsutsugamushi* infection rates in small mammals. Moreover, from the MLST analysis, one of the *O. tsutsugamushi* strains, UM-SNI36 from our study was closely related to strains that have been reported in humans from Laos, Thailand and Papua New Guinea, allele 17 from ST44 (Jolley *et al.*, 2018). The strain also was close to allele 9 from ST22 which was also isolated from humans (Sonthayanon *et al.*, 2010). The discovery of a *O. tsutsugamushi* strain associated with human infections suggests that there it could be potentially transmitted from rodents

to humans via vectors since there was infestation of chiggers on almost all hosts trapped in the current study (Alkathiry *et al.*, 2022). Altogether, these studies imply the risks of contracting scrub typhus among inhabitants or workers in the agriculture sector such as oil palm plantations, where there could be exposure to chiggers and the small mammal hosts.

Incidences of scrub typhus in humans has been associated to the habitat in the 1970s as most reports during that time were from people living in the jungle and suburban areas (Cadigan Jr *et al.*, 1972). A previous study reported that *O. tsutsugamushi* were detected in rodents trapped in large forest cover (Hanifah, 2013). The oil palm plantation study sites for the present study are also located at the forest fringed areas and forest conversion is a risk factor for the emergence of scrub typhus. A high prevalence of *O. tsutsugamushi*-infected chiggers was observed especially in areas with grassland and forest ecotones (Linsuwanon *et al.*, 2021). Our previous ecological analysis of *O. tsutsugamushi* infection in rodents, which included most of the same host specimens analysed in the current study before pathogen genotype data were obtained, concluded that neither habitat nor season was significantly associated with infection, although infection prevalence was highest in oil palm plantations compared with peripheral habitats (Alkathiry et al., 2022). This lack of statistically significant ecological effects may be due to the long duration of *O. tsutsugamushi* infection in small mammals or high reinfection rates coupled with their movement between adjacent habitats (Elliott et al., 2019). In Johor, *O. tsutsugamushi* infection of small mammals was positively associated with a Malaysian endemic vector, *L. arenicola*, although no significant relationship between any chigger species and *O. tsutsugamushi* infection in Perak was apparent (Alkathiry et al., 2022).

5.7 No evidence of *Rickettsia* spp. infection

There was no evidence of *Rickettsia* infection in the small mammals trapped at the study sites in the present study. Even by using two different sets of primers (Labruna *et al.* (2004), we could not amplify any positive specimens. This was plausibly due to the absence of fleas on the hosts, as these ectoparasites are known to be vectors for *Rickettsia* spp. in small mammals. This was congruent to a study reported by Jiang *et al.* (2006). The study reported no infestation of fleas on rats caught in rural areas which was similar to our study. Instead, a higher number of fleas was collected from rodents trapped in suburban and urban areas. Ticks also could be a vector for *Rickettsia* spp. based on a study reported by Minichová *et al.* (2017). The study reported that there was an infection of *R. helvetica* and *R. monacensis* in three *Apodemus flavicollis* rat. Another study reported the presence of *R. typhi* and *R. felis* in rodents trapped in urban areas in Thailand and this was the first detection of rickettsiae in small mammals in Southeast Asia (Rungrojn *et al.*, 2021). This detection of *R. felis* in rodents in Southeast Asia showed that synanthropic rodents are competent in harbouring a pathogen usually harboured by cats and dogs. There is also the possibility that the studied small mammals had recovered from *Rickettsia* infection at the time of capture. This would help explainthe absence of *Rickettsia* sp. in this study. A seroprevalence study might provide insights into the past exposure of the small mammals to *Rickettsia* spp.

There was a possibility of co-infection since *O. tsutsugamushi* and *B. phoceensis* were found in four hosts; THTR010, THTR043, THTR048 and KTR043. This finding however, is not unusual as rodents can harbour different types of pathogens within themselves at the same time (Chareonviriyaphap *et al.*, 2014).

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Major conclusion

There were six species of small mammals identified in the study sites, which were the rodents; *R. tanezumi* R3 mitotype, *R. exulans*, *R. tiomanicus*, *R. argentiventer*, *R. tanezumi s. s.* and the common tree shrew, *T. glis*. The predominant species was *R. tanezumi* R3 mitotype. This group must be further characterised as the abundance of this group was more than half of the total of small mammals caught in oil palm plantation (i. e. Perak and Johor) and has the most infected individuals, implying the important role they play as the potential reservoir for the detected pathogens. There was presence of *O. tsutsugamushi*, LD borreliae, RF borreliae and *B. phoceensis* in five species of the small mammals, namely *R. argentiventer, R. exulans, R. tanezumi* R3 mitotype, *R. tiomanicus* and *T. glis* captured from oil palm plantations in Perak and Johor, Malaysia. Interestingly, an ambiguous borreliae strain was discovered in this study. Strain UM-SNI19 appeared to be separated from the LD borreliae, RF borreliae and even the third group borreliae. Further investigation into genome sequences will be necessary to ascertain the phylogenetic relationship of this specimen and other borreliae group. *Orientia tsutsugamushi* and *B. phoceensis* were detected in the same four *R. tanezumi* R3 mitotype, suggesting co-infection. Findings from this study indicate that *O. tsutsugamushi, Borrelia* spp. and *B. phoceensis* are prevalent among the small mammal populations in the oil palm plantation setting. The fact that these animals can harbour more than one pathogen at the same time increases the risk of disease transmission to other animals and humans in the vicinity.

6.2 Challenges of study

One of the limitations of the study was the limited DNA concentration of the pathogens obtained from the DNA extraction. Even though the protocol of the DNA extraction has been optimised, the quantity or concentration of the bacteria itself could limit the detection rate of the pathogen. Moreover, the tissue specimens from which DNA extraction were performed, could contain on the inhibitors that would interfere in the subsequent PCR amplification. These factors could reduce the PCR detection rate and negatively influence the downstream experiments such as genomic sequencing, MLST and cloning.

During the sampling period, some of the cages were disturbed by monkeys and wild boars, leading to the loss of specimens. In addition, the detection of pathogens was based on the partial sequences of each specific gene marker and this halted further characterisation of unique strain (UM-SNI19). Strain UM-SNI19 which could not be clustered in to any borreliae groups, unfortunately could not be further distinguished. Lastly, the limitation of laboratory working hours due to the various lockdown measured to combat the COVID-19 pandemic, further affected and delayed experimental work.

6.3 Recommendation and future study

From the present study, it is suggested to further investigate the ectoparasites found on the trapped rodents and trees shrews. This is important to link the detected pathogens to the hosts and ectoparasite vectors. Furthermore, a comprehensive data of the arthropods as the vector and rodents as the reservoir allows us to assess the risk and could help to prevent the transmission of *O. tsutsugamushi, Borrelia* spp., *Bartonella* spp. and other undetected pathogens among the oil palm plantation workers. This is because there are more than one type of arthropod infesting the host that could carry different vector-borne

pathogens. The weather data collection should be increased in terms of the collection period in order to get a better correlation between the season and the abundance of hosts and vectors. The environmental factors must also be taken into consideration to get a better perspective on the distribution of small mammals and the associated vectors. This data could help in understanding the influence of vector-host association in pathogen transmission.

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