The vector biology and microbiome of parasitic mites and other ectoparasites of rodents

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

by

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October 2019

Abstract

Background: Rodents have become increasingly recognised as hosts of ectoparasites and reservoirs of numerous vector-borne bacterial zoonoses including scrub typhus (*Orientia* spp.), bartonellosis (*Bartonella* spp.), Lyme disease (*Borrelia burgdorferi* complex), and plague (*Yersinia pestis*). While these diseases are emerging or re-emerging worldwide, the epidemiology and ecology is often poorly described outside Europe and North America, adversely affecting timely surveillance.

Objectives: This study aimed to define the taxonomic diversity and bacterial microbiome of ectoparasites collected from wild rodents in the Asir Region of southwestern Saudi Arabia, with a main focus on chigger mites (family Trombiculidae), the vectors of scrub typhus. The distribution of non-pathogenic bacterial symbionts in ectoparasites that might impact disease transmission was also explored, including in a preliminary study on a laboratory colony of the tropical rat mite, *Ornithonyssus bacoti*.

Methods*:* Wild rodents were trapped in scrubland across one site on the slopes of the Asir Mountains in 2016 (Alous village) and three sites in 2017 (Alous, Alogl and Wosanib). Rodents were euthanized prior to examination and all ectoparasites were collected and stored in absolute ethanol. A 10% subsample of ectoparasites was selected from each rodent for mounting in Berlese fluid and morphometric examination. Following DNA extraction, the v4 region of the bacterial 16S rRNA gene was amplified by PCR, and amplicons were sequenced using Illumina technology. Specific PCRs were used to confirm the presence and strain of selected bacterial pathogens and symbionts. For the *O. bacoti* colony, the mite microbiome was profiled using Illumina and Oxford Nanopore sequencing technologies alongside conventional culture on solid media.

Results: A total of 8,270 ectoparasites were obtained from 74 rodent specimens belonging to five species (*Acomys dimidiatus*, *Myomyscus yemeni*, *Mus musculus*, *Rattus rattus* and *Meriones rex*), comprising 6,774 chigger mites and 1,496 other ectoparasites. Based on the morphology of the scutum, chiggers were assigned to subgenera and provisionally into 19 species, including four newly described species: *Schoutedenichia asirensis* sp. nov*.*, *Schoutedenichia saudi* sp. nov*., Microtrombicula microscuta* sp. nov*.,* and *Microtrombicula muhaylensis* sp. nov*.* Fifteen species were for the first time recorded in Saudi Arabia and

on new host species. The site with the highest mean chigger infestation (97.05%) was Alous, and the host species with the greatest mean infestation rate (60.81%) was the Eastern spiny mouse (*A. dimidiatus*). Three flea species, two louse species and two gamasid mite species were identified with high confidence; whereas immature ticks of the genera *Rhipicephalus* and *Haemaphysalis* were allocated to three and two molecular clades, respectively. Potentially pathogenic bacteria detected in ectoparasites included *Orientia chuto* and a *Coxiella burnetii*-like organism in chiggers, *Bartonella acomydis* in fleas, and organisms related to *Ehrlichia ewingii* and *Anaplasma* spp. in ticks. Symbiotic bacteria with putative mutualistic or parasitic phenotypes were present in fleas (*Wolbachia* clade B, *Candidatus* Cardinium and *Spiroplasma ixodetis*), lice (*Candidatus Legionella polyplacis*), ticks (*Coxiella*- and *Francisella*-like endosymbionts), and chiggers (*Wolbachia* and *Candidatus* Cardinium) as first records for Saudi Arabia. In the *O. bacoti* colony, the mite microbiome included *Staphylococcus*, *Proteus* and *Bacillus*, while *Alcaligenes faecalis* was isolated from mites infected with filarial worms.

Conclusions: This is the first survey of rodent ectoparasite diversity and zoonotic bacterial pathogens performed in the Asir region of Saudi Arabia. The chigger diversity in the region is especially high, and the presence of *Orientia* and *Coxiella* spp. in these mites should be investigated further to determine if they might be vectors of scrub typhus and/or involved in transmission of Q-fever. Other potential pathogens (*Anaplasma* and *Ehrlichia*) were detected in ticks; whereas all ectoparasite groups, including chiggers, contained putative symbionts. In *O. bacoti* mites, the detrimental impact of filarial infection might be associated with *A. faecalis*.

Author's Declaration

The following describes the contributions of other individuals to the work presented in this thesis alongside my own.

Chapter 3

The 2016 fieldwork trip was performed with assistance from Dr Abdulaziz N. Alagaili (Department of Zoology, King Saud University, Riyadh) and my primary supervisor with the rodent trapping and ectoparasite collection. The confirmation of chigger identifications and the formal description of new species was conducted by Dr Alexandr A. Stekolnikov (Zoological Institute of the Russian Academy of Sciences, Saint Petersburg), who also provided the micrographs and drawings of the new species presented in this chapter. Other ectoparasites were identified with training and assistance from my secondary supervisor, Dr John McGarry. The ecological analyses of host-ectoparasite interactions in R were initially conducted by Dr Kittipong Chaisiri (Mahidol University, Bangkok) then repeated by myself, with modification of the figures as required. The phylogenetic trees were produced with assistance from Dr Xiaofeng Dong (Institute of Infection & Global Health, University of Liverpool), except for the *Rhipicephalus* spp. analysis (Figures 3.22 and 3.23), which was performed by Dr Lucas Low (Tropical Infectious Diseases Research & Education Centre, University of Malaya).

For this chapter, I conducted the 2017 field trip, collection and mounting of all ectoparasite material and preliminary identifications, as well as DNA extractions, PCR and initial sequence analyses for both rodents and ectoparasites.

Chapter 4

I performed the DNA extractions on ectoparasite, soil and swab samples, and then I prepared barcoded 16S rRNA amplicon libraries under training from Dr Christina Bronowski (Institute of Integrative Biology, University of Liverpool). Subsequent size selection, pooling and pre-sequencing QC steps, as well as post-sequencing QC, was undertaken by core staff at the Centre for Genomic Research, University of Liverpool. The analyses of microbiome

profiles in QIIME 2 and R were conducted by myself under training from my second supervisor, Prof. Alistair Darby.

Chapter 5

I performed all of the specific PCRs and initial sequence analyses presented in this chapter with assistance from Dr Ana M. Palomar (Center for Biomedical Research, La Rioja) during her research visit to Liverpool. The cloning of the *Bartonella* sequence was conducted by technician Cathy Glover (Institute of Infection & Global Health, University of Liverpool).

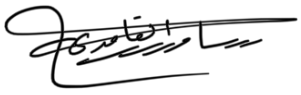
Chapter 6

Dr Coralie Martin's team at the Muséum national d'histoire naturelle, Paris, conducted the antibiotic experiments in jirds and collection of *Ornithonyssus bacoti* mites. I performed the Oxford Nanopore sequencing on the MinION under training from Aleksandra Beliavskaia (Institute of Integrative Biology, University of Liverpool), while Alex conducted the initial assignments of bacterial OTUs. Dr Coralie Martin's team sent fixed bacterial colony material prepared from mite homogenates to Liverpool for DNA extraction.

For this chapter, I conducted DNA extractions, PCR and the initial preparation of mite homogenate cultures during a research visit to the Muséum national d'histoire naturelle in Paris. I also performed the analysis of sequences obtained from bacterial colonies.

I declare that the above is a true and accurate description of how the data presented in this thesis were obtained.

Signed: Samia Alghamdi Date: 31.10.2019



Acknowledgements

It is my pleasure to acknowledge the roles of several individuals who were instrumental in the completion of my PhD research. First and foremost, I would like to thank Allah Almighty for giving me the strength, knowledge, ability, and opportunity to undertake this research and to persevere and complete it satisfactorily. Without His blessings, this achievement would not have been possible.

I would like to express my special appreciation and profound respect to my supervisor Dr Ben Makepeace for all the support, patience, and encouragement he gave me during my PhD project. Without his guidance and constant feedback, I would not have been able to complete this PhD. I also owe a lot of thanks to my two co-supervisors, Prof. Alistair Darby for his help in analysing 16S microbiome data for all ectoparasites and for supporting and helping me perform a comprehensive analysis of an individual ectoparasite microbiome, and Dr John McGarry for his scientific advice and knowledge in the identification of Saudi ectoparasites and UK samples.

I also acknowledge the financial support provided by Al Baha University-Saudi Arabia towards my PhD at the University of Liverpool and the Saudi Arabian Cultural Bureau in London.

I would especially like to thank Dr Alexandr Stekolnikov (Zoological Institute of the Russian Academy of Sciences, Saint-Petersburg) for his help in identifying chigger mites and describing four new species. I am also grateful to Dr Abdul-Aziz Alagaili (Department of Zoology, King Saud University, Riyadh) for his help during our fieldwork in the Asir region of Saudi Arabia.

Heartfelt thanks should also be expressed to a true friend, 'Tonk' (Dr Kittipong Chaisiri, Mahidol University, Bangkok, Thailand), who supported me since the first day of my PhD and was always so helpful. His assistance was invaluable throughout my thesis, particularly in ecological analysis. I want to express my gratitude to Dr Christina Bronowski for her help in the 16S rRNA amplicon sequencing method and Aleksandra Beliavskaia for her assistance in Oxford Nanopore analysis. Huge thanks to the technical team, especially Catherine Hartley and Cathy Glover for their endless assistance and support in the laboratory work. A massive thank you to all my colleagues and staff in the Department of Infection Biology for their encouraging words.

My sincere thanks also goes to my IPAP Members Prof Jane Hodgkinson and Dr Kannan Ganapathy for their supporting and encouraging words throughout the four years of my PhD. Thanks to Dr John Graham-Brown (University of Liverpool) for helping me produce the study location map for Asir region, Dr Ana Maria Palomar (Center for Biomedical Research, La Rioja) for her help in species-specific PCRs, and my other colleagues Dr Xiaofeng Dong, Dr Jordan Clark, Sanaria, Sham and Merit, for their continuous support.

I would like to thank Dr Coralie Martin for her help in sending *Ornithonyssus bacoti* samples from a laboratory colony at Muséum National d'Histoire Naturelle, Paris. Additionally, I would like to thank the CGR team Drs Pia, Anita, Chris, Richie, and Edith for their help in amplicon library preparation, pooling on the 'Mosquito', and QC. I am also grateful to Dr Low Van Lun (Tropical Infectious Diseases Research & Education Centre, University of

Malaya) for providing the phylogenetic tree for three new lineages of *Rhipicephalus* spp. ticks.

My deep appreciation goes out to my family. Words cannot express how grateful I am to my queen-mother, my sister, and brothers for their constant support. Your prayer for me was what sustained me thus far. I would like to thank my late father Qassem who taught me that ventures do not fail and enabled me to face the world. I miss you, father. I would prefer to pay homage by dedicating this work to my late father under whose careful protection I have been able to enjoy my life.

A very special thank you to my beloved husband, Abdulrahman, for always being so supportive towards my work, believing in me, and encouraging me to follow my dreams. Of course, a big thank you to my lovely boys, Mohammed and Mutaz, and my princess Marah for their patience and love while I completed my PhD. I am sure this thesis would never have been completed without you. I thank you all.

Dedication

I dedicate this thesis to my mother and my late father, my husband Abdulrahman, and my beloved children for their constant support and unconditional love.

I love you all dearly.

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List of Abbreviations

%	percentage
16S rDNA	rDNA 16 Svedberg ribosomal deoxyribonucleic acid
16S rRNA	16 Svedberg ribosomal ribonucleic acid
56-KDa TSA	56 Kilodalton type-specific antigen
AG	ancestral group
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide basic local alignment search tool
bp	base pair
CCAC	Canadian Council on Animal Care
CGR	Center for Genomic Research, University of Liverpool
Chao1	Chao1 non-parametric richness estimator
COI	cytochrome oxidase subunit 1 gene
CSR	chigger species richness
cytb	cytochrome b gene
DNA	deoxyribonucleic acid
e.g.	for example
et al.	and others
etc.	and other things
gltA	citrate synthase gene
GPS	Global Positioning System
groEL	chaperonin gene
H’	Shannon diversity index
HTS	high throughput sequencing
HGA	Human granulocytic anaplasmosis
ID	identity
Jack1	first-orderd Jackknife estimator

kDa	kilodalton
km2	square kilometre
KSA	The Kingdom of Saudi Arabia
KW	non-parametric Kruskal-Wallis test
m	metre
M	molar
min	minute
ml	millilitre
ML	Maximum likelihood
MLST	multilocous sequence typing
mm	millimetre
mM	millimolar
n. sp.	specimen new or undescribed
NCBI	National Center for Biotechnology Information
NJ	neighbour joining
ONT	Oxford Nanopore Technologies systems
OTU	operational taxonomic unit
Path	pathogen
PCA	principle component analysis
PCoA	principle coordinate analysis
PCR	polymerase chain reaction
pg	picogram
p-value	calculated value probability
QIIME	Quantitative Insights into Microbial Ecology software package
QP3.0	Quantitative Parasitology 3.0 .
RMSF	Rocky Mountain spotted fever
rpm	round per minute
RT	room temperature
SFG	the spotted fever group
sp.	species (singular form)

spp.	species (plural form)
Sym	symbiont
TG	typhus group
TRG	transitional group
UK	The United Kingdom
UniFrac	unique fraction phylogenetic-based measurement methods
USA	The United States of America
V	voltage
WHO	World Health Organization
wsp	Wolbachia surface protein gene
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	alpha
β	beta
µl	microlitre
µm	micrometre (micron)
µM	micromolar
FLT	Flush Tether
SQB	Sequencing Buffer
LB	Loading Beads
FLB	Flush Buffer

Chapter 1. General Introduction

1.1 The importance of rodent-associated, vector-borne bacterial zoonoses in human history

Zoonoses can be defined as diseases which are transferrable from animals to humans. According to the World Health Organisation (WHO), 75% of human diseases identified as emerging over the past years were acquired from animal sources (WHO, 2005). Wild animals including rodents play a crucial role in maintaining pathogens in the natural environment, and contribute to the discrete distribution and transmission of more than 200 zoonotic infectious diseases (Sharma *et al.,*2018). The mechanism of transfer of these pathogens can be achieved through direct exposure to rodent excreta or bodily fluids/secretions (*e.g*., urine, blood) or indirectly via arthropod vectors such as mites, ticks, fleas, and lice (Telmadarraiy *et al.,*2007; Torres-Mejía. *et al.,*2006).

Some of the most important rodent-associated pathogens that are normally transmitted without the involvement of an arthropod vector are hantaviruses, arenaviruses (*e.g*., Lassa virus) and *Leptospira* spp. (spirochete bacteria). Hantaviruses and arenaviruses can cause hemorrhagic fevers with high fatality rates and are both transmitted via rodent excreta. Korean hemorrhagic fever (now called hemorrhagic fever with renal syndrome, HFRS) was first reported during the Korean War (1950 – 1953) and subsequently in Europe, while in the New World, hantavirus cardiopulmonary syndrome (HCPS) was not recognised until 1993 (Jonsson *et al.,*2010). Whereas HFRS has been reported as endemic in eastern and northern Europe and Asia, HCPS has been described only in the New World (Khan and Khan 2003). Transmission occurs by inhalation of aerosols of virus from rodent feces or urine contamination. Murine rodents, shrews and moles are recorded as natural reservoir hosts for hantaviruses (Olsson *et al.,*2010). Lassa fever was described in the Nigerian town of Lassa in 1969 and is a potentially fatal disease transmitted by excreta of the African multimammate rat (*Mastomys natalensis*), a commensal rodent (Lecompte *et al.,*2006). This virus is restricted to several countries in West Africa, including Nigeria, Guinea, Sierra Leone, Liberia and Ivory Coast. The incidence rate has been estimated as approximately 2-3 million cases per year in West Africa with 5,000 deaths (Goeijenbier *et al.,*2013). Finally, leptospirosis is a globally-distributed bacterial zoonosis that was first described by Adolf Weil in Germany in 1886. It is associated with aquatic environments and warm weather

and cases often increase after heavy rainfall (Bharti *et al.,*2003). Transmission is primarily through contact with soil or water contaminated with rodent urine, although a wide variety of other animals can act as reservoirs (including dogs, horses, cattle, and pigs). Leptospirosis is difficult to diagnose, so cases are probably greatly underestimated, especially in developing countries (Bharti *et al.,*2003).

While hantaviruses, arenaviruses and leptospirosis are important exceptions, most rodent-associated zoonoses either require a haematophagous arthropod vector for transmission, or arthropods constitute an alternative to direct transmission routes. Focusing on bacterial zoonoses, rodents are considered significant reservoirs of the causative agents of numerous human diseases such as Rocky Mountain spotted fever (RMSF), other rickettsioses, Lyme borreliosis, scrub typhus, plague, and tularemia (Dantas-Torres, 2008; Margos *et al.,*2008; Peterson, 2009; Sjöstedt, 2007; Wren, 2003). Examples of pathogens transmitted through rodent/arthropod vector cycles are described in the following sections.

Rocky Mountain spotted fever (RMSF) is a potentially fatal disease caused by *Rickettsia rickettsii*, an obligatory intracellular bacterium, which is transmitted by infected ticks to humans. Historically, the disease was first described by Edward E Maxe in the late 19th century in Idaho, USA (Dantas-Torres, 2008). He observed several signs from patients experiencing a febrile illness called “spotted fever”. Subsequently, these lesions promptly cover all parts of the body (Maxey, 1899). The infected ticks are the primary source for the transmission of this disease to humans (Dantas-Torres, 2007). It was shown that these ticks are able to transmit the pathogen to their offspring through infection of the eggs in the ovary (Ricketts and Gomez, 1908; Ricketts, 1991) but female ticks can also be infected during mating. Humans can only be infected via a tick bite or very rarely through blood transfusion (Johnson and Kadull, 1967; Wells, 1987). Typically, the bacterium infects endothelial cells and it can be found in the nucleus and also in the cytoplasm of the host cell (Harrell, *et al.,*1949; Lacz *et al.,*2006; Wolbach, 1919). In terms of the vectors of *Rickettsia rickettsii* in the USA, the primary species are the American dog tick (*Dermacentor variabilis*) and the Rocky Mountain wood tick (*Dermacentor andersoni*). However, the brown dog tick (*Rhipicephalus sanguineus*) is an important vector the southern USA,

Central America and South America (Bustamante and Varela, 1947) as is the Cayenne tick *(Amblyomma cajennense*) across these same regions (Chapman *et al.,*2006). It is reported that around 250 - 1,200 cases of RMSF are recorded each year in the USA (Labruna, 2009). Clinically, the symptoms include a high fever with purple eruptions in the skin, which are seen first on ankles, wrists, and forehead. While the role of rodents in the epidemiology of RMSF has been underexplored, the tick vectors of this disease feed on rodents as larvae and nymphs. Serological and molecular studies in both North and South America lend support to the idea that rodents may serve as "amplifier hosts" for *R. rickettsii* (Zipser Adjemian *et al.,*2008; Labruna, 2009).

A second major tick-borne disease is Lyme borreliosis, caused by the bacteria *Borrelia burgdorferi* and *Borrelia mayonii* in North America, and *Borrelia afzelii*, *Borrelia garinii* and *Borrelia spielmanii* in Europe and Asia, which are transmitted by infected *Ixodes* spp. Geographical records of this bacteria are widely distributed across Europe and America (Steere, 2001). The disease quickly spread in most of New England and the Midwest, and more than 30 000 cases of Lyme disease annually are recorded in the USA, although its actual occurrence could be ten times greater than this (CDC, 2015). In terms of maintenance in the environment, the disease circulates in an epizootic cycle, in which the bacteria are transmitted between the *Ixodes* tick vector and vertebrate hosts (Spielman *et al.,*1985). The most important maintenance hosts are the white-footed mouse (*Peromyscus leucopus*) in the USA and bank voles (*Myodes glareolus*) in Europe, although many other rodent species and other small vertebrates play roles of varying significance (van Duijvendijk *et al.,*2016). *B. burgdorferi* is the most common tick-borne infection causing human disease in the United States, Europe and also in Asia. This disease clinically was first discovered in Lyme (Connecticut) in the 1970s but the history of its spread is still unclear (Walter *et al.,*2017; Steere *et al.,*2004). One of the most important approaches to enable an understanding of the epizootic distribution and the evolutionary relationships of the Lyme pathogens is the multi-locus sequence typing (MLST) scheme, comprising both cultured isolates and infected ticks. The MLST data suggest that *B. burgdorferi* originated in Europe rather than in North America as proposed previously. Furthermore, MLST may also have the power to identify ecotypes within *B. burgdorferi* and determine their ecological niches (Margos *et al.,*2008). The reason for the increase of human cases of Lyme

disease in the USA has been attributed to several factors such as the expansion tick habitat or geographic ranges of pathogenic genotypes within *B. burgdorferi*, wild host population sizes, climate and ecological change, and evolution of *B. burgdorferi* (Walter *et al.,*2017; Qiu *et al.,*2002).

Another major bacterial disease associated with rodents is scrub typhus, a zoonosis caused by *Orientia* spp. (family Rickettsiaceae). Scrub typhus is transmitted via the bite of chigger mites (larvae of the Trombiculidae) and is maintained in the vector by transstadial and transovarial transmission. The first description of this disease was in Japan in 1899. Its key historic range is the "tsutsugamushi triangle" extending from Pakistan, south-eastern Russia, South-East Asia, Japan, and northern Australia (Peterson, 2009). A human becomes infected by accidental contact with chiggers during outdoor activities and they feed on host tissue fluid (Harwood & James, 1979). The main hosts of chiggers are small mammals (especially rodents) and birds, both of which can become infected by *Orientia* spp. (Traub *et al.,*1975). Larvae of the genus *Leptotrombidium* are the primary vector of the causative bacterium. Scrub typhus came to global attention during World War II due to massive impacts on both sides of the conflict in the Pacific. This was particularly true during the invasion of Sansapor in Dutch New Guinea during July – September 1944. After American troops camped on the ground, almost a 1,000 men were hospitalised with scrub typhus within a few weeks (Peterson, 2009). Overall, although malaria and dengue affected the greatest number of troops during the Second World War, scrub typhus caused the death of as many as malaria (Philip, 1964).

Probably the most famous rodent-borne disease is plague. This is a disease caused by *Yersinia pestis*, a Gram-negative bacterium of the family Enterobacteriaceae, which infects fleas as a primary vector and is maintained in rodents including mice, rats, rock squirrels, wood rats and squirrels. The disease is one of the oldest illnesses ever recorded and killed 75 - 200 million people in Eurasia during the medieval Black Death (CDC.gov, 2019). Six hundred years prior to this, the first plague pandemic (the Plague of Justinian) contributed to the collapse of the Eastern Roman Empire. Although plague epidemics begin with vector-borne transmission of *Y. pestis* by rat fleas, they can be maintained by direct human-to-human transmission via aerosols (pneumonic plague) (CDC.gov, 2019). Direct transmission

from handling infected animal carcasses or aerosol transmission from contact with infected companion animals, especially cats, is also possible (Wren, 2003).

Finally, tularemia or ”rabbit fever’’, is an infectious disease caused by the Gram-negative coccobacillus, *Francisella tularensis* (Staples *et al.,*2006), which was discovered to be the cause of tularemia in the early 20th century by Edward Francis (Sjöstedt, 2007). Four subspecies were determined and classified as *tularensis*, *holarctica*, *mediasiatica*, and *novicida*. However, *F. tularensis* subsp. *tularensis* has only been isolated in North America, and it was the most prevalent strain observed on the continent. It has been estimated to cause 70% of the human cases in North America based on its representation in strain collections (Staples *et al.,*2006). While tularemia has become extremely rare in North America, it still causes periodic outbreaks in Sweden and Finland (Sjöstedt, 2007). Importantly, several transmission routes are possible for this disease, such as contact with dead rodents or rabbits, contact with the faeces of infected small mammals, inhalation of aerosols (contaminated dust), ingestion of contaminated water or undercooked meat, and the bite of vectors such as mosquitoes, deer flies and ticks.

In 1923, tick-borne tularemia was discovered by physicians in Idaho who distinguished enlargement of lymph nodes in response to a tick bite. Moreover, in 1924, Parker was the first scientist to isolate *F. tularensis* from a tick, *D. andersoni,* in Montana (Petersen *et al.,*2009).

During World War II, huge increases in rodent populations in Russia led to tularemia outbreaks affected tens of thousands of people and a large spike in tularemia cases was also observed during the Bosnian wars of the 1990s (Sjöstedt, 2007). *F. tularensis* was the subject of intense research as a biological weapon in both the Soviet Union and the USA during the Cold War, and remains a category A Select Agent in the USA (of the highest concern for bioterrorism).

1.2 The major ectoparasite groups found on rodents

1.2.1 Mites

Mites can be defined as small arthropods belonging to the subclass Acari (class Arachnida) of the phylum Arthropoda, which is divided into two superorders: the Acariformes (including astigmatid and prostigmatid mites) and the Parasitiformes (mesostigmatid mites and ticks) (Brennan, 1957). Although 48,000 species have been described, Acari (mites) are considered to be one of the most varied groups of arthropods, possibly including more than one million species (Chauhan *et al.,*2015; Halliday *et al.,*2009). Mites are found across highly diverse habitats: terrestrial, freshwater, forest, agricultural lands, and even the upper atmosphere (Mullen & Durden, 2009). Additionally, more than 400 described families of mites have increased since 1978 to an incredible 540 recently (Halliday, 2009). The variety of acarine morphology reflects the diversity of ecological niches to which mites have become adapted (Mullen & Durden, 2009). While most species of mites live freely in the environment, some species in multiple independent lineages have developed parasitic lifestyles on animals or plants (Hubert *et al.,*2012). Their morphological and ecological diversity can be considered a measure of their success (Brennan, 1957). They are also regarded as pests of stored food products, feeding on foods including oilseeds, dried fruit, and dried meat; while herbivorous mites feed on over 1,100 plant species (Hubert *et al.,*2012). As a result of their ubiquity in the environment, allergies are caused by dust mites and several other mite species (Basagaña *et al.,*2004).

1.2.2 Ticks

Ticks are also classified in the subclass Acari and are obligatory blood-sucking arthropods found in most areas of the world. Although approximately 1,000 tick species have been described, of which 10% represent vectors of significant medical or veterinary importance (Jongejan & Uilenberg, 2004). Ticks are classified in the order Ixodida within the superorder Parasitiformes (Zhang *et al.,*2019; Cabezas-Cruz *et al.,*2018), and are divided into three families: the Ixodidae (hard ticks) including 694 species, the Argasidae (soft ticks) including 177 species, and the Nuttalliellidae, which contains a single species restricted to southern Africa (Parola & Raoult, 2001) (Figure 1.1). Ticks transmit a large number and variety of

pathogens, threatening humans and other animals worldwide. However, the importance of ticks on public health was more widely recognized with the identification and emergence of *B. burgdorferi* as the causative agent of Lyme disease in 1982 (Parola & Raoult, 2001). Additionally, in Europe, Lyme borreliosis is a widespread tick-borne disease (vectored by *I. ricinus*) in humans (Vayssier-Taussat *et al.,*2015).

Globally, ticks are considered the second most important vectors after mosquitoes for transmitting pathogens, including bacteria, protozoa, helminths, and viruses, to humans and animals (Mediannikov *&* Fenollar, 2014). Furthermore, environmental factors affect the distribution of tick-borne diseases, although the climate is not the only determinant of the geographical distribution of tick species, their population densities and dynamics (Rizzoli *et al.,*2014). In addition to their vector role, ticks are capable of causing severe conditions such as paralysis and toxicities, irritation and allergy (Jongejan & Uilenberg, 2004). Zoonotic diseases worldwide are transmitted by a small number of key species in the genera *Ixodes, Hyalomma, Rhipicephalus, Amblyomma, Haemaphysalis, Dermacentor* and *Ornithodoros*. Most of the important tick vectors use rodents as hosts for the immature stages (larvae and nymphs), with the adults feeding on larger animals such as ruminants, horses and dogs (Schulze *et al.,*2017). Therefore, rodents are important for maintaining populations of ticks that may also bite humans as nymphs or adults, so controlling ticks on rodents could reduce the risk of zoonotic disease (Schulze *et al.,*2017). Rodents also harbour populations of specialist “nidiculous” ticks that live in burrows and nests. Examples of these in Europe are *Ixodes acuminatus* and *Ixodes trianguliceps*, in which the adults as well as the immature stages feed on the rodents. These species are generally not thought to be involved in zoonotic disease transmission because they very rarely bite humans (Jameson & Medlock, 2011).

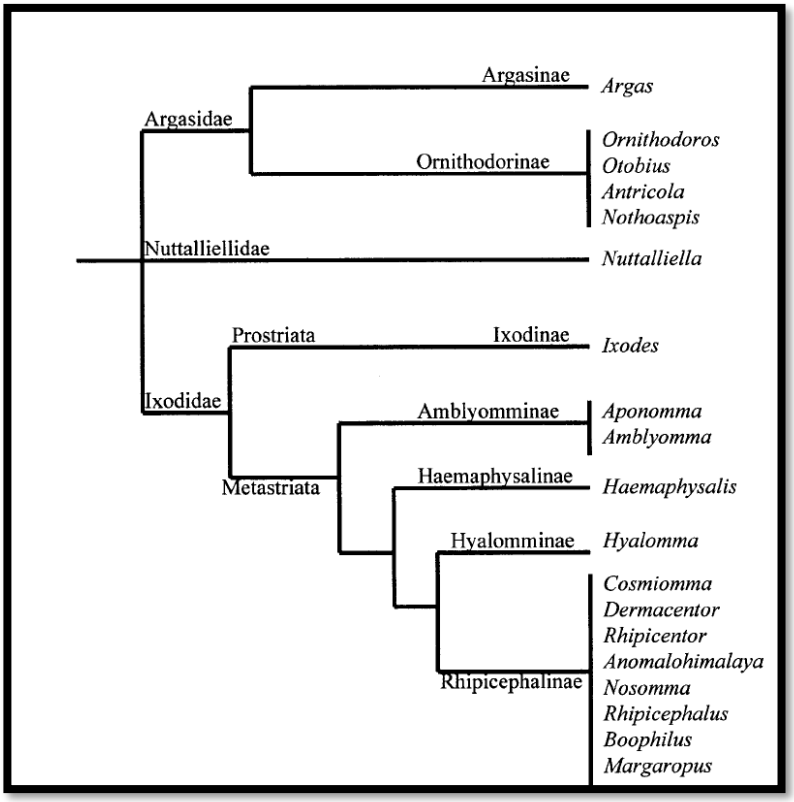


Figure 1.1. Tick classification based on Hoogstraal (Parola & Raoult, 2001).

1.2.3 Fleas

Fleas (order: Siphonaptera) are highly diverse ectoparasites. This order contains approximately 2,500 species and subspecies, grouped into 244 genera in 15 families (Hastriter& Whiting, 2009), while Medvedev (1996) identifies 18 families. Several flea species have been implicated in pathogen transmission. They are small and wingless insects that undergo holometabolous metamorphosis (Torina *et al.,*2013). The lifecycle of fleas consists of egg, larva, pupa, and adult. The larvae are not parasitic but feed on organic debris in the rodent burrow or indoor environment (Krasnov *et al.,*2004). All species in the adult stage are parasitic, and the mouthparts are modified for sucking (Bonnefoy *et al.,*2008). Approximately 94% of the known species occur on mammals; the remaining 6% on birds. In 1898, it been demonstrated that fleas were responsible for transmitting diseases such as murine typhus, which stimulated a group of flea studies (Guernier *et*

*al.,*2014). It has pointed out that less than 100 species of fleas had described by the end of the 19th century, and after that, A. C. Oudemans, Julius Wagner, Nathan C. Rothschild, and Karl Jordan (the "father of flea systematics") made significant contributions. From 1953 to 1971, G. H. E. Hopkins and M. Rothschild published a comprehensive five-volume series on flea systematics, and three additional companion volumes were issued for the remaining families by Mardon in 1981; Traub, Rothschild, and Haddow in 1983; and Smit in 1987. Besides, Hastriter *et al.,*2009 identified 153 flea taxa and provided critical insights into flea phylogeny, convergent evolution, and zoogeography. Fleas are of great medical significance as vectors of the causative agents of several diseases in humans and other animals, and rodents are the principal reservoirs for these pathogens. Globally, the major flea genera on rodents are reported as essential vectors *Y. pestis* and they may play role in the maintenance of *F. tularensis* in nature (Rodríguez-Pastor *et al.,*2019). Fleas also transmit several pathogenic rickettsiae, the most important of which is *Rickettsia typhi*, the causative agent of murine typhus, which is associated with opposums and cats as well as rats (CDC.gov 2019). Recently, *Rickettsia felis* in fleas and other blood-feeding arthropods has been recognised as an emerging zoonosis worldwide, with similar symptoms in humans to murine typhus (Pérez-Osorio *et al.,*2008). Moreover, fleas are the main vectors/reservoirs of *Bartonella* spp.(Chomel *et al.,*2006).

1.2.4 Lice

Lice (order Phthiraptera) are another group of flightless, obligate ectoparasitic insects feeding on blood. Four phthirapteran suborders have identified: the chewing louse suborders (Amblycera, Ischnocera and Rhynchophthirina), and the sucking louse suborder (Anoplura) (Light *et al.,*2010; Johnson *et al.,*2004). Moreover, sucking lice are the more highly widespread taxon on most major groups of eutherian mammals. Although there are up to 540 described species of Anoplura, the potential economic and medical implications of sucking louse infestations are limited to a few species found on humans and domestic animals (Kim, 2007; Light *et al.,*2010). Human body lice (*Pediculus humanus corporis*) are notorious as vectors of epidemic typhus (*Rickettsia prowazekii*), trench fever (*Bartonella* quintana) and relapsing fever (*Borrelia recurrentis*) Warrell, (2019). However, due to the

high host specificity of lice, they are not involved in zoonotic disease transmission; *i.e*., rodent lice will not bite humans and vice-versa.

One of the most widespread genera of Anoplura on rodents is *Polyplax*, for which a substantial monograph has been published (Paterson *et al.,*1953). The best-studied species are *Polyplax serrata* on *Apodemus* spp. mice (Martinů *et al.,*2018) and *Polyplax spinulosa* on *Rattus* spp. worldwide (Frye *et al.,*2015). *Polyplax* spp. lice are common ectoparasites on rodents sold as pets or supplied as food for other pets (Reeves & Cobb, 2005). In the wild, the species diversity of *Polyplax* can be high even within a relatively small area due to host specificity (Krištofík, & Dudich, 2000).

1.3 The Biology of Trombiculid mites

Chigger mites are the larval stage of prostigmatid mites that belong to the family Trombiculidae. Chigger mites can act as vectors of pathogens and often also cause dermatitis and itching. Medically, some species of *Leptotrombidium* are significant as vectors transmitting *Orientia* spp., the causative agent for scrub typhus, also known as "tsutsugamushi fever" (Peng *et al.,*2016). The disease is widespread in northeastern Japan, Southeast Asia, the western Pacific Islands, eastern Australia, China, and parts of south-central Russia, India, and Sri Lanka (Kelly *et al.,*2009).

Chigger mites are recognized as parasites of rodents, other vertebrates and occasionally invertebrates, and they are vectors of other zoonoses in addition to scrub typhus, such as hantavirus disease (Yu and Tesh, 2014). Chiggers are known by different names worldwide, such as redbugs (America), harvest mites (Europe), and scrub itch mites (Australia). Hence, investigating their distribution, diversity, and seasonal abundance is vital for public health. Morphologically, chigger mites are microscopic (approximately <1 mm in length), hairy and round-shaped and differ in colour from white, orange, brown, and red (Nadchatram & Dohany, 1974).

1.3.1 Lifecycle and ecology

The life cycle of chigger mites is comprised of seven stages: egg, deutovum (pre-larva), larva, protonymph, deutonymph, tritonymph, and adult. The adult female mites lay eggs in the soil, which will remain dormant for 4-7 days before becoming a non-feeding deutovum (pre-larva) (Figure 1.2). After around six days, they develop into a larval stage with six legs, and this stage is the only parasitic stage in the life cycle that is living on vertebrates; whereas adults and nymphs are free-living in soil. Then, after feeding (3 - 5 days) on the host, the engorged larva drops down into the soil, then develops into the first stage of the nymph, namely the protonymph, which is inactive. After around ten days, they develop into the eight-legged deutonymph (an active and predatory stage), which feeds on small insects or insect eggs. Finally, they develop into the tritonymph (another inactive phase), which is the transition stage from nymph to adult. These mites are independent predators following their maturation to adulthood. Adult female mites tend to lay their eggs in a single spot (Santibáñez *et al.,*2015).

The whole life cycle of chigger mites typically takes around 150 - 400 days (Shatrov, & Kudryashova, 2006; Nadchatran & Dohany, 1974). Moreover, the duration of the life cycle depends on the chigger species, and indeed environmental factors such as temperature, humidity and the abundance of food. Local climate has an influence on the number of generations in a year. For instance, temperate areas tend to host a single generation of chiggers annually; in contrast, in warmer zones, chiggers have been found to have multi-generations per year (Sasa 1961; Nadchatran & Dohany, 1974). Epidemiologically, only the parasitic larvae are important in scrub typhus, although *Orientia* is transmitted transstadially and transovarially through the free-living stages (Phasomkusolsil *et al.,*2009). The larvae feed through a unique structure, namely the stylostome, which is a tube formed by the parasite in the host skin during feeding (Shatrov, 2009).

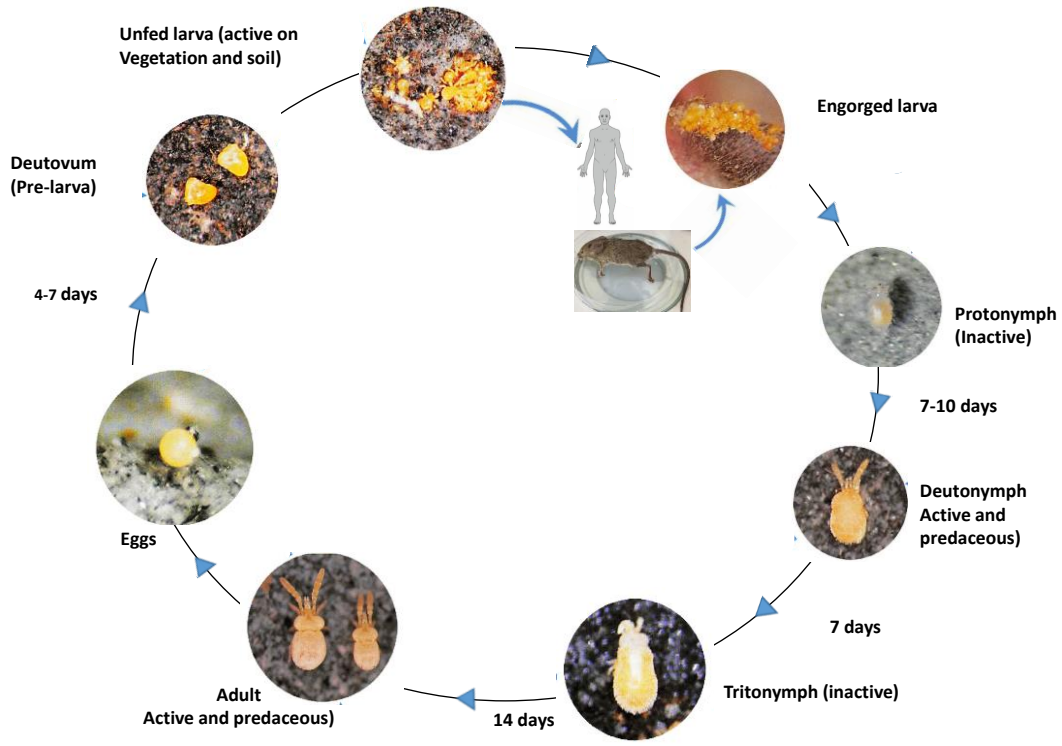


Figure 1.2. The life cycle of trombiculid mites (adapted from Santibáñez *et al.*,2015).

1.3.2 Taxonomy

The taxonomy of the trombiculid mites has mainly been based on the larval stages. Chigger mites belong to the families Leeuwenhoekiidae and Trombiculidae, order Trombidiformes, subclass Acari and class Arachnida. Moreover, *Acarus batatas* (now reclassified as *Eutrombicula batatas*) was the first chigger formally named and is described in 'Systema Naturae' by Linneaus, 1758 (Sasa, 1961). By the late twentieth century, approximately 100 species of trombiculids were placed in seven genera by Goff *et al.,* 1982. The impact of scrub typhus during World War II (see section 1) resulted in the recognition and description of numerous species and genera of chiggers. To differentiate between species, Womersley & Heaslip (1943) presented several morphological features in Trombiculidae that separate

then from the other Acari; *i.e*., measurements of the scutum and other structures, and Wharton *et al.,*1951 assembled the first comprehensive glossary and classification (Goff, 1982). More than 3,000 species of trombiculid mite have been described worldwide, and up to 400 species have been recorded in China alone (Peng *et al.,*2016).

Additionally, the monotypic subfamily Leeuwenhoekiinae was established, which had been included in the Trombiculidae. However, this group was later given the status of a family (Womersley, 1945). Since then, the question about the rank of this taxon has been controversial. For instance, Ewing (1949) included four subfamilies in the Trombiculidae; namely Hemitrombiculinae, Walchiinae, Leeuwenhoekiinae, and Trombiculinae. He proposed terminology for the taxonomic characters based on the external morphology of larvae for the first time. In the following years, a large number of new taxa were described, and the necessity for new generalisations on taxonomy and classification of the family emerged. Wharton *et al.* (1951) presented a new classification of larvae in which Trombiculidae divided into four subfamilies: Leeuwenhoekiinae, Walchiinae, Apoloniinae, and Trombiculinae. Later, the subfamily Walchiinae was renamed Gahrliepiinae (Womersley, 1952) (Figure 1.3). The genus *Leptotrombidium* is the largest in the family Trombiculidae (Nagayo *et al.,*1916).

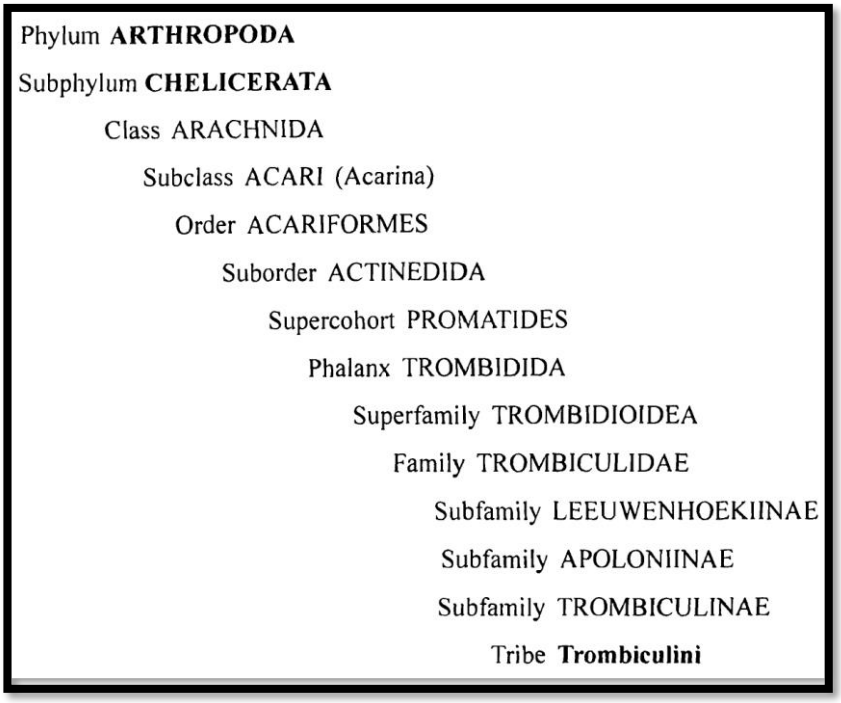


Figure 1.3. Chigger mite classification of the family Trombiculidae (reproduced from Fernandes Stan and Kulkarni, 2003).

1.3.3 Identification

Morphologically, the significant characteristics that have been used for identification of species are the scutum (dorsal shield) and the scutal setae (small hairs), which are used in particular to obtain identification to subgenus level (Nadchatram & Dohany, 1974). Further features used to take identification to the species level are the gnathosome (mouthparts), which contain the palpal coxae, palps, cheliceral blades, and galea (Narang & Lamba, 2010; Nadchatram & Dohany 1974; Goff *et al.,*1982).

1.4 Zoonotic bacteria transmitted by rodent ectoparasites

1.4.1 *Orientia* spp.

Orientia spp., the causative agent of scrub typhus, is an obligate intracellular Gram-negative bacterium that is transmitted to mammalian hosts, including humans, by the bite of larval chigger mites of *Leptotrombidium* spp. (Salje *et al.,*2017).

The *Orientia* genus belongs to the family Rickettsiaceae within the order Rickettsiales (Dumler *et al.,*2001). Until recently, scrub typhus was thought to be confined geographically to the Asia-Pacific and only one species of *Orientia* (*O. tsutsugamushi*) was thought to exist. This opinion was amended with the recent description of an *Orientia* sp. associated with a scrub typhus-like illness in Chile (Balcells *et al.,*2011) and the discovery of a new species, *Orientia chuto*, isolated from a patient who visited Dubai, United Arab Emirates (UAE) (Izzard *et al.,*2010). There are also increasing reports of suspected cases of scrub typhus in Africa (Maina *et al.,*2012). *O. tsutsugamushi* has over 20 types of distinct antigenic strains, of which Gilliam, Kato and Karp were the initially characterised strains (Kelly *et al.,*2009). In the vertebrate host, *O. tsutsugamushi* can be found in endothelial cells as with rickettsiae, but the underlying histopathologic lesions suggest that macrophages might be a more significant target cell than the endothelium (Watt & Parola, 2003; Cho *et al.,*2000). Around one million cases occur in the areas where scrub typhus is endemic each year, and more than a billion people are at risk worldwide (Luce-Fedrow *et al.,*2018). The symptoms of scrub typhus include high-grade fever, headache, and myalgia. The similarity of these *O. tsutsugamushi* symptoms to those of dengue fever, leptospirosis, malaria and murine typhus can have significant consequences. Scrub typhus is responsive to doxycycline; however, if the eschar that is characteristic of the condition is absent, the diagnosis of scrub typhus may be overlooked in favour of these other infections with febrile symptoms. Without early antibiotic intervention, the condition can advance and become a cause of death. The median rate of morbidity for scrub typhus is approximately 6%, but according to Taylor *et al.* (2015), it can reach 40%-50%. Studies have used the presence of eschar to explore the immune responses to *O. tsutsugamushi* early on in infection in

humans. This research has included phenotypic characterisation of infected cells and the mechanisms that the pathogen may use to spread from the site of inoculation (Paris *et al.,*2012).

1.4.2 *Rickettsia* spp.

Rickettsiae are obligatory intracellular Gram-negative bacteria belonging to the order Rickettsiales, which are categorised by complex lifecycles. *Rickettsia* species are transmitted horizontally to vertebrates by different arthropod vectors that feed on various species of animals. To date, lice (Phthiraptera), ticks (Ixodidae), fleas (Siphonaptera) and mesostigmatid mites have been recognised to be vectors of rickettsial agents (Balčiauskas *et al.,*2017). However, the greatest diversity of *Rickettsia* spp. have been found in non-hematophagous arthropods with an unknown secondary host, if any (Weinert *et al.,*2009).

Rickettsia and *Orientia* belong to the family Rickettsiaceae. Rickettsiae *sensu stricto* is are divided into four main groups: the spotted fever group (SFG), typhus group (TG), transitional group (TRG), and ancestral (AG) group (Murray *et al.,*2015; Abdad *et al.,*2018). The SFG is transmitted by ticks and the TG by lice or fleas, while *Rickettsia felis* (flea-transmitted) and *Rickettsia akari* (mite-transmitted) have been placed in the TRG (Gillespie *et al.,*2008). Although this classification was initially controversial, it has been supported as more whole-genome data for rickettsiae has been obtained (Murray *et al.,*2015). The ancestral group contains multiple distinct clades of symbionts of ticks, aquatic organisms and insects, such as the "*Torix* group", which has been identified in leeches and *Culicoides* midges (Pilgrim *et al.,*2017).

More than 30 species of rickettsiae have been described across the world, mainly in the SFG, with new members added annually, and twenty-one species have been identified as pathogens: *R. rickettsii*, *R. parkeri*, *R. africae*, *R. massiliae*, *R. philipii*, *R. conorii*, *R. sibirica*, *R. slovaca*, *R. raoultii*, *R. monacensis*, *R. aeschlimannii*, *R. helvetica*, *R. heilongjiangensis*, *R. japonica*, *R. honei*, *R. tamurae*, *Candidatus* Rickettsia kellyi, *R. australis*, *R. mongolotimonae*, *R. felis* and *R. akari.* Eight species are of unknown pathogenicity: *R. bellii, R. asemboensis*, *R. peacockii*, *R. montanensis*, *R. monteiroi*, *R. rhipicephali*, *R. argasii*, *and R. gravesii* (Fournier and Raoult 2009; Merhej *et al.,*2014). Clinically, most of SFG rickettsial

infections have presented with fever, eschar at the tick-bite site, and widespread cutaneous rash.

Mountain spotted fever and Mediterranean spotted fever are the most severe conditions caused by SFG rickettsia and can be fatal if not treated appropriately. Serological tests using immunofluorescence are usually used to confirm the diagnosis (Fournier & Raoult, 2020).

Very few rickettsial species have been identified from mites. However, *R. akari* is transmitted by the house mouse mite, *Liponyssoides sanguineus*, and is the causative agent of rickettsialpox that has caused hundreds of clinical cases in New York City in the past (Paddock *et al.,*2003). An organism similar to *R. akari* has also been detected in *Leptotrombidium scutellare* chiggers in China and named *Candidatus* Rickettsia leptotrombidium (Huang *et al.,*2017). The various groups of pathogenic rickettsiae are summarised in Table 1.2 (Abdad *et al.,*2018). Members of the genus *Rickettsia* are distinctive among obligate intracellular bacteria in living free in the cytoplasm of eukaryotic cells. Hard ticks (Ixodidae) are recognised as the primary vector of SFG rickettsiae, which are transmitted via the saliva during tick feeding (Parola *et al.,*2013). Several rickettsial species have been isolated from soft ticks (Argasidae) and hard ticks that have not been conclusively linked to human disease (Izzard *et al.,*2018), and some of these [such as *Rickettsia buchneri* in *I. scapularis* (Gillespie *et al.,*2012) and *Rickettsia peacockii* in *Dermacentor* spp. (Felsheim *et al.,*2009) seem incapable of infecting vertebrate cells. Therefore, they are likely to be tick symbionts with a mutualistic role.

Table 1.1. Classification of rickettsiae with details of vectors, reservoirs and geographical distribution.

Rickettsial group	Species	Vector	Disease	Putative reservoir	Geographical distribution	References
Spotted fever group	*R. rickettsii*	Tick (*Dermacentor, Rhipicephalus, Amblyomma*)	Rocky Mountain spotted fever, Brazilian spotted fever, fibre maculosa, São Paulo exanthematic typhus, Minas Gerais exanthematic typhus	Rodents	USA, Mexico, Brazil, Argentina, Colombia	McDade *et al.,*1986; Demma *et al.,*2005; Ogrzewalska *et al.,*2012; Noor *et al.,*2019
	R. parkeri	Tick *Amblyomma* spp.	Maculatum infection, American boutonneuse fever, Tidewater spotted fever	Rodents	US Gulf states, South Atlantic. North and South America	Allerdice *et al.,*2019
	R. africae	Tick (*Amblyomma* spp.)	African tick-bite fever	Ruminants	West Indies, Caribbean, Sub-Saharan Africa	Kelly *et al.,*1996; Kelly *et al.,*2006; Agyemang *et al.,*2005.

R. massiliae	Tick (*Rhipicephalus* spp.)	Mediterranean spotted fever-like disease	Dogs	France, Greece, Spain, Portugal, Switzerland, Sicily, central Africa, Mali, United States Argentina Southern Europe, *e.g.*, Italy	Fernández *et al.,*2006; Beati *et al.,*1993
R. conorii subsp. *caspia*	Tick (*Rhipicephalus* spp.)	Astrakhan fever, Mediterranean spotted fever or Boutonneuse fever	Dogs, rodents	France, Chad	Renvoisé *et al.,*2012; Fournier *et al.,*2003
R. conorii subsp. *conorii*	Tick (*Rhipicephalus* spp.)	Mediterranean spotted fever	Dogs, rodents	Southern Europe, sporadic in northern and central Europe, multiple sub-Saharan countries, Algeria, Turkey, Tunisia	Parola *et al.,*2009; Portillo *et al.,*2015; Parola *et al.,*2006; Kuloglu *et al.,*2012; Sfar *et al.,*2009; Bitam *et al.,*2006
R. conorii subsp. *israelensis*	Tick (*Rhipicephalus*)	Israeli spotted fever	Dogs, rodents	Israeli, Portugal, Sicily	Giammanco *et al.,*2005
R. conorii subsp. *indica*	Tick (*Rhipicephalus* spp.)	Indian tick typhus		India, Pakistan, Sri Lanka, Laos, Italy	Sentausa *et al.,*2012; Robertson *et al.,*1970; Phongmany *et al.,*2006; Jayaseelan *et al.,*1991; Parola *et al.,*2005; Portillo *et al.,*2015

R. sibirica subsp. *amongolotimonae*	Tick (*Rhipicephalus* spp., *Hyalomma* spp.)	Lymphangitis-associated rickettsiosis	Rodents	Southern France, Portugal, China, South Africa	De Sousa *et al.,*2006; Ramos *et al.,*2013; Psaroulaki *et al.,*2005; Fournier *et al.,*2005; Pretorius *et al.,*2004
R. helvetica	Tick (*Ixodes* spp.)	Aneruptive fever	Rodents	Central and northern Europe, Asia	Sprong *et al.,*2009; Fournier *et al.,*2004)
R. raoultii	Tick (*Dermacentor spp.*)	Tickborne lymphadenopathy (TIBOLA), *Dermacent or*-borne necrosis and lymphadenopathy (DEBONEL)	Unknown	France, Slovakia, Poland, Asia	Mediannikov *et al.,*2008; Tian *et al.,*2012; Špitalská *et al.,*2014; Selmi *et al.,*2009; Gaowa *et al.,*2013.
R. slovaca	Tick(*Dermacentor* spp.)	Tibola,debonel	Lagomorphs , rodents, European boar	Europe, China; Japan, Russia, Turkey, Mongolia, Georgia, Thailand	Cazorla *et al.,*2003; Mediannikov *et al.,*2008; Silaghi *et al.,*2011; Tian *et al.,*2012; Fernández-Soto *et al.,*2006; Selmi *et al.,*2009; Parola *et al.,*2005
R. heilongjiangensis	Tick (*Dermacentor* spp. *Haemaphysalis* spp.)	Far Eastern spotted fever	Rodents	Far East of Russia, Northern China, Japan, eastern Asia	Ando *et al.,*2010; Zhang *et al.,*2009; Zhang *et al.,*2000
R. honei	Tick	Flinders Island spotted fever, Thai tick typhus	Rodents, reptiles	Australia, Thailand	Graves and Stenos, 2017; Graves and Stenos, 2003

Typhus group	*R. typhi*	Flea		Rodents	Temperate, tropical and subtropical areas worldwide	Bitam *et al.,*2010; Civen *et al.,*2008
	R. prowazekii	Human body louse	Epidemic typhus, sylvatic typhus	Humans, flying squirrels	Worldwide	McDade *et al.,*1980; Sonenshine *et al.,*1978
Transitional group	*R. akari*	Mite	Rickettsialpox	House mice, wild rodents	Countries of the former Soviet Union, South Africa, Korea, Turkey, North and South America, Balkan countries	Diaz 2009, Choi *et al.,*2007; Ozturk *et al.,*2003; Brezina *et al.,*1973; Eremeeva *et al.,*1995
	R. australis	Tick *(Ixodes holocyclus, Ixodes tasmani)*	Queensland tick typhus	Rodents	Australia, Tasmania	Graves *et al.,*2009
	R. felis	Fleas	Cat flea rickettsiosis	Domestic cats, rodents, opossums	Europe, North and South America, Africa, Asia	Richter *et al.,*2002; Labruna 2009; Leulmi *et al.,*2014; Irwin and Jefferies, 2000

1.4.3 *Ehrlichia* spp.

Ehrlichia is a genus within the Rickettsiales order that resides within cytoplasmic vacuoles of monocytes, granulocytes, and platelets of humans and animals (Wen *et al.,*2003; Dumler *et al.,*2001). This genus currently has been classified into five validly-described species, *E. canis, E. chaffeensis, E. ewingii, E. muris,* and *E. ruminantium* (Pritt *et al.,*2017; Guillemi *et al.,*2019), but several uncultured strains may belong to novel species. *Ehrlichia* spp. cause human monocytic ehrlichiosis (*E. chaffeensis*) and ewingii ehrlichiosis, which are responsible for a vast array of non-specific symptoms including headache, myalgias, fever, and malaise (Moumene *et al.,*2016). This bacterium has the ability to replicate within white blood cells of the mammalian host and the midgut of the tick. The first human case of ehrlichiosis was recognised in the USA in 1986 (Tsai *et al.,*2016). Some *Ehrlichia* species are pathogens of veterinary importance (*e.g*., *Ehrlichia ruminantium* in cattle and small ruminants in sub-Saharan Africa and the Caribbean); while the important zoonotic agents (*E. chaffeensis* and *E. ewingii*) appear to be restricted to the New World, although *E. canis* can cause milder zoonotic infection and is distributed worldwide (Dumler *et al.,*2001). Previous studies characterised human ehrlichioses as tick-borne zoonotic infections; however, three ehrlichial species (*E. chaffeensis, E. ewingii,* and *E. canis*) can also cause disease in dogs (Nagamori, *et al.,*2019; Ndip, *et al.,*2007). Furthermore, one of the most important hosts acting as a reservoir of *E. chaffeensis* are deer; principally *Blastocerus dichotomus* in South America and *Odocoileus virginianus* in North America (Guillemi *et al.,*2019). The main vector of zoonotic ehrlichiae in the USA is the *Amblyomma americanum* tick (Ndip, *et al.,*2007). Infection with *E. chaffeensis* in humans results in moderate to severe febrile illness that is detected frequently in the southeastern and south-central regions of the United States (Sumner *et al.,*1999).

1.4.4 *Anaplasma* spp.

Anaplasma is a genus of the family Anaplasmataceae belonging to the order Rickettsiales. It was originally classified into six species *Anaplasma marginale, A. centrale, A. bovis, A. phagocytophilum, A. ovis* and *A. platys* (Chochlakis *et al.,*2010). In addtion, *A. capra* and *A.*

odocoilei have now been isolated but not yet formally described (Said *et al.,*2018). Moreover, molecularly, *Anaplasma* species and strains have classified in numerous animal and tick species in the north of Africa (Matei *et al.,*2018). *Anaplasma phagocytophilum* is identified as the agent of human granulocytic anaplasmosis disease (HGA) (Dumler *et al.,*2001; Keesing *et al.,*2012). In 1994, the first case of human infection by *A. phagocytophilum* was recorded in the USA and this was followed by a rapid increase in the number of cases reported in the country. This disease can be fatal in the USA (Chen, 1994), while in Europe, the prevalence in humans is very low and the disease symptoms appear to be milder. Zoonotic disease has been rarely reported in the UK (Petrovec *et al.,*1997). *A. phagocytophilum* is known to maintain a tick-rodent cycle in nature. The main vectors of *A. phagocytophilum* are *Ixodes* spp. ticks, *i.e*., *I. pacificus* and *I*. *scapularis* in the USA, and *I. ricinus* in Europe, while *I. persulcatus* is an important vector in Russia (Alekseev *et al.,*2001) and China (Cao *et al.,*2000). *A. phagocytophilum* transmitted mainly by infected tick bites, which is considered the most prevalent route of human infection, but infection via blood transfusion has occurred in rare cases (Bakken *et al.,*1996; Horowitz *et al.,*1998). Genetic analyses of *A. phagocytophilum* have facilitated efforts to identify potential human pathogenicity and enzoonotic cycles (Jaarsma *et al.,*2019). It is now clear that European strains of *A. phagocytophilum* are very different to those circulating in North America (Barbet *et al.,*2013). This leads to different epidemiology on each continent, with a high prevalence of ruminant infection (tick-borne fever) in Europe and little zoonotic disease, whereas the opposite is true in North America (Dugat *et al.,*2015).

1.4.5 *Bartonella* spp.

Bartonella is a genus of Gram-negative, facultative intracellular bacteria, classified in the Alphaproteobacteria, which can invade and maintain infection within erythrocytes and endothelial cells in mammals and therefore chronically infect the blood of their hosts. It belongs to the order Rhizobiales and the family Bartonellaceae, and comprises >30 species or subspecies (Sato *et al.,*2013; Gutiérrez, 2017; Pretorius, *et al.,*2004). These include *B. henselae*, *B. elizabethae, B. alsatica, B. vinsonii* subsp*. arupensis, B. bacilliformis, B. quintana, B. grahamii* that have been associated with human diseases ranging from

subclinical and self-limiting infection to severe, life-threatening disease (Mogollon-Pasapera *et al.,*2009; Regier *et al.,*2016; Ying *et al.,*2002). The genus is named after Alberto Leonardo Barton Thompson, who discovered *Bartonella bacilliformis* in 1905 as the causative agent of Oroya fever (Carrion's disease), which is confined to small hotspots of transmission in South America. Worldwide, *Bartonella henselae*, the agent of cat-scratch disease, is well known as the most prevalent pathogen characteristic of the genus *Bartonella*.

Signs and symptoms of bartonellosis involve chronic bacteraemia, skin lesions, fever, endocarditis, and manifestations of disease of the central nervous system (Ying *et al.,*2002). The cat is the natural reservoir of this pathogen, which is transmitted between them by cat fleas (*Ctenocephalides felis*). Humans become infected when they are scratched or bitten by a cat that has contaminated flea faeces on its claws, in its saliva or between its teeth. Dogs can also become infected with *B. henselae* and can experience endocarditis, fever and *peliosis hepatis* (Regier *et al.,*2019). Several arthropods have been implicated in transmitting different *Bartonella* species; e.g., fleas, sandflies, human body lice, ticks, and vole ear mites (Breitschwerdt and Kordick, 2000). In the wild, the association between rodents and bartonellae is an intimate one, with subclinical bacteremia lasting for months in these mammalian host (Gutiérrez *et al.,*2015). However, as described by Brinkerhoff *et al.* (2010) and Morick *et al.* (2013a), fleas also represent critical players in the *Bartonella* sylvatic cycle, because they harbor a high diversity of *Bartonella* spp. and strains and transmit them with high efficiency among rodents. Therefore, fleas do not only serve as vectors, but also represent additional reservoirs for these bacteria (Birtles 2005, Deng *et al.,*2012). More recently, literature has emerged that offers contradictory findings about whether *Ixodes ricinus* ticks have the capacity to transmit the rodent-associated *B. birtlesii* (Reis *et al.,*2011). Thus, the natural role of ticks in transmission of *Bartonella* in the environment is still under discussion and unresolved (Gutiérrez *et al.,*2015; Reis *et al.,*2011). On the other hand, fleas are undoubtedly the key player in *Bartonella* spp. transmission as arthropod vectors. Currently, more than 20 species of *Bartonella* have been classified and associated with rodents; most of these are recognised as having zoonotic

potential (Nziza *et al.,*2019; Kosoy *et al.,*2018). Table 1.2 summarises a list of *Bartonella* species associated with human disease and transmitted by different arthropods.

Table 1.2. List of *Bartonella* species of medical importance transmitted by different ectoparasites

***Bartonella* spp.**	**Putitive Vector**	**Diseases caused**	**Host reservoir**	**References**
B. quintana	Body louse	Trench fever	Human, macaques	Brouqui, 1996
B. henselae	Cat flea	Cat scratch disease	Domestic cat, dogs	Regnery *et al.,*1992; Welch *et al.,*1992
B. birtlesii	Ticks		Rodents	Reis *et al.,*2011
B. bacilliformis	Sandfly	Carrion's disease		Benson, 1968
B. elizabethae	Rat flea	Endocarditis	Rat	Daly *et al.,*1993
B. grahamii	Rodent flea	Lymphadenopathy	Vole, mouse	Birtles *et al.,*1995
B. tribocorum	Rat	-	Rat	Harrus *et al.,*2009
B. rattimassiliensis	Flea	-	Rat	Gundi *et al.,*2004
B. vinsonii ssp*. arupensis*	Rodent flea	Endocarditis, febrill illness	Mouse, vole	Welch *et al.,*1999
B. vinsonii ssp*. berkhoffii*	Flea	Endocarditis	Dog	Kordick *et al.,*1996
B. taylorii	Fleas	-	Rodents	Birtles *et al.,*1995
B. tamiae	Tick, fleas	Endocarditis	Rodents	Colton *et al.,*2010
B. schoenbuchensis.	Deer ked	Unknown	Cervids	Dehio *et al.,*2004
B. melophagi	Sheep ked	Various clinical manifestation	Sheep	Kumsa *et al.,*2014
B. bovis	Tick	-	Cattle	Brown *et al.,*1999
B. australis	Unknown	-	Kangaroo	Fournier *et al.,*2007
B. koehlerae	Cat flea	Endocarditis	Cat	Droz *et al.,*1999
B. doshiae	Fleas	-	Rodents	Birtles *et al.,*1995
B. alsatica	Rabbit flea	Endocarditis	Rabbit	Heller *et al.,*1999
B. clarridgeiae	Cat flea	Cat scratch disease	Cat	Kordick, 1997
B. ancashensis	Unknown	verruga peruana	Human	Mullins *et al.,*2015
B. rochalimae	Fleas	Endocarditis	Canids	Chomel *et al.,*2004

1.4.6 *Francisella* spp.

Francisella tularensis, a Gram-negative intracellular coccobacillus, is the causative agent of the zoonotic disease, tularaemia. This disease in the northern hemisphere is transmitted to humans via a wide range of different routes, including arthropod bites (ticks and haematophagous insects), infected animals, and food and water contamination (Stephens, 2005; Petersen *et al.,*2009). In 1919, Francis was apparently the first to recognise the transmission of tularaemia by arthropods when he isolated the etiologic agent from a Utah patient with "deer fly fever"; a similar case was obtained in 1911 by Pearse who described "*Bacterium tularense*" as a disease acquired from ground squirrels in California (Petersen *et al.,*2009). Previous studies in Central Europe (Austria, Czech Republic, and Slovakia) have shown that the prevalence of *F. tularensis* was between 0.1–2.8% in ixodid ticks (Kreizinger *et al.,*2013). In the past few years, researchers have discovered new taxa in the genus *Francisella*, which have been described from fish, humans and ticks (Kreizinger *et al.,*2013). Tularemia has been documented as endemic to several European countries, North America, and large parts of Asia (Harrist *et al.,*2019). Two major disease manifestations (ulceroglandular and glandular) should be taken into account when considering the transmission route of tularemia. Ulceroglandular tularemia is considered more likely to be associated with arthropod bites; an ulcer emerges at the bite site and is followed by enlargement of the lymph nodes. Glandular tularemia is identified by regional adenopathy without recognisable skin irritation (Petersen *et al.,*2009).

The ecology of tularemia is complex and not fully understood. However, the type A cycle (*F. t. tularensis*) mostly includes lagomorphs, while the type B cycle (*F. t. holarctica*) commonly involves rodents and other mammals (Whitten *et al.,*2019). Moreover, the primary tick vector for *F. tularensis* in the USA is the dog tick (*Dermacentor variabilis*) (Huntley and Zellne, 2019).

1.4.7 *Coxiella* spp.

Coxiella is a genus of bacteria belonging to the family Coxiellaceae, order Legionellales, and class Gammaproteobacteria (Dehhaghi *et al.,*2019). The primary natural reservoir of the "Q-fever" pathogen, *Coxiella burnetii*, are ticks, in which it colonises the ovaries and has been reported from approximately 40 species (Dehhaghi *et al.,*2019). *C. burnetii* is an obligatory intracellular pathogen that resides in the phagolysosome of infected cells in mammalian hosts. It was previously classified as a *Rickettsia* species based on morphological similarities; however, genetically, it has been placed in the gamma subdivision of Proteobacteria, so has closer affinities to *Legionella* and *Francisella* than to *Rickettsia* (Almeida *et al.,*2012).

Q fever is a zoonotic disease associated with a wide variety of mammalian hosts and has a global distribution. It is recognised that domestic ruminants act as the main reservoir of the bacterium and zoonotic infection results from direct contact with infected animal body fluids or inhalation of airborne, dessication-resistant spores (Duron *et al.,*2015). Although several tick species have been revealed as competent vectors in laboratory experiments *in vivo*, their role as zoonotic vectors in nature is controversial. However, they may play an important role in the transmission of *C. burnetii* between wild vertebrates (Almeida *et al.,*2012).

Numerous *Coxiella*-like bacteria have been discovered in ticks in recent years, but genetic analyses have shown that they are distinct from *C. burnetii* in animals (Gottlieb *et al.,*2015). Therefore, while ticks can carry *C. burnetii*, many prior reports of infection may actually have resulted from misclassification of *Coxiella*-like symbionts (Duron *et al.,*2015). Although the pathogenicity to vertebrates of most *Coxiella*-like symbionts is still unknown, *C. burnetii* itself evolved from one such ancestor (Duron *et al.,*2015), and there has been at least one human case of symptomatic infection caused by a *Coxiella*-like tick symbiont (Angelakis *et al.,*2016).

1.4.8 *Borrelia* spp.

Lyme borreliosis was discussed in the section 1.1. Another important disease caused by *Borrelia* spp. is relapsing fever, which is transmitted primarily by *Ornithodoros* soft ticks (Argasidae) in the case of the tick-borne form or the human body louse (*P. h. humanus*) for louse-borne disease (*Borrelia recurrentis*). Several *Borrelia* species can cause the tick-borne form worldwide, but the best characterised are *B. hermsii* (transmitted by *Ornithodoros hermsi* in western North America), *B. crocidurae* (transmitted by *Carios erraticus* in North and West Africa), and *B. duttoni* (transmitted by *Ornithodoros moubata* in Central, East and South Africa) (Talagrand-Reboul *et al.,*2018). Disease symptoms are similar to those of influenza, with malaise, headache and myalgia. *B. miyamotoi* has been associated with a relapsing-fever-like syndrome in North America, but is transmitted by hard ticks (*Ixodes* spp.) (Cutler *et al.,*2019).

1.5 Arthropod bacterial symbionts and the microbiome of ectoparasites

A microbiome can be defined as a community of commensal, symbiotic, and pathogenic microorganisms (Greay *et al.,*2018). Symbiotic bacteria of arthropods play an important role in the ecology and evolution of their hosts. Many endosymbionts are transmitted vertically from mothers to their offspring (Zchori-Fein, and Perlman, 2004) and can be classified as obligate (primary) or facultative (secondary). One of the most interesting examples is that aphids have an obligate association with the gamma-proteobacterium *Buchnera aphidicola* as the primary symbiont, which has been revealed to contribute directly to aphid fitness by supplying amino-acids that are deficient in the aphid diet (Goodman &Feldman, 2019). In addition, aphids may harbour secondary symbionts, which include other vertically-transmitted bacteria of which the benefits of infection may not be simply nutritional (Oliver *et al.,*2010; Degnan *et al.,*2009; Montllor *et al.,*2002). One example is the gamma-proteobacterium *Hamiltonella defensa*, which protects its aphid host from natural enemies, the parasitoid wasps *Aphidius* spp. (Degnan *et al.,*2009). However, in facultative symbioses, the effects of infection may be facilitated by ecological interactions, such as the food plants or natural enemies of the hosts (Oliver *et al.,*2003).

1.5.1 Mite symbionts

Mites are found in a wide variety of habitats (terrestrial, marine, and freshwater) and some studies on mite microbiomes have been published, although they are less well researched than insects. A previous systematic review highlighted that mite species were associated with 85 bacterial genera and around 150 identified species belonging to seven phyla, with three classes of Proteobacteria and 25 orders (Chaisiri *et al.,*2015). Several bacteria have been reported as potential mite symbionts including *Wolbachia, Cardinium, Acaricomes, Snodgrassella, Spiroplasma, Rickettsiella, Serratia* 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 stimulated by the other potential symbionts are still unknown. *Wolbachia* and *Cardinium* can affect arthropod reproduction by inducing cytoplasmic incompatibility, parthenogenesis, or sex-ratio distortion; for instance, feminization or male-killing (Chaisiri *et al.,*2015). Interestingly, *Wolbachia* and *Cardinium* were recorded in 31 species of mites each, and they were found in five and eight superfamilies respectively. For *Wolbachia*, these were the *Dermanyssoidea, Phytoseioidea, Oppioidea, Cheyletoidea,* and *Tetranychoidea* (Chaisiri *et al.,*2015).

Wolbachia and *Cardinium* can coinfect the same host species, mainly in the spider mites (*Tetranychus* spp.) (Chen *et al.,*2014). Although the reproductive phenotypes induced by *Wolbachia* or *Cardinium* alone have been measured, the impacts of co-infecting the same host by both endosymbionts is still only partly understood. However, it was shown in *Tetranychus piercei* coinfected by both symbionts that *Wolbachia* could stimulate the strength of *Cardinium*-induced CI (Zhu *et al.,*2012).

Pathogenic bacteria have been recorded in mammal-associated mites belonging to the superfamilies *Trombiculoidea, Acaroidea, Dermanyssoidea*, and *Cheyletoidea* (Chaisiri *et al.,*2015; Zeman *et al.,*1982; Brännström *et al.,*2010; Chirico *et al.,*2003*)*. Mites have been found as reservoirs for many pathogenic bacteria that are responsible for disease transmission.

1.5.2 Tick symbionts

The powerful technique of next-generation sequencing has revolutionized the understanding of tick-borne pathogens, endosymbiotic bacteria and other bacteria associated with the tick microbiome. This technology has revealed the complexity of the tick microbiome and also led to its gradual characterisation in terms of the diversity and composition of bacteria that are pathogenic and symbiotic, which may affect ticks' vector competence and vector-reservoir host interactions (Vayssier-Taussat *et al.,*2015; Greay *et al.,*2018). Recent studies of endosymbiotic bacterial species that are transmitted vertically in ticks have categorised them into ten distinct genera *Coxiella*-like endosymbionts (LE)*, Francisella*-LE*, Rickettsiella, Arsenophonus, Spiroplasma, Rickettsia, Lariskella, Cardinium, Candidatus* Midichloria and *Wolbachia* (Ahantarig & Trinachartvanit, 2013; Duron *et al.,*2017). Furthermore, these genera also include species that are pathogenic for vertebrates and humans, which could be transmitted to vertebrates by ticks. For example, the genera of *Hyalomma* and *Haemaphysalis* (ixodid ticks) and an argasid tick of the genus *Ornithodoros* can transmit *C. burnetii*, the Q-fever agent, under laboratory conditions (Duron *et al.,*2015).

Symbionts can play significant roles in their host's physiology, such as reproduction, development, nutritional adaptation to defend against ecological stress, and immunity (Dale & Moran, 2006; Li *et al.,*2018). For example, *Coxiella*-LEs were found to be associated with the reproductive fitness of *Amblyomma americanum* and *Haemaphysalis longicornis* (Zhong *et al.,*2007; Zhang *et al.,*2017). Another example is that *Rhipicephalus microplus* harbours a *Coxiella*-LE mutualist that is vertically transmitted. This bacterium from *R. microplus* is essential for maturation in initial developmental stages (the metanymph to the adult stage), which revealed that this *Coxiella*-LE has a role in tick physiology. The symbiont has been found in high densities in the ovary and Malpighian tubules of the engorged adult female (Guizzo *et al.,*2017).

So far, the role of tick endosymbionts in the transmission of the pathogens that are responsible for severe diseases is not yet clearly understood (Ahantarig & Trinachartvanit, 2013). As described by Macaluso *et al.,*2002, ticks can be infected by SFG rickettsiae by vertical transmission from the mother or by horizontal transmission from an infected host.

In the classic studies of on the effect of the East Side Agent (later identified as *Rickettsia peacockii*) on transmission of *R. rickettsii* by *D. andersoni* ticks, the former rickettsial species, which is benign, appeared to block infection of the tick by the highly virulent *R. rickettsii* (Burgdorfer *et al.,*1981). Accordingly, in laboratory studies using *Dermacentor variabilis*, it was later shown that infection of the ovaries by one rickettsial species, once established, blocks vertical transmission of a second rickettsial species. Therefore, ticks can become refractory to co-infection of the ovaries by two closely-related bacterial species (Macaluso *et al.,*2002). In this context, it is important to note that three groups of tick endosymbionts are closely related, but genetically distinct, to vertebrate pathogens. These are *Coxiella*-LE, which is related to the Q fever agent *C. burnetii, Francisella*-LE, related to the causative agent of tularaemia (*F. tularensis*), and arthropod-specific rickettsial endosymbionts (*e.g.*, *R. buchneri*; Kurtti *et al.,*2015), which are related to SFG rickettsiae.

1.5.3 Lice

Numerous louse species harbour symbiotic bacteria that contribute to nutritional provisioning. Approximately six distinct clades of primary endosymbionts have been investigated from sucking lice, which are essential to synthesise amino acids and vitamins lacking from the louse diet and are therefore required for louse survival (Allen *et al.,*2016). It has been found that some endosymbionts of lice colonise the gut or are housed in mycetomes (Douglas, 1989). Hypša and Křížek 2007 revealed evidence of *Legionella*-like bacterial symbionts in two louse species belonging to the genus *Polyplax* (*P. serrata* and *P. spinulosa*), and this obligate symbiont has been characterised by light and electron microscopy. This bacterium was suggested to be a member of the genus *Legionella* based on the 16S rDNA sequence (Hypsa and Krizek, 2007). In this work, the transition from typical *Legionella* to an obligate symbiont was inferred from the presence of the bacterium in all tested louse individuals, suggesting their transovarial transmission. Moreover, these symbiotic *Legionellaceae* were recognized as monophyletic lineages. Interestingly, recent genome sequencing of the primary symbiont from *P. serrata*, which the authors called *Candidatus* Legionella polyplacis, revealed a laterally-transferred biotin operon that may underlie nutrient provisioning in the symbiosis (Říhová *et al.,*2017).

1.6 The bacterial microbiome of haematophagous arthropods and potential impacts on vector competence.

1.6.1 Ticks

The microbiome can be described as the totality of bacteria and other microorganisms within a multicellular host or environment, including pathogenic, mutualistic and commensal organisms (Finney *et al.,*2015). The structure of a host's microbial community has the potential to affect the ecology and evolution of the host. Reasons for microbiome variation between host individuals of the same species are poorly understood, and this limitation has impeded mechanistic understanding of the functions of microbial communities for pathogen acquisition or resistance (Landesman, 2019). In haematophagous arthropods, variation in the microbiome assemblage might be attributable to differences in degree of engorgement, species, development stage, sex, and geographical locality (Moreno *et al.,*2006; Van Treuren *et al.,*2015; Zolnik *et al.,*2016).

Symbiotic bacterial assemblages within arthropod vectors, including fleas, mites, lice, and ticks, have become an increasing focus of research because they have been found to impact pathogen ecology and transmission (Narasimhan *et al.,*2014; Finney *et al.,*2015; Abraham *et al.,*2017). For example, the presence of endosymbiotic bacterium *Wolbachia* can block the transmission of arboviruses by the mosquito *Aedes aegypti* (Moreira *et al.,*2009), although importantly, this is an artificial symbiont-host relationship (transinfection). In the tick *I. scapularis*, the natural microbiota composition can influence pathogenic spirochete (*B. burgdorferi*) colonization of the tick gut (Narasimhan *et al.,*2014). Conversely, vector-borne pathogens can modify other components of the vector microbiome. In *I. scapularis, A. phagocytophilum* manipulates the tick microbiome to facilitate infection by inducing expression of an antifreeze protein (Abraham *et al.,*2017). In addition to enhancing winter survival of infected ticks, this protein alters biofilm formation in the tick gut, allowing *A. phagocytophilum* to stably infect the vector (Abraham *et al.,*2017). These examples from *I. scapularis* suggest that tick microbiomes could play a significant role in pathogen transmission more widely (Clay & Fuqua, 2010).

The factors determining the origin and maintenance of the tick microbiome are only just beginning to be explored. Ticks come into contact with host blood, skin, and fur, which may influence the microbiome structure, but also have free-living phases in the environment (Barbour *et al.,*2009). Additionally, choice of host and the impact of ingested immune cells and complement may led to alteration of the tick microbiome (Kuo *et al.,*2000; Kurtenbach *et al.,*2002). This potential role of host species on the tick microbiome has been investigated in the context of LD spirochete transmission. For instance, one study performed by Swei and Kwan 2017 found a robust influence of host blood meal identity on *I. pacificus* microbiome composition. In this case, the microbiome composition of ticks that fed on lizards (*Sceloporus occidentalis*) was significantly different compared with the same tick species feeding on mice (*Peromyscus maniculatus*). These differences could potentially be attributed to the specific antimicrobial properties of lizard blood (Lane and Quistad, 1998; Kuo *et al.,*2000). This decrease of microbiome diversity in ticks feeding on lizards is associated with an impaired ability to acquire *B. burgdorferi*. Thus, host identity might be one of the most important factors influencing the composition of the complex tick microbiome community (Landesman *et al.,*2019). On the other hand, Hawlena *et al.* (2013) revealed that differences in microbiome composition between *D. variabili*s and *I. scapularis* ticks and also fleas (*Ctenophthalmus pseudagyrtes* and *Orchopeas leucopus*) were driven by arthropod species and not by different rodent hosts (*Peromyscus leucopus* and *Microtus ochrogaster*) or the environment in southern Indiana, USA. Similarly, a study by Rynkiewicz *et al.* (2015) reported a distinctive microbiome composition between two ticks (*I. scapularis* and *D. variabilis*) and the blood meal of their host (*Peromyscus leucopus),* despite feeding on the same host.

The composition of the microbiome in ticks before and after engorgement has been studied in *I. persulcatus* in China, the main vector of Lyme disease in this region, which is caused by *B. afzelii* and *B. garinii*. This revealed that while the blood meal altered the microbiome composition, it did not change the overall diversity (Zhang *et al.,*2014). Similarly, it has been demonstrated that *I. scapularis* does not acquire the majority of its microbiome diversity during a blood meal, but via environmental factors and from transovarial transmission, which play crucial roles in shaping the bacterial composition in ticks (Zolnik *et al.,*2016). The question of whether ticks have a stable gut microbiota remains

controversial, but a recent study presented evidence against this scenario and suggested that tick microbiome diversity has been exaggerated or misrepresented due to inadequate controls to exclude laboratory contamination (Ross *et al.,*2018). Nevertheless, prior antibiotic treatment of ticks can reduce the colonisation of *B. burgdorferi* in the tick gut, and this was linked to changes in the existing gut microbiota in *I. scapularis* (Narasimhan, *et al.,*2014).

In addition to the competitive interactions between rickettsial species in the tick ovary discussed above (section 1. 4.2), intracellular symbionts may influence the establishment of pathogens in ticks. In *I. scapularis*, the symbiont *R. buchneri* is associated with lower rates of infection with *B. burgdorferi*, but only in adult male ticks (Steiner *et al.,*2014). Additionally, *D. andersoni*, which is a vector of both *R. rickettsii* and the bovine pathogen *Anaplasma marginale,* has a microbiome dominated by three genera of symbionts: *Francisella*-LE*, Arsenophonus*, and *Rickettsia* spp., which colonize the salivary glands and the midgut in adult ticks (Clayton *et al.,*2015). In this vector, laboratory challenge experiments revealed that the abundance of *R. bellii* symbionts was negatively associated with *A. marginale* density. However, in ticks with lower levels of *Francisella*-LE, establishment by pathogenic *Francisella* was also decreased (Gall *et al.,*2016).

1.6.2 Fleas

Similarly to ticks, fleas may transmit multiple bacterial pathogens while also harbouring their own bacterial symbiont communities. Previous studies have documented that *Wolbachia, Rickettsia,* and *Bartonella* were the most common bacteria of cat fleas in northern and southern California (Vasconcelos *et al.,*2018). In this study, *Wolbachia* was one of the most widespread flea endosymbionts found in the reproductive tissue and no significant difference in flea microbiomes were detected between the two geographic sites. In a plague-endemic region of Uganda, microbiome analysis of six species of fleas across an environmental transect revealed that flea species had the greatest impact on microbial composition, although host and environmental factors also had a significant influence (Jones *et al.,*2015). *Bartonella* and *Wolbachia* were the most widespread bacterial species, but there was evidence for potentially novel lineages of symbionts. In Australia, microbiome profiling uncovered that *Wolbachia* was dominant in both cat fleas and

Echidnophaga ambulans, a flea species specific to echidnas, although only the latter harboured *Cardinium* (Lawrence *et al.,2015).* The microbiome of colonised cat flea populations harbouring different infection rates with *R. felis* has also been examined, which showed reduced species richness in infected fleas and impacts on *Wolbachia* prevalence (Pornwiroon *et al.,*2007). Overall, less research on the factors influencing microbiomes, including the impact of the blood meal, have been performed on fleas as compared with ticks. However, an analysis of the blood and gut microbiota of the plateau pika (*Ochotona curzoniae*) in China alongside that of two ectoparasites, the flea *Rhadinopsylla dahurica vicina* and the warble fly *Hypoderma curzonial*, revealed considerable overlap between flea and blood microbial communities, which little impact of environmental or individual host factors (Li *et al.,*2018). The authors concluded that stochastic rather than deterministic processes governed the bacterial community assemblies in blood, warbles and fleas (Li *et al.,*2018).

1.6.3 Diptera

1.6.3.1 *Wolbachia* in *Drosophila* protects against viruses

The influence of vertically-transmitted symbionts and other elements of the arthropod microbiome on pathogen establishment and transmission has been studied in most depth in Diptera (true flies), including haematophagous species. One of the most heavily researched arthropod symbionts is *Wolbachia*, which infects a vast array of arthropods (up to 66% of terrestrial species; Hilgenboecker *et al.,*2008) and also filarial nematodes *Wolbachia* is most famous for inducing cytoplasmic incompatibility in some arthropod hosts, which facilitates its entry into new populations because host embryonic death occurs if an uninfected female mates with an infected male. Some *Wolbachia* strains can also induce different reproductive manipulations in their hosts; *e.g.*, feminization, parthenogenesis induction, and male killing (Stouthamer *et al.,*1999; Hedges *et al.,*2008).

Drosophila melanogaster is usually infected with *Wolbachia*, which represents a powerful model for studying host-pathogen interactions and antiviral responses (Cherry *et al.,*2006). *Drosophila* C virus (DCV) belonging to the Dicistroviridae family, is a natural pathogen of *D. melanogaster*, which is found in wild and laboratory fly populations. Injection of DCV into adult *D. melanogaster* normally causes fly mortality within 4–6 days (Hoffmann, 2003).

Furthermore, *D. melanogaster* is considered a useful tool to analyse resistance to pathogens, with numerous mechanisms of innate immunity pathways conserved between *Drosophila* and mammals. Indeed, it has been shown that all of the genes which have been detected in *Drosophila* host resistance are homologous to genes that have been implicated in mammalian innate immune defences (Hoffmann, 2003).

In 2008, it was first reported that *Wolbachia* infection protects *Drosophila melanogaster* against RNA virus infection (Hedges *et al.,*2008). Thus*, Wolbachia* may play a significant role in defending its host against pathogens. In a much larger subsequent study, 19 strains of *Wolbachia* isolated from 16 *Drosophila* species were injected into *Drosophila simulans* stocks of the same genotype (Martinez *et al.,*2014). Approximately half of the *Wolbachia* strains protected the flies against DCV and another RNA virus, Flock House virus, by reducing viral titres. This protective effect was correlated with the density of the symbiont in the flies' tissues. Interestingly, *Wolbachia* did not protect against a DNA virus in *Drosophila*, insect iridescent virus 6 (Teixeira *et al.,*2008), but the molecular mechanisms underpinning *Wolbachia*-mediated resistance to RNA viruses are still not fully understood. The fact that fruit flies can benefit from increased resistance to viruses might provide a good explanation for the longstanding puzzle as to why *Wolbachia* reaches high prevalence in natural populations of *D. melanogaster* without expressing strong cytoplasmic incompatibility (Teixeira *et al.,*2008).

1.6.3.2 *Wolbachia* and the control of mosquito-borne disease

The yellow fever mosquito, *A. aegypti,* is the primary vector of several arboviruses worldwide, including dengue virus, chikungunya virus and Zika virus (ZIKV). Unlike many other members of the genus *Aedes*, most *A. aegypti* populations worldwide appear to lack *Wolbachia* infections. Currently, dengue represents the most important arboviral disease worldwide, particularly in the tropics, with an annual incidence of almost 400 million infections, of which almost 100 million are clinically relevant (WHO.int, 2019). An initial proposal to control dengue in northern Queensland, Australia, involved introducing a virulent strain of *Wolbachia* (*w*MelPop) into *A. aegypti*, as this strain replicates in somatic tissues and decreases the lifespan of infected hosts (McMeniman *et al.,*2009). This shortened lifespan phenotype had the potential to reduce dengue transmission because of

the relatively long extrinsic incubation period of dengue virus (Walker *et al.,*2011). Unfortunately, it was later found that the strong negative fitness impacts of *w*MelPop prevent it spreading effectively into natural populations of *A. aegypti* (Nguyen *et al.,*2015).

Following the key experiments demonstrating that *Wolbachia* can prevent the dissemination of RNA viruses in *Drosophila*, it was shown that *Wolbachia* in *A. aegypti* reduces vector competence for viruses (Moreira *et al.,*2009). Both the *w*Mel and *w*MelPop-CLA strains could block transmission of dengue serotype 2 in *A. aegypti*, establishing a basic practical approach to suppress dengue (Walker *et al.,*2011). It was particularly important that the *w*Mel strain was equally effective in preventing dengue transmission, because it has minimal fitness impacts on *A. aegypti* and therefore is predicted to spread very efficiently in nature. In 2015, the Zika epidemic in Brazil and surrounding countries was associated with cases of microcephaly and emphasised the importance of mechanisms to combat the Zika vector, *A. aegypti*. It was established that the *w*Mel infection in *Ae. aegypti* significantly inhibited ZIKV infection in mosquito abdomens, and it reduced distributed infection in heads and ZIKV titres in mosquito saliva (Dutra *et al*., 2016). Therefore, release of *Wolbachia*-harboring *A. aegypti* led to an effective mechanism to decrease ZIKV transmission and was used as part of Zika control strategies.

Artificial *Wolbachia* infections such as *w*MelPop have also shown negative impacts on the development of the malaria parasite in the vector; for example, the avian parasite, *Plasmodium gallinaceum* in *A. aegypti* (Moreira *et al.,*2009) and the rodent parasite, *Plasmodium berghei*, in *Anopheles gambiae* (Kambris *et al.,*2010). Moreover, in *Anopheles stephensi*, artificial infection with *Wolbachia* strain *w*AlbB has shown its capability to induce significant levels of cytoplasmic incompatibility and perfect vertical transmission. Remarkably, this strain provides high resistance in the mosquito to the most virulent human malaria parasite, *Plasmodium falciparum* (Bian *et al.,*2013).

However, it is important to note that the effect of *Wolbachia* on pathogen dissemination and virulence in arthropods is complex and most data obtained to date are from artificial *Wolbachia*-mosquito combinations. In the case of natural *Wolbachia* infections in *Culex pipiens* mosquitoes transmitting the avian malarial parasite, *Plasmodium relictum*, the

symbiont not only increased the susceptibility of the vector to the protozoan parasite, but also increased the incidence of salivary gland stage infections (Zélé *et al.,*2014).

1.6.3.3 Symbionts of tsetse flies and vector competence

Tsetse flies (Diptera; Glossinidae) are vectors of pathogenic trypanosomes, the etiological agents of sleeping sickness in humans and domesticated animals, across sub-Saharan Africa (Weiss *et al.,*2013). The primary symbiont, *Wigglesworthia,* and the secondary symbionts, *Sodalis* and *Wolbachia*, are found as the most dominant bacteria in tsetse flies; the former two bacteria are vertically transmitted by maternal milk gland secretions, whereas *Wolbachia* is transmitted directly via infection of germline cells (Attardo *et al.,*2008). It was reported that the gut microbiome of *Glossina pallidipes* was dominated by *Sodalis* and *Wigglesworthia*, which have a key role in the immunological association between tsetse and its microbiome (Maltz *et al.,*2012; Griffith *et al.,*2018).

Wigglesworthia endosymbiotic bacteria are considered critical in providing a trypanosome-immune phenotype for mature tsetse flies. In an indirect manner, these bacteria can regulate transmission of the parasite in their host's gut by responding to the presence of trypanosomes through induction of a “peritrophic matrix mechanism” to contain them (Weiss *et al.,*2013). Furthermore, the *Wigglesworthia* plasmid (pWig1; 5,200 bp) is responsible for synthesising B vitamins that are essential for tsetse metabolic processes, host nutrition and fecundity (Rio *et al.,*2019; Akman *et al.,*2002). Peptidoglycan recognition protein (PGRP-LB) in the testse is vital in the midgut for trypanosome infection processes and is also regulated by the density of *Wigglesworthia* symbionts; this protein appears to have anti-protozoal activity and stimulates resistance to the parasite (Wang *et al.,*2009). Notably, it has been demonstrated that the metabolic capacity of *Wigglesworthia* strains between different tsetse species is not identical. Thus, trypanosomes depend on this bacterium to produce folate (vitamin B_9), but *Glossina brevipalpis* shows a low trypanosome vector competency and houses a *Wigglesworthia* strain incapable of producing folate. Conversely, *Glossina mortisans* exhibits high vector competency and harbours a *Wigglesworthia* strain that can produce folate (Snyder and Rio, 2015). Remarkably, the vector competence of *G. brevipalpis* can be enhanced by supplementing its diet with folate (Rio *et al.,*2019).

Tsetse are naturally resistant to infection with trypanosomes, and this phenotype depends on many biological factors such as host age and density of maternally-transmitted symbionts (Kubi *et al.,*2006). In contrast with *Wigglesworthia*, which has a more restricted distribution within the bacteriome, the facultative symbiont *Sodalis* is widely distributed through the fly tissues, specifically in the gut epithelia and lumen (Maltz *et al.,*2012). This bacterium is recognised as being positively associated with successful colonisation of tsetse by trypanosomes and biological transmission of the parasites (Aksoy *et al.,*2008). Accordingly, in wild-caught flies, the density of *Sodalis* is significantly higher in trypanosome-infected individuals (Griffith *et al.,*2018). Considering the lack of vaccines for trypanosomiasis and the problem of drug resistance in African trypanosomes, manipulation of the tsetse microbiome is a priority research area for control of transmission (Kariithi *et al.,*2018).

Overall aims and objectives of this thesis

The impact of the arthropod microbiome on the biology of the host is becoming increasing recognised, including potentially complex interactions with pathogens, leading to positive or negative effects on vector competence. Unfortunately, most of what we know about microbiome-vector interactions has been derived from mosquitoes and to a lesser extent, ticks, with other taxonomic groups (such as mites) being largely neglected. In addition, the basic taxonomy, ecology and microbiome of arthropod vectors has not been explored in many regions of the world, particularly outside Europe and North America.

Therefore, the aims and objectives of this thesis are:

1. To define ectoparasite diversity (especially chigger mites) on rodents in the Asir region of Saudi Arabia close to areas of human habitation, and describe host-ectoparasite relationships by network analysis (Chapter 3).
2. To define the microbiome of rodent ectoparasites from Asir, including the key differences between and within ectoparasite taxa, as a means to identify potential pathogens and dominant symbionts (Chapter 4).
3. To classify the phylogenetic relationships of dominant arthropod-associated bacterial taxa from Asir in order to separate potential vertebrate pathogens from symbiotic bacteria restricted to the ectoparasite host (Chapter 5).
4. To compare three methodologies for the identification of the dominant members of the tropical rat mite (*Ornithonyssus bacoti*) microbiome from a laboratory colony in Paris and the effects of antibiotic-mediated disruption or filarial infection on bacterial profiles (Chapter 6).

Chapter 2. General Methods & Materials

2.1 Location and habitat

Wild rodents were trapped in scrubland across one site on the slopes of the Asir Mountains of southwest Saudi Arabia in 2016 (Alous, including sub locations A-F, with 23 traps) and three sites in 2017 (Alous = 11, Alogl = 24 and Wosanib = 16 traps). These villages are located between the towns of Muhayil Asir to the northwest and Abha to the southeast on the upper escarpment of the Asir Mountains. Two key biogeographical features nearby are the Jabal Sawdah peak (~3,000 m asl), approximately 10 km to the east/southeast, and the Raydah Sanctuary, 15 km to the southeast. The landscape consists of agricultural land and associated farmsteads, stony wadi beds, and rocky outcrops with scrubby vegetation (dominated by camphor bush, *Tarchonanthus camphoratus*; yielding to African juniper, *Juniperus procera*, at higher elevations) (Miyazaki *et al.* 2007). GPS coordinates were used to record rodent trapping locations; these mentioned geographic coordinates and altitudes were obtained from Google Earth, http:/earth.google.com as shown in (Figure. 2.1.a,b). The trapping sites encompassed two main types of habitat; mountains and agricultural fields (Figure 2.2). Temperature and humidity were recorded using an EzSmart Hygro-Thermometer RHT3 (EXTECH Instruments, U.S.A) at each trapping location.

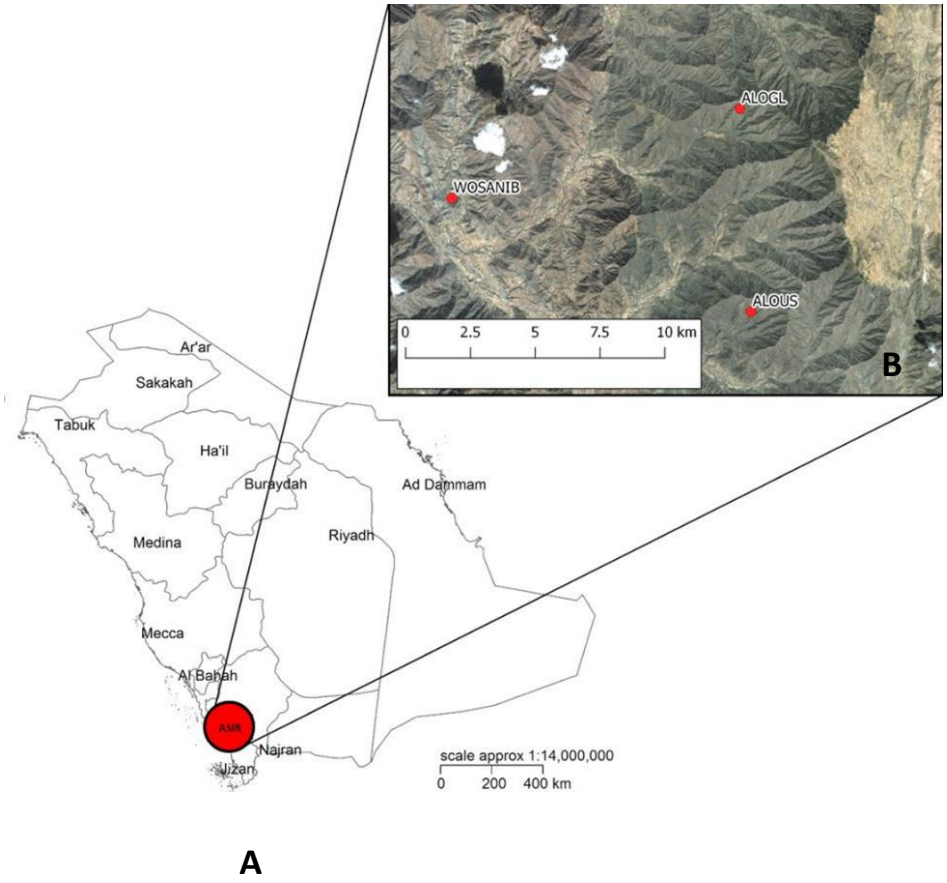


Figure 2.1. Rodent trapping locations in the Asir region, Saudi Arabia. (A) Map of Saudi Arabia. (B) Zoomed region of sample sites on the Asir Mountains.

Each circle indicates the location sites of this study; Alogl, Wosanib, and Alous. https://www.gps-coordinates.net/

Figure 2.2. Rodent habitat in Asir region, Saudi Arabia.

Mountains; (A) Alous, (B) Wosanib, and (C) Alogl. D,E: Detail of agriculture fields near Alogl village.

2.2 Rodent trapping, animal handling, euthanasia and ethics

Trapping rodents was carried out by using locally available live rat traps. Thirty live-traps were set daily at 6:00 pm with bread and peanut butter as bait and left overnight. The collection of the traps was conducted in the early morning of the following day. However, to target other rodent species, additional traps were set at 5:00 am with bread, and peanut butter, as bait for 4-6 hours. After collection, each trap was labelled and placed inside a plastic bag to save the ectoparasites. Traps containing animals were labelled to indicate place and date of capture and then brought from the field to a laboratory within the same day. The trapping protocol used followed Herbreteau *et al.* (2011).

Permission for rodent trapping and euthanasia was obtained from the Saudi Wildlife Authority by the local collaborator, Dr Abdulaziz N. Alagaili (Department of Zoology, King Saud University, Riyadh), before fieldwork commenced. Approval from the University of Liverpool's Animal Welfare and Ethics Review Board was also obtained. Animals were euthanized by inhaled anaesthetic overdose according to guidelines published by the American Veterinary Medical Association Council on Research (Underwood *et al.,*2013) and the Canadian Council on Animal Care (Charbonneau *et al.,*2010). The cage containing the rodent was placed in a sealed bag containing a cotton wool pad soaked in ether. The animals were only handled outside the trap after being completely euthanized and a separate bag was used for each animal. The sex of rodents was determined by the external reproductive organs: the vulva opening and mammary teats for females, and the scrotal sac in males. The tip of the tail was removed and stored in 70% ethanol at 4˚C for molecular barcoding (see section 2.5.1).

2.3 Chigger isolation and preparation of other ectoparasites

2.3.1 Chigger mites isolation

Each rodent cadaver was placed in a steel kidney dish and examined by the naked eye from head to tail by using a fine comb for the existence of chiggers and other ectoparasites. A paintbrush was used to collect chigger mites from four predilection sites on the host (back, chin, ear, and anus) and these were then fixed in absolute ethanol. Other ectoparasites such as fleas, lice, ticks and gamasid mites were isolated from rodents by using fine forceps and brushing, then placed in different tubes, with preservation in absolute ethanol. To minimise loss of chiggers and other smaller ectoparasites, the whole cadaver was also examined under a dissecting microscope to collect any specimens that were missed with the naked eye. Any detached ectoparasites were collected from the bag used for euthanasia and from the kidney dish; the latter was sterilised with ethanol between rodents. In cases of excessive workload, the ear was removed and examined for chigger infestation under a stereomicroscope, and fixed in 70% ethanol within a day for later examination. Example images of chigger infestation in the ear and anus are provided in Figure. 2.3.

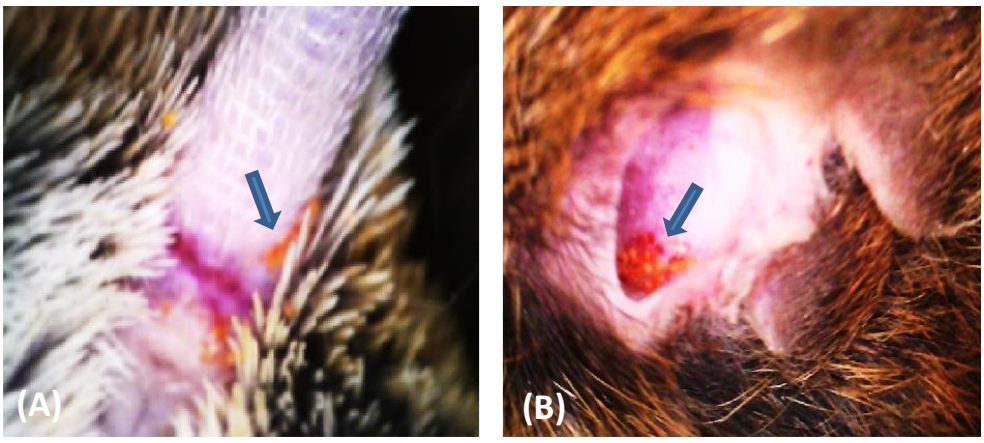


Figure 2.3. (A) chiggers infesting the anus and (B) the ear of a parasitized rodent. (Arrows show chiggers).

2.3.2 Chigger mite identification

Chigger mites were counted to estimate the intensity of infestation per rodent, and a subsample of 10% of the chiggers from each was selected from the four predilection sites on the host (back, chin, ear, and anus) for identification. Prior to examination, fixed chiggers were "relaxed" in water for two hours to reverse clasping of the legs around the body. Chiggers were mounted (dorsal view) on glass slides by using a small drop of Berlese fluid (TCS Bioscience Ltd, UK), and then covered with 13 mm round-coverslips. The specimens were pressed lightly and left overnight to allow the mites to be flattened fully. This is an important and fundamental step for the accuracy of sample morphometrics. The ID number of animal host, study site, sampled chiggers number and date of preparation were included on the label of each slide. Finally, the prepared-slides were incubated for two days at 50°C in a hot air incubator before further examination.

Chiggers were identified to subgenus level following a taxonomic key published by Nadchatram and Dohany, 1974 using measurements of individual mites on a ZEISS Axio Imager M2 microscope through ZEN 2011 imaging software (Carl ZEISS, Germany). Other relevant taxonomic works were consulted, depending on the genus, for identification (Goff *et al.,*1982; Stekolnikov, 2013 and Narang & Lamba, 2010). As chiggers from Saudi Arabia have not been studied before, identification to species (and sometimes even genus) was extremely challenging. Therefore, characteristics of the scutum, sensillae and cheliceral blade were recorded for individual chiggers to classify them into different "morphospecies" (Figure2.4). Representative specimens for each morphospecies were then sent to Dr Alexander Stekolnikov (Zoological Institute, Russian Academy of Sciences, Saint Petersburg, Russia) for definitive identification.

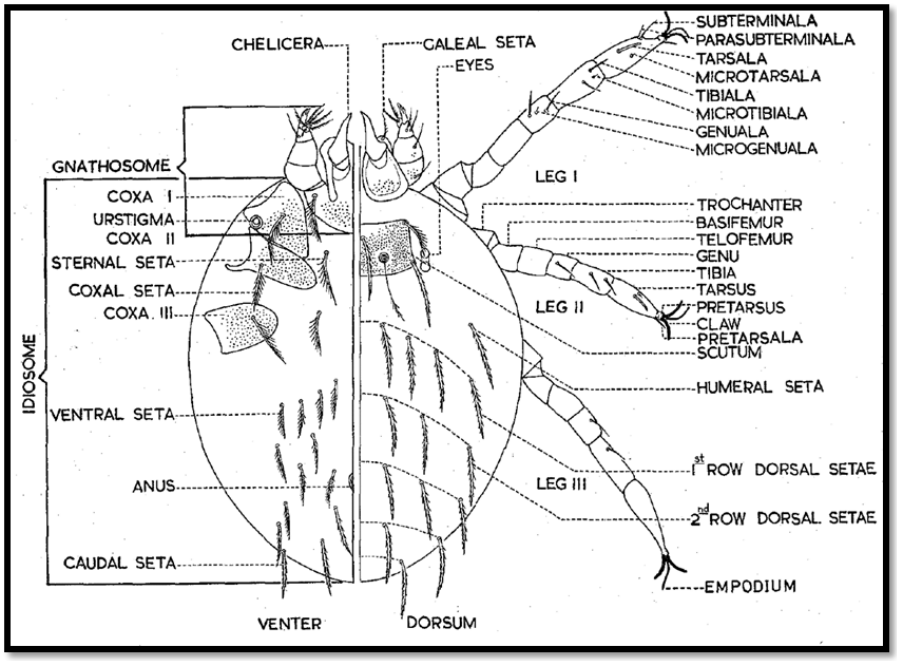


Figure 2.4. Example of essential characteristics for chigger identification to the subgenus level (Nadchatram & Dohany, 1974).

2.3.3 Important taxonomic features for classification of trombiculid mites

The classification of trombiculids is based on parasitic larval stage morphology. This is because they can be found easily on the host compared with the nymphs and adults, which are difficult to sample due to their free-living status in the environment (Kershaw, 2009). The scutum (dorsal shield) is considered the most significant feature for chigger examination along with the scutal setae (tiny hairs), which are used together in order to identify chiggers to genus or subgenus level (Nadchatram & Dohany, 1974). Additional characteristics are essential for identification to the species level (Vercammen-Grandjean & Langston, 1975), such as body shape and appendages (palps and legs) and the chaetotaxy (arrangements of setae). The glossary of terminology and abbreviations published by Goff et al. (1982) and also other established identification keys and criteria by (Nadchatram & Dohany 1974; Vercammen-Grandjean & Langston 1975) have been used in the current work.

2.3.3.1 Gnathosoma

A mite's body is composed of two parts: the gnathosoma and idiosoma. The gnathosome (mouthparts) is divided into three main sections: a pair of palps, galeae and chelicera (teeth or blades), which are all situated on the gnathosome base (fused coxa). Palps of trombiculids are segmented appendages, which consist of the gnathobase coxa, femur, genu, tibia and a tarsus (Figure 2.5). These appendages bear sets of setae that are very important for taxonomic diagnosis. In the dorsal view, the femur and genu each bears a seta. In addition, there are three tibial setae located on the dorsal, dorsolateral and ventral view of the tibia; whereas, the tarsus has a variable number of setae ranging from three to seven (Figure 2.6). The palp setae can be expressed in a formula termed (fPp); for instance, the formula for *Leptotrombidium deliense* is N/N/BNN, where "B" is barbed and "N" is nude.

Galeae are appendages located ventrally to the gnathobase and dorsally around chelicerae. This structure may bear a barbed or nude seta called the galeal seta on the anterior surface. Chelicerae are situated at the perioral region of the mouthparts and are used to penetrate the host skin. They consist of two segments: the cheliceral base, a fixed appendage on the dorsal side of the gnathobase; and moveable structures which are supported by the cheliceral base. The chelostyle (cheliceral blades) has different shapes depending on the genera; in some taxa, the tip of the blade exhibits a unique triangular barb, the so-called "tricuspid". The structure of the chelostyle is also armed or unarmed on the dorsal, ventral or apical surfaces with minute teeth (Figure 2.7).

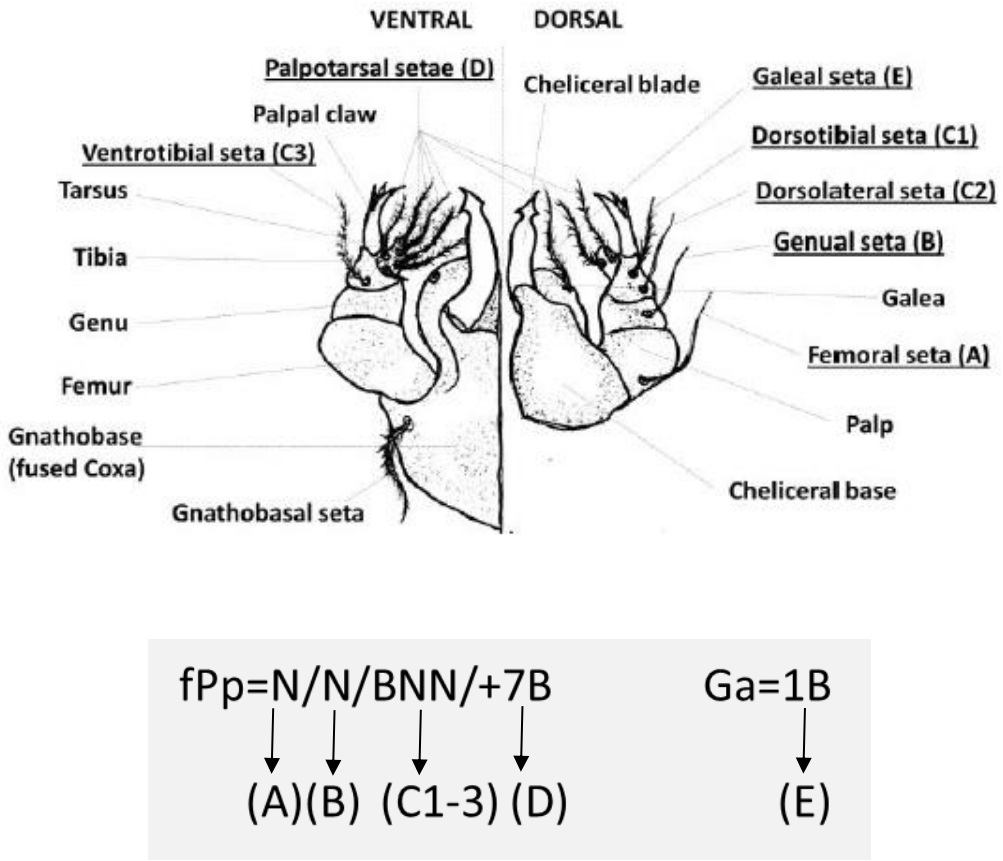


Figure 2.5. Dorsal and ventral view of the gnathosoma.

One femoral seta is nude (A), one genual seta is nude (B), there is one barbed dorso-tibial (C1) and two nude dorsolateral and ventrotibial setae (C2 and C3), and seven spiky palpotarsal setae, which cumulatively create the palpal pilous formula (fPp) above. A single barbed galeal seta (Ga) is shown (Nadchatram & Dohany, 1974; Chaisiri, 2016).

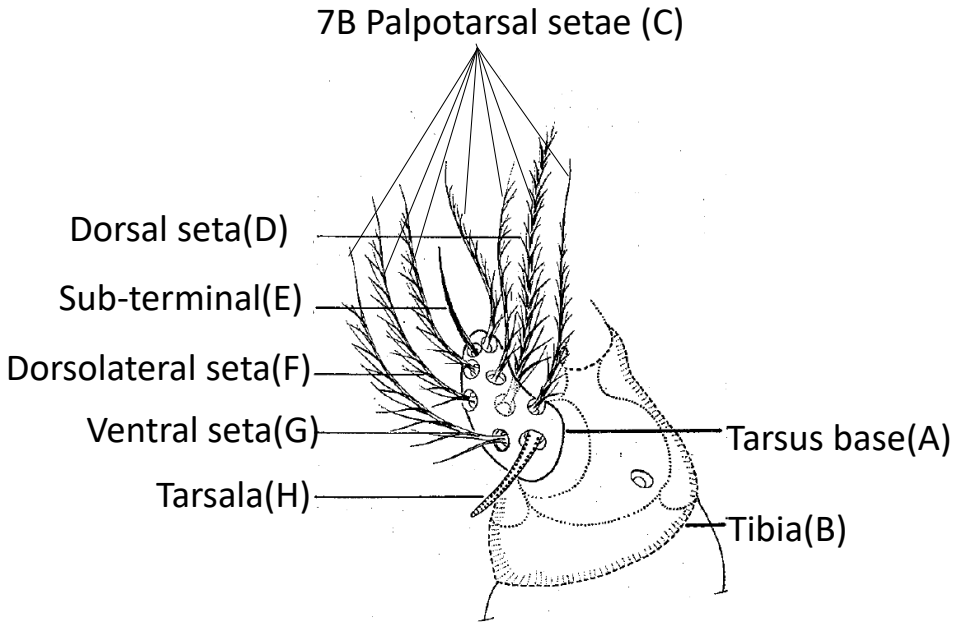


Figure 2.6. Dorsal and ventral view of the palpal tarsus.

Tarsus base (A), tibia (B), seven branched palpotarsal setae (C), dorsal seta (D), sub-terminal seta (E), dorsolateral seta (F), ventral seta (G) and tarsala (H). Modified from Nadchatram & Dohany 1974.

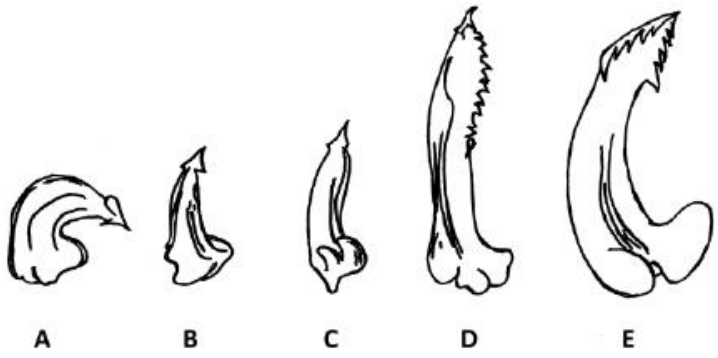


Figure 2.7. Various forms of cheliceral blades *Babiangia*.

(A); *Gahrliepia* (B); *Leptotrombidium* (C); *Shoengastia* (D); *Odontacarus* (E). Adapted from Goff et al. 1982; Nadchatram & Dohany 1974; Chaisiri, 2016.

2.3.3.2 Scutum

The scutum is a dorsal plate located in the anterodorsal part of the chigger body below the gnathosoma. Generally, the scutum is considered the most important part of the chigger used in classification (Vercammen-Grandjean & Langston 1975). The general shape, dimensions and composition of scutal setae play a very important role in determining the various genera, as this feature is usually constant at the genus or subgenus level. The shape of the scutum varies from rectangular, trapezoid, pentagonal or shield-shaped, with a slightly convex or undulated anterior/posterior margin. Moreover, the scutum bears a pair of sensillary bases or pits from which arise a pair of sensillae organs. In addition, the shape of the sensilla can provide the best key character to separate genera with unexpanded sensillae or expanded.

Five scutal setae are typical: two anterolateral (AL), two posterolateral (PL) and one anteromedian (AM). However, in some taxa; *i.e.*, the subfamily Gahrlipiinae, the AM seta is absent; while a pair of AM setae presents in the subfamily Leeuwenhoekiinae. There are some structures of scutal setae which assist to distinguish between the genera. For instance in *L. deliense*, the PL is longer than the AM, and longer than AL; the scutal formula (fSc) = PL>AM>AL. However, in some genera, particularly *Gahrliepia* and *Schoengastiella*, usually two or more extra post-posterolateral setae named “usurped setae” are present. These setae are considered to be the first row of body setae, which are combined into the elongated-posterior part of the scutum (Goff *et al.,* 1982). In addition to the setae, the scutum also contains a pair of specific sensory setae called “sensillae” located in the scutal sensillary bases on the scutum. Sensillae are specifically different from scutal setae and also vary in shape. They may be unexpanded (filamentous) or expanded (lanceolate to globose) at the apical portion. The structure of these sensillae also plays an important role in taxonomic identification (Figure 2.8). Finally, the scutum is always punctate, and the nature of the pits plays a crucial role in diagnostics at the species level.

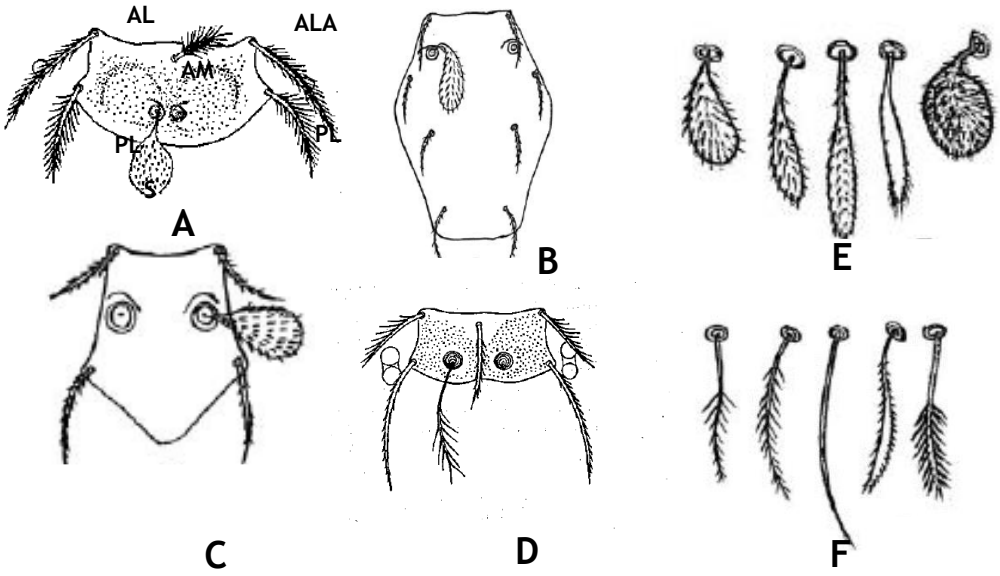


Figure 2.8. Example of Various shapes of scuta in trombiculid genera.

Helenicula (A), *Gahrliepia* (B), *Walchia* (C) and *Leptotrombidium* (D). AL = anterolateral setae, AM = anteromedian setae, PL = posterolateral setae, and S = sensillae. The variation of scutal sensillae shape can be expanded (E) or unexpanded (F). Adapted from Nadchatram & Dohany (1974).

2.3.3.3 Body chaetotaxy

The dorsal and ventral idiosome setae make up the body setae. Chaetotaxy is the word used to refer to the number of and ratio of setae on the arthropod body cuticle in order to allow taxonomy. The setae number can range between 12 and 100 in the dorsal setae; however, the number for ventral setae is usually small and refined.

In order for species identification to occur, establishing the number of body setae is crucial. As far as identification of trombiculid species is concerned, the palpal pilosity formula (fPp) on the gnathosoma is the most significant aspect in defining body chaetotaxy. Multiple body setae are present on the dorsal and ventral sides alike and these come to surface in distinct patterns that form transverse rows. In terms of the dorsal side, the setae begin to emerge behind the scutum, typically beginning with the first pair of dorsal setae on the side of the scutum, which are referred to as “humeral setae”. This is followed by numerous rows of dorsal setae in a pattern from the bottom of the body. Therefore, the “dorsal body setation formula” can be used to calculate the number of humeral setae and dorsal setae

per row, as counted from anterior to posterior (fD); e.g., in *L. deliense*, it is 2. 8. 6. 6. 4. 2 = 28. As opposed to setae on the dorsal surface, setae from the ventral side are often not presented in unique rows, making it very difficult to count and allocate ventral setae into specific rows. The setae shown in the third pair of coxa (legs) is solely present on the ventral body and these make up the ventral setation formula (fV). In terms of the nurogenital pore, "u" is used to represent how many ventral setae are in a row of the fV. To work out the total amount of body setae (NDV), we must add together the number of dorsal (ND) and ventral setae (NV). It is important that the number of ventral setae is less than the dorsal setae. Often, this combined morphological data: fD, fV, ND, NV and NDV is applied to form the species identification standards (e.g., see for *L. deliense* in Figure 2.9).

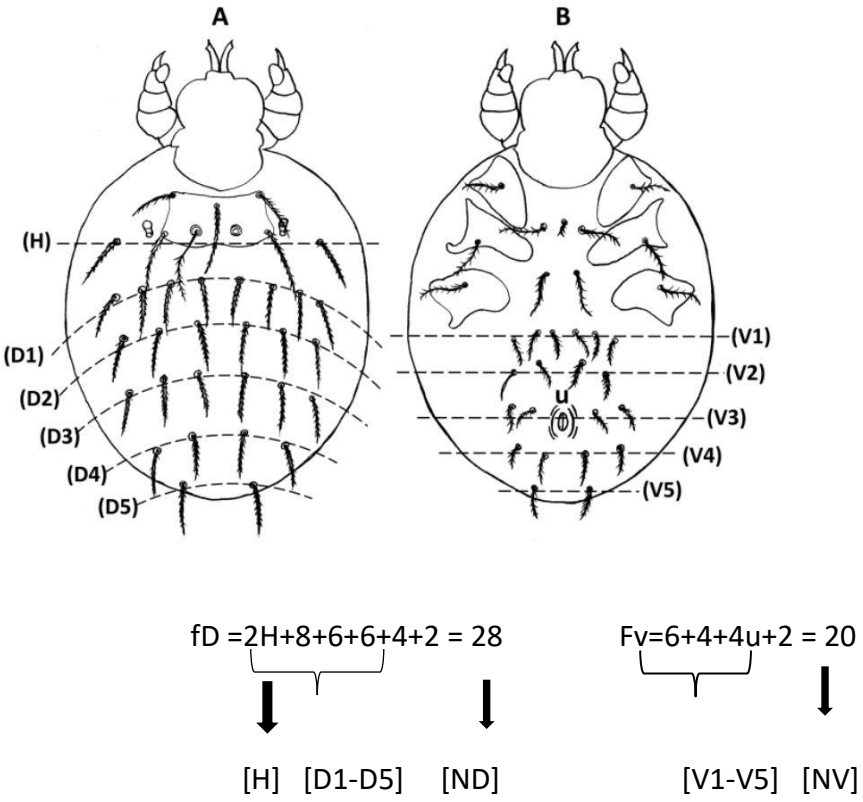


Figure 2.9. Characteristics body chaetotaxy of *L. deliense*.

Dorsal (A) and ventral (B). 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 opening.

2.3.3.4 Legs

There are three pairs of legs possessed by the trombiculid mite, which are known as leg I-pa, leg II -pm and leg III –pp. Post-larval stages, however, have four pairs of legs. There are seven sections to each leg, namely: coxa, trochanter, basifemur, telofemur, genu, tibia and tarsus. Nonetheless, it can be the case that some taxa possess only six segments as the basifemur and telofemur can sometimes be fused together. As for identification, the leg segmentation formula can be used to represent the number of segments on each leg (fsp). For example, there are seven segment of the *Leptotrombidium* genus on leg I, II and III (fsp = 7.7.7). However, the genus *Gahrliepia* possesses only six segments on leg II and III, and

this can be presented as fsp = 7.6.6. Moreover, the ends of the legs have sickle-shaped claws, with a slender claw strengthened by an empodium being present at the centre.

There are two types of setae that are supported by the categorizations of leg segmentations. These are ordinary setae (barbed) and sensory setae (nude). The former is present in every segment, whereas the latter are only found on the genu, tibia and tarsus and thus are known as “genuala”, “tibiala” and “tarsala” setae, respectively. The number of nude leg setae types is determined by their shape, size and characteristics. For instance, microgenualae (a small spur on genua) might be present on leg I, or mastitarsalae (a long whip–like seta on the tarsus). The amount of sensory setae and their types are crucial for the diagnostic process of identification (Figure 2.10). In addition, the number of ordinary setae (barbed) present in coxa I, II and III (shortened to CX, the coxa setation formula) can also be very beneficial for purposes of identification to specific genera; i.e., *Walchia* and *Helenicula.*

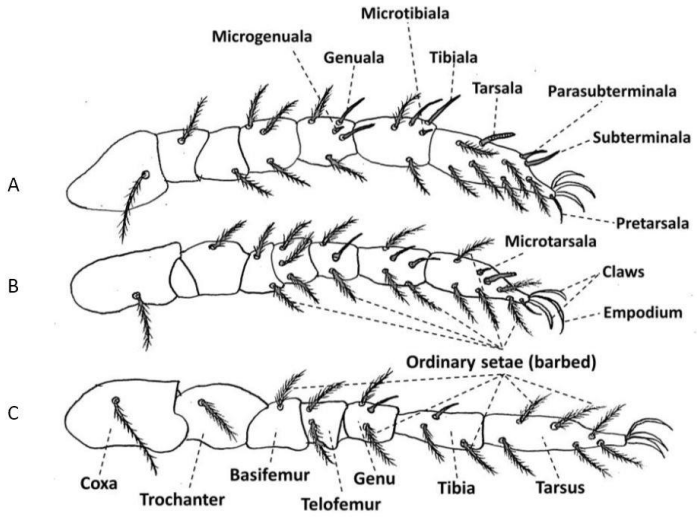


Figure 2.10. Leg segmentation presenting setae of *Leptotrombidium deliense* and bases of branched seta.

Leg 1 (A), leg 2 (B) and leg 3 (C). Adapted from Goff *et al*., (1982) and Chaisiri (2016).

2.3.3.5 Morphometry

The dimensions of the idiosome and its appendages are significant in diagnosis of species. Scutum, setae, sensillae and legs are considered the most important features of trombiculid larvae for definitive taxonomic identification. The following measurements have been demonstrated to be beneficial to taxonomists: the dimensions of the idiosome; the dimensions of the scutum; the dimensions of the sensilla; the lengths of the various body setae; the lengths of legs I, II and III; the length and height of coxa III; and the length and height of tarsus I, II and III (excluding claws). The methods used for measuring the main characteristics of this study are based on the descriptions of Nadchatram and Dohany (1974), Vercammen- Grandjean, and Langston (1975). All measurements are taken in microns, the standard unit generally used in such taxonomic and systematic research of trombiculids (Figure 2.11). Full details of the methods for measurement and relevant abbreviations used here can be found in the (Appendix 1).

There are a limited number of molecular tools available for measuring taxonomic development; therefore, the classification of chiggers has been based on the measurement of larval specimens and general morphology. One possible reason for this lack, and the attendant difficulty of performing molecular work, is linked to the various problems associated with using chiggers as a sample; for instance, they are very small, which can mean that one sample provides insufficient DNA for molecular work to be carried out. In addition, damage to genetic material that may have an effect on PCR amplification can arise as a result of the preparation processes necessary to enable visualisation of the chigger features; for example, using clearing agents containing Berlese fluid (gum chloral). In the case of this research, in order to facilitate further molecular analysis of the chigger microbiome (section 2.5.2.3), a molecular approach that focuses on validating the quality of the DNA extracted from individual chiggers was pursued.

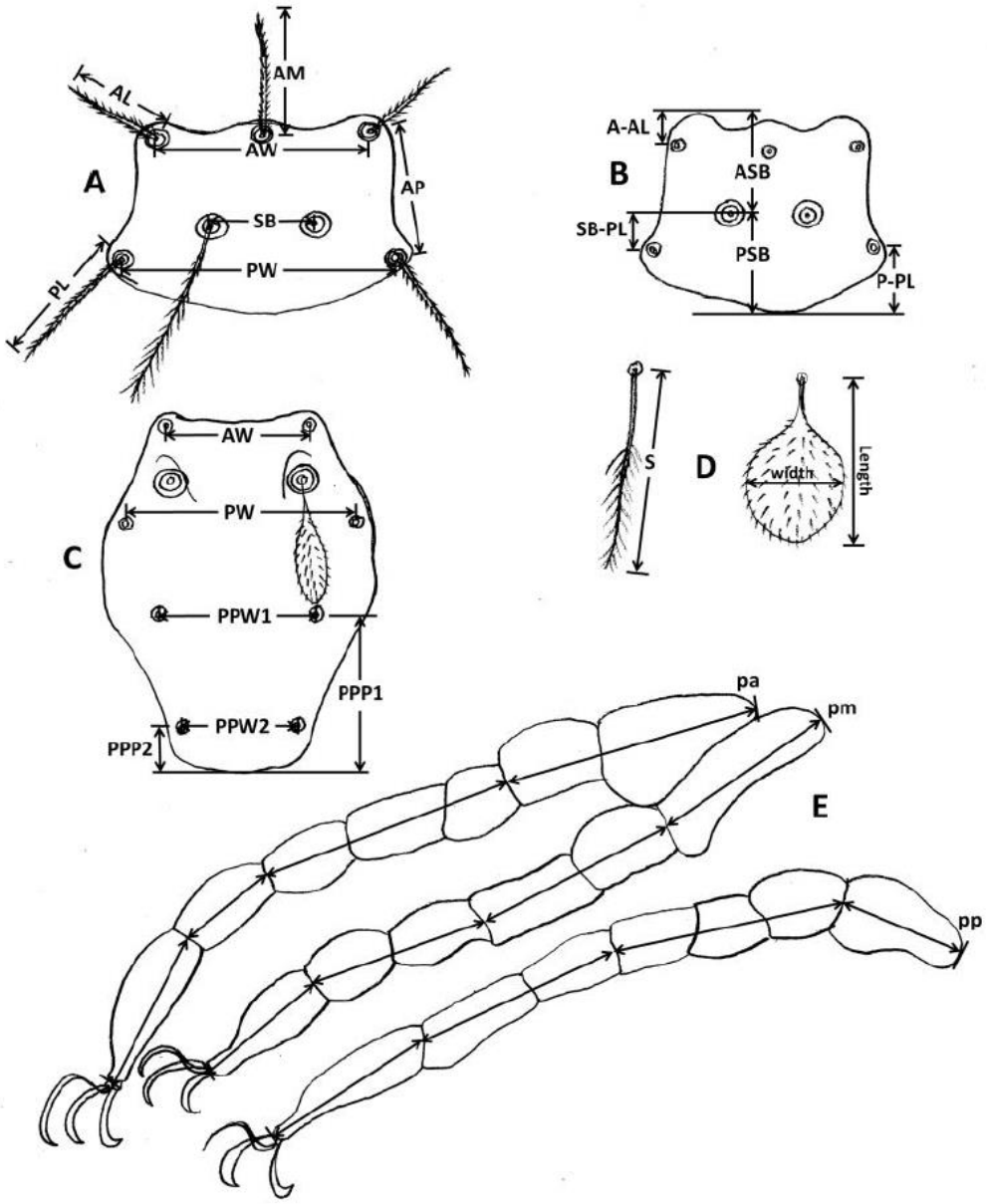


Figure 2.11. Approach of measuring the key characteristics of trombiculid larvae: dimension and length of scutum and its setae.

(A, B and C); dimension and length of sensillae (D); and length of the three legs (E).

2.4 Preparation of non-chigger ectoparasites for identification.

A 5% subsample of ectoparasites was selected from each rodent for mounting in KOH and morphometric examination. Lice, fleas, ticks and gamasid mites were placed in water for 10 min, transferred to 10% potassium hydroxide (KOH) solution and incubated at 37°C for 10 min until the cuticle became clear. The specimens were placed in water for 10 min and then dehydrated in a series using three different alcohol strengths: 50%, 70%, and 100% ethanol (10 min for each stage). Then, the fleas and lice were placed in xylene for 5 min. This process cleared the specimen. Finally, the specimen was transferred to a glass slide with a drop of DPX mountant and covered and labelled. Fleas (Lewis *et al.,*1982; Hopkins *et al.,*1953), ticks (Hoogstraal, 1981), lice (Paterson, *et al.,*1953), and gamasid mites (Tipton *et al.,*1960) were classified with expert assistance from Dr John McGarry from the Institute of Veterinary Science, University of Liverpool. The most important featuers of tick species are presented in Figure 2.12, fleas (Figure 2.13.), lice (Figure 2.14), and gamasid mites (Figure 2.15 and Figure 2.16).

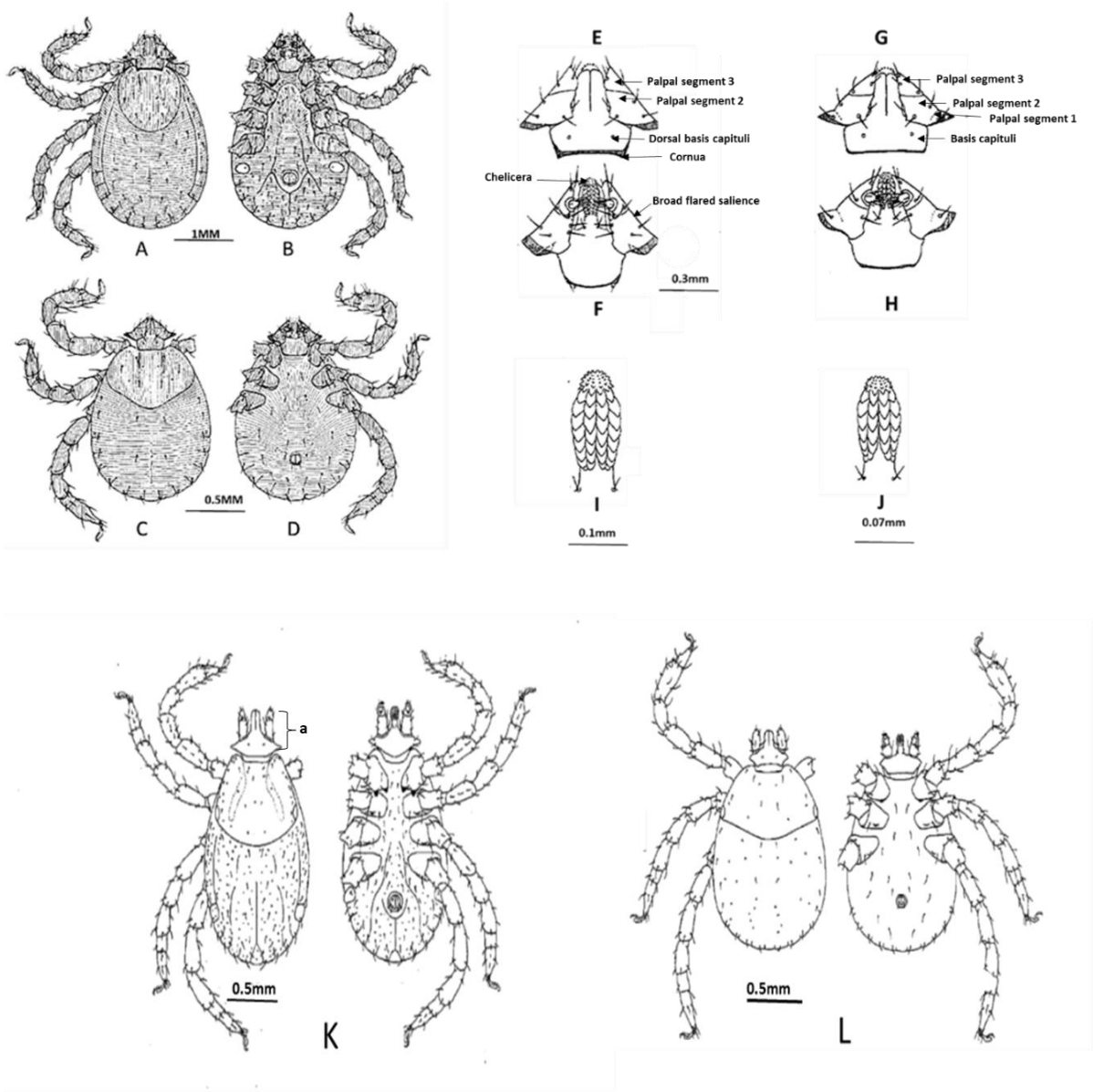


Figure 2.12. Morphology of *Haemaphysalis erinacei* and *Rhipicephalus sanguineus* immature stages.

(A) Dorsal view of *Haemaphysalis erinacei* nymph; (B) ventral view of *Haemaphysalis erinacei* nymph; (C) Dorsal view of *Haemaphysalis erinacei* larva;(D) ventral view of *Haemaphysalis erinacei* Larva; (E) Dorsal view of palps and capitulum *Haemaphysalis erinacei* nymph; (F) ventral view of palps and capitulum *Haemaphysalis erinacei* nymph; (G) Dorsal view of capitulum of *Rhipicephalus sanguineus* larva; (H) ventral view of capitulum *Rhipicephalus sanguineus* larva; (I) Hypostome of *Rhipicephalus sanguineus* nymph; (J) hypostome of *Rhipicephalus sanguineus* larva. (K) Dorsal views and capitulum of *Rhipicephalus sanguineus* nymph(a); (L) ventral views of *Rhipicephalus sanguineus* nymph; (M) Dorsal views of *Rhipicephalus sanguineus* larva; (N) ventral views of *Rhipicephalus sanguineus* larva. Reproduced from (Hoogstraal, 1981).

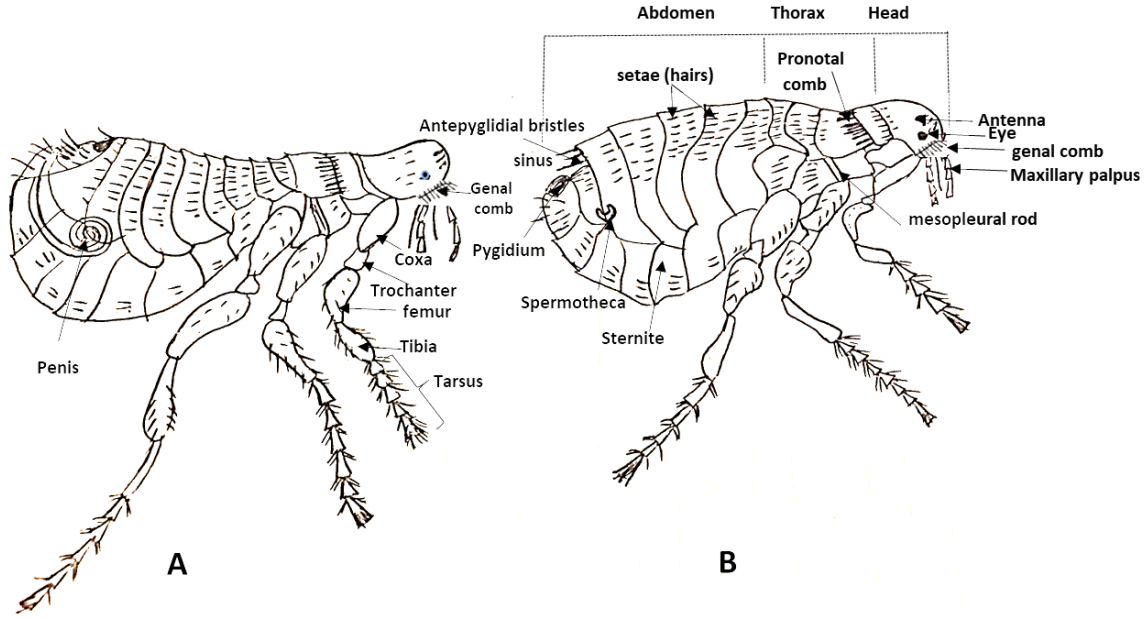


Figure 2.13. General appearance and morphological features of a flea.
(A) Male Flea ; (B) Female flea. Modified from (Mathison and Pritt, 2014).

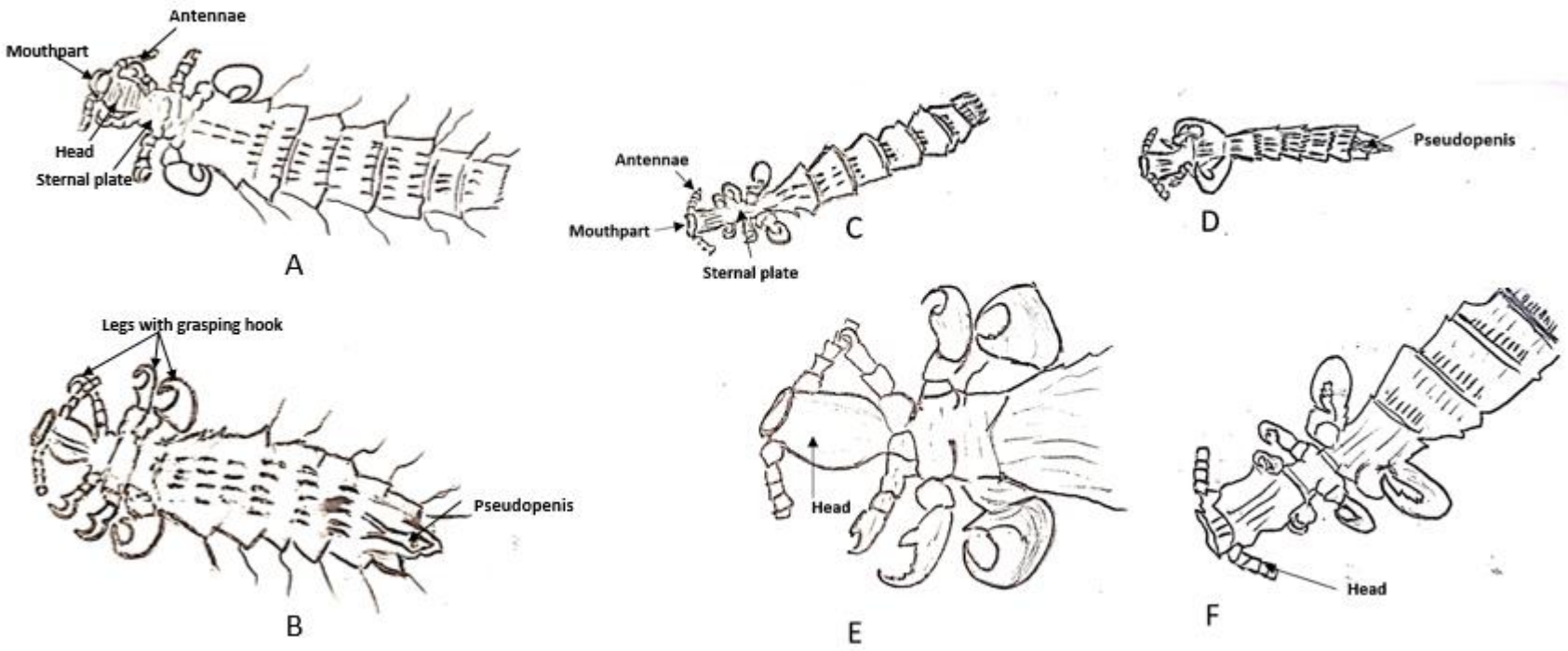


Figure 2.14. General features of a louse species.

(A) *Polyplax oxyrrhyncha* female, (B) *Polyplax oxyrrhyncha* male. (C) *Polyplax brachyrrhyncha* female, (D) *Polyplax brachyrrhyncha* male (E) *Polyplax oxyrrhyncha*, (arrow)length of head at least twice width (F) *Polyplax brachyrrhyncha* (arrow) antennae on head margin.

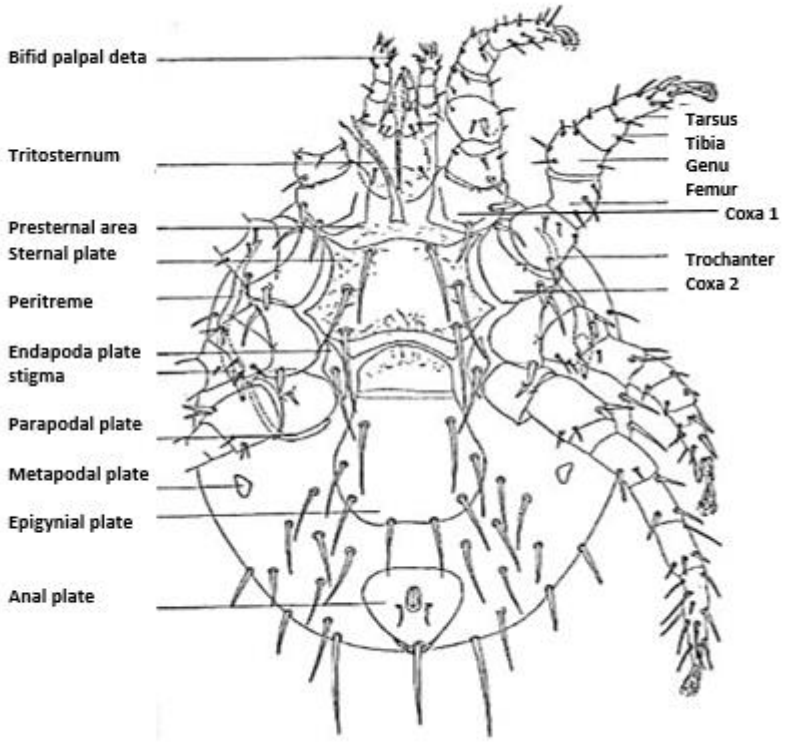


Figure 2.15. General appearance of the ventral view of Laelaps (female). (Modified from Tipton 1960).

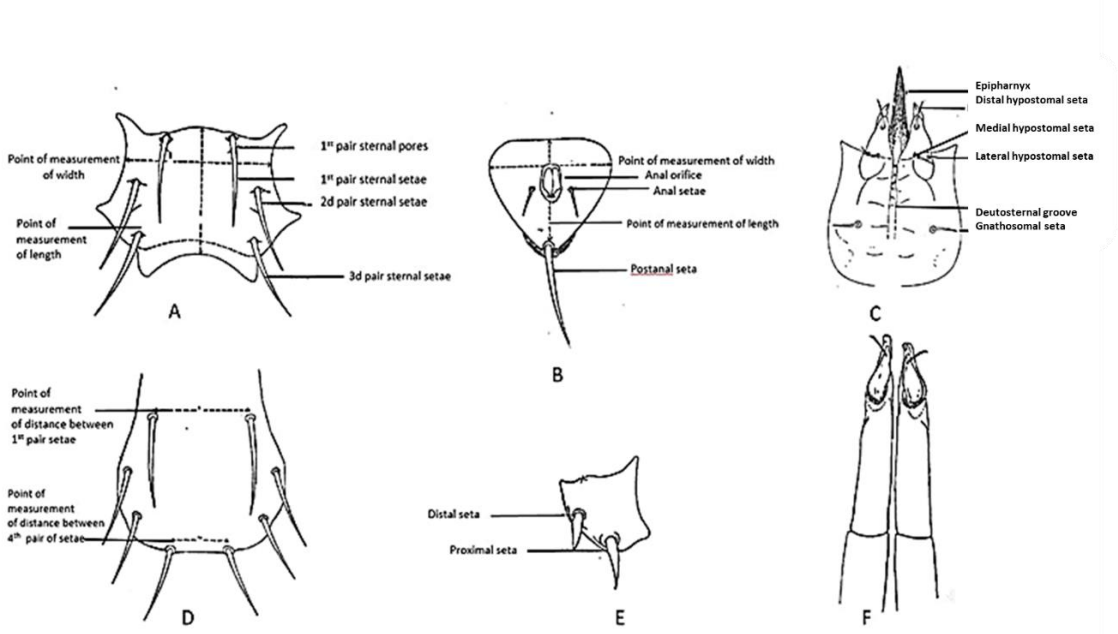


Figure 2.16. Characters of taxonomic importance of *Laelaps*.
(A) Sternal plate, (B) anal plate, (C) gnathosoma, (D) epigynial plate, (E) coxa I, (F) female chelicerae (Modified from Tipton 1960).

2.5 Molecular procedures for identification of rodents, chiggers and other ectoparasites

2.5.1 Rodents

DNA from rodent tails was extracted using the DNeasy Blood & Tissue Kit (QIAGEN). Approximately a 1-cm length of tail tissue was placed into a 1.5 ml microcentrifuge tube. Then 180 µl of ATL buffer and 20 µl of proteinase K solution (600 mAU/ml) were added. The tissue was incubated at 56°C for 6 hours, and then subsequent steps followed the manufacturer's protocol. A minimal volume (200 µl) of nuclease-free water (Ambion) was used in the DNA elution step. The concentration of DNA was determined by Quant-iT Picogreen dsDNA kit (Invitrogen), and read in an Infinite F200 microplate fluorimeter with Magellan™ -Data Analysis Software (TECAN). The DNA concentration was calculated by comparison to a high-range (0 - 1,000 ng/ml) DNA standard curve.

For species identification of the rodents, the mitochondrial cytochrome b (*cytb*) gene was amplified using the primers (L14841- forward: 5'AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA3') and (H15149- reverse: 5'AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3')**.** The cytochrome b (*cytb*) gene is well-recognised as a marker for rodent species identification. The PCR product size is around 290 bp across rodent species and produces reference barcodes (Kocher *et al.,*1989).

PCR amplification was performed in 35-µl reactions containing 3.5 µl of DNA template, 13.2 µl BioMix Red (Bioline), and 0.7 µl of each primer (0.4 µM final concentration). Nuclease-free water was used as a negative control. The PCR was run with 40 cycles as follows: denaturation at 94°C for 4 min, then 94°C for 30 s, 48°C for 30 s, and 60 s at 72°C, with a final extension of 10 min at 72°C. The PCR products were visualized by 1.2 % agarose gel electrophoresis (Bioline) containing SYBR Safe dye (Invitrogen) at 150 V for 30 min in a Bio-Rad gel electrophoresis set. G: Box Gel Documentation System (Syngene) was used to visualised the gels

The DNA samples sent for sequencing with L14841/H15149 forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligned using BIOEDIT software and then analysed by BLAST from the National Centre for Biotechnology Information (NCBI).

2.5.2 DNA extraction from swab samples from different locations on infested hosts

DNA samples were collected from different parts of the body where chiggers attach (ear, back, and anus) on each rodent using PERFORMA gene PG-100 swab kits (DNA Genotek). The DNA samples were collected, inverted ten times with the PERFORMA gene solution and shipped to the UK with dry ice. In the UK, the each sample was defrosted and mixed by shaking vigorously for 5 secs. The samples were then incubated in a heating block (50°C) for a minimum of 2 hr, and 500 µl of the mixed PERFORMAgene sample was transferred to a 1.5 mL microcentrifuge tube. Twenty µL (1/25th volume) of PG-L2P purifier was added to

the microcentrifuge tube and mixed by vortexing for a few seconds. Each sample was incubated on ice for 10 min, then centrifuged at room temperature for 10 min at 15,000 × g. The clear supernatant was transferred carefully with a pipette tip into a fresh microcentrifuge tube and the pellet was discarded. Twenty-five µl (1/20th volume) of 5 M sodium chloride was added to 500 µl of supernatant, followed by 600 µl of 95% to 100% ethanol (room temperature) and mixed gently by inversion 10 times. The sample was allowed to stand at room temperature for 10 min in order to allow the DNA to fully precipitate. The samples were then centrifuged at 15,000 × g at room temperature for 2 min and the supernatant was removed carefully with a pipette tip and discarded without disturbing the pellet. To wash the DNA, 250 µl of 70% ethanol was added and the mixture was allowed to stand for 1 min at room temperature. The ethanol was removed with a pipette tip without disturbing the pellet. To dissolve the DNA pellet, 100 µl of TE buffer was added followed by vortexing for 5 sec, then the samples were incubated at room temperature overnight to ensure complete rehydration of the DNA

2.5.2.1 Testing of DNA yield using different methods for DNA extraction

To obtain the highest yield of DNA, different methods were tested for extraction of DNA from chigger mites: ammonium hydroxide, Chelex DNA extraction (Casquet *et al.,*2012), and the DNeasy Blood & Tissue Kit (QIAGEN). A method was sought that would also work well with ticks. The DNeasy Blood & Tissue Kit (QIAGEN) was found to be the best method (Table 2.1). DNA concentrations were measured by a double-stranded DNA fluorescence-labelling method (Quant-iT Picogreen, Invitrogen) and read in an Infinite F200 microplate fluorimeter (Magellan - Data Analysis Software, TECAN).

Table 2.1. Methods of DNA extraction from chigger mites

DNA extraction methods	Brief protocol
1- DNeasy Blood & Tissue Kit (QIAGEN).	The mite was torn into small pieces using a needle in a 1.7 ml Eppendorf tube comprising 180 µl ATL buffer and 20 µl proteinase K solution. The samples were incubated at 56°C overnight and then the manufacturer's protocol was followed. Thirty µl of nuclease-free water (Ambion) was used in the DNA elution step.
2- Chelex DNA extraction (Casquet *et al.,*2012)	The mite was dried from ethanol and torn into small pieces using a needle in a 1.7 ml Eppendorf tube containing 10 µl of proteinase K solution. Then, 100 µl of 100% Chelex resin [1 gm of ml Chelex Resin in 10 ml of nuclease-free water (Ambion)] was added to the samples. These were incubated for five hours at 56°C, and then centrifuged at 14,000 rpm for 1 min in a benchtop centrifuge. The supernatant containing the DNA was transferred to new 1.7 ml Eppendorf tube.
3- Ammonium hydroxide (NH_4OH)	Chigger mite samples were dried from the 70% ethanol solution and torn into small pieces, and 150 ml of 0.7 M ammonium hydroxide was added to the samples. Then they were heated to 100°C for 18 min. The solution was centrifuged for 10 min at 10,000 × g to remove debris and the lids of the tube were opened at 100°C for 18 min inside a fume hood to evaporate (NH_4OH) to 70-100 µl.

2.5.2.2 Amplification of cytochrome oxidase I gene for DNA quality assessment and barcoding

DNA quality was assessed by amplifying a 710-bp fragment of the cytochrome oxidase, subunit I (*coi*) gene. The mitochondrial *coi* gene has been recognized as a potential molecular marker for phylogenetic analysis of Acari (Cruickshank, 2002; Dabert, 2006). It can be used for DNA quality assessment and also provides insights as a genetic marker for chigger taxonomic studies. Two primers were used to amplify the *coi* target (Table 2.2). The 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). Nuclease-free water was used as a negative control. PCR was run with 40 cycles as follows: initial denaturation at 94°C for 1 min; 5 cycles of 94°C for 1 min, 45°C for 90 sec, 72°C for 90 sec; then 35 cycles of 94°C for 1 min, 50°C for 90 sec, 72°C for 1 min; and concluding with a final extension at 72°C for 5 min. (Folmer *et al.,*1994). The PCR products were visualized by 1.5% agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 minutes in a Bio-Rad gel electrophoresis set. A G:Box Gel Documentation System (Syngene) was used to visualise the gel and PCR products were purified using a QIA-quick PCR Purification Kit.

2.5.2.3 Autofluorescence microscopy for paired-matched morphological and molecular identification of individual chiggers

According to the most common chigger species, 25 chigger mites from four genera, *Ericotrombidium*, *Schoutedenichia, Ascoschoengastia*, and *Pentidionis* were selected for paired *coi* sequence amplification and autofluorescence microscopy. According to a recent study (Kumlert *et al.,*2018), this method enhances visualization of scutum morphology, body setae and leg segmentation for chigger identification to subgenus level when compared to bright field mode. Moreover, the avoidance of maceration (clearing) in Berlese fluid prevents destruction of DNA and allows molecular barcoding of the same individual specimen after micrographs have been obtained. Individual mites preserved in ethanol were aspirated by micropipette, placed on a glass slide with a drop of ATL buffer

(Qiagen) and covered with round coverslips. Scutum imaging with morphometric measurements was obtained using a ZEISS Axio Imager M2 microscope and ZEN 2011 imaging software (Carl ZEISS, Germany). A combination of fluorescence and bright-field microscopy were used with FITC (excitation, 490 nm) filter mode and white light in bright-field mode. Subsequently, individual mite DNA was extracted using the QIAGEN Blood & Tissue kit as described in (section 2.5.2.1).

A PCR assay was performed to amplify an approximately 710 bp fragment of the mitochondrial *coi* as described in section (2.5.2.2). Nuclease-free water was used as a negative control. PCR products were visualized on a 1.2% agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min and purified using a QIAquick PCR Purification Kit. Then the PCR product was sent for Sanger sequencing to Eurofins Genomics. Raw sequence data were aligned using BIOEDIT software tool, analysed by BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi, and sequence alignments were performed using MEGA v7 software to generate phylogenetic trees.

2.6 Other ectoparasites

2.6.1 Ticks.

For tick species barcoding, the mitochondrial *coi* (for *Rhipicephalus* spp. only) and 16S rRNA genes were used with primers and conditions shown in Table 2.2. The 16S PCR was done in 40 µl reactions comprising 20 µl BioMix Red (Bioline), 2 µl of each primer (10 µM) 16S FWD & 16S REV, and 5 µl of DNA template. The amplification of DNA was performed on a thermocycler (BIOMETRA T1 Thermoblock Thermal Cycler), with 10 cycles at 95°C for 3 min, 95°C for 1 min, 48°C for 1 min, and 72°C for 1.5 min; then 32 cycles at 95°C for 1 min, 54°C for 35 s, 72°C for 1.5 min, and a final extension cycle at 72°C for 7 min. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min.

Amplification of the *coi* gene was conducted using a pair of primers specifically designed to amplify a ~550 bp fragment from *Rhipicephalus* spp. mitochondria (Low & Prakash, 2018). PCR was performed in 40 µl reactions comprising 20 µl BioMix Red (Bioline), 1 µl of each

primer (10 µM stock; 0.4 µM final concentration) TICKRSCOI_F and TICKRSCOI_R, and 5 µl of DNA template. The thermocycler conditions included an initial denaturation at 94 °C for 3 min; 35 cycles of 94°C for 30s; 59°C for 30s; 72°C for 45 s; and a final elongation at 72°C for 10 min. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with specific forward and reverse primers to Eurofins Genomics, Germany (Table 2.2).

Aligned sequences were subjected to neighbour-joining (NJ) analysis using PAUP 4.0b10 (Swofford, 2002). The NJ bootstrap values were estimated using 1000 replicates with Kimura's two-parameter model of substitution (K2P distance). Genetic distance analysis was performed with MEGA X (Kumar *et al.,*2018) using the Kimura 2-parameter model with 1,000 replicates (Appendix 36, 37).

Table 2.2. Gene targets and primers used for tick species identification

Target gene	Primer sequence (5'-3')	Product length (bp)	Annealing temperature (°C)	Reference
16S rRNA	5'- CTGCTCAATGATTTTTTAAATTGCTGTGG -3' 5'-CCGGTCTGAACTCAGATCAAGT-3'	460 bp	48°C	(Black & Piesman, 1994)
Rhipicephalus COI	TICKRSCOI_F: 5'-GATTTTGGTTACTTCCTCCTTCTCT-3' TICKRSCOI_R: 5'-GCCTAATAATCCAATTGCTGCT-3'	550 bp	59°C	(Low & Prakash, 2018)
Generic *COI*	LCO1490: 5'GGTCAACAAATCATA AAGATATTGG3' HC02198: 5'TAAACTTCAGGGTGACCAAAAAATCA3'	710 bp	45°C	(Folmer *et al.,*1994)

2.6.2 Statistical analysis

The prevalence, mean intensity, mean abundance and range of ectoparasites on rodent hosts were estimated by using Quantitative Parasitology 3.0. Diversity estimators and indices of ectoparasite infestations on rodents, which includes species richness, Shannon's index (*H'*), Chao, and Simpson, were calculated by using the "BiodiversityR" package (Kindt & Coe, 2005) and applied in the R freeware programming environment (R Core Team 2019). Ectoparasite species accumulation curves were created for assessment of rodent sample size adequacy and to elucidate differences in ectoparsites species richness among different factors (habitat, study sites, host sex, and rodent species). Nonparametric Kruskal–Wallis test with Holm's correction: Wilcoxon test was conducted to study the impact of different factors on ectoparasites species richness.

In terms of analysing ecological networks to study host-ectoparasite interactions, bipartite network analyses were performed on individual hosts from different study sites using "vegan" (Oksanen *et al.,*2015) and "bipartite" packages (Dorman *et al.,*2009) implemented in R freeware. Bipartite networks were transformed to unipartite networks using the "tnet" package (Opsahl 2009). Unipartite network plots show the interaction patterns among hosts and ectoparasite species. An Eigen value of centrality was calculated by using the "evcent" function from the "igraph" package (Csardi & Nepusz, 2006).

Principal coordinates analysis (PCoA) was performed from the weighted UniFrac metric to visualise the bacterial communities among ectoparasite species applied in the R freeware environment (R Core Team 2019).

2.7 Soil samples

Soil samples were collected from three sites in the Asir region: Alogl, Wosanib and Alous during the fieldwork in 2017. One ml of surface soil samples were collected from each site in a 1.7 ml Eppendorf tube, which were stored at -20°C prior to DNA extraction. DNA from the soil samples was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratory

Inc.) following the manufacturer's protocol as follows. Five hundred mg of soil sample was placed in a Powerbead tube and 550 µl of buffer SL was added. The sample was then pulverized followed by centrifugation at 10,000 × g for 10 min. The supernatant was transferred to a 1.5 ml microcentrifuge tube, and 50 µl of buffer RH was added. Three hundred µl of buffer PD was added and mixed well, then the mixture was centrifuged at 10,000 × g for 5 min prior to transfer of the supernatant to a 2.0 ml microcentrifuge tube. Next, 900 µl of buffer TB was added to the samples. The mixture was transferred into a mini spin column and centrifuged at 10,000 × g for 30 sec. Five hundred µl of buffer NW was added to each sample and centrifuged at 10,000 × g for 30 sec. Fifty µl of nuclease-free water was used in the elution step and the sample was allowed to stand for 1 min, then centrifuged at 10,000 × g for 1 min. The DNA samples were stored at -20°C.

2.8 16S rRNA amplicon sequencing for microbiome profiling

2.8.1 Sample selection and preparation for 16S rRNA gene amplicon sequencing

Chigger species and other ectoparasites from Saudi Arabia were selected from different geographic sites, rodent species, and location on the host body. In addition, rodent skin swab and soil DNA extracts were included for analysis to provide insights into the source of the ectoparasite microbiomes. The ectoparasite selection was chosen to provide broad representation of all of the more common species across the chigger mites, gamasid mites, ticks, fleas and lice. Species found only on a single host were not included. Four Illumina sequencing runs were performed, comprising 96 samples per run.

After confirming the species identification for individual chigger mites using the autofluorescence method while they were in ATL buffer, 10 - 50 chigger specimens of the same species from a single host were pooled for DNA extraction using the DNeasy Blood &Tissue Kit (QIAGEN). The specimens were placed in 1.7 ml Eppendorf tubes and crushed with a sterile polypropylene pestle in ATL buffer and proteinase-K, then incubated at 56°C overnight in a heating block; the subsequent steps followed the manufacturer's protocol.

30 µl of nuclease-free water (Ambion) was used in the DNA elution step. Finally, all chiggers pools were concentrated to 10 µl using a 30K Nanosep centrifugal device (PALL). DNA concentrations were measured on a Qubit fluorimeter. Other ectoparasites (ticks, fleas, lice, and gamasid mites) were extracted individually.

2.8.2 Library preparation of 16S rRNA gene amplicon sequencing

2.8.2.1 Dual-index nested PCR amplification

Library preparation of dual-index nested PCR amplification of the 16S rRNA gene for Illumina MiSeq sequencing was performed (Illumina, 2013; and Caporaso *et al.,*2011). Briefly, the general principle of this library preparation approach is that two primers are used in the first round PCR to amplify the V3-V4 region of the 16S rRNA gene, which also include Illumina sequencing adaptors, in which the nucleotide "pads" do not match the 16S rRNA gene template. In the second round, PCR is conducted to attach the barcode dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. This contains eight forward index primers and 12 index primers, which generate 96 samples in different combinations of the tagging process. The dual index nested PCR primers used in the library preparation are presented in (Table 2.3).

The first round PCR was conducted in 25 µl reactions. The master mix contained 125 µl of Q5 High-Fidelity DNA Polymerase (NEBNext Q5 Hot Start HiFi PCR Master Mix, New England Biolabs), 695 µl of microbial DNA-free water(Ultra-Pure, DNA-Free Water), 62.5 µl of each primer [319F forward and reverse 806R (at 0.4 µM final concentration)] and 30 µl of bovine serum albumin; with 4 µl of DNA template per reaction. The PCR comprised initial denaturation at 98°C for 3 min, followed by 18 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 15 sec, extension at 72°C for 40 sec and final extension at 72°C for 5 min .The PCR products were purified using AMPure XP beads following the manufactuer's protocol to use as DNA template for the second round PCR. The second round was performed in a 25 µl reaction containing 12.5 µl of Q5; 2.5 µl of each primer [forward (S501-S508) and reverse (N701-N7012)] and 7.5 µl DNA template. The PCR comprised initial

denaturation at 98°C for 3 min, followed by 20 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 15 sec, extension at 72°C for 40 sec and final extension at 72°C for 5 min .The second-round PCR product was purified using AMPure XP beads following the manufacturer's protocol. The second-round PCR product was measured using the DNA Quant-iT dsDNA Assay kit, High Sensitivity, 1000 before applying the pooling strategy. The workflow diagram of 16S rRNA library preparation is shown in Figure 2.17.

Table 2.3. List of the dual index nested PCR primers used in the library preparation for 16S rRNA gene amplicon sequencing

Index name	Barcode indices	Nested PCR round	Pad/linker	Oligo sequence	Size (bp)
(319F) Forward primer	TGTATAAGAG ACAG	1	ACTCCTACGGGAGGCAGCAG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCCTACGGGAGGCA GCAG	53
(806R) Reverse primer	TAAGAGACAG	1	GGACTACHVGGGTWTCTAAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTW TCTAAT	54
i5 index_S501	TAGATCGC	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTTCCCT ACACGACG	58
i5 index_S502	CTCTCTAT	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTTCCCTA CACGACG	58
i5 index_S503	TATCCTCT	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTTCCCTA CACGACG	58
i5 index_S504	AGAGTAGA	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACAGAGTAGAACACTCTTTCCCT ACACGACG	58
i5 index_S505	GTAAGGAG	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTTCCCT ACACGACG	58
i5 index_S506	ACTGCATA	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACACTGCATAACACTCTTTCCCTA CACGACG	58
i5 index_S507	AAGGAGTA	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACAAGGAGTAACACTCTTTCCCT ACACGACG	58
i5 index_S508	CTAAGCCT	2	ACACTCTTTCCCTACACGACG	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTACACTCTTTCCCTA CACGACG	58
i7 index_N701	TCGCCTTA	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N702	CTAGTACG	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N703	TTCTGCCT	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N704	GCTCAGGA	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66

i7 index_N705	AGGAGTCC	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N706	CATGCCTA	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N707	GTAGAGAG	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTGACTGGAGTTCAGA CGTGTGCTCTTCCGATCT	66
i7 index_N708	CCTCTCTG	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N709	AGCGTAGC	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N710	CAGCCTCG	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N711	TGCCTCTT	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66
i7 index_N712	TCCTCTAC	2	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	66

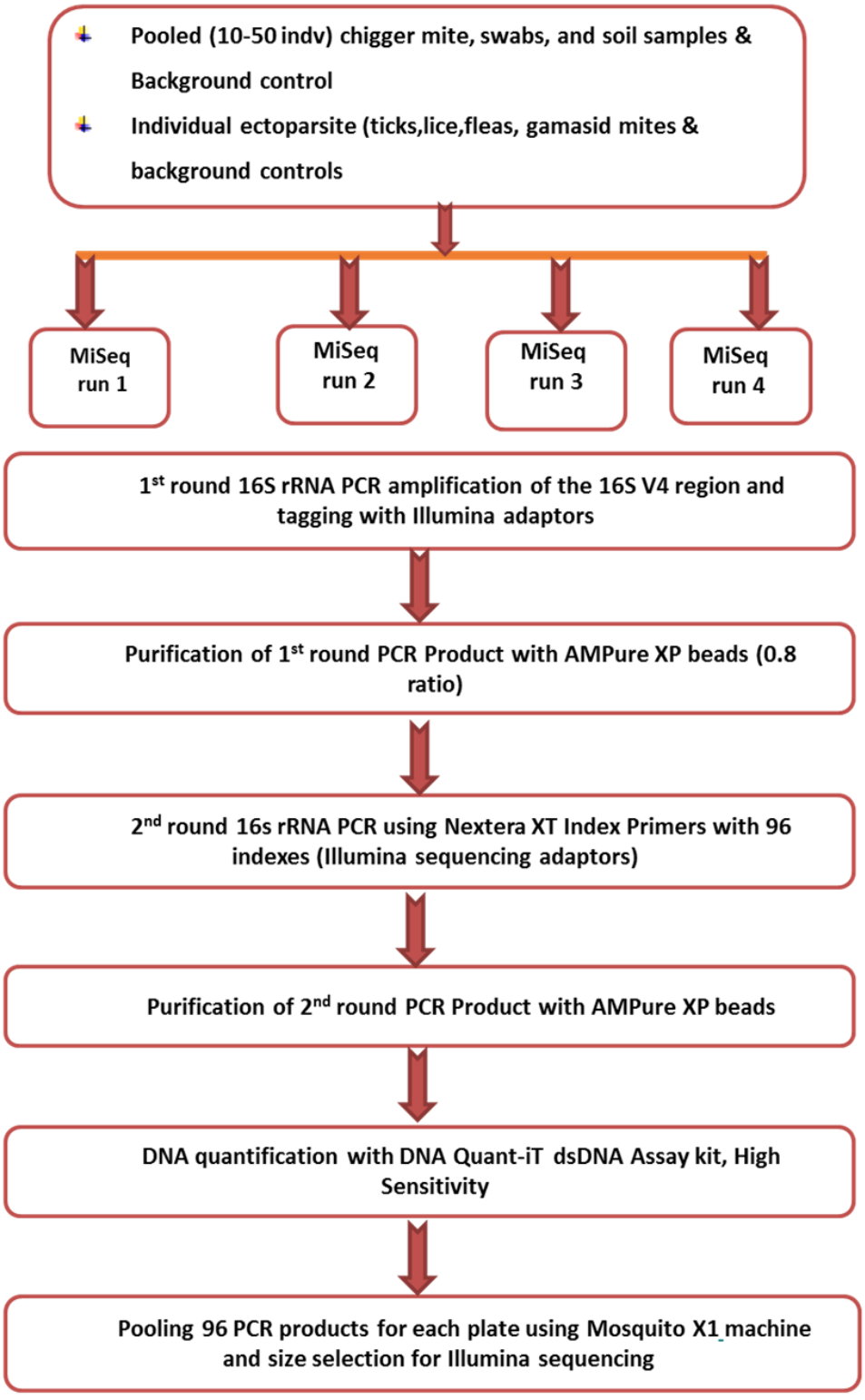


Figure 2.17. The schematic diagram indicates 16S rRNA library preparation of the four runs of Illumina MiSeq/HiSeq sequencing.

2.8.2.2 Pooling strategy for 16S rRNA nested PCR products

Before starting the pooling process using the Mosquito X1 instrument (Nanolitre liquid handling), a spreadsheet was prepared for all DNA volumes of each sample and converted from µl to nl to obtain the final total of demultiplexing samples (around 60 nl). All volumes of DNA were adjusted to be between the range of 25 – 1,200 nL. In the GeneMill at the Centre for Genomic Research (CGR, University of Liverpool), 96-well plates were prepared for the Mosquito X1 robot. The first plate was empty (10 µl of microbial DNA-free water in position A1), and the second plate contained 96 samples of the final purified PCR products. The Mosquito X1 instrument was used to automatically pipette into individual wells in the plate. This process takes 30 min to pool 96 samples in the first well plate in position A1, with a final volume of 70 µl. This volume was transferred to a new Eppendorf tube, and quantified using the Qubit® dsDNA HS Assay Kit.

Finally, 40 µl was send to CGR at University of Liverpool for library preparation and sequencing: https://www.beckmancoulter.com/wsrportal/techdocs?docname=B37419

2.8.3 Post-sequencing bioinformatics

2.8.3.1 Quality filtering of raw paired-end sequence data

The 16S rRNA sequence reads were generated on a MiSeq or HiSeq 2500 sequencer with long read size of the target DNA fragment due to the attachment of the sequencing adaptors and barcode indices on the 3' or 5' end. Therefore, adaptors and primers must be removed from each read prior to downstream analysis. Initially, trimming, quality filtering and de-multiplexing of raw 16S rRNA reads (fastq files) was performed by CGR by using CUTADAPT v.1.2.1 (Martin 2011) and SICKLE v.1.200 (Joshi & Fass 2011), discarding read lengths shorter than 20 or 10 bp. This provided forward (R1) and reverse (R2) reads of the certain read pairs, while the singlet reads (R0) was excluded from the additional steps. Subsequently, SPAdes v.3.1.0 (Bankevich *et al*., 2012) was run with the Bayes Hammer algorithm error correction (Nikolenko *et al*., 2013) of the reads, and all reads were converted to fasta file format. Then, the paired-reads (R1 and R2) were aligned using PEARS, retaining a read size between 270 – 300 bp. Finally, the aligned reads from the two

successful runs on the MiSeq/HiSeq were combined in a single fasta file. This file comprising the whole dataset (millions of reads from 183 samples) was used for further microbiome analyses.

2.8.3.2 Microbiome profiling

To analyses the 16S rRNA microbiome profile, Quantitative Insights into Microbial Ecology (QIIME) software package, QIIME 2 (https://qiime2.org/) (Bolyen *et al.,*2019) was performed to obtain bacterial classification. Reads were clustered into Operational Taxonomic Units (OTUs) units using the open-reference OTU picking approach with taxonomic assignments by the USEARCH61 method (Edgar, 2010). All reads were binned at 98% similarity against the SILVA 16S database release 132 (Quast *et al.,*2013). The post-sequencing bioinformatics workflow is presented in (Figure 2.18).

All OTU read counts were transformed into rows and a sample in columns to percentage using the data filtering option to filter all OTU read counts at <10% (relative abundance) in the samples, and combined into 'Other bacteria OTUs'. Bacterial profiling differed between the individual ectoparasites (fleas, ticks, lice and gamasid mites) and chigger species were classified according to the relative abundance of dominant bacteria OTUs.

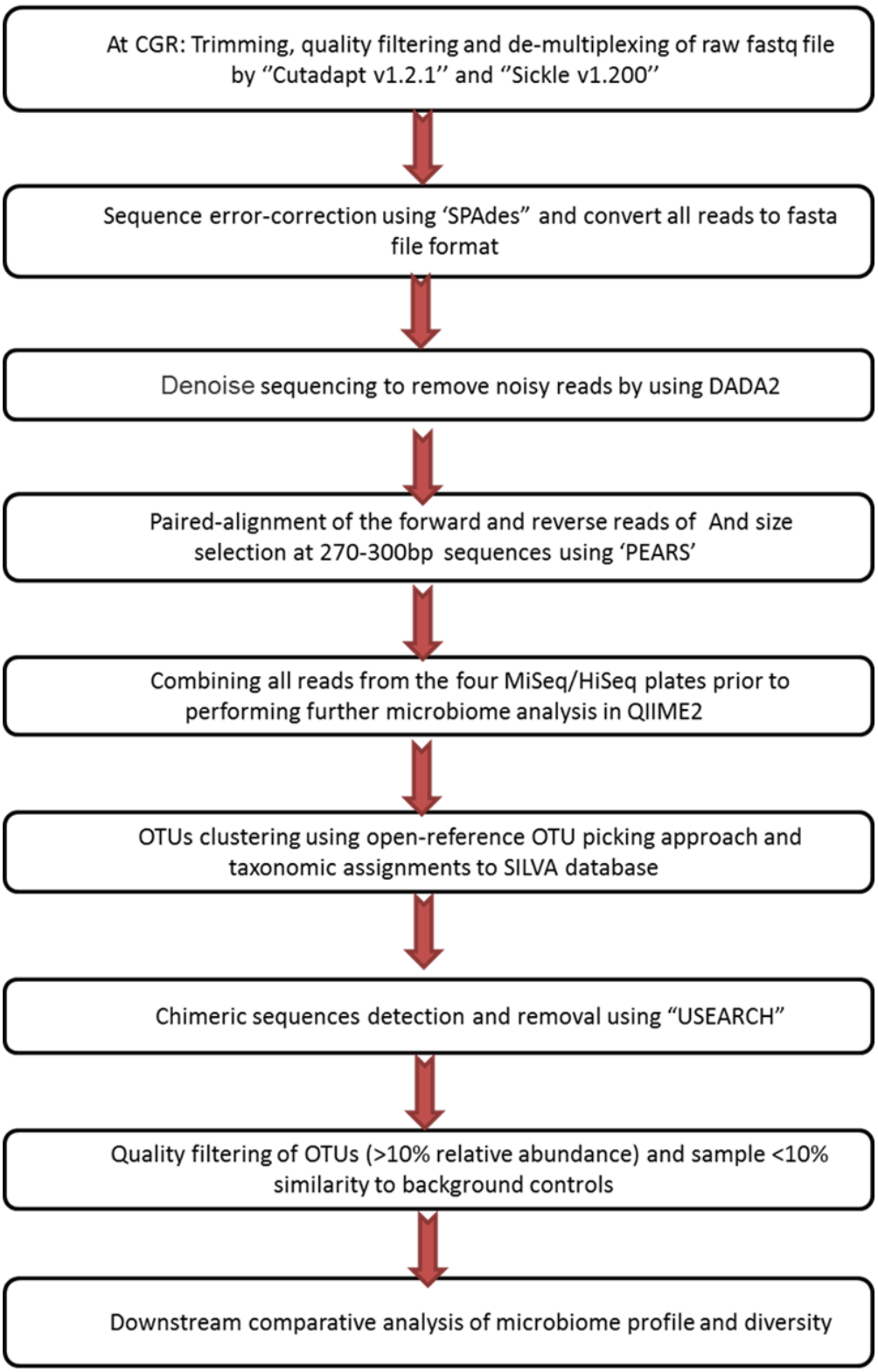


Figure 2.18. Schematic diagram indicates bioinformatics workflow of microbiome profiling using QIIME2 platform.

2.9 Specific PCRs for confirmation of bacterial identity

2.9.1 *Bartonella* spp.

A nested PCR amplification was performed for *Bartonella* spp ,targeting the 16S–23S rRNA intergenic spacer region (ISR) (Telfer *et al.,*2005). Reactions proceeded in a final volume of 25 µl containing 12.5 µl of 2X BioMix Red (BIOTAQ DNA polymerase Bioline, UK), 1 µl of each primer (final concentration, 0.2 µM) as listed in (Appendix 33), and 4 µl DNA template. The PCR amplifications were conducted on a thermal cycler (T3 thermocycler (Biometra). The positive control DNA from *Bartonella bacilliformis* was provided by Prof. Richard Birtles (University of Salford, UK) and nuclease-free water was used as a negative control. The amplification reaction consisted of 3 min at 96°C, then 40 cycles of 96°C C for 10 min, 55°C for 10 min, and 72°C for 50 sec, with a final elongation step of 72°C for 5 min. A semi-nested second-round reaction was performed in which primer big-R was replaced with primer bog-R. The second-round reaction was performed according to the thermal cycle described above with 4 µl of first-round product used in the second-round reaction. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min in a Bio-Rad gel electrophoresis set. The gels were visualised using a G:Box Gel Documentation System (Syngene). A weak PCR amplicon was obtained from female fleas (*Parapulex chephrensis*) and therefore cloning was necessary (section 2.10).

2.9.2 *Rickettsia* spp.

A conventional PCR targeting the *gltA* gene was used to differentiate *Rickettsia* species by sequence analysis. PCR assays were performed in a total reaction volume of 25 µL comprising 12.5 µl of BioMix Red, 4 µl of DNA template, 1 µl of each primer (10 pmol of each primer) (Roux *et al.,*1997) (Appendix 33). Nuclease-free water was used as a negative control. Amplification was carried out using the following conditions: a 3 min denaturation step at 95°C was followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 45°C for 30 sec, and extension at 65°C for 55 sec. Amplification was done by incubation for 7 min

at 72°C to allow complete extension of the PCR products. To confirm the results of amplification, 15 µl of the PCR products were resolved using a 1.5% molecular agarose gel (Bioline) incorporating SYBR® Safe DNA gel stain (Invitrogen) and run at 150 V for 30 min in a Bio-Rad gel electrophoresis set. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with RpCS.409d/RpCS.1258n forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligned using BIOEDIT software and then analysed by BLAST from NCBI.

A conventional PCR targeting a 364-bp fragment of 16S rRNA was used for detection of *Rickettsia* spp. The PCR assay was performed in a total reaction volume of 25 µL comprising 12.5 µl of BioMix Red3 µl of DNA template and 1 µL of each primer (100 pm) *Rickettsia*/16S rRNA as shown in (Appendix 33) (Alberdi *et al.,*2012; Nijhof *et al.,*2007). Amplification was carried out using the following conditions: an initial 3-min denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 70°C for 1 min 30 sec. The amplification was completed by holding the reaction mixture for 7 min at 70°C to allow complete extension. To confirm the results of amplification, 5 µl of the PCR products were resolved using a 1.5% molecular agarose gel (Bioline) incorporating SYBR® Safe DNA gel stain (Invitrogen) and run at 150 V for 30 min in a Bio-Rad gel electrophoresis set. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with *Rickettsia*/16S rRNA forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.9.3 *Coxiella* spp.

A nested PCR amplification was performed for *Coxiella*-like endosymbionts targeting 539 - 542 pb (*Coxiella*-like *rpoB*, Duron *et al.,*2015) in a final volume of 30 µl by using a reaction mixture containing 15 µl of 2X BioMix^{TM} Red (BIOTAQ DNA polymerase, Bioline, UK), 1.2 µl (0.4 µM) each primer as listed in (Appendix 33), and 4 µl of DNA template. The positive control DNA was provided by Dr Lesley Bell-Sakyi (Department of Infection Biology, University of Liverpool, UK) and nuclease-free water was used as a negative control. The PCR amplifications were run on T3 thermal cycler (Biometra). The amplification reaction

consisted of 3-min denaturation at 93°C, followed by 35 cycles of 93°C for 20 sec, annealing at 56°C for 30 sec, and 72°C for 90 sec, with a final elongation step of 72°C for 5 min. A second-round nested reaction was performed with different primers; a final volume of 30 µl with amplification conditions as described above, and 4 µl of first-round product was used in the second-round reaction. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min.

The PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with CoxrpoBF3/CoxrpoBR3 forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.9.4 *Francisella* spp.

Conventional PCR was performed for *Francisella-like* endosymbionts in ticks targeting a 408-bp fragment of a lipoprotein gene (Alberdi *et al.,*2012), using 2X BioMix Red [BIOTAQ DNA polymerase, (Bioline)], 1.2 µl each primer [final concentration, 0.4 µM (Appendix 33)] and extracted DNA (4 µl) to a total reaction volume of 30 µl. The positive control DNA was provided by Dr Lesley Bell-Sakyi (Department of Infection Biology, University of Liverpool, UK) and nuclease-free water was used as a negative control. Reaction mixtures were amplified on a thermal cycler (T3 thermocycler (Biometra) with PCR conditions as follows: activation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec; with a final extension of 72°C for 7 min. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with Francilipo forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.9.5 *Ehrlichia* spp.

Ehrlichia spp. were detected by nested PCR using primers targeting the *groESL* fragment of 1,350 bp (Liz, *et al*., 2002). In the first round, PCR reactions comprised of 15 µl BioMix Red and 1.2 µl each primer [final concentration 0.4 µM) (Appendix 33)] in a final volume of 30 µl. The positive control DNA was provided by Dr Lesley Bell-Sakyi (Department of Infection Biology, University of Liverpool, UK) and nuclease-free water was used as a negative control. PCRs were performed in a thermal cycler (T3 thermocycler, Biometra). Cycling conditions include three preliminary cycles, each consisting of 1 min of denaturation at 94°C, 2 min of annealing at 48°C, and 1.5 min of extension at 68°C, followed by 37 amplification cycles, each consisting of 1 min of denaturation at 88°C, 2 min of annealing at 48°C, and a 1.5 min extension at 68°C. These cycles were followed by an additional extension period of 5 min at 68°C. In the second round amplifications, 4 µl of the primary PCR product was used as a DNA template in a total volume of 30 µl comprising 15 BioMix Red and 0.4 µM of each primer (1.2 µl) used in the second round. Nested cycling conditions were 1 min of denaturation at 94°C, 2 min of annealing at 55°C, and 1.5 min of extension at 72°C, followed by 37 amplification cycles, each consisting of 1 min of denaturation at 88°C, 2 min of annealing at 55°C, and 1.5 min of extension at 72°C. These cycles are followed by an additional extension period of 5 min at 68°C. All amplified products were subsequently maintained at 4°C until they were analyzed by agarose gel electrophoresis. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min in a Bio-Rad gel electrophoresis set.

The PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with HS 43/HSVR forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.9.6 *Orientia* spp.

Amplifications of the TSA47 and TSA56 genes were used for detection of *Orientia* spp. For TSA47, a nested PCR was peformed targeting a 871 bp fragment (Masakhwe *et al.,*2018).

In the first round, reactions contained 10 µl 2X BioMix Red BIOTAQ DNA polymerase (Bioline, UK), 1 µL (final concentration 0.3 µM each primer Ot-145 F and Ot-1780 (Appendix 33)], and 2 µl purified DNA as the template in a total volume of 20 µl. Nuclease-free water was used as a negative control. The positive control DNA (*O. tsutsugamushi* strain UT176 cultured in mammalian cells) was provided by Dr Ivo Elliott (Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit). Cycling conditions involved an initial 2 min denaturation at 95°C, followed by 40 cycles, each comprising a 30 sec denaturation at 94°C, a 30 sec annealing at 54°C, and a 2 min sec extension at 68°C. These 40 cycles were followed by a 7 min extension at 72°C. PCR products maintained at 4°C until they used as templates for the second round of nested PCR reactions. In the second round, PCR amplification conditions and reactions were used same to the first round but used nested primers Ot-263F and Ot-1133R. For the TSA56 gene target, a nested PCR was performed in 25 µL targeting a 620 bp fragment of *Orientia* spp. In the first round, reactions contained of 2X BioMix Red BIOTAQ DNA polymerase (Bioline, UK), 1 µL [final concentration 0.3 M µM of each primer RTS-8 and RTS-9 (Appendix 33)], and 2 µl of purified DNA used as the template in a total volume of 25 µl. Cycling conditions involved an initial 2 min denaturation at 95°C, followed by 30 cycles, each comprising a 1 min denaturation at 95°C, a 1.5 min annealing at 55°C, and a 2 min extension at 72°C. These 30 cycles were followed by a 7 min extension at 72°C. PCR products maintained at 4°C to used as templates for the second round of nested PCR reactions. In the second round, PCR amplification conditions and reactions were similar to the first round but used nested primers RTS-6 and RTS-7. Amplicons were analysed by agarose gel electrophoresis using a 1.5% agarose gel as described above for confirmatory *Bartonella* PCRs, then the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with Ot-263F and Ot-1133R forward and reverse primers and RTS-6 and RTS-7 primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.9.7 *Wolbachia* spp.

Wolbachia spp. were detected using primers targeting the *wsp* gene (Appendix 33). PCR assays were performed with a 40 µL reaction mixture containing 20 µL 2X BioMix Red

BIOTAQ DNA polymerase (Bioline, UK), 1 µl each primer (final concentration, 0.4 µM), and extracted DNA (2 µl). Reaction mixtures were subjected to a thermal cycle of 94°C (activation) for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C annealing for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with wsp 81F/wsp 691R forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from the National Centre for Biotechnology Information (NCBI).

2.9.8 *Anaplasma* spp.

Anaplasma spp. were amplified by nested PCR using primers targeting the *Anaplasma* 16S rRNA (Massung *et al.,*1998). In the first round, reactions comprised of BioMix Red, 0.5 µM each primer [ge3a and ge10r, (Appendix 33)], and 2.5 µl of purified DNA used as the template in a total volume of 25 µl. Nuclease-free water was used as a negative control. Cycling conditions involved an initial 2 min denaturation at 95°C, followed by 40 cycles, each comprising a 30 sec denaturation at 94°C, a 30 sec annealing at 55°C, and a 1 min extension at 72°C. These 40 cycles were followed by a 5-min extension at 72°C. PCR products maintained at 4°C until they were analysed by agarose gel electrophoresis and used as templates for the second round of nested PCR reactions. The final reaction comprised of 1 µl of the primary PCR product used as a template, BioMix Red, 0.2 µM of each primer (ge9Fand ge2R), and nuclease free water. Nested cycling conditions were as described for the primary amplification, using 30 cycles instead of 40 cycles (Massung *et al.,*1998). PCR products then maintained at 4°C until they were analysed by agarose gel electrophoresis using a 1.5% agarose gel as described above for confirmatory *Anaplasma* PCRs. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with ge9F/ge2R forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.9.9 *Spiroplasma* spp.

Touchdown PCR was performed in 30 µL reactions targeting a 1443-bp *rpoB* fragment of *Spiroplasma* (Haselkorn *et al.,*2009). Reactions contained 2X BioMix Red BIOTAQ DNA polymerase (Bioline, UK), 1.2 µl (final concentration 0.4 µM) of each primer, and 2 µl of DNA template (Appendix 33). The positive control DNA was provided by Dr Ana Maria Palomar (Center for Biomedical Research, La Rioja, Spain) and nuclease-free water was used as a negative control. The PCR profile consisted of an initial denaturation for 3 min at 94˚C, followed by 8 cycles of 30 sec at 94˚C, 45 sec at 63˚C, and 45 sec at 72°C, with a reduction in annealing temperature of 1˚C/cycle (63-56˚C). This step was followed by 25 cycles of 30 sec at 94˚C, 45 sec at 53˚C, and 45 sec at 72˚C, with a final extension at 72˚C for 7 min. The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with rpoB forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

2.10 Cloning of genus-specific PCR products using the pGEM®-T Easy Vector System (Promega)

The PCR products were visualized by 1.5 % agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min. A weak PCR amplicon was obtained for some samples, which was excised from the gel and purified using a PureLink® Quick Gel Extraction Kit (Invitrogen, California, USA) according to the manufacturer's instructions, then cloned in the pGEM®-T Easy Vector System (Promega). PCR fragments were ligated overnight at 4°C into the pGEM-T Easy plasmid with a 2:1 insert-plasmid ratio. The ligants were transformed into JM109 *E. coli* competent cells (Promega). Plasmid DNA was extracted from the transformed cell pellets using the Wizard Plus SV Minipreps DNA Purification Kit (Promega), following the manufacturer's protocol. To validate that the

plasmid was cloned successfully, the plasmid was cut by restriction enzyme (*Eco*R1, 20U/ µl) (Thermofisher) at 37°C with a 5-min incubation. Finally, the plasmid DNA samples were excised from the gel and purified using a PureLink® Quick Gel Extraction Kit (Invitrogen, California, USA) according to the manufacturer's instructions and sent for Sanger sequencing with forward primers and reverse primers pUC/M13 to Source Bioscience Ltd, UK. Sequences were identified using the BLASTn online platform (https://blast.ncbi.nlm.nih.gov), which was used to align and compare DNA sequences to the nucleotide collection database.

2.11 16S rRNA profiling of mites using a portable USB sequencer (MinION)

2.11.1 Preparation of *Ornithonyssus bacoti* pools exposed to antibiotics

The tropical rat mite *Ornithonyssus bacoti* is the vector of *Litomosoides sigmodontis*, a popular laboratory model for a helminth infection, filariasis (Hoffmann *et al.,*2000). To determine if the microbiome of *O. bacoti* could be manipulated for subsequent vector competence studies, mites from a laboratory colony at Muséum National d'Histoire Naturelle (MNHN, Paris) were fed on antibiotic-treated jirds (*Meriones unguiculatus*).The protocol followed by staff at MNHN is shown in Table (2.4). Mites (60 per animal) were applied to the jird hosts 3 hr after antibiotic treatment and allowed to feed for 12 hr. This approach was used to test the antibiotic efficacy in depleting the microbiome of *O. bacoti* in comparison to a control group. DNA was extracted from pools of mites (standardised to 30 per treatment group) using the DNeasy Blood & Tissue Kit (QIAGEN) as described in section (2.5.2.1) and subjected to PCR amplification of the 16S rRNA gene prior to sequencing on the Oxford Nanopore Technologies MinION platform.

All animal procedures were carried out in accordance with the EU Directive 2010/63/ UE and the relevant national legislation, namely the French "Decret No. 2013±118, 1er fevrier 2013, Ministère de l'Agriculture, de l'Agroalimentaire et de la Foret". Protocols were approved by the ethical committee of the Museum National d'Histoire Naturelle (Comité

Cuvier, Licence: 68–002) and by the Direction Départementale de la Cohésion Sociale et de la Protection des Populations (DDCSPP) (No. C75-05-15).

Table 2.4. Schedule for antibiotic treatment of jirds prior to the feeding of *O. bacoti* mites

Jird no.	Antibiotic treatment	Route	Schedule	Dose by bodyweight (mg/kg)	Approx. dose per jird (mg)	Delivery time (h)		No. recovered mites
						T-6	T0	
1	Penicillin/streptomycin	SC	Sid	25, 50	1, 2		X	51
2	Gentamycin	SC	Sid	5	0.2		X	50
3	Kanamycin	SC	Bid	5	0.2	X	X	48
4	Penicillin/streptomycin/gentamycin	SC	Sid	25, 50, 5	1, 2, 0.2		X	45
5	Penicillin/streptomycin/kanamycin	SC	Sid/Bid	25, 50, 5	1, 2, 0.2	X	X	36
6	Penicillin/streptomycin/gentamicin/kanamycin	SC	Sid/Bid	25, 50, 5, 5	1, 2, 0.2, 0.2	X	X	47
7	None (vehicle)	SC		-				48

2.11.2 PCR amplification of the 16S rRNA gene, barcoding and pooling

To prepare the DNA library for microbiome profiling, a 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) was used (Kai *et al.,*2019; Leggett & Clark, 2017; Mitsuhashi *et al.,*2017). PCR was carried out in 50 µl reactions containing 14 µl nuclease-free water, 10 µl input DNA (10 ng), 1 µl 16S Barcode, and 25 µl LongAmp Taq 2X master mix (NEB). Amplification was performed using an Applied Biosystems Thermocycler (Thermo Fischer Scientific) with the following PCR conditions: initial denaturation at 95°C for 1 min, then 25 cycles of 95°C for 20 sec, 55°C for 30 sec, and 65°C for 2 min, followed by a final extension at 65°C for 5 min. The following primers were used: [forward] 27F 5-AGAGTTTGATCMTGGCTCAG-3, and [reverse] 1492R 5'-GGTTACCTTGTTACGACTT-3'. There are 12 unique barcodes, allowing the pooling of up to 12 different samples in one sequencing experiment.

The sample was transferred to a 1.5 ml DNA Lo Bind Eppendorf tube. The AMPure XP beads was prepared for use by resuspension using vortex-mixing. Thirty µl of re-suspended AMPure XP beads were added to the reaction and mixed by pipetting, then incubated for 5 min at RT on a rotator-mixer. Next, the sample was spun down and pelleted on a magnetic stand before the supernatant was removed. Beads were washed with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. The 70% ethanol was removed using a pipette and discarded; this step was repeated twice by spinning down the beads and placing the tube back on the magnetic stand before removing any residual ethanol. Then, the beads were dried for ~30 seconds. The tube was removed from the magnetic rack and the pellet resuspended in 10 µl of 10 mM Tris-HCl, pH 8.0, with 50 mM sodium chloride prior to incubation for 2 min at RT. The tube was placed back onto the magnetic rack until the eluate become clear and colourless. Ten µl of eluate was removed and retained in a clean 1.5 ml Eppendorf DNA LoBind tube, and 1 µl of eluted sample was quantified using a Qubit fluorometer. All barcoded libraries were pooled in the desired ratios to a total of 50-100 fmoles in 10 µl of 10 mM Tris-HCl, pH 8.0, with 50 mM sodium chloride. One µl of RAP was added to the barcoded DNA and then the solution was mixed gently by flicking the tube and spun down. The reaction was incubated for 5 min at RT.

2.11.3 Preparing and loading the SpotON flow cell

The library was stored on ice until ready to load. The Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) were thawed at RT. The SQB and FLB, were mixed by vortexing, while the FLT was mixed by pipetting; then all tubes were spun down and returned to ice. After opening the priming port of the flow cell, any small bubbles were removed from the back buffer covering the pore array using a micropipette. The flow cell priming mix was prepared by adding 30 µl FLT directly to the tube of FLB, and mixed by pipetting. Then 800 µl of the priming mix was loaded into the flow cell through the priming port, avoiding the introduction of air bubbles, and incubated at RT for 5 min. The contents of the SQB and LB tubes were mixed by pipetting. The library was prepared in a new tube as follows: 34 µl SQB; 25.5 µl LB (mixed immediately before use), 4.5 µl nuclease-free water, and 11 µl DNA library. Two hundred µl of the priming mix was loaded into the flow cell via the priming port. The prepared library was mixed again gently by pipetting and 75 µl was added to the flow cell via the SpotON sample port in a dropwise fashion. Finally, the sequencing run was initiated using the MinKNOW interface on the computer connected to the MinION sequencer.

2.11.4 Bioinformatics analysis

Raw reads in fast5 format were base-called with Albacore Sequencing Pipeline Software (version 2.2.7). The resulting fastq files were converted into fasta files using *seqtk* software (https://github.com/lh3/seqtk). Downstream analysis was performed at the MG-RAST server (Meyer *et al.,*2008).

2.11.5 Identification of cultivable bacteria from a laboratory colony of *Ornithonyssus bacoti*

2.11.5.1 *Ornithonyssus bacoti* sample preparation

From the laboratory colony, four groups of the tropical rat mite (*O. bacoti*) were prepared under different conditions (*n* = 50 per group; duplicate agar plates per homogenate). In condition A, uninfected mites engorged with blood were crushed with a pestle in 250 µl of PBS. One hundred µl of homogenate was inoculated onto LB agar without vitamins.

Condition B was identical to A, except vitamins B1 (thiamine), B8 (biotin) and B12 (cobalamin) were added to the agar. In condition C, uninfected mites were starved for five days, and the homogenate was inoculated onto LB agar supplemented with vitamins. Condition D resembled condition C, but the starved mites were infected with L3 (infective larvae) of *L. sigmodontis*. All agar plates were incubated at 37°C for two days. Next, single colonies were picked from each plate using a sterile loop and placed in 100 µl of 70% ethanol.

2.11.5.2 DNA extraction and quantification

DNA was extracted from fixed bacterial colonies using the DNeasy® PowerLyzer® Microbial Kit (QIAGEN). 1.8 ml of microbial bacteria was added to a 2 ml Collection tube and centrifuged at 10,000 x g for 30 s at room temperature. The supernatant was transferred and spun at 10,000 x g for 30 s at room temperature and supernatant was then completely removed with a pipette tip. The pellet was re-suspended in 300 µl of PowerBead Solution and gently vortexed to mix, then the resuspended cells were transferred to a PowerBead tube containing 0.1 mm glass beads. Fifty µl of Solution SL was added to the PowerBead tube and then processed with a homogenizer for 5 min at 2,000 rpm before centrifugation at 10,000 × g for 30 sec at RT.

The supernatant was transferred to a clean 2 ml collection tube and 100 µl of Solution IRS was added with vortex-mixing for 5 sec. Samples were incubated at 4°C for 5 min, then centrifuged at 10,000 × g for 1 min at RT. Afterward, the supernatant was completely transferred to a 2 ml collection tube and 900 µl of Solution SB was added before vortex-mixing for 5 sec. Seven hundred µl of the supernatant was loaded into a MB Spin Column and centrifuged at 10,000 × g for 30 s at room temperature. The flow-through was discarded and then the remaining supernatant was added to the MB Spin Column and centrifuged at 10,000 × g for 30 sec at RT. Three hundred µl of Solution CB was added prior to centrifugation at 10,000 × g for 30 sec at RT. Then flow-through was discarded and centrifuged at 10,000 x g for 1 min at room temperature. The MB Spin Column was placed in a new 2 ml collection tube and 50 µl of Solution EB was added to the centre of the white filter membrane before centrifugation at 10,000 × g for 30 sec at RT. Bacterial DNA samples were stored at 20°C. The yield of DNA was calculated using a Qubit 3 Fluorometer with the Qubit dsDNA HS Assay kit following the manufacturer's manual.

2.11.5.3 PCR amplification of 16S rDNA gene

In order to amplify bacterial 16S rDNA, PCR assays were performed with a 40 µL reaction mixture containing 20 µL 2X BioMix Red BIOTAQ DNA polymerase (Bioline, UK), 0.2 µl each primer (final concentration, 0.4 µM) as listed in Appendix 33, and 5 µl of extracted DNA. Reaction mixtures were subjected to a thermal cycle of activation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 60°C annealing for 1 min, and 72°C for 1 min, with a final extension of 72°C for 7 min. The PCR products were visualized by 1% agarose gel electrophoresis containing SYBR Safe dye (Invitrogen) at 150 V for 30 min. Finally, the PCR products were purified using a QIA-quick PCR Purification Kit and sent for sequencing with fD1F/rP2R forward and reverse primers to Eurofins Genomics, Germany. Raw sequence data was cleaned and aligning using BIOEDIT software and then analysed by BLAST from NCBI.

Additionally, to amplify sequences from colonies with fungal morphology, PCR assays were performed with in a 20 µL reaction mixture containing 12.5 µL 2X BioMix Red BIOTAQ DNA polymerase (Bioline, UK), 1 µl each primer(Appendix 33) (final concentration, 0.4 µM), and 3 µl of extracted DNA. Reaction mixtures were subjected to a thermal cycle of activation at 95°C for 3 min, followed by 35 cycles at 95°C for 20 sec, 62°C annealing for 45 sec, and 72°C for 90 min, with a final extension of 72°C for 7 min. Gel electrophoresis, purification of PCR products, sequencing and analysis proceeded as for bacterial colonies above.

Chapter 3. Identification, Diversity and Ecology of Rodents and their Ectoparasites in the Asir region of the Kingdom of Saudi Arabia

3.1 Introduction

The Asir region (عَسِيْر) is located in the southwest of Saudi Arabia and has an area of 76,693 km^2. The region has a stretch of coastline along the Red Sea and a short border with neighboring Yemen. It is characterized by the presence of high mountains, relatively lush vegetation, wadies, and heavy rainfall of up to ~20 inches (500 mm), which is higher than that of any other district in the country. The highest point is Jebel Sawdah peak near the town of Abha, which reaches almost 3,000 m above sea level (Masood, 2012; Miyazaki *et al.,*2007). Much of the terrain and climate are suitable for agricultural activities and are also favorable as rodent habitat. As rodents are the principal carrier of many important vector-borne zoonotic diseases (Mathison & Pritt, 2014) (see Literature Review), humans are likely to be exposed to pathogens from interactions with rodents and from arthropod vectors feeding on these hosts.

In Saudi Arabia, several zoonotic bacterial diseases associated with rodents (or ectoparasites that may feed on rodents) have been reported such as plague, relapsing fever (Al-Gwaiz *et al.,*1994) and Q-fever (Almogren *et al.,*2013). Historically, plague was commonly present during hajj season when the influx of human immigration into the country is very high, although modern public health policies have prevented this (Low, 2008; Niu & Xu, 2019). One particular disease of interest is scrub typhus, caused by *Orientia* spp. and transmitted by chigger mites to humans. *Leptotrombidium* larvae (Trombiculidae) are the primary vector of this bacterium. Although the disease is yet to be reported in Saudi Arabia, a neighbouring country (the United Arab Emirates) has previously reported an autochthonous clinical case, prompting an investigation to be carried out (Izzard *et al.,*2010). To date, no studies on the chigger fauna of Saudi Arabia have been performed. However, given the high abundance of rodent species seen in the country by Al-Mohammed (2008), there is the possibility of a considerable diversity of ectoparasites associated with these hosts, particularly in the Asir region, including chigger mites. Therefore, the present study aimed to: 1) to identify ectoparasites parasitizing wild rodents in Saudi Arabia, with a special focus on chiggers in the Asir region, 2) to investigate the specific interactions between ectoparasites and their rodent hosts, and 3) to evaluate environmental factors driving the diversity of rodents and ectoparasites within particular ecological niches.

3.2 Results

3.2.1 Rodents identified in the Asir region

In total, 74 rodents were captured in Asir region comprising five rodents species: *A. dimidiatus, Meriones rex, Mus musculus, Myomyscus yemeni,* and *Rattus rattus*. The anatomical identification and molecular barcoding agreed in all rodents species. Moreover, the *cytB* barcode sequence was identical within each species of rodent. The data were deposited in the Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org), project code SSS (Table 3.1). Table 3.2 indicates to the number of rodents species found per village in Asir region. The total number of ectoparasites collected from each rodent species from three sites in the Asir region is presented in Table 3.3. The host species with the greatest mean infestation rate (60.81%) was the Eastern spiny mouse (*A. dimidiatus*).

Table 3.1. Representative barcode data for rodent species identified from the Asir Region of Saudi Arabia.

Rodent number	Species	Common name	Product size (bp)	Query Cover (%)	E –Value	Identity (%)	Accession[a]
45	*Acomys dimidiatus*	Eastern spiny mouse	290	100	1e-148	100	KF422691.1
17	*Meriones rex*	King jird	290	100	5e-148	100	AJ851265.1
3	*Mus musculus*	House mouse	280	100	5e-143	100	LC147005.1
8	*Myomyscus yemeni*	Yemeni mouse	290	100	1e-148	100	AF518357.1
1	*Rattus rattus*	Black rat	289	100	2e-146	99	KF282339.1

[a] From the NCBI nucleotide database

Table 3.2. The number of rodents species found per village in Asir region

Village(s)	Rodents species (n)				
	Acomys dimidiatus	***Meriones rex***	***Mus musculus***	***Myomyscus yemeni***	***Rattus rattus***
Alous	33	-		-	1
Wosanib	10	5		2	
Alogl	2	13	3	6	

Table 3.3. Distribution of ectoparasites in the Asir Region by village, year and rodent host.

	Village			
	Alous		Wosanib	Alogl
	Year of collection			
	2016	2017	2017	2017
Rodent species (*n*)	**Total number of ectoparasites (on female/male hosts)**			
***Acomys dimidiatus* (45)**	3,651 (2,349/1,302)	1,917 (270/1,647)	1,002 (316/686)	120 (0/120)
***Meriones rex* (17)**	-	-	367 (21/346)	870 (401/469)
***Mus musculus* (3)**	-	-	-	24 (0/24)
***Myomyscus yemeni* (8)**	-	-	34 (0/34)	199 (17/182)
***Rattus rattus* (1)**	-	86 (86/0)		

3.2.2 Ectoparasites identification in Asir region

3.2.2.1 Chigger species identification in Asir region

In total, 6,774 individual chigger mites were isolated from the body surface of the 74 rodent specimens. Of these, 677 chiggers (approximately 10%) were selected for slide preparation and identification. Variation in scutal morphology, the main criterion for separation into subgenera, is illustrated in Figure 3.1 (Appendix 2-31). Following morphometric analysis in Liverpool and species confirmation at the Zoological Institute of the Russian Academy of Sciences (section 2.3.2), 19 trombiculid species belonging to three tribes (*Gahrliepiini*, *Shoengastiini*, and *Trombiculini*) were identified. Of these, 15 species had been described previously from other countries and four species were new to science (Table 3.4). Dr Alexandr Stekolnikov formally described these novel species as *Schoutedenichia asirensis* sp. nov., *Schoutedenichia saudi* sp. nov., *Microtrombicula microscuta* sp. nov., and *Microtrombicula muhaylensis* sp. nov., which has been published (Stekolnikov *et al.,*2019b).

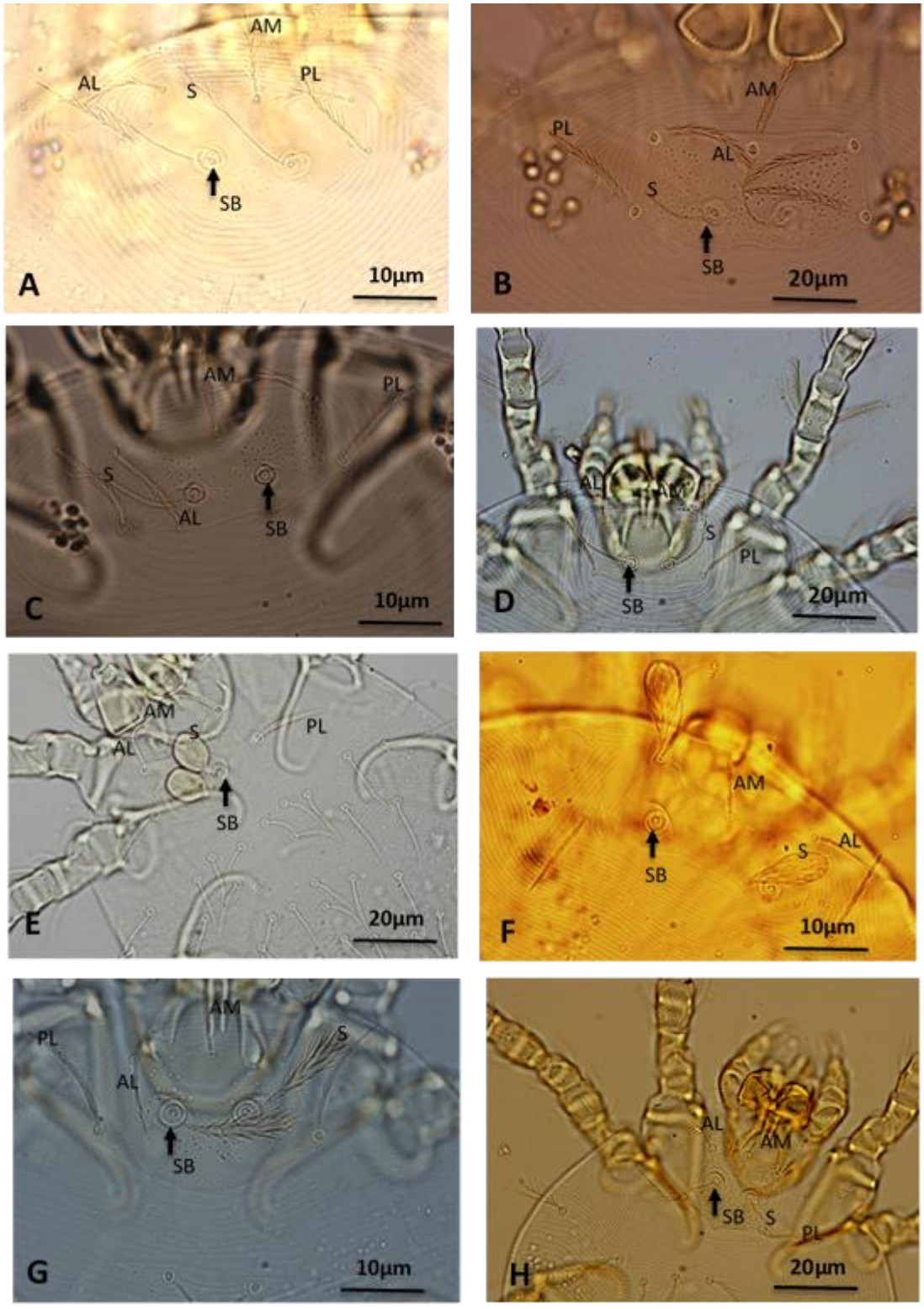


Figure 3.1. Variation in scutal morphology (the main characteristic for subgenus identification. (A) *Pentidionis agamae*; (B) *Ericotrombidium kazeruni*; (C) *Ericotrombidium caucasicum*; (D) *Ericotrombidium galliardi*; (E) *Helenicula lukshumiae*; (F) *Microtrombicula traubi*; (G) *Ascoschoengastia browni*; and (H) *Microtrombicula hoogstraali* . AL—anterolateral scutal seta; AM—anteromedial scutal seta; PL—posterolateral scutal seta; S—sensillum ; SB—sensillary base.

Table 3.4. Species list of chigger mites identified from rodents in the Asir region, Saudi Arabia including four novel species.

No.	*Species*	Distribution	Tribe	*References*	*Host species in this study*
1	*Schoutedenichia asirensis* sp. nov.	Alous, Alogl (Saudi Arabia)	Trombiculini	*Stekolnikov et al.,*(2019b)	*M. yemeni, A. dimidiatus*
2	*Microtrombicula microscuta* sp. nov.	Alous, Wosanib (Saudi Arabia)	Trombiculini	*Stekolnikov et al.,(2019b)*	*A. dimidiatus*
3	*Schoutedenichia saudi* sp. nov.	Alous, Wosanib (Saudi Arabia)	Trombiculini	*Stekolnikov et al.,*(2019b)	*A. dimidiatus*
4	*Microtrombicula muhaylensis* sp. nov	Alous, Wosanib (Saudi Arabia)	Trombiculini	*Stekolnikov et al.,*(2019b)	*A. dimidiatus*
5	*Helenicula lukshumiae*	Iran, Azerbaijan	Trombiculini	*Nadchatram & Traub (1971)*	
6	*Microtrombicula centropi*	Democratic Republic (DR) of Congo	Trombiculini	*Vercammen-Grandjean (1965); Stekolnikov (2018)*	*A. dimidiatus*
7	*Pentidionis agamae*	Israel, Iran	Trombiculini	*André (1929); Vercammen-Grandjean & Loomis (1967); Vercammen-Grandjean et al. (1970); Lucas & Loomis (1968); Stekolnikov et al. (2019a)*	*A. dimidiatus*

8	*Schoengastiella wansoni*	DR Congo, Kyrgyzstan	Gahrliepiini	*Wolfs & Vercammen-Grandjean (1953)*	*A. dimidiatus*
9	*Ericotrombidium caucasicum*	Malta, Italy, Ukraine, Russia	Trombiculini	*Schluger (1967); Vercammen-Grandjean & Langston (1976); Kudryashova & AboTaka (1986); Kudryashova (1998); Stekolnikov et al. (2014)*	*A. dimidiatus*
10	*Schoutedenichia thracica*	Bulgaria, Turkey, Tadjikistan	Trombiculini	*Kolebinova (1966)*	*A. dimidiatus*
11	*Ascoschoengastia browni*	Djibouti	Trombiculini	*Taufflieb, Mouchet & Courtois (1972)*	*A. dimidiatus, M. yemeni*
12	*Microtrombicula traubi*	Azerbaijan, Iran	Trombiculini	*Muljarskaja & Verdieva, (1974); Kudryashova (1998); Kudryashova (1976b); Kudryashova et al. (1978); Stekolnikov et al. (2019a)*	*A. dimidiatus*
13	*Microtrombicula hoogstraali*	Yemen	Trombiculini	*Radford (1954); Vercammen-Grandjean (1965)*	*M. yemeni, M. rex*
14	*Microtrombicula hyraces*	Uganda, Djibouti	Trombiculini	*Vercammen-Grandjean (1965); Vercammen-*	*A. dimidiatus*

				Grandjean (1965); Stekolnikov (2018)	
15	*Schoutedenichia zarudnyi*	Iran	Trombiculini	*Kudryashova (1976); Kudryashova et al. (1978); Kudryashova (1998); Stekolnikov et al. (2019a)*	*A. dimidiatus*
16	*Walchia parvula*	Tadjikistan, Turkmenistan, Azerbaijan	Shoengastiini	*Schluger (1955); Kudryashova (1998)*	*A. dimidiatus*
17	*Gahrliepia lawrencei*	Rwanda	Gahrliepiini	*Jadin & Vercammen-Grandjean (1952); Traub & Morrow (1955)*	*A. dimidiatus*
18	*Ericotrombidium kazeruni*	Iran	Trombiculini	*Kudryashova (1976); Kudryashova et al. (1978); Kudryashova (2004); Stekolnikov et al. (2019a)*	*A. dimidiatus, M. rex*
19	*Ericotrombidium galliardi*	Morocco	Trombiculini	*Vercammen-Grandjean & Taufflieb (1959); Grandjean & Taufflieb, (1959); Vercammen-Grandjean & Langston (1976); Stekolnikov (2018)*	*M. rex, M. yemeni*

The four new chigger species were found on *Acomys dimidiatus, Myomys yemeni*, and *Meriones rex* (Table 3.4). The description of four new species is as follows. *Schoutedenichia asirensis* sp. nov was collected from the anus of *Myomyscus yemeni* from Alogl village and the back and anus of *Acomys dimidiatus* from Alous village (Figure 3.2). This species refers to the type locality ('Asir Region). Morphologically, this species is similar to *Schoutedenichia paulus* Vercammen-Grandjean, 1958 and differs from it by the presence of one pair of eyes vs. two pairs, absence of dorsal teeth of cheliceral blade, larger scutum (AW 48–53 vs. 42, PW 77–86 vs. 56, SD 43–45 vs. 31), longer PL (23–29 vs. 21), fD = 2H-(9–11)-8-8(9)-2(3)+(14–21) vs. 2H-8-6-10-8-10-6-4(Figure 3.3), and by longer legs (Ip 597–647 vs. 486) (Figure 3.4). The second novel species is *Schoutedenichia saudi* sp. nov., isolated from the back and anus of *A. dimidiatus* from Alous and from the back of *A. dimidiatus* from Wosanib villages (Figure 3.5). This species name refers to the ruling dynasty of the Kingdom of Saudi Arabia. This species is similar to *Schoutedenichia geckobia* and differs from it in the presence of subterminala on palpal tarsus (4BS vs. 4B), presence of two genualae I vs. one genuala I, two pairs of humeral setae vs. one pair, shorter AL (28–33 vs. 43; AM > AL vs. AL > AM), fD = 4H-(7–9)-(10–12)+(30–34); DS = 49–57; VS = 66–83; NDV = 116–136 (Figure 3.6). and in longer legs (Ip 885–1000 vs. 757), fsp = 7.7.7; fCx = 1.1.1; fSt = 2.2; fPp = B/B/NNB; fSc: PL > AM > AL; Ip = 885–1000 (Figure 3.7).

***Schoutedenichia asiriensis* Stekolnikov, Al-Ghamdi, Alagaili & Makepeace, sp. nov.(1,4)**

Genus*: Schoutedenichia* Jadin & Vercammen-Grandjean, 1954

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae- Tribe Trombiculini- **Genus** *Schoutedenichia*- species *Schoutedenichia asiriensis*

Hosts*: Acomys dimidiatus* and *Myomyscus yemeni*.

Village: Alous-Alogl

Body site: Ear- Anus

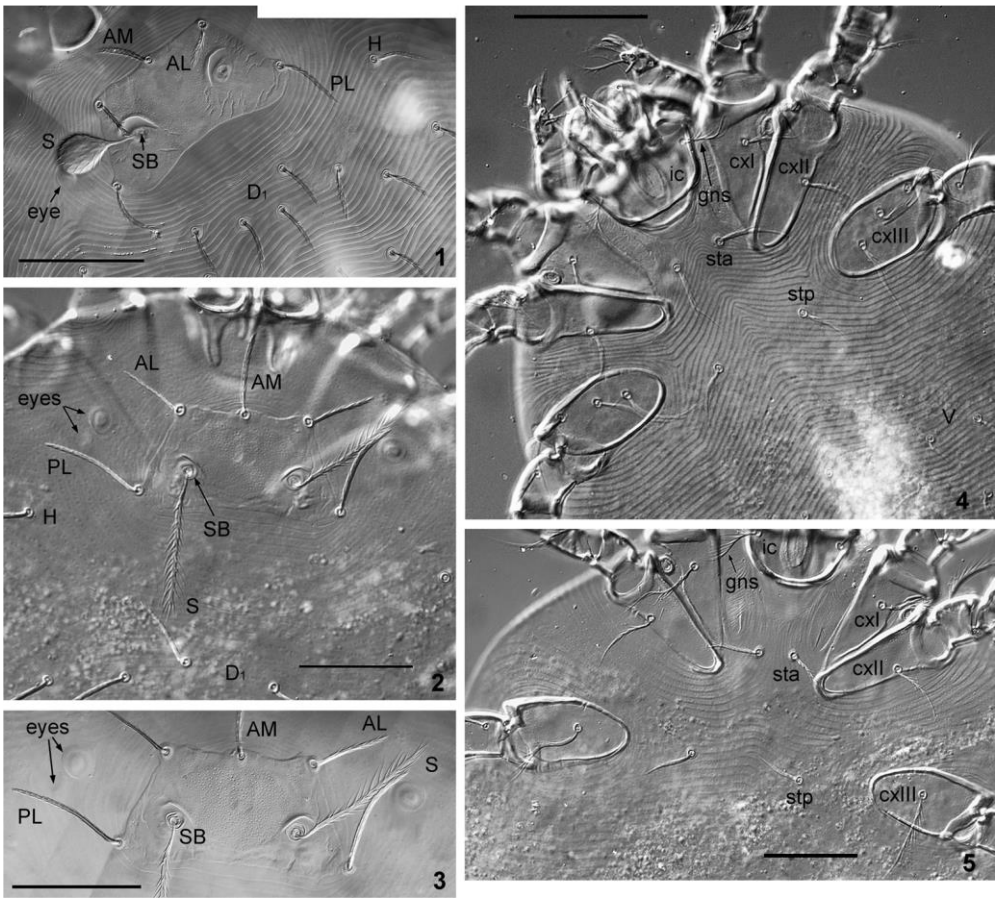


Figure 3.2. *Schoutedenichia asirensis* sp. nov., larva holotype (1,4) and *Schoutedenichia. saudi* sp. nov., larva holotype scutum.

(2, 3, 5): 1, 2—scutum, eyes, and dorsal idiosomal setae. Scale bar 50 µm; 3—scutum and eyes. Scale bar 50 µm; 4, 5—sternal area of idiosoma. Scale bar 50 µm. AL—anterolateral scutal seta (ve); AM—anteromedial scutal seta (vi); cxI—coxa I; cxII—coxa II; cxIII—coxa III; D1—dorsal idiosomal setae of 1st row (C); gns—gnathocoxal seta; H—humeral seta (C antero-marginal); ic—infracapitulum (gnathobase); PL—posterolateral scutal seta (se); S—sensillum (si); SB—sensillary base; sta—anterior sternal seta; stp—posterior sternal seta; V—preanal ventral idiosomal setae.

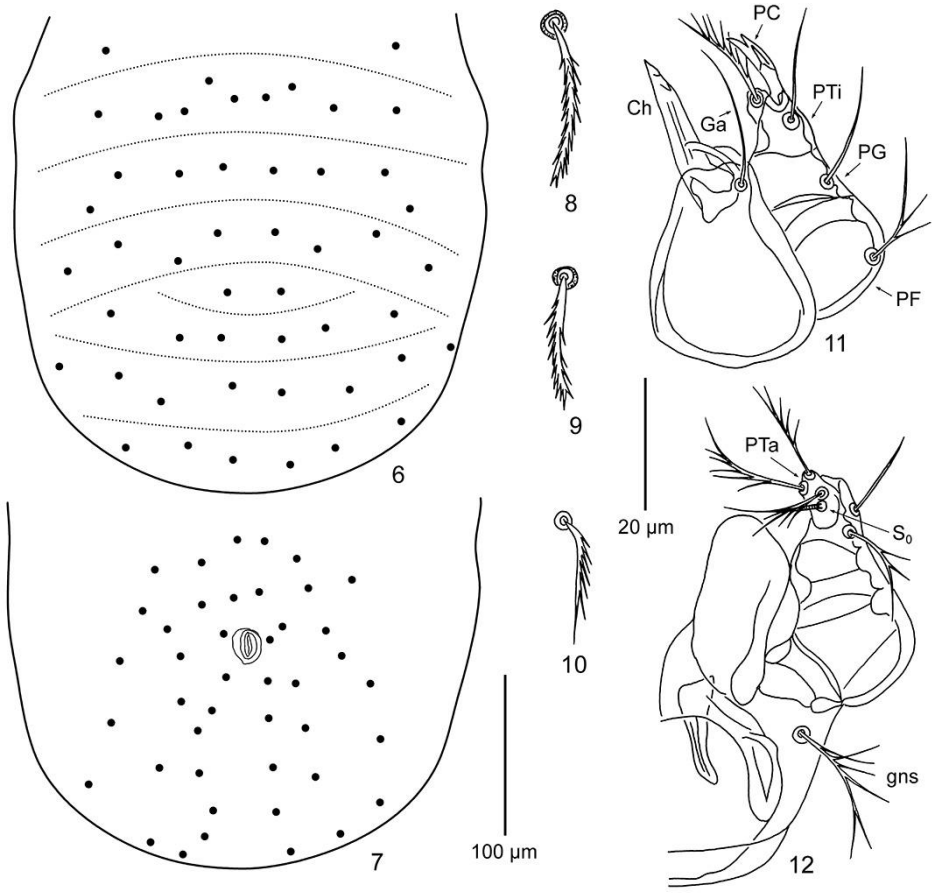


Figure 3.3. *Schoutedenichia asirensis* sp. nov., larva arrangement of dorsal idiosomal setae.

6—arrangement of dorsal idiosomal setae in paratype 10776; 7—arrangement of ventral idiosomal setae in paratype 10776; 8—humeral seta (C antero-marginal) in holotype; 9—dorsal idiosomal seta of 1st row (C) in holotype; 10—preanal ventral idiosomal seta in holotype; 11—dorsal aspect of gnathosoma in holotype; 12—ventral aspect of gnathosoma in holotype. Scale bar 100 µm (6, 7), 20 µm (8–12). Ch—cheliceral blade; Ga—galeal (deutorostral) seta; gns—gnathocoxal seta; PC—palpal claw; PF—palpal femur; PG—palpal genu; PTa—palpal tarsus; PTi—palpal tibia; S_0—palpal tarsala (ω).

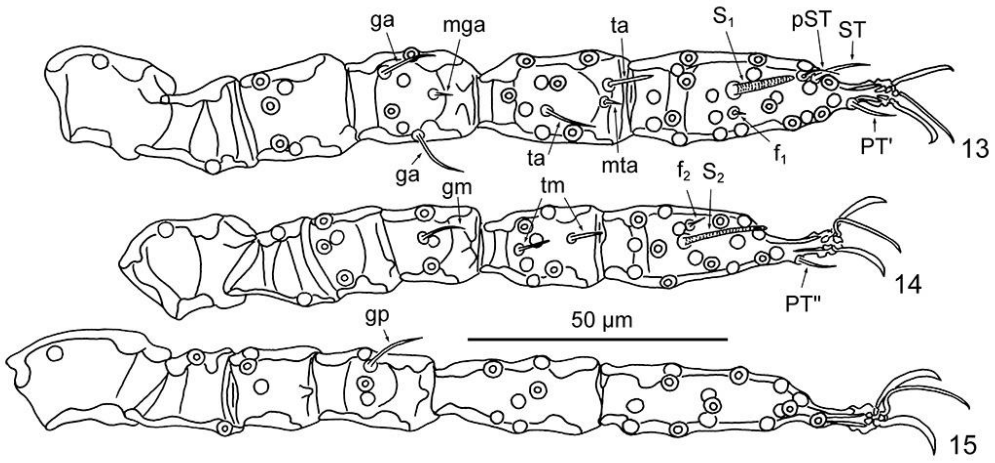


Figure 3.4. *Schoutedenichia asirensis* sp. nov., larva holotype of legs.

13—free part of leg I (trochanter–tarsus); 14—free part of leg II (trochanter–tarsus); 15—free part of leg III (trochanter–tarsus). Scale bar 50 µm. f_1—famulus I (ε); f_2—famulus II (ε); ga—genuala I (σ); gm—genuala II (σ); gp—genuala III (σ); µga—microgenuala (κ); µta—microtibiala (κ); pST—parasubterminala (z); PT'—pretarsala I (ζ); PT"—pretarsala II (ζ); S_1—tarsala I (ω); S_2—tarsala II (ω); ST—leg subterminala (ζ); ta—tibiala I (ϕ); tm—tibiala II (ϕ).

***Schoutedenichia saudi* Stekolnikov, Al-Ghamdi, Alagaili & Makepeace, sp. nov.(2,3,5)**

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae- Tribe Trombiculini- **Genus:** Schoutedenichia- species Schoutedenichia saudi

Host: Acomys dimidiatus

Viallage: Alous-Wosanib

Body site: Back

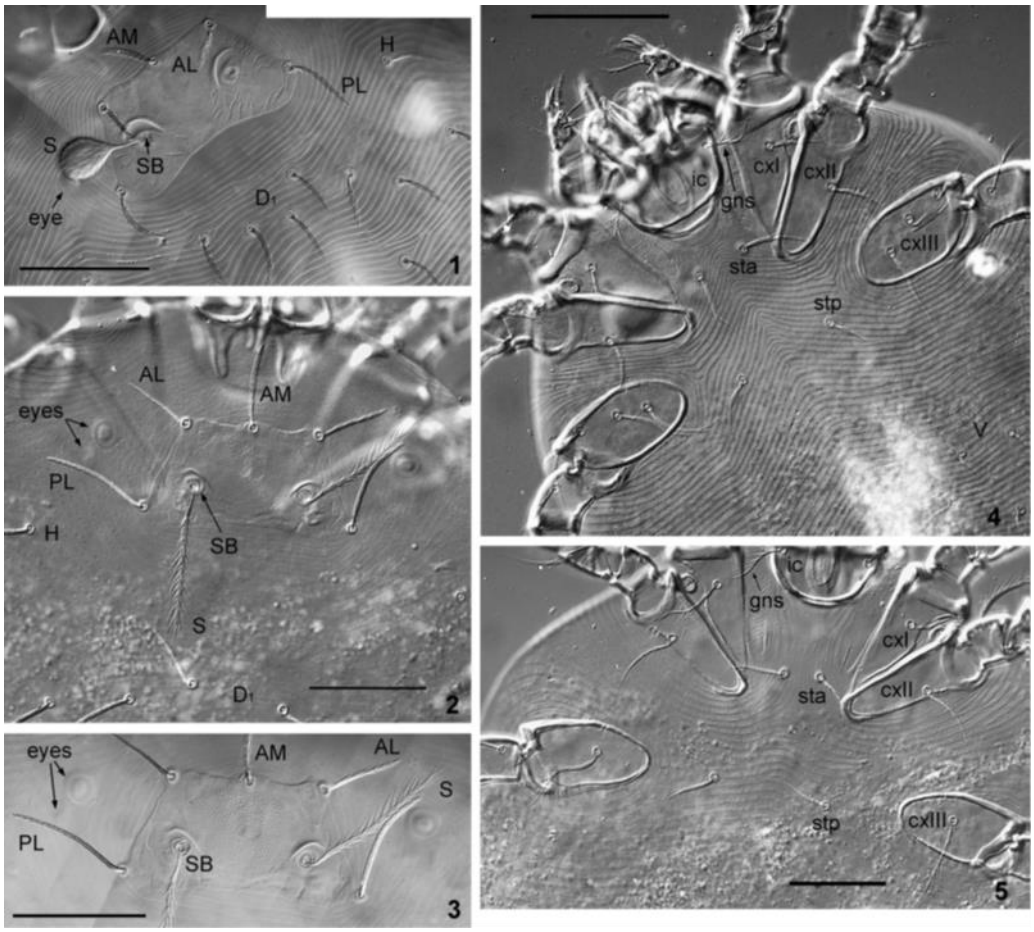


Figure 3.5. *Schoutedenichia. saudi* sp. nov., larva holotype.

(2, 3, 5): 2—scutum, eyes, and dorsal idiosomal setae. Scale bar 50 µm; 3—scutum and eyes. Scale bar 50 µm; 4, 5—sternal area of idiosoma. Scale bar 50 µm. AL—anterolateral scutal seta (ve); AM—anteromedial scutal seta (vi); cxI—coxa I; cxII—coxa II; cxIII—coxa III; D1—dorsal idiosomal setae of 1st row (C); gns—gnathocoxal seta; H—humeral seta (C antero-marginal); ic—infracapitulum (gnathobase); PL—posterolateral scutal seta (se); S—sensillum (si); SB—sensillary base; sta—anterior sternal seta; stp—posterior sternal seta; V—preanal ventral idiosomal setae.

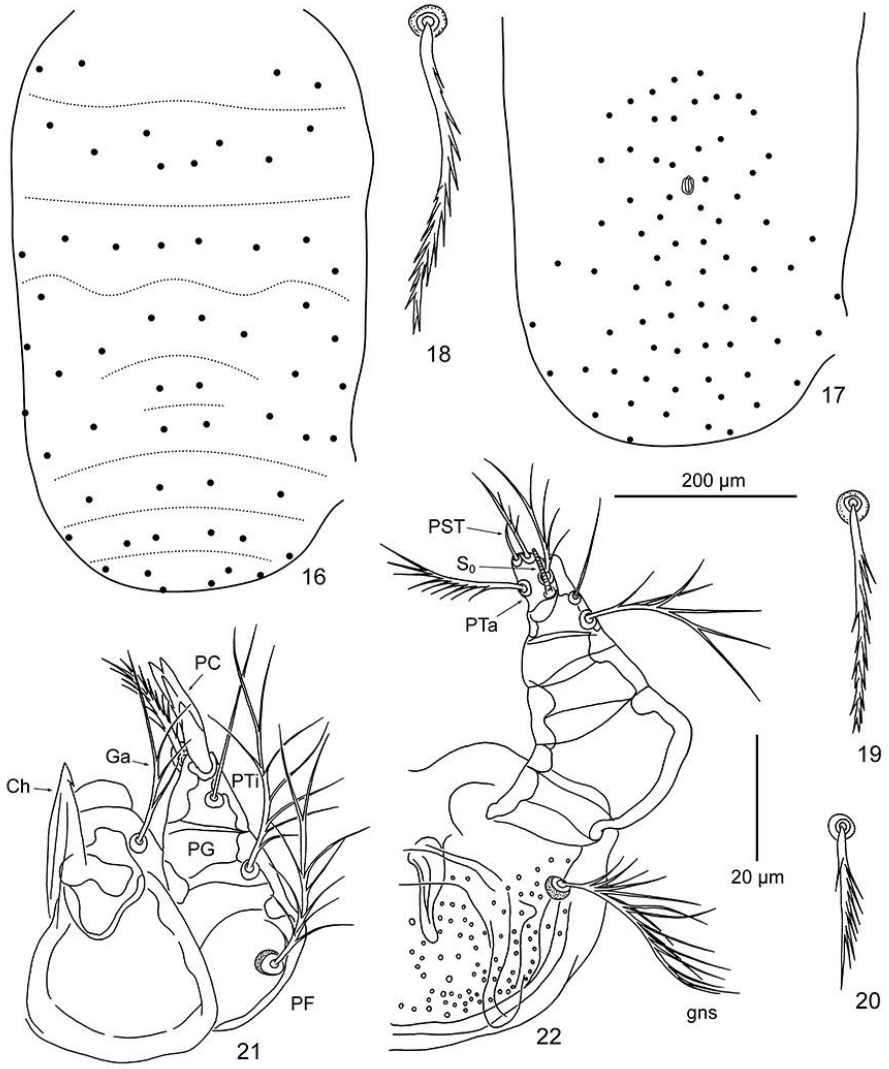


Figure 3.6. S*choutedenichia saudi* sp. nov., larva arrangement of dorsal idiosomal setae.

16— arrangement of dorsal idiosomal setae in paratype 10267; 17— arrangement of ventral idiosomal setae in paratype 10267; 18—humeral seta in holotype (C antero-marginal); 19—dorsal idiosomal seta of 1st row (C) in holotype; 20—ventral preanal idiosomal seta in holotype; 21—dorsal aspect of gnathosoma in holotype; 22—ventral aspect of gnathosoma in holotype. Scale bar 200 µm (16, 17), 20 µm (18–22). PST—palpal subterminala (ζ). Other abbreviations as in Figure 3.3.

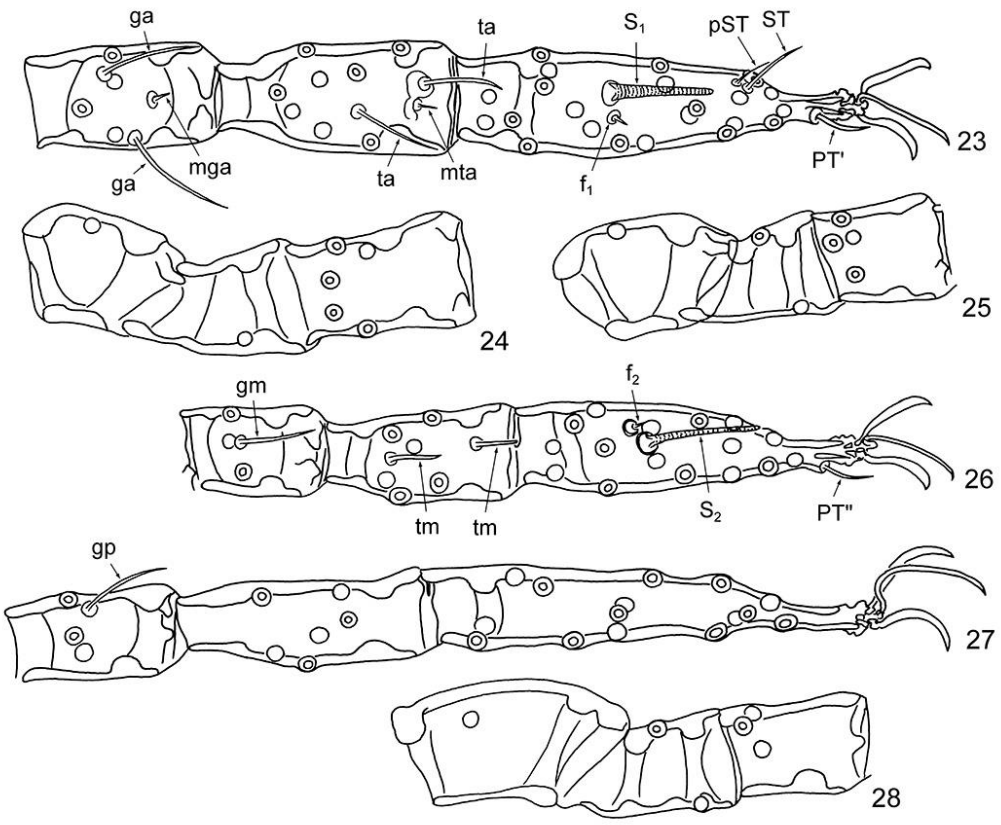


Figure 3.7. *Schoutedenichia saudi* sp. nov., legs.

larva holotype: 23—genu, tibia, and tarsus of leg I; 24—trochanter, basifemur, and telofemur of leg I; 25— trochanter, basifemur, and telofemur of leg II; 26—genu, tibia, and tarsus of leg II; 27—genu, tibia, and tarsus of leg III; 28— trochanter, basifemur, and telofemur of leg III. Scale bar 50 µm.

The third novel species, *Microtrombicula microscuta* sp. nov., was isolated from the ears of *A. dimidiatus* from Alous and Wosanib villages**.** This species name refers to its minute scutum, the size of which is smallest within the genus *Microtrombicula*. This species is similar to *Microtrombicula machadoi* and differs from it in the presence of a three-pronged palpal claw vs. two-pronged, forked sensilla vs. nude, lower number of idiosomal setae (NDV 50–54 vs. 62), presence of a mastitarsala, smaller scutum (AW 16–19 vs. 27, PW 23–30 vs. 34, SB 6–8 vs. 15, SD 30–33 vs. 37), shorter scutal setae (AM 10–13 vs. 17, AL 9–13 vs. 17, PL 12–16 vs. 21(Figure 3.8).), fD = 2H-6-6-4-4-4-(0–4); DS = 26–30; VS = 24–26; NDV = 50–54 (Figure 3.9), and shorter legs (Ip 387–425 vs. 443) (Figure 3.10), SIF = 6BN-N-3-3111.1000; fsp = 7.6.6; fCx = 1.1.1; fSt = 2.2; fPp = B/B/NNB; fSc: PL ≥ AM ≥ AL; Ip = 387–425.

***Microtrombicula microscuta* Stekolnikov, Al-Ghamdi, Alagaili & Makepeace, sp. nov.**

Genus: Microtrombicula Ewing, 1950

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae- Tribe Trombiculini- **Genus** Microtrombicula - species Microtrombicula microscuta

Host: Acomys dimidiatus

Village:Alous-Wosanib

Body site: Ear

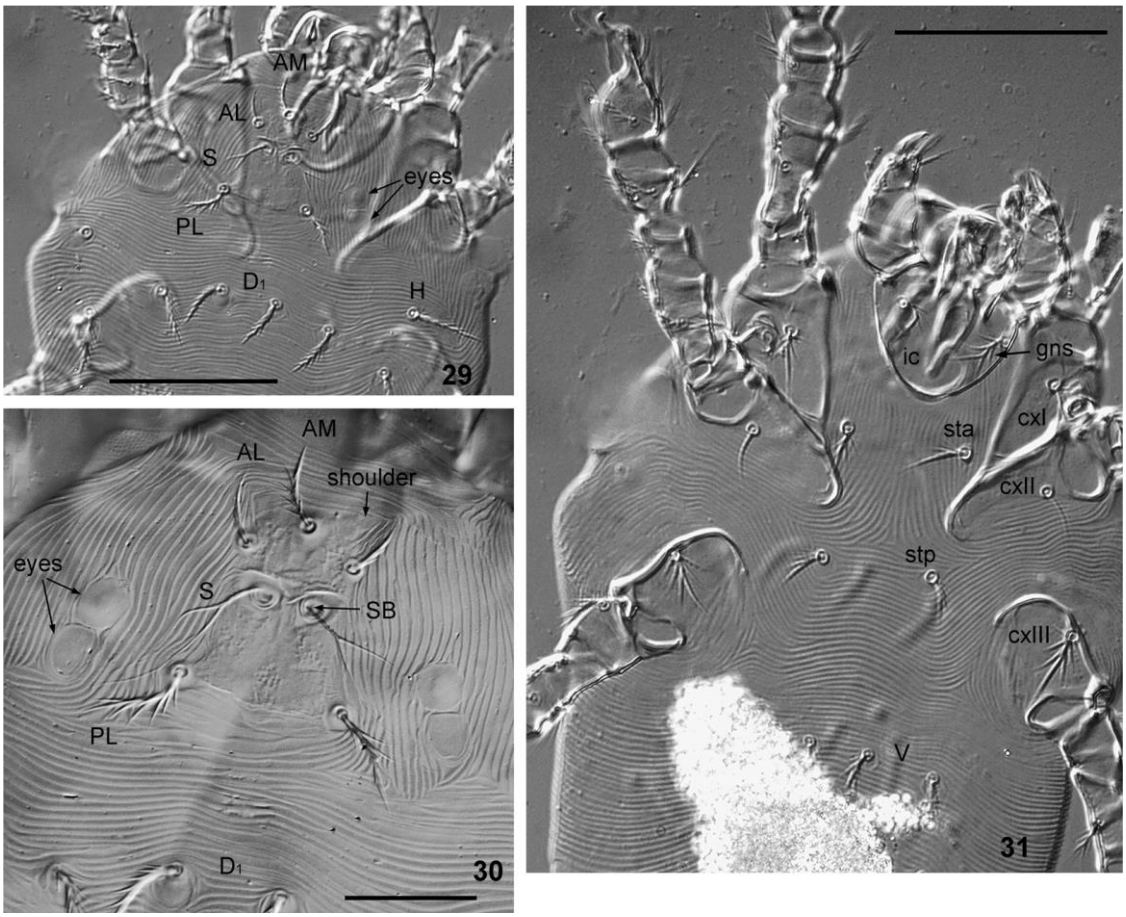


Figure 3.8. *Microtrombicula microscuta* sp. nov., larva holotype scutum.

29—scutum, eyes, and dorsal idiosomal setae. Scale bar 50 µm; 30—scutum and eyes. Scale bar 20 µm; 31—sternal area of idiosoma. Scale bar 50 µm.

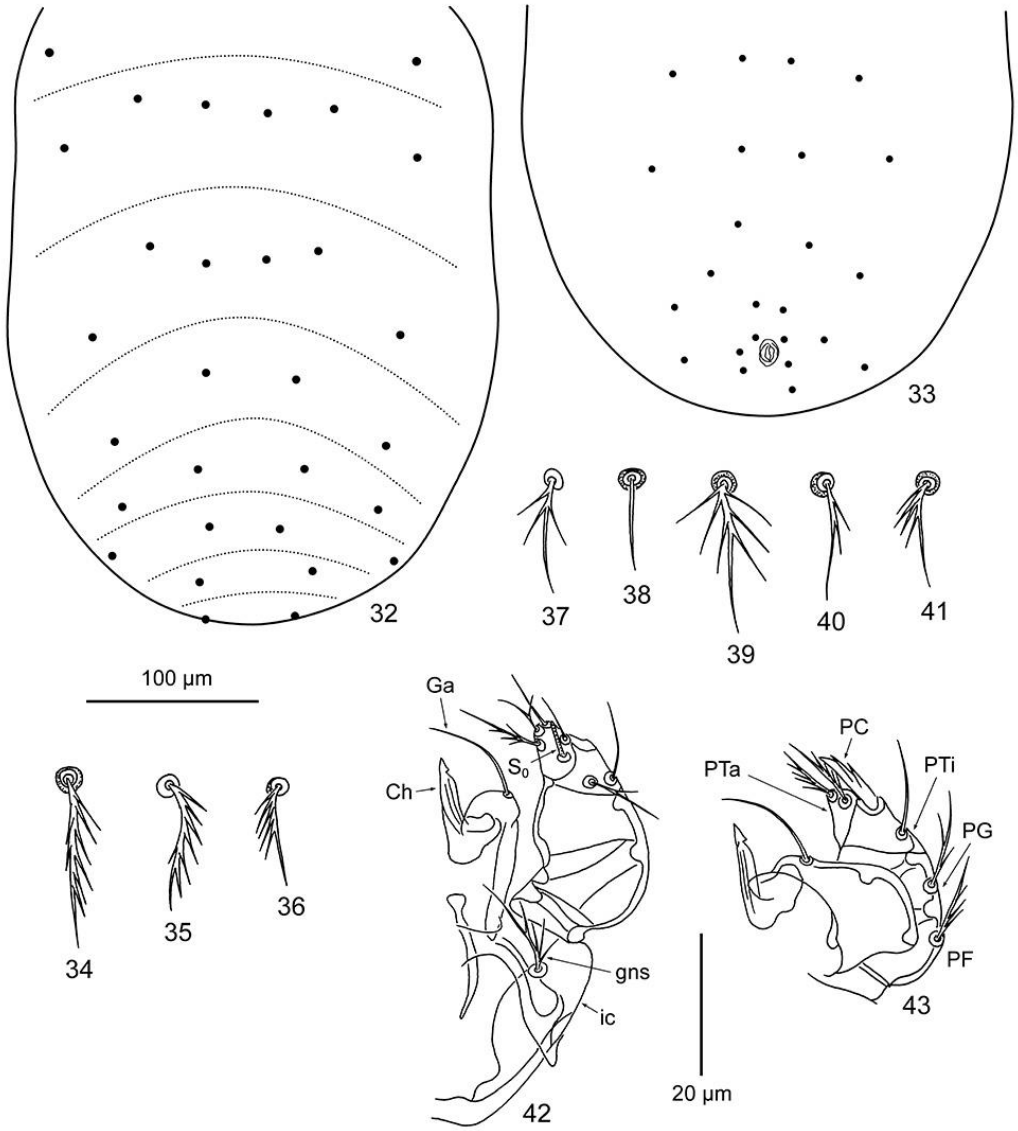


Figure 3.9. ***Microtrombicula microscuta*** **sp. nov., larva arrangement of dorsal idiosomal setae.**

32—arrangement of dorsal idiosomal setae in paratype 10804; 33—arrangement of ventral idiosomal setae in paratype 10804; 34—humeral seta (C antero-marginal) in paratype 10309; 35—dorsal idiosomal seta of 1st row (C) in paratype 10309; 36—preanal ventral idiosomal seta in paratype 10309; 37—coxal seta I in paratype 10309; 38—coxal seta II in paratype 10309; 39—coxal seta III in paratype 10309; 40—anterior sternal seta in paratype 10309; 41—posterior sternal seta in paratype 10309; 42—ventral aspect of gnathosoma in paratype 10309; 43—dorsal aspect of gnathosoma in paratype 10309. Scale bar 100 µm (32, 33), 20 µm (34–43). Abbreviations as in Figure 3.3.

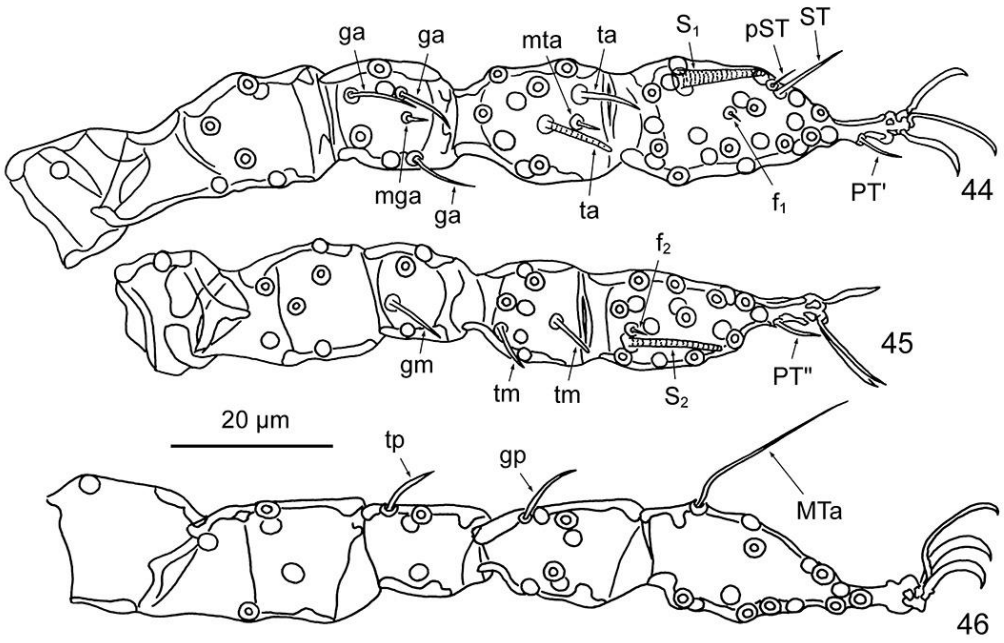


Figure 3.10. ***Microtrombicula microscuta*** **sp. nov., legs.**

larva paratypes: 44—free part of leg I (trochanter–tarsus); 45—free part of leg II (trochanter–tarsus); 46—free part of leg III (trochanter–tarsus). Scale bar 20 µm. MTa—mastitarsala. Other abbreviations as in Figure 3.4.

The fourth new species, *Microtrombicula muhaylensis* sp. nov., was isolated from the back of *A. dimidiatus* from Alous and Wosanib villages and also from the ear of *Meriones rex* from Alogl village(Figure 3.11). This species name refers to Muhayl, a traditional name of the territory within 'Asir Region where the type locality is situated. This species is similar to *Microtrombicula meriones* (Vercammen-Grandjean, Rohde and Mesghali, 1970) and differs from it in the presence of two pairs of eyes vs. one pair, lower number of idiosomal setae (NDV 49–56 vs. 68, fD = 2H-6-6-4-4-2 vs. 2H-6-6-4-4-4-4-2), PL > AM ≥ AL vs. AM ≥ PL > AL, and in smaller scutum(Figure 3.12) (AW 42–47 vs. 57, PW 56–65 vs. 71, PSB 18–20 vs. 27, SD 39–44 vs. 55), SIF = 6BN-N-3-2111.1000; fsp = 7.7.7; (Figure 3.13), fCx = 1.1.1; fSt = 2.2; fPp = B/B/BBB; fSc: PL > AM ≥ AL; Ip = 678–718; fD = 2H-6-6-4-4-2; DS = 22–26; VS = 24–29; NDV = 49–56.

***Microtrombicula muhaylensis* Stekolnikov, Al-Ghamdi, Alagaili & Makepeace, sp. nov.**

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae- Tribe Trombiculini- **Genus** Microtrombicula - species Microtrombicula muhaylensis

Hosts:Acomys dimidiatus and Meriones rex.

Village:Alous- Alogl-Wosanib

Body site: Back-Ear

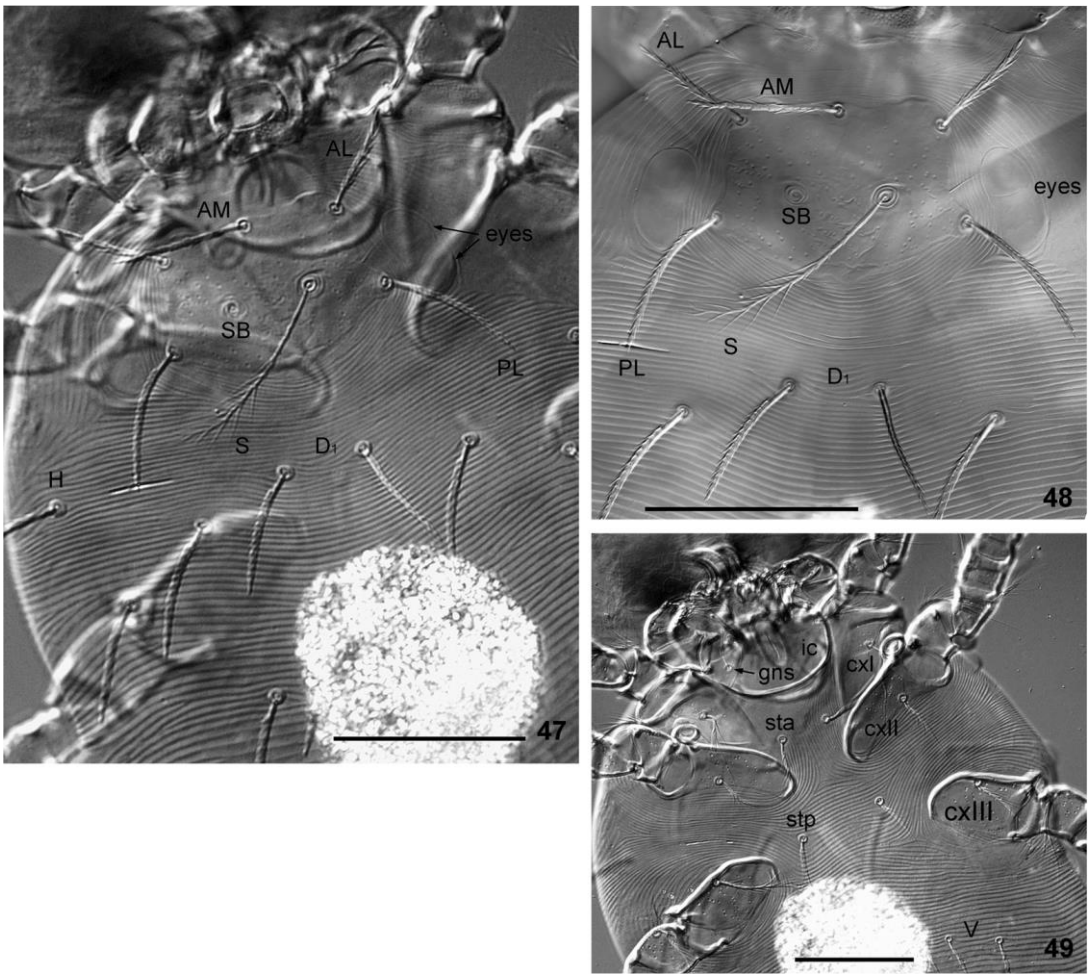


Figure 3.11. *Microtrombicula muhaylensis* sp. nov., larva holotype scutum.

47—scutum, eyes, and dorsal idiosomal setae. Scale bar 50 µm; 48—scutum, eyes, and dorsal idiosomal setae. Scale bar 50 µm; 49—sternal area of idiosoma. Scale bar 50 µm.

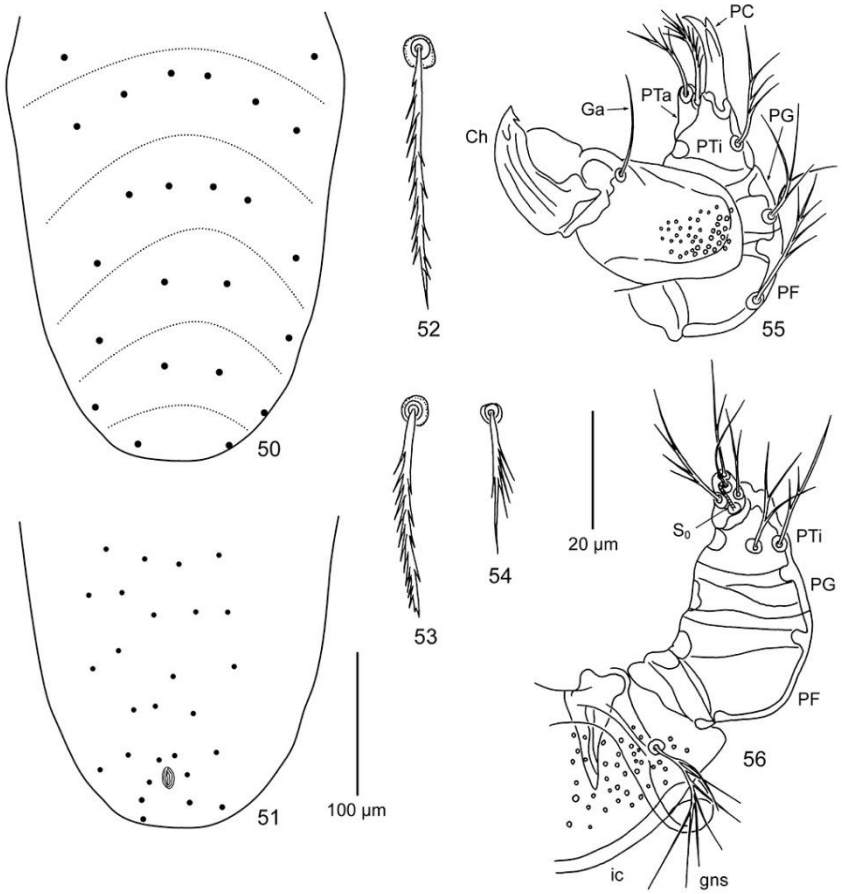


Figure 3.12. ***Microtrombicula muhaylensis*** **sp. nov., larva arrangement of dorsal idiosomal setae.** 50—arrangement of dorsal idiosomal setae in paratype 10841; 51—arrangement of ventral idiosomal setae in paratype 10841; 52—humeral seta (C antero-marginal) in paratype 10840; 53—dorsal idiosomal seta of 1st row (C) in paratype 10840; 54—preanal ventral idiosomal seta in paratype 10840; 55—dorsal aspect of gnathosoma in paratype 10840; 56—ventral aspect of gnathosoma in paratype 10840. Scale bar 100 µm (50, 51), 20 µm (52–56). Abbreviations as in Figure 3.3.

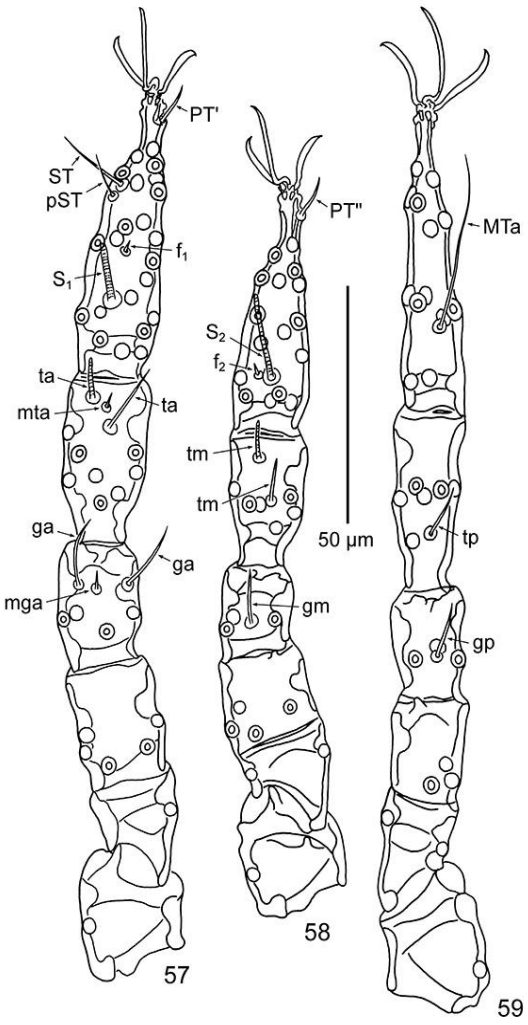


Figure 3.13. ***Microtrombicula muhaylensis*** **sp. nov., legs.**

Larva paratypes 10840: 57—free part of leg I (trochanter–tarsus); 58—free part of leg II (trochanter–tarsus); 59—free part of leg III (trochanter–tarsus). Scale bar 50 µm. MTa—mastitarsala. Other abbreviations as in Figure 3.4.

3.2.2.2 Identification of other ectoparasites from the Asir region

Of 1,496 ectoparasites (excluding chiggers) isolated from the 74 rodent specimens, 31 specimens (2%) were selected for morphological identification using published keys. Only immature ticks (larvae and nymphs) were recovered from rodents and no adult ticks detected. The ticks (order: Ixodida) were placed into two putative species [*Haemaphysalis spp.* and *Rhipicephalus spp.* (Figure 3.14)]; the fleas (Siphonaptera) into three species [*Parapulex chephrensis, Xenopsylla cheopis* (male and female)*,* and *Leptopsylla aethiopica* (female) **(**Figure 3.15)]; the lice (Anoplura) into two species *Polyplax brachyrrhyncha* and *Polyplax oxyrrhyncha* (Figure 3.16); and the gamasid mites (Mesostigmata) into two species *Laelaps lamborni* and *Ornithonyssus bacoti* (Figure 3.17). The key morphological features used to distinguish species of ticks, fleas, lice, and gamasid mite are illustrated in Figures. 3.14 – 3.17. For *Leptopsylla aethiopica,* just a single female was detected, and in the absence of male specimens, confirmation of identification and investigation of subspecies was not possible.

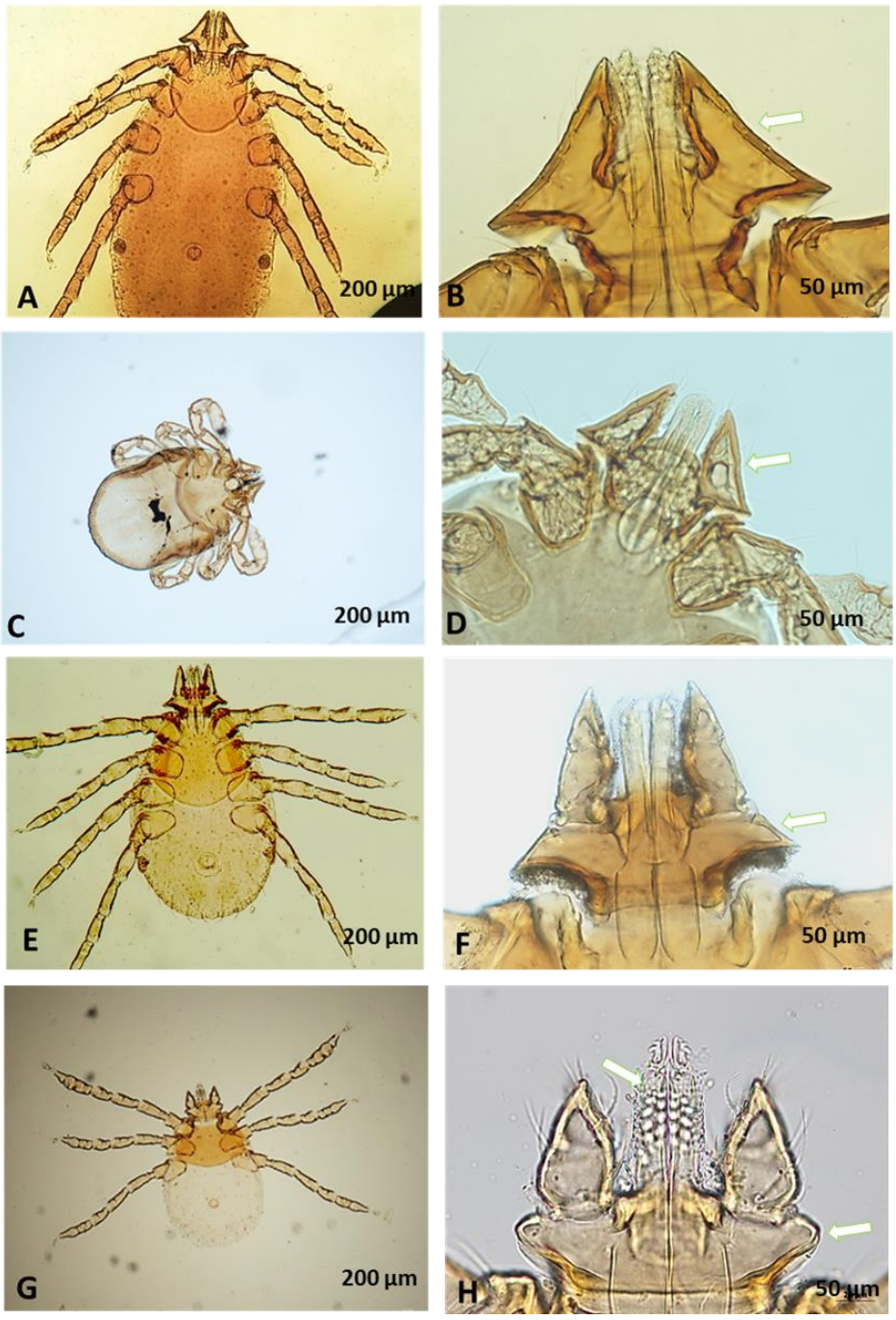


Figure 3.14. Ticks collected from rodents in Saudi Arabia. *Haemaphysalis erinacei* (Alous) and *Rhipicephalus* spp. (Algol) with notes on identification. (A) Nymph of *Haemaphysalis erinacei* (B) high power view of the distinctly flared second palp segment (right arrow), typical of the genus *Haemaphysalis* (C) Larva of *Haemaphysalis erinacei* and (D) high power view of second palp segment in the larval stage(right arrow) (E) Nymphs of *Rhipicephalus* spp. and (F) high power view of the hexagonally-shaped basis capituli (right arrow), associated with the *Rhipicephalus* (G) larva *Rhipicephalus* spp. and (H) high magnification of the expanded basis capituli; Hypostome of *Rhipicephalus sanguineus* nymph (left arrow) (Identifications based on Hoogstraal, 1981).

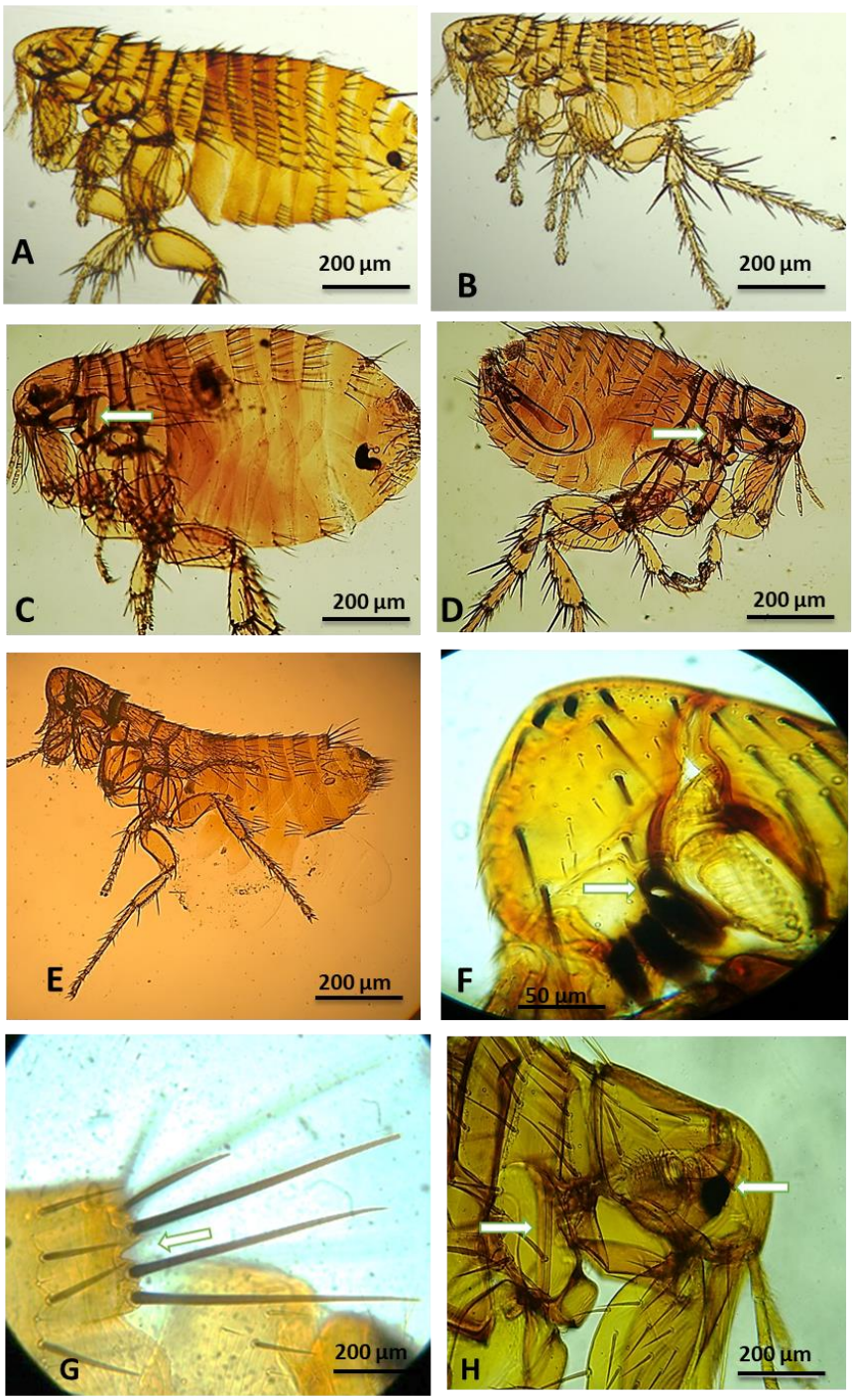


Figure 3.15. Fleas collected from rodents in Asir region, Saudi Arabia, with notes on identification. **(**A) Female *Parapulex chephrensis* from Alous (B) Male. In both sexes the pronotal and genal combs are lacking, and the setae (strong hairs) are dark and very strongly spiniform throughout the body (C) *Xenopsylla cheopis* female (Algol) and (D) male. Pronotal and genal combs are lacking for this species but the presence of a mesopleural rod (arrow) is a defining feature, (E) Female *Leptopsylla aethiopica* (Alogl). There are 3 blunt spines (F) comprising the genal comb in this genus; the third spine (arrow) overlapping the antennal fossa; for the species, the two sets of antepygial bristles not on same podium; there is a clear sinus (arrow, G). (H) *Xenopsylla cheopis:* Arrows shows mesopleural rod on left and the bristle anterior and dorsal to the eye (right arrow). (Identifications based on Lewis, 1982 ; Hopkins *et al.,*1953).

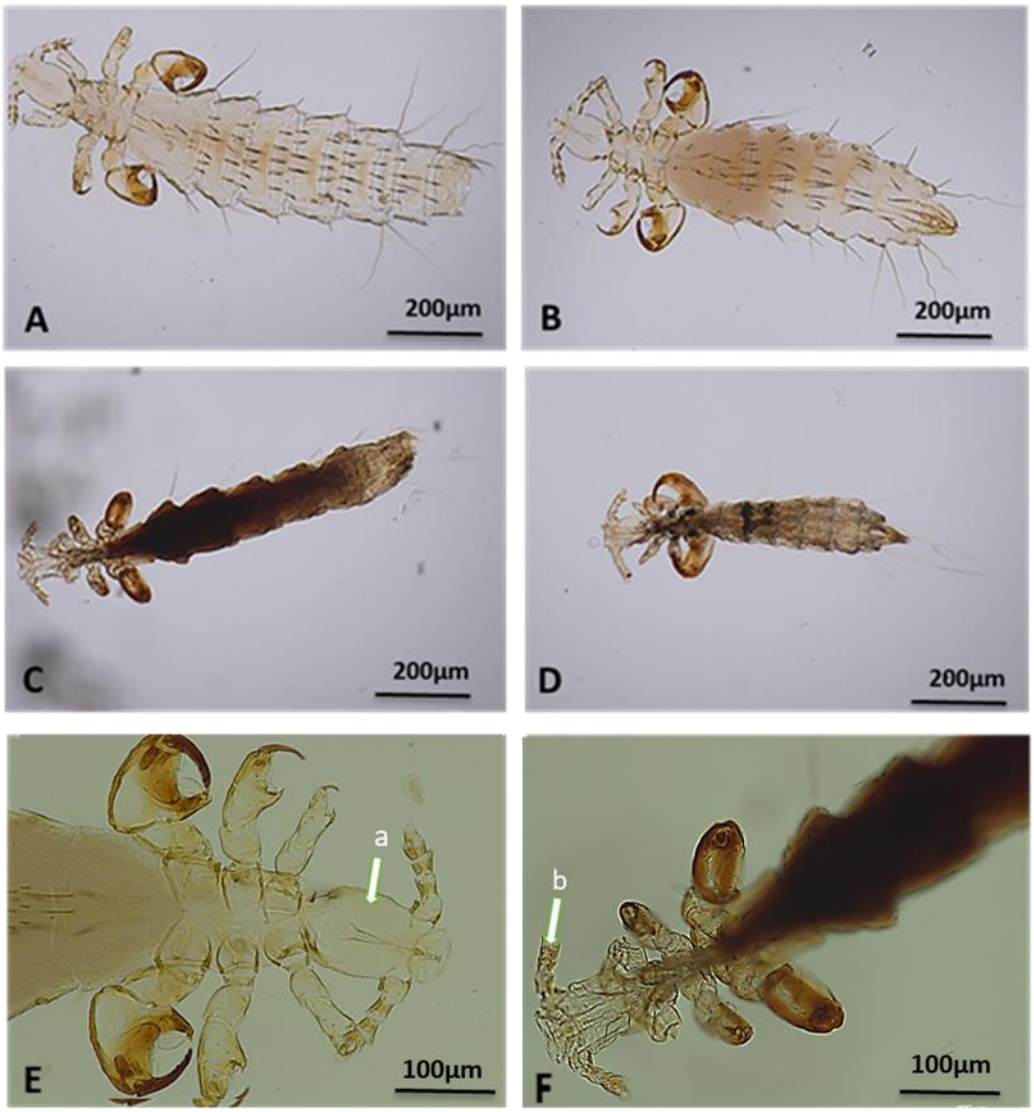


Figure 3.16. Sucking lice from rodents, Asir Region.

(A) *Polyplax oxyrrhyncha* female (Alous), (B) *Polyplax oxyrrhyncha* male, (C) *Polyplax brachyrrhyncha* female, (D) *Polyplax brachyrrhyncha* male (Alous), (E) *Polyplax oxyrrhyncha*, (a)length of head at least twice width (F) *Polyplax brachyrrhyncha* (b) antennae on head margin. (Identifications based on Paterson *et al.,*1953).

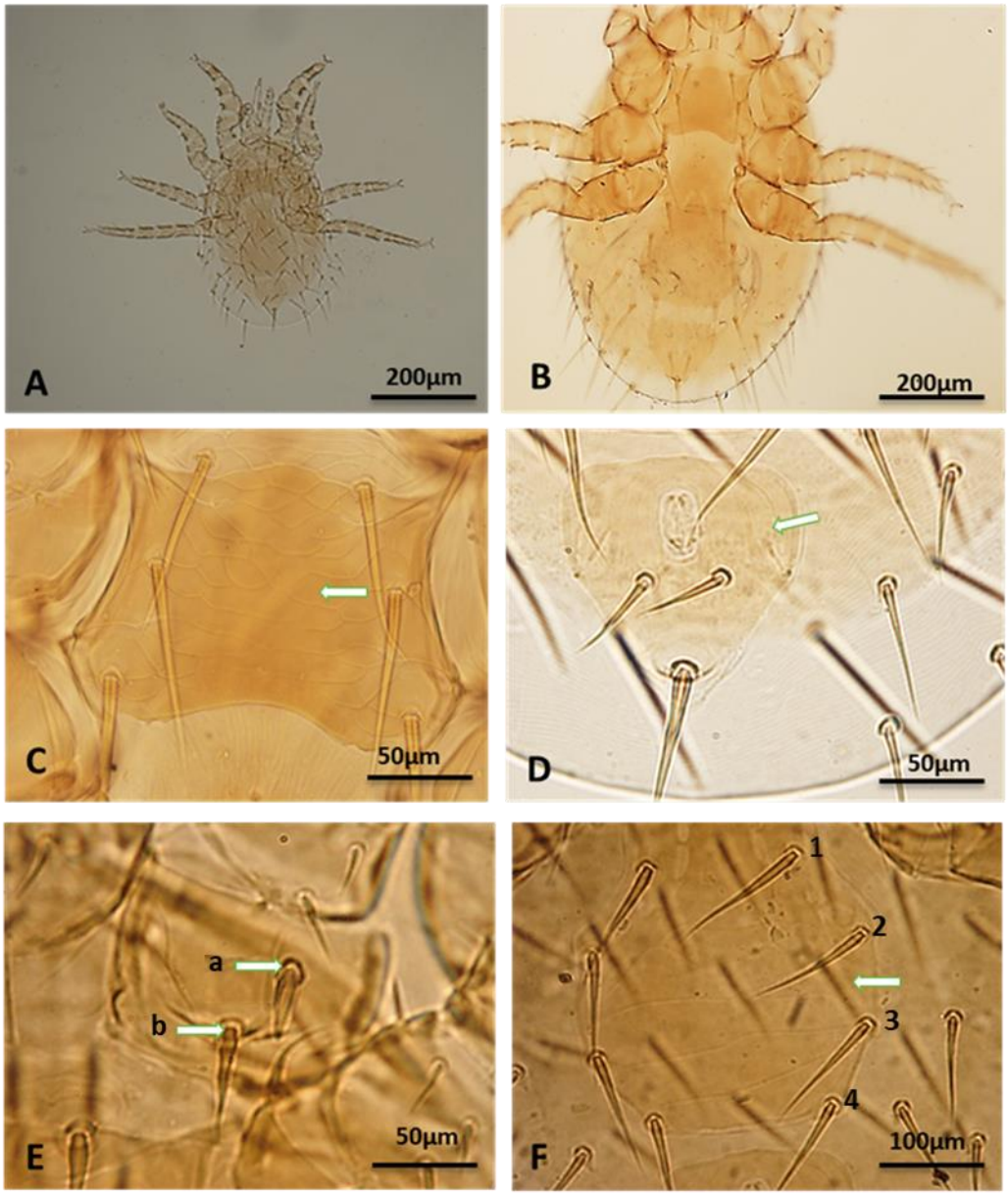


Figure 3.17. A gamasid mite from the Asir Region.
(A,B) *Laelaps lamborni* female (Alogl), (C) high power view of sternal plate of *Laelaps lamborni* (right arrow), (D) high power view of anal plate(right arrow), (E) distal (a), and proximal setae of coxa 1 (b) (left arrows),(F) epigynial plate showing 4 pairs of setae(1-4)(right arrow). Identifications based on Tipton (1960).

3.2.2.3 Autofluorescence microscopy coupled with molecular barcoding of chiggers

Traditional methods for clearing mites before mounting on a slide, using Berlese fluid or lactophenol destroys DNA, so it is not possible to obtain a barcode from specimens prepared in this way. However, ethanol-fixed mites retain natural autofluorescence, enabling a high-resolution image to be obtained prior to DNA extraction (Kumlert *et al.,*2018). Fluorescence microscopy of trombiculid mites to achieve enhanced visualization of scutum morphology was performed for the four novel chigger species and produced green fluorescence alongside bright-field images (Figure 3.18). The same approach was applied to representative specimens of the other chiggers species found at the Alous, Alogl and Wosanib sites in appendix . Following DNA extraction, *coi* PCR and Sanger sequencing, molecular barcodes were obtained from individual chiggers on 80% of occasions. A phylogenetic tree of 16 chigger mitochondrial *coi* sequences 631 bp was then constructed using the maximum composite likelihood method, including NCBI nucleotide reference sequences from chiggers collected from other countries. The Saudi data provided the first barcodes for the genera *Pentidionis* and *Ericotrombidium* (Figure 3.19). The phylogenetic tree showed that in general, each chigger genus separates into a monophyletic clade, albeit with complex branching within genera and sometimes within species. The specimens of *S. saudi* sp. nov. and *A. browni* clustered with congeneric specimens from other parts of the world, but *Microtrombicula traubi* could not be resolved from *Ascoschoengastia* spp. (Figure 3.19). During the original collection of chigger mites off the host, their coloration (orange or white) was noted. Several species appeared to be present in both colour morphs. The mitochondrial *coi* gene was amplified from DNA extracted from orange and white chigger specimens of the same putative species from two tribes (Trombiculini and Shoengastiini). Molecular barcodes were obtained successfully for both colour morphs from three species (*S. zarudnyi*, *P. agamae*, and *S. saudi* sp. nov.) and the sequences for each pair of morphs differed by 0 – 2 SNPs (Table 3.5). The phylogenetic tree (Figure 3.20) constructed from these sequences indicated that the colour morphs were likely to be of the same species.

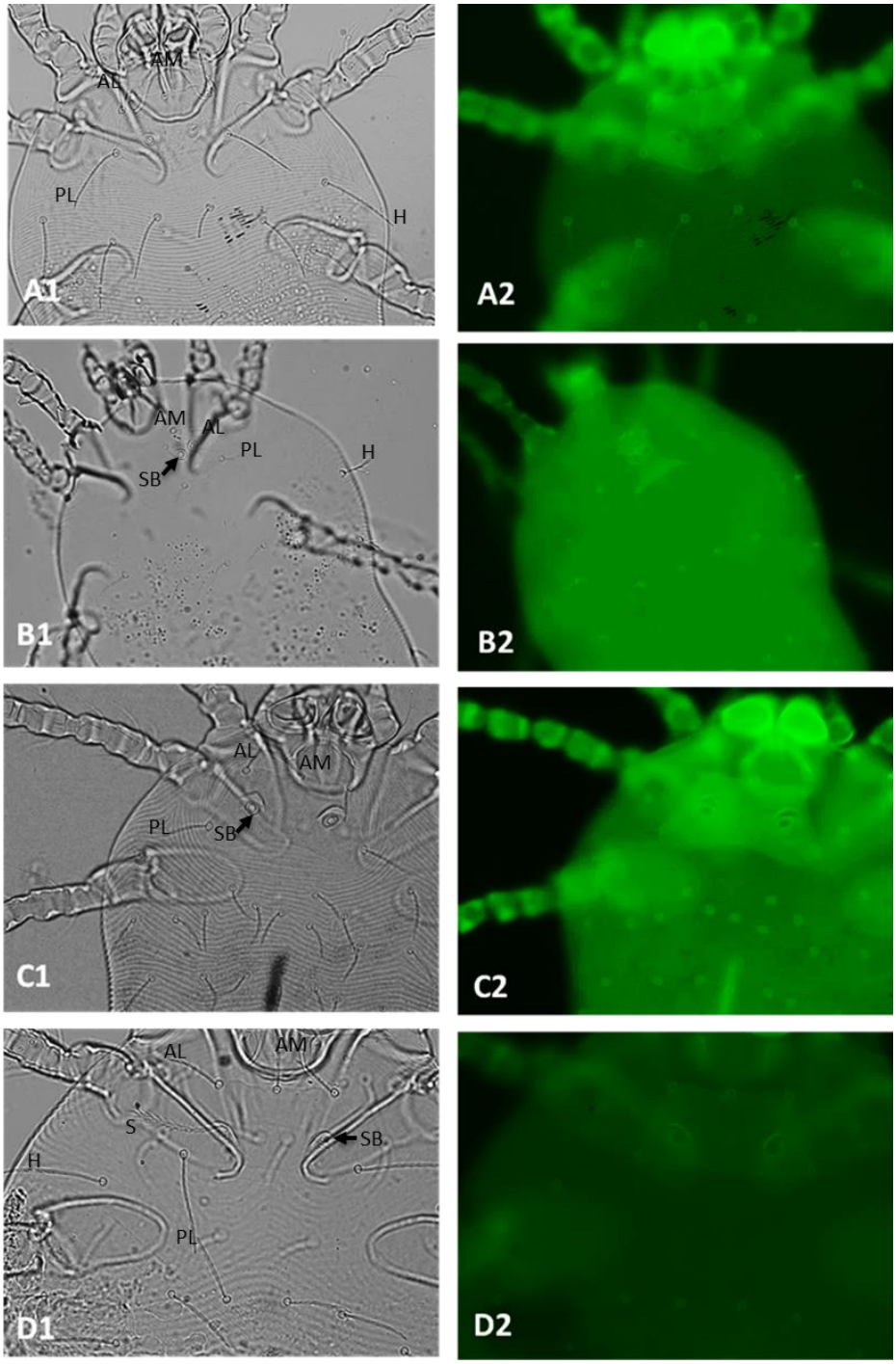


Figure 3.18. Comparison of autoflourescence (right panel) and bright-field (left panel) microscopy of chigger scuta for four new species of chiggers.

Fluorescence microscopy provides enhanced visualization of scutum morphology for (A) *Microtrombicula microscuta sp. nov. (*Alous), (B) *Microtrombicula aff. machadoi (*AS*)*, (C) *Microtrombicula muhaylensis sp. nov. (*Alogl), (D) *Schoutedenichia saudi sp. nov.* (Wosanib). *AL—anterolateral scutal seta; AM—anteromedial scutal seta; H—humeral seta; PL—posterolateral scutal seta (se); S—sensillum; SB—sensillary base.*

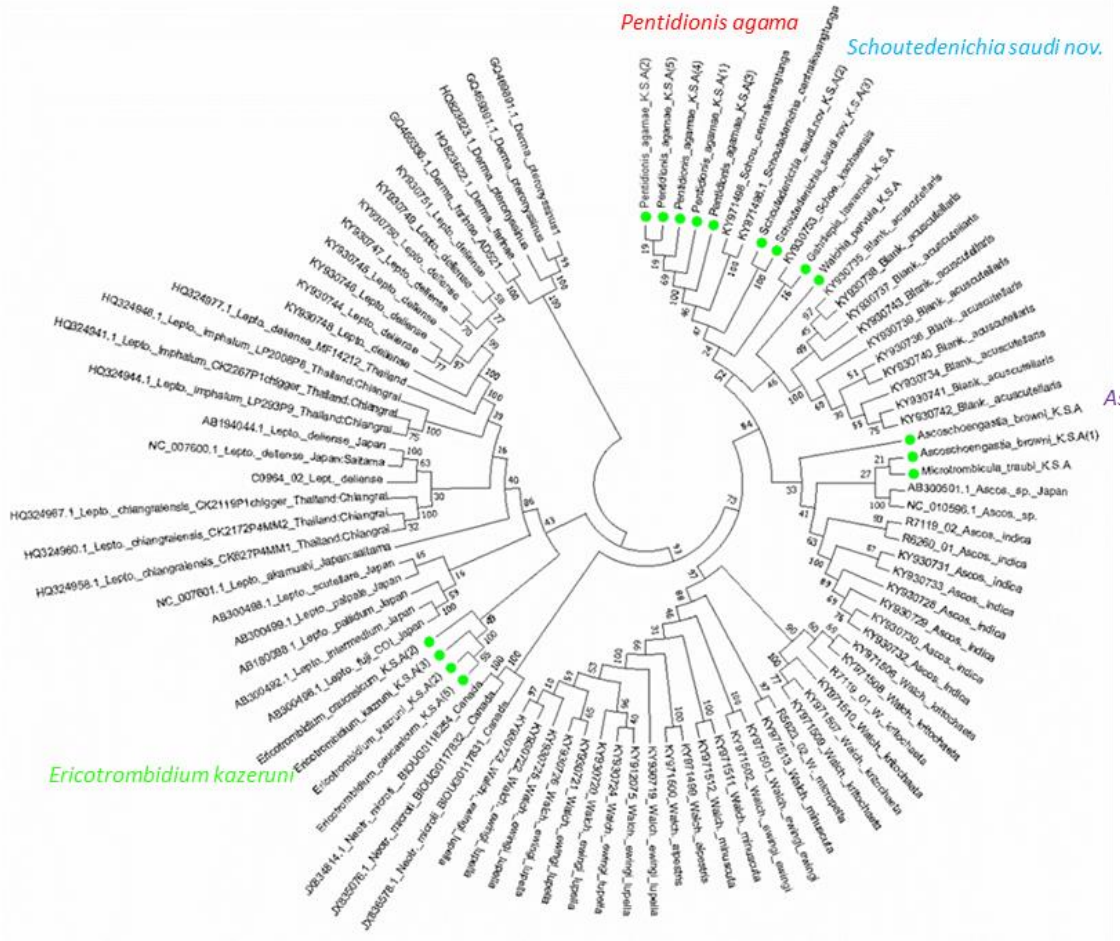


Figure 3.19. A phylogenetic tree of chigger mitochondrial *coi* sequences using the maximum composite likelihood method.

A phylogeny test with bootstrap values based on 1,000 replicates are presented at the nodes. The scale bar measures evolutionary distance indicating substitutions per nucleotide. Samples from the current study are highlighted in green; reference sequences are from NCBI. There were a total of 631 bp positions in the final dataset. Dust mite sequences (*Dermatophagoides* spp.) were used as an outgroup.

Table 3.5. Comparison between *coi* sequences of orange or white colour morphs of three chigger species

Chigger species	Sequence alignment length (bp)	SNPs between morphs	Identity (%)
Schoutedenichia zarudnyi	330 bp	2	99.48
Schoutedenichia saudi. sp. nov.	120 bp	1	99.28
Pentidionis agamae	301 bp	0	100

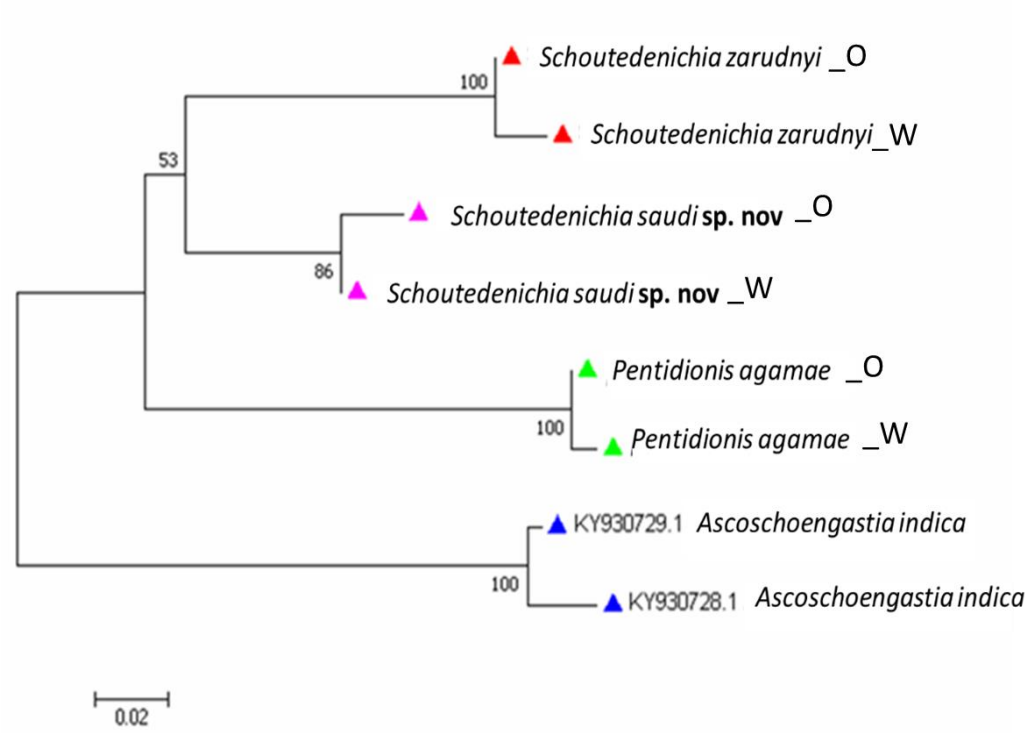


Figure 3.20. A phylogenetic tree of orange (O) and white (W) colour morphs of three chigger species.

Mitochondrial *coi* sequences were analysed using the maximum composite likelihood method. A phylogeny test with bootstrap values based on 1,000 replicates are presented with coloured nodes. The scale bar measures evolutionary distance indicating substitutions per nucleotide. Sequences from *A. indica* from Laos were used as an outgroup. There were a total of 460 positions in the final dataset.

3.2.2.4 Molecular barcoding of immature ticks.

As morphological identification of immature ticks is challenging, molecular barcoding was applied to the specimens provisionally identified as *H. erinacei* and *R. sanguineus* (see section 2.6.1). Mitochondrial 16S rRNA gene sequences of 9 individuals of *Haemaphysalis* nymphs were successfully amplified and sequenced, producing around 550 bp of aligned nucleotides. Specimens from the current study separated into two very distinct clades (Figure 3.21). Although the relationships between *Haemaphysalis* species were not always clearly resolved due to poor bootstrap support for some basal nodes, none of the "*H. erinacei*" samples from the current study clustered with existing *H. erinacei* sequences from other parts of the world (Figure 3.21). While an alternative species identification was not attributable with confidence on the basis of the molecular data, one clade appeared to be

more closely related to *Haemaphysalis spinulosa* than *H. erinacei*. The coi sequences of 18 nymphs of *Rhipicephalus* spp. were successfully amplified and sequenced for the ~550 bp region, and the 16S sequences of all 18 *Rhipicephalus* spp. were also successfully amplified and sequenced for the 456 bp region. From a pool of larvae, only the 16S sequence was successfully amplified. Both the 16S and coi phylogenetic trees showed two *Rhipicephalus* spp genetic lineages, apparently distinct from the previously recognised tropical lineage, temperate lineage and southeast European lineage. While both genes revealed novel lineages A and B, an apparently novel lineage C was discovered just from the pool of larvae in the 16S tree (Figure 3.22, Figure 3.23).

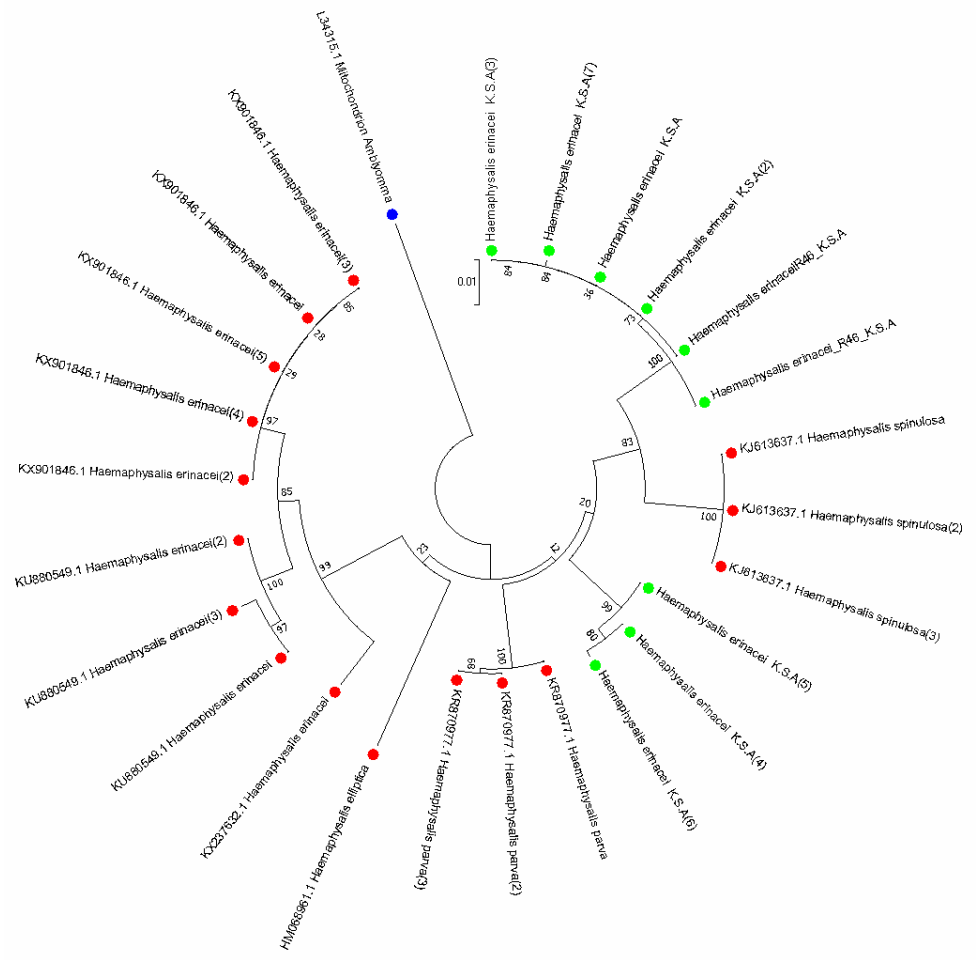


Figure 3.21. **Neighbour joining (NJ) phylogenetic tree of *Haemaphysalis* nymph 16S rRNA sequences using the maximum composite likelihood method.**

Bootstrap values based on 1,000 replicates are presented at the nodes. The scale bar measures evolutionary distance indicating substitutions per nucleotide. Sequences from the current study are shown in green, reference sequences from NCBI are in red, and the outgroup (blue) is from *Amblyomma variegatum*.

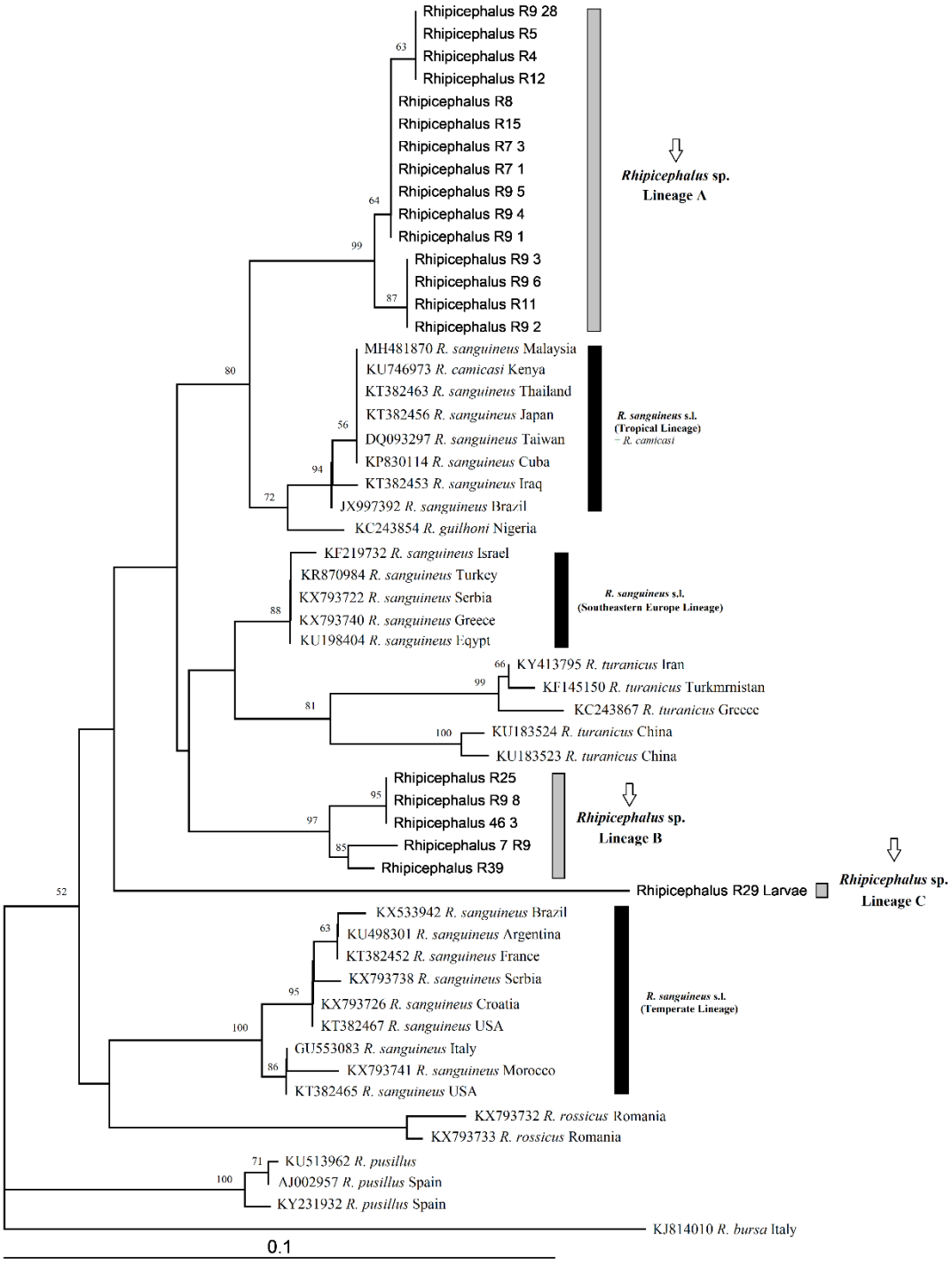


Figure 3.22. Neighbour joining (NJ) phylogenetic tree of *Rhipicephalus* spp. based on 16S rRNA sequences.

Bootstrap values based on 1,000 replicates are presented at the nodes. The scale bar measures evolutionary distance indicating substitutions per nucleotide. New sequences from the current study are shown in bold.

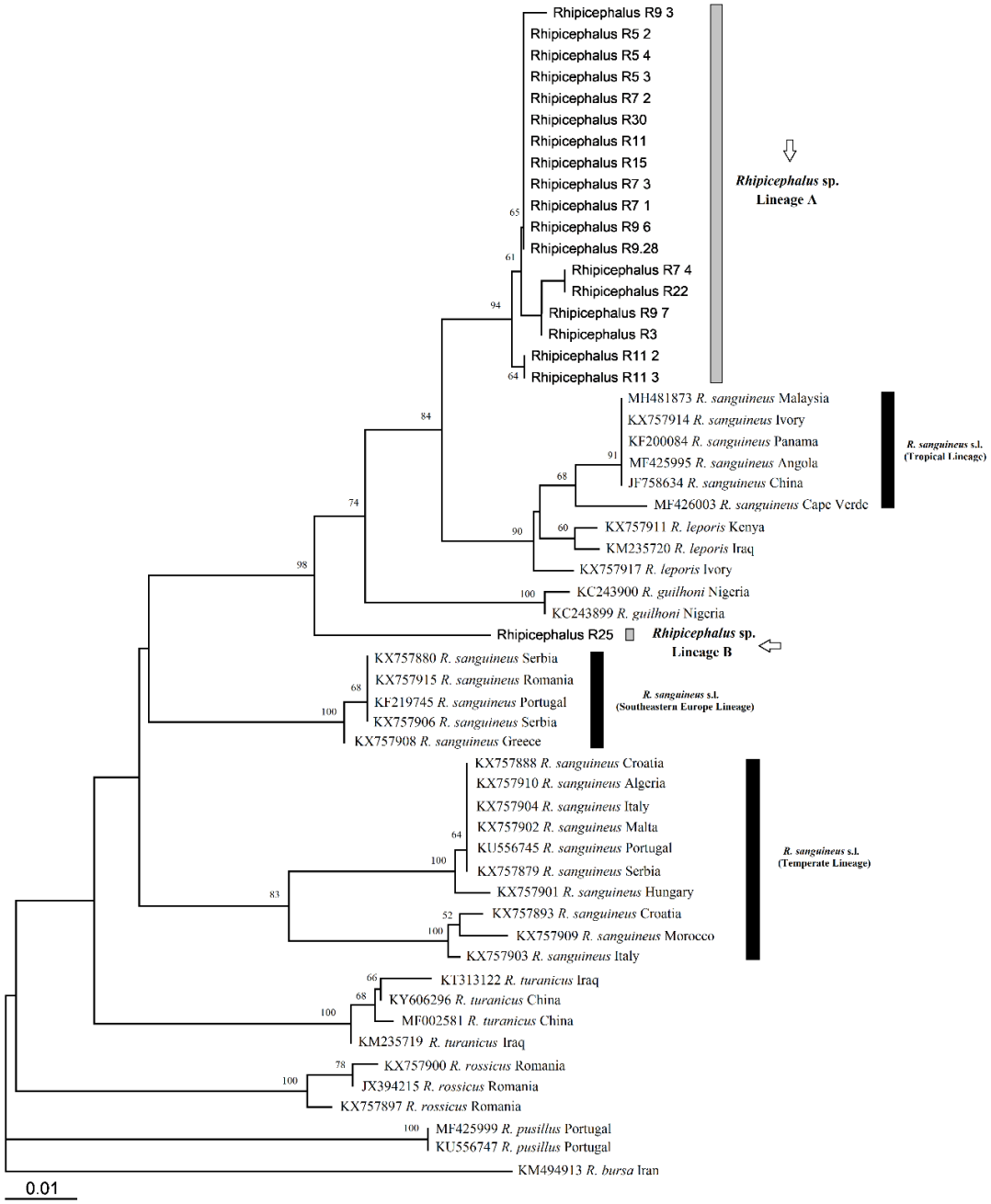


Figure 3.23. Neighbour-joining phylogenetic tree of *Rhipicephalus* spp. based on *COI* gene sequences. New sequences from the current study are shown in bold.

3.2.3 Ectoparasite infestation on rodents in Saudi Arabia

3.2.3.1 Chigger mite infestation on rodents in the Asir region

The highest number of chiggers were collected in Alous, followed by Wosanib and Alogl. The most striking result was that *A. dimidiatus* showed a higher prevalence of infestation with chigger mites (and other ectoparasites) than did *M. rex* and *M. yemeni* (Appendix 35). The overall infestation rate of chigger mites across all species and locations was 82.43%, with the highest rate recorded in Alous at 97.05%, followed by Wosanib (56.25%) and Alogl (79.16%) (Table 3.6).

Ericotrombidium kazeruni was the dominant chigger found on the rodents, with a prevalence of 67.6%, followed by *Schoutedenichia saudi sp. nov* (44.6%), *P. agamae* (37.8%), *A. browni* (33.8%), *E. caucasicum* and *M. muhaylensis* sp. nov. (27%) (Table 3.5). The highest mean intensity was also exhibited by *E. kazeruni* (46.3%), although the greatest number of chiggers of one species found on an individual rodent was 290 *S. saudi* sp. nov. (Table 3.7). Two of the rodent species, *A. dimidiatus* and *M. rex*, were infested by all six chigger species above. Moreover, female mice exhibited a higher mean abundance of chiggers than did males. *Acomys dimidiatus* and *M. rex* demonstrated the greatest chigger species richness.

The distribution of chigger species in each village is presented in (Figure 3.24). In Alous, *E. kazeruni, E caucasicum, P. agamae* and *S. saudi* sp. nov. were the most dominant species (Figure 3.24 A). In Wosanib, the pattern was quite different, as *E. kazeruni* was rare while *Pentidionis agamae, Schoutedenichia saudi sp. nov, S. zarudnyi, and M. muhaylensis* sp. nov. dominated (Figure 3.24 B). In Alogl, *E. kazeruni* and *M. hoogstraali* accounted for the vast majority of all chiggers collected (Figure 3.24C).

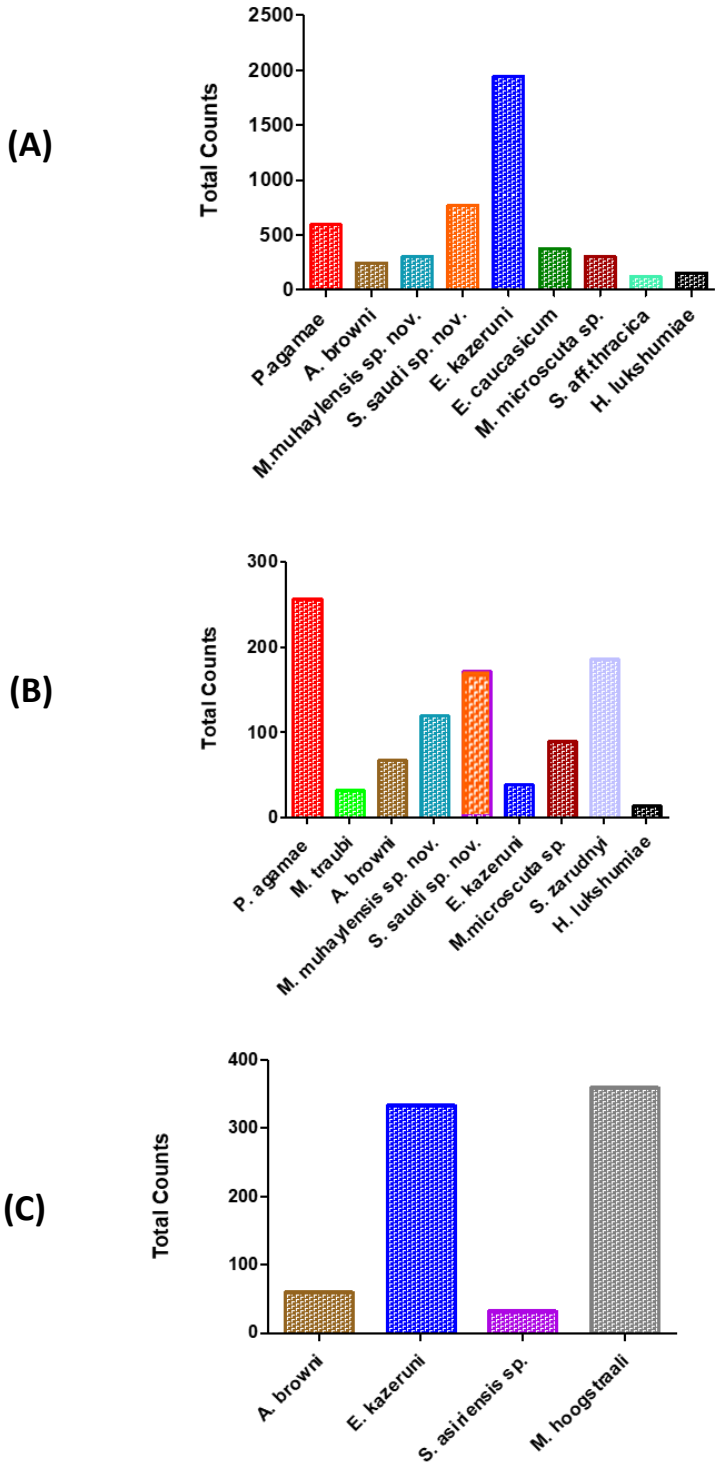


Figure 3.24. The most common chigger mites in the Asir region by village.

(A) Alous, (B) Wosanib, (C) Alogl.

Table 3.6. Infestation of chiggers on rodents in three studied sites in the Asir region during 2016 – 2017.

Village	Year	Month	Latitude	Longitude	Humidity (% RH)	Altitude (m)	Rodents (*n*)	Number infested	Infestation rate (%)
Alous	2016, 2017	July, August	18.27641	42.32061	41	1,594	34	33	97.05
Wosanib	2017	August	18.31564	42.21148	21-75	998	16	9	56.25
Alogl	2017	August	18.34654	42.31654	26-45	2,387	24	19	79.16
Total							74	61	82.43

Table 3.7. Prevalence, mean intensity, mean abundance and range of ectoparasite infestation on rodent hosts from the Asir region (host n = 74).

Parasite taxa	Number of host infested	Prevalence (%)[a] with [95% CI]	Mean intensity [b] [95% CI]	Mean abundance [95%[c] CI]	Range
Ectoparasite group					
Trombiculid mite	60	81.1 [70.4-88.7]	112.9 [88.2-145.8]	91.5 [68.5-120.1]	0-558
Mesostigmatid mite	19	25.7 [16.7-37.1]	10.1 [4.8-23.6]	2.6 [1.2-6.9]	0-83
Hard tick	52	70.3 [58.8-79.8]	13.8 [9.8-20.7]	9.7 [6.5-14.5]	0-104
Flea	42	56.8 [45.3-67.6]	13.9 [10.3-19.3]	7.9 [5.4-11.8]	0-63
Trombiculid mite					
Microtrombicula muhaylensis sp. nov *	20	27.0 [18.1-38.4]	21.3 [12.5-36.3]	5.8 [3.1-10.8]	0-104
Ascoschoengastia browni	25	33.8 [23.5-45.3]	14.9 [10.3-21.4]	5.0 [3.0-7.9]	0-50
Ericotrombidium caucasicum	20	27.0 [18.1-38.4]	19.5 [8.2-43.4]	5.3 [2.1–13.8]	0-142
Ericotrombidium kazeruni	50	67.6 [56.1-77.5]	46.3 [29.3-69.2]	31.3 [19.9-48.4]	0-272
Gahrliepia aff.lawrencei	4	5.4 [1.9-13.3]	3.8 [1.0-7.8]	0.2 [0.1-0.8]	0-10
Helenicula lukshumiae	11	14.9 [8.1-24.9]	15.4 [9.4-27.1]	2.3 [1.0-4.7]	0-53
Microtrombicula hoogstraali	14	18.9 [11.3-29.6]	25.7 [13.6-44.0]	4.8 [2.2-10.5]	0-89
Microtrombicula traubi	9	12.2 [6.3-21.5]	6.8 [4.2-11.0]	0.8 [0.4-1.8]	0-19
Microtrombicula microscuta sp. nov*	15	20.3 [12.4-30.9]	27.4 [17.8-37.7]	5.5 [3.1-9.4]	0-69
Microtrombicula hyraces	7	9.5 [4.5-18.7]	3.7 [2.7-4.6]	0.3 [0.1-0.7]	0-6
Pentidionis agamae	28	37.8 [27.4-49.3]	30.4 [19.6-44.9]	11.5 [6.9-18.8]	0-109
Schoengastiella wansoni	1	1.4 [0.1-7.2]	6 [NA][d]	0.1 [0-0.2]	0-6
Schoutedenichia asirensis sp. nov *	5	6.8 [2.7-15.3]	8.2 [2.6-14.4]	0.5 [0.1-1.6]	0-17
Schoutedenichia saudi sp. nov *	33	44.6 [33.7-56.1]	29.2 [18.8-59.2]	13.0 [7.8-28.7]	0-290

Table 3.7 continued					
Schoutedenichia aff.thracica	14	18.9 [11.3-29.6]	9.3 [6-14.8]	1.7 [0.8-3.2]	0-32
Schoutedenichia zarudnyi	19	25.7 [16.7-37.1]	11.4 [6.7-20.6]	2.9 [1.5-5.8]	0-59
Walchia parvula	4	5.4 [1.9-13.3]	6 [3-7.3]	0.3 [0.1-0.8]	0-8
Mesostigmatid mite					
Laelaps lamborni	15	20.3 [12.4-30.9]	5.5 [3.6-10.5]	1.1 [0.6-2.4]	0-26
Ornithonyssus bacoti	8	10.8 [5.1-20.1]	13.5 [1.5-46.0]	1.5 [0.2-5.9]	0-83
Hard tick					
Haemaphysalis sp.	50	67.6 [56.1-77.5]	12.2 [8.6-19.3]	8.2 [5.5-13.5]	0-104
Rhipicephalus sp.	17	23.0 [14.7-33.7]	6.5 [3.9-11.1]	1.5 [0.8-3.1]	0-25
Flea					
Leptopsylla aethiopica	1	1.4 [0.1-7.2]	1 [NA][d]	0.01 [0-0.04]	0-1
Parapulex chephrensis	20	27.0 [18.1-38.4]	5.8 [3.8-8.9]	1.5 [0.9-2.7]	0-23
Xenopsylla cheopis	5	6.8 [2.7-15.3]	2.8 [1.6-3.6]	0.2 [0.1-0.4]	0-4
Lice					
Polyplax brachyrrhyncha	30	40.5 [29.6-52.0]	9.9 [7.2-14.3]	4.1 [2.7-6.2]	0-40
Polyplax oxyrrhyncha	25	33.8 [23.5-45.3]	6.3 [4.2-10.4]	2.1 [1.3-3.7]	0-32

[a] Prevalence (%) is the percentage of hosts infested

[b] Mean intensity is the mean number of parasite individuals found in infected hosts

[c] Mean abundance is the mean of the number of individuals of a particular parasites species per host examined [Prevalence (%) x mean intensity /100]

[d] Too low sample size for calculation

**Novel chigger species*

3.2.3.2 Infestation with other ectoparasites on small mammals in the Asir region

Haemaphysalis spp. had the highest prevalence of infestation, being found on 50 rodents (67.6%) compared to *Rhipicephalus* spp. at only 23% (Table 3.7). The lice *P. brachyrrhyncha and P. oxyrrhyncha* (40.5%, 33.8%; respectively) were moderately common, as was the flea *Parapulex chephrensis* with a prevalence of 27%. The rarest species were the fleas *Xenopsylla cheopis* and *Leptopsylla aethiopica* at a prevalence of 6.8% and 1.4%, respectively, (the latter represented only by a single specimen). The gamasid mite *Laelaps lamborni* was present on all five rodent hosts (*A. dimidiatus*, *M. yemeni*, *M. rex*, *R. rattus* and *M. musculus*), but at low intensity and abundance (see Table 3.7).

A total of 1496 ectoparasites were acquired from the 74 rodent specimens. Among the five rodent species groups, *Acomys dimidiatus and Meriones rex* had the highest rate of ectoparasite infestation (Table 3.8). The overall infestation rate with ectoparasites was 93.24 %, with the highest rate recorded in Alous at 100%, followed by Wosanib at 93.75% and Alogl at 83.33% (Table 3.9).

Table 3.8. Distribution of non-chigger ectoparasite species by rodent host species.

	Number of ectoparasites			
Rodent species (*n*)	**Ticks**	**Lice**	**Fleas**	**Gamasid mites**
A. dimidiatus (45)	378	365	123	64
M. rex (17)	328	59	-	7
M. yemeni (8)	10	32	4	25
M. musculus (3)	3	-	3	12
R. rattus (1)	-	-	-	83

Table 3.9. Infestation with ectoparasites (excluding trombiculids) on rodents in three studied sites in the Asir region during 2016 – 2017.

Village	Year	Month	Latitude	Longitude	Humidity (% RH)	Altitude (m)	Rodents (*n*)	Number infested	Infestation rate (%)
Alous	2016, 2017	July, August	18.27641	42.32061	41	1,594	34	34	100
Wosanib	2017	August	18.31564	42.21148	21-75	998	16	15	93.75
Alogl	2017	August	18.34654	42.31654	26-45	2,387	24	20	83.33
Total							74	69	93.24

Between the three sampling sites, *Haemaphysalis* spp. ticks dominated in all locations, but *Rhipicephalus* spp. were not found in Alous (Figure 3.25). Regarding fleas, *P. chephrensis* was unique to Alous where *X. cheopis* was absent (Figure 3.25A). Lice were abundant in Alous and Wosanib, whereas they were absent from Alogl (Figure 3.25C). The distribution of *O. bacoti* was highly skewed by the presence of 83 specimens on a single host (a rat) in Alous (Figure 3.25A).

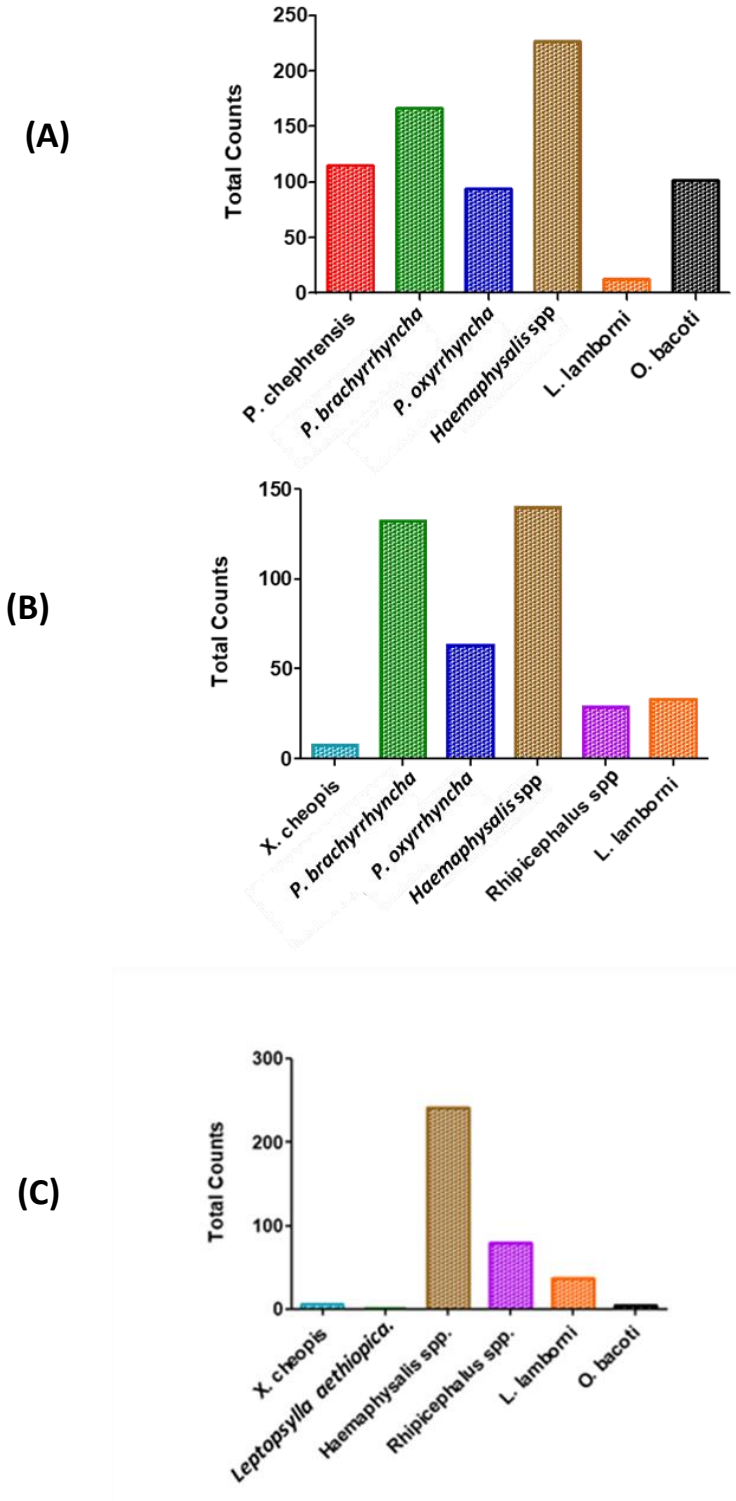


Figure 3.25. Distribution of non-trombiculid ectoparasites in the Asir region.

(A) Alous, (B) Wosanib, (C) Alogl.

3.2.4 Host-ectoparasite relationships in the Asir region

A total of 8,270 ectoparasites were discovered parasitizing five rodent species. The ectoparasite diversity among different habitats, study sites, host species, and host sex are presented (Table 3.10). The cumulative number of ectoparasite species from the sample of 74 rodents is shown as an accumulation curve plot (Figure 3.26). The curve of ectoparasite species richness was found to rise sharply in the first phase up until around 20 rodents examined, then levelled-off in the second phase (between 20 - 40 rodents examined), reaching a plateau in the third phase after 40 rodents were examined. Therefore, despite the small sample size, it appears sufficient to capture ectoparasite diversity in this setting. In addition, when examining accumulation curves separated by host sex, males contributed more information to species richness than did females (Figure 3.27A). This did not appear to be caused solely by the twofold larger sample size of males versus females, because mean ectoparasite abundance was slightly greater on females than on males, Although there was no statistically significant difference (Figure 3.27B).

Additionally, the diversity of ectoparasite species was different among the studied sites. To start with, Alous had the highest species richness (23 species), followed by Wosanib (19 species) and Alogl (15 species) (Table 3.8). Accordingly, the accumulation curve of species richness in Alous was significantly higher in the initial phase (around 10 host specimens) compared to Wosanib and Alogl (Figure 3.28A). The difference in species richness was statistically significant between Alous and Alogl, and Wosanib and Alogl, but not between Alous and Wosanib (Figure 3.28B).

According to host species (Figure 3.29A), *A. dimidiatus* had the highest ectoparasites species richness (25 species), followed by *M. rex* (16 species) and *M. yemeni* (12 species); whereas *M. musculus and R. rattus* had the lowest species richness (7 and 2 species, respectively). However, these latter two hosts had very small sample sizes (only three *M. musculus* and one *R. rattus*). The accumulation curves only reached saturation for *A. dimidiatus* (Figure 3.29A), which had by far the largest sample size. Moreover, there was a

statistically significant difference in mean ectoparasite species richness between the three main host species, confirming the pattern *A. dimidiatus* > *M. rex* > *M. yemeni* (Figure 3.29B).

In terms of habitat types, the ectoparasite species richness differed between mountainous terrain and agricultural fields (Figure 3.30a), reaching saturation only for the former with its much larger sample size. Accordingly, the rodents trapped on mountain slopes harboured a significantly higher species richness than those from agricultural fields (with Wilcoxon test: w = 242.5, p = 0.0018) (Figure 3.30B). However, habitat type, village and rodent species were confounded variables, as Alous was entirely mountainous, whereas all but one rodent trapped in agricultural fields in the other two villages was a king jird (*M. rex*).

Table 3.10. Diversity estimators and indices of ectoparasite infestation on rodents from Saudi Arabia

Group	No. of samples	Species richness	1st order Jacknife[a]	Chao[b]	Bootstrap[c]	Shannon[d]	Simpson[e]
Habitat							
Agricultural field	17	12	16.7	17.88	14.03	2.01	0.83
Mountain	57	26	28.0	26.98	26.79	2.98	0.94
City							
Alogl	24	15	18.8	17.55	16.91	2.27	0.87
Alous	34	23	24.9	23.97	23.84	2.89	0.93
Wosanib	16	19	21.8	21.11	20.39	2.72	0.92
Rodent species							
Acomys dimidiatus	45	25	26.0	25.49	25.61	2.94	0.94
Meriones rex	17	16	20.7	18.94	18.34	2.33	0.87
Mus musculus	3	7	10.3	11.17	8.55	1.89	0.84
Myomyscus yemeni	8	12	18.1	19.15	14.73	2.35	0.89
Rattus rattus	1	2	2.00	2.00	2.00	0.69	0.50
Host sex							
Female	24	23	23.00	23.00	23.65	2.91	0.93
Male	50	26	28.94	30.41	27.28	2.98	0.94
Total	74	26	27.97	26.98	26.77	2.98	0.94

[a] *Jacknife is used to estimate the accuracy of an estimator*

[b] *Chao is an estimator of the abundance in each samples*

[c] *. Bootstrap* is a measure of accuracy *in random samples*

[d] *Shannon is a measure of abundance and evenness of the species diversity in a community*

[e] *Simpson is a measure of the species richness*

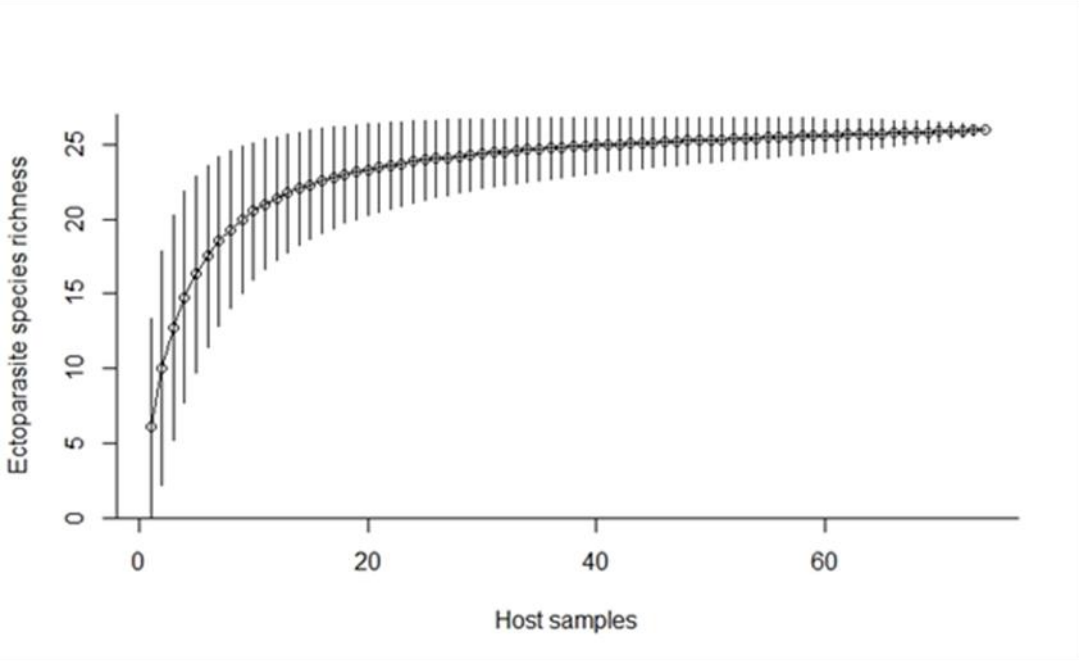


Figure 3.26. Ectoparasite species accumulation curve from 74 rodent specimens.

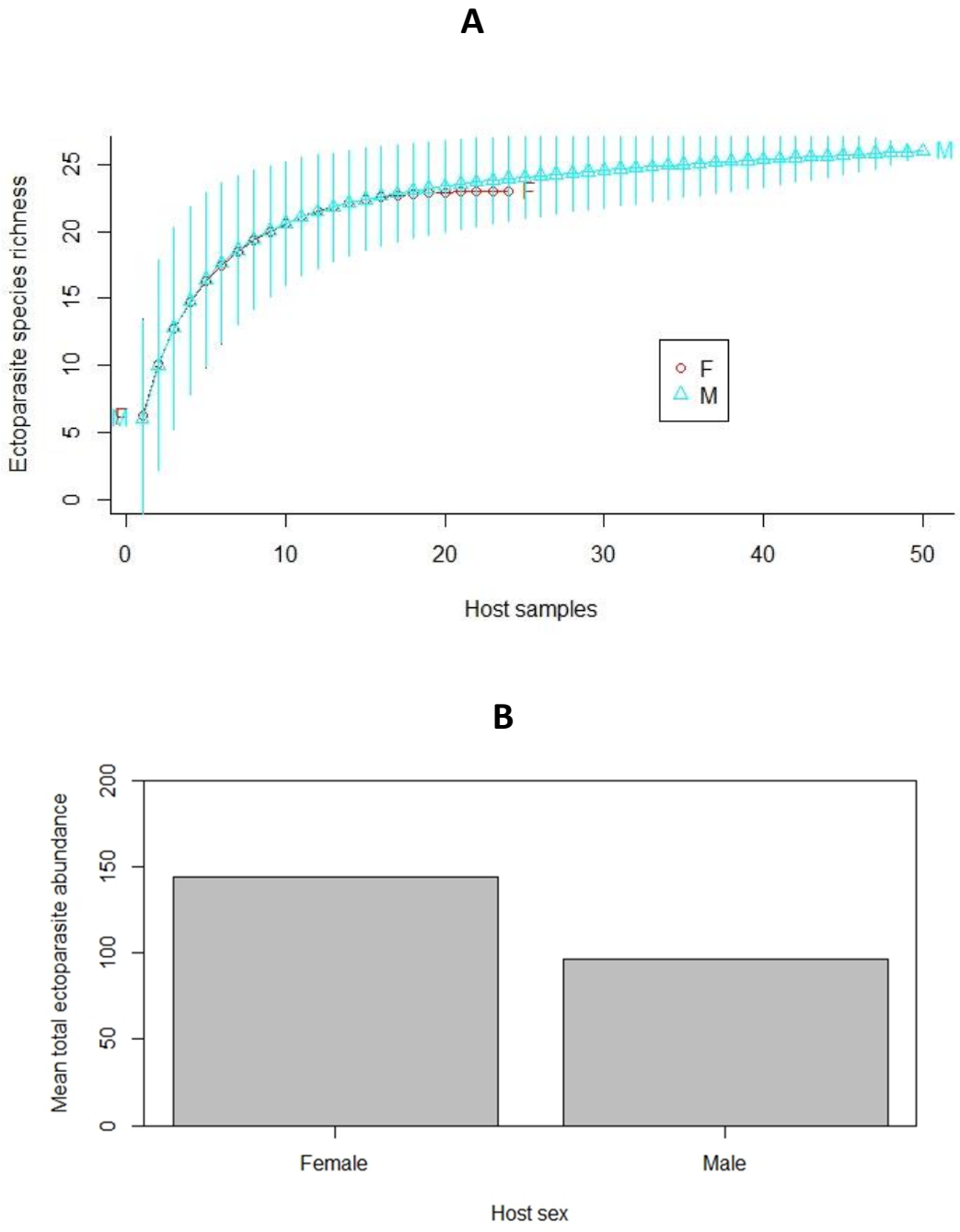


Figure 3.27. Ectoparasite species richness and abundance by host sex.

(A) accumulation curves of ectoparasite species richness by host sex, and (B) Analysis of difference in mean ectoparasite abundance on individual hosts by host sex. Kruskal-Wallis test with Holm's correction: Wilcoxon test: w = 764, p = 0.0582.

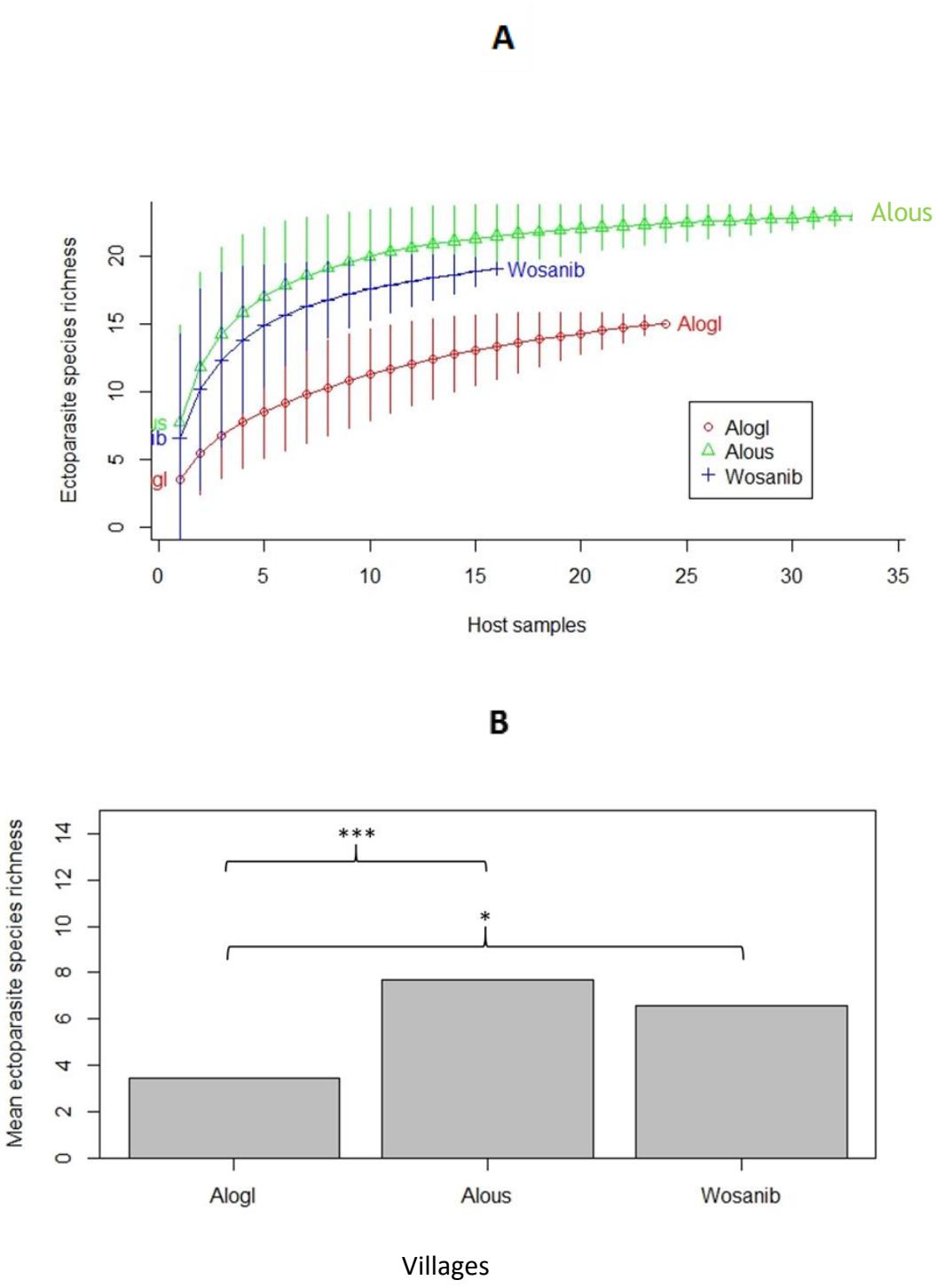
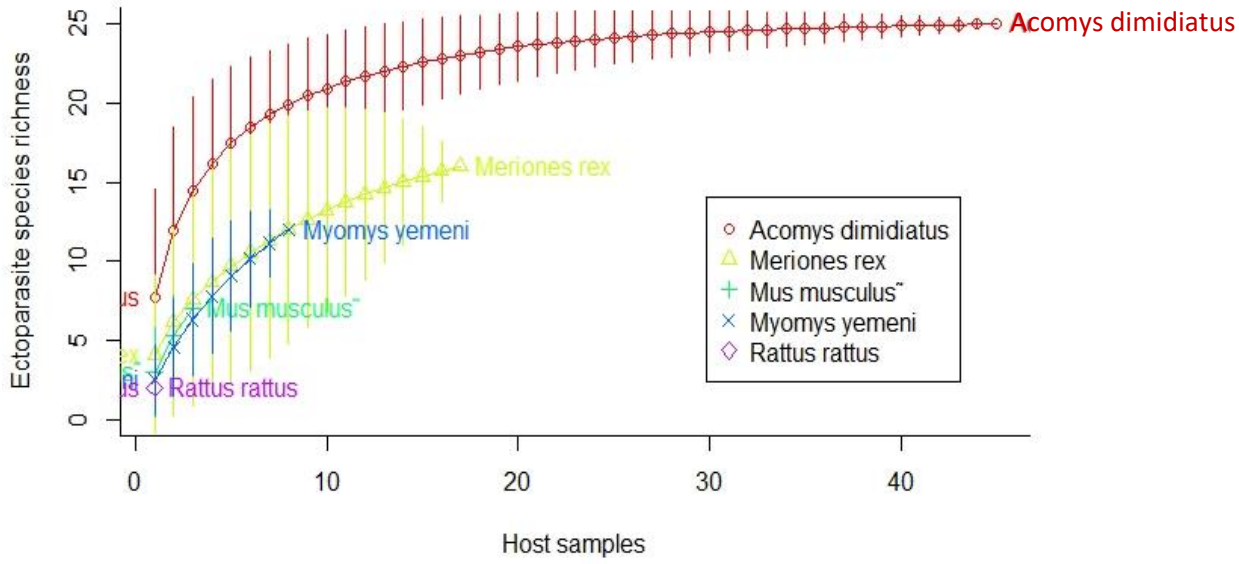


Figure 3.28. Ectoparasite species richness between sample locations.

(A) Accumulation curves of ectoparasite species between villages, and (B) Analysis of difference in mean ectoparasite species richness on individual hosts per site. Kruskal-Wallis test with Holm's correction: Kruskal-Wallis = 20.67, $p < 0.001$. P-value; * = 0.05-0.01, ** = 0.01 – 0.001, *** < 0.001.

A



B

Figure 3.29. Ectoparasite species richness among rodent species.

(A) Accumulation curves of ectoparasite species among rodents species, and (B) Analysis of differences in mean ectoparasite species richness on individual hosts among rodent species. Kruskal-Wallis test with Holm's correction: Kruskal-Wallis = 25.48, $p < 0.001$. P-value; * = 0.05-0.01, ** = 0.01 – 0.001, *** < 0.001.

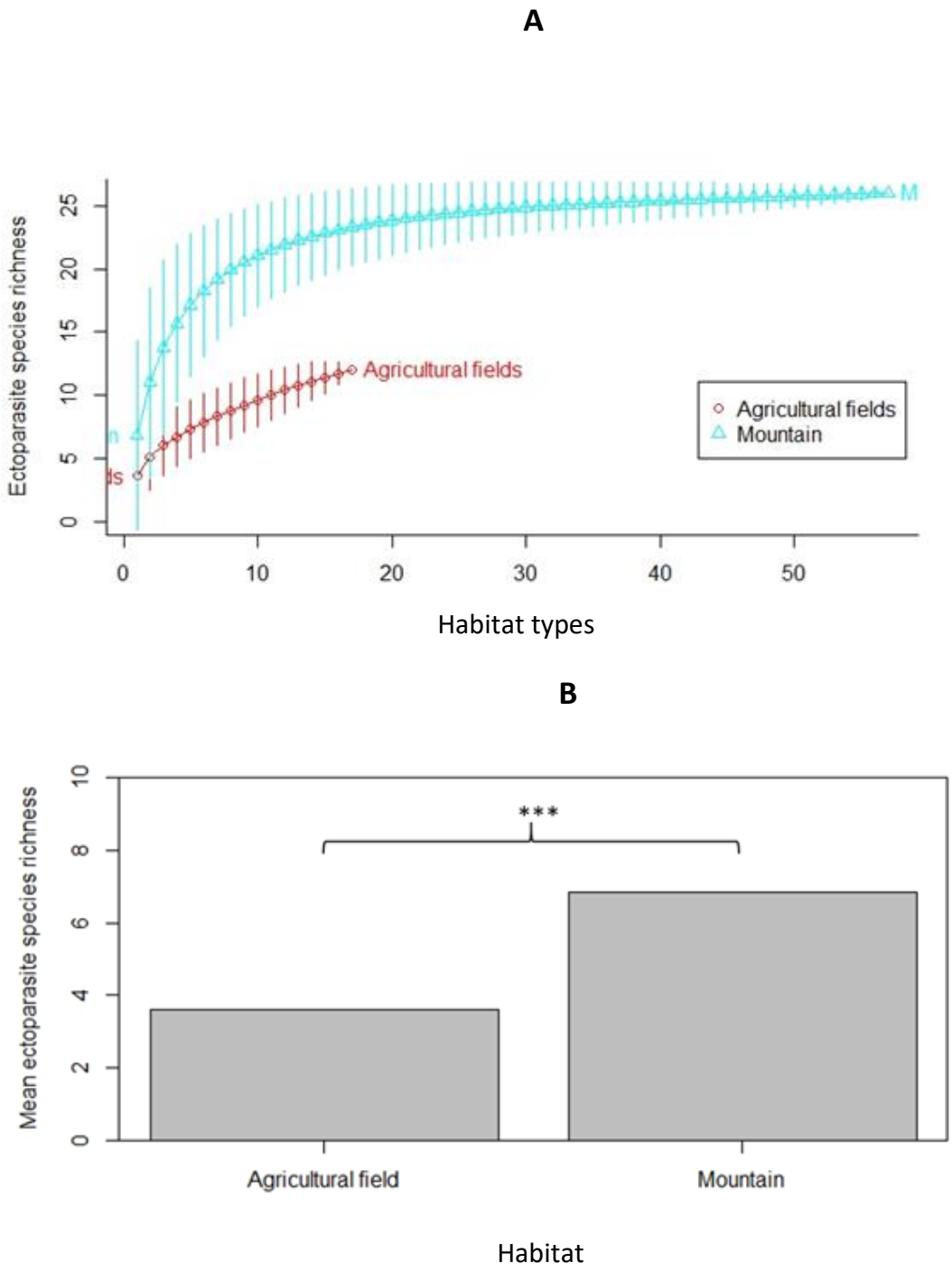


Figure 3.30. Habitat types influence ectoparasites species richness.

(A) Accumulation curves of ectoparasite species between mountainous and agricultural regions (B) Analysis of difference in mean ectoparasite species richness using Wilcoxon test: w = 242.5, p = 0.0018.

3.2.5 Host-ectoparasite network analysis

3.2.5.1 Bipartite network

In order to investigate the host-ectoparasite interaction within study sites, bipartite network and unipartite network analyses were used. The interaction between ectoparasite species and individual hosts was examined in each study site. In Alous, the network was clearly dominated by interactions between *A. dimidiatus* (accounting for all but one rodent host) and a high diversity of chigger species, with the other ectoparasite taxa having a more minor role (Figure 3.31). In other words, chigger mites represent the dominant ectoparasite feeding on rodents in this location. In contrast, mesostigmatid mites (*L. lamborni* and *O. bacoti*) were found on very few *A. dimidiatus* hosts. With the exception of *Schoengastiella wansoni*, chigger mite species were widely distributed between individual hosts, such that every *A. dimidiatus* individual was co-infected with more than one chigger species (Figure 3.31).

In Wosanib (Figure 3.32), the dominance of *A. dimidiatus* as the main host and chiggers as the principal ectoparasites was less pronounced, with a slightly lower species diversity of chiggers. However, rates of coinfection for chiggers and other ectoparasites remained high. Ticks (*Haemaphysalis* spp. and *Rhipicephalus* spp.) and lice (*Polyplax* spp.) were present on most hosts at this location, irrespective of rodent species (Figure 3.32). Additionally, some ectoparasites appeared to have less interaction with few rodents, which means they may be more host specific; for example, mesostigmatid mites (*L. lamborni* and *O. bacoti*) and fleas (*Xenopsylla cheopis)* in Wosanib were restricted to *A. dimidiatus* and fleas (*Leptopsylla aethiopica* and *Xenopsylla cheopis*) in Alogl were restricted to *M. yemeni* and *M. musculus*. Alogl had the least diversity of chiggers with clear dominance by two species, *E. kazeruni* and *M. hoogstraali* (Figure 3.33). At this site, ticks and gamasid mites were more prominent in the host-ectoparasite network, whereas fleas played a minor role and lice were completely absent (Figure 3.33).

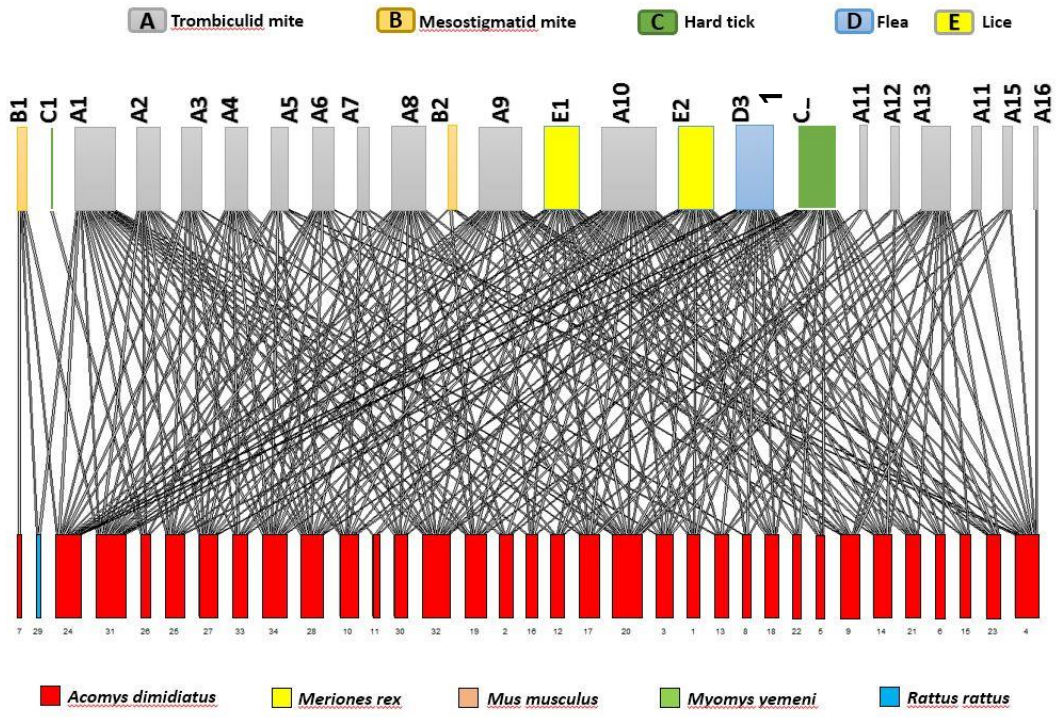
3.2.5.2 Unipartite networks

Unipartite networks of ectoparasite species shared among individual hosts are shown in Figures 3.34 and 3.35. The lines between hosts indicate the connections (shared

ectoparasite species) between individual rodents in the network, while the number in each node is the Eigenvector centrality score, which is proportional to the degree of connectedness.

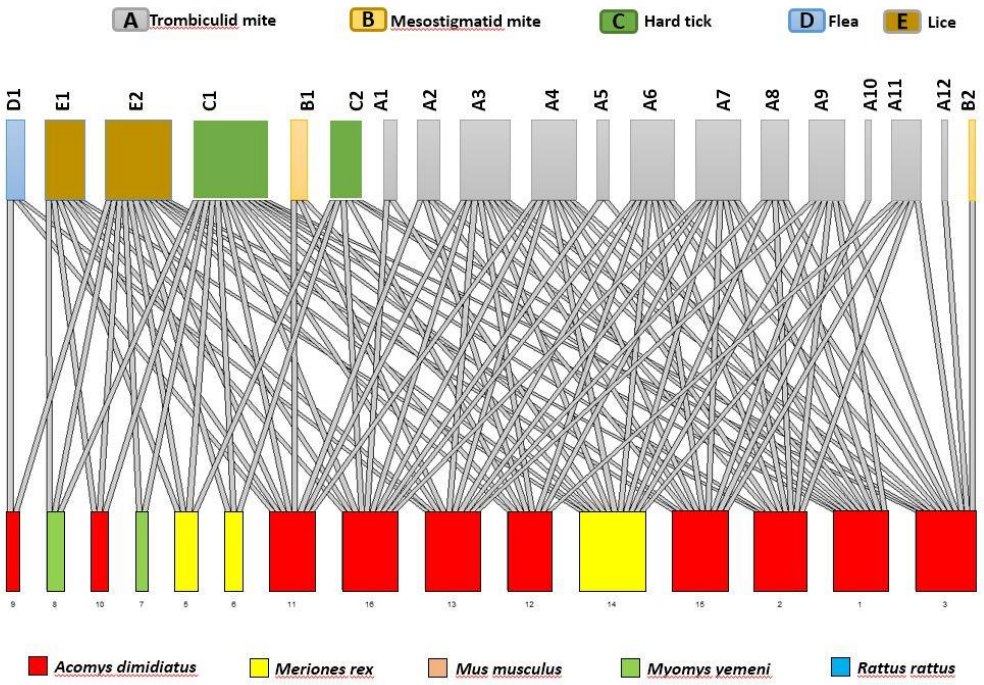
In Wosanib, the highest centrality scores were observed with one individual of *M. rex* and several specimens of *A. dimidiatus* (Figures 3.34). However, in Alogl, one individual each of *M. yemeni* and *A. dimidiatus* had the greatest number of connections, which were more moderate for *M. rex* and very low for *M. musculus* (Figure 3.35). A network is not shown for Alous due to the almost complete dominance by *A. dimidiatus* at that site.

In terms of network analysis, parameters of rodent-ectoparasite interactions from different study sites in the Asir region were calculated, containing network modularity, network connectance, links per species, linkage density, interaction evenness, and nestedness temperature as shown in Table 3.11. These network parameters assisted to distinguish and identify the construction of host-ectoparasite connections in the three sample sites in the Asir region and to visualize overall interactions between hosts and ectoparasites. Among the three cities, the highest linkage density, interaction evenness, and nestedness temperature were present in the Alous network, where the rodent species richness was lower than the other two locations (*i.e*., all but one rodent was *A. dimidiatus*). However, Alogl had the highest network modularity (0.354), which is reflected in the network topology (Figure 3.35), whereas this measure was lowest in Alous (0.238). In contrast, Wosanib showed the highest network connectance (0.368) of the three sites.



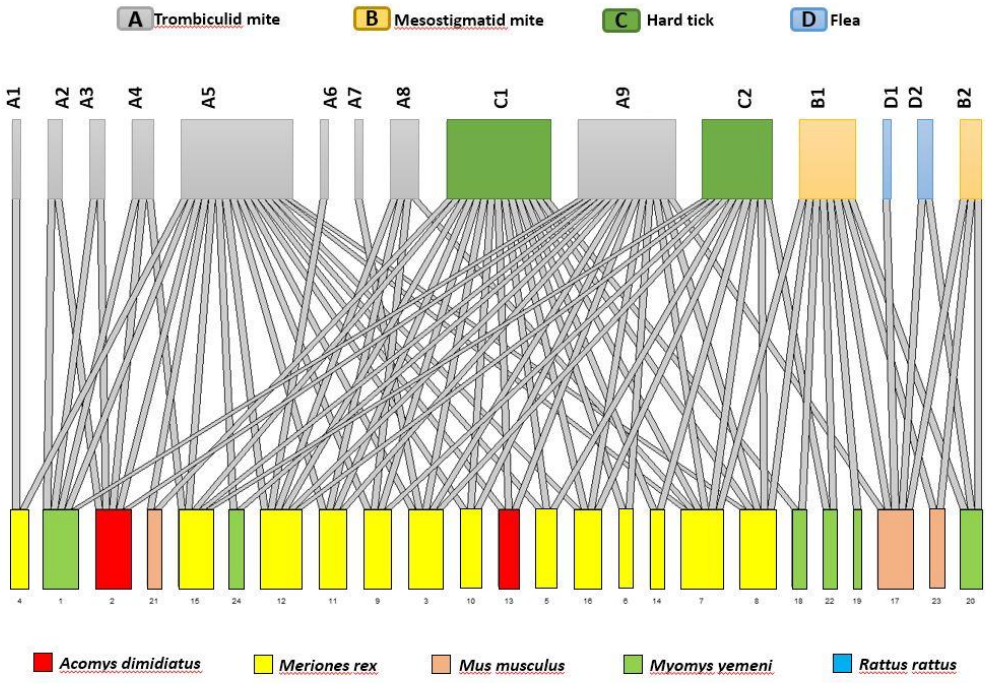
A1	*Pentidionis agamae*	**A14**	*Microtrombicula traubi*
A2	*Schoutedenichia sp., aff. thracica*	**A15**	*Gahrliepia lawrencei*
A3	*Helenicula lukshumiae*	**A16**	*Schoengastiella wansoni*
A4	*Microtrombicula muhaylensis* ***sp. nov***	**B1**	*Ornithonyssus bacoti*
A5	*Microtrombicula microscuta* ***sp. nov***	**B2**	*Laelaps lamborni*
A6	*Schoutedenichia zarudnyi*	**C1**	*Rhipicephalus* spp.
A7	*Microtrombicula hyraces*	**C2**	*Haemaphysalis* spp.
A8	*Ascoschoengastia browni*	**D1**	*Parapulex chephrensis*
A9	*Schoutedenichia saudi* ***sp. nov***	**E1**	*Polyplax oxyrrhyncha*
A10	*Ericotrombidium kazeruni*	**E2**	*Polyplax brachyrrhyncha*
A11	*Microtrombicula centropi*		
A12	*Walchia parvula*		
A13	*Ericotrombidium caucasicum*		

Figure 3.31. **Bipartite interaction network graph illustrating the host-ectoparasite species interactions in Alous.**



A1	*Schoutedenichia* sp., aff. *thracica*	**A10**	*Microtrombicula hyracis*
A2	*Microtrombicula traubi*	**A11**	*Microtrombicula microscuta* ***sp. nov***
A3	*Schoutedenichia saudi* ***sp. nov***	**A12**	*Helenicula lukshumiae*
A4	*Schoutedenichia zarudnyi*	**B1**	*Laelaps lamborni*
A5	*Ericotrombidium caucasicum*	**B2**	*Ornithonyssus bacoti*
A6	*Pentidionis agamae*	**C1**	*Haemaphysalis* spp.
A7	*Schoutedenichia asirensis* ***sp. nov.***	**C2**	*Rhipicephalus* spp.
A8	*Ascoschoengastia browni*	**D1**	*Xenopsylla cheopis*
A9	*Ericotrombidium kazeruni*		
E1	*Polyplax oxyrrhyncha*		
E2	*Polyplax brachyrrhyncha*		

Figure 3.32. Bipartite network graph showing the host-ectoparasite species in Wosanib.



A1	*Microtrombicula microscuta* ***sp. nov***	**A9**	*Microtrombicula hoogstraali*
A2	*Schoutedenichia asirensis* ***sp. nov.***	**B1**	*Laelaps lamborni*
A3	*Schoutedenichia saudi* ***sp. nov***	**B2**	*Ornithonyssus bacoti*
A4	*Ascoschoengastia browni*	**C1**	*Haemaphysalis* spp.
A5	*Ericotrombidium kazeruni*	**C2**	*Rhipicephalus* spp.
A6	*Schoutedenichia* sp., aff. *thracica*	**D1**	*Leptopsylla aethiopica*
A7	*Microtrombicula traubi*	**D2**	*Xenopsylla cheopis*
A8	*Ericotrombidium caucasicum*		

Figure 3.33. Bipartite network chart presenting the host-ectoparasites species interactions in Alogl.

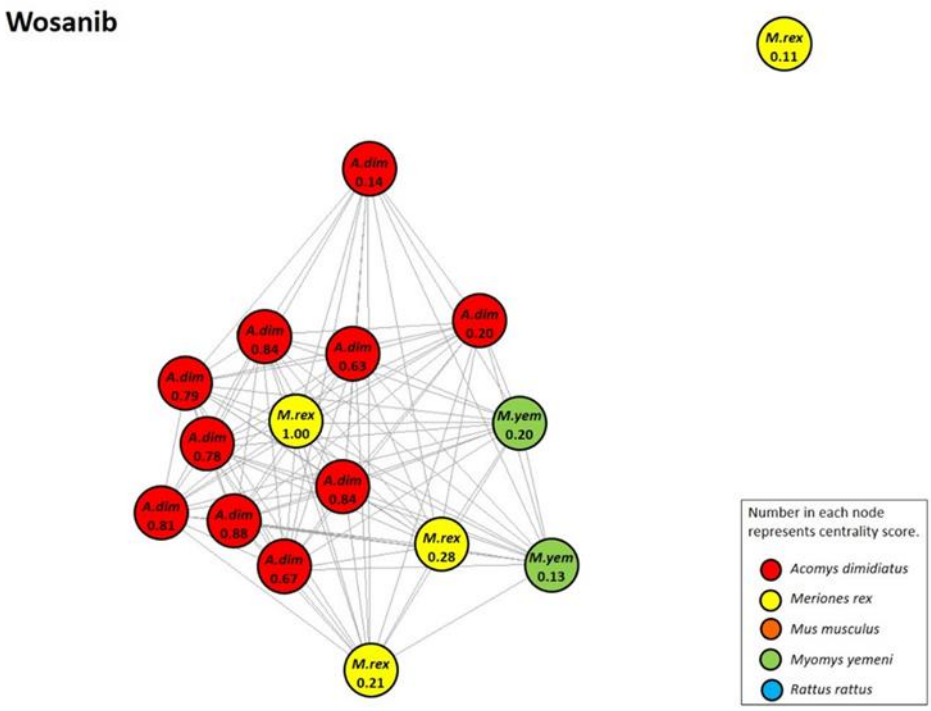


Figure 3.34. Unipartite network illustrating patterns of host-ectoparasite interactions in Wosanib.

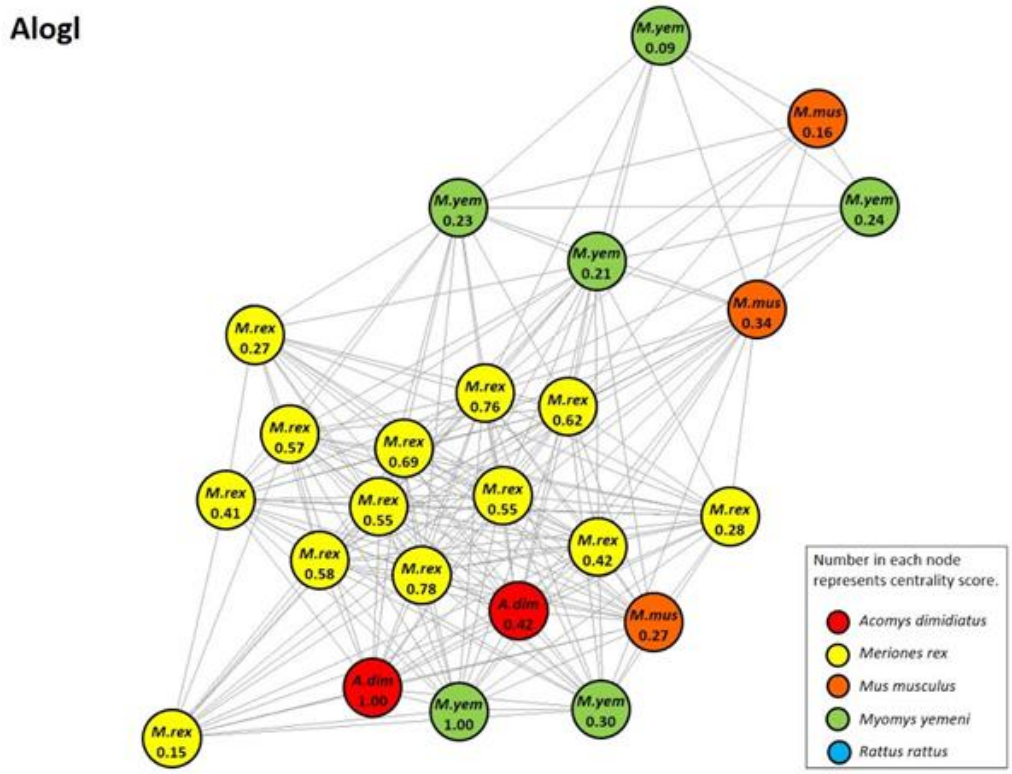


Figure 3.35. Unipartite network illustrating patterns of host-ectoparasite interactions in Alogl.

Table 3.11. Network analysis parameters of rodent-ectoparasite interactions from different study sites in Saudi Arabia.

Parameter	Study site		
	Alogl	**Alous**	**Wosanib**
Number of rodent host	24	34	16
Rodent observed species richness	4	2	3
Ectoparasite observed species richness	15	23	19
Network modularity (likelihood)	0.354	0.238	0.247
Network connectance	0.231	0.335	0.368
Links per species	2.128	4.596	3.088
Linkage density	7.409	12.95	8.352
Interaction evenness	0.751	0.835	0.823
Nestedness temperature	10.818	21.670	7.051

3.2.6 Discussion

3.2.7 Rodent identification

Rodents are key hosts for several groups of ectoparasitic arthropods. The hosts' environment plays a crucial role in ectoparasite survival and therefore richness. The Asir region is situated in the south-western part of the Kingdom of Saudi Arabia between the latitudes of 17°25′ north and 19°50′ north and between longitudes of 50°00′ east and 41°50′ east. Much of region is represented by highlands, which precipitate much more rainfall compared to the other regions of the country, particularly in the summer. This area is rich in wadis, agricultural land and mountains covered with natural vegetation (Jaber & Marzuki, 2018 ; Alzedee, 2004). Because of this, the Asir region tends to have the greatest biodiversity for wild rodents and their ectoparasites. In the current study, a preliminary survey of small mammal hosts was been conducted in the Asir region during the summers of 2016 and 2017. Five rodent species were identified: *A. dimidiatus, R. rattus*, *M. musculus*, *M. yemeni* and *M. rex* (Family Muridae). While *A. dimidiatus* and *M. rex* were reported previously from the Asir region by Al-Mohammed 2008, there seem to be no other reports of other species being identified in Asir**.** However, in the survey conducted on wild rodents from different cities in Saudi Arabia documented by Harrison (1972) and Buttiker and Harrison (1982), 15 rodent species were reported, of which *R. rattus* was the most prevalent, followed by *M. musculus* and *Rattus norvegicus.* Of course, these three rodents are invasive species worldwide associated with urbanisation and human-disturbed habitats. However, Al-Khalili (1984) reported that the most common rodents in the southwest Saudi Arabia were *A. dimidiatus* and Wagner's gerbil (*Gerbillus dasyurus*), and the scarcest species were *M. yemeni,* the Asian garden dormouse (*Eliomys melanurus*), *and Meriones rex*.

Interestingly, *R. rattus* was identified as the most common rodent in previous research in the Ha'il region of northern Saudi Arabia, but in the current study, only one rat was found (in Alous). This may be due to seasonal variations that can influence the population of rats, or perhaps *R. rattus* does not find mountainous habitat suitable to thrive, despite the presence of human dwellings near our study sites (Asiry & Fetoh, 2014; Twigg *et al.,*1992). A study from Egypt identified *R. rattus, R. norvegicus* and Shaw's jird (*Meriones shawi*) as

the most dominant hosts for ectoparasites, while *M. musculus* was infested by the lowest numbers (El-Deeb *et al.,*1999). Furthermore, a similar trend was reported in southern Iran, where 77 rodents (mostly identified as *R. norvegicus, R. rattus, M. musculus*), together with one unidentified hamster species, were infected with ectoparasites (Kia *et al.,*2009).

3.2.8 Ectoparasite identification

The current study is the first to investigate chigger mite diversity in Saudi Arabia; indeed, across the whole Arabian Peninsula, very few records exist for chiggers (Stekolnikov *et al.,*2012; Stekolnikov, 2018). We identified 19 chigger species, including four novel species; moreover, many of the other 15 species were previously known only from their type localities. This reflects insufficiency of studies on the chigger fauna of Africa and Middle and Western Asia. Therefore, our study provides a significant addition to the list of African species found outside the continent (Table 3.4).

In addition to the African species, some chiggers species we identified in Saudi Arabia have been reported previously in neighbouring countries, such as *H. lukshumiae*, *S. zarudnyi*, *E. kazeruni, M. traubi,* and *P. agamae* from Iran (Nadchatram & Traub, 1971, Kudryashova, 1976, Muljarskaja & Verdieva, 1974, and André, 1929). Interestingly*, M. hoogstraali* was originally described from Yemen, which is bordered by the Asir region of Saudi Arabia (Radford, 1954). Thus, the migration of rodents with their ectoparasites is likely across the international border. The presence of "African" chigger species in Arabia is more difficult to explain, although chigger mites (like immature ticks) can travel long distances on migratory birds (Varma 1964). Consistent with this hypothesis, *Microtrombicula centropi* has been reported previously only from the black coucal (*Centropus grillii*) in the DR Congo, which is migratory in some of its range (Erritzøe *et al.,*2012). Overall, we found that two species from the known Iranian fauna (*E*. *kazeruni* and *P. agamae*) were the most abundant species of chiggers at our study sites, although *S. saudi* sp. nov. was locally abundant in Alous. Notably, despite its abundance on rodents in our study, *P. agamae* has been reported previously only from lizards in Israel-Palestine and Iran (Stekolnikov *et al.,*2019).

A minimum of nine non-trombiculid ectoparasite species were observed in our study in Asir, comprising three species of fleas, two species of lice, two species of gamasid mites, and at least two species of ticks. There appears to be only one prior study on rodent

ectoparasites from Asir, which identified *R. sanguineus* and *R. turanicus* ticks after rearing nymphs from wild-caught rodents to adulthood (Al-Mohammed 2008). Our results based on molecular barcoding showed the presence of three novel lineages of *Rhipicephalus* spp. ticks and two distinct clades of *Haemaphysalis* based on phylogenetic analysis, but without obtaining adult specimens, formal descriptions of putative new species or subspecies will not be possible. It is likely that the adults of *Rhipicephalus* spp. will be found on dogs or wild carnivores in the region, while adult *Haemaphysalis* spp. might feed on hedgehogs, dogs or sheep.

The Oriental rat flea *X. cheopis* and *R. sanguineus* have been recorded in Hail city in common with our results (Asiry & Fetoh, 2014). To the best of our knowledge, the flea *L. aethiopica* and the gamasid mite *L. lamborni* and have not been reported previously from Saudi Arabia. Interestingly, we found a single female specimen of *L. aethiopica* on a *M. musculus*, and this flea specimen did not conform to the description of the known subspecies. This could be a new subspecies but cannot be formally described without more materials, particularly male specimens.

3.2.9 Ectoparasite diversity in the Asir region

In this study, we found a relatively high diversity of ectoparasites on rodents. This could be due to several environmental factors such as the humidity, temperature and rainfall distribution within the Asir region. These factors may not only favour larger and/or more diverse rodent populations than the drier regions of Saudi Arabia, but also more conducive microhabitats for ectoparasites that have free-living stages in the environment, such as chiggers, ticks and fleas. The varied vegetation and agricultural areas on the slopes of the Asir Mountains would be a source of food for rodents, as well as shade and a more humid microclimate for ectoparasites in the environment than the barren rocky outcrops also found in the region (Miyazaki *et al.,*2007). There were surprising differences in ectoparasite distribution and diversity between the three sites considering that the distance between each was only ~10 km. The highest diversity of chiggers and other ectoparasites was found in Alous, which lacks agricultural land, suggesting that human disturbance reduced diversity at the other sites, or that *A. dimidiatus* (essentially the only species caught in Alous) is a particularly good host for ectoparasites. Thus, it is difficult to separate the possible effects of habitat (mountain or agricultural land) from host-specific effects on ectoparasite

diversity. Nevertheless, a higher species diversity of chiggers on rodents in mountainous, uncultivated land compared with cultivated flatlands has also been reported from Yunnan, China (Peng *et al.,*2018; Peng *et al.,*2016; Gong *et al.,*2005). Thus, trapping location, the host species, geographical distribution, and the habitat specific may play an important role in determining the level of diversity of rodents-associated arthropods (Maaz *et al.,*2018).

It is possible that altitude has some independent influence on ectoparasite diversity. In the current study, ectoparasite diversity was higher in Alous (at 1,594 m) than in Wosanib (998 m) or Alogl (2,387 m). Unfortunately, with only these three sampling points, it is not possible to draw firm conclusions about the role of altitude. It has been reported from China that flea species richness increased at altitudes ranging from 1,000 to 5,000 m (Gong, *et al.,*2005).

Another potential explanation for the high diversity in Alous is that it was relatively more humid compared to Wosanib and Alogl at the time of sampling, and ambient humidity might impact on ectoparasite species richness. Accordingly, in Alogl (the highest sampled altitude), there was a reduction in species richness alongside the lower humidity recorded at that site. This observation is consistent with previous studies which indicated that low humidity can cause a reduction of ectoparasites (Moyer *et al,.* 2002). Moreover, results from earlier studies have indicated that seasonal changes in temperature and humidity may lead to effects on the prevalence and abundance of nest ectoparasites of birds, although these effects differed between mites, fleas and blowflies (Merino & Potti, 1996). According to Alahmed & Al-Dawood, 2001, the number of rodents and their ectoparasites in Wadi Hanifah in Riyadh was low, which is in agreement with another study in Kuwait by Al-Taqi and Al-Ziady (1982). This is because of the extreme climate in Riyadh, which can reach a maximum temperature of 50°C in the summer (Elhefny, *et al,.* 2012 & Almazroui *et al.,*2012, Alanazi, *et al.,*2018, and Alahmed & Al-Dawood, 2001).

The ectoparasite species richness on male rodents were higher than on females, suggesting that the greater home ranges of males might contribute to more varied ectoparasite infestations (Kowalski *et al.,*2015**;** Krasnov *et al.,*2011). Another explanation could be that higher levels of testosterone in males reduce grooming activity and/or immune function, leading to more niches on the rodent body for different ectoparasites (Morand *et al.,*2004; Hughes *et al.,*2001; Matthee *et al.,*2010). Moreover, it was clear that *A. dimidiatus*

harbored a much greater ectoparasite diversity than the other rodent species sampled in the current study. It has been hypothesised by Morand (2015) that host species with a greater geographical range exhibit higher parasite species richness because they accumulate more parasites than hosts with narrow ranges. This is consistent with the fact that *A. dimidiatus* is found from Sinai to southern Pakistan, whereas *M. rex* and *M. yemeni* are restricted to Saudi Arabia and Yemen. Furthermore, results from a previous study (Bajer *et al.,*2006) have found *P. chephrensis*, *P. oxyrrhyncha* and *P. brachyrrhyncha* on *A. dimidiatus* in Egypt, agreeing with our study. On the other hand, a previous study performed in Ta'if (central western Saudi Arabia) reported a greater abundance and diversity of ectoparasites on *M. rex* than on *A. dimidiatus* (Harrison *et al.,*2015). Our results suggest that *M. rex* appears to be more abundant in agricultural fields compared to mountainous regions, which is important information for this little-studied species (Alagaili *et al.,*2013). However, our findings agree with what has been found before in that *M. rex* is not strictly nocturnal, hence, commonly observed during the daylight. This study of the determination of species richness is important to understand the key drivers, such as geographical features and other abiotic and biotic factors that define the distribution of the ectoparasites (Poulin, 1995; Scott, 1988; Minchella and Scott, 1991; Morand and Arias-Gonzalez, 1997). More significantly, the high abundance of ectoparasites observed in our study close to areas of human activities like farming - and near human residences - suggests significant disease risks if these ectoparasites are carrying zoonotic pathogens (Wei *et al.,*2019).

Considering the small sample size of only 74 rodents, the ectoparasite abundance and diversity in our study was very high for Saudi Arabia. Harrison *et al.* (2015) found only 771 ectoparasites (nine species in total of ticks, fleas and mites) on 161 rodents at two wildlife research centres (Taif and Riyadh cities) in Saudi Arabia. Asiry and Fetoh (2014) obtained 1,287 ectoparasites from 750 rodents in five districts of the Hail region. A total of only seven species of ticks, fleas, lice, and mites were reported. As described by Bajer *et al.* (2006), 162 ectoparasites composed of a total of four species of fleas and lice were found on 168 *A. dimidiatus* specimens from four montane wadis in the St Katherine Protectorate, Sinai, Egypt. One thing all of these prior studies have in common is that chiggers were either not reported to be present (in the Saudi studies) or were noted but not examined further (Bajer

*et al.,*2006). This is likely to have led to a major underestimation of ectoparasite species diversity.

The diversity of chiggers reported on rodents in studies from other countries is very variable, but it is not necessarily higher in the tropics. From 13 sites across Thailand, a subsample from a total of 16,000 chigger mites was isolated and identified to 38 species from 18 host species of rodents (1,574 individuals), which proportional to chigger sample size, is a similar level of diversity compared to our study (Chaisiri *et al.,*2019). In South Korea, 17,457 chigger mites isolated from 1,028 rodents were classified into only 15 species, representing a lower diversity of chiggers than in our study (Kim *et al.,*2019). Conversely, in a vast field study conducted in Yunnan Province, China, 92,990 chiggers collected from 10,222 rodent hosts belonged to 224 species in 22 genera, which is considered the highest diversity of chiggers reported worldwide to date (Zhan *et al.,*2013). A single host species from Yunnan, the red-backed vole (*Eothenomys miletus*), was infested with 111 species of chigger mite; which is also unsurpassed worldwide. Thus, there are various factors that could influence the diversity of chigger mites such as environmental conditions, sample size, host species diversity, and biological habitats.

3.2.10 Host-ectoparasite networks among study sites

3.2.10.1 Bipartite

Epidemiological networks are commonly used to discover the dynamics of parasite transmission and the interactions that may affect transmission patterns (Pilosof *et al.,*2015). An important finding from the bipartite networks between ectoparasite species and host species in the present study is that most chigger species and other ectoparasites showed a low host-specificity index. In Wosanib and Alogl, in which 3 – 4 host species were present, there were few ectoparasite species that were restricted to a single host species, where this occurred, it may have been caused by the scarcity of certain ectoparasite species rather than host specificity. This is supported by the fact that the very low rodent diversity in Alous did not restrict ectoparasite species richness; on the contrary, this was the most diverse site for ectoparasites. This low host-specificity leads to complex interactions between ectoparasite and rodent species. Moreover, our results are consistent with the findings from a previous study in Thailand, which reported that chigger mites show a low species-specificity, with most feeding on many different small mammal species (Chaisiri,

2016). Investigations on chigger mites from other parts of the world have come to similar conclusions (Shatrov & Kudryashova, 2008; Peng *et al. ,*2016; Zhan *et al.,*2013; Jameson, 1999). Interestingly, certain ectoparasites showed a potential for some degree of specificity in individual locations. The fleas *L. aethiopica* and *X. cheopis* only infested *M. yemeni* and *M. musculus* in Alogl, despite the greater availability of *M. rex* (Poulin *et al.,*2006; Olubunmi, 2019). Similarly, in Wosanib, the gamasid mites *L. lamborni* and *O. bacoti* and the flea *X. cheopis* were parasites only on *A. dimidiatus*, which represented 59% of hosts trapped in this area. This is somewhat surprising, as *O. bacoti* and *X. cheopis* are known to exhibit low host specificity (Otubanjo, 2019). However, lice (*P. oxyrrhyncha* and *P. brachyrrhycha*) and the flea (*Parapulex chephrensis*) may be more species-specific to *A. dimidiatus*, as they also dominated on this host in a previous study from Egypt (Bajer *et al.,*2006).

3.2.10.2 Unipartite

To examine host-ectoparasite networks at the level of individual rodents, unipartite analysis was used. This analysis consists of two components: the nodes which represent the hosts, and a link or edge which represents the interactions between two nodes within networks. One advantages of this analysis is that enables measurement of a centrality score between two nodes (hosts) in a linear transformation of hosts' dominance within networks. In other words, the greatest centrality score of hosts (rodent species), the more sharing of ectoparasites between them (Borgatti, 2005). Interestingly, in Alogl and Wosanib, where several different rodent species were present, certain individuals of the three native species (*A. dimidiatus*, *M. rex* and *M. yemeni*) occupied key positions in one or other network. Therefore, no single species dominates in locations where ectoparasites can "choose". In contrast, the invasive species *M. musculus* did not seem to play much of a role in the network at Alogl (the only site where it was present), but the sample size of this species was too small to draw firm conclusions.

The highest linkage density, interaction evenness, and nestedness temperature were found in the Alous network, where the rodent species richness was lower than at the other sites, being almost entirely dominated by *A. dimidiatus.* This suggests that intraspecific interactions form a "tight" network of shared ectoparasites when one host

species is very dominant. The network in Alogl had a higher modularity score and was of a more complex shape, with a nucleus dominated by *M. rex* and one individual each of *A. dimidiatus* and *M. yemeni* displaying very high centrality scores on the periphery. Thus, although *A. dimidiatus,* and *M. yemeni* were less common than *M. rex* at this site, these mice can still play key roles in the network. Network connectance was highest in Wosanib, reflecting a very compact network dominated by a single *M. rex*. The reasons why certain individuals might have disproportionate impacts in networks by sharing the greatest number of parasite species could be due to infections or other conditions than can have detrimental effects on immunocompetence. This was consistent with the previous study reported by (Pilosof *et al.,*2015; Wood & Singleton, 2015).

3.3 Conclusions

In this study, we identified five rodent species infested with a high diversity of ectoparasites, including four novel chigger species, in the Asir region of the Kingdom of Saudi Arabia. In total, 19 chigger species were identified alongside a minimum of nine other ectoparasite species. The host specificity of the ectoparasites was generally low, with *A. dimidiatus* displaying a very high ectoparasite species diversity, especially at the site of Alous where it was the dominant species. Ectoparasite species diversity varied considerably between the three sampling locations and seemed to be associated with higher humidity, moderate altitude and mountainous sites without agricultural disturbance. At the sites with several host species (Alogl and Wosanib), no single host species tended to dominate host-ectoparasite networks. These ecological patterns between host and ectoparasite may have important implications for pathogen transmission by arthropod vectors in Asir.

Chapter 4. Ectoparasite microbiomes from rodents of the Asir region, Saudi Arabia

4.1 Introduction

The most popular method for determining the diversity of the bacterial microbiome is by targeted sequencing of the 16S (small) subunit of ribosomal RNA. This cost of this rapid methodology of 16S rRNA amplicon sequencing is becoming less prohibitive and only a very small sample is required to generate several million reads. Present in all bacteria and archaea, and some membrane-bound organelles of eukaryotes, including mitochondria and chloroplasts, the 16S rRNA gene comprises ~1,500 bp. According to many researchers, including D'Amore *et al.,*2016; Klindworth *et al.,*2013; Sambo *et al.,*2018; Srinivasan *et al.,*2015 and Soergel *et al.,*2012, the hypervariable regions of this gene, of which there are nine, make it a perfect candidate for phylogenetic and classification studies. These <300 bp hypervariable regions denoted V1-V9, are all useful to some extent for taxonomic classification; however, the V4 region is generally found to provide the highest level of discrimination (D'Amore *et al.,*2016).

As described by Fadrosh *et al*. (2014), high-throughput sequencing (HTS) has enabled rapid improvements and transformed our ability in characterizing microbial community composition. Of several currently popular sequencing platforms, including the now-defunct Roche 454 pyrosequencer, Ion Torrent's PGM, Pacific Biosciences' RS, and the MiSeq (Illumina), Rubin *et al.* (2014) identified the MiSeq as the most commonly used for 16 rRNA analysis of microbiome populations from multiple environmental and clinical samples, with analysis based on amplification of the V3-V4 region. Determining variation within microbial populations has benefited greatly from HTS of the 16S rRNA gene, which is important for accurate classification. Nelson *et al.* (2014) highlighted numerous benefits of Illumina MiSeq-based 16S rRNA sequencing, including low cost and high-quality data, greater sample thoughput, and longer sequence reads which provide greater detail of the microbiome composition.

However, a major disadvantage related to the sensitivity of the sequencing is that of contamination, which may lead to incorrect interpretation of microbial profiles within a sample. Specifically in low-biomass samples, contaminants, in the form of bacterial DNA, may be present in sample extraction kits or in general laboratory reagents. Sequencing contaminated samples will substantially influence the results of such studies (Galan *et al.,*2016; Salter *et al.,*2014). Consequently, it is vital that appropriate background (negative)

control samples are prepared and run in parallel with samples. These negative controls will determine the presence of contamination or false-positive samples for other reasons before the researcher dedicates time to the full analysis of the data. In addition to negative controls, it is advisable to include positive controls, which may be a prepared mix of bacterial DNA of known species, such as ZymoBIOMICS Microbial Community Standards (Zymo Research). As described by many researchers, including Galan *et al.,* (2016), Salter *et al.,* (2014) and Caporaso *et al.,* (2011), these positive controls are used to assess the quality of the experimental processes and data; specifically, they can highlight experimental bias and errors such as extraction issues in microbiota studies.

Biologists have often focused on the interaction between hosts and individual symbionts, but the advent of high-throughput 16S rRNA amplicon sequencing has led to an ability to analyse whole symbiotic communities. Symbiotic bacteria in arthropods, particularly heritable species (usually passed from mother to egg) often have major effects on the host phenotype. Non-heritable components of the microbiome can influence the transmission of heritable symbionts through competitive exclusion, as demonstrated in the fruit fly *Drosophila melanogaster* (Brinker *et al.,*2019). On the other hand, the relationship between heritable symbionts and the host can control and shape other non-heritable flora as shown in Lepidoptera, *D. melanogaster*, and *Aedes aegypti* mosquitoes (Brinker *et al.,*2019). Furthermore, an enhanced understanding of the arthropod microbiome and the symbiotic relationship between the host and bacteria may provide information to facilitate the development of solutions for improved ectoparasite control. Consequently, the objective of this chapter is to provide a comprehensive analysis of the whole microbiome of ectoparasites from Saudi Arabia, including both pathogenic and non-pathogenic bacteria.

4.2 Initial data QC

Ectoparasite samples alongside rodent skin swabs and soil samples from Saudi Arabia were sequenced over three runs on the Illumina MiSeq and one run on the HiSeq 2500 platform as described in section 2.8.2.1. Plates 2 and 3, which contained most of the swab and soil samples as well as some ectoparasite samples, showed inadequate sequencing depth compared to plates 1 and 4. Therefore, they were excluded from the analyses presented in this chapter.

Runs 1 and 4 incorporated a total of 192 samples, including 53 pooled chigger samples across six of the most abundant species (*E. caucasicum*, *M. microscuta* sp. nov., *Pentidionis agamae*, *E. kazeruni*, *S. saudi* sp. nov., and *A. browni*), 20 rodent skin swabs, 50 individual male and female fleas (*P. chephrensis* and *X. cheopis*), 19 individual tick nymphs of *Haemaphysalis* spp. and *Rhipicephalus* spp., 28 individual lice (*P. brachyrrhyncha* and *P. oxyrrhyncha*), and three individual gamasid mites (*L. lamborni*). Runs 1 and 4 also incorporated a total of 17 background controls to determine sources of possible contamination. For run 1, the controls included Qiagen kit controls, PERFORMAgene PG-100 (swab) kit controls, water washes from dissecting equipment, extracted microbial DNA -free water (Qiagen), un-extracted microbial DNA-free water, water filtered through Nanosep centrifugal devices (Pall Corporation), ZymoBIOMIC Microbial Community standards, and a negative PCR control. Run 4 used similar controls (Table 4.1).

Following data processing, including filtering, de-multiplexing and error correction, the total number of 16S rRNA reads that obtained for all ectoparasites for runs 1 and 4 were 8.1 million and 14.1 million, respectively.

4.3 Microbiome profile of ectoparasite species

4.3.1 Dominant bacterial OTUs in chigger mites and other ectoparasites

Our results showed a high diversity of bacteria among the ectoparasites, as shown in Appendix 32. Here, some dominant bacteria of interest are highlighted by referring to their maximum OTU proportion (%) in any one sample.

Bartonella occupied up to 72%, *Wolbachia* 84%, and *Spiroplasma* 71% of reads in the *P. chephrensis* flea males and females (*n* = 40), while in *X. cheopis*, *Candidatus* Cardinium (40%) and *Wolbachia* (81%) were particularly dominant (Figure 4.1). *Coxiella, Francisella* and *Anaplasma* were the most dominant bacteria in *Haemaphysalis* spp. ticks (Figure 4.2), whereas *Coxiella* was the only common bacterium in *Rhipicephalus* spp. ticks. A high percentage of *Coxiella* and a *Legionella* symbiont were found in the lice (*P. brachyrrhyncha* and *P. oxyrrhyncha*), as illustrated in Figure 4.3. Furthermore, *Bartonella* was the most common bacterium in the *L. lamborni* mites, and *Actinetobacter* and *Wolbachia* were identified in the *O. bacoti* mites. Additionally, *Weissella, Staphylococcus* and Streptophyta

(probably chloroplasts from pollen) were the most abundant OTUs in the swab samples from different body sites on the rodents, as shown in the Appendix 34.

Interestingly, the dominant bacterial OTUs of the pooled chiggers were Actinobacteria (*Corynebacterium* and *Mycobacterium*), whereas *Staphylococcus* occupied up to 52% of reads and *Candidatus* Cardinium (44%), and *Burkholderiaceae* (31%) were also abundant (Figure 4.4). Notably, *Wolbachia* (80%) comprised a high proportion of OTUs in several chigger species, such as *P. agamae, S*. *saudi* sp. nov., *S. zarudnyi* and *H. lukshumiae.* Moreover, *Candidatus* Cardinium was an abundant bacterium in *S. zarudnyi* and *S. saudi* sp. nov. Moreover, the dominant bacteria in gamasid mites were *Coxiella* and *Staphylococcus.*

4.3.2 Dominant bacterial OTUs in background controls

Arsenophonus and *Sphingomonas* sequences were found to be dominant from the Qiagen DNA extraction kit (Table 4.2). *Corynebacterium, Cutibacterium,* and *Streptococcus* sequences were determined to be common in extracted microbial-DNA free water. Moreover, *Pseudomonas* and *Niveispirillum* were found to be the most abundant bacterial sequences in un-extracted microbial DNA-free water. *Bifidobacterium, Corynebacterium* OTU*s Cutibacterium, Lactobacillus, Staphylococcus,* and *Escherichia-Shigella* were the most sequences in water washes from dissecting equipment. Finally, *Cloacibacterium* and *Pseudomonas* were the most dominant bacterial sequences from the swab DNA extraction kit; whereas *Acinetobacter, Wigglesworthia,* and "Bacterium P201" were the most common sequences from water filtered through Nanosep concentrators**.**

4.4 Diversity measures of ectoparasite microbiomes

4.4.1 Alpha and beta diversity measures among ectoparasite groups

Alpha diversity is a measure of taxonomic diversity in a sample. In other words, in the context of microbiome analyses, it calculates the total number of bacterial taxa present within a sample. Alpha diversity is typically used after OTU counts and taxonomic assignments, such as genus level, have been determined. In this study, several methods were used to measure alpha diversity using QIIME 2; namely, evenness (Pielou's), Shannon diversity index, observed OTUs based on 98% OTU identity, phylogenetic diversity (PD), and

the Chao1 estimator. Multiple rarefaction depths were selected for the OTU table at 100, 1,000, 5,000 and 10,000 reads per sample. Five thousand rarefactions of subsampling reads per sample were chosen as the best value to normalise the data for additional data analysis. Beta diversity was also used, which compares the microbial composition of one community with that of another community. In this study, we used several metrics to measure beta diversity; namely, Bray–Curtis dissimilarity, Jaccard distance, weighted UniFrac and unweighted UniFrac.

In terms of alpha diversity, Figure 4.5A shows the rarefaction curves for all of the ectoparasites, which indicates that the sequencing depth of 5,000 was sufficient to sample the entire bacteria diversity. Moreover, alpha diversity (evenness index) varied among the ectoparasites, as seen in Figure 4.5B, and this variation was driven by the relatively low diversity and evenness for the ticks, lice (*P. brachyrrhyncha* and *P. oxyrrhyncha*) and fleas. However, alpha diversity tended to be higher among the chigger mite species. Weighted UniFrac distance matrices (in which abundance is incorporated) were used to estimate beta diversity among all ectoparasites, as illustrated in Figure 4.6. Moreover, weighted UniFrac distances were compared among ectoparasites groups using a PerMANOVA test, as shown in Table 4.3. Each group of ectoparasites tended to cluster together in the principal coordinates analysis (PCoA) and were separated from the controls, with the exception of the chiggers, which were also located in proximity to the swab samples (Figures 4.6).

Among the fleas, the alpha diversity showed higher values in *P. chephrensis* males compared to females, as shown in Figures 4.7A and 4.7B, which was significant in the case of evenness. An analysis by sex was not performed for *X. cheopis* because only a single male sample was sequenced. Beta diversity was also significantly different between *P. chephrensis* males and females and between females of *P. chephrensis* and *X. cheopis* (Table 4.4). However, segregation between the flea samples was complex because the two sexes of *P. chephrensis* did not cluster perfectly, and there was separation between different sampling locations within Alous (Figure 4.7C).

Alpha diversity in *Rhipicephalus* spp. appeared higher compared with *Haemaphysalis* spp., as shown in Figures 4.8A and 4.8B. However, with only two samples of the former available, it was not possible to perform a statistical comparison between the two genera.

Interestingly, the PCOA revealed that there was extensive variation in *Haemaphysalis* spp. UniFrac distances based on different site locations within Alous village (Figure 4.8C).

Alpha-diversity in the louse species was examined, including evenness and phylogenetic diversity (PD). The *P. brachyrrhyncha* females had lower evenness scores than *P. oxyrrhyncha* males and *P. brachyrrhyncha* males, as shown in Figures 4.9A and 4.9B. However, low sample sizes suggest caution in interpreting these data. Beta diversity using weighted UniFrac distances showed that there was a degree of separation of *P. oxyrrhynchus* male samples from different villages (Alous, Wosanib). There were too few male samples of *P. brachyrrhyncha* to determine sex differences within this species (Figure 4.9C).

Regarding the chiggers, *P. agamae, S. saudi* sp. nov. and *E. kazeruni* had higher evenness relative to the other chigger species (4.10A, and 4.10B). Beta diversity showed a distinguishable community membership and structure across chigger species, as revealed by weighted UniFrac. The *P. agamae* microbiome was much more variable than that of *S. saudi* sp. nov. and *E. kazeruni*, while the latter two species tended to cluster together with *E. caucasicum* (PerMANOVA F = 1.85608, P = 0.001, Figure 4.11). Interestingly, *P. agamae* and *S. saudi* sp. nov. appeared to be more distinct in terms of microbiomes compared with the other chigger species**.**

In terms of bacterial weighted UniFrac distances, PerMANOVA showed significant differences between the chigger species, as illustrated in Figure 4.12. There was a significant difference between *E. kazeruni* and *P. agamae* (*P* = 0.001), and also *E. caucasicum* was significantly different to *M. microscuta* sp. nov and *S. saudi* sp. nov (P = 0.049, 0.023, respectively).

Table 4.1. Background controls performed alongside ectoparasite samples and rodent skin swabs.

Control No.	Description	Number of replicates
1	Water rinse from dissecting equipment	4
2	Extracted microbial DNA-free water (Qiagen kit)	4
3	Un-extracted microbial DNA-free water	4
4	Qiagen kit dummy extraction (no water)	4
5	Microbial DNA-free water filtered through Nanosep concentrator	4
6	PERFORMAgene PG-100 (swab) kit dummy extraction	4
7	ZymoBIOMIC Microbial Community standards	1
8	Negative PCR control	

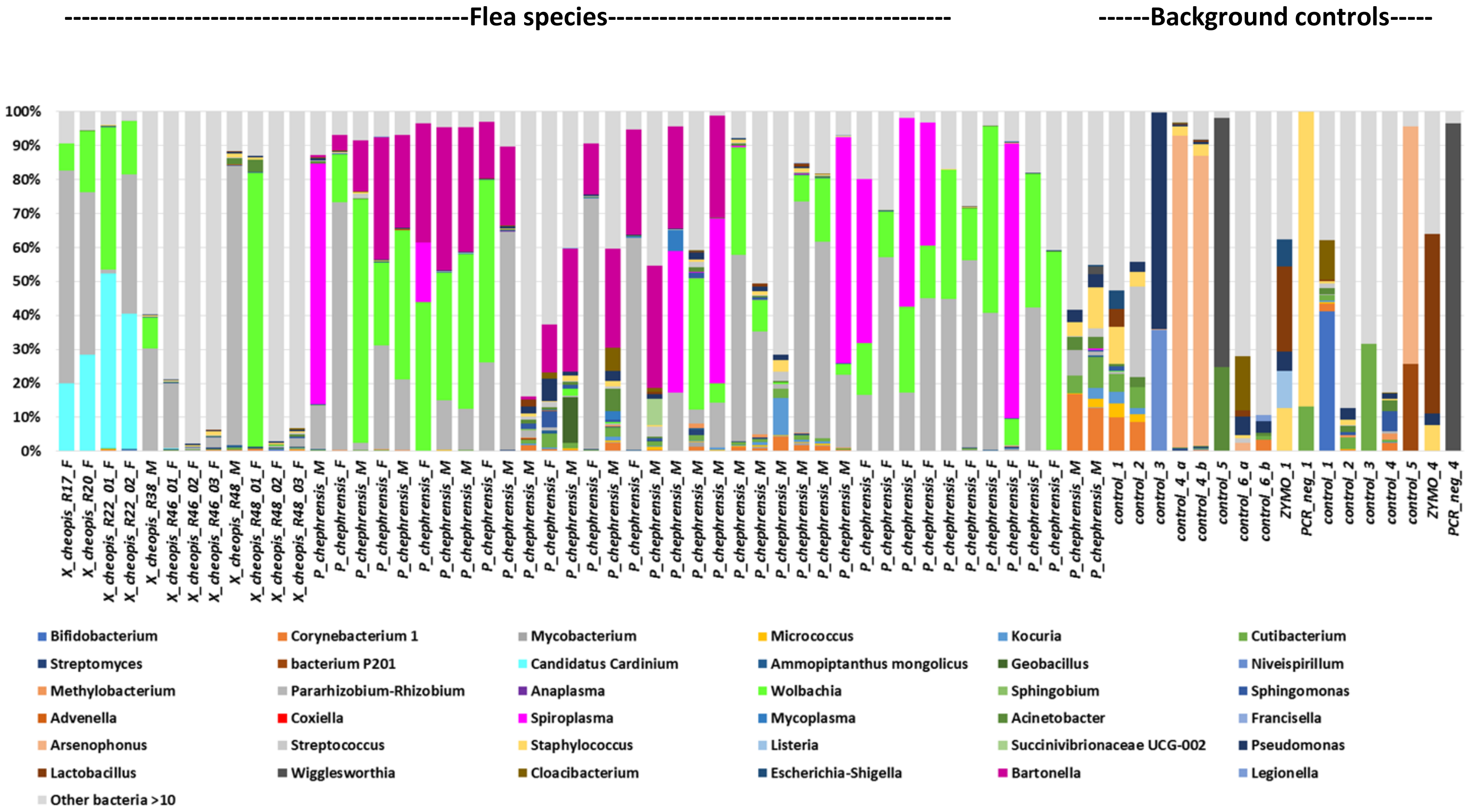


Figure 4.1. Bar charts showing the relative abundance of bacterial OTUs in flea species and background controls.

The data is filtered; OTUs that represented <10% in a sample are combined in “Others” (grey portion).

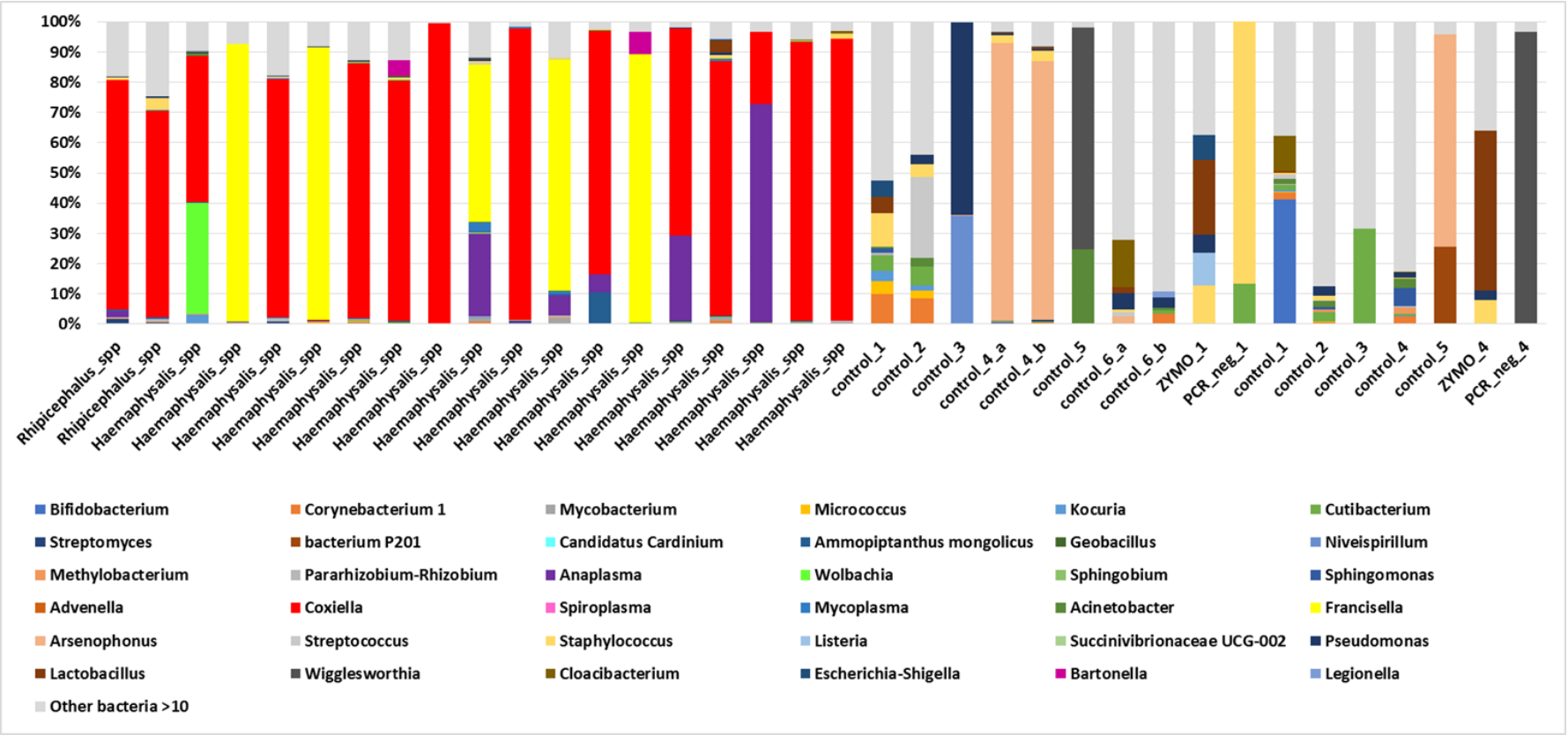


Figure 4.2. Bar charts indicate relative abundance of bacterial OTUs in tick species and background controls.

The data is filtered; OTUs that represented <10% in a sample are combined in “Others” (grey portion).

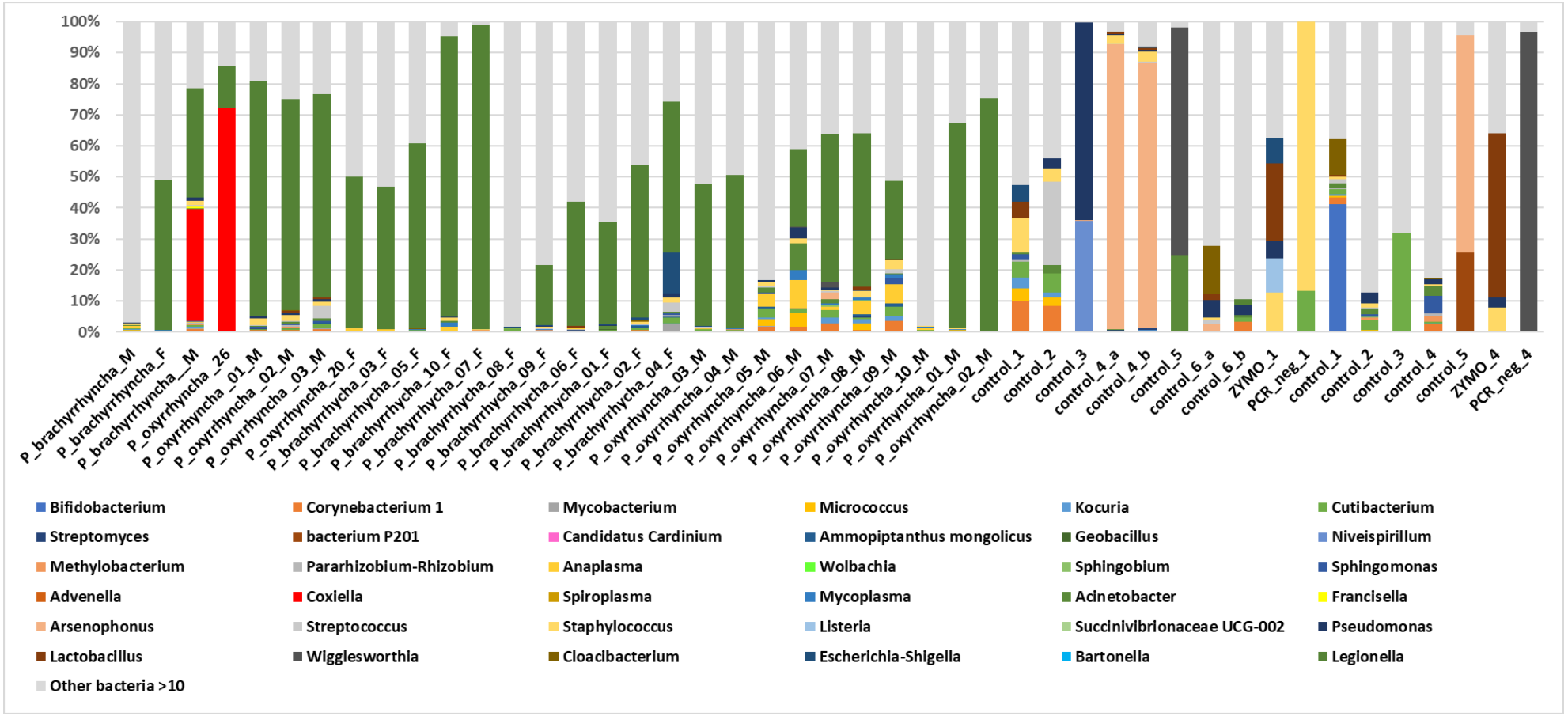


Figure 4.3. Bar charts illustrates relative abundance of bacterial OTUs in individual louse samples, and background controls.
The data is filtered; OTUs that represented <10% in a sample were combined in "Others" (grey portion).

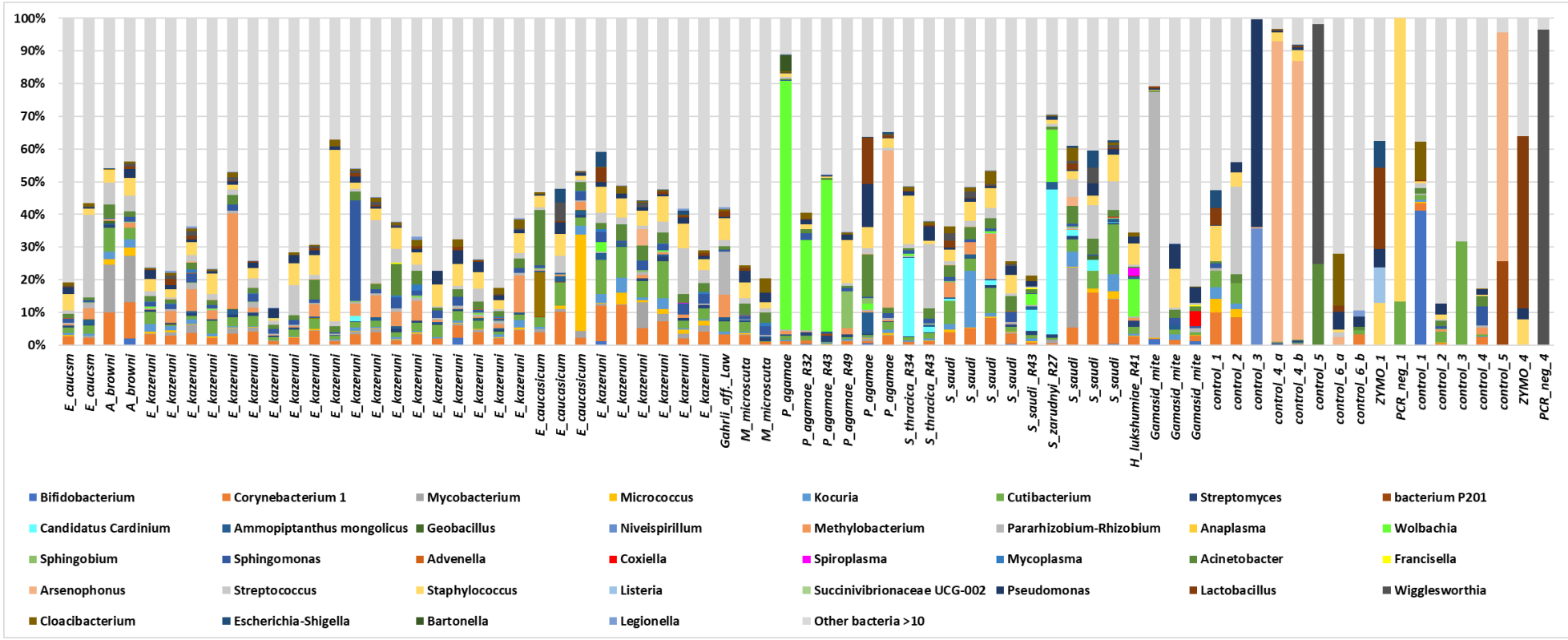


Figure 4.4. Bar charts illustrating the relative abundance of bacterial OTUs in background controls and pooled samples of different chigger species.

The data is filtered; OTUs that represented <10% in a sample are combined in “Others” (grey portion).

Table 4.2. The dominant bacterial OTUs detected in background controls at >5% proportion in one or more controls.

	Maximum percentage (%)											
	Plate 1 controls*							Plate 4 controls*				
Bacterial OUT	**1**	**2**	**3**	**4_a**	**4_b**	**5**	**6**	**1**	**2**	**3**	**4**	**5**
Bifidobacterium	0	0	0	0	0	0	0	**41**	0	0	0	0
Corynebacterium 1	**10**	**9**	0	0	0	0	0	2	0	0	2	0
Micrococcus	4	2	0	0	0	0	0	1	0	0	0	0
Cutibacterium	**5**	**6**	0	0	0	0	0	2	3	**32**	1	0
Bacterium P201	0	0	0	0	0	0	0	0	0	0	0	**26**
Niveispirillum	0	0	**36**	0	0	0	0	0	0	0	0	0
Sphingomonas	1	0	0	0	0	0	0	0	1	0	**6**	0
Acinetobacter	1	3	0	0	0	**25**	0	2	2	0	3	0
Arsenophonus	0	0	0	**92**	**85**	0	2	0	0	0	0	**70**
Streptococcus	0	**27**	0	0	0	0	1	1	0	0	0	0
Staphylococcus	**11**	4	0	3	3	0	1	1	1	0	0	0
Pseudomonas	0	3	**64**	0	1	0	**5**	0	3	0	2	0
Lactobacillus	**5**	0	0	0	1	0	2	1	0	0	0	0
Wigglesworthia	0	0	0	0	0	**73**	0	0	0	0	0	0
Cloacibacterium	0	0	0	0	0	0	**16**	**12**	0	0	0	0
Escherichia-Shigella	**5**	0	0	0	0	0	0	0	0	0	0	0

*See table 4.1 for definitions of each control.

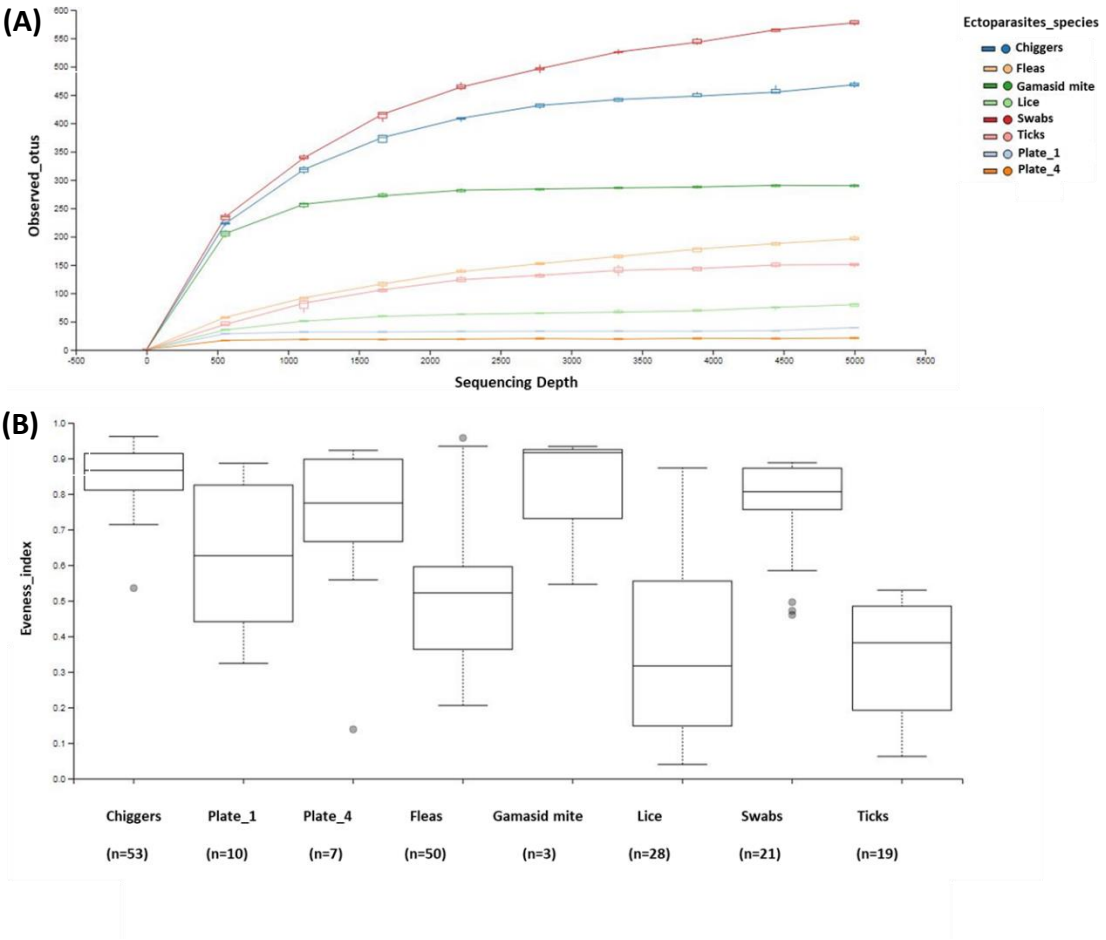


Figure 4.5. Measures of alpha diversity among ectoparasites species.

(A) Observed OTUs number calculated at a rarefaction depth of 5,000 sequences, and (B) eveness diversity index across all sample groups. Plate_1 and plate_4 refer to control samples (see table 4.1).

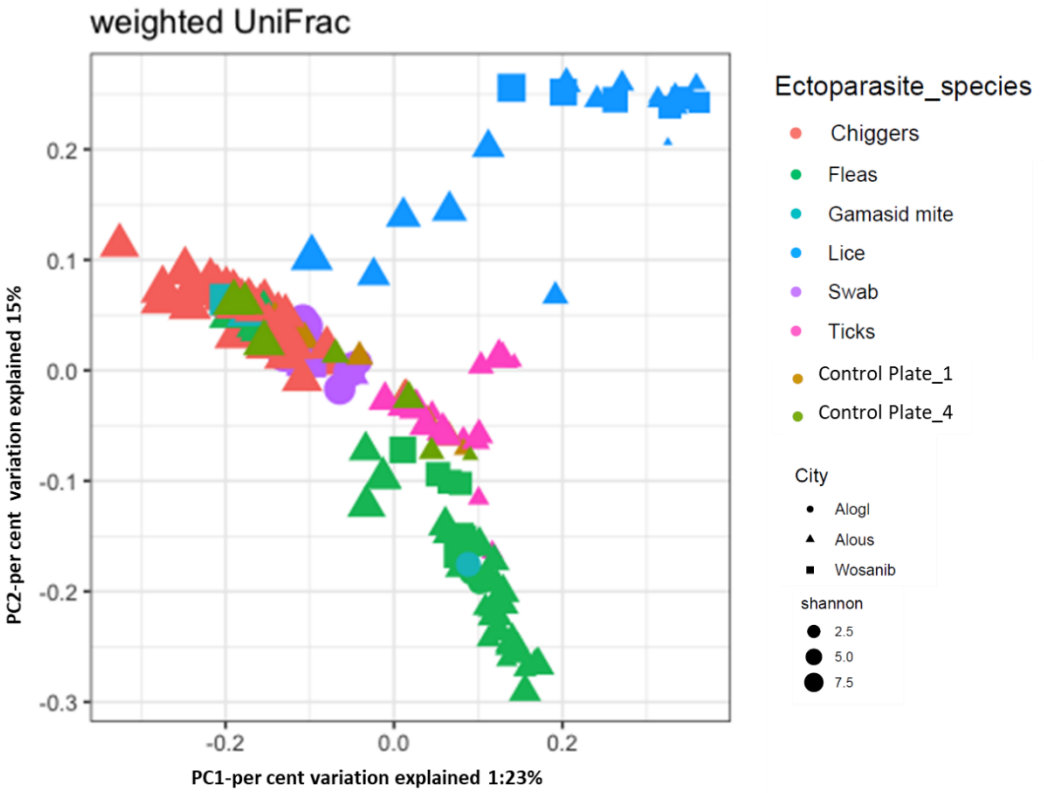


Figure 4.6. Analysis of beta diversity using PCoA of weighted UniFrac distances for all ectoparasite groups.

Table 4.3. Comparison of weighted UniFrac distances among ectoparasites groups and controls using a PerMANOVA test.

Group 1	Group 2	pseudo-F	*p*-value
Chiggers	Control plate 1	5.214846528	0.001*
Chiggers	Control plate 4	3.182494136	0.002*
Chiggers	Fleas	31.97203871	0.001*
Chiggers	Gamasid mite	1.23891055	0.194
Chiggers	Lice	56.11331984	0.001*
Chiggers	Swab	5.837427932	0.001*
Chiggers	Ticks	23.21756335	0.001*
Control plate 1	Control plate 4	0.635879008	0.806
Control plate 1	Fleas	6.413209142	0.001*
Control plate 1	Gamasid mite	1.650123221	0.074
Control plate 1	Lice	21.75289545	0.001*
Control plate 1	Swab	3.587596107	0.001*
Control plate 1	Ticks	6.175808468	0.001*
Control plate 4	Fleas	5.272787984	0.002*
Control plate 4	Gamasid mite	0.720421253	0.706
Control plate 4	Lice	17.53599697	0.001*
Control plate 4	Swab	2.195897636	0.014*
Control plate 4	Ticks	5.797776483	0.001*
Fleas	Gamasid mite	2.291375858	0.044
Fleas	Lice	49.11260255	0.001*
Fleas	Swab	14.77408003	0.001*
Fleas	Ticks	15.90353809	0.001*
Gamasid mite	Lice	13.11634918	0.003*
Gamasid mite	Swab	1.88730785	0.042*
Gamasid mite	Ticks	5.674577879	0.001*
Lice	Swab	40.19958337	0.001*
Lice	Ticks	44.8773678	0.001*
Swab	Ticks	16.05814622	0.001*

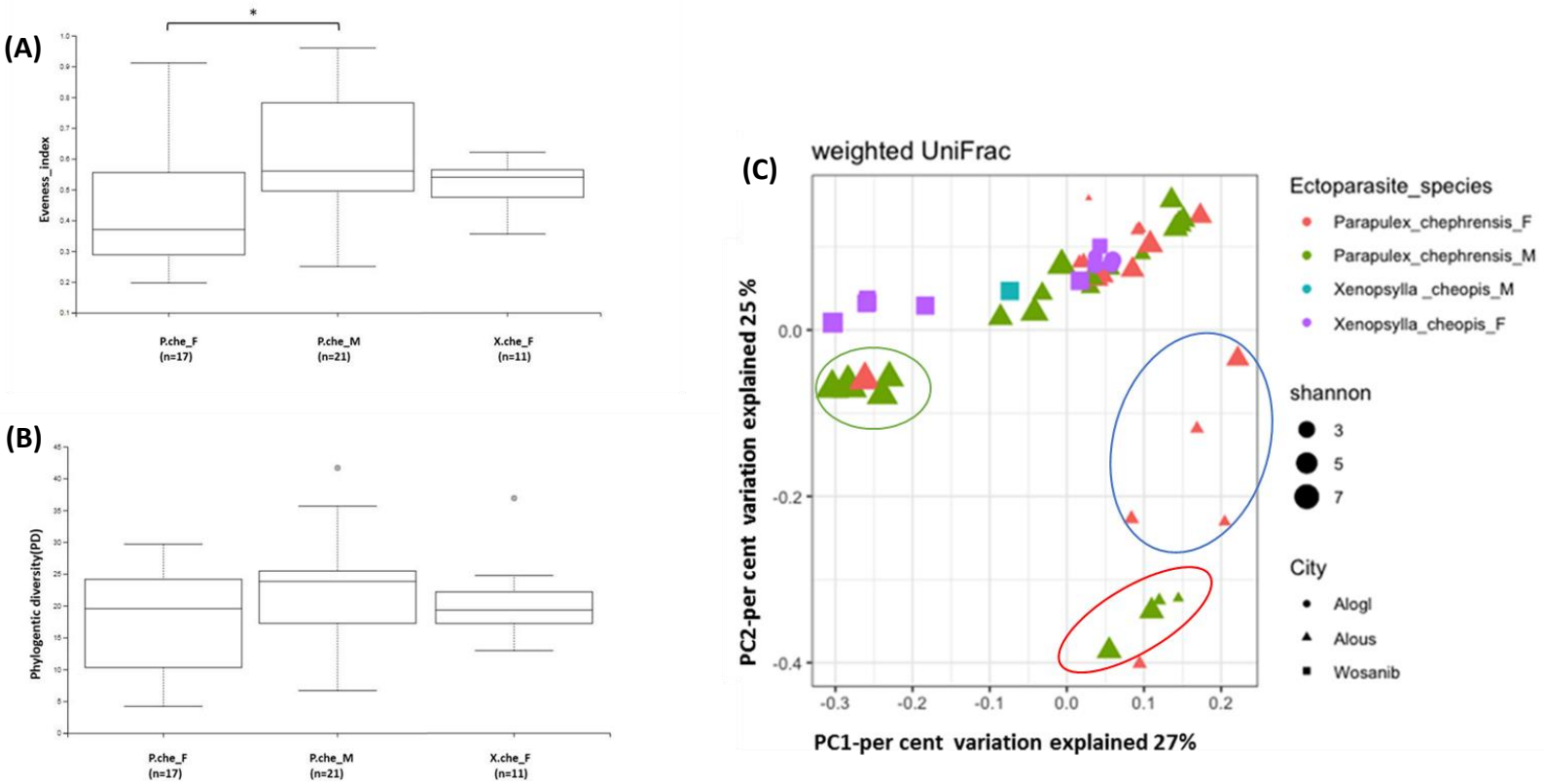


Figure 4.7. Measures of alpha diversity between two species of fleas.

(A) Evenness index, and (B) phylogenetic diversity (PD) after Kruskal-Wallis analysis; *P < 0.01. (C) Measures of beta diversity using PCoA analysis of weighted UniFrac measures. Coloured elipses indicated clustering by sub-location within Alous. (P.che_M= *Parapulex chephrensis male*;P.che_F= *Parapulex chephrensis female*; X.che_F= *Xenopsylla cheopis* female).

Table 4.4. Comparison of weighted UniFrac distances among fleas species using a PerMANOVA test.

Group 1	Group 2	**pseudo-F**	**p-value**
P.chephrensis female	*X. cheopis* famale	5.164019	0.001
P.chephrensis female	*P.chephrensis* male	2.877495	0.03
P.chephrensis male	*X. cheopis* famale	4.675541	0.001

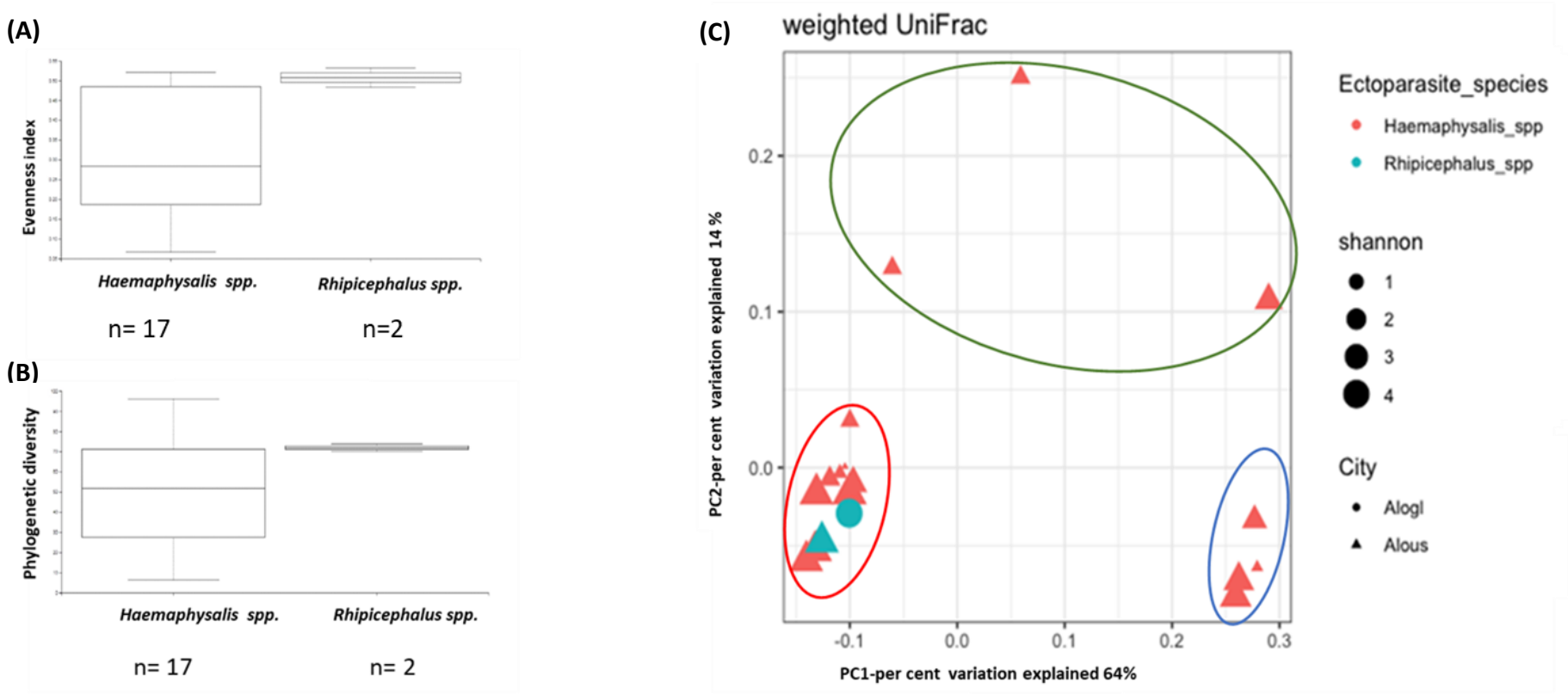


Figure 4.8. Measures of alpha diversity between two genera of ticks.

(A) Evenness index, and (B) phylogenetic diversity (PD). (C) Measures of beta diversity using PCoA analysis of weighted UniFrac metrics. Coloured elipses show clustering by sub-location with Alous.

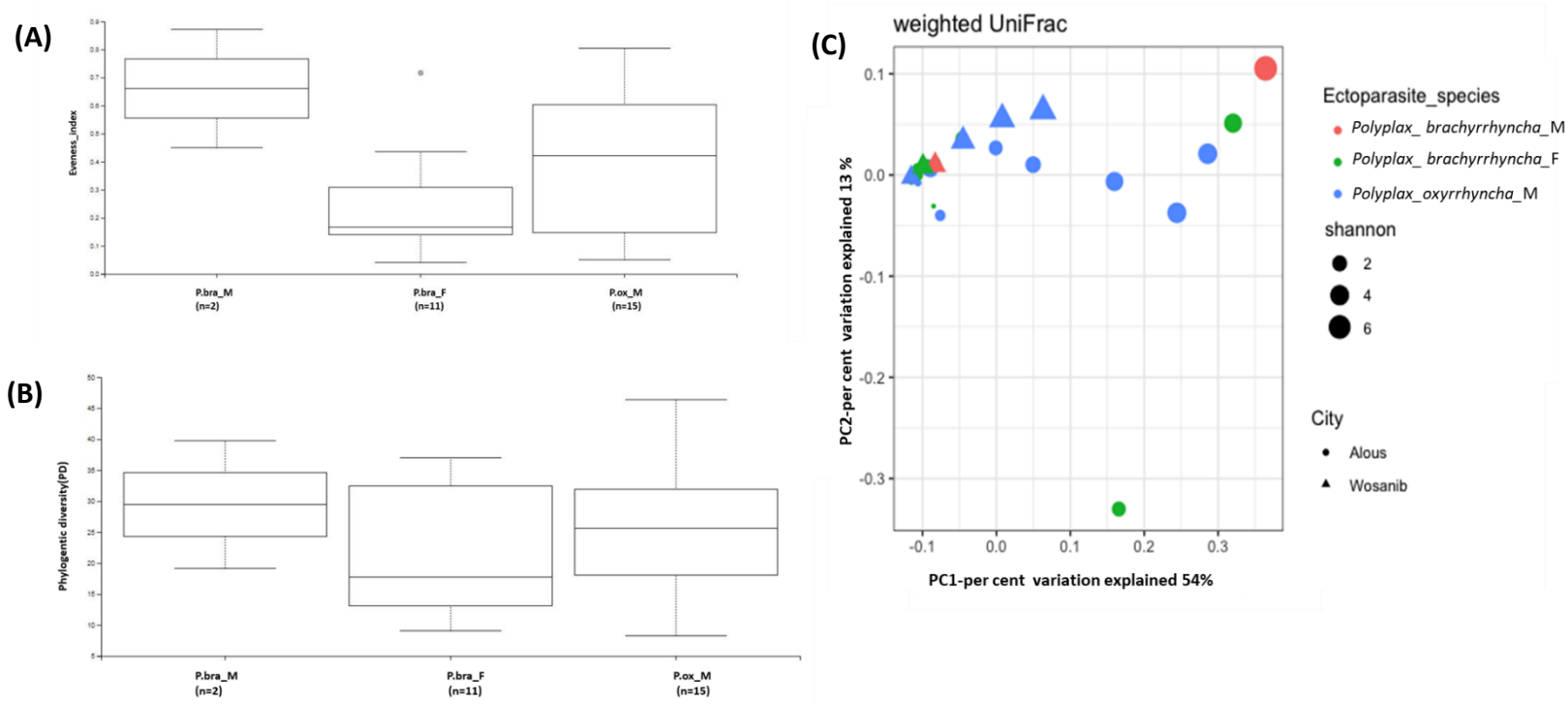


Figure 4.9. Measures of alpha diversity between two species of louse.

(A) Evenness index, and (B) phylogenetic diversity (PD). (C) Measures of beta diversity using PCoA of weighted UniFrac distances. (P.bra_M= (*Polyplax brachyrrhyncha male), P.bra_F= Polyplax brachyrrhyncha* female, and P.ox_M= *Polyplax oxyrrhyncha male*).

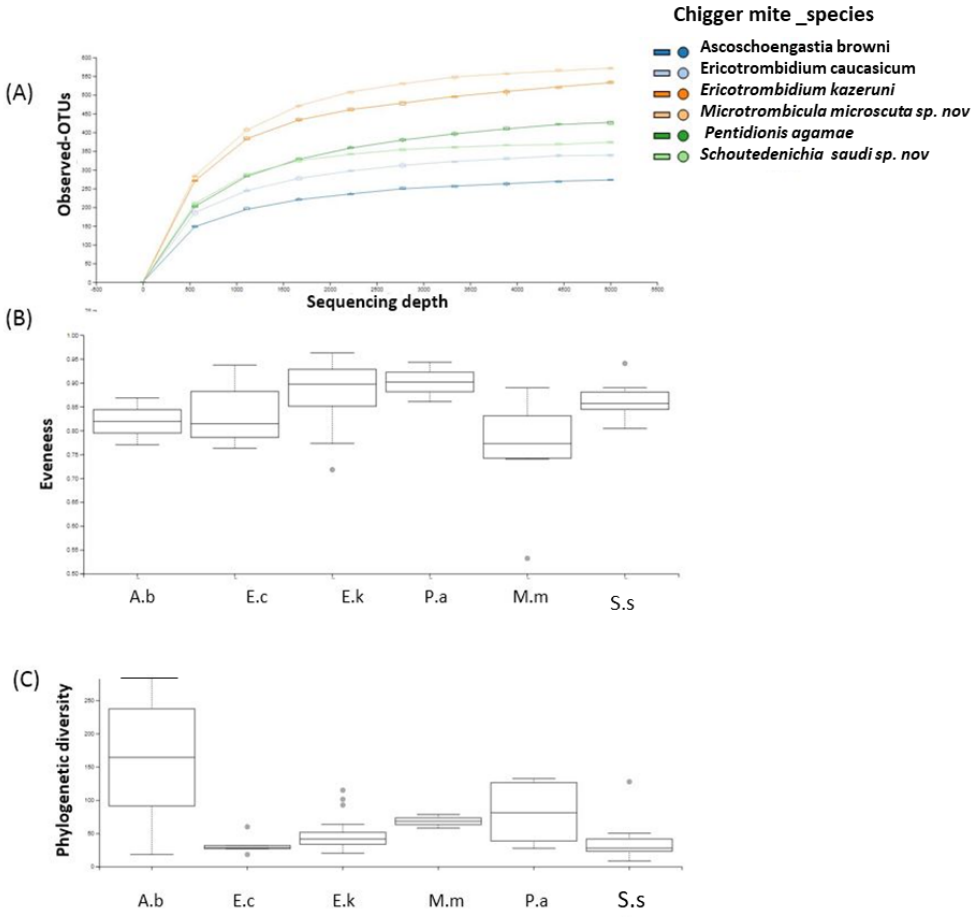


Figure 4.10. Measures of alpha diversity among chigger species.

(A) Observed OTU numbers were calculated at a rarefaction depth of 5,000 sequences. (B) Evenness diversity index and (C) phylogenetic diversity.

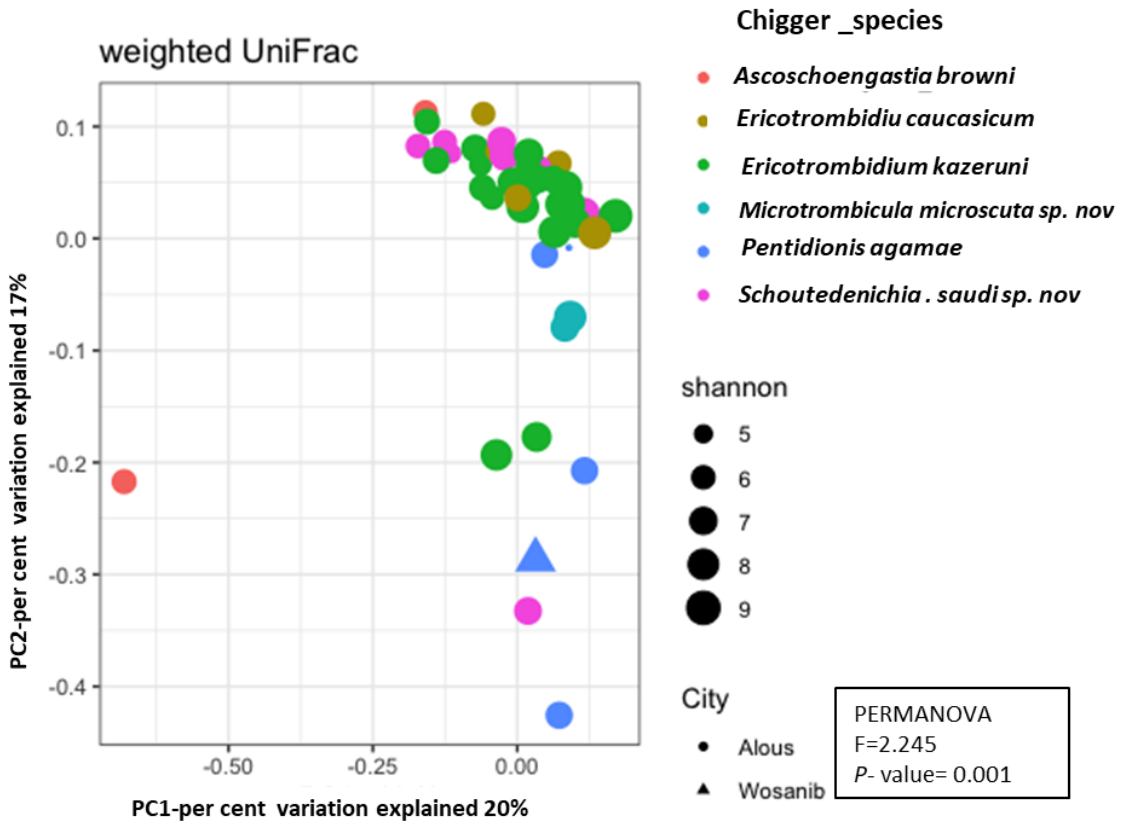


Figure 4.11. Measures of beta diversity using PCoA of weighted UniFrac measures.
P. agamae, S. saudi sp. nov, and *E. kazeruni* clustered separately from all chigger species, showing distinct bacterial communities.

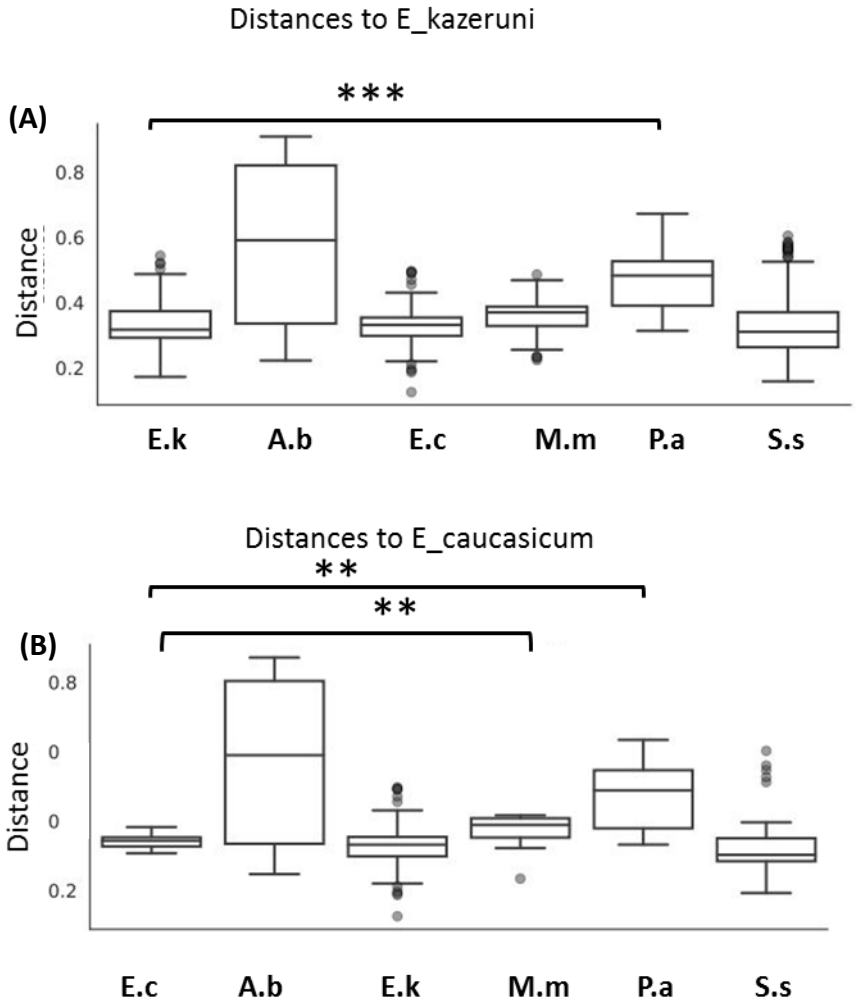


Figure 4.12. Weighted Unifrac distance comparisons among chigger mites species using pairwise PerMANOVA analysis.

(A) *E. kazeruni* is significantly different to *P. agamae* (*P* < 0.001), (B) *E. caucasicum* is significantly different to *M. microscuta sp. nov* (*P* < 0.049) and *P. agamae* (P < 0.023).

4.5 Distribution of *Orientia* in chigger mites from Alous City

Manual inspection of chigger microbiome OTU tables revealed the presence of very low levels of reads matching *Orientia* spp. in two chigger pools. These were an *E. kazeruni* pool from Alous (*Orientia*: 3.21 × 10^{-3} % of reads) on plate 1, and a *P. agamae* pool, also from Alous (*Orientia*: 6.74 × 10^{-4} % of reads), from plate 4. *Orientia* reads were not present in negative control samples and the *Orientia* 16S rRNA sequence in both chigger pools was the same. Raw sequence data was analysed by BLAST from the National Centre for Biotechnology Information (NCBI) to determine the closest hits (Table 4.5).

Table 4.5. NCBI BLASTn results of *Orientia* spp. OTUs sequences indicating top hits.

Bacteria	Hit rank	BLASTn description	Product size (bp)	Query Cover	E-value	Identity (%)	Accession No.
Orientia spp.	1	*Orientia chuto* str. Dubai 16S ribosomal RNA, partial sequence	445	100	0.0	98.88	NR_117903.1
	2	*Orientia tsutsugamushi* isolate TA686 genome assembly, chromosome:I	445	100	0.0	97.98	LS398549.1

4.6 Discussion

This study was the first to examine the ectoparasite-associated bacterial microbiome across ectoparasites in Saudi Arabia; namely, chigger mites, fleas, ticks, lice and gamasid mites, by targeting the V3–V4 hypervariable regions of the 16S rRNA gene. This technique improves our understanding of microbiome profiles and their taxonomic identities.

Four runs were performed on the Illumina MiSeq or HiSeq on ectoparasite, soil and rodent swab samples from the Asir region of Saudi Arabia. However, Plates 2 and 3, which contained additional samples of trombiculid and gamasid mite, as well as swab and soil samples, revealed inadequate sequencing depth compared to plates 1 and 4. Therefore, they were excluded from the analyses presented in this chapter because they could not be processed in an equivalent manner alongside the other plates in QIIME, potentially leading to biased results. There are several possible explanations for this disappointing outcome, but the most likely is a combination of low template DNA concentration and low bacterial content (particularly in the mite samples) coupled with a technical failure on the MiSeq runs. This led the the decision to the use the HiSeq 2500 platform for the fourth plate, which contained a large proportion of chigger samples, and this provided the necessary sequencing depth.

Several authors have emphasised the importance of sequencing background controls when working with samples with low bacterial biomass, as a high similarity in the bacterial community between samples and controls may reflect problems of reagent or environmental contamination (Table 4.1). However, we found a distinct pattern of bacterial OTUs in ectoparasites with low biomass (*i.e*., chiggers) compared with these controls, despite their proximity in the PCoA, which was confirmed in the PerMANOVA test of weighted UniFrac distances (Table 4.3). Moreover, the larger ectoparasites (fleas, lice, and ticks) formed discrete clusters that were more distant from the controls for plates 1 and 4 compared with the chiggers, which is consistent with their higher bacterial load. Importantly, the control samples from plates 1 and 4 did not show any potential pathogenic OTUs such as *Bartonella*, *Anaplasma*, and *Rickettsia*; rather, the dominant contaminating OTUs (Table 4.2) often overlapped with previous studies (Weyrich *et al.,*2019; Xiao *et al.,*2019; Tanner *et al.,*1998; Grahn *et al.,*2003; Eisenhofer *et al.,*2018; Willems, 2014). However, symbiont OTUs (*Arsenophonus* and *Wigglesworthia*) must have originated from

background contamination in the Darby laboratory where the 16S rRNA PCRs were performed, as this lab works on these symbionts. These OTUs were not present above the 1% threshold in the data from actual samples.

The potential causes of OTUs clustering within ectoparasite groups could be due to several reasons. First, location (geographical variation) may play an important role in shaping ectoparasite microbiomes. For example, in the USA, differences in the microbiome of *I. scapularis* ticks collected from four states (North Carolina, Virginia, South Carolina and Connecticut) were clearly apparent. (Van Treuren *et al.,*2015). However, this was over a large geographic scale (hundreds of km) while surprisingly, there were differences in *Haemaphysalis* spp. microbiomes between sub-locations in Alous in the present study (a few hundred metres).Second, several studies have observed a distinct clustering of *I. scapularis* tick microbiomes by sex, with a greater diversity in adult males versus females (Van Treuren *et al.,*2015; Thapa, *et al.,*2019). Unfortunately, as no adult ticks were collected in our study, it was not possible to look at this aspect. Third, Zolnik *et al.,*2016 revealed variation of the tick microbiome among development stages (larvae, nymphs and adult) of *I. scapularis* from Armonk, NY, and between tick organs (salivary gland and midgut). He found a distinct clustering between developmental stages and adult tick male and females, as well as differences between organs. Such a comparison could not be done in the current study because almost tick specimens were exclusively of one stage (nymphs). Fourth, another potential reason for variation in microbiome within an ectoparasite species is choice of host species. Previous studies have demonstrated the impact of host identity on the tick microbiome. For instance, Swei and Kwan, (2017) found a unique clustering of the microbiome of *I. pacificus* nymphs between two host species, a lizard (*Sceloporus occidentalis*), and a mouse (*Peromyscus maniculatus*) using weighted uniFrac distances. Additionally, a recent study showed the role of of the blood meal from the host in shaping the microbiome of *I. scapularis* nymphs, comparing specimens collected from five rodent species: red squirrels (*Tamiasciurus hudsonicus*), raccoons (*Procyon lotor*), gray squirrels (*Sciurus carolinensis*), Virginia opossums (*Didelphis virginiana*), and striped skunks (Mephitis mephitis) (Landesman *et al.,*2019). This factor was not examined in the present study, as almost all of the ticks used for microbiome analysis came from Alous, where the only host was *A. dimidiatus*. Finally, the species of arthropod itself is likely to have the greatest impact on the composition of the microbiome. Although two distinct clades of

Haemaphysalis spp. ticks were identified in the current study (Figure 3.11), only one clade was incorporated into the microbiome analysis.

In the case of the fleas, it was surprising that the analysis of beta diversity using PCoA revealed not only clustering of the two sexes of *P. chephrensis* in Alous village, but also a distinct segregation by sub-location within the village (Figure 4.7 c). For the lice, there was some evidence for clustering of *P. oxyrrhyncha* males between Alous and Wosanib, which might have been caused by the presence of a second host (*M. yemeni* in addition to *A. dimidiatus*) in the latter location (Figure 3.22). Thus, variations in flea and louse microbiomes among different field locations within or between villages may exist owing to differences in environmental or host-associated bacteria. Significant differences in flea microbiomes between villages, as well as influences of other geographic or climatic factors like elevation and temperature, have been reported from a study incorporating an environmental transect in Uganda (Jones, *et al.,*2015).

In terms of the main symbiotic or pathogenic OTUs in each ectoparasite group, several bacterial genera were found to be clearly dominant. In common with several other fleas species such as *C. felis*, *Wolbachia* was very abundant in *P. chephrensis*. The role of *Wolbachia* in fleas has not been explored in detail, but in *Synosternus cleopatrae*, *Wolbachia* has a negative effect on reproductive success in laboratory (but not field) populations (Flatau *et al.,*2018).There is also the possibility that the presence of *Wolbachia* may affect the load of *Bartonella*, as they often coexist in fleas (Flatau *et al.,*2018). More surprisingly, *Spiroplasma* was detected in *P. chephrensis* in the current study. Previous studies have shown that *Spiroplasma* are common symbiotic bacteria in arachnids (including ticks), insects (Diptera, Hemiptera, Hymenoptera and Odonata) and plants. For instance, these bacteria were isolated from 16 of ~200 *Drosophila* species (Watts *et al.,*2009). However, *Spiroplasma* has been rarely reported from fleas, although Hornok *et al.,*(2010) reported this symbiont from *C. felis* in Hungary. By contrast, our results from Alogl and Wosanib showed that *Candidatus* Cardinium was observed as a potential symbiont or pathogen in *X. cheopis* from two host species (*M. musculus* and *M. yemeni*). This bacterium has been reported from *X. cheopis* from Uganda, but as a low OTU proportion, and its effect on fleas (if any) is unknown (Jones, *et al.,*2015).

Coxiella, Francisella, and *Wolbachia* were the key bacterial genera found in ticks in the present study. Previous studies have shown that *Coxiella*–LE are the most widepsread symbionts among tick species (Duron *et al.,*2017). In *R. microplus*, it is essential for tick survival and reproduction (Garcia Guizzo *et al.,*2017), but its role has not been evaluated in detail in other genera. However, *Coxiella*-LE have been reported before from *Haemaphysalis* spp. ticks (Arthan *et al.,*2015; Takhampunya *et al.,*2019; Zhang *et al*. 2019; Zhong *et al.,*2007). These endosymbionts probably play a crucial role in tick survival and reproduction for many tick species because they are widespread across multiple genera, suggesting that the symbiosis dates back to early tick evolution (Duron *et al.,*2017; Bonnet *et al.,*2017). Another symbiotic bacterium that was observed was *Francisella*, which was found in *Haemaphysalis* and has been reported from this genus recently in Thailand, China and Korea (Takhampunya *et al.,*2019; Wang *et al.,*2018; Takhampunya *et al.,*2017). This bacterium is an important endosymbiont in the life cycle of the soft tick *O. moubata*; this species needs *Francisella* to synthesise B vitamins, which are scarce in the blood meal of ticks (Duron *et al.,*2018; Jiménez-Cortés *et al.,*2018). Interestingly, *Wolbachia* was also observed in *Haemaphysalis* spp in the current study. However, detection of *Wolbachia* in *I. ricinus* was found to be due to parasitism by the wasp *Ixodiphagus hookeri* (Plantard *et al.,*2012), which has a global distribution. Thus, this finding from *Haemaphysalis* requires further investigation. In the lice, the presence of *Coxiella* was surprising and does not appear to have been reported before, although the related *Legionella* symbiont (*Coxiella* is in the order Legionellales) was more dominant and widespread in both louse species, suggesting it is the primary symbiont as shown for *P. serrata* (Říhová *et al.,*2017).

Very few data have been published previously on the chigger microbiome. However, in common with a large-scale microbiome study of chiggers in Thailand (Chaisiri *et al.,*2019), the dominant bacterial genera in chiggers in the present study were *Corynebacterium*, *Mycobacterium*, and *Staphylococcus*. Moreover, *Wolbachia* was found in *P. agamae* and *H. lukshumiae* in high proportions in the Alous specimens, whereas *Candidatus* Cardinium was more dominant in *S. saudi* sp. nov. and *S. zarudnyi* from Wosanib. These findings suggest that the existence of endosymbionts associated with chigger mite species could play a significant role in making the bacterial community composition distinctive. In the only wild chigger microbiome studies published previously, both from Thailand (Takhampunya *et al.,*2019; Chaisiri *et al.,*2019) , *Cardinium* was detected in pooled specimens but *Wolbachia*

seemed less prevalent. Notably, the bacterial diversity in the pooled chigger mites was higher than that in the other ectoparasites. This might be because of greater retention of bacteria from the environment than the other ectoparasite groups and less dominance by single endosymbiont taxa, suggesting that chiggers do not have a primary symbiont.

An *Orientia* OTU with a close match to *O. chuto* was discovered in two pooled species of chigger mites (*P. agamae* and *E. kazeruni*) collected from an *A. dimidiatus* host in Alous. The *Orientia* 16S rRNA reads were a very small proportion of the total in these chigger pools, suggesting very few individual mites (possibly only one) was positive in each pool. Therefore, these results require confirmation using specific PCRs targeting other *Orientia* genes. Nevertheless, this is the first preliminary evidence that *O. chuto* is circulating in Saudi Arabia, suggesting that scrub typhus might exist in the country, even though it has never been recognised. Previously, *O. chuto* has only been reported from two other countries. In the United Arab Emirates, it was first isolated from a clinical case but the vector was not identified (Izzard *et al.,*2010); whereas in Kenya, it was detected in a pool of chiggers dominated by *Microtrombicula* spp. that were removed from a Natal multimammate mouse (*Mastomys natalensis*) (Masakhwe *et al.,*2018). Although the occurrence of scrub typhus disease remains to be proven in Saudi Arabia, the threat should nonetheless be taken into consideration. Therefore, future work is needed.

4.7 Conclusion

To our knowledge, this is the first study on rodent ectoparasite diversity and zoonotic bacterial pathogens performed in the Asir region of Saudi Arabia. We performed next-generation sequencing of 16S rRNA gene amplicons, thus providing a global overview of the microbiota of the ectoparasites in this area (chigger mites, fleas, ticks, lice and gamasid mites), which were investigated for the first time in this work. Our findings revealed that the prevalence of bacteria between the chigger mites and the other ectoparasites was significantly different. The dominant bacteria were *Corynebacterium, Staphylococcus, Mycobacterium, Wolbachia* and *Candidatus* Cardinium (in the chiggers); *Coxiella, Francisella, Wolbachia* and *Anaplasma* (ticks); *Wolbachia, Spiroplasma* and *Bartonella* (fleas); and *Legionella* and *Coxiella* (lice). Alpha and beta diversity results showed differences between the chigger mites and the other ectoparasites in terms of bacterial communities. Finally, preliminary evidence for *O. chuto* was discovered for the first time in

Saudi Arabia as a potential pathogen in two species of chigger mites (*P. agamae* and *E. kazeruni*).

Chapter 5. Molecular validation of selected bacteria taxa from Saudi ectoparasite microbiomes

5.1 Introduction

The use of 16S rRNA amplicon sequencing cannot only provide ecological insights into ectoparasite microbiomes, but is also an unbiased method to screen for newly emerging bacterial zoonoses (Lemon *et al.,*2008). Previous studies have used 16S rRNA amplicon sequencing to investigate bacteria circulating among a single ectoparasite species in different locations or have applied the same technology to tissue from rodent hosts on a large scale to identify pathogens that might be novel to a specific geographic area (Galan *et al.,*2016; Carpi *et al.,*2011).

In chapter 4, several potential pathogens or symbionts were identified by 16S rRNA amplicon sequencing, including, *Bartonella, Wolbachia* and *Spiroplasma* in fleas (*Parapulex chephrensis*); and *Coxiella*, *Francisella*, *Ehrlichia* and *Anaplasma* in ticks (*Haemaphysalis* spp. and *Rhipicephalus* spp.). In addition, although MiSeq runs 2 and 3 had insufficient sequencing depth for comprehensive analysis of the microbiome for those samples, some unconfirmed sequences from potential pathogens were apparent in swabs from rodent skin. However, a drawback of the 16S rRNA approach is that this locus is usually too conserved to allow discrimination at the level of bacterial species or strains to separate endosymbionts from pathogenic organisms (e.g., *Coxiella*-LE from *C. burnetii* or *Francisella*-LE from *F. tularensis*) (Martins *et al.,*2012). To confirm the 16S amplicon result, the most popular approach is to use PCR (conventional or nested PCR) to amplify a more variable genetic locus than the 16S rRNA gene and then sequence the product to verify whether a potential pathogenic or symbiotic bacterium is present. PCR has the advantage of being compatible with ethanol-fixed specimens and is more specific than serology (either from rodent hosts or humans), and although culture isolation remains the gold standard for diagnosis or the formal description of a novel pathogen or symbiont, it requires fresh material and can be slow, with low success rates. Another useful molecular approach to capture a vast array of common pathogens is a multiplex PCR panel, which targets the most prevalent pathogens recognised to cause comparable symptoms (Takhampunya *et al.,*2019); however, this often requires extensive optimisation and validation for reliable results.

The interactions between the ectoparasites and their endosymbiotic bacteria, which may be required for normal development and could impact on pathogen transmission, may lead to revealing new insights to control vector-borne diseases. This potential cannot be reached if the distinction between pathogens and endosymbionts is unclear. For instance, previous studies indicated that *Rickettsia* species are mainly endosymbionts, is transmitted vertically within arthropods, and only occur secondarily as vertebrate pathogens (Perlman *et al.,*2006). Moreover, *C. burnetii* is often reported as a pathogenic bacterium in ticks; however, recently, several tick species have been found to harbour maternally-inherited *Coxiella*-LE organisms that are involved in symbiotic interactions. The relationships between these *Coxiella*-LE to the Q fever pathogen remain blurred (Duron *et al.,*2015), but the most likely scenario is that *C. burnetii* evolved from an inherited symbiont of ticks, which succeeded to infect vertebrates cells (Narasimhan *et al.,*2015). The presence of *Coxiella*-LE in the salivary glands of some important tick vectors highlights their potential to interact with tick-borne pathogens (Klyachko *et al.,*2007).

This chapter aimed to confirm the 16S rDNA amplicon sequencing results that were described in the previous chapter by use of species-specific PCR primers. The construction of phylogenetic analysies of sequenced PCR products was then applied to place individual bacterial genera into symbiotic or pathogenic categories where possible.

5.2 Results

5.3 Species-specific PCRs targeting specific genes of ectoparasites in fleas

5.3.1 *Bartonella* in *Parapulex chephrensis*

A nested PCR amplification was performed for *Bartonella* spp. targeting the 16S–23S intergenic spacer region (ISR) as described in section 2.9.1. A total of 20 fleas of both sexes of *P. chephrensis in A. dimidiatus* from Alous were found positive for *Bartonella*. The PCR product was cloned from three samples and DNA from two colonies was sent for Sanger sequencing, but only one high-quality sequence was obtained. By BLASTn, this sequence obtained displayed very high identity (99.41%) to a strain of *Bartonella acomydis* from Egypt (Table 5.1).

Table 5.1. Results of specific PCR assays to confirm infection with bacterial pathogens and symbionts in *P. chephrensis* fleas.

Bacterial genus (sequences obtained)*	Rodent host	Village	Gene target	Hit rank[#]	Query length	Query cover (%)	Identity (%)	E-value	Accession	Source	Location
Bartonella (1)	*A. dimidiatus*	Alous	Intergenic spacer region	1	659	76	99.41	0.0	AB602564	*Bartonella acomydis* strain KS7-1 (from *Acomys russatus*)	Egypt
				2		76	99.01	0.0	AB602563	*Bartonella acomydis* strain KS2-1 (from *Acomys russatus*)	Egypt
Wolbachia (5)	*A. dimidiatus*	Alous	*Wsp*	1	497	100	100	0.0	KY363325	*Wolbachia* endosymbiont of *Ctenocephalides felis felis*	Kazakhstan
				4		100	99.8	0.0	JN601163	*Wolbachia* endosymbiont of *Musca domestica*	Switzerland
Spiroplasma (1)	*A. dimidiatus*	Alous	*rpoB*	1	1,382	100	99.42	0.0	DQ313832	*Spiroplasma ixodetis* strain Y-29 (from *Ixodes pacificus*)	Oregon, USA
				2		100	99.13	0.0	MG859278	*Spiroplasma* sp. strain DMAR11 (from *Dermacentor marginatus*)	Spain

*Results listed on the same row were identical where they overlapped (the length of high-quality sequence varied – results displayed are for the longest sequence obtained).

[#]The highest ranked unique matches are shown in each case, ignoring intervening hits where they were identical to the top hit.

5.3.2 *Wolbachia* endosymbiont in *Parapulex chephrensis* and *Haemaphysalis* spp.

Conventional PCR amplification targeting the *Wolbachia* surface protein (*wsp*) was used to detect *Wolbachia*. Of 10 flea samples (*P. chephrenis*), three males and five females produced positive PCR results for *Wolbachia* DNA as shown in Figure 5.1. Although this was not a quantitative assay, the intensity of PCR products suggested that the *Wolbachia* load was higher in female compared with male fleas. High-quality sequences were obtained from the female specimens only. The sequence of the obtained PCR product showed 100% identity to a *wsp* sequence from the cat flea, *C. felis* (Table 5.1). Moreover, Sanger sequencing of *wsp* from a tick specimen (*Haemaphysalis* spp.) produced a sequence that was also 100% identical to the same *Wolbachia* symbiont of *C. felis* sequence (Table 5.2).

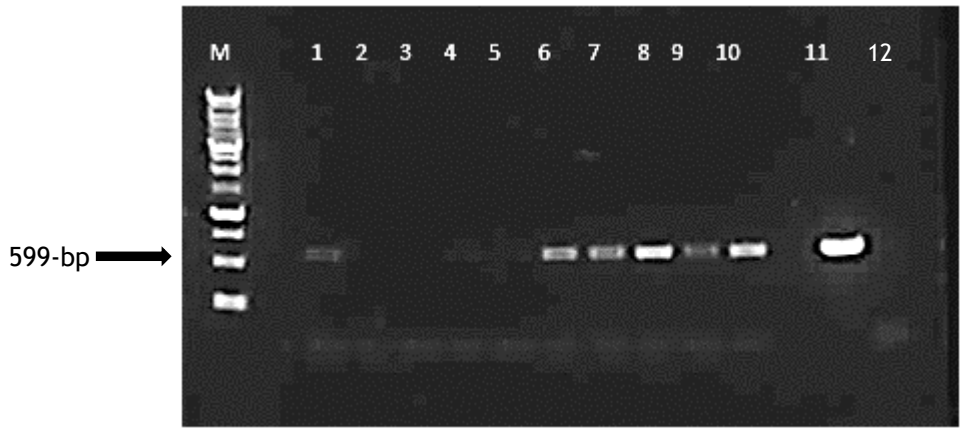


Figure 5.1. Amplification products of a fragment of the *Wolbachia wsp* gene in *P. chephrensis* specimens collected from rodents.

Lane M, DNA ladder. Lanes 1–5, male samples, and 6-10 female samples that were positive in 16S rDNA screening. Lane 11, positive control; lane 12, negative control.

5.3.3 *Spiroplasma* in *Parapulex chephrensis*

The existence of *Spiroplasma* DNA in *P. chephrensis* fleas was verified by touchdown PCR amplification of the *rpoB* fragment. A positive female flea sample was visualised by agarose gel electrophoresis with the expected product size of 1,382 bp (Figure 5.2). Sanger

Table 5.2. Results of specific PCR assays to confirm infection with bacterial pathogens and symbionts in ticks.

Bacterial genus (sequences obtained)*	Tick genus#	Rodent host	Village	Gene target	Hit rank^	Query length	Query cover (%)	Identity (%)	E-value	Accession	Source	Location
Coxiella (6)	*R*	*A. dimidiatus, M. rex, M. yemeni*	Alogl, Wosanib	*rpoB*	1	222	100	100	9^{-111}	KY678179	*Coxiella* endosymbiont of *Rhipicephalus* sp. isolate Tchien14	Ivory Coast
					7		100	99.55	4^{-109}	CP024961	*Candidatus* Coxiella mudrowiae isolate CRS-CAT (from *Rhipicephalus sanguineus*)	Israel
Wolbachia (1)	*H*	*A. dimidiatus*	Alous	*wsp*	1	487	97	100	0.0	KY363325	*Wolbachia* endosymbiont of *Ctenocephalides felis felis*	Kazakhstan
					3		97	99.16	0.0	KX385020	*Wolbachia* endosymbiont of *Xenopsylla* sp. strain AL03	Xinjiang, China
Francisella (4)	*H*	*A. dimidiatus, M. rex*	Wosanib	17 kDa lipoprotein gene	1	348	100	99.14	4^{-176}	AY375423	*Francisella* endosymbiont of *Ornithodoros porcinus* clone 02-52/53	Southern Africa
					3		100	98.85	6^{-174}	GU968877	*Francisella*-like endosymbiont of *Dermacentor albipictus* haplotype 5	Alberta, Canada
Anaplasma (2)	*H*	*A. dimidiatus*	Alous	16S rRNA gene	1	451	100	99.11	0.0	JX846966	Uncultured *Anaplasma* sp. clone TN6SP12GE (from *Bandicota indica*)	Taiwan
					2		100	98.67	0.0	MN795628	*Anaplasma phagocytophilum* isolate Ap005R (from cattle)	Uganda
Ehrlichia (2)	*H/R*	*M. rex*	Alogl	*groESL*	1	702	100	96.58	0.0	KJ410291	*Ehrlichia* sp. BL116-7 (from *Hyalomma asiaticum*)	Xinjiang, China
					3		100	93.87	0.0	KJ410295	*Ehrlichia* sp. TC249-2 (from *Dermacentor nuttalli*)	Xinjiang, China

*Results listed on the same row were identical where they overlapped (the length of high-quality sequence varied – results displayed are for the longest sequence obtained).

#*R* = *Rhipicephalus* spp.; *H* = *Haemaphysalis* spp.

^The highest ranked unique matches are shown in each case, ignoring intervening hits where they were identical to the top hit.

sequencing showed that the DNA amplicon had closest sequence identity (99.42%) to a *rpoB* fragment from *Spiroplasma ixodetis* strain Y29 (Table 5.1), a symbiont of the Western black-legged tick, *I. pacificus* (Tully *et al.,*1995).

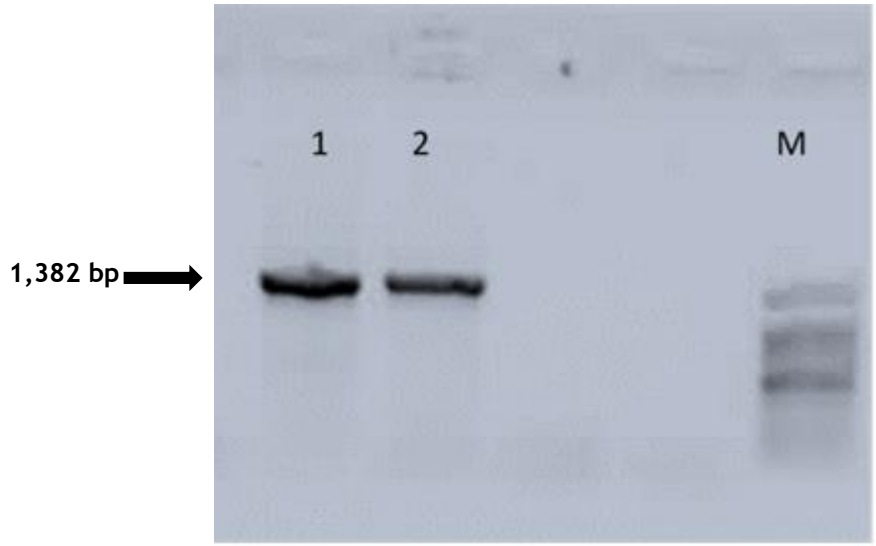


Figure 5.2. **Gel electrophoresis showing PCR amplification of the *Spiroplasma rpoB* gene in a female *P. chephrensis*.**

Lane M, DNA ladder, lanes 1, positive control; lane 2, flea samples.

5.4 Detection of *Coxiella, Francisella, Ehrlichia*, and *Anaplasma* in tick, louse and chigger species

5.4.1 *Coxiella*-like endosymbionts in ticks and chiggers

Tick DNA samples (*Haemaphysalis spp.* and *Rhipicephalus spp.*) and chigger mite samples (*S. saudi* sp. nov. and *P. agamae*) were tested for *Coxiella* presence using a nested PCR assay and sequencing of the *rpoB* gene using *Coxiella*-specific primers targeting 539 - 542 bp (Duron *et al.,*2015). After *rpoB* amplification (Figure 5.3a) and BLAST analysis, the DNA sequencing results from the *Rhipicephalus* spp. were shown to be 100% identical to a *Coxiella*-like endosymbiont from a *Rhipicephalus* sp. from Ivory Coast, although only ~200 bases of good sequence were obtained (Table 5.2). The identity of the *Coxiella* sequence from *Haemaphysalis* spp. could not be confirmed due to inadequate PCR product sequence

quality. *Coxiella rpoB* amplicons were generated from the chigger samples (Figure 5.3B), but sequence quality was poor due to multiple peaks in the chromatograms. No reliable sequence was obtained from the *P. agamae* sample, but short forward and reverse sequences

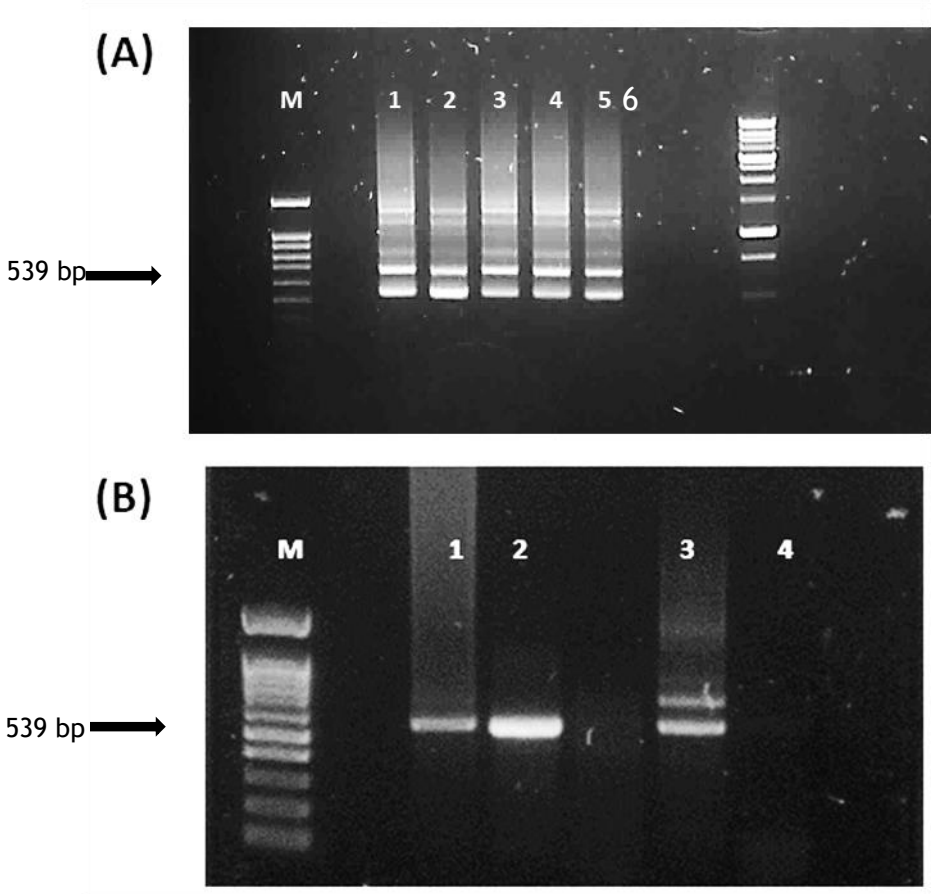


Figure 5.3. 1.5% gel electrophoresis of *rpoB* gene revealing PCR amplification of *Coxiella*-like endosymbionts of ticks.

(A) Lane M, 100-bp marker. Lane 1-3, *Rhipicephalus* spp.; lane 4, *Haemaphysalis* spp.; lane 5, positive control and lane, 6 negative controls. (B). Lane M, 100-bp marker. Lane 1-2, chigger samples; lane 3, positive control; lane 4, negative control.

Table 5.3. Results of specific PCR assays to confirm infection with bacterial pathogens and symbionts in *S. saudi sp. nov*. chiggers.

Bacterial genus (sequences obtained)	Rodent host	Village	Gene target	Sequence direction	Hit rank[#]	Query length	Query cover (%)	Identity (%)	E-value	Accession	Source	Location
Coxiella (1)	*A. dimidiatus*	Alous	*rpoB*	F	1	170	98	97.02	1^{-72}	CP040059	*Coxiella burnetii* strain RSA439 (from *Dermacentor andersoni*)	Montana, USA
					30		98	95.24	1^{-67}	KP985291	*Coxiella* endosymbiont of *Ornithodoros rostratus* isolate Orost4	Brazil
				R	1	126	95	92.50	2^{-39}	CP040059	*Coxiella burnetii* strain RSA439 (from *Dermacentor andersoni*)	Montana, USA
					28		95	91.67	1^{-37}	CP000733	*Coxiella burnetii* Dugway 5J108-111 (rodent isolate)	Utah, USA

[#]The highest ranked unique matches are shown in each case, ignoring intervening hits where they were identical to the top hit.

(>100 bases) containing single peaks were produced from the *S. saudi* sp. nov. sample. These did not overlap; however, analysis by BLASTn was consistent for the forward and reverse sequences, with closer matches to the Q-fever pathogen *C. burnetii* than to *Coxiella*-LE from ticks (Table 5.3).

5.4.2 *Francisella* endosymbiont of *Haemaphysalis* spp.

A conventional PCR was performed for *Francisella* in ticks targeting a 408-bp fragment of a lipoprotein gene (Alberdi *et al.,*2012). The primers amplified the target gene from a *Haemaphysalis* spp. and produced the expected 408-bp bands on an agarose gel (Figure 5.4). Sequencing of the PCR products revealed a match of 99.14% identity to *Francisella*-LE from the argasid tick, *Ornithodoros porcinus* (Table 5.2).

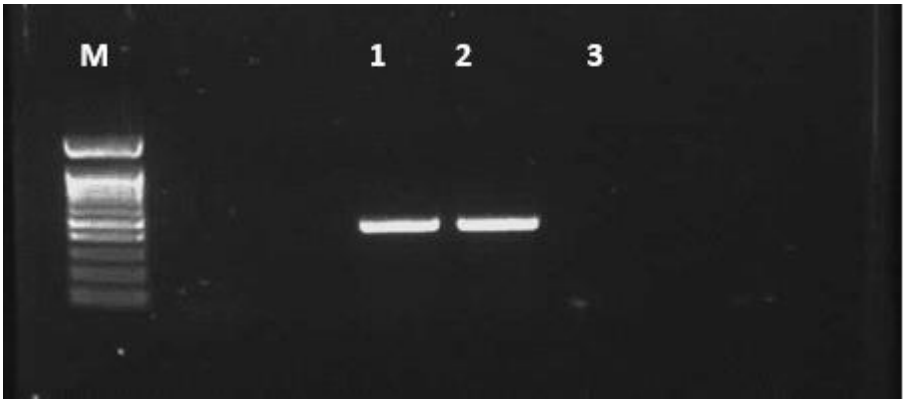


Figure 5.4. Gel electrophoresis illustrating PCR amplification of Francisella endosymbionts in Haemaphysalis spp. tick samples.

Lane M, 100-bp marker. Lane 1, *Haemaphysalis* spp.; lane 2, positive control and lane, 3 negative control.

5.4.3 *Anaplasma* spp. in *Haemaphysalis* spp. ticks and *P. oxyrrhyncha* lice

Anaplasma in ticks (*Haemaphysalis* spp.) and lice (*P. oxyrrhyncha*) were amplified by nested PCR using primers targeting the *Anaplasma* 16S rRNA and produced the expected 500 bp bands on an agarose gel (Figure 5.5). Sequencing of the PCR products from Haemaphysalis

spp. followed by BLASTn analysis revealed a best match (99.11%) to an uncultured Anaplasma sp. from a spleen sample from a greater bandicoot rat (Bandicota indica) in Taiwan (Table 5.4). One sequence from louse was identical to the tick sequence, whereas a second sequence from a louse differed by one SNP (Table 5.4). However, the top hit remained the Taiwanese sequence (accession no. JX846966).

Table 5.4. Results of specific PCR assays to confirm infection with bacterial pathogens and symbionts in *P. oxyrrhyncha* lice.

Bacterial genus (sequences obtained)*	Rodent host	Village	Gene target	Hit rank[#]	Query length	Query cover (%)	Identity (%)	E-value	Accession	Source	Location
Anaplasma (1)	*A. dimidiatus*	Alous	16S rRNA gene	1	453	100	98.90	0.0	JX846966	Uncultured *Anaplasma* sp. clone TN6SP12GE (from *Bandicota indica*)	Taiwan
				2		100	98.45	0.0	MN795628	*Anaplasma phagocytophilum* isolate Ap005R (from cattle)	Uganda
Anaplasma (1)	*A. dimidiatus*	Alous	16S rRNA gene	1	456	100	99.12	0.0	JX846966	Uncultured *Anaplasma* sp. clone TN6SP12GE (from *Bandicota indica*)	Taiwan
				2		100	98.68	0.0	MN795628	*Anaplasma phagocytophilum* isolate Ap005R (from cattle)	Uganda

*Results listed on the same row were identical where they overlapped (the length of high-quality sequence varied – results displayed are for the longest sequence obtained).

[#]The highest ranked unique matches are shown in each case, ignoring intervening hits where they were identical to the top hit.

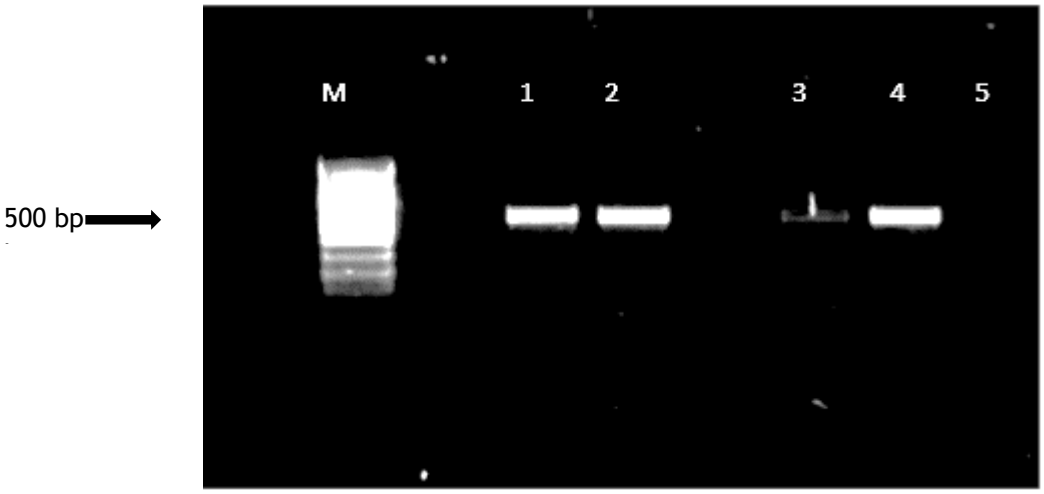


Figure 5.5. Gel electrophoresis of DNA PCR amplification of Anaplasma gene in tick and louse specimens collected from rodents.

Lane M, 500-bp marker. Lanes 1–2 tick samples; lanes 3-4 louse samples that were positive in 16S rDNA screening; lane, 5 negative control.

5.4.4 *Ehrlichia* spp. in *Haemaphysalis* spp., *Rhipicephalus* spp., and rodent skin swab samples

Ehrlichia spp. in *Haemaphysalis* spp. and *Rhipicephalus* spp. ticks were detected by nested PCR using primers targeting the *groESL* fragment, which amplified and produced the expected 1,350 bp bands on an agarose gel (Figure 5.6). The PCR products from both genera were identical, the longest consensus of forward and reverse sequences being 702 bp. This had a best match (96.58% identity) to "*Ehrlichia* sp. BL116-7" from a *Hyalomma asiaticum* tick from China (Table 5.2; Kang *et al.,*2014). In addition, an *M.rex* rodent skin swab sample with traces of *Ehrlichia* reads in the 16S rRNA amplicon sequencing was positive by PCR. Sequencing of the PCR products in both directions produced 523 bp of consensus sequence with 96.94% identity to the same *Ehrlichia* strain as from the ticks (Table 5.5).

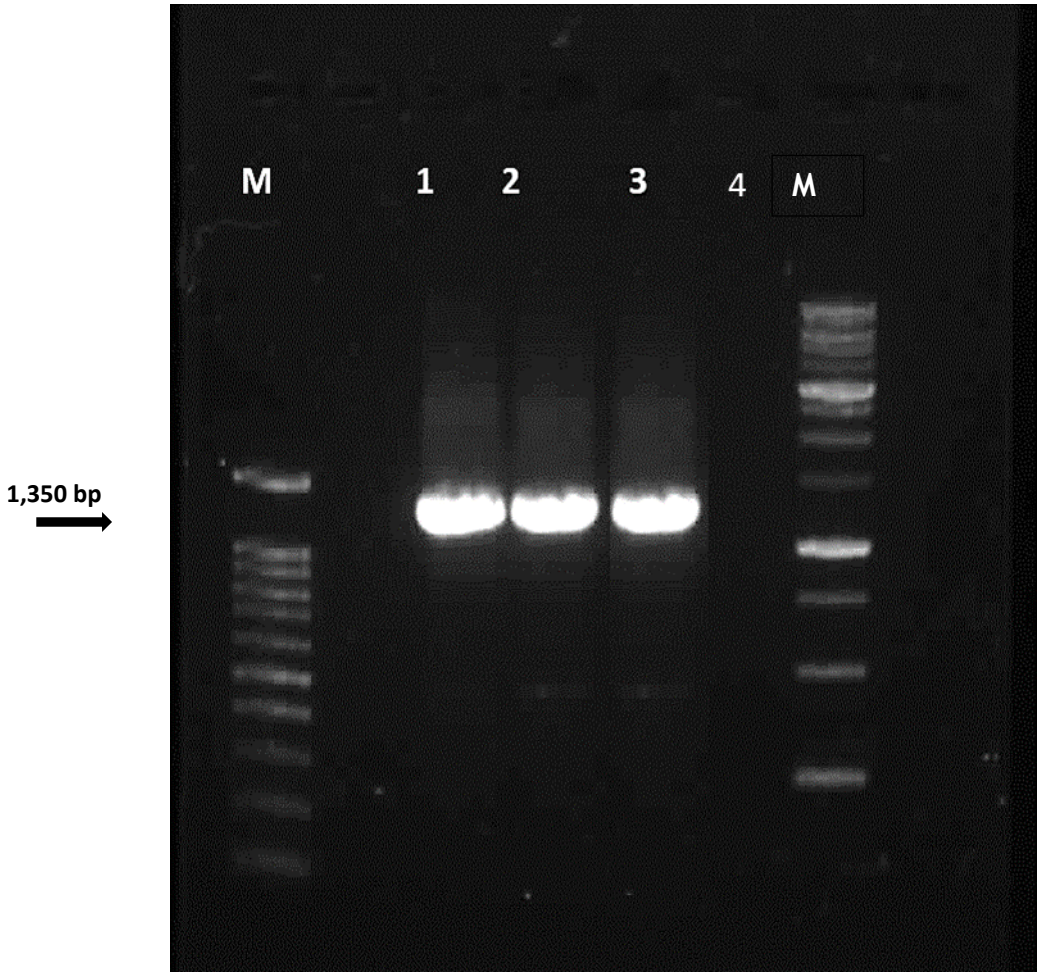


Figure 5.6. Gel electrophoresis indicates nested-PCR amplification of the Ehrlichia groESL fragment in ticks and a rodent skin swab sample.

Lane M, 100-bp and 1 kb markers; lane 1-2, tick samples; lane 3 swab sample; lane 4, negative control.

Table 5.5. Results of specific PCR assays to confirm the presence of bacterial pathogens and symbionts in rodent skin swabs.

Bacterial genus (sequences obtained)*	Rodent host (body site)	Village	Gene target	Hit rank[#]	Query length	Query cover (%)	Identity (%)	E-value	Accession	Source	Location
Ehrlichia (1)	*M. rex* (ear)	Alogl	*groESL*	1	523	100	96.94	0.0	KJ410291	*Ehrlichia* sp. BL116-7 (from *Hyalomma asiaticum*)	Xinjiang, China
				3		98	94.58	0.0	KX987380	*Ehrlichia* sp. strain WHHFZY-12 (from *Haemaphysalis flava*)	Hubei, China
Rickettsia (4)	*A. dimidiatus* (back or chin)	Alous	*gltA*	1	659	100	99.54	0.0	JQ354961	Uncultured *Rickettsia* sp. (from *Anopheles melas*)	Gabon
				3		100	99.39	0.0	KY488187	Uncultured *Rickettsia* sp. clone 19-S-2014 (from *Apodemus flavicollis*)	Poland

*Results listed on the same row were identical where they overlapped (the length of high-quality sequence varied – results displayed are for the longest sequence obtained).

[#]The highest ranked unique matches are shown in each case, ignoring intervening hits where they were identical to the top hit.

5.5 *Rickettsia* in rodent skin swab samples

In addition to the *Ehrlichia* sequences described above, some rodent skin swab samples from *A. dimidiatus* contained rickettsial sequences in the 16S rRNA amplicon sequencing analysis. A conventional PCR targeting the *gltA* gene was used to differentiate *Rickettsia* species by sequence analysis. The primers amplified the target gene and produced the expected 630 bp bands on an agarose gel (Figure 5.7). Sequencing of the PCR products from four samples produced identical sequences with a 99.54% match to *gltA* from an uncultured *Rickettsia* sp. found in an African mosquito, *Anopheles melas* (Table 5.5).

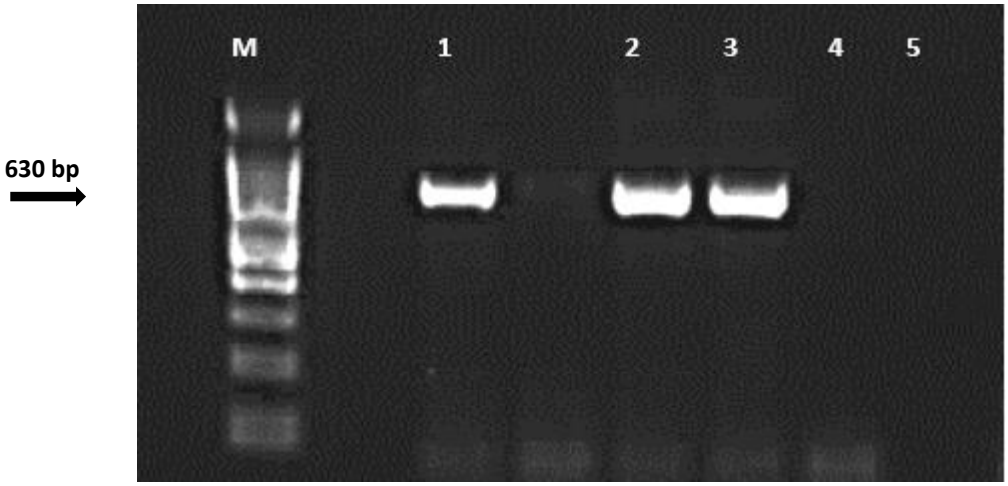


Figure 5.7. **Gel electrophoresis illustrating PCR amplification of a Rickettsia gltA sequence from rodent skin swab samples.**

Lane M, 100-bp marker; lane 1-2, swab sample from rodent back; lane 3, a swab from rodent chin; lane 4, negative control.

5.6 *Orientia* spp.

Attempts to amplify *Orientia* spp. DNA in a pool of chiggers (*Pentidionis agamae*) that was positive by 16S rRNA amplicon sequencing (see section 4.5) were made using nested PCR with primers targeting the TSA47 and TSA56 genes. Bands of the expected size were generated using these primers, but sequencing of the PCR products (both directly and after cloning) produced poor quality sequences with no matches by BLASTn. The positive control (*O. tsutsugamushi* strain UT176) produced a clean TSA47 sequence by direct Sanger sequencing.

5.7 Discussion

To our knowledge, this is the first study that has examined multiple rodent ectoparasite groups for zoonotic and symbiotic bacteria in Saudi Arabia. In this chapter, the bacteria genera that were identified by 16S rRNA amplicon sequencing were identified to species level by conventional or nested PCR targeting specific microbial genes. *Bartonella, Wolbachia, Spiroplasma, Coxiella, Francisella, Anaplasma, Ehrlichia,* and *Rickettsia* were all confirmed to be present and important information on the strains/species in the ticks, fleas, lice, chigger mites, and rodent skin swab was obtained.

Bartonella acomydis was detected in fleas *(P. chephrensis*) from the host *A. dimidiatus* in Alous village. The phylogenetic analysis of *Bartonella* based on the sequence of the ISR showed that the species of *Bartonella* was *B. acomydis* at 99.41% identity. This species has been isolated previously from the blood of wild-captured golden spiny mice (*Acomys russatus*) imported into Japan as pets from Egypt (Sato *et al.,*2013) and so our finding is consistent with the previous study, as the flea host in our case was also from the genus *Acomys*. However, to the best of our knowledge, this is the first record worldwide for this *Bartonella* sp. in a flea and the first record for Saudi Arabia. The pathogenicity of this species to humans or other animals is unknown.

Another dominant bacteria taxa found in *P. chephrensis* fleas was *Wolbachia* spp., which is the most prevalent symbiont of arthropods worldwide and well recognised as a reproductive manipulator in many species (McGraw *et al.,*1999). The *wsp* gene sequence from flea specimens showed that the symbiont was closely related to clade B, which is one of several clades of *Wolbachia* reported from other flea species (Casiraghi *et al.,*2005; Tay, 2013). To the best of our knowledge, *Wolbachia* has not been reported from *P. chephrensis* before. In common with other studies in flea such as *C. felis*, we found *Wolbachia* to be more common in female versus male fleas (Zurita *et al.,*2016). There are very few data on the impact of *Wolbachia* on the reproductive biology of fleas (if any), but in *C. felis*, a negative impact on reproduction was reported form a laboratory colony but not in wild specimens (Flatau *et al.,*2018).

A second potential reproductive manipulator, *Spiroplasma,* was also detected in female fleas (*P. chephrensis*) from Alous. The BLASTn analysis placed this symbiont within the *ixodetis* clade, which was first reported from several ixodid tick genera. It has a wide

geographic distribution in ticks, with reports from *I. pacificus* from Oregon (Tully *et al.,*1995), *I. ricinus* from Slovakia (Bell-Sakyi *et al.,*2015)., and an unspecified *Ixodes* sp. from Germany (Henning *et al.,*2006). However*,* the *ixodetis* clade is not restricted only to ticks but has been described in many other groups of arachnids and insects*,* including both acariform and parasitiform mites, Diptera (true flies), Hemiptera (true bugs), Lepidoptera (butterflies and moths) and beetles (Coleoptera) (Sanada-Morimura *et al.,*2013; Lindh *et al.,*2005; Fukatsu and Nikoh, 2000). In some insect species, such as the ladybird beetle *Anisosticta novemdecimpunctata*, *S. ixodetis* is a male-killing parasite (Tinsley and Majerus, 2006). However, the question of mammalian infections with *Spiroplasma* remains controversial (Bastian *et al.,*2007; Binetruy *et al.,*2019). There appears to be only one prior report of *Spiroplasma* infection in fleas previously, which was from *C. felis* in Hungary (Hornok *et al.,*2010). Thus, the present study extends the evidence for *S. ixodetis* infection in yet another order of insect hosts.

In the present study, we showed that ticks carried several non-pathogenic and potentially pathogenic bacteria within an individual sample. All *Rhipicephalus* spp. individuals screened by PCR and sequencing were positive for *Coxiella*-LE similar to other *Rhipicephalus* spp. worldwide (Gottlieb *et al.,*2015; Guizzo *et al.,*2017). However, the presence of this symbiont in *Haemaphysalis* spp. could not be confirmed by Sanger sequencing. The high prevalence of *Coxiella*-LE suggests they are transmitted by the transovarial route, which is considered as confirmed if the symbionts are detected in eggs (Klyachko *et al.,*2007). In the adult female, the density of *Coxiella*-LE tends to be higher compared to males due to the high concentrations in the ovaries, as reported for *Haemaphysalis longicornis* (Ruiling *et al.,*2019*),* but we could not investigate this in the current study as we only obtained immature specimens. It has been documented that these symbionts are essential in some tick species for normal development (Guizzo *et al.,*2017), and can rarely infect humans via tick bites (Angelakis *et al.,*2016). Previous studies have demonstrated that *Coxiella*-LE are found in several organs of ticks such as midgut, Malpighian tubules and ovaries. Although there is evidence that these symbionts contribute to numerous aspects of host fecundity and survival, the underlying molecular mechanisms are not yet fully defined (Wang *et al.,*2018). However, genomic analyses suggest it is very likely that *Coxiella*-LE provide an essential source of B-vitamins for their tick hosts (Rio *et al.,*2016; Gottlieb *et al.,*2015; Guizzo *et al.,*2017). Notably, *Coxiella*-LE are

not only important symbionts of the *Rhipicephalus* and *Haemaphysalis* genera, because treatment with antibiotics eliminated the *Coxiella*-LE from *Amblyomma americanum*, leading to decreased larvae hatching and reproductive fitness (Zhong *et al.,*2007).

Surprisingly, a *Coxiella* infection was also confirmed in chigger mite pools, which appeared to be closely related to *C. burnetii* (Q fever agent). Other researchers have previously theorised about *C. burnetii* in tick species and have demonstrated that tick-associated *Coxiella*-LE are closely related to *C. burnetii*. Indeed, prior research has revealed that *C. burnetii* evolved from a *Coxiella*-LE ancestor that acquired the ability to infect vertebrate cells (Duron *et al.,*2015). To the best of our knowledge, *Coxiella* has only been reported once before from chigger mites, in which a positive pool obtained from a rodent in Thailand appeared to be infected with a *Coxiella*-LE strain related to those found in hard ticks (Takhampunya *et al.,*2019). For the present study, future work should investigate whether *C. burnetti* is present in chiggers using additional genetic loci for strain characterisation.

Another dominant bacterium confirmed from ticks (in this case, only *Haemaphysalis* spp.) was *Francisella.* It has been shown that *Francisella*-LE are present in many tick genera, for example, *Haemaphysalis, Rhipicephalus*, *Amblyomma*, and *Dermacentor*, and they are particularly widespread in ticks obtained from a wide variety of animal hosts (such as wild boar, chicken, and snakes) in Asia (Koh *et al.,*2019; Sumrandee *et al.,*2016; Sumrandee *et al.,*2014; Rakthong *et al.,*2016). Surprisingly, the *Francisella*-LE from the Saudi *Haemaphysalis* spp. appeared to be most closely related to a symbiont from an argasid tick, *Ornithodoros porcinus*, from Southern Africa (Scoles, 2004). It seems likely that mammalian hosts could play a role in spreading *Francisella*-LE between ticks, as unlike *Coxiella*-LE, they appear to have evolved recently through "domestication" of the mammalian pathogen, *F. tularensis* (Gerhart *et al.,*2016; Gerhart *et al.,*2018; Duron *et al.,*2018). Interestingly, in an experiment in which the microbiome of *D. andersoni* ticks was disrupted using an antibiotic treatment, reduction in the native *Francisella*-LE population was associated with reduced susceptibility to *Francisella novicida*, which was used as a model for vector-borne *F. tularensis* (Gall *et al.,*2016).

Potential pathogens from the family Anaplasmataceae found in ticks comprised *Ehrlichia* (in both *Haemaphysalis* spp. and *Rhipicephalus* spp.) and *Anaplasma* (in *Haemaphysalis* spp. ticks and *P. oxyrrhynchus* lice only in Alous village). Interestingly, there was also

evidence for *Ehrlichia* in skin swab samples taken from the ear of an *M. rex* trapped in Alogl. The most likely source for this sequences in the swab was ectoparasite faeces, which has recently been considered as a material for monitoring transmission of vector-borne diseases (Pilotte *et al.,*2016), as well as a potential source of disease transmission itself (*e.g.*, *Bartonella* spp., *R. typhi* etc.). The closest matches (~97% identity) to these *Ehrlichia* spp. *groESL* sequences from ticks and swabs were to uncultured *Ehrlichia* spp. from hard ticks in China (Kang *et al.,*2014), which are of unknown pathogenic potential. However, the best match to a characterisied *Ehrlichia* spp. (~94%) was to *E. ewingii*. It has been recorded that *E. ewingii* is pathogenic in humans and dogs and infects granulocytes; moreover, it may be fatal in humans, especially if not diagnosed promptly. Several *E. ewingii*-like organisms have been recorded in ticks and wild animals outside North America, including Japan and Argentina (Matsumoto *et al.,*2011; Kawahara *et al.,*2006; Monje *et al.,*2019). In addition, our results are quite comparable with previous study that identified a strain related to *E. ewingii* in *H. longicornis* ticks from China (Luo *et al.,*2016). In this respect, further research is needed to reveal the role of rodent species as potential reservoirs of *Ehrlichia* in the Asir region and the possible risk to public and veterinary health. For the *Anaplasma* spp., the closest 16S rRNA sequence matches (~99% identity) were to an uncultured strain from rodent spleen in Taiwan (unpublished data). The second hit (98.6% identity) was to an uncultured "*A. phagocytophilum*" strain from cattle in Uganda (unpublished data), although hits of identical confidence were made to several isolates of *A. bovis*, a non-zoonotic pathogen of domestic cattle that infects mononuclear cells and has a wide distribution globally (Guo *et al.,*2019). Thus, as 16S rRNA discriminates poorly between *Anaplasma* spp., additional gene targets will need to be sequenced from the positive samples in order to verify the species.

Finally, rickettsial DNA was detected in rodent skin swab samples taken from the back and chin of the animals. The best match (99.5% identity) for these rickettsial *gltA* sequences was to uncultured *Rickettsia felis*-like organisms from African mosquitoes (Socolovschi *et al.,*2012). However, the match to a cultured rickettsial species from argasid ticks, *R. lusitaniae*, was also very high (~99% identity). This species has been reported previously in *Ornithodoros erraticus* from pigpens in the south of Portugal (Milhano *et al.,*2014) and also from *Ornithodoros yumatensis* from two bat caves in Yucatan, Mexico (Sánchez-Montes *et al.,*2016). It has not been associated with human disease. Argasid ticks only feed for brief

periods and do not remain attached to mobile hosts, usually remaining hidden in burrows, nests or in the cracks and crevices of buildings. Therefore, it is possible that the rickettsiae or rickettsial DNA from soft ticks was excreted onto rodent fur in an infested burrow. Equally, if mosquitoes were the source of these rickettsial sequences, excretion of rickettsial material in their faeces is also likely to occur after blood-feeding (Dieme *et al.,*2015; Pilotte *et al.,*2016).

5.8 Conclusion

In this first survey of potentially pathogenic bacteria and symbionts from rodent ectoparasites in the Asir region of Saudi Arabia, the presence of *Wolbachia*, *Spiroplasma* and *Bartonella* (in fleas); *Coxiella*, *Francisella*, *Anaplasma* and *Ehrlichia* (in ticks); *Anaplasma* (in lice); and *Coxiella* (in chiggers) was confirmed. In addition, rodent skin swabs were shown to contain DNA sequences from *Ehrlichia* and *Rickettsia*. Two bacteria with pathogenic potential for domestic animals (related to *A. phagocytophilum* or *A. bovis* and *E. ewingii*) were detected; these could also be potentially zoonotic pathogens. The *Coxiella* in chiggers and *B. acomydis* from fleas should also be characterised further to determine if they might be transmitted to humans or domestic animals to cause disease. The presence of symbionts co-existing with pathogenic bacteria in arthropods may also influence pathogen transmission. Therefore, this study provides essential baseline information for further research in the Asir region in future.

Chapter 6. The microbiome of a laboratory colony of the tropical rat mite (*Ornithonyssus bacoti*)

6.1 Introduction

Helminth parasites infect more than two billion people globally. Onchocerciasis and lymphatic filariasis are two neglected, vector-borne tropical diseases caused by filarial nematodes. Onchocerciasis (or river blindness) is caused by *Onchocerca volvulus* and affects about 20 million people in sub-Saharan Africa. The disease manifests as severe dermatitis and visual impairment (Who.int, 2019), and is transmitted by blackflies (*Simulium* spp) that breed in fast-flowing rivers. Lymphatic filariasis (or elephantiasis) is caused by *Wuchereria bancrofti* (>90% of cases) or *Brugia* spp. and affects about 36 million people across the tropics. The disease symptoms can include massive swelling of the legs due to lymphoedema, as well as hydrocele and deformities of the breasts (Who.int, 2019). The infection can be transmitted by a wide variety of mosquito species.

Research on filarial diseases, particularly vaccine research, has been hampered by failure of the human parasites to develop in immunocompetent mice. To address this gap, the *Litomosoides sigmodontis* model was developed in laboratory mice. This is a New World parasite that naturally infects the cotton rat, *Sigmodon hispidus,* but will complete its lifecycle in immunocompetent BALB/c mice (Hoffmann *et al.,*2000). Importantly, *L. sigmodontis*, in common with many filarial nematodes, contains a *Wolbachia* endosymbiont. This means that the model system can be used to test the efficacy of antibiotics targeting this symbiont, which is possible control option for human filarial diseases that may be rolled out soon (Hoerauf *et al.,*1999; Specht *et al.,*2018). The tropical rat mite, *O. bacoti* (Hirst, 1913), is the vector of *L. sigmodontis* and belongs to the family Macronyssidae (Hoffmann *et al.,*2000). This mite has a cosmopolitan distribution on rodents worldwide and was present on several rodents sampled in the current study in Asir (see Chapter 3).

The purpose of this chapter was to determine the microbiome of *O. bacoti* in a laboratory colony maintained at the Muséum national d'histoire naturelle, Paris, which is used for experiments on *L. sigmodontis*. The feasibility of manipulating the microbiome by antibiotic treatments of the jird host prior to mite feeding was also explored as a pilot study for future work on how the microbiome might affect the vector competence of *O. bacoti*. The

microbiome of *O. bacoti* was investigated using two culture-independent methods [16S rRNA amplicon sequencing using the Illumina MiSeq and Oxford Nanopore Technologies (ONT) systems], as well as traditional bacterial culture on solid media.

6.2 The life cycle of *O. bacoti*

The *O. bacoti* life cycle is consists of five stages; larvae, nymph, protonymph, deutonymph, and adults. Firstly, the eggs take 1-4 days to hatch into larvae with six legs, and larvae take 24 hours to moult into eight-legged protonymphs, which need a blood meal before developing into deutonymphs. This stage does not require a blood meal; they take 24-36 hours to moult into the adult. Moreover, after the fertilisation which takes from 24-48 hours, the female adult needs a blood meal to lay their eggs. Both sexes feed intermittently (every 48-72 h) on the host and do not remain attached for long periods (Diagnosis, Tropical rat mite (*Ornithonyssus bacoti*) acariasis. (2005). The whole life cycle thus takes up to two weeks (Figure 6.1).

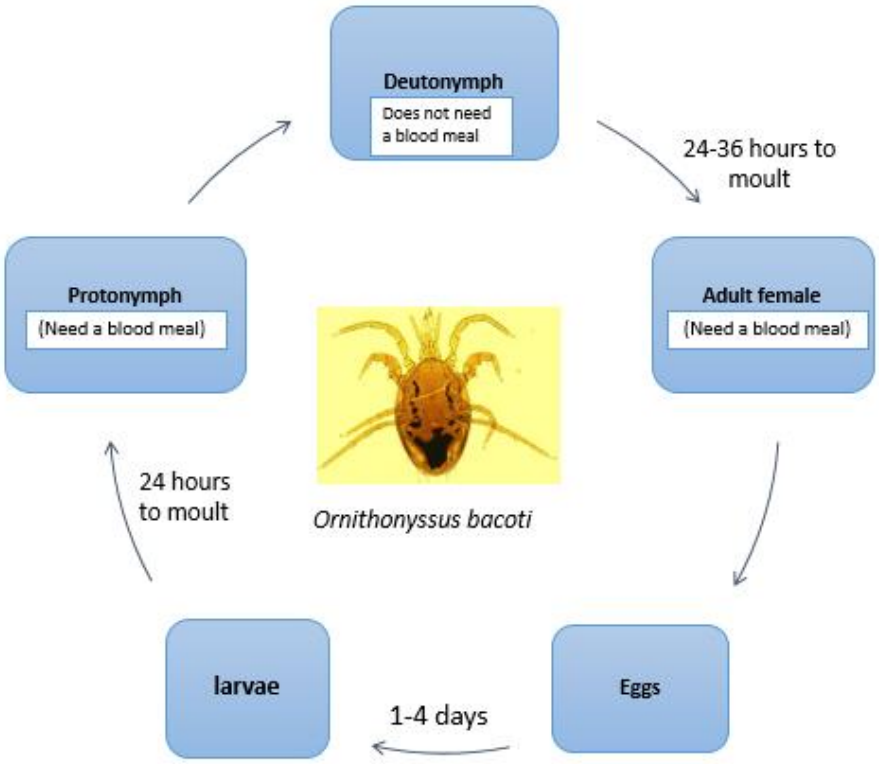


Figure 6.1. Life cycle of *O. bacoti* (the tropical rat mite).

6.3 Life cycle of *Litomosoides sigmodontis*

In common with all filarial nematodes, *L. sigmodontis* has a complex life cycle. Mites are infected when they ingest peripheral blood containing first-stage larvae (microfilariae) from the rodent host. The nematodes undergo two moults in the vector, which takes approximately 10 days in total (Figure 6.2). The infective larvae (L3) are transferred to the definitive host during a blood meal and develop through two further moults into male and female adults, which reside in the pleural cavity. The sexually mature adults mate at around 30 days post-infection and embryogenesis takes another 18 days, so microfilariae are not found in the blood until >50 days post-infection (Figure 6.2). Generally, *L. sigmodontis* is maintained in its natural host (*S. hispidus*) or jirds (*Meriones unguiculatus*) because it achieves higher and longer-lasting microfilarial densities in these species compared with mice (Fulton *et al.,*2018). Housing and maintenance of the mites is challenging, as they need high humidity (~70%) and a temperature of 27-28˚C; great care must also be taken

to prevent bites to animal facility staff or infestation of other parts of the facility (Hübner *et al.,*2009; (Fulton *et al.,*2018). Depending on the laboratory, the infection of gerbils or cotton rats may not proceed by natural transmission but by dissection of mites to acquire L3; these are then injected subcutaneously at a dose of 70-100 L3 per rodent (Fulton *et al.,*2018) (Figure 6.2).

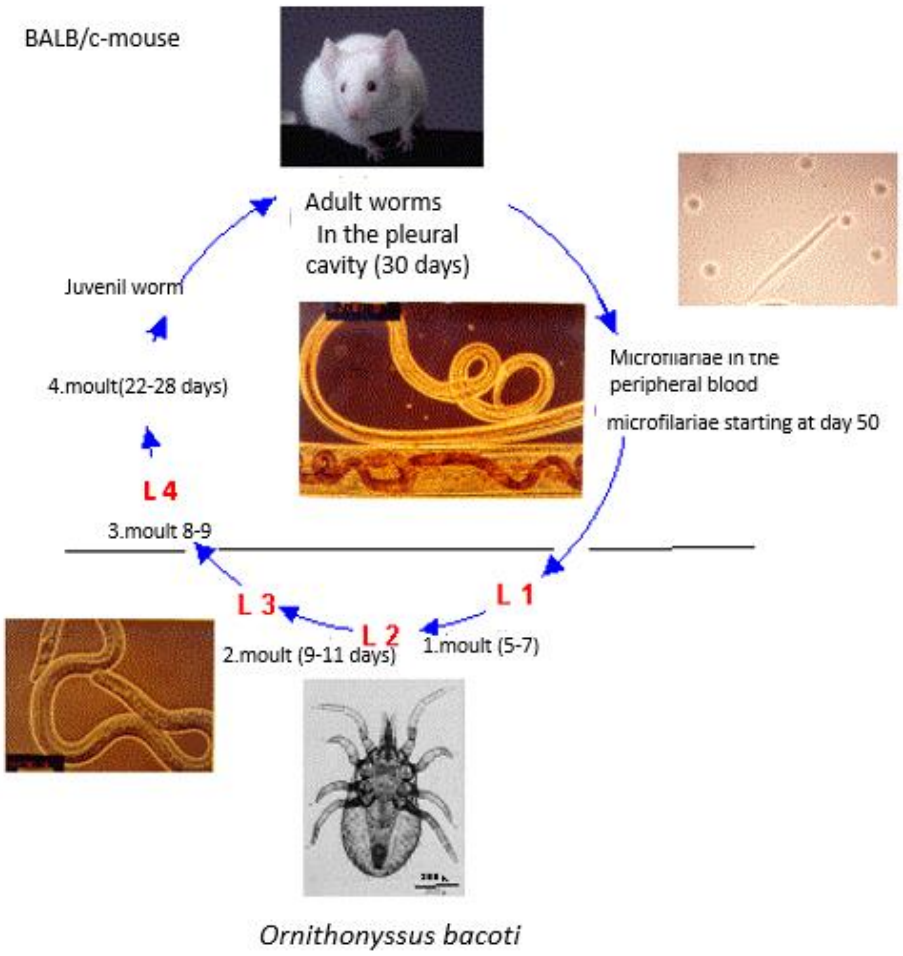


Figure 6.2. Life cycle of *Litomosoides sigmodontis*

Adapted from *http://www.ra.cs.uni-tuebingen.de/tuebingenome/subprojects/sp3/sp3b.html*

6.4 Identification of the microbiome profile of *O. bacoti* using three different methods

6.4.1 Identification of microbiome profile in *O. bacoti* using 16S rRNA amplicon sequencing on the Illumina MiSeq

The *O. bacoti* samples from Paris were sequenced on Illumina MiSeq plate 2, which unfortunately had shown poor sequencing depth (see Chapter 4). However, some patterns in the data could still be discerned. Mites fed on untreated jirds had a microbiome heavily dominated by *Staphylococcus*, with smaller contributions of *Corynebacterium*, *Pseudomonas* and *Acinetobacter* (Figure 6.3). Surprisingly, most of the antibiotic combinations (with the exception of penicillin-streptomycin duotherapy) did not markedly change this profile, although the proportion of *Lactobacillus* increased. However, penicillin-streptomycin duotherapy, but not other antibiotic combinations containing these two drugs, had a dramatic effect on the mite microbiome, in that it became dominated almost entirely by *Proteus*. In background controls, *Acinetobacter*, *Staphylococcus* and *Corynebacterium* were all present, but *Propionibacterium* tended to be more dominant (Figure 6.3).

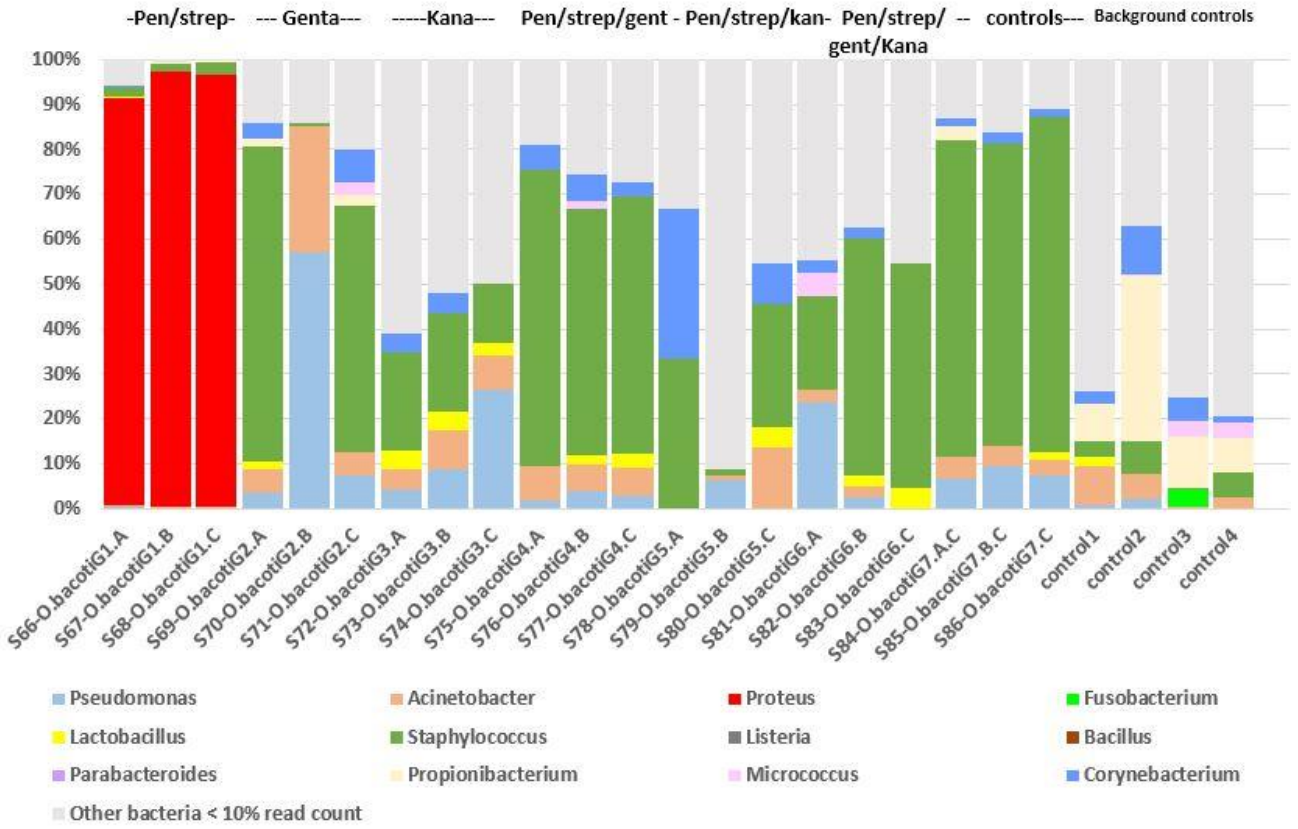


Figure 6.3. Stacked bar charts show the relative abundance of bacterial OTUs in *O.bacoti* mitestreated with different antibiotics using 16S rRNA amplicon sequencing on the Illumina MiSeq. The data are filtered; minority OTUs contributing <10% are combined in "other bacteria" (grey). Key to antibiotics: pen, penicillin; strep, streptomycin; gent, gentamycin; kana, kanamycin.

6.4.1.1 Identification of microbiome profile in *O. bacoti* using 16S rRNA amplicon sequencing on the MinION (ONT)

A conventional PCR amplification of the 16S rRNA gene in *O. bacoti* samples was performed to produce the full-length gene (1,500 bp) required for amplicon sequencing on the MinION system. Amplification was successful in all the pools of *O. bacoti* (Figure 6.4). According to the MinION sequencing results, the microbiome of untreated mites was heavily dominated by *Candidatus* Blochmannia, with smaller contributions from *Staphylococcus, Pseudomonas, Massilia* and *Nitrosomonas* (Figure 6.5). The most dramatic microbiome disruption was caused by penicillin-streptomycin duotherapy, which led to almost complete dominance by *Proteus* in agreement with the Illumina MiSeq data. The other antibiotic treatments had a less marked effect, but *Stenotrophomonas* and *Synechococcus* became more abundant in some pools of mites treated with penicillin-streptomycin-kanamycin, and one of the pools of mites treated with gentamycin displayed much greater proportions of *Pseudomonas*, *Acinetobacter* and *Massilia* at the expense of Blochmannia (Figure 6.5). The background controls could not be sequenced for this experiment due to insufficient DNA yield.

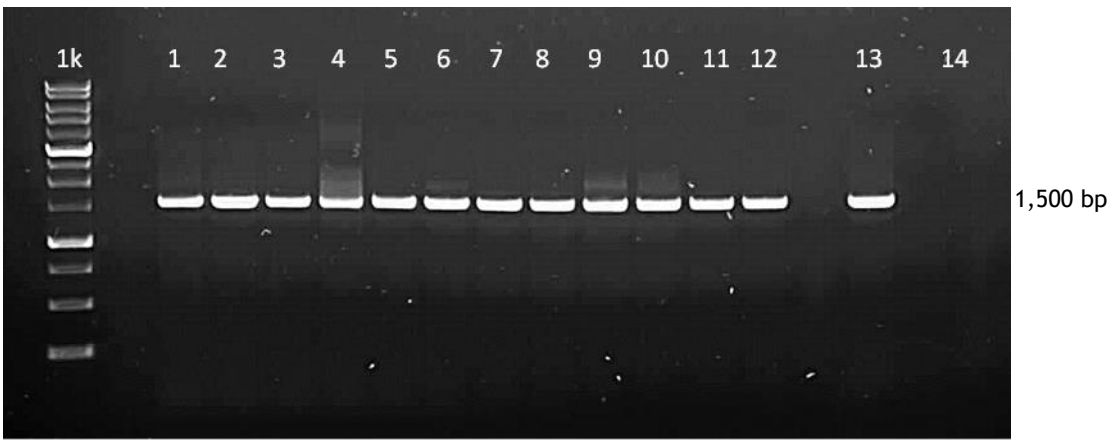


Figure 6.4. Gel electrophoresis illustrating PCR amplification of full-length 16S rRNA gene in *O. bacoti* samples.
Lane M, 500-bp marker (1K). Lane 1-12 *O. bacoti* samples; lane 13; positive control; lane 14, negative control.

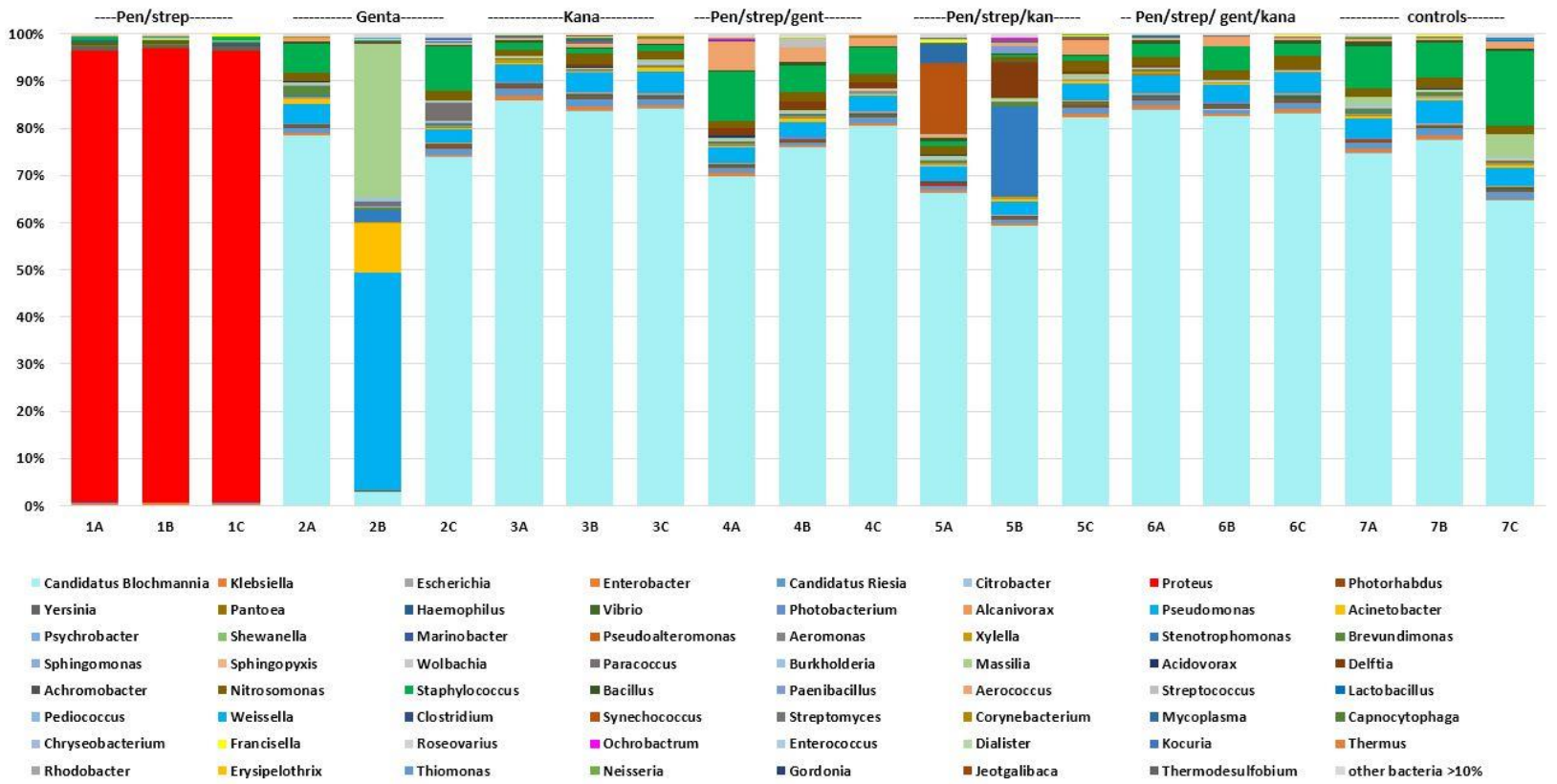


Figure 6.5. Stacked bar charts show the relative abundance of bacterial OTUs in *O. bacoti* mites treated with different antibiotics using 16S rRNA amplicon sequencing on the MinION (ONT).

The data are filtered; minority OTUs contributing <10% are combined in "other bacteria" (grey). Key to antibiotics: pen, penicillin; strep, streptomycin; gent, gentamycin; kana, kanamycin.

6.4.2 Identification of cultivable bacteria from a laboratory colony of *O. bacoti*

It was not possible to attempt bacterial culture from the antibiotic-treated mite samples as they had been stored in ethanol for shipment to the UK. However, culture was performed on untreated mite homogenates, including a batch of *O. bacoti* infected with *L. sigmodontis* (see section 2.11.5.2). Individual colonies from each batch of mites was assayed by a PCR targeting the 16S rRNA gene (Figure 6.6) and the products were subjected to Sanger sequencing. The length of high-quality sequence from several colonies was shorter than expected (Table 6.1). However, colonies from *O. bacoti* homogenates yielded high-confidence BLAST hits to *Alcaligenes faecalis* (Burkholderiales: *Alcaligenaceae*), especially from infected mites (Table 6.1). Interestingly, no colonies were obtained from starved, uninfected mites. Some additional colonies were identified as *Staphylococcus lentus, Yersinia mollaretii*, and species close to *Pseudogracilibacillus* sp. SW110 (Bacillales: *Bacillaceae*), *Weissella thailandensis* (Lactobacillales: *Leuconostocaceae*), *Bacillus* sp. strain SAB-2 MA (Bacillales: Bacillaceae), and *Proteus mirabilis* (Enterobacterales: *Morganellaceae*). A comparison of the OTUs identified between the conventional culture method and the next-generation 16S rRNA amplicon sequencing methods (Illumina and ONT) is presented in Table 6.2.

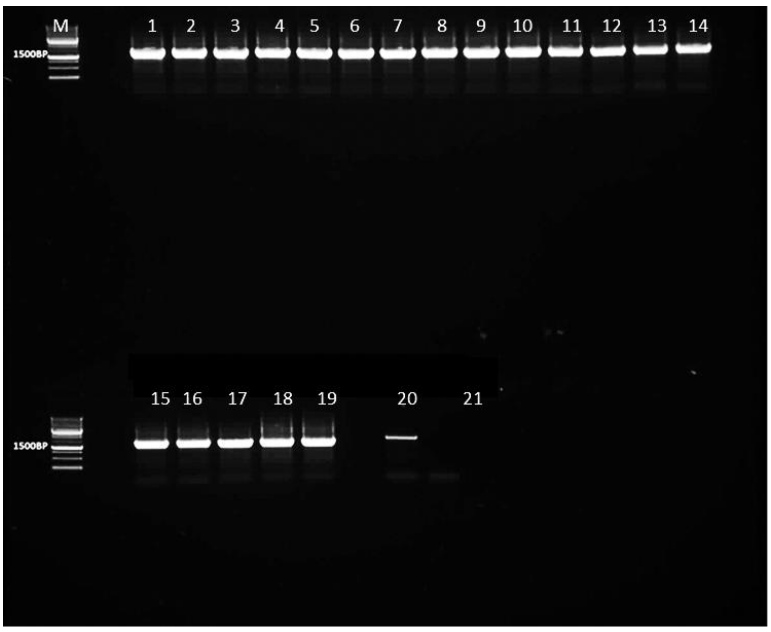


Figure 6.6. Gel electrophoresis illustrating PCR amplification of the full-length 16S rRNA gene from colonies obtained from *O. bacoti* homogenates.

Lane M, 500-bp marker (1K). Lanes 1-19, colony samples; lane 20, positive control; lane 21; negative control.

Table 6.1. NCBI BLASTn results of 16S rRNA gene sequences obtained from colonies from *O. bacoti* homogenates.

Group*	BLASTn description	Sequence length	Max score	Query cover	E-value	Identity (%)	Accession no.
A	*Yersinia mollaretii* strain MCM, isolate 4A	107	172	92%	1e-39	98%	LT745988.1
A	*Alcaligenes faecalis* strain GX19 ,	964	1707	99%	0.0	99%	KU937382.1
B	*Pseudogracilibacillus* sp. SW110	104	169	99%	2e-38	96%	KR611042.1
B	*Weissella thailandensis* strain CAU3107	125	196	100%	1e-46	95%	MF429421.1
B	*Bacillus* sp. strain SAB-2 MA ,	125	196	100%	1e-46	95%	MH707127.1
B	*Proteus mirabilis* strain NY-201801	152	259	100%	2e-65	97%	MK392127.1
D	*Alcaligenes faecalis* strain B17	772	1410	99%	0.0	99%	MG234449.1
D	*Alcaligenes faecalis* strain BS13	114	211	100%	3e-51	100%	MK377187.1
D	*Alcaligenes faecalis* strain UMAGOD12	643	211	100%	1e-180	99%	MH091063.1
D	*Alcaligenes faecalis* strain UMAGOD12	606	1096	99%	0.0	99%	MH091063.1
D	*Alcaligenes sp*. strain CM_L_7716,	285	527	100%	8e-146	100%	MK397528.1
D	*Alcaligenes faecalis* strain B17	912	1677	99%	0.0	99%	MG234449.1
D	*Alcaligenes faecalis* strain SCNAH10	186	333	100%	1e-87	99%	KT327202.1
D	*Alcaligenes faecalis* strain UMAGOD12	283	521	99%	4e-144	100%	MH091063.1
D	*Staphylococcus lentus* strain FC3104 ,	274	507	100%	1e-139	100%	MK439492.1

*A, engorged mite extract with plating on agar without vitamins; B, engorged mite extract with plating on agar with vitamins; D, extract from starved, L. sigmodontis-infected mites with plating on agar with vitamins.

Table 6.2. Comparison of bacterial OTUs found in *O. bacoti* using three different methods.

Bacterial OUT	Illumina MiSeq	MinION (ONT)	Culture of bacteria and Sanger sequencing
Proteus	X	X	X
Staphylococcus	X	X	X
Corynebacterium	X	X	
Lactobacillus	X	X	
Pseudomonas	X	X	
Candidatus Blochmannia		X	
Massilia		X	X
Bacillus sp.	X	X	X
Alcaligenes faecalis			X
Yersinia mollaretii			X
Acinetobacter	X	X	

6.5 Discussion

Overall, the evaluation of the three different technology methods revealed some similar findings of OTUs at the genus level such as *Proteus, Bacillus,* and *Staphylococcus.* However, as expected, the culturing method did not provide a large diversity of bacteria compared to the two culture-independent techniques. Even some of the culturable bacteria that consistently appeared as OTUs in the Illumina and ONT sequencing data (*e.g.*, *Corynebacterium*, *Pseudomonas* and *Acinetobacter*) were not seen as colonies. This may have been due to competition from the other cultivable species or because the DNA signal from these organisms was from dead rather than viable bacteria. Conversely, most colonies obtained from untreated mites, especially those infected with *L. sigmodontis*, were identified as *Alcaligenes faecalis*, which was not observed in the 16S rRNA amplicon sequencing data. It is possible that *A. faecalis* was misidentified in the ONT data as *Delftia*, which is another member of the Burkholderiales, as ONT sequencing has a high error rate (Cuscó *et al.,*2018). On the other hand, *Weissella* and *Yersinia* were identified in the ONT data and were also isolated as colonies.

Alcaligenes faecalis is an environmental species that most likely would have outgrown many other types of bacteria. Supporting our data, Smrž (2003) grew *A. faecalis* from homogenates of the mould mite, *Tyrophagus putrescentiae* (Acari: Acaridida), which is an acariform mite unrelated to *O. bacoti.* The latter may have acquired *A. faecalis* from the fur of jirds contaminated with faeces or from the environment. Jird skin or fur is also a likely source of *Staphylococcus*, *Yersinia* and *Proteus*, and the lack of colony growth from starved mites supports this interpretation. It is unclear from the amplicon sequencing data if *O. bacoti* also has an uncultivable primary or secondary endosymbiont, as Blochmannia reads were very dominant in the ONT data but were not observed in the Illumina data. Blochmannia is an obligate endosymbiont of carpenter ants (Williams & Wernegreen., 2015) and some true bugs (Wernegreen *et al.,*2009), but is also a member of the Enterobacterales, which contains a large variety of other arthropod symbionts. The Darby laboratory where the PCR amplicons were prepared, performs culture of *Arsenophonus* and *Sodalis* (both Enterobacterales), so contamination of the ONT libraries with DNA from these species is possible. Unfortunately, since the negative controls for this experiment had too little DNA available to sequence, further work will be required to resolve this issue.

The results of the antibiotic treatment of jirds on the *O. bacoti* microbiome were surprising, as only penicillin-streptomycin duotherapy, not triple or quadruple combinations containing penicillin-streptomycin, had a radical effect. Both Illumina and ONT sequencing showed that *Proteus* completed dominated the *O. bacoti* microbiome when jirds received penicillin and streptomycin only. This suggests that *Proteus* was resistant to these drugs but not to gentamicin or kanamycin, perhaps allowing it to outgrow other bacteria in the mite gut when the two other antibiotics were used only. Complex effects of different antibiotic treatments on the arthropod microbiome have been reported before, showing that competitive interactions can exist between symbionts like *Wolbachia* and the gut microbiome, which can even kill *Anopheles* mosquitoes if they are infected with *Wolbachia* and then take a blood meal (Hughes *et al.,*2014). Interestingly, in mites infected with *L. sigmodontis* and subsequently starved, isolation of *A. faecalis* was very common. In future, it should be investigated it *A. faecalis* has a role in the reduced fecundity reported from *L. sigmodontis*-infected mites compared with uninfected females (Nieguitsila *et al.,*2013).

There have been few prior studies on the impact of symbionts on the competence of filarial vectors. Instead, there has been more of a focus on *Wolbachia* symbionts of filarial worms and their importance during parasite development in the vector. It was shown that if *Simulium* blackflies feed on onchocerciasis patients who have received doxycycline therapy targeting *Wolbachia*, development of *O. volvulus* within the vector is partially arrested (Albers *et al.,*2012). A similar effect was reported in *O. bacoti* feeding on *L. sigmodontis*-infected jirds that had been treated with tetracycline. Moreover, surviving *Wolbachia*-depleted L3 that infected new jirds via feeding by *O. bacoti* showed a reduced ability to mature if they were female worms, with no detrimental effect seen in males (Arumugam *et al.,*2008). The impact of *Wolbachia* infection in the vector itself has been little investigated for filarial worms, but if the pathogenic strain *w*MelPop is introduced into *A. aegypti*, development of *Brugia* is inhibited in the mosquito (Kambris *et al.,*2009). There was some provisional evidence for natural *Wolbachia* infection in *O. bacoti* in the ONT data (Figure 6.5), but this requires verification.

The potential impact of alteration of the vector microbiome on disease transmission extends beyond just *Wolbachia*. In *D. andersoni* ticks exposure to antibiotic treatment could a decrease in the density of *R. bellii* symbionts increased susceptibility to *A. marginale* infection, but the decrease in *Francisella*-LE in the same species reduced establishment by

pathogenic *Francisella* (Gall *et al.,*2016). Altering the microbiome by antibiotic treatment can also reduce tick reproductive capacity and this might have indirect effects on vector competence (Zhong *et al.,*2007). However, not all microbiome disruption by antibiotics is detrimental to vector competence. In the malaria vector *Anopheles gambiae*, ingestion of blood from antibiotic-treated patients increased the susceptibility of the mosquitoes to *P. falciparum* and increased vector fecundity and lifespan (Gendrin *et al.,*2015). Very little work on the effect of the microbiome on vector competence in mites has been performed to date, but it was noted that in a laboratory colony of the chigger *Leptotrombidium imphalum*, infection with *O. tsutsugamushi* was positively associated with a novel Amoebophilaceae OTU in adult female mites (Ponnusamy *et al.,*2018).

6.6 Conclusions

A comparison of three methods to investigate the laboratory colony of *O. bacoti* showed some similarities and differences (Table 6.2). *Staphylococcus*, *Proteus* and *Bacillus* were seen with all three methods, but unsurprisingly, few of the OTUs observed with the 16S rRNA amplicon sequencing methods were successfully cultured. Therefore, the diversity of the cultured bacteria was very low. The most dominant cultured bacterium, *A. faecalis*, was not evident as a prominent OTU in the sequencing data. However, it was most commonly seen in extracts from *L. sigmodontis*-infected mites, and the mites used for Illumina and ONT sequencing were uninfected. A key possible source of error is the existence of contaminating OTUs in the laboratory environment, suggesting that the bacterial biomass in *O. bacoti* was low (Salter *et al.,*2014). Microbial DNA in extraction kits, laboratory reagents or equipment might explain the unexpected Blochmannia OTU in the ONT data that was not corroborated by Illumina. Differences in Illumina and ONT sequencing technologies (*e.g.*, error rate and read length) and the bioinformatic pipelines used to assign OTUs will also be responsible for differences between the two datasets.

Chapter 7. Final discussion, conclusion, and future work

7.1 Final discussion and conclusion

This is, to our knowledge, the first study to discover a high diversity of ectoparasites from a small area of Saudi Arabia. Mainly, a large number of chigger specimens was collected from the area that leads us to contribute significantly to the knowledge of the chigger fauna of the Arabian Peninsula. Our detailed research on chigger mites and other ectoparasites provided baseline information for further experimental investigation and for public health research on the potential importance of pathogens such as *O. chuto.*

Geographically, Saudi Arabia is bordered by Yemen, Jordan, Kuwait, Qatar, the United Arab Emirates, Oman and Iraq, and separated from the African continent by the Red Sea. For most of these countries, there are no records of chigger mites or the fauna is poorly described. However, there were similarities in the trombiculid fauna of Asir with that described from Central Asia (including southern Turkey and northern Iran) and from Central and East Africa. Notably, Asir is situated at the border between the Ethiopian and Palearctic zoogeographical regions; therefore, a mixture of African and Asian species is not wholly unexpected. A connection with the Iranian chiggers is *M. muhaylensis* sp. nov., which is similar to *M. meriones* described in Iran. This raises a further research question about the identification of some chigger species closely related to each other with a few differences in morphometric formulae such as scutum, seta, legs, palpal pilous formula, and coxal setation formula. Thus, future work should concentrate on investigating new approaches that may help to differentiate between chigger species accurately. Other species represents a connection of Saudi Arabian chiggers with the fauna of Sub-Saharan Africa. *Gahrliepia lawrencei* and *M. centropi* were known only from Central Africa. In addition, *S. asiriensis* sp. nov. is similar to a Central African species, *S. paulus*; and *M. microscuta* is similar to *M. machadoi* described from Angola. One species, *M. hyracis*, was recorded both in Central and Eastern Africa (Uganda and Djibouti), and *A. browni* was previously known only from its type locality in Djibouti; *i.e*., ~650 km south of the 'Asir Region across the Red Sea. Therefore, our data reveal that many chigger species could have wide ranges extending from Central Africa to Western and even Central Asia, and from south of the Arabian Peninsula to Eastern Europe. Previously, only eight African species have been

recorded outside the continent (Stekolnikov 2018); thus, our collection adds five more such species (*G. lawrencei, A. browni, E. galliardi, M. centropi,* and *M. hyracis*).

Overall in this study, we found impressive ectoparasite diversity on small rodents, particularly chigger species, but also three novel lineages of *Rhipicephalus* spp. ticks and two distinct clades of *Haemaphysalis* based on phylogenetic analysis. The genetic diversity of the *R. sanguineus* (brown dog tick) complex worldwide has been investigated in some detail. Prior to our study, the use of molecular barcoding (predominantly targeting mitochondrial rRNA genes) had delineated three clades within the complex: the temperate lineage in Western Europe, North America and temperate regions of South America; the tropical lineage in tropical South America, Africa (including North Africa) and the Asian tropics; and the Southeastern European lineage in Romania, the Balkans, Greece, Turkey, the Middle East and North Africa (Hekimoğlu *et al.,*2016; Chitimia-Dobler *et al.,*2017; Low & Prakash, 2018). In a recent redescription of *R. sanguineus*, the species *sensu stricto* was defined as the temperate lineage only (Nava *et al.,*2018). The tropical lineage also encompasses *R. camicasi* and *R. guilhoni*, but has not been formally redescribed as a distinct species. Finally, the Southeastern European lineage overlaps with the tropical lineage in North Africa (Egypt), but little sampling from the Arabian Peninsula had taken place before our study was performed.

It is remarkable considering the global distribution of the *R. sanguineus* complex and the extent of prior phylogenetic analyses of this group that three novel clades were discovered within such a small geographic area in Saudi Arabia. The evolutionary origin of the complex is uncertain, but ancestral area reconstruction suggests either a single origin in Western Europe, or a wider distribution across Europe and the Middle East (Hekimoğlu *et al.,*2016). In both scenarios, the Middle East was a key staging post in the colonisation of Africa by *R. sanguineus sensu lato*. Our findings in Saudi Arabia suggest that this region is a diversity hotspot for the complex and more sampling from the Arabian Peninsula is likely to change the current view on how it spread across the globe. Recently, *Rhipicephalus* spp. ticks were collected from dogs and camels in Riyadh province and subjected to morphometric and molecular analyses (Chandra *et al.,*2019). Their phylogenetic analyses used three mitochondrial markers (16S and 12S rRNA genes and *coi*) and they placed a specimen from

dogs and a subset of the camel samples as "*Rhipicephalus* cf. *camicasi*", which formed a sister group to the subtropical lineage of *R. sanguineus* s.l. The remaining specimens from camels were identified as *Rhipicephalus turanicus*, a species that can be very difficult to distinguish from *R. sanguineus* morphologically (Hekimoğlu *et al.,*2016). Sequencing of additional loci from the Asir samples and incorporation of the sequences from the study of Chandra *et al*., (2019) into phylogenetic trees will be important to determine if there is any overlap in the clades identified in both studies.

To investigate the microbiome of chigger mites, a 16S rRNA gene amplicon sequencing approach was used for the first time in Saudi Arabia to identify potential symbiotic and pathogenic bacteria in pooled chiggers and individuals of the larger ectoparasites. In chapter 4, we presented a comprehensive analysis of the microbiome of ectoparasites from the Asir region, revealing a higher bacteria diversity in chigger mites compared with other ectoparasites; *i.e*., fleas, ticks, and lice. In agreement with a previous study from Thailand, *Actinobacteria*, *Corynebacterium, Staphylococcus, Burkholderiaceae* and *Mycobacterium* were found as the dominant bacterial OTUs of the pooled chiggers, which may acquire these bacteria from the environment or rodent skin (Chaisiri *et al.,*2019). In the current study, two research questions regarding the chigger microbiome had been planned. (1) How do chigger mites acquire their microbiome, whether from the environment or their relationship with their host; and (2) how does their microbiome vary depending on their feeding site on the host (*i.e*., ear, anus, chin, or back) to identify patterns of OTU clustering between chiggers and their site of attachment. Both local soil samples and skin swabs from different locations on each host had been collected and DNA extracted, but unfortunately, sequencing problems with Illumina MiSeq plates 2 and 3 prevented these questions from being addressed at this stage.

Some of the most striking results in this study are the presence of *Wolbachia* bacteria in several chigger species such as *P. agamae, S*. *saudi* sp. nov., *S. zarudnyi* and *H. lukshumiae.* These bacteria have been reported previously in a number of mite species such as *Leptotrombidium scutellare* chiggers (Ogawa *et al.,*2019), spider mites (*Tetranychus* spp.; Zhao *et al.,*2013), and *Tyrophagus putrescentiae*;; (Erban *et al.,*2016; Hubert *et al.,*2018), which is consistent with our findings. However, *Wolbachia* was very rare in the microbiome

of chigger species in Thailand (Chaisiri *et al.,*2019). A recent interesting study reported by Ogawa *et al.* (2019), identified three genera of symbiotic bacteria (*Wolbachia, Rickettsia*, and *Rickettsiella*) in unfed, wild-caught *L. scutellare* larvae by 16S rRNA amplicon sequencing, indicating that they acquire these symbionts by vertical transmission. In addition to *Wolbachia*, *Candidatus* Cardinium was detected in chigger species such as *S. zarudnyi* and *S. saudi* sp. nov, and this findings are in line with previous studies reported by Takhampunya *et al.,* 2019 and Chaisiri *et al*., 2019 from Thailand. In common with *Wolbachia*, this bacterium has the capacity to alter the reproduction of their arthropod hosts via cytoplasmic incompatibility, feminization, or male-killing (Zhang *et al.,*2016). However, it is not known if any of these effects are caused by *Wolbachia* and/or *Cardinium* in trombiculid mites, although both symbionts induce cytoplasmic incompatibility in spider mites (Xie *et al.,*2016), which are closely related to chiggers.

Importantly, some evidence of infection by the mite symbiont and vertebrate pathogen, *Orientia,* was found in two pools of chigger mites (*P. agamae* and *E. kazeruni)*. Unfortunately, attempts to confirm this by specific PCR and cloning were not successful probably because the infection was extremely low density in the pools. However, the *Orientia* OTU in these two pools displayed ~99% identity with an isolate of *O. chuto* from Dubai, was not found in background controls, and could not have originated from the laboratory environment, as *Orientia* (a BSL3 pathogen) is not handled anywhere on the University of Liverpool campus. Although spurious detection of *Orientia* therefore seems unlikely, confirmation of chigger infection by specific PCR and/or metagenomic sequencing is required before any further investigations of potential circulation of scrub typhus in Saudi Arabia are attempted.

The rodent skin swabs were collected in this study to compare with the chigger microbiome, but instead provided some additional general insights into the symbionts and potential pathogens circulating in the Asir region. The presence of *Ehrlichia* DNA in the swabs probably reflected contamination of rodent skin with tick excreta; however, *Ehrlichia* has not been reported to be transmitted via this route. The detection of DNA from a *Rickettsia* spp. in a rodent skin swab that was not seen in the ectoparasite data was intriguing. The most likely explanation for this is the presence of excreta on rodent skin

from transient ectoparasites like soft ticks. As interest in vector excreta for disease surveillance increases (Pilotte *et al.,*2016), this finding opens up the possibility of using skin swabs from animal hosts to provide additional information on the zoonoses circulating in a particular region. Although there have been several recent reports of the detection of *Rickettsia* spp. in chiggers (Ogawa *et al.,*2019; Huang *et al.,*2017; Jacinavicius *et al.,*2019), their potential importance as symbionts and/or vertebrate pathogens is unknown, and this genus was absent from chiggers in our dataset.

According to our findings in chapter 5, we also validated a list of potential symbionts and pathogenic bacteria of fleas, ticks, and lice using species-specific PCR gene targets. A bacterium related to *E. ewingii,* an important pathogen of humans and dogs (Matsumoto *et al.,*2011; Kawahara *et al.,*2006; Monje *et al.,*2019), was detected in both *Haemaphysalis* spp. and *Rhipicephalus* spp. ticks. It will be important to attempt to isolate this potential pathogen in culture and animal models, and also determine if it is associated with particular genetic lineages of the two tick vectors. Moreover, an organism closely related to the Q fever pathogen, *C. burnetii*, was detected in the chigger mite, *S. saudi* sp. nov. Additional genetic loci should be sequenced from this sample to determine if there is a potential for Q fever transmission in Asir.

7.2 Future work

As far as we are aware, this was the first study of rodent-associated bacterial zoonoses and ectoparasite diversity conducted in the Asir region of Saudi Arabia. The results so far have been encouraging and we propose that further research should be undertaken in the following areas:

A potentially important finding in this study was the detection of *O. chuto*-like 16S rRNA sequences in two pools of chigger mites. If confirmed, this would be only the third time that this particular scrub typhus pathogen has been detected worldwide after prior reports from the United Arab Emirates (Izzard *et al.,*2010) and Kenya (Masakhwe *et al.,*2018). Although we attempted verification of this results using species-specific primers for the 47-

kDa protein (TSA47) and the 56-kDa type-specific antigen (TSA56), we did not obtain sufficient PCR products for sequencing and cloning attempts failed. Therefore, more chigger material (especially *P. agamae* and *E. kazeruni*) from Alous should be collected and screened using specific PCRs for *O. chuto* to confirm this result. To determine if the pathogen is circulating in the area, domestic dogs could be used as "sentinels" for serological screening as was done recently in Chile. Detection of a significant seroprealence in dogs in Asir could provide justification to extend serological testing to the human population of the region to ensure that scrub typhus is considered as a differential diagnosis for febrile illnesses (Weitzel *et al.,*2018).

Second, species-specific PCR should be used to confirm and classify the symbiotic bacteria such as *Wolbachia* detected in several chigger species (*P. agamae, S*. *saudi* sp. nov., *S. zarudnyi* and *H. lukshumiae*). In the longer term, it might be possible to establish colonies of Saudi chiggers and determine if there are interactions between *Orientia* and other symbionts within the mites and/or effects on mite reproduction, as has been done in Thailand (Ponnusamy *et al.,*2018; Takahashi *et al.,*1997; Phasomkusolsil *et al.,*2014).

Third, further investigations are required on the microbiome of the tropical rat mite (*O. bacoti*), using species-specific PCR to confirm the identity of the many OTUs in the Paris colony that could not be cultured. The impact of *L. sigmodontis* infection on the microbiome of *O. bacoti* should be examined in more detail, especially the potential for *A. faecalis* to contribute to the detrimental effects on mite fecundity caused by filarial infection (Nieguitsila *et al.,*2013). Moreover, the use of penicillin-streptomycin treatment of jirds could be extended to animals infected with *L. sigmodontis* to determine if the native *O. bacoti* microbiome is important for filarial development in the vector.

Finally, to formally describe potential new species of *Rhipicephalus* and *Haemaphysalis* ticks, adult samples would be required. A likely source of adult *Rhipicephalus* spp. in the *R. sanguineus* complex would be domestic dogs and wild carnivores to compare with the immature stages collected from rodents, from which three novel tick lineages were identified. Adult *Haemaphysalis* may also be present on carnivores or possibly erinaceids like hedgehogs (Khaldi *et al.,*2011). In addition, it would be valuable to collect more

material of the flea *L. aethiopica* from Alogl to determine if a novel subspecies exists in this region.

7.3 Conclusion

The present study provides a detailed account of a surprising variety of rodent ectoparasites carrying a high diversity of endosymbionts and potentially pathogenic bacteria in the Asir region of Saudi Arabia. Particularly in the case of chigger mites, more studies are warranted on whether they transmit scrub typhus in this region. Our research provided important insights into rodent ectoparasites and their microbiomes in an understudied region of the world and provides baseline information on potential diseases (including zoonoses) circulating in a peridomestic setting. Thus, this work opens many doors for further epidemiological and experimental investigations in Asir.

Appendices

Appendix 1: Abbreviations and morphometric formulae used for chigger mite taxonomy in this thesis (adapted from Chaisiri. 2016).

Abbreviations	Description
The 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
PSB	Distance between a line connecting sensillae bases to posterior-most
SD	Scutal depth or ASB + PSB
AP	Distance between bases of anterolateral and posterolateral setae
Seta	
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
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 Of margin

PPP2 Distance from base of the 2st pair of usurped setae to posterior Of margin

S Length of sensillae

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)

Other formulae

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

Appendix (2): *Ascoschoengastia browni* Taufflieb, Mouchet & Courtois, 1972

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae- Tribe **Trombiculini-** Genus *Ascoschoengastia* - species *Ascoschoengastia browni*

Hosts. *Acomys dimidiatus* and *Myomyscus yemeni*.

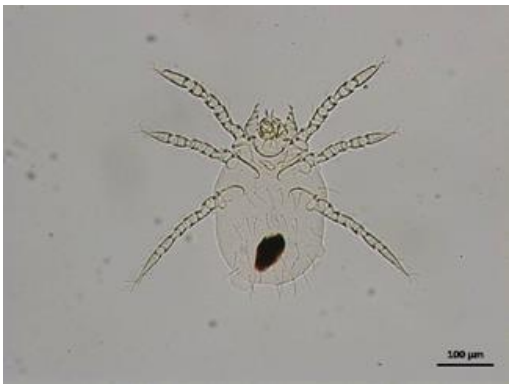
Village: Alous-Alogl

Body site: Back-Anus-Ear

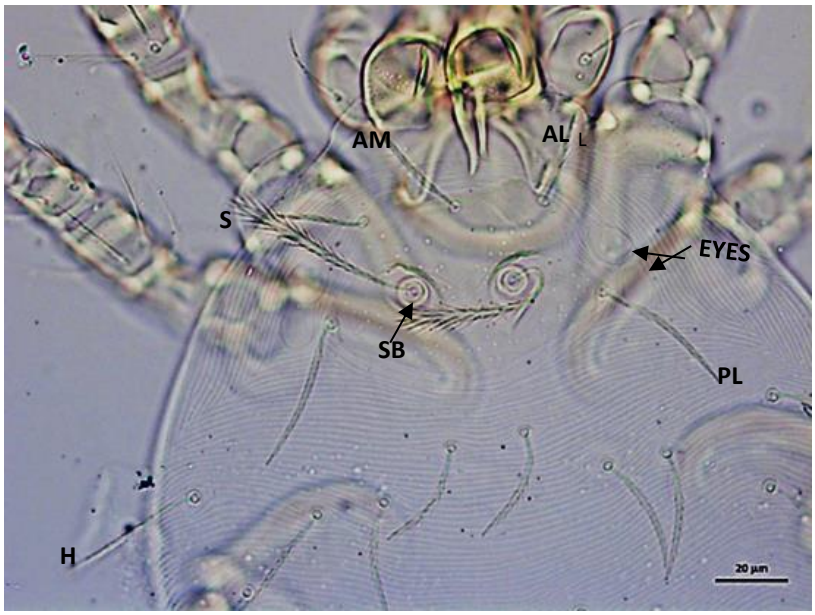
Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
29	39.5	15.5	14.5	14.5	28.5	16.5	17.5	15.5	23	23	141	124	141	406

Diagnosis	
fPp	B/B/BNB
fD	2H+8-6-6-4-4-1
DS	31
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	56
Ga	1B



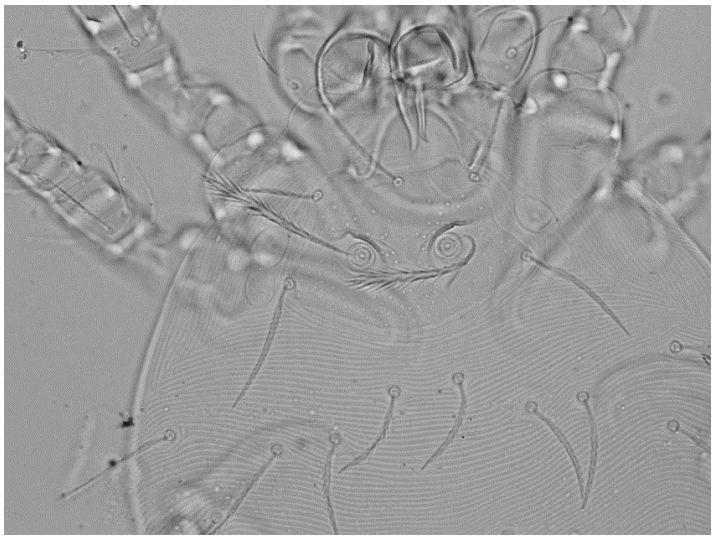
Chigger body

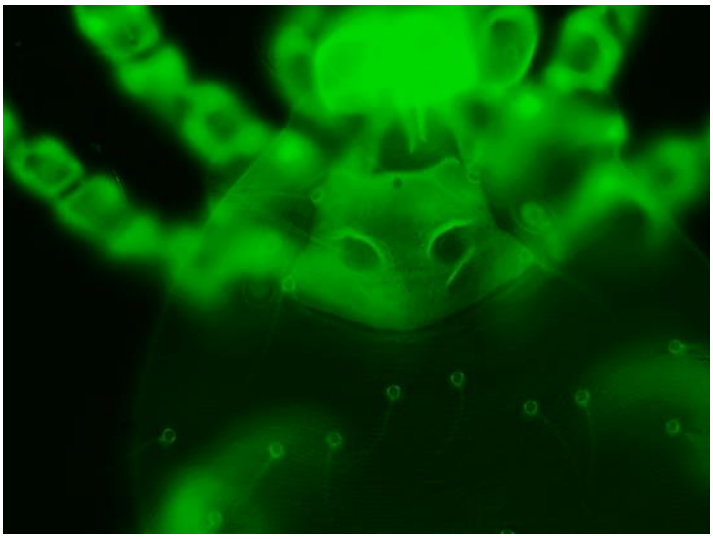


Scutum photo

Appendix (3): A combination of autofluorescent and brightfield microscopy-

Ascoschoengastia browni





Appendix (4): *Helenicula lukshumiae* Nadchatram & Traub, 1971

Classification: Family Trombiculidae - Subfamily Trombiculinae - Tribe Schoengastiini - Genus *Helenicula*- Species *Helenicula lukshumiae*

Hosts*: Acomys dimidiatus*.

Village:Alous

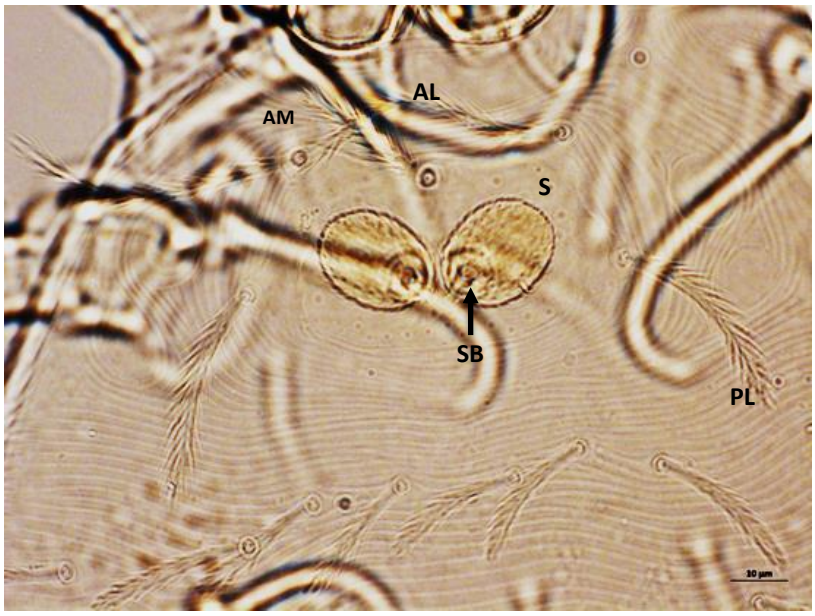
Body site: Back

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
26	37	55	15	7	22	14	15	30	22.5	30	156	127	152	784

Diagnosis	
fPp	4H+6-6-8-8-8-6
fD	B/B/BBB
DS	46
fcx	1.1.2
fsp	7.7.7
fst	2.2
NDV	92
Ga	1B

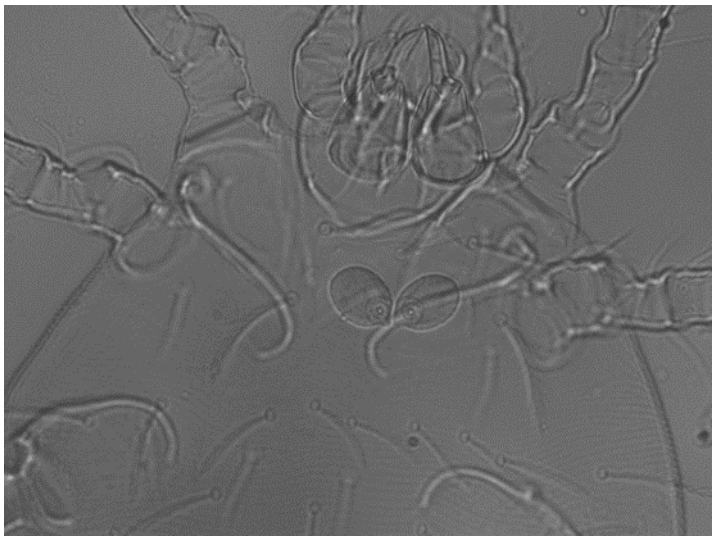
Chigger body

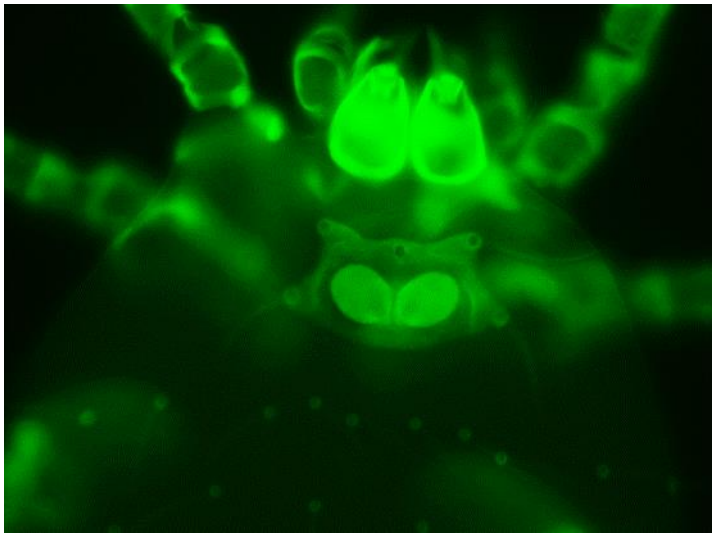


Scutum photo

Appendix (5): A combination of autofluorescent and brightfield microscopy-

Helenicula lukshumiae





Appendix (6): ***Schoutedenichia thracica*** Kolebinova, 1966

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae- Tribe Trombiculini- Genus *Schoutedenichia* - species *Schoutedenichia thracica*

Hosts*: Acomys dimidiatus*.

Village:Alous

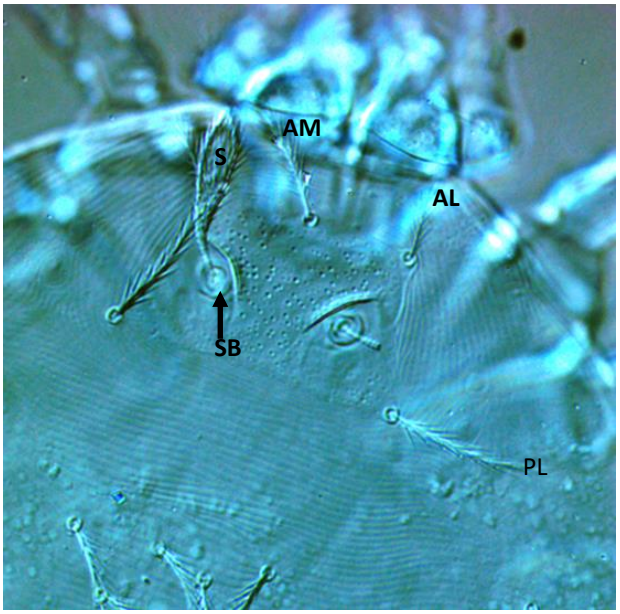
Body site: Back

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
30	41	20	14	11.5	25.5	22.5	19	13	23	22	55	124	151	758

Diagnosis	
fPp	B/B/BBB
fD	4H+12-12-18-17-11-9-6-3
DS	22
fcx	1.1.1
fsp	7.7.7
fst	2
NDV	190
Ga	1B

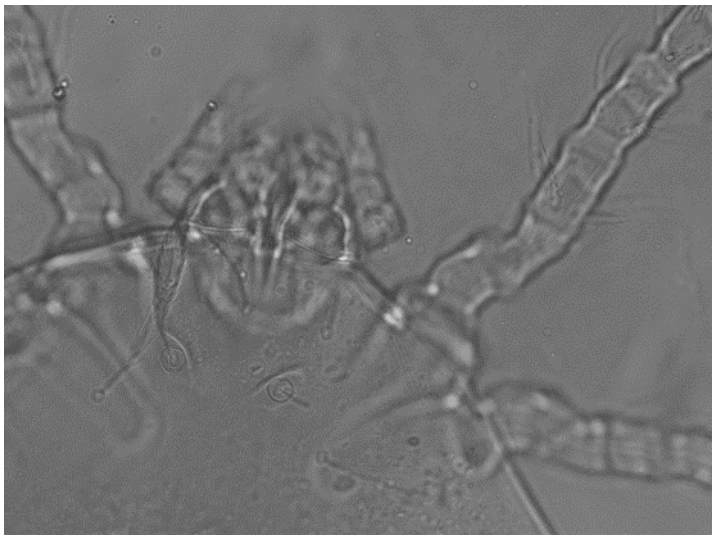
Chigger body

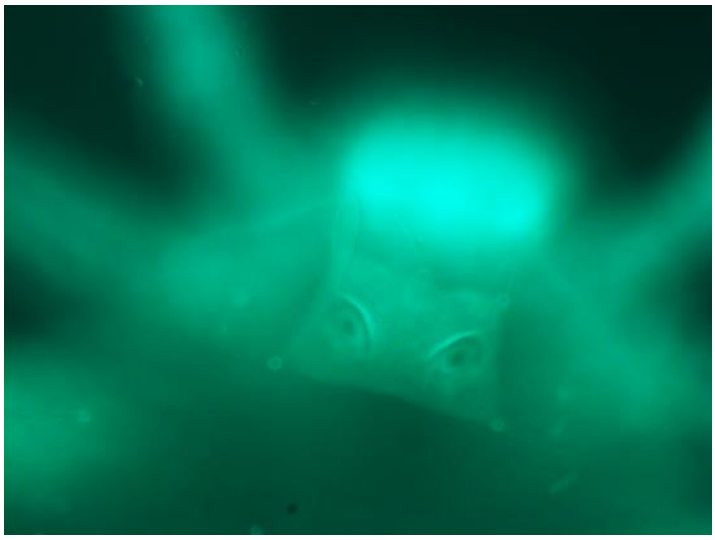


Scutum photo

Appendix (7): A combination of autofluorescent and brightfield microscopy-

Schoutedenichia thracica





Appendix (8): *Ericotrombidium caucasicum* (Schluger, 1967)

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini

Genus: *Ericotrombidium* Vercammen-Grandjean, 1966

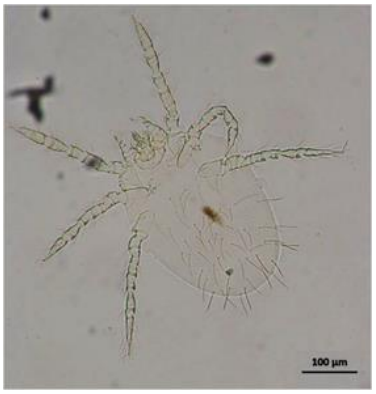
Hosts: *Acomys dimidiatus*.

Village: Alous

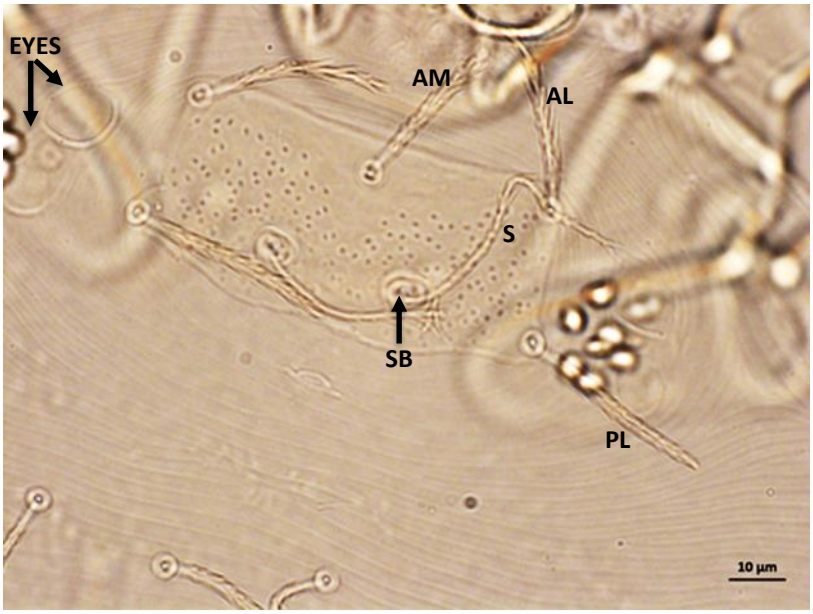
Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
60	65	20	23	12	35	23	34	40	41	36	272	232	257	761

Diagnosis	
fPp	B/B/NBB
fD	2H+8-6-6-6 -4-9
DS	41
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	85
Ga	1B



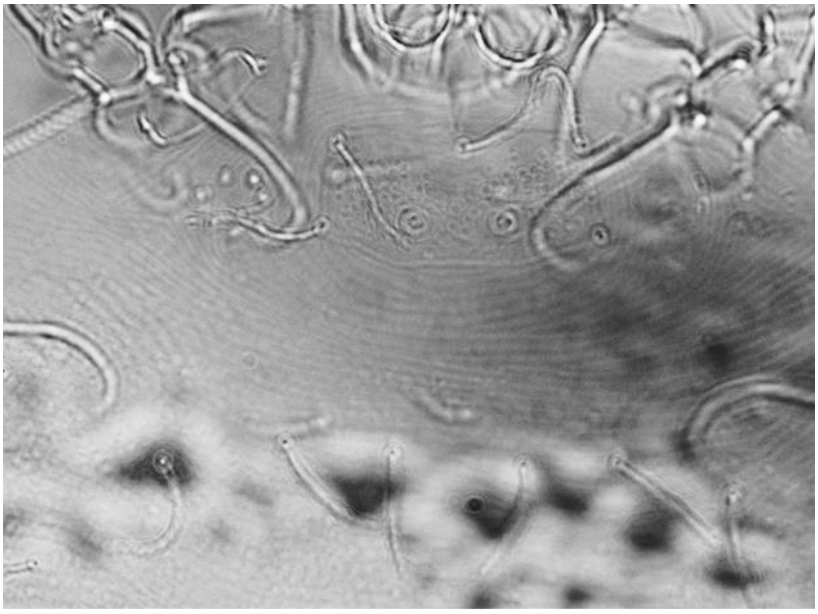
Chigger body

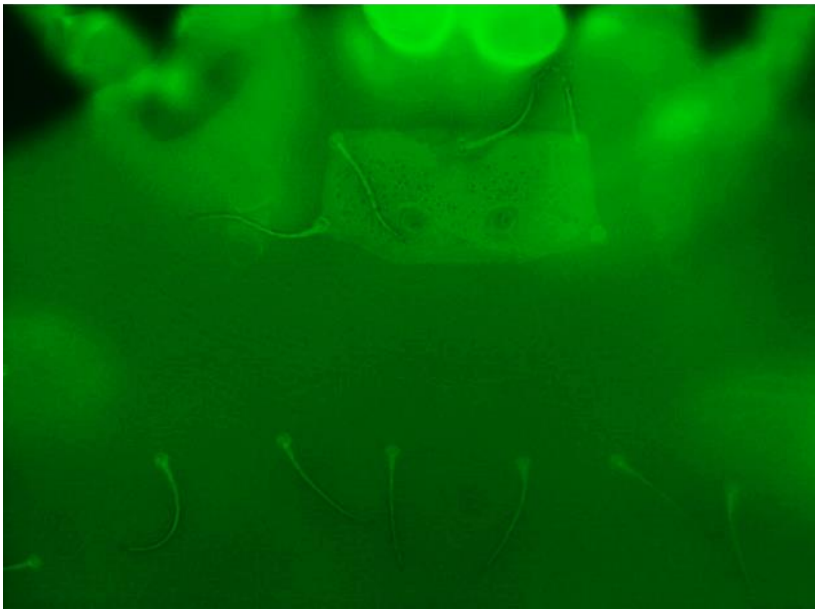


Scutum photo

Appendix (9): A combination of autofluorescent and brightfield microscopy-

Ericotrombidium caucasicum





Appendix (10)*: Gahrliepia lawrencei* Jadin & Vercammen-Grandjean, 1952

Taxonomy: Family Trombiculidae - Subfamily Gahrliepiinae - Tribe Gahrliepiini - Genus *Gahrliepia* - Species *Gahrliepia lawrencei*

Hosts: *Acomys dimidiatus*.

Village: Alous

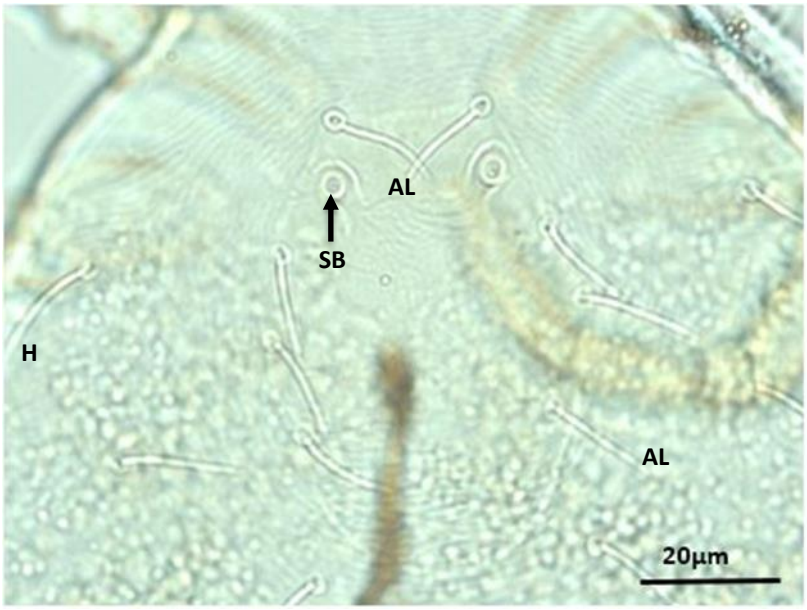
Body site: Back

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
23	42.5	25	12	62	74	21	30	16	17	20	128	113	137	560

Diagnosis	
fPp	N/N/NNN
fD	2H+2-4-6-6-2-6
DS	28
fcx	1.1.2
fsp	6.6.6
fst	2.2
NDV	77
Ga	1N

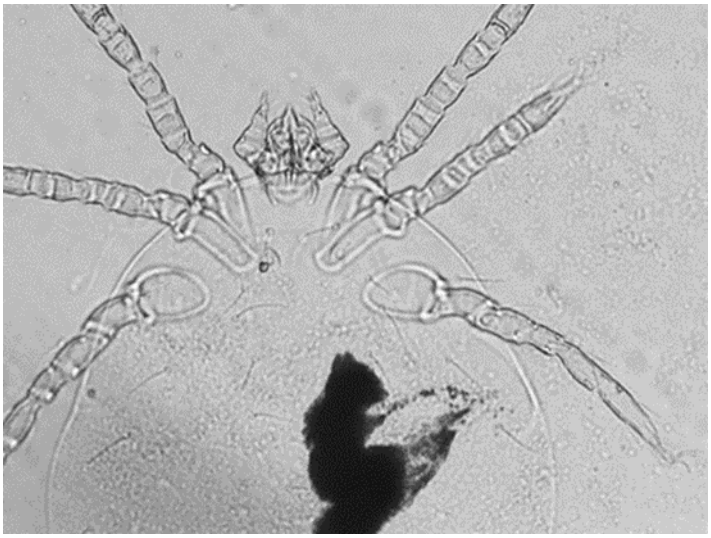
chigger body

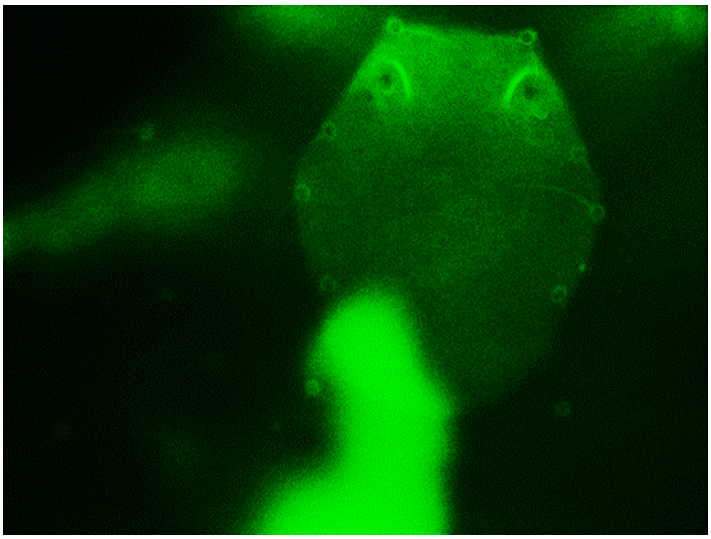


Scutum photo

Appendix (11): A combination of autofluorescent and brightfield microscopy-

Gahrliepia lawrencei





Appendix (12)*: Schoengastiella wansoni* Wolfs & Vercammen-Grandjean, 1953

Taxonomy: Family Trombiculidae - Subfamily Gahrliepiinae - Tribe Gahrliepiini - Genus *Schoengastiella* - Species *Schoengastiella wansoni*

Hosts: *Acomys dimidiatus*.

Village:Alous

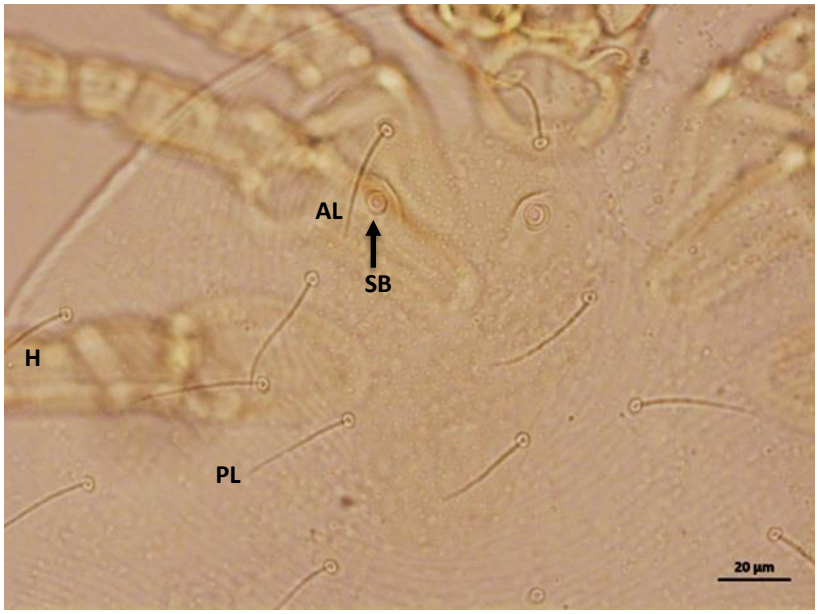
Body site: Anus

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
24	43	24.5	13	52	65	25.5	31	18.5	19	21	128	113	127	368

Diagnosis	
fPp	N/N/NNN
fD	2H+4-6-6-4-2-2
DS	26
fcx	1.1.2
fsp	6.6.6
fst	2.2
NDV	71
Ga	1N

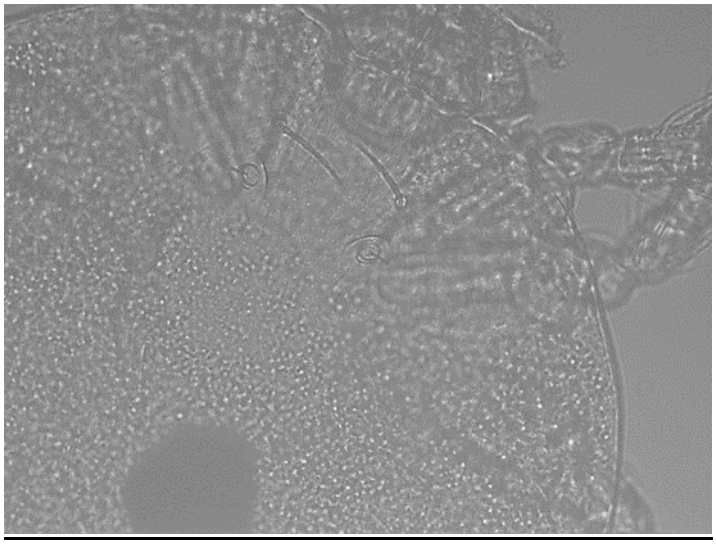
Chigger body

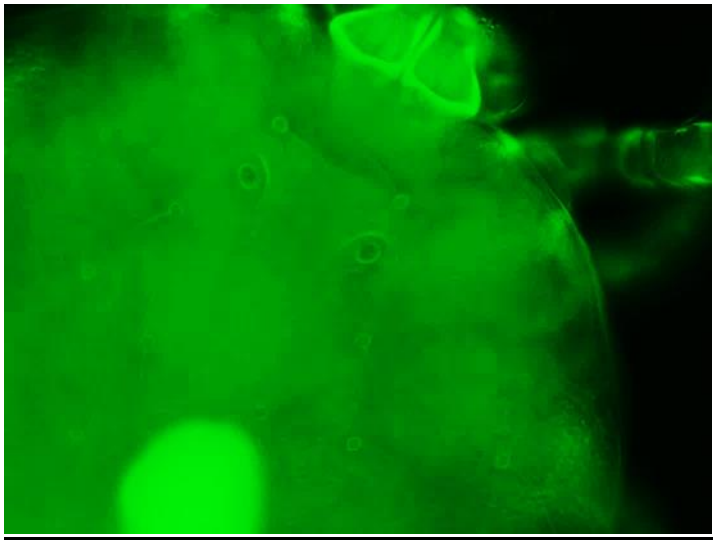


Scutum photo

Appendix (13): A combination of autofluorescent and brightfield microscopy-

Schoengastiella wansoni





Appendix (14): *Walchia parvula* Schluger, 1955

Taxonomy: Family Trombiculidae - Subfamily Gahrliepiinae - Tribe Gahrliepiini - Genus *Walchia* - Species *Walchia parvula*

Hosts: *Acomys dimidiatus*.

Village:Alous

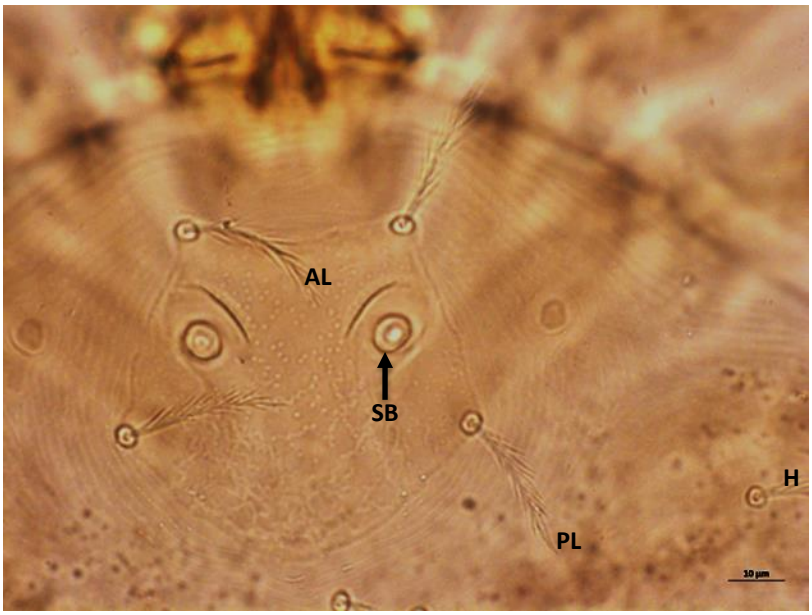
Body site: Anus-chin

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
22	35	19.5	12	22	34	21	-	18	16	15	120	101	115	540

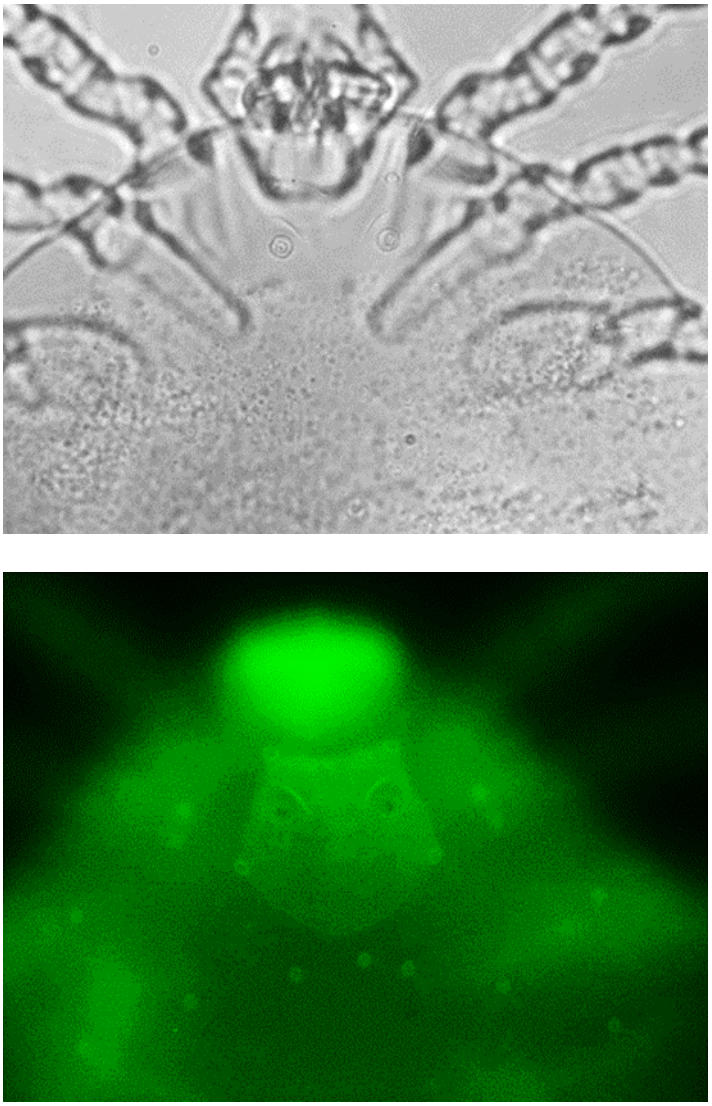
Diagnosis	
fPp	N/N/NNN
fD	2H+7-10-6-6-3-2-2
DS	38
fcx	1.1.2
fsp	4.6.6
fst	2.2
NDV	93
Ga	1N

Chigger body



Scutum photo

Appendix (15): A combination of autofluorescent and brightfield microscopy- *Walchia parvula*



Appendix (16): *Schoutedenichia zarudnyi* Kudryashova, 1976

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Schoengastiini - Genus *Schoutedenichia* - Species *Schoutedenichia zarudnyi*

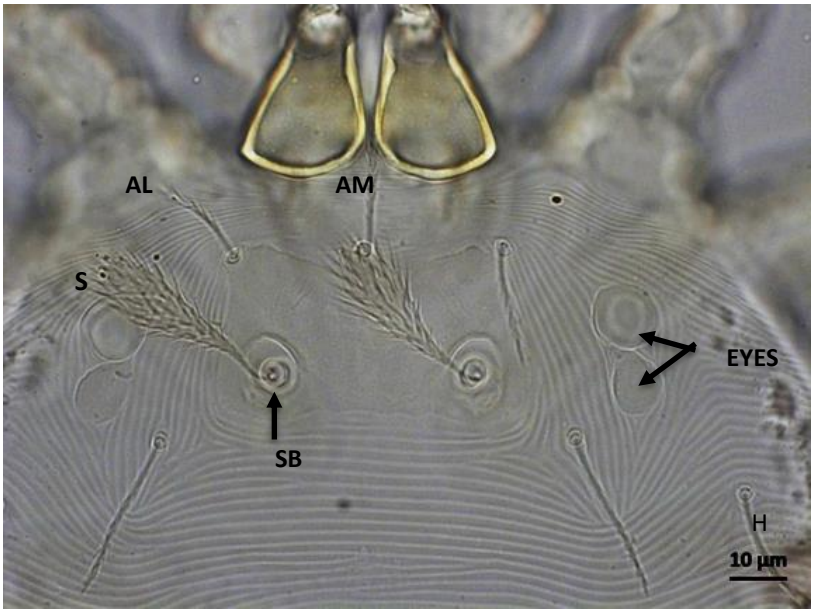
Hosts: Acomys dimidiatus.
Village: Alous- Wosanib
Body site: Back

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
27	56.5	215	13.5	5	18.5	26	14	13.5	17	18	147	123	142	74 2

Diagnosis	
fPp	B/B/NNB
fD	4H+6-2-8-8-2-6-4-4
DS	44
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	83
Ga	1B

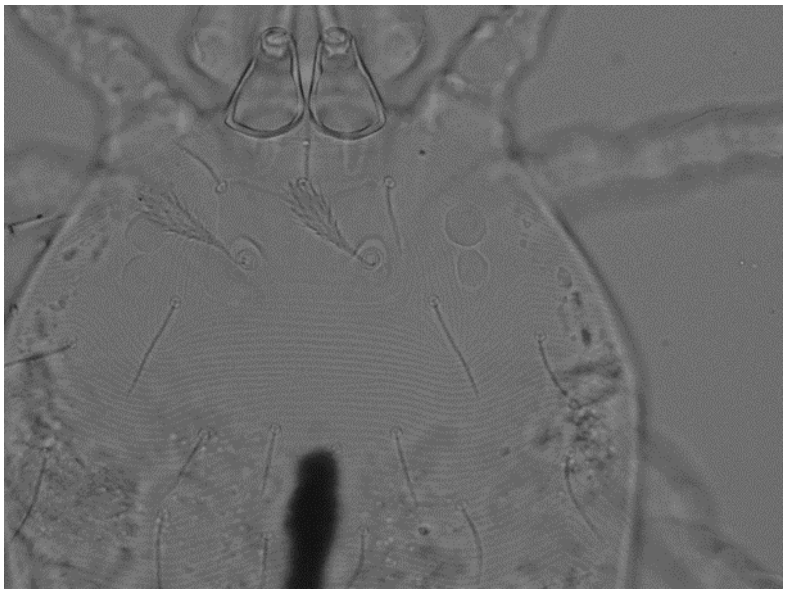
Chigger body

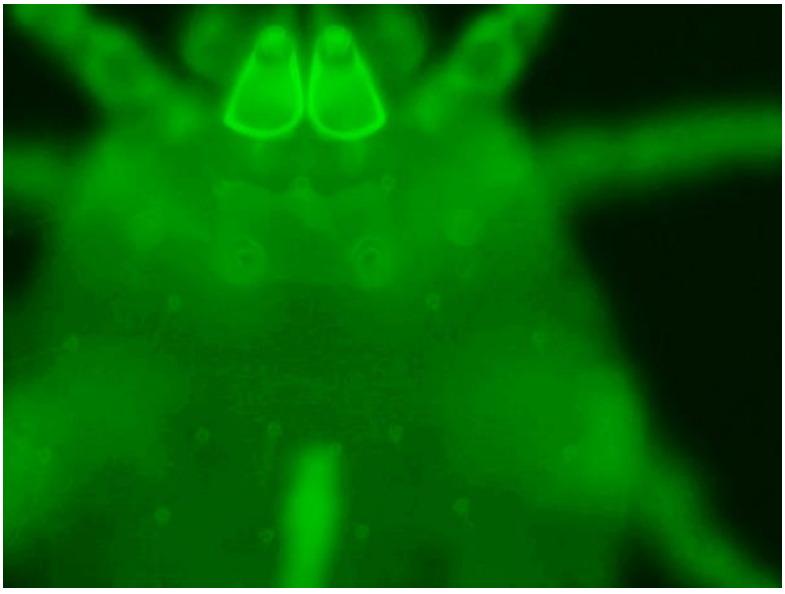


Scutum photo

Appendix (17): A combination of autofluorescent and brightfield microscopy-

Schoutedenichia zarudnyi





Appendix (18): *Ericotrombidium galliardi* (Vercammen-Grandjean & Tauffliеb, 1959)

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini - Genus *Ericotrombidium* - Species *Ericotrombidium galliardi*

Myomyscus yemeni and *Meriones rex*

Hosts: *Myomyscus yemeni and Meriones rex*

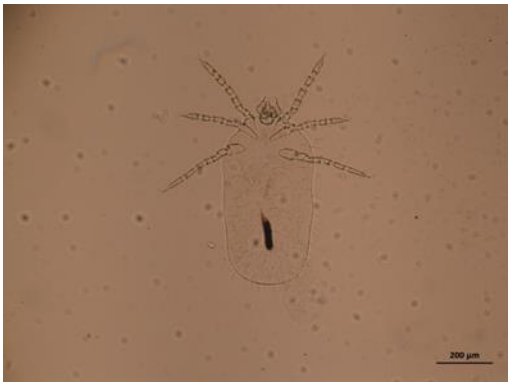
Village: Alogl

Body site: Anus-Ear

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
27	30.5	11	14.5	5	19.5	15.5	17	21	18.5	18.5	163	141	161	840

Diagnosis	
fPp	B/B/NBB
fD	2H+8-6-6-4 -2-2
DS	30
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	57
Ga	1B

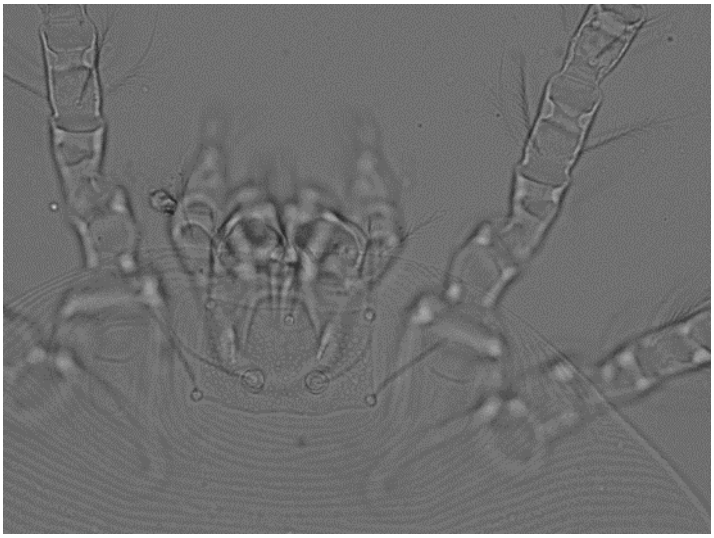


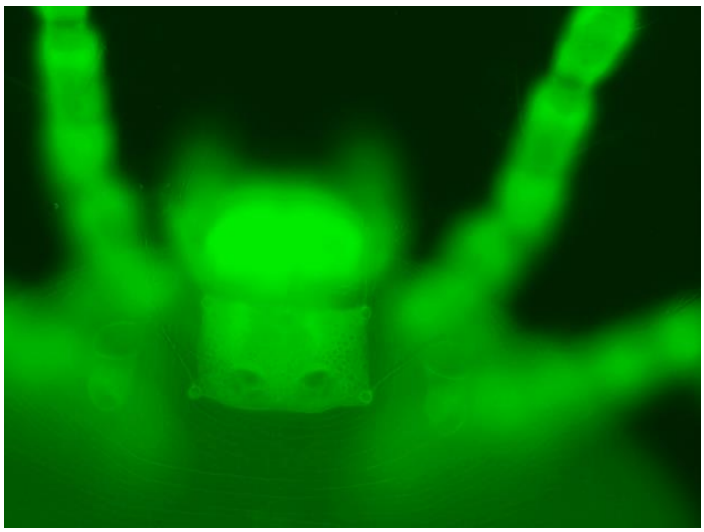
Chigger body

Scutum photo

Appendix (19): A combination of autofluorescent and brightfield microscopy-

Ericotrombidium galliardi





Appendix (20): *Ericotrombidium kazeruni* (Kudryashova, 1976)

Taxonomy: *Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini*

Genus: *Ericotrombidium - Species Ericotrombidium kazeruni*

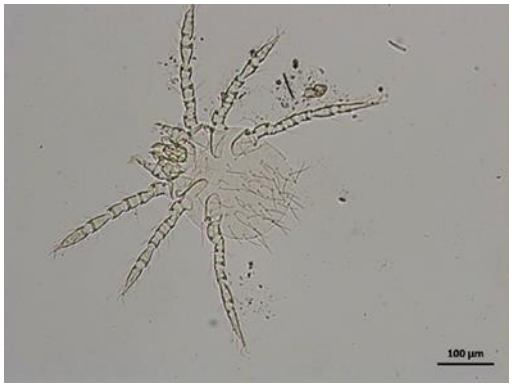
Hosts*:* *Acomys dimidiatus and Meriones rex.*

Village*:* Alous-Wosanib-Alogl

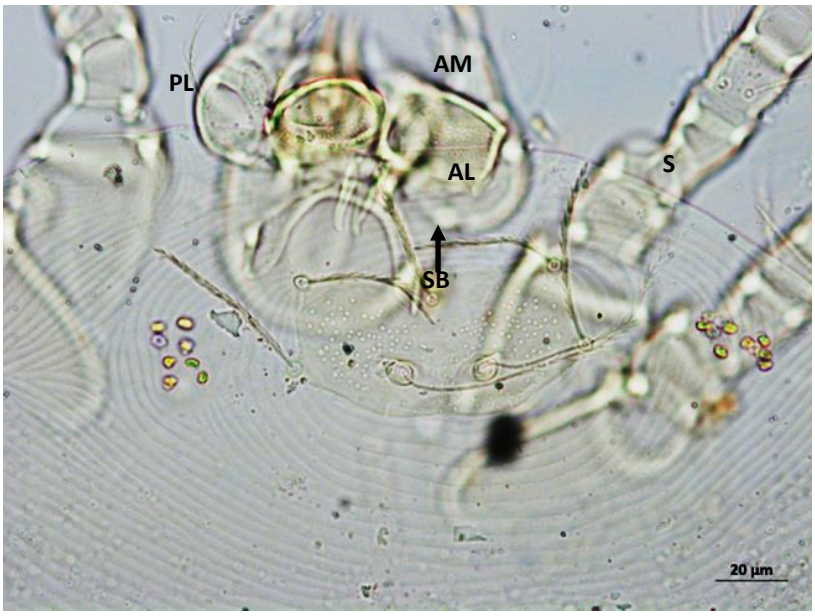
Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
68	80	23	29	14	43	24	34	40	50	42	272	236	270	778

Diagnosis	
fPp	B/B/NBB
fD	2H+8-6-6-6 -4-9
DS	41
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	85
Ga	1B



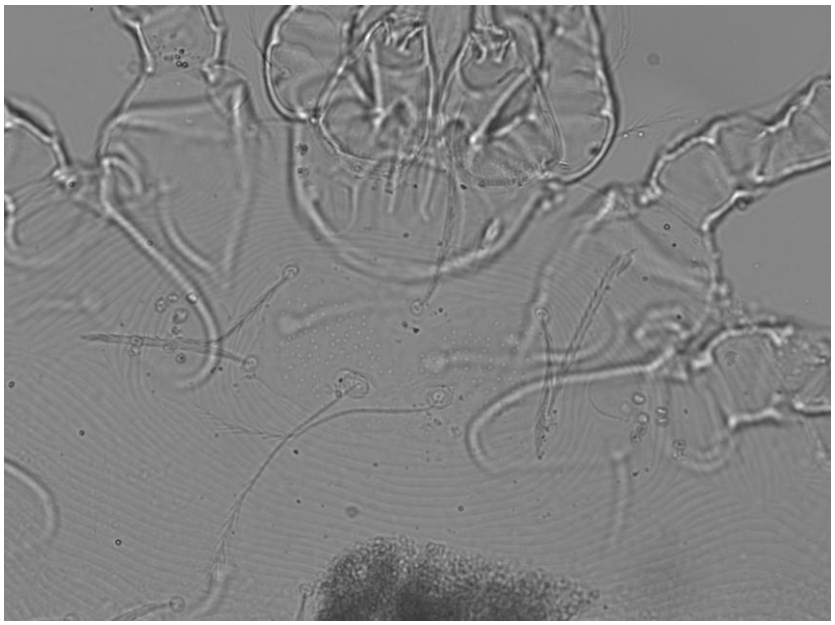
Chigger body

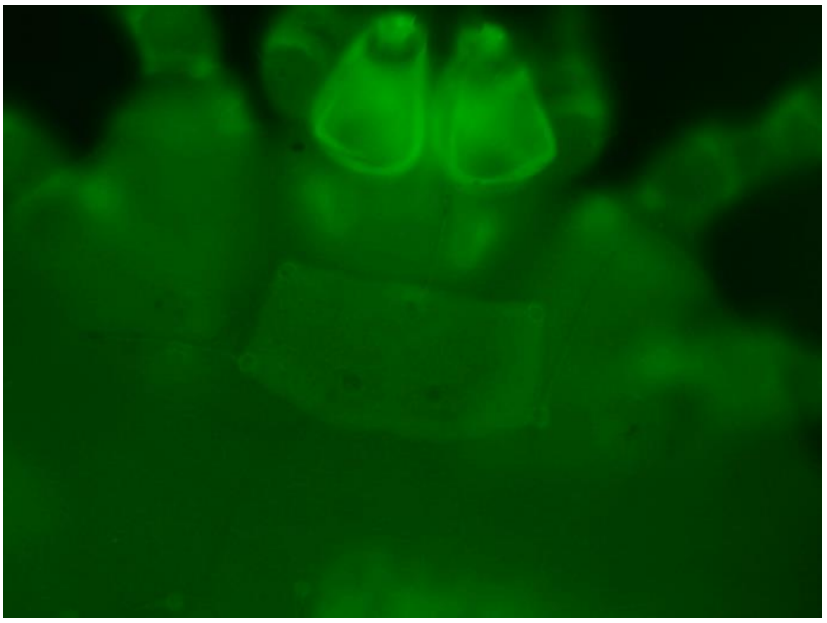


Scutum photo

Appendix (21): A combination of autofluorescent and brightfield microscopy-

Ericotrombidium kazeruni





Appendix (22): ***Microtrombicula centropi (Vercammen-Grandjean, 1965)***

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini **-** ***Genus*** *Microtrombicula - Species Microtrombicula centropi*

Hosts: *A. dimidiatus*

Village: Wosanib

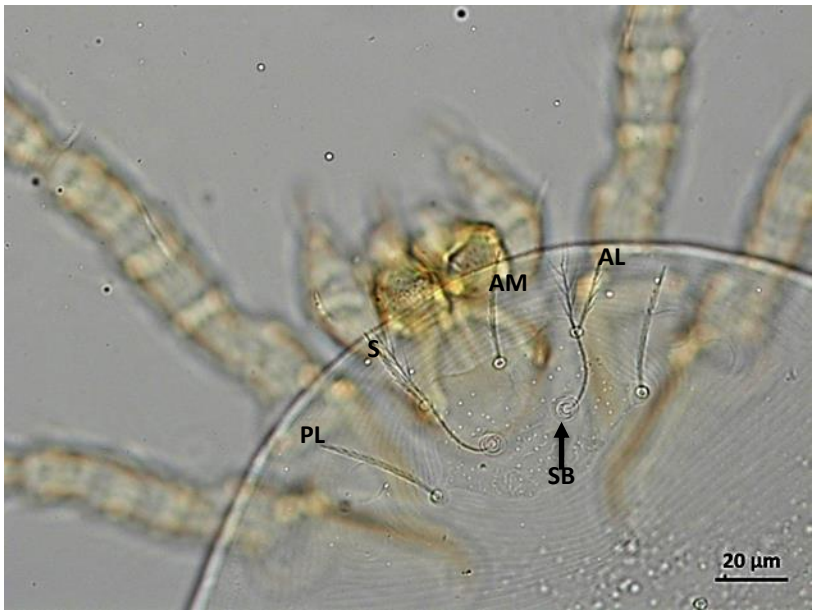
Body site: Back

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
33	38.5	12	13.5	15.5	29	14	15.5	16	18	19	135	115	131	682

Diagnosis	
fPp	B/B/NNB
fD	2H+8-8-7-4-3
DS	32
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	66
Ga	1B

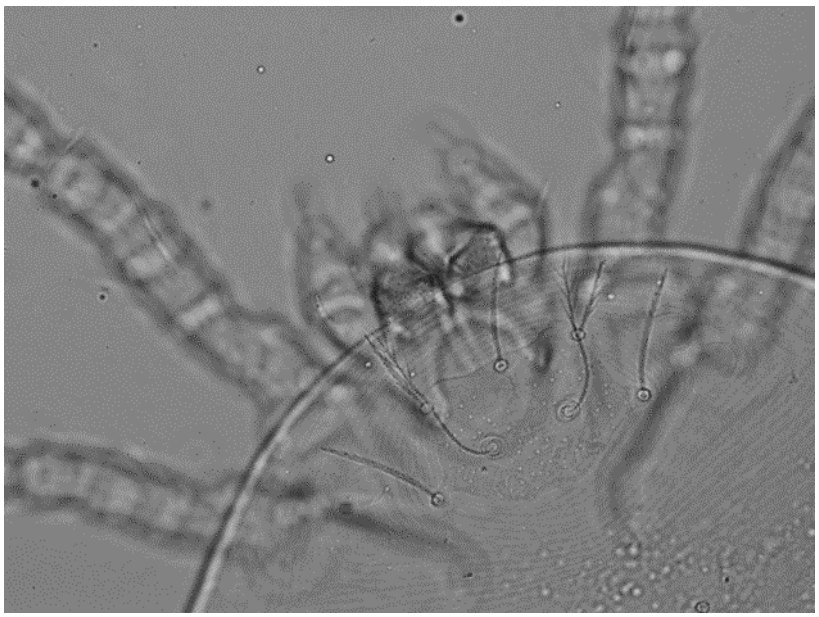
Chigger body

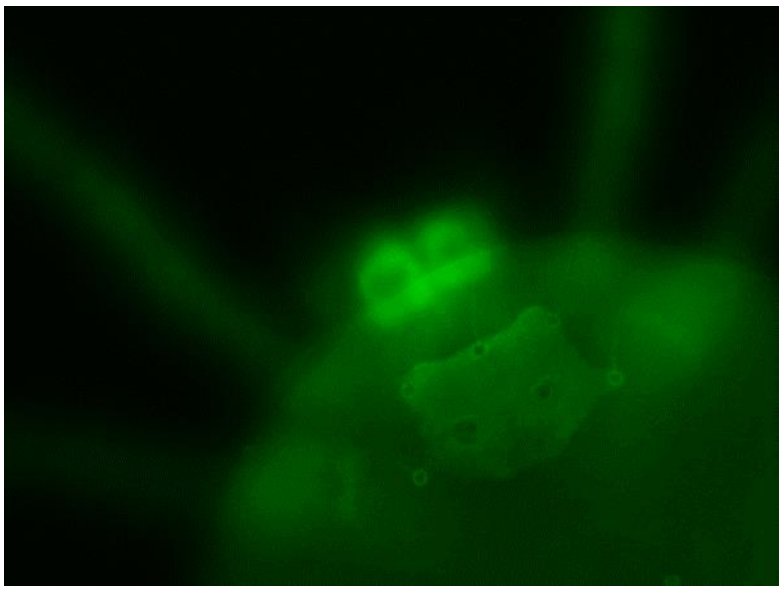


Scutum photo

Appendix (23): A combination of autofluorescent and brightfield microscopy-

Microtrombicula centropi





Appendix (24): *Microtrombicula hyracis (Vercammen-Grandjean, 1965)*

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini - ***Genus*** *Microtrombicula - Species Microtrombicula hyracis*

Hosts: *Acomys dimidiatus.*

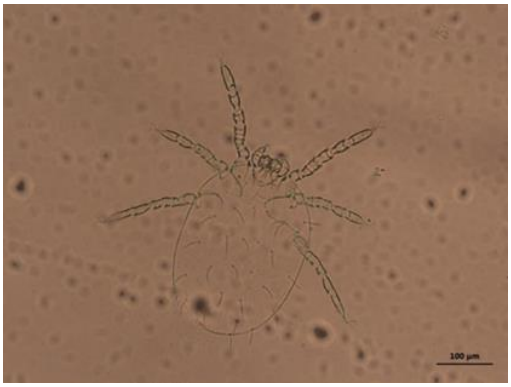
Village:Alous

Body site: Anus- chin

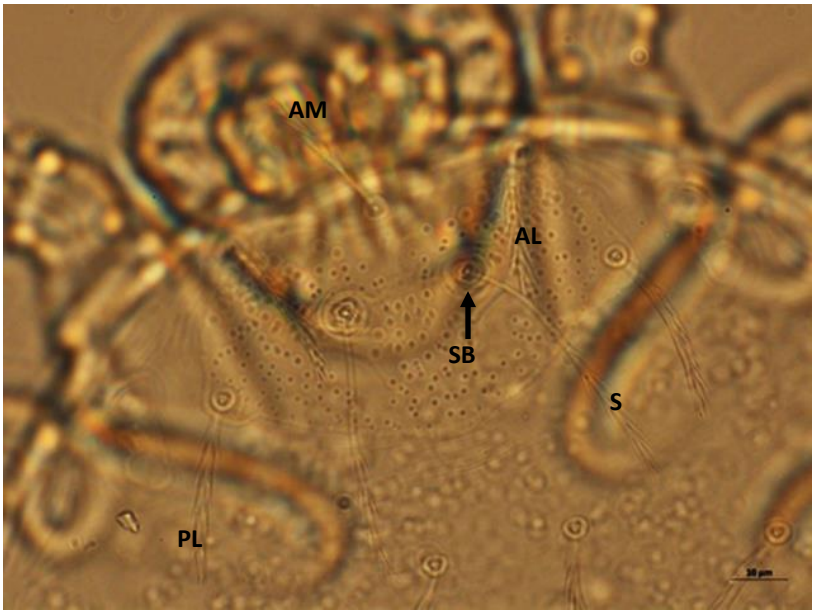
Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
30	41	12	13.5	15	28.5	16	16	17.5	18.5	19.5	130	111	127	663

Diagnosis	
fPp	B/B/NBB
fD	2H+8-6-6-6 -4-9
DS	41
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	85
Ga	1B



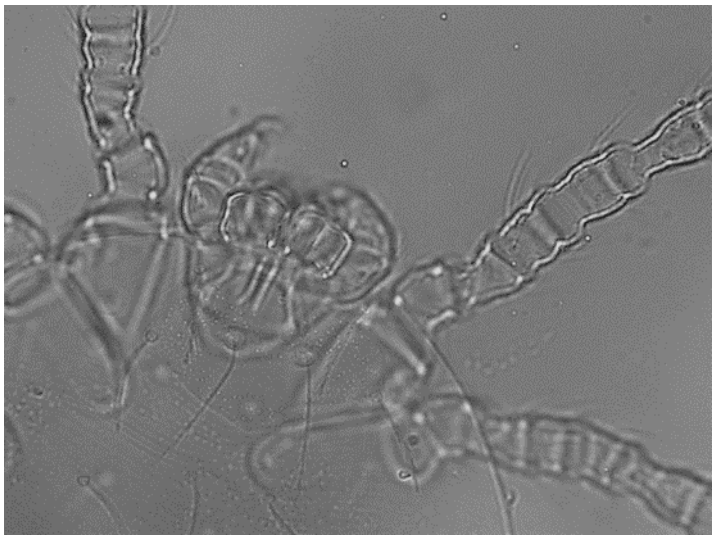
Chigger body

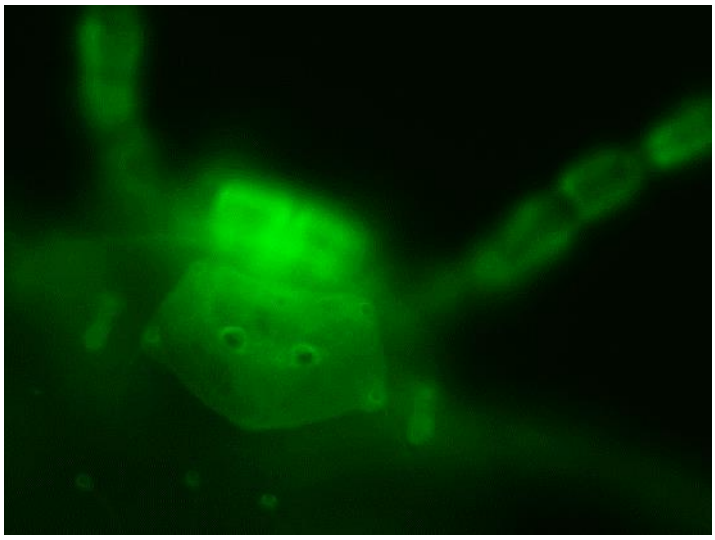


Scutum photo

Appendix (25): A combination of autofluorescent and brightfield microscopy-

Microtrombicula hyracis





Appendix (26): *Pentidionis agamae (André, 1929)*

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini

Genus: *Pentidionis* - Species *Pentidionis agamae*

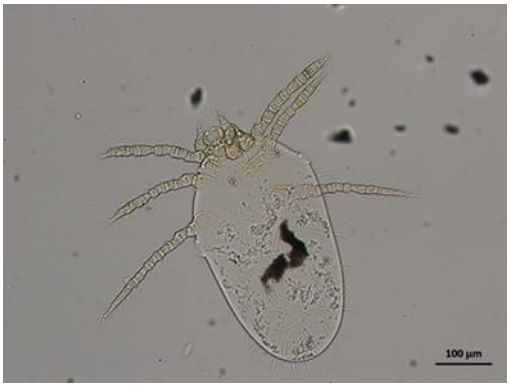
Hosts: *Acomys dimidiatus.*

Village: Alous- Wosanib

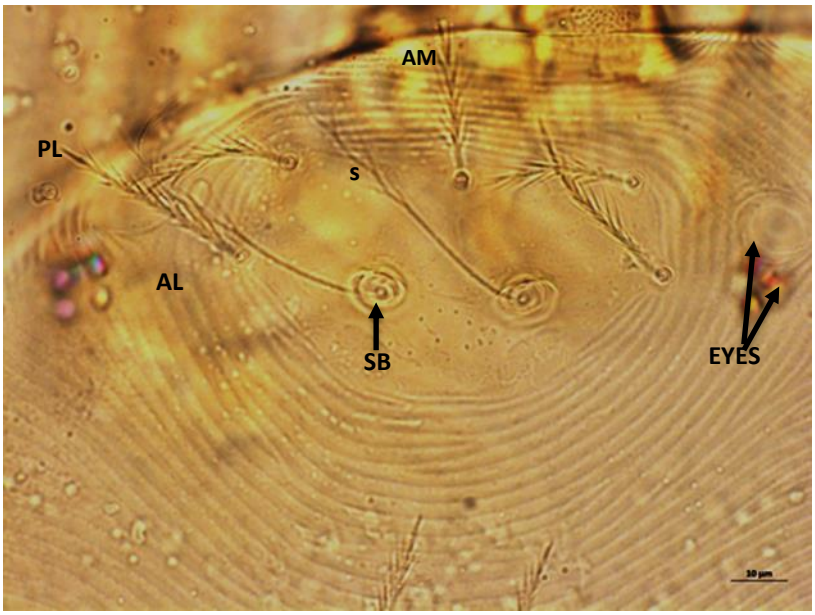
Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
31.5	39	13	13.5	10	23.5	10.9.5	15	18	25	21	152	136	164	452

Diagnosis	
fPp	B/B/NBB
fD	2H+8-6-6-6 -4-9
DS	41
fcx	1.1.1
fsp	7.7.7
fst	2.4
NDV	85
Ga	1B



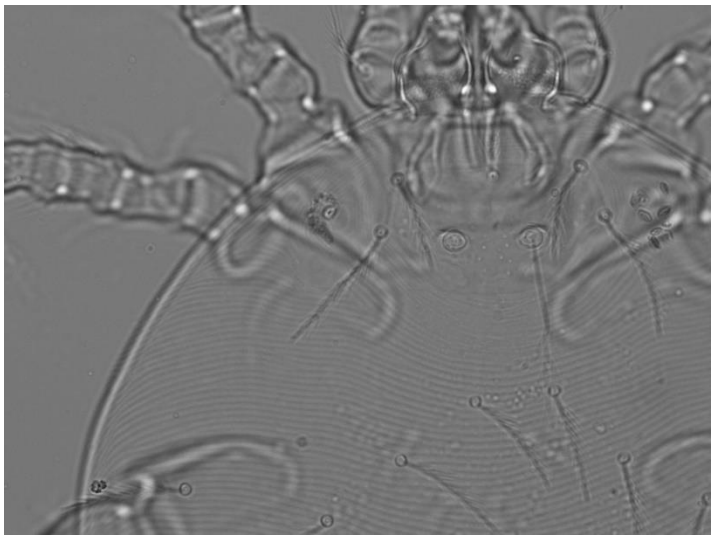
Chigger body

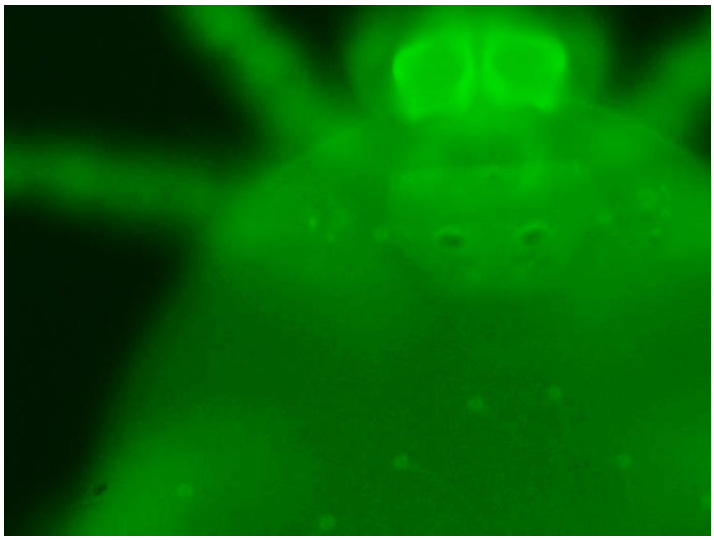


Scutum photo

Appendix (27): ***A combination of autofluorescent and brightfield microscopy-***

Pentidionis agamae





Appendix (28): ***Microtrombicula hoogstraali (Radford, 1954)***

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini - Genus *Microtrombicula* - Species *Microtrombicula hoogstraali*

Hosts: *Myomyscus yemeni and Meriones rex*

Village: Alogl

Body site: Ear

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
16	24	85	12	11	23.5	14.5	10	10.5	15	21	106	88	99	525

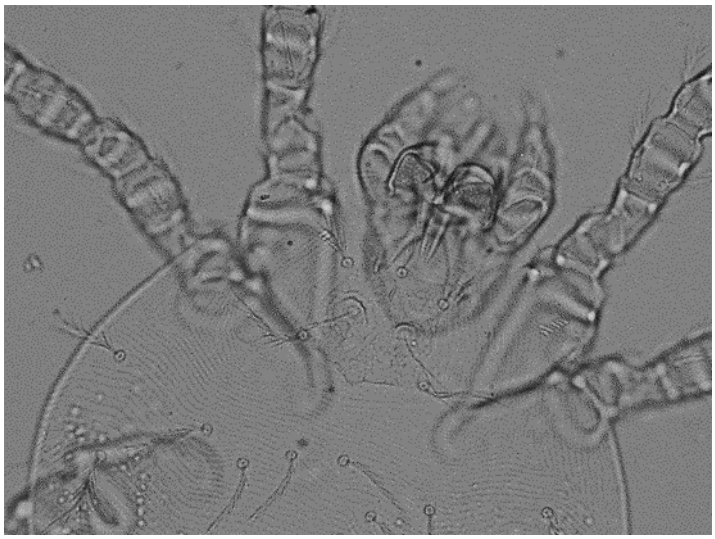
Diagnosis	
fPp	B/B/BBB
fD	2H+6-2-6-4 -6-4-2
DS	32
fcx	1.1.1
fsp	7.7.7
fst	2.2
NDV	61
Ga	1B

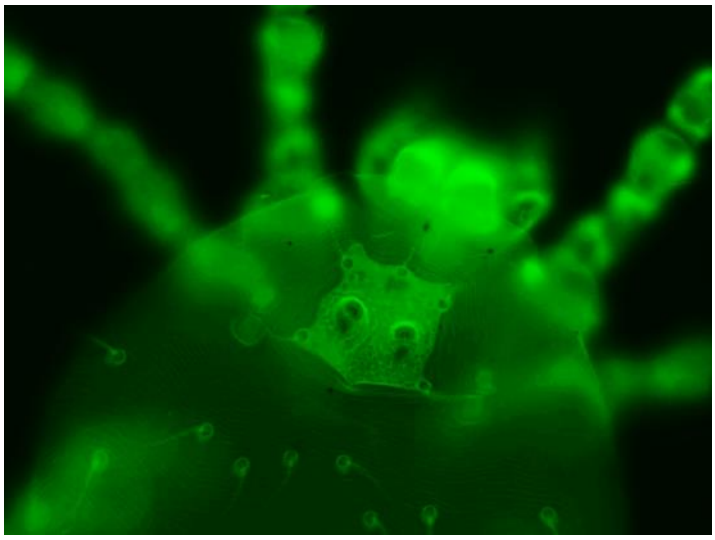
Chigger body

Scutum photo

Appendix (29): A combination of autofluorescent and brightfield microscopy-

Microtrombicula hoogstraali





Appendix (30): ***Microtrombicula traubi (Muljarskaja & Verdieva, 1974)***

Taxonomy: Family Trombiculidae - Subfamily Trombiculinae - Tribe Trombiculini - Genus *Microtrombicula* - Species *Microtrombicula traubi*

Hosts: *Acomys dimidiatus.*

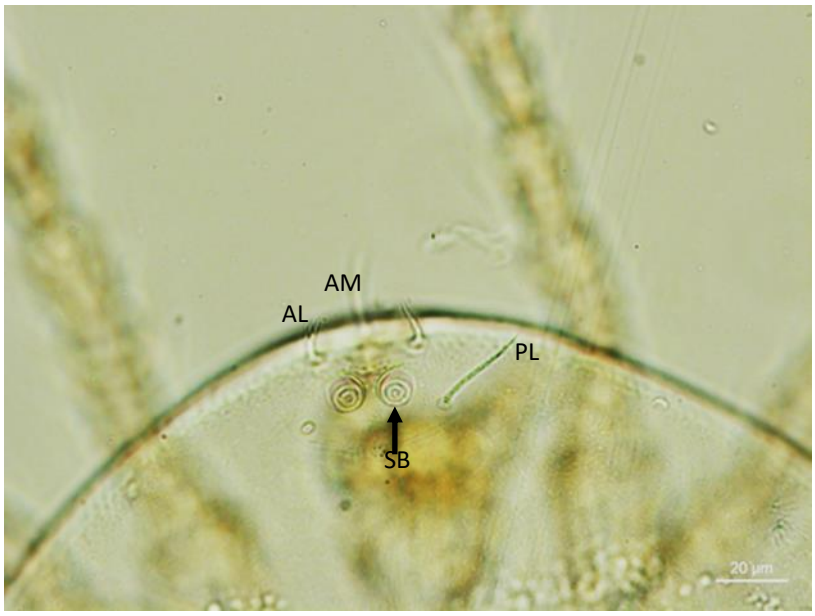
Village: Alous- Wosanib

Scutum measurements

AW	PW	SB	ASB	PSB	SD	AP	AM	AL	PL	H	PA	PM	PP	IP
16	23	7	14.5	10	24.5	13	13	10	19	23	132	108	126	695

Diagnosis	
fPp	B/B/BBB
fD	2H+4-4-6-2 -6-2
DS	32
fcx	1.2.1
fsp	7.7.7
fst	2.4
NDV	65
Ga	1B

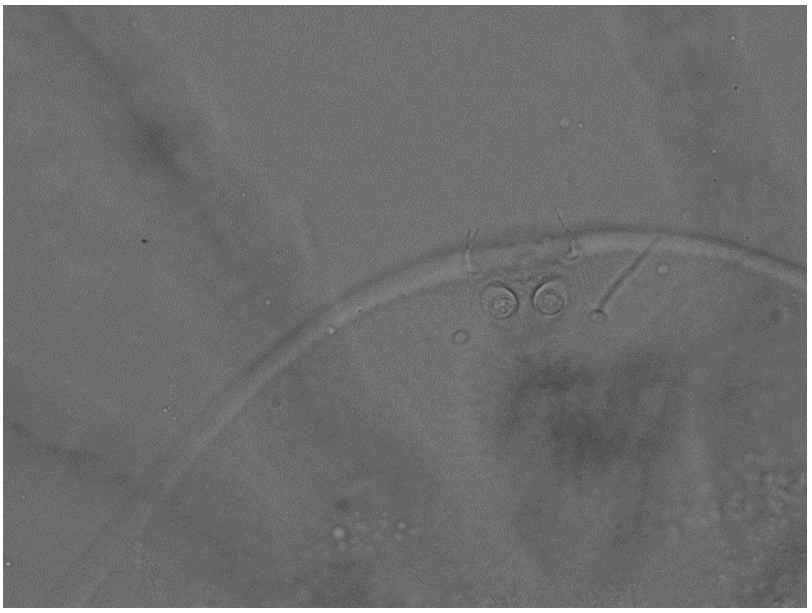
Chigger body

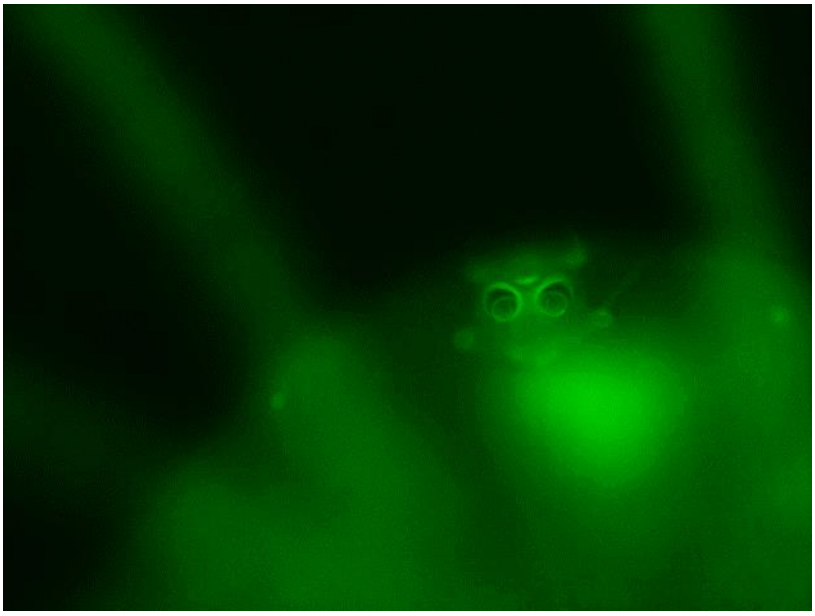


Scutum photo

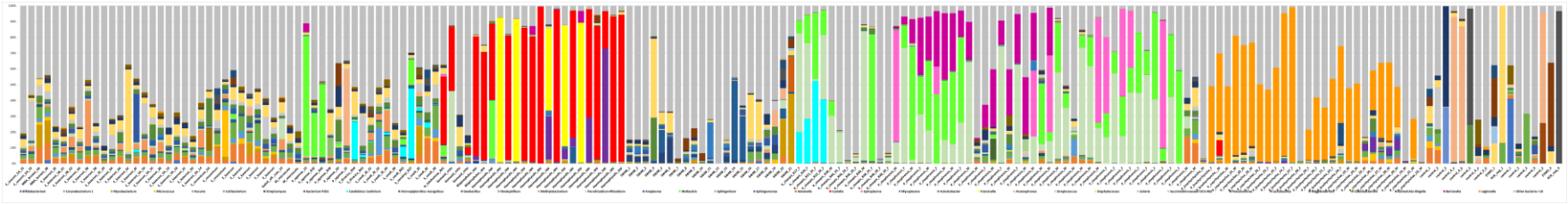
Appendix (31): A combination of autofluorescent and brightfield microscopy-

Microtrombicula traubi





Appendix (32): Bar charts show relative abundance of bacterial OTUs (genus level) of whole ectoparasites and background controls.The data is filtered; OTUs that represented <10% in a sample are combined in “Others” (grey portion).

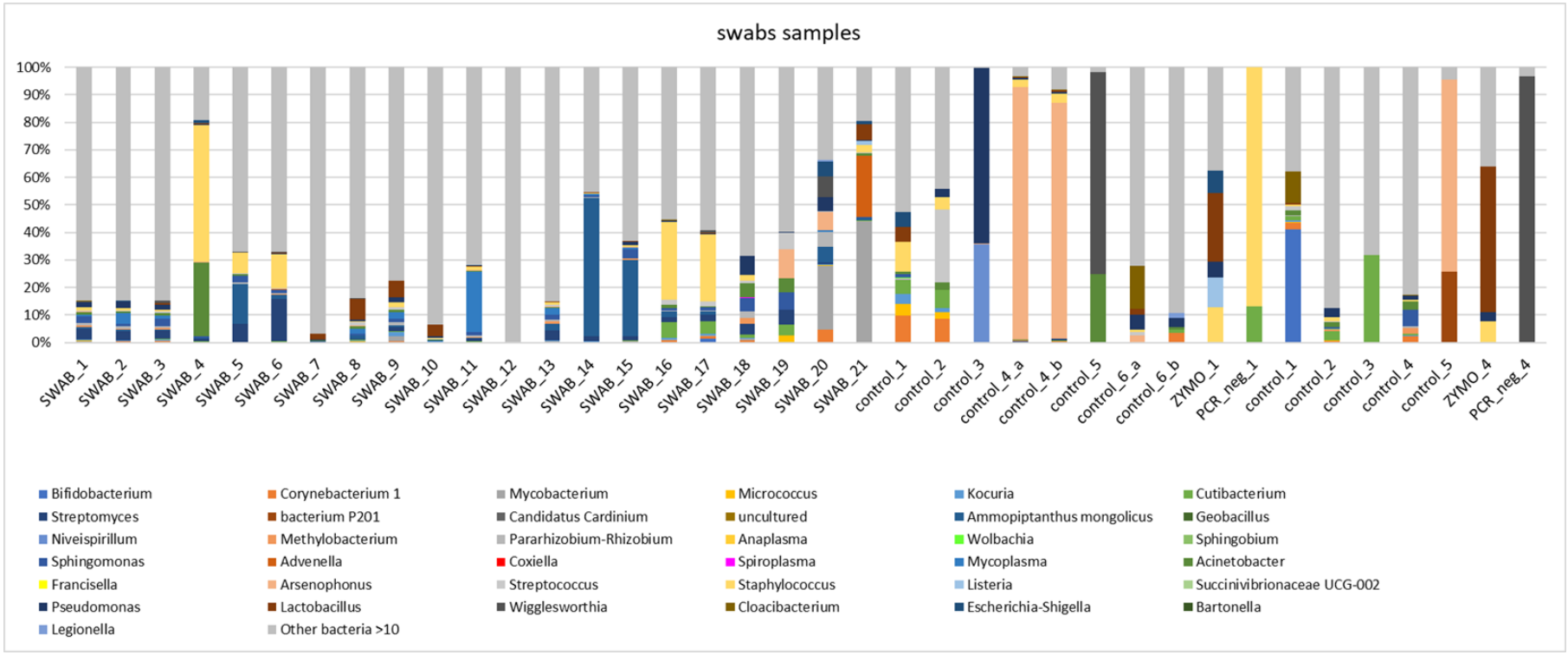


Appendix (33): species –specific primers peformed of PCR amplification of bacterial genes target of ectoparasites on rodents

Bacteria sp.	Ectoparasites	PCR type	Target gene	Primer name	Oligonucleotide sequence ('5-3')	Product length (bp)	Reference
Bartonella	Fleas	Nested (first round	16S ISR	Big (forward) Big (reverse)	5'TTGATAAGCGTGAGG TC'3 5'TCCCAGCTGAGCTACG'3	400 bp	(Telfer *et al.,*2005)
		Nested (second round		Big (forward) Bog (reverse)	5'TTGATAAGCGTGAGG TC'3 5'TGCAAAGCAGGTGCT CTCCCA3'		
Rickettsia	Swab back,chin	Conventional PCR	*gltA*	RpCS.409d (forward) RpCS.1258n (reverse)	5'CCTATGGCTATTATGCTTGC3' 5'ATTGCAAAAAGTACAGTGAACA3'	600 bp	Roux *et al.,*1997
	Laelaps agilis	Conventional PCR	16s rRNA	16srRNA (forward) 16SrRNA (revearse)	5'GAACGCTATCGGTATGCTTAACAA3' 5'CATCACTCACTCGGTATTGCTGGA3'	364 bp	Alberdi *et al.,*2012)
Coxiella-like	Ticks and chigger *spp.*	Nested (first round)	*rpoB* gene	CoxrpoBF2(forward) CoxrpoBR1(reverse)	5'GGGCGNCAYGGWAAYAAAGGSGT3' 5'CACCRAAHCGTTGACCRCCAAATTG3'	607-610 bp	(Duron *et al.,*2015)
		Nested (second round)		CoxrpoBF3(forward) CoxrpoBR3(reverse)	5'TCGAAGAYATGCCYTATTTAGAAG3' 5'AGCTTTMCCACCSARGGGTTGCTG3'	539-542 bp	
Francisella		Conventional PCR	*lipoprotein*	Francilipo (forward) Francilipo (reverse)	5'GTAAGACATATAACTTACTTGT3' 5'AGCTAGTATCATGACACTTA3'	408 bp	(Palomar *et al.,*2019)
Ehrlichia	Tick –lice-swab	Nested (first round)	*groESL*	HS 1a (forward) HS6a (reverse)	5'AITGGGCTGGTAITGAAAT'3 5'CCICCIGGIACIAIACCTTC'3	1,350 bp	(Liz, *et al.,*2002)
Ehrlichia	Tick –lice-swab	Nested (second round)	*groESL*	HS 43 (forward) HSVR (reverse)	5'AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC'3 5'CTCAACAGCAGCTCTAGTAGC'3		

Orientia	Chigger mites	Nested (first round)	TSA47	Ot-145 (forward) Ot-1780 (reverse)	5'ACAGGCCAAGATATTGGAAG'3 5' AATCGCCTTTAAACTAGATTTACTTATTA'3	871 bp	(Masakhwe *et al.,*2018).
		Nested (second round)		Ot-263 (forward) Ot-1133 (reverse)	5'GTGCTAAGAAARGATGATACTTC'3 5'ACATTTAACATACCACGACGAAT'3		
		Nested (first round)	TSA56	RTS-8 (forward) RTS-9 (reverse)	5'AGGATTAGAGTGTGGTCCTT'3 5'GTTGGAGGAATGATTACTGG'3	620 bp	
		Nested (second round)		RTS-6 (forward) RTS-7 (reverse)	5'GTTGGAGGAATGATTACTGG'3 5'AGCGCTA GGTTTATTAGCAT'3		
Wolbachia	Fleas-Ticks	Conventional PCR	Wsp	wsp81F(forward) wsp691R (reverse)	5'TGGTCCAATAAGTGATGAAGAAAC'3 5'AAAAATTAAACGCTACTCCA3'	500bp	Plichart *et al.,*2005
Anaplasma	Ticks	Nested (first round)	16S ribosomal RNA	ge3a(forward) ge10r(reverse)	5'CACATGCAAGTCGAACGGATTATTC3' 5'TTCCGTTAAGAAGGATCTAATCTCC3'	500bp	(Massung *et al.,*1998)
		Nested (second round)		ge9 (forward) ge2(reverse)	5'AACGGATTATTCTTTATAGCTTGCT3' 5'AACGGATTATTCTTTATAGCTTGCT3'		
Spiroplasma	Fleas	Touchdown PCR	*rpoB gene*	*rpoB(*forward) rpoBR2(reverse)	5'GGNTTTATTGAAACACCATAYGCTC'3 5'GCATGTAATTTATCATCAACCATGTGTG'3	1443 bp	(Haselkorn *et al.,*2009)
bacterial 16S rDNA	*Ornithonyssus bacoti*	Conventional PCR	16S Ribosomal DNA	fD1(forward) rP2(reverse)	5'AGAGTTTGATCCTGGCTCAG'3 5'ACGGCTACCTTGTTACGACTT'3	1500 bp	(Weisburg, 1991)
Fungal	*Ornithonyssus bacoti*	Conventional PCR		FungiQuant(forward) FungiQuant(reverse)	5'GGRAAACTCACCAGGTCCAG3' 5'GSWCTATCCCCAKCACGA3'	1400 bp	(Weisburg, 1991)

Appendix (34): Bar charts show relative abundance of bacterial OTUs (genus level) of swabs samples and background controls.The data is filtered; OTUs that represented <10% in a sample are combined in "Others" (grey portion).



Appendix (35)*: Table 3.4 Prevalence, mean intensity of chiggers species infestation on rodent hosts from the Asir region (host n = 74).*

Parasite taxa	Number of host infested	Prevalence (%)[a] with [95% CI]	Mean intensity [b] [95% CI]	Rodent species
Trombiculid mite				
Microtrombicula muhaylensis ***sp. nov***	20	27.0 [18.1-38.4]	21.3 [12.5-36.3]	*A.dimidiatus, M,rex*
Ascoschoengastia browni	25	33.8 [23.5-45.3]	14.9 [10.3-21.4]	*M. yemeni, A. dimidiatus*
Ericotrombidium caucasicum	20	27.0 [18.1-38.4]	19.5 [8.2-43.4]	*A. dimidiatus*
Ericotrombidium kazeruni	50	67.6 [56.1-77.5]	46.3 [29.3-69.2]	*A. dimidiatus, M,rex*
Gahrliepia aff.lawrencei	4	5.4 [1.9-13.3]	3.8 [1.0-7	*A. dimidiatus*
Helenicula lukshumiae	11	14.9 [8.1-24.9]	15.4 [9.4-27.1]	*A. dimidiatus* M .rex, M.yemeni
Microtrombicula hoogstraali	14	18.9 [11.3-29.6]	25.7 [13.6-44.0]	*A. dimidiatus*
Microtrombicula traubi	9	12.2 [6.3-21.5]	6.8 [4.2-11.0]	
Microtrombicula microscuta **sp. nov***	15	20.3 [12.4-30.9]	27.4 [17.8-37.7]	*A. dimidiatus*
Microtrombicula hyraces	7	9.5 [4.5-18.7]	3.7 [2.7-4.6]	*A. dimidiatus*
Pentidionis agamae	28	37.8 [27.4-49.3]	30.4 [19.6-44.9]	*A. dimidiatus*
Schoengastiella wansoni	1	1.4 [0.1-7.2]	6 [NA][c]	*A. dimidiatus*
Schoutedenichia asirensis ***sp. nov** *	5	6.8 [2.7-15.3]	8.2 [2.6-14.4]	*M. yemeni, A. dimidiatus*
Schoutedenichia saudi ***sp. nov** *	33	44.6 [33.7-56.1]	29.2 [18.8-59.2]	*A. dimidiatus*
Schoutedenichia aff.thracica	14	18.9 [11.3-29.6]	9.3 [6-14.8]	*A. dimidiatus*
Schoutedenichia zarudnyi	19	25.7 [16.7-37.1]	11.4 [6.7-20.6]	*A. dimidiatus*
Walchia parvula	4	5.4 [1.9-13.3]	6 [3-7.3]	*A. dimidiatus*

Appendix (36): Table 1 Average 16S rRNA genetic distances among *Rhipicephalus* taxa based on the Kimura-2-parameter model.

	1	2	3	4	5	6	7	8	9	10
***R.* sp. (Lineage A)**	0.56									
***R. sanguineus* (Tropical)**	4.64	0.34								
R. guilhoni	4.49	2.19	-							
***R. sanguineus* (S. Europe)**	6.74	4.62	4.21	0.18						
R. turanicus	10.50	9.06	8.82	6.18	4.37					
***R.* sp. (Lineage B)**	7.81	7.19	6.17	5.89	9.50	1.40				
***R.* sp. (Lineage C)**	14.33	14.10	14.18	13.81	16.70	14.90	-			
***R. sanguineus* (Temperate)**	10.94	9.73	9.80	6.87	9.57	8.30	14.64	1.31		
R. rossicus	12.55	12.17	10.69	9.21	13.50	13.59	18.44	10.03	1.35	
R. pusillus	11.41	11.82	15.79	10.33	13.03	13.86	15.79	12.36	12.19	0.59

Appendix (37): Table 1 Average COI genetic distances among *Rhipicephalus* taxa based on the Kimura-2-parameter model.

	1	2	3	4	5	6	7	8	9	10
***R.* sp. (Lineage A)**	0.29									
***R. sanguineus* (Tropical)**	4.85	3.40								
R. leporis	3.37	3.39	1.09							
R. guilhoni	5.04	6.54	5.87	0.33						
***R. sanguineus* (S. Europe)**	8.63	8.93	8.61	8.04	0.13					
R. turanicus	10.09	10.87	11.80	10.95	8.44	0.65				
***R.* sp. (Lineage B)**	5.44	6.78	6.73	6.32	7.49	10.52	-			
***R. sanguineus* (Temperate)**	10.48	9.36	10.58	11.10	7.84	10.31	10.17	2.76		
R. rossicus	12.80	11.46	12.39	10.83	8.35	9.85	10.47	10.68	0.88	
R. pusillus	13.58	13.99	13.52	11.84	11.29	10.73	10.06	11.85	10.79	0.00

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