Title

MOLECULAR ECOLOGY OF CHIGGER MITES (ACARI: TROMBICULIDAE) AND ASSOCIATED BACTERIA IN THAILAND

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

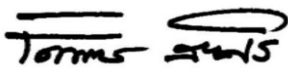
by

Kittipong Chaisiri

November 2016

AUTHOR'S DECLARATION

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author



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This research was carried out in the Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool

ABSTRACT

Molecular ecology of chigger mites (Acari: Trombiculidae) and associated bacteria in Thailand

Kittipong Chaisiri

Chiggers are the tiny six-legged larval stage of mites in the family Trombiculidae. These mites, particularly the genus *Leptotrombidium*, act as important vectors of *Orientia tsutsugamushi,* the causative agent of scrub typhus disease in the Asia-Pacific region (including Thailand). Although the medical impact of these mites has been recognized in the country due to the increasing incidence of the disease in humans, knowledge of the ecology and epidemiological role of these mites is still very limited to date.

A systematic review of mite-associated bacteria was conducted from 193 publications (1964 - January 2015) providing a reference database of bacteria found in mites of agricultural, veterinary and medical importance. Approximately 150 bacterial species were reported from 143 mite species with *Cardinium, Wolbachia* and *Orientia* as the dominant genera.

Nationwide field sampling of small mammals from 13 locations in Thailand revealed a high diversity of chigger mites. From approximately 16,000 mites isolated from 18 host species examined (1,574 individual animals), 38 chigger species were found including three species new to science (*i.e*., *Trombiculindus kosapani* n. sp., *Helenicula naresuani* n. sp. and *Walchia chavali* n. sp.) and 10 new records for the first time in the country. Brief taxonomic information for the morphological identification of chiggers is provided. A combination of autofluorescent and brightfield microscopy was demonstrated to be a novel approach to study both the morphology and DNA profile of the same individual chigger.

Most chigger species showed low host specificity. The diversity of chiggers on hosts was influenced by host intrinsic (*i.e.*, host phylogeny and maturity) and extrinsic factors (*i.e.,* habitat and geographical location). Chigger species richness and host-chigger network connectance were found to be interrelated variables explaining human scrub typhus incidence in Thailand.

Chigger-associated bacteria were investigated for the first time using an Illumina MiSeq 16S rRNA amplicon sequencing approach. DNA of *O. tsutsugamushi* was detected in the chigger population as expected. In addition to *O. tsutsugamushi*, *Borrelia* and *Mycobacterium* were identified aspotential pathogens of human and animals. Potential symbiotic bacteria of arthropods; *e.g*., Candidatus *Cardinium, Pseudonocardia, Rickettsiella* and *Wolbachia* were also discovered for the first time in chiggers. An important technical limitation was that chigger DNA starting quantity (individual specimens *versus* pooled mites) was found to have a significant impact on the apparent microbiome profile.

These outcomes from the studies of chigger taxonomy and the ecology of host-chigger interactions, as well as the composition of the microbiome in chiggers, are of key importance to the chigger research field, providing essential information for disease epidemiology with vector control implications.

Keywords: Chiggers, Trombiculidae, molecular ecology, microbiome, bacteria, 16S rRNA amplicon sequencing, Illumina MiSeq, Thailand

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DEDICATION

This work is dedicated to my parents, wife and children for their love and patience

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LIST OF ABBREVIATIONS

AICc	Akaike's information criterion adjusted for sample size
ANOSIM	analysis of similarity
ANOVA	analysis of variance
BioDivHealthSEA	Local impacts and perceptions of global changes: Health, biodiversity and zoonoses in Southeast Asia project
biom	biological observation matrix
BLASTn	Nucleotide basic local alignment search tool
bp	base pair
C	central
CA	correspondence analysis
CEGMA	core eukaryotic genes mapping approach
CERoPath	Community ecology of rodents and their pathogens in Southeast Asia project
cf.	compare or see also; commonly used for open nomenclature
CGR	Center for Genomic Research, University of Liverpool
Chao1	Chao1 non-parametric richness estimator
CO_2	carbon dioxide
COI	cytochrome oxidase subunit 1 gene
CSR	chigger species richness
ddH_2O	double-distilled water
df	degree of freedom
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dsDNA	double stands deoxyribonucleic acid
E	east
e.g.	for example
EM	electron microscope

epank1	*Ehrlichia phagocytophila* protein gene in Ankyrins family
et al.	and others
etc.	and other things
E-value	expected value (in BLAST)
F1	filial generation 1
F2	filial generation 2
FITC	fluorescein isothiocyanate
ftsZ	filamenting temperature-sensitive mutant Z gene
GC content	guanine-cytosine content
GenBank	National genetic sequence data base
GLM	Generalized linear models
gltA	citrate synthase gene
GPS	Global Positioning System
groEL	chaperonin gene
gyrb	DNA gyrase subunit B
H'	Shannon diversity index
HTS	high throughput sequencing
IBM	International Business Machines Corporation
ID	identity
i.e.	that is
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	intergenic spacer
Jack1	first-orderd Jackknife estimator
K	the number of estimated variables in AICc model selection
kDa	kilodalton
km^2	square kilometre
KOGs	key orthologs for eukaryotic genomes
KW	non-parametric Kruskal-Wallis test
Lao PDR	Lao People's Democratic Republic
LB	Luria-Bertani medium

Log-like	log likelihood function
MaIT	Model-averaged importance of term
m	metre
M	molar
min	minute
ml	millilitre
ML	Maximum likelihood
MLST	multilocous sequence typing
mm	millimetre
mM	millimolar
MPR	minimum positive rate
N	north
NCBI	National Center for Biotechnology Information
NE	northeast
ng	nanogram
NJ	neighbour joining
nm	nanometre
nmol	nanomole
No.	number of
NODF	Nestedness metric based on overlap and decreasing fill
n. sp.	specimen new or undescribed
OpPath	opportunistic pathogen
opt	optimal
OTU	operational taxonomic unit
Path	pathogen
PCA	principle component analysis
PCoA	principle coordinate analysis
PCR	polymerase chain reaction
pg	picogram
Polymerase LD	DNA polymerase enzyme with low bacterial DNA
PPLO	pleuropneumonia-like organisms (mycoplasma)

p or p-value	calculated value probability
QIIME	Quantitative Insights into Microbial Ecology software package
qPCR	quantitative polymerase chain reaction
RDP	Ribosomal Database Project
rpm	round per minute
RT-PCR	real-time polymerase chain reaction
S	south
Sap	saprophyte
SEA	Southeast Asia
SEATO	Southeast Asian Treaty Organisation
sec	second
SEM	scanning electron microscope
SMRT	single molecule real-time sequencing
SNPs	single nucleotide polymorphisms
sp.	species (singular form)
spp.	species (plural form)
STRs	short tandem repeats
SymA	symbiont (arthropod)
SymN	symbiont (nematode)
TEM	transmitted electron microscope
TSA	Trypticase soy agar
TSB	Tyrpticase soy broth
W	west
WGS84	World Geodetic System 1984
WHO	World Health Organization
Wr	Akaike's weight
wsp	*Wolbachia* surface protein gene
UK	The United Kingdom
UniFrac	unique fraction phylogenetic-based measurement methods
USA	The United States of America

V	voltage
VIF	variance inflation factor
vs	versus
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
16S rDNA	16 Svedberg ribosomal deoxyribonucleic acid
16S rRNA	16 Svedberg ribosomal ribonucleic acid
56-KDa TSA	56 Kilodalton type-specific antigen
α	alpha
β	beta
γ	gamma
µl	microlitre
µm	micrometre (micron)
µM	micromolar
^{o}C	Celsius degree
%	percentage

Abbreviations of chigger morphometry methods are given separately in CHAPTER 3

CHAPTER 1

GENERAL INTRODUCTION: CHIGGER BIOLOGY AND MEDICAL IMPORTANCE

CHAPTER 1

General Introduction: Chigger Biology and Medical Importance

Chigger is the common colloquial term used in parts of Asia for the tiny larval stages of trombiculid mites, also known as 'redbugs' (America), 'harvest mites' (Europe), 'scrub itch mite' (Australia), and 'tsutsugamushi' or 'kedani' (Japan). Chiggers have been recognised as vectors of *Orientia tsutsugamushi* (formerly known as *Rickettsia tsusugamushi*), causative agent of 'scrub typhus disease' in the Asia-Pacific region since the 1930s (Rapmund 1984; Kelly et al. 2009), and their bites result in intense irritation and dermatitis in humans and animals. They are important vectors of serious human disease and thus the subject of medical entomology research, and the nature of their enigmatic life cycle - existing as a parasite in the larval stage only, with other stages predating on other arthropods also makes these mites fascinating topics of ecological and behavioural studies.

1.1 General morphology

When unfed, the chigger is very small (<1 mm in length), hairy, oval to round-shaped, and varies in colour from white, cream, orange, brown to red (Nadchatram & Dohany 1974). The exoskeleton is thin and soft. Its body can be divided into 2 main parts: (1) The gnathosoma or head/mouthparts comprises specialized appendages (chelicera, palp and galea) for feeding and with a sensory function; and (2) idiosoma (abdomen and thorax) which is the major part of the

body supporting the eyes, scutum (antero-dorsal plate), body setae (hair) and legs (Vercammen-Grandjean & Langston 1975), (Figure 1.1). Larvae possess 3 pairs of legs, and other instars 4 pairs, starting with the deutonymph stage. Combinations of characteristic details and their measurements are very important for taxonomic classification and identification; more details are described in CHAPTER 3.

1.2. Life cycle and behaviour

The general life cycle of chiggers was firstly postulated by Nagayo et al. (1917), subsequent to detailed life cycle studies being possible when laboratory rearing of colonies become practicable decades later (Wharton & Carver 1946). The mites have 7 life cycle stages: egg, deutovum (pre-larval), larva, protonymph, deutonymph, tritonymph and adult (Shatrov & Kudryashova 2006). Only the larval instar is parasitic, feeding on a wide range of vertebrates and incidentally on humans; whereas adults and nymphs live in soil and are predators of soft-bodied arthropods (Figure 1.2). Life cycle duration of chiggers depends on the species, and is influenced significantly by environmental factors, *i.e.* temperature, humidity or abundance of food and nutrition in their territory (Nadchatram & Dohany 1974). Local climate influences the number of chigger generations in a year. For example, a single generation is usual in chigger populations in temperate areas (North America, Europe or East Asia), but in warmer zones as found in tropical countries, there tend to be multiple generations, with up to 5 or more within each year (Jones 1951; Sasa 1961; Hahn & Ascerno 2008). The life cycle of trombiculid mites is briefly described below.

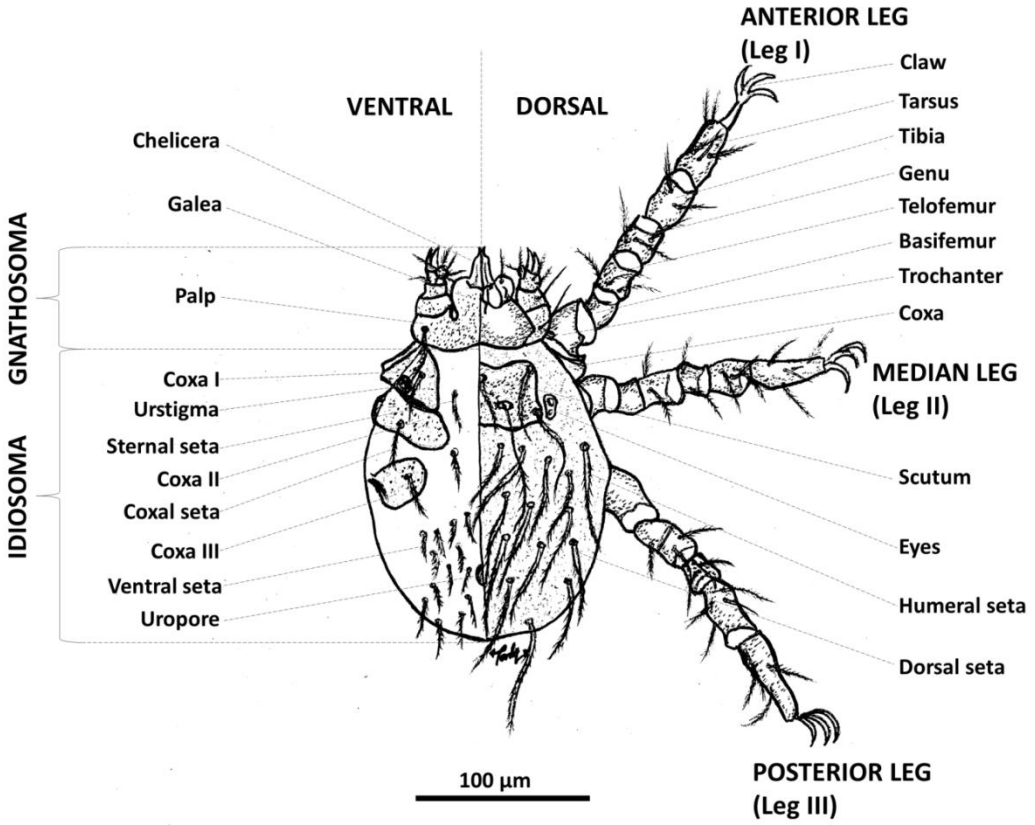

Figure 1.1 General appearance of dorsal and ventral views of chigger larva (modified from Nadchatram and Dohany, 1974)

Focussing in tropical areas, nymphal and adult stages comprise much of the lifespan duration, averaging 400-600 days (99%) as free-living in the environment, while the larval stage appears to occupy normally around 1% of life cycle time as an ectoparasite (Shatrov 1992). However, in temperate zones with harsher seasonal conditions, a recent study reported evidence of *Hirsutiella zachvatkini* in prolonged contact on rodent hosts through autumn and winter months until environmental conditions favour dropping-off and further development (Moniuszko & Mąkol 2016). Adults and nymphs hunt arthropods and their eggs; *i.e.*, Collembola, Diptera, Hemiptera, and Lepidoptera (Lipovsky & Schell 1951; Lipovsky 1954), as well as feeding on soil nematodes for food (Morishita & Nakamatsu 1958). Nymphs are

morphologically similar to adults but smaller in size, and sexual organs are not fully developed. Adult sexes can be recognized by appearance of specialized setae around the genital area of the male which are completely absent in females. The mites reproduce sexually with indirect copulation; the unique insemination process of some mite and tick taxa (Lipovsky et al. 1957; Oldfield et al. 1970; Alberti et al. 2000). However, there is evidence for parthenogenesis (asexual reproduction whereby the embryo can develop directly from unfertilized eggs in the absence of males) inpopulations of *Leptotrombidium arenicola* and *L. deliense* (Kaufmann & Traub 1966; Liu & Hsu 1985 cited in Stekolnikov 2013). The process of sexual reproduction described by Lipovsky et al. (1957) starts when the male deposits a spermatophore (a sac of sperm) onto a substrate, and when the female encounters the spermatophore she pushes it through her ventral genital opening, so that fertilisation occurs.

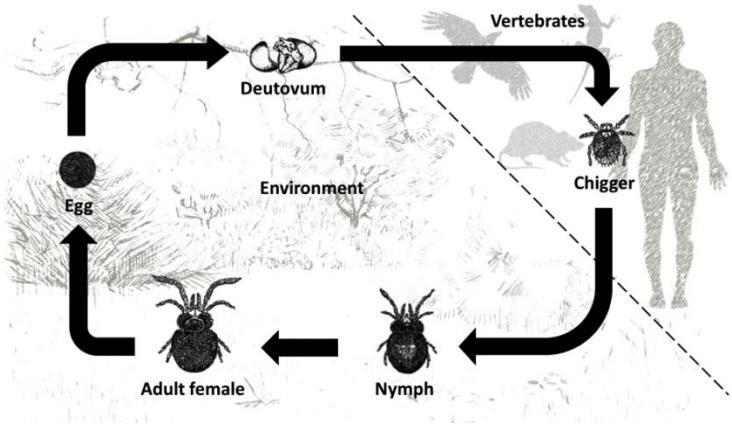

Figure 1.2 Parasitic and free-living life cycle of different stages of chiggers

A couple of weeks after fertilization, the female starts to lay eggs in soil or vegetation and continues to oviposit for a month, producing up to 400 eggs in a lifetime (Nadchatram & Dohany 1974). The deutovum (pre-larval stage) develops

inside the egg for 6-10 days before the active larva, the “chigger”, hatches out. Its parasitic life begins at this point. Unfed chiggers are usually found as motionless clusters or “chigger islands” waiting for their host in shaded areas on the ground or a few inches above the ground; *i.e.*, on vegetation, dry leaves, grasses, twigs or soil debris. Their static behaviour, waiting for an opportunity to bite a host, was realized as the most important bionomic aspect of public health importance (Sasa 1961).

Several publications have proved that chiggers exhibit diurnal activity, being most active in the day time, particularly in the morning or evening when the ground temperature is not as high as in midday or early afternoon (Audy 1961; Kohls et al. 1954). Chiggers detect the approach of a vertebrate host in their area by reacting to air movements and the level of CO_2 in the air current, and then immediately crawl up onto the top of an object for a chance to attach onto the host. However, Sasa et al. (1957) proposed that CO_2 acts as a chemical stimulant, which awakes their parasitic instinct from the resting stage, rather than a guide to navigate direction towards the host.

There appears to be some misunderstanding of chigger feeding behaviour on mammals. They are neither blood sucking nor burrowing mites. Rather, chiggers cause a serious bite by using strong blade-liked mouthparts, the chelicerae, to pierce the host’s skin. During feeding, extra-oral digestion is a key process of the feeding behaviour of the trombiculid mites. A feeding tube-like cavity called the “stylostome” is developed from chigger saliva, constructing a complex glycoprotein structure from the stratum corneum into the host’s epidermis or dermis tissue (Hase et al. 1978; Shatrov 2009). Powerful enzymes from the salivary gland are

released into the wound, and the larvae are then able to feed on the digested tissue, lymphoid and cellular components by pumping the lysate into their body. Shatrov (2015) found that the salivary glands of newly hatched chiggers contain efficient secretory granule enzymes, suggesting the potential ability to feed on a host very soon after hatching.

In general, small terrestrial mammals (rodents and insectivores) are the most preferred hosts for chiggers. Nevertheless, some other vertebrates; *i.e.*, bats, birds, amphibians or reptiles that live in the same microhabitat can be attacked, as low host specificity is commonly found in trombiculids (Loomis 1956; Shatrov & Kudryashova 2008). With their wide-host feeding habit, this could be one of explanations for the occurrence of huge diversification of *Orientia tsutsugamushi* strains in nature (Kelly et al. 2009; Paris et al. 2013).

Typically, larvae attach and feed for about 3-5 days on their host (Shatrov 1992). However, the length of feeding time can be different depending on host type. There is some evidence revealing different periods of chigger feeding on reptiles (up to 30-65 days on horned lizards), birds (a week on wild birds or chickens), and mammals (a couple of days on rodents and humans) (Melvin et al. 1943; Harrison 1953; Sasa et al. 1957). In a situation where hosts are scarce or unavailable, unfed larvae can survive more than 200 days without food and are able to feed again when hosts become available (Shatrov & Kudryashova 2006).

Engorged chiggers detach from their host after feeding, and become quiescent for 2 - 3 days under protected objects in the environment. The larvae

develop into a quiescent pupating stage or “protonymph” (similar to nymphochrysalis in other mites) inside the old larval cuticle, and then “8-legged deutonymphs” develop soon after within period of a month. Deutonymphs become active again, hunting soil arthropod eggs and larvae for food, and then re-enter another quiescent stage. Lastly, the tritonymph (imagochrysalis) emerges as the final transitional stage before developing into an adult to complete the life cycle. The whole life cycle (from egg-to-egg) can take around 150 – 400 days in chiggers from the northern hemisphere (Shatrov & Kudryashova 2006), whereas tropical species tend to exhibit a shorter period, on average 72 days (Womersley & Heaslip 1943; Neal & Barnett 1961; Nadchatram & Dohany 1974).

1.3 Ecology and geographical distribution

Chiggers are cosmopolitan mites, and are highly diversified across various habitat types throughout the world; *i.e.*, forest, woodland, grassland, scrub vegetation, agricultural land, swamp and seacoast. Besides these biotypes, some chigger species may be found in extreme environments such as at high altitude levels (Himalayas and Andes), desert biomes, or extremely cold regions in Alaska, Russia, Korea and the north of Japan (Traub et al. 1967; Traub & Wisseman 1968; Kudryashova 1998; Takahashi et al. 2012).

Considering the Trombiculidae as a whole, chiggers are best represented in the Oriental, Australasian and Afrotropical regions compared to the other areas (Wharton 1952). Some chigger genera are widely represented in several zoogeographical regions; *e.g*., *Leptotrombidium* is widespread (Oriental, Palearctic,

Australasia, Afrotropical and Nearctic) but not in Neotropical, Antarctic, Madagascan and Oceania regions; *Blankaartia* is found in the Nearctic, Neotropical, Palearctic, Afrotropical and Oriental regions; *Eutrombicula* is found in the Western hemisphere, Nearctic and Neotropical regions; *Neotrombicula* is found in temperate zones, the Nearctic and Palearctic; and *Ascoshoengastia* in the Oriental, Australasian and Afrotropical regions (Wharton 1952; Halliday 1998; Brown 2002; Daniel & Stekolnikov 2004; Stekolnikov 2013; Stekolnikov et al. 2014). Other genera are restricted in distribution; *e.g.*, *Trombiculindus* and *Lorillatum* have been reported only in the Oriental region; and *Womersia* is limited to the Americas (Wharton 1947; Vercammen-Grandjean & Langston 1975).

1.4 Taxonomy and classifications

The systematics and taxonomy of chiggers are almost entirely based on morphology, with the sclerotized structure of larvae having a specific terminology, “nepophylogeny” (Vercammen-Grandjean et al. 1973). The mites are classified in the family Trombiculidae of the subclass Acari. Details of their present taxonomic position are showed in Figure 1.3.

The number of newly discovered species has increased greatly to around 3,000 species, particularly after World War II (Sasa 1961; Brennan & Goff 1977). In parallel with other complex taxa in the Acari, the classification system within Trombiculidae is still unclear and largely debatable due to their morphological variation, high diversity, worldwide distribution and low host specificity. The most recent phylogeny of Trombiculidae was proposed by Shatrov & Kudryashova (2008)

comprising 4 subfamilies: (1) Leeuwenhoekiinae; (2) Apoloniinae; (3) Gahrliepiinae and (4) Trombiculinae (Figure 1.4).

PHYLUM: Arthropoda

CLASS: Arachnida

SUBCLASS: Acari

SUPERORDER: Acariformes

ORDER: Trombidiformes

SUBORDER: Prostigmata

COHORT: Parasitengonina

SUBCOHORT: Trombidiae

SUPERFAMILY: Trombiculoidea

FAMILY: Trombiculidae

Figure 1.3 Taxonomic ranking of Family Trombiculidae (Krantz & Walter 2009)

Terminology and morphological characters of trombiculid larvae were initially described by Ewing (1949), and continually improved thereafter (Wharton et al. 1951; Vercammen-Grandjean & Langston 1975; Brennan & Goff 1977; Goff et al. 1982). A number of morphological characteristic features are used in combination to discriminate Trombiculidae from the other Acari; *i.e.* the presence of a single dorsal scutum (with a pair of sensillae) on the anterior part of the idiosoma; coxae I and II close together; urstigma located on the postero-lateral margin of coxa I; two claws and empodium on the tip of each leg; constant setation formula or hair number as 1-1-3 on the palp segments: femur, genu and tibia,

respectively; and setation pattern on the idiosoma usually organized in regular rows (A B Shatrov & Kudryashova 2008).

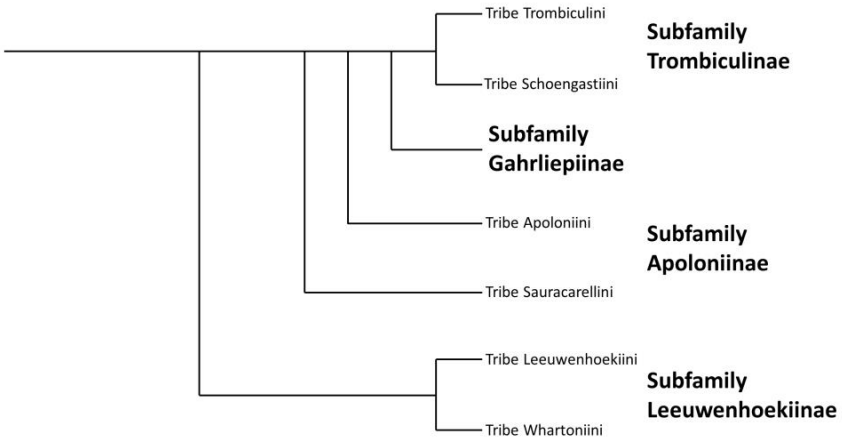

Figure 1.4 Phylogeny of tribes within the family Trombiculidae following Shatrov and Kudryashova (2008).

1.5. Chigger diversity in Southeast Asia

Southeast Asia (SEA) comprises 11 countries located in two different geographical sub-regions: mainland or Indo-China Peninsula (Cambodia, Lao PDR, Myanmar, Thailand, Vietnam, and West Malaysia); and the Malay Archipelago (Brunei, East Malaysia, Indonesia, Philippines, Singapore and Timor). The region has been realized as one of the world's hotspots of biodiversity, particularly for mammals (Myers et al. 2000; Schipper et al. 2008). In addition, parasite diversity and emerging infectious diseases are also prevalent in accordance with the high diversity of available hosts in this region (Jones et al. 2008; Morand et al. 2014).

Undoubtedly, a diversity of chigger species in SEA is also highly evident and this part of the world was hypothesized as a focus of speciation of some trombiculid

species (Stekolnikov 2014). Nadchatram & Dohany (1974) summarized practical information on diversity and keys to the subgenera of Trombiculidae in SEA, with approximately 350 species from over 40 subgenera found to parasitize different vertebrate host groups (reptiles, birds, bats and small mammals). Subsequently, a number of continually updated works on the descriptions of new species/genera of Trombiculidae in the region were published (Hadi & Carney 1977; Suzuki 1980; Nadchatram 1989; Tanskul 1991; Brown 1992; Tanskul & Linthicum 1997; Nadchatram 2006; Stekolnikov 2014; Chaisiri et al. 2016). Details of Trombiculidae diversity are shown in Table 1.1.

Table 1.1 List of chigger genera reported on different vertebrate host groups in Southeast Asia (Nadchatram & Dohany 1974).

Subfamily	**Genus**	**Small mammal**	**Bat**	**Bird**	**Reptile**
Leeuwenhoekiinae	*Odontacarus*	X		X	
	Whartonia		X		
Gahrliepiinae	*Gahrliepia*	X			
	Schoengastiella	X			
	Walchia	X			
Trombiculinae	*Ascoschoengastia*	X			
	Babiangia				X
	Blankaartia	X		X	
	Cheladonta	X			
	Chiroptella		X		
	Cricacarus	X			
	Diplectria		X		

Table 1.1 (continued)

Subfamily	Genus	Small mammal	Bat	Bird	Reptile
	Doloisia	X			
	Eltonella				X
	Eutrombicula	X		X	X
	Fonsecia				X
	Guntherana	X			
	Heaslipia	X		X	
	Helenicula	X		X	
	Herpetacarus	X			X
	Kayella	X			
	Laotrombicula	X			
	Leptotrombidium	X		X	
	Lorillatum	X			
	Neoschoengastia			X	X
	Neotrombicula			X	
	Mackiena			X	
	Microtrombicula	X	X		X
	Myotrombicula		X		
	Riedlinia		X		
	Rudnicula		X		
	Sasatrombicula		X		
	Schoengastia	X		X	
	Schoutedenichia	X			
	Siseca	X		X	X
	Susa	X			
	Toritrombicula			X	
	Trisetica		X		
	Trisetoisia	X			

Table 1.1 (continued)

Subfamily	Genus	Small mammal	Bat	Bird	Reptile
	Trombicula		X		
	Trombiculindus	X			
	Trombigastia		X		
	Vatacarus				X
	Vercammenia				X
	Walchiella	X			

1.6 The public health importance of chiggers

In relation to human public health, there are 2 main problems of medical importance: (1) The mites cause skin irritation and intense itching through their bite and (2) they are vectors of microbial pathogens.

In the first aspect, trombidiosis or trombiculiasis is an itching dermatitis condition due to feeding by trombiculid chiggers (Krantz & Walter 2009). The victims are usually bitten during outdoor activity; *e.g.*, when resting on grass, working on farmland or walking past bushes where a cluster of chiggers has gathered. A number of cases has been reported in Europe, America and occasionally in Asia during summer and autumn when the larval chiggers become active (Pogacnik & Kansky 1998; Schulert & Gigante 2014). *Neotrombicula autumnalis* and *Eutrombicula alfreddugasi* are the important chigger species known to bite humans and cause trombidiosis in Europe and America, respectively. More than other 50 species have been reported to attack humans, such as *Eushoengastia*

serothermobia, *E. koreaensis*, *Hirsutiella zachvatkini*, *Kepkatrombicula desaleri*, *Leptotrombidium akamushi, L. deliense*, *Neotrombicula japonica* and *Trombicula toldti*, along with unspecified species of various chigger genera: *Blankaartia, Leptotrombidium*, *Gahrliepia* and *Schoengastia* (Finnegan 1945; Wharton 1952; Sasa 1961; Pogacnik & Kansky 1998; Shatrov & Stekolnikov 2011; Santibáñez et al. 2015). These researchers pointed out that the species attacking humans normally parasitized a broad host range (*i.e.*, they exhibit low host specificity) and affect mammals, birds and reptiles.

An itchy rash consisting of maculo-papular lesions develops at the point of attachment, and is caused by host hypersensitivity responses to the chiggers' salivary antigens. The itchy spots start from minute red macules and develop into itchy-watery papules within 24 - 48 hours. The period of irritation can remain for up to a week, whereas skin lesions with a dark pigmentation scar present for longer, up to a month. In some cases, severe scratching, particularly at the papule stage, may lead to secondary bacterial infection (Pogacnik & Kansky 1998; Juckett 2013).

The second public health impact of chiggers relates to their role as potential vectors of human pathogens. Apart from the well-known bacterium, *Orientia tsutsugamushi*, transmitted by chiggers, the mites have also been reported to bear some other microbial pathogens; *e.g.*, *Bartonella spp.* (Kabeya et al. 2009; Loan et al. 2015), *Rickettsia spp.* (Choi et al. 2007) and Hantaan virus (Zhang et al. 2003; Yu & Tesh 2014). As the most notable role that chiggers play as vectors is in the transmission of *O. tsutsugamushi*, causative agent of scrub typhus, this review will focus primarily on this disease field.

Scrub typhus or Tsutsugamushi disease has been recognised as one of the most under-diagnosed and under-reported febrile illnesses in the Asia-Pacific region (WHO 1999; Paris et al. 2013). It had a dramatic impact during World War II and the Vietnam conflict, when the disease became better known to the Western world. There were several thousand cases of the disease in the Allied forces and in Japanese soldiers with hundreds of deaths: mortality rates ranged from 1 - 33.5% after troops had landed and settled in scrub-vegetation areas in Southeast Asia and the Pacific islands (Peterson 2009; Kelly et al. 2009). The outbreak was reported as a mysterious and fearsome phenomenon to the operating soldiers; indeed, “They were often more afraid of clumps of grass than they were of the enemy" (Traub & Wisseman 1968).

The geographical distribution of the disease covers approximately 13,000,000 km^2 ranging from Afghanistan in the west, China and Siberia in the north, Korea and Japan in the east; alongside all of the Southeast Asian countries in the south and down to the northern part of Australia (Kelly et al. 2009). This area is the so-called “Tsutsugamushi Triangle” (see Figure 1.5), where over a half of the World’s population lives at risk inside this endemic zone, with one million cases reported every year (Zhang et al. 2010; Watt & Parola 2003). In addition to the endemic areas, there are sporadic reports of scrub typhus cases in Africa (People’s Republic of Congo, Cameroon) and South America (Chile) (Osuga et al. 1991; Ghorbani et al. 1997; Balcells et al. 2011). Furthermore, a new *Orientia* species, *O. chuto*, was also discovered in the Middle East (Dubai) (Izzard et al. 2010), reflecting that the distribution range may be even larger than previously thought.

The scrub typhus agent, *O. tsutsugamushi* is a Gram-negative alpha-proteobacterium, closely related to *Rickettsia*, the intracellular pathogenic or invertebrate symbiotic bacteria in the family Rickettsiaceae. The bacterium was separated from the genus *Rickettsia* based on differences in the ultrastructure of the outer membrane envelope, and the organism lacks peptidoglycan and lipopolysaccharide components. These characteristics led to re-classification in a novel separate genus "*Orientia*" (Tamura et al. 1995). However, the bacterium still exhibits conserved properties similar to other *Rickettsia*, such as obligate intracellular parasitism and the ability to survive in either vertebrate or arthropod hosts.

Leptotrombidium spp. are the important vectors transmitting the scrub typhus agent to humans under natural conditions (Traub & Wisseman 1968; Stekolnikov 2013). The two species, *L. akamushi* and *L. deliense* and the other morphologically related species (the *akamushi-deliense* complex) have been recognized as the major vectors in the Asia and Pacific regions (Sasa 1961). The first species probably occupies the temperate region of the endemic zone comprising Eastern China, Japan, Korea and the northern islands of the Philippines, whereas the latter is responsible for the disease transmission in tropical areas represented by the South and Southeast Asian countries. Although over 45 chigger species have been reported to harbour *O. tsutsugamushi*; *e.g.*, *Ascoshoengastia indica, Blankaartia acuscutellaris, Gahrliepia saduski, Helenicula miyagawai, Leptotrombidium arenicola, L. fletcheri, L. imphalum, L. pallidium, L. palpale, L. scutellare, Shoengastiella ligula* and *Walchia pacifica* (Frances et al. 2001; Fournier

et al. 2008; Ogawa & Ono 2008; Kuo et al. 2011; Lee et al. 2011; Tilak et al. 2011; Phasomkusolsil et al. 2012; Zhang et al. 2013; Shin et al. 2014; Santibáñez et al. 2015; Park et al. 2015), *Leptotrombidium* spp. is the only taxon proven to transmit *O. tsutsugamushi* (Santibáñez et al. 2015), and further investigations on the vectorial role of the other chigger genera are required. In addition, Nadchatram & Dohany (1974) noticed that the scrub typhus agent is usually transmitted by orange-coloured species of chiggers rather than the white or yellow species.

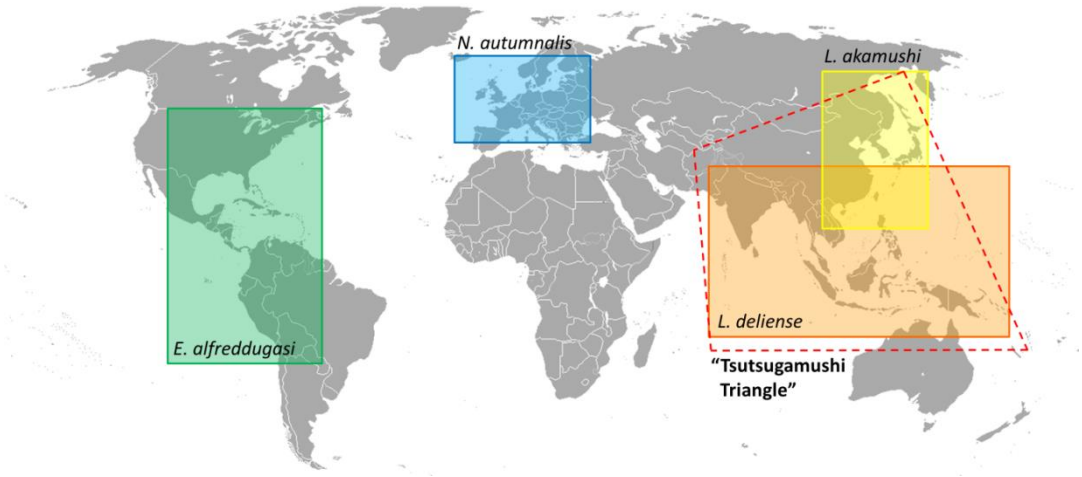

Figure 1.5 Endemic area of scrub typhus disease, also known as the "Tsutsugamushi tringle" (dashed line), and geographical distribution of the four main trombiculid species of medical importance.

Orientia is maintained and circulated in the environment through chiggers and several vertebrate hosts, particularly in rodents and other small mammals that chiggers feed on. The majority of publications reported *Orientia* in association with chiggers parasitizing small mammals, *i.e.*, rodents and insectivores, whereas the status of chiggers and *Orientia* infection on some other host groups are less well defined. This reflects a potential knowledge gap regarding to the role of other

vertebrates; *e.g*., birds, reptiles, bats and large mammals as a host for chiggers and potential reservoirs of *O. tsutsugamushi* in the wild.

Similar to chiggers, rodents can act as an appropriate reservoir for *Orientia* as they can maintain infection for a long period, and also boost the chigger population by providing food for the larval mites (Frances et al. 2000). This is probably the reason why a huge diversification of the bacteria occurred, with more than 20 serotypes and over 70 antigenic strains discovered in human and reservoir animals (Kelly et al. 2009; Santibáñez et al. 2015). The massive antigenic strain diversity of *O. tsutsugamushi* (*i.e.,* based on the 56-KDa type specific antigen gene) has been linked with evolutionary processes resulting from selective mutation driven by the complexity of either the chiggers' or rodents' host immune responses to the bacterial infection (Ohashi et al. 1992; Takhampunya et al. 2014). The bacteria use not only transovarial or vertical transmission, passing from infected females to offspring as an essential route to maintain their infection in mites, but also the transtadial route, so that the organism remains within the mites throughout their different life stages (Traub & Wisseman 1974; Frances et al. 2001; Phasomkusolsil et al. 2009; Shin et al. 2014). Thus, using transmission electron microscopy, *O. tsutsugamushi* was observed in various tissues of *L. pallidum*: the reproductive organs of male and female adults; *i.e.*, spermatogonia, spermatocyte, spermatid, oogonia and oocyte; the rudimentary reproductive organs of larvae; and the somatic cells and eggs after oviposition (Urakami et al. 1994).

Based on different molecular detection methods such as conventional PCR, nested PCR and RT-PCR of specific gene targets (16S rRNA and the 56 kDa type-

specific antigen), the rate of *Orientia* infection in field-collected mites ranged from 0.2 – 5.9% (Lee et al. 2011; Seto et al. 2013; Zhang et al. 2013). This is in accordance with the data of Audy (1958) who previously estimated that the bacterial infection rate of *L. deliense* in the field was low, at only 0.1 – 10%. In laboratory-reared colonies, vertical transmission rates declined gradually from the parent generation (wild collection) to the F1 and F2 generations (Phasomkusolsil et al. 2009).

There is some evidence of the potential effect of *O. tsutsugamushi* infection on the mite's development and reproduction by delaying generation time of their metamorphoses and reducing egg production (Roberts et al. 1977; Phasomkusolsil et al. 2012); nevertheless no developmental effects were found in *L. deliense* colonies (Frances et al. 2001). In some cases, *O. tsutsugamushi* infection can produce sex-ratio distortion; males were rarely found in infected colonies of *L. arenicola* and *L. fletcheri* (Roberts et al. 1977; Rapmund et al. 1972). Takahashi et al. (1997) suggested that the appearance of *O. tsutsugamushi* in the mite's gonad may suppress male development, and a sex ratio closer to parity can be rescued by application of antibiotic treatment. In conclusion, *O. tsutsugamushi* appears to be a symbiont of trombiculid mites that can mediate reproductive phenotypes.

The occurrence of scrub typhus disease and its widely diversified strains in endemic areas are assumed to be associated with the four main players which are (1) the bacterial agent *Orientia* itself, (2) chiggers as vector of the disease, (3) vertebrate animals as hosts for chigger feeding and vehicles for bacterial horizontal transmission, and (4) humans as accidental host of chiggers and the role of humans in habitat alteration, which could increase the risk of exposure to chiggers.

However, there are still many unresolved points in the research field, with a number of research questions that could be raised, particularly interactions among the bacteria, vectors, reservoir hosts and humans on the basis of the ecology and epidemiology of the disease.

1.7 General aim and objectives of the thesis

The main aim of this thesis is to define chiggers and their microbiome diversity in relation to ecology and scrub typhus epidemiology in Thailand. To address the main aim, the study can be divided into two primary research objectives.

The first objective is to understand the diversity and ecology of chiggers in relation to scrub typhus epidemiology. This includes research questions such as what is the diversity of chigger species on wild small mammals in Thailand? How are chigger species distributed across the country? What are the ecological factors influencing chigger diversity? Are there specific host-chigger interaction patterns? How does host-chigger parasitism impact on the ecology and epidemiology of scrub typhus disease?

The second objective is to characterize chigger-associated bacteria. A number of questions are relevant here, such as what is the diversity of the microbiome of chiggers? Do chiggers harbour other potential pathogens or bacterial symbionts apart from *Orientia*? Is *Orientia* infection limited only to known vector species? Does the chigger microbiome vary between chigger species, studied sites or habitat?

This thesis is conducted under the context of “from-field-to-lab”, and spans local field studies, specimen collection and transportation, classical systematic and taxonomic studies, database management, molecular biology and next generation sequencing to bioinformatics. In CHAPTER 2, a systematic review of the literature regarding mite-associated bacteria worldwide is provided with a compilation of bacterial genera found in different mites of medical, veterinary and agricultural importance. Biological characteristics of the bacteria such as saprophytic, pathogenic and symbiotic bacteria associated with mites are discussed. In CHAPTER 3, I introduce the systematics and taxonomy of trombiculid chiggers, including morphological identification criteria. The diversity of chiggers on small mammals in Thailand is also reported within this chapter. In CHAPTER 4, an ecological study of chiggers, host-chigger interaction networks and the implications for the epidemiology of scrub typhus in our studied sites are analysed and discussed. In CHAPTER 5, a microbiome analysis of chiggers using the 16S rRNA amplicon sequencing approach is presented for both individual and pooled chiggers. Preliminary works that set the scene for future studies, including whole genome sequencing of *L. deliense*, characterization of microsatellite markers for *L. deliense* population genetic studies, and *in situ* hybridization with immunogold-TEM to detect intracellular bacteria in chiggers are presented in CHAPTER 6. Finally, an overall conclusion and potential perspectives for future studies are provided in CHAPTER 7.

CHAPTER 2

SYSTEMATIC REVIEW OF MITE-ASSOCIATED BACTERIA

CHAPTER 2

Systematic review of mite-associated bacteria

This chapter presents the systematic review of mite-associated bacteria, which has been published in Chaisiri et al. (2015). A dataset of bacterial diversity found in mites was compiled from 193 publications (from 1964 to January 2015). A total of 143 mite species belonging to the 3 orders (Mesostigmata, Sarcoptiformes and Trombidiformes) were recorded and found to be associated with approximately 150 bacteria species (in 85 genera, 51 families, 25 orders and 7 phyla). From the literature, the intracellular symbiont *Cardinium*, the scrub typhus agent *Orientia* and *Wolbachia* (the most prevalent symbiont of arthropods) were the dominant mite-associated bacteria, with approximately 30 mite species infected by each. Moreover, a number of bacteria of medical and veterinary importance were also reported from mites, including species from the genera *Rickettsia*, *Anaplasma, Bartonella, Francisella, Coxiella, Borrelia, Salmonella, Erysipelothrix* and *Serratia*. Significant differences in bacterial infection patterns among mite taxa were identified. These data will not only be useful for raising awareness of the potential for mites to transmit disease, but also enable a deeper understanding of the relationship of symbionts with their arthropod hosts, and may facilitate the development of intervention tools for disease vector control. This review provides a comprehensive overview of mite-associated bacteria and is a valuable reference database for future research on mites of agricultural, veterinary and/or medical importance.

2.1 Introduction

Mites are classified in the subclass Acari (class Arachnida) of the phylum Arthropoda. Although approximately 48,200 species have been described (Halliday et al. 2000), a further half-million species are believed to exist worldwide (Kettle 1984). More so than any other arthropod group, mites are found in highly diverse habitats: terrestrial, marine, freshwater and even in the upper atmosphere due to dispersal through aerial currents (Krantz & Walter 2009). Whereas most mite species live freely in the environment, some species have evolved to be parasitic on other animals or on plants and are therefore of great agricultural and veterinary importance, although their medical impact is generally more modest. Some species are significant destructive pests of stored food products; while others (such as house dust mites) produce faecal allergens, inducing asthma in human. Mites can also produce serious skin conditions by feeding on the skin of domestic animals (mange) and can cause dermatitis in humans. Finally, some species act as important vectors of pathogenic microorganisms of medical and veterinary importance (Arlian et al. 2003; Brouqui & Raoult 2006; Valiente Moro et al. 2009).

The relationship between bacteria and arthropods can be divided into 2 main aspects, which are not mutually exclusive: (1) bacteria recognized as pathogens transmitted by an arthropod vector, and (2) bacteria residing as symbionts within their arthropod host. The study of the first aspect usually concerns surveillance for emerging or re-emerging diseases and interactions between the arthropod vector, environment, wildlife, domestic animals and humans. In contrast, the second research area concerns other bacteria that may influence the physical,

ecological and evolutionary traits of their arthropod host, usually without transmission of these organisms to a second host in which disease may occur. These studies are often designed to characterize and define symbiont–arthropod interactions. For example, the nutritional mutualist, *Buchnera aphidicola*, synthesizes essential amino acids for its aphid host (*Acyrthosiphon pisum*) that feeds on plant phloem, which has a very low essential amino-acid content (Gunduz & Douglas 2009). Pea aphids also harbour defensive mutualists such as *Regiella insecticola*, which protects the host population from a natural enemy (the pathogenic fungus, *Pandora neoaphidis*) by reducing the sporulation rate in aphid cadavers, thus reducing the probability of pathogen transmission to other aphids (Scarborough et al. 2005). Other bacterial genera are capable of manipulating their hosts' reproduction: *Wolbachia, Cardinium, Spiroplasma* and *Rickettsia* induce detrimental phenotypes in their arthropod hosts such as cytoplasmic incompatibility, parthenogenesis induction, feminization and male killing (Stouthamer et al. 1999; Tinsley & Majerus 2006; Enigl & Schausberger 2007; Giorgini et al. 2009). These findings may be utilized to enhance prospects for biological control since there is the potential to manipulate arthropod populations of agricultural, medical or veterinary importance.

Recently, the number of publications on arthropod-associated bacteria has substantially increased, particularly for the Diptera, Hemiptera and Hymenoptera (Baumann 2005; Crotti et al. 2010; Martinson et al. 2011; Taylor et al. 2011; Martin et al. 2012; Zucchi et al. 2012; Skaljac et al. 2013). In parallel, studies on mite-bacterial relationships have also increased, but to a lesser extent compared with the

insect orders above. Moreover, sources of information with respect to mites and their pathogens and symbionts are widely distributed in the literature, and the compilation of data in terms of review publications is still very limited. Accordingly, the aims of this literature review are (1) to obtain an overview of bacterial diversity in mites and its potential applications, and (2) to provide comparative data for mite-associated bacteria of agricultural, veterinary and medical importance to stimulate hypothesis driven research.

2.2 Materials and methods

2.2.1 Literature search

This review focuses on reports of bacteria found in mites across the world. The publications were extracted by searching from 2 major scientific literature databases, PubMed (www.ncbi.nlm.nih.gov/pubmed) and Web of Knowledge (www.webofknowledge.com). Three main mite orders (Mesostigmata, Sarcoptiformes, Trombidiformes), some common names of mites (*e.g.,* gamasid mite, dust mite, itch mite, spider mite, harvest mite, free-living mite, chigger etc.) or scientific names (*e.g.*, *Dermanyssus*, *Dermatophagoides*, *Leptotrombidium* or *Tyrophagus*), were used in combination with the term “bacteria” or “bacterium” as the keywords and applied to the title field or abstracts in those databases. From the obtained literature, the number of bacterial taxa (genus and species level) was recorded for each mite species. Only the publications reporting a minimum of genus-level identifications of bacteria were included in the database for statistical analyses. Bacterial scientific names obtained from the literature were checked for

taxonomic assignment following the NCBI Taxonomy Browser (http://www.ncbi.nlm. nih.gov/Taxonomy/Browser/wwwtax.cgi), while for the latest mite taxonomic classifications, Krantz & Walter (2009) was consulted.

2.2.2 Mite classification

Taxonomically, the mites were classified into 3 orders and 14 superfamilies. In addition, for the purposes of the present investigation, mites were also grouped into 4 types based on life history (Krantz & Walter 2009): (1) “Vertebrate parasite” was defined as a mite species which at some lifecycle stage feeds on vertebrate animals or are confirmed as disease vectors; (2) “Invertebrate predator” was defined as a mite species which at some lifecycle stage hunts or feeds upon other invertebrates (some of which are used as biological pest control in agricultural practice); (3) “Plant parasite” was defined as a mite species that feeds on live plant tissues (with some species responsible for economic losses in agricultural products); and (4) “House pest and allergen” are those mites which spoil stored foodstuffs or contain powerful allergens that induce detrimental immune responses in humans and/or animals (Table 2.1).

2.2.3 Bacterial classification

For bacteria, apart from taxonomic classification, 4 bacterial groups were categorized due to their biological characteristics. Following the scheme of Valiente Moro et al. (2009), the different categories were defined as: (1) “Saprophyte” – examples are bacteria which have not been described as being pathogenic; (2) “Opportunistic pathogen” – species in this category cause disease in compromised

vertebrate hosts but not in healthy hosts; (3) “Pathogen” – most species in the genus are pathogens of vertebrates; and (4) “Symbiont” – bacteria that strictly live in association with an arthropod host.

2.2.4 Statistical analysis

In order to visualize the distribution of the bacteria found in each mite superfamily, a correspondence analysis (CA) was performed using R freeware (R Core Team 2015) with the “ade4” package (Dray & Dufour 2007). The CA was calculated by counting the number of bacterial genera positively reported in each mite taxon. Before starting the analysis, data from 4 mite superfamilies (Oppioidea, Rhodacaroidea, Erythraeoidea and Eviphidoidea) were removed due to only one record of bacteria each that could cause analysis bias (outliers).

To investigate the difference of the 4 biological types of mite on bacterial diversity, the species number of bacteria (species richness) in each order was recorded across the 4 mite categories. This was analysed using the non-parametric Kruskal–Wallis test, and multiple pairwise comparison tests were performed with SPSS version 21.0 software (IBM Corporation, Armonk, New York, USA), applying 95% confidence intervals. P-values were adjusted for multiple comparisons.

Table 2.1 Summary of selected mite taxa extracted from the literature review of mite-associated bacteria. The Oppioidea (*) are exclusively free-living, and therefore lie outside our mite life history classification scheme. Abbreviation: VP = Vertebrate parasite, IP = Invertebrate parasite, PP = Plant parasite and HA = House pest & allergen

Order	Superfamily (number of species)	Mite species	Importance
Mesostigmata	Dermanyssoidea (46)	*Andreacarus* sp., *A. petersi, Androlaelaps casalis, A. fahrenholzi, A. jamesoni, Cameronieta strandmanni, Chiroptonyssus hematophagus, Dermanyssus sp., D. gallinae*, *D. hirundinis, Eugamasus* sp., *Eulaelaps stabularis, Haemogamasus* sp., *H. ambulans, H. criceti, H. hirsutus, H. nidi, H. reidi, Haemolaelaps* sp.,*H. glasgowi, Hirstionyssus isabellinus, H. musculi, Hyperlaelaps amphibious, H. arvalis, Ichoronyssus miniopteri, Laelaps* sp., *L. agilis, L. dearmasi, L. hilaris, L. multispinosus, L. muris, L. pavlovskyi, Liponyssoides sanguineus, Myonyssus gigas, Ophionyssus natricis, Ornithonyssus bacoti, O. sylviarum, Periglischrus iherngi, Pneumonyssus sp., Raillietia caprae, Spinturnix* sp., *S. myoti*, *S. psi*, *Steatonyssus* sp., *S. furmani, S. occidentalis*	VP, IP
	Eviphidoidea (1)	*Macrocheles subbadius*	IP
	Phytoseioidea (10)	*Euseius finlandicus*, *Galendromus annectens, Metaseiulus occidentalis, Neoseiulus barkeri, N. bibens, N. californicus, N. cucumeris, Phytoseiulus longipes, P. persimilis, Proprioseiopsis lenis*	IP
	Rhodacaroidea (1)	*Euryparasitus emarginatus*	VP
Sarcoptiformes	Acaroidea (7)	*Acarus siro, Aleuroglyphus ovatus, Rhizoglyphus robini, Sancassania berlesei, Tyroborus lini, Tyrophagus* sp., *T. putrescentiae*	VP, PP, HA
	Analgoidea (2)	*Dermatophagoides farina, D. pteronyssinus*	HA

Table 2.1 (continued)

Order	Superfamily (number of species)	Mite species	Importance
Sarcoptiformes	Glychyphagoidea (5)	*Aeroglyphus robustus, Blomia tropicalis, Chortoglyphus arcuatus, Glycyphagus domesticus, Lepidoglyphus destructor*	HA
	Hemisarcoptoidea (1)	*Carpoglyphus lactis*	HA
	Oppioidea* (1)	*Oppiella nova*	
	Sarcoptoidea (2)	*Psoroptes cuniculi, P. ovis*	VP
Trombidiformes	Cheyletoidea (8)	*Cheyletus eruditus, Demodex* sp., *Syringophilopsis turdi*, *S. sturni Torotrogla cardueli*, *T. lusciniae*, *T. merulae*, *T. rubeculi*	VP, IP
	Erythraeoidea (2)	*Leptus lomani, L. sayi*	IP
	Tetranychoidea (23)	*Brevipalpus* sp., *B. californicus, B. obovatus, B. phoenicis, Bryobia berlesei, B. kissophila, B. praetiosa, B. rubrioculus, B. sarothamni, Eotetranychus suginamensis, E. uncatus, Oligonychus gotohi, Panonychus mori, Petrobia harti, Schizotetranychus cercidiphylli, Tetranychus cinnabarinus, T. kanzawai, T. parakanzawai, T. phaselus, T. piercei, T. pueraricola, T. truncates, T. urticae*	PP
	Trombiculoidea (34)	*Aschoshoengastia* sp., *A. indica, Blankaartia acuscutellaris, Eushoengastia* sp., *E. koreaensis, Gahrliepia saduski, Leptotrombidium* sp., *L. akamushi, L. arenicola, L. chiangraiensis, L. deliense, L. fletcheri, L. fuji, L. himizu, L. imphalum, L. intermedium, L. kawamura, L. kitasatoi, L. linhuaikongense, L. orientale, L. palladium, L. palpale, L. pavlovskyi, L. scutellare, L. taishanicum, L. umbricola, Miyatrombicula kochiensis, Neotrombicula* sp., *N. autumnalis, N. japonica, Schoengastia* sp., *Schoengastiella ligula, Trombicula gaohuensis, Walchia pacifica*	VP

2.3 Results & Discussions

2.3.1 Methodological approaches to the identification of bacteria in mites

A total of 193 publications were included in this systematic review from 1964 until January 2015. The number of publications reporting mite-associated bacteria was found to increase gradually over this period (Figure 2.1). However, we estimated that the total volume of literature describing mite-bacteria associations is 5 times smaller than that for ticks and 20 times smaller than that for insects (data not shown).The eligible publications covered 143 mite species belonging to 3 orders and 14 superfamilies (Table 2.1). The most studied mite taxon was Dermanyssoidea (46 species), followed by Trombiculoidea (34 species), Tetranychoidea (23 species) and Phytoseioidea (10 species).

Clearly, prior to the early 1990s (Figure 2.1), analyses of bacteria in mites were restricted to non-molecular methods such as conventional bacterial cultures with biochemical characterization, inoculations of laboratory animals and serological tests. For example, *Mycoplasma* spp. were isolated from goats' ear mites, *Psoroptes cuniculi* and *Raillietia caprae*, by culturing the crushed mites in PPLO agar supplemented with pig serum, and then the bacteria were identified by biochemical characteristics (Cottew & Yeats 1982). Similarly, the red poultry mite, *Dermanyssus gallinae*, was studied for its potential vectorial role for *Salmonella gallinarum* and *Erysipelothrix rhusiopathiae* transmission in the poultry industry by culturing mite extracts in selective enrichment media, selenite broth (Zeman et al.

1982) and crystal-violet sodiumazide broth (Chirico et al. 2003), respectively. In the scrub typhus research field, a number of studies have used mouse passages to amplify *Orientia tsutsugamushi* from wild chigger mites fed on the rodents, and different strains of the bacterium were indirectly detected by various serological methods (*e.g.*, fluorescence antibody assays, immunoperoxidase staining and complement fixation tests (Kitaoka et al. 1974; Roberts et al. 1977; Dohany et al. 1978; Shirai et al. 1982; Ree et al. 1992; Frances et al. 2001; Lerdthusnee et al. 2002; Phasomkusolsil et al. 2009). Of course, such specific methods allow the identification of the target organism only, and unculturable bacteria would not be detected.

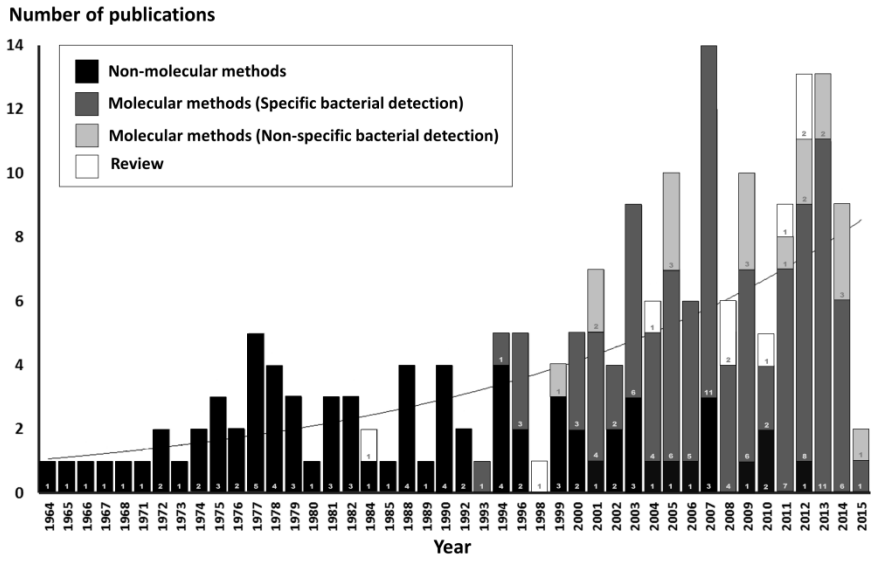

Figure 2.1 The number of publications reporting mite-associated bacteria. Numbers within bars refer to a breakdown of publications by methodology. Curve represents an exponential line of best fit.

With the advent of the molecular era, the development of specific PCR assays and conventional and next-generation sequencing techniques revealed a

significantly higher microbial diversity than was previously estimated by culture-dependent approaches (Hugenholtz et al. 1998; Yun et al. 2014; Hubert et al. 2015). In particular, use of 16S rDNA PCR with bacterial species-specific primers has been widely used for bacterial taxonomic studies in mites (Figure 2.1). Additionally, several publications used specific PCR to amplify other bacterial genes of interest. For example, the protein-coding genes: *ftsZ, groEL, wsp* and citrate synthase (*gltA*) were used in *Wolbachia* studies (Hong et al. 2002; Gotoh et al. 2005; Yu et al. 2011; Zhu et al. 2012; Ros et al. 2012; Suh et al. 2015; Glowska et al. 2015; Zhang et al. 2015); outer membrane protein B gene, 17 KDa antigenic gene and *gltA* were used for *Rickettsia spp*. (Reeves et al. 2006; Reeves et al. 2007; Choi et al. 2007; Tsui et al. 2007); the 16S–23S intergenic spacer (ITS) and *gltA* were used for *Bartonella spp.* (Kabeya et al. 2009; Kamani et al. 2013); *gyrB* was used for *Cardinium* (Ros et al. 2012; Zhu et al. 2012); the 5S–23S ITS was used for *Borrelia spp*. (Literak et al. 2008); the *epank1* gene was used for *Anaplasma phagocytophilum* (Literak et al. 2008); and the 56-kD type-specific antigen gene was used extensively for *O. tsutsugamushi* (Tamura et al. 2000; Pham et al. 2001; Khuntirat et al. 2003; Lee et al. 2011; Liu et al. 2013; Seto et al. 2013; Shin et al. 2014; Takhampunya et al. 2014).

An alternative approach has been the use of conserved primers to amplify 16S rRNA products in an unbiased fashion (Figure 2.1), followed by cloning and sequencing of selected clones for taxonomic assignment (Hogg & Lehane 1999; Hogg & Lehane 2001; Hoy & Jeyaprakash 2005; Hubert et al. 2012; Tang et al. 2013; Murillo et al. 2014; Hubert et al. 2015). However, to the best of our knowledge, only one publication has used the Roche 454 pyrosequencing platform targeting 16S

rRNA amplicons to reveal the bacterial community of a mite species (in this case, the bulb mite, *Rhizoglyphus robini*; Zindel et al. 2013). In a more recent study, bacterial genomic sequences from 100 species (predominantly enterobacteria) were identified during assembly of the *Dermatophagoides farinae* (dust mite) genome (Chan et al. 2015).

2.3.2 Bacterial diversity in mites

Mite species were found to be associated with 85 bacterial genera (approximately 150 identified species) belong to 7 phyla (plus 3 classes of Proteobacteria) and 25 orders (Table 2.2). *Cardinium* (in 31 mite species), *Wolbachia* (31 hosts) and *Orientia* (32 hosts) were the most prevalent bacteria; followed by *Bartonella*, *Anaplasma* and *Rickettsia*, with 16, 14 and 11 mite species reported, respectively (Figure 2.2). Among the 7 bacterial phyla and the 3 classes of Proteobacteria (α, β and γ), Bacteriodetes, Firmicutes, Tenericutes and Actinobacteria were reported in all 3 mite orders (Figure 2.3). However, Chlamydiae were reported only in mites from the order Mesostigmata, and Spirochaetes were found in the Mesostigmata and Trombidiformes, but not in the Sarcoptiformes (Figure 2.3).

Symbionts can be obligatory or facultative, live inside or outside host cells, and can affect their host negatively, positively, or have no discernible phenotype. Some symbiotic bacteria may provide benefits to the host in particular environments, but can be disadvantageous under different circumstances (Hoy & Jeyaprakash 2008). A number of bacteria were reported as potential mite symbionts

in this literature survey, including *Wolbachia, Cardinium, Acaricomes, Spiroplasma, Snodgrassella, Serratia, Rickettsiella* and *Schineria*. *Wolbachia* and *Cardinium* have been relatively well studied in terms of effects on their mite hosts, which manifest as reproductive alterations. However, the phenotypes (if any) induced by the other potential symbionts remain unknown. *Wolbachia* and *Cardinium* manipulate mite reproduction by inducing cytoplasmic incompatibility, parthenogenesis, sex-ratio distortion (*e.g*., male-killing and feminization), and an increase in female fecundity (Breeuwer & Jacobs 1996; Weeks & Breeuwer 2001; Chigira & Miura 2005; Gotoh et al. 2005; Groot & Breeuwer 2006; Gotoh et al. 2007; Novelli et al. 2008; Zhu et al. 2012; Zhao, et al. 2013; Suh et al. 2015; Zhang et al. 2015). These reproductive manipulation strategies facilitate vertical transmission through the female line and drive the spread of the symbionts into mite populations (Zhao, et al. 2013).

Wolbachia is the most prevalent arthropod symbiont (infecting approximately 40% of terrestrial species; Zug & Hammerstein 2012) and is also found in some species of filarial nematodes (Ferri et al. 2011). In mites, although 31 species were positively reported for *Wolbachia* infection, the bacteria occurred only in 5 of 14 studied superfamilies: the Dermanyssoidea (various parasitic mites of vertebrates), Phytoseioidea (fungivorous, pollenophagous and predatory mites), Oppioidea (in an oribatid free-living mite, *Oppiella nova*), Cheyletoidea (parasitic mites of birds, but not in *Demodex spp*.), and Tetranychoidea (phytophagous mites). Interestingly, *Cardinium* was also found in 31 mite species but these were distributed across 8 superfamilies, representing a much broader host range than *Wolbachia* (Table 2.2). According to these findings, *Cardinium* appears to be a more

important symbiont for mites than it is for other arthropods (Zug & Hammerstein 2012).

Apart from these reproductive symbionts, another symbiotic bacterium, *Acaricomes phytoseiuli*, has been isolated from the predatory species, *Phytoseilus persimilis*, which is widely used for biological control of spider mites (major agricultural pests), (Pukall et al. 2006). Plants damaged by feeding spider mites release volatiles to attract predacious mites when hunting their prey. Schütte et al. (2008) reported that *A. phytoseiuli* caused *P. persimilis* to become refractory to plant volatile attraction, leading to a high tendency to miss their prey (the so-called "non-responding syndrome"). Moreover, infected mites developed symptoms such as body shrinkage, cessation of oviposition and even death. Accordingly, the bacterium was realized as a potential pathogen of predatory mites (Schütte & Dicke 2008; Schütte et al. 2008).

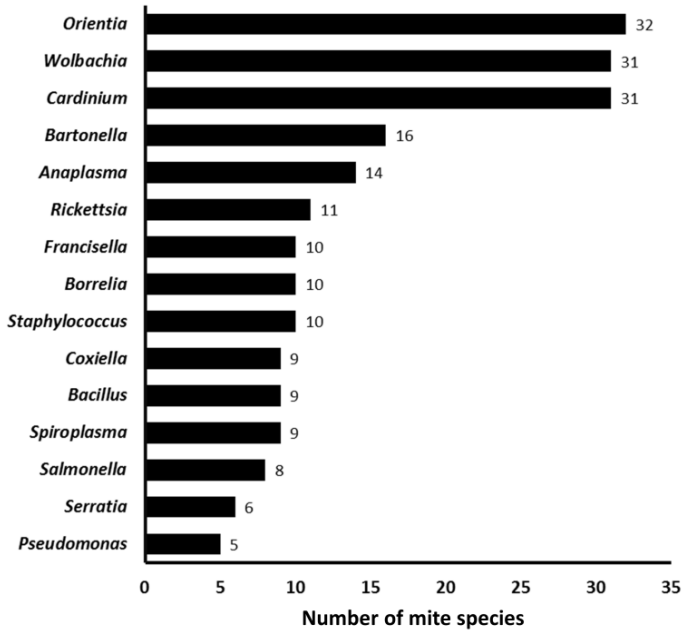

Figure 2.2 The top-ranked 15 bacterial genera detected in mites.

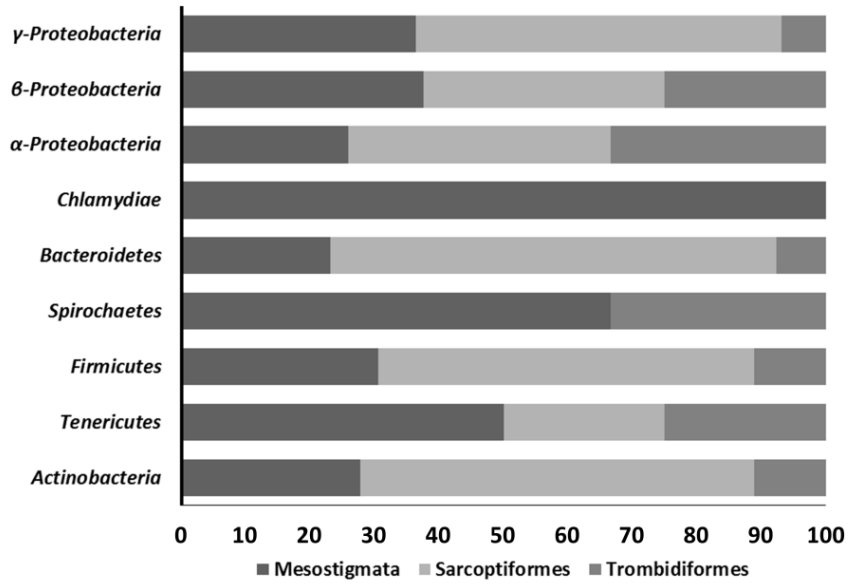

Figure 2.3 Proportions (%) of the most abundant bacterial groups in the 3 principal mite orders

Mites are often overlooked as vectors of diseases when compared with ticks or haematophagous insects, but a number of pathogenic bacteria have been reported in the vertebrate-parasitic mite superfamilies Dermanyssoidea, Acaroidea, Cheyletoidea and Trombiculoidea. In terms of veterinary importance, mites have been reported as potential vectors and reservoirs of several pathogenic bacteria of livestock. *E. rhusiopathiae*, the causative agent of erysipelas, and *S. gallinarum*, causing fowl typhoid, were reported in the poultry red mite, *D. gallinae* (Zeman et al. 1982; Chirico et al. 2003; Wales et al. 2010; Brännström et al. 2010; Valiente Moro et al. 2011). These diseases rapidly spread in infected flocks with moderate to high morbidity, resulting in significant economic damage (Takahashi et al. 2000; Shah et al. 2005). With respect to mammalian livestock, *Anaplasma spp*., such as *A. phagocytophilum* (causing tick-borne fever in ruminants) were found in various mite species of the superfamily Dermanyssoidea (Fernandez-Soto et al. 2001; Reeves et

al. 2006); whereas the opportunistic pathogen *Serratia marcescens* was found in the scab mites, *Psoroptes ovis* and *P. cuniculi*, although a role for this bacterium in the pathogenesis of psoroptic mange has not been demonstrated (Mathieson & Lehane 1996; Hogg & Lehane 1999; Perrucci et al. 2005).

For human public health, the most researched mite-associated bacterium is the scrub typhus agent, *O. tsutsugamushi*. This Rickettsia-like bacterium has been mainly found in chiggers (the larval stage of trombiculid mites), with more than 30 species reported as hosts (Kitaoka et al. 1974; Shirai et al. 1982; Ree et al. 1992; Kelly et al. 1994; Urakami et al. 1999; Frances et al. 2001; Jensenius et al. 2004; Tilak et al. 2011; Phasomkusolsil et al. 2012; Seto et al. 2013). Thus, in contrast with *Cardinium* and *Wolbachia* (Zug & Hammerstein 2012), *Orientia* appears to be a highly specialized symbiont of a single mite superfamily. Three genera of chiggers, *Leptotrombidium, Schoengastia* and *Blankaartia*, were also implicated in having a vectorial role for *Bartonella tamiae*, one of several *Bartonella spp.* that cause illness in Asian populations (Kosoy et al. 2008; Kabeya et al. 2009). Moreover, *Bartonella spp.* have been detected in other mite taxa; *i.e.*, the Dermanyssoidea, Acaroidea, Glycyphagoidea and Cheyletoidea (Reeves et al. 2006; Kopecký et al. 2014; Murillo et al. 2014). Bradley *et al* (2014) reported strong evidence of the tropical rat mite (*Ornithonyssus bacoti*) in *Bartonella* transmission, as two dogs and their owner were infected by *B. henselae* after being exposed to the mite. Thus, we suggest that parasitic mites could play an important role as vectors or reservoirs of human bartonellosis in addition to the main blood-feeding arthropod vectors such as sandflies and fleas.

Table 2.2 Phylogenetic distribution of bacteria detected in mite taxa

Bacterial Taxons			Bacterial biology*	No. Mite spp	No. Ref	Mite Taxa (number of mite species in literatures)													
						Mesostigmata					Sarcoptiformes					Trombidiformes			
Phylum	Order	Genus				Dermanyssoidea (46)	Phytoseioidea (10)	Rhodacaroidea (1)	Eviphidoidea (1)	Acaroidea (7)	Analgoidea (2)	Glycyphagoidea (5)	Hemisarcoptoidea (1)	Oppioidea (1)	Sarcoptoidea (2)	Cheyletoidea (8)	Erythraeoidea (2)	Tetranychoidea (23)	Trombiculoidea (34)
Actinobacteria	Actinomycetales	*Brevibacterium*	Sap.	3	3	X				X			X						
		Corynebacterium	Sap., Path.	2	2								X			X			
		Acaricomes	SymbA.	1	2		X												
		Kocuria	Sap.	4	3					X	X	X	X						
		Micrococcus	Sap.	1	1						X								
		Sanguibacter	Sap.	1	1								X						
		Microbacterium	Sap.	1	1								X						
		Mycobacterium	OpPath.	1	1	X													
		Propionibacterium	OpPath.	2	2										X	X			
		Streptomyces	Sap.	1	1	X													
		Tsukamurella	OpPath.	1	1	X													
Tenericutes	Entomoplasmatales	*Spiroplasma*	SymbA., OpPath.	9	7	X	X		X	X							X	X	
	Mycoplasmatales	*Mycoplasma*	Sap., OpPath.	2	1	X									X				
Firmicutes	Erysipelotrichales	*Erysipelothrix*	Path.	1	6	X													
	Clostridiales	*Eubacterium*	Sap., OpPath.	1	1	X													
	Lactobacillales	*Aerococcus*	Path.	1	1	X													
		Alloiococcus	Sap., OpPath.	1	1										X				
		Enterococcus	Op.Path	3	3	X					X		X						
		Vagococcus	Sap., OpPath.	1	1	X													
		Lactobacillus	Sap.	1	1	X													
		Leuconostoc	Sap.	1	1								X						
		Weissella	Sap.	1	1	X													
		Lactococcus	Sap.	1	1					X									
		Streptococcus	Op.Path.	3	3						X		X			X			

Table 2.2 (continued)

Bacterial Taxons			Bacterial biology*	No. Mite spp	No. Ref	Mite Taxa (number of mite species in literatures)													
						Mesostigmata					Sarcoptiformes					Trombidiformes			
Phylum	Order	Genus				Dermanyssoidea (46)	Phytoseioidea (10)	Rhodacaroidea (1)	Eviphidoidea (1)	Acaroidea (7)	Analgoidea (2)	Glycyphagoidea (5)	Hemisarcoptoidea (1)	Oppioidea (1)	Sarcoptoidea (2)	Cheyletoidea (8)	Erythraeoidea (2)	Tetranychoidea (23)	Trombiculoidea (34)
Firmicutes	Bacillales	*Bacillus*	Sap.	9	12	X				X	X	X	X			X		X	
		Lysinibacillus	Sap.	1	1					X									
		Oceanobacillus	Sap.	2	2					X			X						
		Virgibacillus	Sap.	1	1					X									
		Listeria	Path.	1	1	X													
		Sporolactobacillus	Sap.	1	1								X						
		Jeotgalicoccus	Sap.	1	1	X													
		Staphylococcus	Sap., OpPath.	10	7	X				X	X	X	X		X	X			
Spirochaetes	Spirochaetales	*Borrelia*	Path.	10	5	X		X											X
Bacteroidetes	Bacteroidales	*Bacteroides*	Sap., OpPath.	1	1								X						
		Prevotella	Op.Path	1	1							X							
	Cytophagales	*Cardinium*	SymbA.	31	26	X	X			X	X	X	X	X				X	
		Ohtaekwangia	Sap.	1	1					X									
	Flavobacteriales	*Myroides*	Sap., OpPath.	2	2	X				X									
		Elizabethkingia	Op.Path.	1	1								X						
Chlamydiae	Chlamydiales	*Chlamydia*	Path.	2	2	X													
Proteobacteria (α)	Rhizobiales	*Bartonella*	Path.	16	12	X				X	X	X				X			X
		Afipia	Sap.	1	1								X						
		Ochrobactrum	Sap.	1	1											X			
		Devosia	Sap.	1	1	X													
		Rhizomicrobium	Sap.	1	1					X									
		Phyllobacterium	Sap.	1	1										X				
		Rhizobium	Sap.	3	3					X	X		X						
	Rhodobacterales	*Paracoccus*	Sap.	1	1								X						
	Rickettsiales	*Anaplasma*	Path.	14	2	X										X			

Table 2.2 (continued)

Bacterial Taxons			Bacterial biology*	No. Mite spp	No. Ref	Mite Taxa (number of mite species in literatures)													
						Mesostigmata					Sarcoptiformes					Trombidiformes			
Phylum	Order	Genus				Dermanyssoidea (46)	Phytoseioidea (10)	Rhodacaroidea (1)	Eviphidoidea (1)	Acaroidea (7)	Analgoidea (2)	Glycyphagoidea (5)	Hemisarcoptoidea (1)	Oppioidea (1)	Sarcoptoidea (2)	Cheyletoidea (8)	Erythraeoidea (2)	Tetranychoidea (23)	Trombiculoidea (34)
Proteobacteria (α)	Rickettsiales	*Ehrlichia*	Path.	1	2														X
		Rickettsia	Path., SymbA.	11	13	X	X												X
		Orientia	Path.	32	61														X
		Wolbachia	SymbA., SymbN.	31	38	X	X							X		X		X	
	Sphingomonadales	*Sphingomonas*	Sap.	1	1								X						
Proteobacteria (β)	Burkholderiales	*Alcaligines*	Sap., OpPath.	2	2	X				X									
		Delftia	Sap.	1	1	X													
		Pelomonas	Sap.	1	1					X									
		Polaromonas	Sap.	1	1	X													
		Duganella	Sap.	1	1											X			
		Massilia	Sap.	1	1								X						
	Neisseriales	*Snodgrassella*	SymbA.	1	1											X			
Proteobacteria (γ)	Chromatiales	*Curacaobacter*	Sap.	1	1										X				
	Enterobacteriales	*Enterobacter*	OpPath.	2	2		X				X								
		Erwinia	Sap.	1	1								X						
		Escherichia	OpPath.	4	4	X					X					X			
		Ewingella	OpPath.	1	1								X						
		Klebsiella	Sap., OpPath.	1	1						X								
		Morganella	OpPath.	1	1								X						
		Proteus	Sap., OpPath.	1	1	X													
		Pantoea	Sap., OpPath.	3	3					X			X		X				
		Providencia	Sap., OpPath.	3	2	X					X	X							
		Salmonella	Sap., Path	8	8	X					X								
		Serratia	SymbA, OpPath.	6	10		X			X			X		X				
		Xenorhabdus	SymbN,	2	1						X	X							

Table 2.2 (continued)

Bacterial Taxons			Bacterial biology*	No. Mite spp	No. Ref	Mite Taxa (number of mite species in literatures)													
						Mesostigmata					Sarcoptiformes					Trombidiformes			
Phylum	Order	Genus				Dermanyssoidea (46)	Phytoseioidea (10)	Rhodacaroidea (1)	Eviphidoidea (1)	Acaroidea (7)	Analgoidea (2)	Glycyphagoidea (5)	Hemisarcoptoidea (1)	Oppioidea (1)	Sarcoptoidea (2)	Cheyletoidea (8)	Erythraeoidea (2)	Tetranychoidea (23)	Trombiculoidea (34)
Proteobacteria (γ)	Enterobacteriales	*Yersinia*	Path.	1	1	X													
	Legionellales	*Coxiella*	Path.	9	5	X													
		Rickettsiella	SymbA.	2	5	X	X												
	Pasteurellales	*Haemophilus*	OpPath.	1	1											X			
		Pasteurella	Path.	1	3	X													
	Pseudomonadales	*Acinetobacter*	Sap.	5	4	X					X		X			X			
		Psychrobacter	Sap.	2	2	X							X						
		Pseudomonas	Sap., OpPath.	5	5	X				X	X		X						
	Thiotrichales	*Francisella*	Path.	10	7	X													
	Xanthomonadales	*Stenotrophomonas*	Sap., OpPath.	1	1					X									
		Schineria	SymbA.	1	1	X													

*Bacteria were grouped according to their biological characteristics: OpPath. = Opportunistic Pathogen (vertebrate), Path. = Pathogen (vertebrate), Sap. = Saprophyte, SymbA = Symbiont (Arthropod), SymbN = Symbiont (Nematode).

In addition to the poultry pest *D. gallinae*, members of the superfamily Dermanyssoidea that may feed on humans have been found to be infected with pathogenic bacteria of medical importance. For example, the intracellular pathogens, *Rickettsia akari* (causing rickettsial pox) was isolated from the mouse and rat mites, *Liponyssoides sanguineus* and *Ornithonyssus bacoti* (Jensenius et al. 2004; Brouqui & Raoult 2006; Reeves et al. 2007); *Rickettsia typhi* (causing murine typhus) was also found in *O. bacoti* (Grabrarev et al. 2009); and *Rickettsia prowazekii* (the causative agent of epidemic typhus) was isolated from *Androlaelaps fahrenholzi* and *Haemogamasus reidi* in addition to the main louse vector of the disease (Kettle 1984; Jensenius et al. 2004; Bitam 2012). Moreover, another intracellular pathogen, *Coxiella burnetii* (the causative agent of Q-fever) was detected in *L. sanguineus, O. bacoti, D. gallinae, Eulaelaps stabularis, Androlaelaps spp.* and *Haemogamasus spp.* (Zemskaya & Pchelnika 1968; Kettle 1984; Kocianova 1989; Reeves et al. 2007); the spirochete, *Borrelia burgdorferi* (a causative agent of Lyme disease) was found in *O. bacoti, Myonyssus gigas, Laelaps agilis, E. stabularis, Euryparasitus emarginatus, Eugamasus* sp. and *Haemogamasus spp.* (Lopatina et al. 1999; Netusil et al. 2005; Netusil et al. 2013); and a further highly-virulent pathogenic bacterium in humans and other mammals, *Francisella tularensis* (causative agent of tularaemia), was isolated from *O. bacoti, Hirstionyssus spp., Haemogamasus spp*. and *Laelaps spp.* (Timofeeva 1964; Petrov 1971; Zuevskii 1976; Lysy et al. 1979).

2.3.3 Patterns of mite-bacterial association

The correspondence analysis revealed well-separated associations between the mite taxa (10 superfamilies) and bacterial types, with the first and second dimensions explaining 96% of the total variance (Figure 2.4). Trombiculoidea were reported to be strongly associated with pathogenic bacteria, whereas symbiotic bacteria were clustered with mites from the Phytoseioidea and Tetranychoidea. However, the remainder of mite taxa (Dermanyssoidea, Acaroidea, Analgoidea, Glycyphagoidea, Hemisarcoptoidea, Sarcoptoidea and Cheyletoidea) were less strongly associated with opportunistic pathogens and saprophytes. Undoubtedly, in part these data reflect important biases related to bacteria-specific studies, such as the exclusive focus on the Trombiculoidea as vectors of *O. tsutsugamushi*; or the fact that the relationship between symbiotic bacteria and mites has been better studied in the Phytoseioidea and Tetranychoidea than in the other mite taxa.

Of 25 bacterial orders, only 7 taxa showed significant differences in bacterial species richness among the 4 biological mite groups: Actinomycetales (Chi-square = 24.97, $p < 0.0001$), Bacillales (Chi-square = 30.64, $p < 0.0001$), Cytophagales (Chi-square = 79.21, $p < 0.0001$), Entomoplasmatales (Chi-square = 19.33, $p < 0.0001$), Pseudomonadales (Chi-square = 24.77, $p < 0.0001$), Rhizobiales (Chi-square = 17.69, $p = 0.001$) and Rickettsiales (Chi-square = 21.82, $p < 0.0001$) (Figure 2.5). However, there were no significant differences in total bacterial species richness among the 4 mite groups (Chi-square = 5.72, $p = 0.126$). Mites in the ‘pest and allergen’ group showed higher total bacterial richness than mites in the other groups (significantly so for Actinomycetales, Bacillales, Cytophagales, Pseudomonadales and

Rhizobiales); however, Rickettsiales were completely absent there (Figure 2.5H). Mite species in this category are well recognized as generators of allergens and carriers of some pathogenic fungi in human stored food products (Franzolin et al. 1999; Hubert et al. 2004; Hubert et al. 2012). Invertebrate-predator mites harboured a high number of Entomoplasmatales (Figure 2.5E), although these were exclusively derived from the genus *Spiroplasma.* The bacteria in this genus are known as reproductive manipulators of insect predators and plant pests (Enigl & Schausberger 2007; DiBlasi et al. 2011; Rivera et al. 2013), demonstrating that predacious and phytophagous mites and their plant hosts form an important habitat for maintaining *Spiroplasma* in nature.

2.3.4 Outcomes and perspectives

This systematic review of the literature suggests that important differences in bacterial flora may exist between mites with different lifestyles, since "house pests and allergens" displayed a particularly diverse microbiome enriched for several of the bacterial orders included in the analysis (with the notable exceptions of the Entomoplasmatales and Rickettsiales). A key priority for allergy research will be to determine whether these apparent associations are confirmed by further unbiased, high-throughput sequencing methods; and if so, the extent to which the bacterial flora of mite pests may modulate conditions such as atopic dermatitis (Sonesson et al. 2013). It would also be interesting to investigate the putative absence of Rickettsiales from this group of mites to reveal any potential barriers to colonization, especially as the Rickettsiales are clearly widespread in other mite categories.

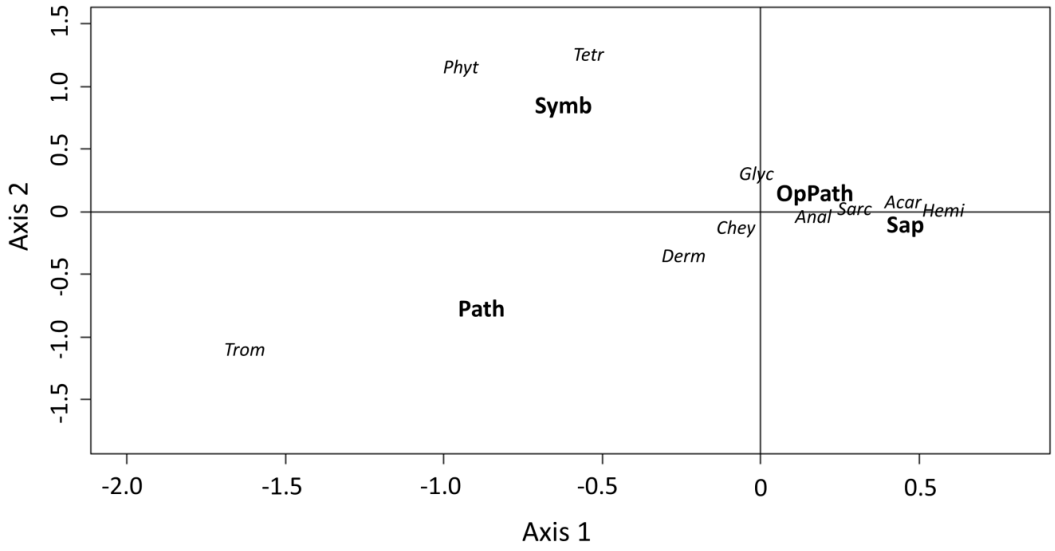

Figure 2.4 Correspondence analysis of 10 mite superfamilies (Acar, Acaroidea; Anal, Analgoidea; Chey, Cheyletoidea; Derm, Dermanyssoidea; Glyc, Glycyphagoidea; Phyt, Phytoseioidea; Hemi, Hemisarcoptoidea; Sarc, Sarcoptoidea; Tetr, Tetranychoidea and Trom, Trombiculoidea) associated with the categorized bacterial groups (Sap, Saprophytes; OpPath, Opportunistic Pathogens; Path, Pathogens; Symb, Symbionts).

On the basis of the mite literature published to date, very few bacterial species have become uniquely adapted to mites, with only *Orientia spp., R. akari* and *A. phytoseiuli* contending as mite-specific symbionts. For the former 2 species, the possibility that they are not restricted to mites with a vertebrate parasite lifestyle should be considered. Indeed, other arthropod-transmitted human pathogens, such as *Rickettsia felis*, have been detected in non-biting arthropods (Thepparit et al. 2011). Our review of the literature also raises the hypothesis that *Cardinium* is so widely distributed in mites (Weeks et al. 2003) that it may be better adapted to this taxon [and perhaps other arachnids, Duron et al. (2008)] than it is to insects.

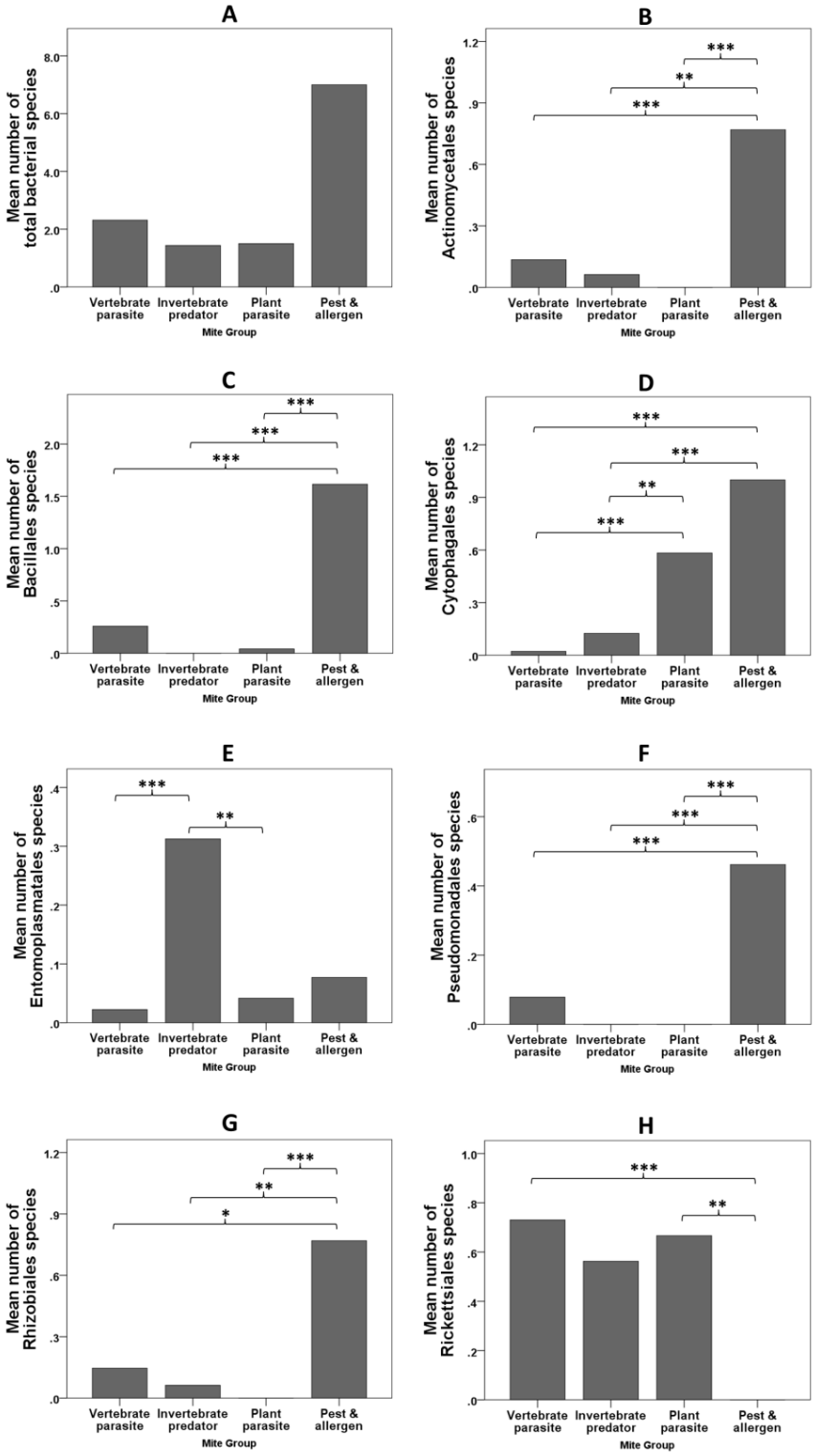

Figure 2.5 Analysis of differences in bacterial species richness among mite taxa with multiple pairwise comparisons after Kruskal–Wallis test (*p < 0.05, **p < 0.01, ***p < 0.001) for all bacterial species combined (A), Actinomycetales (B), Bacillales (C), Cytophagales (D), Entomoplasmatales (E), Pseudomonadales (F), Rhizobiales (G) and Rickettsiales (H).

In conclusion, this review provides useful reference data of mite-associated bacteria for further research, with the intention to increase awareness of the potential for mites to transmit disease. A deeper understanding of the impact of symbionts on their arthropod hosts may also facilitate the development of intervention tools for vector and pest control, for which precedents for insects already exist (Jeffery et al. 2009; Iturbe-Ormaetxe et al. 2011). Manipulation of the microbiome could lead to future opportunities to decrease the medical, veterinary and agricultural impact of mites, although major challenges in the handling and colonization of many species lay ahead.

CHAPTER 3

CHIGGER IDENTIFICATION AND DIVERSITY IN THAILAND

CHAPTER 3

Chigger identification and diversity in Thailand

3.1 Introduction

3.1.1 Diversity of chiggers in Thailand

Thailand is located on the Indo-Chinese Peninsula, Southeast Asia - a region that is well recognised as an important biodiversity hotspot on the planet (Myers et al. 2000). A high chigger density and diversity is predicted and expected in this country. Trends in chigger research and published outputs for Thailand are linked to the vector role of chiggers for scrub typhus disease, with a focus in this area developing in the 1960s, primarily by researchers from the US Army Medical Research and Development Command and the Southeast Asian Treaty Organisation (SEATO) Medical Research Laboratory (Nadchatram & Lakshana 1965; Traub & Lakshana 1966; Traub et al. 1968). The first checklist of chiggers in Thailand was published several decades ago by the latter organisation (which has now been superseded by the Armed Forces Research Institute of Medical Sciences in Bangkok) and lists 85 recorded species (Gould et al. 1966; Lakshana 1973). Subsequently, the lists of chigger species in Thailand were updated to 147 species by Tanskul (1993). However, the checklist still contained dubious information, and the presence of several species could not be confirmed in the territory of Thailand, although they have been reported from neighbouring countries such as Malaysia and Myanmar.

Accordingly, to update information on the diversity of chigger mites in Thailand as the main objective of this chapter, data obtained from the literature were re-examined. Following this, an intensive field collection across the country under CERoPath (Community Ecology of Rodents and their Pathogens in Southeast Asia: www.ceropath.org) and BioDivHealthSEA (Local impacts and perceptions of global changes: Health, biodiversity and zoonoses in Southeast Asia: www.biodivhealthsea.org) was organised, and using both approaches, the chigger status for Thailand was reassessed.

To ensure high accuracy and reliability during diversity studies of chiggers, taxonomic skills; *i.e.*, sample handling, slide preparation and recognition of key characteristic features are essential to analyse the chigger samples from field collections. Accordingly, we provide here taxonomic information and a basic morphological method for chigger identification, particularly to subgenus level.

3.2 Important taxonomic characters for identification

Taxonomic classification of trombiculid mites is based on larval morphology because only this stage is parasitic and easily found on hosts, compared to the nymphs or adults, which being free-living, are difficult to sample. Indeed, descriptions of nymphs and adults in the wild are very limited to date, and much of our understanding of chigger ecology is based on the larval stage. Chigger identification requires microscopic observation of morphology and morphometric analysis of external characteristics. In this respect, the most important feature for chigger examination is the scutum (a dorsal shield) and the scutal setae (tiny hairs),

particularly to obtain identifications to genus or subgenus level (Nadchatram & Dohany 1974). Observations on additional characteristics such as body shape and appendages (palps and legs) and on the chaetotaxy (the arrangements of the body and appendage setae) are essential for identification to the species level (Vercammen-Grandjean & Langston 1975). For the present work, morphological identification used the glossary of terminology and abbreviations published by Goff et al. (1982) alongside other established identification keys and criteria (Nadchatram & Dohany 1974; Vercammen-Grandjean & Langston 1975).

3.2.1 Gnathosoma

The gnathosoma comprises the head and mouthparts, located anteriorly to the body (the idiosoma). It consists of three main parts formed of paired segmented appendages: (1) palps (pedipalps), (2) chelicerae (teeth or blades) and (3) galeae; all located at the base of gnathosoma (gnathobase). Importantly, these appendages bear sets of setae whose number and type (pilosity: whether the setae are barbed or nude) are very important for taxonomic diagnosis.

Palps of trombiculids are segmented appendages each consisting of a coxa (fused to form the gnathobase), femur, genu, tibia and a tarsus. Viewed dorsally, the femur and genu each support a seta. In addition, there are usually three setae located on the dorsal, dorsolateral and ventral surface of the tibia; whereas the tarsus has a variable number of ranging from three to seven. A combination of these setations, the specific arrangement of seta outlined above, can be expressed in the so-called “palpal pilous formula (fPp)” (Figure 3.1).

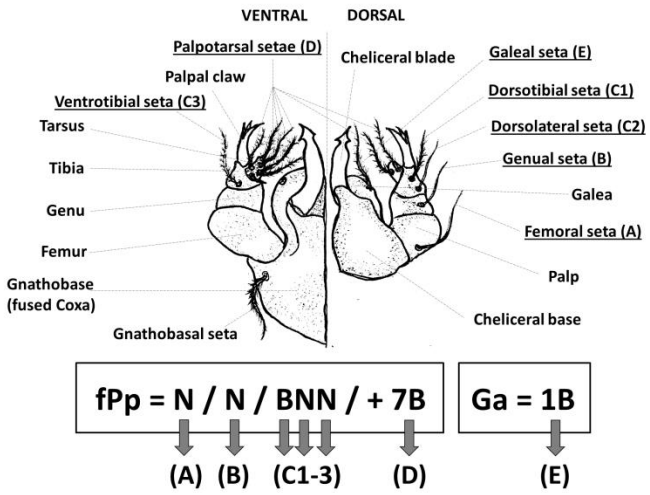

Figure 3.1 Dorsal and ventral view of the gnathosoma of *Leptotrombidium deliense*. The species has a single nude femoral seta (A), one nude genual seta (B), one barbed dorso-tibial (C1) and two nude dorsolateral and ventro-tibial setae (C2 and C3), and seven barbed palpotarsal setae, which collectively generate the palpal pilous formula (fPp) above. A single barbed galeal seta (Ga) is presented (E).

Galeae are appendages that extend ventrally from the gnathobase and curl dorsally around chelicerae. There is usually a barbed or nude seta called the galeal seta or galeala presented on each anterior surface.

Chelicerae are located at the perioral area of the mouthparts and are used to pierce the host skin. They consist of two parts: the cheliceral base, a fixed appendage attached on the dorsal of gnathobase; and a pair of chelostyles, blade-liked moveable organs which are held by the cheliceral base. There are several differences in the shape of the chelostyle. The structure can be armed or unarmed with dorsal, ventral or apical minute teeth. In some genera, the tip of the blade always bears a unique triangular spike, called the “tricuspid” (see Figure 3.2).

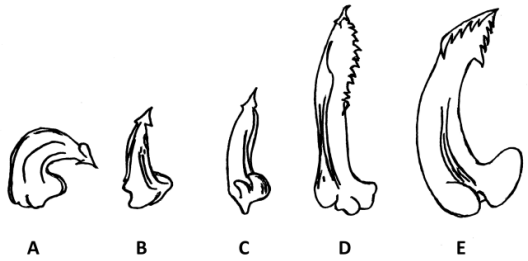

Figure 3.2 Various forms of cheliceral blades: *Babiangia* (A); *Gahrliepia* (B); *Leptotrombidium* (C); *Shoengastia* (D); *Odontacarus* (E). Redrawn from Goff et al. (1982); Nadchatram & Dohany (1974).

3.2.2 Scutum

The scutum is a sclerotized plate located on the anterodorsal part of chigger body below the gnathosoma. In theory, the scutum acts as a dorsal shield protecting the area of the central nervous system of the mite (Vercammen-Grandjean & Langston 1975). The general shape, dimensions and composition of scutal setae are characters of great diagnostic importance as they are usually constant at the genus or subgenus level. The shape of the scutum can be rectangular, trapezoid, pentagonal or shield-shaped with a slightly convex or undulated anterior/posterior margin. The scutum of Leeuwenhoekiinae and Apoloniinae possesses a unique antero-median appendix (nasus), which is absent in both Gahrliepiinae and Trombiculinae.

A set of scutal setae (usually five) is evident: two anterolateral (AL), two posterolateral (PL) and one anteromedian (AM). However, there are exceptions in some taxa; *i.e.*, the AM seta is absent in the subfamily Gahrlipiinae, whereas a pair of AM setae presents in the subfamily Leeuwenhoekiinae. Comparison of these scutal setae lengths can be expressed in a scutal formula (fSc). For example in *L.*

deliense, the PL is longer than the AM, which in turn is longer than AL; the scutal formula (fSc) in this case will be fSc = PL>AM>AL. In some genera, particularly *Gahrliepia* and *Schoengastiella*, there are usually two or more extra post-posterolateral setae called “usurped setae” present. These usurped setae are thought to be the first row of body setae that are incorporated into the elongated-posterior part of the scutum (Goff et al. 1982).

In addition to the setae described above, the scutum also bears a pair of specialized sensory setae called “sensillae” planted in the scutal sensillary bases. Sensillae appear distinctly different from scutal setae, and also vary in shape. They may be unexpanded (filamentous) or expanded (lanceolate, clavate or globular) at the apical portion. The characteristic of these sensillae also play an important role in taxonomic identification (see Figure 3.3).

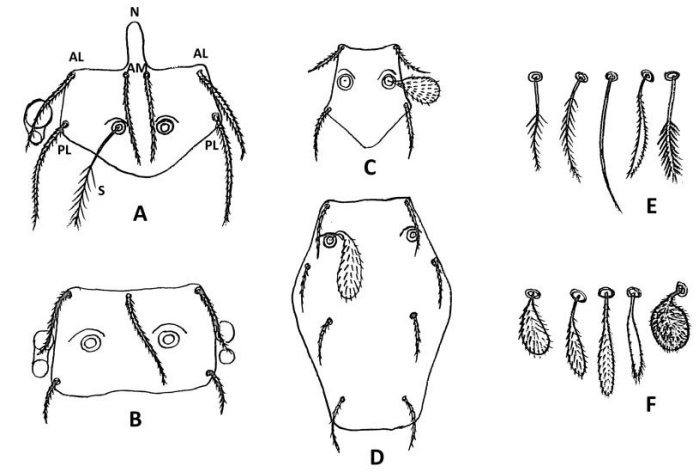

Figure 3.3 Example of scutal variation in trombiculid genera: *Odontacarus* (A), *Walchiella* (B), *Walchia* (C) and *Gahrliepia* (D); N = nasus, AL = anterolateral setae, AM = anteromedian setae, PL = posterolateral setae, and S = sensillae. The variation of scutal sensillae shape can be unexpanded (E) or expanded (F). Redrawn from Nadchatram & Dohany (1974).

3.2.3 Body Chaetotaxy

The number and pattern of the arrangement of setae on the arthropod body cuticle is called “chaetotaxy”. In terms of trombiculid species identification, the body chaetotaxy is as important as the palpal pilosity formula (fPp) on the gnathosoma described earlier. There are numerous body setae on both the dorsal and ventral sides which are arranged in distinctive patterns of transverse rows. On the dorsal surface, the setae start posterior to the scutum, usually beginning with the first pair of dorsal setae on each side of the scutum called “humeral setae”, and followed by several rows of dorsal setae sequentially down to the posterior end of the body. The number of humeral setae and dorsal setae in each row, counting from anterior to posterior gives the “dorsal body setation formula” (fD). On the ventral side, the setae are sometimes not well arranged in distinct rows as are those on the dorsal surface, and sometimes it is difficult to count and assign ventral setae into specific rows. Ventral body setation formula (fV) involves only the setae presented after the third pair of coxa (legs). At the level of urogenital pore, the number of ventral setae on this row is indicated by “u” in the fV. The total number of body setae (NDV) is the sum of the number of dorsal (ND) and ventral setae (NV). This combined morphological data: fD, fV, ND, NV and NDV is commonly used as species identification criteria, shown below for *L. deliense* (Figure 3.4).

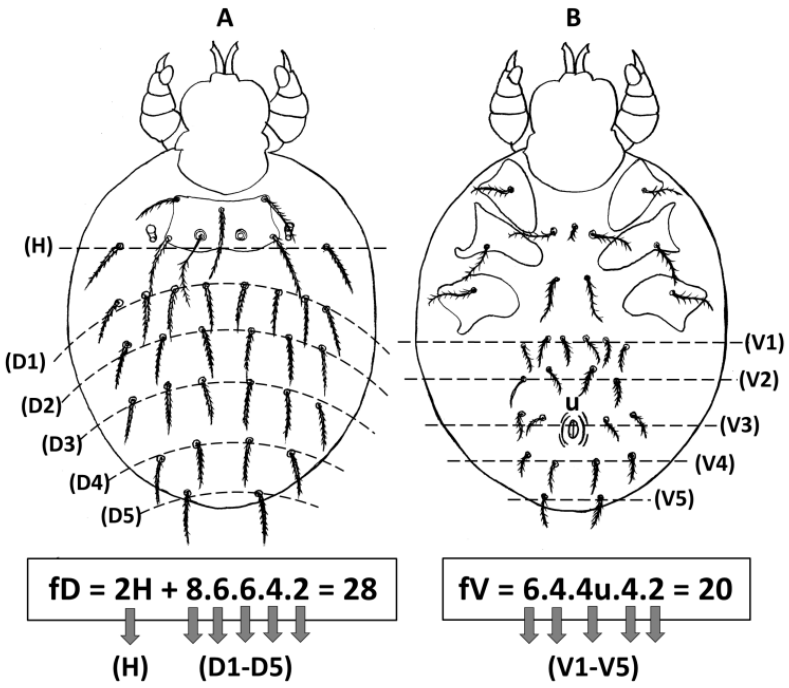

Figure 3.4 Dorsal (A) and ventral (B) aspects of L. deliense showing body chaetotaxy: dorsal body setation formula (fD), ventral body setation formula (fV), number of dorsal setae (ND = 28), number of ventral setae (NV = 20) and total number of body setae (NDV = ND + NV = 46); u = urogenital pore.

3.2.4 Legs

Trombiculid larvae possess three pairs of legs (*i.e.*, they are hexapods), nominated as leg I (anterior), leg II (median) and leg III (posterior). The fourth pair of legs is developed after the deutonymph stage. Legs are usually articulated by seven segments: coxa, trochanter, basifemur, telofemur, genu, tibia and tarsus. However, the number of segments can be six in some taxa, if the basifemur and telofemur are fused. Towards identification, the number of segments on each leg can be expressed in a leg segmentation formula (fsp). For example, genus *Leptotrombidium* has seven segments equally on leg I, II and III (fsp = 7.7.7), whereas genus *Gahrliepia*

has only six segments on leg II and III which is expressed by the fsp = 7.6.6. Additionally, the tip of the legs bears a pair of sickle-shaped claws with an empodium in the middle.

There are two types of setae on the leg segments: ordinary setae (barbed) and sensory setae (nude). The former appear on every segment, whereas the latter are usually located on the genu, tibia and tarsus: the so called “genuala”, “tibiala” and “tarsala” setae, respectively. A number of sensory leg setae types are named according to their shape, appearance or size; *e.g.* microgenualae (a small spur on genu), or mastitarsalae (a long whip-liked seta on the tarsus). The type and number of these nude sensory setae are of importance for systematic identification (Figure 3.5). The number of ordinary setae on coxa I, II and III (fCx = coxal setation formula) are also helpful for diagnosis in certain specific genera; *i.e.*, *Walchia* and *Helenicula*.

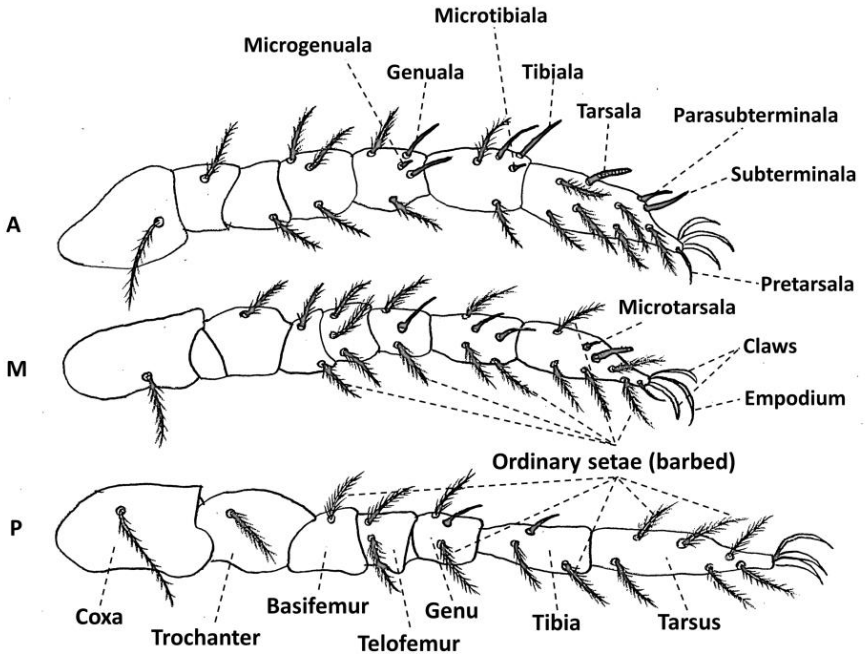

Figure 3.5 Leg segmentation and setae of *Leptotrombidium deliense*: anterior (A), median (M) and posterior legs (P). Redrawn from Goff et al. (1982).

In addition, the overall length of each leg: leg I (pa), leg II (pm) and leg III (pp), as well as the sum of the three legs' length (index pedibus, Ip = pa + pm + pp) are furthermore used in taxonomic identification.

3.3 Morphometry

Morphometry or morphometric analysis of the aforementioned important features of trombiculid larvae - scutum, setae, sensillae and legs are used for definitive taxonomic identification. In this section, the measurement methods of the key characteristics used in this thesis are followed mainly according to the description of Nadchatram & Dohany (1974) and Vercammen-Grandjean & Langston (1975). The measurements are given in microns, the standardised unit universally used in taxonomic and systematic studies of trombiculids. Methods of measuring and abbreviations are described in Figure 3.6 and Table 3.1.

As stated previously, chiggers have been identified based only on morphology and measurement of larval specimens, as development of molecular tools for taxonomic development is still very limited. This might be because there are several difficulties and limitations of the chigger sample itself, such as its miniscule size, which could potentially lead to too little DNA obtained from one individual sample to do molecular work. Moreover, the specimen requires preparation processes such as clearing in chemical agents to facilitate visualization of the chigger features. The clearing agents mainly used are lactophenol and Berlese's fluid (gum chloral), which can damage the genetic material and subsequently might affect PCR amplification. Here, a molecular approach was

preliminarily applied, which aimed to validate the quality of extracted DNA from individual chiggers for further molecular analyses of the chigger microbiome (CHAPTER 5) and to introduce molecular identification methods to the chigger taxonomic research field.

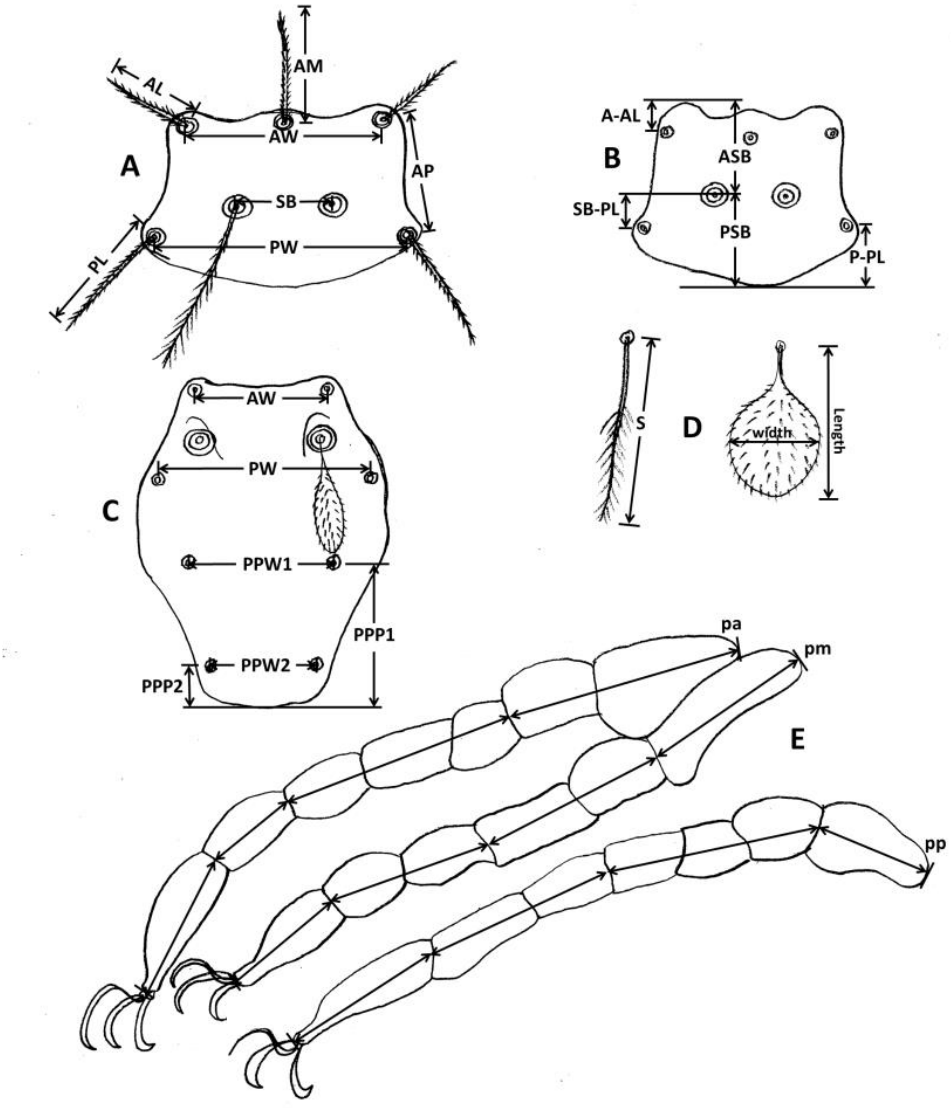

Figure 3.6 Measuring approach for the main features of trombiculid larvae: dimension and length of scutum and its setae (A, B and C); dimension and length of sensillae (D); length of the three legs (E).

Table 3.1 Abbreviations of taxonomic terms and morphometric formulae used in this thesis

Abbreviation	Description
On Scutum	
AW	Distance between bases of anterolateral setae
PW	Distance between bases of posterolateral setae
SB	Distance between bases of sensillae
ASB	Distance between a line connecting sensillae bases to anterior-most margin of scutum
PSB	Distance between a line connecting sensillae bases to posterior-most margin of scutum
SD	Scutal depth or ASB + PSB
AP	Distance between bases of anterolateral and posterolateral setae
AM	Length of anteromedian seta
AL	Length of anterolateral seta
PL	Length of posterolateral seta
A-AL	Distance from base of anterolateral seta to anterior lateral margin of scutum
P-PL	Distance from base of posterolateral setea to posterior margin of scutum
SB-PL	Distance between a line connecting sensillae bases and base of posterolateral seta
PPW1	Distance between the bases of 1st pair of usurped setae
PPW2	Distance between the bases of 2nd pair of usurped setae
PPP1	Distance from base of the 1st pair of usurped setae to posterior margin of scutum
PPP2	Distance from base of the 2st pair of usurped setae to posterior margin of scutum
S	Length of sensillae
On legs	
Pa	Length of anterior leg (leg I)
Pm	Length of median leg (Leg II)
Pp	Length of posterior leg (Leg III)
Ip	Index pedibus (pa + pm + pp)
Formulae and others	
fPp	Palpal pilous formula
fSc	Scutal formula
fD	Dorsal body setation formula
fV	Ventral body setation formula
Fsp	Leg segmentation formula
fCx	Coxal setation formula
ND	Number of dorsal setae
NV	Number of ventral setae
NDV	Total number of body setae (ND + NV)
B or N	Barbed (plumose) or nude

3.4 Materials and methods

3.4.1 Animal trapping, location and habitat categorization

Through collaboration with the two mentioned projects above (CERoPath and BioDivHealthSEA), chiggers were collected from wild small mammal hosts: rodents (Muridae and Sciuridae) and insectivores (Erinaceomorpha and Scandentia). The animals were caught alive using live-traps or Sherman traps with choices of bait; *e.g*., maize, banana or cassava, as they are good attractants and can last for long periods when traps are set for a couple of days. Trapped animals were brought immediately to the mobile laboratory unit for processing. In addition, animals were also collected with the help of local hunters, and their experience in hunting skills increased the number of sampled rodents. Local hunters were also well informed about the objectives of the present research study. They were asked to provide information on the location of animal capture including habitat and type of land use, and Global Positioning System (GPS) locations were taken afterwards by a field team member. In the case of accidentally trapped animals unrelated to the study such as protected species, animals were released immediately at the captured place within the same day.

The trapping protocol followed that of Herbreteau et al. (2011). During 2008 - 2015, animals were collected from 13 locations throughout Thailand: Buriram, Chantaburi, Chiangrai, Kalasin, Kanchanaburi, Loei, Nakornsawan, Nan (Pua, Tha Wang Pha and Bo Klue districts), Prachuab Kirikhan, Songkhla and Tak (Figure 3.7 and Table 3.2). Within each location, 10 to 12 trapping days at a minimum of 100

traps per day (10 lines of 10 traps) were set within an area of approx. 10 km^2. Each study site was sampled once during the dry (November – March) or wet season (June – July). At the level of the trapping line, GPS coordinates were recorded in the WGS84 datum system (as used in Google Earth) by Garmin Montana 600 (Garmin International Inc., Olathe, Kansas), except in Chantaburi and Nakornsawan, where the coordinates were determined through Google Earth software v. 7.1.2.2041 (Google Inc., Mountain View, California).

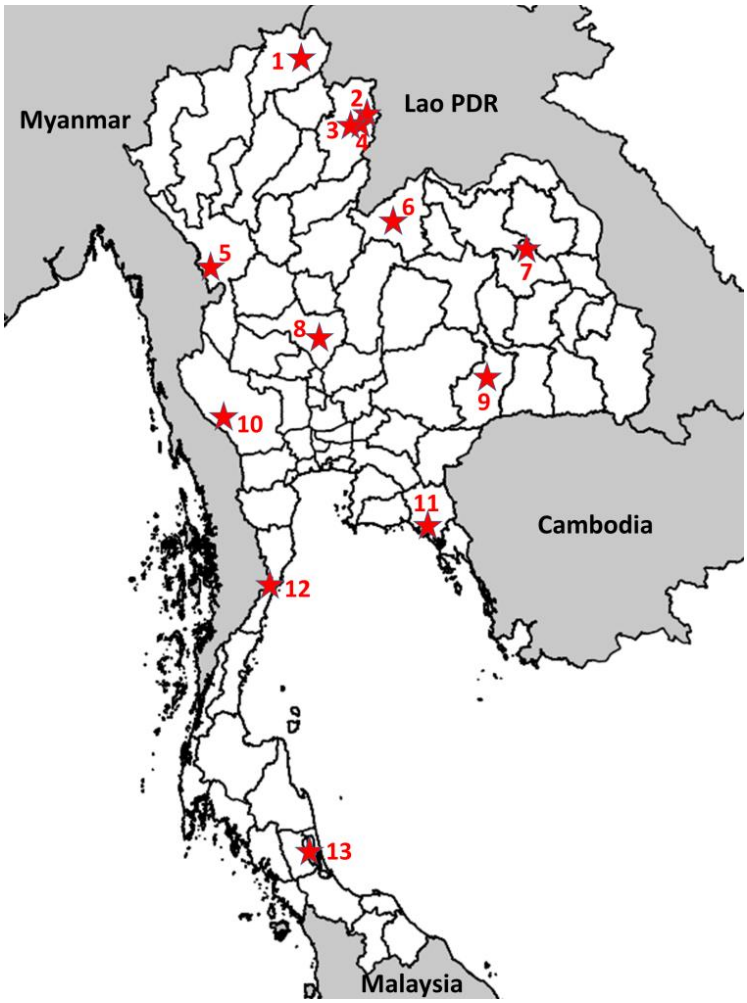

Figure 3.7 Map of Thailand showing the 13 study locations (1) Chiangrai, (2) Nan: Bo Klua, (3) Nan: Tha Wang Pha, (4) Nan: Pua, (5) Tak, (6) Loei, (7) Kalasin, (8) Nakornsawan, (9) Buriram, (10) Kanchanaburi, (11) Chantaburi, (12) Prachuab Kirikhan and (13) Songkhla

For ecological analysis, trapping sites were divided equally into 4 different types of habitats with respect to human land use or human disturbed habitat (anthropization index in ecology), spanning low to high levels of disturbance (Herbreteau et al. 2011; Blasdell et al. 2015; Chaisiri, Siribat, et al. 2015). These were:

1. Forest: primary, secondary or community forest including mature plantations of timber woods (*e.g.*, teak, rubber tree or eucalyptus)

2. Dry land: non-flooded agricultural land (*e.g.*, cassava, maize, pineapple or dry rice) including fallow, grassland, dry field and shrub

3. Rain-fed land: flooded farm and cultivated land (*e.g.*, rice field, legume, shrimp or fish farm) including other types of floodable land, swamp or marsh

4. Settlement: human built-up area (*e.g.*, an isolated house, factory, market, village or city)

3.4.2 Animal handling and euthanasia

Traps containing animals were carefully labelled to indicate place and date of capture, and then brought immediately from the field to a mobile laboratory unit within the same day. Consequently, animals were euthanized by cotton wool soaked in chloroform and placed in a closed container. The animals were only handled outside the trap after being completely euthanized. All the procedures on animal handling followed the guidelines of the American Society of Mammalogists and were also compliant with the European Union legislation guidelines (Directive

86/609/EEC). Local ethic approval was obtained from the Ethical Committee of Mahidol University, Bangkok, Thailand, No. 0517.1116/661.

Animal attributes such as weight, sex and maturity were collected. Gender was determined by the presentation of external reproductive organs: vulva opening and mammary teats for females; and the scrotal sac in males. In the case of juvenile animals, gender is unreliably discriminated from external appearance, so internal reproductive organs; *i.e*., ovary, uterus, testes or seminal vesicle were examined during dissection. Although the true age of animals could not always be determined, observations of sexual organs (*e.g*., prominent mammary teats, vaginal opening, the testes position inside or outside the body and development of the seminal vesicle) were highly informative for evaluation of animal maturity (juvenile or adult).

Measurements of individual animals (*i.e*., head-body, hind foot, ear, tail and skull length) were recorded for morphological identification using available identification keys (Lekagul & Jeffrey 1977; Aplin et al. 2003). For the problematic or cryptic species, molecular DNA barcoding (COI gene) was applied with the aid of "Rodent SEA Identification Tool" (www.ceropath.org/barcoding_tool/rodentsea).

3.4.3 Chigger isolation and specimen preparation

The animal bodies were primarily checked by the naked eye for the presence of chiggers and other ectoparasites (Figure 3.8). Subsequently, the ears were removed and examined for chigger infestation under a stereomicroscope. The chigger samples collected from the ear and elsewhere on the body (particularly the

axillary and perianal region) were fixed in 70 – 95% ethanol. In cases of excessive work load within a day, ears were preserved whole in 70 - 95% ethanol for later microscopic examination.

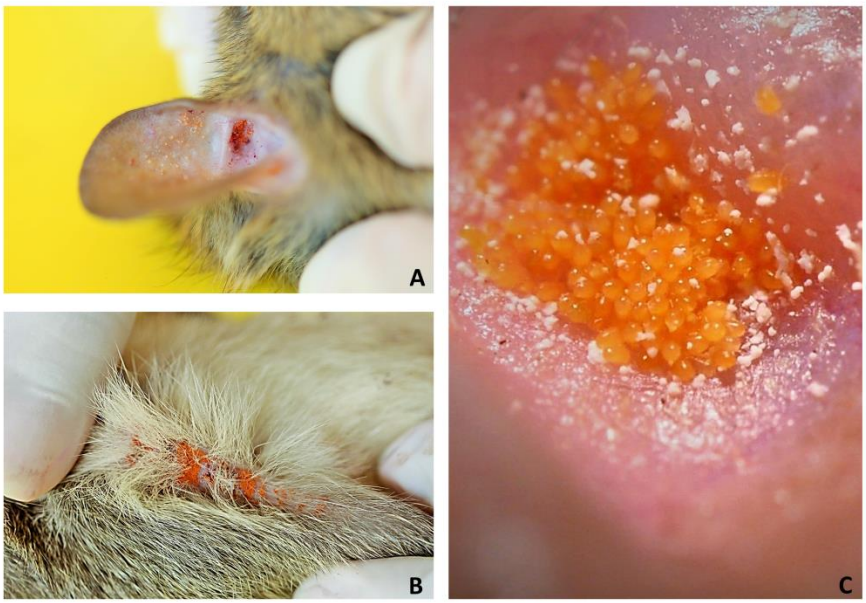

Figure 3.8 Cluster of chiggers infesting the inside the ear (A), on the body (B), and macro photography of a chigger cluster in the inner ear pinna of an infested rodent (C).

Chiggers from the same animals were counted to estimate intensity of infestation and were harvested within the same tube. To identify and estimate chigger species richness, 10 - 20% of chiggers from each infested animal were intentionally selected by differences in observed sizes and microscopic appearance as subsamples representative for a specific animal. Selected fixed chiggers were relaxed in small petri-dishes containing ultra-pure water (PureLab Option-Q, ELGA, UK) for at least one hour, mounted (dorso-ventral orientated position) on glass slides with a small droplet of clearing agent, Berlese's fluid (TCS Bioscience Ltd, UK), and then covered with 13 mm round-coverslips. Specimens were lightly pressed in

order to allow the mite to be stretched and flattened fully. This is a significant and essential step for the accuracy of sample morphometrics. Animal host ID number, location, number of sampled chiggers and date of preparation were carefully labelled. The prepared-slides were finally incubated for two days at 50^{o}C in a hot air incubator before further examination.

3.4.4 Chigger identification

In Liverpool, the main features of the specimens on slides were checked and measured as described previously using a ZEISS Axio Imager M2 microscope through ZEN 2011 imaging software (Carl ZEISS, Germany). Firstly, chiggers were identified to subgenera level following the pictorial identification key published by Nadchatram & Dohany (1974), whereas species identification was putatively achieved by following several original descriptions of particular genera or species (Traub & Morrow 1955; Traub & Nadchatram 1966; Traub et al. 1968; Nadchatram & Traub 1971; Vercammen-Grandjean & Langston 1975; Nadchatram & Dohany 1980; Stekolnikov 2013). These chigger specimens were provisionally classified to different morphospecies and sent to Dr Alexandr Stekolnikov (Academy of Sciences, Universitetskaya Embankment, Saint Petersburg) for second opinion and confirmation of identification.

3.4.5 Scanning electron microscopy to visualise ultrastructure of chiggers

Chigger samples collected in absolute ethanol from field collections were re-fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer

overnight, and adhered to 13 mm round-coverslips using Poly-L-Lysine solution. The samples were washed two times in 0.1 M phosphate buffer, pH 7.4, for 3 min. Subsequent fixation and staining were carried out in water using 2% osmium tetroxide for 1 hour, 1% tannic acid for 30 min, and 2% osmium tetroxide for 1 hour. Between each fixation and staining step, the samples were washed three times thoroughly with double-distilled water (ddH2O) for 3 min. Then, the mites were stained overnight with 1% uranyl acetate in water at 4°C. After staining, the samples were washed five times with ddH2O for three min, and progressively dehydrated with a series of ethanol concentrations in water; *i.e*., 30%, 50%, 70%, 90% and 100% for five min in each step. After dehydration, the samples were critical-point dried in CO_2 (Quorum Technologies K850) and sputter-coated with 10 nm of gold-palladium particles (Quorum Technologies Q150T). The samples on coverslips were adhered to SEM stubs with conductive silver epoxy and left to dry overnight. Finally, sample imaging was done at 10 kV with a FEI Quanta FEG 250 electron microscope operating under xT microscope control software (v.6.2.7). All sample preparation processes and SEM imaging were done by Dr Alison Beckett (Biomedical EM Unit, School of Biomedical Sciences, University of Liverpool).

3.4.6 Molecular procedures for individual chigger samples

3.4.6.1 Microscopic observation of chigger specimens

Different chigger genera/species were selected from ethanol-preserved stocks. The chiggers were relaxed in ultra-pure water at least an hour before mounting on glass slides and covered with 13 mm round-coverslip. To avoid DNA

degradation, the specimens were quickly photographed and measured under the ZEISS Axio Imager M2 microscope using bright field mode and for a second time using a fluorescein isothiocyanate (FITC) filter without any fluorescent labelling. Using these images, the specimens were identified to subgenus level and then the same individuals were subsequently subjected to DNA extraction.

3.4.6.2 Genetic analysis

DNA was extracted from whole individual chigger specimens using the DNeasy Blood & Tissue Kit (QIAGEN). The mites were crushed with pellet pestles (polypropylene sticks) in a 1.7 ml Eppendorf tube containing ATL buffer and proteinase K solution. The mixtures were incubated at 56°C overnight, and then subsequent steps followed the manufacturer's protocol. A minimal volume (30 µl) of nuclease-free water (Ambion™) was used in the DNA elution step. DNA concentrations were quantified by a double-stranded DNA fluorescence-labelling method (Quant-iT Picogreen, Invitrogen™) read in an Infinite F200 microplate fluorimeter (Magellan™ - Data Analysis Software, TECAN).

To assess DNA quality of the mite samples, the mitochondrial cytochrome oxidase I (*COI*) gene was selected for polymerase chain reaction (PCR) to amplify the gene target. The mitochondrial *COI* gene has been well-recognized as a potential molecular marker for phylogenetic analysis of Acari (Cruickshank 2002; Dabert 2006). This mitochondrial gene is not only used for DNA quality assessment, but can also give insights as a genetic marker for chigger taxonomic studies. The primers used to amplify the *COI* gene were derived from Folmer et al. (1994), which yields a

PCR product size around 710 bp across invertebrate species (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). PCR amplification was carried out in 50-µl reactions containing 5 µl of DNA template, SensiMix SYBR mastermix (Bioline), and 2 µl of each primer (0.4 µM final concentration). PCR was run with 40 cycles as follows: initial denaturation at 94^{o}C for 1 min; 5 cycles of 94^{o}C for 1 min, 45^{o}C for 90 sec, 72^{o}C for 90 sec; 35 cycles of 94^{o}C for 1 min, 50^{o}C for 90 sec, 72^{o}C for 1 min; concluding with a final extension at 72^{o}C for 5 min. The PCR products were visualized by 1.2% agarose gel electrophoresis containing SYBR Safe dye (Invitrogen^{TM}) at 120 V for 40 min.

PCR amplicon bands were excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN), then subjected to cloning in the pGEM-T Easy Vector System (Promega). PCR fragments were ligated overnight at 4$^{\circ C}$c into the pGEM-T Easy plasmid with a 1:1 insert-plasmid ratio. The ligants were transformed into JM109 *E. coli* competent cells (Promega) by heat shock at 42^{o}C for 45 sec, then supplemented with SOC medium and incubated at 37^{o}C in a 200 rpm shaking incubator for two hours. The transformants were plated on LB/ampicillin/IPTG/X-gal agar, and incubated overnight at 37^{o}C. After overnight incubation, the plates were stored at 4^{o}C for three days allowing better recognition between blue and white colonies. White colonies (recombinant clones) were inoculated in LB ampicillin broth and incubated again overnight at 37^{o}C in a 200 rpm shaking incubator. Plasmid DNA was extracted from the transformed cell pellets using the Wizard Plus SV Minipreps DNA Purification Kit (Promega), following the manufacturer's

protocol. Finally, the plasmid DNA samples were sent for Sanger sequencing with pUC/M13 forward and reverse primers to Source Bioscience Ltd, UK.

Nucleotide Basic Local Alignment Search Tool with BLASTn online platform (https://blast.ncbi.nlm.nih.gov) was used to align and compare DNA sequences to the nucleotide collection database. DNA sequences were aligned with ClustalW multiple alignments and a phylogenetic tree was created with the Neighbor Joining (NJ) method using Mega software version 6.06 (Tamura et al. 2013). The mitochondrial *COI* sequence of *Haemaphysalis flava* (GenBank accession no AB075954.1) was used as an outgroup for the phylogenetic tree construction.

3.4.7 Statistical analysis

Mean intensity or the mean number of conspecific chigger species living on an infested host and the range of chigger infestation on the small mammals were estimated. Chigger species richness (CSR; computed for observations of chigger diversity on each animal species) as well as Shannon's index (*H'*) were calculated as chigger diversity estimators by using "BiodiversityR" package (Kindt & Coe 2005) implemented in the R freeware programming environment (R Core Team 2015).

3.5 Results

3.5.1 Chigger infestation on small mammals in Thailand

In total, 1,574 small animals were examined for chiggers from 13 locations (11 provinces) in Thailand. The host species were: *Bandicota indica, Bandicota savilei, Berylmys berdmorei, Berylmys bowersi, Leopoldamys edwardsi, Leopoldamys*

sabanus, Maxomys surifer, Mus caroli, Mus cervicolor, Mus cookii, Mus sp., *Niviventer fulvescens , Rattus andamanensis, Rattus argentiventer, Rattus exulans, Rattus sakaeratensis, Rattus nitidus, Rattus tanezumi, Rattus* sp. (Rodentia: Muridae), *Hylomys suillus* (Erinaceomorpha: Erinaceidae) and *Tupaia glis* (Scandentia: Tupaiidae). The overall infestation rate was 23.8% (ranging from 5.7 to 95%) with the highest rate reported in Bo Kleu district (Nan) at 95%, followed by Hat Yai district (Songkhla), Tak Fah district (Nokornsawan) and Laem Singh district (Chantaburi) at 64.5, 48.2, and 46.1%, respectively. The lowest infestation rate (5.7%) was found in animals from Sai Yok district, Kanchanaburi (Table 3.2).

Oriental house rats, *Rattus tanezumi* and greater bandicoot rats, *Bandicota indica*, demonstrated the greatest chigger species richness (21); followed by Indochinese forest rats, *Rattus andamanensis* (12,) and common tree shrews, *Tupaia glis* (11). Greater white-toothed rats, *Berylmys bowersi* were parasitized with the highest mean chigger intensity (113.3) followed by *T. glis* (87.6) and *R. andamanensis* (62.7). Chigger infestation status and estimation of diversity indices are shown in Table 3.3. Among the infested animals (375 individuals), approximately one-half (190 individuals, 50.7%) were infested by a single chigger species, while the rest was infested with two (125 individuals, 33.3%) or multiple chigger species (60 individuals, 16%). Furthermore, large numbers of chiggers (up to 645 individuals) were found parasitizing one animal (*R. tanezumi*), and as many as seven chigger species were recovered from an individual host (*T. glis*).

Of 16,761 isolated chiggers, 2,519 specimens (about 15%) were selected for slide preparation and identification, resulting in 38 trombiculid species belonging to

12 genera and three tribes being discovered. *Ascoshoengastia indica* was the dominant chigger found on the small mammals, with a prevalence of infestation of 7.31%, followed by *Leptotrombidium deliense* (5.22%), *Walchia micropelta* (5.16%) and *Walchia pingue* (3.85%). *W. micropelta* and *W. micuscuta* parasitized the widest host range. Twelve host species were infested by these two chigger species, followed by *W. kritochaeta, L. deliense* and *A. indica* which were found on 11, 10, and 8 different host species, respectively. In addition, three new chigger species were found as well as 10 species recorded for the first time in Thailand (Table 3.4). Ultrastructure illustrations of selected chigger species are presented in Figure 3.9.

Three new chigger species from rodents were described: *Helenicula naresuani*, *Trombiculindus kosapani* and *Walchai chavali*. Formal descriptions of the three species were prepared by Alexandr Stekolnikov (see Chaisiri et al. 2016). The first one, *H. naresuani*, was found on *B. indica* and *T. glis* collected from a rubber tree plantation in Prachuab Kirikhan province and the backyard of a house close to a forest edge in Bo Kleu district, Nan province, respectively. The species has been named after King Naresuan of Ayutthaya Kingdom in A.D. 1590–1605, one of the most glorious Thai kings. This species is morphologically similar to *Helenicula mutabilis*, but differs by a larger scutum (AW = 58–59 vs. 45–50, PW = 75–79 vs 68–72, and PSB = 16–18 vs 12–14) but shorter scutal setae (AM = 31–34 vs 36–42, AL = 50–52 vs 55–64 and PL = 41–45 vs 50–56), longer legs (Ip = 801–806 vs 740– 745) and a fPp = B/B/BBB vs. B/B/BNB.

Table 3.2 Infestation status for chiggers on small mammals in 13 studied locations in Thailand during 2008 - 2015.

Province	District	Year	Season	Latitude	Longitude	Number examined animal	Number infested animal	Infestation rate (%)
Nan	Pua	2008, 2010	Dry & Wet	19.12545	100.86202	298	32	10.7
Buriram	Muang	2009	Wet	14.90311	103.11365	110	25	22.7
Loei	Muang	2009	Wet	17.45114	101.64634	232	46	19.8
Kalasin	Sahatsakhan	2010	Dry	16.29887	103.55315	186	29	15.6
Kanchanaburi	Sai Yok	2011	Wet	14.01667	99.53333	226	13	5.7
Chiangrai	Wiang Chai	2011	Dry	19.88956	99.95113	71	20	28.2
Prachuap Khirikhan	Muang	2012	Dry	11.76527	99.65642	130	39	30.0
Nakhonsawan	Tak Fah	2013	Wet	15.34976	100.49193	87	42	48.2
Songkhla	Hat Yai	2013	Wet	7.00201	100.52691	76	49	64.5
Tak	Mae Sot	2013	Dry	16.80552	98.74550	37	16	43.2
Nan	Tha Wang Pha	2013	Dry	19.13926	100.71925	25	10	40.0
Nan	Bo Klua	2014	Dry	19.14333	101.15395	20	19	95.0
Chantaburi	Laem Singh	2015	Wet	12.50766	102.13257	76	35	46.1
Total						**1,574**	**375**	**23.8**

Table 3.3 Infestation status, observed and estimated diversity (CSR = Chigger Species Richness, *H'* = Shannon's index) of chigger on small mammal species

Small mammal Host	Number infested host	Chigger intensity	Mean intensity	Range	CSR	*H'*
Bandicota indica	87	3,297	37.9	2-238	21	2.62
Bandicota savilei	3	180	60.0	32-109	6	1.74
Berylmys berdmorei	6	141	23.5	3-76	7	1.83
Berylmys bowersi	3	340	113.3	11-317	4	0.69
Hylomys suilus	3	49	16.3	8-32	5	1.56
Leopoldamys edwardsi	1	12	12.0	12	1	0
Leopoldamys sabanus	2	30	15.0	3-27	2	0.63
Maxomys surifer	20	615	30.6	3-82	9	1.69
Mus caroli	8	422	52.6	15-156	2	0.58
Mus cervicolor	14	703	50.2	7-266	3	0.67
Mus cookie	10	292	29.2	3-73	4	1.26
Mus sp.	2	120	60.0	24-96	1	0
Niviventer fulvescens	7	103	14.7	2-34	4	1.31
Rattus andamanensis	7	439	62.7	8-141	12	2.26
Rattus argentiventer	6	165	27.5	10-46	4	1.33
Rattus exulans	5	83	16.6	2-26	4	1.28
Rattus sakaeratensis	25	921	36.8	4-115	10	1.87
Rattus sp.	2	90	45.0	2-88	1	0
Rattus tanezumi	161	8,496	52.7	1-645	21	2.34
Tupaia glis	3	263	87.6	20-135	11	2.29

Table 3.4 The prevalence (%) and infestation details of 38 trombiculid species found on small mammals in Thailand

Chigger species	Number host species infested	Number host individual infested	Prevalence (%)
Tribe Gahrliepiini			
Gahrliepia elbeli	3	6	0.39
Gahrliepia fletcheri	1	4	0.26
Gahrliepia sp., cf. *orientalis*	2	2	0.13
*Gahrliepia xiaowoi**	2	3	0.20
Schoengastiella ligula	3	18	1.17
Walchia chavali n. sp.**	3	9	0.59
Walchia dismina	7	13	0.85
Walchia kritochaeta	11	45	2.94
Walchia lupella	5	49	3.20
Walchia micropelta	12	79	5.16
*Walchia minuscuta**	12	48	3.13
Walchia pingue	7	59	3.85
Walchia rustica	6	23	1.50
*Walchia ventralis**	1	1	0.07
Tribe Shoengastiini			
Ascoschoengastia indica	8	112	7.31
Helenicula kohlsi	5	12	0.78
Helenicula naresuani n. sp.**	2	2	0.13
*Helenicula pilosa**	3	11	0.72
Helenicula simena	3	8	0.52
Schoengastia propria	1	3	0.20
Schoutedenihia centralkwangtunga	1	2	0.13
Tribe Trombiculini			
Blankaartia acuscutellaris	3	24	1.57
Leptotrombidium deliense	10	80	5.22
Leptotrombidium elisbergi	1	1	0.07
Leptotrombidium imphalum	4	8	0.52
Leptotrombidium macacum	1	1	0.07
*Leptotrombidium sialkotense**	1	1	0.07
Leptotrombidium sp., cf. *guzhangense*	1	5	0.33
Leptotrombidium sp., cf. *maccacum*	1	1	0.07
*Leptotrombidium subangulare**	1	1	0.07
*Leptotrombidium tenompaki**	2	6	0.39
*Leptotrombidium turdicola**	3	7	0.46
*Leptotrombidium yunlingense**	1	1	0.07
*Lorillatum hekouensis**	1	1	0.07
Microtrombiula munda	1	3	0.20
Trombiculindus kosapani n. sp.**	1	2	0.13
Trombiculindus paniculatum	1	1	0.07
Trombiculindus variaculum	1	1	0.07

*new record in Thailand, **newly described species

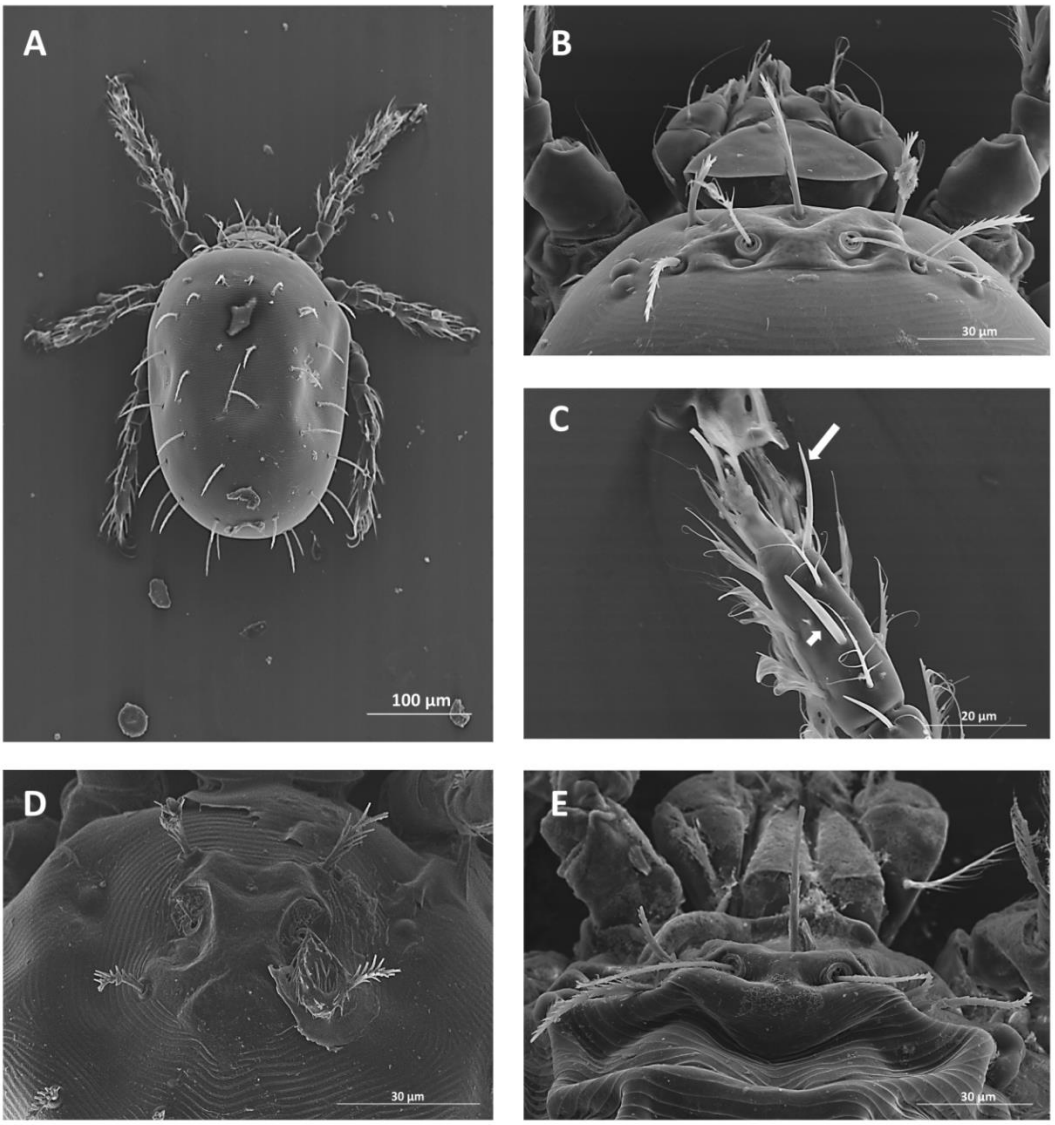

Figure 3.9 SEM micrographs show ultrastructural morphology of (A) whole aspect, (B) scutum and gnathosoma, (C) tarsus of anterior leg with tarsala, microtasala (short arrow) and subterminala (long arrow) of *Leptotrombidium deliense*; and scutum of (D) *Walchia lupella* and (E) *Blankaartia acuscutellaris*.

The second species, *T. kosapani*, was isolated from *T. glis* collected around a house in Bo Kleu district, Nan province. The species was dedicated to Mr. Kosa Pan or Foreign Minister Pan, a famous Siamese diplomat and minister, who headed the Thai embassy in France (A.D. 1686). This species is closely related to *Trombiculindus*

paniculatum, but the new species differs in fSc, (AM=PL >AL vs PL>AM>AL), shape of ALs and PLs (not expanded vs stout lanceolate) and fPp = N/N/BNN vs N/N/BNB.

The third new species, *W. chavali* was named after Mr. Yannick Chaval, a wildlife expert who contributed greatly to the CERoPath and BioDivHealthSEA projects and field studies. The species was found on several rodents, *Maxomys surifer, Leopoldamys sabanus* and *Rattus tanezumi* from evergreen forests in Hat Yai district, Songkhla province. *W. chavali* is similar to *W. pingue*, but can be easily differentiated by the palpal claw (two-pronged vs three-pronged) and peniscutum (PLs off to cuticle when scutum reduced) presented in the new species.

3.5.2 Genetic analysis of mitochondrial *COI* from individual chigger DNA

From 18 DNA extracts from 3 chigger genera (*Leptotrombidium, Ascoschoengastia* and *Walchia*), there were 15 samples positive for *COI* PCR yielding an 83.3% PCR success rate. Six PCR positive samples (*L. deliense, L. imphalum*, two *A. indica, W. kritochaeta* and *W. micropelta*), which were able to be identified to species level using flourescence and bright field microscopy, were selected for gene cloning and Sanger sequencing. Five sequences showed BLASTn results similar to trombiculid mites and tick sequences deposited in the NCBI nucleotide database, whereas another sequence from *L. imphalum* was matched to the *COI* DNA of *Bandicota indica*, the same rodent host species that the mite actually fed on (see Table 3.5). This demonstrates that PCR can amplify host tissue contaminating the chigger sample.

A phylogenetic tree of the five chiggers based on the mitochondrial *COI* gene sequence is presented in Figure 3.10. The tree clearly separated the three mite genera, *Ascoschoengastia, Walchia* and *Leptotrombidium* into different groups, whereas the outgroup, the tick *Haemaphysalis flava*, was more distant from the five chiggers.

Table 3.5 NCBI BLASTn results of mitochondrial *COI* sequences of individual chigger species, only the best matching are presented.

Chigger sample	Product size (bp)	BLASTn description	E-value	Identity (%)	Accession no.
A. indica 01	709	*Ascoschoengastia* sp. TATW-1 mitochondrial DNA, complete genome, strain: TATW-1	1.00E-170	82%	AB300501.1
A. indica 02	709	*Neotrombicula microti* voucher BIOUG01178-31 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	3.00E-147	82%	JX836578.1
L. deliense	703	*Leptotrombidium deliense* mitochondrial DNA, complete genome	0	85%	AB194044.1
L. imphalum	709	*Bandicota indica* voucher BI-1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondria	0	99%	JQ307468.1
W. kritochaeta	709	*Walchia hayashii* mitochondrial DNA, complete genome, strain: TWHW-1	9.00E-167	82%	AB300500.1
W. micropelta	709	*Rhipicephalus turanicus* isolate Xinjiang cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	2.00E-108	77%	JQ737086.1

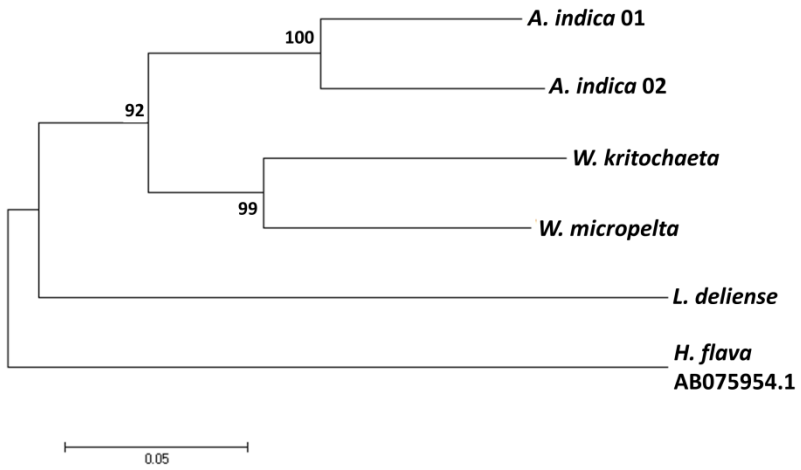

Figure 3.10 Neighbour joining (NJ) phylogenetic tree of chigger mitochondrial *COI* sequences using the maximum composite likelihood method. A phylogeny test with bootstrap values based on 1,000 replicates are presented at the nodes. The scale bar measures evolutionary distance indicating substitution per nucleotide.

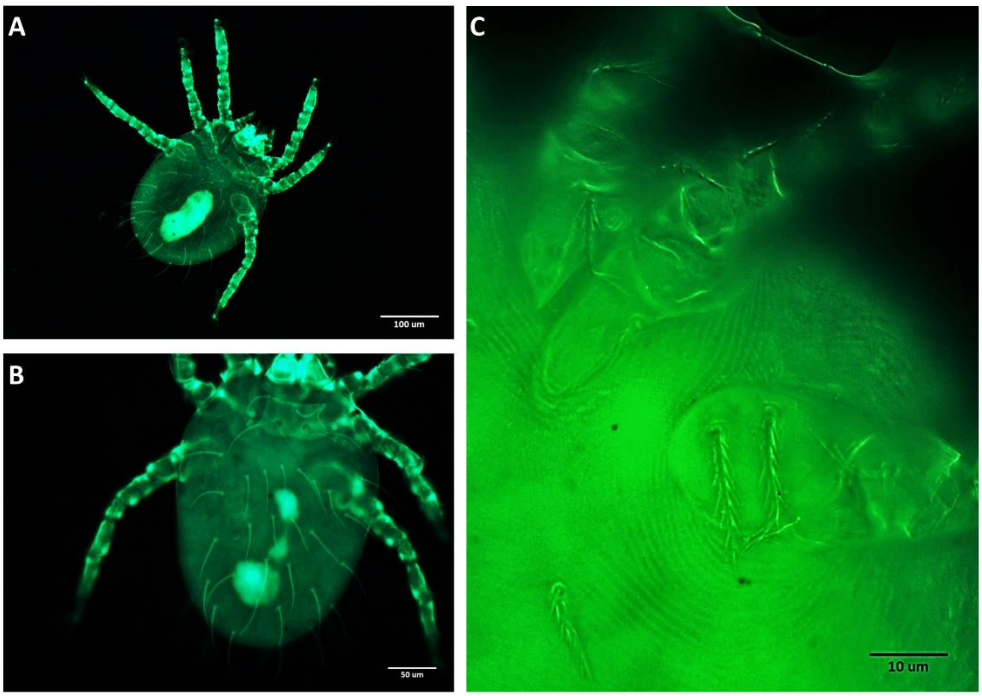

Figure 3.11 Fluorescence microscopy of whole body and legs of *L. deliense* (A), dorsal setae of *L. deliense* (B), and coxal setae of *Walchia micropelta* (C).

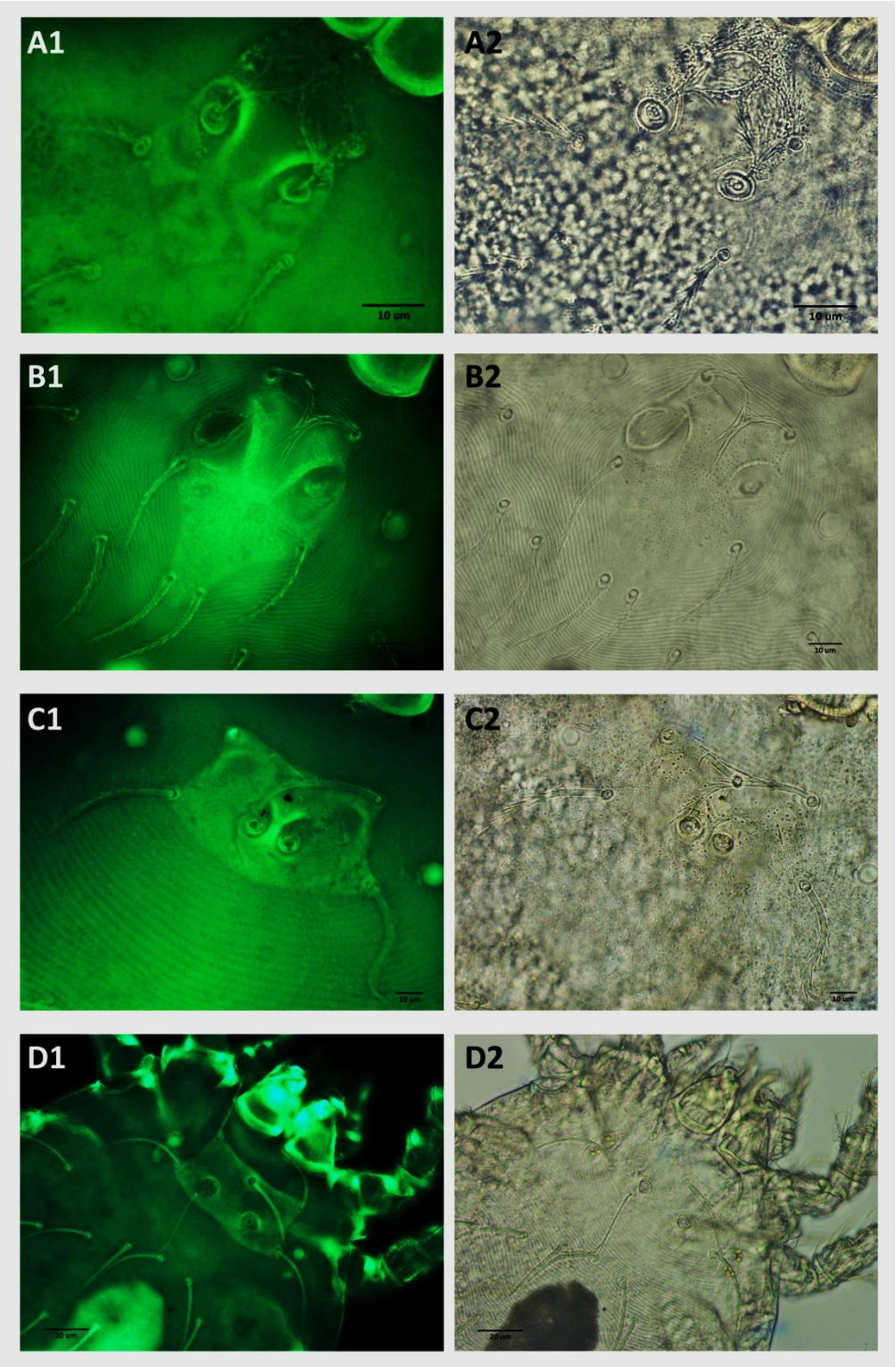

Figure 3.12 Comparison of flourescence (left panel) and bright-field (right panel) microscopy of chigger scuta. Fluorescence microscopy enables enhanced visualization of scutum morphology for *Walchia* sp. (A), *Schoengastiella* sp. (B), *Helenicula* sp. (C) and *Leptotrombidium* sp. (D).

Table 3.6 List of trombiculid species and their geographical distribution in Thailand (N = Northern, NE = North-eastern, E = Eastern, C = Central, W = Western and S = Southern). The species that potentially found on small mammals are indicated with asterisk (*), (Traub & Morrow 1955; Nadchatram 1963a; Nadchatram 1963b; Lakshana 1969; Nadchatram & Traub 1971; Vercammen-Grandjean & Langston 1975; Nadchatram et al. 1980; Suzuki 1980; Stekolnikov 2013) .

Species	Geographical region					
	N	**NE**	**E**	**C**	**W**	**S**
Subfamily Trombiculinae						
Tribe Trombiculini						
Babiangia parmifera	No information					
*Blankaartia acuscutellaris**	X	X	X	X	X	
Chiroptella sandoshami	X					
Grandjeana calva						X
Leptotrombidium Andrei	X					
*Leptotrombidium arvinum**	X	X			X	
*Leptotrombidium binbium**	X			X		
*Leptotrombidium burmense**	No information					
*Leptotrombidium deliense**	X	X		X	X	X
Leptotrombidium dendrium		X				
*Leptotrombidium elisbergi**	X					
*Leptotrombidium fulleri**	X	X				
Leptotrombidium sp., cf. *guzhangense**		X				
*Leptotrombidium hanseni**	X					
Leptotrombidium harrisoni	X					
*Leptotrombidium imphalum**	X				X	
*Leptotrombidium macacum**	X					
Leptotrombidium sp., cf. *macacum**					X	
*Leptotrombidium peniculatum**		X				
*Leptotrombidium pilosum**		X				
*Leptotrombidium scanloni**	X	X				
*Leptotrombidium scutellare**	X	X				
*Leptotrombidium sialkotense**	X					
*Leptotrombidium subangulare**	X					
*Leptotrombidium tenompaki**					X	X
*Leptotrombidium turdicola**	X					
*Leptotrombidium yunlingense**	X					
*Lorillatum hekouensis**		X				
*Lorillatum kianjoei**		X				X
*Lorillatum mastigophorum**		X				
*Lorillatum panitae**	X					
Microtrombicula chamlongi		X		X		

Table 3.6 (continued)

Species	Geographical region					
	N	**NE**	**E**	**C**	**W**	**S**
*Microtrombicula munda**		X			X	
Miyatrombicula benensoni	X					
Myotrombicula vercammeni		X				
Neotrombicula scorpionis		X				
Sasatrombicula siamensis	X					
Toritrombicula densipiliata				X		
*Trombiculindus armatum**			X			
*Trombiculindus atractimorphe**		X				
*Trombiculindus gateri**		X				
*Trombiculindus imbricatum**			X			
*Trombiculindus macrosphenum**			X			
*Trombiculindus manooni**	X					
Trombiculindus kosapani sp. nov.*	X					
*Trombiculindus paniculatum**	X	X			X	
*Trombiculindus santasirii**	X					
*Trombiculindus sibynatum**				X		
*Trombiculindus thurmani**		X				
*Trombiculindus variaculum**	X					
Tribe Schoengastiini						
*Ascoschoengastia audyi**	X	X		X		
*Ascoschoengastia indica**	X	X	X	X	X	X
Ascoschoengastia kittii	X					
Ascoschoengastia leechi	X					
*Ascoschoengastia lorius**				X		
*Ascoschoengastia tafia**	X					
*Cheladonta gouldi**		X	X			
*Helenicula kohlsi**	X	X			X	
*Helenicula mutabilis**	X					
Helenicula naresuani sp. nov.*	X				X	
*Helenicula pilosa**	X				X	
*Helenicula scanloni**	X					
*Helenicula simena**	X					
Herpetacarus cadigani		X				
Herpetacarus leprochaeta		X				
Neoschoengastia longipes	X					
Neoschoengastia solitus	X					
*Schoengastia huxsolli**					X	
*Schoengastia kanhaensis**		X		X		
*Schoengastia propria**					X	
*Schoengastia vieta**	No information					
*Schoutedenichia centralkwangtunga**	X				X	
*Susa prachongae**	X					

Table 3.6 (continued)

Species	Geographical region					
	N	**NE**	**E**	**C**	**W**	**S**
*Susa traubi**	X					
*Walchiella hanseni**	X					
*Walchiella harinastai**				X		
Subfamily Gahrliepiinae						
*Gahrliepia elbeli**	X	X			X	
*Gahrliepia fenestrulata**	X				X	
*Gahrliepia fletcheri**						X
*Gahrliepia marshi**		X				
Gahrliepia sp., cf. *orientalis**	X					
*Gahrliepia starki**						X
*Gahrliepia tylana**					X	
*Gahrliepia xiaowoi**	X					
*Schoengastiella ligula**	X				X	
*Walchia dismina**	X	X				
*Walchia disparunguis**					X	
*Walchia khunyingi**	X					
*Walchia kritochaeta**	X	X		X	X	
*Walchia lupella**	X	X	X	X	X	
*Walchia micropelta**	X	X		X	X	
*Walchia minuscuta**	X	X			X	
*Walchia pingue**	X	X		X	X	X
*Walchia rustica**	X	X	X		X	X
*Walchia suvajrai**	X					
Walchia chavali sp. nov.*						X
*Walchia ventralis**	X					
Subfamily Leeuwenhoekiinae						
Odontacarus audyi	X					
Whartonia dewitti	X					
Total	**60**	**37**	**8**	**15**	**26**	**10**

3.6 Discussion

The content of this chapter has been mainly published in Chaisiri et al. (2016), which includes a newly revised checklist of chigger species in Thailand, descriptions of the three new species and 10 records of endemic species found for the first time in the country. In addition to results published in the article, details of chigger infestation in each geographical location and host as well as a preliminary

application of a molecular taxonomic approach to this mite group are reported here. Alongside this data, micrographs of the chigger species from the field collections are given in the Appendix, providing taxonomic features, chigger images, and geographical distribution.

Considering previous chigger records as evidence for the existence of certain species in the territory of Thailand, which have been published in scientific journals or monographs (Nadchatram & Lakshana 1965; Gould et al. 1966; Traub & Lakshana 1966; Traub et al. 1968; Lakshana 1973; Vercammen-Grandjean & Langston 1975; Tanskul 1993), and the data obtained from the field surveys in the present work, 99 chigger species are listed in the revised checklist of Thailand. The complete list of chigger species distributed in six geographical regions of Thailand (the Northern, North-eastern, Western, Central, Eastern and Southern; National Geographical Committee of Thailand) is shown in Table 3.6.

The intensive surveys of small mammal hosts described here revealed a considerable diversity of chiggers comprising a total of 38 species (35 identifiable species and 3 unidentified: *Leptotrombidium* sp., cf. *guzhangense, Leptotrombidium* sp., cf. *macacum*, and *Gahrliepia* sp., cf. *orientalis*). There were 10 chigger species (*Leptotrombidium sialkotense, Leptotrombidium subangulare, Leptotrombidium tenompaki, Leptotrombidium turdicola, Leptotrombidium yunlingense, Lorillatum hekouensis, Helenicula pilosa, Gahrliepia xiaowoi, Walchia minuscuta* and *Walchia ventralis*) reported for the first time in Thailand after they were reported previously in neighbouring countries. For example, *L. tenompaki* from Songkhla, a border province between Thailand and Malaysia, was reported previously in Sabah State of

Malaysia (Vercammen-Grandjean & Langston 1975; Stekolnikov 2013); while *G. xiaowoi* and *W. minuscuta* found earlier in Yunnan province of south-western China (Wen 1984; Peng et al. 2015; Peng et al. 2016), were also discovered here in Chiangrai, the northernmost province of Thailand, which is not a long distance from Yunnan.

The most dominant genus is *Leptotrombidium*, for which at least 21 species were recorded. *Leptotrombidium* has been realized as the largest genus in the trombiculid family, containing around 340 estimated species worldwide except in South America (Stekolnikov 2013), and many of them are of importance as vectors of scrub typhus (Strickman 2001). The other prominent genera in Thailand were *Trombiculindus* and *Walchia* presenting 12 species each. The occurrence of several species that has never been found anywhere else; *e.g*., several species in *Helenicula* and *Trombiculindus*, suggests a high level of endemism. Moreover, co-existence of closely related species in the "mutabilis" group: *H. mutabilis, H. scanloni, H. simena* and *H. naresuani* n. sp., is potential evidence of a speciation origin of this group within Thailand.

In terms of the six geographical regions of Thailand, chigger diversities in the Northern (60 species), North-eastern (37 species) and Western (26 species) regions were rather high compared to the other three regions: Central (15 species), Southern (10 species) and Eastern (8 species). This might be because of biased sampling within the regions; the Central, Eastern, and Southern being examined with less rigour. In addition, some chigger species were ubiquitously found almost in all geographical regions such as *Ascoshoengastia indica, Leptotrombidium*

deliense (but still no evidence from the Eastern regions), *Walchia lupella* (absent in the Southern regions), *Walchia pingue* (absent in the Eastern) and *Blankaartia acuscutellaris* (absent in the Southern). Accordingly, further investigation in the Eastern and Southern regions may be particularly informative for updating the checklist.

Focusing on scrub typhus, certain chigger species from the list, notably *A. indica, B. acuscutellaris, L. deliense, L. imphalum* and *Shoengastiella ligula*, have been strongly implicated as potential vectors of the disease (Tanskul et al. 1994; Tanskul et al. 1998; Frances et al. 1999; Phasomkusolsil et al. 2009; Tilak et al. 2011; Phasomkusolsil et al. 2012; Vikrant et al. 2013; Takhampunya et al. 2014; Takhampunya et al. 2016). However, examination for the presence of *Orientia tsutsugamushi* infection in the other species is urgently required to identify the other potentially important scrub typhus vectors in order to gain a better understanding of the epidemiology of the disease in Thailand.

In the present study, the field surveys investigated chigger infestation only on small mammal hosts, whereas chigger communities on the other host groups such as birds, reptiles and large mammals, and their potential role in harbouring *Orientia* were not examined. Of course, birds could be a very important host contributing to spread of chiggers across a wide geographical distribution. Indeed, birds potentially have a wider foraging and roaming area than do terrestrial animals, particularly in the case of migratory species. They may act as a vehicle for chigger transportation, carrying a mite colony from one place to a new environment. For example, *Blankaartia acuscutellaris* is a chigger species that is

widely distributed across many continents (America, Europe, Africa and Asia), except Antarctica and Australia. In order to achieve this global distribution, migratory birds may be hypothesized as a potential host carrying and spreading *B. acuscutellaris* across these continents. In addition, we can also hypothesize that other potential hosts such as reptiles, bats and large mammals probably harbour their own chigger communities, and these have not been the subject of investigations for *Orientia* infection to date. Therefore, extensive surveys of chigger infestation on hosts other than small mammals are important for understanding chigger diversity, ecology and the epidemiology of scrub typhus disease in the future.

Autofluorescence imaging (FITC filter mode) using the ZEISS Axio Imager microscope enabled enhanced visualization of some important characteristics for chigger identification, particularly the shape of the scutum, body setae and leg segmentation when compared to normal bright field mode (Figure 3.11 and 3.12). The novel idea that autofluorescence might accentuate certain morphological aspects for chigger imaging without using lactophenol or Berlese's fluid to clarify the specimen was first raised by Dr Daniel H. Paris (Mahidol-Oxford Research Unit, Bangkok). The potential utility of this approach now forms a substantial part of the thesis of Rawadee Kumlert, a PhD student in the Paris laboratory, and the work on this aspect presented here represents an exploratory collaboration led by Dr Paris. The specimen preparation for autofluorescent imaging is very simple, requiring only that the sample is mounted in a drop of sterile water and covered with a coverslip. This offers considerable advantages to examine and measure the chigger specimen

without using any clearing agent that could affect genetic material, and the same individual specimen is able to be used for subsequent molecular taxonomic studies. Here, we identified chigger specimens with this method, and DNA of those same individuals was obtained, leading to successful amplification of the chiggers' mitochondrial *COI* gene by PCR. The method could be applied further for molecular taxonomic studies of this mite family, enabling the development of a molecular barcoding tool, which would undoubtedly benefit the chigger biology and scrub typhus epidemiology research fields.

Finally, with the well-designed field study protocol of CERoPath and BioDivHealthSEA, chigger infestation data was collected systematically with the inclusion of different variables; *i.e*., hosts, geographical locations, habitats, season and human scrub typhus incidence. The findings described in this chapter formed the basis for an investigation into chigger parasite ecology, host-parasite infestation patterns and diversity of chigger species which may be used in relation to scrub typhus epidemiology in Thailand (CHAPTER 4). The chigger specimens obtained from our field collections were also subjected to molecular analysis and detection of *O. tsutsugamushi* and other chigger-associated bacteria, which are the subject of CHAPTER 5 of this thesis.

CHAPTER 4

ECOLOGY OF CHIGGERS AND EPIDEMIOLOGY OF SCRUB TYPHUS IN THAILAND

CHAPTER 4

Ecology of chiggers and epidemiology of scrub typhus in Thailand

4.1 Introduction

Parasite ecology is an integrative trend of modern ecology, which considers parasitism as one type of biological symbiosis in the environment, along the continuum of mutualism, commensalism and parasitism (Douglas 2010). This research field has gradually grown in the past decades from parasite systematics and taxonomy describing lifecycles and morphology, to understanding host-parasite interactions and evolution within ecological perspectives (Jaramillo 2011). Here, we used the latter approach to understand patterns in ecological parasitism between small mammals (the host) and their chiggers (the ectoparasite), together with links with human scrub typhus epidemiology in Thailand.

The first basic research question on chigger parasitism that we addressed was the species number and diversity of parasitic chigger species in Thailand, and to define ecological factors potentially driving the diversification of the ectoparasite. For diversity measurement, parasite species richness has been widely used as a fundamental parameter to determine the parasite burden in different types of hosts (Walther & Morand 1998; Morand & Poulin 2000; Poulin 2004; Bordes et al. 2011; Cooper et al. 2012; Kamiya et al. 2014). Parasite species richness can vary by its host species in terms of phylogenetic relationships. If we imagine that host species act as “Biological Islands” for parasitic exploitation, then two nearby

"islands" or the two closely-related host taxa, are more likely to harbour similar parasite fauna than are more distant taxa. In other words, two closely related host taxa tend to be parasitized similarly in parasite assemblages than two less connected hosts (Poulin 2014). However, parasite species richness could be determined by some other host individual or population traits; *i.e.*, body mass, immunity, longevity, home range, and density, as well as host-related biogeographical factors such as geographical distribution , latitudinal gradients or habitat types (Krasnov 2008; Kamiya et al. 2014; Poulin 2014; Morand 2015). These factors could be linked together with other abiotic or biotic environmental conditions. Focussing on the ectoparasite, determinant factors influencing ectoparasite diversity were mainly discovered in flea and mosquito models (Krasnov et al. 2004; Alfonzo et al. 2005; Schäfer et al. 2006; Krasnov 2008; Beketov et al. 2010). Krasnov (2008) summarized in their review that flea assemblages on their small mammal hosts (mainly rodents) was influenced by determinants including host intrinsic factors, off-host abiotic factors and host-flea community structure. For example, in Central Europe, flea species on small mammals showed greater diversity on male hosts than onfemale hosts (Morand et al. 2014); flea assemblages of rodents were found to be positively correlated with the size of host geographical range in different host species (Krasnov et al. 2014); species richness of mosquito larvae assemblages depended on habitat and aquatic vegetation characteristics (Beketov et al. 2010); and diversity of mosquitoes was positively correlated with water permanence (area of permanent water bodies) and forest cover (Schäfer et al. 2006). For mites, very scarce information on species richness determinants are available, even for those mite vectors of medical importance, such as chiggers.

Network analysis is becoming an alternative approach to study applied ecology in host-parasite interactions. There are two types of network analysis commonly used in parasite ecology: (1) in bipartite networks, hosts and parasites are treated as the interaction between two distinct types of nodes (hosts on one side and parasites on the other); whereas in (2) unipartite networks, only hosts or parasites are assigned as nodes which connect each other with interaction links (Poulin 2010); *i.e.,* the same parasite species is shared between host nodes or the same host species is parasitized between parasite nodes. Host-parasite network analysis can be applied to study several epidemiological aspects, such as to examine the structure of host-parasite interactions through network topology parameters; *i.e.,* nestedness, connectance and modularity; to visualize overall interactions between hosts and parasites; or to understand parasite transmission by identifying key host species in the population (Dormann et al. 2009; Godfrey 2013; Morand, McIntyre, et al. 2014). Here, both aspects of bipartite and unipartite network analyses were used to study interactions between small mammal hosts and their chigger ectoparasites in population study sites and at the whole country-wide community level in Thailand.

Geographical information for Thailand obtained from the Thai Meteorological Department (2014) and United Nations Thailand (2016) is summarized as follows: the northern and western parts are the most mountainous area of the country, with high forest density; the north-eastern is the largest and the most dry area comprising two main mountain ranges on the east and south of the region; the central part is mainly flatland, with the capital city, Bangkok, and

several other developed cities located in the bottom part of the region; the eastern part comprises short mountain ranges alternating with flatlands, and the coastal line in the south part of the region where trading ports and important industrial areas of the country are located; and the southern part is a narrow peninsula with tropical forest, bearing the Siam gulf on the east and Indian ocean on the west, descending to the equator line which experiences the highest rainfall and humidity compared to the other regions. Upper regions of Thailand; *i.e*., the northern, north-eastern, western and central, usually experience subtropical inland climatic conditions, and are drier than the eastern and southern regions where tropical maritime characteristics generate a higher amount of rainfall and humidity, as well as lower seasonal and diurnal temperature variation. During the winter period, the effect of cool breezes from China occasionally reduces the temperature of the upper region, particularly in the north and north-eastern parts, to a very low level around 0°C.

Deforestation and human land use alteration has rapidly increased during the past decades in Southeast-Asian countries, including Thailand, in line with national development strategy and population growth (Fox & Vogler 2005; Trisurat et al. 2010). This human disturbance has been blamed as the major threat to biodiversity loss in the region. Habitat changes in Thailand are usually associated with agricultural purposes, and therefore occur primarily in the areas of lower altitude closer to available water sources (streams, rivers or irrigation systems) and which are readily accessible from main roads (Trisurat et al. 2010). Therefore, human settlements are generally established in the lowland areas together with

short-lived annual crops such as legumes and rice, whereas land at higher elevations is used for dry land agricultural crops (*e.g.,* cassava and maize) and some perennial plantations (*e.g.*, teak and rubber tree) in the foothills. This generates human land-use transects or urbanization degrees from low human pressure (forests at high altitude) to agricultural land and human settlements at the lower elevations.

Scrub typhus is recognized as a tropical neglected disease, with its epidemiology linked with environmental factors for the chigger vector and wildlife reservoirs. Human cases are commonly reported from rural areas, particularly from areas of scrub vegetation and new agricultural land after forest clearing (Lerdthusnee et al. 2008; Tsai & Yeh 2013). In Thailand, the disease incidence has gradually increased and is drawing more national public health attention. The patient number rose from less than 100 cases annually before the 1980s to 750 - 900 cases/year between 1988 – 1991 and has been as high as 5,000 cases/year after 2001 (Suputtamongkol et al. 2009). Several serotypes based on serological assays; *e.g.,* Karp, Kato, TA678, TA686, TA716, TA763 and TA1817, as well as the 56-KDa type-specific antigen (TSA) genotypes; *e.g.*, SEA1, SEA2, SEA3, LA, TA, TH1, TH2, Gilliam-liked, Karp-like and Kato-like have been discovered in animals and humans in Thailand (Manosroi et al. 2006; D J Kelly et al. 2009; Ruang-areerate et al. 2011; Wongprompitak et al. 2015). Despite the fact that Thailand was reported as having the highest number of human scrub typhus cases recorded in Asian countries (Kelly et al. 2015), the majority of publications concerning scrub typhus in Thailand focused mainly on disease surveillance, clinical case reports, diagnostic features and

genotyping of the aetiological agent *Orientia tsutsugamushi* itself (Watt et al. 1996; Manosroi et al. 2003; Fournier et al. 2008; Rodkvamtook et al. 2013; Sriwongpan et al. 2013); hence, the epidemiological determination of factors explaining the occurrence of the disease are largely unstudied (but see Suputtamongkol et al. 2009). In the final part of this chapter, we attempt to use the relevant parameters from an ecological study of chigger parasitism and host-chigger network analyses, as well as local environmental information obtained from field studies, to identify potential factors that explain human scrub typhus epidemiology in Thailand.

Here, the objectives of this chapter can be summarized as follows: (1) to determine chigger diversity on small mammal hosts in different biotypes (habitat, study site and season), and with respect to host traits (gender and maturity); (2) to examine patterns in habitat preference of the dominant chigger species; (3) to explore host-chigger interactions through ecological network analysis; and (4) to investigate potential determinant factors which may explain chigger species richness on the small mammal hosts and human scrub typhus epidemiology in Thailand.

4.2 Materials and methods

Data obtained from the field survey of 13 sites in Thailand (as presented in CHAPTER 3), together with environmental data and scrub typhus incidence from Ministry of Public Health, Thailand, were linked in order to conduct an ecological and epidemiological study of chiggers and scrub typhus disease in the country.

4.2.1 Analysis of chigger diversity according to different habitats, season, site and host attributes

To estimate chigger diversity in Thailand, chigger species richness (CSR), first-ordered Jackknife (Jack1) and Shannon index (*H'*) were calculated across different habitat, season, study site and host attributes (sex and maturity) using "BiodiversityR" package (Kindt & Coe 2005) implemented in the R freeware (R Core Team 2015). CSR is the number of chigger species found on a given host species or individual, representing the actual observation of chigger species by simply counting the species number. The other two indices, Jack1 and *H'*, are used as the species richness and diversity estimators, respectively. The estimators help to reduce bias that may occur from sampling effort or the effect of under-sampling of the rare species (Smith & van Belle 1984; Colwell & Elsensohn 2014). Jack1 is a non-parametric estimator producing the most precise and less biased estimation of species richness, realized as more suitable for analysing parasite data compared to other estimators (Walther & Morand 1998). *H'* is widely used to estimate the diversity index in ecological research fields including parasite diversity; *e.g.*, endo- and ectoparasite diversity of fish and small mammals (Korallo et al. 2007; Ponlet et al. 2011; Chapman et al. 2015).

Chigger species accumulation curves were generated for evaluation of rodent sample size adequacy, as well as to illustrate differences in chigger species richness among different factors. Nonparametric Kruskal–Wallis and multiple pairwise comparison tests were performed to investigate the effects of habitat on chigger species richness.

4.2.2 Association between dominant chigger species and habitat

The twelve selected chigger species that were included in this analysis were selected from chigger species which infested ≥10 individual hosts. To visualize the association between the chigger species and habitat types, correspondence analysis (CA) was performed by using "FactoMineR" package in R freeware (Husson et al. 2007). CA is similar to principle component analysis (PCA), a statistical method to simplify the two categorical variables (chigger species and habitats in this case) in the dataset by reducing dimensionality of the dataset and to visualize the association of two particular variables through a two-dimensional CA plot, whereas PCA is used to analyse numerical variables (Lê et al. 2008).

4.2.3 Network analysis of host-chigger interaction

Host-chigger network analysis was conducted to explore interactions through assessment of network architectures (properties) by focusing at both the level of host species (pooled host species and locations) and the host individual.

4.2.3.1 Bipartite network analysis

To study the community ecology of host-chigger interactions, bipartite network analyses were conducted on both the community and individual basis of the host-ectoparasite interactions using "vegan" (Oksanen et al. 2015) and "bipartite" packages (Dormann et al. 2009) implemented in R freeware. A host-chigger interaction matrix (presence/absence) of the pooled 13 study sites was created with host species as rows and chigger species as columns. The matrix was

visualized for bipartite network and nestedness patterns using “visweb” and “plotweb” functions, respectively. The chigger species specificity index was also computed by the “specieslevel” function to identify specialists or species with few links (fewer share the same chigger species with the others) and generalists or species with many links (sharing of several chigger species with the others) in the community.

Meanwhile, on the host individual basis, the same type of matrices was generated for each studied site/community. Subsequently, a number of network properties were estimated: (1) Nestedness is the degree of how many interactions realized by specialists belong to subset of those realized by generalists in the particular community. The NODF (Nestedness metric based on overlap and decreasing fill) was computed using “nestednodf” functions. The NODF ranges from 0 - 100, where a value of 100 indicates perfect nestedness and 0 represents an absence of nestedness (Almeida-Neto et al. 2008). (2) Connectance is defined as the proportion of possible links between the realized species (Dunne et al. 2002); in other words, this can be described as the proportion of established interactions or network complexity in a particular community. (3) Links per species is the mean number of the same chigger species shared (links) per host. (4) Modularity is a measure of community structure/compartmentalization of a particular network. The higher the modularity, the more sub-communities dependently clustered in the network. In other words, sub-communities consist of species with many links among themselves and sparsely interact with species in other sub-communities (Fortuna et al. 2010). Network modularity of the 13 sites was computed by the

"computeModules" function, where each matrix of bipartite weighted graph is taken into account to compute the parameter. A repeat of 100,000 computational steps was applied by default to confirm that no better clustering than the current one could be identified (Dormann et al. 2009).

4.2.3.2 Unipartite network analysis

Bipartite networks were transformed to unipartite networks using the "tnet" package (Opsahl 2009). Unipartite network plots illustrate the relative interaction patterns among hosts regarding the co-occurrence of chigger species shared within the particular community. An Eigen value of centrality was calculated using the "evcent" function from the "igraph" package (Csardi & Nepusz 2006). This centrality measurement was used to estimate the role of each host as a connector to other hosts with respect to the same shared chigger species. A higher value of centrality of a node (host) is associated with a higher connection to the other nodes (other hosts), suggesting a high number of parasite species co-occurred in the network (Morand, McIntyre, et al. 2014).

4.2.4 Multiple regression models of independent variables explaining chigger species richness and scrub typhus incidence in the human population

Generalized linear models (GLM) were constructed in order to identify potential effects of host attributes (species, sex, maturity and body mass) and ecological factors (habitat, site and season) to explain chigger species richness at

the level of individual hosts. Linear regression models with “Poisson” family for chigger species richness count (skewed-data containing many zeros) were modelled in the “lme4” package (Bates et al. 2015) embedded in R freeware. A selection of models based on the likelihood-based method, Akaike’s Information Criterion (AICc), was adjusted for sample size using the “gmulti” package (Calcagno & de Mazancourt 2010) in R freeware. Model selection can sometimes produce an uncertain fit or implausible model in the output. Accordingly, the quality of various models was quantified by the model weight value (Akaike’s weight: *Wr*), which can be realized as the probability that a particular model is the best selection from the available data (Burnham & Anderson 2002). Delta AICc (ΔAICc), the difference between the AICc value of a given model and a model with minimum AICc, was also calculated in order to facilitate the best model selection. Model-averaged Importance of Term (MaIT) was illustrated through histogram plotting to identify the significance of particular variables. MaIT is defined as the proportion of the best models in which each given candidate variable term appears after all possible models are computed (*i.e.*, 150 models for chigger species richness and 250 models for scrub typhus incidence in the present analysis) (Sackton & Hartl 2013). The variables with an importance score with >80% proportion support (default in the “gmulti” package) were included in the final model.

The best selected model was evaluated from following criteria: low AICc score, clear ΔAICc difference from the substitute models, high *Wr* score, and explicative variables passing with 80% MaIT support. The best model was subsequently designated for an Analysis of Deviance table (ANOVA type II test) in

order to emphasize the role of explicative independent variables on individual chigger species richness. To identify the degree of multicollinearity among explicative variables, the variance inflation factor (VIF) was computed in the "car" package (Fox & Weisberg 2011) in R freeware. The higher the VIF value, the stronger the collinearity, and a VIF score >10 was used as a common cut-off threshold to indicate strong multicollinearity in a model (O'brien 2007).

4.2.4.1 Data source

Data for scrub typhus human case numbers from the 13 studied sites was obtained from the Bureau of Epidemiology, Ministry of Public Health, Thailand (unpublished data). The human case data were selected for the particular districts and years when the field surveys of small mammals had been conducted. Geographical and environmental information; *i.e.*, GPS coordinates (latitude-longitude), elevation, and annual mean temperature of the studied sites were derived from the CERoPath project. The environmental information above and host-chigger network properties were assigned as candidate independent variables to explain scrub typhus human case number across the country.

To examine pairwise relationships between those variables above and scrub typhus case number, the non-parametric Spearman's rank with significance test was conducted in R freeware. Finally, in order to evaluate the best fit model and initially identify constitutive variables accounting for scrub typhus epidemiology in Thailand, GLM with "Poisson" family and model selection with AICc were applied as described above.

4.3 Results

4.3.1 Chigger diversity in Thailand

High diversity of chiggers was discovered with 38 chigger species parasitizing 18 small mammal species captured across Thailand. Information on chigger diversity among different habitats, seasons, study sites and host attributes are given in Table 4.1. The cumulative number of chigger species in all examined hosts is illustrated through a species accumulation curve plot (Figure 4.1). The curve of chigger species richness was found to sharply increase in the initial phase (around the first 200 hosts examined), then the rate of increase declined in the middle phase (between 200 - 1,000 host examined), and finally stayed steady in the plateau phase (after 1,000 host examined). This demonstrates that the host sample size is sufficient for chigger diversity estimation within the whole population.

There were differences in chigger diversity among the studied sites. Small mammal hosts from Nan-Bo Kleu, a site in northern Thailand, had the highest chigger species richness (20 species), followed by Chiangrai (northern), Nan-Pua (northern), Kalasin (north-eastern) and Tak (western), with 12, 12, 11 and 11 chigger species, respectively. In contrast, hosts from Chantaburi (eastern) and Nakornsawan (central) were infested by the lowest number of chigger species, only 4 chigger species were equally discovered from both sites (Table 4.1). At the whole country level, chigger species richness showed a positive significant correlation with the latitudinal gradient of the studied sites (Spearman's rank correlation = 60.81, p = 0.0023); hosts from the upper regions of the country tend to be infested with a

higher chigger species richness than those from the other locations further south (Figure 4.2).

In term of habitat categorization focusing at an individual host level, chigger species richness varied significantly among the four habitat types: forest, dry land, rain-fed land and human settlement (Kruskal-Wallis chi-square = 91.29, df = 3, $p < 0.0001$). At the whole population level, chigger species richness also differed among the habitats; hosts trapped in forest harboured significantly higher chigger species richness than those trapped in other habitats (Figure 4.3).

In term of habitat association, some chigger species appeared to be habitat generalists, tending to be present in many habitat types, such as some species in the genus *Walchia* (*W. micropelta, W.rustica, W. pingue*) and *Ascoshoengastia indica*, while some others were habitat specialists, occurring only in particular habitats. These included *Helenicula kohlsi* in human settlements, *W. minuscuta, Schoengastiella ligula* and *Blankaartia acuscutellaris* in rain-fed lowland, and *H. pilosa* in dry landscapes. *Leptotrombidium deliense*, the main vector of scrub typhus disease in Thailand, was present medially between dry land and forest; thus both are potential habitats for humans and biting chiggers which can transmit the causative agent of this disease. Details of habitat associations for the dominant chigger species are shown in Figure 4.4.

Table 4.1 Diversity observation and estimation of chigger infestation on small mammals in Thailand. Abbreviation: N = number of host examined, CSR = Chigger species richness, Jack1 = First-ordered Jackknife estimator, *H'* = Shannon diversity index

Parameter	N	CSR	Jack1	*H'*
Habitat				
Forest	122	29	39.9	2.8
Dryland	288	20	22.9	2.4
Rain-fed land	360	18	20.9	2.4
Settlement	371	16	20.9	2.1
Season				
Wet	832	15	15.9	2.4
Dry	563	36	45.9	2.9
Location				
Buriram	131	5	5.9	1.3
Chantaburi	76	4	4.9	0.6
Chiangrai	70	12	15.9	2.1
Kalasin	185	11	13.9	1.9
Kanchanaburi	214	6	7.9	1.5
Loei	206	10	10.9	1.9
Nakornsawan	87	4	4.0	1.0
Nan (Bo Kleu)	20	20	30.5	2.7
Nan (Pua)	138	12	13.9	2.2
Nan (Tha Wang Pha)	25	10	12.8	2.2
Prachuab Kirikhan	130	9	10.9	1.6
Songkhla	76	7	7	1.7
Tak	37	11	15.8	2.1
Host gender				
Female	717	28	33.9	2.7
Male	673	35	45.9	2.8
Host maturity				
Adult	955	30	35.9	2.7
Juvenile	431	29	38.9	2.8

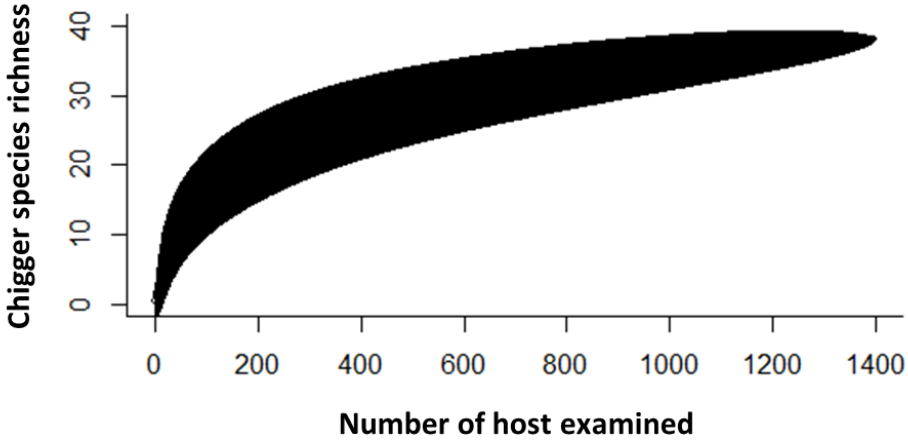

Figure 4.1 Chigger species accumulation curve of an overall 1,395 examined small mammal hosts

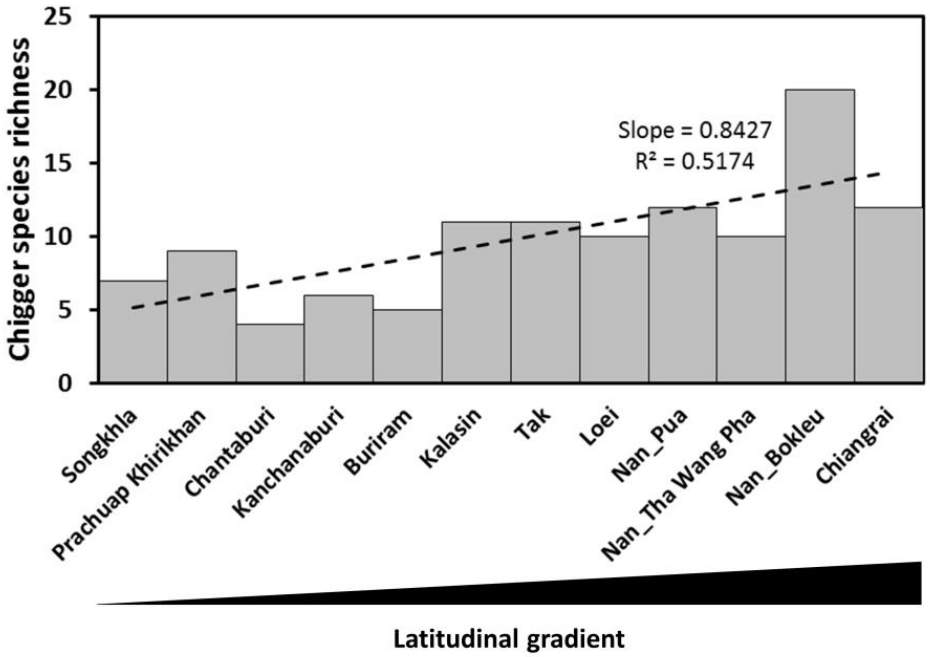

Figure 4.2 Bar plot showing positive correlation between chigger species richness in the 13 studied sites and latitudinal gradients in Thailand.

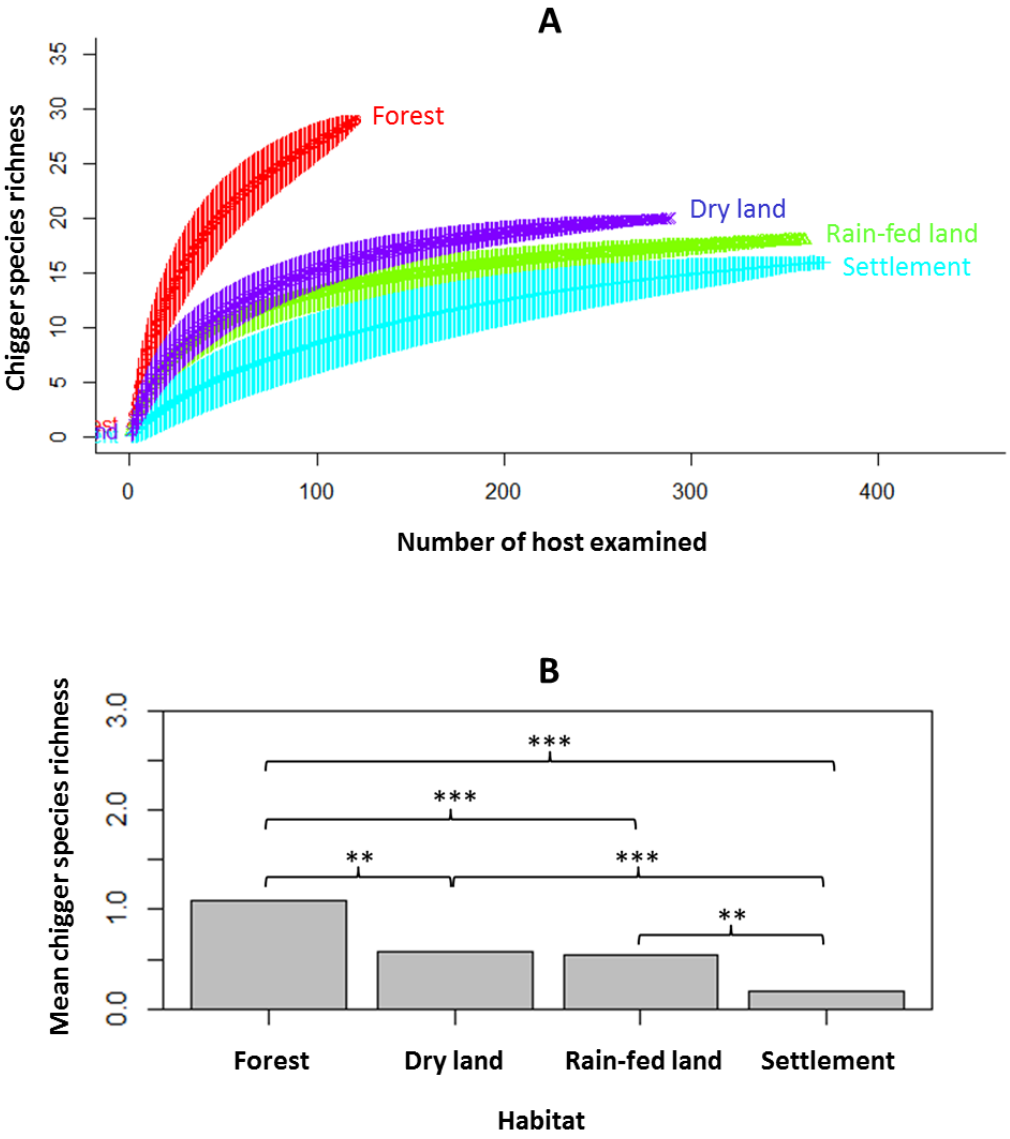

Figure 4.3 Effect of habitat types to chigger species richness: (A) species accumulation curves among different habitats and (B) Analysis of difference in mean chigger species richness on individual hosts among each habitat with multiple pairwise comparisons after Kruskal-Wallis with Bonferroni post hoc test. (**) $p < 0.01$, (***) $p < 0.001$

Regarding the effect of season at individual host level, there was no significant difference in either chigger species richness (Mann-Whitney U test = 165510, p = 0.421) or chigger abundance (Mann-Whitney U test = 163340, p =

0.757) when wet and dry seasons were compared. However, at the level of the whole country population, hosts captured in the dry season (November – March) were clearly infested with a higher chigger species richness than those from the wet season (April – October) (Table 4.1 and Figure 4.5).

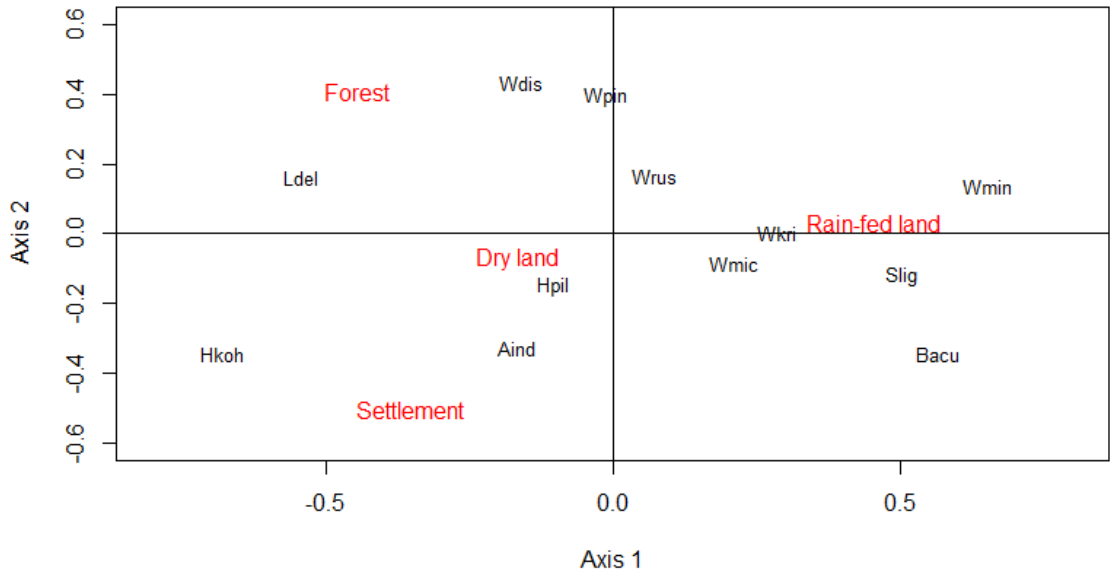

Figure 4.4 Correspondence Analysis (2D Plot) showing the association between the 12 dominant chigger species (Aind = *Ascoschoengastia indica*, Bacu = *Blankaartia acuscutellaris*, Hkoh = *Helenicula kohlsi*, Hpil = *Helenicula pilosa*, Ldel = *Leptotrombidium deliense*, Slig = *Schoengastiella ligula*, Wdis = *Walchia dismina*, Wkri = *Walchia kritochaeta*, Wmic = *Walchia micropelta*, Wmin = *Walchia minuscuta*, Wpin = *Walchia pingue*, Wrus = *Walchia rustica*) within the four categorized habitats. The first and second dimensions explain 87% of the total variance.

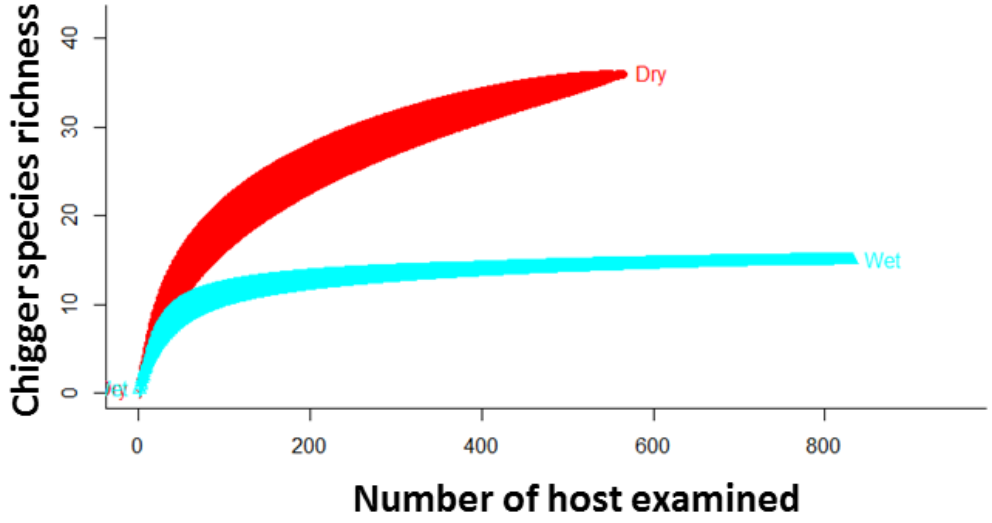

Figure 4.5 Chigger species accumulation curves between dry (red) and wet season (blue)

4.3.2 Host-chigger network analysis

Host-chigger interaction within population (study sites) and community (country) was explored through bipartite and unipartite network analysis approaches. At a whole community level, interactions between 18 small mammal hosts and 38 chigger species was determined for community structure, host specificity and species co-occurrence pattern through nestedness. While, at the population level, the interaction between individual hosts and chiggers was examined, and network properties as well as architecture within each studied site were reported.

4.3.2.1 Network analysis in community level

Bipartite network of interaction between small mammal hosts and chiggers in Thailand was visualized through a matrix grid with host species in rows and chigger species in columns (Figure 4.6A), and the bipartite graph shows highly complex interactions between the host and parasite species (Figure 4.6B).

On the host side, *Bandicota indica* and *Rattus tanezumi* were placed at the top rows of the bipartite matrix (Figure 4.6A), harbouring a great chigger species assemblage in the community. The two non-rodent small mammals, *Hylomys suillus* (Erinaceomorpha: Erinaceidae) and *Tupaia glis* (Scandentia: Tupaiidae) showed segregated species co-occurrence patterns, and were infested by different chigger species from the other murid rodent hosts.

On the chigger side, some chigger species (those in the leftmost columns) were broadly found on several host species (Figure 4.6A); *e.g., Walchia micropelta, W. minuscuta, W. kritochaeta* and the main vector of scrub typhus, *L. deliense*, could be determined as generalist species which were able to feed on a wide host range. More than half of chigger species were found on more than one host species, suggesting low host-specificity of their feeding habit. In addition, the species-specificity index was calculated in order to quantify the degree of host specificity in each chigger species (Table 4.2). In the table, sampling effort bias was minimised by focusing only on the chigger species that infested >10 host individuals, and most of those chiggers showed a low species-specificity index (ranging from 0.171 – 0.542).

Unipartite network of chigger species co-occurrence among the hosts is presented in Figure 4.7. Again, the two rat species, *B. indica* and *R. tanezumi* appeared to be the most central hosts sharing chigger species with the other hosts in the network.

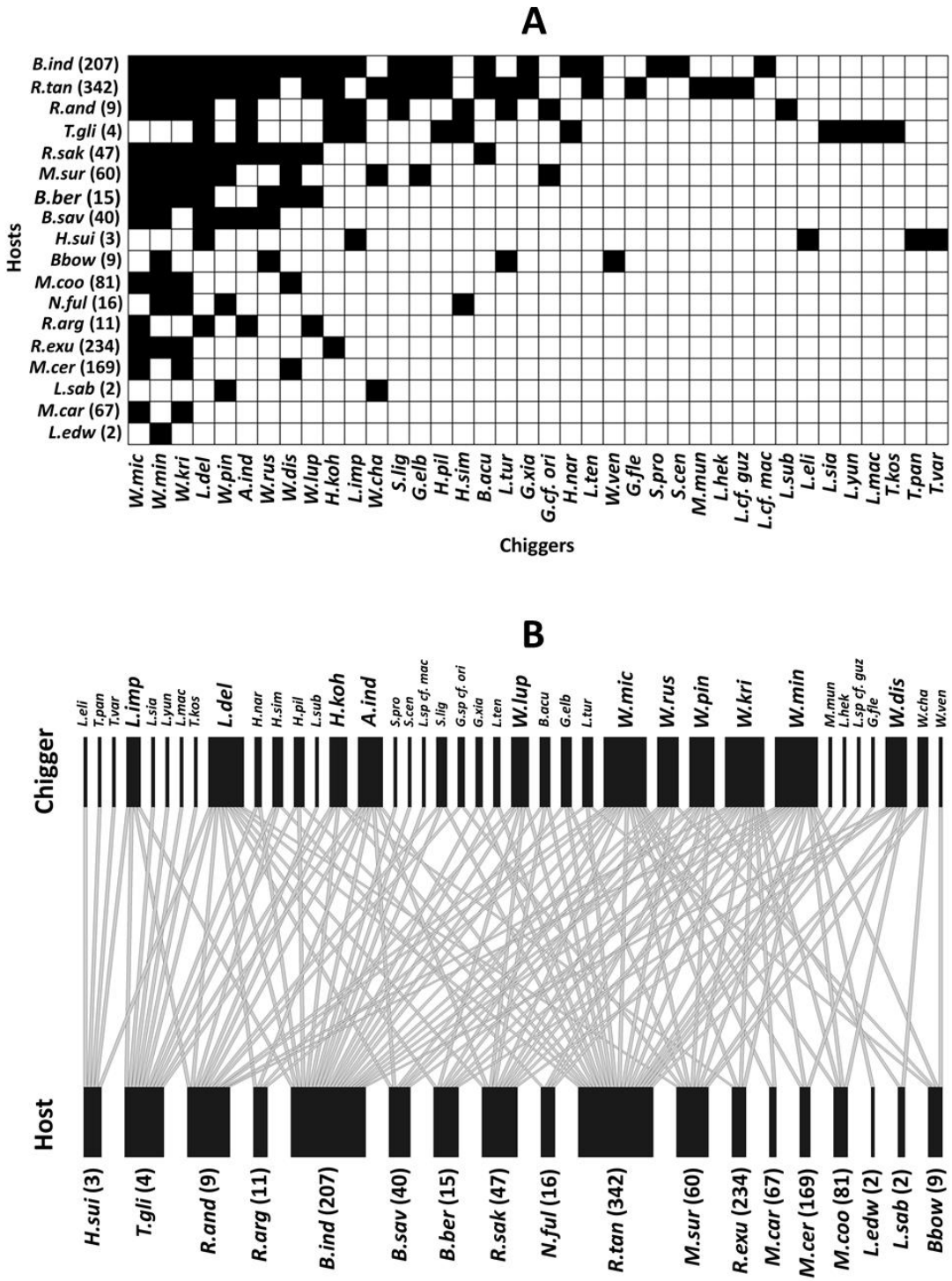

Figure 4.6 Nestedness matrix (A) and bipartite graph (B) of host-chigger associations based on presence-absence data. The number of individual hosts examined is shown in brackets. Color labelling in panel A indicates the presence (black) or absence (white) of interactions between animal hosts and chiggers.

Table 4.2 Species specificity index of trombiculid chiggers on small mammal hosts in Thailand

Chigger	No. host individuals infested	Species specificity index
Ascoschoengastia indica	112	0.304
Blankaartia acuscutellaris	24	0.542
Gahrliepia elbeli	6	0.542
Gahrliepia fletcheri	4	1
Gahrliepia sp., cf. *orientalis*	2	0.685
Gahrliepia xiaowoi	3	0.685
Helenicula kohlsi	12	0.391
Helenicula naresuani (n. sp.)	2	0.685
Helenicula simena	8	0.542
Helenicula pilosa	11	0.542
Leptotrombidium deliense	80	0.216
Leptotrombidium elisbergi	1	1
Leptotrombidium imphalum	8	0.453
Leptotrombidium macacum	1	1
Leptotrombidium sialkotense	1	1
Leptotrombidium sp., cf. *guzhangense*	5	1
Leptotrombidium sp., cf. *maccacum*	1	1
Leptotrombidium subangulare	1	1
Leptotrombidium tenompaki	6	0.685
Leptotrombidium turdicola	7	0.542
Leptotrombidium yunlingense	1	1
Lorillatum hekouensis	1	1
Microtrombiula munda	3	1
Schoengastia propria	3	1
Schoengastiella ligula	18	0.542
Schoutedenihia centralkwangtunga	2	1
Trombiculindus kosapani (n.sp.)	2	1
Trombiculindus paniculatum	1	1
Trombiculindus variaculum	1	1
Walchia chavali (n.sp.)	9	0.542
Walchia dismina	13	0.342
Walchia kritochaeta	45	0.193
Walchia lupella	49	0.391
Walchia micropelta	79	0.171
Walchia minuscuta	48	0.171
Walchia pingue	59	0.304
Walchia rustica	23	0.342
Walchia ventralis	1	1

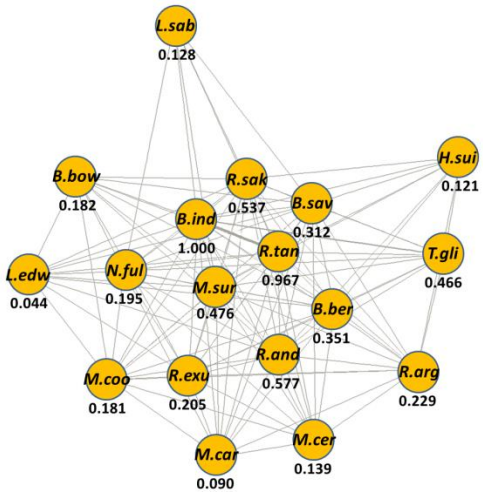

Figure 4.7 Unipartite network graph and Eigenvector centrality scores illustrate pattern of chigger sharing among 18 small mammal hosts in Thailand.

4.3.2.2 Network analysis in population level

In each studied site, bipartite network properties of host-chigger interaction including NODF, network connectance, links per species and network modularity were computed and are shown in Table 4.3. These network parameters allow us to differently investigate the structure of host-chigger interactions in the 13 study sites in Thailand. Among the sites, the highest NODF and connectance were found in the Nakhonsawan network, where chigger species richness was as low as four species; meanwhile the Chiangrai network showed rather high chigger species richness (12 species), but was the lowest in NODF and connectance. In contrast, Chiangrai showed the highest modularity within the network, whereas the least network modularity was found in Prachuab Kirikhan.

After testing the relationship among network parameters, there was a significant negative correlation between chigger species richness and connectance (Spearman rank correlation = 558.91, $p < 0.0001$). The lower the chigger species

richness in a network, the higher the level of host-chigger network connectance (proportion of realized interactions) (Figure 4.8A). In addition, network modularity showed a significant negative correlation with NODF (Spearman rank correlation = 462, p = 0.037); that is, when nestedness decreased the network tended to increase its modularity (becoming highly structured in compartments) (Figure 4.8B).

Table 4.3 Bipartite network parameters of host-chigger interaction in the 13 studied sites in Thailand (Abbreviation: CSR = Chigger species richness; NODF = Nestedness metric based on overlap and decreasing fill)

Location	No. host examined	No. host infested	CSR	NODF	Connectance	Links per species	Modularity
Buriram	131	25	5	32.97	0.296	1.233	0.468
Chantaburi	76	35	4	15.16	0.271	0.974	0.263
Chiangrai	70	20	12	14.97	0.129	0.968	0.646
Kalasin	185	29	11	30.61	0.175	1.4	0.456
Kanchanaburi	214	13	6	16.66	0.231	0.947	0.617
Loei	206	46	10	32.81	0.176	1.446	0.372
Nakhonsawan	87	42	4	56.18	0.416	1.521	0.319
Nan (Bokleu)	20	19	20	22.36	0.131	1.282	0.536
Nan (Pua)	138	32	12	22.09	0.158	1.386	0.492
Nan (Tha Wang Pha)	25	10	10	25.1	0.21	1.05	0.517
Prachuap Khirikhan	130	39	9	44.82	0.199	1.458	0.249
Songkhla	76	49	7	40.74	0.259	1.589	0.293
Tak	37	16	11	24.9	0.176	1.148	0.553

Additionally, we present a unipartite network plot based on individual hosts' data in order to illustrate architectural patterns of host-chigger interaction in each of the studied sites (Figure 4.9A-4.9K). Some networks showed a clustering pattern (high modularity) in the population; *e.g.*, the networks of Chiangrai, Kanchanaburi and Nan-Bo Kleu (Figure 4.9B, 4.9D, 4.9F), whereas some others showed a rather uniform network structure (lower modularity); *e.g.*, the networks of Prachuab Kirikhan and Songkhla (Figure 4.9I, 4.9J).

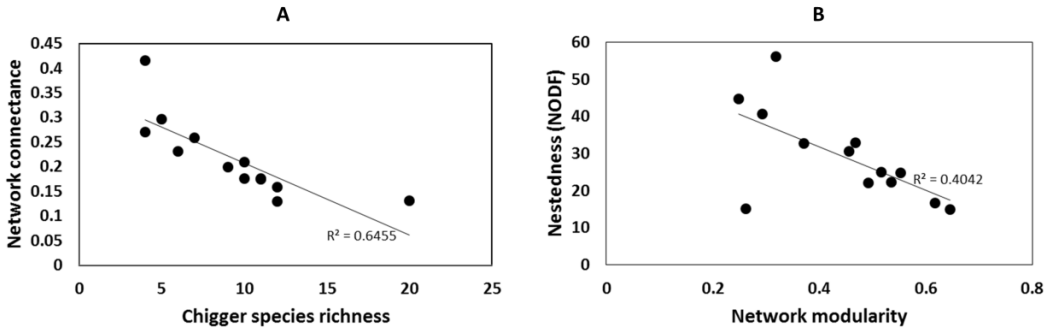

Figure 4.8 Correlation plots showing relationship between: chigger species richness and network connectance (A); network modularity and nestedness NODF (B)

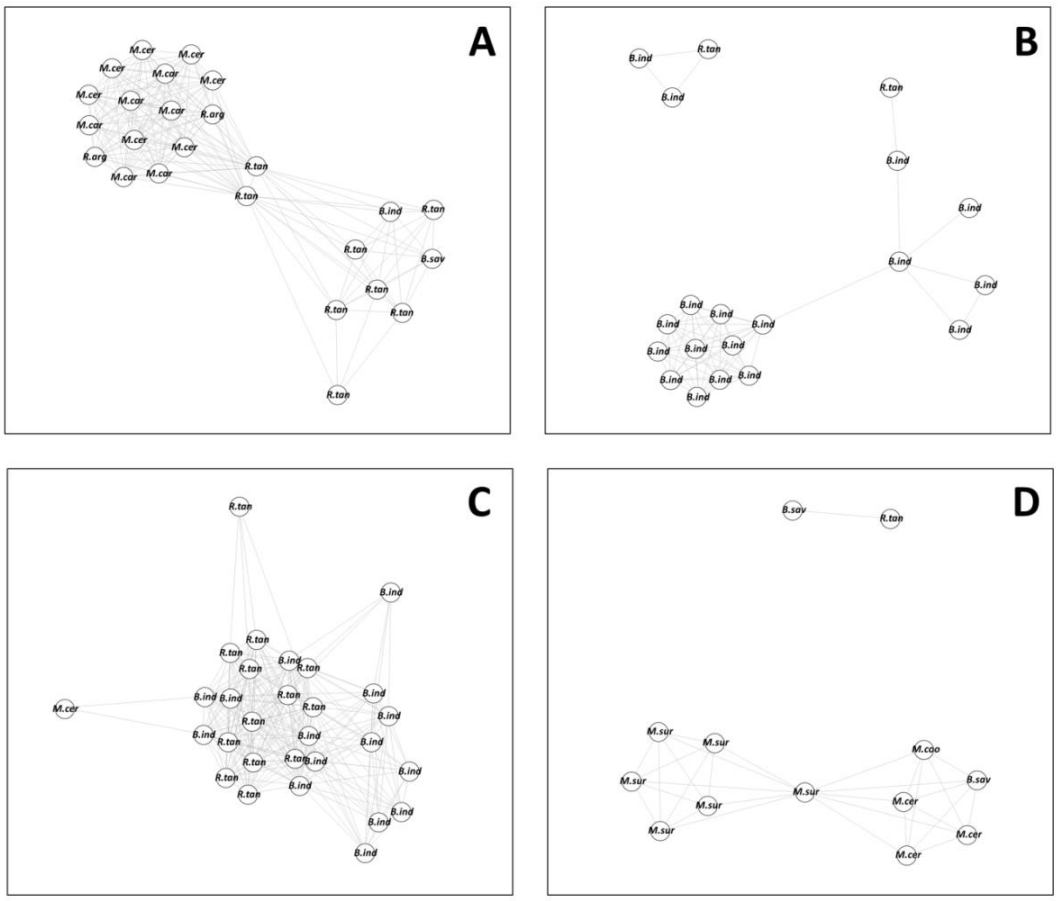

Figure 4.9 Unipartite network graphs illustrate pattern of host-chigger community (individual host level) in the 11 studied sites in Thailand: Buriram (A), Chiangrai (B), Kalasin (C) and Kanchanaburi (D). Only the sites where hosts infested with ≥ 5 chigger species are included.

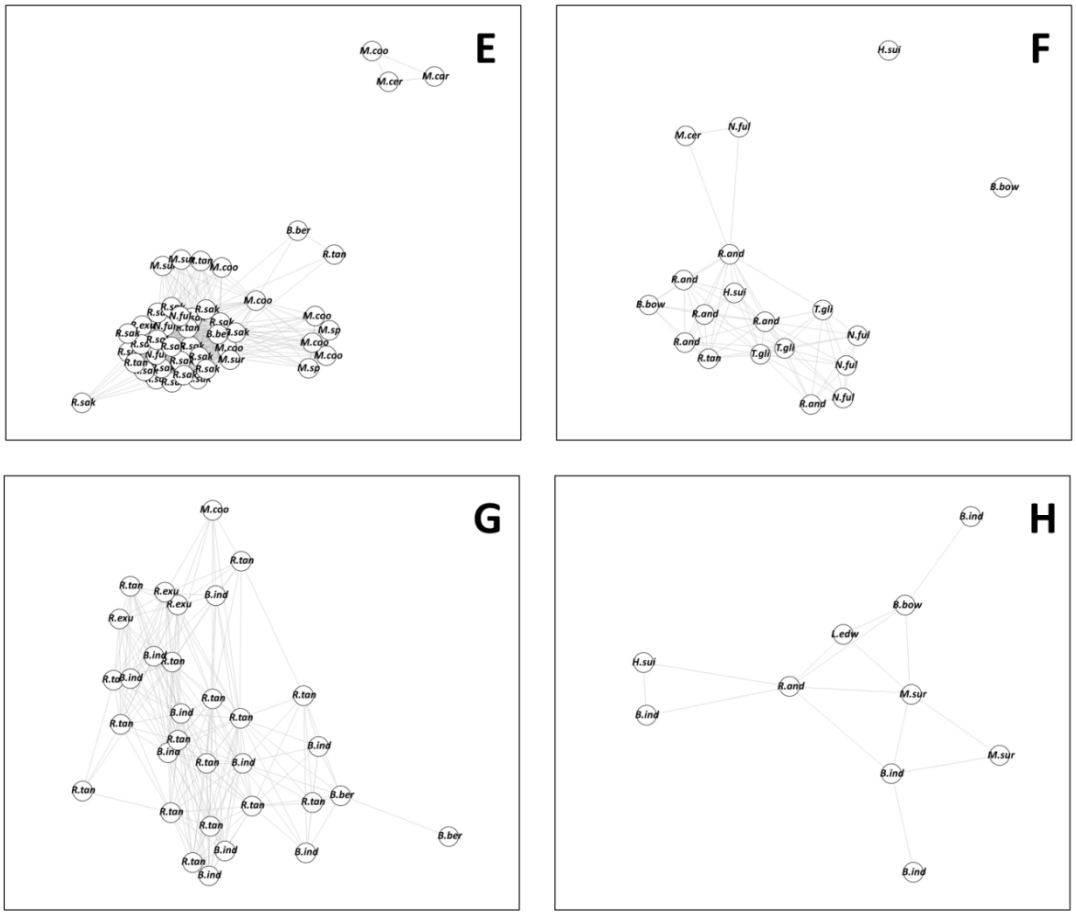

Figure 4.9 (continued) Loei (E), Nan-Bo Kleu (F), Nan-Pua (G) and Nan-Tha Wang Pha (H)

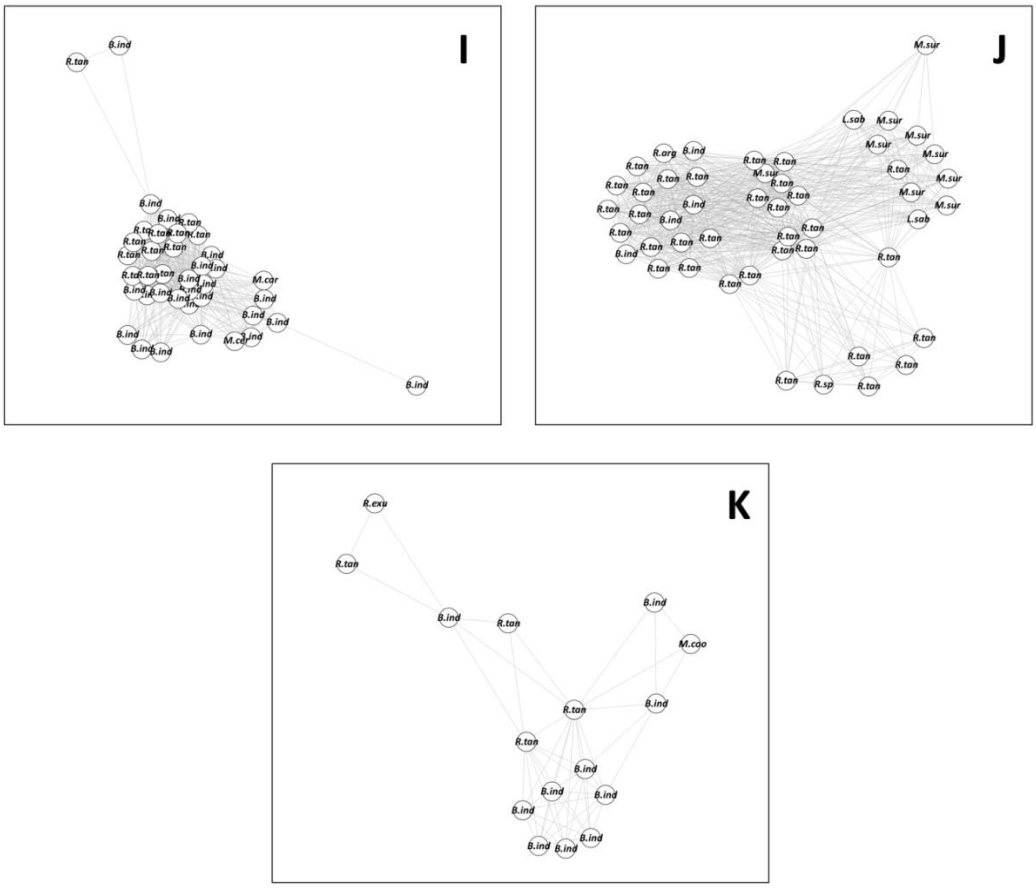

Figure 4.9 (continued) Prachub Kirikhan (I), Songkhla (J) and Tak (K)

Table 4.4 Comparison of the generalized linear models (GLM) testing the effect of various independent variables on individual chigger species richness (GLM with Poisson distribution). Selection of the models was done using Akaike's Information Criterion corrected for sample size (AICc). Only the first 10 models are showed. The initial model for AICc selection was Chigger species richness ~ Rodent Species + Sex + Maturity + Weight + Site + Habitat + Season. Abbreviations: *K* = the number of estimated variables, Log-like = maximized value of the logarithm of the likelihood function, ΔAICc = the difference between AICc value of a given model and the model with minimum AICc and W_r = Akaike weights. Analysis of Deviance (ANOVA type II test) significant level (* <0.05, ** < 0.01, *** <0.001).

Model	Dependent variable ~ Independent variables	*K*	Log-like	AICc	ΔAICc	W_r
1	**Chigger species richness ~ Rodent Species*** + Maturity* + Site*** + Habitat***	**5**	**-1302.503**	**2683.754**	**0**	**0.32932**
2	Chigger species richness ~ Rodent Species*** + Maturity* + Site*** + Habitat*** + Body mass	6	-1302.291	2685.476	1.722	0.13921
3	Chigger species richness ~ Rodent Species*** + Maturity* + Site*** + Habitat*** + Season	6	-1302.446	2685.786	2.032	0.11920
4	Chigger species richness ~ Rodent Species*** + Sex + Maturity* + Site*** + Habitat***	6	-1302.483	2685.86	2.106	0.11487
5	Chigger species richness ~ Rodent Species*** + Maturity* + Site*** + Habitat*** + Season + Body mass	7	-1302.228	2687.502	3.748	0.05054
6	Chigger species richness ~ Rodent Species*** + Sex + Maturity* + Site*** + Habitat*** + Body mass	7	-1302.271	2687.588	3.834	0.04842
7	Chigger species richness ~ Rodent Species*** + Sex + Maturity* + Site*** + Habitat*** + Season	7	-1302.425	2687.896	4.142	0.04150
8	Chigger species richness ~ Rodent Species*** + Site*** + Habitat*** + Body mass	5	-1304.674	2688.094	4.34	0.03759
9	Chigger species richness ~ Rodent Species*** + Site*** + Habitat***	4	-1305.758	2688.119	4.365	0.03712
10	Chigger species richness ~ Rodent Species*** + Sex + Maturity* + Site*** + Habitat*** + Season + Body mass	8	-1302.208	2689.618	5.864	0.01755

Table 4.5 Generalized linear models of individual chigger species richness (with Poisson distribution). The best fit models were selected, and Analysis of Deviance Table (Type II tests) on the explicative independent variables was given. Only significant values are included. VIF = Variance Inflation Factor

Dependent variable	Explicative variables	Category	Log ratio Chi-square (df, P-value)	Estimate	Std. Error	P-value	VIF
Individual chigger species richness	**Rodent Species**		**273.900 (21, < 0.0001)**				1.051
	B. indica vs	*B. savilei*		-0.916	0.46	0.046	
		B. bowersi		-1.825	0.73	0.013	
		M. caroli		-1.726	0.34	< 0.0001	
		M. cervicolor		-2.159	0.27	< 0.0001	
		M. cookii		-1.445	0.34	< 0.0001	
		N. fulvescens		-1.172	0.42	0.005	
		R. exulans		-2.509	0.42	< 0.0001	
		T. glis		1.069	0.43	0.014	
	Maturity		**8.179 (1, 0.004)**				1.052
	Adult vs	Juvenile		-0.283	0.10	0.004	
	Site		**50.443 (11, < 0.0001)**				1.203
	Buriram vs	Chantaburi		-0.702	0.32	0.033	
		Nan (Bo Kleu)		1.006	0.39	0.010	
		Nan (Tha Wang Pha)		0.711	0.31	0.023	
		Songkhla		0.456	0.21	0.035	
	Habitat		**55.420 (3, < 0.0001)**				1.159
	Forest vs	Settlement		-1.074	0.17	< 0.0001	

4.3.3 Independent variables explaining chigger species richness and scrub typhus epidemiology in Thailand

4.3.3.1 Explicative variables for chigger species richness at the host individual level

For AICc model selection, seven independent variables derived from either small mammal host traits or environmental data (rodent species, sex, maturity, body mass, site, habitat, and season) were used in the initial (global) model determining chigger species richness at the individual host level. After 150 models fitted by AICc, the top 10 best selected models with relevant parameters are presented in Table 4.4.

Rodent species, site and habitat were the three variables which appeared in all of those 10 models, which implies they have a strong influential effect explaining chigger species richness. After examining the computed parameters (AICc, ΔAICc and Wr) and MaIT plot, the first model (Chigger species richness ~ Rodent species + Maturity + Site + Habitat) was picked for further discussion (Table 4.5), as the model showed a combination of lowest AICc score, an acceptable ΔAICc and Wr difference gap between the model itself and the runner-up, and all four independent variables passed the default 80% threshold cut-off in MaIT plot (Figure 4.10A). In addition, calculation of the variance inflation factor (VIF) gave a score lower than 10 (common cut-off) for all of the variables (VIF: Rodent species = 1.051; Maturity = 1.052; Site = 1.203; Habitat = 1.159), which means there is no serious multicollinearity effect produced among the variables.

Bandicota indica was infested with significantly higher chigger species richness than the three *Mus* species (log odd estimate values less than 0); *i.e., M. caroli* (estimate = -1.726, $p < 0.0001$), *M. cervicolor* (estimate = -2.159, $p < 0.0001$) and *M. cookii* (estimate = -1.445, $p < 0.0001$); and also exceeded that of the other hosts in different genera: *Berylmys bowersi* (estimate = -1.825, $p = 0.013$), *Niviventer fulvescens* (estimate = -1.172, $p = 0.005$) and *Rattus exulans* (estimate = -2.509, $p < 0.0001$). The chigger species richness was not quite as high (but still significantly higher than rodents from other genera) in *B. savilei* (estimate = -0.916, $p = 0.046$). Only a non-rodent host, *Tupaia glis* (Scandentia: Tupaiidae) harboured a significantly higher chigger species richness than *B. indica* (log odd estimate value more than 0; *i.e.,* estimate = 1.069, $p = 0.014$), (Table 4.5). In terms of host attributes, adult hosts harboured a significantly greater chigger species richness than juveniles (estimate = -0.283, $p = 0.004$). However, there was no significant effect of gender; chigger species richness was not significantly different between male and female hosts.

In terms of impact of the environment, there were significant differences in chigger species richness between the studied sites. Also, we found again that chigger species richness was affected by habitat; animals captured in forest demonstrated significantly higher chigger species richness than those animals from human settlement habitats (estimate = -1.074, $p < 0.0001$), (Table 4.5).

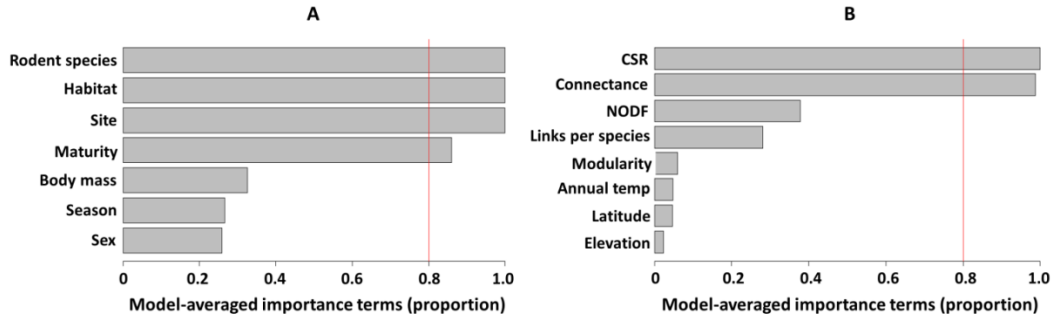

Figure 4.10 Model-averaged importance terms of independent variables used to explain: (A) chigger species richness and (B) scrub typhus human cases.

4.3.3.2 Explicative variables for human scrub typhus epidemiology in Thailand

Data on human scrub typhus cases were extracted at district level for each of the 13 studied sites (unpublished data; Bureau of Epidemiology, Ministry of Public Health Thailand). These human case data were taken from the same year in which each small mammal field survey was conducted, aiming to get as precise contemporary data as possible for epidemiological analysis. In addition, some environmental factors related to each site (latitude, elevation and annual mean temperature) were derived from the CERoPath project (Table 4.6). At the population (site) level, those environmental parameters in Table 4.6 and host-chigger network properties (NODF, connectance, links per species and modularity) in Table 4.3 were included as candidate variables in an initial (global) model to predict human scrub typhus case number. Here, we took host-chigger network parameters into account, because small mammals are important maintenance hosts for chiggers. Thus, our hypothesis was that the pattern of host-chigger interaction within the same population where humans can be accidental hosts could potentially contribute as explicative variables for scrub typhus epidemiology.

After 250 models were fitted by AICc, the top 10 best selected models with relevant parameters are presented in Table 4.7. From our data, chigger species richness and network connectance strongly influenced scrub typhus case number in the studied sites, as the two variables appeared in all top 10 selected models. Model 1 (Scrub typhus human case number ~ Chigger species richness + NODF + Network connectance) and Model 2 (Scrub typhus human case number ~ Chigger species richness + Network connectance) were apparently almost equivalent, with only slight differences in ΔAICc and *Wr* between the two models. Chigger species richness and network connectance passed the MaIT threshold, but not NODF (Figure 4.10B). Thus, we decided to pick Model 2 for further discussion, as including NODF does not much improve model performance. There was not a serious multicollinearity effect, as VIF of the two variables was lower than the threshold cut-off (VIF: Chigger species richness = 4.051 and Network connectance = 4.234).

In addition to the GLM, univariate analysis was also conducted and we found again that scrub typhus case number positively correlated with chigger species richness (Spearman rank correlation = 45.71, p = 0.0006) and negatively correlated with host-chigger network connectance (Spearman's rank correlation = 485.45, p = 0.011), (Figure 4.11).

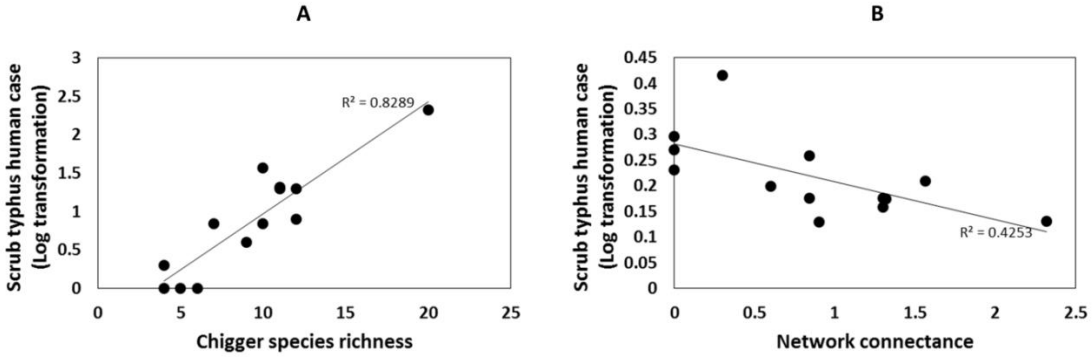

Figure 4.11 Correlation plots showing relationship between scrub typhus human case and chigger species richness (A); network connectance (B).

Table 4.6 Human scrub typhus case number and environmental information at the district level for 13 studied sites in Thailand

Location	District	Latitude	Elevation (m)	Annual mean temp (°c)	Scrub typhus case number (year)
Buriram	Muang	14.90311	141.9	25	1 (2009)
Chantaburi	Laem Singh	12.50766	25.73	26	1 (2015)
Chiangrai	Wiang Chai	19.88956	111.7	30	8 (2011)
Kalasin	Sahatsakhan	16.29887	279.5	27	21 (2010)
Kanchanaburi	Sai Yok	14.01667	279.5	26	1 (2011)
Loei	Muang	17.45114	56.26	28	7 (2009)
Nakornsawan	Tak Fah	15.34976	Na	Na	2 (2013)
Nan	Bo Kleu	19.14333	447.3	20	209 (2014)
Nan	Pua	19.12545	144	26	20 (2010)
Nan	Tha Wang Pha	19.13926	176.3	27	37 (2013)
Prachuap Khirikhan	Muang	11.76527	111.7	26	4 (2012)
Songkhla	Hat Yai	7.00201	62.9	28	7 (2013)
Tak	Mae Sot	16.80552	371.6	23	20 (2013)

Table 4.7 Comparison of the general linear models (GLM) to test the effect of various independent variables to scrub typhus human case number (GLM with Poisson distribution). Selection of the models was done using Akaike's Information Criterion corrected for sample size (AICc). Only the top 10 models are shown. The initial model for AICc selection was Scrub Typhus Human Case Number ~ CSR + Latitude + Elevation + Annual Mean Temp + Network Modularity + NODF + Links per species + Network Connectance. Abbreviations: *K* = the number of estimated variables, Log-like = maximized value of the logarithm of the likelihood function, ΔAICc = the difference between AICc value of a given model and the model with minimum AICc, W_r = Akaike weights, CSR = Chigger Species Richness, NODF = Nestedness. Analysis of Deviance (ANOVA type II test) significant level (* <0.05, ** < 0.01, *** <0.001).

Model	Dependent variable ~ Independent variables	*K*	Log-like	AICc	ΔAICc	W_r
1	Scrub typhus human case number ~ CSR*** + NODF* + Network Connectance***	4	-47.289	114.578	0	0.3244
2	**Scrub typhus human case number ~ CSR*** + Network Connectance*** **	**3**	**-50.626**	**114.966**	**0.388**	**0.2672**
3	Scrub typhus human case number ~ CSR*** + Links per species* + Network Connectance***	4	-47.639	115.279	0.701	0.2285
4	Scrub typhus human case number ~ CSR*** + Annual Mean Temperature +Network Connectance**	4	-49.775	119.55	4.972	0.027
5	Scrub typhus human case number ~ CSR*** + Network Modularity + Links per species** + Network Connectance***	5	-45.43	119.661	5.083	0.0255
6	Scrub typhus human case number ~ CSR*** + Network Modularity + NODF** + Network Connectance***	5	-45.679	120.159	5.581	0.0199
7	Scrub typhus human case number ~ CSR*** + Latitude + NODF** + Network Connectance***	5	-45.78	120.36	5.782	0.0181
8	Scrub typhus human case number ~ CSR*** + Elevation + Network Connectance***	4	-50.349	120.699	6.121	0.0152
9	Scrub typhus human case number ~ CSR*** + Latitude + Links per species** +Network Connectance***	5	-46.006	120.12	5.542	0.0143
10	Scrub typhus human case number ~ CSR*** + Network Modularity + Netwrok Connectance***	4	-50.565	121.13	6.552	0.0122

4.4 Discussion

4.4.1 Chigger diversity in different habitats, season, site and host attributes

In intensive field surveys conducted by the CERoPath project in 13 sites across Thailand, high chigger diversity was discovered (38 species discovered on 18 small mammal species in different habitats and seasons), yielding over one-third of the diversity reported in the revised chigger species checklist of Thailand (Chaisiri et al. 2016).

Chigger species richness differed among different geographical locations in Thailand; for example hosts from northern (Chiangrai and Nan) and western (Tak) provinces, where the sites include mountain ranges and higher forest density, tend to have the greatest chigger diversity compared to hosts from the eastern and central sites (Chantaburi and Nakhonsawan), which are mainly flatland with less vegetation. Accordingly, parasite diversity (chiggers in this case) could be mainly determined with regard to biogeographical conditions that change with different sites or regions (Morand 2015).

Of the biogeographical factors, latitudinal gradients have been hypothesized as the major determinant factor for parasite diversity because key bioclimatic conditions change with latitude; *i.e.,* mean temperature, humidity and rainfall (Lindenfors et al. 2007; Bordes et al. 2010; Morand 2015). Parasite species richness is expected to follow the hypothesis that free-living host species are more

diversified at the lower latitude, closer to equator. Some evidence for this trend has been reported, for example in parasites and infectious disease of humans (Guernier et al. 2004), protozoans of primate species (Nunn et al. 2005), and microparasites (bacteria and viruses) of wild rodents (Bordes et al. 2011). But in contrast, our findings showed the opposite trend, and are in agreement with the result of other macroparasite studies: flea species richness on rodent hosts increased with an increase in latitude (Krasnov et al. 2004) and helminth diversity of carnivores increased with distance from the equator line (Lindenfors et al. 2007). Interestingly, chigger diversity in China showed a somewhat similar pattern with the present study in Thailand, as chigger diversity in Yunnan province increased gradually from the lower latitudes (21 - 24^{o}N) until reaching a peak at around 25 - 26^{o}N and then decreased (Peng et al. 2016). Perhaps because our study was conducted at lower latitudes (7 - 19^{o}N), the trend of chigger diversity continually increased. While Peng et al. (2016) reported strikingly high chigger diversity; *i.e*, 274 species in small mammal hosts from Yunnan province, the diversity of chiggers in the same host group gradually decreases in more southery countries such as Thailand and Malaysia, with 79 and 65 species, respectively (see CHAPTER 3; Nadchatram 1970; Mohd Zain et al. 2015; Chaisiri et al. 2016). Thus, the results of these studies imply that chiggers are more diversified in temperate or sub-temperate than tropical zones. This pattern of increasing parasite diversity in upper latitudes still does not have a clear explanation. However, Morand (2015) provided a hypothesis linking geographical range of hosts and their parasite species richness. The size of host geographical range positively correlates with parasite species richness, because large geographical ranges offer a higher probability for a host to be infected by

many parasite species (Morand 2000). Accordingly, host species living at higher latitudes may have larger geographical distribution ranges, which may facilitate the accumulation of several parasite species compared with hosts that have smaller geographical distributions at lower latitudes. In addition, the effect of host population density on parasite species richness cannot be ruled out, and needs to be investigated in future studies.

In term of habitat, chigger species richness decreased gradually from forest, agricultural lands to human settlement habitats, suggesting that urbanization has a negative effect on chigger diversity. This finding is similar to that of McKinney (2008), and confirms the trend of diversity loss of both invertebrates and vertebrates when urbanization has taken place. Urbanization and deforestation for agricultural use result in a high rate of habitat fragmentation, which affects ecological processes such as animal movement patterns, plant dispersal, community dynamics, and reproduction of animal and plants as well as nutritional flows in food webs (Andreassen et al. 1998; Dooley & Bowers 1998; Collinge 2000). These changes potentially act as perturbations within a community, and may lead directly or indirectly to species loss in the community.

At the whole country level, hosts in dry season were clearly infested with higher chigger species richness than those captured in wet season. Seasonal distributions of chiggers, temperature and precipitation have been previously recognised as important factors determining chigger activity in temperate and tropical zones (Sasa 1961). Nevertheless, there is no clear pattern of seasonal occurrence in trombiculid mite species; some species being highly abundant in

summer whereas others appear in the autumn-winter period (Frances et al. 1999; Traub & Wisseman 1968; Kim et al. 2010; Lee et al. 2012). With respect to the findings presented here, we initially discovered that more chigger species infested small mammals during the dry period than in the rainy season. The majority of chigger species populations in Thailand are probably more active during the dry period (Lerdthusnee et al. 2008), as wet conditions may inhibit or delay activity in some chigger species. Environmental factors related to seasonal change; *e.g.*, temperature, rainfall, humidity or soil condition could play a crucial role in shaping chigger behaviour, reproduction and population dynamics. Although there was an obvious difference between the two seasons regarding the results in the present study, a seasonal effect was not selected to explain chigger diversity in the multivariate GLM analysis. Our field studies were not designed to control the sampling strategy of the two seasons within the same studied site. Thus, additional data collection from the field is needed in order to answer the research questions on seasonal effects on chigger species richness.

Regarding to GLM analysis, chigger species richness could be strongly shaped by host intrinsic factors (*i.e.,* host species and maturity) and extrinsic factors (*i.e.,* habitat and geographical location). The effect of different host species on parasite species richness has been well documented, as it is a primary and intuitive observation unit to study variation of parasite species richness (Krasnov 2008; Poulin 2014). The variation could be due to various host traits of different taxa such as body mass, diet range, metabolic rate, immunogenetics, longevity or geographical distribution size (Nunn et al. 2003; Lindenfors et al. 2007; De Bellocq

et al. 2008; Kamiya et al. 2014; Morand 2015). Effects of maturity could be due to the fact that adult hosts live longer and roam further searching for food; therefore there is the opportunity for increased exposure to chigger foci in the environment compared to juvenile animals. Another consideration is that adults have a larger body mass than juveniles, thereby providing a larger surface area for different chigger species to feed on. This possibility has previously been suggested for fleas and their hosts (Krasnov 2008).

Host environmental factors could play important role in chigger species richness on small mammal hosts in Thailand. Unlike endoparasites which live inside their host, and whose species composition and richness tends to vary due to internal host factors (*e.g.*, development of immunity; competition for nutrients), arthropod ectoparasites live outside on the fur or on the skin of the host's body. As such, the species richness of ectoparasites is affected not only by host factors but also by off-host environmental conditions: landscape habitat and climate in particular also have an important impact (Krasnov 2008; Morand 2015). The ectoparasites in our study, the chigger larvae, were found mainly in the ear and occasionally on other parts of the host body. They have a short association with their host, as they feed once in their larval life remaining attached for just a few days to do so, before leaving to develop into the free-living stages in environment (Shatrov 1992). This suggests that chigger species richness may be rather influenced more by host environmental parameters than by host traits, in accordance with what was previously found in chiggers from China (Zhan et al. 2013) and in the case of the flea study by Krasnov et al. (2004). However, host intrinsic factors cannot be

ruled out, particularly host immune responses to ectoparasite infestation, as chiggers attach to the host skin for many days while feeding on host tissue. Studies of the effects of host immunity on chigger infestation at a population or community level would constitute a valuable contribution to further research on the ecology of chigger parasitism.

4.4.2 Host-chigger interaction through ecological network analyses

4.4.2.1 host-chigger interaction at community level

At the whole country level, bipartite network analysis exhibited complex interactions between host and chigger species. A network parameter, nestedness, was computed. Nestedness has become a parameter of choice now widely used to report species co-occurrence or species distribution patterns and network of species interactions in ecological studies (Almeida-Neto et al. 2008; Krasnov 2008; McQuaid & Britton 2013). To date, contradictory results on nested patterns have been reported in ecological network studies, particularly in host-parasite communities (Krasnov 2008). The definition of nestedness given by Wright & Reeves (1992) is the pattern that "the sites inhabited by fewer species tend to be subsets of the biotas of richer sites". In our case, the hosts infested by fewer chigger species are subsets of the species assemblages of the hosts infested by richer chigger species. Our result showed a nestedness NODF = 45.17, which reflects a moderate level of chigger species nestedness pattern among the host species in the community (Figure 4.6A). In other words, this can be interpreted as the bipartite matrix revealing 45.17% co-occurrence of specialist species, with chiggers in the

right-hand columns and hosts in the lower rows overlapping with the generalist species (chiggers in leftmost columns and hosts in the upper rows, thus following (Ulrich et al. 2009).

There was a trend of segregation of chigger species assemblages between murine rodents and the two non-rodent small mammals, *Hylomys suillus* (Erinaceomorpha: Erinaceidae) and *Tupaia glis* (Scandentia: Tupaiidae) (Figure 4.6A). Perhaps this could be explained by differences in their biology; *e.g.* activity patterns (nocturnal in murid rodents and *H. suillus* but diurnal in *T. glis*), foraging habitat or shelter type.

Most of the chigger species found in the present study parasitized a wide host range and showed a low species-specificity index (Figure 4.6B and Table 4.2). This confirms the low host-specificity of chigger feeding as previously reported (Shatrov & Kudryashova 2008; Zhang et al. 2013; Peng et al. 2015). This evidence of low host-specificity is important with respect to scrub typhus epidemiology, since chiggers may perhaps attach to and feed on any animals that occupy or pass through their territory, including humans.

Unipartite network of chigger species co-occurrence among the hosts is presented in Figure 4.7. This analysis allows computing of Eigenvector centrality, a mathematic vector in linear transformation that characterizes the whole projection of vertices (corners or points where lines meet) in a network graph (Borgatti 2005). In a general sense and for our application, this method computes the centrality of a node (host) as a function of the centralities of the other nearby nodes (other hosts).

The higher centrality score of a node, the more connections to the other nodes regarding parasite sharing. This concept can be applied to determine the role of each host species sharing their parasites within a network (Morand, McIntyre, et al. 2014; Pilosof et al. 2015). Similar to previous findings using the bipartite matrix, *B. indica* and *R. tanezumi* appear to be the most central hosts in the network (Figure 4.7), suggesting that the two rodent species play a greater role in host-chigger interaction as well as chigger distribution in the community.

Although *Bandicota indica* is reported as a rodent species with specialized habitat preferences, and is fairly prevalent in rain-fed lowland fields (Herbreteau et al. 2005; Palmeirim et al. 2014; Blasdell et al. 2015), this particular rodent is recognised as a main reservoir host for several pathogens and parasites in Southeast Asia. These include hantavirus, herpes viruses, *Bartonella* spp.*, Leptospira* spp.*, Orientia tsutsugamushi, Babesia* spp., *Toxoplasma gondii, Trypanosoma* spp., several intestinal helminths and various ectoparasites including lice, ticks, and mites (including species other than chiggers) (Tangkanakul et al. 2000; Hugot et al. 2006; Jittapalapong et al. 2010; Herbreteau et al. 2012; Chaisiri, Siribat, et al. 2015; Klangthong et al. 2015; Chaisiri et al. 2016). This reflects the possibility of substantial horizontal transmission between other hosts and *B. indica* to reach such high parasite diversity in this habitat type. For example, horizontal transmission of *O. tsutsugamushi* from one host to another probably occurs less frequently compared with transovarial and transstadial transmission within the mite (Phasomkusolsil et al. 2009; Kumar et al. 2010). One possibility is that chiggers might drop off a host due to movement or the grooming process, and then switch

feeding to a new host, which potentially could allow cross-transmission between hosts. In addition, bacterial transmission between mites might occur during co-feeding of chiggers on a host (Frances et al. 2000), which could be hypothesized as one of the horizontal transmission routes. However, the precise mechanism needs to be clarified in further studies.

Rattus tanezumi was also found as another very central host in the network. This rat has been reported as a habitat generalist species (low habitat preference) which occurs in all types of habitat (Palmeirim et al. 2014; Blasdell et al. 2015). Because the species has adapted well to the changing environment of Southeast Asia (Morand et al. 2015), *R. tanezumi* may potentially act as a "bridge species" linking host-parasite communities, including chiggers, from forest to agricultural land and human household settings.

4.4.2.2 host-chigger interaction at population level

Bipartite network properties of host-chigger interaction including NODF, network connectance, links per species and network modularity were computed in each studied site. Our result found that chigger species richness was negatively correlated with network connectance; lower chigger species richness in the network tended to have higher level of host-chigger network connectance (proportion of realized interactions). This pattern has been reported previously in studies on flea-mammal networks, which means the diversity (phylogenetic effect) might influence in some way their interactions (Mouillot et al. 2008; Poisot et al. 2013).

In addition, we also found a negative correlation between network modularity and nestedness; which means when nestedness decreased, the network tended to increase its modularity, becoming highly structured in compartments. Fortuna et al. (2010) studied the relationship between nestedness and modularity in plant-pollinator, plant-seed dispenser and host-parasite communities, and they found a significant correlation between the two network indices only in the plant-pollinator model. They found also that network connectance affected the correlation trend (switching from a positive to negative trend and vice versa) of nestedness and modularity. In communities with low connectance, the higher the apparent nestedness and the stronger the modularity (positive trend); whereas in the high connectance community the opposite was seen: the higher the nestedness, the lower the modularity (negative trend). Our result was in accordance with the latter trend, suggesting that host-chigger communities in Thailand may interact at a high level of connectance.

Unipartite network plots were generated in each studied site illustrating architectural patterns of host-chigger interaction. Focusing at nodes or individual hosts in the networks, again, we found evidence that the role of *R. tanezumi* is one of a central species (connector) in the network of Buriram, Songkhla and Tak (Figure 4.9A, 4.9J, 4.9K). This confirms and reinforces the importance of *R. tanezumi* in host-chigger interactions.

4.4.3 Effect of independent variables explaining scrub typhus epidemiology in Thailand

Human scrub typhus case numbers in Thailand were influenced by chigger species richness and host-chigger network connectance. Chigger species richness positively correlated with the disease case number, such that the higher the chigger species richness, the more human scrub typhus cases were registered in the area (Figure 4.11A). Chigger diversity has never been previously included in the scrub typhus epidemiological research field. Although a number of publications reported chigger diversity in their studies, none has been linked with human scrub typhus incidence within the areas (Lerdthusnee et al. 2008; Zhan et al. 2013; Lin et al. 2014). Thus, our finding appears to be the first time that a relationship between species richness of chigger vectors and scrub typhus incidence has been reported. An increase in chigger species richness perhaps increases the chance for human exposure to several chigger species, which may include vectors of the causative scrub typhus agent, *O. tsutsugamushi*.

Network connectance, another variable explaining occurrences of the disease, correlated negatively with scrub typhus case number. The region with a lower network connectance tends to exhibit a higher number of disease cases (Figure 4.11B). One of many possible associations to explain this relationship is that high interactions among small mammal species or individuals themselves regarding chigger sharing could divert or reduce the risk of human exposure in such a community to the vectors of the disease, acting like a “dilution effect”. This is similar to the concept of “zooprophylaxis”, the diversion of disease arthropod

vectors from human to domestic animals or wildlife for feeding (WHO 1982; Ault 1994), which could be applied as an optional strategy and ecological and epidemiological tool for disease vector control programmes (Saul 2003; Donnelly et al. 2015).

Alternatively, the influence of chigger species richness and network connectance to scrub typhus incidence could be explained if the sites where higher chigger species richness occurred have a greater diversity of small mammal hosts and less man-made environmental changes, or fewer humans present in that particular area. This might be another reason that humans are less likely to be exposed to the chigger vector of scrub typhus disease.

4.5 Conclusion

In summary, chigger diversity on small mammals in Thailand differed among rodent species, geographical location, latitude, habitat, and urbanization. There is a habitat preference found in some chigger species, which facilitates not only to understand more about their ecology but also enabling identification of suitable habitats which support potential scrub typhus disease vectors. Most chigger species infest a wide host range, showing low host specificity. This catholic feeding habit may increase the probability of people being bitten. In host-chigger network analysis, *B. indica* and *R. tanezumi* was identified as key host species that share and distribute chigger species to the other hosts in the community. Finally, chigger species richness and host-chigger network connectance were identified as the main variables to explain human scrub typhus incidence in Thailand.

CHAPTER 5

INVESTIGATION OF MICROBIOME PROFILE IN CHIGGERS USING A 16S RRNA AMPLICON SEQUENCING APPROACH

Chapter 5

Investigation of microbiome profile in chiggers using a 16S rRNA amplicon sequencing approach

5.1 Introduction

Scrub typhus is a neglected tropical disease that can cause severe fever and is potentially fatal if not treated promptly. The disease is caused by a bacterium, *Orientia tsutsugamushi*, which is transmitted by chiggers, the parasitic larval stage of trombiculid mites (Kelly et al. 2009; Santibáñez et al. 2015). The mites are also known to cause trombiculiasis, an itching dermatitis condition in humans and other animals due to the host's allergic response to the chiggers' saliva (Krantz & Walter 2009). To date, the known vectorial role of chiggers for bacteria is still largely limited to scrub typhus disease, as almost all studies were conducted using *Orientia*-specific detection (Chaisiri, McGarry, et al. 2015), whereas other bacteria have been largely ignored.

With the advent of modern molecular technology, the development of high throughput sequencing (HTS) has facilitated studies of the microbiome, allowing insights into the entire microorganism community from a particular sample (Turnbaugh et al. 2007; Hamady & Knight 2009). This allows bacteriologists to elucidate both culture-dependent and culture-independent bacteria in samples within days and within an affordable budget (Loman & Pallen 2015). HTS

technologies give effective and rapid generation of data by sequencing large numbers of samples in parallel.

Bacterial 16S ribosomal RNA (16S rRNA) gene amplicon sequencing has become a popular approach for microbiome studies, as it offers substantial advantages: it is fast, relatively inexpensive, reads are produced from a single gene target, and downstream analysis workflows are available and well standardized (Hamady & Knight 2009; Sanschagrin & Yergeau 2014). The 16S rRNA gene is a sequence of DNA that encodes the rRNA material of the small subunit of bacterial ribosomes in the cytoplasm. The gene is present in all bacteria, around 1,500 bp in length containing nine hypervariable regions (V1 – V9), which differ in discriminatory power for taxonomic classification (Chakravorty et al. 2007). With this characteristic, the gene has been recognized as the ideal molecular marker for taxonomic and phylogenetic studies, particularly the V4 region which has been identified as the most informative (Soergel et al. 2012; D'Amore et al. 2016).

A number of HTS bench-top sequencers for microbiome investigations are available on the market; *e.g.*, Illumina MiSeq and HiSeq, Life Technologies Ion Torrent and Pacific Bioscience RSII. The Illumina Miseq is currently the most popular platform for microbiome analyses of various sample types; *e.g.*, water, soil, arthropods as well as clinical samples of domestic animals and human (Kennedy et al. 2014; Rubin et al. 2014; Nelson et al. 2014; Cong et al. 2015; Jervis-Bardy et al. 2015; Zhou et al. 2016). Several advantages have been recognised regarding Illumina MiSeq performance, such as data quality, low error rate, long read length (2 x 300 bp), ease of 16S rRNA gene PCR preparation, and costs are manageable for

small- to medium-scale projects (Kozich et al. 2013; Nelson et al. 2014). Common principles of Illumina Miseq sequencing can be briefly outlined as follows: (1) DNA template library preparation involves the addition of MiSeq sequencing nucleotide adaptors to template fragments in order to immobilize the DNA templates onto solid surfaces of the sequencing flow cell; (2) sequencing reactions are performed under clonal amplification (PCR-based method) by DNA polymerase and ligase enzymes, generating batches of clonal amplicons; and (3) nucleotide base-calling is performed in a nucleotide-by-nucleotide stepwise fashion, leading to emission of fluorescence signals labelled on deoxynucleotide triphosphates (dNTPs) (Rizzo & Buck 2012; Illumina 2013; Loman & Pallen 2015).In light of the advantages above and as this platform is available at the in-house facility (Center for Genomic Research, University of Liverpool), the Illumina MiSeq was selected for use in the present study.

While deep sequencing of 16S rRNA gene amplicons is powerful for microbiome studies, there are important limitations to the technology which could result in microbiome profile distortion. Microbiome data producing from by HTS is unavoidably affected by contamination from laboratory procedures and molecular laboratory reagents (Salter et al. 2014). Accordingly, background controls from various stages of the experiment should be set up and sequenced alongside the samples to account for any contaminants or false-positive signals prior to data interpretation (Galan et al. 2016; Jousselin et al. 2016). In addition, positive controls such as an artificial microbial community (a “mock” community of known composition) becomes another recommended strategy used to reduce noise and

identify potential biases in microbiome studies (Misic et al. 2015; Tremblay et al. 2015; D'Amore et al. 2016), which have recently become commercially available from companies; *e.g.*, ZymoBIOMICS (Zymo Research 2016).

Recently, research on symbiotic bacteria in arthropods have gradually attracted more biologists' attention because these bacteria have been recognized as major players driving their hosts' traits, ecology and evolution (Moran et al. 2008; Ferrari & Vavre 2011). Thus, a microbiome investigation of chiggers will not only be useful to increase awareness of the potential for the mites to transmit disease pathogens, but the identification of associated symbionts may also facilitate deeper understanding of the symbiont-chigger relationship, leading to further development of intervention tools for vector control. Due to their small size and the limited taxonomic descriptions available, the study of the chigger microbiome is extremely challenging and few molecular protocols have been published. Therefore, we initiated a microbiome study of chiggers focusing on the 16S rRNA gene with the HTS approach.

The objectives of this thesis chapter are: (1) to investigate for the first time the microbiome profile of chiggers parasitizing small mammals from Thailand; (2) to identify potentially pathogenic bacteria and arthropod symbionts in addition to *O. tsutsugamushi*; (3) to compare diversity and community composition of the microbiome among different chigger species and between environmental conditions.

5.2 Materials and methods

5.2.1 Genomic DNA preparation of chigger samples

5.2.1.1 Chigger sample selection and process

As described in CHAPTER 3 regarding chigger identification, a subsample of 10 - 20% of the chiggers was selected from each small mammal host for identification. As reported in CHAPTER 3, hosts were infested with single, double or multiple chigger species. To ensure genetic material was obtained from the correct chigger species, only samples from single-species-infested hosts were selected for genomic preparation. The chigger specimens were microscopically checked again by mounting in ATL buffer (QIAGEN), confirming that the correct species materials were selected before continuing the further steps of DNA extraction.

Individual chiggers were picked from different species, habitats and sites in Thailand (see details of habitat and seasonal classification in CHAPTER 3). In addition, 50 individuals were pooled as representative of species, habitat (mixed species) and study site (mixed species). A pooled sample of 50 harvest mites, *Neotrombicula autumnalis*, collected on rodents from Preston Montford Field Studies Centre, Shrewsbury, UK, was included as an outgroup representing a different geographical region. The workflow diagram of sample selection for chigger genomic materials is presented in Figure 5.1.

Several studies applied surface sterilization of arthropod samples prior to DNA extraction (Ponnusamy et al. 2014; Sanders et al. 2014; Lawrence et al. 2015).

The sterilization step usually involves the usage of 0.1 - 0.5% bleach (sodium hypochlorite) to treat specimens, resulting not only in removal of contaminating bacteria on the surface, but potentially external symbiotic bacteria too. Hammer et al. (2015) reported that there was no significant effect of surface sterilization on community structure and the relative abundance of microbiome between sterile and non-sterile insects. On the other hand, too aggressive sterilization (perhaps due to damage of the arthropod's external structures caused by handling) could reduce the abundance of internal microbiota, leading to biases in microbiome composition between samples. Therefore, because chigger specimens are very small and delicate, and might harbour both external and internal symbionts, surface sterilization was omitted in this study.

5.2.1.2 DNA extraction and quantification

After species confirmation in ATL buffer, chigger individuals or pools were subsequently subjected to DNA extraction using the DNeasy Blood & Tissue Kit (QIAGEN). For mechanical disruption, the specimens were dried in 1.7 ml Eppendorf tubes and crushed with a sterile polypropylene pestle in ATL buffer and proteinase-K. The mixtures were incubated at 56^{o}C overnight (in a water bath or heating block; see below for the rationale for modifying the protocol) for enzymatic digestion, and then DNA preparation continued according to the manufacturer's protocol. The DNA was finally eluted in 30 µl of nuclease-free water and stored at -20^{o}C.

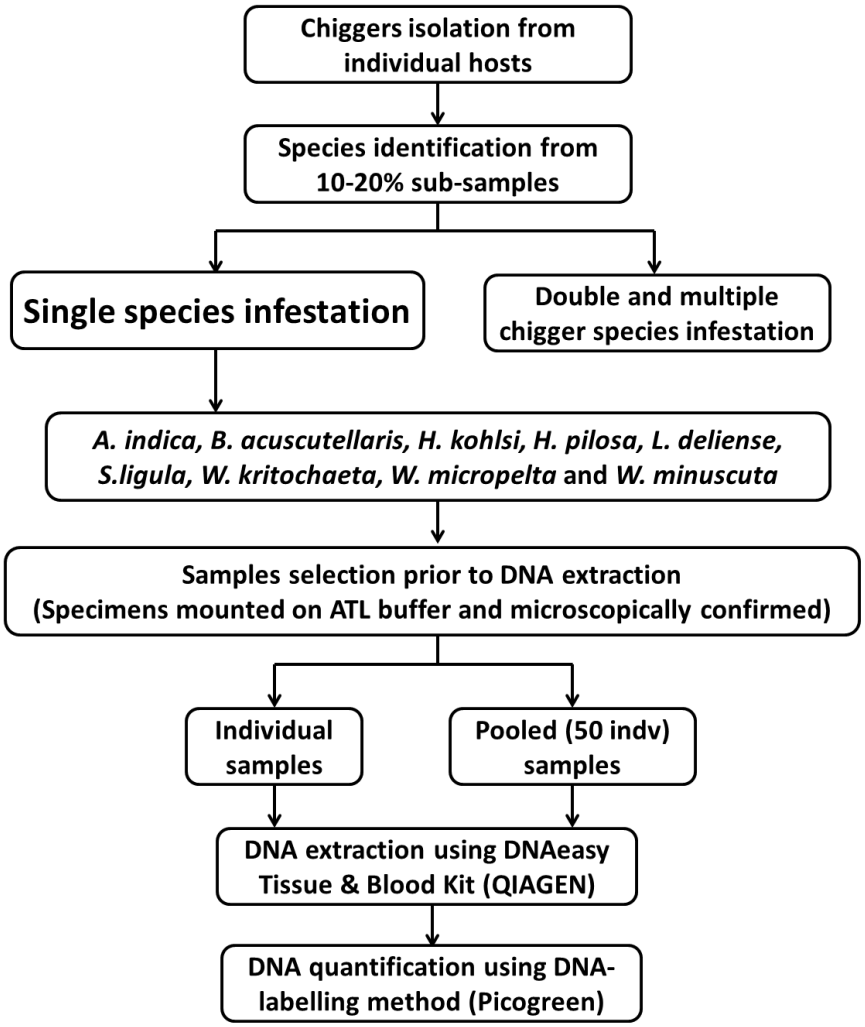

Figure 5.1 Schematic diagram shows selection of individual and pooled chigger samples for DNA extraction.

Soil samples were collected from two different provinces in Thailand, Chantaburi and Udonthani, during the CERoPath fieldtrip in 2015. In each site, the soil samples were collected from three different depths: surface, 25 cm and 50 cm. DNA from soil samples was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratory Inc.) following the manufacturer's protocol. In addition, some soil DNA samples from Lao PDR provided by Dr Sabine Dittrich (Oxford-Mahosot Wellcome Trust Unit in Laos) were included in the study.

All DNA samples were quantified using a fluorescent labelling method, the Quant-iT^{TM} Picogreen dsDNA kit (Invitrogen), and read in an Infinite F200 microplate fluorimeter with Magellan™ -Data Analysis Software (TECAN). DNA concentration was calculated by comparison to high-range (0 - 1,000 ng/ml) and low-range (0 - 25,000 pg/ml) DNA standard curves.

5.2.2 Library preparation and next generation sequencing of 16S rRNA gene for microbiome investigation

5.2.2.1 Dual index nested PCR amplification of 16S rRNA gene for Illumina MiSeq sequencing

To prepare the DNA library for microbiome sequencing, a 16S rRNA dual-index nested PCR protocol for MiSeq Illumina sequencing was applied (Caporaso et al. 2011; Kozich et al. 2013; Illumina 2013). The general concept of this library preparation method is that the first round PCR amplifies the V4 region of the 16S rRNA gene and incorporates Illumina sequence adaptors and nucleotide pads that do not match any 16S rRNA gene sequence at the position; these pads are also used to prevent hairpin formation of product amplicons. Subsequently, a second round PCR is performed to attach the barcode indices (Nextera XT DNA protocol, Illumina) and Illumina sequencing adaptors to the first-round amplicon products. There are eight forward index primers and 12 reverse index primers containing their own unique barcodes, which create 96 different combinations of the tagging process. This allows pooling of up to 96 samples to be read in a single Illumina MiSeq

sequencing run, increasing cost-effectiveness. The PCR primers used in the library preparation are presented in Table 5.1.

In this study, four Illumina MiSeq sequencing lanes were generated, comprising 96 samples in each run. The first three lanes aimed to investigate the microbiome profile of individual chiggers, whereas the final lane was used for the microbiome study of pooled chigger samples. Three types of negative controls were included on every lane in order to identify potential background contamination from sample manipulation equipment, DNA extraction kits and PCR reagents used in the library preparation steps. The background controls were: water in contact with equipment (*i.e.*, glass slides, coverslips, paintbrushes, dissecting needles, gloves and working area) followed by DNA extraction (Control 1); nuclease-free water followed by DNA extraction (Control 2); and nuclease-free water without DNA extraction (Control 3).

Table 5.1 Primers for 16S rRNA nested PCR library preparation for microbiome investigations. The first round primers contain a conserved region of the 16S (V4) rRNA gene (in green), Illumina overhang adaptors (in blue) and nucleotide pad linkers (in yellow). The primers for the second round contain Illumina overhang adaptors (in blue) incorporated with products from the first round and eight unique nucleotides of barcode indices (in red) attached to Illumina sequencing adaptors (in purple).

Primers	Nested PCR stage	Oligo sequence	Size (bp)
16sv4forward	1	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3'	57
16sv4reverse	1	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3'	54
DI_N501For	2	5'-AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTTCCCTACACGACGCTC-3'	61
DI_N502For	2	5'-AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTTCCCTACACGACGCTC-3'	61
DI_N503For	2	5'-AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTTCCCTACACGACGCTC-3'	61
DI_N504For	2	5'-AATGATACGGCGACCACCGAGATCTACACAGAGTAGAACACTCTTTCCCTACACGACGCTC-3'	61
DI_N505For	2	5'-AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTTCCCTACACGACGCTC-3'	61
DI_N506For	2	5'-AATGATACGGCGACCACCGAGATCTACACACTGCATAACACTCTTTCCCTACACGACGCTC-3'	61
DI_N507For	2	5'-AATGATACGGCGACCACCGAGATCTACACAAGGAGTAACACTCTTTCCCTACACGACGCTC-3'	61
DI_N508For	2	5'-AATGATACGGCGACCACCGAGATCTACACCTAAGCCTACACTCTTTCCCTACACGACGCTC-3'	61
DI_N701Rev	2	5'-CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N702Rev	2	5'-CAAGCAGAAGACGGCATACGAGATCTAGTACGGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N703Rev	2	5'-CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N704Rev	2	5'-CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N705Rev	2	5'-CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N706Rev	2	5'-CAAGCAGAAGACGGCATACGAGATCATGCCTAGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N707Rev	2	5'-CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N708Rev	2	5'-CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N709Rev	2	5'-CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N710Rev	2	5'-CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N711Rev	2	5'-CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTGACTGGAGTTCAGACGTGTGCTC-3'	57
DI_N712Rev	2	5'-CAAGCAGAAGACGGCATACGAGATTCCTCTACGTGACTGGAGTTCAGACGTGTGCTC-3'	57

For the individual chigger samples (the first three lanes), the first round PCR was conducted in a 25 µl reaction containing 17.7 µl of nuclease-free water; 2.5 µl of GeneAmp 10X PCR buffer I; 1.2 µl of GeneAmp dNTPs (0.5 mM final concentration); 1.2 µl of each 16sv4forward and reverse primers (0.5 µM final concentration); 0.2 µl of AmpliTaq DNA Polymerase LD (1.25U); and 1 µl of DNA template. The PCR comprised initial denaturation at 94^{o}C for 5 min, followed by 18 cycles of denaturation at 94^{o}C for 1 min, annealing at 56^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 5 min. The whole amplicon products including the target gene and Illumina linker sequences were around 300 - 350 bp in size. Subsequently, PCR products were size-selected and purified using a Chroma Spin^{TM}-200 column (Takara-Clontech) following the manufacturer's protocol. Only DNA fragments >150 bp were retained and primer-dimers <50 bp were removed by this step after elution with 50 µl nuclease-free water. The size-selected PCR products were then used as the DNA templates for second round PCR. This round was performed in a 25 µl reaction containing 15.7 µl of nuclease-free water; 2.5 µl of GeneAmp 10X PCR buffer I; 1.2 µl of GeneAmp dNTPs (0.5 mM final concentration); 1.2 µl of each forward and reverse index barcoding primers (0.5 µM final concentration); 0.2 µl of AmpliTaq DNA Polymerase LD (1.25U); and 3 µl of DNA template. The PCR comprised initial denaturation at 94^{o}C for 5 min, followed by 25 cycles of denaturation at 94^{o}C for 1 min, annealing at 65^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 5 min. Both PCR amplification rounds were conducted in a T3 thermocycler (Biometra).

For the pooled chigger samples in the final Illumina MiSeq sequencing lane, the protocol of 16S rRNA library preparation was slightly adapted from thatdescribed above. As the GeneAmp dNTPs (Applied Biosystems) was no longer supplied from the company, the PCR components used in the nested PCR were inevitably changed. PCR thermocycling conditions remained the same in both PCR amplification rounds as described above. For the PCR components, the first round PCR was conducted in 25 µl reactions containing 12 µl of microbial DNA-free water (QIAGEN); 10 µl 2X Sensimix^{TM} SYBR No-Rox kit (Bioline); 1 µl of each 16sv4forward and reverse primers (0.4 µM final concentration); and 1 µl of DNA template. The products were size-selected using a Chroma Spin^{TM}-200 column (Takara-Clontech) following the manufacturer's protocol and used as DNA template for the second round PCR. This round was conducted in a 25 µl reaction containing 10 µl of microbial DNA-free water (QIAGEN); 10 µl 2X Sensimix^{TM} SYBR No-Rox kit (Bioline); 1 µl of each forward and reverse index barcoding primers (0.4 µM final concentration); and 3 µl of DNA template.

5.2.2.2 Pooling strategy of 16S rRNA nested PCR products for Illumina MiSeq sequencing

The final PCR products (5 µl) were visualised on a 1.2% agarose gel incorporating SYBR Safe DNA gel stain and run at 120 V for 45 min in a Bio-Rad gel electrophoresis set. The gels were visualised using a G:Box Gel Documentation System (Syngene). The PCR products were purified individually using a QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's protocol, with 30 µl of nuclease-free water used the in elution step.

For each sequencing lane, the 96 purified PCR products were pooled by adjusting the DNA volume on the basis of the band density in the gel images. A volume of 5, 10 or 15 µl was taken from the samples with high, medium and low band densities, respectively (see example in Figure 5.2). From the DNA pools, 3 aliquots (30 µl each) were quantified for DNA concentration using the Quant-iT^{TM} Picogreen dsDNA kit (Invitrogen), and the aliquots with the highest DNA concentration were submitted for Illumina MiSeq sequencing at the Centre for Genomic Research (CGR), University of Liverpool. A diagram of the library preparation and sequencing workflow is presented in Figure 5.3.

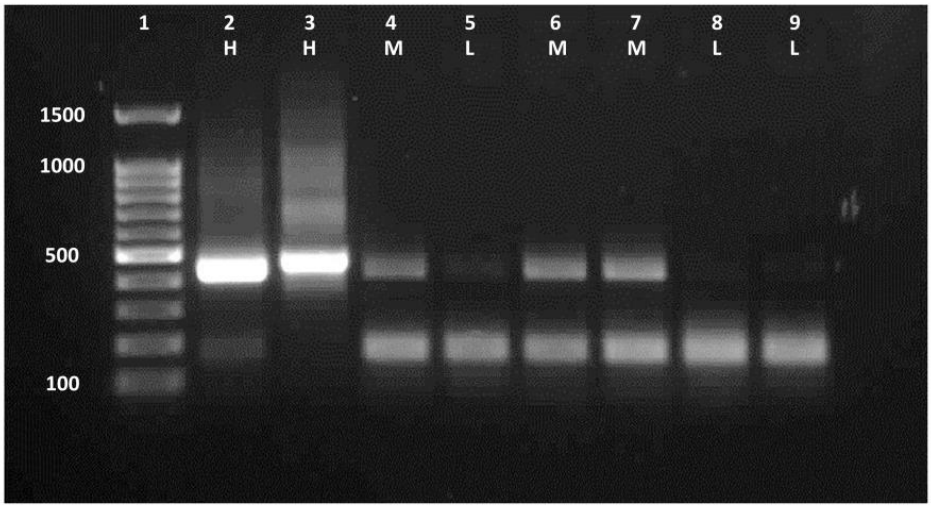

Figure 5.2 Image of agarose gel electrophoresis presenting an example of variation of 16S rRNA product densities after the second round PCR in the library preparation step. H, M and L indicate high, medium and low band densities, respectively, for normalization strategy of amplicon pooling prior to Illumina MiSeq sequencing.

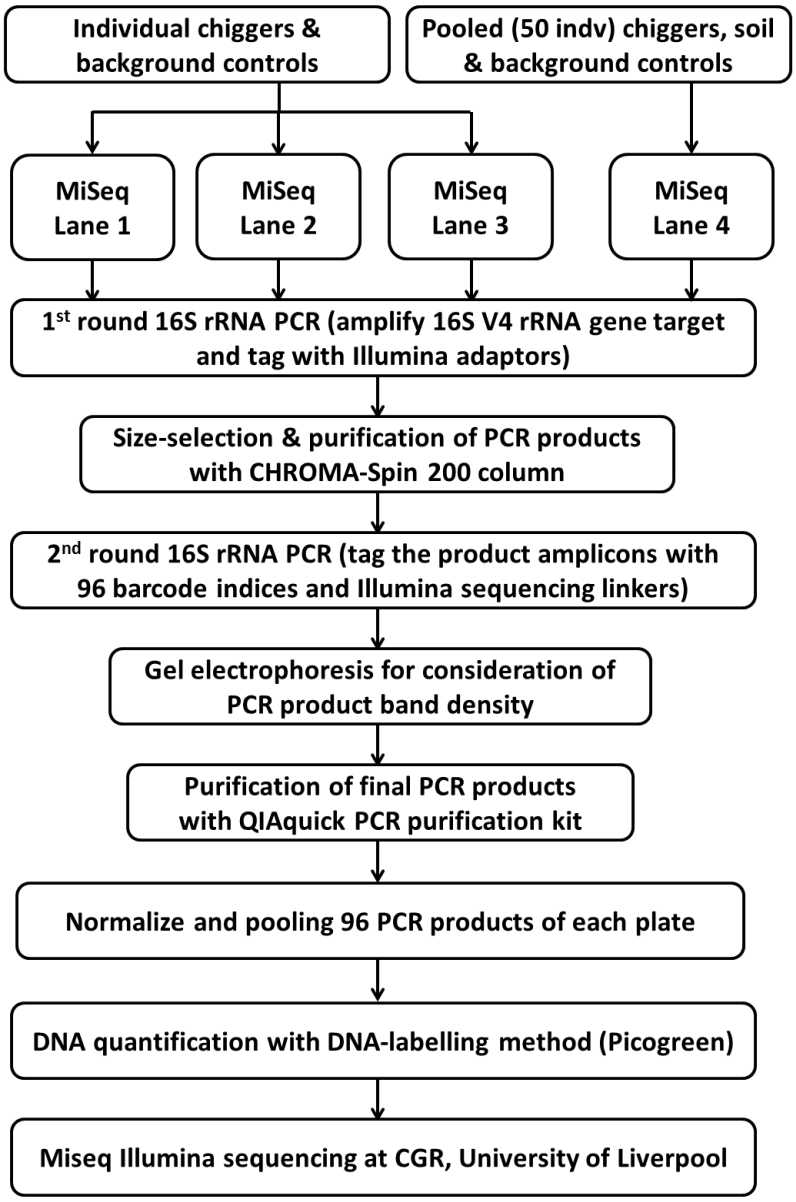

Figure 5.3 Schematic diagram shows 16S rRNA library preparation workflow of the 4 plates of Illumina MiSeq sequencing.

5.2.3 Post-sequencing bioinformatic processes

5.2.3.1 Quality filtering of raw paired-end sequence data

In general, individual nucleotide sequences (reads) generated from next generation sequencing are longer than the expected size of the target fragment due

to the attachment of the sequencing adaptors and barcode indices on the 3' or 5' end. They should be removed from each read prior to downstream analysis, otherwise analysis errors will result. Here, quality filtering of the raw 16S rRNA reads was initially done by CGR. The reads in fastq format were trimmed with CUTADAPT v.1.2.1 (Martin 2011) and SICKLE v.1.200 (Joshi & Fass 2011), and the sequences with an average nucleotide base quality score lower than 20, as well as a length shorter than 10 bp after trimming, were discarded. This finally gave forward (R1) and reverse (R2) reads of the certain read pairs, whereas the singlet reads (R0), the only one of a read pair that passed the filtering, were excluded from the further steps. Subsequently, error correction of the reads was performed using BayesHammer algorithm in SPAdes v.3.1.0 (Bankevich et al. 2012; Nikolenko et al. 2013). Then, the paired-reads (R1 and R2) were aligned using PANDAseq (Masella et al. 2012), generating assembled reads representative for the certain pairs. Only reads with a size between 270 - 300 bp were retained. Finally, the aligned reads from the four lanes of MiSeq sequencing were combined in a single fasta file. The file containing the whole dataset (millions of reads from 377 samples) was used for further microbiome analyses. The LINUX command line script for the quality filtering processes above was developed by A. Christina Gill (Institute of Infection & Global Health, University of Liverpool).

5.2.3.2 Microbiome profiling

Analyses of 16S rRNA microbiome profile were performed using the Quantitative Insights into Microbial Ecology (QIIME) software package, version 1.8.0, http://qiime.org (Caporaso et al. 2010). To define bacterial taxonomy, reads

were grouped by sequence similarity called “OTUs” or Operational Taxonomic Units using an open-reference OTU picking approach with USEARCH61 method (Edgar 2010). All reads were binned at 97% similarity against the Greengene database v.13_8 (McDonald et al. 2012). Any reads that did not match the reference database were subsequently clustered *de novo* against each other with the same similarity threshold. These steps were done through “pick_open_reference_otus.py”, in which bacterial taxonomic assignment with UCLUST against the Greengene database v.13_8 (Edgar 2010), sequence alignment with PyNAST (Caporaso et al. 2009), and tree-building with FastTree v.2.1.3 (Price et al. 2010) were also generated in the outputs. Within an OTU, the most abundant read was selected as a representative sequence for that particular OTU. The OTU table (providing the taxonomic assignment and read count of 16S rRNA gene sequences in each OTU and sample) was created in biom format (Biological Observation Matrix).

Chimeric sequences were identified using “ChimeraSlayer” (Haas et al. 2011) and removed from the OTU table. Chimeras have been recognised as a common artefact produced from confounding processes during 16S rRNA gene amplification (Acinas et al. 2005; Ashelford et al. 2005; Haas et al. 2011). The chimeric molecules are created when a PCR amplicon is incompletely formed during the extension stage and the aborted product is incorporated in subsequent PCR cycles prior to annealing and synthesis of a new amplicon from the second DNA strand parent. If this chimeric sequence passes through subsequent taxonomic classification steps without checking, it may be identified as a novel sequence by the reference

database, causing a false positive bias. A new OTU table and phylogenetic tree were regenerated again after removal of chimeras.

To facilitate a manual investigation of microbiome profiles, the OTU table was filtered, aiming to discard any OTUs with a relative abundance (proportion of total read count) of <1% across samples, whereas the OTUs represented by ≥1% of reads were retained for further analyses. The OTUs with fewer than five read counts were identified as singletons and then removed from the OTU table.

There are several potential sources of errors and biases during the sample preparation and generation of 16S rRNA library; *e.g.,* equipment and reagents used in specimen manipulation and DNA extraction kit and water contamination; producing substantial challenges for high-throughput sequencing experiments, particularly when dealing with low biomass microbiota samples (Salter et al. 2014; Galan et al. 2016). As our study dealt with such challenging specimens, very small DNA yields from individual chiggers were obtained, and these low biomass samples could fail to outcompete contaminating 16S rRNA gene sequences. Accordingly, a sample-control similarity check using the β-diversity approach (Bray-Curtis dissimilarity) was applied using the "ecodist" package (Goslee & Urban 2007) implemented in R freeware (R Core Team 2015). Any samples that exhibited a microbiome pattern (OTUs) to the background controls of >20% similarity were excluded, as about a 20 - 30% cut-off seemed acceptable in discriminate sample-control similarity according to previous studies (Minard et al. 2015; Jervis-Bardy et al. 2015). We decided to remove low quality samples before conducting comparative analyses rather than subtracting contaminant OTUs of likely

background control origin, as the latter could affect the relative abundance in the samples. A summary of the post-sequencing bioinformatics workflow is presented in Figure 5.4.

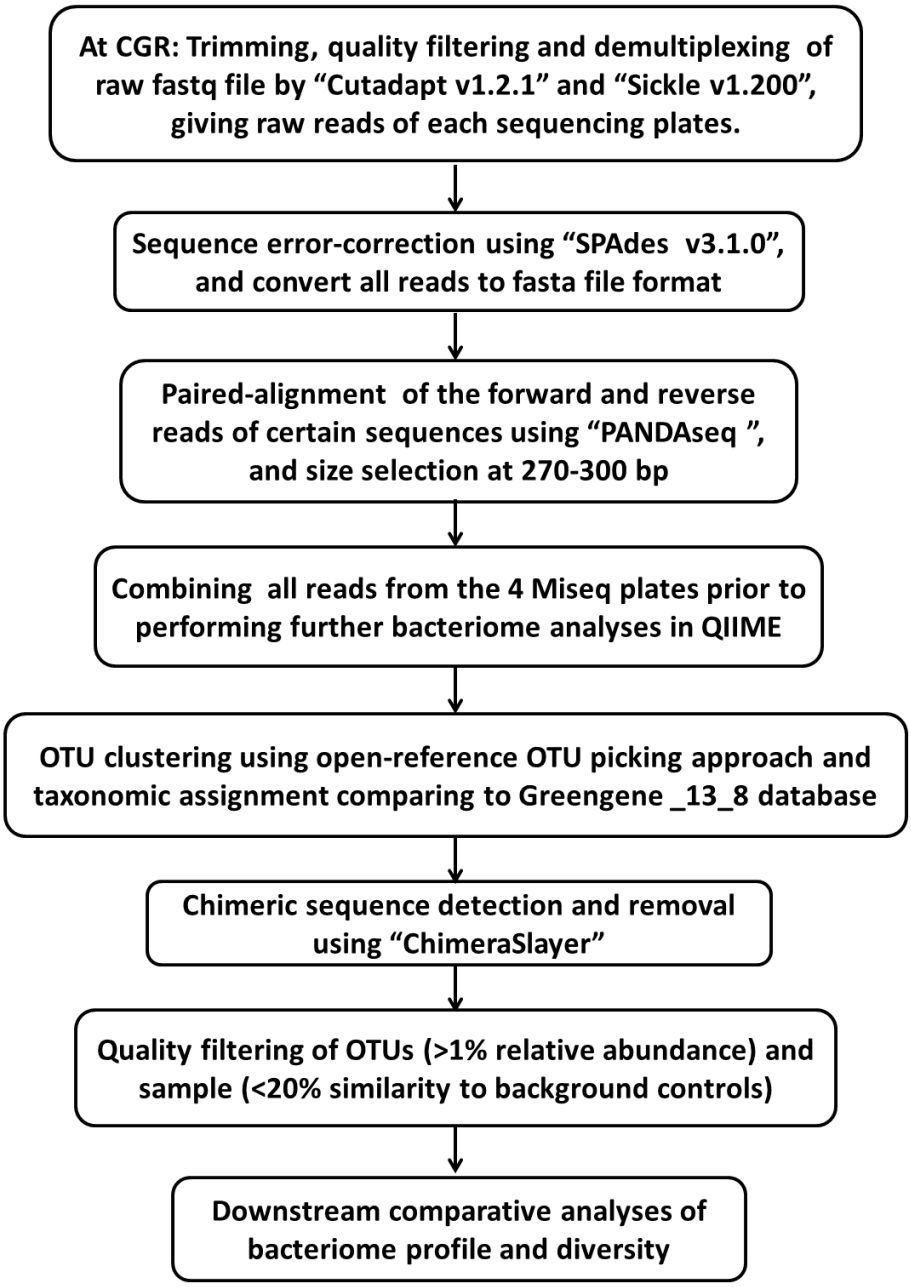

Figure 5.4 Schematic diagram showing post-sequencing bioinformatics workflow of data filtering and microbiome profiling with QIIME platform.

5.2.4 Comparative analyses of microbiome data

5.2.4.1 Summary of bacterial communities by relative abundance

The OTU table was transformed to a bacterial taxonomy table with raw read counts of each OTU presented in columns and samples in rows. The read count data were normalized to relative abundance, using total sum scaling method or proportion. Bacterial communities were summarized with regard to sample groups (individuals and pools), selected chigger species and studied sites (mixed species), as well as soil samples from Thailand and Lao PDR. The dominant OTUs (the OTUs that presented proportionally ≥10% within a sample) were plotted as stack-bar charts; whereas the OTUs that showed <10% in a sample were combined together in "others". This helped to visualize bacterial communities represented by the dominant OTUs within the samples.

5.2.4.2 Alpha-diversity of the chigger-associated microbiome

First, the read count data in the OTU table was normalized by different rarefaction depths at 100, 1,000, 10,000 and 100,000 reads per sample in order to optimize the best value for data normalization prior to further comparative analyses. Rarefaction subsampling at 10,000 sequences depth was then applied as it showed the highest percentage of samples and OTU recovery compared to the others (Table 5.2). The diversity of bacterial OTUs among different sample groups (individual chiggers, pooled chiggers and soil) and categories (chigger species, habitats and study sites) was determined through richness and diversity estimators available as default in QIIME; *i.e.,* observed richness, chao1 non-parametric richness

estimator, and whole-tree phylogenetic diversity (PD_whole_tree). Non-parametric Kruskal-Wallis (KW) tests with Bonferroni post-hoc comparisons were performed to compare the alpha-diversity of bacterial OTUs among the sample groups.

5.2.4.3 Beta-diversity of bacterial composition

Similar to the alpha-diversity analysis, a rarefaction depth at 10,000 reads per sample was first applied in the data normalization step prior to beta-diversity analysis of bacterial composition among the sample groups. At the level of individual and pooled chiggers, beta-diversity of bacterial composition among different categories (chigger species, habitat and study site) was computed by QIIME defaulted unweighted and weighted UniFrac (Unique Fraction) phylogenetic-based measurement methods. These methods take phylogenetic distance (that is, the fraction of tree length between sets of bacterial taxa) into account for calculation between pairs of samples in beta-diversity metrics (Lozupone & Knight 2005). Principle Coordinate Analysis (PCoA) was used to transform complex multidimensional data in the metrics to a new set of data regarding different orthogonal axes, and plotted them to visualize clustering patterns of bacterial composition in the samples. Nonparametric ANOSIM method (Analysis of Similarity) with 1,000 permutations was used to test whether the clustering pattern of bacterial composition in the samples was statistically significant.

Table 5.2 Comparison of rarefaction statistics among different sequence depths (100 – 100,000 reads/sample); the asterisk (*) indicates the sequence depth selected for data normalization in further analyses in this study.

Sequence depth (reads/sample)	No. of sample	Sample recovery (%)	No. of OTUs	OTUs recovery (%)	Total read	Table density
Original OTU table	217	100	995	100	23,732,857	0.143
Subsample 100 even	217	100	579	58.19	21,700	0.034
Subsample 1,000 even	217	100	740	74.37	217,000	0.07
Subsample 10,000 even*	**208**	**95.41**	**817**	**82.11**	**2,080,000**	**0.114**
Subsample 100,000 even	102	47.01	779	78.29	10,200,000	0.165

5.2.5 Verification of *Geobacillus* OTUs in chiggers and background controls

As *Geobacillus* spp., a genus of Gram-positive thermophilic bacteria, were dominant in individual chigger samples (see Results), quantitative PCR (qPCR) prior to Sanger sequencing of the PCR products was performed in order to investigate whether these *Geobacillus* were genuinely associated with the chiggers or acquired from some sort of contamination during laboratory procedures.

5.2.5.1 Geobacillus specific primer design

A pair of PCR primers was designed aiming to specifically amplify a 16S rRNA gene portion for the genus *Geobacillus*. Ten representative sequences of *Bacillales* and *Geobacillus* OTUs derived from MiSeq sequencing lanes and additional sequences of *Bacillus* spp. (KC443093.1, AB501343.1, DQ207730.2, AF058766.1, HM470251.1, DQ906100.1 and AF233579.1) from the NCBI nucleotide database were aligned with ClustalW multiple alignments. To differentiate between

Geobacillus and *Bacillus* sequences, the primers were picked from regions containing sets of single nucleotide polymorphisms (SNPs) using Primer 3 v.4.0.0 (Untergasser et al. 2012). The following primers, 16SGbF (GTCCGGAATTATTGGGCGTA) and 16SGbR (TACGCATTTCACCGCTACAC) were used in qPCR and conventional PCR, amplifying 150 bp of the DNA fragment. The full-length *Geobacillus* amplicon product was synthesized by Eurogenetec Ltd. as a single-stranded oligonucleotide (Southampton, UK) and used as a standard control in the qPCR assay.

5.2.5.2 Molecular laboratory procedures

For the qPCR assay, different types of DNA samples [individual, 25-pooled and 50-pooled chiggers, as well as water samples from the laboratory water bath and QIAGEN microbial DNA-free water (negative control)] were used in the experiment. DNA from chiggers and 10 µl of water bath samples were extracted using the DNeasy Blood & Tissue Kit (QIAGEN) as previously described in Chapter 3. Serial dilutions of DNA standard control from 5 x 10^6 to 10^{-1} copies were prepared for use on each plate. The qPCR was carried out in 20 µl reactions containing 1 µl of DNA template, 10 µl of 2X SensiMix SYBR (Bioline), 1 µl of each primer (0.2 µM final concentration), and 7 µl of microbial DNA-free water. The qPCR was run with 35 cycles as follows: initial denaturation at 95^{o}C for 10 min; 35 cycles of 95^{o}C for 15 sec, 55^{o}C for 30 sec, 72^{o}C for 15 sec; and finally melting curve analysis, increasing the temperature from 50^{o}C to 95^{o}C in 0.5^{o}C increments. The PCR cycles were run in a MiniOpticon Real-Time PCR System (Bio-Rad), and quantitative data analysis was performed by CFX Manager Software v.3.1 (Bio-Rad). An analysis of variance

(ANOVA) with post-hoc Tukey HSD correction was performed in order to test differences in 16S rRNA gene sequence copies among the sample types.

To verify specificity of the primers in amplifying *Geobacillus* sequences, conventional PCR and Sanger sequencing of the products were applied in this step. In addition to the samples above, extracted DNA from soil (as previously described) was included in the experiment. The PCR components and cycle conditions were exactly the same as for qPCR, but without melting curve analysis. The amplicons were visualized by 1.2% agarose gel electrophoresis, incorporating SYBR Safe (Invitrogen), at 120 V for 40 min. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN), then subjected to a cloning experiment in the pGEM-T Easy Vector System (Promega). PCR fragments were ligated overnight at 4^{o}C into the pGEM-T Easy plasmid with 2:1 insert-plasmid ratio. The ligants were transformed into JM109 *E.coli* competent cells (Promega) by heat shock at 42^{o}C for 45 sec, then supplemented with SOC medium and incubated at 37^{o}C in a 200 rpm shaking incubator for 2 hours. The transformants were plated on LB/ampicillin/IPTG/X-Gal agar, and incubated overnight at 37^{o}C. After overnight incubation, the plates were stored at 4^{o}C for 3 days to allow better differentiation between blue and white colonies. Ten white recombinant colonies of each sample were inoculated in LB ampicillin broth and grown overnight at 37^{o}C in a shaking incubator at 200 rpm. Plasmid DNA was extracted from the transformed-cell pellets using Wizard Plus SV Minipreps DNA Purification Kit (Promega) following the manufacturer's protocol. Finally, the plasmid DNA samples were sent for Sanger

sequencing with pUC/M13 forward and reverse primers at the Source Bioscience Company, UK.

DNA sequences were assigned for bacterial taxonomy using RDP Naive Bayesian rRNA Classifier Version 2.10 (Wang et al. 2007) on the website https://rdp.cme.msu.edu. The taxonomical hierarchy was accepted at a >80% confidence threshold (Soergel et al. 2012). DNA sequences were aligned with ClustalW multiple alignments, and phylogenetic tree construction was performed with the Maximum likelihood (ML) method using Mega software version 6.06 (Tamura et al. 2013). To investigate the pattern of bacterial sequences in different sample types, a bicolor heat map based on positive/negative data was created using the "gplots" package (Warnes et al. 2016) in R freeware.

5.2.6 *Geobacillus* culture from chigger specimens

To attempt to validate the *Geobacillus* 16S rRNA amplicon data, we attempted to grow the bacterial cells or spores that may exist in or on chiggers using TSB (Trypticase soy broth) and TSA (Trypticase soy agar) media with the aid of high-temperature incubation. Twenty mite samples (mixed species) freshly isolated from small mammal hosts in the field study (Tha Wang Pha, Nan) were submerged in TSB (1 mite in 150 µl) and incubated for five hours at room temperature. The samples were heated to 55^{o}C for 10 min to reduce the viability of non-thermophilic bacteria, then stored at 4^{o}C and transported to the Faculty of Tropical Medicine, Mahidol University, Bangkok. The bacteria in TSB samples were grown once again on TSA plates and incubated at either 37^{o}C or 55^{o}C overnight.

5.2.7 Determination of GC content in 16S rRNA sequences

Percentage of GC content in DNA template has been hypothesized as one of several sources of biases in HTS data from 16S rRNA-based microbiome studies (Pinto & Raskin 2012; Kennedy et al. 2014). Accordingly, we evaluated whether the influence of GC content differential affected data obtained from individual and pooled chiggers, which were the low and high DNA concentration templates, respectively. Representative sequences of the dominant bacterial OTUs from both individual and pooled chiggers were checked for GC content using "Oligo Calc", an oligonucleotide properties calculator available at http://biotools.nubic.northwestern.edu/OligoCalc.html (Kibbe 2007). The mean GC contents of the dominant OTUs were compared between individual and pooled chiggers by a parametric two-sample T-test.

5.3 Results

5.3.1 Sequencing results

The total number of 16S rRNA reads from the whole set of 377 samples (275 individual chiggers, 69 pooled chiggers, 18 soil samples and 15 background controls) after quality filtering, de-multiplexing and error correction was 51,896,654 (mean reads/sample = 137,656.91; SD = 69,521.87). After R1 - R2 paired alignment and size selection at 270 - 300 bp, the read number was reduced to 49,635,427 (mean reads/sample = 131,658.96; SD = 69,921.81), yielding around 94% average sequence recovery.

Following several steps of OTU clustering, OTU quality filtering at >1% relative abundance, bacterial composition similarity check between background controls and samples with the Bray-Curtis method, and chimera sequence removal, 995 bacterial OTUs was identified from 217 retained samples with a total of 23,732,857 reads. Regarding control-sample similarity checking using the 20% cut-off, almost a half of samples (42.2%) was excluded. In particular, 56.4% of individual chigger samples failed to pass the cut-off, and were discarded from the dataset (Figure 5.5). This could be explained by the issue of low DNA template concentration of individual chiggers (mean = 0.026 ng DNA template added to the PCR reaction, *n* = 256 samples) compared to pooled chiggers and soil samples, where the mean DNA template added to the PCR was much higher at 1.136 ng (*n* = 84 samples) and 6.983 ng (*n* = 12 samples), respectively. Thus, the low biomass individual chigger samples provided too little DNA template to compete with DNA in kit reagents and the water used in PCR library preparation (Tanner et al. 1998; Salter et al. 2014), resulting in a high similarity in bacteria composition between many of the samples and the background controls. However, although excluded samples represented a sizeable proportion of the total set, the retained data after this stringent filtering step were of higher quality for further downstream analyses.

5.3.2 Microbiome profiling

5.3.2.1 Dominant bacterial OTUs in chiggers

Bacterial profiles between individual and pooled chiggers differed regarding the relative abundance of dominant bacterial OTUs. Several *Geobacillus*

(Fermicutes: Bacillales) and Comamonadaceae (Betaproteobacteria: Burkholderiales) OTUs appeared as dominant bacteria in individual chiggers, occupying up to 80% and 60%, respectively, in a sample. The other subdominant OTUs of individual chiggers were *Sphingobium* (Alphaproteobacteria: Sphingoonadales) and *Brevibacillus* (Firmicutes: Bacillales), (Figure 5.6).

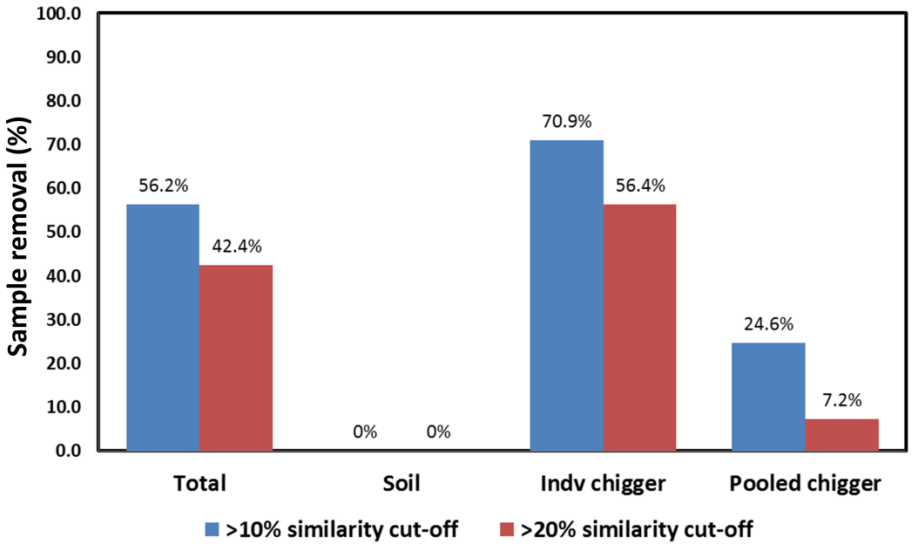

Figure 5.5 The bar chart shows a comparison of sample removal among different sample groups (individual, pooled chiggers and soil samples) after sample-control similarity check (Bray-Curtis dissimilarity) at 10% and 20% cut-offs.

The dominant bacterial OTUs of pooled chiggers were the family Neisseriaceae (Betaproteobacteria: Neisseriales), genus *Corynebacterium* (Actinobacteria: Actinomycetales) and *Staphylococcus* (Firmicutes: Bacillales), which occupied up to 93%, 83%, and 53% of OTUs, respectively, in a sample (Figure 5.7 and 5.8). Bacterial profiles of soil samples collected in Thailand and Lao PDR were clearly different. The dominant OTUs of soil from Thailand were Betaproteobacteria, Deltaproteobacteria, Nitrospirales and Bradyrhizobiaceae; whereas Acidobacteria, Actinocorallaria, Koribacteriaceae and

Thermogemmatisporaceae were the dominant bacteria found in soil samples from Lao PDR (Figure 5.9).

5.3.2.2 Bacterial OTUs of public health importance, arthropod symbionts and potential contaminant OTUs from background controls

After data filtering, a number of potential pathogenic bacteria of public health importance; *e.g*., *Borrelia, Mycobacterium* and *Orientia tsutsugamushi*, as well as arthropod symbionts; *e.g.*, *Cardinium, Pseudonocardia, Rickettsiella* and *Wolbachia* were selected for discussion. *O. tsutsugamushi*, the causative agent of scrub typhus disease was found in the chiggers. The bacterial sequences were detected in 13 pooled and three individual samples yielding a 20% and 2.75% prevalence rate, respectively, and occupying up to 18% of OTU proportion in a sample. Apart from *O. tsutsugamushi,* the other bacteria appear to be reported for the first time in trombiculid mite taxa. The spirochete *Borrelia* was detected in 32 pooled chigger samples (49.23% positive rate), with a relative abundance of up to 28.15% in a sample. *Mycobacterium* was found in 48 pooled chigger samples (73.85% positive rate), and also presented in all soil samples (100% positive rate) from Thailand and Lao PDR. The full list of the other selected OTUs is showed in Table 5.3. We were also able to count the number of infections and estimate prevalence rates of these bacteria in chigger populations. The number of positive samples for each bacterium was counted only for the samples that possessed more than five read counts (Razzauti et al. 2015). As the results show in Table 5.3, almost OTUs from individuals were a subset of OTUs from the pooled chiggers but not for *Geobacillus* and *Brevibacillus.*

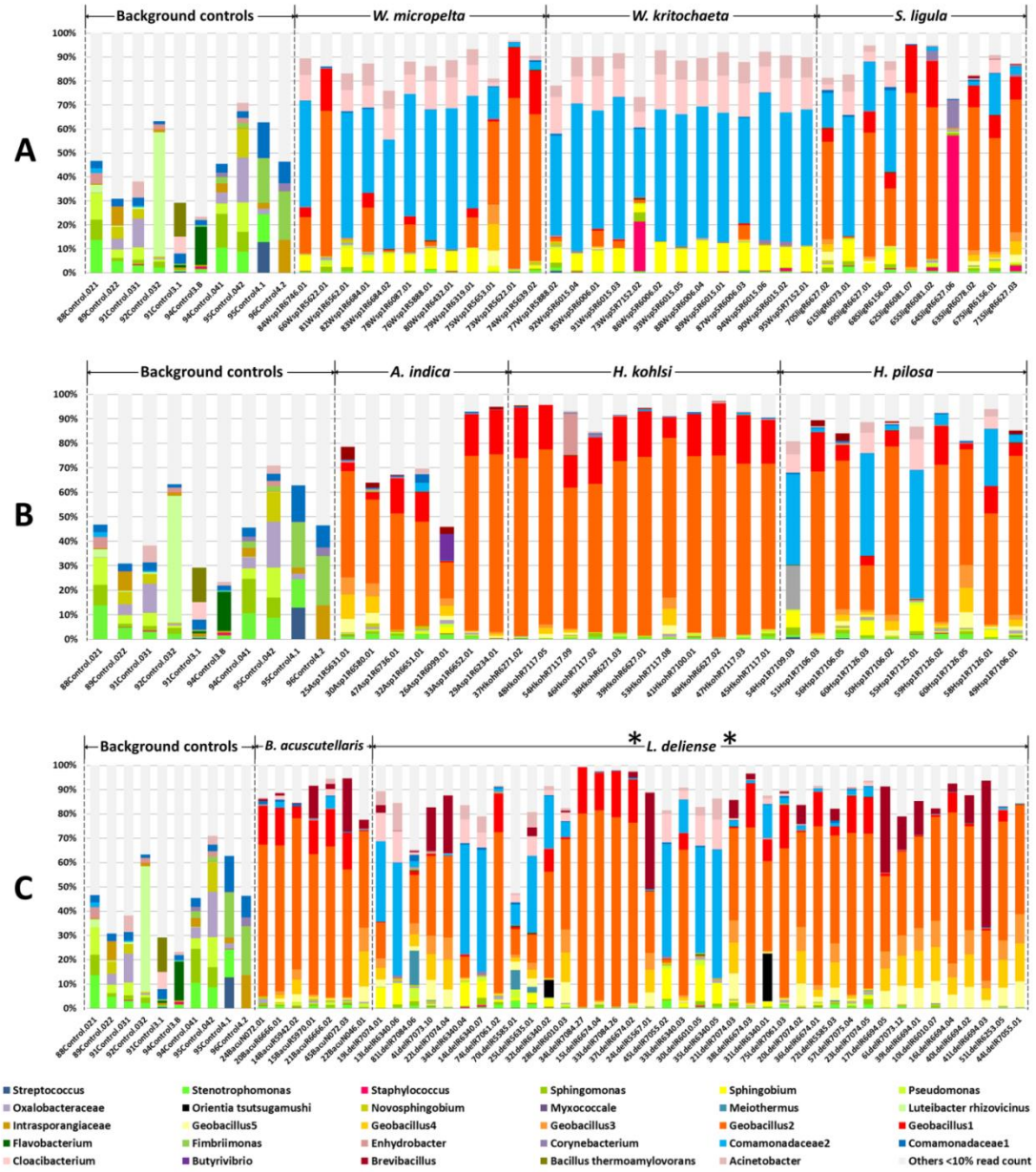

Figure 5.6 Stacked bar charts show the relative abundance of bacterial OTUs in background controls and individual chiggers from (A) subfamily Gahrliepiinae and subfamily Trombiculinae: (B) tribe Schoengastiini and (C) tribe Trombiculini. The data is filtered; OTUs that represented <10% in a sample were combined in "Others" (grey portion). *Orientia tsutsugamushi* (black portion) was found only in *L. deliense,* marked with asterisks (*).

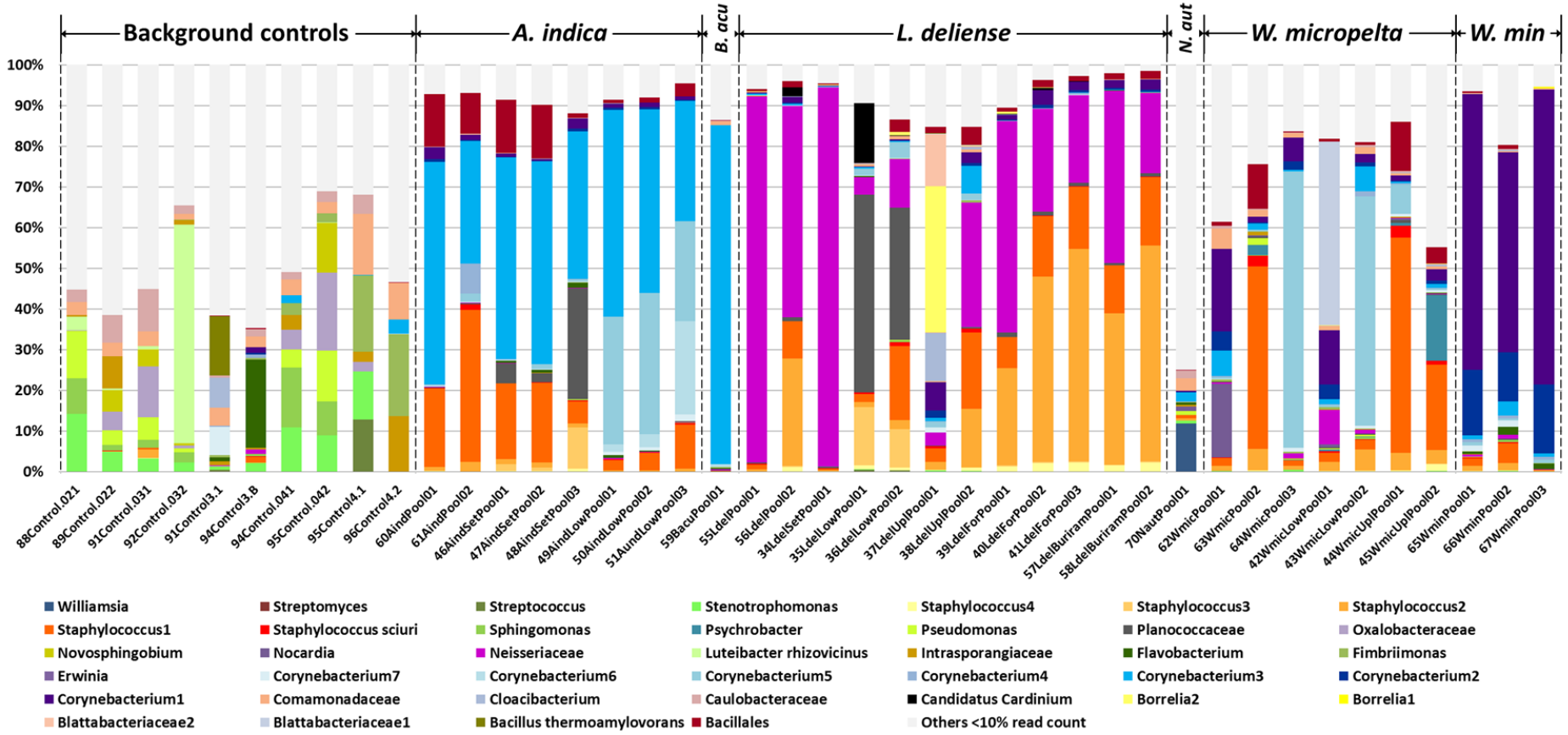

Figure 5.7 Stacked bar charts show relative abundance of bacterial OTUs in background controls and pooled samples of different chigger species. The data is filtered; OTUs that represented <10% in a sample were combined in “Others” (grey portion).

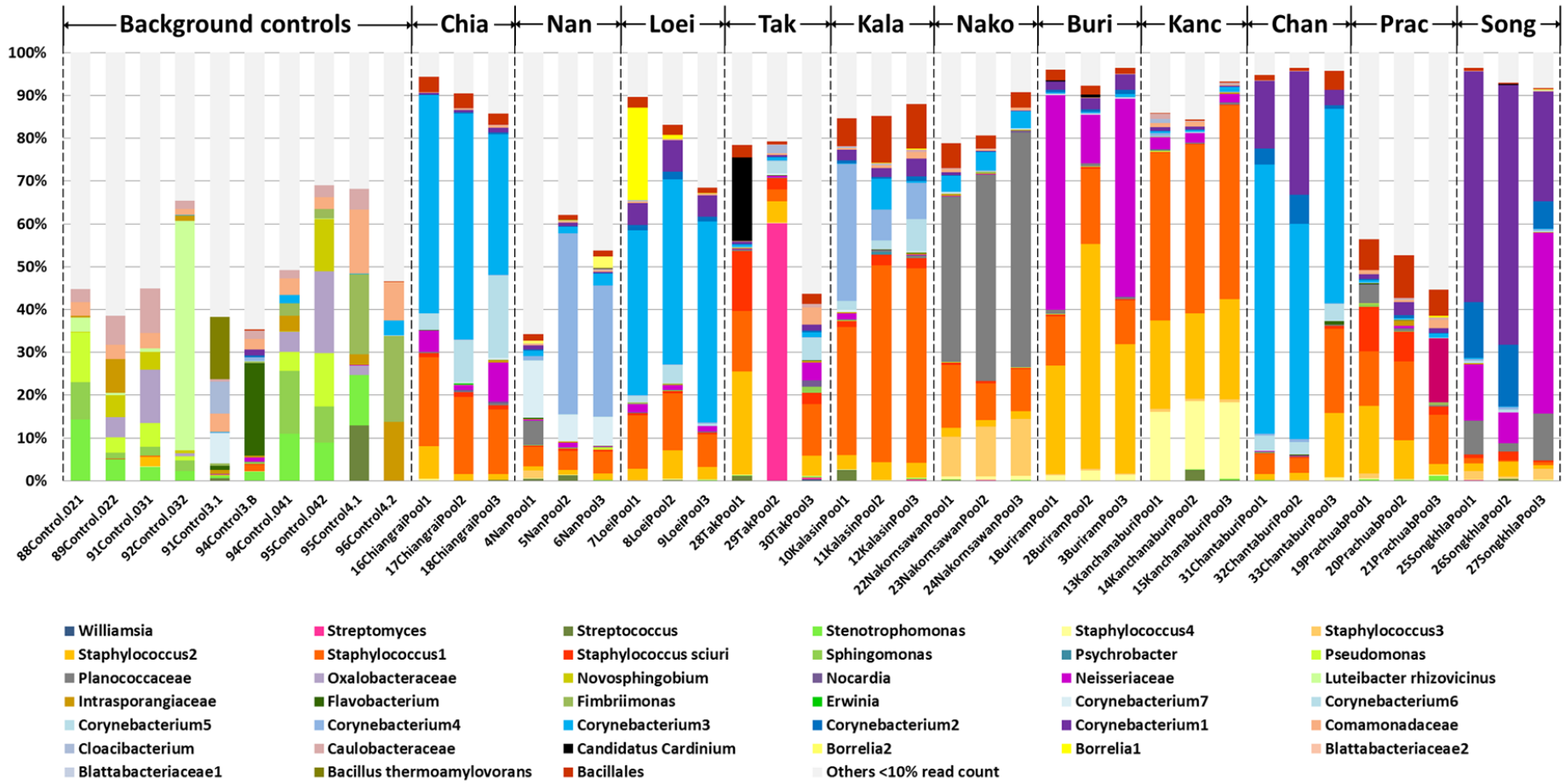

Figure 5.8 Stacked bar charts show relative abundance of bacterial OTUs in background controls and pooled samples of different study sites. The data is filtered, OTUs that represented <10% in a sample were combined in “Others” (grey portion).

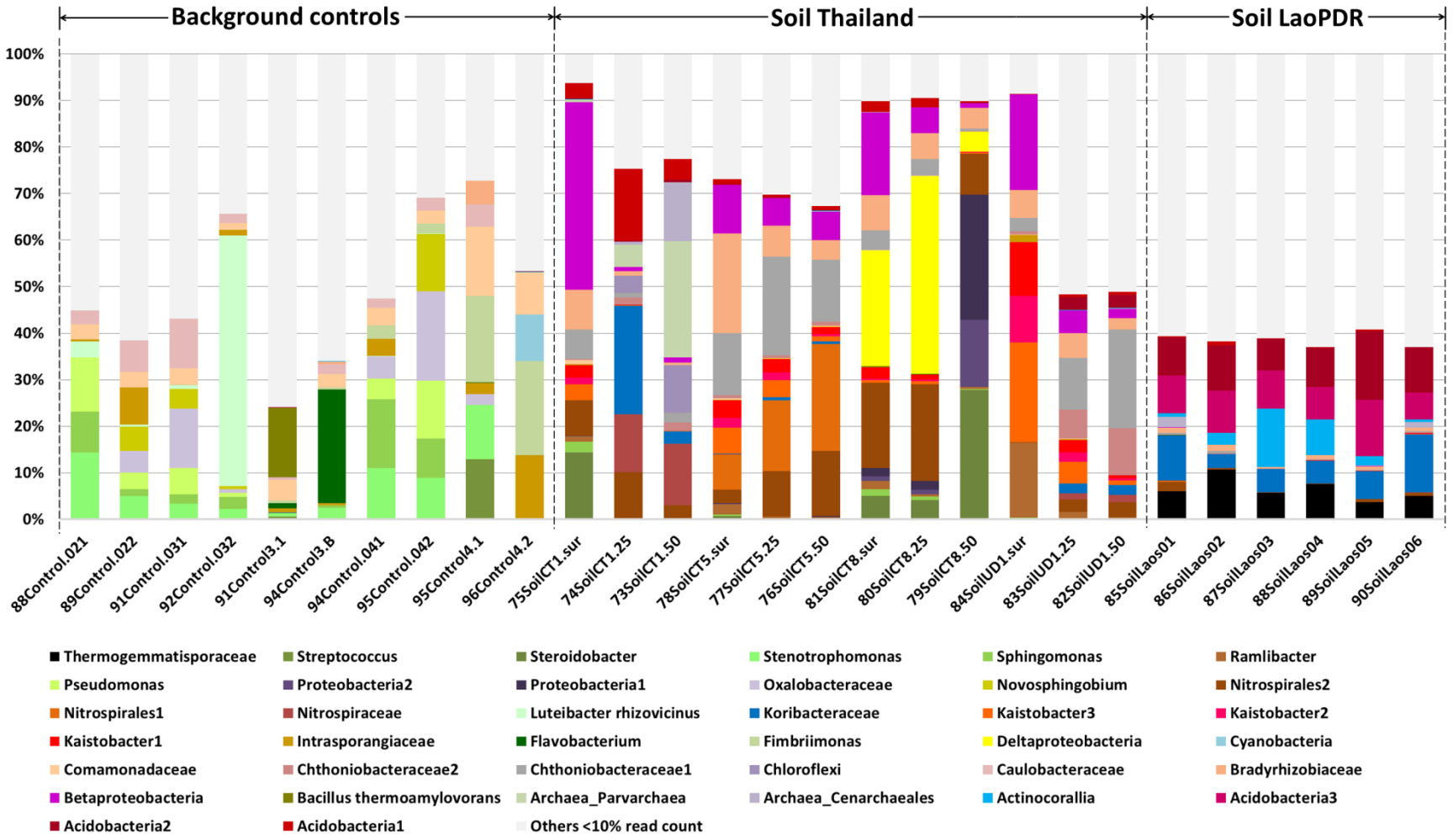

Figure 5.9 Stacked bar charts show relative abundance of bacterial OTUs in background controls and soil samples from Thailand and Lao PDR. The data is filtered, OTUs that represented <10% in a sample were combined in “Others” (grey portion).

Table 5.3 Selected bacterial taxa of public health importance, potential symbionts of arthropods, and other dominant OTUs detected in individual and pooled chiggers in comparison to soil and background controls. Only the OTUs that presented with more than five reads and identified minimally to genus level are included.

Bacterial taxa (OTU identify)	Maximum OTU Proportion (%)	Individual chigger		Pooled chigger		Soil		Control	
		No. of Samples	(%)	No. of samples	(%)	No. of samples	(%)	No. of samples	(%)
Opportunistic/Potential pathogens									
Anaerococcus (504674)	1.85	5	(4.59)	3	(4.62)	-	-	-	-
Arcobacter (4455962)	1.17	2	(1.83)	4	(6.15)	-	-	-	-
Bacillus cereus (4463224)	1.37	4	(3.67)	31	(47.69)	11	(61.11)	-	-
Borrelia (New.Reference OTU7)	28.15	1	(0.92)	32	(49.23)	-	-	-	-
Campylobacter (New.CleanUp.ReferenceOTU30)	1.07	-	-	3	(4.62)	-	-	-	-
Clostridium (New.ReferenceOTU2470)	2.48	-	-	6	(9.23)	-	-	-	-
Corynebacterium (13485)	14.19	1	(0.92)	51	(78.46)	-	-	2	(13.33)
Haemophilus parainfluenzae (4473129)	1.92	8	(7.34)	11	(16.92)	-	-	1	(6.67)
Helicobacter (New.ReferenceOTU397)	4.53	-	-	5	(7.69)	-	-	-	-
Moraxella (1127280)	1.27	-	-	4	(6.15)	-	-	-	-
Mycobacterium (4448095)	3.35	13	(11.93)	48	(73.85)	18	(100)	-	-
Nocardia (102163)	17.41	5	(4.58)	35	(53.84)	1	(5.55)	-	-
Orientia tsutsugamushi (301131)	18.02	3	(2.75)	13	(20)	-	-	-	-
Staphylococcus (4446058)	55.99	9	(8.25)	62	(95.38)	-	-	6	(40)
Potential arthropod symbionts									
Candidatus Cardinium (New.ReferenceOTU10)	16.16	2	(1.83)	18	(27.69)	-	-	-	-
Pseudonocardia (4435518)	1.32	1	(0.92)	29	(44.62)	3	(16.67)	-	-
Rickettsiella (8028)	1.06	2	(1.83)	12	(18.46)	-	-	-	-
Wolbachia (New.ReferenceOTU2936)	1.78	-	-	2	(3.08)	-	-	-	-

Table 5.3 (continued)

Bacterial taxa (OTU identify)	Maximum OTU Proportion (%)	Individual chigger		Pooled chigger		Soil		Control	
		No. of Samples	(%)	No. of samples	(%)	No. of samples	(%)	No. of Samples	(%)
Other outstanding OTUs									
Acinetobacter rhizosphaerae (4334053)	6.13	4	(3.67)	33	(50.77)	-	-	-	-
Brevibacillus (3307468)	60.39	61	(55.96)	-	-	-	-	-	-
Burkholderia bryophila (4320353)	1.27	38	(34.86)	2	(3.07)	-	-	-	-
Geobacillus (New.ReferenceOTU5884)	10.73	82	(75.23)	-	-	-	-	-	-
Methylobacterium adhaesivum (4303249)	3.96	11	(10.09)	12	(18.46)	-	-	-	-
Nevskia (516554)	2.06	27	(24.77)	3	(4.61)	7	(38.88)	-	-
Sphingobacterium multivorum (4423201)	1.15	39	(35.78)	1	(1.54)	-	-	1	(6.67)
Sphingobium (4393057)	14.69	83	(76.14)	11	(16.92)	1	(5.55)	-	-
Streptomyces (821185)	59.26	2	(1.83)	14	(21.54)	1	(5.55)	-	-

In addition, contaminant OTUs were identified from background controls which were processed alongside chigger samples in 16S rRNA gene library preparation. Thirty-four bacterial OTUs were possibly background contaminants based on more than 5% relative abundance (proportion) present in at least one control sample (Table 5.4).

Table 5.4 The 34 dominant OTUs (more than 5% proportional read count in a single control) present in background controls. The highest OTU proportions in particular controls are showed in bold. The following OTUs were previously reported as potential contaminants in [a]Tanner et al. (1998); [b]Grahn et al. (2003); [c]Barton et al. (2006); [d]Salter et al. (2014) and Galan et al. (2016).

Bacterial taxa (OTU identify)	Maximum proportion (%)			
	All controls	Control 1	Control 2	Control 3
Species *Luteibacter rhizovicinus* (177555)	41.59	24.73	**41.59**	0.07
Genus *Flavobacterium* (4438548)[d]	22.86	0.00	**22.86**	0.03
Genus *Fimbriimonas* (New.ReferenceOTU3)	20.36	0.00	3.54	**20.36**
Family Oxalobacteraceae (4476547)	19.15	4.64	12.50	**19.15**
Genus *Stenotrophomonas* (2806353)[a,b,d]	18.02	14.22	3.30	**18.02**
Species *Bacillus thermoamylovorans* (21214)	14.97	-	**14.97**	-
Family Comamonadaceae (4396454)	14.92	3.31	9.08	**14.92**
Genus *Sphingomonas* (4423410)[a,d]	14.59	8.72	2.47	**14.59**
Family Intrasporangiaceae (4432889)	13.81	7.95	1.22	**13.81**
Genus *Streptococcus* (4473883)[d,e]	12.89	-	0.56	**12.89**
Genus *Hydrogenophilus* (4317875)	12.85	-	**12.85**	0.03
Genus *Pseudomonas* (4456891)[b,d,e]	12.49	11.64	5.59	**12.49**
Genus *Novosphingobium* (581019)[d]	12.28	5.70	4.21	**12.8**
Family Cytophagaceae (737260)	10.67	-	**10.67**	0.02
Species *Methylobacterium organophilum* (New.ReferenceOTU2)	10.56	**10.56**	10.15	0.03
Family Caulobacteraceae (4353264)	10.41	6.76	**10.41**	4.80
Phylum Cyanobacteria (98258)	10.06	-	0.04	**10.06**
Genus *Ochrobactrum* (4388385)[d]	9.84	**9.84**	1.25	9.53
Genus *Rhodococcus* (4468125)[d]	9.44	3.42	7.32	**9.44**
Genus *Wautersiella* (New.ReferenceOTU2903)[d]	8.92	-	**8.92**	-
Genus *Cloacibacterium* (4154872)	8.64	-	**8.64**	-
Family Methylophilaceae (101445)	7.87	1.85	0.41	**7.87**
Genus *Lactobacillus* (619224)	7.32	-	-	**7.32**
Family Caulobacteraceae (4339358)	7.23	6.74	**7.23**	0.01
Genus *Thermicanus* (439457)	7.14	-	**7.14**	-

Table 5.4 (continued)

Bacterial taxa (OTU identify)	Maximum proportion (%)			
	All controls	Control 1	Control 2	Control 3
Genus *Corynebacterium* (942245)[d,e]	7.13	-	**7.13**	-
Genus *Acinetobacter* (4482374)[a,c,d,e]	7.11	0.03	**7.11**	3.56
Genus *Fluviicola* (646052)	6.91	-	**6.91**	0.03
Class Alphaproteobacteria (New.CleanUp.ReferenceOTU210)	6.07	-	**6.07**	0.03
Family Microbacteriaceae (1109043)	6.06	-	**6.06**	-
Family Caulobacteraceae (810889)	5.84	-	**5.84**	0.61
Species *Kocuria rhizophila* (4477552)	5.49	3.35	**5.49**	2.00
Family Bradyrhizobiaceae (4475561)	5.14	-	0.45	**5.14**
Family Polyangiaceae (New.ReferenceOTU9)	5.03	-	0.03	**5.03**

5.3.2.3 GC content of 16S rRNA gene sequences between individual and pooled chigger samples.

The microbiome results showed a substantial difference in dominant bacterial OTUs between individual and pooled chigger samples. Probably, DNA template concentration and GC content, or combination of these, might play a critical role in PCR amplification bias. Here, after excluding the OTUs from the negative controls, the GC content (%) of dominant OTUs from individual (15 OTUs) and pooled chiggers (26 OTUs) were calculated and compared. The mean GC content of OTUs in individual samples was significantly higher than in pooled chiggers [two sample t-test (equal variance): $t = 2.45$, $df = 39$, $p = 0.0094$] (Figure 5.10).

5.3.3 Verification of *Geobacillus* in individual chiggers

Due to their thermophilic characteristics, *Geobacillus* vegetative cells grow optimally at a temperature between 45^{o}C to 70^{o}C (Nazina et al. 2001). During

chigger DNA preparation, individual samples were lysed in proteinase-K at 56^{o}C in a water bath; a potential source of contaminating thermophilic bacteria. To determine if the water bath contained *Geobacillus* DNA that may have contaminated the chigger specimens, further controls were conducted as follows.

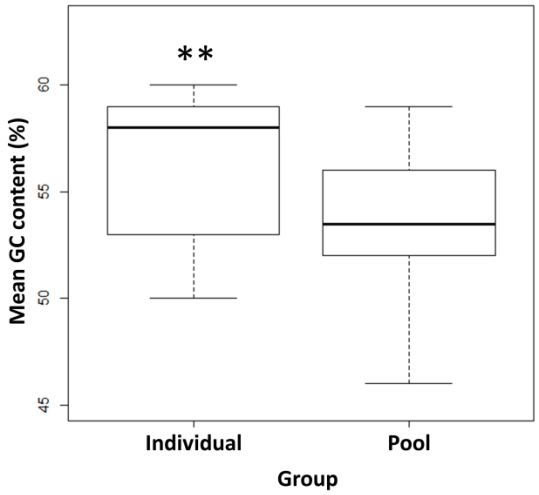

Figure 5.10 Boxplot shows significant difference (two sample t-test) in mean GC content of 16S rRNA gene sequences between individual and pooled chigger samples. (**) $p < 0.01$

A pair of *Geobacillus*-specific primers targeting the 16S rRNA gene were designed and applied to both individual and pooled chiggers, as well as soil and water bath samples, by qPCR. The result showed 16S rRNA gene sequence copies significantly increased from the individual, 25-pooled and 50-pooled chigger samples, whereas water bath samples also yielded a high amount of sequence copies; ANOVA ($F = 34.37$, $df = 4$, $p < 0.0001$, with post hoc Tukey HSD correction) (Figure 5.11). However, although qPCR signals were obtained from water bath samples, it was not clear if the signals arose from *Geobacillus* or other bacteria until the specificity of the *Geobacillus*-specific primers was tested. To verify the primer

specificity, the PCR products (150 bp) were cloned and sent for Sanger sequencing. The sequencing results showed that not only *Geobacillus* was amplified by the primers but also several other taxa; *e.g.*, Actinobacteria, Firmicutes and Proteobacteria, suggesting low primer specificity for genus *Geobacillus* amplification.

Regarding the amplicon sequences, bacterial communities among chigger, soil, and water bath samples were completely distinct from each other. *Geobacillus* clones were found only in individual chiggers, not pooled chigger samples or from the water bath. This is in accordance with the previous results from 16S rRNA gene Illumina MiSeq sequencing lanes. In addition, only the 22 sequences assigned to Phylum Firmicutes (to which *Geobacillus* belongs) were selected and analysed once again with multiple sequence alignment prior to phylogenetic construction using the Maximum Likelihood method. The tree clearly separated *Geobacillus* in chiggers from Bacillales and *Paenibacillus* in water bath samples (Figure 5.12), confirming that *Geobacillus* signals from chiggers did not originate from the water bath in the DNA preparation step.

In addition, a *Geobacillus* culture experiment was preliminarily conducted aiming to grow the bacteria directly from chigger samples collected in Nan province, one of the studied sites in Thailand. This could produce strong evidence to confirm if *Geobacillus* exists in chiggers. However, the culture experiment of 20 chigger samples failed to generate bacterial colonies on TSA media after incubation at 37^{o}C and 55^{o}C.

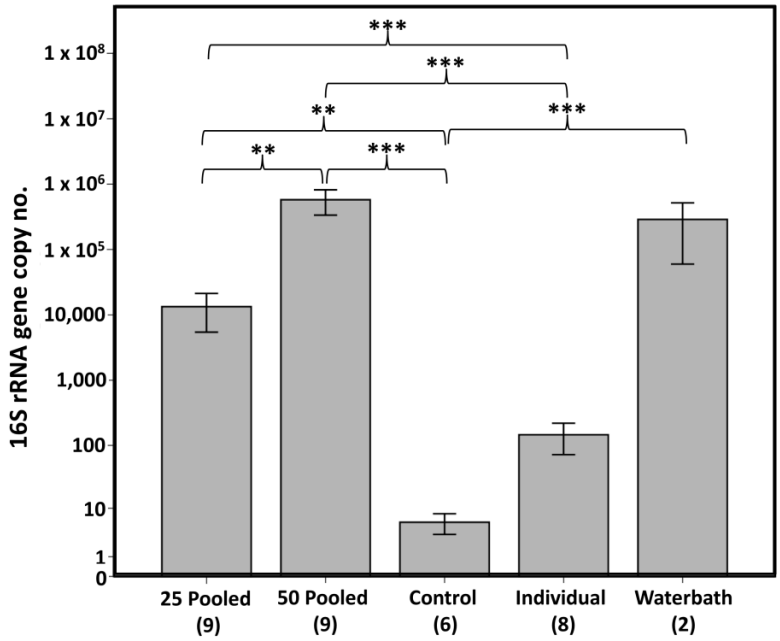

Figure 5.11 Boxplot showing analysis of differences in mean 16S rRNA gene copy among different sample groups as determined by qPCR (multiple pairwise comparisons after ANOVA with Tukey HSD correction post-hoc test). Numbers in brackets indicate the sample size of each group. (**) $p < 0.01$, (***) $p < 0.001$.

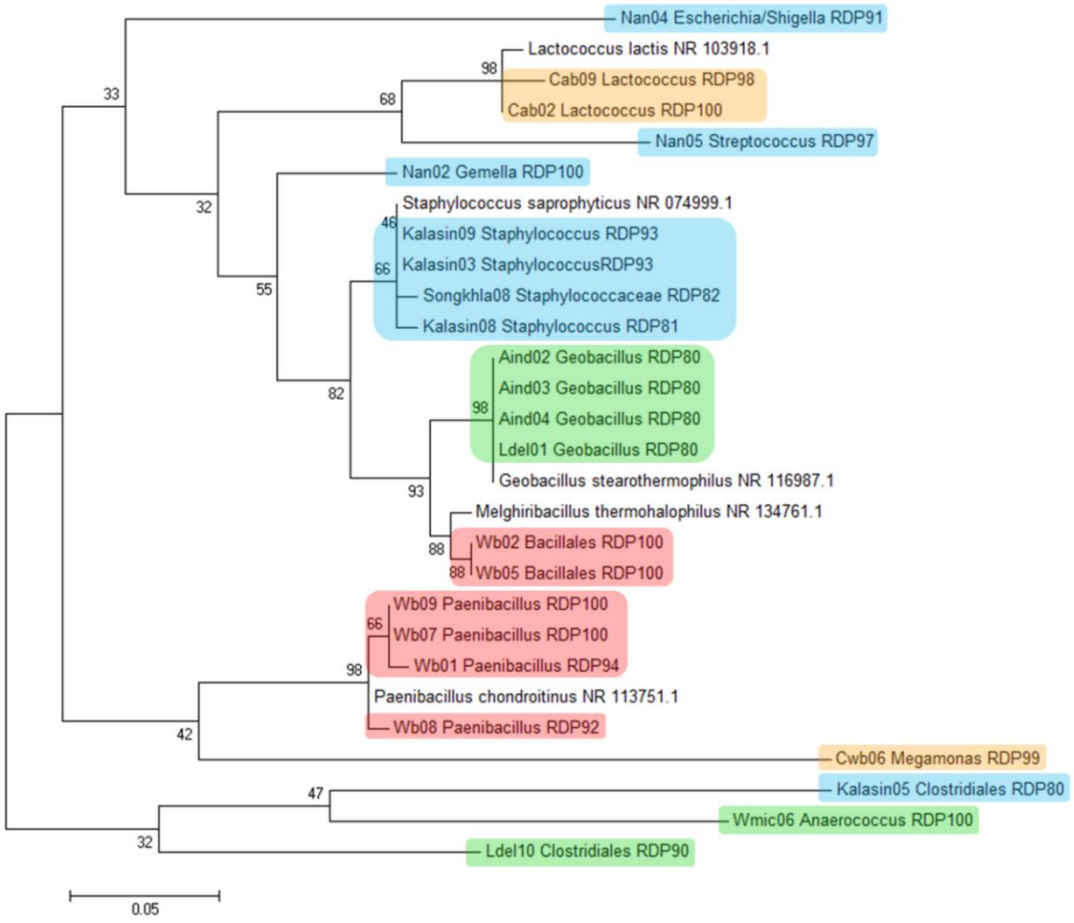

Figure 5.12 Maximum likelihood (ML) tree of partial 16S rRNA sequences among different sample groups (Red = waterbath samples, Green = individual chiggers, Blue = pooled chiggers and Orange = negative controls). Bacterial taxonomic assignment was given in each sequence with a confidence threshold of >80%. Sequences of *Geobacillus stearothermaphilus* (NR116987.1), *Lactococcus lactis* (NR103918.1), *Melghiribacillus thermohalophilus* (NR134761.1), *Paenibacillus chondroitinus* (NR113751.1) and *Staphylococcus saprophyticus* (NR074999.1) obtained from the nucleotide database (NCBI) were included for comparison. Phylogeny test with bootstrap values based on 1,000 replicates are presented at the nodes. The scale bar measures evolutionary distance indicating substitutions per nucleotide.

5.3.4 Bacterial diversity analyses

5.3.4.1 Alpha-diversity among sample groups and categories

Alpha-diversity of bacterial OTUs among different sample groups (individual chigger, pooled chiggers and soil) and categories (chigger species, habitat and study site) was determined through the richness estimator, chao1, and whole-tree phylogenetic diversity index, the PD_whole_tree (Table 5.5). Bacterial diversity was significantly different among the sample groups (KW chi-square = 38.65, df = 3, $p < 0.0001$). Individual chiggers showed significantly lower bacterial diversity than pooled chiggers and soil samples, but were not significant different when comparing to background controls (Figure 5.13). As stated previously, DNA template concentrations in individual samples were much lower than pooled chiggers and soil samples, even as low as the template of background controls in some cases. This could differential affect 16S rRNA PCR efficiency as pooled chiggers and soil potentially provided more diverse DNA templates in the PCR amplification. At both the individual and pool level, the results failed to show any significant difference in bacterial diversity among chigger species, habitats and sites.

5.3.4.2 Beta-diversity: bacterial community clustering among sample groups and categories

In comparisons among sample groups, although some slight overlap in bacterial community clustering occurred between background controls and individual chigger samples, the bacterial compositions in almost of the samples were significantly separated (ANOSIM: R = 0.7997, p =0.001) into their own clusters

(Figure 5.14). This shows low background contamination in the data after the data filtering step (Bray-Curtis similarity check between samples and controls) was applied.

Again, we found that pooling of chigger samples showed a better relative effect on the bacterial community component among different sample categories than the individuals (see ANOSIM results in Figure 5.15). Bacterial communities were significantly clustered with respect to chigger species and study sites in both individual and pooled chiggers, whereas habitat failed to show a significant effect (Figure 5.14). Study site and chigger species had similar effects on bacterial composition, implying that geographical differences and host phylogeny might be correlated factors determining bacterial communities in chiggers.

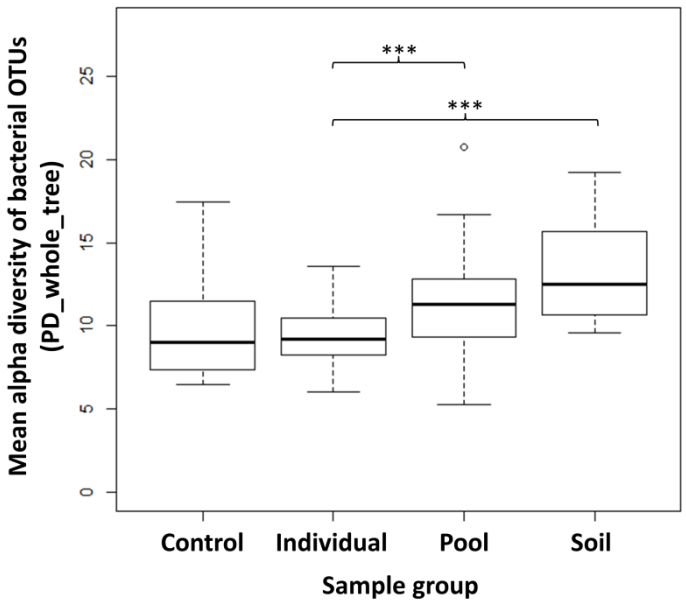

Figure 5.13 Boxplot shows analysis of difference in mean alpha diversity of bacterial OTUs (whole-tree phylogenetic diversity index) among different sample groups after non-parametric Kruskal-Wallis test with post-hoc Bonferroni correction. (***) $p < 0.001$

Table 5.5 Alpha-diversity estimation of bacterial OTUs among sample groups and categories

Sample Group (n)	Sample Categories (n)	Observed (SD)	Chao1 (SD)	PD_whole_tree (SD)
Individual chigger (109)		79.6 (14.7)	95.7 (18.7)	9.3 (1.5)
Chigger species:	*A. indica* (7)	90 (14.7)	111.1 (12.6)	10.1 (2.0)
	B. acuscutellaris (7)	71.1 (8.9)	83.7 (12.5)	8.2 (1.1)
	H. kohlsi (11)	73.5 (12.8)	89.4 (20.5)	9.3 (1.5)
	H. pilosa (10)	85.3 (9.6)	102.0 (15.6)	9.9 (1.4)
	L. deliense (39)	78.0 (16.2)	94.1 (20.1)	9.1 (1.4)
	S. ligula (10)	78.0 (11.6)	92.1 (16.7)	8.4 (0.8)
	W. kritochaeta (12)	76.8 (16.4)	96.2 (23.1)	8.9 (1.7)
	W. micropelta (13)	87.9 (12.2)	101.6 (11.5)	10.7 (1.4)
Habitat:	Forest (9)	74.2 (8.1)	93.8 (14.8)	8.7 (0.7)
	Dry land (48)	80.3 (15.2)	96.6 (19.2)	9.5 (1.5)
	Rain-fed land (28)	78.4 (11.9)	93.7 (17.1)	9.0 (1.4)
	Settlement (18)	79.9 (17.6)	96.0 (21.6)	9.3 (1.7)
Site:	Buriram (9)	89.6 (16.2)	103.3 (18.7)	10.5 (1.7)
	Chiangrai (7)	78.1 (13.2)	91.6 (21.3)	8.6 (0.9)
	Kalasin (12)	85.1 (14.1)	103.4 (20.9)	9.6 (1.2)
	Kanchanaburi (2)	83.0 (1.4)	90.5 (0.4)	10.0 (0.1)
	Loei (17)	80.9 (15.4)	95.7 (19.3)	9.5 (1.5)
	Nakhonsawan (3)	67.0 (6.2)	82.2 (5.2)	7.8 (0.5)
	Nan (10)	81.1 (16.8)	98.5 (20.5)	9.2 (2.3)
	Prachuab Kirikhan (17)	73.0 (15.2)	92.7 (17.1)	8.9 (1.5)
	Songkhla (15)	73.8 (13.2)	89.7 (17.1)	8.6 (1.1)
	Tak (17)	82.2 (12.0)	97.6 (19.3)	9.9 (1.4)
Pooled chigger (65)		108.8 (29.3)	132.2 (31.6)	11.3 (2.8)
Chigger species:	*A. indica* (8)	101.7 (33.7)	128.3 (38.9)	11.3 (3.8)
	L. deliense (12)	87.9 (22.2)	114.9 (16.2)	9.7 (1.8)
	W. micropelta (8)	124.2 (9.9)	151.2 (9.4)	12.7 (1.4)
	W. minuscuta (2)	178.5 (48.8)	195.7 (47.1)	18.2 (3.4)
Habitat:	Forest (3)	74.0 (13.8)	104.7 (6.1)	8.2 (1.3)
	Dry land (4)	130.0 (11.7)	147.4 (8.9)	13.2 (1.0)
	Rain-fed land (7)	102.1 (18.1)	131.0 (19.7)	11.0 (1.6)
	Settlement (4)	121.7 (29.5)	146.7 (30.7)	13.3 (3.2)
Site:	Buriram (5)	73.6 (13.7)	106.2 (24.5)	8.2 (1.3)
	Chantaburi (3)	117.0 (12.1)	168.1 (23.2)	13.0 (1.5)
	Chiangrai (3)	101.3 (18.1)	125.3 (23.2)	10.3 (2.2)
	Kalasin (3)	97.6 (12.0)	108.4 (24.5)	9.8 (1.0)
	Kanchanaburi (3)	90.3 (16.0)	102.8 (20.6)	9.5 (1.8)
	Loei (3)	113.6 (11.0)	129.0 (10.8)	11.1 (0.3)
	Nakhonsawan (3)	110.6 (13.7)	125.2 (18.2)	10.6 (1.3)
	Nan (3)	165.6 (11.0)	182.1 (9.7)	16.1 (0.8)
	Prachuab Kirikhan (3)	124.0 (13.0)	149.9 (7.8)	12.6 (1.3)
	Songkhla (3)	97.3 (8.5)	123.2 (20.2)	10.1 (1.1)
	Tak (3)	122.3 (9.5)	148.9 (16.9)	12.3 (0.9)
Soil (12)		109.8 (34.3)	150.6 (38.6)	13.1 (3.0)
Background control (15)		80.5 (33.7)	103.4 (40.9)	10.1 (3.8)

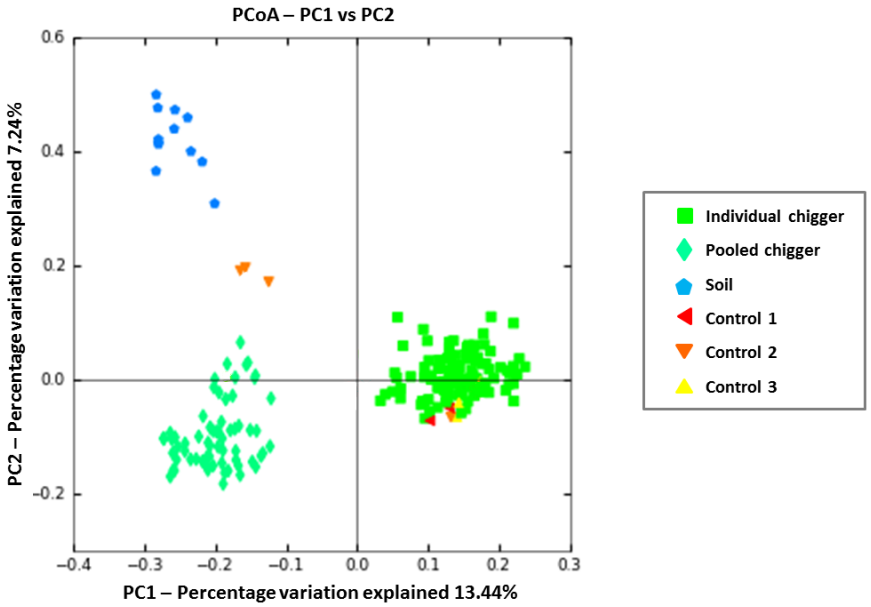

Figure 5.14 The PCoA plot was calculated from weighted UniFrac metric, and shows significant separation among sample groups (ANOSIM: R = 0.7997, p =0.001).

5.3.5 Visualisation of bacteria on chiggers using scanning electron microscopy

As a preliminary method to visualise chigger-associated bacteria, scanning electron microscopy (see materials and methods described previously in CHAPTER 3) was applied to 12 samples each of several chigger species (*i.e.*, *A. indica, B. acuscutellaris, L. deliense*, *W. lupella and W. pingue*). There was some evidence of vegetative cells and clusters of bacteria on *L. deliense*, *B. acuscutellaris* as well as unidentified putative bacterial spores on the surface of *W. pingue* (Figure 5.16). These images demonstrate that a variety of bacteria are present externally on chiggers and that the adherence to the cuticle was sufficiently strong to withstand washing and fixation procedures.

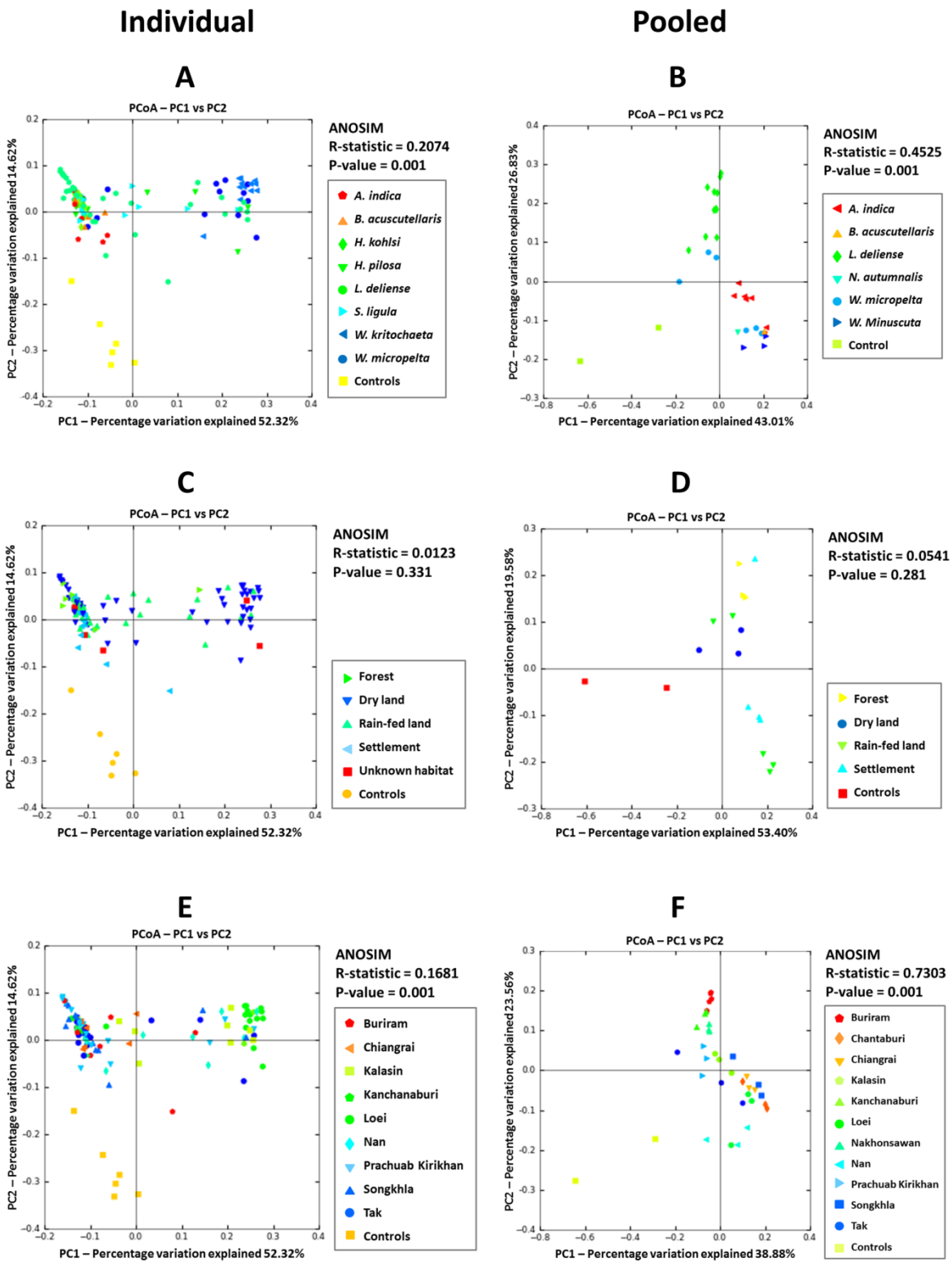

Figure 5.15 The PCoA plots were calculated from the unweighted UniFrac metric showing bacterial community clustering of individual (left panels) and pooled chiggers (right panel) among different sample categories: (A - B) chigger species, (C - D) habitat and (E - F) study site.

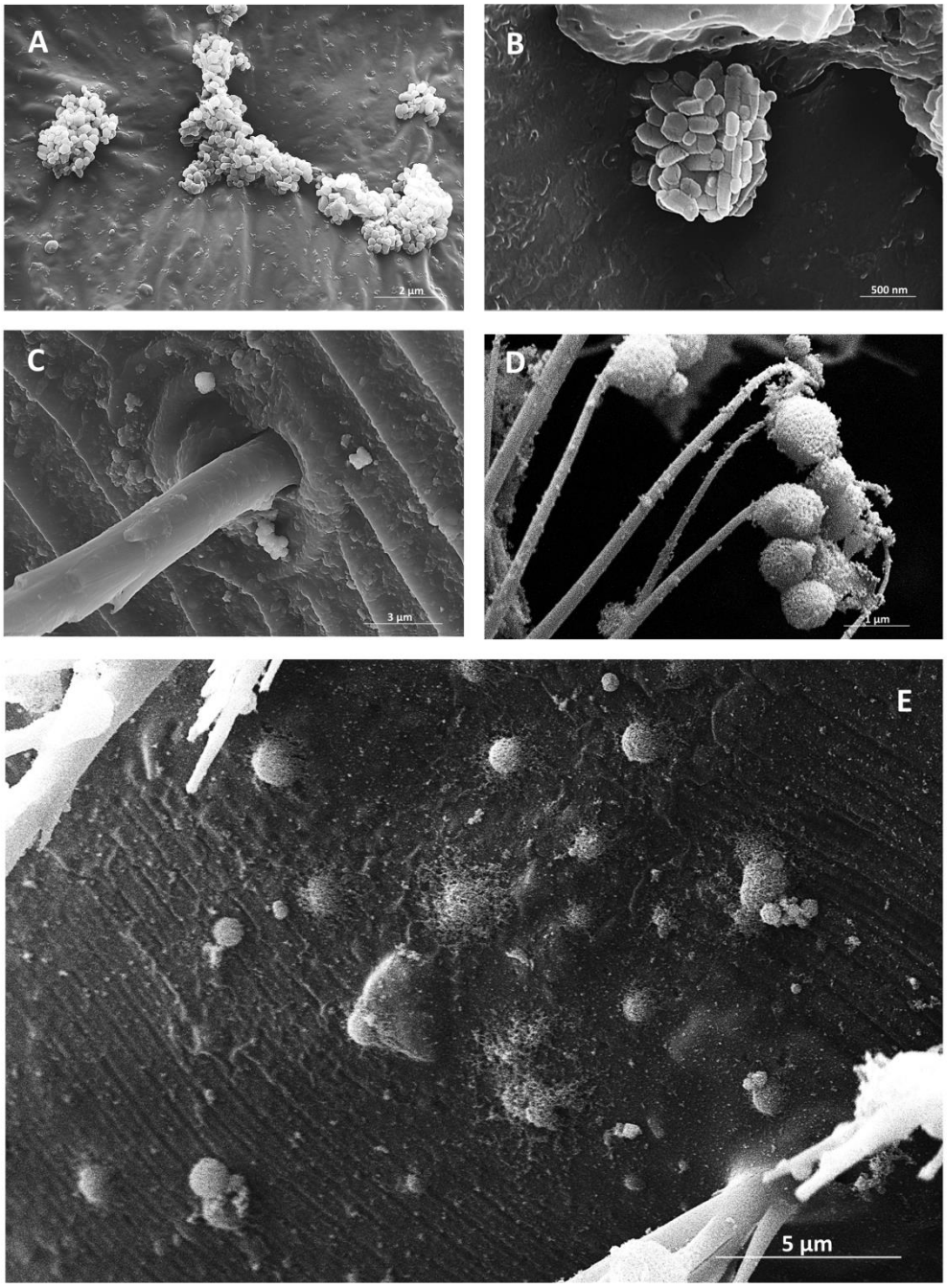

Figure 5.16 SEM micrographs showing evidence of vegetative cells of bacteria on the body surface of *L. deliense* (A - B) and *B. acuscutellaris* (C); and putative bacterial spores on leg setae (D) and the postero-dorsal part of the idiosoma (E) of *W. pingue*.

5.4 Discussion

5.4.1 Bacterial profiles between individual and pooled chigger species

The microbiome profile of trombiculid mites is poorly understood, as most of the bacteriological research on this mite taxon was focused only on the agent of scrub typhus disease, *O. tsutsugamushi* (Chaisiri, McGARRY, et al. 2015). Here, to investigate intensively the chigger-associated bacteria and their OTU structure for the first time, analyses of both individual and pooled chigger samples were carried out and compared (Figure 5.6 to 5.8). In addition, as the chigger lifestyle off the host is largely associated with the ground surface and deeper soil niches, a bacterial investigation of soil samples from Thailand and Laos was performed alongside the chigger samples (Figure 5.9).

5.4.1.1 Dominant bacteria in individual chiggers

In individual chiggers, several bacterial OTUs; *i.e.*, *Geobacillus, Sphingobium, Brevibacillus* and Comamonadaceae were dominantly represented in the samples. These bacteria are apparently the free-living bacteria which chiggers may acquire from the environment. The role of these bacteria in internal or external symbiotic relationships with arthropods or other invertebrates is still unknown. However, these bacteria were reported in several arthropod-associated microbiome studies as follows. Jousselin et al. (2016) found OTUs assigned as *Geobacillus, Brevibacillus* and Commamonadaceae in a study of the aphid genus *Cinara,* and suggested that these water- and soil-borne bacteria are probably contaminants from the environment. *Geobacillus* were also reported as associated bacteria in wild sandflies

Lutzomyia longipalpis and ants *Tetraponera penzigi* (McCarthy et al. 2011; Seipke et al. 2013). *Brevibacillus* was found in the honey bee *Apis mellifera* and parasitoid wasp *Asobara tabida* (Evans & Armstrong 2006; Zouache et al. 2009), and some species in this genus; *e.g*., *Brevibacillus laterosporus*, are known as entomopathogenic bacteria used in insect pest control against house flies, black flies and mosquitos (Rivers et al. 1991; Ruiu et al. 2006; Ruiu et al. 2013). *Sphingobium* were reported in plant-feeding insects; *e.g*., the walking stick *Ramulus artemis* and the beetle *Saperda vestita*, and is thought to play a role in digestion of plant cellulose in the insect gut (Delalibera et al. 2005; Schloss et al. 2006; Shelomi et al. 2013).

Geobacillus is a genus of Gram-positive spore-forming thermophilic bacteria firstly described from a high-temperature oil field (Nazina et al. 2001). According to a review of the genus in Zeigler (2014), these bacteria are incredibly abundant and occur worldwide across all of the seven continents, as well as the Pacific Ocean and Mediterranean Sea. Moreover, there is evidence for *Geobacillus* spores from >10,000 m height in the upper troposphere (DeLeon-Rodriguez et al. 2013) and as low as 3,000 m deep in oil wells and gold mines (Wang et al. 2006; Rastogi et al. 2009). These bacteria are capable of metabolizing long-chain hydrocarbons, and play a role in opportunistic decomposition of polysaccharide organic substances in the environment (Zeigler 2014). Therefore, *Geobacillus* spores are potentially found almost everywhere on Earth, including at high densities in soil worldwide (Marchant et al. 2002; Al-Hassan et al. 2011; Zeigler 2014), perhaps explaining their association with trombiculid chiggers. On the basis of *Geobacillus* evidence in our chigger

samples (see Results), we can conclude that the appearance of *Geobacillus* OTUs in individual chiggers originated in Thailand, but further investigations are required to determine whether the interaction between these bacteria and chiggers is genuinely symbiotic or rather results from environmental contamination.

As our experiment failed to culture *Geobacillus* from chigger samples, this unsuccessful result could be interpreted as either: (1) the culture conditions; *e.g.*, the temperature and duration of the incubation step were not suitable for the growth of the bacterial cells or spores; or (2) there was no *Geobacillus* in the chiggers collected from this particular location.

There were discernible patterns in bacterial OTUs between different chigger taxa. *Geobacillus* OTUs tended to be associated with chigger species from the subfamily Trombiculinae; *i.e.*, *Ascoschoengastia indica, Helenicula kohlsi, Blankaartia acuscutellais* and *Leptotrombidium deliense* (Figure 5.6B and 5.6C); whereas chiggers in the subfamily Gahrliepiinae, particularly the two species in the genus *Walchia* (*W. micropelta* and *W. kritochaeta*), were associated with bacteria in the Comamonadaceae (Figure 5.6A). This could be due to two different scenarios as follows: (1) if the bacterial taxa are actually closely associated with their chigger hosts, the symbiotic relationships could diverge selectively by evolutionary process; or (2) there is no enduring symbiotic relationship, but the bacteria occurred differentially on the chigger host taxa due to geographic variation in the sources of environmental contamination.

5.4.1.2 Dominant bacteria in pooled chiggers

In pooled chiggers, the dominant bacterial OTUs were Neisseriaceae, *Corynebacterium* and *Staphylococcus* (Figure 5.7 and 5.8). Both *Staphylococcus* spp. and *Corynebacterium* spp. are widely recorded in the microbiota of animals, particularly on the skin and mucosal membranes (Rasmussen et al. 2000; Callewaert et al. 2013; Rodrigues Hoffmann et al. 2014; Misic et al. 2015), and also they are occasionally present as a small microbial component in the environment (Reche & Fiuza 2005; Hanson et al. 2016). Chiggers may have acquired these two bacterial genera from the skin of the small mammal host. Another interesting dominant OTU was the Neisseriaceae, as several genera from this family are known to associate with animals as normal flora or pathogens (Todar 2012). *Snodgrassella alvi,* a newly described bacterial symbiont discovered in bees, is also a member of the Neisseriaceae (Kwong & Moran 2013). Following this precedent, it could be hypothesized that the Neisseriaceae OTUs may represent a strong candidate for chigger symbionts, and need to be investigated in further studies.

There were differences in bacterial profiles both between chigger species and study sites. In terms of chigger species, Neisseriaceae clearly presented in *L. deliense* whereas different *Corynebacterium* OTUs were apparent in *A. indica* and *Walchia minuscuta* (Figure 5.7). Hence, host phylogeny might play some role in chigger-microbe interactions. Regarding geographical differences, the bacterial profile in harvest mites (*Neotrombicula autumnalis* from the UK) was completely distinct from that of the Thai chigger specimens. Conversely, along the 11 provinces of Thailand, the bacterial composition tended to be similar within the same study

sites (Figure 5.8). This indicates once more that apart from the effect of host phylogeny, the bacterial profile of chiggers could be influenced by geographical distribution.

From the observations above, bacterial profiles showed important differences between individual and pooled samples. Several sources of bias could explain this discrepancy, particularly during the PCR amplification step in 16S rRNA library preparation such as: (1) DNA template concentrations (Wu et al. 2010; Kennedy et al. 2014) – individual chiggers being much lower than pools; (2) PCR polymerases from different companies as stated in Materials & Methods (Wu et al. 2010; Ahn et al. 2012); and (3) the GC content of the DNA template inducing preferential amplification (Pinto & Raskin 2012; Benítez-Páez et al. 2016). This makes the results difficult to compare across individuals and pools, but comparative analyses of the microbiome among categories (chigger species, habitat and site) should rather be performed separately within individual and pooled groups.

5.4.1.3 Effect of GC content bias in microbiome data between individual and pooled samples

PCR amplification of high-GC DNA templates is usually less efficient compared to lower-GC targets. This amplification bias results from competitive annealing of primers to the templates (McDowell et al. 1998; Mamedov et al. 2008). The nucleotide bases G and C are paired with three hydrogen bonds, which are strongly stiffened and more difficult to separate during denaturation than the two hydrogen bonds between A and T. As showed in the results, the GC content of OTUs

from individual chiggers was higher compared to those from pooled chiggers; for example, the GC content between *Geobacillus* (59%) or Comamonadaceae (57%) from individuals was clearly higher than *Staphylococcus* (52%) or Neisseriaceae (53%). In pooled samples with high DNA template concentration, *Geobacillus* and Comamonadaceae probably failed to compete with the other lower-GC templates, but succeeded in individual samples with lesser competitors in the reaction.

5.4.2 Potential pathogenic and symbiotic bacteria in chiggers

In addition to *O. tsutsugamushi*, some other potentially pathogenic bacteria; *i.e.*, *Borrelia* and *Mycobacterium* were presented in chigger samples. In both individual and pooled samples*, O. tsutsugamushi* was strictly found in *Leptotrombidium deliense*, the main vector of scrub typhus in Thailand. Previous results on 56 kDa gene detection indicated that *O. tsutsugamushi* was widely distributed in various trombiculid genera *e.g. Eushoengastia, Helenicula, Leptotrombidium, Neotrombicula* and *Walchia* (Pham et al. 2001; Liu et al. 2004; Lee et al. 2011; Park et al. 2015), which is in contrast to our result. This might be because different molecular detection methods (gene targets 56 kDa versus 16S rRNA genes) were applied. In this case, it might be that either (1) The *Orientia*-specific gene target (56 kDa) performs better in *Orientia* detection than universal 16S rRNA gene targeting and/or (2) the *O. tsutsugamushi* infection status in Thai chiggers outside the genus *Leptotrombidium* was too low to be detected with the sample sizes we attained here.

In addition, assessing the true prevalence of *O. tsutsugamushi* infection in chiggers is challenging, as almost all previous studies detected DNA of the bacterial infection from pooled chiggers (mass detection), with individual samples providing too little DNA. However, the number of chigger specimens testedvaried in different studies (*e.g*., 30, 50, or 100 chiggers in a pool), and could lead to biased calculations of prevalence. Therefore, the minimum positive rate (MPR), the number of mites in positive pooled samples divided by the total number of mites examined in all pooled samples, was then used to estimate *O. tsutsugamushi* infection rate in pooled chiggers (Tanskul et al. 1994; Shim et al. 2009). In the present study, from 65 chigger pools (3,250 individuals) examined, *O. tsutsugamushi* was detected in 13 pools (650 individuals). Thus, the MPR of *O. tsutsugamushi* infection was 0.2, slightly lower than previous studies, which varied from 0.5 to 1 in China, Korea, Malaysia and Thailand (Roberts et al. 1977; Tanskul et al. 1994; Shim et al. 2009; Zhang et al. 2013).

Apart from its pathogenicity causing disease in vertebrates, *Orientia* associates with their chigger hosts as a bacterial symbiont. There is strong evidence of vertical transmission from parent to the next generations (S P Frances et al. 2001; Shin et al. 2014). In addition, there are some reports showing the bacteria can act as reproductive manipulators by distorting sex-ratio (male-killing) and reducing egg productivity in female chiggers (Roberts et al. 1977; Phasomkusolsil et al. 2012).

Here, we report first time the evidence of *Borrelia* in trombiculid mites from Southeast Asia, although the bacteria have been reported previously in harvest mites (*Neotrombicula autumnalis*) from Germany and the Czech Republic (Kampen

et al. 2004; Literak et al. 2008). Several species of this genus are known as the causative agents of borrelliosis such as Lyme disease; *e.g*., *B. burgdorferi* and relapsing fever; *e.g*., *B. hermsii* (Schwan & Piesman 2002; Tilly et al. 2008). Small mammals, birds and lizards have been known as important reservoirs for these bacteria, and the knowledge of potential arthropod vector of these zoonotic disease agents was limited to ticks and lice (Masuzawa 2004; Norte et al. 2015). Although *Borrelia* infection is globally distributed in America, Africa, Europe, Eastern Asia and Australia, research information on these bacteria and borreliosis in Southeast Asia is still very limit (Masuzawa 2004; Lindgren & Jaenson 2006; Qiu et al. 2008; Mayne 2011; Trape et al. 2013). Thus, our results indicate that chiggers could potentially play some role in *Borrelia* transmission, causing uncharacterized borrelliosis or febrile illness to humans and animals in Thailand and other countries in the region.

Mycobacterium spp. was another potential pathogenic bacterium found in chigger and soil samples in the present study. The bacteria in this genus are widespread in the environment (air, water and soil), and some of them are known as causative agents of serious diseases in humans and animals; *e.g*., *M. tuberculosis*, *M. bovis* and *M. leprae* causing human tuberculosis, cattle tuberculosis and leprosy, respectively (LoBue et al. 2010; Hruska & Kaevska 2012; Bratschi et al. 2015). Moreover, several species of non-tuberculous mycobacteria from the environment; *e.g*., *M. avium* complex, *M. abscessus, M. kansasii,* and *M. simiae* have been increasingly recognized worldwide as atypical mycobacteria causing pulmonary disease (Chan & Iseman 2013; Johnson & Odell 2014). Here, *Mycobacterium* was found to be highly abundant in all soil samples (100%) from Thailand and Lao PDR,

and was also well represented in 48 pooled chigger samples (73.85%). This result strongly suggests that chiggers gained *Mycobacterium* spp. from soil. Although the type of relationship between chiggers and *Mycobacterium* is unknown and further investigations are required, chiggers appear to be reservoirs of *Mycobacterium* and might play some role in bacterial transmission in the environment.

Similar to *O. tsutsugamushi,* these two bacterial genera, *Borrelia* and *Mycobacterium,* could be hypothesized to be potential symbionts of chiggers as they were highly abundant and prevalent in chigger samples (see Table 5.3). However, further studies are still needed to assess the effect of these bacteria on their chigger hosts.

For other potential arthropod symbionts, several bacteria were found in chiggers including: Candidatus *Cardinium*, *Pseudonocardia, Rickettsiella* and *Wolbachia* (Table 5.3). *Cardinium* and *Wolbachia* are the very well-studied symbionts of arthropod hosts (see more details in CHAPTER 2). These two bacteria have been known to alter their host reproduction in several ways; *e.g*., inducing cytoplasmic incompatibility, parthenogenesis, male killing and feminization (Gotoh et al. 2006; Hoy & Jeyaprakash 2008; Werren et al. 2008; Zhang et al. 2015). *Pseudonocardia* is the external symbiont associated with the complex symbiosis system of fungus-growing ants. The bacteria are housed in a specialized structure on the cuticle of the worker ants. The ants cultivate fungi in a specialized "garden" as their primary food source. At the same time, they have to compete with their natural enemy, microfungi *Escovopsis*, which is a fungal parasite exploiting the ant's fungal cultivar. In this event, *Psudonocardia* provide antimycotics to supress the

parasitic fungus and thus help in garden defence, whereas the ants appear to provide nutrition for bacteria through glandular secretion (Cafaro et al. 2011; Marsh et al. 2013). The genus *Rickettsiella* is an intracellular bacterium associated with several arthropods such as ticks, mites and insects (Schütte & Dicke 2008; Simon et al. 2011; Bouchon et al. 2012; Leclerque & Kleespies 2012). Although the bacteria have been mostly recognized as an arthropod pathogen, there is a somewhat mutualistic interaction of a *Rickettsiella* with the pea aphid, *Acyrthosiphon pisum*, which has been reported to induce a change in the aphid's body colour (Tsuchida et al. 2010).

The results of potential pathogenic bacteria reported alongside arthropod symbionts in this study shed new light on chigger-associated bacteria, and several of these bacterial taxa may become the focus of further studies. We suggest that apart from *O. tsutsugamushi,* chiggers could play a role as a vector or reservoir of *Borrelia* transmission in Thailand. As *Borrelia* can cause febrile symptoms similar to the other endemic bacterial pathogens; *e.g*., *Orientia* (scrub typhus) and *Leptospira* (leptospirosis) in Thailand, future epidemiological research should consider *Borrelia* as a potential agent for emerging febrile illness in humans. In addition to *Cardinium, Pseudonocardia, Rickettsiella* and *Wolbachia,* the other dominant bacterial OTUs; *e.g*., *Geobacillus* and *Neisseriaceae*, could also be candidates for characterization of chigger-symbiont relationships and perhaps application to a chigger vector control strategy in the future.

5.4.3 Potential contaminating OTUs from background controls

Potentially thirty-four bacterial contaminants were identified from the background controls as shown in Table 5.4. About one-third of those have been reported previously as potential contaminants in water and reagents used in DNA extraction and PCR amplification processes (Tanner et al. 1998; Grahn et al. 2003; Barton et al. 2006; Salter et al. 2014; Galan et al. 2016). The OTUs of genera *Fimbriimonas, Streptococcus* and *Lactobacillus;* families Bradyrhizobiaceae and Polyangiaceae; and Phylum Cyanobacteria were likely the source of nuclease-free water and PCR reagent contaminants, as they were dominant in control 3 (nuclease-free water not passed through the DNA extraction process). Whereas, OTUs of the species *Bacillus thermoamylovorans* and *Luteibacter rhizovicinus;* genera *Cloacibacterium*, *Corynebacterium, Flavobacterium, Fluviicola, Hydrogenophilus, Thermicanus* and *Wautersiella;* and family Cytophagaceae were the potential source of contamination from the DNA extraction kit in control 2 (DNA extraction without chigger sample). However, control 1 (equipment and lab bench-washed water through DNA extraction process) did not show an informative result, as no OTUs uniquely presented in this control, although some of them (*e.g.*, *Methylobacterium organophilum* and *Ochrobactrum*) rather appeared alongside control 2 and 3.

5.5 Conclusions

In summary, the result of this microbiome investigation in trombiculid larvae showed different dominant bacteria between individual chiggers; *i.e.*, *Geobacillus* and Comamonadaceae, and pooled chiggers; *i.e.*, *Corynebacterium* and

Neisseriaceae. A substantial difference in DNA template concentrations between individual and pooled samples probably played a crucial role for this bias in PCR amplification of 16S rRNA library preparation prior to next generation sequencing. The GC content of the bacterial DNA template in samples was another important factor driving the bias between individuals and pools. Although pooled samples seem to exhibit potentially more reliable microbiome data, particularly in comparative analyses, some informative results from individuals should not be ignored. Here, we suggest that our studies combining individual and pooled samples benefited the microbiome survey by characterizing the full picture of chigger-associated bacteria, whereas data from pooled samples are recommended for use in comparative analyses. In terms of bacterial communities in chiggers, we found that they appeared to differ due to influences from host phylogeny and geographical factors, but the relative contribution of each was difficult to separate. For the other chigger-associated bacteria, the causative agent of scrub typhus disease, *O. tsutsugamushi* was present in the samples as expected, whereas *Borrelia* and *Mycobacterium* appeared as the potential pathogens of humans and animals. Some other symbiotic bacteria of arthropods were also found in chiggers; *e.g.*, Candidatus *Cardinium*, *Pseudonocardia*, *Rickettsiella* and *Wolbachia*. These preliminary findings should assist further surveillance of other potential emerging diseases transmitted by trombiculid chiggers in the region, and deeper understanding of chigger-symbiont relationships may also facilitate in development of biological tools for vector control.

CHAPTER 6

PRELIMINARY STUDIES AND DIRECTIONS FOR FURTHER RESEARCH

CHAPTER 6

Preliminary studies and directions for further research

There are some unfinished and inconclusive works that could be followed-up in the future, as well as other suggested perspectives for further studies after the thesis is completed. The three following works: (1) whole genome project of *L. deliense*, (2) DNA microsatellite characterization of *L. deliense* and (3) modified-in situ hybridization with TEM to detect intracellular bacteria have been started at the late stage of my PhD thesis. Unfortunately, it was not possible to complete these projects in the available timeframe.

6.1 The draft genome of *Leptotrombidium deliense*

The whole genome project of *L. deliense* has been initially established as a collaborative work between Dr Ben Makepeace (my primary supervisor) and Dr Alistair Darby (Institute of Integrative Biology, University of Liverpool). In September 2015, *L. deliense* were isolated from ground squirrels (*Menetes berdmorei*), captured in an extra field survey of the CERoPath project at Udonthani province, Thailand. Genomic DNA was extracted from 50 pooled chiggers using the DNeasy Blood & Tissue Kit (QIAGEN), and quantified using a fluorescent-labelling method, Quant-iT^{TM} Picogreen dsDNA kit (Invitrogen) as described in CHAPTERS 3 and 5. Subsequently, the DNA sample was checked for size and intactness by running a 2% agarose gel at 100V for 1 hour (Figure 6.1) prior to submission for whole genome sequencing at the Centre for Genomic Research (CGR), University of Liverpool.

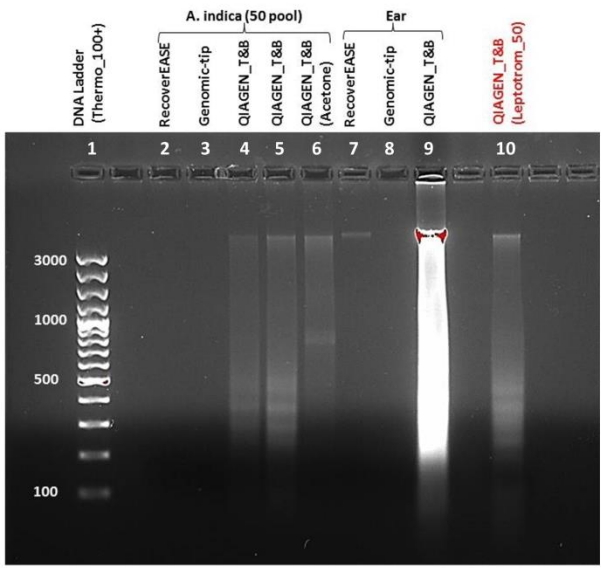

Figure 6.1 Gel electrophoresis illustrates genomic DNA of a 50-pooled *L. deliense* sample extracted using the DNeasy Blood & Tissue Kit (Lane 10). Lane 1 = DNA ladder; Lane 2 to 6 = 50-pooled *Ascoschoengastia indica* samples extracted using different DNA extraction kits. Lane 7 to 9 = rodent ear samples extracted using different DNA extraction kits.

Whole genome sequencing procedures and post-sequencing bioinformatic analyses were entirely done by CGR staff (Dr Luca Lenzi, Dr Anita Lucaci and Charlotte Nelson). The chigger DNA material was subjected to sequencing library preparation using the NEBNext Ultra DNA library Preparation Kit (Illumina). The sequencing was carried out on the Illumina MiSeq with 2 x 250 bp paired-end sequencing. After trimming, quality filter and error correction, the paired-end reads were assembled *de novo* using the Discovar platform, and the contigs shorter than 500 bp in length were excluded. The genome of the thirteen-lined ground squirrel, *Ictidomys tridecemlineatus* (from http://www.ensembl.org/index.html), was used to screen potential DNA contamination from the host. The completeness of the final

genome assembly was assessed using CEGMA version 2.5 (Core Eukaryotic Genes Mapping Approach), in order to evaluate whether the genome is completely assembled by aligning the Key Orthologs for eukaryotic Genomes (KOGs) onto the assembly.

6.2 DNA microsatellite characterization of *Leptotrombidium deliense*

DNA microsatellites or short tandem repeats (STRs) are nucleotide repeating units of 2 - 6 bp in length, widely present throughout the nuclear genome of eukaryotic organisms. Because of high DNA polymorphism and variation in repeat number (allele polymorphism), microsatellites have become a major group of genetic markers for population genetic inferences in a variety of species (Bhargava & Fuentes 2010; Putman & Carbone 2014; Queiros et al. 2015). As the chigger, *L. deliense,* has been recognized as the main vector of scrub typhus disease in Thailand and countries of Southeast Asia, understanding the population genetics of this mite species; *e.g*., genetic diversity, population structure, ecological speciation, phylogeography and population gene flow would be of major benefit to epidemiological studies of scrub typhus disease in the region.

Draft genome data from high throughput sequencing has been recognized as a potential source to identify microsatellite loci for population genetic studies (Castoe et al. 2012; Cwiklinski et al. 2015). To characterize DNA microsatellite markers, first STRs (di-, tri-, tetra- and penta-nucleotide repeats) were searched and identified throughout the 68 Mb draft genome sequence data of *L. deliense* using MSATCOMMANDER software (Faircloth 2008). In total, 5,501 tandem repeats were

initially identified in the whole genome data. To select microsatellites for further characterization, the tandem repeats were filtered following these criteria: (1) only tri- and tetra-microstaellites were included to avoid strand slippage problems; (2) only >10 repeat copies were included; (3) only perfect microsatellites (with a repeat sequence that is not interrupted by any base not belonging to the motif) were included; and (4) any compound/imperfect microsatellites and microsatellites that were located very close to each other (<1,000 bp) on the same scaffold were excluded. With these filtering criteria, seventeen microsatellite loci were retained, and primer design aiming to amplify the flanking regions of the 17 microsatellites was done using Primer 3 web version 4.0 on the website http://primer3.ut.ee (Untergasser et al. 2012). Primer selection criteria were set to select the product size at Min = 150, Opt = 200, Max = 350 and the primer size at Min = 18, Opt = 20 and Max = 23. Primer sequences of the 17 microsatellite loci are presented in Table 6.1.

6.3 Modified *in-situ* hybridization detecting intracellular bacteria of *Leptotrombidium deliense* with transmission electron microscopy

A combination of a modified *in-situ* hybridization technique and immunogold-labelling with transmission electron microscopy (TEM) were applied, aiming to detect intracellular bacteria in *L. deliense* (Lherminier et al. 1999; Thimm & Tebbe 2003; Kennaway et al. 2004; Anantasomboon et al. 2008). Chiggers were isolated from host skin and preserved immediately in a pre-cooled mixture of 4% paraformaldehyde and 0.1% glutaraldehyde dissolved in 0.2 M Millonig's buffer at pH 7.4. Briefly, the preparation for ultrathin sectioning was as follows: the specimens were dehydrated in ethanol concentration gradients (*i.e.*, 30%, 50%,

70%, 90% and 100%) and embedded in LR White Resin prior to sectioning at 75 nm using a Leica UC6 UltraMicrotome (Leica Microsystems). Sections were collected on formvar coated 300 mesh copper grids. The sections were pre-digested with 50 µg/ml pepsin glycine buffer for 5 min and hybridized in a hybridization buffer containing formamide (30%), 40 mM PIPES buffer, 0.1% FicoII 400, 0.2% SDS, 10 mM EDTA, 0.1% bovine serum albumin, 0.14 M NaCl and 20 nmol of universal bacterial 16S rRNA oligonucleotide probe-labelled with biotin [Btn-GUACAAGGCCCGGGAACGUAUUCACCG-Btn (Greisen et al. 1994)] (Sigma-Aldrich) for 2, 6, 8 or 12 hours in a 37^{o}C hybridizing chamber. A random biotin-labelled oligonucleotide, not matching any 16S rRNA gene on the Ribosomal Database Project (RDP), was used as negative control probe (Btn-ACCGAGACACACGCGACUGGUGACGUU-Btn). Following *in-situ* hybridization, the grids were immunogold-labelled. Grids were first washed in 0.1 M sodium phosphate buffer, blocked for 20 minutes with 0.1% BSA followed by a 4 hour incubation in goat anti-biotin 20 nm gold conjugate (BBI Solutions, Cardiff, UK) diluted 1:4 and 1:6 with 0.1% BSA at 36^{o}C. The grids were washed in sterile water to remove unbound gold conjugate and subsequently stained with 4% aqueous uranyl acetate and Reynolds lead citrate. Finally, the sections were imaged using a FEI Tecnai G2 Spirit 120KV bioTWIN TEM equipped with a SIS Megaview III camera. A pellet of *E. coli* JM109 competent cells was processed alongside the chigger samples, and used as a positive control (with 16S rRNA oligonucleotide probe hybridization) and negative control (with random probe hybridization). Ultrathin sections of chigger and *E. coli* samples, immunogold labelling and TEM were

performed by Dr Alison Beckett at the Biomedical EM Unit, School of Biomedical Sciences, University of Liverpool.

6.4 Results and Discussions

For the whole genome of *L. deliense*, the CEGMA result showed 73.39% and 83.87% of complete and partial KOGs successfully aligned to the assembly, respectively. The final assembly was annotated using MAKER version 2.31.8 with transcripts and protein sequences of a bee mite (*Tropilaelaps mercedesae*), a spider mite (*Tetranychus urticae*), and a velvet mite (*Dinothrombium tinctorium*), as well as the Uniprot/Swissprot protein database as a reference for gene identification. In summary, the total size of the draft genome of *L. deliense* is around 68 Mb containing 28,287 scaffolds (size range, 500 - 66,176 bp). Protein analysis showed that 12,521 transcripts were predicted to contain known protein family domains.

In order to further optimise the assembly, the whole genome is currently being re-analysed by a PhD student, Xiaofeng Dong, under the supervision of Dr Alistair Darby, to improve the discrimination between contigs of mite and host origin. This ongoing project is the first to sequence the genome of a trombiculid mite, the vector of scrub typhus disease, and the genome will substantially benefit the future of chigger mite research.

For the characterization of microsatellite primers, PCR conditions of each primer set were initially optimised to amplify those microsatellite loci from individual *L. deliense* samples. However, preliminary attempts to amplify these loci failed and further optimisation was not possible within the available timeframe.

This is probably due to the challenges of the specimen itself; *i.e*., the very low DNA template concentration of individual mites (mean = 0.025 ng/µl, N = 125) or the quality of DNA templates, such as degradation and PCR inhibitor issues.

After an initial attempt to conduct *in situ* hybridization of 16S rRNA probe detecting universal bacteria on *E. coli* samples, immunogold labelling failed to produce an unambiguous bacterial signal. Gold beads were equally present in the bacteria cells and the background of both *E. coli*-positive (16S rRNA probe) and negative (random probe) controls. Also, we found that prolonged incubation during in situ hybridization (>6 hours) damaged cytomorphological structures of chiggers and *E. coli* samples. It was unclear whether the problem was caused by the incubation time or concentration and composition of reagents in the hybridization buffer. Accordingly, several steps obviously needed optimization, such as pepsin pre-digestion to facilitate probe accession into the samples; in-situ hybridizing conditions (*e.g*., concentration of formamide, salt and detergent), as well as temperature and time of incubation; immunogold labelling conditions; and all associated washing steps.

These three projects above produced important preliminary information after the initial attempts described here, but obtaining definitive results was not possible within the time constraints of this thesis. However, these three areas represent priorities for further exploration and set the scene for chigger research in the years ahead.

Table 6.1 Primer sequences of 17 *L. deliense* microsatellite loci designed from *L. deliense* draft whole genome sequencing data.

Locus name	Scaffold ID|size(bp)	Repeat	Copy	Product (bp)	FW primer (5'-3')	Size (bp)	Tm (°C)	RV primer (5'-3')	Size (bp)	Tm (°C)
Ldemic01	scaffold4838|3280	(GCT)	10	180	TGACAATGTGATACAGCAGCAG	22	59	GAATGATGTTGACCGATGTTGC	22	58.5
Ldemic02	scaffold278|19996	(AAC)	15	183	AGGCAGCATCAAAACGAACA	20	58.6	CATCGTAGTTGTTGCCGTGT	20	58.8
Ldemic03	scaffold213|22487	(AAC)	16	202	ACCGTGGGATCATACGTTGT	20	59.1	TTCTGCGTCGTTCAACATTG	20	57.3
Ldemic04	scaffold38|39157	(AAC)	12	210	TGATGCGCGTATTGGAGAAC	20	58.1	TGCTGTTTATGTTGCTGCTGT	21	59
Ldemic05	scaffold278|19996	(AGC)	11	216	ATTCGACGTAACGCAGCAAG	20	59.2	TGTTGTTGTGGTTGTGGCAA	20	59
Ldemic06	scaffold373|17549	(AAC)	10	219	CGTGTCAACTCAGCACCAAT	20	58.7	TCTGCAATTTAGTGGTTGTTGC	22	58
Ldemic07	scaffold1845|7216	(GTTT)	11	222	TCCAGCTGTTCGAACCTCAT	20	59	CGTTCCGTTCGCACTTATTG	20	57.8
Ldemic08	scaffold73|32327	(AAG)	12	238	TGCGAAACGACCATGTTCAA	20	58.7	GCTCATCACACAACGAAAGC	20	58
Ldemic09	scaffold8009|2050	(AAG)	12	255	GAGAAACGTTGCTGCAGTGT	20	59.3	GGTCCTCGATCAGCAGGTAA	20	58.8
Ldemic10	scaffold819|12062	(GTT)	16	257	ATCTCATCGTTTCGGGCTTC	20	58	AGCTGCGCATTGAACAATCA	20	59.1
Ldemic11	scaffold1029|10562	(ATC)	12	263	CAAAGCAACGCATTTCAGCA	20	58.1	AATGAAATTCGAACCGCCGT	20	58.8
Ldemic12	scaffold385|17449	(GTT)	14	268	GGTGACGTCATTTGCGAAGT	20	59.1	TCACCGTCGGACAATTGGTA	20	59
Ldemic13	scaffold985|10812	(GAT)	14	270	TGTGTGCGTGTGTGTTTGAA	20	59.1	AATCGTGTAACTCCATCGCA	20	57.3
Ldemic14	scaffold23672|686	(ATT)	13	270	ACACACGACTAGAATCAACACG	22	58.6	AATGAGCAACGACGGAATGG	20	58.9
Ldemic15	scaffold8009|2050	(AGC)	11	295	GGCAAACGCTACTACTTCGG	20	59	ACTTGCGTGCACTGTACTTG	20	59
Ldemic16	scaffold278|19996	(GAT)	16	319	AAGCTCCGTTGAAAGCGTTT	20	58.9	CAACGTCGAAGCTTACGTGT	20	58.8
Ldemic17	scaffold15490|1201	(AAT)	11	350	GCCAGCTTTATTTGTCGTGC	20	58.3	CCACAAAAGGCGGCATATCA	20	58.9

CHAPTER 7

FINAL CONCLUSION, PERSPECTIVES AND FUTURE WORK

CHAPTER 7

Final conclusion, perspectives and future work

This final chapter aims to synthesise the findings of this comprehensive research on trombiculid chiggers in Thailand. This thesis includes a variety of topics ranging across the biology and ecology of the parasitic larval mites on small mammal hosts, the host-chigger interaction, the role of chiggers in scrub typhus epidemiology and profiling the microbiome of chigger populations. These outcomes will be of benefit for the chigger research field, providing fundamental information for vector control and further epidemiological studies. The limitations and challenging points of the present study are noted and discussed.

7.1 Final discussion and conclusions

7.1.1 Diversity of chiggers in Thailand

In a combination of a literature review starting from the 1960s, and extensive field surveys of trombiculid mites parasitizing small mammals across Thailand, a high diversity of chiggers was reported in the country. The list of trombiculid species was updated to 99 species (two subfamilies and 27 genera), including 10 new records for the country, and three new species (*i.e.*, *Helenicula naresuani*, *Trombiculindus kosapani* and *Walchia chavali*) were also described from materials obtained from the field surveys (CERoPath and BioDivHealthSEA project). Some dominant species such as *Ascoschoengastia indica, Blankaartia acuscutellaris, Leptotrombidium deliense, Walchia lupella* and *Walchia pingue* were widely

distributed in several geographical regions of Thailand. *Leptotrombidium* appeared to be the dominant genus with 21 recorded species, and several species in this genus have been recognized as the main vectors of scrub typhus in the disease-endemic areas (Strickman 2001; Stekolnikov 2013). There was potential evidence of speciation of *Helenicula* originating in Thailand, as several closely related species in the genus showed a high level of endemism, and have never been reported outside the country (Chaisiri et al. 2016).

Although the present study provides a comprehensive species checklist of chiggers, the data are mainly focussed on the chiggers parasitizing small mammals; *e.g*., rodents and insectivores, whereas chigger communities on the other animal hosts such as birds, reptiles and large mammals as well as unfed chiggers in the environment are scarcely reported (but see Nadchatram & Lakshana 1965; Nadchatram 1967; Nadchatram & Kethley 1974; Tanskul 1991). Another important point is that the systematics and classification of chiggers is entirely based on the morphology of the larval stage (Vercammen-Grandjean et al. 1973), and adults and nymphs are rarely examined. A potential research question regarding phenotypic plasticity can be raised about the accuracy of the reported diversity of the mite taxa; that is, whether slight differences in morphology of some closely-related larval species (*e.g*., >300 species in the genus *Leptotrombidium*) develop to distinct or the same species at the adult stage. Thus, future surveys need to design research strategies covering those aspects in order to assess more accurately the diversity of chiggers in the region.

7.1.2 Towards a combined morphometric and molecular approach to chigger identification

Information about the genetics of chiggers is very limited, particularly for specimens collected from the wild. A challenging point of chigger taxonomy is to link together phenotypic and genetic information. This is due to the limitations inherent in the nature of the chigger specimen itself. Due to their tiny size, specimens require microscopic examination to obtain a species identification. Slide preparation and specimen clearing in mounting medium are therefore inevitably involved prior to morphological identification. This causes difficulties in extracting genetic material from the same individual on the slide, as chemical agents used in the clearing step can damage genetic material.

Here, we found that using a fluorescent light source with a FITC filter during microscopy could be an alternative method enhancing visualization of chigger specimens in water without the use of any clearing agents. It was a great advantage to get genomic DNA from the same individual specimen, facilitating PCR amplification of chigger gene targets. Genomic data obtained from this promising procedure, with the aid of chigger genomic information on the NCBI database, *e.g.*, mitochondrial and rRNA gene sequences, could lead to the rapid adoption of molecular approaches for chigger identification and population genetic studies, including DNA barcoding, multilocus sequence typing (MLST), and DNA microsatellite analysis.

7.1.3 Ecology of chigger parasitism and scrub typhus epidemiology in Thailand

In the present study, chigger diversity (species richness) was used as the main dependent variable to study the influence of ecological factors affecting chigger communities on small mammal hosts in Thailand. Here, we found that host phylogeny (host species) and some ecological factors (habitat, geographical location, latitudinal gradient and season) affected the diversity of trombiculid chiggers across the 13 study sites of the country. Chigger species richness significantly differed among the host species. Non-rodent species; *e.g*., the tree shrew (*Tupaia glis*) harboured a higher number of chigger species compared to rodents. Host maturity positively affected chigger species richness; that is, mature animals were infested with more diverse chigger species than the younger ones, whereas there was no effect of host sex. Habitat showed a strong effect on chigger diversity. While hosts from forest were infested with the highest chigger species richness, hosts in urban settlements harboured the least, reflecting that urbanization influences chigger diversity by reducing their species richness. There was a trend of habitat preference found in some chigger species; *e.g.*, *Blankaartia acuscutellaris, Schoengastiella ligula* and *Walchia minuscuta* were habitat specialists found mainly in rain-fed lowland; *Helenicula pilosa* was found on dry lands; and *Leptotrombidium deliense* was found either in forest or on dry land. Chigger diversity was also determined by geographical locations with regard to different latitudinal gradients. Hosts from the study sites in the north at high latitudes harboured significantly greater chigger species richness than the sites in

the south. In addition, hosts trapped during the dry season were infested with much higher chigger species richness than hosts from the wet season. It seems that chigger communities in Thailand are more diversified in the upper latitude zones, where climatic conditions are drier, comparing to the lower zone, which is close to the equatorial line with higher humidity and more rainfall. These results suggest that diversification of chiggers in Thailand was determined by both biotic factors (*i.e*., host phylogeny and attributes) as well as abiotic factors (*i.e*., climatic and environmental conditions).

Host-chigger interactions were studied using an ecological network analysis approach. At the population level (the whole country), chigger species assemblages among the small mammal hosts showed a moderate nestedness pattern; that is, a moderate level of co-occurrence among specialist and generalist chigger species in the population. Bipartite network analysis illustrated complex interactions between chigger assemblages and hosts. Almost all chigger species infested more than one host species, suggesting low host-specificity in host-chigger interactions. This is a crucial aspect in scrub typhus epidemiology, since low host-specificity allows chiggers to attack any animals that pass through their territory, including humans. In a unipartite network analysis, *Bandicota indica* (greater bandicoot rat) and *Rattus tanezumi* (oriental house rat) were identified as the central nodes (hosts) sharing chigger species to the other hosts in the network. As the latter species, *R. tanezumi,* is known as a habitat generalist, able to thrive in all habitat types (Palmeirim et al. 2014; Blasdell et al. 2015), it could potentially play a key role in parasite dispersal from one habitat to the other communities. To investigate structure of host-chigger

interactions among the 13 study sites, a number of network properties; *i.e*., NODF (nestedness), connectance and modularity were computed. There were some associations between chigger diversity and network properties. For example, chigger species richness showed a negative correlation with network connectance (proportion of realized interactions within a network); that is, host-chigger interactions will increase when there is a decrease in chigger species richness in the community. Also, we found that nestedness negatively correlated with network modularity, which means that decreasing nestedness leads to increased clustering structures (modules/compartments) within the host-chigger network.

Epidemiological determination factors of scrub typhus incidence in Thailand were characterized using a generalized linear model approach, taking into account the chigger diversity, host-chigger network properties, environmental parameters and human case numbers of scrub typhus disease in the 13 study sites in the analysis. With these available data, we found that chigger species richness and host-chigger network connectance were the main variables to explain human scrub typhus incidence in Thailand. Higher scrub typhus incidence occurred in the sites that showed higher chigger diversity and lower connectance in small mammal host-chigger interactions. High chigger diversity probably enhances in some way the circulation or transmission of scrub typhus agent, *Orientia tsutsugamushi,* in the communities; whereas high connectance or chigger sharing among small mammal species or individuals themselves may act like a "dilution effect", potentially diverting or reducing the risk of human exposure to chiggers in such communities.

7.1.4 Chigger-associated microbiome

The microbiome of trombiculid chiggers was studied for the first time using a 16S rRNA gene amplicon sequencing approach in order to find other potential pathogenic bacteria or bacterial symbionts apart from *Orientia tsutsugamushi*. The investigation of chigger-associated microbiome was conducted based on both individual and pooled samples.

The microbiome profile of chiggers seemed to be largely associated with several dominant bacterial OTUs from the environment or host skin; *i.e*., *Brevibacillus, Corynebacterium, Geobacillus, Sphingobium, Staphylococcus*, Neisseriaceae and Comamonadaceae. The true symbiotic relationship between chiggers and these bacteria is unknown, and further investigations are still required to confirm whether there is a significant relationship between chiggers and the bacteria, or chiggers only acquired environmental bacteria by chance when traversing different environments. Apart from *Orientia tsutsugamushi,* some potential pathogenic bacteria were identified; *e.g., Borrelia* and *Mycobacterium.* These two genera were abundantly present in chigger samples and have been recognized as potential causes of borreliosis (Lyme disease and relapsing fever) and several types of tuberculosis in animals and humans, respectively (Tilly et al. 2008; Johnson & Odell 2014; Bratschi et al. 2015). Bacterial symbionts of arthropod also appeared in chigger samples; *e.g*., two potential reproductive manipulators of several arthropod species, *Cardinium* and *Wolbachia* (Gotoh et al. 2006; Werren et al. 2008)*;* an external symbiont of fungus-growing ants, *Pseudonocardia* (Cafaro et al. 2011)*;* and a potential intracellular pathogen of arthropods, *Rickettsiella*

(Bouchon et al. 2012). Other than pathogenicity in mammals, *Orientia* could play another role as a symbiont of trombiculid mites, as there are some reports showing effects on the mite's development and reproduction, such as delayed metamorphosis period, reduce fecundity and distorted sex-ratios with male killing (Roberts et al. 1977; Frances et al. 2001; Phasomkusolsil et al. 2012). Those bacteria listed above could be prime candidates for future studies of interactions in chigger-bacteria symbioses, and deeper understanding of symbiotic relationships might ultimately lead to development of novel vector control strategies.

In bacterial diversity analyses, the alpha-diversity of bacterial OTUs significantly differed among sample groups (individuals, pools and soil samples). The lowest bacterial diversity was observed in individuals, in accordance with the issue of small biomass compared to the other sample groups. On the other hand, we failed to see any significant difference in bacterial alpha-diversity among sample categories; *i.e*., chigger species, habitats and study sites. In terms of beta-diversity, there were significant differences in bacterial composition among sample categories; *i.e*., chigger species and sites, but not among habitats, suggesting that geographical conditions and host phylogeny might play some role in determining bacterial communities in chiggers.

There were contrary results between microbiome structures of individuals and pooled samples. The conflicting patterns are probably due to biases in 16S rRNA gene PCR amplification, when the sequencing libraries were generated. We found that dominant bacterial OTUs from individuals were significantly richer in GC content than the dominant OTUs from pools. DNA template concentrations

between individuals and pools were substantially different, being much lower in individuals due to their small size and very low biomass. Also, different sources of DNA polymerases were used between individuals and pools in PCR amplification. With those reasons, we can surmise that several sources of bias were associated with the PCR amplification; *i.e*., differences in concentration and GC content of DNA templates and DNA polymerase enzymes, potentially explaining the occurrence of contrary microbiome structures in the present study. These have been noted previously in several microbiome studies (Mamedov et al. 2008; Wu et al. 2010; Pinto & Raskin 2012; Kennedy et al. 2014; D'Amore et al. 2016). Mitigating the sources of biases associated with PCR is essential to ensure high quality and reliable microbiome results.

Another issue largely discussed in relation to 16S rRNA microbiome studies is the DNA contamination of laboratory kits and reagents in sample library preparation procedures. These contaminants affect the results of microbiota studies by producing false positive signals, particularly when dealing with low microbial biomass samples (Salter et al. 2014); *e.g*., chiggers. In order to identify contaminating signals, background controls representative for water, PCR reagents, DNA extraction kits and dissecting equipment were sequenced alongside chigger samples. In the present study, nearly half of the samples, particularly the individual chiggers, showed a bacterial composition similar to background controls (Bray-Curtis dissimilarity), and were subsequently removed from the downstream analyses. Although a large number of samples were discarded, this ensured that only high-quality data were retained.

With regard to the trombiculid's life stages; *i.e*., free-living adults and nymphs versus parasitic larvae, differences in the microbiome of these stages could be hypothesized and investigated in future studies. Adult and nymph samples could be collected from the environment to determine if their microbiome composition and diversity are very different to those of larvae. In addition, the microbiome profile of chiggers could be studied regarding to their position on a host. Thus, the feeding position of chigger samples; *e.g*., within the ears, axillary region or the urogenital area, could be recorded in order to determine whether microbiome profiles differ depending on where they originate on the body. For example, chiggers collected around the urogenital area might be more associated with the host's gut microbiota, whereas chiggers from the ear perhaps correlate with the bacterial flora from the upper respiratory tract.

Another interesting research question could be raised asking about the influence of *Orientia* on the other bacteria within their chigger host; that is, whether the microbiome varies in terms of diversity and composition between *Orientia*-positive and -negative mites. Unfortunately, from our microbiome results in CHAPTER 5, we had low number of *Orientia*-positive samples (only 13 pools and three individual chiggers), which is definitely insufficient to provide statistical power for comparative analysis. Therefore, more *Orientia*-positive samples obtained in future studies would help to answer this research question.

7.2 Other future works and potential implications

Although systematics and taxonomy of trombiculid mites (Oudemans 1912) have been established over many decades and the mites are widely known as

vectors of scrub typhus disease, particularly since World War II (Browning & Raphael 1945; Kelly et al. 2009; Peterson 2009), classification of this mite taxon still depends on morphology (of larvae), while molecular taxonomy is definitely limited. Therefore, the development of molecular identification tools; *i.e*., DNA barcoding, would be of benefit not only to facilitate chigger identification, but also to significantly improve our understanding of the taxonomic arrangement and evolution of this mite taxon.

In terms of the epidemiology of scrub typhus in Southeast Asia, *O. tsutsugamushi* infection in human patients has been shown to have tremendous strain variability; *i.e*., high antigenic diversity of the 56 KDa surface protein gene and a high rate of genetic recombination (Kelly et al. 2009; Paris et al. 2013). Several human cases (approximately 8 - 25%) were infected by multiple *O. tsutsugamushi* strains, which may be because the patients were either bitten by several chiggers, or there were multiple strains in an individual chigger (Sonthayanon et al. 2010; Duong et al. 2013; Phetsouvanh et al. 2015). It is still unclear how the bacterial strains have become so diversified, and highly genetically recombined. Reservoir hosts (*i.e*., small mammals, birds and reptiles) could potentially play some role in boosting strain diversity and the recombination process of the bacterium. Also, multiple strain infections in chiggers perhaps occur when the mites feed on the same small mammal host bearing different *O. tsutsugamushi* strains. An even more complicated situation that the strain variation in hosts could involve accumulation of various strains transmitted to a host from different generations or populations of chiggers (Duong et al. 2013). It could be hypothesized that *O. tsutsugamushi*

infection and circulation in chiggers and vertebrate hosts are a critical force driving strain diversification and genetic recombination. Therefore, a comparison of *O. tsutsugamushi* strain/genotype variation among those main players within a local scale, together with the aid of population genetic or network analysis approaches; *i.e*., unipartite network analysis, could help deepen our understanding of the interactions and population structure of the bacterial strains circulating in vectors and hosts in a population.

From the microbiome results, a number of bacterial symbionts of arthropods; *e.g*., *Cardinium*, *Pseudonocardia*, *Rickettsiella*, *Wolbachia* and other dominant bacteria; *e.g*., *Geobacillus,* Neisseriaceae, *Borrelia* and *Mycobacterium* were initially discovered in chiggers. These provide a list of potential chigger-associated bacteria for further experiments to deeper characterize the relationships, and to determine whether those bacteria have some phenotypic effect on their chigger hosts. Longer-read sequencing systems such as the Pacbio SMRT sequencing (Single Molecule Real-Time sequencing: Pacific Bioscience) could be applied to generate whole-length 16S rRNA sequences (~1,500 bp) of particular bacterial OTUs of interest. This would be a complementary approach to confirm the taxonomic assignment of certain bacteria in chigger samples. Experiments designed to assess the effect of these bacteria in laboratory-reared chiggers (*i.e*., reproductive and other physiological functions) as well as –omic approaches (*e.g*., transcriptomics and proteomics) could help deepen our understanding of how the bacteria interact with the chigger hosts. Finally, fluorescence *in-situ* hybridization of the bacteria in chiggers could be another technique to illustrate and localize

chigger-symbiont relationships. Potential effects of symbionts on chigger biology, for instance in reproductive manipulation or impacts on the transmission of *O. tsutsugamushi* by competition or facilitation, could ultimately lead to novel control strategies for scrub typhus vectors.

The findings of this study provide some useful information for implementation of scrub typhus prevention strategies. Hence, we found some other potential factors driving scrub typhus incidence in Thailand, such as higher chigger diversity in the area and low host-parasite network connectance, which are in addition to previously known epidemiological factors [*i.e*., most patients were found during the transitional time between late rainy to early winter season (October - December); and the majority of the cases were associated with occupational exposure to rodents or other animals in peridomestic habitats such as ricefields, gardens and plantations (Suputtamongkol et al. 2009)]. Those associations above could be applied to establish a prevention strategy and health educational campaign to facilitate control of scrub typhus disease in Thailand. In addition, our results revealed potential symbiotic bacteria of arthropods in the chigger microbiome study. The impact of bacterial symbionts on pathogen susceptibility is an emerging field which has led to many studies on the interaction between the arthropod microbiome and vectored pathogens. Several hypotheses have been put forward for whysymbionts might directly or indirectly reduce pathogen susceptibility in a host such as (1) symbionts release substances that have a negative effect on pathogen growth; and (2) symbionts compete in nutrient consumption, leading to poor growth of the pathogen (Gall et al. 2016). This idea

could be applied in the chigger research field, and a deeper understanding of symbionts and *O. tsutsugamushi* interactions might provide an alternative way to reduce the scrub typhus vector competence of chiggers using microbiome manipulation as a form of biological control.

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APPENDIX

Ascoschoengastia indica (Hirst, 1915)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Schoengastiini > Genus *Ascoschoengastia* > Species *Ascoschoengastia indica*

Diagnosis:

fPp = B/B/NBB + 6B Ga = 1N fSc = PL > AM > AL

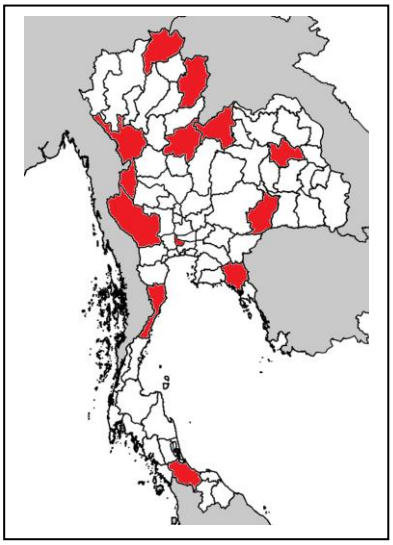
fD = 2H + 8.6.6.6(5).4.2.(2); ND = 33-36 fV = varied; NV = 32-40 NDV = 65-76

fsp = 7.7.7 fCx = 1N.1N.1N Sensillae dimension = 32 (29-36) x 10 (9-10)

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	Pa	Pm	pp	lp
Min	36	55	22	21	19	40	22	22	18	27	29	29	177	151	172	502
Max	52	66	27	25	23	47	28	31	22	34	36	36	200	174	203	571
Mean	46	60	24	23	21	44	25	27	19	46	32	34	191	164	189	544

Based on 15 specimens

Distribution:



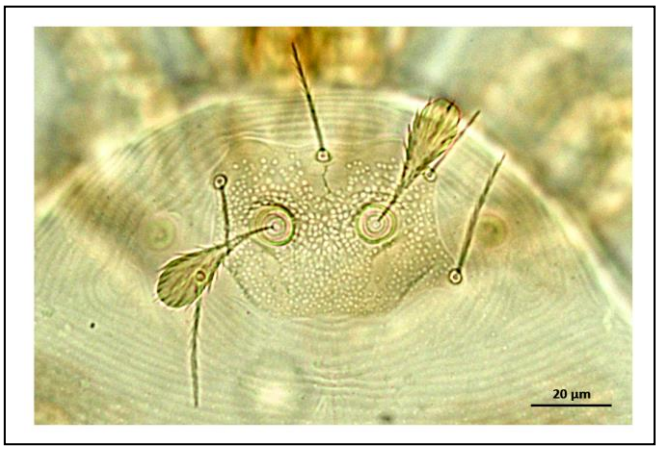
Australia, Car Nicobar Island, Guam, India, Indonesia, Malaysia, Maldives, Myanmar, Papua New Guinea, Philippines, Sri Lanka

In Thailand: Bangkok, Buriram, Chanthaburi, Chiangrai, Kalasin, Kanchanaburi, Loei, Nan, Nonthaburi, Phitsanulok, Prachuap Khirikhan, Songkhla, Tak (see map).

Host in this study:

Bandicota indica, B. savilei, Rattus andamanensis, R. argentiventer, R. sakaeratensis, R. tanezumi, Tupaia glis

Scutum photo:



Blankaartia acuscutellaris (Walch, 1922)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Blankaartia* > Species *Blankaartia acuscutellaris*

Diagnosis:

fPp = B/B/NNB + 7B Ga = 1N fSc = PL > AM > AL

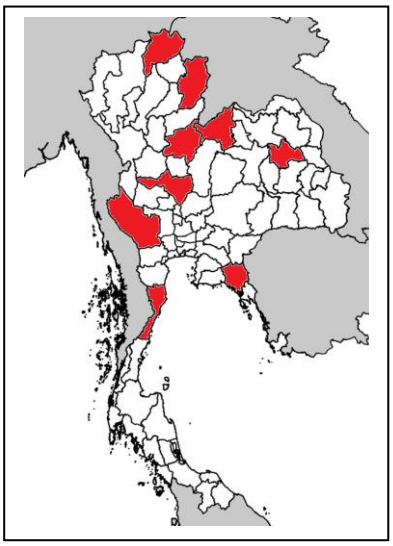
fD = 2H + 6.6.6.4.2; ND = 26 fV = 6.2.2.2U.4(2).2; NV = 18(16) NDV = 44(42)

fsp = 7.7.7 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	Pa	pm	pp	Ip
Min	73	75	29	28	38	67	25	49	32	70	71	64	309	287	353	949
Max	79	85	34	36	47	81	31	59	44	82	85	81	371	334	396	1,091
Mean	76	80	32	33	43	76	28	54	38	77	77	74	338	314	373	1,025

Based on 12 specimens

Distribution:



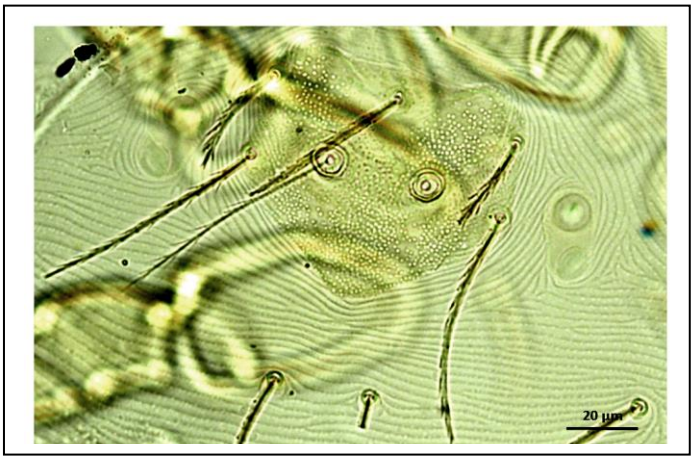
Azerbaijan, Cameroon, Central African Republic, China, Congo, Guam, Hungary, India, Indonesia, Kazakhstan, Malaysia, Maldives, Myanmar, Moldova, Philippines, Russia, Spain, Sri Lanka, Ukraine

In Thailand: Chanthaburi, Chiangrai, Kalasin, Kanchanaburi, Loei, Nakhonsawan, Nan and Phitsanulok, Prachuap Khirikhan (see map).

Host in this study:

Bandicota indica, R. sakaeratensis, R. tanezumi

Scutum photo:



Gahrliepia elbeli Traub and Morrow, 1955

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Gahrliepia* > Species *Gahrliepia elbeli*

Diagnosis:

fPp = B/B/NNN + 4B Ga = 1N fSc = PL ≥ AL Usurped setae = 4

fD = 2H + 6.8(6).8.6.4.4(2).2; ND = 38-40 fV = varied; NV = 44-50 NDV = 82-90

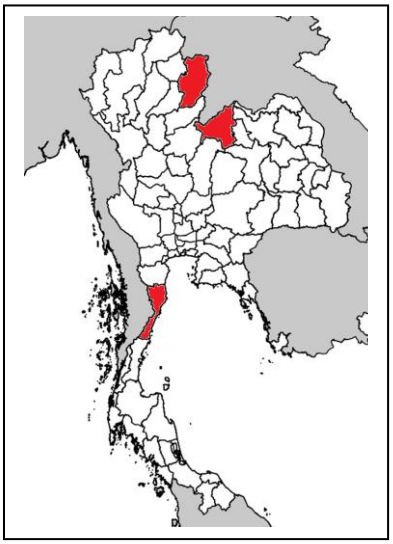
fsp = 7.6.6 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AL	PL	PPW1	PPW2	H	pa	pm	pp	lp
Min	45	75	46	21	113	135	36	30	35	53	43	36	215	191	228	634
Max	50	78	48	23	123	146	37	33	38	55	47	39	238	208	244	681
Mean	48	76	47	22	119	141	37	32	36	54	45	38	228	201	237	666

Based on 6 specimens

Distribution:

In Thailand: Loei, Nan, Prachuap Khirikhan (see map).



Host in this study:

Bandicota indica, Maxomys surifer, Rattus tanezumi

Scutum photo:

Gahrliepia fletcheri Gater, 1932

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Gahrliepia* > Species *Gahrliepia fletcheri*

Diagnosis:

fPp = B/B/NNN + 4B Ga = 1N fSc = PL ≥ AL Usurped setae = 8-12

fD = 2H + 4.4.6.6.then varied; ND = 30-34 fV = varied; NV = 50-75 NDV = 80-109

fsp = 7.6.6 fCx = 1B.1B.1B

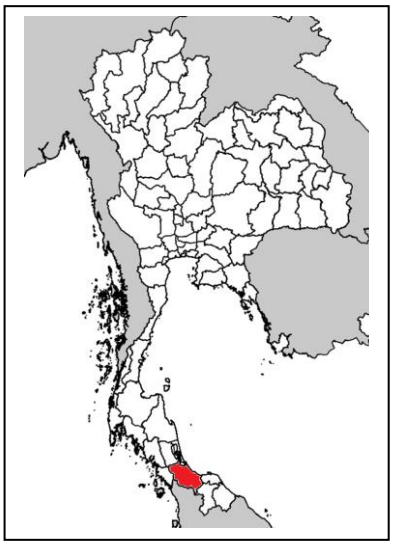
	AW	PW	SB	ASB	PSB	SD	AP	AL	PL	PPW1	PPW2	H	pa	pm	Pp	lp
Min	47	75	43	20	153	176	38	34	35	-	-	33	214	190	238	647
Max	51	81	48	27	180	208	42	40	42	-	-	46	245	218	261	725
Mean	49	78	46	24	161	185	40	37	38	-	-	42	232	202	244	679

Based on 8 specimens

Distribution:

India, Malaysia, Myanmar

In Thailand: Songkhla (see map).



Host in this study:

Rattus tanezumi

Scutum photo:

Gahrliepia sp., cf. *orientalis* Wen and Xiang, 1984

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Gahrliepia* > Species *Gahrliepia* sp., cf. *orientalis*

Diagnosis:

fPp = B/B/NNN + 4B Ga = 1N fSc = PL > AL Usurped setae = 6

fD = 2H + 8.6.8.8.6.4.2; ND = 44 fV = varied; NV = 48-68 NDV = 92-112

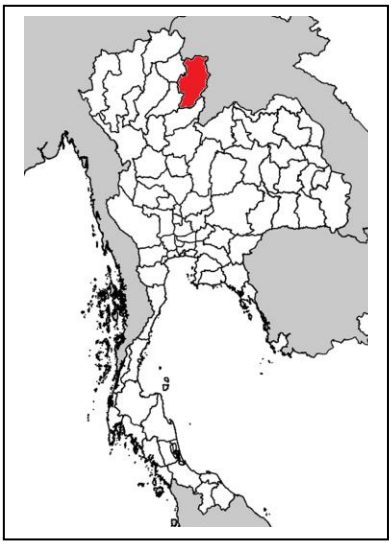
fsp = 7.6.6 fCx = 1B.1B.1B Sensillae dimension = 35 (34-38) x 11 (10-11)

	AW	PW	SB	ASB	PSB	SD	AP	AL	PL	PPW1	PPW2	H	pa	pm	Pp	lp
Min	45	68	41	21	121	142	37	32	41	46	26	40	185	160	218	583
Max	46	70	45	23	124	147	38	33	43	47	26	47	211	185	238	621
Mean	45	69	43	22	122	144	37	33	41	46	26	44	200	177	227	605

Based on 3 specimens

Distribution:

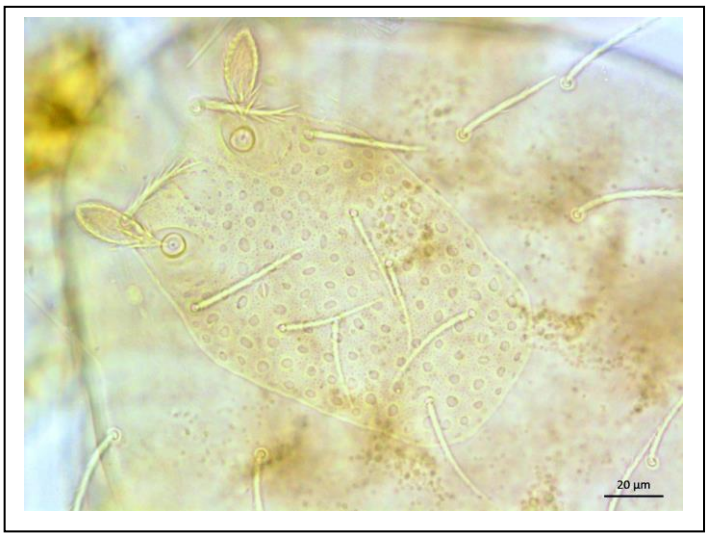
In Thailand: Nan (see map).



Host in this study:

Maxomys surifer, Rattus andamanensis

Scutum photo:



Gahrliepia xiaowoi Wen and Xiang, 1984

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Gahrliepia* > Species *Gahrliepia xiaowoi*

Diagnosis:

fPp = B/B/NNN + 4B Ga = 1N fSc = PL > AL Usurped setae = 8

fD = 2H + 10.6.8.8.then varied; ND = 30-38 fV = varied; NV = 58-72 NDV = 88-110

fsp = 7.6.6 fCx = 1B.1B.1B

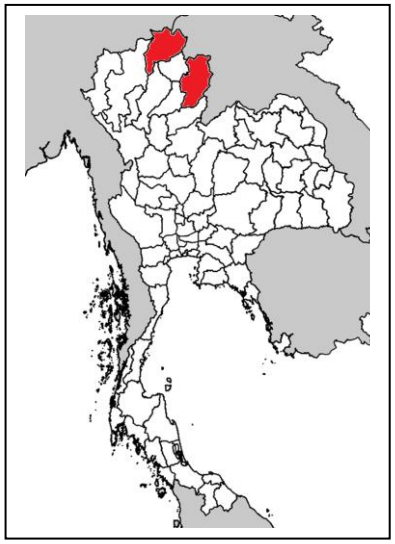
	AW	PW	SB	ASB	PSB	SD	AP	AL	PL	PPW1	PPW2	H	pa	pm	Pp	lp
Min	45	77	43	21	112	133	41	36	37	53	26	32	202	180	212	596
Max	49	80	47	24	122	145	42	39	45	55	27	44	245	209	252	706
Mean	47	78	46	22	117	140	42	37	41	54	27	41	225	199	239	664

Based on 4 specimens

Distribution:

China

In Thailand: Chiangrai and Nan (see map).



Host in this study:

Bandicota indica, Rattus tanezumi

Scutum photo:

Helenicula kohlsi (Philip and Woodward, 1946)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Schoengastiini > Genus *Helenicula* > Species *Helenicula kohlsi*

Diagnosis:

fPp = B/B/BNB + 4B Ga = 1B fSc = AL > PL > AM

fD = 14.12.10(11).10.10.then varied; ND = 65-87 fV = varied; NV = 44-64

NDV = 109-151 fsp = 7.7.7 fCx = 1B.1B.1B

Sensillae dimension = 20 (19-21) x 16 (14-17)

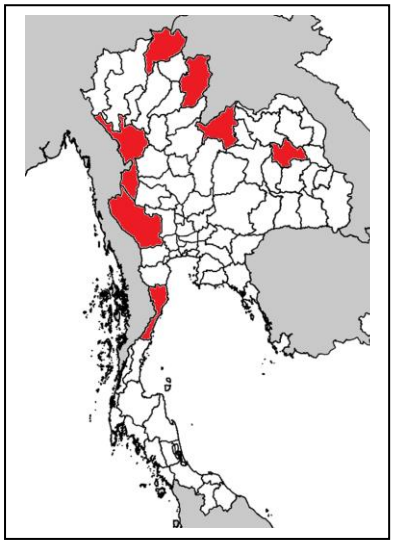
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	49	59	8	26	8	37	17	22	41	31	34	219	177	215	612
Max	54	72	12	31	12	42	21	28	53	38	42	244	201	237	682
Mean	51	66	10	28	11	40	19	24	47	34	37	234	193	229	657

Based on 8 specimens

Distribution:

Australia, China, India, Iran, Nepal, Pakistan, Philippines, Vietnam

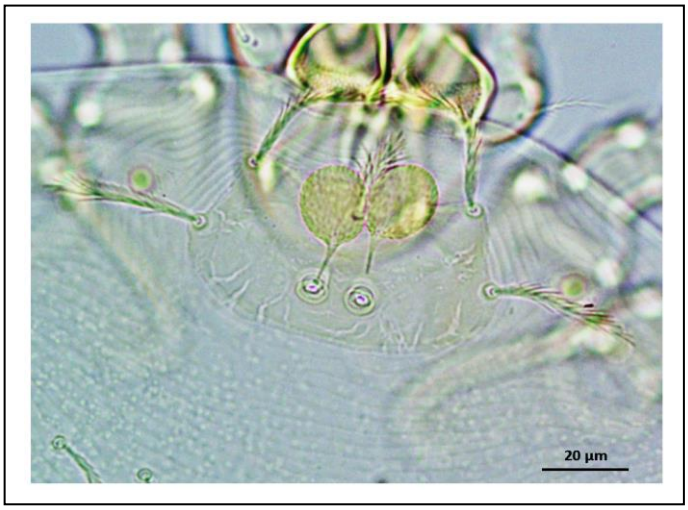
In Thailand: Chiangrai, Kalasin, Kanchanaburi, Loei, Nan, Prachuap Khirikhan, Tak (see map).



Host in this study:

Bandicota indica, Rattus andamanensis, R.exulans, R. tanezumi, Tupaia glis

Scutum photo:



Helenicula naresuani n. sp. Stekolnikov, 2016

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Schoengastiini > Genus *Helenicula* > Species *Helenicula naresuani*

Diagnosis:

fPp = B/B/BBB + 5B Ga = 1N fSc = AL > PL > AM

fD = 4H+6(5).6.8.2 then varied; ND = 38-42 fV = varied; NV = 40 NDV = 78-82

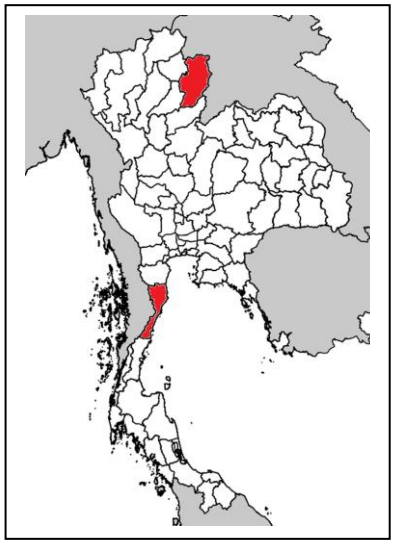
fsp = 7.7.7 fCx = 1B.1B.4B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	57	75	11	27	17	45	30	31	47	44	41	279	229	256	756
Max	61	82	13	31	20	51	31	35	58	47	44	300	239	287	822
Mean	59	78	12	28	19	48	30	34	53	46	42	291	236	272	797

Based on 3 specimens

Distribution:

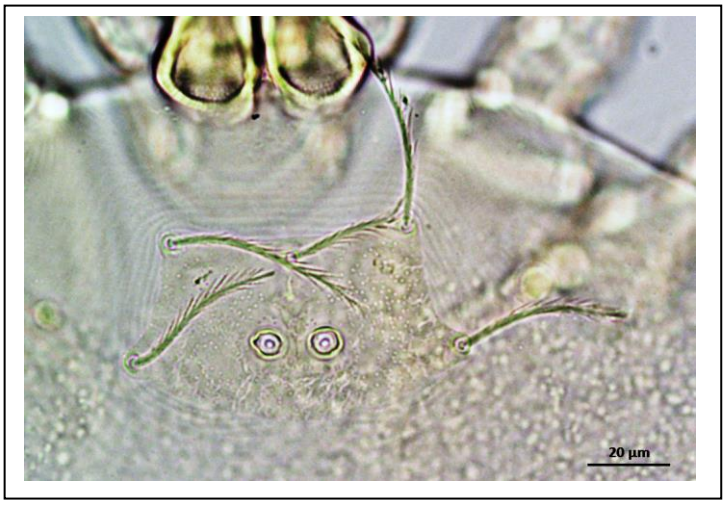
In Thailand: Nan, Prachuap Khirikhan (see map).



Host in this study:

Bandicota indica, Tupaia glis

Scutum photo:



Helenicula pilosa (Abonnenc and Taufflieb, 1957)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Schoengastiini > Genus *Helenicula* > Species *Helenicula pilosa*

Diagnosis:

fPp = B/B/BBB + 4B Ga = 1B fSc = AL > PL > AM

fD = varied; ND = 98-110 fV = varied; NV = 81-94 NDV = 179-204

fsp = 7.7.7 fCx = 1B.1B.1B Sensillae dimension = 22 (19-26) x 17 (16-18)

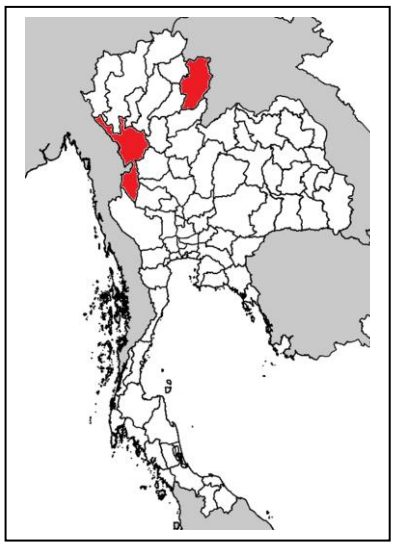
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	51	67	10	29	14	44	23	22	56	36	40	241	203	245	691
Max	56	75	13	34	17	49	27	30	64	42	46	273	227	274	760
Mean	53	70	11	31	15	46	25	26	60	39	43	257	216	261	735

Based on 11 specimens

Distribution:

Chad, Nepal

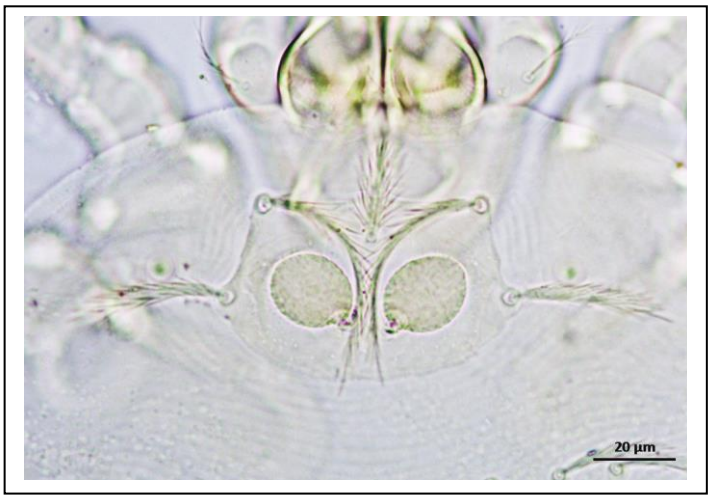
In Thailand: Nan, Tak (see map).



Host in this study:

Bandicota indica, Rattus tanezumi, Tupaia glis

Scutum photo:



Helenicula simena (Hsu and Chen, 1957)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Schoengastiini > Genus *Helenicula* > Species *Helenicula simena*

Diagnosis:

fPp = B/B/BBB + 5B Ga = 1N fSc = AL > PL > AM

fD = 10.(9-11).(9-10) then varied; ND = 53-61 fV = varied; NV = 52-60

NDV = 105-121 fsp = 7.7.7 fCx = 1B.1B.5(6)B

Sensillae dimension = 28 (25-31) x 17 (16-18)

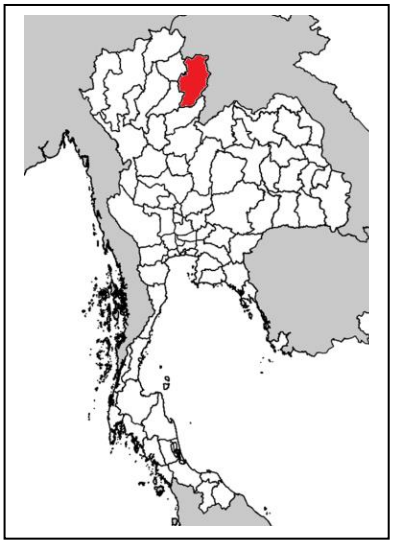
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	55	69	11	28	12	41	27	24	62	45	40	244	209	250	704
Max	58	72	13	30	18	47	28	32	66	51	45	259	221	259	738
Mean	56	69	12	29	15	44	27	29	63	47	42	253	214	253	721

Based on 5 specimens

Distribution:

China, Hong Kong, Vietnam

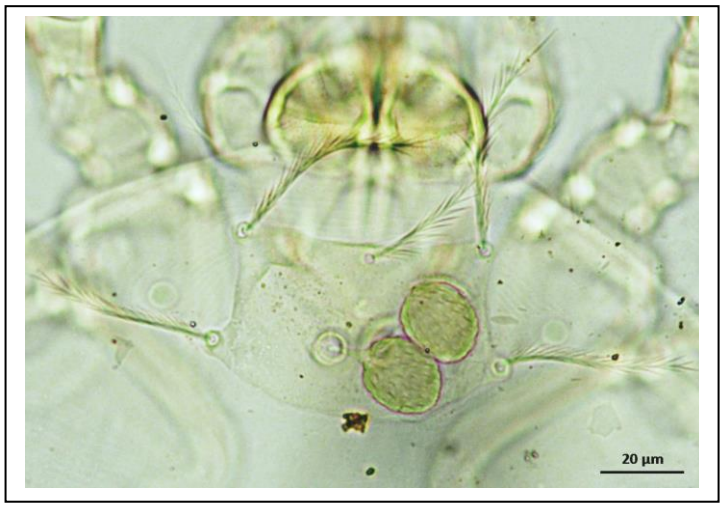
In Thailand: Nan (see map).



Host in this study:

Niviventer fulvescens, Rattus andamanensis, Tupaia glis

Scutum photo:



Leptotrombidium deliense (Walch, 1922)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium deliense*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = PL ≥ AM > AL

fD = 2H + 8.6.6.4.2; ND = 28 fV = 6.4.4u.4.2(4); NV = 20 (22) NDV = 48 (50)

fsp = 7.7.7 fCx = 1B.1B.1B

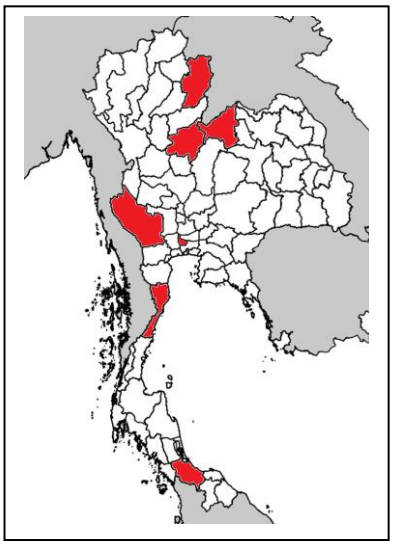
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	56	66	26	23	13	37	24	42	34	44	52	43	194	190	225	609
Max	64	78	34	29	16	44	32	52	43	53	72	56	256	219	256	731
Mean	60	72	29	26	15	40	28	47	39	48	59	48	233	208	243	685

Based on 52 specimens

Distribution:

China, India, Indonesia, Laos, Malaysia, Maldives, Papua New Guinea, Philippines, Solomon Islands, Taiwan

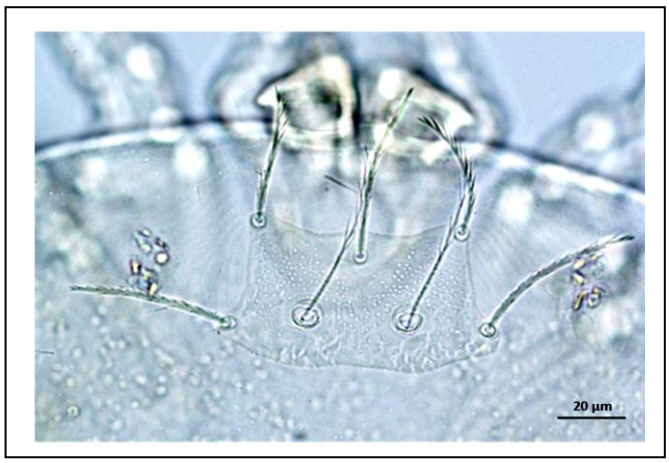
In Thailand: Kanchanaburi, Loei, Nan, Nonthaburi, Prachuap Khirikhan, Phitsanulok and Songkhla (see map).



Host in this study:

Bandicota indica, B. savilei, Berylmys berdmorei, Hylomys suillus, Maxomys surifer, Rattus andamanensis, R. argentiventer, R. sakaeratensis, R. tanezumi, Tupaia glis

Scutum photo:



Leptotrombidium elisbergi Traub and Lakshana, 1966

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium elisbergi*

Diagnosis:

fPp = B/B/BBN + 7B Ga = 1B fSc = PL ≥ AM >> AL

fD = 2H + 8.6.6.4.2; ND = 28 fV = 8.6.4u.4.2; NV = 24 NDV = 52 (50)

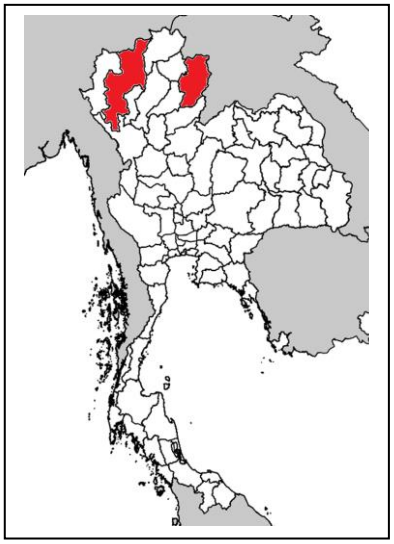
fsp = 7.7.7 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	70	87	39	29	16	46	24	57	40	56	-	59	260	232	267	762
Max	77	90	41	30	18	48	29	58	41	58	-	63	265	238	273	772
Mean	74	88	40	29	17	47	26	57	40	57	-	60	262	235	270	768

Based on 3 specimens

Distribution:

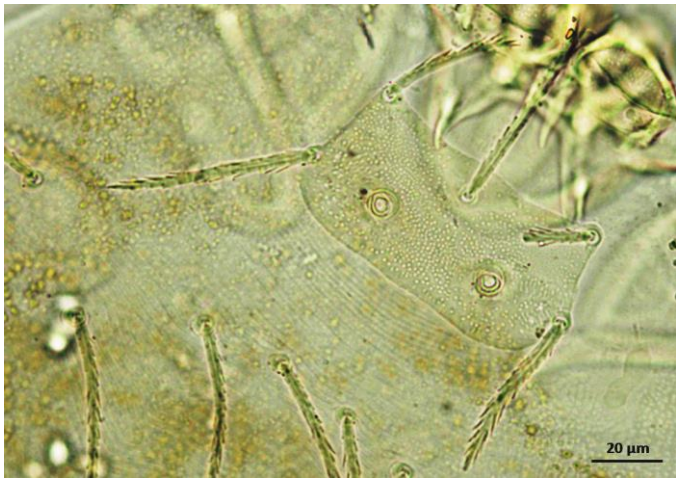
In Thailand: Chiangmai, Nan (see map).



Host in this study:

Hylomys suilus

Scutum photo:



Leptotrombidium imphalum Vercammen-Grandjean and Langston, 1976

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium imphalum*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = PL ≥ AM > AL

fD = 2H + 8.6.6.4(6).2; ND = 28(30) fV = 8(6).4(6).4u.4.4.2; NV = 26 (24-28)

NDV = 54 (52-58) fsp = 7.7.7 fCx = 1B.1B.1B

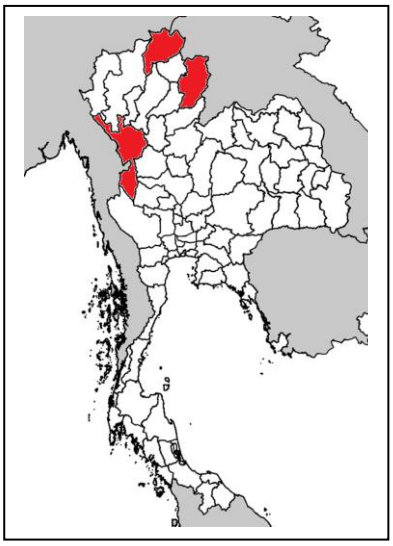
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	56	66	25	25	13	39	28	43	41	50	57	51	244	215	264	735
Max	66	76	29	30	15	45	32	58	49	57	68	56	270	246	293	810
Mean	59	70	27	28	14	42	30	52	44	53	61	53	255	231	276	763

Based on 14 specimens

Distribution:

India, Malaysia, Myanmar, Taiwan

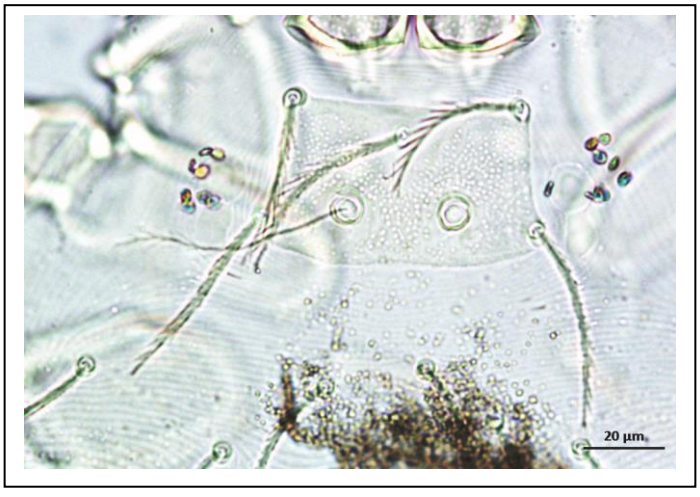
In Thailand: Chiangrai, Nan, Tak (see map).



Host in this study:

Bandicota indica, Hylomys suilus, Rattus andamanensis, Tupaia glis

Scutum photo:



Leptotrombidium macacum (Womersley, 1952)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium macacum*

Diagnosis:

fPp = N/N/BNB + 7B Ga = 1B fSc = AM > PL ≥ AL

fD = 2H + 10(11).10.10(11).8.6.4; ND = 50 (52) fV = 8.6.6.6u then varied; NV = 40-44

NDV = 94 (90-96) fsp = 7.7.7 fCx = 1B.1B.1B

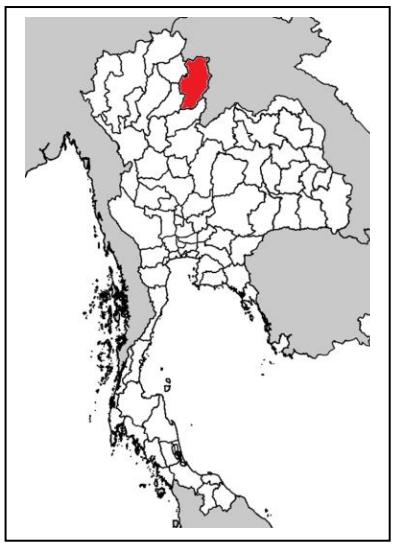
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	62	77	32	27	13	41	27	46	39	37	65	41	230	200	241	671
Max	65	81	32	29	14	42	28	51	41	40	65	42	235	208	248	692
Mean	64	79	32	28	13	41	27	48	40	39	65	41	233	205	244	684

Based on 3 specimens

Distribution:

India, Nepal

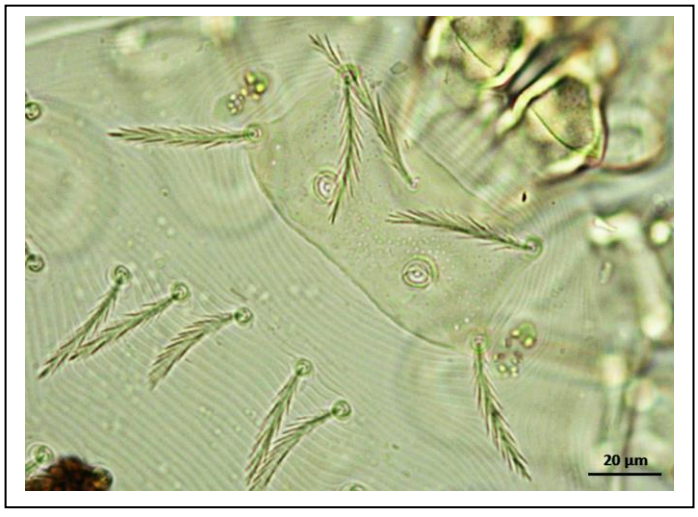
In Thailand: Nan (see map).



Host in this study:

Tupaia glis

Scutum photo:



Leptotrombidium sialkotense Vercammen-Grandjean and Langston, 1976

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium sialkotense*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = AM > PL > AL

fD = 2H + 8.6.6.6.2; ND = 30 fV = varied; NV = 40 NDV = 70

fsp = 7.7.7 fCx = 1B.1B.1B

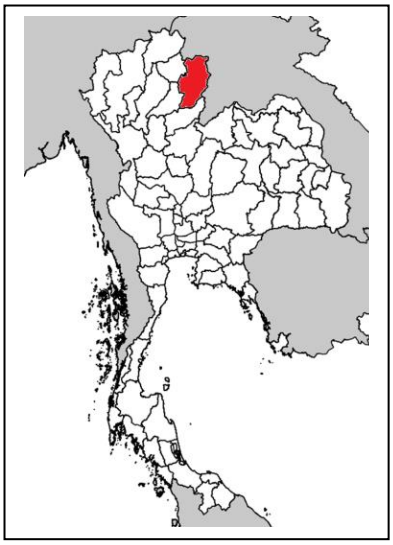
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Max	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean	61	71	27	29	14	43	31	52	43	48	-	49	266	218	265	749

Based on 1 specimens

Distribution:

Pakistan

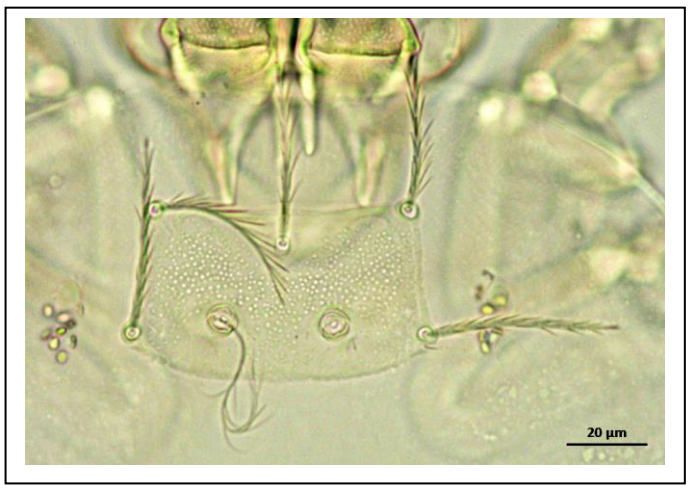
In Thailand: Nan (see map).



Host in this study:

Tupaia glis

Scutum photo:



Leptotrombidium sp., cf. *guzhangense* Wang, Li and Tian, 1985

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium* sp., cf. *guzhangense*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = PL > AM > AL

fD = 2H + 8.6.6.4.2; ND = 28 fV = 8(10).8.4u.4.2.2; NV = 28 (30) NDV = 56 (58)

fsp = 7.7.7 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	58	63	25	22	12	35	23	39	34	48	44	45	208	199	222	634
Max	66	71	27	25	14	39	26	43	37	52	55	47	230	219	238	688
Mean	60	66	26	24	13	37	24	42	35	49	50	46	221	206	229	657

Based on 10 specimens

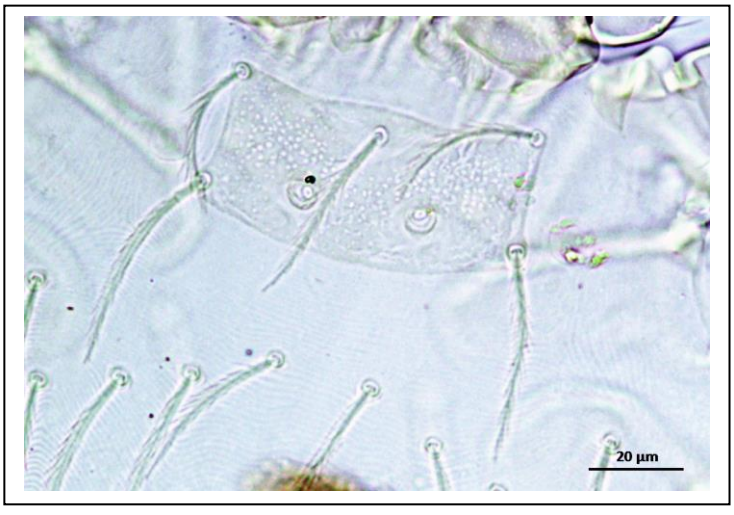
Distribution:

In Thailand: Kalasin (see map).

Host in this study:

Rattus tanezumi

Scutum photo:



Leptotrombidium sp., cf. *macacum* (Womersley, 1952)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium* sp., cf. *macacum*

Diagnosis:

fPp = N/N/BNB + 7B Ga = 1B fSc = AM > PL ≥ AL

fD = 2H + 10(11).8.8.10.4.2; ND = 44 (45) fV = varied; NV = 44-46

NDV = 88-90 fsp = 7.7.7 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	63	77	28	28	13	42	29	54	41	45	57	49	240	221	262	730
Max	70	85	40	31	15	44	31	56	45	48	75	51	258	229	272	760
Mean	65	80	32	29	14	43	30	56	43	46	64	50	249	225	267	742

Based on 4 specimens

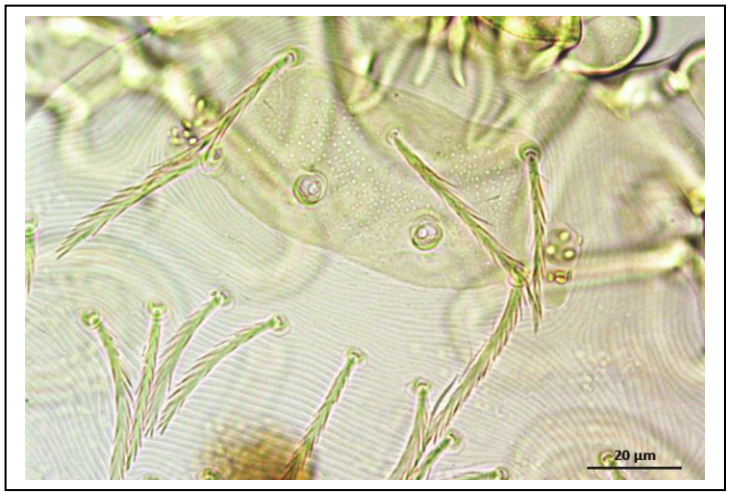
Distribution:

In Thailand: Tak (see map).

Host in this study:

Bandicota indica

Scutum photo:



Leptotrombidium subangulare Wen and Xiang, 1984

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium subangulare*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = PL > AM > AL

fD = 2H + 8.6.6.4.2; ND = 28 fV = 6.4.4u.4.2; NV = 20 NDV = 48

fsp = 7.7.7 fCx = 1B.1B.1B

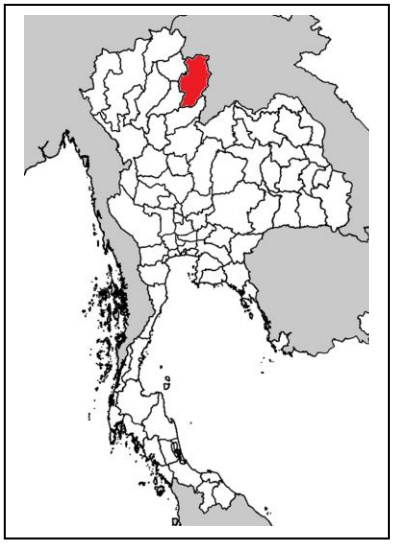
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	66	74	30	30	13	43	25	59	41	64	93	63	283	250	292	828
Max	66	77	30	30	14	44	28	60	45	66	95	65	297	250	294	839
Mean	66	75	30	30	13	42	26	59	43	65	94	64	290	250	293	833

Based on 2 specimens

Distribution:

China

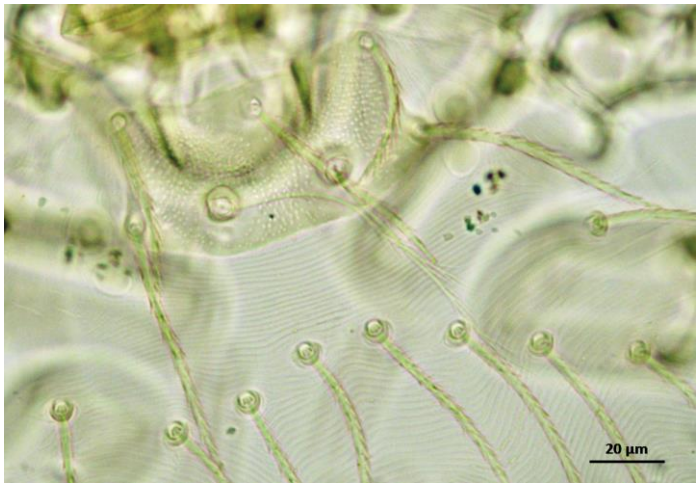
In Thailand: Nan (see map).



Host in this study:

Rattus andamanensis

Scutum photo:



Leptotrombidium tenompaki Stekolnikov, 2013

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium tenompaki*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = PL ≥ AM > AL

fD = 2H + 8.6.6.4.2; ND = 28 fV = 6.4.4u.4.2; NV = 20 NDV = 48

fsp = 7.7.7 fCx = 1B.1B.1B

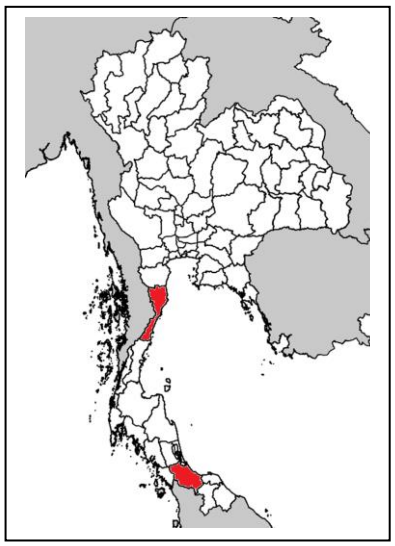
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	63	74	29	27	14	42	29	52	46	55	64	53	250	213	253	724
Max	66	78	31	30	15	45	30	58	48	60	80	62	260	230	269	771
Mean	65	77	30	28	14	43	30	55	47	56	73	57	257	222	258	739

Based on 2 specimens

Distribution:

Malaysia

In Thailand: Prachuab Kirikhan, Songkhla (see map).



Host in this study:

Bandicota indica, Rattus tanezumi

Scutum photo:

Leptotrombidium turdicola Vercammen-Grandjean and Langston, 1976

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium turdicola*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = PL ≥ AM > AL

fD = 2H + 10.10.10.8.4.2; ND = 46 fV = varied; NV = 38-40 NDV = 84-86

fsp = 7.7.7 fCx = 1B.1B.1B

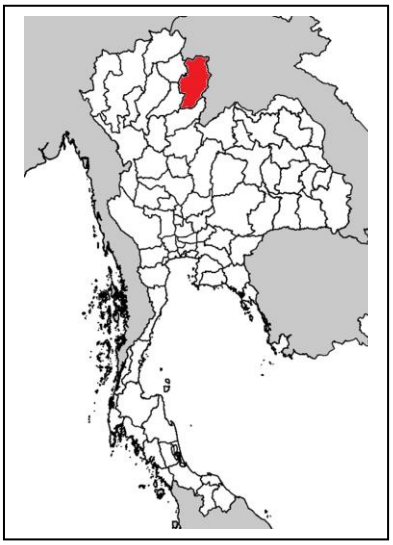
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	68	79	30	32	14	47	30	47	45	56	75	54	253	233	285	771
Max	79	94	36	35	19	52	34	65	54	72	83	66	273	251	301	818
Mean	74	85	32	33	16	49	31	58	49	63	80	61	266	241	292	801

Based on 5 specimens

Distribution:

Malaysia

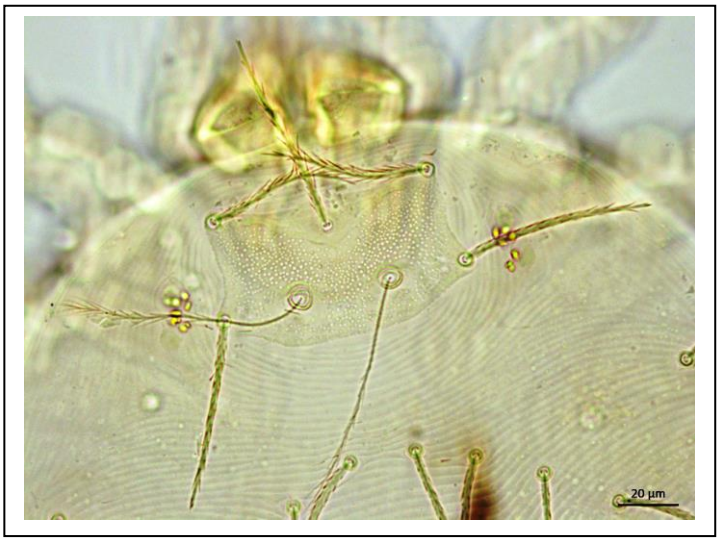
In Thailand: Nan (see map).



Host in this study:

Berylmys bowersi, Rattus andamanensis, R. tanezumi

Scutum photo:



Leptotrombidium yunlingense Yu, Yang, Zhang and Hu, 1981

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Leptotrombidium* > Species *Leptotrombidium yunlingense*

Diagnosis:

fPp = N/N/BNB + 7B Ga = 1B fSc = AM ≥ PL > AL

fD = 2H + 13.10.8.11.10; ND = 54 fV = varied; NV = 63 NDV = 117

fsp = 7.7.7 fCx = 1B.1B.1B

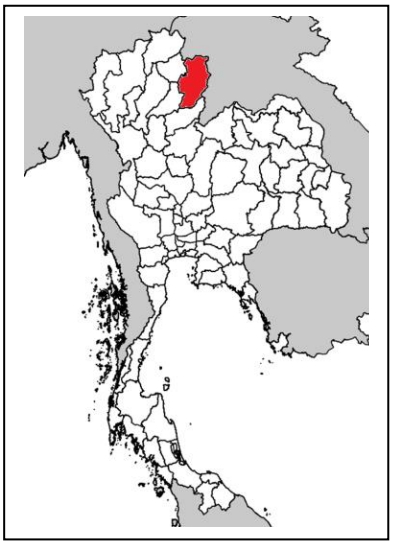
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Max	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean	70	83	33	32	16	48	33	50	45	49	-	52	263	232	270	765

Based on 1 specimens

Distribution:

China

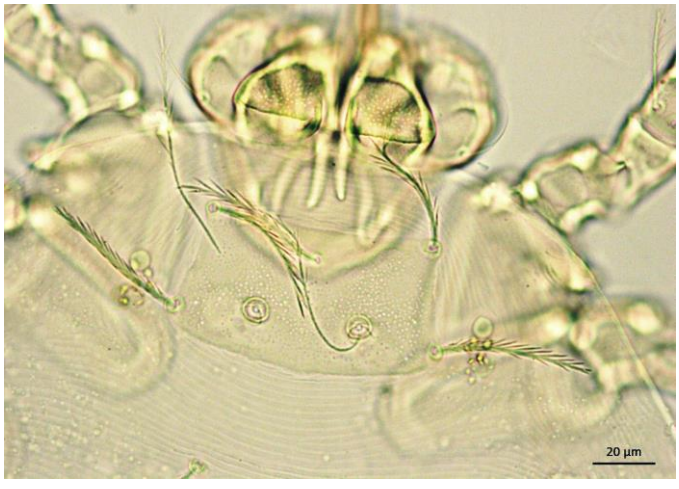
In Thailand: Nan (see map).



Host in this study:

Tupaia glis

Scutum photo:



Lorillatum hekouensis Yu, Chen and Lin, 1996

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Lorillatum* > Species *Lorillatum hekouensis*

Diagnosis:

fPp = N/N/NNB + 7B Ga = 1B fSc = PL >> AM > AL

fD = 2H + 8.8.8.6.4.4; ND = 40 fV = varied; NV = 38 NDV = 78

fsp = 7.7.7 fCx = 1B.1B.1B Leg sensillae: Mastitibiala & Mastitarsala

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Max	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean	69	84	29	41	14	55	32	56	45	86	80	59	441	360	423	1224

Based on 1 specimens

Distribution:

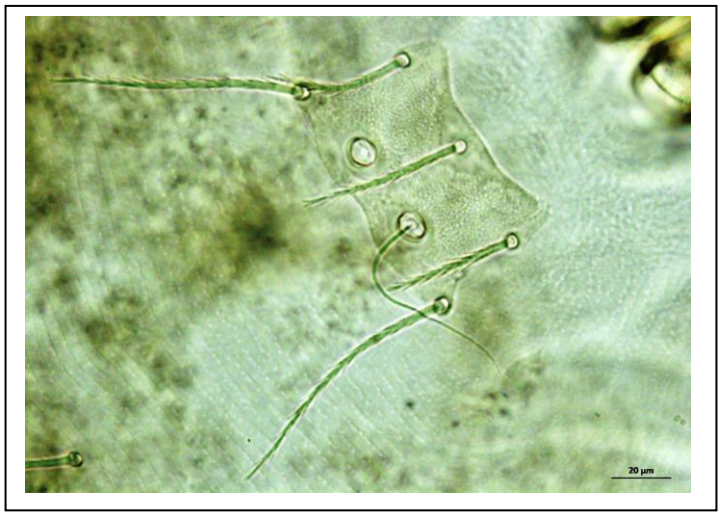
China

In Thailand: Kalasin (see map).

Host in this study:

Rattus tanezumi

Scutum photo:



Microtrombicula munda (Gater, 1932)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Microtrombicula* > Species *Microtrombicula munda*

Diagnosis:

fPp = B/B/BBB + 6B Ga = 1N fSc = PL > AM > AL

fD = 2H + 6.6.4.4.2(3); ND = 24 (25) fV = 4.4.2.4.4U.4.2(4); NV = 24 (26)

NDV = 48-51 fsp = 7.7.7 fCx = 1N.1N.1N

Leg sensillae: Mastitibiala & Mastitarsala

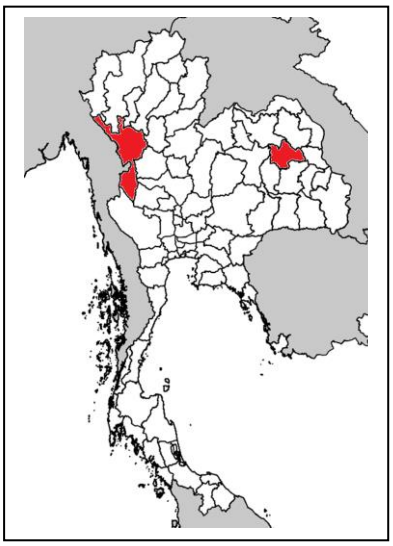
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	31	43	10	18	19	38	19	20	17	27	29	29	172	146	166	488
Max	32	45	12	20	23	43	20	21	23	28	29	30	176	155	173	501
Mean	31	44	11	19	20	40	19	21	20	28	29	29	174	152	170	498

Based on 3 specimens

Distribution:

Malaysia, Sri Lanka

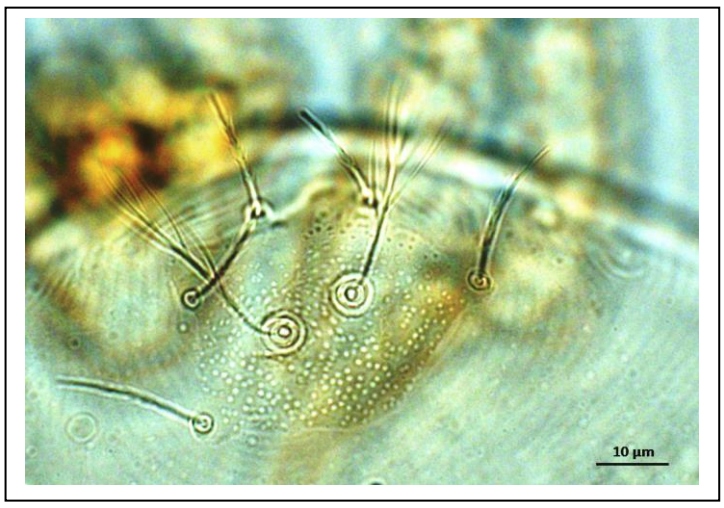
In Thailand: Kalasin, Tak (see map).



Host in this study:

Rattus tanezumi

Scutum photo:



Schoengastia propria Audy and Womersley, 1957

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Schoengastia* > Species *Schoengastia propria*

Diagnosis:

fPp = B/B/NNB + 7B Ga = 1N fSc = AL > PL >> AM

fD = 2H + 10(9-11).8.6.4.4.2; ND = 36 (35-37) fV = varied; NV = 40-43

NDV = 75-80 fsp = 7.7.7 fCx = 1B.1B.1B

Sensillae dimension = 31 (31-32) x 16 Cheliceral blade with row of 5 denticles

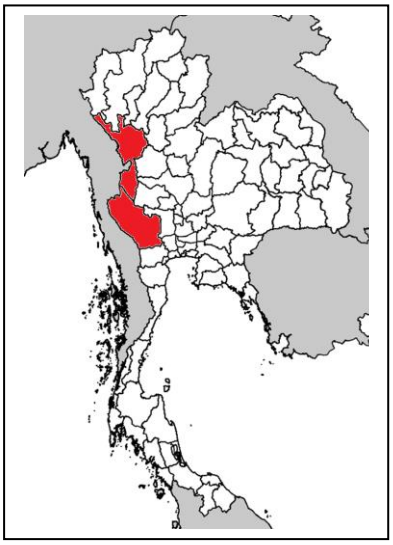
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	46	68	14	28	24	54	24	23	61	56	41	312	272	296	883
Max	51	73	17	31	26	55	25	25	70	61	44	324	274	320	917
Mean	49	71	15	29	25	54	24	24	66	58	43	319	273	310	903

Based on 3 specimens

Distribution:

India

In Thailand: Kanchanaburi, Tak (see map).



Host in this study:

Bandicota indica

Scutum photo:

Schoutedenichia centralkwangtunga (Mo, Chen, Ho and Li, 1959)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Schoutedenichia* > Species *Schoutedenichia centralkwangtunga*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AM > AL

fD = 4H + 4(5).10(8-9).8 then varied; ND = 48 (47-50) fV = varied; NV = 40

NDV = 87-90 fsp = 7.7.7 fCx = 1B.1B.1B

Sensillae dimension = 28 (26-31) x 12

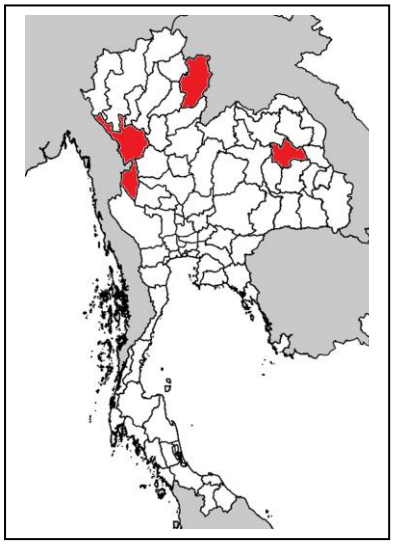
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	52	75	42	23	17	41	37	23	18	29	34	204	175	191	559
Max	55	79	44	25	18	43	39	25	19	30	37	212	178	200	587
Mean	53	77	43	24	17	42	38	24	18	30	35	206	177	196	576

Based on 3 specimens

Distribution:

China, Vietnam

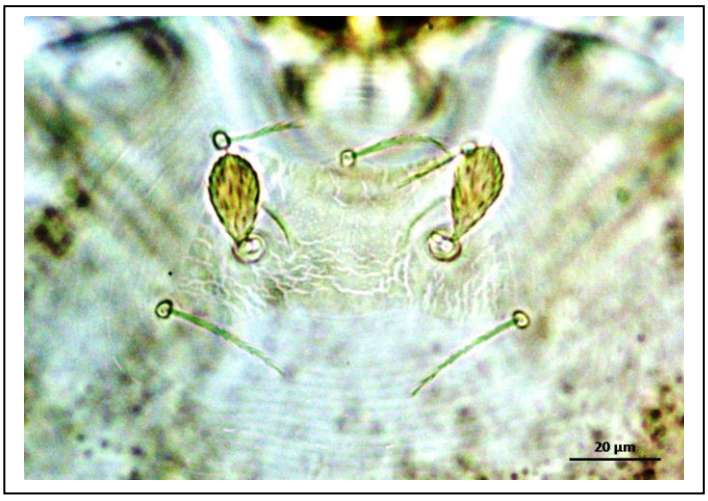
In Thailand: Kalasin, Nan, Tak (see map).



Host in this study:

Bandicota indica

Scutum photo:



Schoengastiella ligula Radford, 1946

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Schoengastiella* > Species *Schoengastiella ligula*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = AL > PL

fD = 2H + 6(8).8.then varied; ND = 48-52 fV = varied; NV = 48-56 NDV = 96-108

fsp = 7.6.6 fCx = 1B.1B.1B Sensillae dimension = 31 (30-32) x 14 (13-14)

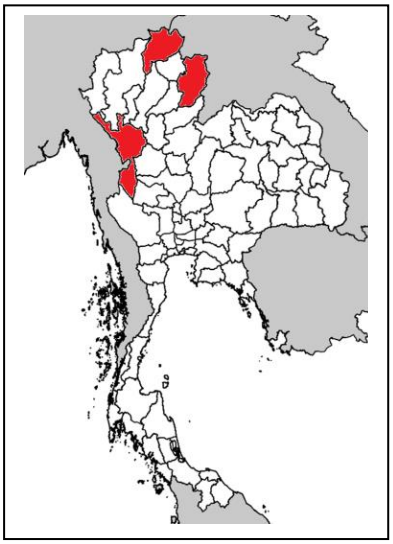
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	36	50	33	25	56	81	42	-	38	36	30	38	216	197	234	648
Max	38	57	37	27	69	96	47	-	45	41	32	45	241	213	243	691
Mean	37	52	35	26	61	87	44	-	41	39	31	42	230	207	239	676

Based on 11 specimens

Distribution:

China, India, Myanmar, Nepal

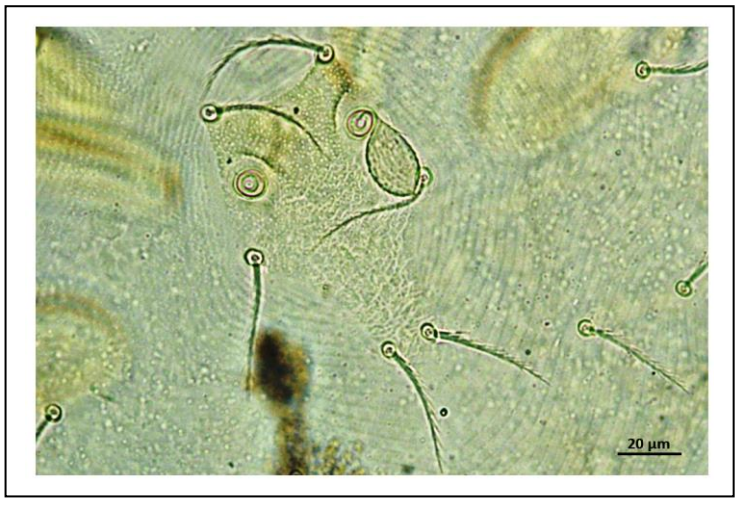
In Thailand: Chiangrai, Nan, Tak (see map).



Host in this study:

Bandicota indica, Rattus andamanensis, R. tanezumi

Scutum photo:



Trombiculindus kosapani n. sp. Stekolnikov, 2016

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Trombiculindus* > Species *Trombiculindus kosapani*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = AM = PL > AL

fD = 2H + 8.6.6.4.2; ND = 28 fV = 8(9).4.4u.4.2; NV = 22 (23) NDV = 50 (51)

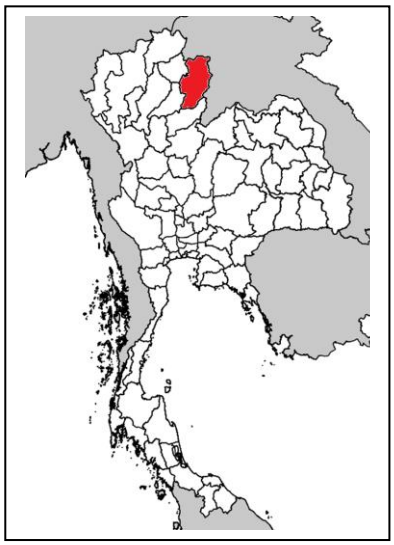
fsp = 7.7.7 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	63	74	32	26	14	41	17	43	35	44	-	46	216	200	235	652
Max	65	76	33	28	15	43	20	48	39	48	-	46	236	214	241	691
Mean	64	75	32	27	14	42	19	45	37	46	-	46	228	208	238	676

Based on 3 specimens

Distribution:

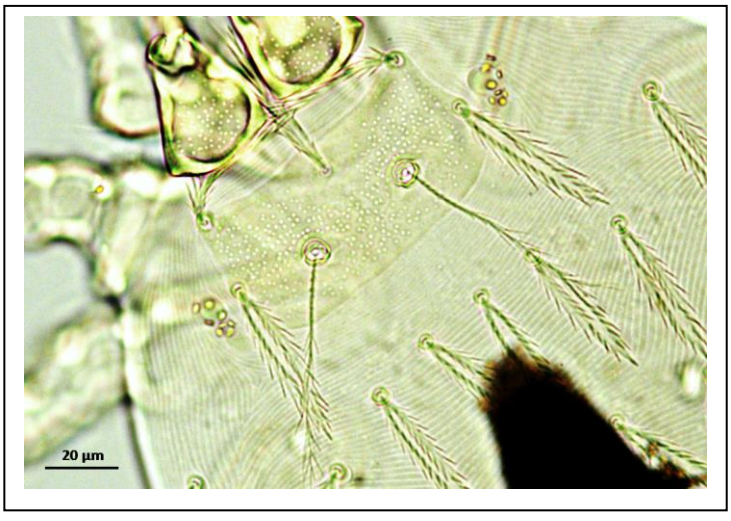
In Thailand: Nan (see map).



Host in this study:

Tupaia glis

Scutum photo:



Trombiculindus paniculatum (Traub, Nadchatram and Lakshana, 1968)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Trombiculindus* > Species *Trombiculindus paniculatum*

Diagnosis:

fPp = N/N/BNB + 7B Ga = 1B fSc = PL > AL > AM

fD = 2H + 8.6.6.4.2; ND = 28 fV = 8.4.4u.4.2; NV = 22 NDV = 50

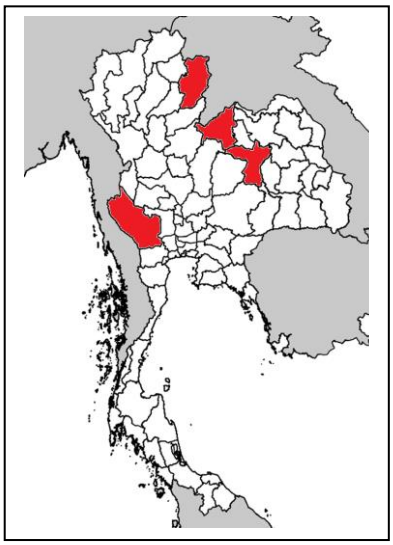
fsp = 7.7.7 fCx = 1B.1B.1B

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	65	77	35	28	16	44	15	38	41	55	-	54	242	220	256	721
Max	67	78	36	31	17	47	16	41	45	58	-	61	255	233	271	759
Mean	65	77	35	29	16	45	15	39	43	56	-	56	248	224	261	734

Based on 3 specimens

Distribution:

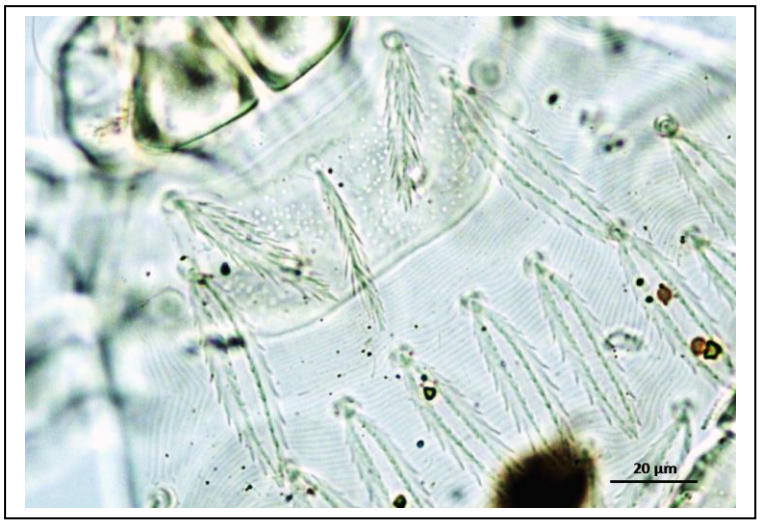
In Thailand: Kanchanaburi, Khon Kaen, Loei, Nan (see map).



Host in this study:

Hylomys suillus

Scutum photo:



Trombiculindus variaculum (Traub and Nadchatram, 1967)

Classification: Family Trombiculidae > Subfamily Trombiculinae > Tribe Trombiculini > Genus *Trombiculindus* > Species *Trombiculindus variaculum*

Diagnosis:

fPp = N/N/BNN + 7B Ga = 1B fSc = n/a

fD = 2H + 8.6.6.4.2; ND = 28 fV = 6.4.4.4u.2; NV = 22 NDV = 48

fsp = 7.7.7 fCx = 1B.1B.1B

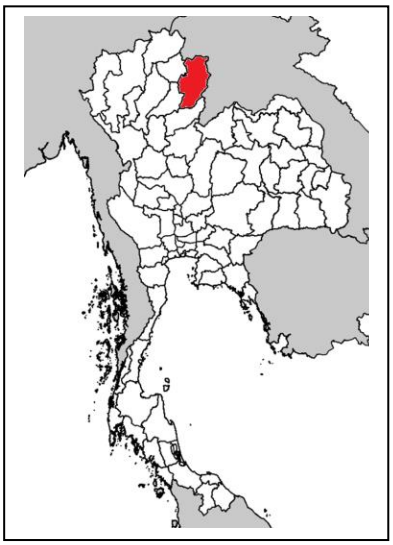
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	S	H	pa	pm	pp	lp
Min	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Max	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean	64	75	40	32	14	46	16	-	-	64	-	56	212	214	245	671

Based on 1 specimens

Distribution:

Malaysia

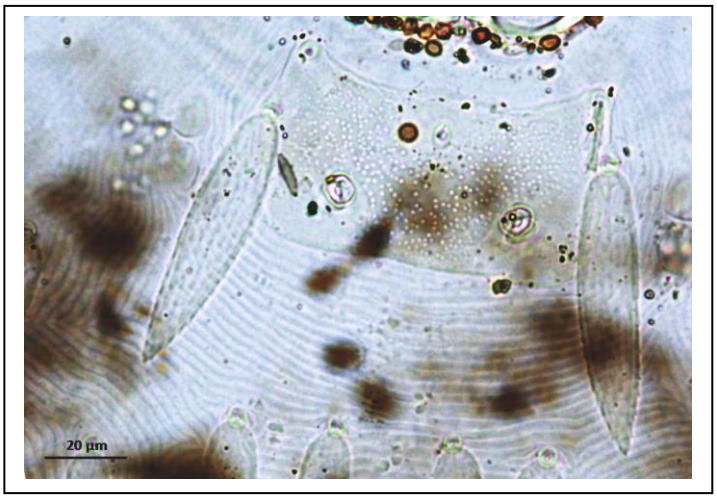
In Thailand: Nan (see map).



Host in this study:

Hylomys suillus

Scutum photo:



Walchia chavali n. sp. Stekolnikov, 2016

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia chavali*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AL (peniscutum)

fD = 2H + 6.6.(6–8).6.4.2.2; ND = 33-36 fV = varied; NV = 40-51 NDV = 74-87

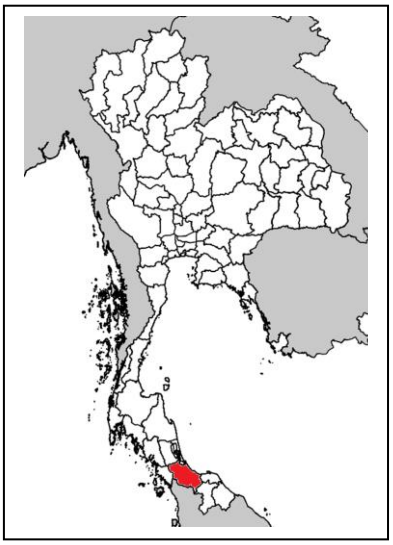
fsp = 7.6.6 fCx = 1B.1B.3B Sensillae dimension = 31 (28-33) x 13 (12-13)

	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	30	56	23	20	32	53	36	-	19	30	28	230	200	234	669
Max	34	83	27	23	40	64	46	-	23	34	34	247	213	254	715
Mean	32	67	25	21	37	58	41	-	21	32	30	238	208	242	689

Based on 15 specimens

Distribution:

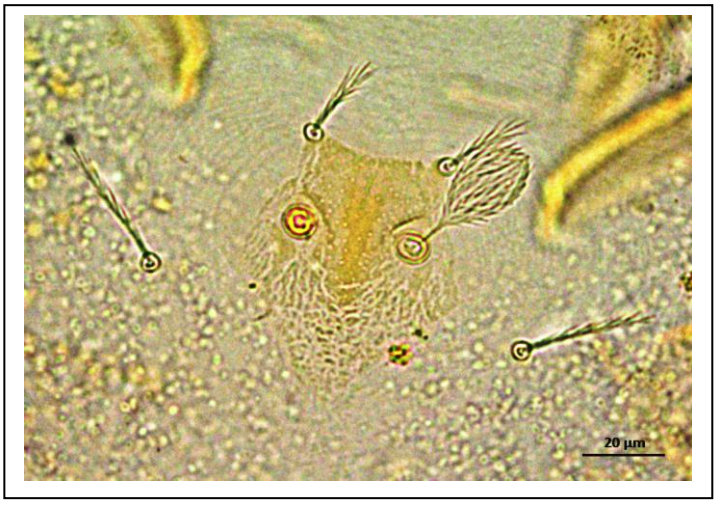
In Thailand: Songkhla (see map).



Host in this study:

Leopoldamys sabanus, Maxomys surifer, Rattus tanezumi

Scutum photo:



Walchia dismina (Schluger, Grochovskaja, Ngu, Hoe and Tung, 1960)

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia dismina*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AL

fD = 2H + 6.6.6.6.4 then varied; ND = 34-38 fV = varied; NV = 44-60 NDV = 78-98

fsp = 7.6.6 fCx = 1B.1B.2B Sensillae dimension = 29 (28-30) x 12

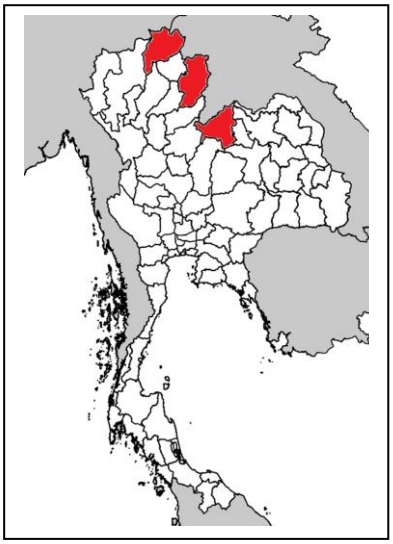
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	31	38	21	19	34	54	31	-	20	27	28	240	202	236	641
Max	35	44	24	21	41	62	37	-	24	32	32	257	224	257	738
Mean	33	41	23	20	37	58	34	-	22	30	30	248	216	245	702

Based on 6 specimens

Distribution:

Vietnam

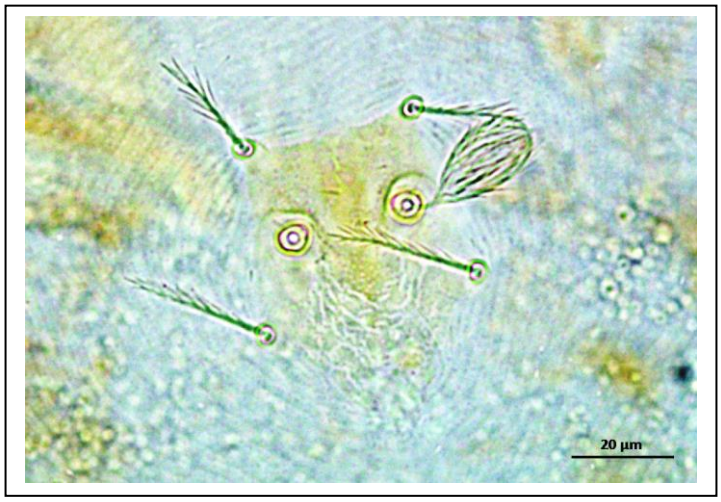
In Thailand: Chiangrai, Loei, Nan (see map).



Host in this study:

Bandicota indica, Berylmys berdmorei, Maxomys surifer, Mus sp., *M. cervicolor, M. cookii, Rattus sakaeratensis*

Scutum photo:



Walchia kritochaeta (Traub and Evans, 1957)

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia kritochaeta*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AL

fD = 2H + 6.6.6(4) then varied; ND = 35-40 fV = varied; NV = 54-62 NDV = 89-102

fsp = 7.6.6 fCx = 1B.1B.1B Sensillae dimension = 31 (30-32) x 12 (11-12)

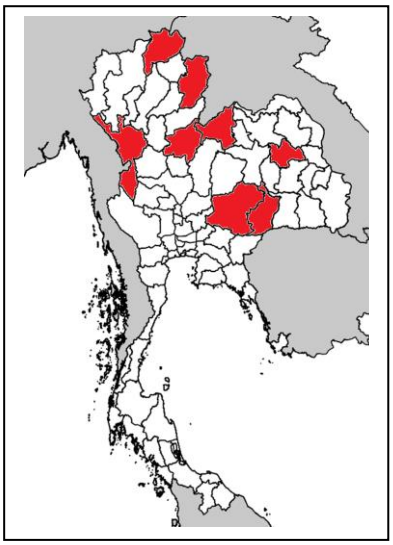
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	29	40	22	18	35	53	32	-	19	24	29	198	174	204	580
Max	33	49	25	20	42	61	34	-	22	28	32	215	188	216	617
Mean	31	44	24	19	38	58	33	-	20	26	31	205	181	211	598

Based on 7 specimens

Distribution:

China

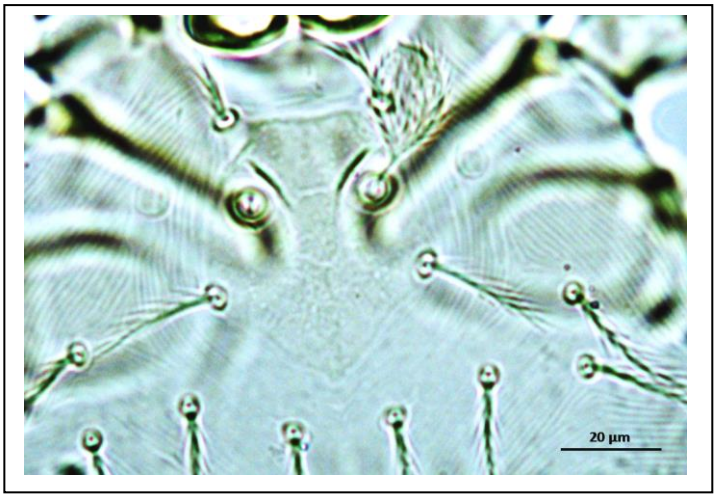
In Thailand: Buriram, Chiangrai, Kalasin, Loei, Nakhon Ratchasima, Nan, Phitsanulok, Tak (see map).



Host in this study:

Bandicota indica, Berylmys berdmorei, Maxomys surifer, Mus caroli, M. cervicolor, M. cookii, Niviventer fulvescens, Rattus andamanensis, R. exulans, R. sakaeratensis, R. tanezumi

Scutum photo:



Walchia lupella (Traub and Evans, 1957)

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia lupella*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL ≥ AL

fD = 2H + 6.6.6 then varied; ND = 36-40 fV = varied; NV = 50-56 NDV = 86-96

fsp = 7.6.6 fCx = 1B.1B.2B Sensillae dimension = 26 (24-28) x 12 (11-12)

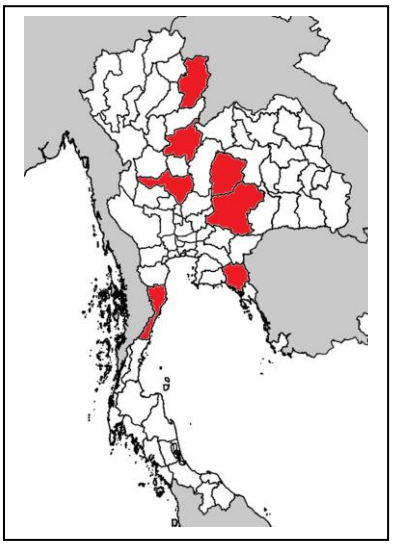
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	31	48	26	19	33	53	34	-	22	24	27	166	149	175	494
Max	34	55	30	22	43	64	37	-	27	27	31	185	170	194	542
Mean	32	52	28	21	39	60	35	-	24	26	29	176	158	187	522

Based on 7 specimens

Distribution:

India

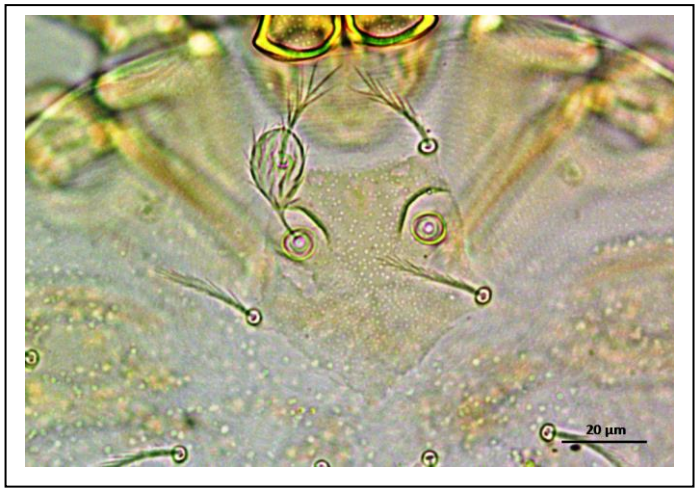
In Thailand: Chaiyaphum, Chantaburi, Nakhon Ratchasima, Nakhonsawan, Nan, Phitsanulok, Prachuab Kirikhan (see map).



Host in this study:

Bandicota indica, Berylmys berdmorei, Rattus argentiventer, R. sakaeratensis, R. tanezumi

Scutum photo:



Walchia micropelta (Traub and Evans, 1957)

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia micropelta*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AL

fD = 2H + 6.6.6.6.4(5-6).2.2; ND = 34-36 fV = varied; NV = 46-54 NDV = 80-90

fsp = 7.6.6 fCx = 1B.1B.2B Sensillae dimension = 18 (16-19) x 12 (12-13)

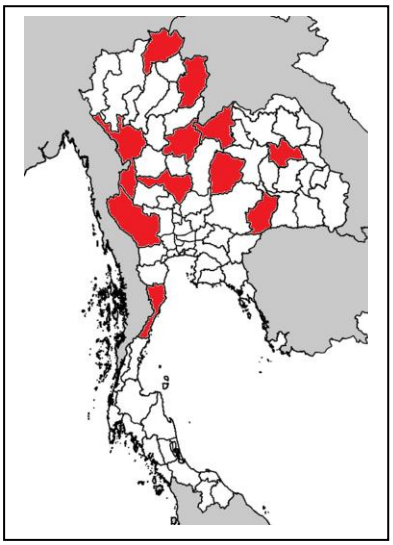
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	20	22	17	14	25	40	25	-	13	17	19	121	110	133	372
Max	24	29	21	16	33	48	31	-	17	19	23	144	117	142	402
Mean	23	25	19	15	28	44	28	-	14	18	22	135	114	137	387

Based on 19 specimens

Distribution:

China, Vietnam

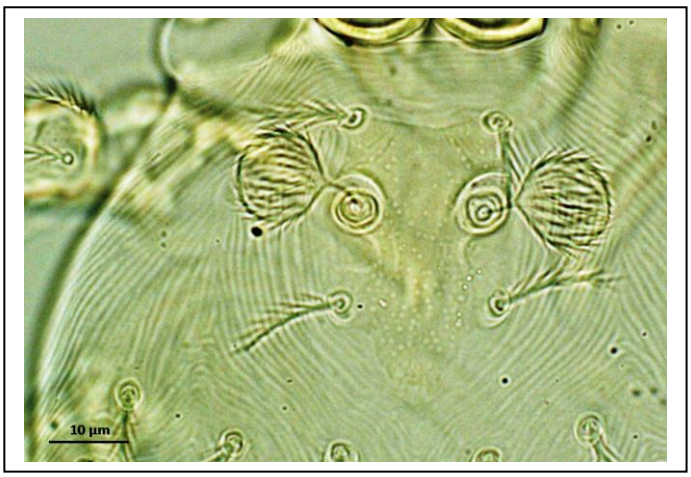
In Thailand: Buriram, Chaiyaphum, Chiangrai, Kalasin, Kanchanaburi, Loei, Nakhonsawan, Nan, Phitsanulok, Prachuab Kirikhan, Tak (see map).



Host in this study:

Bandicota indica, B. savilei, Berylmys berdmorei, Maxomys surifer, Mus caroli, M. cervicolor, M. cookii, Rattus andamanensis, R. argentiventer, R. exulans, R. sakaeratensis, R. tanezumi

Scutum photo:



Walchia minuscuta Chen, 1978

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia minuscuta*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AL

fD = 2H + 6.6.6.8(6-7).4(5-6).2.2; ND = 34-38 fV = varied; NV = 48-54 NDV = 82-92

fsp = 7.6.6 fCx = 1B.1B.2B Sensillae dimension = 23 (19-26) x 13 (12-14)

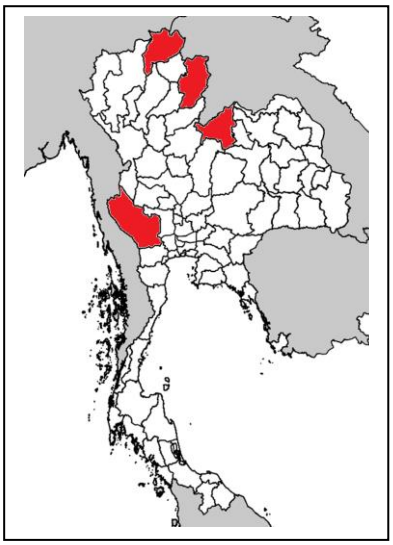
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	25	30	22	17	33	50	32	-	17	21	22	160	138	167	471
Max	30	39	24	20	38	57	35	-	20	25	28	185	163	189	536
Mean	28	34	23	18	35	53	33	-	18	22	26	172	150	177	500

Based on 10 specimens

Distribution:

China

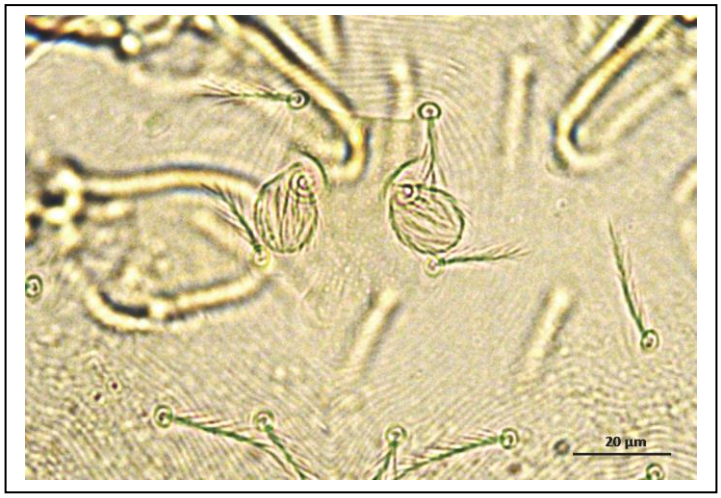
In Thailand: Chiangrai, Kanchanaburi, Loei, Nan (see map).



Host in this study:

Bandicota indica, B. savilei, Berylmys berdmorei, B. bowersi, Leopoldamys edwardsi, Maxomys surifer, Mus cookii, Niviventer fulvescens, Rattus andamanensis, R. exulans, R. sakaeratensis, R. tanezumi

Scutum photo:



Walchia pingue Gater, 1932

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia pingue*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL ≥ AL

fD = 2H + 6.6.6.8(6).4.2.2; ND = 34-36 fV = varied; NV = 40-57 NDV = 74-93

fsp = 7.6.6 fCx = 1B.1B.2B Sensillae dimension = 26 (26-27) x 11 (11-12)

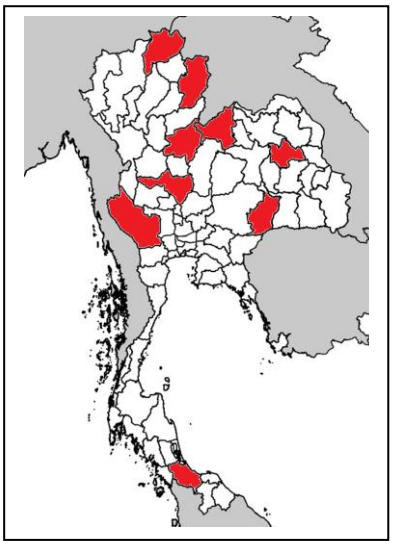
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	25	41	23	19	38	57	33	-	20	21	24	167	145	169	482
Max	35	48	31	23	42	64	39	-	25	26	28	191	160	196	547
Mean	32	45	29	20	40	60	37	-	23	24	26	178	154	180	513

Based on 9 specimens

Distribution:

Malaysia

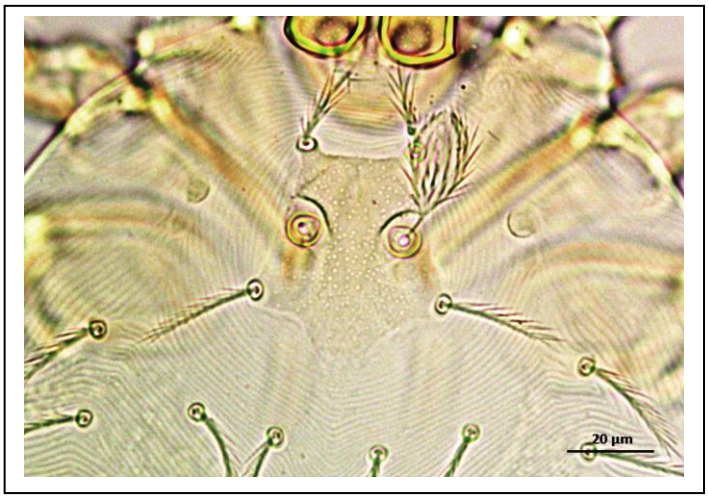
In Thailand: Buriram, Chiangrai, Kalasin, Kanchanaburi, Loei, Nakhonsawan, Nan, Phitsanulok, Songkhla (see map).



Host in this study:

Bandicota indica, B. savilei, Leopoldamys sabanus, Maxomys surifer, Niviventer fulvescens, Rattus sakaeratensis, R. tanezumi

Scutum photo:



Walchia rustica (Gater, 1932)

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia rustica*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = AL > PL

fD = 2H + 6.6.6.6(8).4.4.2; ND = 36-38 fV = varied; NV = 42-50 NDV = 78-88

fsp = 7.6.6 fCx = 1B.1B.1B Sensillae dimension = 30 (26-33) x 11 (11-12)

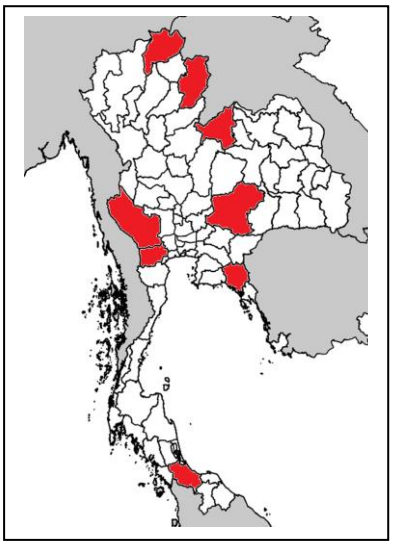
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	47	59	38	24	55	80	39	-	31	29	34	195	171	202	571
Max	50	66	41	26	64	89	46	-	36	32	39	217	179	213	607
Mean	48	62	39	25	59	84	42	-	33	31	37	203	174	207	585

Based on 6 specimens

Distribution:

China, India, Malaysia, Myanmar, Vietnam,

In Thailand: Chantaburi, Chiangrai, Kanchanaburi, Loei, Nakhon Ratchasima, Nan, Ratchaburi, Songkhla (see map).



Host in this study:

Bandicota indica, B. savilei, Berylmys berdmorei, B. bowersi, Rattus sakaeratensis, R. tanezumi

Scutum photo:

Walchia ventralis (Womersley, 1952)

Classification: Family Trombiculidae > Subfamily Gahrliepiinae > Tribe Gahrliepiini > Genus *Walchia* > Species *Walchia ventralis*

Diagnosis:

fPp = N/N/NNN + 4B Ga = 1N fSc = PL > AL

fD = 4H + 6.8.6 then varied; ND = 35-36 fV = varied; NV = 41-46 NDV = 76-82

fsp = 7.6.6 fCx = 1B.1B.1B

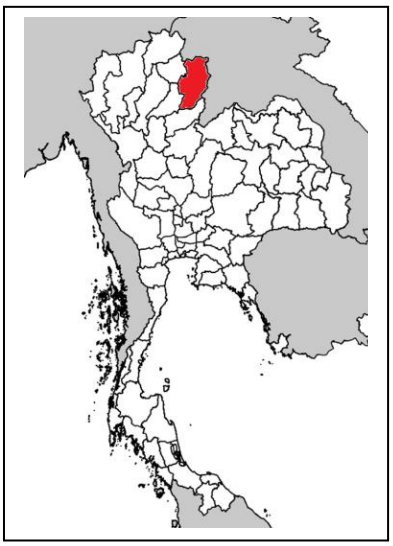
	AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	pa	pm	pp	lp
Min	31	41	29	23	47	70	40	-	29	37	39	237	216	244	699
Max	32	44	31	25	51	75	44	-	31	39	40	259	231	260	751
Mean	31	42	30	24	49	73	41	-	30	37	39	251	224	254	730

Based on 4 specimens

Distribution:

Malaysia

In Thailand: Nan (see map).



Host in this study:

Berylmys bowersi

Scutum photo: