The Clinical Epidemiology of Scrub Typhus in Humans, Chiggers and Rodents

Thesis submitted for the degree of Doctor of Philosophy
Trinity Term 2019

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ABSTRACT

Research on the natural history of scrub typhus took place predominantly before the 1970s. This project developed modern techniques to revisit fundamental aspects of the epidemiology and ecology of the disease in Thailand and Laos. Three sites of high scrub typhus disease transmission were identified. Vector chiggers and small mammal hosts were collected, identified and tested for *Orientia tsutsugamushi*, the causative agent and mapped using GIS. The complex ecological interactions of infected and uninfected vectors and hosts with habitats and seasons were investigated. A low-input targeted enrichment sequencing method was developed and applied to a subset of positive samples. Over 18 months, 244 small mammals and ~17,000 chiggers were tested resulting in 279 *O. tsutsugamushi* PCR positive samples. Sixty-nine positive human samples were collected. Overall 8.6% of individual chiggers and 25.9% of chigger pools tested positive. At most sites <1% of individual chiggers tested positive, whereas at the highest-risk site 18% were positive. No consistent high-risk area of infection was identified within our study sites (~9km^2). High-risk sites were associated with a lower diversity of chigger species, higher proportion of recognized vector species and a higher mean number of chiggers attached to hosts. The end of the dry season was most strongly associated with *O. tsutsugamushi* positivity. The extremely low quantities of DNA in these samples combined with the complex genome assembly created numerous challenges for sequencing and bioinformatics analysis. Phylogenetic clustering was evident among samples collected from the same sites, although strains with greater genetic differences also appear to co-exist even on the same host. In Thailand and Laos, human infections rise dramatically during the rainy season. However, corresponding proportions of infected chiggers remained stable, suggesting that human behaviour plays a critical role. Improving our understanding of risk behaviour could yield relatively simple interventions to reduce disease acquisition through public health education.

ACKNOWLEDGMENTS

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I am particularly indebted to Liz Batty and Serge Morand. Liz took on an IT novice and patiently and expertly guided me through bioinformatics training and supported me throughout the analysis. In Serge, I could not have had a more experienced and knowledgeable ecologist and field scientist to guide me. Many enjoyable hours were spent together in the field discussing a rich variety of topics. I am also very grateful to Ben Makepeace who agreed to be a collaborator back in 2014 and has been a wonderful source of advice. The scrub typhus world is small and having such enthusiasm and expertise to drawn on has been incredibly helpful.

I was extremely lucky to find and recruit Ms. Neeranuch Thangnimitchok, recently graduated from university in Bangkok. Nuch soon found her feet in

Vientiane and proved a fast learner, reliable and well-organised laboratory technician. Her sharp eyes and steady hand allowed us to process an enormous number of field samples.

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Finally, I would like to thank my wife, Cat for the sacrifices she made to her career to join me in Laos together with little Alexia and later Tristan who arrived safely, albeit unexpectedly during this project! Thank you for supporting me and putting up with my frequent travels and endless work. It was a wonderful adventure.

DEDICATION

This thesis is dedicated to my father, who passed away in April 2018

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General introduction, burden of scrub typhus in Thailand & Laos and thesis aims & objectives

1 Chapter 1: General introduction, burden of scrub typhus in Thailand & Laos and thesis aims & objectives

1.1 Introduction

Scrub typhus is a vector-borne zoonotic disease with the potential of causing life-threatening febrile infection in humans. The disease is caused by a Gram-negative bacterium, *Orientia tsutsugamushi*, and is transmitted by trombiculid mites (chiggers). Scrub typhus has an expanding known distribution, with most disease occurring across South and East Asia and parts of the Pacific Rim. It is present in a large variety of habitats from semi-urban parks and gardens to plantations and forest edges. Rural workers are typically most at risk, but cases are increasing among city dwellers visiting the countryside for recreation. An enormous population is thus potentially at risk and the disease goes unrecognized in many areas. The lack of lasting immunity contributes to the burden of disease, with rural workers in endemic areas probably being infected several times in their lives. As an obligate intracellular organism and Biosafety Level 3 pathogen in cell culture, many barriers exist to understanding its biology and pathogenesis. The organism has an unusual genome among the Rickettsiaceae, consisting of a very high proportion of repeats and proliferation of mobile genetic elements that present challenges for whole genome sequencing and phylogenetic analysis. Scrub typhus has a complex ecology with multiple vector/reservoir chigger species and an even broader range of hosts. The interaction between these, the environment and humans remains only partially understood. Much of existing scrub typhus dogma originates from research during and immediately after World War II and only in the past decade has there been a gradual resurgence in investigation. Numerous unanswered questions remain and critical areas for study employing modern tools such whole-genome sequencing, geographical information systems and computational infrastructure exist. In this introductory chapter I provide a general overview of scrub typhus including its pathogenesis, clinical features, diagnosis and treatment. I discuss the global burden of disease and then focus on Thailand and Laos. Finally I summarise the aims and objectives of my thesis.

Throughout this thesis both old and new country names are used. When referencing information from an article using on old name, this was not changed. For example, Burma (now Myanmar), Malaya (now Malaysia) and West Pakistan (now Pakistan).

1.2 Taxonomy and naming

Orientia tsutsugamushi is an obligate intracellular coccobacillus belonging to the family Rickettsiaceae, in the order Rickettsiales, within the class α-proteobacteria, phylum Proteobacteria and domain Bacteria.

Controversy surrounds the first identification of the organism in Japan in 1920 by Hayashi, who misidentified it as a protozoan parasite and named it *Theileria tsutsugamushi* [1]. Nagayo et al. provided the first detailed description of the organism, renaming it *Rickettsia orientalis* [2]. Ogata and other Japanese investigators at this time reached a consensus to use the name *Rickettsia tsutsugamushi* [3]. However *R. orientalis* and other names including *Rickettsia tamiyai* and *R. tsutsugamushi* var. *tropica* were in use until at least the 1960s, creating additional confusion [4] [5]. In 1995, following sequencing of the 16 S rRNA gene, the organism was renamed as *Orientia tsutsugamushi* [6].

The disease, now generally known as scrub typhus, has received a huge variety of names, reflecting its geographical range and the challenges with identifying the pathogen. In Japan it was known as tsutsuga-mushi (literally "dangerous bug"), shima-mushi, Shichito fever and kedani (flood or river) fever. In Southeast Asia, frequently used terms included: tropical typhus, rural typhus, mite fever, "K" or "OXK" type typhus and mite-borne or chigger-borne typhus. In Sumatra it was briefly known as pseudotyphoid fever of Deli; in North Queensland, Australia as Mossman fever, endemic glandular fever, pseudotyphus of sugar cane cutters and Sarina fever; and in the Republic of China (hereafter Taiwan) it was referred to as Batran or Mokkui fever [7-14]. In 1940, Lewthwaite & Savoor suggested that on the basis of precedence 'tsutsugamushi disease' should be used [15]. Traub & Wisseman argued that the name scrub typhus was misleading, as the disease also occurs in habitats that cannot be described as "scrub". They proposed 'chigger-borne typhus', but the name did not stick and scrub typhus remains the commonly used term today [14].

1.3 Historical perspective

Scrub typhus was probably first described in an ancient Chinese text named 'Zhouhofang' produced in 313 AD [16]. The first account of 'Japanese river fever' in western medical literature was published as a letter from Dr Theodore Palm, a medical missionary in Japan, in 1878 [17]. However, the disease was already well established in Japanese folklore and clinical accounts date back to at least 1810 in Japanese literature [18]. By 1920, Japanese investigators had confirmed the transmission of scrub typhus by the mite *Trombicula akamushi* and had gathered evidence that rodents and birds may act as reservoirs [19-21]. A number of typhus-like illnesses were being investigated in Malaya, the Dutch East Indies and Formosa during the 1920s, often as a result of outbreaks in rubber and oil palm plantation workers. With the discovery and subsequent confirmation that *Proteus mirabilis* OXK antigen agglutinates in scrub typhus, it was concluded by WW2 that scrub typhus, tsutsugamushi disease and the many other varieties were one and the same [7,22,23].

Scrub typhus proved to be third only to malaria and dengue fever (second to and more feared than malaria by troops in the Pacific Theatre) as a cause of fever, morbidity and mortality during WW2. More than 16,000 cases and 600 deaths occurred in Allied troops alone [8]. Explosive outbreaks were reported across the region. At Sansapor and on the islands of Owi and Biak in New Guinea, more than 150,000 man-hours were lost to the infection, rendering some units ineffective [8]. Mortality rates varied widely from 0.6% on Owi to 33.5% in Finschhafen, New Guinea [8,24]. As a result typhus commissions were set up in 1943 by the United States at Buna in Oro Bay, New Guinea and then in North Burma. The British had already begun studying the disease at the Institute for Medical Research (IMR) in Kuala Lumpur, Malaya in 1924. During the war, British-led research halted at the IMR and continued as the Scrub Typhus Research Laboratory at Imphal, Manipur, India from 1945-6. From 1948, for several decades, British and American research continued at the IMR [13,25-28]. Scrub typhus was also a leading cause of fever in the Vietnam War [29,30]. Much of the current knowledge on the ecology of scrub typhus acquired during this period remains established doctrine and has not been revisited using modern techniques.

1.4 Global epidemiology and burden of disease

Scrub typhus in humans has been reported from a vast area from Pakistan in the west to Japan and the Pacific Rim in the east, the Primorsky Krai of southern Siberia to the north and south to the Chagos archipelago in the Indian Ocean and Tropical North Queensland in Australia.

Historical reports from the 1950s using unreliable serological tests suggested evidence of scrub typhus in Ruanda-Urundi (now Rwanda and Burundi) [31] and Yemen [31,32]. In the 1990s there were two case reports of travellers to Cameroon [33] and the Republic of Congo [34] with serological evidence consistent with scrub typhus. Recently publications from Kenya [35] and Djibouti [36] report serological evidence of the disease in the local population. Most intriguingly of all, molecular evidence of infection in a patient in the United Arab Emirates [37] was reported as a possible new species *Orientia chuto* sp. nov. in 2010 and in 2016 molecular and serological evidence was published on scrub typhus cases from Chiloe Island, Chile [37,38]. Xu et al. reviewed in detail the epidemiology of scrub typhus in humans in 2017 [39].

In 1999, the World Health Organization (WHO) recognized the importance of scrub typhus by stating 'scrub typhus is probably one of the most under diagnosed and under reported febrile illnesses requiring hospitalization in the region [Southeast Asia] [40]. Rural and semi-urban populations across South and East Asia are at risk, a total of at least 2 billion people. In some areas of Southeast

Asia, scrub typhus causes up to 23% of febrile hospital admissions [41-44]. Recent publications have reviewed the epidemiology [39], burden of disease [45] and untreated mortality for scrub typhus in humans [46]. In prospective studies in six countries across South and East Asia published after 2001, seroprevalence ranged from 9.3 to 27.9% with a median of 22.2% [45]. In all studies examining seroprevalence in a range of populations and scenarios a much broader range is seen from 0-73% [39]. There appears to be an increase in minimum disease incidence reported by countries with passive national surveillance systems (South Korea, Japan, China and Thailand) with a median of 4.6/100,000/10 years [45]. Mortality rates also range widely with a median of 6% untreated, falling to 1.4% for treated cases [45].

1.5 Pathogenesis

Following the bite of an infected chigger, *O. tsutsugamushi* infection is mainly associated with dendritic cells, monocytes and the vascular endothelium. The process of dissemination is not fully understood but probably occurs via direct haematogenous and lymphatic spread of the cells infected at the inoculation site [47,48]. In cultured dendritic cells, *O. tsutsugamushi* appears capable of escaping autophagy and replicates rapidly in the cytosol [49]. The disease is characterised by a vasculitis-like pattern as the bacteria invades and proliferates in vascular endothelial cells of small and medium-sized blood vessels. Disease severity is associated with massive cytokine release (TNF-α, IL-8, IL-10 and IFN-γ-producing NK cells) [50]. In animal models, the lungs are the major target organ for *O. tsutsugamushi* infection [51]. Two recent reviews focus on advances in our understanding of scrub typhus pathogenesis and biology [50,52].

1.6 Clinical features

The incubation period for scrub typhus is approximately 6-21 days, with most cases becoming symptomatic within 14 days following the bite of an infected trombiculid mite. Patients typically present with several days of fever, headache, myalgia and a dry cough. Lymphadenopathy is common. A maculopapular rash is infrequent and an eschar is an important clue to the diagnosis. Eschars are black cigarette burn-like necrotic lesions occurring at the site of the bite. Although not pathognomonic, if found they are highly suggestive of the disease. Studies report the presence of eschars in anywhere from 7-97% of cases [53]. Their frequency is probably less in those with recurrent infection in endemic settings, but the lesion is often overlooked as it frequently occurs in the inguinal, genital or axillary regions. Deafness, tinnitus and conjunctival suffusion may also be present. Severe disease with multiorgan failure and death can occur. The most common complications include pneumonitis, acute respiratory distress syndrome, meningoencephalitis, acute renal failure, myocarditis and severe hepatitis [54-56].

Particularly in the absence of an eschar, the disease presents as an undifferentiated febrile illness and thus may be readily confused with malaria, typhoid fever, dengue, leptospirosis, murine typhus, infectious mononucleosis, HIV seroconversion illness, Q fever and others.

1.7 Diagnosis

Laboratory diagnosis of scrub typhus remains challenging. Koh et al. published a review of scrub typhus diagnosis in 2010 [57]. During the early era of research, xenodiagnosis, microscopy and the serological Weil-Felix OX-K agglutination test were the mainstays of diagnosis. Xenodiagnosis, usually by rodent intraperitoneal inoculation (although many species, from rabbits to primates have been used) and serial passage proved to be relatively sensitive and specific and continued to be used until recently. The technique is, however, costly, time consuming, prone to contamination and should be performed in a Biosafety Level 3 facility. The method has now been superseded by *in vitro* cell culture, though this too suffers from the same limitations. The Weil-Felix OX-K agglutination test has been in use for nearly a century. It suffers from poor sensitivity and specificity and paired samples are needed for confirmation. Other serological tests have been widely employed in diagnostics, particularly the indirect immunofluorescence assay (IFA), indirect immunoperoxidase assay (IIP) and enzyme-linked immunosorbent assays (ELISA). IFA is sensitive and rapid, but costly and requires experienced technicians to perform. There has been a rise in point-of-care immunochromatographic tests (ICT), often used as first-line screening tools. All serological assays are beset by the need to define titre cut-offs for positive results and these can vary dramatically between endemic and non-endemic settings. Acute and convalescent results are also usually needed for robust diagnosis.

Several molecular techniques are in use including real time and conventional PCR and the loop-mediated isothermal PCR assay. These target a range of outer membrane proteins including 56 kDa, 47 kDa, 16s and GroEL. Specificity is very high, but sensitivity varies, primarily due to the variable quantity of *O. tsutsugamushi* in the specimen. Rickettsial load in blood is highly variable and often very low, depending on the timing of sample collection. In a study of 155 acutely unwell scrub typhus patients, a median of 13 copies/ml of *O. tsutsugamushi* DNA in whole blood was reported [58]. Buffy coat may be a better sample type and eschar crust, biopsy and even swabs can yield good quantities of *O. tsutsugamushi* DNA. Due to strain variation and genetic diversity the optimal PCR target remains unclear. Whole genome sequencing methods are in development, but no antigen or protein based diagnostics have so far been discovered.

1.8 Treatment

Treatment is relatively simple with the vast majority of clinical cases responding well to doxycycline within 48 hours. Azithromycin, chloramphenicol and rifampicin are alternatives. A course of 7 days treatment is recommended, but shorter courses may be sufficient. In severe disease, parenteral and nasogastric delivery of these antibiotics is advised. In pregnancy, azithromycin or chloramphenicol (except in the final trimester) is most suitable and in children a short course of doxycycline probably outweighs any risks associated with the antibiotic [59]. Reports of doxycycline and chloramphenicol resistance in Northern Thailand in the 1990s are a concern, though poorly substantiated [60]. Recent work on antimicrobial susceptibility testing of *O. tsutsugamushi* isolates, found no evidence to support the existence of resistance [61].

1.9 Immunity, strain diversity and virulence

Natural immunity to *O. tsutsugamushi* in humans requires both Th1 cell-mediated and humoral responses. Studies in Malaya soon after WW2 demonstrated that previously infected and treated patients were susceptible to reinfection with heterologous strains (the usual scenario in such an antigenically heterogeneous organism) within a few months. Homologous protection may last for 2 or 3 years [62]. Similar findings have been reported more recently using non-human primate studies [63].

Various immunological techniques have been used to define the immunogenic strains of scrub typhus. Three major strains: Karp (New Guinea), Kato (Japan) and Gilliam (Assam) together with an ever-expanding list of antigenically classified isolates have been used in an attempt to categorize strain diversity. With the advent of molecular tools and sequencing of genes, the impressive heterogeneity of *Orientia* isolates has been further revealed. The geographic distribution of genotypic variants was reviewed in 2009 [56]. This heterogeneity has been a major factor in the lack of progress developing improved diagnostic tests and an effective vaccine. Strain diversity is discussed in more detail in Chapter 5.

Since the disease was first described, the clinical severity has been reported to vary geographically. Low virulence (0.6% mortality) was seen in American troops on Owi and Biak islands, New Guinea; 1.5% mortality in British troops on Ceylon and 6% on the Pescadores islands, Taiwan. This rose to 10% on mainland Taiwan, as high as 35% in Finschhafen, New Guinea and 60% in Kochi, Japan [9,11,27]. Mortality rates seem to stay consistent in a given area in spite of fluctuations of incidence [8]. There is some evidence that the clinical picture can vary between strain type, as reported for Karp and Boryong strains in South Korea [64].

1.10 Scrub typhus in Thailand and Laos

1.10.1 Thailand

Thailand is a large country of 69 million people in Southeast Asia. The country is approximately 1,700km from its northern-most point (20°N) to its southern extreme (5°N). The weather is dominated by the tropical southwest monsoon, but the country is affected differently. The north and central regions are dry from November to February, with the pre-monsoon starting in March and full monsoon from June to October. In southern Thailand, most rainfall occurs in October and November. Southern Thailand also has less seasonal variation in temperature. The number of people involved in agriculture is gradually falling and now thought to be 33%, though this varies regionally and is probably higher in Northern Thailand [65].

1.10.1.1 National Data

Scrub typhus is a nationally reportable disease in Thailand and the data are publically accessible from the National Disease Surveillance System (R506), Bureau of Epidemiology, Ministry of Public Health (http://www.boe.moph.go.th/boedb/surdata/disease.php?ds=44). Robust criteria, combining clinical features and laboratory diagnostic tests were used to define a case of scrub typhus disease. The highest incidence rates per 100,000 population are seen in Mae Hong Son with 168 (standard deviation (SD) 86), Tak 108 (66), Nan 99 (62), Chiang Rai 56 (29) and Phang Nga in the south of Thailand with 49 (28) (Figure 1).

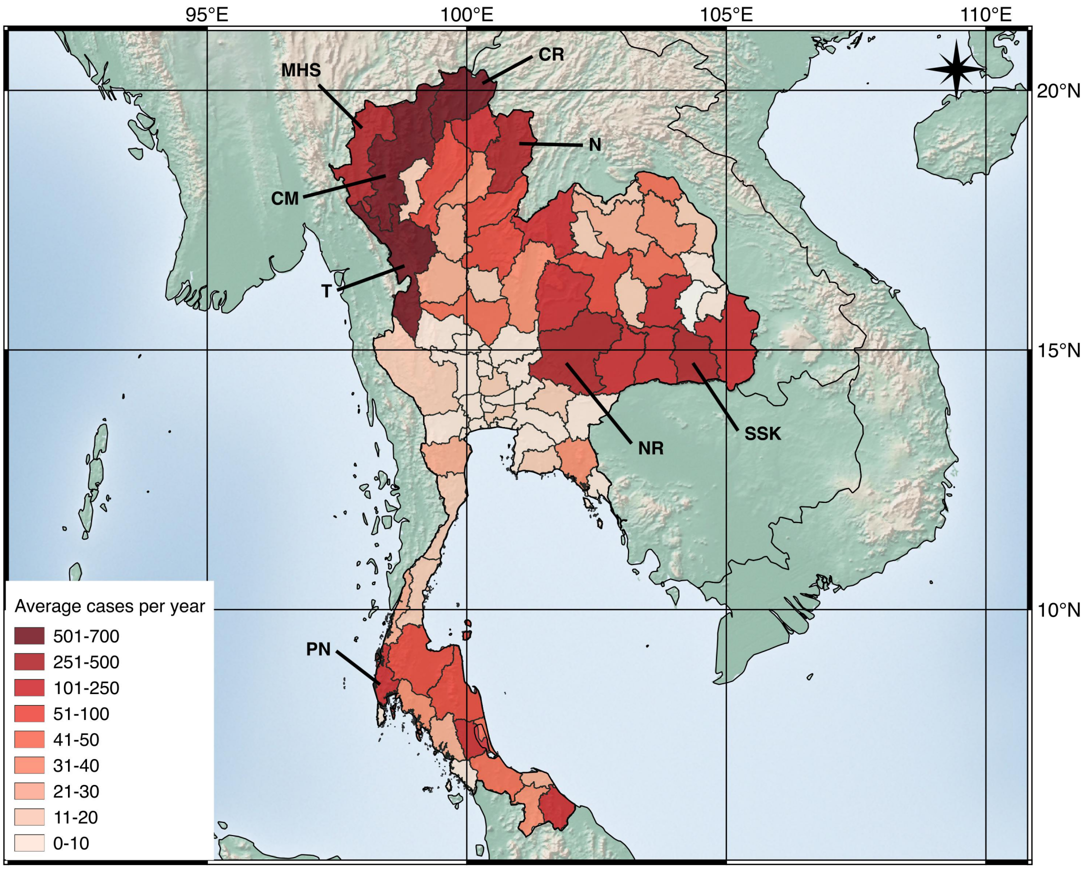


Figure 1 Mean number of human scrub typhus cases per year between 2003 and 2017 by province. CR = Chiang Rai, N = Nan, SSK = Si Sa Kaet, NR = Nakorn Ratchasima, PN = Phang Nga, T = Tak, CM = Chiang Mai, MHS = Mae Hong Son.

A total of 93,589 cases of scrub typhus were reported between 2003 and 2017. The annual incidence rate/100,000 population peaked in 2013 at 10,592 (Figure 2). The greatest burden of disease in Thailand is seen in the north. Five provinces (Chiang Rai, Chiang Mai, Tak, Nan and Mae Hong Son) account for 42,104 (45%) cases in the 15-year period reviewed. These 5 provinces comprise just 7% of the entire population.

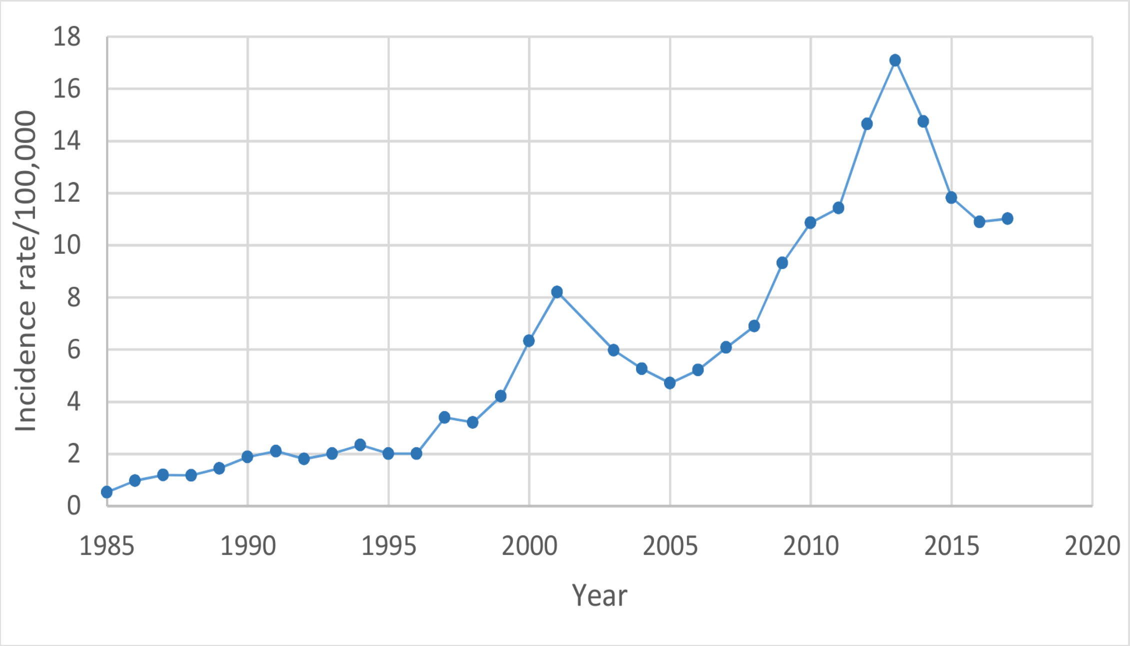


Figure 2 Scrub typhus incidence rate per 100,000 population/year for Thailand

Among occupational groups reported, 45% (inter-quartile range 42-46) were involved in agriculture. A very similar annual incidence of cases was seen among the 25-34, 35-44 and 45-54 age groups. In 2017, 1,154/7,211 (16%) of cases were in children under the age of 15 years.

The seasonality of scrub typhus in tropical and sub-tropical latitudes is well recognized (see Chapter 2). Nationally the highest number of cases is seen in October, followed by July and August. The majority present between June and November, but a small number occur throughout the year. The seasonality is most marked in the north and east, with much less fluctuation (and numbers) in the south and central regions (Figure 3).

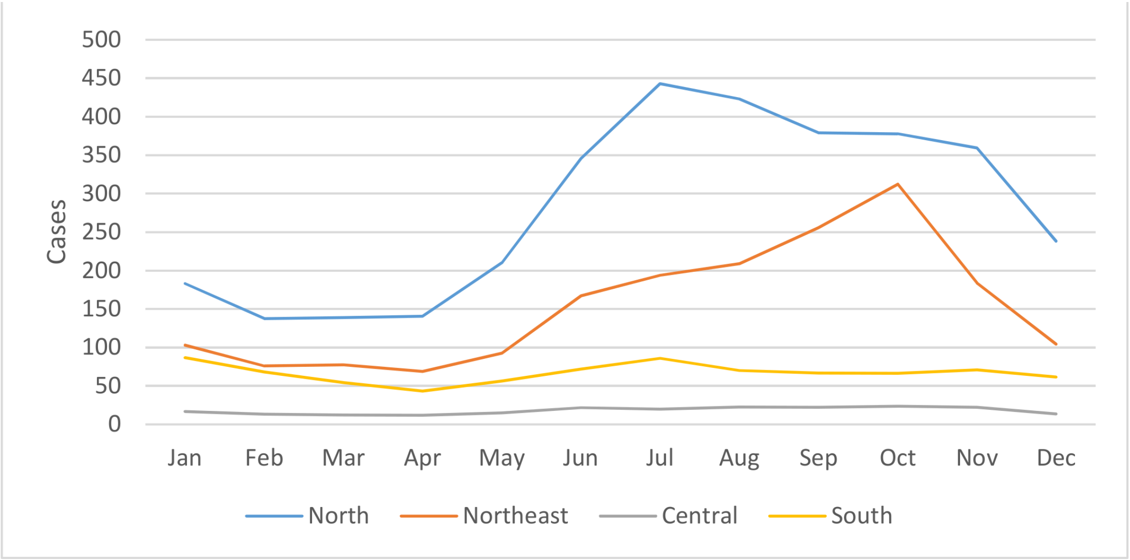


Figure 3 Scrub typhus mean number of human cases by month per region, 2003-2017

1.10.1.2 Chiang Rai Province

Chiang Rai Province is officially divided into 18 districts, 124 sub-districts and 1,816 villages and has 1.3 million inhabitants. Although the majority of the population is ethnic Thai, 12.5% are of hill tribe origin.

During the period 2008 to 2017, 8,722 cases were reported province-wide. The highest incidence was in July, August and June similar to overall data for Northern Thailand. Scrub typhus case distribution is shown across the province at the sub-district level in Figure 4.

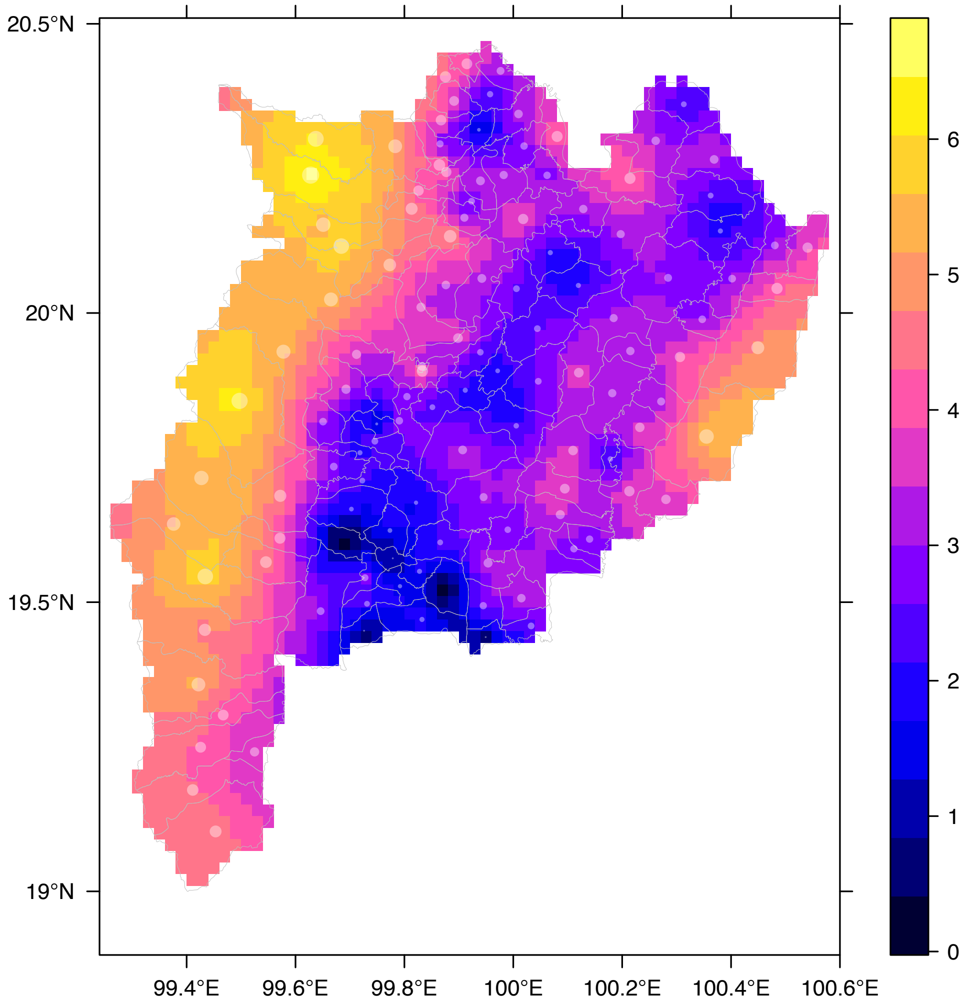


Figure 4 Distribution of scrub typhus cases in Chiang Rai Province at the subdistrict level (performed using kriging interpolation on a log scale)

Mae Fahluang District has both the highest burden (1,746 reported cases) and incidence of 218 per 100,000 (Table 1).

Table 1 Thai national data for selected sub-districts of Chiang Rai Province where attempted vector/host collections took place 2016-18

District	Sub-district	Population	No. cases 2008-17	Mean annual incidence/100,000	Notes
Meuang	Mae Yao	17,854	216	121	Location of Ban Song Kwair
Meuang	Huai Chomphu	11,642	240	206	Location of Ban Mae Mon
Meuang	Mae Khao Tom	12,626	30	24	Single trapping visit
Meuang	Tha Sai	9,898	9	9	Single trapping visit
Meuang	Huai Sak	19,703	32	16	Single (unsuccessful) tapping visit
Mae Suai	Wawi	35,128	522	149	Single (unsuccessful) tapping visit
Mae Fah Luang	Thoet Thai	21,593	471	218	Location of Ban Thoet Thai

For the 3 study sites selected for repeated collection of vectors and hosts (see Chapter 3), the village population, number of reported cases and incidence rate are shown in Table 2.

Table 2 Population size and number of cases for the 3 selected study sites where repeated vector/host collections took place. Note that Ban Huay Khom consists of 4 small villages several kilometres apart; one of these is Ban Song Kwair (population ~150)

Village	Population	No. of cases 2008-17	Mean annual incidence/100,000
Mae Mon	1089	43	395
Huay Khom	1610	25	155
Thoet Thai	5447	74	136

Dr Tri Wangrangsimakul performed data extraction of the Thai data and created Figure 2 & Figure 3. Dr Serge Morand created Figure 4.

1.10.2 Laos

Laos is about half the size of Thailand, but has a fraction of the population at just 7 million. It is much lower on the human development index (HDI), a composite measure of per capita income, life expectancy and education at 139th compared to Thailand (83rd) [66]. Around 70% of the population is involved in agriculture. The climate is very similar to Northern and Eastern Thailand.

The only national data on human scrub typhus comes from the Lao-Oxford-Mahosot Hospital Wellcome Trust Research Unit (LOMWRU), in the capital Vientiane. Two prospective fever studies between 2001 and 2010, identified scrub typhus as the cause of 15% of fevers [41,43]. Scrub typhus testing has been prospectively performed on patients with fever >38°C, meeting certain inclusion criteria since 2003. All cases were screened with an IgM rapid diagnostic test (RDT). A total of 1,325 patients have tested positive (Figure 5).

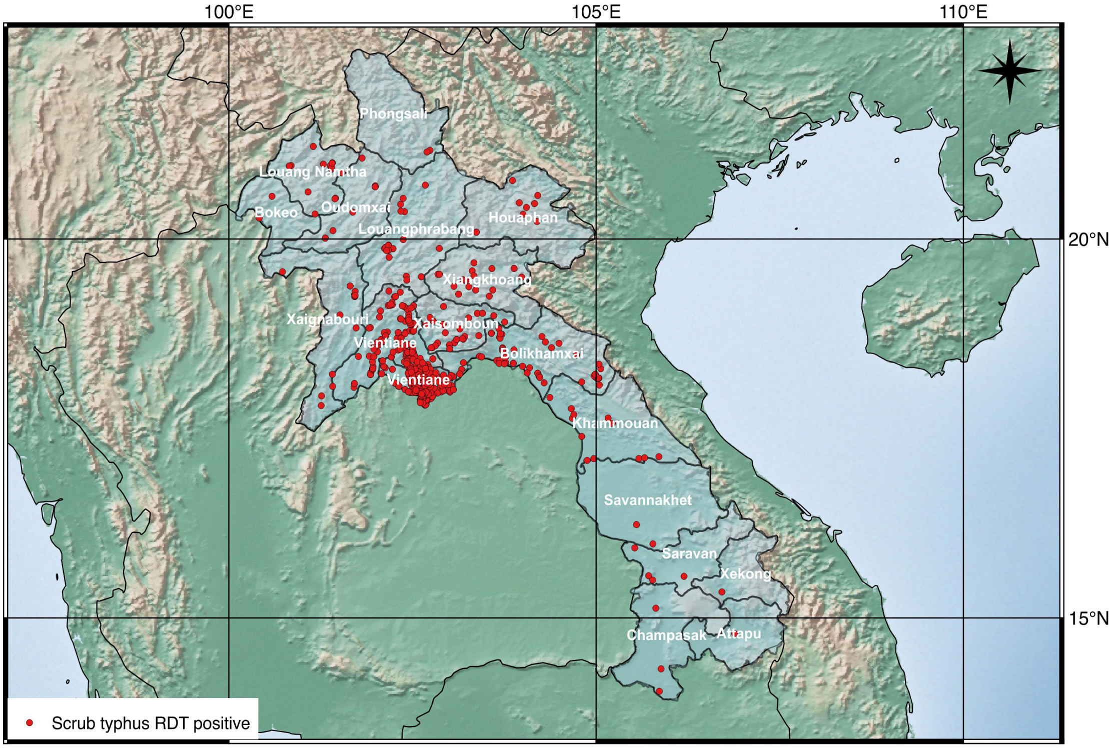


Figure 5 Distribution of scrub typhus Rapid Diagnostic Test (RDT) positive human cases in Laos 2003-2017

The data shown in Figure 5 illustrates that human cases were concentrated around Vientiane Capital and Vientiane Province. This reflects the fact that patients with fever in that region will frequently present to Mahosot Hospital, a national tertiary referral hospital. More detailed inspection of the case distribution also shows that outside the capital, the majority of the cases' villages are along major roads, perhaps reflecting easier access to the capital.

Cases show the same overall seasonal pattern observed in Northern Thailand, with most cases during the rainy season from June to October and the pattern repeating itself annually (Figure 6). The seasonal pattern is illustrated more clearly using cumulative monthly cases (Figure 7).

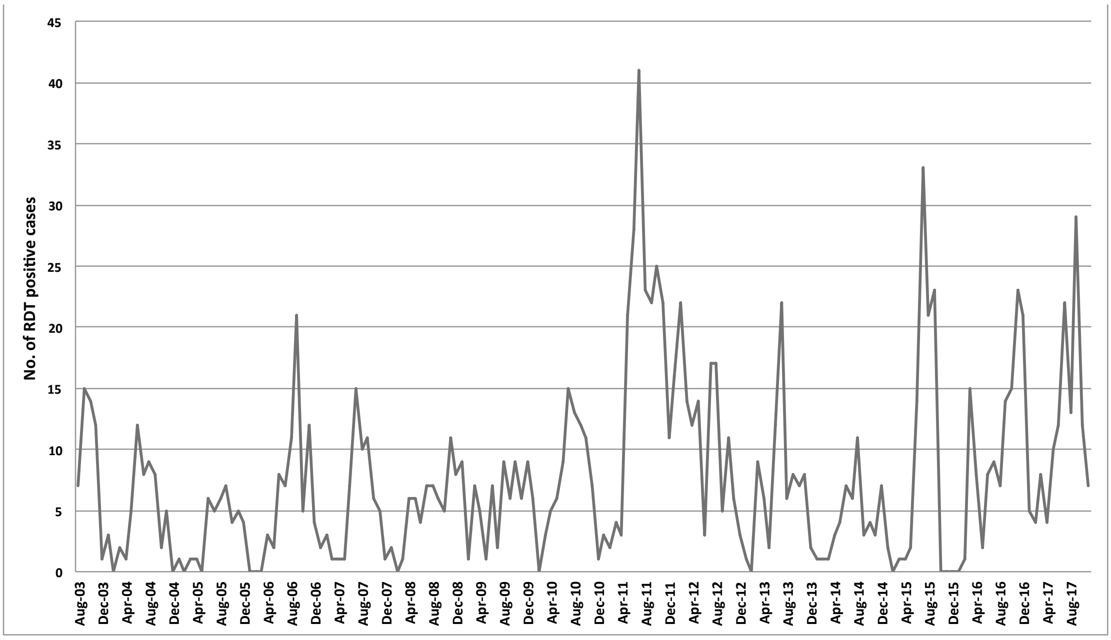


Figure 6 Scrub typhus cases presenting to Mahosot Hospital, Vientiane 2003-2017

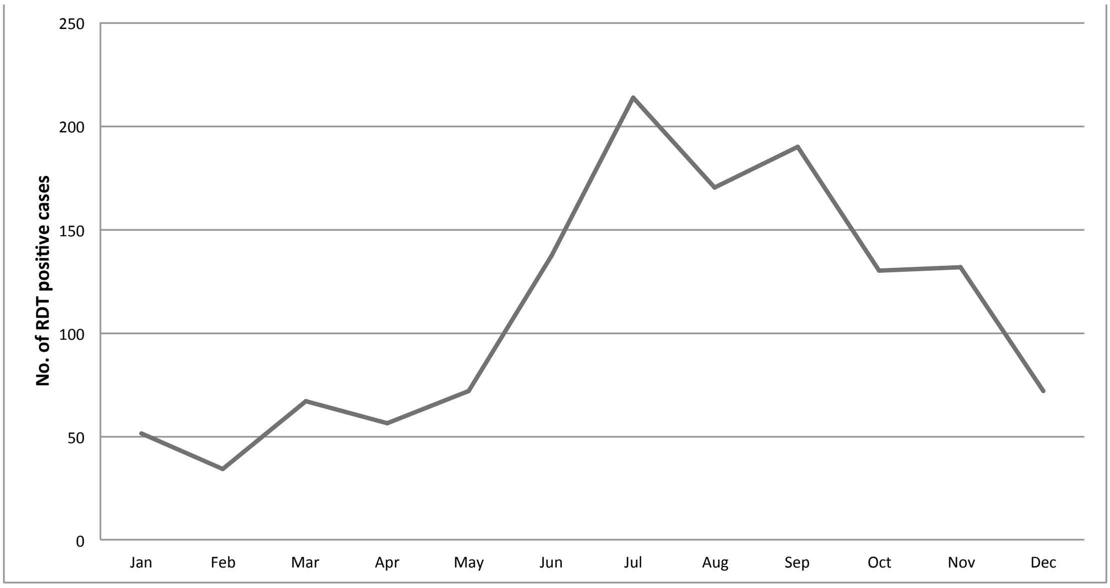


Figure 7 Cumulative monthly scrub typhus RDT positive cases

1.11 Aims and objectives of the thesis

The main objective of this thesis is to revisit the clinical epidemiology and ecology of scrub typhus in humans, chiggers and rodents using cutting-edge modern technologies. To address this main objective, the study is divided into three primary research objectives.

The first objective is to systematically review the literature on scrub typhus ecology. I analyse and map studies investigating scrub typhus in vectors and non-human hosts. Key research questions include: what are the main vector and host species and how are they distributed? What should the minimum reporting criteria be for studies investigating a vector-borne pathogen? I go on to discuss existing knowledge of the major themes in scrub typhus ecology and highlight gaps in our understanding.

The second objective is to identify one or more sites of high scrub typhus transmission and to investigate these repeatedly at different times of the year. Relevant research questions include: what are the dynamics and rates of *O. tsutsugamushi* in vectors and hosts? What impact does season, habitat, rainfall, vector and host species diversity have on *O. tsutsugamushi*-positivity rates? How do these findings link to human scrub typhus risk and seasonality?

The third objective is to develop targeted low-input enrichment sequencing methods to be performed directly on DNA extracted from individual chiggers, rodent tissues and human samples. Important questions include: can sufficient sequence coverage be achieved directly on field samples? Are the sequencing data sufficient to begin to define genomic relationships between *O. tsutsugamushi* in chiggers, rodents and humans? Can these relationships be linked to geographical location and ecological parameters (such as habitats and seasons)?

This work begins in the field, identifying areas of high human scrub typhus incidence, collecting and storing specimens and recording locations and ecological parameters. In the laboratory, taxonomic and molecular analyses are performed. Data management systems are created and maintained. Geospatial mapping and next generation sequencing and bioinformatics and statistics are employed in the analysis. In CHAPTER 1, scrub typhus is introduced and the burden of disease in Thailand and Laos presented. In CHAPTER 2, a systematic review of scrub typhus ecology is reported. CHAPTER 3 describes the investigation of scrub typhus in humans, chiggers and rodents. I explain the process of study site selection and discuss field and laboratory methods. I present results of the collection and laboratory testing of chiggers, small mammals and human samples. In CHAPTER 4, I assess the ecology of vectors and hosts at high transmission sites and attempt to link *O. tsutsugamushi* positive patients to habitat, season, rainfall and other ecological parameters. I also compare and contrast the disease ecology in Northern Thailand with the Penghu Islands in Taiwan. In CHAPTER 5, I present the methodological development of low-input targeted enrichment sequencing of *O. tsutsugamushi* directly from field samples. I develop and improve the methods using chigger DNA spiked with *O. tsutsugamushi* DNA and colony-bred chiggers. I go on to sequence chiggers, rodents and human samples and begin to look at phylogenetic relationships at different geographic scales. Finally in CHAPTER 6, I discuss overall conclusions and potential directions for future research. During the course of this research I also introduced whole-genome sequencing (WGS) capacity in Laos using the portable Oxford nanopore MinION and report the first ever WGS in the country, performed on *Rickettsia typhi* that is sympatric and has a similar clinical presentation to scrub typhus. This work is presented elsewhere [67].

CHAPTER 2

Scrub typhus ecology with systematic review of *Orientia tsutsugamushi* in vectors and non-human hosts

2 Chapter 2: Scrub typhus ecology with review of *Orientia tsutsugamushi* in vectors and non-human hosts

2.1 Introduction

Scrub typhus can be acquired in both rural and semi-urban environments and thus an enormous population is likely to be at risk. The ecology of scrub typhus covers a multitude of topics from the life cycle and biology of the vector, to its interaction with hosts and the environment. Much of our existing knowledge of these topics is based on research carried out during WW2 and until 1970s. Traub & Wisseman published the last comprehensive review on the subject in 1974 [14]. Scientific and technological advances now provide the opportunity to revisit many of these critical topics. The many gaps in our knowledge act as barriers to our ability to make breakthroughs in diagnostics and vaccine development and ultimately public health interventions to reduce the burden of the disease in poor rural communities across Asia and potentially further afield.

Data were systematically reviewed from all accessible articles using aetiological diagnostic tests to identify *Orientia* sp. infection in vectors and non-human hosts and map the location of these studies. The major themes in the ecology of the disease are then reviewed. The relationship between human infection and disease ecology is examined and the limitations of the existing literature are discussed and minimum reporting criteria proposed. Finally, the key gaps in our understanding are reviewed and available tools identified to begin to unravel the details of this complex tropical disease.

2.2 Materials and Methods

2.2.1 Eligibility criteria

Articles were selected with two separate aims. Firstly, all articles using any aetiological laboratory test to detect *Orientia* infection in any potential vector or non-human animal host were included. Secondly, any article not included in the first selection, but containing information broadly encompassing the term "ecology" was reviewed. In this review the term "ecology" describes vector-host-pathogen interactions in the context of their environment and evolution. Although the focus of the systematic review is on non-human hosts, the review of ecology includes detailed human interactions. There were no restrictions based on year of publication or language.

2.2.2 Information sources

Articles were identified through electronic resources and by scanning reference lists of relevant articles. The electronic search was performed using Embase (1974-present), Medline (1950-present), CAB Abstracts (1910-present) and Web of Science (1900-present). Additionally, an unpublished list of scrub typhus articles produced by Michael W. Hastriter in 2012 was scanned for relevant articles (previously, but no longer, accessible at: http://www.afpmb.org/sites/default/files/whatsnew/2012/Hastriter_Complete.pdf). The first search took place on 26th October 2015. I continued to receive alerts for additional articles using the same search terms from all 4 electronic databases on a weekly basis until 20th November 2018.

2.2.3 Search strategy

The electronic databases were searched using the following terms: scrub typhus or *Orientia tsutsugamushi* or *Rickettsia tsutsugamushi* or *O tsutsugamushi* or *Orientia tsu** or akamushi disease or Japanese river fever or Nippon river fever or mite typhus or mite-borne typhus or tropical typhus or tsutsugamushi disease or Kedani fever or akamushi or shimamushi or shichito fever or XK typhus. These terms were combined with at least 1 of the following terms: ! mite* or chigger* or trombicul* or Leptotromb* or rodent* or rats or mammal* or animal* or ecolog* or epidemiolog* or vector* or 'natural history'. Duplicate search results were removed using Endnote X7. Articles were searched in all languages. No unpublished literature or conference abstracts were included.

This review followed the PRISMA statement for systematic reviews (Appendix A-1). The review was not eligible for registration with the international prospective register of systematic reviews (PROSPERO) as it does not have a health related outcome directly relevant to human health.

2.2.4 Study selection

The author reviewed titles and abstracts for all articles for inclusion. If there was any doubt regarding inclusion, then the full article was obtained for assessment. A native speaker in collaboration with the author reviewed articles in languages other than English, French, German or Dutch for inclusion (see acknowledgments).

2.2.5 Data extraction

The author and two other investigators extracted data for year of study, dates of sample collection, location, host and vector species collected, numbers tested and numbers positive, whether samples were pooled or tested individually, sample type, vector infestation rate and index, vector collection method, habitat description, rainfall during study, minimum, maximum and mean temperature during study and laboratory test used. For Chinese, Japanese, Russian, Korean and Thai languages a native speaker extracted data using the same template, crosschecking with the author for consistency. Data was entered into a pre-designed Microsoft Access database.

2.2.6 Planned analysis

2.2.6.1 Descriptive summaries

The distribution and accuracy of each study site location was recorded and all laboratory tests used to identify *O. tsutsugamushi* were noted and classified into groups (Appendix A-2, Table 1). The distribution of key vector species is described together with all reported vector species. Key themes in the ecology of scrub typhus are reviewed in detail. The risk of bias was high due to many missing data, particularly denominator values for number of tested vectors and hosts.

2.2.6.2 Statistics

The primary outcome of the systematic review was the median (range) positivity of *O. tsutsugamushi* in diverse mites and vertebrates. Analysis was performed using Stata v15 (StataCorp, College Station, Tx) and the R statistical software (R Core Development Team, 2018).

2.2.7 Geocoding

All study site locations were geocoded with the aim of creating a single location for each study site. Where exact coordinates were provided, these were used. For all other locations, the “Geocode csv with Google/Open street map”, MMQGIS plugin for QGIS was used to geocode sites (QGIS Development Team (2018), QGIS Geographic Information System. Open Source Geospatial Foundation Project (http://qgis.osgeo.org). Any available combination of address, city, state, province and country was entered. Where geocoding failed, several solutions were explored. Firstly, the site was searched for on the internet using numerous resources and then geocoded manually using Google Maps. Secondly, spelling variation of place names (e.g. for Korean sites) was frequently inconsistent with Google Maps, and variations were tried with input from a native speaker where possible. Finally, if no location could be found, then the next administrative level up was selected by using the geocoding method above.

A number of additional situations arose. 1) Where samples were collected from multiple locations and pooled such that it could not be determined from where the samples originated, the mean latitude and longitude of these sites was used to generate a single point. 2) Where samples were collected from multiple contiguous administrative areas, these areas were combined in QGIS using the ‘Vector - Geoprocessing – Dissolve’ function and a polygon centroid generated to create a single point for the study site. 3) Where samples originated from multiple non-contiguous sites, these areas were selected using QGIS and converted from singlepart to multipart and then a centroid created to give a single point. In the latter case the point could be outside the actual administrative zones where the study took place.

2.2.7.1 Geocoding accuracy measure

All study sites were classified into 1 of 6 administrative divisions. Administrative levels 1 to 4 were based on those listed in the International Organization for Standardization codes ISO 3166-1 and ISO 3166-2 [68]. Where further detail for a particular country was required, this information was obtained through the country's Wikipedia page for administrative divisions. Some extinct historical administrative divisions were encountered, and here the closest match or next administrative level up was selected. Two additional administrative divisions were included: Level 0 for the country alone and level 0.5 for a well defined region of a country e.g. Peninsular Malaysia, Kyushu or Kanto regions of Japan.

2.3 Results

2.3.1 Systematic review of scrub typhus in vectors and non-human hosts

2.3.1.1 Study characteristics

A total of 276 articles were included in the systematic review, with a further 145 reviewed for information on a general discussion of scrub typhus ecology. Only 6 articles were excluded, as the full text could not be obtained (Figure 8). Systematic review articles were published between 1924 and 2018, and other articles reviewed dated back to 1878. Systematic review papers included 198 in

English, 39 in Standard Chinese (Mandarin), 19 in Japanese, 9 in Russian, 8 in Korean, 2 in Dutch and 1 in Thai (Figure 9).

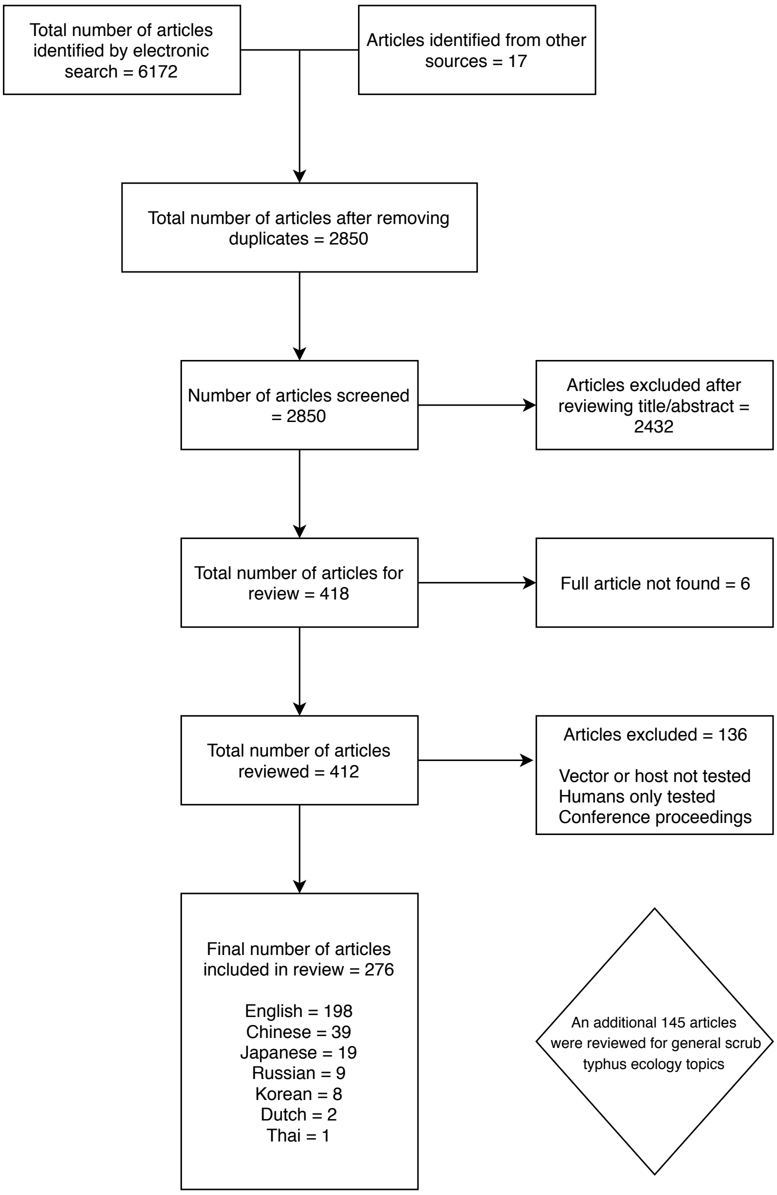


Figure 8 Study selection strategy flowchart

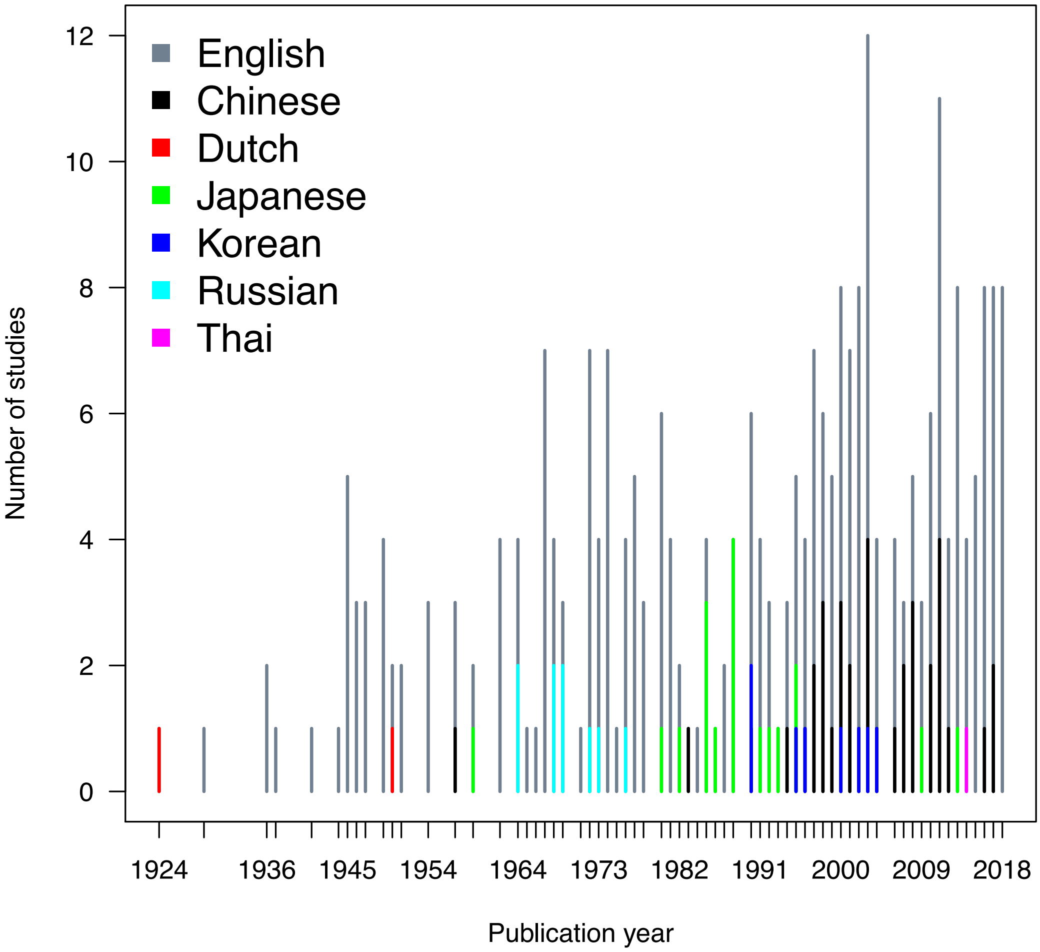


Figure 9 Number of included studies published in different languages over time

The number of published articles has gradually increased over time. Some of the earliest work was published in Dutch, reflecting the Dutch presence in the East Indies (now Indonesia). From the mid-1960s to the mid-1970s there were a number of papers in Russian. There has been little published investigation in Russia since then. Articles in Japanese were more frequent in the 1980s, followed by Korean in the 1990s to 2000s. Articles from these 2 countries are now mostly published in English. A surge in Mandarin Chinese articles is seen from the mid-90s.

2.3.1.2 Geography

Historically scrub typhus was thought to be present across a large swathe of South and East Asia, known as the “tsutsugamushi triangle”. In vectors and non-human hosts, the pathogen has been identified from as far north as the Russian Kuril Islands, north of Japan and Inner Mongolia (~49°N) [69,70]. The most easterly record comes from the Eastern Solomon Islands (~167°E) [71]. To the south there is evidence from North Queensland, Australia (~21°S) and in the west from eastern Iran (~59°E) [72,73]. In 1946 Baker published a study suggesting a rickettsial species consistent with scrub typhus that was detected in Canadian voles trapped on Grosse Isle in the St. Lawrence River near Quebec City [74]. More recently 2 studies using 16s rRNA sequencing of blood samples from rodents in the Ardennes, France and in Senegal [75] and from dogs near Kruger National Park, South Africa [76], identified organisms with close sequence homology to *O. tsutsugamushi*. In 2006, serological evidence of human scrub typhus was reported from Chiloe Island in Chile [77]. In 2016, molecular testing confirmed further cases [38] and then in 2018, serological evidence of *O. tsutsugamushi* was demonstrated in dogs on Chiloe Island (~42°S and 73°W) [78]. In the same year, an organism with close homology to *O. chuto* was detected in pooled *Microtrombicula* and *Neotrombicula* species chiggers in Baringo County, Kenya [79]*.* This follows the identification of a human case of *O. chuto* infection in the United Arab Emirates in 2010 [37]*.* Other human serological evidence from Africa and the Middle East is discussed in Chapter 1. *O. tsutsugamushi* has been identified from as high as 3,200 m above sea level in the Kaghan valley of West Pakistan [80].

The disease, in *sensu lato*, has an expanding known geographical distribution and much remains to be understood of its distribution across tropical and subtropical regions, its presence in vectors and hosts and role in causing human disease.

2.3.1.2.1 Studies sites per country

Articles were included in this review spanning publication across 94 years. Studies for which laboratory tests were performed on vectors and non-human hosts to identify *O. tsutsugamushi* took place at 793 sites in 30 countries (Appendix A-2, Table 6). South Korea and Japan had by far the most study sites recorded at 183 and 144, respectively (accounting for 42.1% of all study sites). Thailand had 87 sites, China 66, Taiwan 63, Russia 53, India 44 and Malaysia 43. Thirteen countries had 3 or less study sites (Figure 10).

It is worth noting that individual studies varied enormously in the number of collection sites, with some having just 1 and others 30 or more. Additionally, more study sites than those reported here exist in practice, but where data could not be separated by site some were amalgamated following the strategy described above.

Apart from an early inconclusive investigation in Canadian voles by Baker in 1946 [74], it is only since 2015 that investigations into *Orientia* infection in vectors and hosts have taken place outside the Asia-Pacific region.

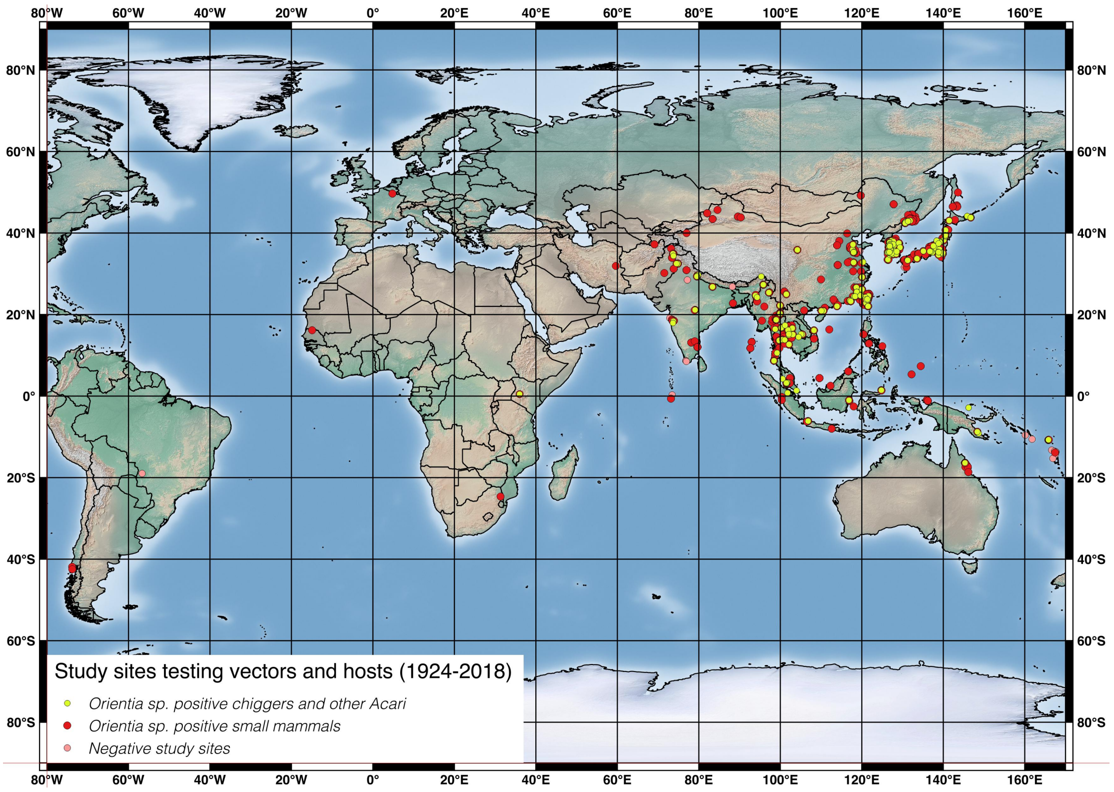


Figure 10 Location of study sites investigating *O. tsutsugamushi* in vectors and hosts. [One study identifying *O. tsutsugamushi*-like organisms in small mammals in Quebec Province, Canada in 1946 is omitted here [74]].

2.3.1.2.2 Negative study sites

Of all 793 study sites, 53 (6.7%) reported no positive vectors or hosts. These were located in 12 countries across the Asia Pacific region and Brazil (Figure 10). Twenty-one of these sites were from a single study of ports and harbours in Taiwan [81].

2.3.1.2.3 Geocoding accuracy

Only 12/793 sites (1.5%) were geocoded to administrative level 0 (corresponding to an unknown point in the country) and a further 12 sites to administrative level 0.5. 100 sites were geocoded at level 1, 124 sites at level 2, 209 sites at level 3 and 336 (42%) were geocoded most accurately at level 4 (either an exact site was provided or the village or equivalent given). On the other hand, 456 sites (58%) were geocoded at administrative level 3 or less, indicating that the majority of reported sites were no more accurate than the district or equivalent level (Appendix A-2, Table 6).

2.3.1.3 Laboratory tests and sample types

Given the 94-year period from which included studies were drawn and the many countries in which studies were performed, it is unsurprising that a large range of laboratory tests and combinations of tests were used. More than 40 tests and combinations of tests were recorded (Appendix A-2, Table 1). These include some broad categories (serology, antigen tests and molecular tests) for which further details were not provided. Four studies did not clearly state the laboratory test used. Two of these were review articles that contained data not published elsewhere [82,83], 1 was a short report [84] and the fourth paper was on studies of transovarial transmission in chiggers collected from the wild [85].

To aid analysis, these tests were grouped into 8 categories, including 1 category "unknown" (Appendix A-2, Table 1). Serological tests, (which include direct and indirect immunofluorescence (DIF, IIF), indirect immunoperoxidase (IIP), complement fixation (CF), enzyme-linked immunosorbent assays (ELISA) and the Weil-Felix (OXK) test), were performed in 121/275 (44%) articles. Next most commonly used was culture (with or without microscopic confirmation) with 72/276 (26%) articles. All but one of these used the xenodiagnosis method of animal inoculation and passage. Only 3 such studies were reported since the start of the 21st century. Molecular methods were used in 63 articles from 14 countries. The first of these was published in 1995. A range of *O. tsutsugamushi* PCR targets were used including 47 and 56 kDa, GroEL, OmpB, in-house targets and nested PCRs. Two of the studies used 16s rRNA sequencing [75,76]. Microscopy alone ("organ impression smears") was performed in 3 studies, 2 prior to 1950 and 1 from India in 2012 [86-88].

Combinations of tests were used in 71/276 (26%) studies. Fifty-three studies used a combination of culture (xenodiagnosis) and one of several serological tests. Less frequently, 11 studies (all published since 2000) reported combinations of culture and molecular diagnosis. Four of these studies used L929 cell culture [89-92]. Seven studies used a combination of molecular (PCR) and serological (ELISA or IIF) tests to report *O. tsutsugamushi* testing of vectors and hosts. Figure 11 shows the use of test groups over time.

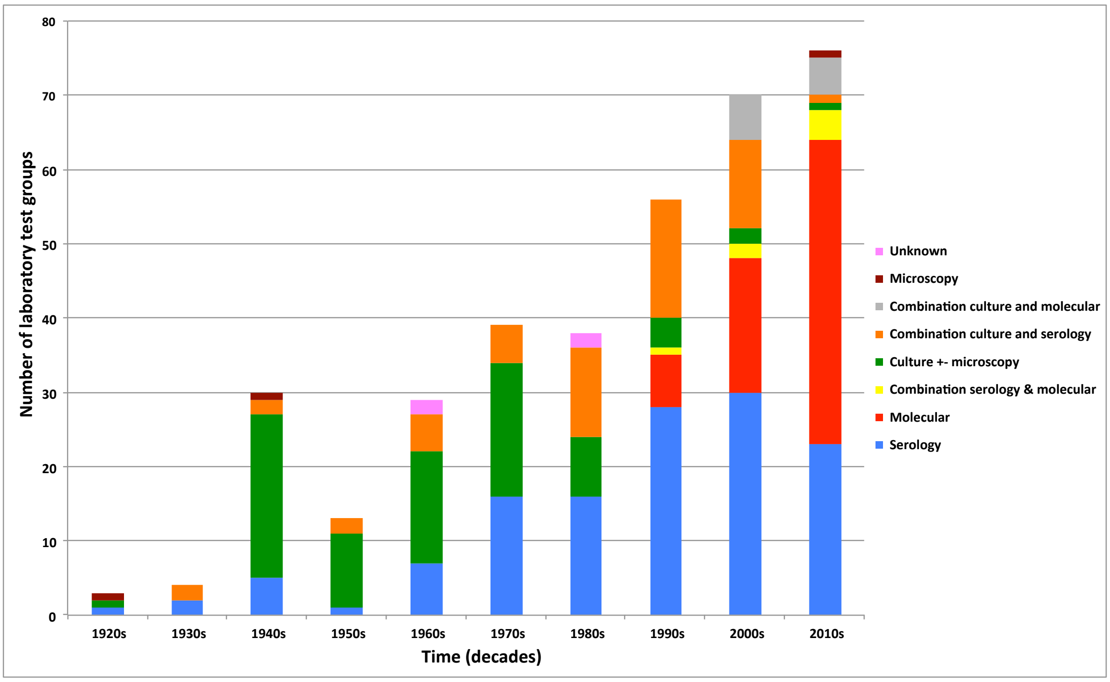


Figure 11 Use of different categories of laboratory test over time

Different sample types were used from host animals. Reflecting the frequency of serological studies, 99/276 (36%) articles used serum specimens. Whole blood was analysed in 23/276 (8%) studies. Single organ types were tested in 69/276 (25%) studies, with spleen predominating in 62 studies, kidney in 2 and brain in 5 (from the 1940s). A high proportion of studies, 91/276 (33%) used a combination of tissues (spleen, liver, kidney, lung, brain or whole blood). In only 3 studies was the specimen not recorded, 2 of these were review articles [82,83] and the other Audy's WW2 report [87].

2.3.1.4 Vectors

2.3.1.4.1 *Orientia tsutsugamushi* testing of individual and pooled vectors collected from hosts

A total of at least 74 "vector" species were tested for *O. tsutsugamushi* using a laboratory test. Sixty of these were trombiculid mites and the rest were other members of the Acari: Ixodida, Laelapidae or Macronyssidae. Of the Trombiculidae, 46 species tested positive for *O. tsutsugamushi* at least once (Appendix A-2, Table 2). Vectors were tested either individually or as pools of individuals. Pool size varied enormously, from less than 10 to over 1000 chiggers. A total of over 123,000 individuals and 8,000 pools (accounting for over 1 million chiggers) were tested.

In some studies, the species were listed but details on the numbers tested (denominator) of each were not specified. In other studies the overall number of different species tested were listed, but the data was not divided between two or more collection sites. In these cases, the species was reported as "not identified' in favour of the location which was deemed a more useful data record. For pools of vectors, many were of mixed species or unidentified, and in many studies vectors were identified only to genus rather than species.

Table 3 Summary of number of tested "vectors" and number of positives of all species combined. Data is divided into individual vectors or pooled (multiple) individuals and subdivided by laboratory test category.

	Total individuals tested	Total individuals positive (%)	Total pools tested	Total pools positive (%)
Culture +- microscopy	1,286	35 (2.7%)	2,919	688 (24%)
Combination culture and serology	37,603	244 (0.6%)*	1,928	389 (20%)
Combination culture and molecular	-	-	229	22 (9.6%)
Combination serological and molecular	8,019	90 (1.1%)	347	195 (56%)
Serological	45,439	2,260 (5%)	1,170	123 (11%)
Molecular	30,767	588 (1.9%)	1,343	411 (31%)
Unknown	?	9	80	24 (30%)

* 1 study reported 75% *O. tsutsugamushi* infection rate in pools made up of 20,700 *L. deliense*, but the number of pools tested and number positive were not reported [93]
Percentages shown in parentheses were pooled by giving equal weight to all studies.
? = unknown

Percentage infection rates among individual trombiculid mites range from 0.6% to 5% depending on the laboratory tests employed. The highest rates of infection were observed using immunofluorescence techniques that are sensitive but can suffer from false positives (see Chapter 1). The same problems are seen with culture with or without microscopic confirmation. Molecular methods gave an overall infection rate of 1.9%. Pools of vectors gave expectedly higher positivity rates ranging from 9.6 to 56%. The highest rate was seen for combined molecular and serological methods, although only a small number of pools were tested in this manner. Molecular techniques gave a positivity rate of 31% and surprisingly using serological tests only, 11% were reported positive.

2.3.1.4.2 *Orientia tsutsugamushi* testing of key vector species by laboratory test category

A summary of *O. tsutsugamushi* testing of vectors using different categories of laboratory test are shown in Table 4 for the three most frequently reported species of chigger. The median positive per site varies significantly dependent on the number of studies and size of studies.

Table 4 Summary of total and median tested and *O. tsutsugamushi* positive for the 3 most frequently reported *Leptotrombidium* chigger species, subdivided into laboratory test categories

Lab method	Vector species name	Total Tested	Median tested	Min	Max	Total positive	Median positive/ study site	Median positive (%)
Combination culture & molecular								
	L. deliense	0*	0	0	0	0	0	0
Pools	*L. scutellare*	10[§]	10	10	10	8	4	40%
	L. pallidum	?[§]	?	?	?	3	3	-
Combination culture & serology								
	L. deliense	?[§]	?	?	?	3	3	-
Pools	*L. deliense*	?[§]	?	?	?	2	2	-
	L. scutellare	10	10	10	10	4	4	40%
Pools	*L. scutellare*	15	4	1	10	5	2.5	63%
	L. pallidum	1811[§]	66	23	734	75	12	18%
Pools	*L. pallidum*	115[§]	15	1	52	68	5	33%
Combination culture +- microscopy								
	L. deliense	7[§]	4	1	6	1	1	25%
Pools	*L. deliense*	398[§]	7	1	131	193	3	43%
Pools	*L. scutellare*	183[§]	24	11	148	5	1	4.2%
	L. pallidum	17[§]	8.5	1	16	8	4	47%
Pools	*L. pallidum*	36[§]	3	1	30	13	1	33%
Serology								
	L. deliense	1874	314	285	1275	51	4	13%
Pools	*L. deliense*	665	333	5	660	18	9	27%
	L. scutellare	1242	131	1	1110	6	3	2.3%
	L. pallidum	1900	42	12	1263	202	10	24%
Pools	*L. pallidum*	?[§]	?	7	?	73	4	-

Molecular								
	L. deliense	44	22	15	29	3	1.5	6.8%
Pools	*L. deliense*	515	6	1	315	124	3	50%
	L. scutellare	3053	54	11	1907	57	5	9.3%
Pools	*L. scutellare*	127[§]	4	1	105	35	1	25%
	L. pallidum	1357	38	1	474	57	7	18%
Pools	*L. pallidum*	4	2	1	3	0	0	0%
Combined molecular & serology								
Pools	*L. deliense*	42	42	42	42	25	25	60%
	L. scutellare	2050	119	6	579	22	2	1.7%
	L. pallidum	2735	80	1	1420	31	3	3.8%

* 1 study reported 75% *O. tsutsugamushi* infection rate in pools made up of 20,700 *L. deliense*, but the number of pools tested and number positive were not reported [93]

[§] Includes studies where number of individuals/pools tested not given (i.e. no denominator)

? = unknown

2.3.1.4.3 *Orientia tsutsugamushi* testing of free-living vectors

A separate analysis of free-living trombiculid mites (larvae (chiggers), nymphs and adults) was carried out. In view of our current understanding of the life cycle of trombiculid mites, *O. tsutsugamushi*-infected free-living larvae should be considered potential vectors, though not necessarily to humans. In total 40,995 individual and 266 pools of trombiculid mites were tested. Infection rates for individuals were: 413/18,945 (2.2%) with culture alone, 380/15,852 (2.4%) with serological tests, 304/6,125 (5%) using combined culture and serology and 1/27 (3.7%) with molecular tests.

Table 5 Summary of free-living trombiculid mites (larvae, nymphs and adults) tested by different laboratory categories. All species testing positive for *O. tsutsugamushi* at least once are shown with total and median numbers tested and testing positive.

Lab method	Vector species name[i]	Total tested	Median tested	Min	Max	Total positive	Median positive /study site	Median positive (%)
Combination culture & serology	*L. intermedium*	3237	3237	3237	3237	3	3	0.1%
	L. pallidum	1879	940	53	1826	288	144	15.3%
	L. deliense	570	570	570	570	10	10	1.8%
	L. palpale	177	177	177	177	0	0	0.0%
	L. scutellare	113	56	19	94	2	1	1.8%
	L. vivericola	80	80	80	80	1	1	1.3%
Pools	*L. intermedium*	2	2	2	2	1	1	50.0%
Combination culture +- microscopy	*L. deliense*	7060	3530	1180	5880	413	413	11.7%
	G. cassiope	6120	6120	6120	6120	0	0	0.0%
	A. indica	4930	4930	4930	4930	0	0	0.0%
	L. scutellare	65	65	65	65	0	0	0.0%
Pools	*L. deliense*	2	2	2	2	1	1	50.0%
Pools	*L. pavlovskyi*	?	?	?	?	1	1	-
Serology	*L. scutellare*	8444	591	235	2443	41	1	0.2%
	L. deliense	3030	289	41	949	132	16	5.5%
	L. intermedium	1208	1208	1208	1208	2	2	0.2%
	L. pallidum	743	743	743	743	134	134	18.0%
	L. keukenshrijveri	646	646	646	646	15	15	2.3%
	L. fletcheri	404	202	13	391	14	7	3.5%
	L. vivericola	358	358	358	358	15	15	4.2%
	L. arvinum	181	90	58	123	9	4.5	5.0%
	Leptotromb. sp	127	127	127	127	9	9	7.1%
	Odontacarus sp	81	81	81	81	3	3	3.7%
	L. bodense	77	21	9	47	2	2	9.5%
	L. peniculatum	67	67	67	67	1	1	1.5%
	E. wichmanni	23	23	23	23	2	2	8.7%
	M. chamlongi	5	5	5	5	1	1	20.0%
Molecular	*L. scutellare*	27	27	27	27	1	1	3.7%
Pools	*L. scutellare*	242	121	8	234	7	4	3.3%
Pools	*L. fuji*	14	14	14	14	1	1	7%
Pools	*N. japonica*	1	1	1	1	1	1	100.0%

L = Leptotrombidium, G = Guntheria, A = Ascoschoengastia, E = Eutrombicula, M = Microtrombicula, N = Neotrombicula

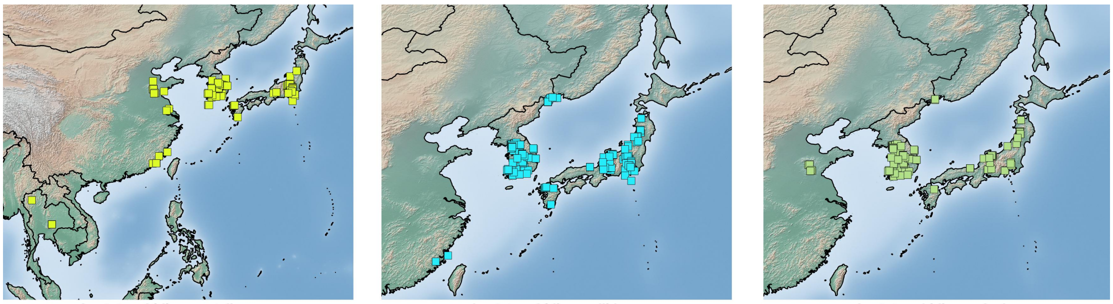
[i] *Leptotombidium akamushi*, *L. pavlovskyi* & *Helenicula miyagawai* were also reported as testing positive, but without a denominator.

Thirty-one species of trombiculid mite were tested for *O. tsutsugamushi* and at least 23 species were positive (Table 5). All species were of the genus

Leptotrombidium apart from *Eutrombicula wichmanni*, *Odontacarus* sp. and *Microtrombicula chamlongi* all of which were reported positive from a single study in Thailand using immunofluorescence [94], *Neotrombicula japonica* from 1 study in Japan [95] and *Helenicula miyagawai* from Mt. Gwanak, outside Seoul, South Korea [96]. Of *Leptotrombidium* species tested in the greatest numbers: *L. pallidum*, *L. deliense*, *L. scutellare and L. fletcheri* were the most frequently positive.

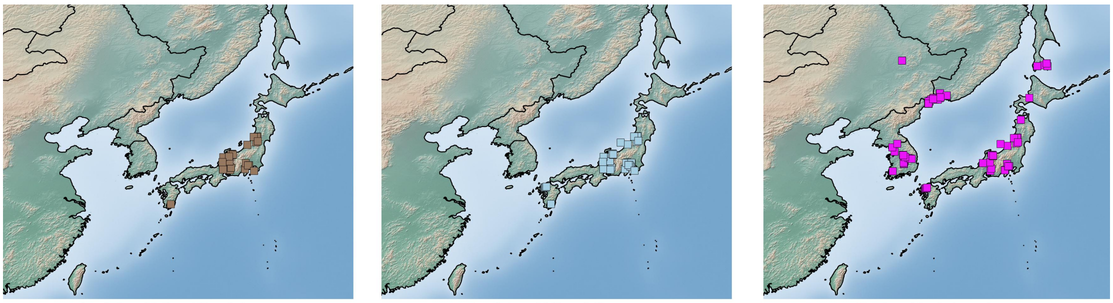
2.3.1.4.4 Distribution of key vector species

There is very little published information summarising the distribution of chigger species considered important human vectors of scrub typhus. Kim et al. recently reported the distribution of 9 representative *Leptotrombidium* species [97]. The locations of the 16 most frequently positive trombiculid species from articles included in this review are shown in Figure 12.

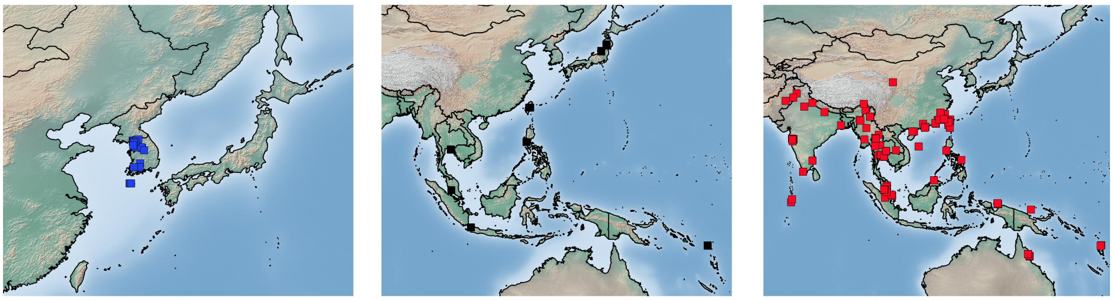


Leptotrombidium scutellare *Leptotrombidium pallidum* *Leptotrombidium palpale*

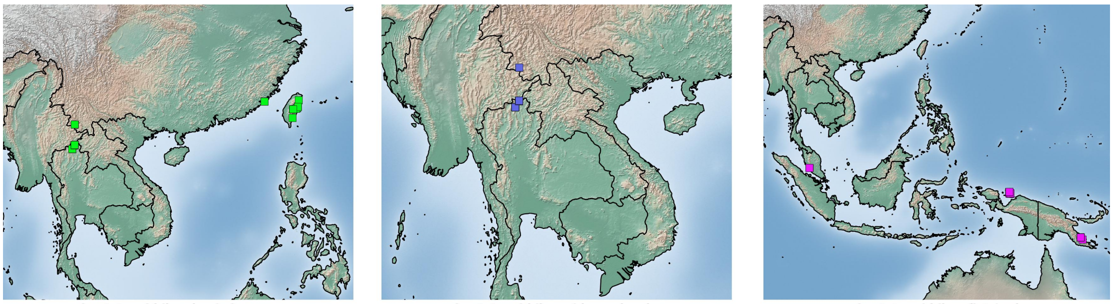
Leptotrombidium orientale *Leptotrombidium pavolvskyi* *Leptotrombidium intermedium*



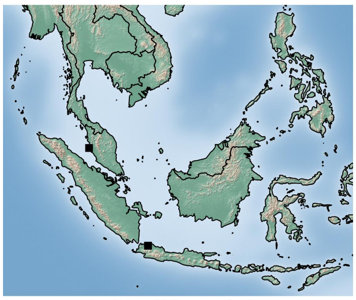
Leptotrombidium kitasatoi *Leptotrombidium fuji* *Neotrombicula japonica*



Euschoengastia koreaensis *Leptotrombidium akamushi* *Leptotrombidium deliense*



Leptotrombidium imphalum *Leptotrombidium chiangraiensis* *Leptotrombidium fletcheri*



Leptotrombidium arenicola

Figure 12 Distribution maps of the 16 most frequently reported *O. tsutsugamushi*-positive chigger species from all studies included in this review.

2.3.1.4.5 Other positive trombiculid species

Orientia sp. positive tests have been reported from a further 17 genera of trombiculid mites, made up of at least 32 species (Figure 13). Figure 13 includes chiggers reported to genus only (including *Leptotrombidium*) and unidentified *O. tsutsugamushi* positive chiggers to provide a complete map of positives. It is likely that some of these will be among the 16 species shown above. These are distributed across the Asia-Pacific region, with the exception of the recent report of an organism close to *O. chuto* in either *Microtrombicula* or *Eutrombicula* species of chiggers in Kenya [79]. The robustness of these data are variable, with many different laboratory tests, of variable specificity, used to identify the presence of *Orientia* in vectors. Furthermore, for a number of species, trombiculid mites were pooled and the possibility of mixed-species pools remains. A full analysis of *O. tsutsugamushi*-positive species is given in Appendix A-3.

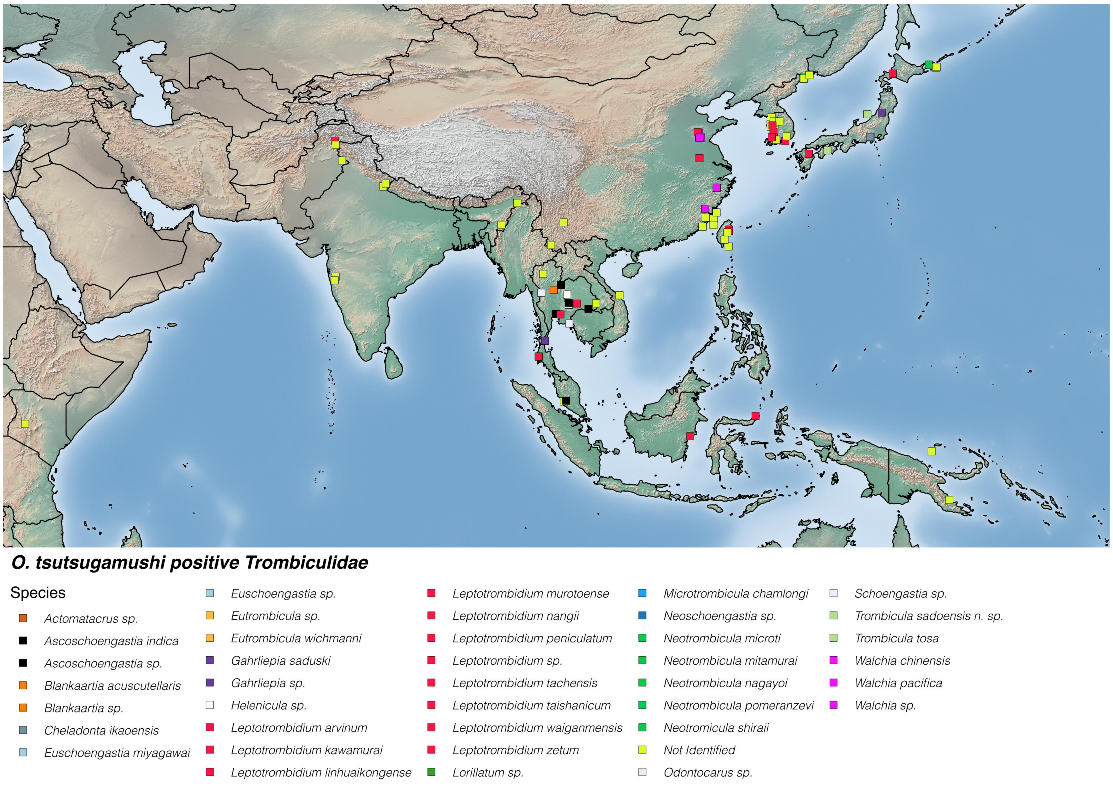


Figure 13 Location of all other trombiculid mite species not listed in Figure 12 testing positive for *O. tsutsugamushi* including those identified to genus level only and unidentified chiggers.

2.3.1.5 Hosts

2.3.1.5.1 *Orientia tsutsugamushi* testing of major host animal groups

A total of 234 species of “host” vertebrates (excluding humans) were tested for *O. tsutsugamushi*, with 122 species testing positive (Appendix A-2, Table 3). In Table 6 all different forms of laboratory tests are combined. A large number of hosts were reported here as either: ‘species not identified’ or ‘multiple species listed’ (as for vectors above).

Table 6 Summary of number of tested hosts and positives of all species, subdivided by laboratory test category

Laboratory test category	Total individuals tested	Total positive	Percent positive
Culture +- microscopy	16,486	2,943	18%
Combination culture and serology	14,195	2,761	19%
Combination culture and molecular	389	91	23%
Microscopy alone	250	6	2%
Combination serological and molecular	3,443	1,387	40%
Serological	36,089	10,874	30%
Molecular	12,198	1170	10%
Unknown	169	10	6%

Serological tests were performed most frequently, with 36,089/83,219 (43%) hosts being tested using these methods. These were also most frequently *O. tsutsugamushi* positive at 10,868/35,960 (30%). Culture with or without microscopy and culture with serological confirmation were next most frequent with 16,486/83,219 (20%) and 14,195 (17%) tests performed and similar rates of positivity at 2,943/16,486 (18%) and 2,761/14,195 (19%), respectively. Although rarely performed, microscopy alone expectedly had the lowest rates of positivity at 6/250 (2%). Molecular methods were used to test 12,198 (15%) of hosts with 1,170/12,198 (10%) positive (Table 6).

To assist with summarising the results, the 234 species tested were classified into 21 groups. The testing of non-human hosts for *O. tsutsugamushi* has been performed primarily on small mammals, long considered the major hosts for vector trombiculid mites. The Muridae (rats and mice) included the major part of identified species tested at 52,670/62,726 (84%). The Cricetidae (voles, hamsters etc.), Soricidae (shrews) and Sciuridae (squirrels) constituted just 4%, 2% and 2.7% respectively. Birds (Aves) constituted 0.5%.

Table 7 Summary of percentage of hosts testing *O. tsutsugamushi* positive, subdivided into taxonomic groups

Group	Major species tested	Total individuals tested	Total positive (all test types)	Percent positive
Artiodactyla[i] (even-toed ungulates)	Cow, sheep, goat, pig	1568	54	3.4%
Aves (birds)	Chicken, *Passer domesticus, Motacilla cinerea*	293	16	5.5%
Canidae[ii] (canid)	Dog*, Cerdocyon thous*	1826	325	17.8%
Chiroptera[iii] (bats)	*Rhinolophus ferrumequinum, Eptisicus serotinus*	797	99	12.4%
Cricetidae (voles, hamsters)	*Cricetulus triton, Microtus fortis, Myodes glareolus*	2516	308	12.2%
Echimyidae[iv] (spiny rats)	*Thrichomys fosteri*	85	0	0
Erinaceidae (hedgehogs)	*Echinosorex gymnura*	8	0	0
Felidae[iv] (cats)	*Leopardus pardialis*	7	0	0
Herpestidae (mongooses)	*Herpestes javanicus*	1	0	0
Lagomorpha (pika, rabbits)	*Ochotona roylei*	6	0	0
Marsupialia (marsupials)	*Isoodon macrourus, Thylamys macrurus*	285	37	14.5%
Muridae (rats, mice)	*Apodemus agrarius, Rattus rattus, Rattus norvegicus, Rattus tiomanicus, Bandicota indica*	52,670	13,419	25.5%
Mustelidae (badgers, weasels)	*Melogale personata*	7	1	14.2%
Reptilia	Lizards*, Physignathus*	61	0	0

(reptiles)	*lesuerii*			
Sciuridae (squirrels)	*Callosciurus notatus,* *Tamias sibiricus*	1692	105	6.2%
Simiformes (monkeys)	*Macaca fascicularis*	27	12	44%
Soricidae (shrews)	*Suncus murinus,* *Crocidura lasiura*	1247	165	13.2%
Talpidae[v] **(moles)**	*Urotrichus talpoides*	13	3	23%
Tupaiidae (tree shrews)	*Tupaia glis*	333	49	14.7%
Viverridae[vi] **(civets)**	*Paradoxurus hermaphrodites*	6	0	0
Multiple or unidentified species	-	20,056	4649	23.2%

[i] Only performed in China, Taiwan & Russia
[ii] Only dogs positive
[iii] Single study from South Korea using serology
[iv] Only tested in Brazil
[v] Only tested in Japan
[vi] Only tested in Vietnam

Of the major vertebrate groups tested, combining all test types, the Muridae had the highest proportion of positive tests at 25.5%. Of the other major groups of small mammal, the Cricetidae and Soricidae had similar rates of positivity at 12.2 and 13.2%, respectively. Six per cent of the mainly arboreal Sciuridae were positive. The Canidae tested 19% positive, with the majority, 307/319 (96%) tested using serological methods. Among the Artiodactyla, cows, goats and pigs were tested by serological methods only, testing 3.6% positive overall. The group listed as “multiple or unidentified species” had a similar positivity rate to the Muridae, most likely because the species composition was similar to that presented overall, with most being Muridae (Table 7).

2.3.1.5.2 *Orientia tsutsugamushi* testing of key host species by laboratory test category

Table 8 presents data for the 5 most frequently tested species subdivided by laboratory test category. The major species tested and positivity rates are broadly consistent with the overall data presented above. Muridae account for most of the species listed. A large number of dogs were tested serologically in several studies with a median positive rate per site of 5.5%. Two Chinese studies from Bole region of Xinjiang, China account for the surprisingly large number of sheep testing positive by PCR [98,99]. Whole blood was collected and tested by 56 kDa PCR.

Table 8 Total and median tested and testing *O. tsutsugamushi* positive for the 5 most frequently tested host species, subdivided by laboratory test category

Lab method	Host species name	Total Tested	Median tested	Min	Max	Total positive	Median positive/study site	Median positive (%)	Minium (%)	Max (%)
Combination culture & molecular	*Rattus norvegicus*	224	112	12	212	38	19	20.5	16	25
	Apodemus agrarius	30	15	3	27	13	4	30	0	33
	Bandicota indica	15	15	15	15	8	2	0	0	13
	Rattus rattus	15	15	15	15	1	0.5	0	0	0
	Cricetulus triton	13	13	13	13	5	5	38	38	38
Combination culture & serology	*Rattus rattus*	5626	284.5	12	1643	1316	21	16	0	100
	Bandicota indica	1561	75	1	885	127	17	12	0	29
	Apodemus speciosus	1435	9	1	263	438	2	20	0	100
	Rattus losea	1335	163	49	411	213	29.5	22	0	30
	Rattus tiomanicus	1171	585.5	234	937	54	27	10.5	0	21
Culture +- microscopy	*Rattus tiomanicus*	1503	115.5	1	303	355	24.5	24	0	30
	Rattus sabanus	1084	72	2	362	251	19.5	18.5	0	60
	Rattus argentiventer	497	23.5	0	150	182	7	34	0	100
	Rattus exulans	377	6	0	152	56	1	0	0	50
	Apodemus agrarius	296	8.5	3	109	38	2	13	0	86

Serological	*Apodemus agrarius*	8991	19	1	1094	3250	4.5	22.5	0	88
	Rattus tiomanicus	1413	2	1	327	573	1	26	0	100
	Dog	1523	20	6	238	307	5.5	24.5	0	84
	Rattus sabanus	1375	14.5	2	748	644	4	30.5	0	63
	Rattus flavipectus	1081	21.5	7	769	870	6	44	4	100
Molecular	*Rattus norvegicus*	2414	25	1	1261	113	1	2	0	57
	Apodemus agrarius	1410	16	2	370	304	3	27	0	88
	"Rodent"	927	230	201	266	60	12.5	5	4	12
	Mus musculus	557	20	1	183	25	1	5	0	100
	Sheep	510	255	210	300	16	8	3.5	3	4

2.3.1.5.3 *Orientia tsutsugamushi* testing of other host species

Mus species have long been considered as unimportant host species for *O. tsutsugamushi*-carrying chigger species [14]. However in culture-based studies a median of 16% positive was seen for wild *M. caroli* and (13.5/34.5) 40% for *M. musculus*.

Many species of Cricetidae have been tested. Using combination culture and serology, a median of 28% (7.5/27) *Microtus montebelli* and 5% (0.5/10) *M. fortis* were positive. By serology, 17% (1/6) of *Cricetulus triton* and using molecular techniques 3% (1/33) of *Cricetulus migratorius* tested positive.

Suncus murinus was the most frequently tested of the Soricidae and a median of 6% (1/15.5) was positive among all studies. Of the Sciuridae, *Callosciurus notatus* was positive with a median of 2% (5/243.5) serologically and 13% (1/8) using combined culture and serology. Chiroptera were tested only by serological methods and 12% (38/308) of *Eptesicus serotinus* and 11% (7/66) *Rhinolophus ferrumequinum* were positive.

For birds, using PCR, 17% (2/12) of *Motacilla cinerea* (grey wagtail) and 10% (1.5/15) of *Passer domesticus* (house sparrow) were positive in China (see below).

2.3.1.6 Ecological relationships of vectors and hosts

Bipartite network analysis is becoming increasingly used to understand host-parasite interactions [100]. These networks help reveal the importance of certain species in the transmission ecology of a disease [101]. Bipartite network figures depicting the relationship between host animal species and vectors were constructed for selected country groups. A quantitative depiction was made for all host/vector species interactions (shown by line thickness). A sub-analysis is also shown for interactions where either a vector or host tested positive for *Orientia*. Dr Prabin Dahal created Figures 14 to 19.

Networks for Southeast Asia clearly show *L. deliense* and *Ascoschoengastia indica* as key chigger species whether testing *O. tsutsugamushi* positive or not (Figures 14 & 15). Among those testing positive, the pattern of host species was different with little overlap. In the China/Taiwan analysis, *L. deliense* is again the key species and associated with a wide host range (Figures 16 &17). Networks for Japan, Korea and far-eastern Russia reveal a different pattern of host-vector interactions. Overall *L. pallidum* and *G. saduski* interacting with *Apodemus* spp. and *Microtus* spp. hosts were by far the most frequent (Figures 18 & 19). However, among *O. tsutsugamushi*-positive species, *L. pallidum*, *L. orientale* and *Neotrombicula japonica* appear to be key species.

2.3.1.6.1.1 Southeast Asia

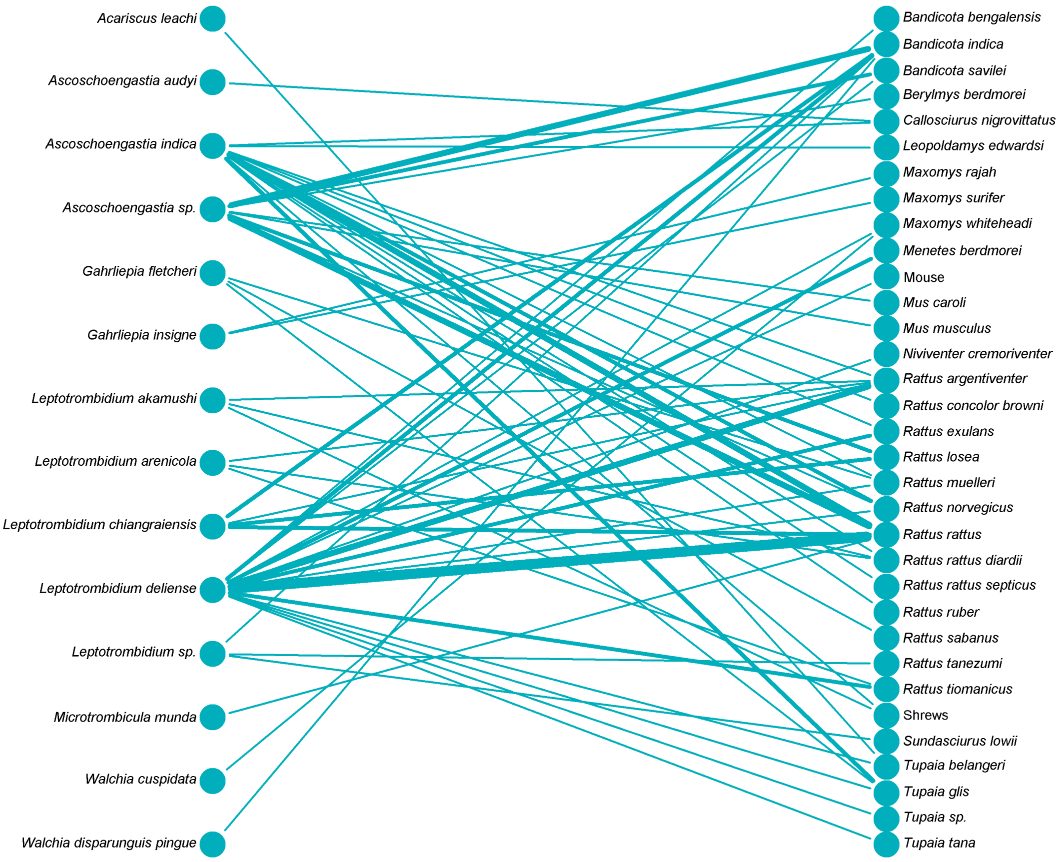


Figure 14 Network analysis of small mammal and chigger species for studies from Southeast Asian countries (Thailand, Vietnam, Malaysia, Indonesia and Myanmar)

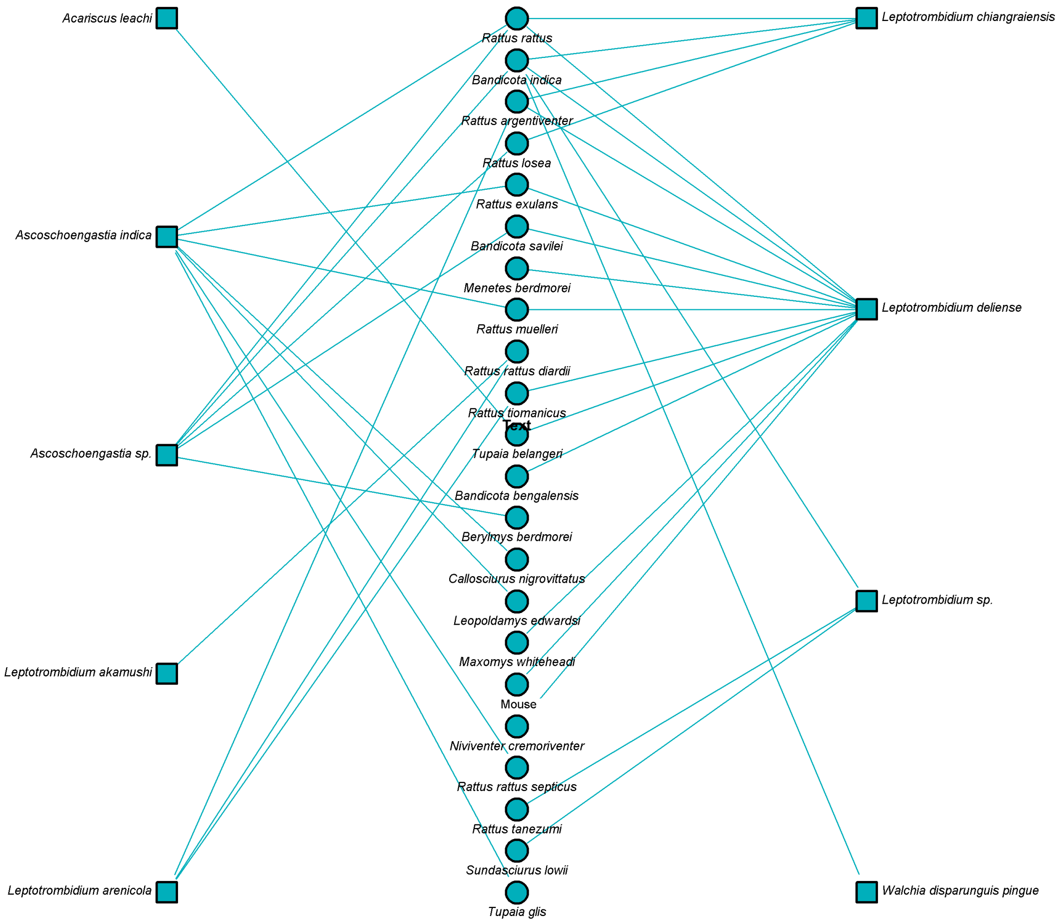


Figure 15 Network analysis of small mammal and chigger species testing positive for *O. tsutsugamushi* by any laboratory test for studies from Southeast Asian countries.

2.3.1.6.1.2 China and Taiwan

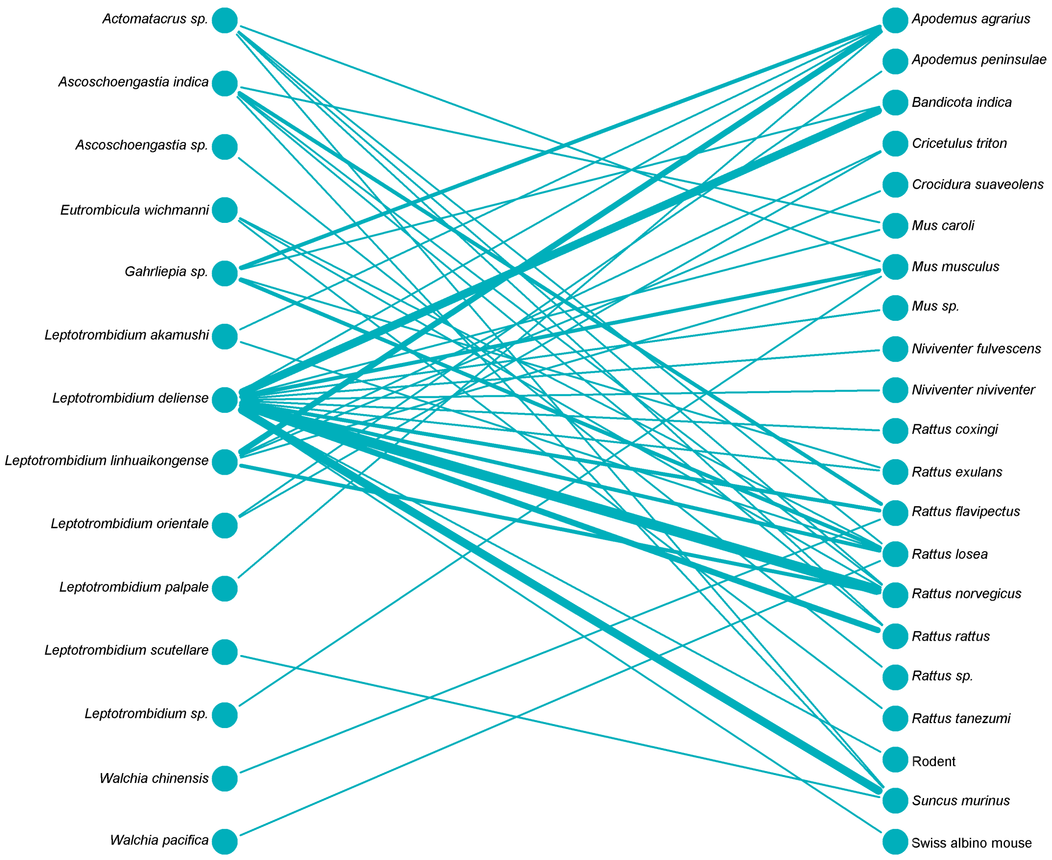


Figure 16 Network analysis of small mammal and chigger species for studies from China & Taiwan. Swiss albino mouse is included where it was used as a bait animal to collect chiggers.

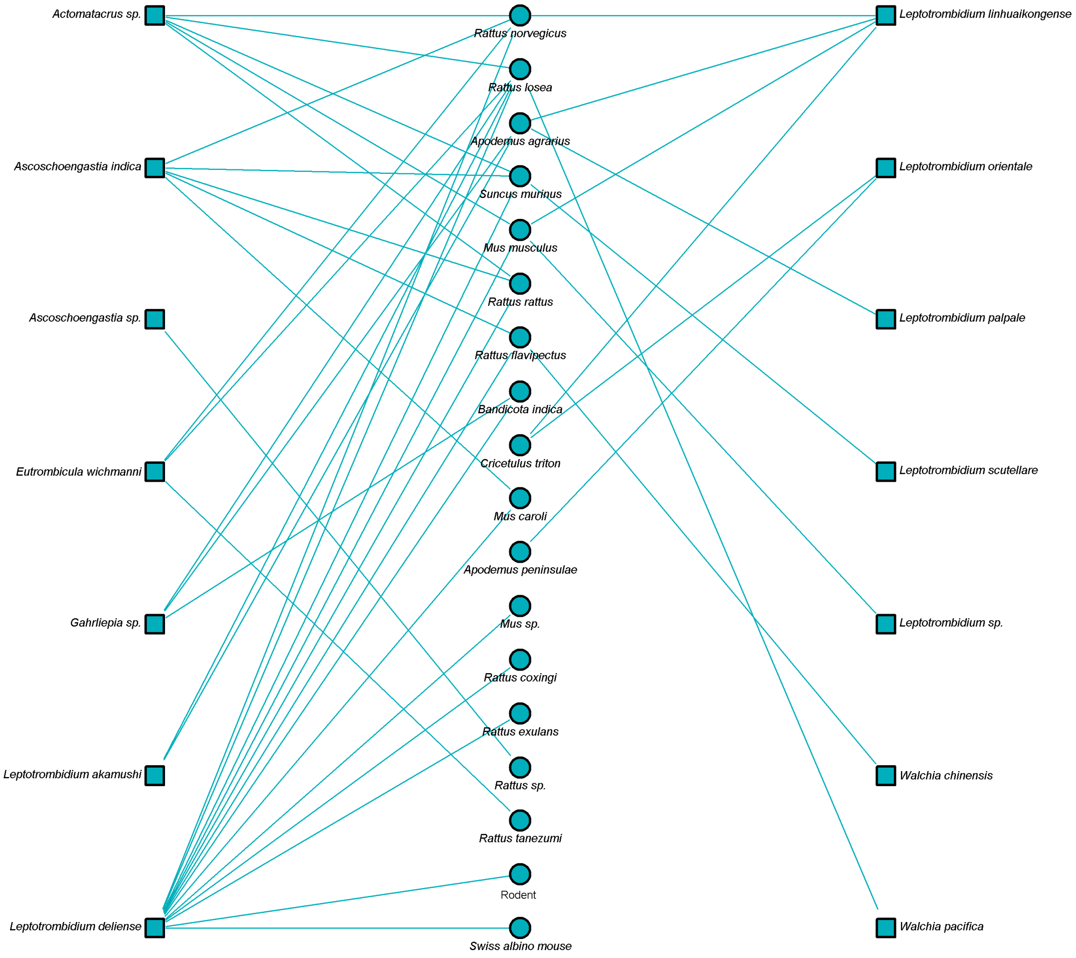


Figure 17 Network analysis of small mammal and chigger species testing positive for *O. tsutsugamushi* by any laboratory test for studies from China and Taiwan

2.3.1.6.1.3 Japan, South Korea and Russia

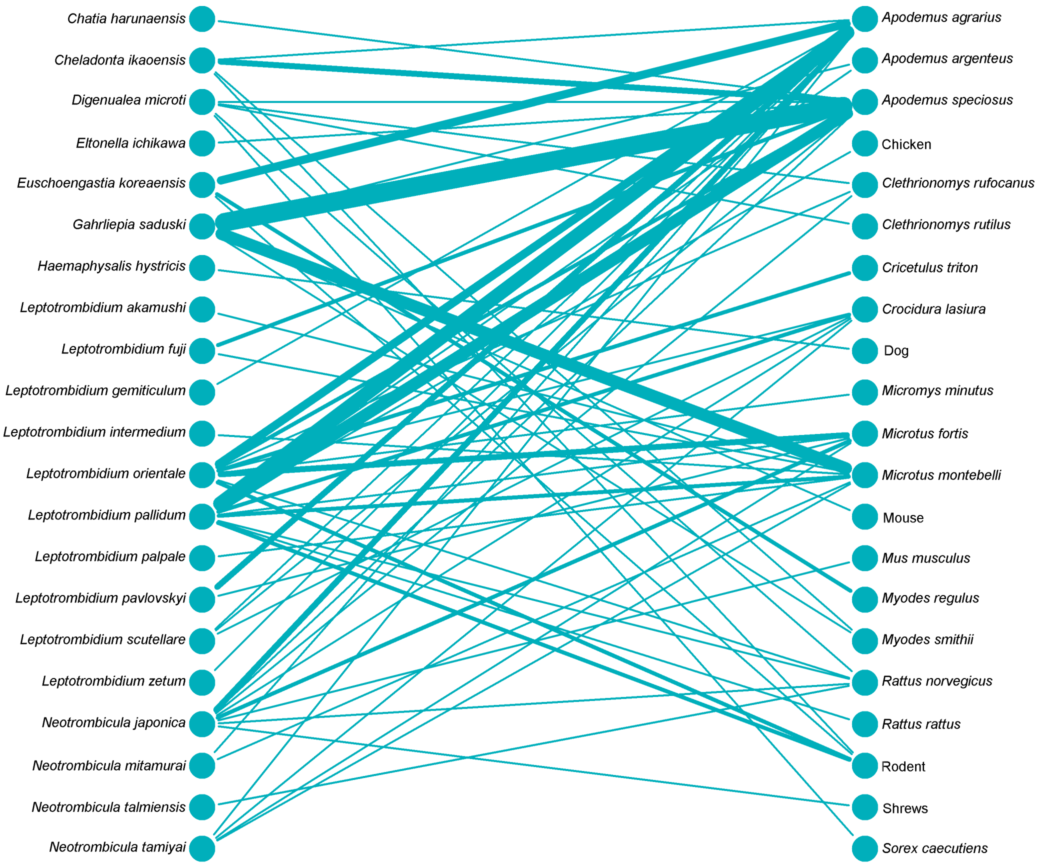


Figure 18 Network analysis of small mammal and chigger species for studies from Japan, South Korea and Russia

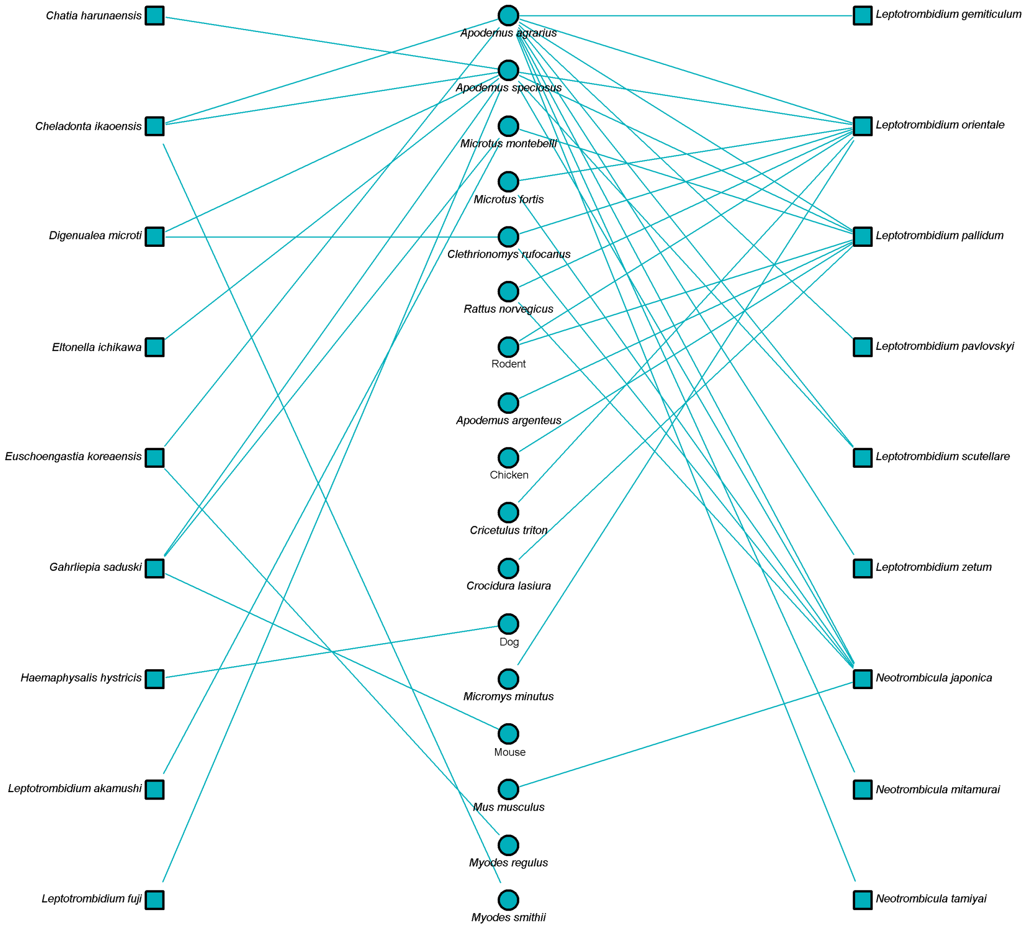
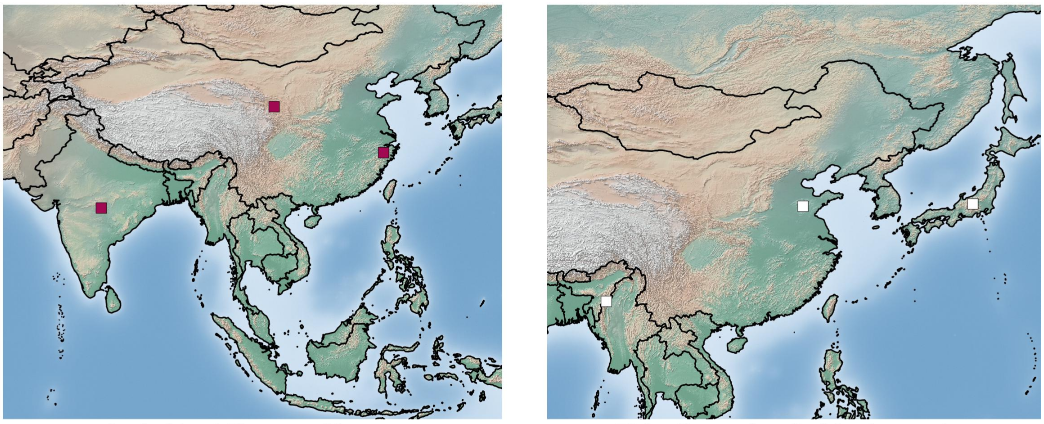


Figure 19 Network analysis of small mammal and chigger species testing positive for *O. tsutsugamushi* by any laboratory test for studies from Japan, South Korea and Russia

2.3.1.6.2 Non-chigger species

Few non-trombiculid mites have been reported as testing positive for *O. tsutsugamushi*. In an unspecified site in Japan, several *Haemaphysalis* sp. ticks removed from scrub typhus-infected dogs tested positive by PCR [102]. A section from Audy's WW2 report also recorded scrub typhus in the same genus of ticks using xenodiagnosis [87]. Two of 12 pools of *Ixodes* spp. ticks removed from rodents tested positive for *O. tsutsugamushi* by PCR in Shandong Province, China [103]. *Ornithonyssus bacoti* (Macronyssidae) removed from rodents in Nagpur, India, tested positive by PCR (1 of 5 pools) [104] and in the review of Rickettsial disease in China by Fan et al., *Echinolaelaps echidninus* and *Laelaps turkestanicus* also tested positive, but further details are not provided [82] (Figure 20).



Laelapidae & Macronyssidae

Ticks: Haemaphysalis & Ixodes species

Figure 20 Location of *O. tsutsugamushi*-positive non-trombiculid mite species

2.3.1.6.3 Chigger indices, percentage host infestation and *Orientia* positivity

Only 43/276 (16%) studies provided data on the chigger index (mean number of chiggers per host species) of collected animals. Even fewer studies, just 34 (13%), reported the percentage infestation rates of collected animals (percentage of host species with 1 or more chiggers found attached). Chigger index was reported for 47 host species and percentage infestation for 52 species. Consistent with overall data on hosts tested, the major genera and chigger indices reported were: *Apodemus* spp. (66), *Bandicota* spp. (54), *Mus* spp. (4), *Rattus* spp. (50) and *Suncus murinus* (29) (Appendix A-2, Table 4). The median percentage infestation rates were: *Apodemus* spp. (38%), *Bandicota* spp. (95%), *Mus* spp. (27%), *Rattus* spp. (59%) and *Suncus murinus* (59%) (Appendix A-2, Table 5).

Chigger index and small mammal infestation rates were compared to *O. tsutsugamushi* positive and negative test results across all species and locations using the Mann-Whitney U test. There was a significant association of chigger index with positive detection of the pathogen ($p<0.005$) (Figure 21). This relationship was not seen with percentage infestation. It should be noted there were very few *O. tsutsugamushi* positive samples where either parameter was recorded.

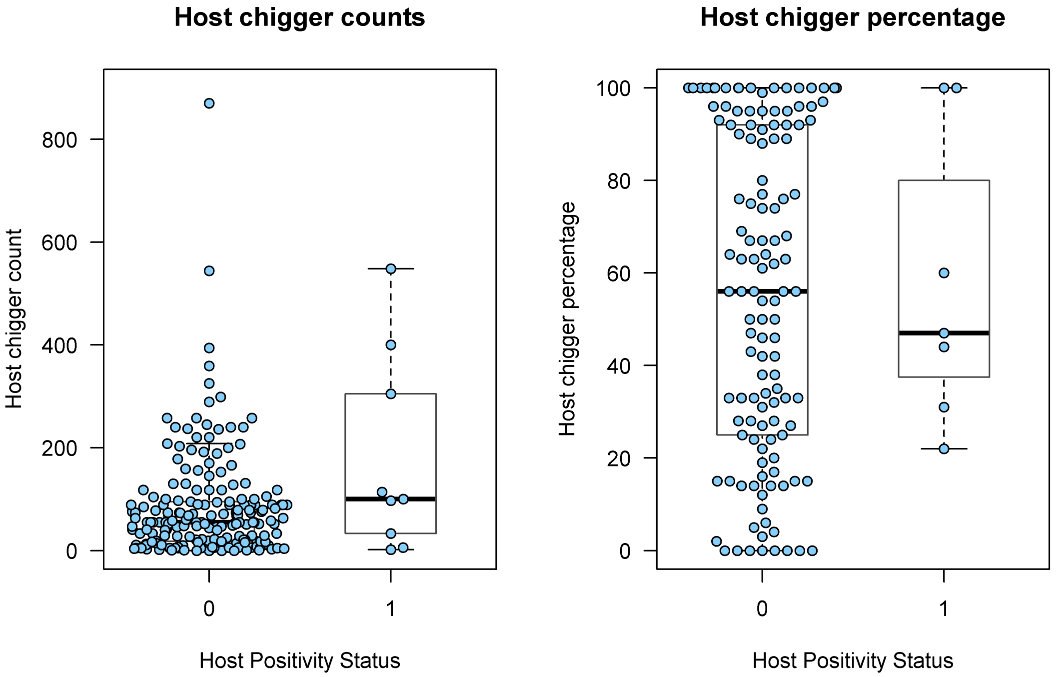


Figure 21 Box plots showing small mammal host chigger index (count) and percentage for *O. tsutsugamushi* positive and negative test results

2.3.1.7 Summary of ecological data

For 610/793 (77%) study sites no descriptive information on the habitat or other local ecological features was given. At 91 (12%) study sites, only the very briefest and most basic habitat classification was given. Frequently used terms included: scrub, fallow, fields, agricultural land, mountainous, forest and forest edge, rice fields, grass, parks, riverside, orchards and plantations, settlements, urban and rural. More detailed description was provided for 57 (7%) study sites. In these cases the above terms were usually used with additional, non-scientific habitat description. Common examples included: crop names (banana, tea, sugarcane and sweet potato); forest types (coniferous, deciduous, broadleaf, evergreen and bamboo), general plant types (*Miscanthus*, lalang, palm, *Pandanus* and *Lantana*).

A small minority of just 31 (4%) study sites provided detailed scientific habitat and or ecological description, including soil type. To be classified as such, at least one plant must have been identified to the species level. Of the 31 sites, 20 were sites where *O. tsutsugamushi* was identified in vectors or non-human hosts. Ten sites were in Japan where commonly reported plants included *Phragmites communis*, *Quercus serrata*, *Cryptomeria japonica*, *Phyllostachys pubescens*, *Artemisia* sp. and others. Of 4 sites in Malaysia, *Imperata cylindrica*, *Paspalum* and *Melastoma* were reported. Additional reports from 2 sites in Tajikistan (*Populus pruinosa*, *Tamarix* sp. and *Salix* sp.), 2 sites in Taiwan (*Bidens pilosa*, *Miscanthus* sp. and *Leucaena* sp.), and single sites from Russia and the Philippines were found. No clearly dominant plant species were reported among the sites, even in the same country. Soil type (loam, red clay, humus etc.) was recorded at only 5 sites. No analysis of soil was performed in any of the included studies.

A very small number of studies reported rainfall either as an annual figure or total precipitation during the study period. Of the 13 studies, 10 were in China, 2 in India and 1 in Russia. Twelve studies reported average, minimum and maximum temperatures at the study sites. The average temperatures reported ranged from 12 to 26°C and maximum of 42°C and minimum of -2°C.

2.4 General ecology themes

2.4.1 Trombiculid mite life cycle

Many aspects of the life cycle of chiggers are well understood from laboratory colony studies, though how this may vary in nature is not. Neal & Barnett provided a detailed account of the life cycle of *Trombicula akamushi* in laboratory conditions [105]. Males produce stalked spermatophores that are deposited in the environment and taken up by females to fertilise their eggs. Egg deposition begins between 6 and 21 days post insemination. Egg laying continues for as much as 253 days in one case, with mean daily egg production ranging from 2.4 to 21.7 and a maximum of 41 recorded. After 7-11 days the ovum ruptures to produce a quiescent deutovum. A further 5-7 days later the 6-legged larva emerges. These larvae remain within a few centimetres of their birthplace and after 2 days may start to display host-seeking behaviour by forming clusters on leaves, grasses and twigs above the soil surface. Larvae can survive for many months awaiting the opportunity to feed. The larvae feed on digested tissue fluid from a vertebrate host, becoming engorged and increasing in size by several fold. Larvae will feed from anything between 2 to 12 days or longer, depending on the chigger species. They then detach and return to the soil surface. Over about 3 days they develop into another quiescent phase – the nymphophane or protonymph. After a further 7-10 days the 8-legged deutonymph emerges. This, and the adult stage, feed on arthropod eggs (e.g.

Culex mosquito eggs), or recently deceased or quiescent soft-bodied insects such as Collembola [106,107]. Two weeks later the nymph develops into the tritonymph (teleiophane), lasting about 2 weeks from which the adult finally emerges [9,10,14,25,27,105,108,109] (Figure 22). Adults may survive for 15 months or more. Their food preferences in the wild for different species remain unknown. In some tropical species of *Leptotrombidium*, the entire cycle may take just 2 or 3 months, allowing at least 2 generations per year. Only a single generation is possible in more temperate zones [14]. It can frequently be seen that groups of attached chiggers are of similar size, suggesting that opportunities may arise for batches of chiggers, possibly from the same brood, to latch onto a host [14].

This detailed understanding of the trombiculid mite life cycle is almost entirely derived from studies on mites kept in laboratory colonies, due to the challenges involved in observing mites in their natural habitat. It is thus unknown how these stages may vary in nature.

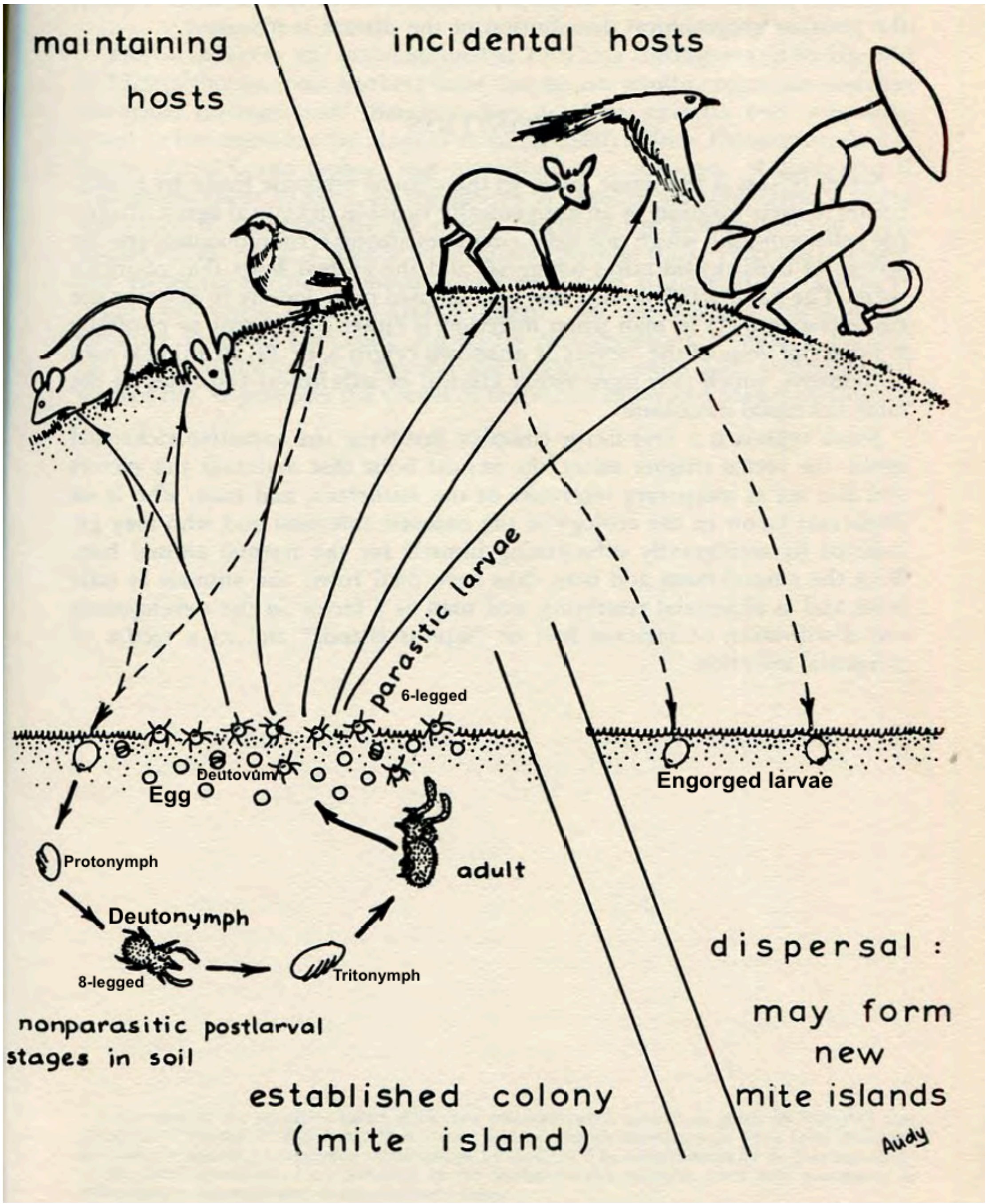


Figure 22 Chigger and scrub typhus life cycle (adapted from Audy, 1961 [9])

Comparisons in the life cycle between *O. tsutsugmaushi* infected and uninfected chigger lines of *L. imphalum* and *L. changraiensis* have suggested developmental stages are delayed in infected lines, but host-feeding time is reduced. A shorter feeding time may confer a survival advantage [110]. From laboratory studies, typical female to male ratios of offspring range from 5:1 to 2:1. However in infected chiggers, 95-100% are female [13,85,110-113]. The occurrence of infected males has been reported only in *L. pallidum* and *L. imphalum* [114,115]. However, *O. tsutsugamushi* is not found in spermatophores [116]. Parthenogenesis has been demonstrated in *L. arenicola*, but to what extent this occurs in nature and in other species is not known [117].

2.4.2 Transovarial and transstadial transmission

Transmission of *Orientia tsutsugamushi* via the ovum is known as transovarial transmission and through the various stages of the life cycle as transstadial transmission. Nagayo & Kawamura first recognized this in the 1920s [20,118]. In adults the ovaries appear to most frequently harbour *O. tsutsugamushi* compared with other organs [119]. In larvae a higher incidence of *O. tsutsugamushi* in the salivary organs was seen in unengorged larvae than in engorged [120,121].

Transovarial transmission of *Orientia* has shown to be effective in chiggers kept in the laboratory. Rapmund et al. reported close to 100% efficiency over 4 generations in *L. akamushi* [85]. In *L. deliense* transmission to the F2 generation was approximately 95% and 92.6% in *L. arenicola*. However a marked decline in transmission was seen after 17 generations with fewer eggs produced [122,123]. Similarly rates of 93-100% were reported from Malaysia and Thailand in *L. chiangraiensis*, *L. fletcheri* and *L. arenicola* [13,124]. Transstadial transmission must clearly be present if transovarial transmission rates are high, however evidence of *O. tsutsugamushi* in different life stages has been difficult to demonstrate. Several studies showed lower rates of *O. tsutsugamushi* isolation in stages other than engorged larvae, particularly in eggs, deutova and adults [85,111,119,125]. This was also noted when free-living adults and nymphs were collected from a hyperendemic area in Malaya and all tested negative [126]. These findings led investigators to postulate whether *O. tsutsugamushi* are in some way reactivated during the feeding stage and become occult in other stages [14,119].

Whether small mammals or other hosts can act as a reservoir of *O. tsutsugamushi* infection is of crucial importance in understanding the ecology and population genetics of this pathogen. To date there has been relatively little investigation of this, all performed in the laboratory setting. In one study 3 different species of chigger were allowed to feed on experimentally infected wild-caught rodents. After 10 days, 9.1% of *L. fuji*, 5% of *L. pallidum* and 0% of *L. deliense* were infected and at 20 days all were negative [127]. In a further study Takahashi et al. were able to demonstrate transstadial transmission but no transovarial transmission [128]. Walker et al. carried out a similar study with *L. deliense* and *L. arenicola*. No *L. arenicola* acquired *O. tsutsugamushi*, however 7.5% of *L. deliense* did, with evidence of transstadial transmission to the adult stage, but no transovarial transmission could be demonstrated [112]. Toyokawa was also able to demonstrate that several typical vector species including *L. akamushi* and *L. scutellare* were able to acquire *O. tsutsugamushi* during feeding [129]. Traub et al. also investigated this question and showed 60-100% of engorged larvae (tested in pools) were *O. tsutsugamushi* -positive 1-4 days after detaching. This figure fell to 10-27% after 5-15 days. A single case (1/43) of transovarial transmission was reported [130]. In 2009 Frances re-examined this subject and again demonstrated that *L. deliense* feeding on a rickettsaemic host will acquire *O. tsutsugamushi* and transmit it transstadially. Indeed infection could be demonstrated in all life stages, but no vertical transmission occurred. A single case of transovarial transmission in *Blankaartia acuscutellaris* occurred, despite the fact that the engorged larvae tested negative. This case was hard to explain, but could reflect very low levels of *O. tsutsugamushi* post-feeding [131]. The possibility remains for *O. tsutsugamushi* to be boosted in the chigger population from wild small mammals.

The existing evidence suggests that chiggers probably act as both host and reservoir of the disease. There is much uncertainty about the interaction between *Orientia* and transovarial and transstadial transmission. The lack of evidence of vertical transmission of *O. tsutsugamushi* following acquisition from an infected host in the laboratory, led to speculation that this event may be so rare as to be probably irrelevant in nature. However, evidence is growing that for many vector-borne pathogens, the prevalence in vectors is very low and transmission is inefficient. For example, tick-borne encephalitis virus prevalence was 0.5-2% in *Ixodes ricinus* in endemic areas [132] and 1.7% of the same species of tick were infected with *Anaplasma phagocytophilum* in Switzerland [133]. Even if transmission is infrequent in nature, this may be sufficient to maintain and spread the pathogen in the population.

2.4.3 Chigger behaviour

Larval chiggers are present in a huge range of habitats, depending on species, where they await the opportunity to attach to a suitable host to feed. During this time, chiggers remain very still, probably moving less than 45 cm [134]. Chiggers are primarily stimulated by carbon dioxide, exhaled from an approaching host. They display negative geotaxis and phototaxis, and neither sound, heat, vibration, human sweat nor saliva, nor various other chemicals could induce a clear questing response [135-138].

Chiggers tend to be inactive until the temperature rises above 10°C and begin to crawl above 12°C. Chiggers can crawl at approximately 10 cm per minute at 28°C [139]. They are surprisingly hardy, surviving for 60 days at 1-2°C and even -20°C for a month [139]. Chigger survival submerged in water for 2 weeks (with subsequent normal continuation of the life cycle) has been recorded, with important implications in its ecology [109,130].

2.4.4 Chigger feeding

Once aboard a host, chiggers may move around for some time, before attaching at a suitable site to feed [14]. The larva attaches by means of its sharp mouth parts (chelicerae) and develops a characteristic feeding tube (stylostome) over several hours. The stylostome is formed by the chigger's salivary enzymes, which contain homologues of tick cement proteins and probably form the structure of the tube on contact with host tissues [140]. The stylostome may extend for 120μm or more. Below the distal end of the stylostome a pool of digested epithelioid and lymphoid tissues is created and this is "sucked" up by the chigger [141-143]. There is little evidence that chiggers routinely ingest blood, particularly as the stylostome probably only extends a little beyond the epidermis. Traub, however, reported visualising red blood cells within the stylostome [130,144]. Presumably, *O. tsutsugamushi* is usually acquired from lymphoid tissue.

It is widely reported in the literature that chiggers feed only once during their life cycle, attaching from anywhere between 2 days and several weeks depending on the species [5,8,27,28,109,145-147]. In temperate climates, some species of chigger appear to overwinter on the host and detach once conditions are suitable for development into the next instar [148]. If this is the case then axiomatically chiggers must be able to act as both vector and host. Traub & Wisseman reported that when a host was killed once chiggers had begun to engorge, 250/1,000 individuals detached and reattached to a new host. Transmission of *O. tsutsugamushi* to the second host could not be demonstrated [130]. They went on to claim that 5% of chiggers feeding on a host will voluntarily detach and after regressing in size, one third reattached to a new host [14]. However, Kohls et al. could not elicit any reattachment of chiggers to a second host [149]. The lack of studies in this area reflects the challenges involved. Traub postulated that reattachment might be a more important phenomenon in nature, with frequent small mammal deaths [144]. Live-trapped animals examined immediately after death frequently have a large proportion of unattached chiggers present on the animal's body, together with clusters remaining attached (pers. obs.). To what extent these represent detached chiggers, or chiggers in the process of finding a site for attachment is unknown.

The phenomenon of co-feeding chiggers and the role this plays in exchanging *O. tsutsugamushi* between individual vertebrates is a much-overlooked subject. Chiggers tend to aggregate into tight clusters to feed, very often consisting of the same species and same age. Frances et al. were able to show transmission of *O. tsutsugamushi* to uninfected *L. deliense* (1.6%) and *B. acuscutellaris* (2.5%) co-feeding with infected chiggers [150]. This may not only act as an important method of horizontal transfer of *O. tsutsugamushi* between individuals of the same and other species, but also account for some of the enormous strain diversity and existence of multiple strains in the same individual vertebrates [151,152]. The mechanisms and processes driving the evolution of strain diversity and the interaction of the pathogen between chiggers and mammal hosts (including humans) is an important area for future research.

2.4.5 Criteria for vectorship

To definitively establish vectorship, several criteria should be met. The vector must be naturally infected with the pathogen and it must be able to transmit it to a host. The vector should be prevalent in the place where infection occurs and naturally infected hosts should be confirmed. Finally, evidence should be found of the vector feeding on a host, including man [14]. Due to the size of chiggers, the latter can be challenging, though has been reported [134,153].

It is possible that different species of chigger have different propensities for biting certain vertebrates. Traub suggested that intrazootic chigger species may exist that maintain *O. tsutsugamushi* among, for example rodents, but are not predisposed to bite humans [14]. In a human volunteer study, *L. fletcheri*, a well-established vector species in Malaysia, readily attached to humans, whereas *L. arenicola* did not, even when kept on the skin in a capsule for 24 hours [154].

2.4.6 Habitats and microhabitats

The Oxford English Dictionary defines scrub as: "[ME, var. of shrub], a) vegetation consisting mainly of brushwood or stunted forest growth, b) land covered with this". Evidence suggests that scrub typhus is present in a far greater range of habitats than that described by the word "scrub". *O. tsutsugamushi* in vectors and hosts has been found on sandy beaches in Malaya [155], in deep jungle [156], in semi-urban or peri-urban environments [96,157], in localized areas within semi-desert, alpine meadows and subarctic glacial moraine at 3,200m above sea level in West Pakistan [80].

Chiggers appear to be relatively habitat specific, though some key vector species, such as *L. deliense*, seem to be able to colonise a greater range of habitats. *L. deliense* is frequently found in scrub and forest, while *L. fletcheri* may be collected from certain grassy areas and *L. arenicola* from vegetation alongside beaches [158,159].

At the larger scale, certain general types of habitats seem to favour the presence of scrub typhus. However, it must be remembered that the presence of sufficient numbers of a maintaining host animal is inextricably linked to the presence of the vector and therefore the disease. Neither chiggers nor vertebrates they feed on are uniformly distributed in any environment. During WW2 Audy and colleagues conducted detailed investigations into the association of both human cases and vector chigger species with different habitats. This is chronicled in the 403-page War Office report of the Scrub Typhus Research Laboratory, South East Asia Command published in 1947 [87]. Three key risk habitats were identified: 1) man-made wasteland as a result of a) rural abandoned clearings due to shifting cultivation practices, b) domestic or suburban neglected areas or c) neglected gardens and plantations. 2) Water meadows including the grassy edges of water bodies and seepages in drier areas. 3) Hedgerows or fringe habitats, typically where two types of habitat meet such as forest edges (ecotones) [25,26]. It might be expected that areas with chiggers would become gradually confluent, except for unsuitable lacunae of terrain, thus forming an endemic area or region [159]. In a recent extensive survey in Northwest Yunnan, China, chigger diversity was lower in the flatlands, but mean abundance and intensity was higher than in the mountains. *L. deliense* predominated in flatlands, and *L. scutellare* in mountains [160].

Ecotones may provide the conditions to allow both rodents and chiggers to thrive. One study of forest and open scrubland transects found the highest numbers of chiggers attached to rodents trapped in ecotones (three times more *L. deliense* than away from the ecotone) [145,159]. Goff in Papua New Guinea reported an abundance of *L. deliense* in disturbed habitat but not in undisturbed areas [161]. A more detailed assessment collecting free-living chiggers using black plates placed on the ground, found chiggers to be more commonly associated with cleared areas in scrub habitat, along paths, fringes of scrub habitat and under trees and bushes [162]. Using a similar method on Hachijo Island, Japan, chiggers were located in damp areas in the transition between hills and flat areas and in forests near flat areas [139]. In Taiwan, a detailed assessment of ploughed and fallow habitats found three times more chiggers in the fallow fields. There was no association with rodent density or species, suggesting that the microhabitats of the fallow field, with more shade, leaf litter and shrubs provided more suitable habitat for chigger survival [163]. Porous, well drained, moist soil appears to be most suitable, but no detailed studies have been performed [10].

Certain plant species have often been cited as associated with scrub typhus, such as grasses including *Imperata cylindrica* (kunai grass in New Guinea, kogan grass in the Philippines, lalang grass in Malaya), *Saccharum spontaneum, Eleusine indica, Cyperus iria* and *Paspalum conjugatum* [5,8,27,28,164]. In the "*yudokuchi*" or noxious areas of northwest Honshu along riverbanks, *Miscanthus sinensis* and *Phragmites communis* are common [165]. However, Audy concluded that detailed botanical surveys did not prove useful as no clear correlation between plant species and the disease emerged. He proposed a more synecological picture using the broader habitat groups described above and it is from here that the term "scrub" became synonymous with the disease [159].

Whether scrub typhus occurs in primary forest and has a sylvatic cycle that can "escape" to infect bordering chigger-mammal-chigger cycles and man has been much debated [25]. Almost no studies of vectors and hosts have been carried out in what can be described as true primary forest. Traub et al. reported positive isolation of *O. tsutsugamushi* from three rodents (*Rattus mulleri*, *R. edwardsi* and *R. rajah* group) as well as a pool of *Ascoschoengastia audyi* chiggers collected from a *Callosciurus* squirrel in primary Malayan jungle [156]. Muul & Liat also reported *O. tsutsugamushi* by isolation and serologically in forest rodents (*R. sabanus*) and a single squirrel *C. notatus*, however the forest at Bukit Lanjan near Kuala Lumpur was not strictly primary [166-168]. Even within rainforests, habitat can vary with small clearings due to fallen trees, paths and along the banks of rivers and streams [144]. These may provide the opportunity for increased densities of rodents and chiggers [159]. Forests tend to have greater small mammal diversity, but lower density, with each mammal associated with particular chigger species [9]. There is no definite evidence of humans acquiring scrub typhus in primary forest, given that very few humans live completely in undisturbed forest without altering it. A study of antibodies to scrub typhus in the Orang Asli tribes of Malaysia, found higher levels in those living in deep forest clearings compared to those on the forest edge or in villages [169]. However, it was impossible to say where the disease was acquired and whether the fringe habitats within the forest were important.

2.4.7 Seasonality: temperature, rainfall and humidity

The seasonality of human scrub typhus has been well described in several countries across Asia. In Japan, South Korea, Taiwan and northern parts of China the infection presents almost exclusively from spring until early winter. In Thailand, Burma and India disease has been described as most common from June until November, but present throughout the year. In Malaysia, New Guinea and the Pacific Islands seasonality has been less clearly defined. To what extent this pattern is dependent on temperature, rainfall, humidity and resultant numbers of vector chiggers remains unclear. Traub & Wisseman noted that all cases have occurred in either tropical or subtropical conditions and that no significant outbreaks have been reported during the dry season in India or central/southern Burma [14].

In more northern latitudes, some seasonality is to be expected, with winter temperatures dropping too low for chiggers to feed or chiggers overwintering on their hosts [148]. Several studies have linked the disease to the presence of different species of chiggers at different times of the year, in different parts of a particular country. In South Korea, for example, *L. scutellare* numbers peak in autumn corresponding to the highest rates of human cases [170-175], although this was not seen in all investigations [176]. *L. pallidum* is more common in northern and eastern areas, where fewer cases are seen and thus *L. scutellare* is believed to be the key vector [177]. A similar pattern is reported from Japan, with *L. scutellare* and *L. pallidum* causing autumn-winter cases and *L. akamushi* summer cases [178-182]. Outbreaks in soldiers training on Mt. Fuji's foothills were seasonal, despite the soldiers' presence year round [157]. A recent summary of human scrub typhus in Japan over a 59-year period demonstrated major outbreaks in October to December, with smaller case numbers in May to June in southern prefectures, whereas northern and northeastern areas had highest rates in May-June and moderate numbers in October to December [183]. Similarly, in Shandong, China peak human cases corresponded to peak *L. scutellare* numbers [184]. In the Primorye region of Russian Siberia, *L. pavlovskyi* peaked in summer and is implicated in human cases [185]. In lower Burma and Manipur (India) the seasonal variation in numbers of *L. deliense* was reportedly similar to that of human cases [25,27,186] and in Thailand *L. deliense* was most abundant during the rainy season from April to December [187]. In Tropical North Queensland cases peaked from March to July, coinciding with the rainy season and the period immediately after the rains [188]. In Tamil Nadu, India, the highest incidence occurred from October to December, coinciding with peak chigger numbers, though chigger numbers did not fall greatly at other times of the year [189]. In Maharashtra, only minor seasonal variation was seen in *Suncus murinus* and *Rattus blandfordi* chigger indices, whereas *Rattus rattus rufescens* had lower rates overall and near absence of chiggers from April to June [190]. In Malaysia, however, no marked seasonality in either human or rodent infections was seen, with only a small decrease during the dry season [86,167]. In the Pescadores Islands, Taiwan, many cases presented in military personnel from April until November. Here *L. deliense* is the vector and chigger numbers fall to nearly zero in winter and a close correlation of chigger abundance with human infection was reported [191,192]. Olson et al. estimated a minimum requirement of 0.69 chiggers per shrew as the critical abundance needed to result in 1 human case per month [191].

The importance of temperature was investigated on the Pescadores Islands. Chiggers were recorded on rodents 12 days after the first 30°C daytime temperature in the year and the first human case occurred 10 days later, though this varied year-to-year dependent on cold spells [193]. Others also reported a close correlation on the Pescadores between mean monthly temperature, chigger abundance and human cases, but not so with rainfall [194,195]. In a more widespread study across Taiwan, human disease incidence correlated well with overall chigger abundance, though surprisingly not with *O. tsutsugamushi* infected chigger abundance [196]. In Guangzhou, China, each 1°C temperature rise corresponded to a 14.98% increase in the monthly number of human cases [197] or an odds ratio of 3.8 [198]. However a clear correlation of cases with temperature was not seen in two studies from South Korea and India [199,200]. In both Japan and

South Korea it has been clearly demonstrated that the monthly distribution of cases becomes more evenly distributed at more southerly latitudes [11,173].

Audy's extensive investigations in India and Burma revealed that chigger abundance falls during the dry season [186]. The proportion of rodents carrying *L. deliense* rose after rains began, but the mean number per rodent (chigger index) lagged behind by a few weeks [9]. In Malaya, using bait animals in a hyperendemic area, 10 times fewer chiggers attached to rodents during dry periods than wet [126]. In the same study, but using human volunteers, 70% became infected during wet periods compared to 5-29% in dry spells. However the picture was somewhat confused as more chigger pools tested positive during the period with the lower chigger index (of 12) compared to a high index of 304 [126]. In Thailand, chigger species diversity was higher in the dry season and human scrub typhus incidence correlated strongly with chigger diversity [201]. In more tropical climates, annual temperature variation is less marked and here rainfall may be more critical to chigger abundance and human disease [135,192]. Where and how these two factors converge and interact in different regions is not fully understood. In Tropical North Queensland all but 1 human case was reported east of the 60 inch isohyet (where 1500mm or more rain falls annually) [188,202] and in Thailand chigger abundance and human cases are highest during the rainy season [111]. In a study transect in Malaya, chigger abundance could be maintained by sprinkling the ground with water after the rains had ceased [203].

Temperature and humidity are certainly important factors in chigger development. A minimum temperature is required for eggs to hatch and in hotter climates, chiggers are more active in cooler damp morning conditions and seek refuge from very high temperatures by entering soil as deeply as 18 cm below the surface [9,135,204-206]. Scrub typhus risk has also been associated with hours of sunshine, lower atmospheric pressure (associated with rainfall) and in some studies humidity [184,197,207].

The importance of a time lag between weather events (such as exceptionally heavy monsoonal rains) and human cases should not be underestimated and may reflect both rodent breeding success and the chigger life cycle. In Guangzhou, after 4 months lag, every 10% increase in relative humidity was associated with 8.5% [95% CI: 2.7% to 14.5%] increased odds for infection, and a 1-unit increase in multivariate El Niño Southern Oscillation (ENSO) index between 2006 and 2014 was associated with a 23.6% increased odds scrub typhus with a 5-month lag [198].

2.4.8 Vectors

Trombiculid mites are considered to be the major vector of scrub typhus. The term chigger probably derives from the Spanish *chico* meaning small, and initially referred to the scrub-itch trombiculid mites of North America. Later the term became synonymous with all trombiculid mite larvae [208]. Chiggers belong to the family Trombiculidae, subclass Acari, class Arachnida and phylum Arthropoda [108]. There are over 3,000 species of Trombiculidae present across almost the entire world. The identification of chiggers to the species level is technically challenging given their small size and the lack of accessible and updated taxonomic keys. Indeed, there has been much confusion over identification, with many genera and species names changing over time. Recent advances in identification using autofluorescence techniques and genetic barcoding of conserved 18s rDNA or mitochondrial cytochrome c oxidase subunit 1 genes may pave the way to greater taxonomic clarity [96,209]. Over 50 species of chiggers are known to bite man and of these 10 species have good evidence of transmitting *O. tsutsugamushi* to man and a further 5 are possible vectors [108]. Only members of the genus *Leptotrombidium* are confirmed vectors to humans; among these *L. deliense*, *L. akamushi*, *L. arenicola*, *L. imphalum*, *L. scutellare*, *L. pallidum* and *L. pavlovskyi* are the most important [108].

Chiggers may be collected from hosts, using black plates or other objects placed on the ground, from the surface of boots of a standing person and from soil surface matter using Berlese or Tullgren funnels [10,139,204].

The prevalence of *O. tsutsugamushi* in chiggers is low, with a median per site reaching 18% in *L. pallidum*, but less than 10% in other key species using molecular diagnostic tools (Table 5). Free-living chiggers generally have lower percentage infection rates compared to engorged chiggers (Table 5). In one study, for example, 2.6 % of engorged *L. deliense* collected from wild rodents were positive by DIF while just 1.1% of offspring from the same collection were positive (i.e. naturally infected) [111].

Santibáñez provides a recent detailed update on the role of chiggers as vectors of human pathogens [108] and Stekolnikov published an updated key to the genus *Leptotrombidium* in 2013 [210].

It is likely that trombiculids are divided into the following groups: those that do not bite humans or carry *Orientia*, those that bite man but do not carry *Orientia* (scrub-itch mites), those that carry *Orientia* but do not bite man (possibly intrazootic), and those that both carry *Orientia* and bite humans [11]. Of the latter, there are few common species, perhaps due to their habitat preferences and likelihood of encountering man. Nadchatram attempted to classify chiggers into 7 ecological groups, of which group 1 are the red-orange coloured soil surface dwellers with a broad range of hosts including man [211].

The possibility of other vectors of scrub typhus should not be ignored, particularly in areas distant to the classical scrub typhus "triangle", including the United Arab Emirates and Chile. Several reports of ticks testing positive for *O. tsutsugamushi* have been published [102,103] and recently for a non-chigger mite, *Ornithonyssus bacoti*, too [104]. *O. tsutsugamushi* has been shown to multiply in inoculated ixodid ticks [212]. Traub & Wisseman cited Russian research that suggested *O. tsutsugamushi* survived in fleas for 11 days and could be transmitted by the flea bite, but details were lacking [14]. The history of leech bites at the site of eschars in scrub typhus cases in Chile has prompted further investigation but no current evidence of leeches being vectors exists [77,213]. Chiggers have been found to carry novel *Rickettsia*, *Anaplasm*a and *Borrelia* species, but to what extent these are pathogens or transmitted to humans is unknown [214,215]. Evidence from China implicates trombiculid mites in the transmission of Hanataan virus [216]. It seems probable that arthropods that feed on rickettsiaemic hosts may be able to acquire *Orientia*, but the bacteria probably cannot cross the gut wall and onward transmission has not been documented.

2.4.9 Hosts

There are two major groups of vertebrates that host larval chiggers; the "maintaining hosts" which comprise small mammals (rodents, shrews, ground-dwelling birds) and "incidental hosts" (other birds and larger mammals including humans). Several reports provide detailed lists of animal hosts that reveal the enormous range of species that can be parasitized [9,12,14,217]. Harrison reported *L. deliense* from 87 species of host [217]. Only monkeys, gerbils, hamsters and humans are thought to suffer clinically with scrub typhus [9,22].

The patchy distribution of chiggers in the environment has already been alluded to, and will be discussed further. Maintaining hosts are able to acquire chiggers and either re-deposit them at the same site or a nearby site, whereby the intensity of this interaction (i.e. number of hosts) contributes to the abundance of mites and where *O. tsutsugamushi* is present, the risk of disease [9,186]. Incidental hosts, such as birds and monkeys, may play a role in transporting chiggers to more distant sites and setting up new foci of infection.

It has been estimated that in ideal conditions, a single small mammal host could support four generations of chiggers per year, with a total of 10,000 feeding on that individual [9]. In Peninsular Malaysia, one *R. argentiventer* was estimated to support 4,000 chiggers/month [218]. Individual rodents can host over 1,000 chiggers at one time, tree shrews 5,000 and birds as many as 11,000 [9]. The rate of turnover of chiggers is an important factor for vector competence [145].

The pattern and degree of infestation of hosts is dependent on several factors. Host behaviour and habitat exploitation is of great importance. Terrestrial and scansorial mammals are more heavily infested and frequently infected with *O. tsutsugamushi* than arboreal mammals [14,168,219,220]. More generalist rodents that exploit different types of habitats, such as *Rattus* species are often implicated in scrub typhus. Indeed, Traub et al. postulated that scrub typhus distribution closely mirrors the distribution of *Rattus* and *Leptotrombidium* species, with the place of *Rattus* taken by voles in Korea and Japan [144].

Vertebrate home range size may be important, with greater exposure to chiggers and the ability to host large numbers of ectoparasites with more extensive ranging [25,111,218]. However, data on home range sizes for rodents are relatively few. In Taiwan studies of larger species (*Bandicota indica* and *Rattus losea*) reported not more than 500 m ranges [163]. At Changi Camp, Singapore, as much as 700 yards was recorded for *Rattus* species [221]. On Shichito Island, Japan, re-trapping of *R. norvegicus* took place up to 50 m away, 50-60 m for mice and 25-30 m for voles [222]. In Malaysia, 1 *R. tiomanicus* was re-trapped 560 m away [223]. Vertebrate body size may also be important but data are inconsistent. Traub reports less than 20 chiggers attached to individual tigers, leopards, civets and deer, whereas small rodents and shrews may have thousands [109]. Small terrestrial mammals may be best suited to acquiring chiggers, as they forage on and in the ground where their backs and ears are at the height of many species of questing chiggers. Small mammal burrows and nests may also be suitable locations for chiggers to attach.

The site of chigger attachment varies in different hosts. In rodents the ear fossae are typically parasitized, in Tupaidae the midline and inguinal region, in shrews the perianal area and in macaques the eyelids and eyebrows [109]. Traub suggested that host grooming efficiency may explain why mice frequently have few chiggers, whereas shrews have many [109]. However, there appears to be very little study on grooming efficiency between different small mammal species. Other possible factors affecting attachment site include skin thickness, hair type, skin immune response and microclimatic conditions [211,224,225]. Some chigger species attach to specific areas of a host's body, for example *Schoengastia schuffneri* has a predilection for rat groins [161,164]. In man, the attachment site is often associated with areas of pressure, for example the axillae or around the waistband [14].

There is some degree of host specificity among chiggers, though to what extent this is dependent on host contact with habitat-specific chigger species is unclear [226]. Some chigger species when presented with a human finger refuse to attach, but when presented with a bird readily attach and engorge [135].

Rodents, like humans in endemic areas, probably become infected repeatedly with *O. tsutsugamushi* during their lifetime. In the wild, captured *R. tiomanicus* were rickettsaemic for a mean of 97 days [223]. In the laboratory, rickettsaemia was recorded in *R. rattus* after a *Leptotrombidium* bite from day 7 until 8 weeks later [227]. In another study viable *O. tsutsugamushi* were recovered from the kidney of a *R. annandalei* 4 months after being infected [220]. In an endemic setting, at least a 50% lifetime infection rate in rodents has been estimated [223]. *O. tsutsugamushi* yields from tissue are greatest from spleen, followed by liver and kidney. Lung tissue is also frequently positive, but results are inconsistent [124,220,228,229].

2.4.9.1 Role of birds

The role of birds in the ecology and distribution of scrub typhus has been almost completely ignored. Several bird species have been reported as efficient hosts of vector chigger species. Ground-living birds such as quail are frequently heavily colonised, with 18,500 *T. akamushi* removed from 9 birds in Malaysia [25]. Others reported *L. deliense* and *T. pseudoakamushi* on Chinese quail (*Coturnix chinensis)*, Greater coucal (*Centropus javanicus* or *C. sinensis*), Raffles' malkoha (*Rhinortha chlorophaea*) and Oriental reed warbler (*Acrocephalus orientalis*) [230,231]. In Japan the green pheasant (*Phasianus colchicus tohkaidi* or *P. versicolor*) harboured *L. pallidum* [232]. Japanese scientists reported 72 species of birds carrying trombiculid mites, including *L. pallidum* and *L. scutellare*, but all attempts at isolating *O. tsutsugamushi* from these failed [233].

Kitaoka showed that chickens and pigeons could both be experimentally infected and remain rickettsaemic for as long as 42 days [233]. The domestic chicken was reported to harbour both *L. scutellare* and *L. pallidum*. Kitaoka also exposed chickens to areas of high disease endemicity and was able to recover *O. tsutsugamushi* from a liver and spleen pool of 1 chicken and from *L. pallidum* attached to the chickens [234]. The only records of wild birds carrying *O. tsutsugamushi* come from Khasansky District, Primorye, Russia and Xinjiang Province, China. In the Russian Far East, these were black-faced bunting (*Emberiza spodocephala*) and long-tailed rosefinch (*Carpodacus sibiricus*) tested using xenodiagnosis [235]. In China 5/91 house sparrows (*Passer domesticus*) and 3/16 grey wagtails (*Motacilla cinerea*) tested positive from spleen tissue by PCR [98,99].

These species (except the resident *Passer domesticus*) breed in Russia and Northern China and migrate to South Korea, southern Japan, southern China, South and East Asia depending on the species [236]. Long-distance migrants arriving in Europe from Africa have been found to be carrying non-European mites [237]. Chiggers may be transported by birds in both local areas (ground-living birds) and over long distances (migrants). These could potentially set up new foci of disease and explain the extension of scrub typhus along island chains, for example East to the Solomon Islands and southwest to the Chagos Islands. This also raises the intriguing potential for further extension towards Madagascar and the African continent. However, recent reports of humans and dogs infected with *O. tsutsugamushi* in Chile would suggest a different mechanism of spread, as there are no known bird migration routes between Asia/Australia and Chile. Human transportation of rodents through international trade and shipping has been implicated in the spread of many infectious diseases.

2.4.10 Immunity and strain diversity

There has been very little research into the dynamics of antibodies to scrub typhus in either animal or human hosts. One study of rodents in Thailand demonstrated that IgG persisted for 10 months in animals removed from the wild and kept in captivity [111]. IgM was detected until week 6 in naturally infected *Rattus rattus* [227]. Studies in humans in the early 1950s clearly showed that even in those with recent natural scrub typhus infection, there was no significant protective effect to re-exposure by a different strain [238]. Homologous immunity was stronger, providing complete protection for at least 3 years [62]. Similarly, in cynomolgus macaques *(Macaca fascicularis)* homologous immunity was evident for at least 8 months, but not after 5-6 years [63].

Multiple strains may be present in a single colony of chiggers (*L. pallidum*) established from one individual [239]. Additionally multiple strains can be maintained in an *L. imphalum* colony by transovarial transmission [110].

In humans, mixed infections identified by MLST comprised as much as 25% of cases in Thailand and 8.6% in Laos [240,241]

Laboratory studies showed that transmission of strains from chiggers to rodents was variable, in that only the Karp strain was found in rodents fed upon by Karp and Gilliam-infected chiggers [242]. In Shandong Province, China, sequencing of the 56 kDa gene from rodents, chiggers and humans in one area suggested some consistency in key genotypes [243]; whereas in a study from 6 Thai provinces, a large diversity of genotypes was seen in humans with only 2 genotypes commonly distributed throughout the country [244].

2.4.11 Mite islands

The concept of "mite islands" was first put forward by Audy in the 1940s. The idea was based on the patchy distribution of human cases and of chiggers in the environment (Figure 23). The size of mite islands should be considered on two scales. At the largest scale, islands consist of endemic areas, ranging from a few square miles, to thousands of square miles, often bounded by major ecological barriers. At the small scale, an "island" of mites may be within less than 1 square foot and patchy over an area of hundreds of metres. The Japanese were well aware of this, describing localised high-risk areas as *yudokuchi* ("noxious area"). The patchiness may be more marked in drier climates, where mites are associated with damper areas. The islands may expand and contract over time, dependent on a myriad of factors [9,11,26,159,245]. Mite islands have been recorded in an enormous range of habitats from man-made wasteland, neglected cultivation and plantations to water meadows and forest edge [9,26]. More unusual habitats include parks [246], walled vegetable gardens [247] and subalpine meadows [80]. There have not been reports from very arid habitats or deep forest.

Figure 23 Map showing patchy distribution of human scrub typhus cases in the Assam & Burma regions in 1944-45, from Audy, Red mites and typhus, 1968 [208].

The presence of a mite island is probably dependent on factors such as suitable climatic conditions, species composition and stability of the vertebrate population, intensity of the vertebrate-chigger-vertebrate interaction, food supply (for larvae and adults), soil, microclimate and chigger predators [9,25]. The role of small mammals in the small-scale dynamics of mite islands is of importance. Rodents probably forage on similar routes and thus chiggers can be picked up and returned to suitable habitats on a regular basis. Even within the same areas, two rodents of the same species can have markedly different numbers of chiggers attached. Additionally, a rodent removed from its home range for three days and then replaced will very quickly acquire the chiggers that would have attached in the intervening time [248]. Rodent burrows have been reported to yield large numbers of free-living chiggers [10].

Several studies demonstrate the patchy distribution of chiggers in nature. In a transect through *Imperata cylindrica* grass in Malaysia using rabbits or rats as bait animals or black plates to collect free living chiggers, numbers varied at different points along the transects. Variation also occurred over time and the highest numbers were seen at the start of the rainy season [126,134,249]. In these studies, no clear link to habitat type was identified, though in another study negative areas were more frequently on clay soil [250]. Another study collecting wild rodents in similar habitat in an area of 1,300m^2 could find no clustering of *O. tsutsugamushi* infected rodents [251]. In Japan, studies using sentinel voles *(Microtus montebelli)* also reported the patchy distribution of *O. tsutsugamushi* infected voles and a changing pattern over time [246,252,253]. Most positive sites were positive on more than one occasion over the course of 3 years [254]. In chemoprophylaxis studies with humans, individuals seated on grass 45 cm apart had significantly differing numbers of chiggers attached, which was consistent to the location if two people changed position [134].

The differences between endemic and non-endemic areas have also been examined in a few cases. In Japan, 50% *L. pallidum* tested positive for *O. tsutsugamushi* in an endemic area (of human disease) and only 3.8% in a non-endemic area [255]. At the site of an intense outbreak of disease in plantation workers on the Goodyear Estate, Deli, Sumatra, 50.5% of rats were infested with mites with a chigger index of 104, whereas on two nearby estates without outbreaks, 6.3% and 2.3% of rats were infested with indices of 7 [231]. The study by Ishikura et al. certainly suggests that even areas thought to be non-endemic for scrub typhus, but occurring within an endemic region, may not be completely free of risk [255]. Our understanding of this remains poor.

The dynamics of mite island size and distribution over time are also not well understood. Some hyperendemic sites have become low to no-risk sites over the course of several years [25,256]. It is likely that the factors mentioned above as important for the establishment of a mite island, may change as, for example, a plantation matures resulting in less rodent food availability and different microclimatic conditions less suitable for vector chiggers species [14].

2.4.12 Land use, climate change and disease risk

The importance of changing climate, habitats and environment on the risk of infectious diseases is becoming increasingly recognised [257-260]. There has been little research into these impacts for scrub typhus. Man's multiple impacts on habitat, such as through shifting cultivation and clearing of forests has been linked to the creation of fringe and scrub type habitats that are thought to favour more intense host-chigger-host cycles. Some countries with the infrastructure to record scrub typhus incidence have reported declines in human cases (Japan and Taiwan), whereas others have seen increases (South Korea and China). The reasons behind these changes are not well understood. Saito et al. suggested that the fall in incidence in Japan could be partly due to less river flooding as a result of widespread dam building [261]. Kuo et al. in Taiwan reported that the abandonment of rice growing fields following Taiwan's joining of the World Trade Organisation in 2001, led to an increase in suitable chigger habitat which had been controlled by regular ploughing. Surprisingly, however the rates of *O. tsutsugamushi* in small mammals in fallow and ploughed fields were not statistically different despite much higher numbers of chiggers in fallow areas [163]. In South Korea, a recent study suggested that climate change might be partly responsible for the northern expansion of the key vector *L. scutellare* [96].

2.4.13 Humans

2.4.13.1 Focalization of human cases

The concept of mite islands as described above first came about as a result of many observations of focal outbreaks among soldiers in WW2. Classic examples include an outbreak of 756 cases in Ceylon after 4 days' exposure [157] and in Sansopor, Dutch New Guinea, in which 931 cases with 34 deaths occurred over 1 month, with almost all cases linked to abandoned village gardens and plantations [164,262]. In Finschhafen, New Guinea, reoccupation of a camp abandoned just 6 weeks previously resulted in 16 cases and 6 deaths, all of who were involved in clearing grass to make an outdoor cinema [8]. Plantation workers were also affected in well-described focal outbreaks. Cases often occurred in groups of workers detailed with clearing specific areas of the plantation [23,230,263]. The importance of shifting cultivation and the resultant exposure to potentially high risk habitats was also noted [159]. There has been a steep rise in reports published of scrub typhus outbreaks in the last decade, particularly in India and China [39]. This is most likely due to a combination of increasing awareness, land use changes from expanding populations and climate change with more extreme weather events. A striking aspect of many of the reports on outbreaks is that those exposed (e.g. military personnel, plantation workers) were immunologically naïve to scrub typhus [263-266].

2.4.13.2 Human behaviour and disease risk

In scrub typhus endemic areas, disease risk varies over the course of the year, with fewer human cases during winter periods in northern latitudes and dry periods in tropical latitudes. To acquire a chigger bite, exposure to suitable habitat is required. Occupational risk is important, with the main burden of disease in farmers, plantation workers and the military [9,23,27,188]. There have been increasing cases due to recreational exposure in suburban parks and gardens [96,267].

Certain activities, such as lying or sitting directly on the ground are particularly hazardous [26]. A recent detailed analysis of farming practice between a high incidence area in South Korea and a low incidence area of Japan with similar environmental and climatic features and the same vector (*L. scutellare*) identified certain risk factors. Working longer hours in the field, more bending forward or squatting at work, resting on the ground, lack of protective clothes and less washing of clothes and skin were all statistically significantly associated with scrub typhus infection [268]. A separate study of farmers in South Korea identified water around the home, dry field farming and working with livestock as significant risk factors [269]. Ecological niche modelling using maximum entropy algorithm has recently been used to predict scrub typhus occurrence across China, based on data from Jiangsu Province [270].

2.4.13.3 Interventions to reduce risk

No vaccine currently exists to prevent scrub typhus. Vaccine development was first attempted and trialled at the end of WW2, with some protection provided to naturally acquired scrub typhus in a small number of test cases [271,272]. However, fully vaccinated military personnel were not prevented from acquiring severe disease [273]. Further attempts in Japan in the 1940s also failed to provide lasting immunity [274]. The Japanese successfully used the mild Pescadores strain of *O. tsutsugamushi* to demonstrate protection from a virulent strain [275]. Smadel et al. went on to work on a similar strategy in the early 1950s (see Chapter 1). Valbuena et al. provide a more detailed account of the history of vaccine development [276]. However, a number of other preventive measures can be taken and these have been summarised recently by Xu et al. [39]. Avoiding high-risk areas and reducing the chance of a mite bite by avoiding sitting or lying directly on the ground are important. The use of insecticides on skin and clothing has been shown to be effective in several studies during WW2 [5,10,26,28,139]. Physical removal of attached mites is impractical due to their small size, though Malay mothers were reported to remove chiggers from their children with a needle [22]. Thorough washing of skin and clothes with a detergent after outdoor activities is recommended. Clearing suitable habitat for chiggers may reduce risk in the long term. Similarly, rodent control, although challenging to accomplish in practice, can reduce disease risk, although conversely the immediate disease risk may increase, with a large population of chiggers suddenly without their usual food source. For short-term, high-risk activities such as soldiers on operation or field workers, there is some evidence that weekly doxycycline may be effective prophylaxis [277]. Public engagement with regard to the measures described above is likely to be very useful. The application of mathematical modelling to strategies to reduce scrub typhus risk in humans by Min et al. suggested that reducing contact between humans and chiggers is more effective than attempting to either control rodents or chiggers [278].

2.5 Discussion

2.5.1 Introduction

Studies investigating *O. tsutsugamushi* in vectors and non-human hosts span almost a century and nearly 30% of the literature is published in a language other than English. Prior to 1924, there were a few studies in Japan where mites were allowed to feed on monkeys and an eschar and illness compatible with scrub typhus was recorded, however no laboratory tests were performed to identify the pathogen in these studies [21,279,280].

Studies were performed in 30 countries and at least 793 locations. Approximately 40 different types of laboratory tests were used to identify *O. tsutsugamushi*. These test were combined into 8 categories to analyse available data (Appendix A-2, Table 1). Seventy-four “vector” species were tested and 46 were positive, including Ixodida (ticks), Macronyssidae and Laelapidae (mites). Depending on the lab test category, overall positivity ranged from 0.6% to 5% for individual vectors and 9.6 to 56% for pooled vectors. *L. deliense* was most frequently positive across South and Southeast Asia, while *L. pallidum* and *L.*

scutellare were key vector species in South Korea and Japan. Among free-living chiggers, 31 species were tested with 23 species positive. Almost all of these were *Leptotrombidium* species. It is assumed that many species of ectoparasite that feed on a host with active *O. tsutsugamushi* infection may be capable of acquiring the pathogen. However, as trombiculid mites usually feed just once in their life cycle, successful transovarial and transstadial transmission is required to act as a vector. No evidence exists to suggest that Ixodida or other non-trombiculid mites are capable of onward transmission. Thus, *Leptotrombidium* species appear to be best equipped to act as vectors, though the reasons for this are unknown. A large number of hosts were tested, amounting to 234 species that included mostly small mammals, but also Artiodactyla, birds and lizards. In all 122 species tested positive, ranging from 2 to 40% positive overall depending on the laboratory test category. The Muridae were both the most frequently tested and *O. tsutsugamushi* positive.

In addition to the 276 articles included in the systematic review, 145 other articles were reviewed to summarise knowledge of key themes in scrub typhus ecology. Topics reviewed include the chigger life cycle, feeding behaviour and role of hosts including birds in the disease ecology. Although the disease is termed “scrub” typhus, data is lacking to clearly link the risk of human infection to this somewhat vague habitat type. Higher risk areas within endemic areas probably exist and are likely to be related to the presence of suitable habitat for chigger species that transmit the pathogen to humans together with sufficient maintaining hosts. Our understanding of the extent of such areas and dynamics over time is very limited.

2.5.2 Study limitations

Articles included in this review are of widely varying quality. Very few articles provide a comprehensive account of all potentially useful data collected (Table 9). Some studies chose to focus on either vector or host, providing minimal details on the group not under investigation. Trombiculid mites are notoriously difficult to identify to species level, with limited and often out-of-date taxonomic keys available. Many authors identified trombiculid mites to genus only, and it is likely that misidentification was frequent. Furthermore, much of the taxonomy of vectors (and some hosts) has changed over the long time course of scrub typhus research. For this review, species names known to be the same were combined and the current taxonomy used.

An enormous number of combinations of data were presented in the literature. For example: vectors and hosts collected from different locations were combined for laboratory analysis; multiple host and vector species were pooled for testing; several laboratory tests and multiple samples types were combined for diagnosis. It was not practical to extract and analyse the same data in different combinations. Many studies presented some results without a denominator (i.e. number positive given without number tested). In the analysis, the total number tested obviously excludes studies where the number was not provided, however the number testing positive was included. Therefore slightly higher positivity rates should be expected. Where data was available for study location, but species not provided, this was collected in favour of the species names if the locations were combined. The location of positives was considered a more useful outcome measure as almost any animal host may become infected when bitten by an infected chigger.

In order to identify articles for inclusion, very broad search terms to account for the terminology of scrub typhus and ecology were required. Additionally, many references were cross-checked and the Michael Hastriter list of scrub typhus literature was also reviewed, but unfortunately is no longer available on-line. It is possible that some articles were not captured. Only a single person performed article selection and data extraction for most articles. For non-English articles, extraction was done together with native speakers, to ensure all data was extracted as accurately as possible. Only 6 articles were excluded, as the full-text could not be found. An article reviewing Chinese research into scrub typhus and two papers from Japan, including Tamiya's 300-page "Recent advances in studies on Tsutsugamushi disease in Japan" reveal that there are additional local, governmental and military reports and monographs that were not identified through our extensive literature search [82] [178,281].

2.5.3 Proposed minimum data recording and reporting standards

As described in previous sections, many articles reporting the presence of scrub typhus in vectors and non-human hosts fail to provide many useful data and other key pieces of information. It is acknowledged that for some studies, certain information may not be available, or may be beyond the scope of the study.

As with other branches of medical research (https://www.ndorms.ox.ac.uk/research-groups/equator-network), in order to improve our understanding of the ecology of scrub typhus it will be vital that future research becomes more standardised in design and reporting. Table 9 below lists all recommended information to be recorded in all studies, where relevant. This checklist may also be applicable to studies collecting vectors and hosts for laboratory testing of other pathogens.

Future studies should also ensure that they make use of the "Strengthening of reporting of observational studies" (STROBE) checklist and "Checklist for One Health Epidemiological Reporting of Evidence" (COHERE) [282,283].

Table 9 Summary of proposed minimum data recording and reporting standards for studies investigating *O. tsutsugamushi* in vectors and non-human hosts

Topic	Checklist item	Notes
Dates	Vector and host collection dates	Month(s) & year(s) Where collections are made monthly, data should be reported monthly to inform seasonality of disease
Location	Study site GPS coordinates and administrative divisions 1 to 4 *	Spelling, transliteration and changes over time can be avoided by using GPS coordinates
Sample collection & storage method	Hosts trapping technique. Vectors collection method. Sample storage medium and conditions.	For collection of free-living vectors, provide details on methods [$]
Host species	All hosts should be identified to genus and species	Reference up to date nomenclature
Vector species	All vectors should be identified to genus and species where possible [#]	Record identification method & reference up to date nomenclature
Laboratory methods	Provide details of laboratory methodology	Where several methods are used, ensure data clearly separated by test
Sample type	List	Where several sample types tested, separate results. Where tissues are pooled, provide details

Testing hosts	Numbers tested and numbers positive	Avoid pooling samples from different hosts (and species) Avoid pooling samples from different sites
Testing vectors	Numbers tested and numbers positive. If pooled give approximate number or range per pool.	Avoid pooling samples from different sites
Host vector infestation rate	Proportion of hosts infested by the vector. Divide by host species and location.	If possible provide vector species composition
Host vector index (chigger index – average number of chiggers/host)	Vector (chigger) index. Divide by host species and location	If possible provide vector species composition
Ecology	Habitat description; mean, minimum and maximum temperature; rainfall; humidity; soil type	Note differences between study sites where applicable
Study ethics	Provide details of appropriate animal handling & euthanasia (if used) protocol & approval	Local, national and international standards may apply

[*] Administrative division categorization varies widely from country to country. The equivalent of province, district, sub-district and town or village should be given.

[#] At minimum, genus must be recorded. For pooled vectors, the majority of constituent genus/species should be recorded.

[$] Black plate, Berlese or Tullgren funnels or other techniques

2.5.4 Major gaps in our knowledge of scrub typhus ecology

Although useful research and important advances in our understanding of the ecology of scrub typhus has taken place, much of this took place prior to the 1970s and little research has re-examined existing dogma.

The life cycle of trombiculid mites suggests that chiggers act as both vector and host. However, whether these laboratory studies are applicable in nature is not well understood. How *O. tsutsugamushi* may influence the biology of the vector and the dynamics of the pathogen through the life stages are similarly poorly understood. That chiggers infected by feeding on bacteraemic hosts apparently do not effectively pass on the pathogen transovarially raises important questions about the reservoir of the disease, the population genetics and impressive strain diversity that is well recognized. Co-feeding chiggers may go some way to explaining this, but very few studies have investigated this phenomenon. Further attempts at the challenging studies required to establish whether chiggers feed more than once in nature during their life cycle are also much needed. The mechanisms behind the apparent ability of *O. tsutsugamushi* to manipulate the sex ratio of offspring, with very high proportions being female allowing onward transovarial transmission is poorly understood. Could *O. tsutsugamushi* be improving its chances of survival by increasing the proportion of females in the population? This phenomenon is well recognised in *Wolbachia* and in some *Rickettsia* species (all α-proteobacteria) [284].

Leptotrombidium species appear to be the major vector of scrub typhus, but to what extent other genera or even other ectoparasites could be involved is unknown. Why are *Leptotrombidium* species the key vector? Recent identification of scrub typhus in Chile, parts of Africa and the Middle East suggest that *Orientia* species are present across much of the tropics and subtropics. Why is human disease limited to a few case reports across this region? The disease frequently presents as an undifferentiated fever and is probably overlooked, particularly in regions where it is little known. In areas where the disease is emerging, local strains may cause mild or sub-clinical illness or perhaps competent vector species are not present.

The term “scrub typhus” suggests a particular habitat type. However, it is clear that the infection can be found in many different habitat types. Mite islands were well described during WW2, but this was mostly based on human outbreaks rather than systematic survey of vectors and hosts. As revealed above, there were very few study sites reporting no positives, and most of these (except Brazil) were close to sites with positives. What factors govern the presence of the disease in an area? Is the disease widely distributed in endemic areas with hot spots related to suitable habitat for vector chigger life cycle and a sufficiently high small mammal population? The scale and dynamics of any such hotspots or "mite islands" is not understood. What is the role of birds in scrub typhus ecology? Birds are known to harbour vector chigger species and wild birds have tested positive for *O. tsutsugamushi*. It seems possible that birds may allow the disease to be distributed over wider areas and potentially for new hot spots to be set up. Which bird species are most important? It is likely that ground dwelling birds with large home ranges or migrant species that spend time foraging on the ground are most suited to distributing infected chiggers. The role of migratory birds in distributing ticks carrying infectious diseases has been widely reported [285,286].

The seasonality of the disease in humans is well described. The factors that control this are less clear. It is expected that beyond an uncertain northern latitude, the influence of temperature is most important, with winters too cold for chiggers to be active. In more southern latitudes, rainfall is probably more critical with chigger numbers being higher after the rains have started. Human activity and exposure risk should not be underestimated. Chiggers will not attach to humans unless given sufficient opportunity. Some recent studies have begun to investigate farming practices and behaviours associated with the risk of disease.

2.5.5 Possible tools to begin to unravel these

Advances in molecular tools over the past 3 decades, including quantitative PCR and nested PCR, allow more robust diagnostics with high specificity. Whole-genome sequencing and low-input enrichment sequence capture (as attempted in this study for the first time) will provide invaluable insight into the complex population genetics and structure of *O. tsutsugamushi* in vectors, hosts and humans that may help understand both the pathogenicity and ecological interactions. The same technologies could also much improve our taxonomic understanding of trombiculid mites. Regrettably there are very few and decreasing number of experts in the morphological taxonomy of trombiculid mites. To our knowledge only a single functional chigger colony currently exists for the laboratory study of many of these important and unanswered questions and developing new colonies would be of great benefit.

Access to powerful geospatial data to map infected and uninfected vectors and hosts and link these with habitat, land use and the environment will help develop our understanding of the geographical extent of the disease and the factors that influence it. Niche modelling methods could be used to predict regions where O. tsutsugamushi may be unrecognised as has been performed for other infectious diseases [287]. Long-term climate and climate change data may allow us to predict the risk of disease and the factors that govern this. Studies on migrating birds could provide useful data on the dynamics of infection and establishment of new risk areas. All these in combination with computational power and statistical modelling techniques will advance our understanding of the ecology and disease risk.

CHAPTER 3

Investigation of *Orientia tsutsugamushi* in chiggers and small mammals in Northern Thailand and Laos

3 Chapter 3: Investigation of *Orientia tsutsugamushi* in chiggers and small mammals in Northern Thailand and Laos

3.1 Introduction

One of the primary aims of this project was to identify a site of high human scrub typhus transmission in order to study the ecology of the disease in chiggers, rodents and sympatric humans over a period of time. Many fundamental questions of scrub typhus ecology remain incompletely understood. Few studies have investigated the dynamics of scrub typhus over time in vectors and hosts using molecular tests, particularly from the same locations. This project investigated sites in Vientiane Capital and Vientiane Province of Laos and Chiang Rai and Nan Provinces of Northern Thailand (Figure 24).

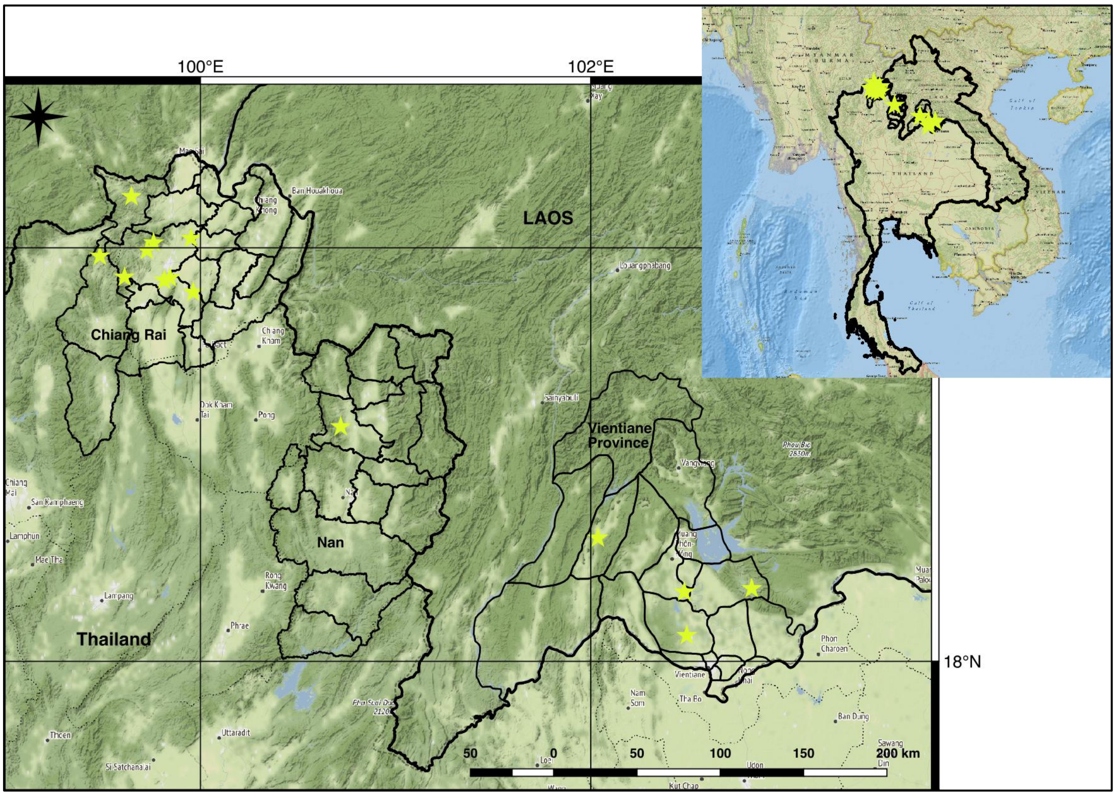


Figure 24 Overview map showing all field sites (yellow star) in Chiang Rai & Nan Provinces, Thailand and Vientiane Province, Laos

Both areas are known to have a high incidence of scrub typhus (see Chapter 1). Sites varied from riverbanks close to Chiang Rai city centre to remote hill tribe villages (Figure 25). Both regions are tropical and characterised by the southwest monsoon, described in more detail in Chapter 4. In the lowlands of both regions, 2 wet rice crops are planted annually. The first is planted June/July and harvested in November/December, soon after which planting is repeated, to be harvested in May/June. Dry (hill) rice is planted and harvested as for the first crop of wet rice. There are 2 to 3 corn (maize) crops annually.

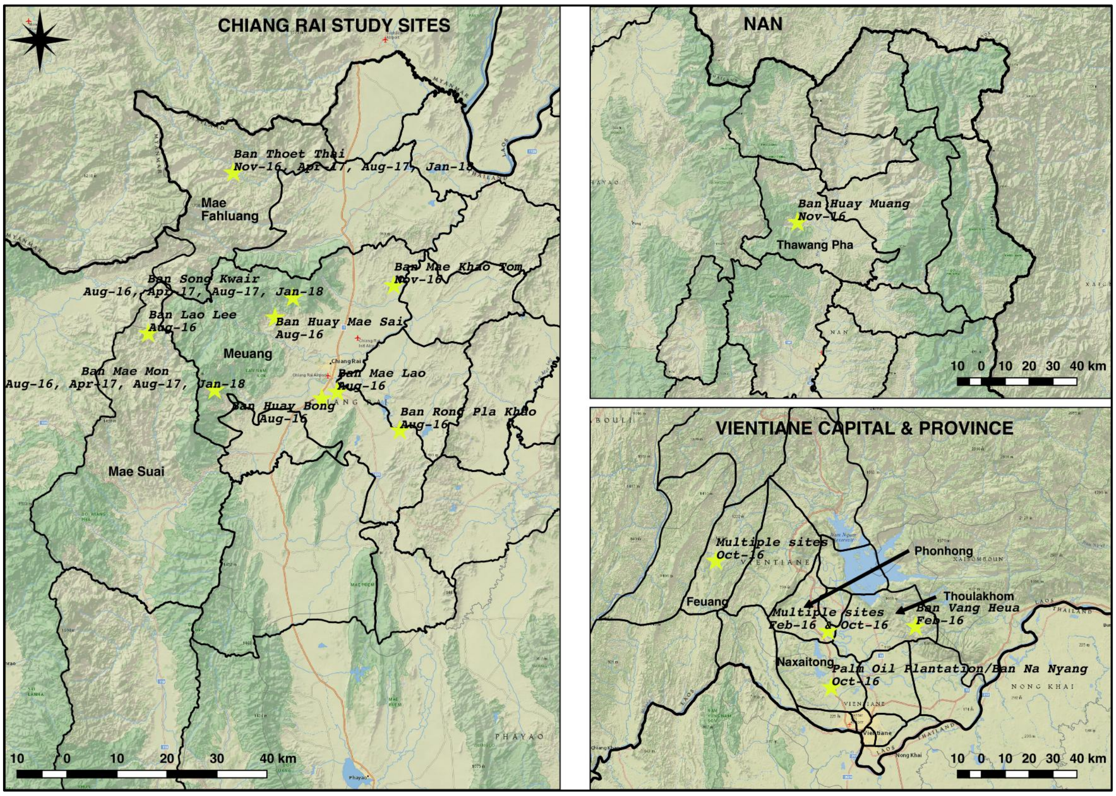


Figure 25 Detailed map showing location and visit dates of all vector/host collection sites

The challenges of identifying a site with high human scrub typhus transmission are described in this chapter. Field methods employed to collect small mammal hosts and chiggers are presented, and the mitigation of risks working in the field discussed. The techniques used to process field samples and the challenges of morphometric speciation of chiggers are explained. Laboratory methods to test human, chigger and rodent samples for the presence of *O. tsutsugamushi* are given together with the results of experiments to optimise the methods. A significant proportion of field samples tested low positive by quantitative PCR (qPCR) and these were further investigated for the possibility of false positives. Results, with a focus on the three villages ultimately selected for repeated sampling, are presented. Results range from rodent and chigger species diversity to *O. tsutsugmaushi* infection rates in vectors and small mammal hosts and chigger infestation rates of small mammals. These data are divided into seasons (corresponding to field visits) and mapped using Geographical Information Systems (GIS) tools. Ecological and habitat analyses are presented in Chapter 4 and whole-genome sequencing of positive samples in Chapter 5.

Findings from this project are compared with studies from Thailand and other countries. The rates of scrub typhus in vectors and hosts are compared and contrasted with sympatric human disease rates and the factors likely to be associated with human infection are explored together with potential public health interventions.

3.2 Study site selection

3.2.1 Identifying high risk sites in Laos

In Laos the only existing data on human scrub typhus cases comes from our laboratory, based in the main hospital (Mahosot Hospital) in the capital city, Vientiane. As part of on-going fever studies, patients presenting with fever and meeting study inclusion criteria were tested for scrub typhus using an IgM-based rapid diagnostic immunochromatographic test (RDT). The home village of RDT positive cases were recorded and mapped using QGIS (QGIS Development Team 2017. QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org). Over 1,300 cases have been recorded since August 2003. Villages were selected for attempted small mammal trapping based on their cumulative number of cases, their location and accessibility, the range of habitat types and the availability of local hunters to assist with trapping.

3.2.1.1 Pilot studies in Laos

A pilot visit took place prior to my arrival in Laos, between 12th and 20th May 2015 during the hottest period of the year and the early rainy season.

A second collection took place in November 2015 (end of the rainy season) at 6 sites, selected using the same criteria described. On the second visit only free-living chiggers were collected using black-plates and from soil using Berlese funnels (see methods).

3.2.2 Case finding and site investigation in Thailand

Unfortunately all vectors and hosts collected in Laos up to May 2016 tested PCR negative for *O. tsutsugamushi* and alternative sites were investigated. At this time a study was taking place in Chiang Rai Province, Thailand at a sister research unit (Chiang Rai Clinical Research Unit, CCRU). The study was investigating paediatric scrub typhus cases and by June 2016 had recruited over 50 patients with strong evidence (many with eschars and testing PCR positive) of recent infection. It was decided to investigate the epidemiology of these cases to identify potential high-risk sites.

Together with a research nurse from CCRU, selected patients were visited at home, often in relatively remote and difficult to access sites to the north and west of Chiang Rai. We met with the patient's family, and where possible the child, in an attempt to understand possible exposure sites during the 2-week period prior to symptom onset. This necessitated a careful review of the child's movements including route to school, areas where he/she played, accompaniment of adults to fields/plantations, weekend excursions and other activities. Time was taken to visit exposure sites by car or on foot and GPS points recorded. The identification of overlapping exposure sites and places with strong evidence of exposure was of particular epidemiological interest.

3.3 Field materials and methods

3.3.1 Field work health and safety

Careful planning and risk assessment was performed prior to each field visit, in accordance with the University of Oxford policy statement S5/07 "Safety in Fieldwork". All field trips were made together with local staff and necessary permissions sought from appropriate government departments in advance.

All members of the field team were fully informed of the nature of the work and appropriate training provided. Any medical problems that could affect their ability to carry out their work were identified and risks of the work discussed.

General fieldwork risks associated with travel and working in rural areas and rough terrain were reviewed. In Laos areas northeast of Vientiane Province were avoided due to an unstable security situation.

Specific fieldwork related risks include infection from ectoparasites (including chiggers), snake bites and other envenomation, rodent bites, accidental contamination from small mammal blood and urine, accidental inhalation of inhalational anaesthetics used to euthanize small mammals, sharps risk from needles used for rodent cardiac blood collection and cold injury from dry ice.

To reduce the risk of ectoparasite bites, DEET was provided and team members advised to thoroughly wash their skin and hair with detergent every evening. Long boots were worn in the field. Rodent traps were handled with care and euthanized animals handled with gloves and while wearing a mask. Isoflurane was used only in open, well-ventilated areas. Needles were transferred directly into a sharps bin after use. Dry ice was handled with gloves and when transported in the vehicle the boxes were carefully sealed and sufficient ventilation maintained in the vehicle.

3.3.2 Small mammal trapping

Live trapping of small mammals took place at all study sites. Live trapping is essential, as many ectoparasites will rapidly detach from an animal once dead. Additionally, the collection of blood by cardiac puncture can only be performed immediately after the animal is killed.

Sturdy, locally made wire-mesh traps of 2 sizes (30x15x15cm and 40x20x20cm) were purchased and transported to field sites (Figure 26). Traps were baited with pieces of corn, cassava or green banana, as these tend not to attract ants and last 3 trapping nights. Hunters used many other types of bait including dried fish, snails and sticky (glutinous) rice. Traps were placed at intervals of about 10m in lines of 10 traps across the trapping site. Trap lines were placed in an attempt to cover the range of habitats present at each study site and in areas of likely rodent activity. Some sites covered areas of 9 square kilometres. Traps were deployed in late afternoon and checked daily soon after dawn, as most target animals are nocturnal. Traps were set for 3 nights and when sites were revisited, attempts were made to place traps in the same locations. Approximately 130 traps were set for each 3-day collection. When non-target animals were accidentally trapped, these were released immediately at the same location. During this study one large-toothed ferret-badger, *Melolgale personata* and one bird were accidentally trapped.

At several study sites we engaged local hunters and villagers to assist with trapping. Hunters are skilled in identifying areas of small mammal activity, thus much increasing trap success rates. At the majority of the study sites, villagers regularly hunt and consume larger rodent species, tree shrews and squirrels. Willing hunters were provided with a small number of traps and asked to trap in the vicinity of the village. Hunters were paid for successful capture, with prices varying depending on the size of the species. At some sites villagers also set traps around their homes and food stores.

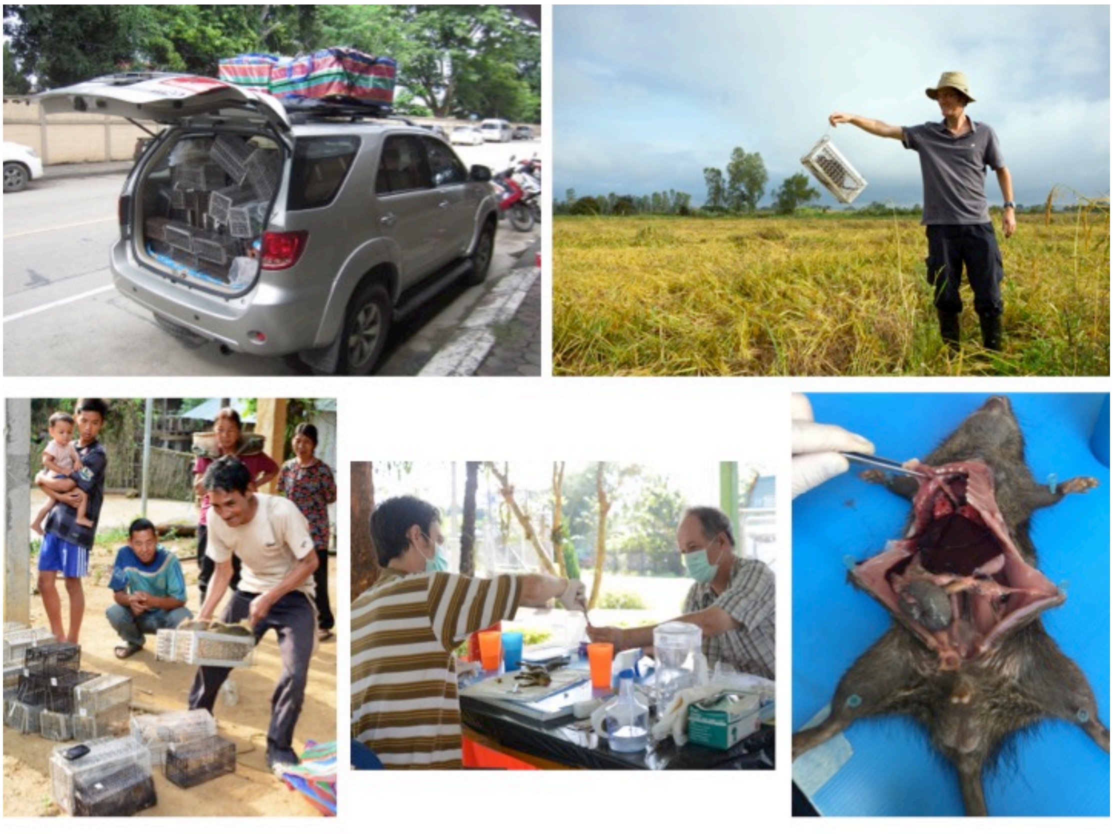


Figure 26 Clockwise from top left: vehicle for fieldwork loaded with equipment and traps; author with wire mesh trap with *Bandicota indica* in rice fields, Chiang Rai Province; *B. indica* dissection; field laboratory with author (left) and Dr Serge Morand (right); hunter presenting trapped animals in village.

3.3.3 Location recording and habitat description

The location of all collected field specimens (small mammals and free-living chiggers) and trap lines were recorded using a Garmin Oregon 650 (Garmin International, Olathe, Kansas) global positioning system (GPS) device. Device accuracy was usually to 3 metres, but in some steep-sided valleys decreased to 7-10m.

Every trap was tagged with a unique number and this was recorded with the GPS waypoint. The device also contains an in-built camera that allows photographs of the immediate area to be taken and linked to the waypoint. Free-living chiggers and soil samples were collected into numbered tubes/bags and similarly recorded.

Successful trapping sites were recorded as accurately as possible. Hunters and villagers were asked to mark the position of trapped animals. We then attempted to return to those places, accompanied by the hunter and record the GPS point as accurately as possible. Accuracy was recorded on a scale: 1 = <10m, 2 = <100m, 3 = <1,000m and 4 = >1,000m.

Habitat description was made at every study site and is described in Chapter 4.

3.3.4 Small mammal euthanasia, identification and processing

Traps containing small mammals were brought to a mobile laboratory directly from the field. This was set up in a suitable covered but well ventilated area, away from any public thoroughfare. In Thailand, arrangements were made with the local Primary Care Unit to provide some space with tables and chairs. In Laos, we used secluded areas of guesthouses by arrangement. Animals were kept in a quiet, shady place to minimize stress.

Traps containing small mammals were placed in an airtight box with the addition of cotton wool soaked with 10ml of the inhalational anaesthetic isoflurane [288]. Animals were only removed from the trap once dead.

All animal-handling procedures followed the American Society of Mammologists, 2016 guidelines [289]. Procedures were additionally compliant with the 2010 EU directive on the protection of animals used for scientific purposes (2010/63/EU). Small mammal euthanasia followed the 2013 guidelines of the

American Veterinary Medical Association Council on Research [288]. All field and mobile laboratory protocols and procedures followed the "Protocols for field and laboratory rodent studies" 2011 guide [290].

This study was approved by the Oxford Tropical Research Ethics Committee (OxTREC 48-15 for Laos and 52-14 for Chiang Rai) and Lao National Ethics Committee for Health Research (039/2016 NEHCR).

All small mammals were weighed in grams and measured in millimetres using a ruler or Vernier calipers. The following length measurements were made: head and body, tail, hind foot, ear and skull. The animal was sexed and for females the pattern of mammary teats recorded. The stage of sexual maturity was noted. For males the length of the testes and degree of seminal vesicle development and for pregnant females the number of embryos was also recorded [290].

Using a combination of morphological features and measurements, small mammals were identified to species level wherever possible following the keys in Chaval 2011 [291] and Francis, 2008 [292]. Photographs and key features of species trapped in this project are given in Appendix B-1. Some juvenile specimens and some individuals of certain genera (e.g. *Maxomys*, *Niviventer* and *Rattus*) that have complex and incompletely understood taxonomy could only be recorded to the genus level. Research is continuing to better understand their taxonomy, and skulls were preserved for morphometric analysis and liver tissue for DNA barcoding by sequencing the conserved mitochondrial cytochrome c oxidase 1 (COI) gene. Unfortunately the results of this work, being undertaken by collaborators, were not available at the time of writing.

Full details of the dissection protocol are available at http://www.ceropath.org/FichiersComplementaires/Herbreteau_Rodents_protocols_2011.pdf. Briefly, the animal was pinned ventral side up and cardiac puncture performed using a 26G needle, passed under the sternum in the midline. Blood was transferred into an EDTA tube and stored at +4°C. The animal was then carefully examined for ectoparasites and these collected as detailed below.

The ventrum was cleaned with 70% ethanol and then the body cavity opened using blunt-ended scissors from just anterior to the genital opening as far as the anterior end of the thoracic cavity and the skin pinned open. Prior to harvesting tissues, scissors and forceps were thoroughly washed consecutively in Dettol, water and then 70% ethanol. Small pieces (>100mg) of lung, liver and spleen were collected into pre-labeled cryotubes and these stored on dry ice in the field and subsequently at -80°C in the laboratory.

Other samples were collected for separate projects including kidney and the gastrointestinal tract. The skull was removed and preserved in ethanol for morphometric analysis. Any endoparasites were also stored for identification.

All surfaces were thoroughly decontaminated prior to dissecting the next animal.

3.3.5 Chigger removal from small mammals

Each animal was carefully inspected with the naked eye for chiggers, usually seen as 2-3mm clusters deep in the conchae of the ears (Figure 27). Both ears were detached as close to the skull as possible using scissors and stored in 70%

ethanol at +4°C, for examination in the laboratory. In certain species, chiggers are more frequently found on other parts of the body. On *Suncus murinus* (house shrew) hairless parts on the posterior legs, whereas in tree shrews, ground squirrels and arboreal squirrels, the anogenital region and along the ventral midline are often heavily infested. The skin containing attached chiggers was carefully dissected away and placed into 70% ethanol.

Figure 27 Clockwise from top left: Orange cluster of chiggers in ear of *Maxomys surifer*; cluster of feeding chiggers (scale bar 0.5mm); single chigger on the tip of cactus spine attached to stick used to remove attached chiggers (x10); rodent ear held open with needles for examination of presence of chiggers under stereo microscope.

In the laboratory the ears or skin was removed from the tubes and placed on a thin foam block wrapped in clean tissue paper. The ears were carefully cut open to expose the conchae and crevices where chiggers are typically located. The ears were then pinned open using 30G needles attached to 2ml syringes (Figure 27).

Ears were then examined under a Brunel IMXZ stereo microscope (Brunel Microscopes Ltd., Chippenham, U.K.) at 15x to 100x magnification. The total number of chiggers per animal were counted, the number of separate clusters counted and the colours of chiggers recorded.

Where numbers allowed, a representative set of 3 chiggers per animal were collected into separate, labelled Eppendorf tubes containing 70% ethanol. These were selected from different clusters and of different colours in an attempt to capture the diversity of species present on the host.

To facilitate collection, homemade devices consisting of a thin stick (3-4mm in diameter) with a single toothbrush bristle or very fine cactus spine glued to the tip were used (Figure 27). Chiggers not firmly attached to the host were dislodged using the bristle; those well attached were removed with the aid of the cactus spine. Care was taken not to damage the chigger as this could leak body cavity contents and reduce the opportunity to detect *O. tsutsugamushi* and allow species identification.

The remainder of the chiggers attached were collected into pools of ~30 individuals in separate Eppendorf tubes. The blunt side of a sterile fine scalpel blade was sometimes used to dislodge groups of well-attached chiggers.

All individuals in pools were counted to record the total number present.

3.3.6 Free-living chigger collection

Two techniques were used to collect free-living chiggers in the environment.

1. Black plate method [293,294]. Small formica plates 10x30cm and ~0.5cm thick were placed on the ground or inserted into holes and possible animal burrows (Figure 28). Plates were placed in groups of 10 to 20, each separated by a few centimetres. The field worker then exhaled over the area for about 1 minute to stimulate chigger questing activity as recommended by Uchikawa et al. [294]. After 5 minutes, plates were examined carefully. A hand lens was used when needed. The size, colour, rate and pattern of movement help the experienced observer determine if a chigger is present. Suspected chiggers were killed with a drop of 70% ethanol and transferred to an Eppendorf tube using any fine tipped object e.g. fine brush or blade of grass.
2. Berlese or Tullgren funnel method [295]. Soil samples consisting of the top 5-10cm of soil in a 20x20cm quadrat were collected together with overlying leaf litter. These were placed into sturdy plastic bags and transported to the mobile laboratory. Locally made Berlese funnels were used and 12 positioned together on a supporting platform (Figure 28). Soil was placed carefully onto the fine mesh base and a 50ml conical tube containing 70% ethanol attached underneath. All joins were sealed with tape and the lid attached containing the light source. Lights were kept on for 72 hours, at which point the 50ml tubes were removed. The area was roped off with warning signs. Under the stereo microscope, the tubes were poured into glass petri dishes and examined for 5-10 minutes each to identify any chiggers among the rich diversity of soil fauna present.

Figure 28 Clockwise from top left: Examining black plates for free-living chiggers with the aid of a hand lens; black plate inserted into rodent burrow; Berlese funnels containing soil samples in operation; single chigger crawling over 1 Baht coin.

3.3.7 Human samples

PCR positive scrub typhus EDTA buffy coat and whole blood samples stored in Laos and paediatric cases recruited in the Chiang Rai study were retrieved and sent to the Wellcome Centre for Human Genetics, Oxford. These were selected if: the sample could be found with sufficient volume, the Ct value was less than 40, and where possible paired blood and eschar samples were available. Results are discussed in Chapter 5.

3.4 Laboratory materials and methods

3.4.1 Chigger identification

The identification of chiggers is a complex and challenging task. The chigger is first removed from the tube using a Pasteur pipette with the naked eye or with the use of a hand lens. The chigger is placed between two cover slips on a glass slide and examined at low power (100x) to confirm the presence of a trombiculid mite. Key features include the presence of 3 pairs of legs and a scutum or dorsal shield-like structure.

Identification to species was performed using a compound microscope - the Nikon Eclipse 80i and images viewed and saved using the Nikon NIS Elements D 4.13.05 software package. A scale bar was applied to each image. All morphometric measurements and image manipulation was performed using the open-source software ImageJ (https://imagej.net/ImageJ). Magnification lenses of 400x and 600x were used. All images were obtained using both a mercury lamp UV light (without a filter) to make use of the autofluorescent properties of the chigger scutum, integument and appendages or using a bright-field halogen lamp.

Recent work by Kumlert et al. describes methods for autofluorescence and bright-field microscopy to identify chiggers [209]. To fully identify and document the species a set of 16 photographs is required, however some of these are multi-layer photographs that must be stitched together using photographic software.

The shape of the scutum and scutal setae (sensory "hairs") allows the subfamily and tribe of chigger to be identified. With some simple measurements and reference to Nadchatram & Dohany's 1974 key to Southeast Asian chigger genera the genus can usually be reliably identified [296]. To identify the chigger to species level a further set of characteristics must be recorded. These vary between genera, but typically include scutal and sensillary measurements, dorsal and ventral setae number and pattern, leg length, the number and pattern of coxal setae, specialised leg setae, palpal chaetotaxy and chelicerae [209]. Some of these features are more easily recorded whilst examining the specimen under the microscope. As a minimum, the scutum, legs and dorsal/ventral setae were photographed.

Unfortunately resources for the taxonomic identification of trombiculid mites are relatively few, and focused entirely on the larval stage with no existing keys for Southeast Asian nymphs and adults. Several different keys are available; many are decades old and frequently out-dated and incomplete. An exception is Stekolnikov's 2013 key to the *Leptotrombidium* of the world [210].

In this study identification of chigger species was made using defining characteristics and reference keys listed in Appendix B-2. Morphometrics were also compared with data presented by Dr Kittipong Chaisiri, for chiggers species collected in Thailand as part of his PhD thesis [297]. Where identification was inconclusive, a full set of photographs were taken and second opinions sought from Dr Rawadee Kumlert or Dr Chaisiri, both of Mahidol University, Bangkok. Suspect adult trombiculids were photographed and the images reviewed by Professor Joanna Mąkol, Wroclaw University, Poland.

3.4.1.1 Taxonomic characteristics

The Trombiculidae are one of the largest families in the Acari group, with at least 3,000 species. In Southeast Asia there are over 37 genera and 13 subgenera and in Thailand alone 93 species are currently recognized [296,298]. To morphologically identify chiggers, an understanding of taxonomic characteristics is required and described briefly herewith. More detailed descriptions can be found in Vercammen-Grandjean, 1968 and Nadchatram & Dohany, 1974 [296,299]. A list of common abbreviations is provided in the glossary of abbreviations in Appendix B-2.

3.4.1.1.1 Idiosome

The subspherical body or idiosome has a soft external integument capable, in some species, of expanding by up to 1,000 times during engorgement. Inserted into the integument on both dorsal and ventral surfaces are setae in a variety of forms. One or more pairs of humeral setae are present anteriorly on the dorsum, followed by rows of dorsal setae. The number and pattern of dorsal and ventral setae can be converted into a formula. For example in *Leptotrombidium deliense*, the following pattern is seen: fD = 2H (humeral) + 8.6.6.4.2 (28) and fV = 6.4.4.4.2 (20) giving a total number (NDV) of 48 (Figure 29). Rudimentary eyes are often visible (particularly under UV light) immediately lateral to the scutum. A urogenital opening can also be observed, though this has no taxonomic use.

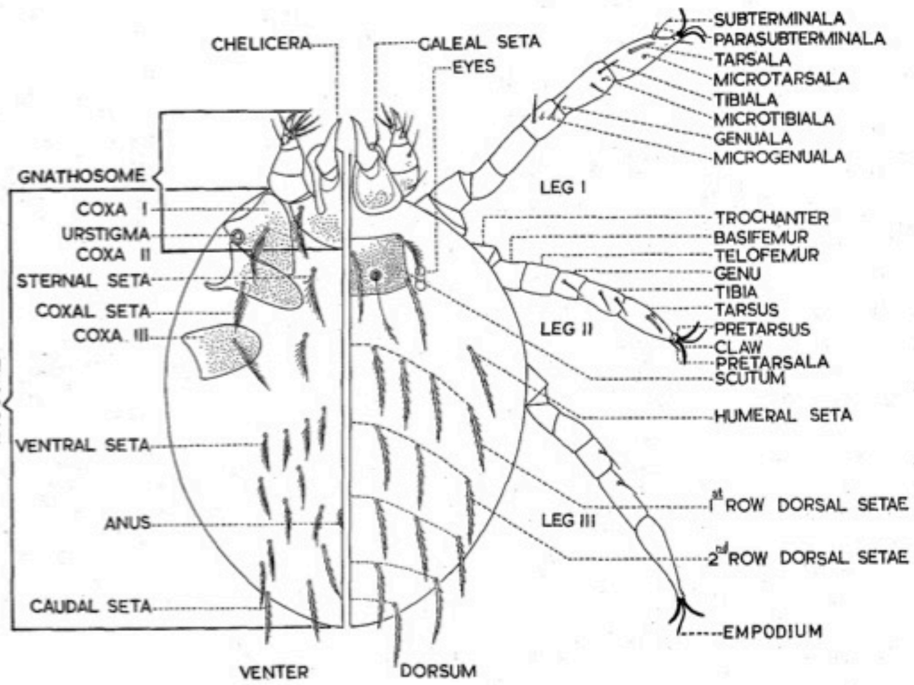


PLATE 1. Dorsal and ventral aspects of *Leptotrombidium deliense* (Walch).

Figure 29 Dorsal and ventral features of a chigger. Taken from Nadchatram & Dohany, 1974

3.4.1.1.2 Scutum

The scutum is the most important anatomical feature for taxonomic identification. The shape and dimensions of the scutum and anterior and posterior setae must be recorded. One pair of pseudostigmatic organs or sensillae is attached to sensillary bases. These organs may be slender or expanded. A scutal formula (fSc) compares the length of anterolateral (AL), anteromedian (AM) and posterolateral (PL) setae. For example, in *L. deliense* the pattern fSc = PL>AM>AL is seen. In some groups such as the *Gahrliepiinae* with elongated scuta, additional "usurped" setae may be present on the scutum, thought to have become incorporated from midline dorsal setae (Figure 29, Figure 31).

3.4.1.1.3 Gnathosome

The gnathosome comprises the head and mouthparts of the chigger (Figure 30). The pedipalp or "palp" is a sensory organ at the tip of which palpal claws are present for gripping the host. The cheliceral blades are inserted into the host prior to feeding. The galea is situated ventral to the chelicerae. The pattern of setae attached to the palps and galea and whether they are barbed or nude allows the construction of a palpal formula fPp. For example in *L. deliense* the fPp = N/N/BNN + 7B and Ga =1B (where N = nude, B = barbed and Ga = galea). The order in the formula runs: femoral, genual, dorsotibial, dorsolateral, tibial, ventrotibial and the galeal setae (Figure 30). The calculation of this formula is particularly challenging and often hampered by poor microscopic visualisation, especially in feeding mites where tissue may obscure these structures. The shape, presence of "teeth" and nature of the cap of the cheliceral blades are also important features.

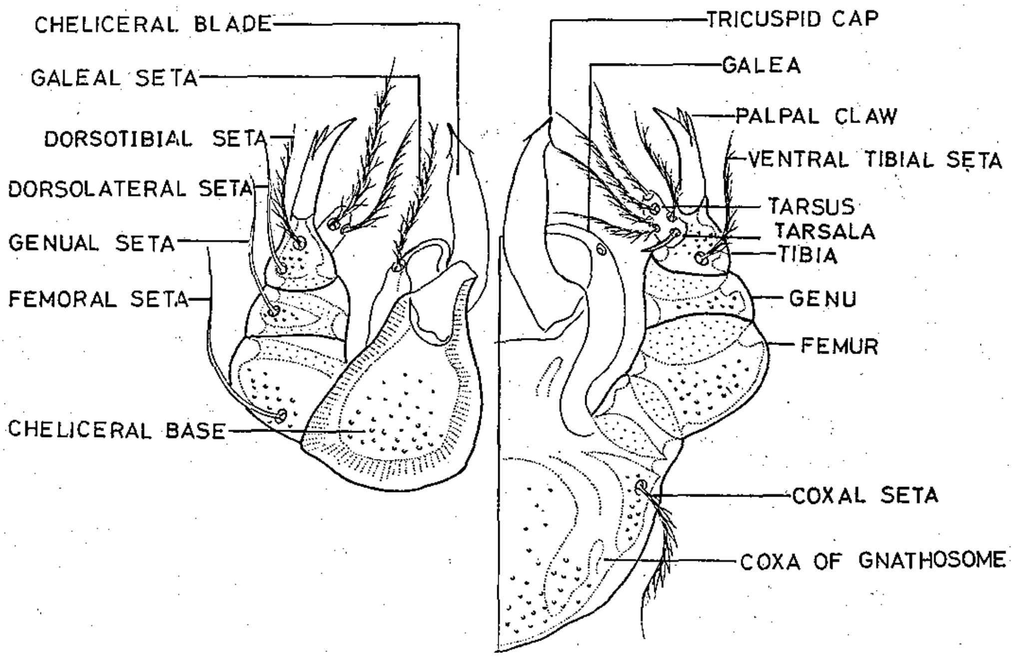


Figure 30 Anatomical details of the chigger gnathosome. Taken from Nadchatram & Dohany, 1974

3.4.1.1.4 Legs

Trombiculid larvae have 3 pairs of legs (while nymphs and adults have 4). These are divided into leg 1 (anterior or Pa), leg 2 (median or Pm) and leg 3 (posterior or Pp). Legs are usually 7-segmented but in the subfamily Leuwenhoekiinae legs are 6-segmented. Legs have 2 types of setae: barbed ordinary setae present on each segment and specialised sensory (nude) setae. The latter are given specific names such as “genuala” and “tibiala” and can be useful to determine the species. The legs end with a pair of claws and a median slender claw-like empodium. The relative sizes of the 2 true claws help distinguish certain species, particularly in the genus *Walchia*. At the articulating end of the leg a coxal segment is present. The pattern and number of coxal setae can also be crucial diagnostic information in the genera *Walchia* and *Helenicula*.

3.4.1.1.5 Morphometry

The morphometric analysis of certain features of chiggers is critical to identifying the species. The distance between the AL setae (AW), the PL setae (PW), between AL and PL (AP) and between the sensillary bases (SB) are measured together with the distance from the anterior scutal margin to the mid-sensillary base (ASB) and the from the mid-sensillary base to the posterior margin (PSB) (Figure 31). The length of scutal setae and width of sensillae (if expanded) should also be measured. The length of each leg and combined leg length (Ip) should be recorded. Note that a range of measurements is found between individuals of the same species.

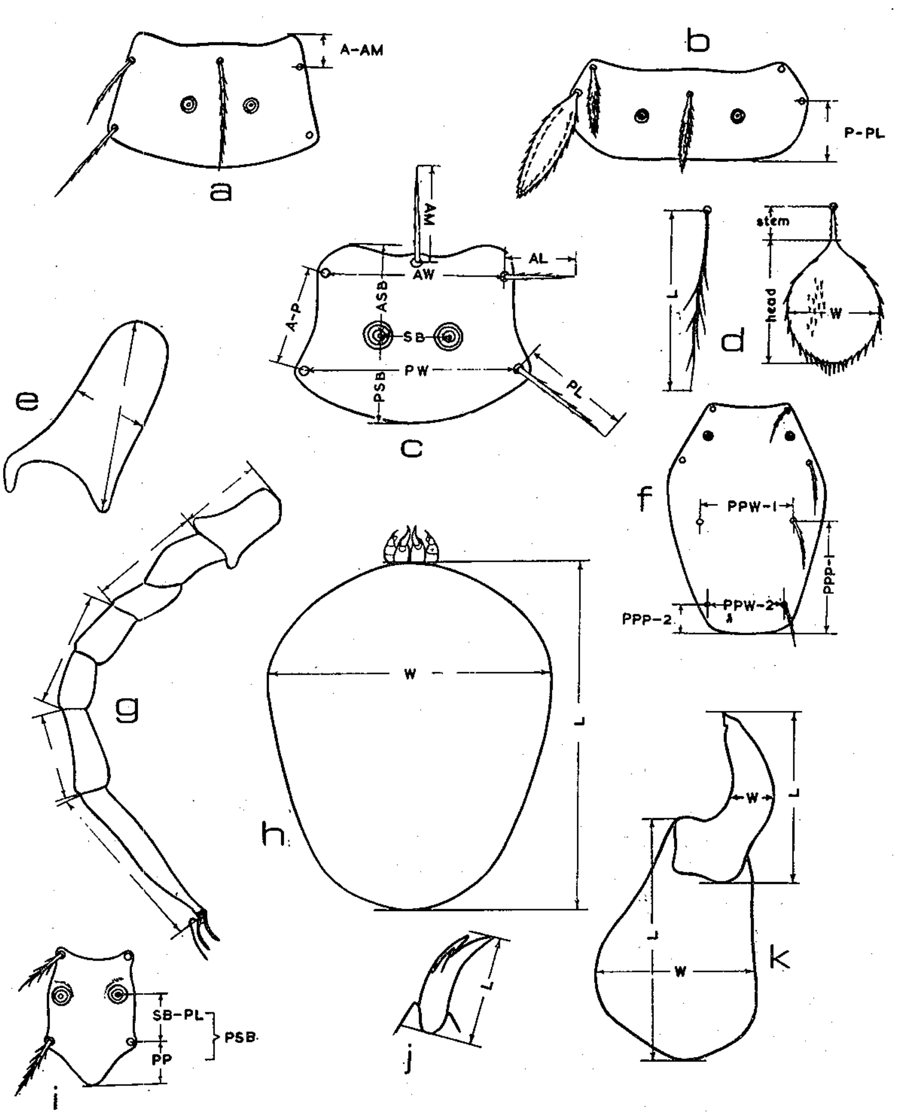


PLATE 6. Method of Measuring

Figure 31 Chigger morphometry. Scutum (a,b,c,f,i); sensilla (d); coxa (e); leg (g); body (h); chelicera (j); palp (k). Taken from Nadchatram & Dohany, 1974.

3.4.2 Chigger, rodent and human tissue DNA extraction

DNA was extracted from individual chiggers, pools of chiggers, rodent tissues and human samples using the Qiagen Blood and Tissue Kit (Qiagen, USA). The following procedures were followed prior to protein digestion.

Individual chiggers were removed with a pipette and placed on a glass slide with a black background to aid visualization. The ethanol was soaked up with a tissue paper and the chigger rinsed twice with distilled water, soaking up the fluid with tissue paper each time. The chigger was then resuspended in 90 µl buffer ATL (Qiagen). Under a stereo microscope, the chigger was cut through the mid-gut using a sterile 30G needle attached to a syringe.

For pools of chiggers, the tube was briefly centrifuged and as much ethanol removed as possible. The tube was the allowed to air dry for several minutes before being washed twice with distilled water, each time the tube was air-dried using a heating block at 50°C. Then 30 µl of ATL buffer was added and chiggers crushed using a sterile polypropylene motorized pestle (Motorized pellet pestle Z35991, Sigma Aldrich, USA). The pestle was then rinsed with 60 µl of buffer ATL.

Rodent tissues were cut into a small piece (≤10mg of spleen or ≤25mg of liver or lung) and weighed on a precision balance (0.1 mg accuracy) before adding 180 µl of buffer ATL. The tissue was cut into multiple pieces using sterilised fine tipped dissecting scissors.

Buffy coat or whole blood was extracted from a starting volume of 200 µl. Eschars were collected either as pieces of crust in 70% ethanol or swabs. Crusts were transferred into 200 µl ATL buffer. Swabs were thoroughly mixed with the ethanol, centrifuged at 20,000 rpm for 20 minutes and the supernatant removed, and the pellet was resuspended in ATL buffer.

Thereafter 20 µl of proteinase K was added and the samples thoroughly mixed. Chigger, rodent and eschar swabs were incubated at 56°C for 3 hours, ensuring all tissue was completely digested. Whole blood and buffy coat was incubated for

1 hour and eschar crust was incubated overnight. The rest of the steps followed the manufacturer's protocol. Chigger samples were eluted in 45 µl, while rodent and human samples in 100 µl of buffer AE. Samples were stored at -20°C before PCR.

3.4.3 Real-time, conventional and nested PCR for *O. tsutsugamushi*

Quantitative real-time PCR targeting the 47 kDa *O. tsutsugamushi* outer-membrane protein was performed on all rodent, chigger and human samples [300]. The primer and probe sequences are shown in Table 10.

Table 10 *Orientia tsutsugamushi* forward and reverse primer and probe sequences for the 47 kDa outer membrane protein target

Name	**Sequence (5'->3')**
OtsuFP630	AACTGATTTTATTCAAACTAATGCTGCT
OtsuRP747	TATGCCTGAGTAAGATACRTGAATRGAATT
OtsuPR665	6-FAM-TGGGTAGCTTTGGTGGACCGATGTTTAATCT-TAMRA

A PCR master mix was prepared by combining the following reagent volumes per sample: 15 µl of Platinum PCR Supermix UDG (Sigma Aldrich, USA), 0.25 µl each of Forward and Reverse Primers (10 µM) and 0.5 µl of Probe (10 µM). For chigger samples 4 µl of sterile water and 5 µl of DNA was added. For rodent and human samples 8 µl of sterile water and 1 µl of DNA added to complete the Master Mix. PCR was run with the following conditions: 2 minutes at 50°C, then denaturation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Real-time PCR was performed on a Bio Rad CFX96 (Bio Rad, USA). Pre-prepared in-house quantitative standards were used. Duplicate 10-fold concentrations from 10^0 to 10^6 (1 µl each) and two no-template controls were included on every run.

Nested PCR was performed on a small number of samples investigated for low positive qPCR results. Outer and inner primers of the 56 kDa outer membrane protein were used and the PCR products run on 1.2% agarose gel by electrophoresis. Ethidium bromide was used to stain DNA for visualization.

3.4.4 Rodent serum immunofluorescence antibody testing

Scrub typhus antigen-coated Teflon-slides were purchased from the Australian Rickettsial Reference Laboratory (ARRL), Geelong, Australia. The slide was allowed to dry at room temperature whilst two-fold serial dilutions of rodent serum from 1:16 to 1:4096 were prepared in 2% skim milk in phosphate buffered saline (PBS) diluent. 2 µl of diluted serum was dropped onto each well and incubated at 37°C for 30 minutes. The slide was then washed four times with PBS, dried and 2 µl of diluted FITC anti-mouse conjugate added to each well. The incubation and washing steps were then repeated and fluorescence mounting medium added to the slide. An experienced technician examined the slide to determine the titre of positive samples.

3.4.5 Human scrub typhus IgM rapid diagnostic testing

Several different rapid diagnostic tests were used for the detection of scrub typhus IgM in this project (Access Bio *CareStart* Scrub Typhus test (Somerset, NJ, USA), SD BIOLINE Tsutsugamushi test (Kyonggi-do, South Korea) and InBios Scrub Typhus Rapid Detect (Seattle, WA, USA). An aliquot of serum is added to the test strip and a positive result read after 10-15 minutes. The exact protocol varies between manufacturers, as does the sensitivity and specificity of the test. All tests used followed the manufacturer's protocol.

3.4.6 Sanger sequencing of PCR products

For the investigation of low positive, high cycle threshold (Ct) real-time qPCR results, a small subset of samples were sent for Sanger sequencing to a commercial company, Macrogen Korea https://dna.macrogen.com. The nested 56 kDa PCR products were sequenced and the sequence files delivered electronically for analysis.

3.4.7 Data management and analysis

Most field and all laboratory data was entered on to a purpose-built Microsoft Access database. Data analysis and statistical tests were performed using Stata v15 (StataCorp, College Station, TX).

3.5 Results

3.5.1 Pilot field studies in Laos (2015)

In the first pilot study of May 2015, 15 villages were selected across Vientiane Province in Phonhong and Thoulakhom Districts (Figure 32). All selected villages had at least 3 reported human scrub typhus cases. All trapping was performed using local hunters. A total of 71 small mammals of 9 species were trapped and spleen tissue alone tested for *O. tsutsugamushi* by PCR. All tested negative. 26/71

(37%) animals were infested with chiggers. In total 288 chiggers were removed for identification and 125 of these were tested individually for *O. tsutsugamushi* – all testing negative. Chiggers were of 13 species in 7 genera. Of the total number of chiggers, 50/288 (17%) were identified as *L. deliense* and 17 of these were tested, and all were negative.

The second visit, collecting free-living chiggers at 6 sites resulted in 110 chiggers, 109 of which were identified as *Schoengastia kanhaensis* and all tested negative. This work was carried out as part of the PhD thesis of Dr Rawadee Kumlert, Mahidol University, Bangkok [301].

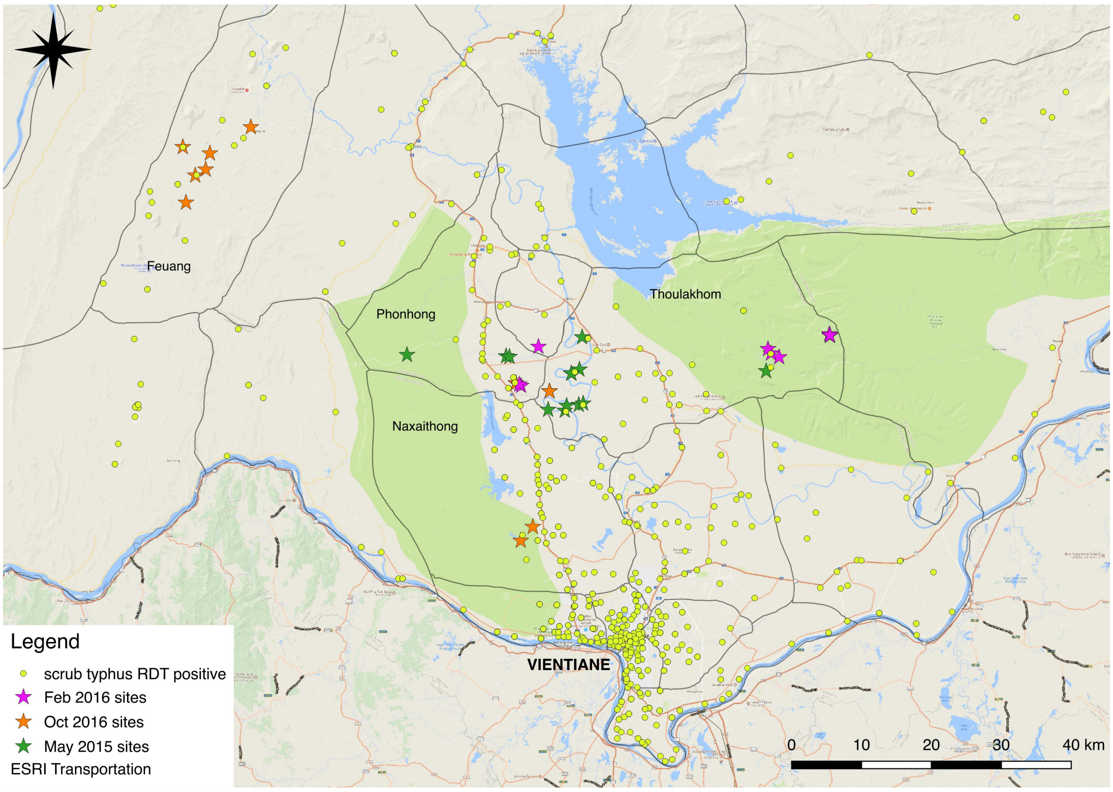


Figure 32 The home village of human scrub typhus RDT positive cases (2003-17) and vector/host trapping sites 2015-16.

3.5.2 Further investigations in Laos (February 2016)

Following my arrival in Laos in January 2016, with no *O. tsutsugamushi* positive rodents or chiggers to date, a further field trip took place in late February 2016. On this occasion, I selected two areas based on successful rodent trapping in May 2015, recent human scrub typhus cases from the villages and the presence of *Leptotrombidium* chiggers (known scrub typhus vectors) on rodents trapped there previously. Additionally, the 2 sites differed in habitat – one being in hills with degraded forest between 740 and 840m above mean sea level (asl) and the other a flat area of rice fields in a river flood plain at 175m asl (Figure 32).

A total of 44 small mammals of 12 species were trapped, 26 from the upland and 18 from the lowland. Lung, spleen and liver tested negative for *O. tsutsugamushi* by PCR for all animals. 34 (79%) of the animals were infested with chiggers, with an overall chigger index (mean number of chiggers per animal) of 33 (range 0-126). Chiggers of 13 species were recorded, 6 of which were *Walchia* species. Only a single *L. deliense* was identified from a *Niviventer* sp. in the upland site. 88 individual chiggers and 30 pools (a total of 614 chiggers) tested negative. Attempts at collecting chiggers with black plates and from soil were unsuccessful.

3.5.3 Study site selection in Thailand (June 2016)

Between July 2015 and June 2016, 53 scrub typhus patients had been enrolled in the study at CCRU. Of these, 45 were successfully contacted and 31 were investigated for possible follow-up. Twenty patients were selected for follow up at home on the basis of: willingness to participate, more than 1 case in the village and likely exposure close to home. Following these epidemiological investigations, 8 sites were selected for small mammal and/or free-living chigger collection (Figure 33).

Following the first field collection in Chiang Rai Province in August 2016, laboratory testing of rodent spleen and a proportion of chiggers yielded no positives (although subsequent completion of sample testing resulted in a small number of positives).

As a result, on the next field visit in November 2016, we visited 2 new sites. The village of Thoet Thai in Mae Fahluang District was the home of a very recent patient and had been a site of successful rodent trapping for the provincial Vector Borne Disease Control Unit, whom we hired to assist with trapping on this occasion. Mae Fahluang District also has one of the highest incidence rates of human scrub typhus in Chiang Rai Province (and in the whole of Thailand) according to national statistical data (see Chapter 1). The second site was Ban Huay Muang, Tha Wang Pha District, Nan Province, some 3 hours drive to the southeast of Chiang Rai. This village has been a study site used previously by colleagues Serge Morand and Kittipong Chaisiri and was reported to have 17% (51/285) *O. tsutsugamushi* positive chiggers from rodents trapped in 2012-13 (unpublished data) (Figure 33, Figure 25).

By January 2017, the results of laboratory analyses over preceding months had identified positive vectors and/or hosts at 4 sites in Chiang Rai Province: Ban Thoet Thai, Mae Fahluang District and Ban Mae Mon, Ban Song Kwair and Ban

Mae Khao Tom, Meuang District. Samples from Ban Huay Muang, Tha Wang Pha District, Nan Province were also positive.

Three sites in Chiang Rai Province were ultimately selected as they were all reasonably accessible from CCRU and each had different ecological characteristics.

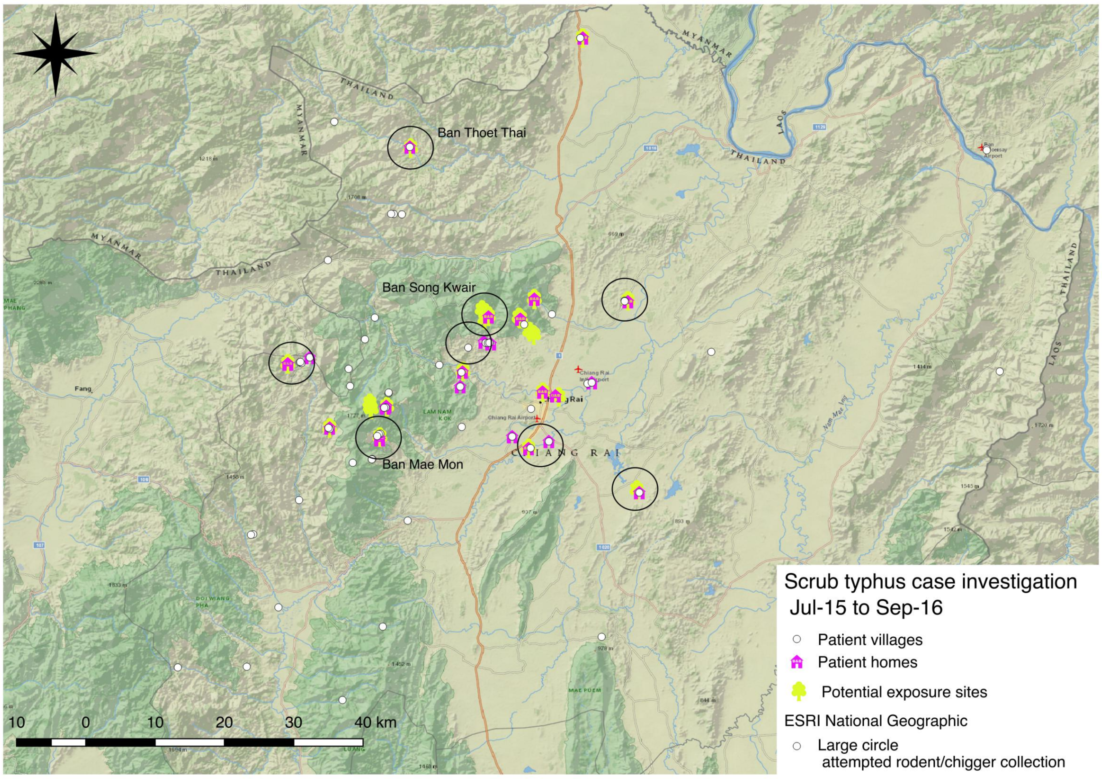


Figure 33 Paediatric scrub typhus epidemiological investigation and study site selection. The 3 main study sites: Ban Thoet Thai, Ban Song Kwair and Ban Mae Mon are labelled.

3.5.3.1 Study site description

Ban Thoet Thai (BTT) is a village of ~5,000 people located in the northwest of Chiang Rai Province, a few kilometres from the Myanmar border. The town has a mix of hill tribe ethnicities including Shan, Akha, Lahu and Lisu. The village was famous for being the home of Khun Sa, a Shan warlord involved in the heroin trade during the 1970s and 80s. The study site (550m asl) lies on the edge of the village and encompasses a small shallow river, an area of rice fields about 500m wide, vegetable gardens and an isolated building. There are fallow areas and a patch of secondary forest rising up the hillside that also houses a number of graves.

Ban Mae Mon (BMM) is a village of ~1,500 people at 1,200m asl. A steep road climbs from the valley floor to the southwest of Chiang Rai. This relatively prosperous Akha and Lisu village is focused on growing coffee. Three parts of the village were selected for trapping. Just below the village in coffee and vegetable plantations and fallow areas, among fruit tree plantations and fallow areas and above the village in a community reforestation project.

Ban Song Kwair (BSK) is a relatively poor and isolated Akha village at 650m asl to the northwest of Chiang Rai city. There are ~150 inhabitants living at the end of the valley surrounded by steep hillsides with degraded secondary forest, bamboo and teak plantations. A stream flows along the edge of the village. Dry rice, corn and pineapple fields are planted above and below the village.

3.5.4 Final field investigations in Laos (October 2016)

In light of lessons learned over the preceding 18 months, a further attempt to locate a site of scrub typhus transmission in Laos was made in October 2016. Using the same methods employed in Chiang Rai, 4 PCR positive cases close to Vientiane were investigated. Two sites with strong epidemiological evidence were selected for trapping. At both sites (Palm oil plantation and small village) we failed to trap any rodents. A new site in Feuang District, Vientiane Province was selected for trapping. This relatively isolated rice-growing valley to the northwest had produced 16 cases over the preceding 3 years. Again all our own trap lines were unsuccessful and only 4 *Rattus exulans* were collected from 2 villages. We also returned to Phonhong District, Vientiane Province where rodents had been successfully trapped on 2 previous occasions (Figure 32).

3.5.5 Laboratory methods optimization

A series of experiments were designed to test existing laboratory standard operating procedures (SOP) and investigate specific aspects of these SOPs.

A number of *O. tsutsugamushi*-infected chiggers from the only active chigger colony in existence at the Armed Forces Research Institute for Medical Sciences, Bangkok were kindly provided by Dr Silas Davidson. These included 3 species: *L. deliense*, *L. imphalum* and *L. chiangraiensis*.

3.5.5.1 Standard operating procedure optimization

The optimal method of extracting DNA from an individual chigger is not known. The quantity of DNA in an individual chigger is likely to be low, given the tiny size of a chigger. Two methods were tested in parallel using known positive chiggers of the same species in triplicate. Method 1 punctured the mid-gut with a fine-tipped needle and method 2 crushed the chigger with a pestle. Method 1 gave a mean cycle threshold (Ct) of 29.77 (23,256 copies/μl) and method 2 a Ct of 30.43 (7,024 copies/μl). Analysed with an unpaired *t*-test, $p=0.05$ (CI 0.0003-1.74). The difference between methods was non-significant. As the needle method was practically easier it was used throughout the study.

The optimal elution volume for chiggers was investigated using: 45 µl, 2 x 25 µl (total 50 µl) and the same 45 µl eluted twice. The single elution of 45 µl gave the lowest Ct value of 29.13 (32,220 copies/µl), compared to 30.27 and 30.07 respectively. This was also non-significant. All chigger samples were eluted in 45 µl.

The effect of UV (520 nm wavelength) on *O. tsutsugamushi* DNA in a chigger being examined under a microscope for 5 minutes (during identification) was investigated, as UV is known to damage DNA. One of two 5 µl aliquots of DNA extracted from the same chigger was exposed to UV light for 5 minutes and then both tested by qPCR in parallel. The Ct value of UV exposed DNA was 31.85 versus 31.28 for unexposed. Therefore, UV light was not thought likely to impact the chance of identifying positives.

3.5.6 Investigation of low-positive PCR results

A cut off for qPCR positives was set locally at a Ct of 40.4. Rodent tissues and chiggers (particularly individuals) frequently had Ct values close to the cut off. In order to investigate the potential for false positives due to non-specific PCR binding, a recognized pitfall of qPCR, additional investigation was carried out.

A nested 56 kDa PCR was performed on 2 individual chigger samples (Ct 39.22 & 38.19), 1 rodent spleen tissue (Ct 39.49) and 1 chigger pool (Ct 40.2). The PCR products were sent for Sanger sequencing.

Returned sequences were viewed, trimmed and errors resolved using Applied Biosystems software. Nucleotide sequences were blasted for *O. tsutsugamushi* (TaxID 784) using NCBI BLASTn. Results are shown in Table 11.

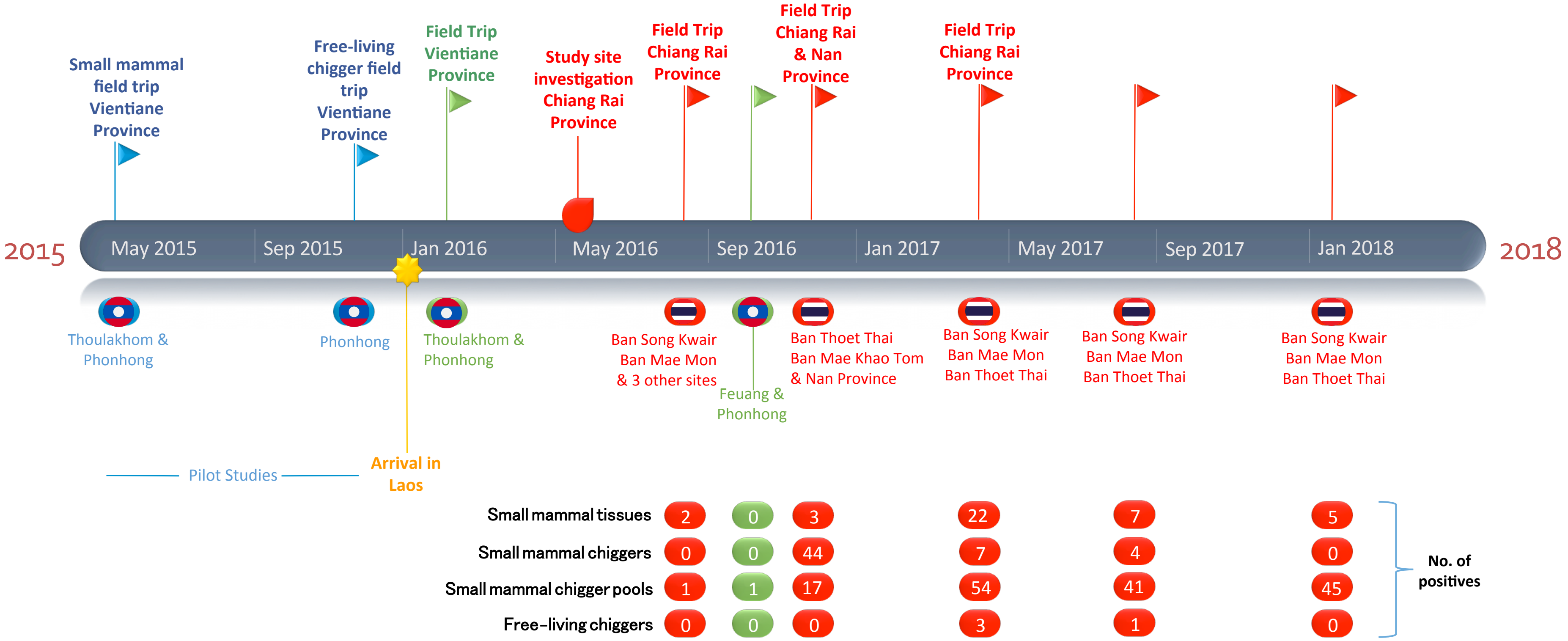
Table 11 Results of nucleotide blast of Sanger sequencing of PCR products from 2 individual chiggers, a rodent spleen and chigger pool samples

Sample name	**Sequence size (bp)**	**Result**
C0307_OTSU_FP630_CLN	237	No match
C0317_OTSU_FP630_CLN	275	14% coverage (39bp) at 97% identity to *O. tsutsugamushi* 47 kDa gene
R178POOL1_OTSU_FP630_CLN	294	5% coverage (15bp) at 100% identity to str. Gilliam
R0135S_OTSU_RP747_CLN	242	6% coverage (16bp) at 100% identity to str. UT76

The sequences were of low quality and 1 individual chigger did not result in a match (the sequence was shorter than the other samples). Although only a small part of the sequences matched exactly to *O. tsutsugamushi,* this provides some evidence against these very low-positive samples being falsely positive due to non-specific PCR binding. Additional evidence is presented in Chapter 5.

3.5.7 Field collections

Seven field trips took place between February 2016 and January 2018. Five of these were in Northern Thailand and two in Laos. These trips were designed to take place during different seasons and weather conditions (Figure 34).



Small mammal tissues	2	0	3	22	7	5
Small mammal chiggers	0	0	44	7	4	0
Small mammal chigger pools	1	1	17	54	41	45
Free-living chiggers	0	0	0	3	1	0

Figure 34 Timeline of field investigations in Laos and Thailand, 2015 to 2018 with number of positive samples collected at each visit

3.5.8 Small mammals

3.5.8.1 Trap success rates

A total of 244 small mammals were trapped over the course of this study. Trapping success varied from 0 to 12.5% depending on the site. At 2 of the 8 sites investigated in Chiang Rai Province (Ban Lao Lee and Ban Rong Pla Khao), we failed to trap any small mammals. In Laos hunters trapped all but a single rodent. Traps lines set without the help of hunters at 6 locations yielded no small mammals. The highest trap success rate of 12.5% was at Ban Huay Muang, Nan Province, where several hunters were employed to trap small mammals. At our 3 main study sites, trap success rates ranged from 1.2 to 6.2% (overall mean 3.8%) (Table 12). Within some sites, certain areas were much more productive than others, for example in BTT we caught repeatedly close to the river but only succeeded in trapping two animals in the forested area.

Table 12 Trap success rate (number of successful trappings/total traps set) for each visit at the 3 main study sites.

Study Site	Aug ‘16	Nov ‘16	Apr ‘17	Aug ‘17	Jan ‘18
BTT	-	2.3%	4%	2.7%	3.7%
BSK	5%	-	6.2%	1.2%	5.3%
BMM	5%	-	3.1%	3.7%	3.1%

Summary GIS maps showing the exact location of all traps and collected hosts/vectors over time are depicted below in Figure 36, Figure 37 and Figure 38. Note that for BTT, the area around one paediatric case’s home and some nearby paddyfields were also investigated on all but 1 visit and yielded a small number of positives (data included in results but not shown in Figure 36).

3.5.8.2 Trapping accuracy

Trapping accuracy was scored at 1 (<10m) for 184/244 (75%) of animals. From the 3 main study sites, 11/155 were scored at accuracy 2 (<100m), 8/155 at level 3 (<1000m) and 1/155 at level 4 (>1000m). Hunters trapped all the 20 animals with accuracy level 4, where the exact location could not be determined.

3.5.8.3 Species diversity

A total of 19 species of small mammals were trapped from 10 sites across Northern Thailand and Laos. Among the 244-trapped animals, 15 could only be identified to genus level (2 *Rattus*, 1 *Mus*, 10 *Niviventer* and 3 *Maxomys*). The 19 species were composed of 3 orders within the class Mammalia: Rodentia (17 species); Soricomorpha – *Suncus murinus*, the Asian house shrew and Scandentia – *Tupaia glis/belangeri*, the Northern tree shrew. Within the Rodentia, 16 species were of the family Muridae – the typical rats and mice and 1, *Callosciurus erythraeus* (Pallas's squirrel) belonging to the Sciuridae.

The most frequently trapped species was *Rattus tanezumi* (55 individuals), a highly adaptable species found in numerous habitat types and *Bandicota indica* (46 individuals), a rice paddy specialist. *R. andamanensis* (23) and *R. exulans* (25) typical of secondary forests and settlements respectively were also caught in large numbers (Table 13).

Table 13 Numbers of small mammal species trapped on different field visits. # Denotes Lao field visits.

Small mammal species	**Feb-2016#**	**Aug-2016**	**Oct-2016#**	**Nov-2016**	**Apr-2017**	**Aug-2017**	**Jan - 2018**	**Total**
Bandicota indica	7	0	11	12	2	6	8	46
Berylmys berdmorei	1	0	0	6	0	0	0	7
Berylmys bowersi	0	0	0	1	2	0	2	5
Callosciurus erythraeus	0	0	0	0	1	0	0	1
Leopoldamys edwardsi	4	0	0	1	0	0	0	5
***Maxomys* sp.**	3	0	0	0	0	0	0	3
Maxomys surifer	3	0	0	6	1	0	2	12
Menetes berdmorei	0	1	0	0	0	0	3	4
Mus caroli	3	0	0	0	0	2	0	5
Mus cervicolor	2	0	0	0	0	0	0	2
Mus cookii	0	1	0	0	2	6	1	10
***Mus* sp.**	0	0	1	0	0	0	0	1
Niviventer fulvescens	0	0	0	2	1	0	0	3
Niviventer langbianis	1	0	0	0	0	0	0	1
***Niviventer* sp.**	10	0	0	0	0	0	0	10
Rattus andamanensis	4	0	0	0	9	3	7	23
Rattus exulans	0	9	4	4	2	1	5	25
Rattus losea	5	0	0	0	0	0	0	5
Rattus nitidus	0	3	0	1	4	1	1	10
***Rattus* sp.**	0	0	0	0	2	0	0	2
Rattus tanezumi	0	11	0	3	16	12	13	55
Suncus murinus	0	1	0	0	0	0	1	2
Tupaia glis	1	1	0	0	0	0	5	7
Total	**44**	**27**	**16**	**36**	**42**	**31**	**48**	**244**

Species distribution was primarily related to the habitats present at trapping sites. At the 3 main study sites, 8 to 9 species were trapped. At BTT species that favour rice fields and cultivation were predominant with 38/47 (81%) being either *B. indica* or *R. tanezumi*. In BSK with its high proportion of forest cover, *R. andamanensis* was frequent 19/64 (30%) and *R. exulans* trapped around homes comprised 16/64 (25%). In BMM with its patchwork of habitats, *R. tanezumi* was commonest at 20/43 (47%) followed by *Mus cookii* at 8/43 (19%) from fallow grassland and cultivation. A hunter shot the single arboreal squirrel *C. erythraeus*. Seven animals were collected dead (from hunters).

3.5.8.4 Orientia tsutsugamushi PCR results

Lung, liver and spleen tissue was tested from all 244 animals. All but one *O. tsutsugamushi* positive animal were from Chiang Rai Province sites. At least 1 tissue tested positive from 39/244 (16%) small mammals. Eight species from 5 genera tested positive. *R. tanezumi* and *R. andamanensis* were most frequently positive with 16/55 (29%) and 6/23 (26%), respectively, followed by 6/25 (16%) *R. exulans* and 5/45 (11%) *B. indica*. Although numbers were smaller, 2/8 *M. cookii*, 2/8 *R. nitidus*, 1/11 *Maxomys surifer* and 1/4 *Berylmys bowersi* also gave positive results.

Of the three study sites BTT had the highest proportion of positive animals with 15/45 (33%), BSK had 16/64 (25%) and BMM 7/43 (16%) (Table 14).

Table 14 Numbers of small mammal species collected at each study site with *O. tsutsugamushi* PCR results.

	Main study sites						Chiang Rai – other sites						Nan		Laos					
Small mammal species	**Ban Song Kwair**		**Ban Mae Mon**		**Ban Thoet Thai**		**Ban Huay Bong**		**Ban Mae Lao**		**Ban Mae Khao Tom**		**Ban Huay Muang**		**Thoulakhom**		**Phonhong**		**Feuang**	
PCR result	+	–	+	–	+	–	+	–	+	–	+	–	+	-	+	–	+	–	+	–
Bandicota indica	-	-	-	-	5	16	-	-	-	-	-	4	-	3	-	-	-	18	-	-
Berylmys berdmorei	-	-	1	1	-	1	-	-	-	-	-	-	-	5	-	-	-	1	-	-
Berylmys bowersi	-	-	-	-	-	2	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Callosciurus erythraeus	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leopoldamys edwardsi	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	4	-	-	-	-
***Maxomys* sp.**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-
Maxomys surifer	-	3	-	-	-	-	-	-	-	-	-	-	1	5	-	3	-	-	-	-
Menetes berdmorei	-	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mus caroli	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	3	-	-
Mus cervicolor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-
Mus cookii	-	-	1	7	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

***Mus* sp.**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Niviventer fulvescens	-	-	-	1	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Niviventer langbianis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
***Niviventer* sp.**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-
Rattusandamanensis	6	13	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-
Rattus exulans	3	13	-	-	1	1	-	-	-	1	-	-	-	2	-	-	-	-	-	4
Rattus losea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-
Rattus nitidus	2	6	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Rattus* sp.**	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rattus tanezumi	3	10	5	15	8	9	-	1	-	2	-	-	-	2	-	-	-	-	-	-
Suncus murinus	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tupaia glis	-	1	-	5	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Total	16	48	7	36	15	33	0	1	0	3	0	4	1	20	0	26	0	30	0	4

The total proportion of positive animals at each field collection varied over time (Figure 35). In August 2016, 2/23 were positive (BSK & BMM only), in November 2016, 2/11 from BTT only. A peak of 22/42 (52%) was recorded in April 2017 with 13/22, 6/10 and 3/10 positive animals at BSK, BTT and BMM respectively. In August 2017, 7/31 tested positive and were fairly evenly distributed between sites. In January 2018, 5/48 tested positive, all from BTT. Note that in August and November 2016 not all the main study sites were visited.

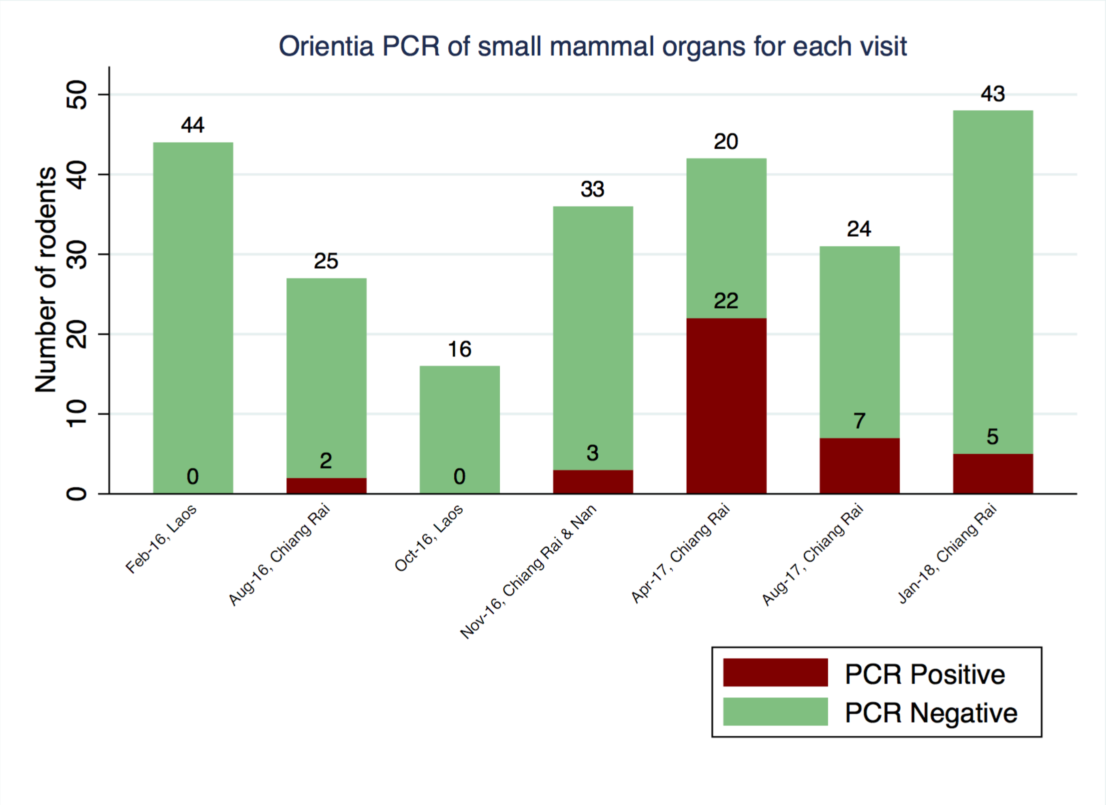


Figure 35

3.5.8.5 Organ positivity

Three different tissue types were tested for the presence of *O. tsutsugamushi* by PCR. Spleen and lung were equally frequently positive with 22 each, whereas 9 liver samples tested positive. In only 2 of the 39 positive animals were all 3 organs positive and in 10/39, two organs were positive. In four cases, liver was the only positive organ. The mean Ct value for all organs was 37.56 (range 29.5 to 40.3). The association between organ type and PCR positivity was analysed using a Generalised Estimating Equation (GEE) population averaged model with the *logit* link function. The odds ratio for lung or spleen positivity compared to liver was 2.59 (95% CI 1.25-5.3) with *p*-value = 0.01.

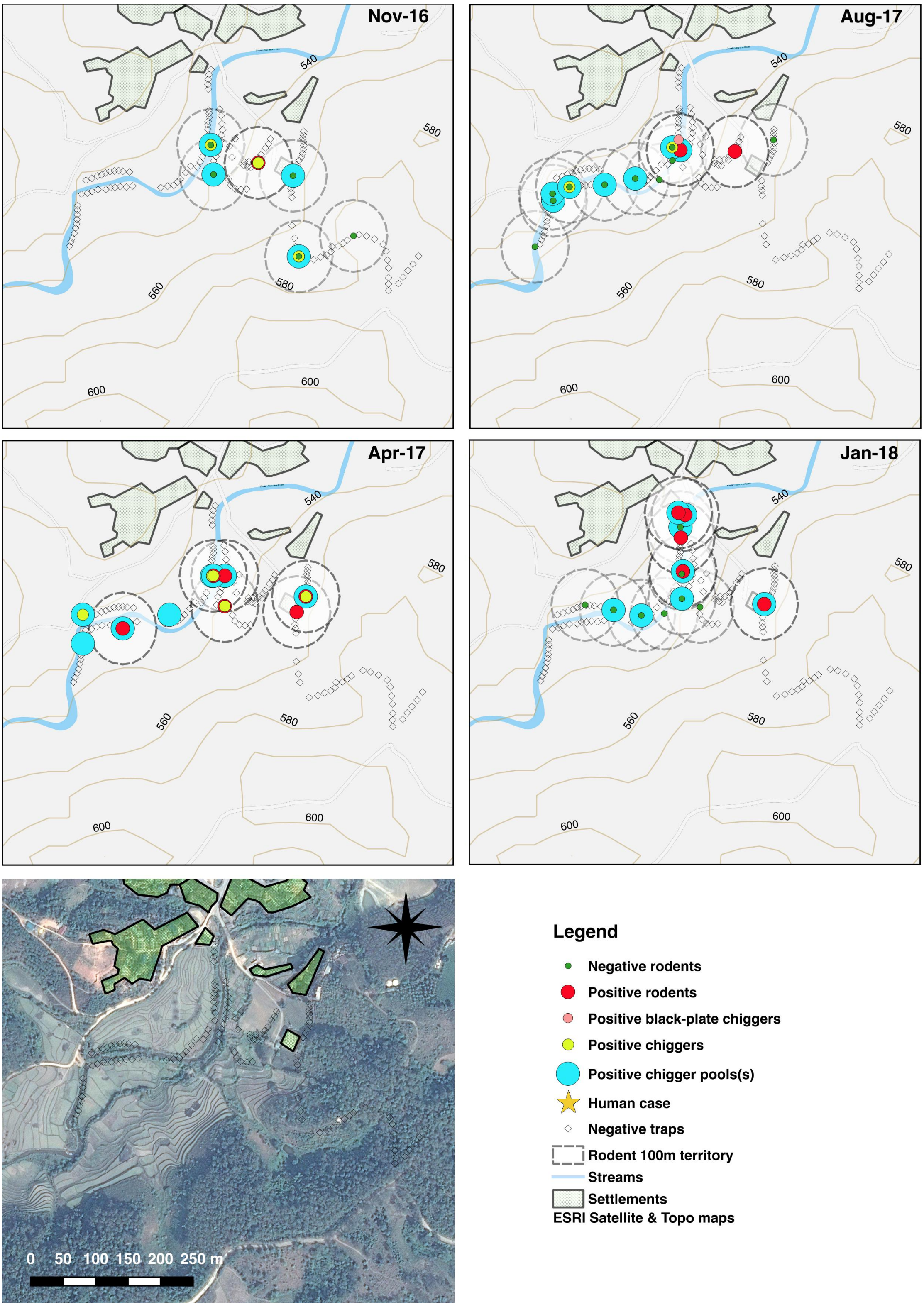


Figure 36 Ban Thoet Thai, Mae Fahluang District, Chiang Rai Province. The locations of all trap sites are shown, together with the position of *O. tsutsugamushi* positive rodents; chiggers collected from rodents and free-living chiggers. Each rodent is marked with a 100m zone, an estimate of territory size. Results are shown over 4 time points. This study site consists of a small stream, rice paddies, vegetable gardens, fallow areas and a small patch of degraded secondary forest. One nearby Paediatric case presented to the Provincial hospital in 2016. Note 2 additional sites ~3km away were also investigated.

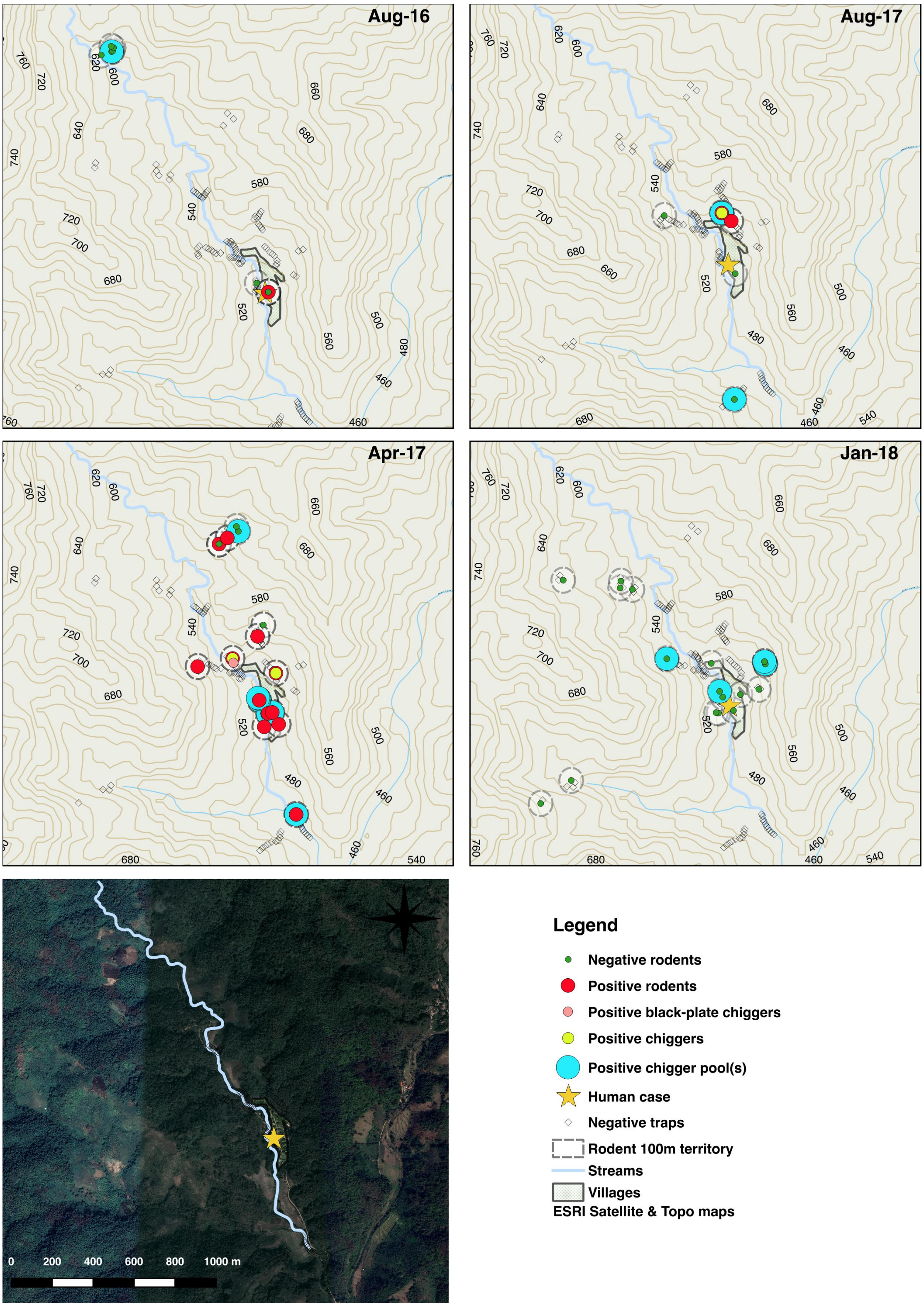


Figure 37 Ban Song Kwair, Meuang District, Chiang Rai Province. The locations of all trap sites are shown, together with the position of *O. tsutsugamushi* positive small mammals; chiggers collected from rodents and free-living chiggers. Each rodent is marked with a 100m zone, an estimate of territory size. Results are shown over 4 time points. This Akha village is in a steep sided valley at ~600m asl surrounded by degraded secondary forest with bamboo, teak plantations, dry rice and maize fields. Three paediatric cases from this area presented in 2016.

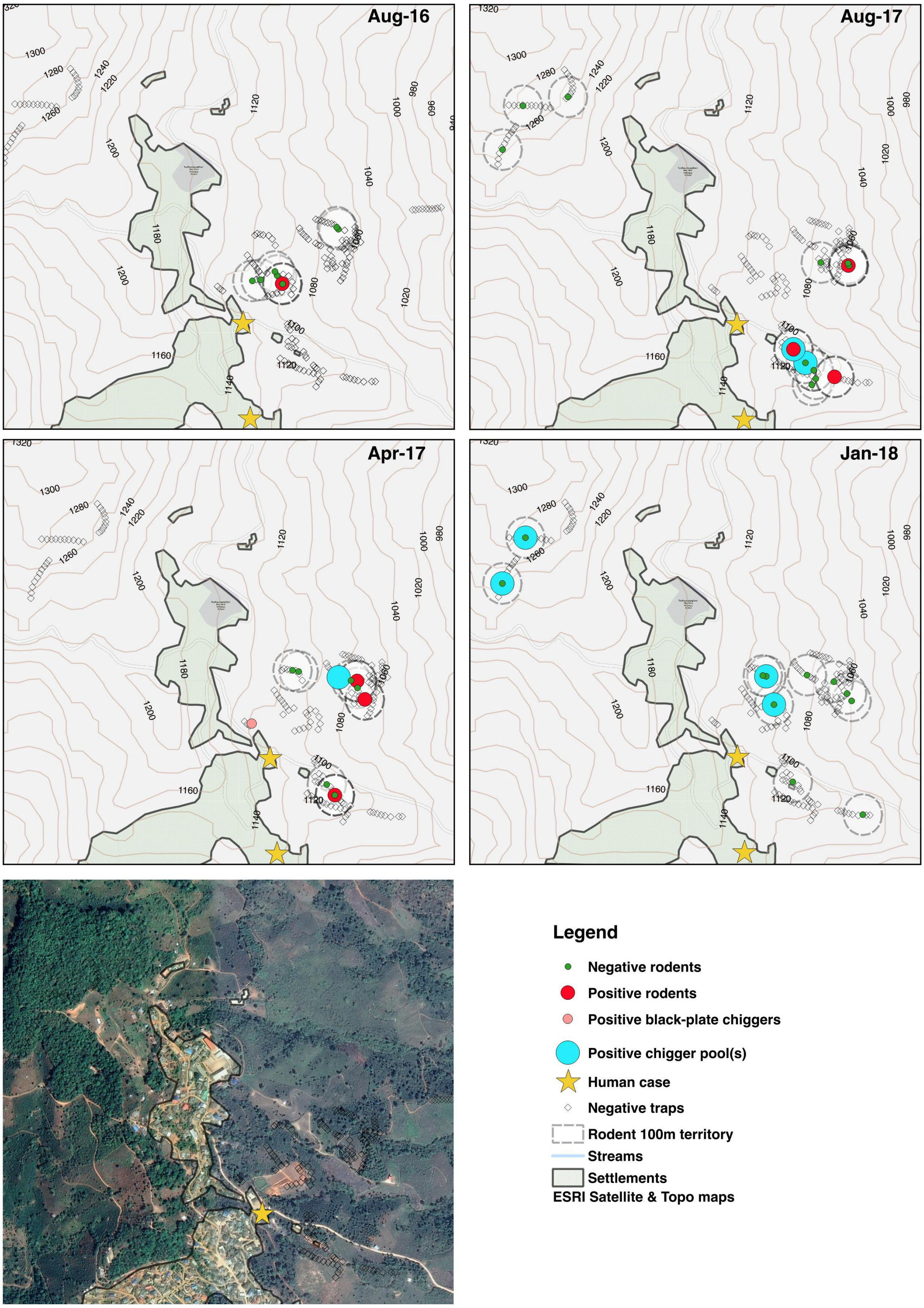


Figure 38 Ban Mae Mon, Meuang District, Chiang Rai Province. The locations of all trap sites are shown, together with the position of *O. tsutsugamushi* positive rodents; chiggers collected from rodents and free-living chiggers. Each rodent is marked with a 100m zone, an estimate of territory size. Results are shown over 4 time points. This study site (at 1100-1300m asl) consists of steep sided hills where coffee, fruit and some vegetables are cultivated. There are fallow areas and a reforestation project just above the village. Two paediatric cases were referred to the Provincial hospital from this village in 2016.

3.5.9 Chiggers infesting small mammals

Chiggers present on all animals were removed, counted and a subset identified. Chiggers were tested both individually and as pools for *O. tsutsugamushi*.

3.5.9.1 Chigger indices and infestation rates

Infestation of an animal was defined as the presence of at least 1 chigger. Of the 244 animals, 212 (87%) were infested. One *Tupaia glis* was infested with 444 chiggers. Another measure is the chigger index or mean number of chiggers per individual. Chigger indices for small mammal species are given in Table 15.

Table 15 Chigger indices for small mammal species

Rodent species	Number of small mammals	Chigger Index (Mean/ rodent)	Maximum	Minimum	Standard deviation
Bandicota indica	46	110	411	0	117
Berylmys berdmorei	7	44	172	5	59
Berylmys bowersi	5	4	7	0	3
Callosciurus erythraeus	1	2	2	2	.
Leopoldamys edwardsi	5	9	25	0	12
***Maxomys* sp.**	3	51	126	8	65
Maxomys surifer	12	21	104	0	32
Menetes berdmorei	4	115	360	7	167
Mus caroli	5	33	142	0	62
Mus cervicolor	2	12	23	0	16
Mus cookii	10	16	57	0	18
***Mus* sp.**	1	25	25	25	.
Niviventer fulvescens	3	2	4	0	2
Niviventer langbianis	1	18	18	18	.
***Niviventer* sp.**	10	8	42	0	13
Rattus andamanensis	23	54	297	0	75
Rattus exulans	25	23	170	0	46
Rattus losea	5	5	23	0	10
Rattus nitidus	10	114	183	10	54
***Rattus* sp.**	2	15	21	8	9

Rattus tanezumi	55	114	421	0	103
Suncus murinus	2	10	17	3	10
Tupaia glis	7	138	444	0	162
Total	**244**	**41**	**444**	**0**	**54**

The highest chigger index was seen for the scansorial *T. glis* (162), albeit from only 7 individuals. *R. tanezumi* and *B. indica* were also heavily infested with chigger indices of 117 and 110 respectively. A number of species had low chigger indices, e.g. the *Mus* species ranged from 12 to 33.

Mean chigger indices per site were calculated irrespective of the vertebrate species or season of collection. BTT had by far the highest mean chigger index of 151, followed by BMM with 86 and BSK with 62. Ban Huay Muang, Nan Province had a mean index of 32 from a single visit. The two Lao sites, Phonhong and Thoulakhom Districts also had relatively low mean indices of 27 and 13 respectively.

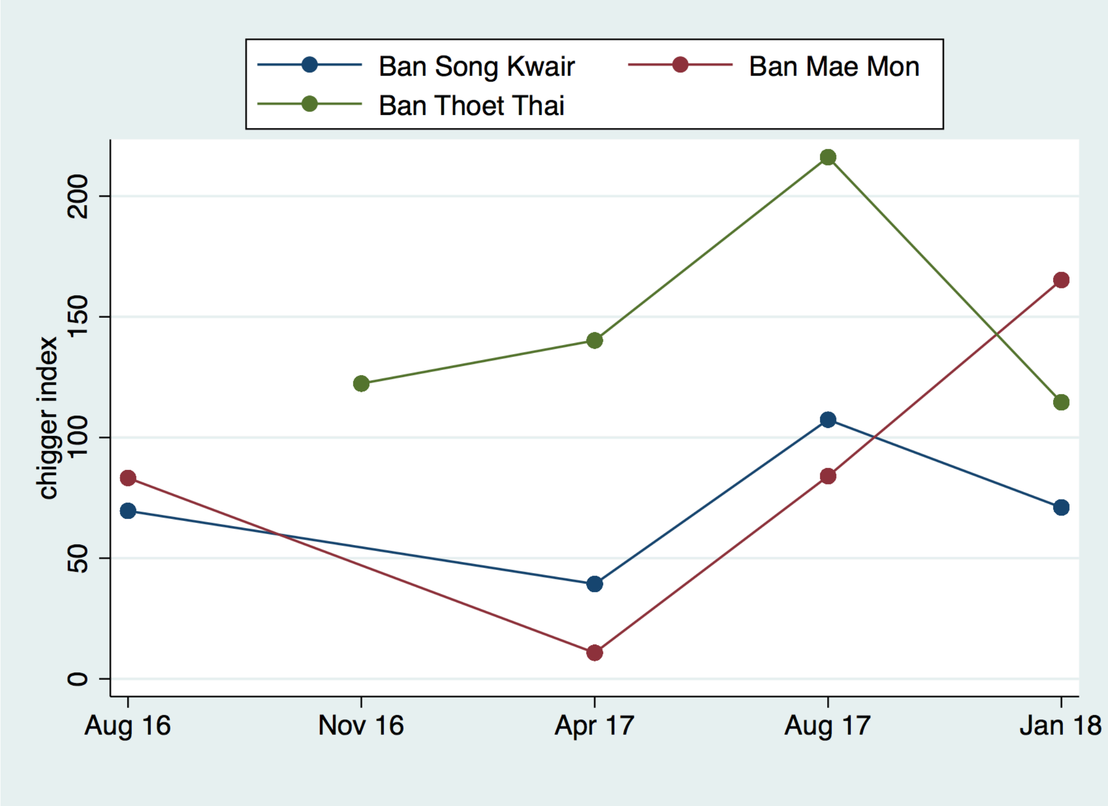


Figure 39 Mean chigger index for all trapped animals at each field visit, shown for each of the main study sites

Chigger indices were observed to vary over the seasons. Although not entirely consistent between sites, for BTT and BSK, the chigger index peaked during the rainy season in August and was lowest at the end of the dry season (April) in BSK and BMM, whilst being lowest at BTT during the mid-dry season in January (Figure 39).

The chigger indices over time for the species trapped at the 3 main study sites are shown in Figure 40, Figure 41 & Figure 42. The graphs highlight the trapping success rates and irregularity with which different species were collected. At BTT the chigger index of *B. indica* follows the overall pattern seen for BTT, whereas *R. tanezumi* showed little fluctuation in chigger index over the seasons. In BMM, the *R. tanezumi* chigger index was lowest at the end of the dry season and in BSK only *R. andamanensis* showed a rise in chigger index during the rainy season.

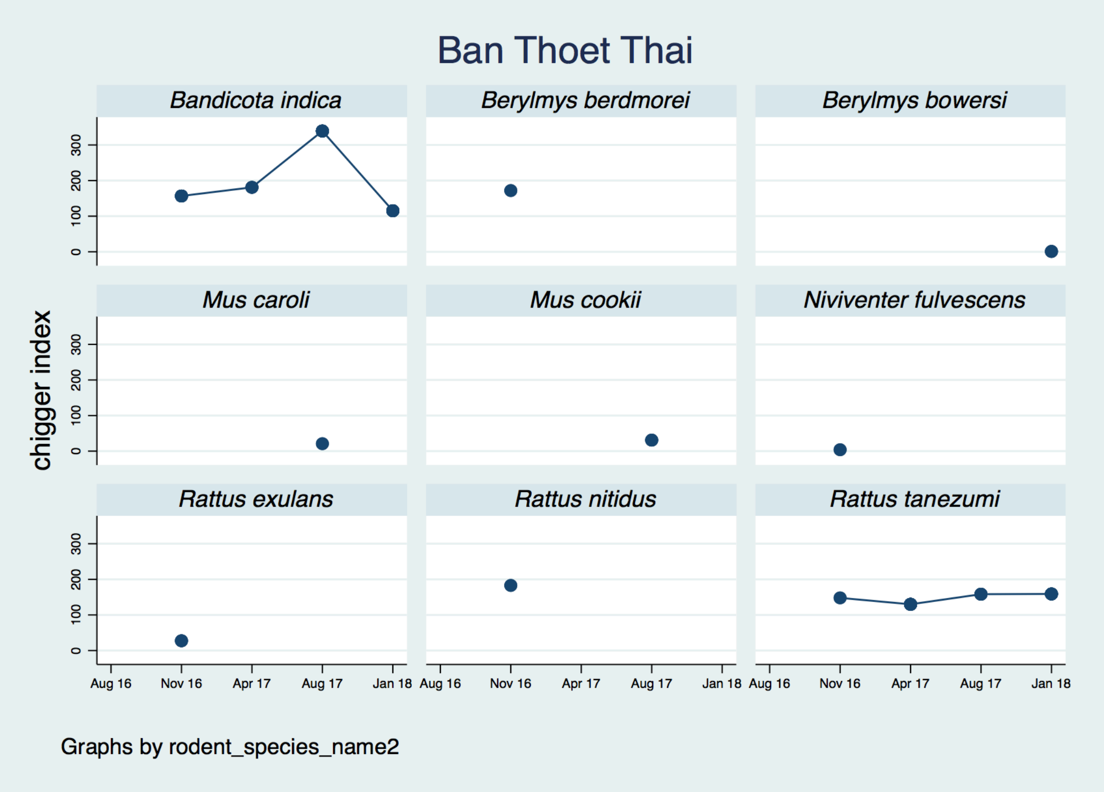


Figure 40 Chigger index for trapped small mammal species at each field visit

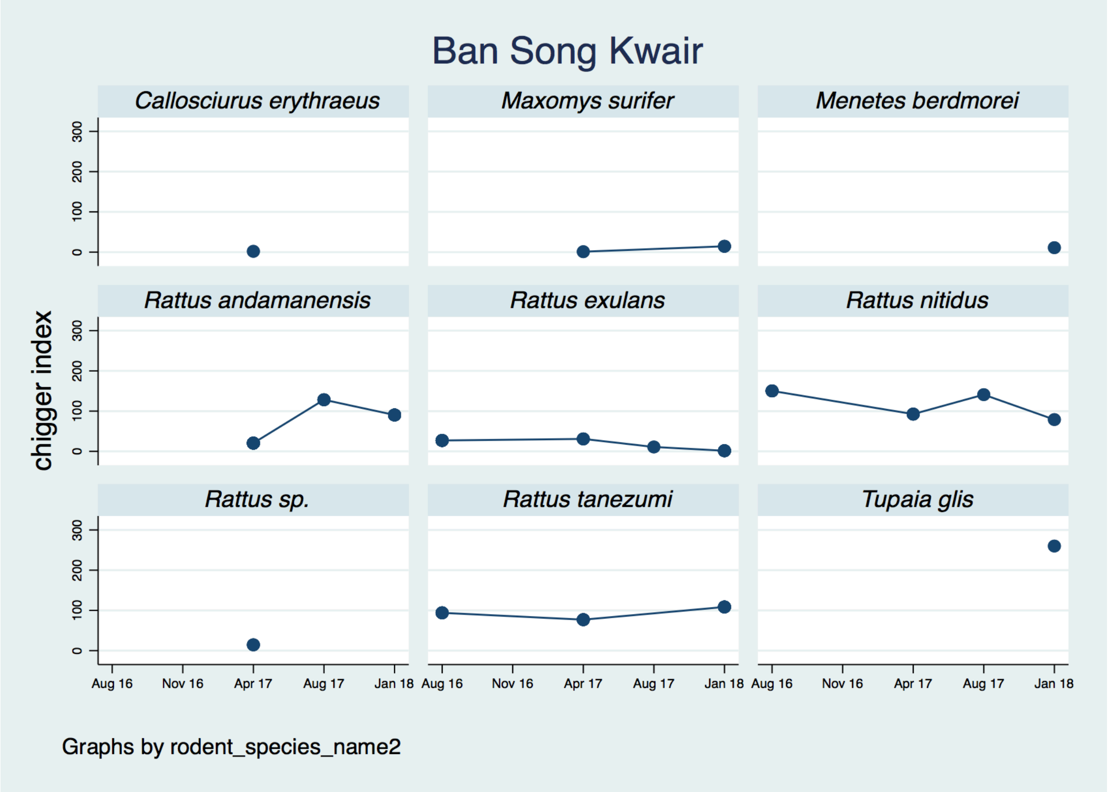


Figure 41 Chigger index for trapped small mammal species at each field visit

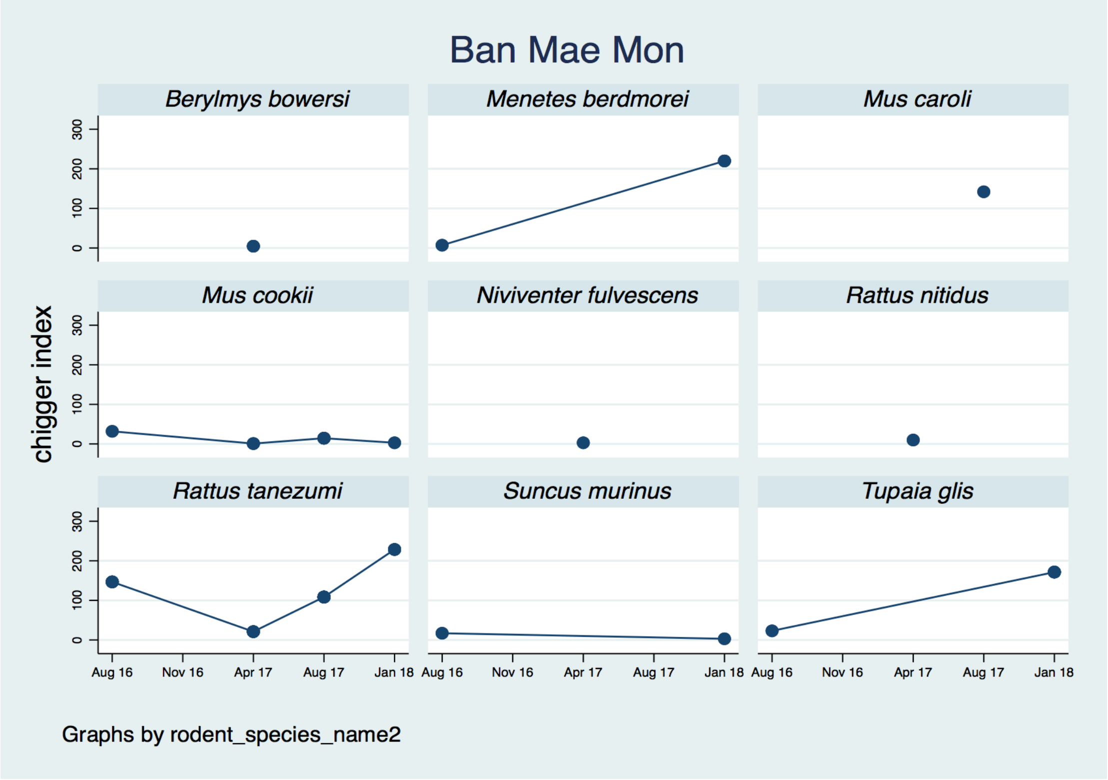


Figure 42 Chigger index for trapped small mammal species at each field visit

3.5.9.2 Chigger species diversity

A total of 803 chiggers were collected for identification, 785 of which were identified and tested (18 were lost during processing). Chiggers removed from rodents totalled 637 and 148 were free-living. When possible, 3 chiggers were identified from each animal.

46 species of chigger in 15 genera and 2 subfamilies were identified. 7 species (57 individuals) were close matches to described species (represented by "cf" *conferatur* = compared to [Latin] e.g. *Gahrliepia* cf. *tylana*). Additionally 33 chiggers could only be identified to genus level, either due to damage to key morphological features or lack of suitable identification keys. 11 individuals proved to be non-chiggers after microscopic examination (see below).

Three chigger species were recovered only as free-living. From rodents *Leptotrombidium* species were most frequently identified, particularly the vector species *L. deliense* and *L. imphalum* that accounted for 134/637 (21%) and 89/637 (14%), respectively. *Walchia* spp. (at least 11 species in all) and *Ascoschoengastia* spp. comprised the other 2 major groups of chiggers with 248/637 (39%) and 68/637 (11%) of all chiggers parasitizing rodents.

Chigger diversity varied between different study sites. At BMM and BSK 25 and 23 species were identified respectively. However, at BTT only 15 species were recorded. In Phonghong and Thoulakhom Districts, Laos, 13 and 8 species were identified, respectively. This is discussed further in Chapter 4. A summary of diversity for a selection of sites is shown in Table 16. Diversity did not vary appreciably at different times of the year.

Table 16 Number of individuals of selected chigger genera/species collected at the 3 main sites in Thailand and 2 sites in Laos. The overall species diversity is also shown.

Chigger genus/species	Ban Song Kwair	Ban Mae Mon	Ban Thoet Thai	Phonhong	Thoulakhom
***Ascoschoengastia* sp.**	39	10	2	-	7
***Blankaartia* sp.**	-	-	-	2	-
***Gahrliepia* sp.**	4	12	1	-	-
***Helenicula* sp.**	10	15	2	-	-
Leptotrombidium deliense	79	36	33	3	1
Leptotrombidium imphalum	8	6	83	-	-
***Leptotrombidium* sp. (other)**	3	21	2	4	-
***Lorillatum* sp.**	-	-	-	-	5
***Schoengastia* sp.**	-	-	-	3	-
***Schoengastiella* sp.**	8	-	-	-	-
***Schoutedenichia* sp.**	1	-	1	-	-
***Susa* sp.**	1	-	-	-	-
***Trombiculindus* sp.**	1	18	1	-	-
***Walchia* sp.**	35	36	33	59	36
***Walchiella* sp.**	1	-	-	-	-
Total no. of species	**23**	**25**	**15**	**13**	**8**

Considering only the 2 confirmed human vector species *L. deliense* and *L. imphalum*, these comprised just 1/49 (2%) and 3/71 (4%) at the two Lao sites. In BTT the proportion was highest with 116/163 (71%), followed by BSK with 87/197 (44%) and BMM with 42/156 (27%).

The chigger species diversity and number hosted by different small mammal species is listed in Appendix B-2. *L. deliense* was recorded from 11 species and *L. imphalum* from 4 species. *Rattus* species and *B. indica* hosted 85% of *L. deliense* and 99% of *L. imphalum*.

3.5.9.3 *Orientia tsutsugamushi* PCR of individual chiggers

In total 637 chiggers removed from rodents were tested for *O. tsutsugamushi* by PCR. Eleven species from 7 genera had at least 1 individual positive (Table 17). 55/637 (8.6%) of individual chiggers were positive. Four species comprised 78% of all positives: *L. deliense* 17/134 (13%), *L. imphalum* 6/89 (7%), *Walchia kritochaeta* 13/70 (19%) and *W. micropelta* 7/45 (16%).

The majority of positives were collected in November 2016 (44/55), with very small numbers in April 2017 (8/55), August 2017 (3/55) and none in January 2018. Of those collected in November 2016, 21 positives were from rodents in Nan Province and 23 from BTT.

From the 3 main study sites, the vast majority of *O. tsutsugamushi* positive chiggers from small mammals were from BTT 26/143 (18%), with only 3 from BSK and none at BMM (Table 17).

Table 17 Chigger species numbers collected from all sites (combined chiggers from small mammals and free-living chiggers), with *O. tsutsugamushi* PCR results[i] *Denotes sites where only free-living chiggers were collected

Chigger species	Ban Song Kwair		Ban Mae Mon		Ban Thoet Thai		Ban Huay Mae Sai*		Ban Mae Lao		Ban Mae Khao Tom		Ban Huay Muang		Thoulakhom		Phonhong		Feuang		Palm oil plantation*	
PCR Result	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
Ascoschoengastia indica	-	39	-	10	-	1	-		-	-	-	-	1	3	-	7	-	-	-	6	-	-
***Ascoschoengastia* sp.**	-	-	-	1	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Blankaartia acuscutellaris	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	2	-	-	-	-
***Cheyletus* sp.**	-	2	-	1	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Eutrombicula* sp.**	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	7	-	1
Eutrombicula wichmanni	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gahrliepia cf. tylana	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gahrliepia cf. xiaowoi	-	3	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gahrliepia elbeli	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Gahrliepia tenella	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Gahrliepia xiaowoi	-	1	1	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Helenicula kohlsi	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Helenicula naresuani	-	6	-	4	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-

Helenicula pilosa	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Helenicula scanloni	-	-	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Helenicula* sp.**	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Laelapidae	-	2	-	1	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptotrombidium arvinum	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptotrombidium cf. fletcheri	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptotrombidium deliense	$\boxed{3}$	76	-	36	$\boxed{13}$	20	-	-	-	-	-	-	$\boxed{3}$	3	-	1	-	3	-	-	-	45
Leptotrombidium dendrium	-	-	-	-	$\boxed{4}$	-	-	-	-	-	-	-	-	23	-	-	-	-	-	-	-	-
Leptotrombidium elisbergi	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptotrombidium harrisoni	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptotrombidium imphalum	-	8	-	6	-	76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Leptotrombidium* sp.**	-	-	-	1	-	2	-	-	-	-	-	-	-	1	-	-	-	4	-	-	-	-
Leptotrombidium turdicola	-	1	-	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lorillatum kianjoei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-
***Notoedres* sp.**	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Odontacarus audyi	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Schoengastia kanhaensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-
Schoengastiella ligula	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Schoutendichia* sp.**	$\boxed{1}$	-	-	-	$\boxed{1}$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Susa traubi	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Trombiculindus cf. atractimorphe	-	-	-	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trombiculindus cf. variaculum	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trombiculindus paniculatum	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trombiculindus variaculum	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Walchia alpestris	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-
Walchia cf. micropelta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	-	5	-	-	-	-
Walchia cf. minuscuta	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-
Walchia dismina	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Walchia disparunguis	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
W. disparunguis disparunguis	-	4	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Walchia ewingi ewingi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-
Walchia ewingi lupella	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	30	-	1	-	-
Walchia isonychia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	1	-	-	-	-
Walchia kritochaeta	1	22	-	-	6	16	-	-	-	4	-	-	6	4	-	8	-	3	-	-	-	-
Walchia micropelta	-	1	-	18	-	7	-	-	-	-	1	1	6	3	-	3	-	9	-	3	-	-
Walchia minuscuta	-	-	-	1	-	-	-	-	-	-	-	1	3	7	-	-	-	-	-	-	-	-
Walchia rustica	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Walchia* sp.**	-	-	-	-	-	2	-	-	-	-	1	-	-	6	-	3	-	2	-	-	-	-
Walchia turmalis	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Walchia ventralis	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***Walchiella* sp.**	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**5**	**192**	**1**	**155**	**26**	**143**	**0**	**1**	**0**	**4**	**2**	**2**	**21**	**55**	**0**	**49**	**0**	**71**	**0**	**17**	**0**	**46**

[i] Table of *O. tsutsugamushi* positive chigger species with strength of evidence, and new additions from this project is shown in Appendix A-3

3.5.9.4 Orientia tsutsugamushi PCR of chigger pools

Throughout the whole study, 659 pools were prepared and tested for *O. tsutsugamushi*, consisting of a total of 16,941 chiggers. The mean pool size was 26 (range 3-63).

Three species of small mammal had insufficient chiggers to make pools. An additional 5 species had 5 or less pools tested. As the most numerous and heavily infested rodent species, *R. tanezumi* and *B. indica* accounted for the largest proportion of pools with 249/659 (38%) and 182/659 (28%) respectively (Table 18).

Table 18 Number of individual small mammal species with chigger pools collected and *O. tsutsugamushi* PCR results

Small mammal species	Mammals with pools/Total	Total pools	PCR positive	Percentage positive
Bandicota indica	38/46	182	63	34.6
Berylmys berdmorei	5/7	14	4	28.6
Berylmys bowersi	0/5	0	0	0
Callosciurus erythraeus	0/1	0	0	0
Leopoldamys edwardsi	2/5	2	0	0
***Maxomys* sp.**	1/3	5	0	0
Maxomys surifer	5/12	13	6	46.2
Menetes berdmorei	4/4	14	0	0
Mus caroli	2/5	6	0	0
Mus cervicolor	1/2	1	0	0
Mus cookii	6/10	8	0	0
***Mus* sp.**	1/1	2	0	0
Niviventer fulvescens	0/3	0	0	0
Niviventer langbianis	1/1	1	0	0
Niviventer sp.	3/10	5	0	0
Rattus andamanensis	17/23	51	9	17.6
Rattus exulans	9/25	27	1	3.7
Rattus losea	1/5	1	0	0
Rattus nitidus	10/10	46	9	19.6
***Rattus* sp.**	2/2	3	0	0

Rattus tanezumi	51/55	249	71	28.5
Suncus murinus	1/2	1	0	0
Tupaia glis	6/7	28	8	28.6
Total	**166/244**	**659**	**171**	**25.9**

Chigger pools were positive for *O. tsutsugamushi* in 171/659 (25.9%). Eight species had at least one positive pool, however *R. tanezumi* and *B. indica* made up 78% of positives (Table 18). *M. surifer* had the highest proportion of positives with 6/13 (46%), followed by *B. indica* with 63/182 (35%) and *R. tanezumi* and *T. glis* with 29%.

A single positive pool was detected from a *B. indica* trapped in October 2016 in Phonhong District. This is the first and only positive vector or host sample ever recorded from Laos. In Ban Mae Khao Tom, Chiang Rai, 2/4 pools tested positive from just 4 collected animals and in Nan Province 10/31 (32%) of pools were positive (Table 19).

Table 19 Number of individual small mammal with chigger pools collected from each study site and *O. tsutsugamushi* PCR results

Site	Mammals with pools	Total pools	PCR positive	Percentage positive
Ban Song Kwair	47/64	162	19	11.7
Ban Mae Mon	35/43	140	35	25
Ban Thoet Thai	44/47	257	104	40.5
Ban Huay Bong	0/1	0	0	0
Ban Mae Lao	1/3	1	0	0
Ban Huay Muang	11/21	31	10	32.3
Ban Mae Khao Tom	2/4	4	2	50
Thoulakhom	7/26	13	0	0
Phonhong	17/30	40	1	2.5
Feuang	2/4	11	0	0

Of the 3 main study sites, BTT had consistently the highest proportion of positives (41% overall) through different seasons. Excluding August 2016 (when only 1/64 pools was positive), the proportion of positive pools combined from the 3 main study sites ranged from 27% during the rainy season of August 2017 to 41% at the end of the dry season in April 2017 (Figure 43).

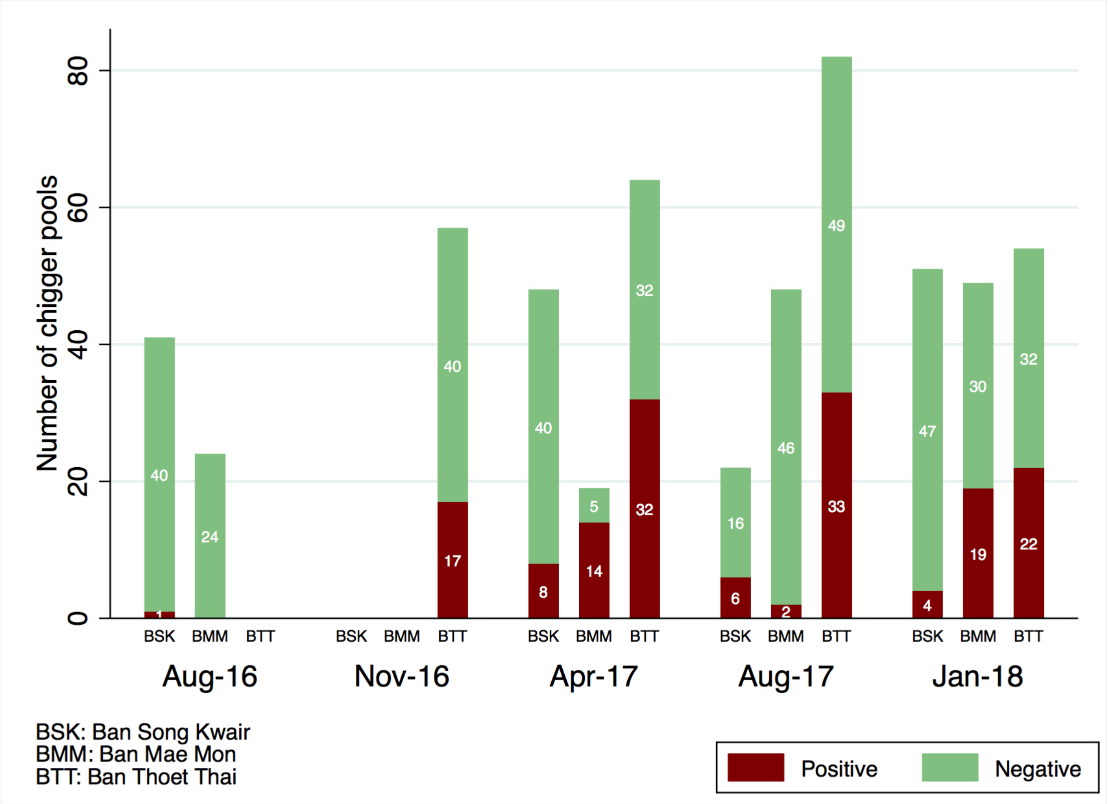


Figure 43 Proportion of *O. tsutsugamushi* PCR positive chiggers pools collected at different times of the year from the 3 main study sites

3.5.10 Free-living chiggers

Two methods were used to collect free-living chiggers: black plates and Berlese funnels (for soil samples). Berlese funnels were used once in Laos (February 2016) and once in Chiang Rai Province (January 2018). Black plate collections were attempted at 11 sites. This included 4 sites (a palm oil plantation 22km north of Vientiane capital; Ban Lao Lee, Ban Rong Pla Khao and Ban Huay Mae Sai in Chiang Rai Province) where no small mammals were trapped. One site, Ban

Huay Mae Sai, was investigated as the site of a scrub typhus case 2 weeks previously, however rodent trapping did not take place.

A total of 150 free-living chiggers were collected from 7 sites. Only two *L. turdicola* were collected using Berlese funnels in January 2018 from the reforestation area of BMM. From black plates, 13 species were identified from 6 genera, however 32 individuals were not conclusively identified (Table 20).

Table 20 Free-living chigger species and *O. tsutsugamushi* PCR results[i]

Chigger species	**PCR negative**	**PCR positive**
***Eutrombicula* sp.**	8	0
Eutrombicula wichmanni	2	0
Gahrliepia cf. xiaowoi	7	0
Gahrliepia xiaowoi	6	**1**
Leptotrombidium deliense	67	**2**
Leptotrombidium dendrium	23	0
Leptotrombidium imphalum	7	**1**
***Leptotrombidium* sp.**	1	0
Leptotrombidium turdicola	4	0
Odontacarus audyi	2	0
Trombiculindus cf. atractimorphe	15	0
Trombiculindus cf. variaculum	1	0
Walchia turmalis	1	0
Total	**144**	**4**

[i] Table of *O. tsutsugamushi* positive chigger species with strength of evidence, and new additions from this project is shown in Appendix A-3

In Laos 7 *Eutrombicula* sp. and 45 *L. deliense* were collected from 2 sites. All *L. deliense* were collected from a 2x2m square area in ~20cm tall grass, weeds and leaf litter in the shade of a moderate sized tree, with damp soil and <10m from a large swampy area.

At Ban Huay Mae Sai a single *E. wichmanni* was collected and from Nan Province, 23 *L. dendrium* from several possible rodent burrow entrances in an area of degraded secondary forest.

The 3 main study sites were investigated in detail, with black plates attempted on each visit in all the major habitats. From BSK 7 chigger species (32 individuals) were collected, of which 21 were *L. deliense* and 4 *L. imphalum.* These were collected from an open area of tall weeds (cut every year), among leaf litter under trees beside the river and around an isolated rice-field hut. In BMM, 8 species (34 individuals) were collected. The majority of these came from a single rodent burrow on a hillside of small coffee bushes and comprised *Gahrliepia*, *Trombiculindus* and *Walchia* species. Three *L. deliense* were collected from an open area of grass and weeds. In BTT, 4 *L. imphalum* were collected from an area of sparse weeds bordering a vegetable plot alongside the river.

Free-living chiggers were collected on all field trips apart from February 2016 in Laos. In January no *L. deliense* or *L. imphalum* were collected and in April only 2 *L. deliense* were found at BSK. In August larger numbers were collected with 15 in both 2016 and 2017. In November chiggers were collected only from two presumed rodent burrows in Nan Province.

PCR testing took place for 148/150 chiggers (2 were lost) and 4 were positive (2.7%). Of these 1 was a *Gahrliepia xiaowoi* collected in April 2017 from a presumed rodent burrow at BMM, 2 were *L. deliense* from BSK in April 2017 and 1 was *L. imphalum* from BTT in August 2017.

3.5.11 Small mammal serology

Blood was collected from 205 animals for IgG serological analysis using immunofluorescence antibody (IFA) testing. Animals collected dead and some *Mus* spp. or juveniles could not be bled by cardiac puncture. IFA was reported as follows: "negative" with a titre of <1:16, "weak" 1:16 to 1:64, "moderate" 1:128 to 1:512 and "strong" >1:512. Nine species had positive titres recorded, with a total of 99/205 (49%) animals testing positive. The highest proportion of positives was seen for *R. tanezumi* 36/51 (71%) and *B. indica* 26/38 (68%). High proportion of positives was also seen in *R. andamanensis* 11/20 (55%) and *R. nitidus* 6/10. At least 50% of *Berylmys* sp. and *Leopoldamys edwardsi* were also positive, but collected in low numbers. 3/16 *R. exulans* were positive. No *Mus* spp., shrews or squirrels tested positive and all 5 *R. losea* were negative (Figure 44).

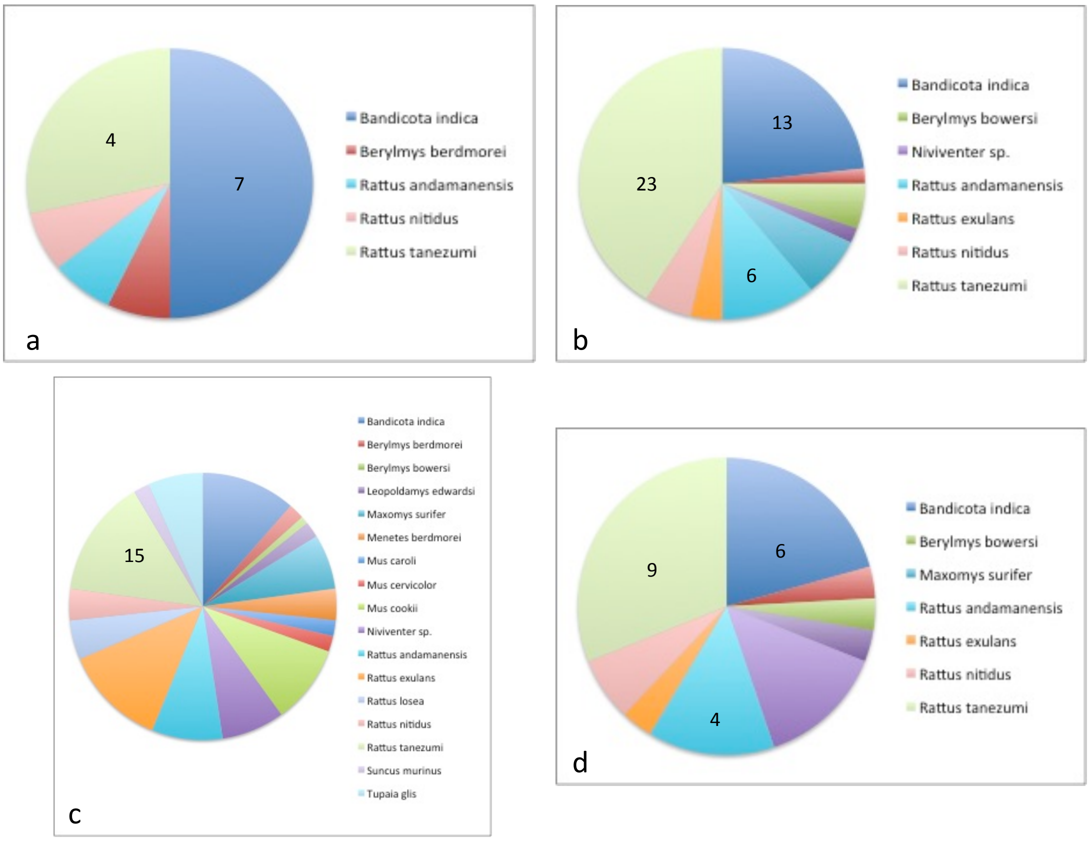
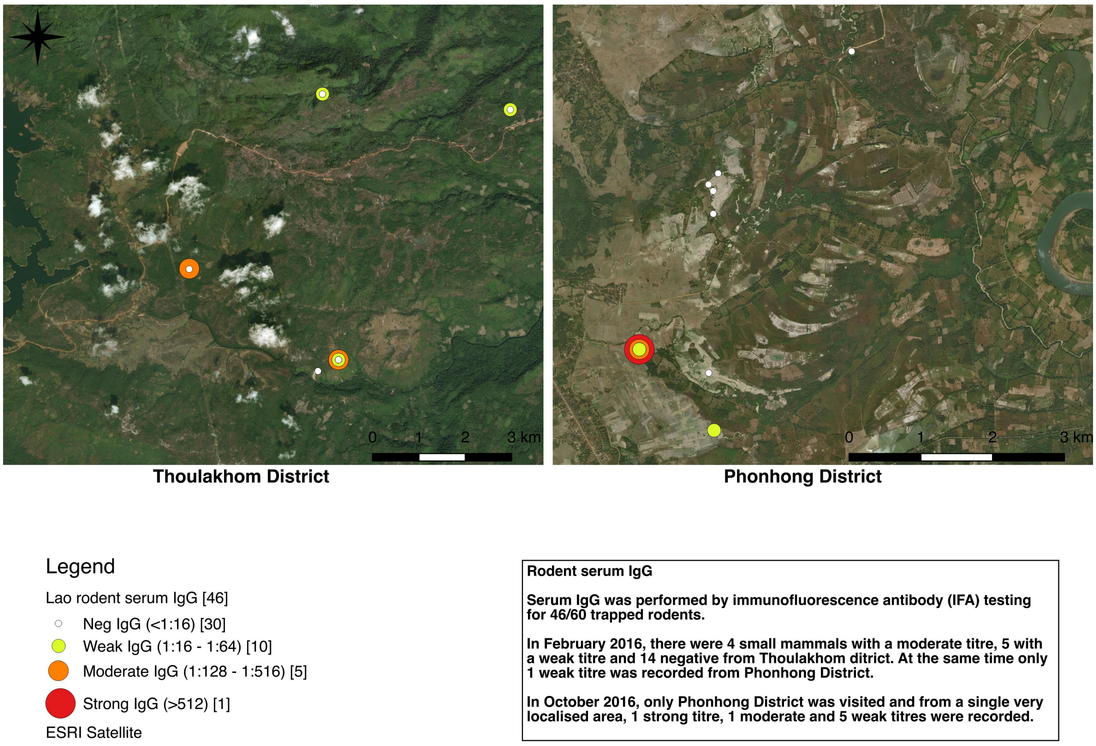


Figure 44 IgG immunofluorescence antibody (IFA) testing of small mammal species, divided into a) strong IgG (>1:512), b) moderate IgG (1:128 to 1:512), c) weak IgG (1:16 to 1:64) and negative. Negative titres are fairly evenly spread among species.

In Laos in February 2016 (when all rodent tissues and chiggers tested PCR negative), all 16 rodents trapped in the lowlands of Phonhong District were IgG negative, whereas 9/26 tested positive from the upland Thoulakhom district. However in October 2016, all 6 tested animals were positive (Figure 45).



Rodent serum IgG

Serum IgG was performed by immunofluorescence antibody (IFA) testing for 46/60 trapped rodents.

In February 2016, there were 4 small mammals with a moderate titre, 5 with a weak titre and 14 negative from Thoulakhom ditrict. At the same time only 1 weak titre was recorded from Phonhong District.

In October 2016, only Phonhong District was visited and from a single very localised area, 1 strong titre, 1 moderate and 5 weak titres were recorded.

Figure 45 Location of small mammals trapped in 2 districts in Laos, with serum IgG results

At the three main study sites, 18/43 (42%) at BMM, 24/64 (38%) at BSK and 39/48 (81%) at BTT were positive (Figure 46)

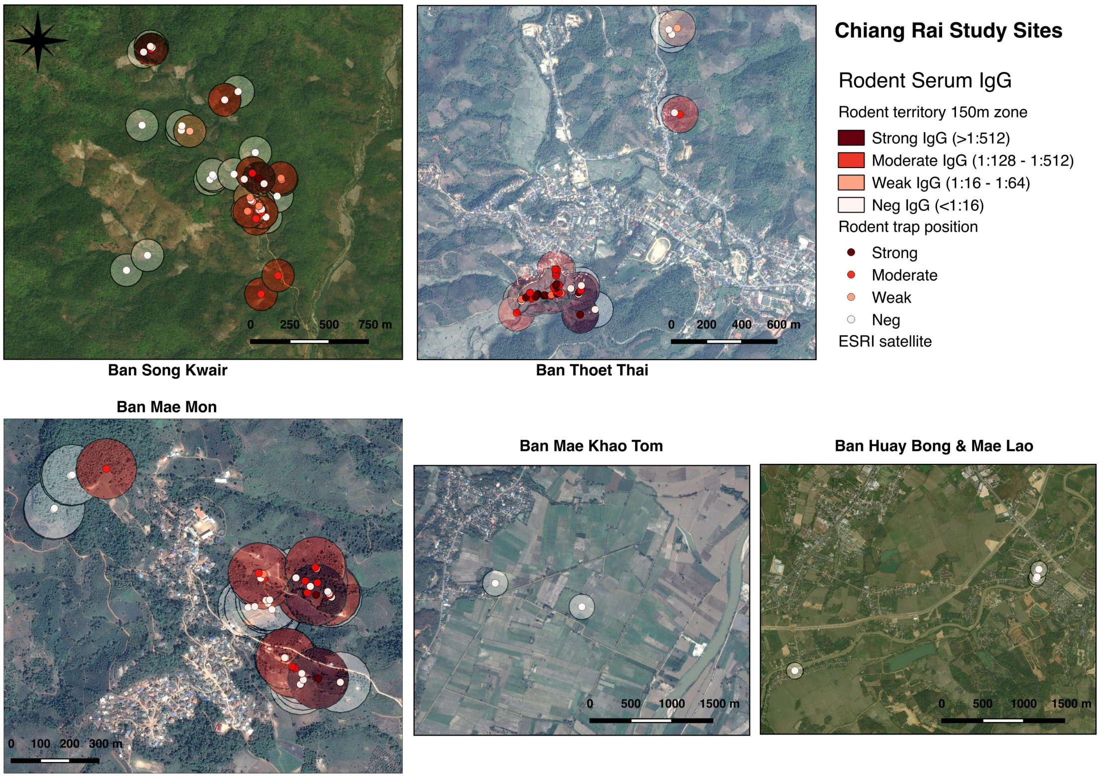


Figure 46 Location of all small mammals trapped in Chiang Rai Province showing serum IgG results. Each animal is marked with a 150m buffer, an estimated territorial range (for larger species)

There was little difference in IgG positivity during different seasons with 33/84 (39%) in the dry season, 27/38 (71%) at the end of the dry season, 29/62 (47%) in the wet season and 9/20 (45%) at the end of the wet season.

3.5.12 Other parasites

Three additional genera of mite were collected from 5 animals that proved not to be trombiculids: *Laelapidae*, *Notoedres* (Sarcoptidae) and *Cheyletus* (a predatory mite species). These mites were also tested for *O. tsutsugamushi* by PCR and all tested negative.

Ticks of 2 species (*Dermacentor* sp. and *Ixodes granulatus*) were found on 8 animals. Several fleas (unidentified) and lice were also collected.

During animal dissection a number of endoparasites were regularly observed including: Pentastomida, Filarioidea, Angiostrongylidae and liver cysts.

3.6 Discussion

Although a site of high human scrub typhus transmission with *O. tsutsugamushi* positive chiggers and rodents could not be identified in Laos, we succeeded in identifying 4 new sites of transmission in Chiang Rai Province, Thailand and in confirming the existence of a site in Nan province. A single positive chigger pool from Laos confirms the presence of the pathogen in chiggers. Three high-risk villages with different environmental and ecological profiles and recent human cases were selected for intensive study. Ban Thoet Thai (BTT) proved to be a hot spot of infection in vectors and hosts (and humans). A high density of rodents, infested with a high chigger index of 151 was recorded. Chigger species diversity was relatively low at 15 (compared to ~25 species at the other main sites). However, 71% consisted of the 2 major known vector species *L. imphalum* or *L. deliense* and 18% of individual chiggers and 41% of pools removed from rodents were positive for *O. tsutsugamushi*. Although these two *Leptotrombidium* species were most frequently positive with 23/55 (42%), it was surprising that *W. kritochaeta* and *W. micropelta* accounted for 20/55 (36%) of all individual positives. *Walchia* species are not known human vectors.

Identifying and studying a high transmission site proved challenging, particularly in Laos. With widespread human cases, it was expected that infected chiggers would be readily identified. Existing knowledge suggests that for scrub typhus cases to occur the following are required: habitat and conditions suitable for the chigger-small mammal-chigger cycle and human exposure to these sites. The extent of areas of infection risk within an endemic area remains poorly understood.

Several problems arise when attempting to locate a high transmission site. Most Lao patients were assigned to their home village. However farmers, for example, are probably exposed around their farms rather than at home, which may be several kilometres away. Several potential exposure sites may exist, especially for mobile adults. Due to difficult logistics and lack of time, we did not attempt to follow up large numbers of adult cases in Laos to locate high-risk exposure areas, as we succeeded in doing with children in Chiang Rai.

Trapping rodents is not always successful due to low rodent density and/or their wariness of traps. This may be due to population fluctuations as a consequence of climate patterns, food availability and human hunting pressure. In many parts of Southeast Asia, larger rodent species are hunted for food and hunting pressure is high in Laos [302]. However, it is unlikely that hunting pressure alone significantly reduces the overall population, but perhaps makes small mammals more wary of live traps and may explain our very low trap success rates in Laos. Even if we had clearly identified high-risk human exposure sites in Laos, our reliance on hunters to trap small mammals gave little control over selecting trapping sites. Hunters focus on areas they know to have animal activity. An additional problem encountered was that during busy farming periods, hunters were unavailable (October 2016 field trip). The low density of rodents at some sites was also evident in Chiang Rai Province, where we failed to successfully trap rodents at two epidemiological promising sites.

Why did we succeed in finding only 1 positive chigger pool in Laos? Unfortunately the first 71 small mammals trapped in 2015 had only their spleens tested and positives from other organs may have been missed. Only a subset of individual chiggers were tested and pools were not made to test all chiggers infesting these animals. Interestingly, the proportion of known vector species was also very low among the chiggers identified, with 17% being *L. deliense* in May 2015 (not all were tested) and 2 to 4% in February and October 2016 respectively. This compares starkly with BTT where 71% of chiggers were known vector species. The reasons for this are not understood. Furthermore, the first 3 field trips in Laos all occurred during the dry season, when chigger indices are lower, though *O. tsutsugamushi* positive chiggers remain high. Serological analysis of small mammals trapped in Laos revealed 31% of animals tested had evidence of past exposure, suggesting the disease is present.

Another important and unresolved difficulty is the collection of free-living chiggers. We focused on black plates as processing soil with Berlese funnels is labour intensive and allows only 12 samples to be investigated every 3 days. On most field visits, at least 1 person was dedicated to this work, usually doing 2-3 hours work after dawn (chiggers are apparently less active during the heat of the day). Although 150 chiggers were collected, two thirds of these were collected from under 1 tree in Laos and 2 presumed rodent burrows in Thailand. In BTT, where the chigger index was 151, we collected only 4 chiggers in 12 field days. A rough estimate gives 1 chigger collected for every 2.3 person-hours of work from every 137 black plates! Although rodent burrows have provided some success, locating them is difficult.

Relatively few studies have collected free-living chiggers (Chapter 2). In some studies low numbers were collected e.g. 200 in Thailand and 87 from 15 sticky traps left in 3 locations for 2 months each in Korea [96,303]. However, in others from Malaysia, Japan, Australia and other areas, as many as 10,000 larvae were collected from certain spots [254,304,305]. The distribution and location of free-living chiggers in the environment and their determinants remains poorly described.

The overall trap success rate of 3.8% was similar to that seen in other regions of Indochina (Pers. Comm., Dr Serge Morand). A total of 244 small mammals were trapped, 155 from the three main study sites. Through the course of the project 17,894 chiggers were collected, processed and tested. 785 were identified, the major bottleneck to the laboratory workflow. It was only with the assistance of a full-time laboratory technician, that this work could be completed. 279 samples tested *O. tsutsugamushi* positive: 53 small mammal tissues, 51 individual chiggers from rodents, 171 pools of chiggers and 4 free-living chiggers. Targeted enrichment sequence capture was performed on a proportion of these samples (Chapter 5). The majority of samples contained low quantities of *O. tsutsugamushi* DNA. Small mammal tissue mean Ct value was 37.56 (range 29.5 to 40.3) and the importance of testing several organs is noted. Spleen and lung were significantly more likely to be positive than liver (2.59 (95% CI 1.25-5.3) with p-value = 0.01). Mouse-model studies suggest the lungs are an important site of pathology [51]. Only two studies have compared PCR detection rates in different organs, and both assessed liver, spleen and kidney. Spleen proved most frequently positive, but kidney alone was positive in 3 rodents [229,306]. For individual chiggers the mean Ct was 37.44, median 38.38 and range 30.27 to

40.25. The mean corresponds to approximately 100 copies/µl of *O. tsutsugamushi*, range 11 to 2,850. This was lower than the mean of 20,000 copies/µl for the colony-bred chiggers, tested during laboratory optimization work. However, as the PCRs were run at different times, with different batches of standards and no chigger gene was quantified, direct comparisons are difficult. Only one recent study documents quantitative PCR results on individual (wild) chiggers with a range of 13.8 to 2,251.6 copies/µl in Thailand [307].

Two different laboratory tests were used to detect positive small mammals – PCR and IFA IgG. Using IFA as the "gold standard" test, positive predictive value (PPV) of PCR was 74% (95% CI 57 to 88) and negative predictive value 57% (95% CI 49 to 65) and a sensitivity of 26% (95% CI 18 to 36) and specificity of 92% (95% CI 84 to 96). Comparing the two methods directly using McNemar's Test gives a *p*-value = 0.132, suggesting there is insufficient evidence to conclude a difference between PCR and IFA at the 0.05 significance level. The dynamics of rickettsaemia in rodents is not known, but rodents presumably are repeatedly infected over their lifetimes. A single study demonstrated the persistence of IgG for up to 10 months in rodents removed from their habitat and kept in the laboratory [111]. Both methods provide useful but different information.

There have been 22 published reports on scrub typhus investigation in vectors and hosts in Thailand since 1952 (see Chapter 2 for additional data). Two reports consider only seroprevalence in dogs [308,309]. Eight studies investigate seroprevalence in small mammals using immunofluorescence antibody testing [91,92,111,310-314]. A wide range of positivity rates were reported from 0% in rodents trapped in Bangkok Metropolitan markets [312], to 27/27 (100%) of *R. rattus* in

Chonburi Province [311]. *B. indica* and *R. rattus* were most frequently positive. The findings in this study were similar with *R. tanezumi* (*R. rattus* is frequently misidentified as *R. tanezumi*) and *B. indica* most frequently positive with an overall seropositivity of 49%.

Nine studies, mostly pre-2000, used xenodiagnosis (often combined with other tests) to investigate the presence of *O. tsutsugamushi* in small mammals [124,242,244,315-320]. Positivity rates reached 33% in some studies, again with *Bandicota* species, *R. rattus* and *R. losea* being most frequently positive. Two studies also used cell culture, with 13% of *B. indica* testing positive [91,92].

Five studies report testing vectors and hosts using molecular methods. Khuntirat et al. reported 2/31 individual chiggers and 2-10% rodent tissues positive by 56 kDa PCR, from 2 villages in Chiang Rai Province [306]. Another study reported 1/3 *R. exulans* and 1/1 *Bandicota savilei* positive also by 56 kDa PCR from Chonburi Province [311]. Finally, using 16s amplicon sequencing, positive rodents were found from 3 sites in Thailand, *R. tanezumi* was most frequently infected (5/67) [75]. Recently Linsuwanon et al. reported 20/1,594 individual chiggers positive by PCR from military training sites around Thailand. Positive chiggers came from 5 genera including 6 *Gahrliepia* species from 6 Provinces [307]. In another study, 3/66 (4.6%) rodents (2 *R. tanezumi* and 1 *B. indica*) from Phang Nga Province tested positive, as did 26/1259 (2.1%) of chiggers from the same site. In Sisaket Province, 2/613 chiggers were also positive [321]. PCR results of small mammal testing in this study found higher rates of positivity, with 16% overall, with 16-33% positive at the 3 main study sites. These results are similar to those of some studies using xenodiagnoses.

Six studies tested chiggers from rodents [111,124,310,313,317,318]. These used both IFA and xenodiagnosis. One study reported 0/77 *Leptotrombidium* sp. positive [310]. In Nonthaburi Province 19/974 (2%) *L. deliense* and 10/473 (2%) *Ascoschoengastia* sp. were positive by IFA [111]. In Phitsanulok Province, 66/898 (7.3%) *Blankaartia acuscutellaris*, 49/1560 (3%) *L. deliense* and 2/162 *Ascoschoengastia* sp. were IFA positive and at 3 villages in Chiang Rai 48/202 (24%), 68/425 (16%) pools of *L. chiangraiensis* and *L. imphalum* respectively were positive by xenodiagnosis [313,318]. Although not directly comparable, in this study 8.6% of individual chiggers and 26% of pools were positive. The identification of several *Walchia* species, *Gahrliepia elbeli*, *Helenicula naresuani* and *Schoutedenichia* sp. positive for *O. tsutsugamushi* in this study has not been reported previously in Thailand or elsewhere.

In Thailand there have only been 2 previous studies investigating free-living chiggers for *Orientia*, published in 1966 and 1981. In the first study, 6/17 (35%) of *L. deliense* pools were positive by xenodiagnosis from 2 sites near Bangkok [303]. In the second study, 3764 chiggers were collected from 6 sites across Thailand [94]. Nine species tested positive by direct IF from 3 sites. *L. deliense* was by far the most frequently positive, with 87/2125 (4%) [94]. This was slightly higher than the 2.7% in our study and moreover, a number of species with reported positives are not recognised human scrub typhus vectors (e.g. *Eutrombicula* and *Odontacarus* species), but may be vectors to small mammals or birds. False positives are a concern with IFA [57]. Among the 4 positive free-living chiggers identified in this study, 1 was *Gahrliepia xiaowoi*. This is the first known record of *O. tsutsugamushi* in this species, which has not been recorded as biting man. Given the chigger life cycle, the presence of *O. tsutsugamushi* in the unfed larval form suggests the species is capable of transmitting the pathogen.

Although most positive small mammal species had positive chigger pools, there were 2 surprising discrepancies. *Tupaia glis* had 8 positive pools, but completely negative serology and PCR. Conversely, 2 *Mus cookii* tested PCR positive, but all chigger pools and serology were negative. Traub et al. have suggested that certain species (arboreal and scansorial Sciurids and Tupaiids) could be more resistant to *O. tsutsugamushi* infection [14] or may have much lower exposure if there is no arboreal cycle to the infection.

The chigger index has been widely regarded as a proxy for scrub typhus risk. In more northern latitudes chigger indices fall markedly over winter, as does human scrub typhus incidence [170,191,192]. In tropical latitudes chigger numbers fall during the dry season when conditions are less suitable for the chigger life cycle to complete [187,189]. Chiggers have been shown to be closely associated with damp soil during dry periods [9]. In this study, chigger indices were similarly lower during the early and late dry season compared to the wet season. This corresponds to the pattern of human scrub typhus in the region where cases rise at the start of the rainy season and drop off at the end of the rains. However, in all 3 main sites, and particularly in BTT, the proportion of *O. tsutsugamushi* positive chigger pools (perhaps the best available proxy for scrub typhus risk) ranged from 41 and 50% in the dry season and 30 and 37% in the wet season. This does not correlate with human risk. There is very little available data on local human ranging patterns during different seasons. Traub made similar observations in Malaysia [126]. That the seasonal pattern of chigger indices between small mammal species is not consistent was also observed in central India [190].

There are several weaknesses inherent in this field study. Firstly, not all the main study sites were visited at the same time of year, with both BSK and BMM not visited in November 2016. Furthermore, longer-term data over several years would improve the strength of the findings. Secondly, chigger species diversity is probably under-represented with the identification of 3 individuals from each animal. Unfortunately, due to the complexity of identification and available time, this was the limit of our capacity. Some misidentifications were probably made, particularly for genera and species with fewer available taxonomic keys.

It is likely that human behaviour is a more critical risk factor for acquiring scrub typhus than is widely credited in the literature. Although other factors are probably important too, including rodent density, chigger index, habitat type and suitability for key human vector species. Some old human volunteer studies, suggested chiggers are somewhat reluctant to bite humans. Chiggers probably do not bite humans without sufficient opportunity to attach. This would not regularly happen when simply passing through a high-risk area. In this region, farming behaviour is very seasonal and most activity occurs between May/June and November/December. Very little takes place from January to April. During peak planting and harvesting periods many rural people are involved and spend long periods in the field, working, eating and resting and often taking young children with them. These are the periods when most human cases are recorded. Infected chiggers are present throughout the year but human cases wax and wane, most likely corresponding to the risk of exposure.

CHAPTER 4

Scrub typhus ecology in Northern Thailand

4 Chapter 4: Scrub typhus ecology in Northern Thailand

4.1 Introduction

Natural systems are extremely complex, consisting of an assemblage of populations sharing a common environment. These populations of different species form a community that interact with each other and the environment in many different ways. These interactions are dynamic and may be influenced by a multitude of factors including climate and disturbance by human activity or natural events. Host-parasite interactions are a critical factor in the ecology and evolution of infectious diseases [322,323]. These interactions influence host biology, behaviour, sex ratio variation, population regulation, invasion dynamics, genetic diversity and immunity [100,322,324].

Parasite fitness is dependent on its ability to infect and reproduce in a host. In the case of scrub typhus, the "parasite" is the bacterium *Orientia tsutsugamushi* and its host is believed to be the trombiculid mites. These mites in turn interact with their small mammal hosts (and humans) and their environment ("host" here refers to both the trombiculid mites, which in turn are mainly hosted by small mammals). The ecology of the disease is complex and challenging to study, particularly in its natural environment. The majority of field studies have investigated the rates and dynamics of *O. tsutsugamushi* in the vector mites and their typical small mammal hosts. Extremely few have attempted to link these findings to the environment.

The ecological analyses attempted here are comprised of the concepts of species richness and diversity, community structure and interactions. These are investigated using different statistical tools including network analysis and generalized linear modelling.

There are two aspects to the measure of diversity in a community: the first is the number of species present (species richness) and the second, how the abundances are distributed among species (diversity and community structure). Statistical methods can be used to estimate these and make comparisons with those measured in the field. These methods are, however, beset by statistical problems. Small sample size and the inevitable under-sampling of rare species are frequent issues [325].

Network analysis is becoming increasingly used to understand host-parasite interactions [100]. The method examines the relational data between species and constructs models. In this case, the interactions between trombiculid mites, small mammals, habitat types and the presence of *O. tsutsugamushi*.

A matrix of association can be created between species pairs as either a binary (presence/absence) or quantitative measure. This measure of association can be calculated as the Checkerboard, or C-score [326]. Higher C-scores have lower co-occurrence of species in the matrix and low C-scores suggest high co-occurrence or a more aggregated matrix. Another measure of structure in an ecological system is nestedness. Nestedness is a measure of the pattern and structure of subsets within a community. Greater nestedness tends to be found in sites with lower species richness that constitute subsets of those present in species-rich sites [327,328].

Bipartite networks examine the interaction between compartments within a community such as hosts and parasites or vectors and habitats. Using mathematical models, the importance of particular species or habitats in the transmission ecology of a disease can be estimated [101]. Ecological communities are typically composed of several sub-communities and improving the understanding of these may assist with investigating functional overlap and specialization [329]. This modularity can be detected in binary or quantitative networks but is probably more accurate in quantitative networks [329].

The relationship between species and ecological niches has great relevance to disease ecology. Species can be broadly classified into a continuum between habitat specialists and generalists. Understanding the factors that drive this for species involved in transmitting infectious diseases can be vital to preventative interventions. The degree of habitat specialization of species can be mathematically estimated using the Paired Difference Index (PDI) [330].

The transformation of bipartite networks into unipartite networks allows the interaction of species (or habitats) in one compartment or sub-community to be analysed. These "nodes" interact with each other and further measures and models of community structure can be made.

The degree of centrality of a node as measured using the Eigenvector centrality score, helps elucidate the network architecture [101,331]. In this analysis the sharing of chigger species by small mammal hosts with or without infection with *O. tsutsugamushi* and habitat types make up the nodes. For example, small mammal species with stronger interaction ("connectance") share more of the same chigger species, and those with higher centrality scores should be considered more important nodes in the network.

Generalized linear models (GLM) were used to investigate associations between *O. tsutsugamushi* PCR positive and negative small mammals, individual chiggers and chigger pools with transect location, forest cover, trap location and season.

This study is the first attempt to perform detailed ecological analyses of scrub typhus at the scale of a single study site using GIS and statistical tools. Data collected at 4 intervals over an 18-month period at just 3 sites in Chiang Rai Province, Thailand was used for more detailed analysis.

A field visit to a scrub typhus research site on the Penghu (Pescadores) Islands in Taiwan provided the opportunity to compare and contrast the ecology of these two geographically diverse sites of high human scrub typhus incidence.

This work follows closely the methods employed by Bordes et al. in their study of zoonotic disease in Southeast Asia and by Chaisiri et al. in the analysis of ecological and microbiological diversity of chigger mites and small mammals in Thailand [101,201,297]. Dr. Serge Morand kindly provided assistance with analysis including the use of R scripts and statistical support.

4.2 Methods

4.2.1 Habitat and climate description

Habitat was described at all field sites. At the lowest resolution sites were divided into four broad categories: upland (higher elevation of > ~400m above sea level (asl) with sloping cultivated or fallow ground), lowland (lower elevation of <400m asl) with flatter cultivated or fallow ground usually in a river alluvial plain, forest (of any type) and settlement (any built up area). At medium resolution a list of 14 commonly encountered habitat types was developed: coffee, corn (maize), fallow area, fruit plantation, mixed secondary forest, rice paddy field, rice paddy field bordering a riverbank, river bank, swamp, teak (*Tectonia grandis*) plantation, vegetable garden either bordering a riverbank or not, village or riverbank beside a village. Unfortunately no widely accepted medium-resolution habitat classification scheme exists. At the highest resolution the major plant types were recorded using terms including: deciduous, evergreen, bushes, weeds, bamboo and cultivated plant/tree varieties. The average height of trees and proximity of settlements and water sources were also recorded.

At the three main study sites an attempt was made to perform a more detailed botanical survey. Each site was surveyed and photographs and specimens of leaves and flowers taken and catalogued. With the kind assistance of an experienced Lao botanist familiar with the regional flora, Mr Bounsamay Soulileuth, working with the Institut de Recherche pour le Développement (IRD), these were identified to species level where possible.

Climate data for the study period was taken from https://en.climate-data.org and included maximum, minimum and mean temperature and rainfall.

4.2.2 Taiwan case study

Live rodent trapping using Sherman traps baited with sweet potato and peanut butter was performed at 6 sites during my visit between 9th and 17th October

2017. The sites selected were all within ~5 kilometres of Huxi Township on the main island of the Penghu Islands (Figure 49, 23.6°N, 119.6°E). This area has the highest reported cases of human scrub typhus across the Islands. Trapping took place in semi-urban areas, vegetable gardens and abandoned/overgrown plots of land. A few chiggers were collected for identification and PCR analysis in Laos and subsequent sequence capture (see Chapter 5). I also attempted to collect free-living chiggers using the black plate method (Chapter 3).

4.2.3 Ecological analysis

Ecological data comprised small mammal (hosts) and chigger (vectors) species diversity, habitat and season linked to the presence or absence of *O. tsutsugamushi*. Analysis was performed for 10 sites for hosts and 9 sites for vectors (at 1 site, trapped hosts carried no chiggers). Detailed analyses were restricted to the 3 main study sites in Chiang Rai Province, Thailand.

4.2.3.1 Species richness

Species richness is defined as the actual number of chigger or small mammal species found at different study sites. Two statistical estimates were made for species richness: first-order Jackknife (Jack1) and Bootstrap using the *vegan* package v2.5-3 [332] in R freeware (R Development Core Team 2018). The Jack1 method is based on the presence or absence of a species in a given area, rather than species abundance [333,334]. The bootstrap method estimates quantities in the population (at different locations) by averaging estimates from multiple small data samples [334,335]. Both these non-parametric estimators aim to provide an accurate assessment of total species richness with the least possible bias [334,336].

Jack1 performs best with low sampling size and bootstrap outperforms other methods with increased sampling efforts [336]. Estimates using both these methods are presented, together with rarefaction curves (number of species as a function of the number of samples) for chigger and small mammal species for different collection sites.

Species diversity has two separate components: species richness and relative abundance (evenness or dominance). There are many different measures of diversity [337]. The Shannon index (H') was used here as it incorporates both components of diversity and provides a simple summary [338]. This was calculated using the *vegan* package in R [332]. Confidence intervals were calculated using the *boot* and *mosaic* packages in R. Typical values range between 1.5 and 3.5 with the index increasing as both the richness and evenness of the community increase. All measures of diversity are influenced by statistical sampling, difficulties of accurately identifying all the species present and delineating ecological communities at different scales. The Shannon index is unsuitable for comparing sites with large differences in species richness [337].

4.2.3.2 Network analysis

Host-ectoparasite bipartite networks were investigated using either quantitative (i.e. total number of each small mammal or chigger species) or binary matrices.

The following quantitative networks were investigated:

1) All small mammal and chigger species (interactions with less than 2 chigger species among all hosts were excluded)

2) Small mammal (n=8-9) and chigger species (n=12-21) at each of the 3 main study sites
3) Small mammal species (n=21) and habitat
4) Most common chigger species (n=13) and habitat at all study sites

The following binary (presence/absence) networks were investigated:

1) *O. tsutsugamushi* positive small mammals (n=7) and *O. tsutsugamushi* positive chigger species (n=11)
2) *O. tsutsugamushi* positive small mammals and chigger species (n=20) with habitat (n=13).

4.2.3.2.1 Bipartite network

The C-score was calculated for each bipartite network [326]. This gives a number between 0 and the total number of rows on each side of the bipartite network multiplied together. The C-score was generated using the R package *bipartite* [329].

A measure of nestedness, the NODF formula, was calculated for both binary and quantitative matrices using the *vegan* package. The degree of nestedness ranges from 0 to 100 [327].

The function *computeModules* in the R package *bipartite* was used to calculate different sub-communities (modules) within the bipartite network [329].

4.2.3.2.2 Habitat specialization

The degree of habitat specialization for chigger and rodent species overall and for those species testing *O. tsutsugamushi* positive for the 14 habitat types used was tested using PDI in the R package *bipartite.* The PDI is measured on a continuous scale from 0 to 1, where a species testing >0.5 is a habitat specialist and <0.5 is a habitat generalist [330].

4.2.3.2.3 Unipartite network

Bipartite networks where separate nodes connecting hosts and chiggers or either hosts or chiggers with habitat were transformed into unipartite networks using the *tnet* package in R.

The Eigenvector centrality (EC) was calculated using the *evcent* function from the *igraph* package in R. The EC measures the influence quantitatively of a particular node (i.e. small mammal, chigger or habitat category) in the unipartite network being considered. The nodes with the highest EC (measured between 0 and 1) are most likely to be connected to other nodes and play a more important role in this interaction. Chiggers or small mammal-habitat networks with individual species EC scores closest to 1 are likely to be most able to occupy multiple habitats and be generalists, whilst those with EC scores closer to 0 are likely habitat specialists.

The *igraph* function *cluster_louvain* was used to model unipartite network modularity structure [339]. The size of the interaction between unipartite nodes is shown by the thickness of connecting lines. Different coloured nodes represent unipartite modules. These differ from bipartite modules, discussed earlier. These two additional measures of community structure are not discussed further in the text.

4.2.3.3 Generalised Linear Modelling

GLM was used to investigate associations between *O. tsutsugamushi* PCR positive small mammals, chiggers and chigger pools and transect location, forest cover, habitat type (trap location) and season. Model selection was performed using the machine learning technique of generalized boosted regression modelling using the *dismo* and *gbm* packages in R [340-342]. The relative quality of these models was estimated using receiver operating characteristic (ROC) curves, where the area under the curve is measured. A range from 1 (perfect discrimination) to 0.5 (no discriminatory ability) is generated. Akaike information criterion (AICc) for model strength was also reported. These were calculated using the *ROCR* package in R. Mixed effects models were tested for the different variables to assess their influence on the best-fit model for *O. tsutsugamushi* PCR positivity using the *arm* package in R.

4.3 Results

4.3.1 Habitat and climate description

Fourteen habitat types were created to describe those encountered at all 10 study sites (Table 21).

Table 21 Number of small mammals trapped (n=244) in different habitats (medium resolution classification) in Thailand and Laos with approximate total trap nights (habitats in which hunters set traps was not always known)

Habitat type	Approx. total trap nights	No. trapped	Habitat type	Approx. total trap nights	No. trapped
Coffee plantation	420	5	**River bank**	270	5
Corn field	200	15	**Swamp**	30	3
Fallow area	810	39	**Teak plantation**	250	5
Fruit tree plantation	380	4	**Vegetable garden**	360	5
Mixed secondary forest	1380	68	**Vegetable garden/river bank**	280	4
Rice paddy field	600	32	**Village**	300	25
Rice paddy field /river bank	240	27	**Village/river bank**	430	6

Twenty-one of the commonest plant species were identified (Table 22). The majority of these (13) were members of the grasses and bamboos (*Graminae*). Wild sugarcane *Saccharum spontaneum*, 3 species of invasive African grasses of the genus *Pennisetum*, *Imperata cylindrica*, a common grass of East Asia and 2 bamboo species dominated. Two *Compositae*, *Ageratum conyzoides* (chick-weed) and *Bidens pilosa* (Black-jack) were also very widespread and are both invasive.

Table 22 Most commonly encountered plant species at the three main study sites

Field Site	**Plant species**	**Plant family**
1,2,3	*Ageratum conyzoides* L.	**COMPOSITAE**
1,2,3	*Bidens pilosa* L.	**COMPOSITAE**
1,2,3	*Microstegium ciliatum* (Trin.) A.Camus	**GRAMINAE**
1,2,3	*Microstegium ciliatum* (Trin.) A.Camus	**GRAMINAE**
1,2,3	*Bambusa tulda* Roxb.	**GRAMINAE**
1,2,3	*Thysanolaena latifolia* (Roxb.) Honda	**GRAMINAE**
3	*Scleria terrestris* (L.) Fass.	**CYPERACEAE**
3	*Anomianthus dulcis* (Dun.) Sincl.	**ANNONACEAE**
3	Genus unknown	**ANNONACEAE**
3	*Dalbergia foliacea* Wall.	**LEGUMINOSAE, PAPILIONOIDAE**
1,2,3	*Dendrocalamus barbatus* Hsueh & D.Z. Li	**GRAMINAE**
2,3	*Alpinia malaccensis* (Burm.f.) Roscoe	**ZINGIBERACEAE**
1,2,3	*Pennisetum polystachion* (L.) Schult.	**GRAMINAE**
1,2,3	*Saccharum spontaneum* L.	**GRAMINAE**
1,3	*Thyrsostachys siamensis*	**GRAMINAE**
1,2,3	*Imperata cylindrica* (L.) Raeusch.	**GRAMINAE**
1	*Pennisetum purpureum* Schumach.	**GRAMINAE**
1,3	*Pennisetum setaceum*	**GRAMINAE**
1	*Rottboellia exaltata*	**GRAMINAE**
1	*Senna siamea*	**LEGUMINOSAE, PAPILIONOIDAE**

1=Ban Thoet Thai, 2=Ban Song Kwair, 3=Ban Mae Mon

The climate is dominated by the southwest monsoon that brings heavy rains from May to October, peaking in August and September. From November to April it is dry, with just the occasional heavy shower during March and April. Temperatures peak at over 40°C in April/May, cool to the low 30s with the onset of the rains and may fall as low as 0°C at night in higher elevations during December and January (Figure 47 & Figure 48).

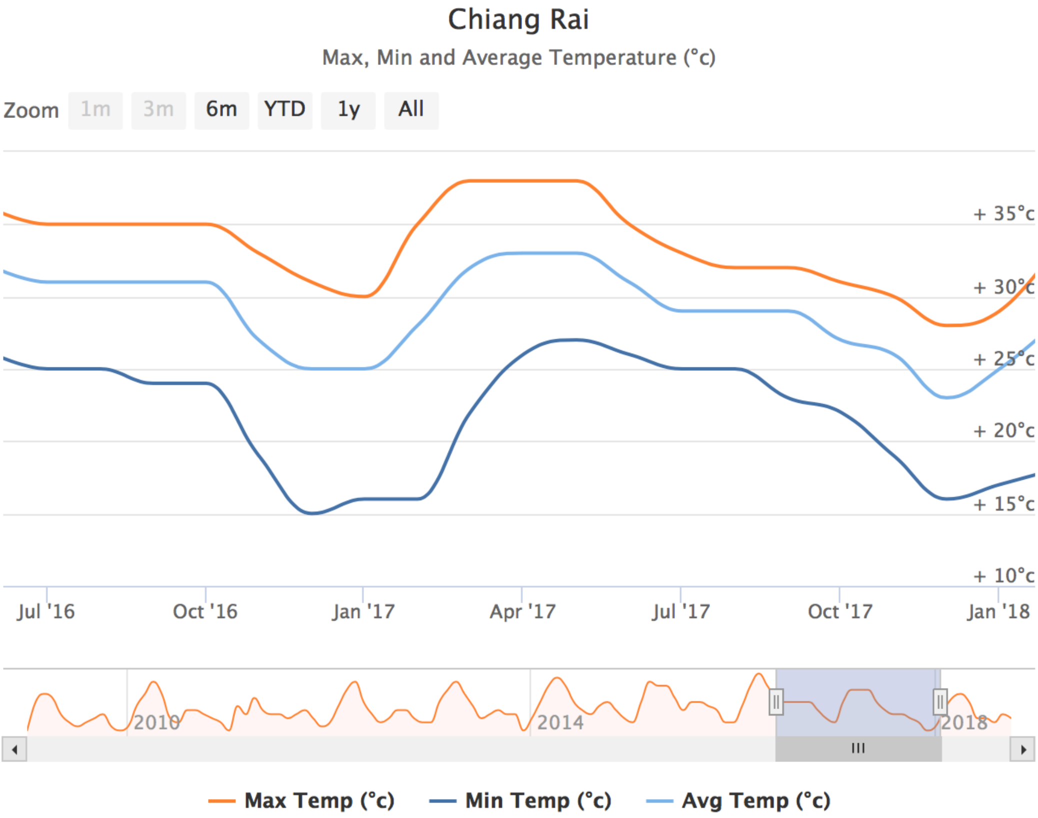


Figure 47 Maximum, minimum and average temperature recorded in Chiang Rai city during the course of the study (July 2016 to January 2018) (taken from https://en.climate-data.org)

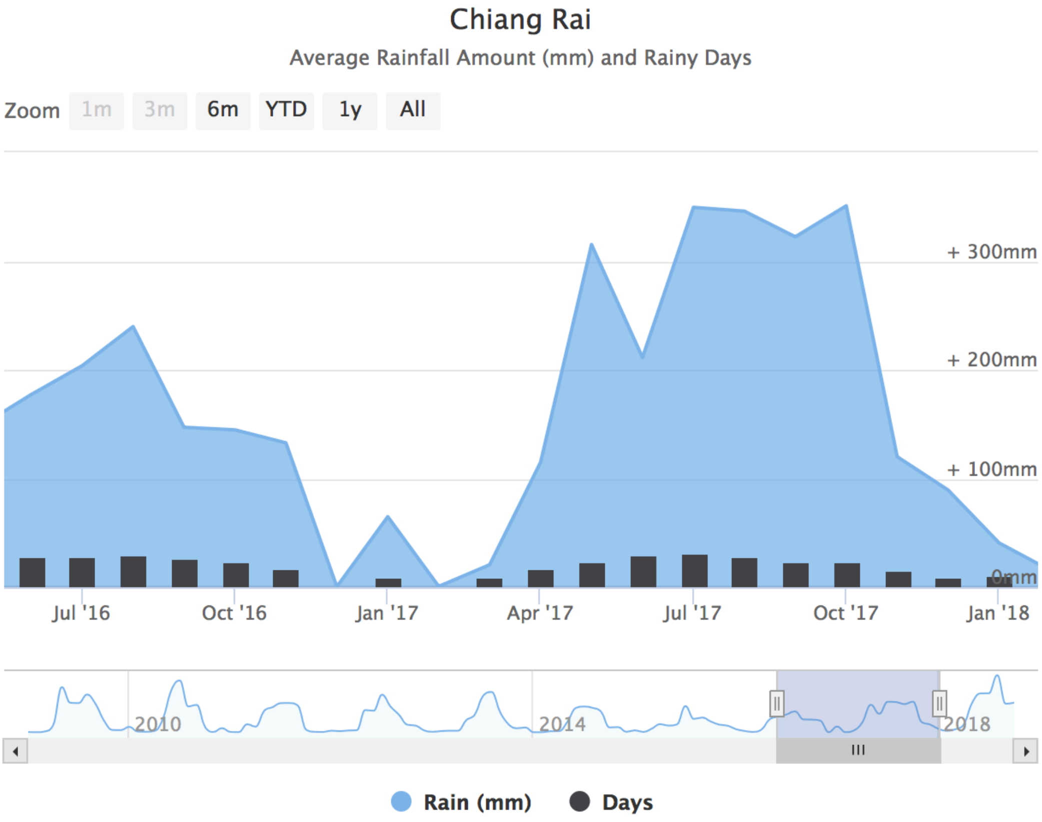


Figure 48 Cumulative monthly rainfall recorded in Chiang Rai city during the course of the study (July 2016 to January 2018) (taken from https://en.climate-data.org)

4.3.2 Taiwan case study

Trap success rates were very high at ~30% during my visit, compared to ~5% in Thailand. Four species of small mammal are regularly trapped: *Rattus losea*, *R. norvegicus*, *Mus musculus* and the shrew *Suncus murinus*. Chigger indices were high, with 200-400 individuals found on many of the *Rattus* species. Lower numbers were seen on *S. murinus* and *M. musculus*. Almost all chiggers reported from the islands are *L. deliense*.

I collected 83 chiggers from 2 *R. losea*, in a fallow area of grasses and low trees. Twenty were randomly selected for species identification, and all identified as *L.*

deliense. A total of 19 individuals and 7 pools were tested (Chapter 3). One individual and 1 pool tested positive for *O. tsutsugamushi* by PCR.

Approximately 4 hours of black plating was carried out at two sites where rodents were trapped with high chigger indices. No chiggers were collected.

During my visit, the mean temperature was in the high 20s centigrade, with high humidity, occasional rain showers and frequent high winds.

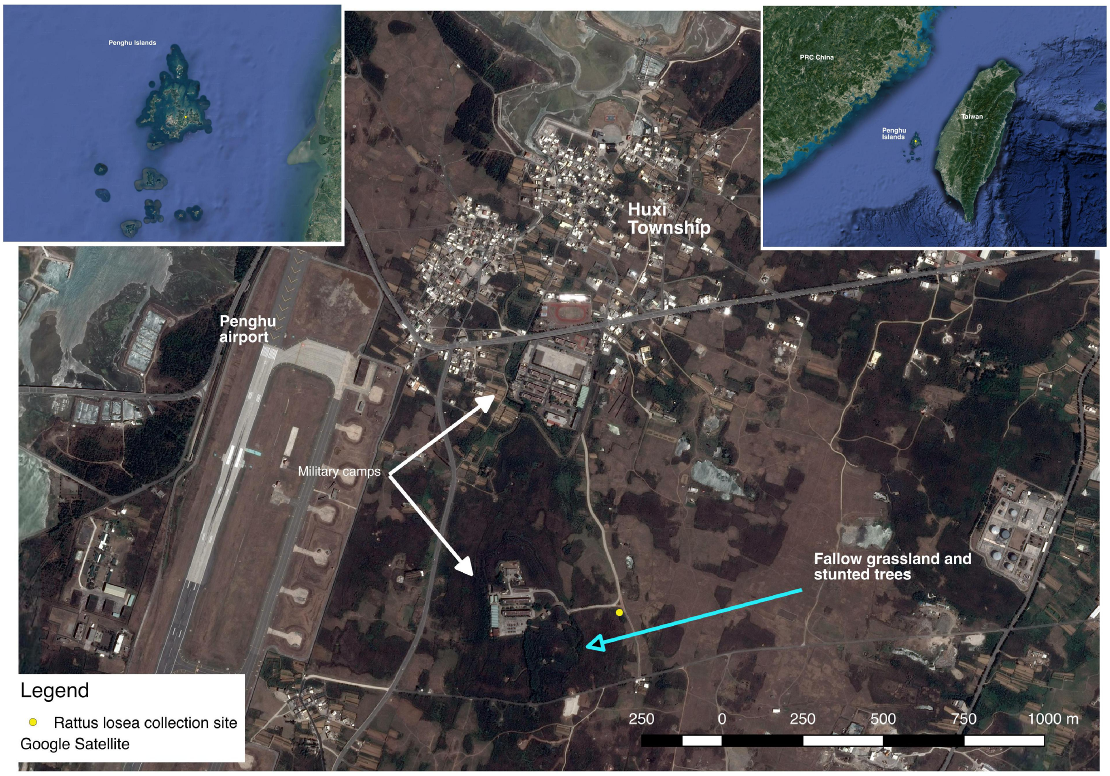


Figure 49 Map of Taiwan showing Penghu Islands (top right), the Penghu Islands (top left) and rodent trapping site near Huxi Township (October 2017)

4.3.3 Ecological analysis

4.3.3.1 Species richness

A total of 19 species of small mammal were positively identified in this study, although several animals could only be identified to genus. These were excluded from the estimates. For all study sites combined, an estimate of small mammal species richness during this study using Jack1 was 19.3 (standard error (se) 0.9) and bootstrap 19.8 (se 2.1). Rarefaction curves that begin to plateau, suggest that actual species richness is reaching the estimated. The rarefaction curve (Figure 50) is very close to plateauing, suggesting that sampling saturation for small mammal species was close to being achieved.

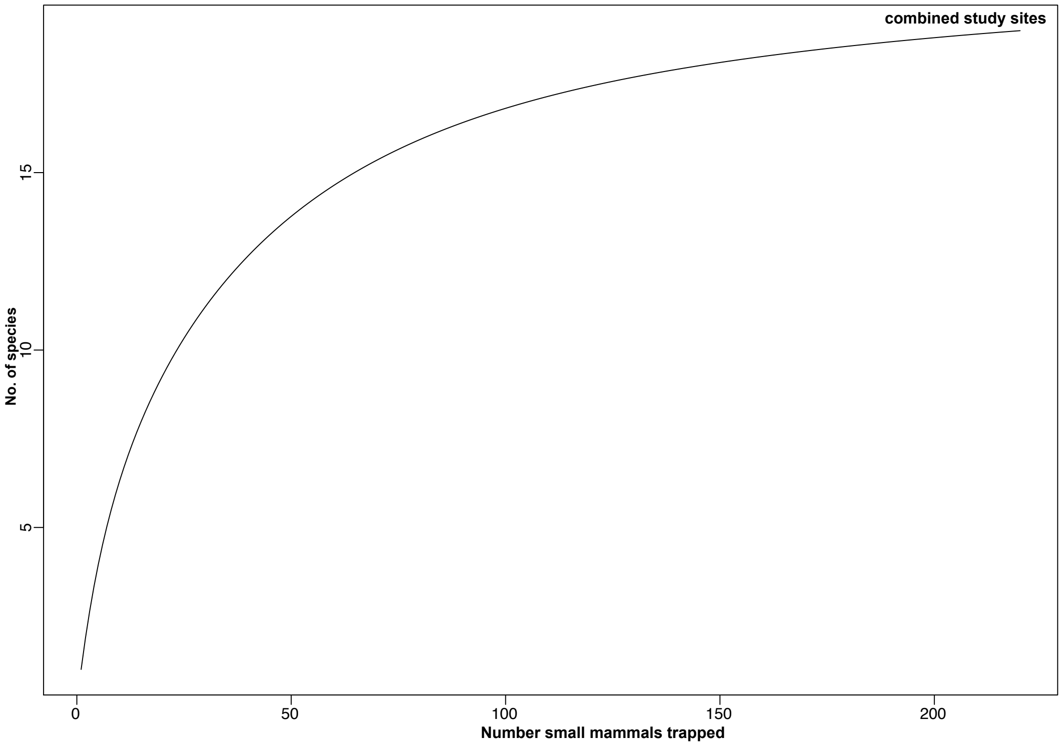


Figure 50 Rarefaction curve for small mammal species trapped at all study sites combined

A total of 45 chigger species were identified during the course of this study. An overall estimate of chigger species richness using Jack1 was 53 (se 2.9) and bootstrap 56 (se 3.5). The rarefaction curve for all sites combined (Figure 51) suggests sampling saturation had not yet been achieved.

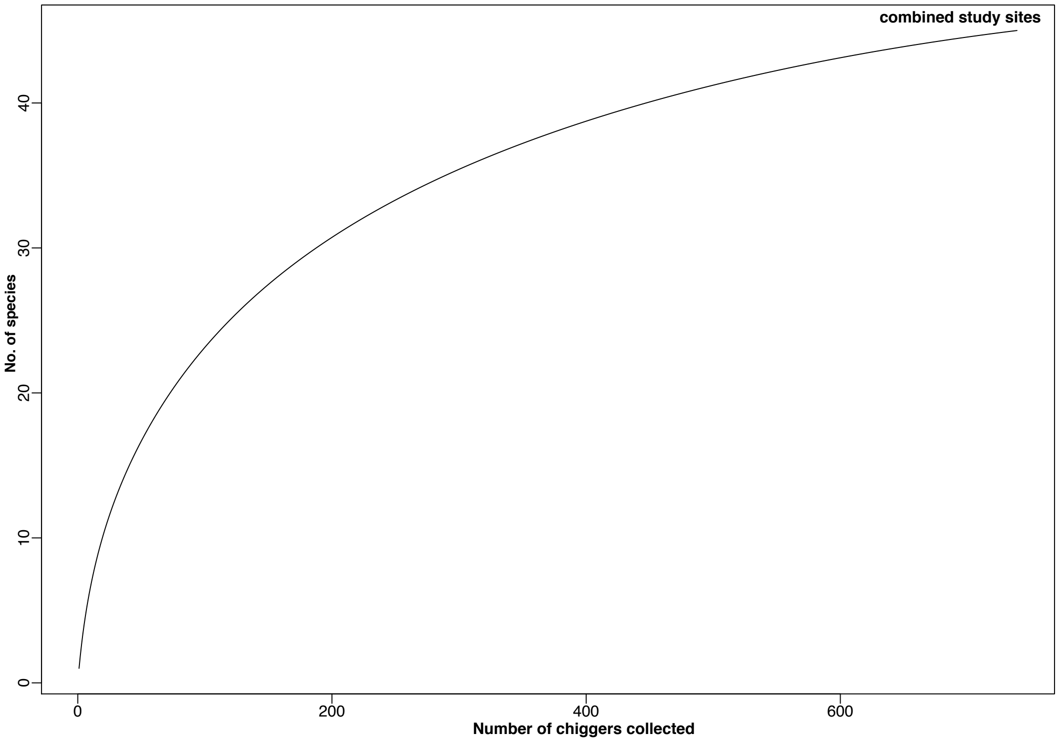


Figure 51 Rarefaction curve for chigger species collected from all study sites combined

A separate analysis was performed for individual study sites to illustrate sampling saturation at each location. The higher estimates reflect the range of subsampling at different sites. An estimate of small mammal species richness using Jack1 was 23.5 (se 2.4) and bootstrap 21.3 (se 1.8). This was most evident in Ban Song Kwair (BSK) and Phonhong District, followed by Ban Thoet Thai (BTT) and Ban Mae Mon (BMM) (Figure 52).

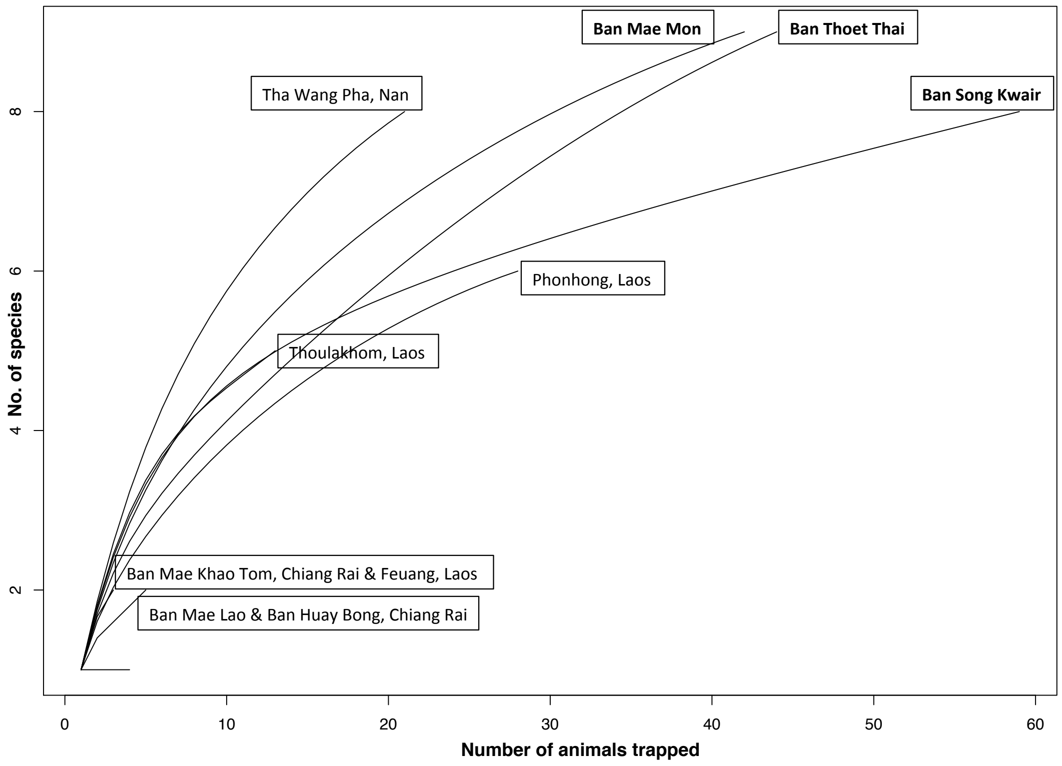


Figure 52 Small mammal species richness rarefaction curves for all study sites

Forty-five chigger species were recorded from the 10 field sites in Laos and Thailand. Jack1 estimate was 68.6 species (se 11.7) and bootstrap 55.36 (se 7). As for small mammals, numbers at some locations was very small. Rarefaction curves, however, suggest that for the 3 main sites in Chiang Rai Province, Phonhong and Thoulakhom in Laos and Nan Province were reaching the estimated number of species (Figure 53).

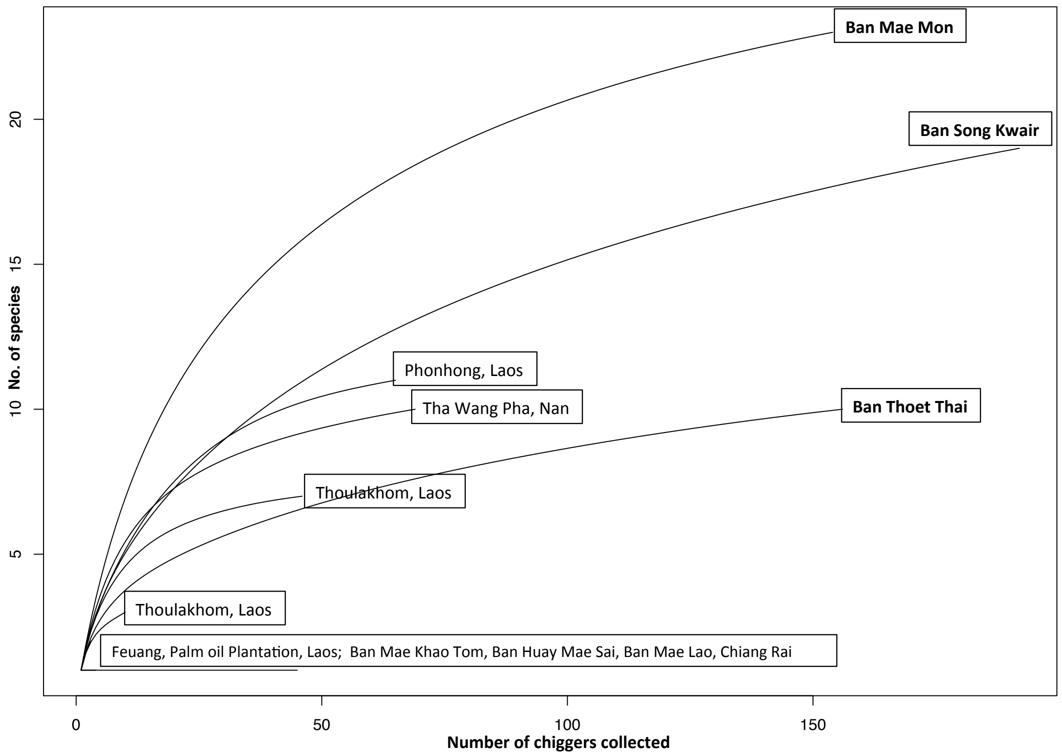


Figure 53 Chigger species richness rarefaction curves for all study sites

4.3.3.2 Species diversity

The Shannon index was measured for the three main sites for chiggers and small mammals. At BMM, *H'* was 1.66, 95% confidence intervals (CIs) [1.37, 19.2] and 1.41 [0.98, 1.78] for chiggers and small mammals respectively. At BSK, *H'* was lower at 1.57, 95% CIs [1.28, 1.86] (chiggers) and 1.3 [0.9, 1.67] (small mammals). Diversity was lowest at BTT with *H'* 1.39, 95% CIs [0.99, 1.71] for chiggers and 1.02 [0.64, 1.31] for small mammals.

4.3.3.3 Network analysis

4.3.3.3.1 Small mammal species and chiggers

The interactions between small mammal species and chiggers across all sites were investigated. Species interactions of only 1 individual were excluded. These data are uneven across sites, with most concentrated on 3 sites (see below) and sparse data with low numbers of some species at other sites. Figure 54 and Figure 55 show that a small number of small mammal-chigger interactions occurred frequently. The C-score for chigger-small mammal co-occurrence was 12.1 (p=0.01), suggesting moderate co-occurrence of species across sites. Examining the matrix for small mammal-chigger co-occurrence gave a C-score of 4.55 (p=0.01). Nestedness was relatively low with a NODF of 34. Analysis of modularity among this data, suggested that *L. deliense* was grouped with *R. tanezumi*, *R. nitidus*, *N. fulvescens* and *M. berdmorei*, whilst *L. imphalum* was associated with *B. indica* (Table 23). EC scores placed *L. deliense* (1.0) and *W. kritochaeta* (0.91) centrally, while *L. imphalum* had a score of 0.38. For small mammals, *R. tanezumi* (1.0) was central, with *B. indica* and *M. surifer* at 0.68.

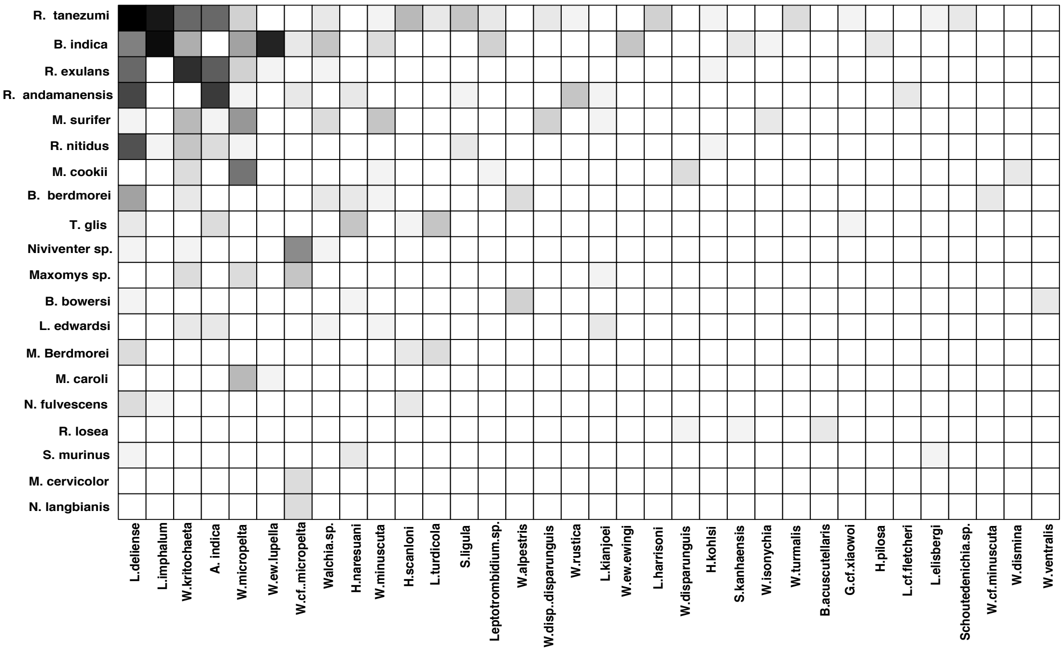


Figure 54 Nestedness matrix showing the quantitative interaction between small mammal and chigger species collected at all sites (excluding interactions of only 1 individual from a species).

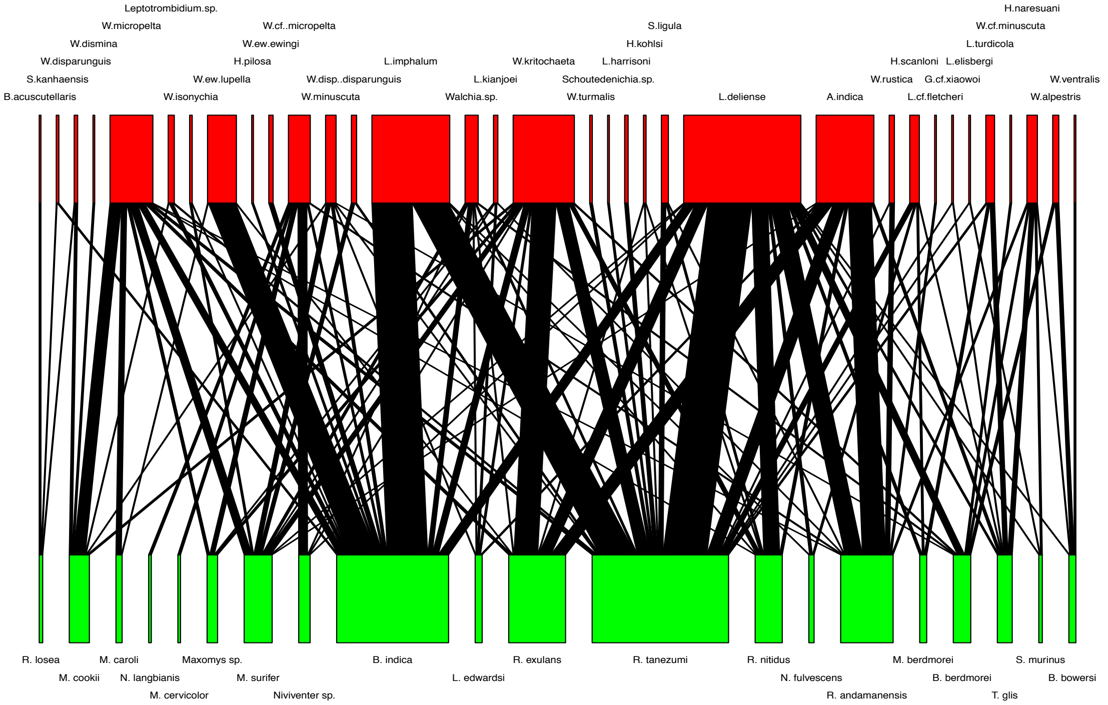


Figure 55 Quantitative bipartite network graph for small mammal-chigger interactions across all study sites. The thickness of the connecting lines reflects the size of the quantitive interaction between any given host-chigger species pair.

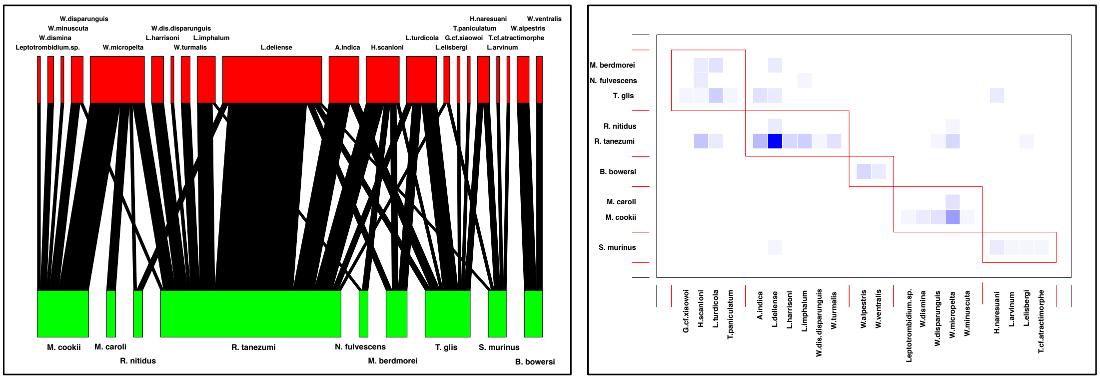
4.3.3.3.2 Small mammal species and chiggers at Ban Thoet Thai, Ban Song Kwair & Ban Mae Mon

Bipartite network analysis of small mammal-chigger interactions at the 3 main study sites revealed different patterns (Figure 56 & Figure 57). For BMM, the small mammal-chigger C-score was low (1.5, *p*=0.07) suggesting high co-occurrence, while the chigger-small mammal C-score was higher at 8.6 (*p*=0.03). Small mammals at this site were trapped in the broadest range of habitat types and this is clearly reflected by the chigger species richness recorded (21 species, compared to 16 at BSK and 12 at BTT). Sub-community analysis grouped *L. deliense*, *L. imphalum* and *L. harrisoni* with *R. tanezumi* and *R. nitidus*. The *Mus* species were grouped with several *Walchia* species (Table 23). *R. tanezumi* and *L. deliense* were central in the unipartite network with EC of 1.0.

BSK, an area with a high degree of secondary forest with some clearing for cultivation, showed low C-scores of 1.5 and 4.9 (*p*=0.01). *L. deliense* and *L. imphalum* were grouped with *R. tanezumi*, *R. nitidus* and *M. berdmorei*, although *R. andamanensis* was also closely associated with *L. deliense* (Table 23). EC scores were 1.0 for *R. tanezumi* and *A. indica*, 0.94 for *L. deliense* and 0.77 for *W. kritochaeta*.

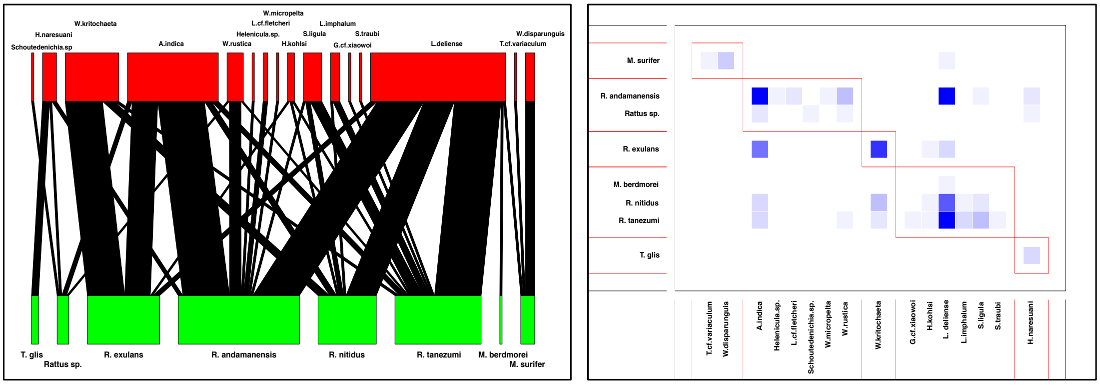
Habitat diversity and chigger species richness were lowest at BTT. Here the C-scores were very low, suggesting a highly aggregated matrix (0.98 and 1.8, *p*=0.01). Nestedness was also highest (NODF =45, compared to 41 at BSK and 30 at BMM). *B. indica* and *R. tanezumi* were grouped with several species, including *L. imphalum* (Table 23). At this site, *L. deliense* was grouped with several other species. *B. indica* was central (EC = 1). Despite its abundance, *L. impahlum* was not central (0.46), with *W. kritochatea* at 1 and *L. deliense* 0.72.

Ban Mae Mon



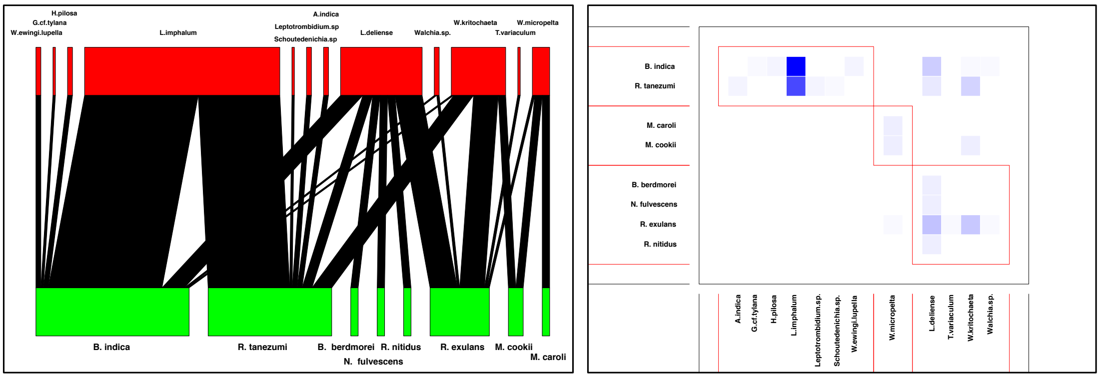
C-score = 1.5, p=0.07 (Rodents vs chiggers)
C-score = 8.6, p=0.03 (Chiggers vs rodents)

Ban Song Kwair



C-score =1.5 , p=0.01 (Rodents vs chiggers)
C-score = 4.9, p=0.01 (Chiggers vs rodents)

Ban Thoet Thai



C-score =0.98 , p=0.01 (Rodents vs chiggers)
C-score = 1.8, p=0.01 (Chiggers vs rodents)

Figure 56 Bipartite network figures and sub-community modules (with C-scores) for small mammal and chigger interactions at the 3 main study sites. The degree of shading of the module blue boxes reflects the size of the host-chigger species pair interaction.

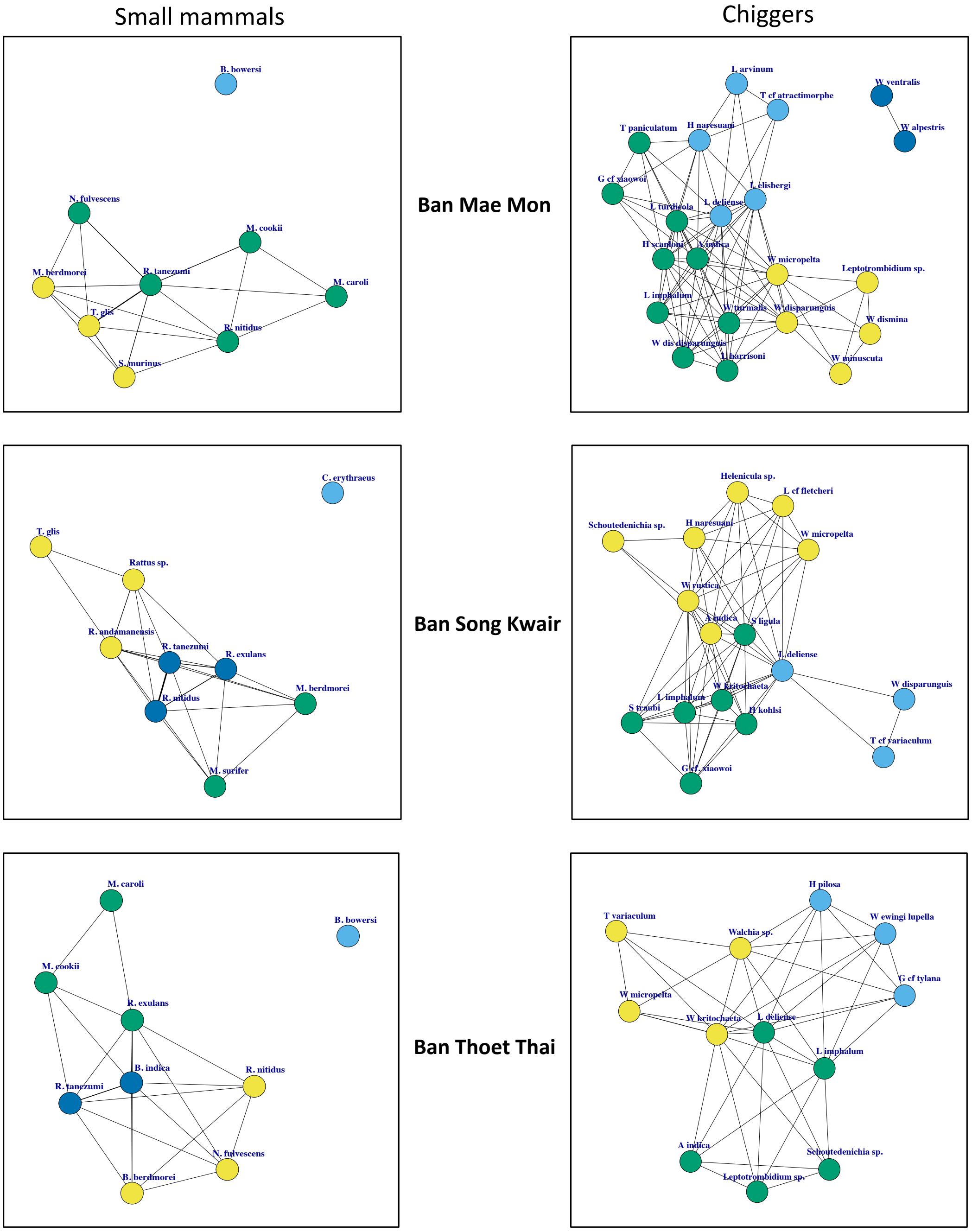


Figure 57 Unipartite network models for small mammals and chiggers at the 3 main study sites. The strength of the interaction between "nodes" is indicated by the thickness of the connecting line. The most centrally located nodes have Eigenvector scores closest to 1. The colours of the nodes reflect the unipartite modules (another measure of community structure) generated after transforming bipartite networks into unipartitie networks.

4.3.3.3.3 Chiggers and habitats

Examination of the interaction between chigger species and habitat types showed that *L. deliense* was least specialist with a PDI of 0.86, whilst for *L. impahlum* the PDI was 0.95 and *W. kritochaeta* 0.83. Nestedness was relatively low (NODF 71). Fallow and mixed secondary forest was most central (EC 1 and 0.87 respectively), while *L. deliense* and *W. kritochaeta* was also most central (1 and 0.82). C-scores were low at 2.53 and 2.96 (p=0.01). *L. imphalum* was not found in association with mixed secondary forest, but perhaps more closely associated with proximity to water (Figure 58).

It is worth noting here the differences in these finding when data is reanalysed (below) for the presence of *O. tsutsugamushi*.

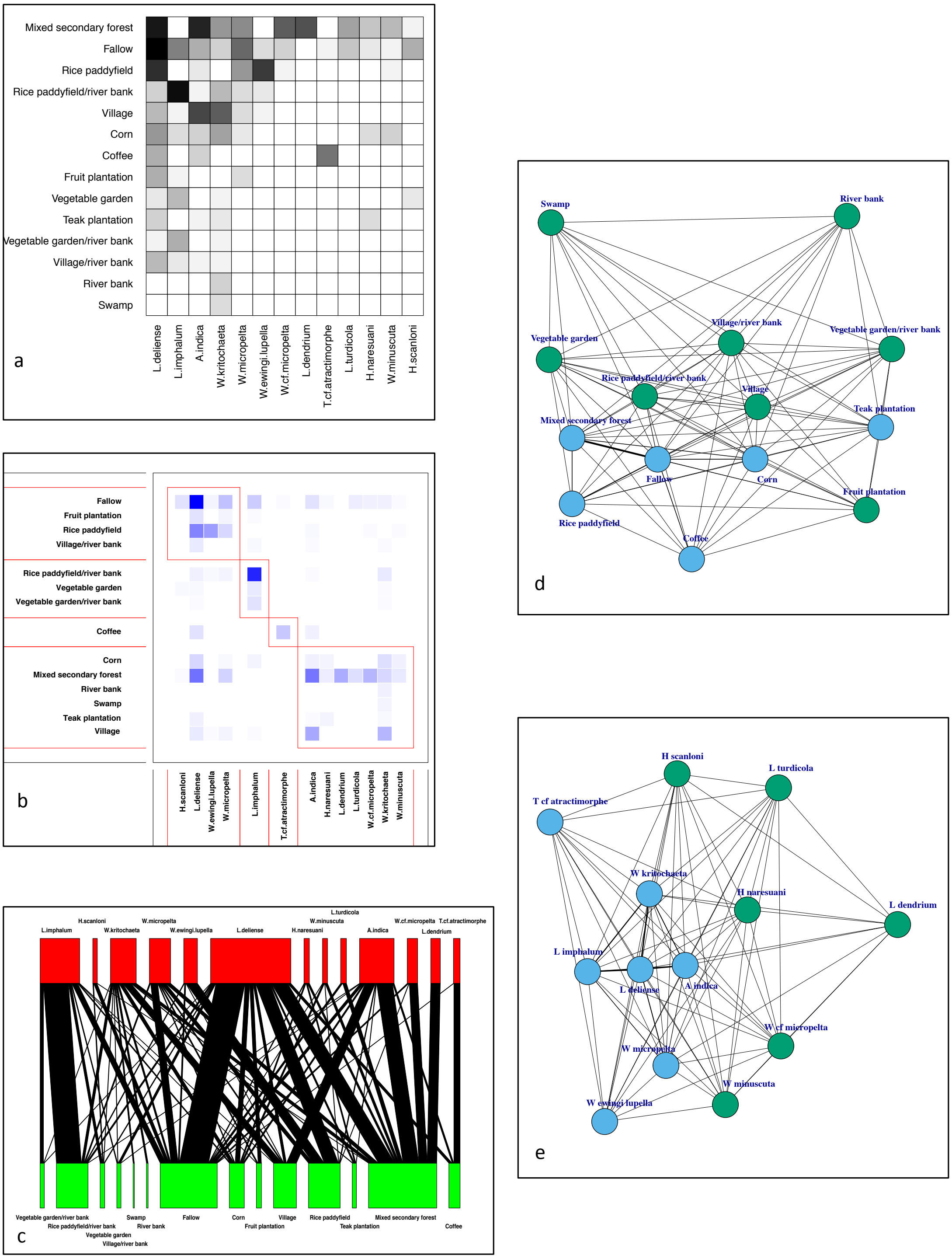


Figure 58 Chigger-habitat interactions for all sites combined: (a) Nestedness matrix, (b) sub-community modules, (c) bipartite network, (d) unipartite network for habitat and (e) chiggers. The degree of shading of boxes & line thickness reflect the size of the interaction of habitat-chigger species interactions. Unipartite network colours reflect unipartite modules.

4.3.3.3.4 Small mammals and habitats

Small mammal-habitat C-scores were 2.86 and 6.6 (p=0.01). Nestedness was lower with a NODF score of 42. Of the common species, *R. tanezumi* was more generalist (PDI = 0.71), with *B. indica* more specialist at 0.92. *R. tanezumi* was also most central with an EV of 1. Of the habitat types, fallow was most central.

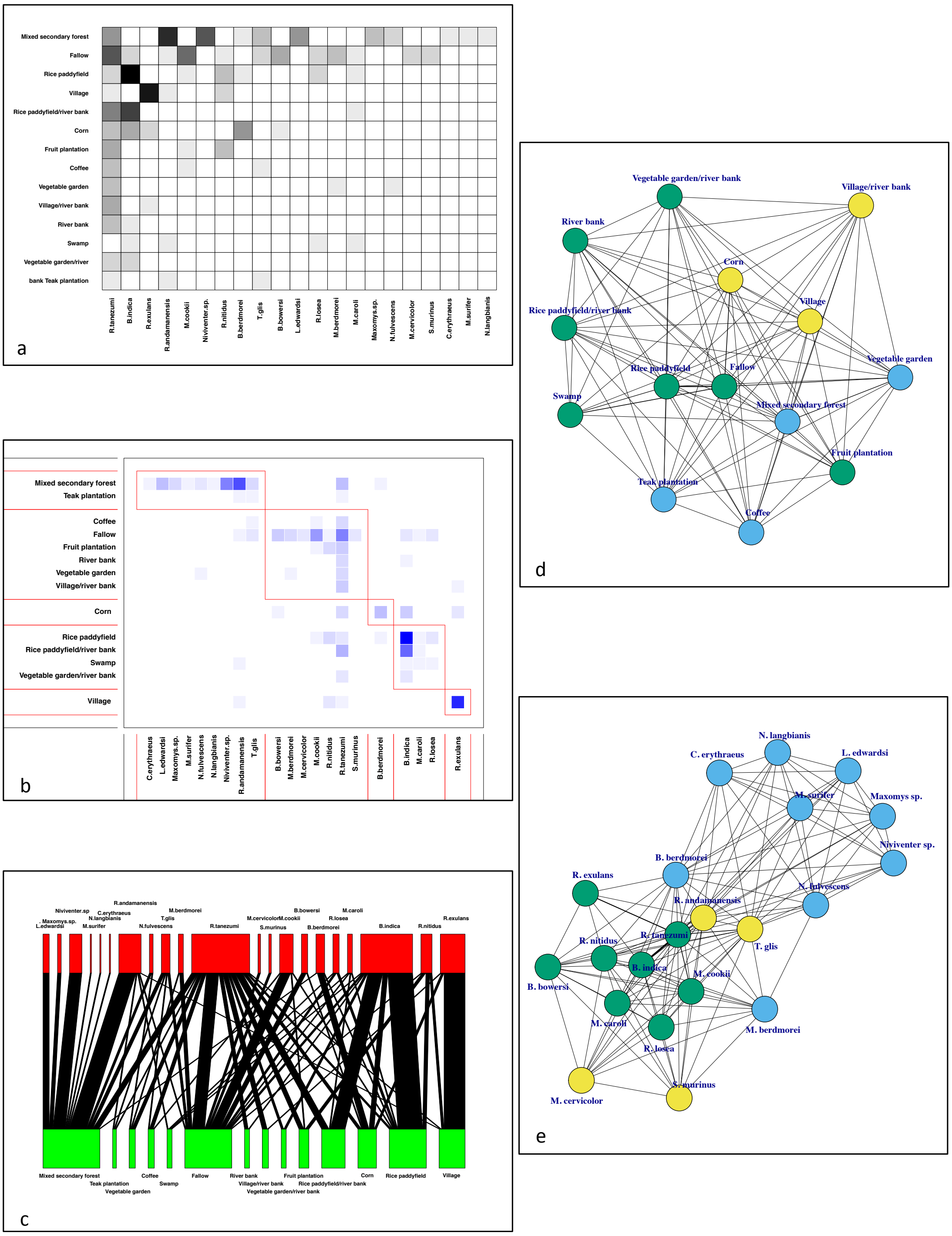


Figure 59 Small mammal-habitat interactions for all sites combined: a) Nestedness matrix, (b) sub-community modules, (c) bipartite network, (d) unipartite network for habitat and (e) chiggers. The degree of shading of boxes & line thickness reflect the size of the interaction of habitat-chigger species interactions. Unipartite network colours reflect unipartite modules.

4.3.3.3.5 *O. tsutsugamushi* positive vectors and hosts

A non-quantitative network analysis was used to analyse the interactions between *O. tsutsugamushi* positive chiggers and their host species. Low C-scores of 0.09 and 0.32 (p=0.01) were seen for both directions of interaction, confirming high co-occurrence of the species involved. The NODF score was very low at 3. Sub-community analysis revealed *L. imphalum* to be associated with *R. tanezumi*, while *B. indica* and *M. surifer* were grouped with *W. micropelta* and *W. minuscuta* and *L. deliense* and *W. kritochaeta* with *R. andamanensis*, *R. exulans* and *B. berdmorei* (Table 23). Centrality scores placed *B. indica* and *W. kritochaeta* centrally, with *L. deliense* at 0.93, *L. imphalum* 0.42, *R. tanezumi* 0.83 and *R. andamanensis* at only 0.21 (Figure 60).

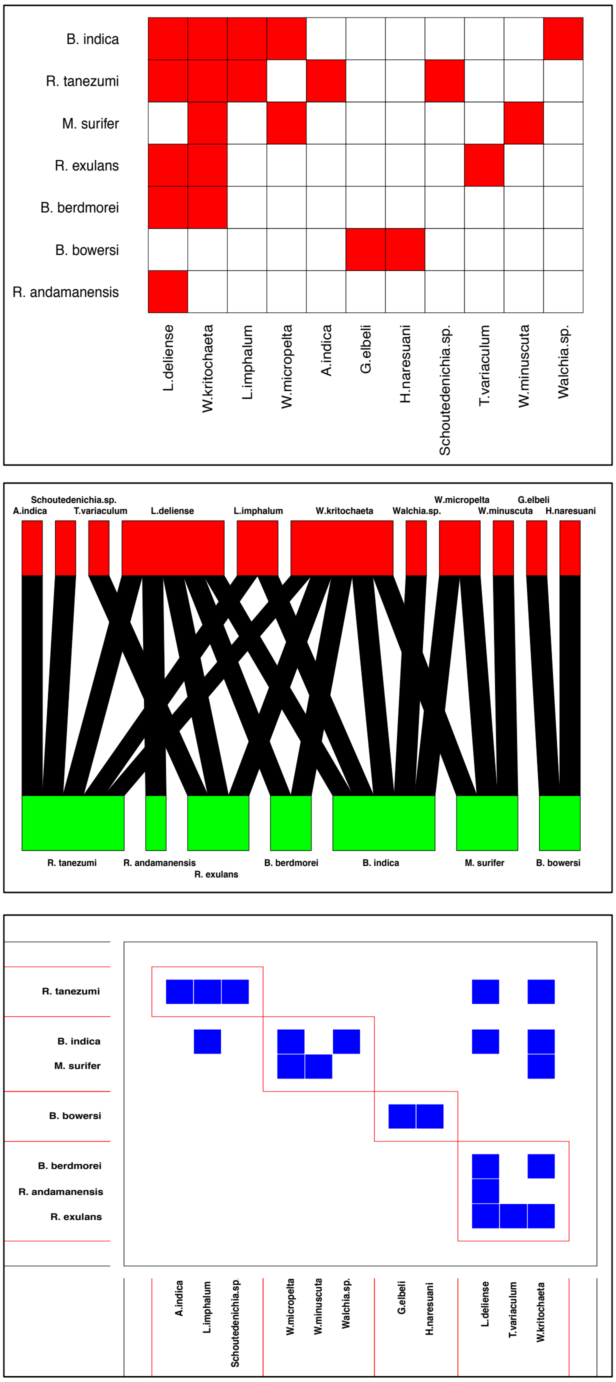


Figure 60 Non-quantitative nestedness matrix, bipartite network and sub-community modules for chigger-small mammal interactions where either species tested PCR positive for *O. tsutsugamushi*

4.3.3.3.6 *O. tsutsugamushi* positive vectors/hosts and habitats

A non-quantitative matrix was also used to analyse the interaction between *O. tsutsugamushi* infected vectors and hosts with habitat types (Figure 61). C-scores were 3.8 and 9.2 (*p*=0.01). Nestedness was relatively low (NODF 38). Sub-community analysis grouped *R. tanezumi*, *L. imphalum* and *S. ligula* with river edge habitat and several food source habitats (vegetable gardens) as seen at BTT (Table 23). A number of species including *L. deliense* are grouped with forested habitat (and village), although the latter probably refers to BSK, where the village is closely associated with forest. *L. deliense* and *R. tanezumi* are true generalists with a PDI of 0.33 and 0.42, respectively, whilst for *L. imphalum* the PDI is 0.5 and *B. indica* is 0.83. Paddy field/riverbank (EC 1), corn field (0.91) and mixed secondary forest (0.83) were most central in the unipartite network (Table 24). The village and coffee plantation were least represented in the network. That riverbank and vegetable garden alone had low centrality scores may reflect the lower number of animals trapped in that habitat type.

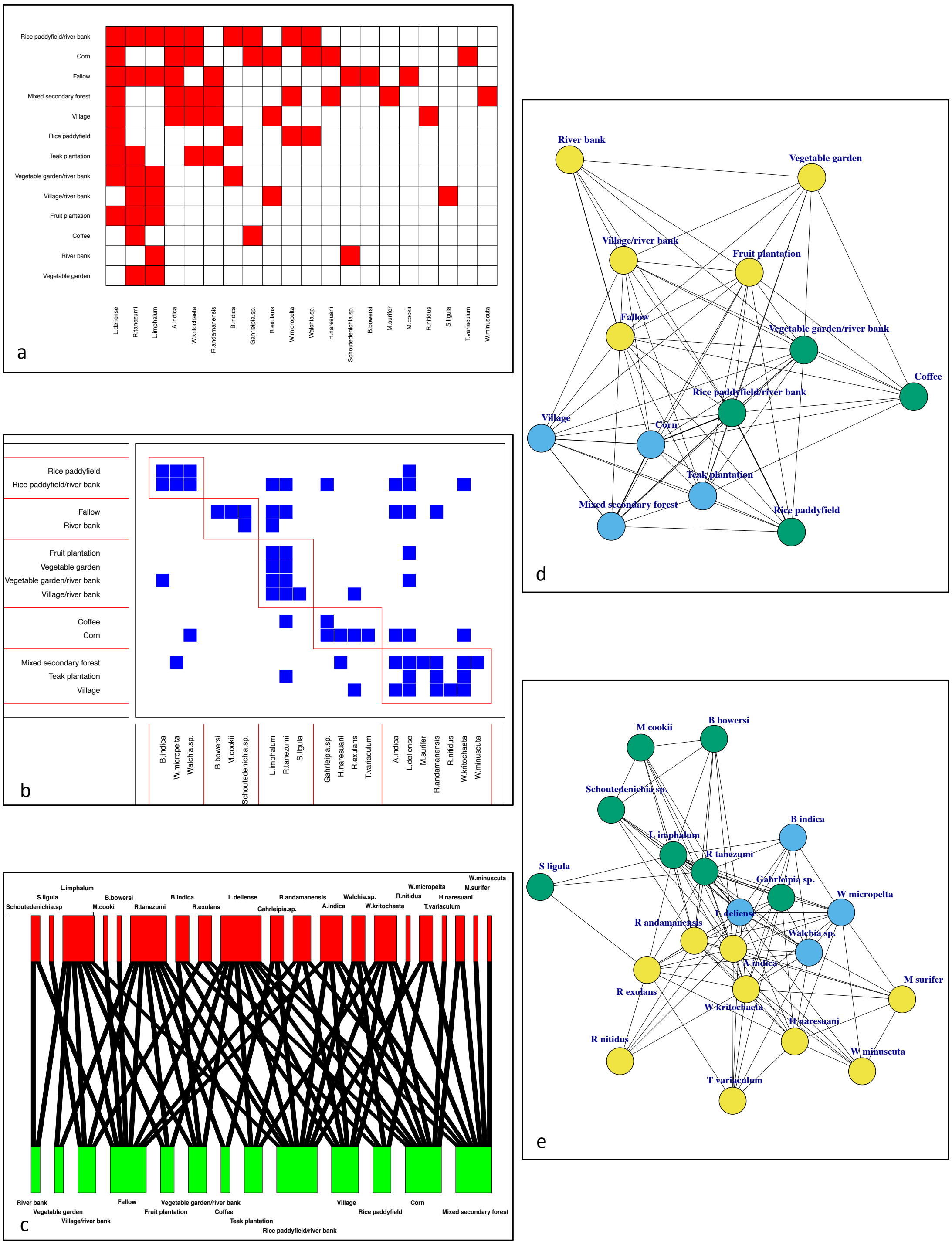


Figure 61 ***O. tsutsugamushi*** **positive small mammal/chigger & habitat interactions: a) Nestedness matrix, (b) sub-community modules, (c) bipartite network, (d) unipartite network for habitat and (e) chiggers. The degree of shading of boxes & line thickness reflect the size of the interaction of habitat-chigger species interactions. Unipartite network colours reflect unipartite modules.**

Table 23 Summary of bipartite sub-community analysis for small mammal-chigger interactions at the 3 main study sites, for habitat and either small mammals or chiggers and for habitat and *O. tsutsugamushi* PCR positive samples

	MODULE 1		**MODULE 2**		**MODULE 3**		**MODULE 4**		**MODULE 5**	
Ban Mae Mon	*M. berdmorei* *N. fulvescens* *T. glis*	*G. cf. xiaowoi* *H. scanloni* *L. turdicola* *T. paniculatum*	*R. nitidus* *R. tanezumi*	*A. indica* *L. deliense* *L. harrisoni* *L. imphalum* *W. disp. disp.* *W. turmalis*	*B. bowersi*	*W. alpestris* *W. ventralis*	*M. caroli* *M. cookii*	*Leptotrombidium* sp. *W. dismina* *W. disparunguis* *W. micropelta* *W. minuscuta*	*S. murinus*	*H. naresuani* *L. arvinum* *L. elisbergi* *T. cf. atractimorphe*
Ban Song Kwair	*M. surifer*	*T. cf. variaculum* *W. disparunguis*	*R. andamanensis* *Rattus sp.*	*A. indica* *Helenicula* sp. *L. cf. fletcheri* *Schoutedenichia* sp. *W. micropelta* *W. rustica*	*R. exulans*	*W. kritochaeta*	*M. berdmorei* *R. nitidus* *R. tanezumi*	*G. cf. xiaowoi* *H. kohlsi* *L. deliense* *L. imphalum* *S. ligula* *S. traubi*	*T. glis*	*H. naresuani*
Ban Thoet Thai	*B. indica* *R. tanezumi*	*A. indica* *C. cf. tylana* *H. pilosa* *L. imphalum* *Leptotrombidium* sp. *Schoutedenichia* sp. *W. ewingi lupella*	*M. caroli* *M. cookii*	*W. micropelta*	*B. berdmorei* *N. fulvescens* *R. exulans* *R. nitidus*	*L. deliense* *T. variaculum* *W. kritochaeta* *Walchia* sp.				

Habitat & chiggers	-Fallow -Fruit plantation -Rice paddy -Village/river bank	*H. scanloni* *L. deliense* *W. ewingi lupella* *W. micropelta*	-Rice paddy/ riverbank -Veg garden -Veg garden/ river bank	*L. imphalum*	Coffee	*T. cf. actractimporphe*	-Corn -Mixed forest -River bank -Swamp -Teak	*A. indica* *H. naresuani* *L. dendrium* *L. turdicola* *W. cf. micropelt* *W. kritochaeta* *W. minuscuta*		
Habitat & small mammals	-Mixed forest -Teak	*C. erythraeus* *L. edwardsi* *Maxomys* sp. *N. fulvescens* *N. langbianis* *Niviventer* sp. *R. andamanensis* *T. glis*	-Coffee -Fallow -Fruit plantation -River bank -Veg garden -Village/ riverbank	*B. bowersi* *M. berdmorei* *M. cervicolor* *M. cookii* *R. nitidus* *R. tanezumi* *S. murinus*	Corn	*B. berdmorei*	-Rice paddy -Rice paddy/ riverbank -Swamp -Veg garden/ riverbank	*B. indica* *M. caroli* *R. losea*	Village	*R. exulans*
Habitat and PCR positive	*R. tanezumi*	*A. indica* *L. imphalum* *Schoutedenichia* sp.	*B. indica* *M. surifer*	*W. micropelta* *W. minuscuta* *Walchia* sp.	*B. bowersi*	*G. elbeli* *H. naresuani*	*B. berdmorei* *R. andamanensis* *R. exulans*	*L. deliense* *T. variaculum* *W. kritochaeta*		

Table 24 Eigenvector (EV) centrality scores for different habitat types in association with small mammal and chigger species and for any sample testing *O. tsutsugamushi* positive

Small mammals		Chiggers		*O. tsutsugamushi* positive	
Habitat	**EV**	**Habitat**	**EV**	**Habitat**	**EV**
Fallow	1	Fallow	1	Rice paddy/river bank	1
Rice paddy	0.81	Mixed forest	0.87	Corn	0.91
Corn	0.59	Corn	0.61	Mixed forest	0.83
Swamp	0.54	Rice paddy	0.61	Village	0.66
Mixed forest	0.53	Rice paddy/river bank	0.49	Rice paddy	0.60
Village	0.43	Village	0.49	Fallow	0.59
Vegetable garden	0.40	Teak plantation	0.36	Teak plantation	0.49
Fruit plantation	0.38	Vegetable garden	0.36	Vegetable garden/river bank	0.44
Rice paddy/river bank	0.36	Village/river bank	0.31	Village/river bank	0.32
Coffee	0.36	Coffee	0.31	Fruit	0.30
Teak plantation	0.34	Fruit plantation	0.25	Coffee	0.27
Vegetable garden/river bank	0.21	Vegetable garden/river bank	0.22	River bank	0.20
River bank	0.21	River bank	0.07	Vegetable garden	0.18
Village/river bank	0.19	Swamp	0.07	-	-

A summary of the centrality of different habitat types when associated with small mammals, chiggers and those testing positive does not reveal any consistent findings. Rice paddy, fallow areas, corn and mixed secondary forest are generally most central (Table 24).

4.3.3.4 Generalised Linear Modelling

GLMs were constructed for *O. tsutsugamushi* PCR positive/negative vector and small mammal samples combined with the following factors: site code, transect location, habitat type (trap location), forest cover and season. Analysis of the correlation among the different explanatory variables revealed season to be strongly correlated with PCR positivity (relative influence of 87%), followed by trap location (relative influence 8.9%). Transect location and forest cover were dropped from the analysis. The area under the curve (AUC) was 0.726 (se = 0.038), suggesting a good model fit.

Repeat assessment of the 2 best-fit models showed season to have a relative influence of 64.4% and the second best-fit model was trap location with a relative influence of 35.6%. The AICc for the best–fit model was 279.

The seasons were classified into: dry, end of the dry, wet and end of the wet. The predicted probabilities of PCR positivity were most strongly observed during the end of the dry season and end of the wet season, with lower probabilities during the wet and dry seasons (Figure 62).

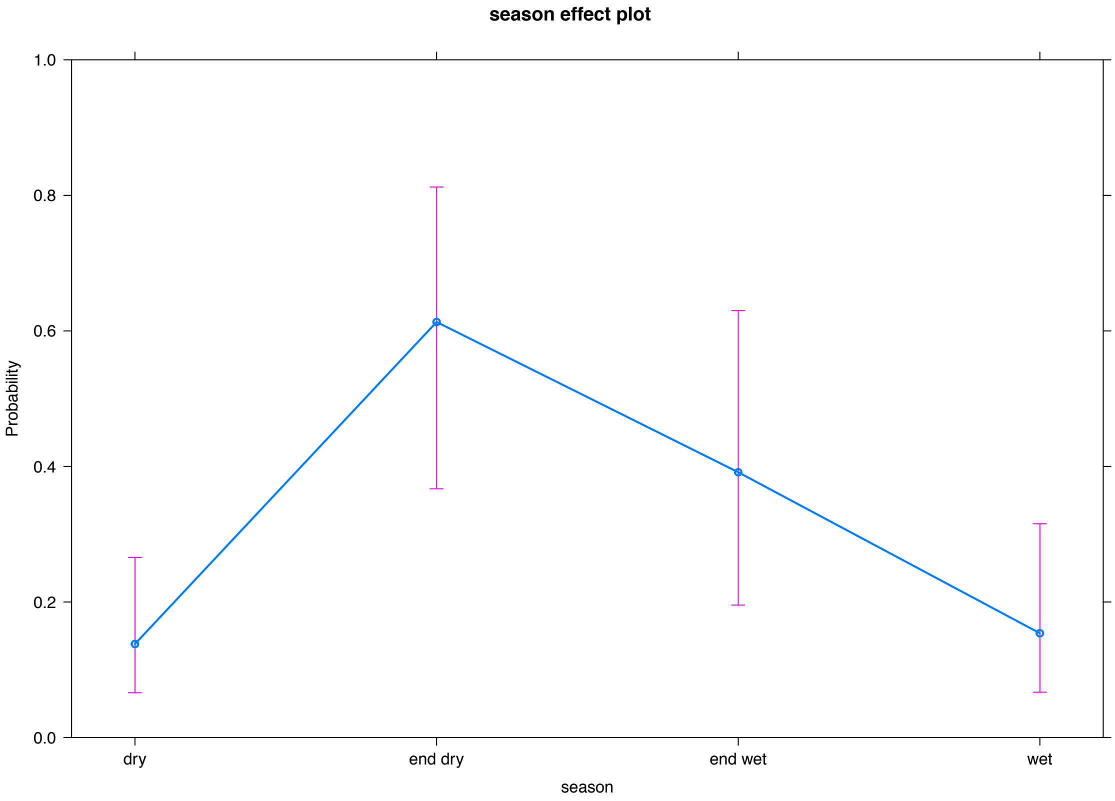


Figure 62 Predicted probabilities of *O. tsutsugamushi* PCR positivity during different seasons

The odds ratios (OR) for *O. tsutsugamushi* PCR positivity for different seasons are shown in Figure 63. The end of the dry season had the highest OR of 7, compared to end wet (OR 3) and wet season (OR ~1) (Figure 63).

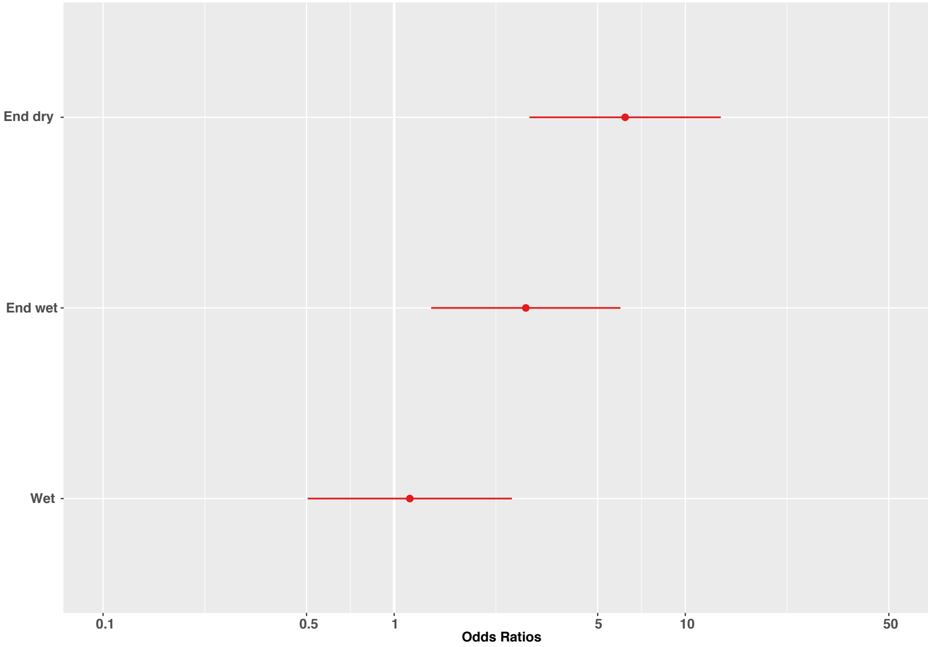


Figure 63 Odds ratios for season as predictor of *O. tsutsugamushi* positivity

Analysis of the fixed effects of different rodent species on the best-fit model of *O. tsutsugamushi* PCR positivity and season is shown in (Figure 64). Some species such as *R. tanezumi*, *R. andamanensis* and *B. indica* have a positive effect, while *R. exulans* and many of the *Mus* species have a negative effect.

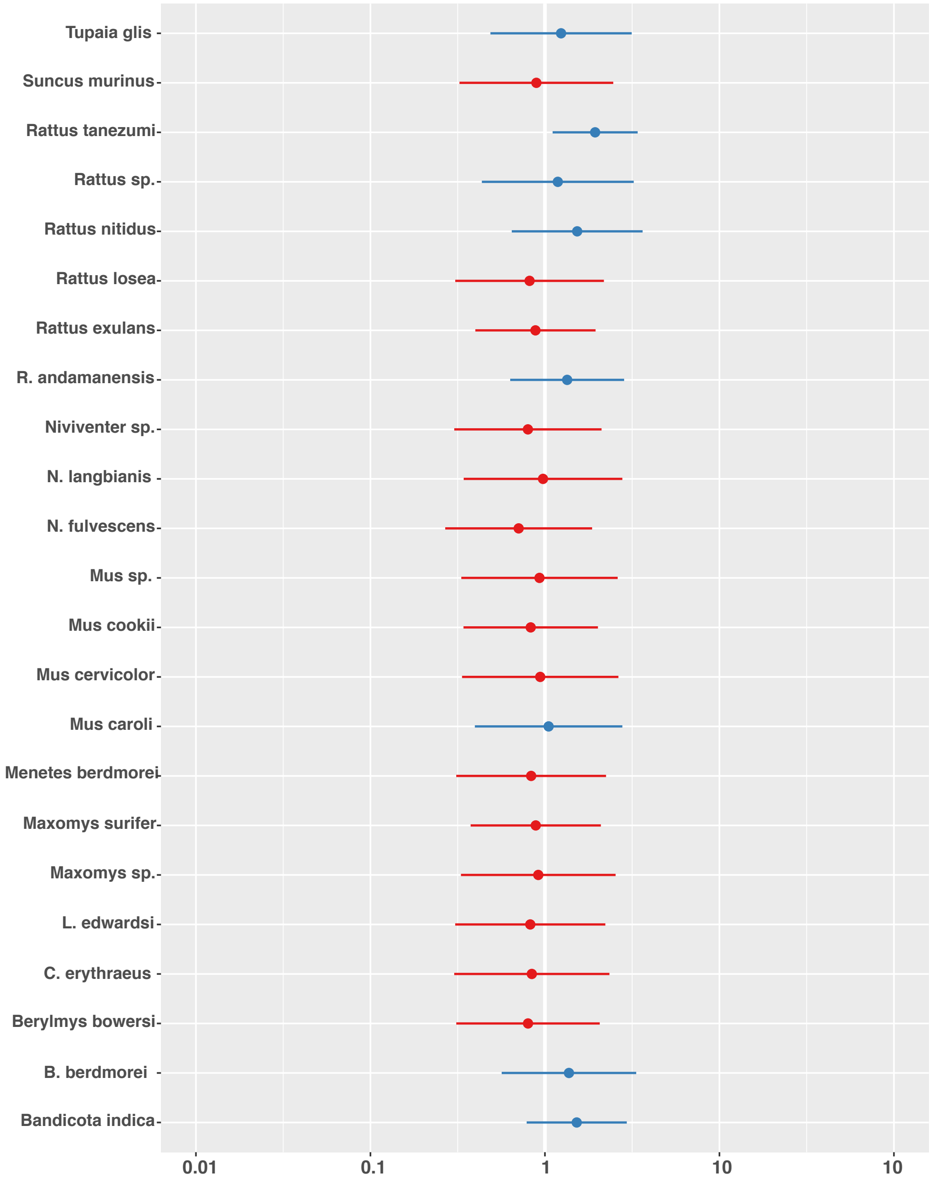


Figure 64 Fixed effects of different rodent species on best-fit model of *O. tsutsugamushi* PCR positivity and season. Positive effects are shown in blue and negative effects in red.

The same analysis was performed for habitat type (trap location). This shows the positive effect of rice paddy field, teak plantation and other forest or fallow habitats. Other plantations and farmed areas (vegetables, corn, rubber) showed negative effects (Figure 65)

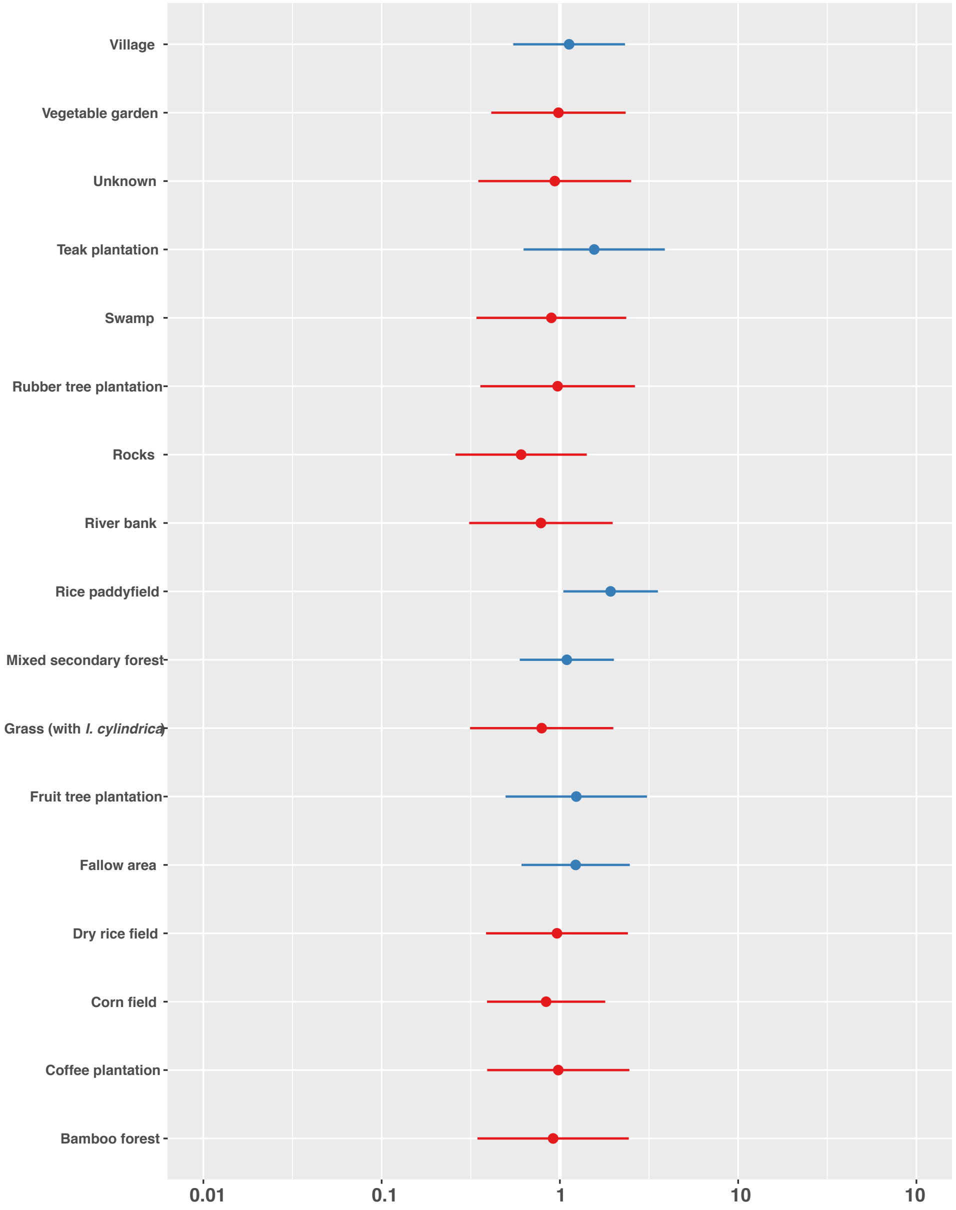


Figure 65 Fixed effects of different habitat types (trap location) on best-fit model of *O. tsutsugamushi* PCR positivity and season. Positive effects are shown in blue and negative effects in red.

The area under the ROC curve for season and *O. tsutsugamushi* PCR positivity was 0.82 suggesting a good model fit.

4.4 Discussion

This is the first apparent attempt in scrub typhus research to map the spatial distribution of *O. tsutsugamushi* positive and negative chiggers and their small mammal hosts at a high-resolution scale of just a few square kilometres in an endemic area. Ecological analyses were performed to compare the species and their interactions at sites of varying scrub typhus risk and to link these to habitats and seasons. Very little is currently understood about the factors that determine an area to be high risk for scrub typhus and what the size and temporal dynamics of such a site are. Improving our understanding of this could lead to important practical public health interventions.

Three districts with scrub typhus incidence ranging from 218 to 121/100,000 population were investigated in Chiang Rai Province. The district with the highest incidence was Mae Fahluang (study site BTT) and lowest Meuang (study site BSK). Note, however, that village level data reports BTT as the lowest incidence of the 3 villages. This is probably due to the much larger population of BTT and proportionately higher number of people not regularly exposed to the disease. The ecology of scrub typhus clearly varies across the enormous endemic region and comparisons with the Penghu Islands in Taiwan provide useful insights.

Species richness estimates suggest that the total number of species present at the main study sites were close to that recorded in the study. BTT had consistently the highest proportion of *O. tsutsugamushi* PCR positive chigger pools at all sampling times, regardless of season. A total of 41% of pools tested positive, compared to 25% in BMM and 12% in BSK. Similarly 18% of individual chiggers at BTT tested positive, with <1% at BSK and BMM testing positive. Chigger indices were also highest at almost all collections in BTT. Chigger diversity was lowest at BTT with *H'* of 1.39, compared to *H'* 1.57 and 1.66 at the other sites. Whilst having the lowest chigger diversity, BTT had the highest proportion of the known vector species *L. deliense* and *L. imphalum* at 71% of all species, whereas these made up 44% and 27% at BSK and BMM respectively.

The species matrix was highly aggregated at BTT with C-scores of 0.98 and 1.8 ($p = 0.01$) and also the greatest degree of nestedness (NODF 45). Among the species interactions, *B. indica* and *W. kritochaeta* appeared to be most central with EC scores of 1. *L. deliense* was also centrally placed with an EC of 0.76 and *L. imphalum* less so (EC 0.46).

In the few other studies of small mammal-ectoparasite interactions in Southeast Asia, *B. indica*, *R. tanezumi*, *B. savilei* and *R. exulans* were consistently centrally placed in network analyses [101,201]. The central role of *W. kritochaeta* and the identification of several positive individuals of this species suggest it may play a more important role in scrub typhus than currently believe, though possibly more as an intrazootic vector.

Specific *O. tsutsugamushi* positive chigger-host interactions showed a high co-occurrence of species (C-scores of 0.07 and 0.32, p = 0.01), suggesting few species are important in disease transmission. Comparisons between positives and habitat types identified *R. tanezumi* and *L. deliense* as true habitat generalists (PDI 0.33 and 0.42 respectively), perhaps confirming why *L. deliense* is such a widespread and important vector of scrub typhus. In this analysis paddy field/riverbank habitat was most centrally placed with EC of 1. Habitat centrality scores are inconsistent in the different analyses (Table 24) suggesting no clear association between habitat type and chigger/small mammal species and *O. tsutsugamushi*-positivity.

GLM of different ecological factors identified only season as strongly associated with *O. tsutsugamushi* PCR positivity, with the highest odds ratio of 7 for the end of the dry season.

Taiwan has a long history of scrub typhus research, dating back to the 1930s. The Penghu Islands, formerly the Pescadores Islands, consist of a low-lying group of around 90 coralline islands, with a population of ~100,000 in an area of 54sq. miles. The islands are home to several Taiwanese military bases, and depend largely on fishing and tourism during the summer months.

The Penghu Islands have a somewhat unique situation, in which cases of scrub typhus frequently occur in women and children as well as visitors and stationed military personnel. This has been attributed to the close geographical association of homes with gardens and fields, the high density of rodents and chiggers and that most men are fishermen. There are just 4 species of small mammals reported and *L. deliense* is almost exclusively the chigger species parasitizing small mammals [194].

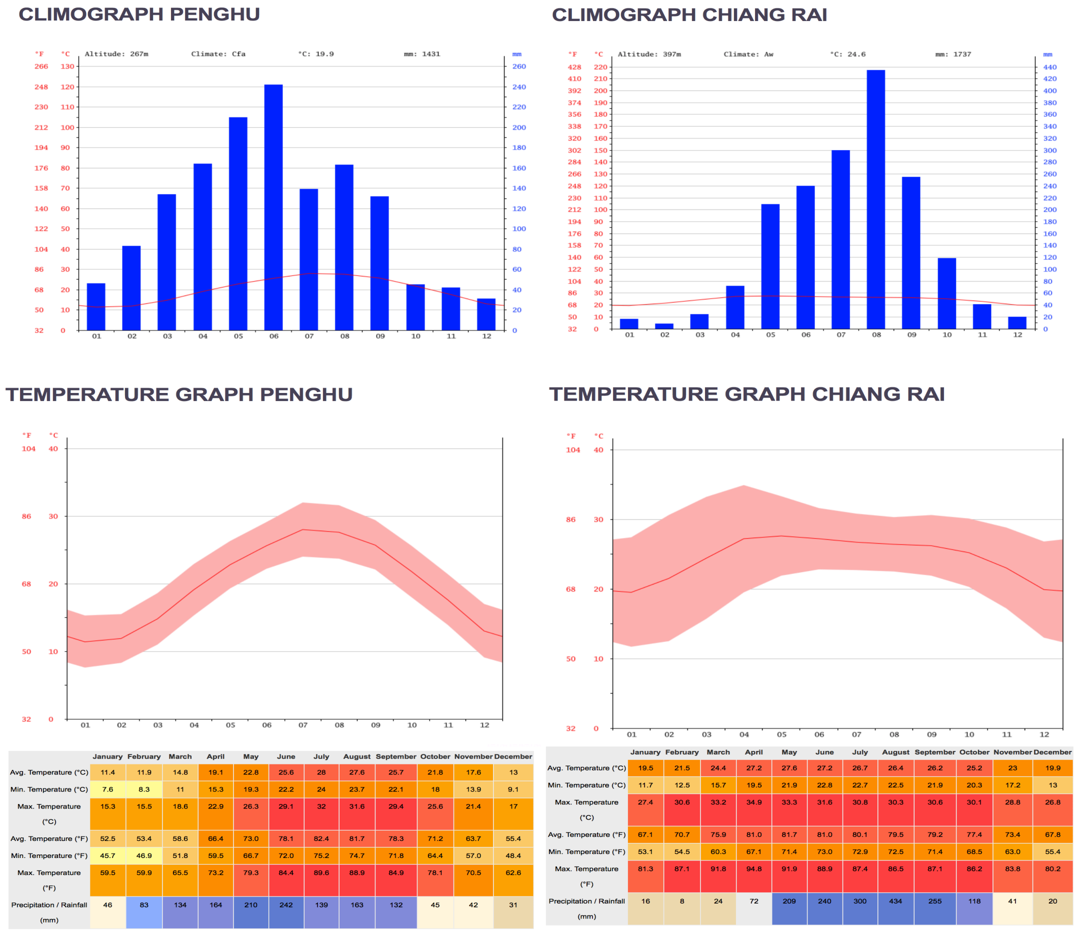
Scrub typhus in humans has a strong seasonal pattern with cases reported between late April and November. This appears to be closely linked to chigger indices on rodents, which drop to very low levels in winter months [191,194,196].

There appears to be a strong correlation with temperature, but none to rainfall, perhaps related to a minimum temperature required for mite eggs to hatch, and for larvae to become active in questing for a host [193,195].

The habitat is dominated by *Miscanthus* grass; an introduced mimosa *Leucaena leucocephala* and small trees (~4m tall). Walls made of coral divide vegetable gardens and homes, providing ideal rodent breeding habitat.

Coral wall and *Miscanthus* grass, followed by *Leucaena* were the key habitats with positivity rates of 32%, 36% and 14% respectively in chigger pools [247]. One study in 2011 reported 20/21 pools of chiggers positive for *O. tsutsugamushi* and in a further investigation in 2013, 22% of chigger pools and 17% of individual chiggers were PCR positive, while 33% of rodents and 30% of shrews were seropositive [343,344]. In 2015 Kuo et al. reported 95% of rodents to be seropositive and 59.5% of chigger pools (~100 individuals/pool) positive by PCR [196].

Dynamics of chigger indices are different between Penghu and Chiang Rai. In Penghu, indices fall to 0 in January/February [191,194,196]. At the main study sites in Chiang Rai, indices tended to fall during the dry, cooler periods but were often still above 100. Mean winter temperatures in Penghu are markedly lower; averaging from 11-14°C between December and March with lows of 7°C. In Chiang Rai, temperatures average between 19 and 24°C during the same period with lows of 12°C. In Japanese studies, chiggers did not begin to crawl until temperatures exceeded 12°C [345]. Total rainfall is comparable between the two regions, although Chiang Rai is drier from January to March (Figure 66).



	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	11.4	11.9	14.8	19.1	22.8	25.6	28	27.6	25.7	21.8	17.6	13
Min. Temperature (°C)	7.6	8.3	11	15.3	19.3	22.2	24	23.7	22.1	18	13.9	9.1
Max. Temperature (°C)	15.3	15.5	18.6	22.9	26.3	29.1	32	31.6	29.4	25.6	21.4	17
Avg. Temperature (°F)	52.5	53.4	58.6	66.4	73.0	78.1	82.4	81.7	78.3	71.2	63.7	55.4
Min. Temperature (°F)	45.7	46.9	51.8	59.5	66.7	72.0	75.2	74.7	71.8	64.4	57.0	48.4
Max. Temperature (°F)	59.5	59.9	65.5	73.2	79.3	84.4	89.6	88.9	84.9	78.1	70.5	62.6
Precipitation / Rainfall (mm)	46	83	134	164	210	242	139	163	132	45	42	31

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	19.5	21.5	24.4	27.2	27.6	27.2	26.7	26.4	26.2	25.2	23	19.9
Min. Temperature (°C)	11.7	12.5	15.7	19.5	21.9	22.8	22.7	22.5	21.9	20.3	17.2	13
Max. Temperature (°C)	27.4	30.6	33.2	34.9	33.3	31.6	30.8	30.3	30.6	30.1	28.8	26.8
Avg. Temperature (°F)	67.1	70.7	75.9	81.0	81.7	81.0	80.1	79.5	79.2	77.4	73.4	67.8
Min. Temperature (°F)	53.1	54.5	60.3	67.1	71.4	73.0	72.9	72.5	71.4	68.5	63.0	55.4
Max. Temperature (°F)	81.3	87.1	91.8	94.8	91.9	88.9	87.4	86.5	87.1	86.2	83.8	80.2
Precipitation / Rainfall (mm)	16	8	24	72	209	240	300	434	255	118	41	20

Figure 66 Comparison of rainfall, maximum, minimum and average temperature between Chiang Rai, Thailand and Penghu Islands, Taiwan (taken from https://en.climate-data.org)

A complete absence of scrub typhus cases occurs on the Penghu Islands from November to March [346]. By contrast Chiang Rai (and Thailand) sees a lower but continuous number of cases through the dry season (Chapter 1).

Small mammal and chigger species diversity is very low on Penghu. The habitats are less diverse than those in Northern Thailand. This would not be unexpected for a small, featureless island and probably results in many fewer ecological niches. Presumably these niches favour *L. deliense*, though it is clearly a generalist, adaptable species.

There are a number of limitations to these analyses. Firstly the sampling effort was limited to four visits (12 days in total) at each site in Thailand over the course of 18 months. Of the total 5 sampling visits, on two occasions not all the 3 main sites were sampled. Sampling success (rodent capture rates) also varied between sites and visits. Secondly, only 3 chiggers per host (~5% of total) were identified to species level. It is likely that the overall diversity was underreported, however identification of all chiggers attached to several hosts revealed that the technique captures the majority of diversity present. Thirdly, relatively high-resolution classification of habitat types was made with 14 recorded. It is acknowledged that for some small mammals species, particularly the larger species that were trapped in edge or fringe habitats, their home ranges probably encompassed more than 1 of the classified habitats.

There has been very little research into scrub typhus ecology in recent decades and most current dogma is based on either laboratory-based research or field observations correlating human disease with the environment, vectors and hosts. One study sampled thirteen $10km^2$ sites in 11 provinces in Thailand on 2 occasions (once in the dry and once in the wet season) [201]. Chigger species richness was positively associated with human scrub typhus incidence. This is the converse to what was identified in this study. The chigger species richness at a single $10km^2$ site when correlated to province-wide or even district-wide human incidence data could be misleading. Indeed, the same study reports species richness was significantly higher in forest-captured animals compared to those from human settlements, which was also seen in this study. However, human risk might be more associated with cleared and disturbed habitats than exposure to forest. The study by Chaisiri et al. also did not test chiggers collected for *O. tsustugamushi* to better understand the interactions of positive chiggers with habitats.

At the scales investigated in this study, higher risk sites appear to be associated with lower chigger species diversity, higher chigger indices and greater proportion of known vector species. Small mammal host density is undoubtedly important, to maintain high chigger numbers locally, and the density was high in BTT. The importance of the end of the end of the dry season and end of the wet season identified through GLM is of interest but harder to explain. The end of the dry season is typically associated with the "mango showers" and this first rainfall may be a strong stimulus for eggs to hatch into larvae. *O. tsutsugamushi* is transovarially transmitted, and makes up a very low proportion of overall total positive larvae. It would thus be expected that the proportions of positives should remain relatively constant throughout the year. It is interesting that the seasons associated with PCR positivity also correspond to those of intensive farming activity and high human incidence. Much further research is required to help build our understanding of the complex ecology of this important pathogen.

CHAPTER 5

Sequencing of *Orientia tsutsugamushi* from humans, chiggers and small mammals

5 Chapter 5: Sequencing of *Orientia tsutsugamushi* from humans, chiggers and small mammals

5.1 Introduction

Several factors combine to make genomic studies of *Orientia* infection challenging. The bacterium is an obligate intracellular pathogen, necessitating cell culture for laboratory propagation, and it is present in highly variable but typically low levels in naturally infected samples. The *O. tsutsugamushi* genome is relatively poorly defined, with just eight published full sequences, which illustrate the high density of repetitive elements and the extreme rate of genomic rearrangement, added challenges that make innovative approaches to sample preparation, sequencing and analysis essential.

Orientia is typically collected from a range of specimen types including human whole blood, buffy coat and eschar tissue, rodent blood and organs, and chiggers. The absolute quantity of *O. tsutsugamushi* DNA present in these specimen types is variable, but frequently low. In one study of 155 infected human blood samples tested by 16s PCR, the median was 13 copies/ml, the interquartile range 0-334 and the maximum 310,253 [58]. Very little data is available for the quantity of *O. tsutsugamushi* in individual chiggers. A recent study from Thailand reported a range of 13.8 to 2,252 copies/µL [307]. There is no published data from rodents.

Several methods have been utilised to unravel the strain diversity and begin to understand the population genetic structure of *O. tsutsugamushi* among infected humans, rodent hosts and vectors across different geographical regions.

Since WW2, several serological typing methods have been employed including complement fixation, immunofluorescence and immunoperoxidase assays, monoclonal antibody typing and immunoblot assays [56]. The prototype strains of Karp (New Guinea), Gilliam (Assam, India) and Kato (Japan) were described between 1943 and 1955. Subsequently, more than twenty antigenically distinct strains have been reported. They reveal a great degree of phenotypic diversity across South and East Asia. Several antigenic types appear to be widely present throughout the region, with one (TA716) making up over 70% of isolates from several countries [56]. However the data is geographically patchy and to what extent genetically distinct strains may cross-react (and thus may be difficult to distinguish antigenically) is not well understood.

More recently, genetic analysis of highly variable single genes for outer membrane proteins such as the 56 kDa and 47 kDa antigens or more conserved genes (e.g. GroEL) have been used to define genotypic variation. A recent detailed analysis of 56 kDa sequences from across South and East Asia identified at least 17 clusters of genotypes belonging to 5 identifiable groups [97]. This study also suggested that genetic diversification was attained by point mutations and recombination among diverse genotypes and that the identification of identical or near-identical 56 kDa genes in geographically distant sites suggests that bird migration, perhaps via an East Asia/Australasia Flyway, may play an important role [97]. Multi-locus sequence typing (MLST) is a method that measures the sequence variation in a set of housekeeping genes to characterize strains by their profile of distinct alleles (an allele is a unique DNA sequence for one of the amplified fragments). Several schemes have been proposed, though no single scheme has been universally accepted [240,241,347-349]. One MLST scheme compared human isolates from 3 regions of Laos with a strain from nearby Udon Thani in Northeast Thailand. Low levels of population differentiation were reported between geographically close (Vientaine and Udon Thani) strains, while isolates from southern Laos formed a distinct population [241]. In this study, 8% of isolates appeared to represent mixed infection, and in Thailand 25% of infections were reportedly mixed [240]. Recent whole-genome phylogenetic comparisons between 8 well-characterised strains revealed relationships that were significantly different from phylogenies created from single-gene or MLST schemes, illustrating the increased resolution achievable from whole-genome sequencing [350]. At the single-gene level, such as 56 kDa, enormous genetic variability is seen, while at the MLST level only a few clonal clusters are evident.

Next-generation sequencing (NGS) techniques have become the gold standard for revealing the genetic variation of organisms [351]. Culture of *O. tsutsugamushi* in eukaryotic cells can increase the quantity and concentration of DNA available for downstream whole-genome sequencing by thousands of fold. This technique, which commonly uses mouse fibroblast L929 cells, is technically demanding, costly, time-consuming and prone to contamination. Handling infected-cell cultures is also hazardous and carries a risk of infection in those accidentally exposed [352]. The entire process must be undertaken in Biosafety level 3, with all its associated costs and complications. Additionally, cell culture risks the selection of a single strain present in a mixed-strain sample and thus it may not reveal true levels of diversity.

Recent whole-genome sequencing (WGS) studies using short- and long-read technology to sequence a diverse group of strains reveal very little synteny between even closely related strains. These studies show that gene order varies substantially and that rearrangements and gene duplication rates are high, suggesting that recombination between genomes in an infected cell may be a key mechanism driving genetic diversity [351,354-7,359].

In summary, the many difficulties associated with conducting a large-scale study at the whole-genome level of *O. tsutsugamushi* in human, vector and small mammal samples prompted the investigation of novel approaches. A probe-based targeted enrichment sequencing strategy was developed, tested and improved to generate genome-scale data where possible from these sample types at low cost and high throughput.

The project followed four distinct phases (Figure 67). 1) A set of hybridization probes targeting the coding parts of the *O. tsutsugamushi* genome (a method akin to exome sequencing) was designed to capture the likely diversity of the organism. 2) A low-input library enrichment method (Illumina's Nextera XT) was initially tested on a set of 69 human samples collected in Laos and Northern Thailand, together with 2 rodent tissues and 3 species of colony-bred *O. tsutsugamushi*-infected chiggers. 3) Motivated by high read-duplication rates in initial sequencing experiments, the low-input method was compared with a whole-genome amplification (WGA) step prior to standard library preparation, on a set of chigger DNA samples spiked with a range of *O. tsutsugamushi* concentrations together with individual infected colony chiggers. 4) The WGA-based method was selected and used to sequence a subset of chigger and small-mammal field samples and a small number of human samples.

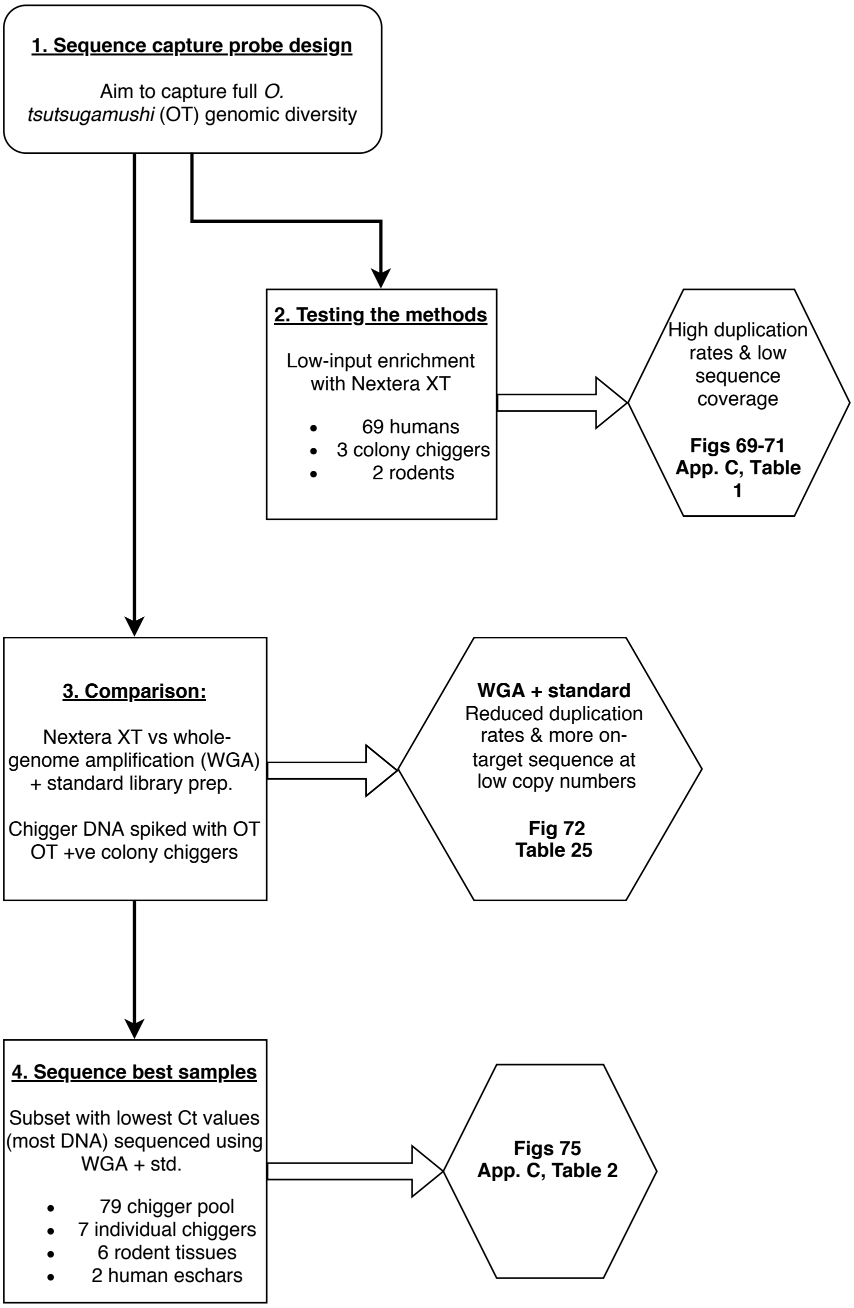


Figure 67 Flow diagram showing steps in method development and reference to figures and tables

I hypothesise that 1) genomics techniques can be developed to improve the efficiency and sensitivity with which genome scale sequence data can derived from *O. tsutsugamushi* containing natural samples and 2) such data can reveal genetic structuring at local levels, such as a single study site or even an individual host animal, in a large-scale study. The methodology developed and tested during this study aimed to investigate these hypotheses.

5.2 *Orientia tsutsugamushi* genome

Orientia is in the family Rickettsiaceae, a member of the order Rickettsiales. Two species of *Orientia* are currently recognised - *O. tsutsugamushi* and *O. chuto*, the latter known solely from a patient infected in the United Arab Emirates [37]. The heterogeneity of strains, coupled with the recent molecular identification of *O. tsutsugamushi* in humans in Chile [38] and 16s sequences with close homology to *O. tsutsugamushi* in dogs in South Africa [76] and in small mammals in Senegal and France [75], suggest the possibility of further species and future taxonomic re-evaluation.

Many intracellular pathogens including most of the Rickettsiaceae tend to have small, stable genomes with few repetitive sequences and mobile genetic elements [353]. The trend towards a reduced genome is probably an outcome of small, isolated bacterial populations and repeated population bottlenecks [354]. On the other hand, recent sequencing of the *Rickettsia felis* genome identified a plasmid [355] whose function is presumably to facilitate the exchange of genetic material with other individuals of the same or different species, suggesting that rickettsial population genetics may be more complex than previously thought.

Compared to other members of the family, *O. tsutsugamushi* has a unique and unusual genome. At 2.1Mbp it is nearly double the size of that of its closest relative, *Rickettsia prowazekii*, with an average GC content of 30.5% that is similar to other Rickettsiaceae [356,357]. There are no known plasmids or prophages. The first complete genome sequence of *O. tsutsugamushi* was that of the Korean strain Boryong, published in 2007 [356] and followed by the Japanese strain Ikeda a year later [358]. The *O. tsutsugamushi* genome is one of the most repetitive of all bacterial genomes [356]. Analysis of the Boryong strain identified 2,179 potential protein-coding sequences, 963 of which represented fragmented genes, and a core of 512 genes recognizably shared with other Rickettsiaceae [356]. A recent attempt to define the functional genome of 8 strains of *O. tsutsugamushi* identified a relatively small set of 657 core genes [350]. The non-coding regions consisted of an extraordinary number of longer repetitive sequences (operationally defined as sequences >1000bp in length, matching another 1000bp region with up to 100 differences (mismatches, insertions and deletions)), accounting for between 33 and 51% of the six genomes assessed [350]. There is a remarkably high number of *tra* genes that code for the conjugative Type 4 secretion system (TFSS), which usually mediates horizontal gene transfer between bacteria, including for virulence genes [359]. Comparison between the two reference strains and other Rickettsiaceae demonstrates that the core gene set is highly conserved, but that extensive genome reshuffling, duplications and deletions occur [353,360].

The reasons behind the high proportion of repetitive and mobile genetic elements in *O. tsutsugamushi* are not fully understood. *Orientia* is a host-switching pathogen with both effective transovarial transmission and horizontal transmission to a huge diversity of host species including man [85,119]. There are at least 10 trombiculid mite species that act as vectors to humans [108] and possibly many more that act only as intrazootic vectors. Unlike other vectors of Rickettsiaceae, chiggers tend to co-feed in large groups, sharing a pool of digested tissues [150], enabling *Orientia* co-infection and providing a potential opportunity for transfer of DNA between bacteria.

5.3 Targeted enrichment sequencing

Targeted enrichment sequencing is a tool whereby certain pre-selected regions of the genome are targeted for sequencing, via hybridisation with a set of probes corresponding to the sequences of interest. The method is akin to, and works similarly to, whole-exome sequencing where just the “exome” or coding portions of the human genome is sequenced. Targeted enrichment can be a useful sequencing tool where the whole genome is not required, or a particular genome of interest is selected from contaminating DNA [361,362].

Targeted enrichment sequencing has a number of potential advantages over whole-genome sequencing. The method is suitable for very low quantity and quality of input DNA, while generating sufficient coverage for downstream analysis [363]. Bioinformatic effort and cost are also frequently reduced [361]. In the case of *Orientia*, the approach enables the targeting of the regions of the genome likely to be of most analytic interest by excluding the extensive repetitive regions. Targeted enrichment sequencing also provides for the sequencing of much larger regions, with fewer constraints on sequence conservation, than typical PCR-based methods.

Among pathogens, the technique has been employed in metagenomic analysis of multiple virus species, where culture is difficult and costly [364-366]. Few reports exist in bacteria. An example is *Neisseria meningitidis* which has been sequenced directly from clinical samples, specifically cerebrospinal fluid, where culture often fails due to prior antibiotic treatment [367].

A custom panel of probes was designed for targeted enrichment of *Orientia* and details of the methods are provided in the following section. Agilent Technologies, following our design input, manufactured the probes.

5.4 Methods

5.4.1 Chigger, rodent and human tissue DNA extraction and PCR

Methods are provided in Chapter 3.

5.4.2 Probe design

Targeted enrichment sequencing used a set of Agilent SureSelect probes designed by Dr Liz Batty, Bioinformatician at the Wellcome Centre for Human Genetics in Oxford, for this project. The probes were designed in the following way, aiming to ensure that the full diversity of the *O. tsutsugamushi* genome would be successfully captured. Two finished reference strains (Boryong and Ikeda) plus six other available assemblies were used to select probes. The complete Boryong reference strain was used as the initial sequence. To cover genes not found in the Boryong genome, or which had high levels of divergence from the Boryong genome, predicted open reading frames from all eight genomes were clustered into groups based on >=80% identity at the protein sequence level. Sequences were added to the probe design from each cluster until all the sequences representing each protein cluster had <5% divergence at the DNA sequence level from any included sequence. Probes are able to capture sequences with this level of divergence from the probe sequence, so this method ensured that the probe set would capture all known *O. tsutsugamushi* gene diversity. The reference Boryong gene sequence was always included if it had a representative in the cluster under consideration. This process generated a total sequence length of 4.7Mb. Sequences that would capture human and rodent genome (*Rattus norvegicus*) were removed. Probe design and sequence capture was performed using Agilent SureSelect technology.

5.4.3 Library preparation

Library preparation, WGA, sequence capture and sequencing was performed by Dr Mariateresa de Cesare and colleagues of the High-throughput Genomics team at the Wellcome Centre for Human Genetics in Oxford.

5.4.3.1 Standard + whole genome amplification

In order to concentrate the DNA in the specimens, a Speed-Vac (Eppendorf, Hamburg, Germany) was used which vaporises the solvent without affecting the DNA. Input samples varied from 40 µL (chiggers) to ~50 µL (human samples) and 95 µL for small mammal samples. WGA was performed following the manufacturer's protocol for the REPLI-g Single Cell Kit (Qiagen, Hilden, Germany). Briefly, the dried genomic DNA was resuspended in 2.5 µL of TE and subsequently mixed with 2.5 µL of Buffer D1 and incubated for 3 minutes at 15-25°C. 5 µL of Buffer N1 was added and the sample mixed. To this, 40 µL of Master

Mix (H_20, REPLI-g sc Reaction Buffer and REPLI-g sc DNA Polymerase) was added and the mixture was incubated at 30°C for 8 hours. Finally, the REPLI-g sc DNA Polymerase was inactivated by heating the sample at 65°C for 3 minutes.

The concentration of the amplified DNA was assessed using a Qubit dsDNA HS Assay (Thermo Fisher, MA, USA). Samples were normalized to 500 ng mass in 34 μL. DNA was fragmented using an Episonic instrument, following the manufacturer's protocol (EpiGentek, NY, USA). Post-fragmentation an Ampure XP bead clean-up was performed by adding 34 μL of Ampure XP to the DNA, mixed thoroughly and incubated for 5 minutes at room temperature. The sample was transferred to a magnet and incubated for 8 minutes and the supernatant removed and kept. Finally the beads were washed twice with 200 μL of 80% ethanol, removing and discarding the supernatant each time. Beads were air-dried and then resuspend in 35 μL of EB buffer.

For library preparation the following were combined (per reaction): NEBNext End repair reaction buffer (6.5 μL), NEBNext End prep enzyme mix (0.75 μL) and water (24.25 μL) to create the End repair/dA-tailing Mastermix. The DNA was then added and incubated at 20°C for 30 mins, 65°C for 30 minutes and then held at 4°C. Next the Adapter ligation mastermix was prepared using the following volumes per reaction: 3.75 μL of Blunt/TA Ligase master mix, 1 μL of Ligation enhancer, 1.5 μL of 15 μM index adapter and 12.25 μL of nuclease free water. This was then incubated for 15 minutes at 20°C, followed by an Ampure XP bead clean-up using 86.5 μL of beads and finally eluted into 100 μL EB buffer.

Size-selection for the Illumina HiSeq4000 was then performed by adding 52 µL of Ampure XP to the DNA, mixing, incubating for 5 minutes at room temperature and then transferring to a magnet for 8 minutes. The supernatant was then transferred to a fresh plate and the process repeated using 25 µL of Ampure XP. Finally the beads were washed with ethanol as previously described and resuspended in 20 µL of EB buffer.

PCR was then performed on the library using 10 µL of Pre-PCR library, 5 µL of indexed primer i5 and i7, 10 µL water and 25 µL NEBNext Q5 PCR Master Mix. The following conditions were used: 98°C for 30secs, 98°C for 10secs, 65°C for 30secs, 72°C for 30secs, 72°C for 5mins and 10 cycles performed.

A final Ampure XP bead clean-up was carried out using 37.5 µL of beads and eluted in 30 µL of EB buffer. Qubit and Tapestation DNA analysis was performed for all libraries prior to sequencing.

5.4.3.2 Nextera XT

DNA was normalized for an input of 1 ng in 5 µL across all samples. Nextera Tagmentation Master Mix was prepared with the following volumes per reaction: Tagment DNA Buffer (10 µL) and Amplicon Tagment Mix (5 µL). Samples were then mixed and incubated at 55°C for 5 minutes, and held at 10°C. Then 5 µL of Neutralize Tagment Buffer was added, mixed and incubated for a further 5 minutes.

PCR on the library was performed by preparing a Master Mix with the following volumes per reaction: 15 µL Nextera PCR Master Mix, 5 µL i5 Indexed Primer and

5 µL i7 Indexed Primer. The following cycle conditions were set: 72°C for 3 minutes, 95°C for 30 seconds, 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes.

An Ampure XP bead clean-up was carried out on the post-PCR library using 30 µL of beads, following the sample protocol described above. Beads were resuspended in 52.5 µL of EB buffer, mixed for 2 minutes and then placed on a magnet for 2 minutes. A final recovery of 50 µL of eluent was used for Qubit and Tapestation DNA analysis.

5.4.4 Sequence capture

Paired-end DNA libraries prepared using either WGA followed by a standard in-house library preparation, or Nextera XT, were enriched for capture using pre-designed Agilent SureSelectXT probes.

The pool of indexed libraries was first normalized to 750 ng in 3.4 µL. A Master Mix containing 2.5 µL of SureSelect Indexing Block #1, 2.5 µL SureSelect Block #2, 3 µL IDT xGen Blocking Oligos was prepared. This was added to the sample, mixed and placed on a thermocycler at 95°C for 5 minutes and then 65°C for 5 minutes.

Next the Hybridization Buffer Master Mix (SureSelect Hyb #1 to #4 and RNase Block) in a total volume 13.5 µL was prepared. 5 µL of baits were aliquoted and added to the Hybridization Buffer Master Mix. This was then transferred to the samples held at 65°C and incubated for 24hrs.

Dynabeads MyOne Streptavidin T1 beads were prepared using the manufacturer's standard protocol. The PCR plate was maintained at 65°C while moving the samples to the bead plate and pipette mixing. Samples were then incubated on a mixer at 1100 rpm for 30 minutes at room temperature. Samples were then spun briefly, place on a magnetic rack and the supernatant removed and saved. The beads were resuspended in 200 µL of SureSelect Wash Buffer 1 and incubated for 15 minutes at room temperature, replaced on the magnetic rack and the supernatant discarded. The procedure was repeated with SureSelect Wash Buffer 2, incubated for 10 minutes at 65°C and discarding the supernatant as before. The process was repeated 3 times. The beads were then resuspended in 30 µL of distilled water.

14 µL of this was transferred to undertake a post-hybridization PCR using the following PCR Master Mix (Herculase II Reaction buffer, 100mM dNTP Mix, qPCR Library Quantification Primer Premix, nuclease free water and Herculase II Fusion DNA Polymerase), with the cycle parameters of: 98°C for 2mins, 98°C for 30secs, 57°C for 30secs, 72°C for 1 min, 72°C 10 minutes. 14 cycles were performed.

5.4.5 Sequencing

Sequencing was performed on the Illumina HiSeq4000 with paired-end 150 bp reads.

5.4.6 Bioinformatics pipeline

Dr Liz Batty designed the bioinformatics pipeline. The steps were performed by the author under supervision, except for the calculation of pairwise differences, creation of the coverage plot (Figure 74), UPGMA tree and Figure 78 comparing the two trees, which were performed by Dr Batty.

Raw reads generated from Illumina HiSeq4000 were mapped to the UT76 reference genome using BWA MEM v0.7.12 [368]. Samtools flagstat v1.8 was used to summarise the total number of reads and the proportion mapping to the reference. The reads were then deduplicated using Picard MarkDuplicates v2.0.1 and the same statistics recalculated, along with the total number of fragments present in the library. Depth of coverage across the whole genome and the proportion of the core genome represented at 1x, 5x and 10x minimum per-base coverage was calculated using GATK v3.7 [369].

Taxonomic read classification was performed using Centrifuge [370] for a small number of *O. tsutsugamushi* positive colony chiggers. Visualization of species classification was carried out with Pavian [371].

Haploid variant calling and core genome alignment was performed using snippy [372]. The method identified single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) between the sequence reads and the reference genome. A matrix of pairwise sequence comparisons, recording the number of positions at which each pair of sequences differed, was constructed using only the positions in the reference sequence that were called in all of the highest-performing samples [373]. This set of sequence positions called in all samples was treated as a multiple sequence alignment for constructing maximum-likelihood (ML) phylogenetic trees using iqtree v1.3.11 [374]. The most suitable model was selected using ModelFinder Plus which computes the log-likelihoods of an initial parsimony tree for many different models and the Akaike information criterion (AIC), corrected AIC and Bayesian information criterion (BIC) [375]. To estimate branch supports of the generated phylogenetic tree inferred from the multiple sequence alignment, ultrafast bootstrap approximation was used [376].

To validate the phylogeny generated above, all sites called as homozygous in each sample was used to create an unbiased pairwise distance matrix containing the largest number of sites for each pair of sequences. Pairs of samples, compared at all of the positions called in both members of the pair (a substantially larger number of (different) positions than were called in all of the selected high-performing samples) were used to construct a further distance matrix. This data was then used to create an unweighted pair group method with arithmetic mean (UPGMA) tree using the phangorn package in R [377]. The phylogenies generated by the ML and UPGMA methods were compared using the phytools package in R [378].

5.5 Results

The first step in validating the targeted enrichment sequencing was performed by testing a set of retrospectively collected human samples. The Illumina Nextera XT low-input library preparation method was used.

5.5.1 Human samples

In total 69 human samples were selected, covering a wide geographical range: 33 from Chiang Rai Province, 39 from Laos and 1 from Taiwan (Figure 68). Among these, 31 were buffy coat samples, 18 whole blood and 20 eschars. The median Ct was 34.8 (range 27.3 to 41.3). The median Ct was similar for buffy coat samples (34.7), eschars (35.2) and whole blood (36.5). There were 11 paired whole-blood/buffy coat and eschar samples from patients collected in Chiang Rai and 2 from Laos.

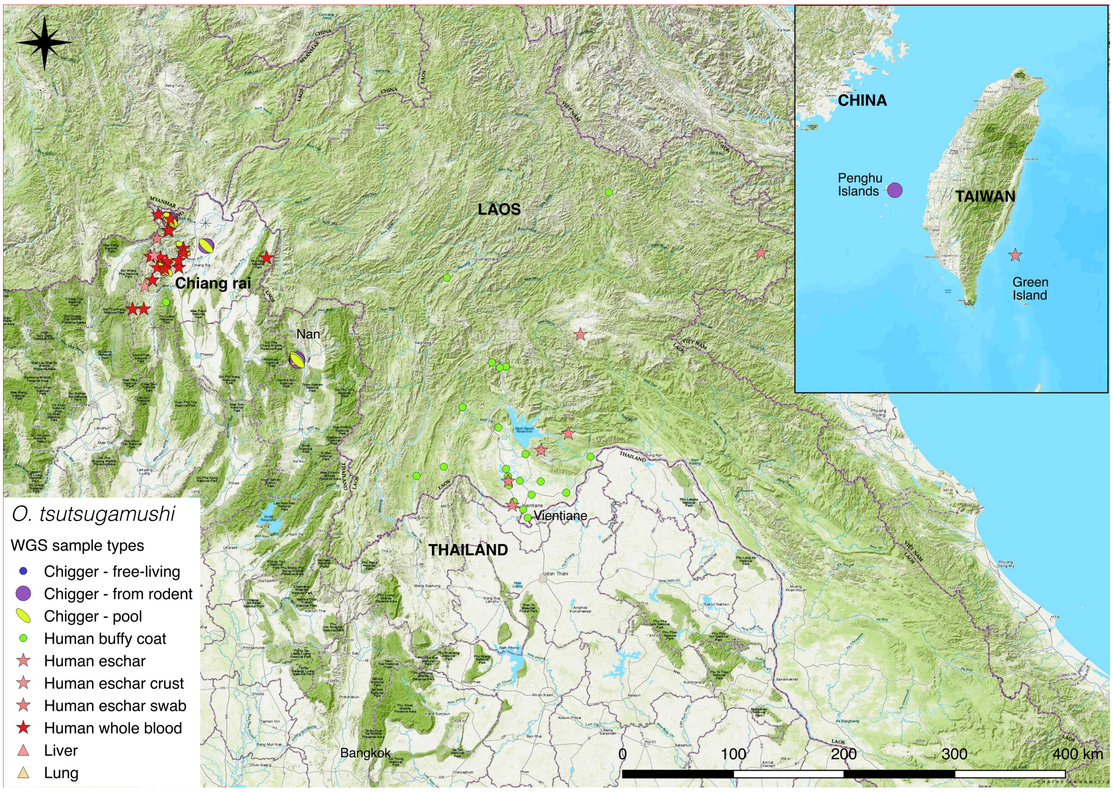


Figure 68 Summary map of human, chigger and small mammal *O. tsutsugamushi* positive samples. Chigger and small mammal samples were collected during fieldwork between 2015-18, human samples between 2006-2018.

5.5.1.1 Sequencing statistics

This first sequencing run contained eschar crusts (pieces of eschar tissue), eschar swabs, buffy coat, whole blood, colony chiggers and rodent lung tissue. The Armed Forces Research Institute for Medicine (AFRIMS) in Bangkok, Thailand kindly provided *O. tsutsugamushi*-infected colony chiggers. This is the only active chigger colony in existence. Negative controls were also included. The mean library fragment size varied greatly: some samples, particularly the colony chiggers, had libraries of larger fragments, while many of the human samples had much smaller-insert libraries. Library size tends to reflect the input concentration of DNA (a lack of DNA makes shorter-insert libraries) but it can also reflect DNA quality (sequencing statistics are shown in Appendix C, Table 1).

5.5.1.2 Duplication rates

Sequencing read duplication rates (the proportion of sequence reads that represent an extra copy of a read that is already in the dataset) were >90% for almost all samples (Figure 69), limiting the information content of sequencing data from the low-input, no-pre-amplification method. These results led to re-evaluation of the library preparation methods and the comparison of an alternative method, WGA and standard library preparation with Nextera XT.

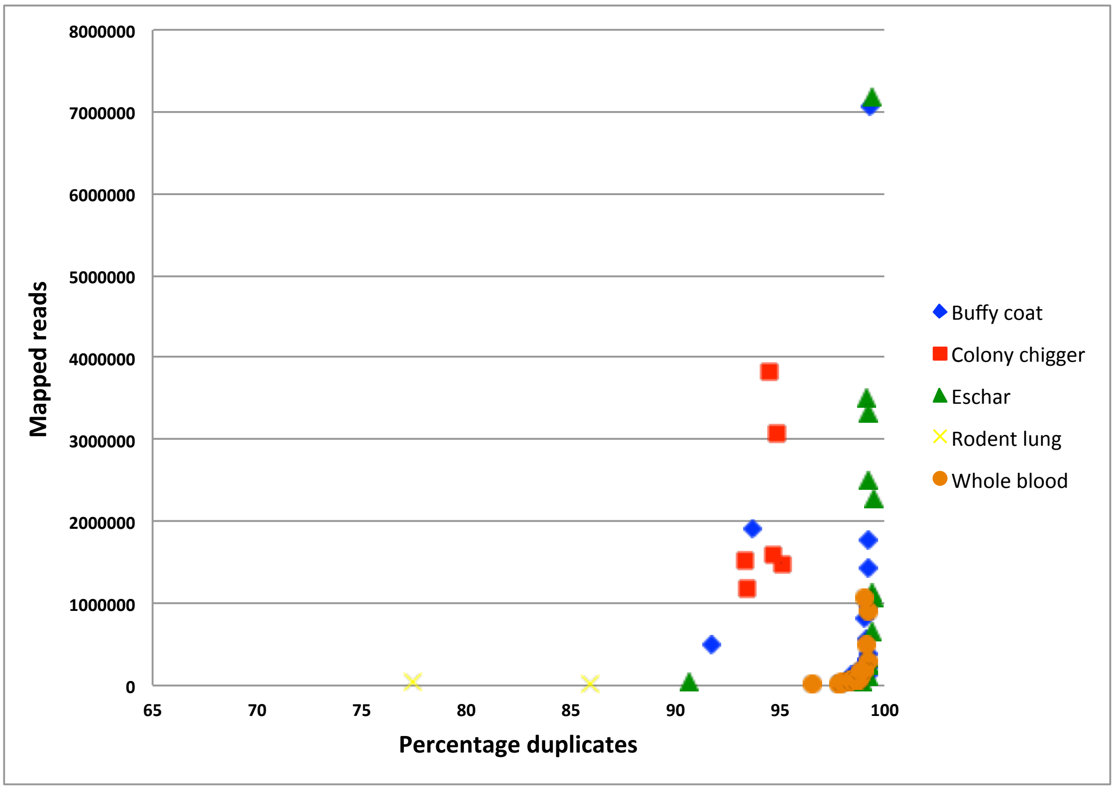


Figure 69 Read duplication rates among reads mapping to reference genome

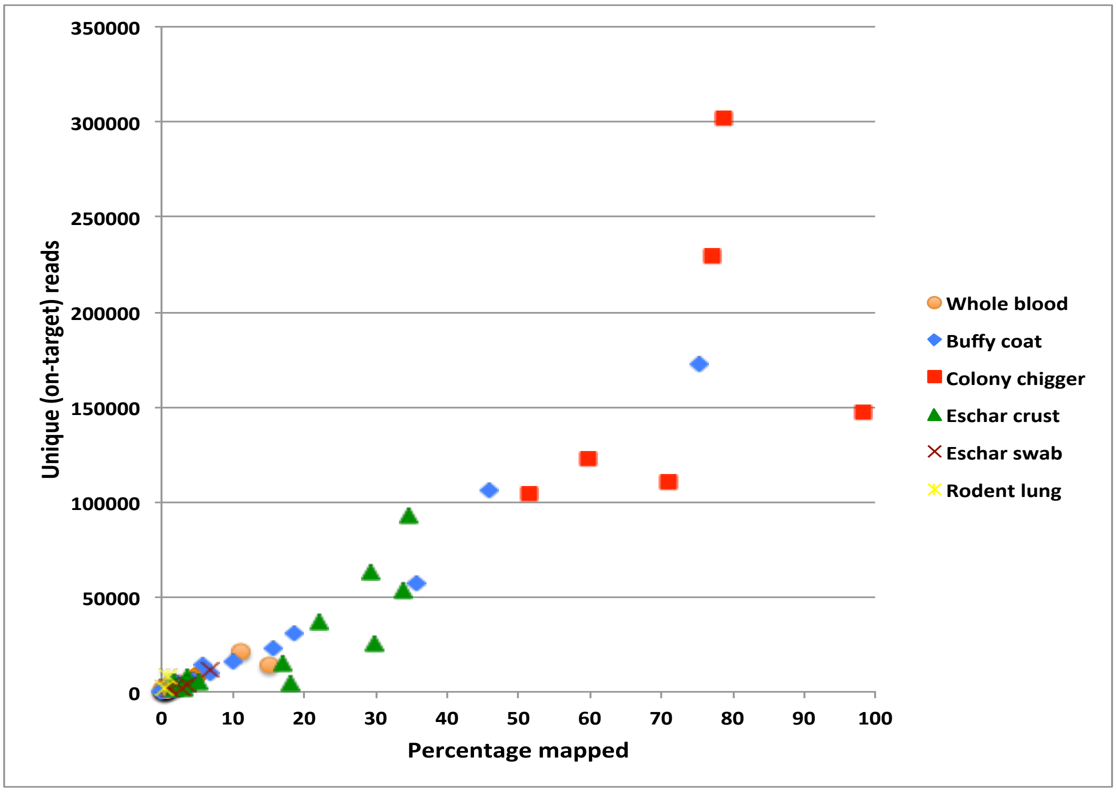
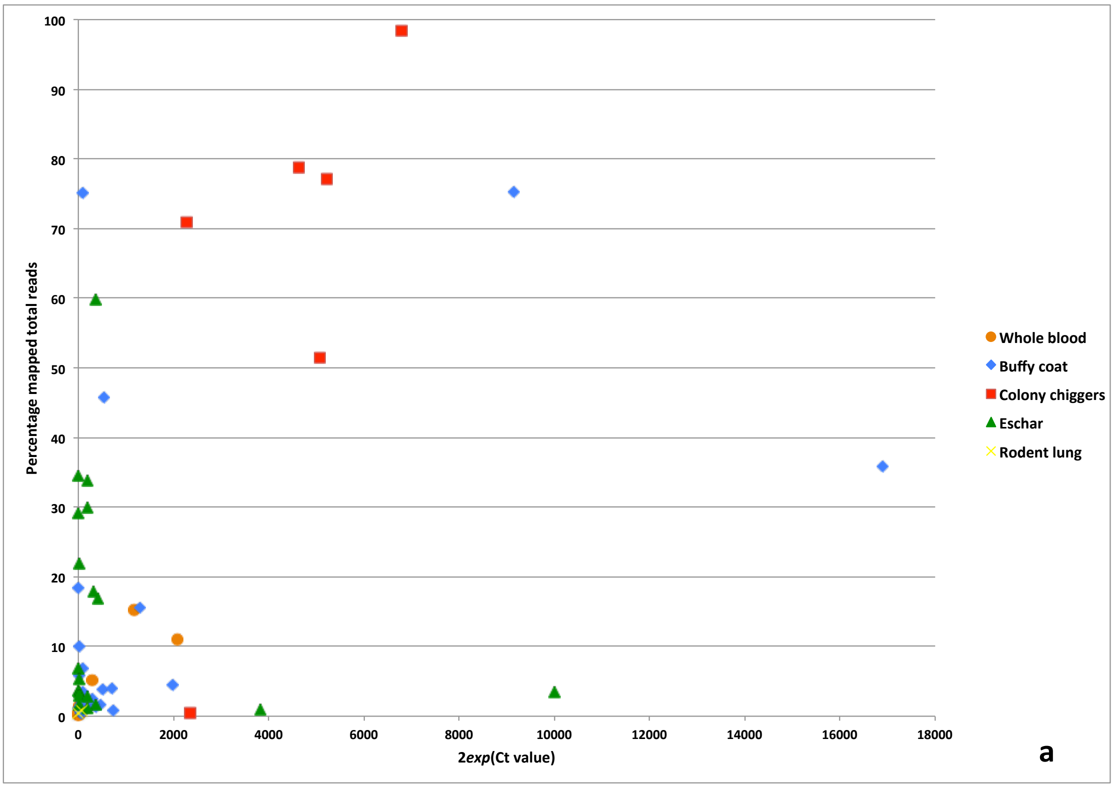


Figure 70 Number of unique, on-target reads versus rate of successful mapping to the reference genome.

In most samples, only a small proportion of reads mapped to the reference genome (Figure 70), reflecting the performance of the methodology on samples that in general had very small amounts of *O. tsutsugamushi* sequences. The near-proportionality between unique on-target read number and rate of mapping reflects the library normalisation step, where equal amounts of raw library containing variable amounts of *O. tsutsugamushi* sequence are pooled for the joint enrichment step. Colony chiggers performed best, with mean core genome coverage of 108-fold (42-205) and mean read mapping rate of 73% (51-98%) reflecting their higher input total copy number and corresponding lower Ct (mean 29.4, range 28.6-30.2). Buffy coat and eschar samples were more variable, with mean coverage 71 and 52 respectively and mean percentage mapped of 11 and 12%. Whole blood performed least well with mean coverage of 9 and mean mapping rate of just 2.5%. De-duplicated coverage of the core genome at 10x of more than 50% was seen for only 1 buffy coat, 1 eschar and colony chigger samples. At 5x coverage, 3 buffy coat, 1 eschar and colony chiggers had 50% coverage.

5.5.1.3 Comparison with Ct value

The majority of sample types investigated contain very low quantities of *O. tsutsugamushi* DNA and this was clearly reflected here in the sequencing of human samples. We would expect a positive association between the rate of reads matching *Orientia* sequences and the number of *Orientia* genome copies detectable by qPCR, implied by Ct. We simplified this expected relationship by comparing read mapping rate to the quantity 2^{Ct} (2exp(Ct)). The samples with the highest mapping rate tended to come from colony chiggers and buffy coat, and to have a lower Ct (higher input number of genomes), but the relationship was not very precise, probably because of a variety of factors. Different types of sample had different ranges of estimated genome copies but also different properties such as total DNA content. In addition, many samples fell near the lower limits of detection of the qPCR assay, while some had anomalously low Cts. As a rough guide, a Ct of ≥35 (~100 copies/ µL or lower) results in poor coverage and low percentage mapping to the reference, making later phylogenetic comparisons difficult.



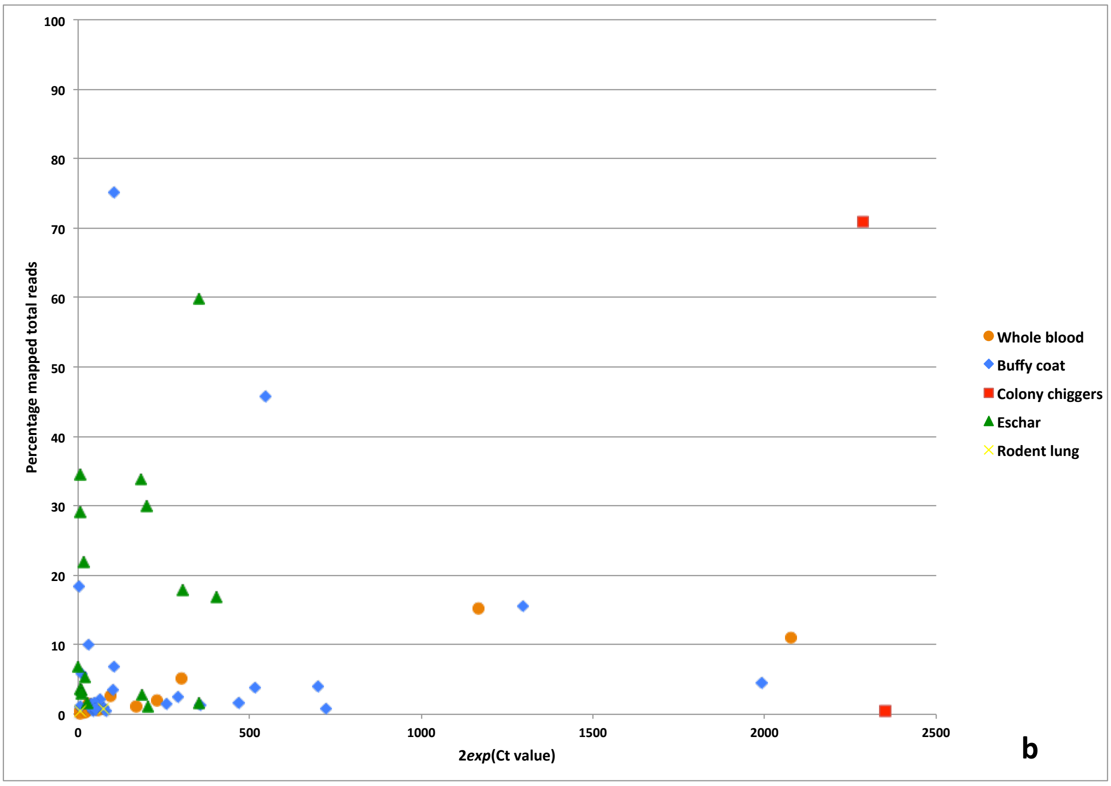


Figure 71 2^{Ct} (2exponent(Ct)) scatter plots for different sample types. b) magnifies the x-axis near the origin.

5.5.1.4 Coverage across genome

The proportion of the genome covered at least 10x (10 reads covering each base position) was generally very low among these human *O. tsutsugamushi*-positive samples. Most colony chiggers achieved coverage of >90% and just 4 buffy coat samples and 4 eschars exceeded 20% coverage. The highest coverage was seen for buffy coat. At 5x coverage depth, 4 eschar samples and 5 buffy coat samples exceeded 20% genome representation.

5.5.2 Spike-in experiment and *O. tsutsugamushi*-infected colony chiggers

High duplication rates and relatively low coverage in many samples prompted further evaluation of the methods. In the absence of *O. tsutsugamushi*-positive field samples at this stage, a spike-in experiment was designed. A range of chigger DNA samples spiked with *O. tsutsugamushi*, to simulate the estimated maximum range of concentrations likely to be found in naturally infected individual chiggers was created. *O. tsutsugamushi* infected colony-bred chiggers of 3 species species (*Leptotrombidium deliense*, *L. chiangraiensis* and *L. imphalum*) were included. A new method, WGA with standard library preparation, was compared with the previous method, low-input Nextera XT library preparation, in parallel.

5.5.3 Spike-in experiment design

The following estimate for the number of cells in an individual chigger was based on the average quantity of DNA extracted from single chiggers, measured using the Qubit fluorometric high sensitivity DNA quantification device (Thermo Fisher

Scientific, USA). For convenience, we then assumed that each infected chigger contained 100,000 *O. tsutsugamushi* genomes in order to estimate the amount of bacterial DNA we would expect to find if we extracted DNA from a naturally infected chigger. This was based on quantitative real-time PCR results on individual field collected and laboratory bred chiggers (Linsuwanon, pers. comm.).

Assuming 1 chigger approximately = 100 ng of DNA

1 pg of DNA = 978 Mb
1 ng of DNA = 978,000 Mb
100ng of DNA = 97,800,000 Mb

Chigger genome is approximately 117 Mb and diploid

97,800,000 / (117 x 2) = **418,000 cells** [1]

Assume upper limit of number of genome copies of *Orientia tsutsugamushi* in 1 chigger is 100,000

$$\text{1 genome copy} = \frac{\text{no. of base pairs x weight in grams/mole (Daltons)}}{\text{no. of molecules in a mole}}$$

$$\text{1 genome copy} = \frac{2.1 \times 10^{6} \times 650}{6.02 \times 10^{23}} = 2.27 \times 10^{-15}$$

Therefore 100,000 genomes = **0.227 ng** [2]

To create the spike-in solution, DNA was extracted from 20 chiggers of the genus *Walchia* that had previously tested negative for *O. tsutsugamushi* using the 47 kDa real-time PCR. DNA extraction was performed using the methods described previously. The 20 extracted DNA samples (40µL each) of negative chiggers were

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[1] Number of base pairs in 1 picogram of DNA from Dolezel et al. (ref 260) & Dong et al. (ref 280)

[2] Number of *O. tsutsugamushi* genome copies in individual chiggers from qPCR results

pooled and then split into 20 tubes, such that the sample was equivalent to the mean amount of DNA extracted from a chigger.

O. tsutsugamushi (strains UT76 and CRF136) DNA extracted from cell culture was used to create the dilution series. The concentration was 838 ng/µL with 82% of the DNA being from *O. tsutsugamushi* and 18% from contaminants, (as estimated by qPCR and bulk sequencing of the isolate) giving a starting concentration of *O. tsutsugamushi* of 687 ng/µL.

- 100,000 copies of *O. tsutsugamushi* = 0.227 ng of DNA
- 100,000 copies/µL = 0.42 ng/µL of UT76 stock solution (assuming DNA is 82% *O. tsutsugamushi*)
- To create a final concentration of 0.42 ng/µL equivalent to 100,000 copies/µL: 2 µL of *O. tsutsugamushi* DNA was added to 18 µL of water, mixed thoroughly and 5 µL of this removed and added to 45 µL of water, mixed again and then 2 µL added to 38 µL water

The following concentrations were made following a dilution series using the prepared *O. tsutsugamushi* and chigger solutions: 100,000, 50,000, 25,000, 10,000, 5,000 and 1,000 copies.

Dried DNA was resuspended in 2.5 µL for WGA preceding standard library preparation or 6 µL for NexteraXT (see below).

5.5.3.1 Sequencing statistics

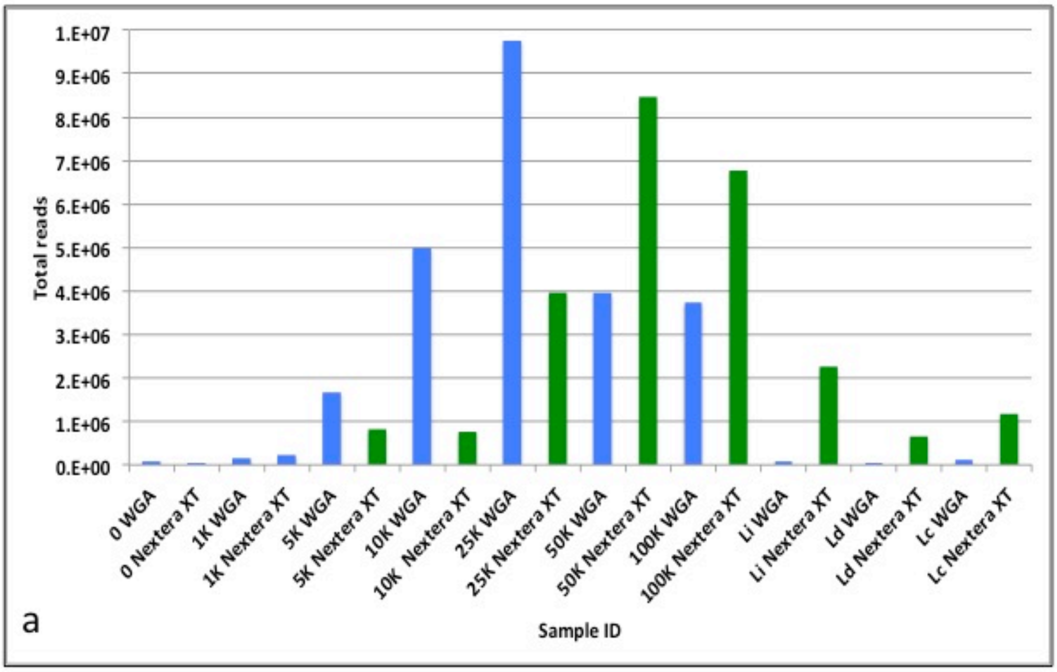
Sequencing libraries were successfully made for 7 dilutions of *O. tsutsugamushi* spiked into chigger DNA from 0 copies to 100,000 copies. Three individual colony-bred chiggers of 3 species were also sequenced (Table 25).

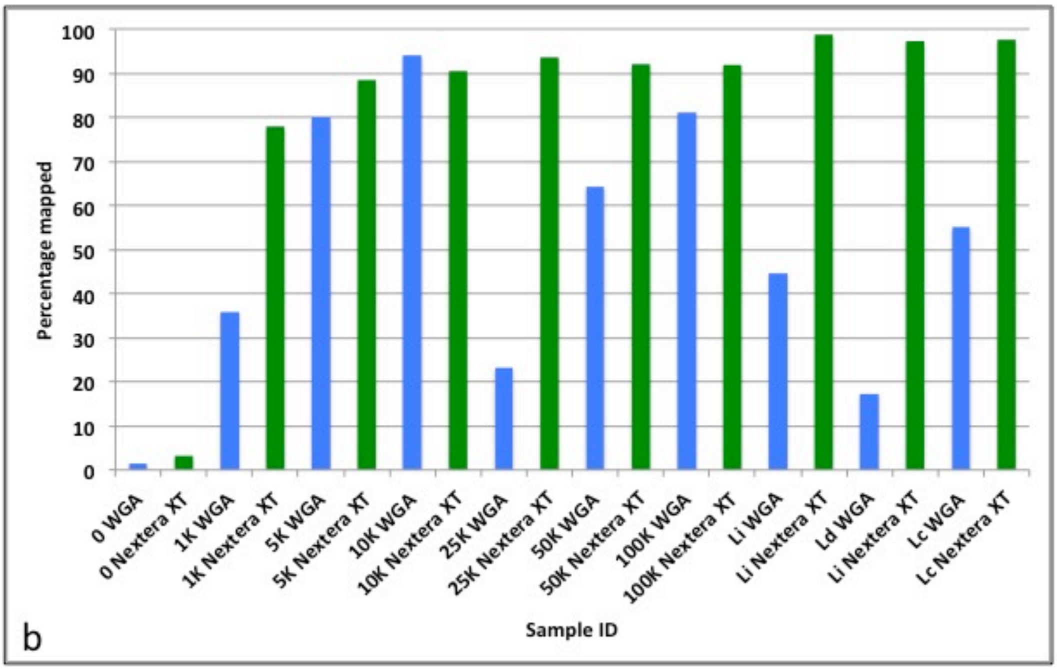
Table 25 Sequencing statistics generated from the spike-in experiment for chigger DNA spiked with *O. tsutsugamushi* at varying concentrations and individual colony-bred chiggers. WGA followed by standard library preparation (blue) was compared with Nextera XT (green).

Sample	Total reads	Mean coverage of core genome	On-target unique reads	Percent mapped to reference genome	% Dups[$]	1x[#]	5x[#]	10x[#]
100K copies	3734072	324.5	2847907	78.7	4.3	99.9	99.7	99.1
100K copies	6771472	225.0	3262599	91.5	56.6	99.9	99.8	99.7
50K copies	3957156	378.1	2329214	60.9	4.6	99.9	99.6	98.5
50K copies	8465760	235.6	3417053	91.7	65.2	99.9	99.8	99.8
25K copies	9749230	369.7	1945949	20.8	6.2	99.8	98.3	93.9
25K copies	3961434	98.4	1596399	93.3	65.8	99.9	99.8	99.7
10K copies	4993842	121.0	4170102	93.2	15.2	95.9	83.3	71.2
10K copies	760490	26.7	351222	90.1	57.8	99.8	98.5	93.6
5K copies	1662208	99.1	1201168	76.2	7.4	87.8	67.4	54.3
5K copies	819912	22.3	310365	88.0	65.9	99.7	98.2	90.8
1K copies	156072	3.8	48193	31.7	3.4	28.9	13.4	9.4
1K copies	224270	5.5	74465	77.0	65.5	94.2	52.8	14.3
0 copies	65410	0	1290	0.6	2.0	0.1	0	0
0 copies	32388	0	265	3.9	30.1	0.2	0	0
L. imphalum	64770	3.2	54510	33.3	4.9	27.3	9.4	5.7
L. imphalum	2260824	95.5	584665	98.8	55.7	99.9	99.8	99.7
L. deliense	44662	0.7	5901	13.6	3.6	11.3	3.7	1.9
L. deliense	654368	32.6	366249	97.2	51.1	99.9	99.5	97.6
L. chiangraiensis	116144	5.9	20793	49.3	6.7	16	7.9	5.4
L. chiangraiensis	1172430	42.8	1192501	97.6	58.0	99.8	99.5	99

[$] Percentage duplications
[#] Percentage of core genome covered at 1x, 5x & 10x

The libraries from the NexteraXT and WGA followed by conventional library prep were constructed, enriched and sequenced separately, generating between 10^5 and 10^6 reads for all copy-number standard samples and for individual colony chiggers (Figure 72a). The total number of reads generated for each copy number varied inconsistently between the two workflows.





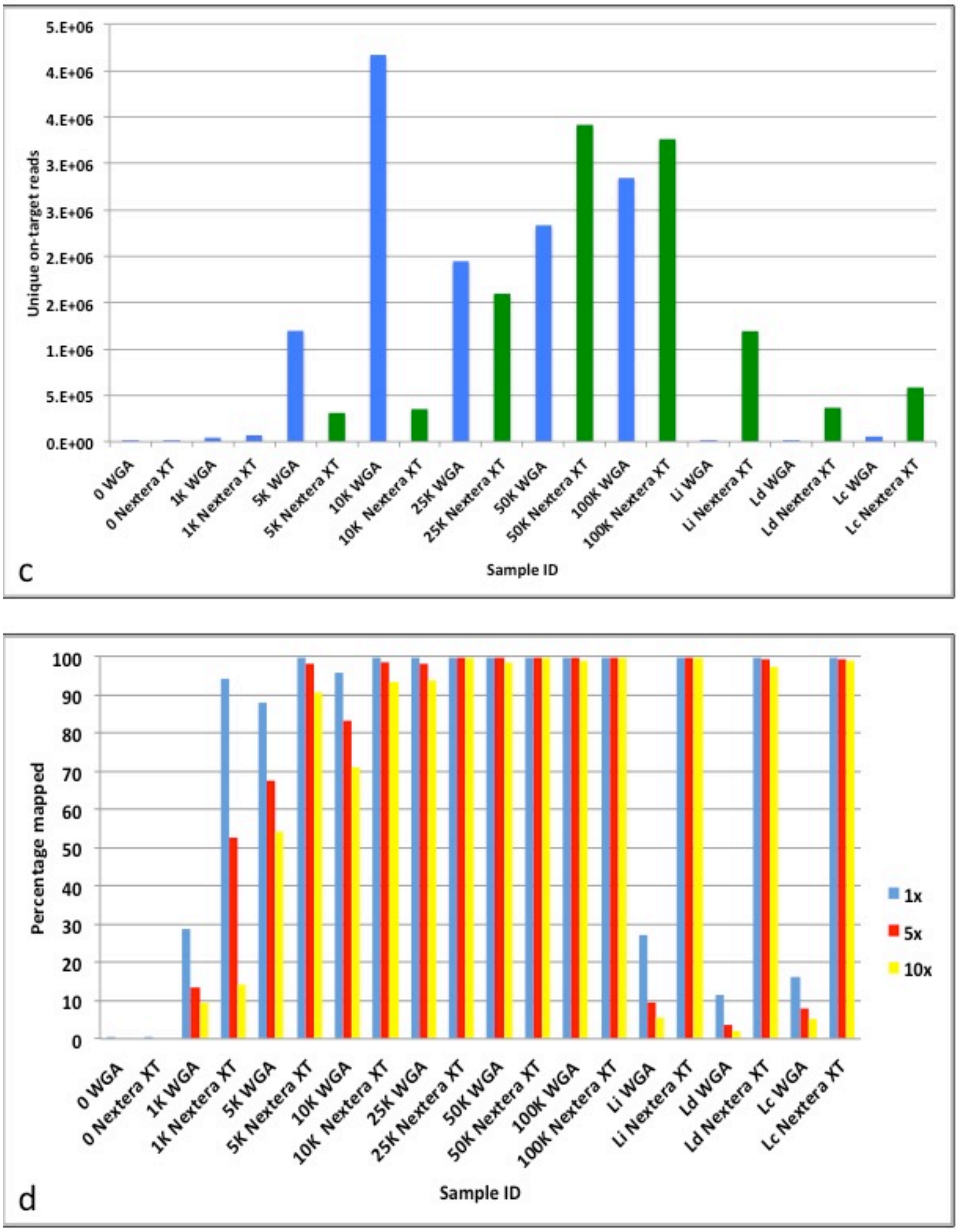


Figure 72 Comparison of WGA and standard library prep (Blue) and Nextera XT (green) for different quantities of *O. tsutsugamushi* (0 to 100,000 copies) and 3 species of colony-bred chiggers. a) Total number of reads generated, b) Percentage mapped to reference genome, c) Yield of unique (de-duplicated) reads, d) Proportion of the core genome covered at >=1x. Two zero-copy (negative control) samples 0_a and 0_b were included. Li = *Leptotrombidium imphalum*, Ld = *L. deliense*, Lc = *L. chiangraiensis.*

Based on theoretical considerations and past experience, we would expect the proportion of reads mapping to the reference to increase with the number of *O.*

tsutsugamushi input genome copies, with a plateau near 100% achieved as the proportion of target in the sample reaches ~1%. Therefore we were surprised to observe variable and hard-to-interpret results (Figure 5b).

For the WGS + standard library preparation, the proportion of reads mapping to the UT76 reference sequence varied inconsistently between 93.2% for 10,000 copies to 20.8% for 25,000 copies. Higher numbers of mapped reads were seen for the Nextera XT library as a consequence of the higher total number of reads and therefore the proportion of reads that are *O. tsutsugamushi* (rather than contamination) (Figure 72b). These results are probably additionally affected by inconsistent pooling of libraries.

The duplication rate of WGA + conventional libraries was substantially improved compared with the original Nextera XT approach (3.4-15.2% compared with 51-66% duplicates) with the result that the number of unique, on-target reads was in general increased in comparisons of the same number of input copies, and the total yield of on-target reads reflected input amounts relatively well (Figure 72c).

For the colony chiggers in the comparison experiment, fewer (13.6 to 49.3%) reads mapped for libraries made using the WGA method than with for those made with Nextera XT, a result that, in view of the overall success of the method, perhaps represented an issue with the (WGA) amplification step. Coverage of the genome at 1x ranged from 11.3 to 27.3 and fell to 1.9-5.7 at 10x (Figure 72d). The 1,000 copy WGA + standard library was re-sequenced in a subsequent run and results were similar. The inconsistency between Nextera and WGA methods in the percentage on-target rate made it difficult to judge where among our copy-number standards real (albeit colony-infected) chiggers appear, but the results overall suggest that the chosen standards were biologically relevant and that many natural samples have relatively low *O. tsutsugamushi* DNA content.

5.5.3.2 Metagenomic read classification in colony chiggers

The species of origin of reads from each enriched sample were checked by taxonomic read classification using Centrifuge [370] and visualization using Pavian [371]. Consistently high proportions of reads were assigned to *O. tsutsugamushi* (Figure 73). A small minority of reads were assigned to other organisms for reasons that remain unclear, but misassignment of sequences or low-level contamination by organisms during sample collection or sequence preparation seem the most likely reasons [379,380].

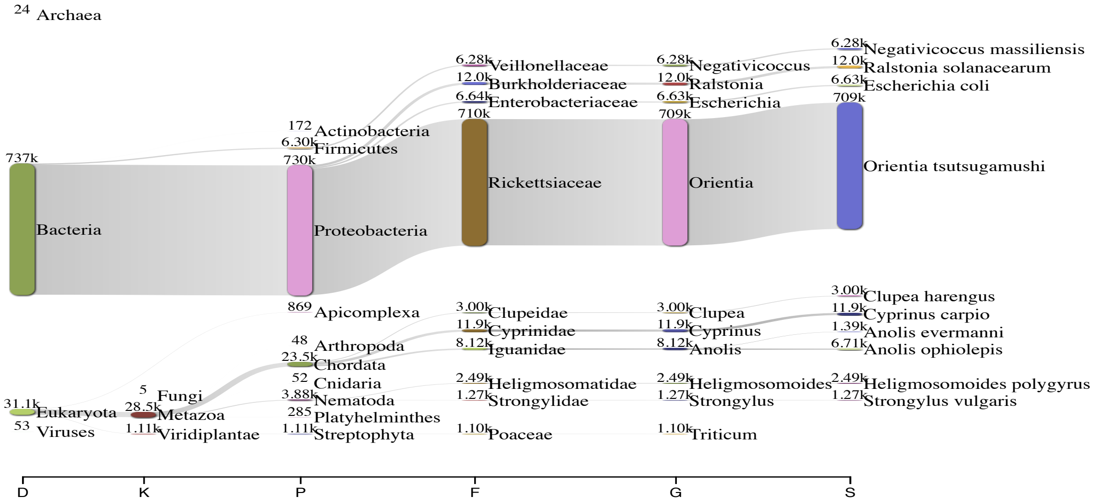


Figure 73 Sankey diagram of reads from sequence capture of a *Leptotrombidium imphalum* colony-bred chigger classified using Centrifuge and visualized with Pavian.

5.5.3.3 Coverage across genome

Figure 74 shows coverage plot for 1,000 to 100,000 copy-dilutions normalized to bulk sequencing of an *O. tsutsugamushi* reference isolate. The pattern of relative coverage across the genome is consistent among samples with different input amounts, reflecting the relatively consistent technical performance of the enrichment method and that the unique coding sequences in the probe panel represent only a fraction of the reference isolate's total sequence, the rest of which is poorly captured. More detailed analyses would be required to ascertain whether all sequences included in the panel performed equally well when present. The GC plot in Figure 74 confirms that GC coverage does not appear to influence the pattern of relative coverage by sequence capture across the genome.

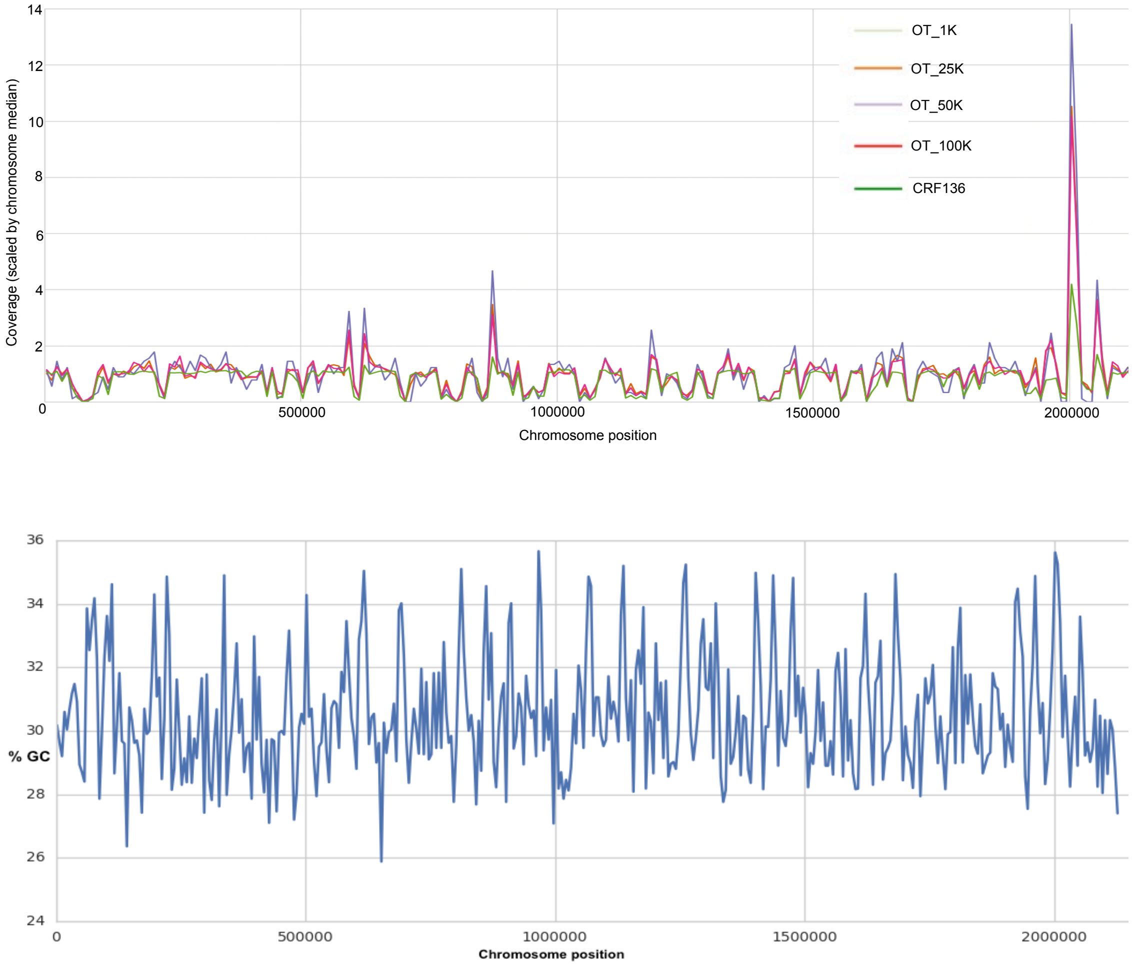


Figure 74 Above: coverage plot for 1,000 to 100,000 copies versus bulk sequencing of *O. tsutsugamushi* reference genome. Below: the GC coverage for the same reference genome.

5.5.4 Chiggers and small mammals

Targeted sequence capture was performed on a selection of individual chiggers, chigger pools, free-living chiggers and rodent tissues collected from field trips. As a consequence of the results of the spike-in experiment, WGA plus standard library preparation was chosen for subsequent experiments.

The selection was based on samples with the lowest Ct values (i.e. with the highest quantity of input DNA) and that provided a representation of sample types and locations. The mean Ct value was 30.6, median 30.7 and range 24.6 to 35.

Six individual chiggers were included of 3 species in 2 genera. These included 2 *L. deliense*, 4, *L. imphalum* and 1 *A. indica.* These were collected from Ban Thoet Thai, Chiang Rai Province and 1 from the Penghu Islands, Taiwan (Figure 68). A single free-living chigger (*L. imphalum*) from Ban Thoet Thai was included. Colony chiggers of 3 species were also included.

A total of 79 chigger pools were included from 29 different small mammals. Of these, a single pool was sequenced from 14 small mammals. In one case as many as 10 pools were sequenced from a single animal. Pools came from the 3 main study sites.

Lung and liver tissue was included from 4 small mammals of 2 species. Three were *R. tanezumi* and 1 *R. exulans.* Both liver and lung from the same animal were tested in 2 cases.

5.5.4.1 Sequencing statistics

The sequencing results were highly variable across the sample types (Appendix C, Table 2). The mean Ct of samples used in phylogenetic comparisons below was 29 (range 24.6-33). Total reads generated varied enormously from 9,538 to 219,953,112, as did mean genomic (un-deduplicated) coverage from 0 to 2309.

Three samples generated very high read counts. Although they came from the set of samples processed using the WGA method, their duplication rates remained relatively high (range 40-89%).

Some individual chiggers and many pooled chiggers performed well. A single free-living chigger had coverage of 36.7%, 17.8% and 11.7% at 1x, 5x and 10x respectively. Rodent tissues performed least well. The mean Ct for these samples was 33.6 (32.14 to 34.8). Mean read-mapping proportion was very low at 0.95% and the percentage coverage of the core genome at 1x ranged from 0.6 to 4.4%. A scatter plot of % reads mapping to reference vs 2^{Ct} illustrates a general trend towards higher percentage mapped with increasing estimated amount of *O. tsutsugamushi* DNA, but with large variability (Figure 75).

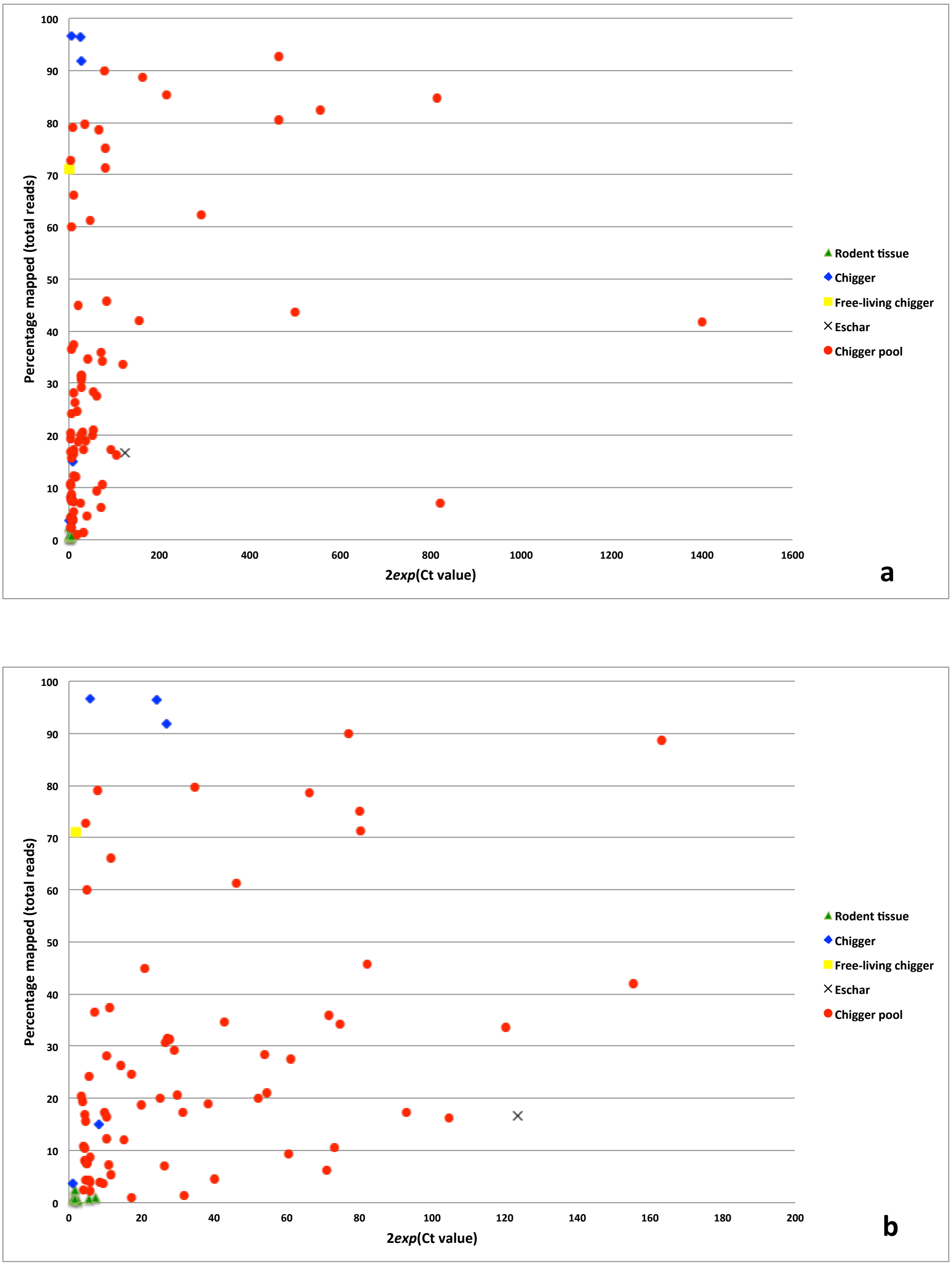


Figure 75 2^{Ct} (2exponent(Ct)) scatter plots for different sample types. b) magnifies the x-axis near the origin.

5.5.5 Phylogenetic analysis

Variant calling was performed on the entire set of sequenced samples using a pipeline. Of the 183 samples, 118 samples had >=20 bases called. However, due to the low sequence coverage for many samples, phylogenetic comparisons were attempted only for a limited set of 17 samples with >50,000 bases called (Table 26). The median Ct value for these samples was 28.8 (range 25.4-32.6). The addition of further samples close to this threshold generated a phylogeny with bootstrap values of <70 at several branches, suggesting unreliable branch supports.

Table 26 Individual chiggers and chigger pools used in the phylogenetic analysis

ID	Small mammal host	Predominate chigger species	Location
R129Pool6	*R. tanezumi*	*L. imphalum & W. kritochaeta*	Thoet Thai, Chiang Rai
R133Pool6	*R. tanezumi*	*L. imphalum*	Thoet Thai, Chiang Rai
R172Pool2	*B. indica*	*L. imphalum*	Thoet Thai, Chiang Rai
C0593	*B. indica*	*L. imphalum*	Thoet Thai, Chiang Rai
R173Pool10,11&12	*B. indica*	*L. imphalum & W. ewingi lupella*	Thoet Thai, Chiang Rai
R198Pool2	*B. indica*	*L. imphalum*	Thoet Thai, Chiang Rai
R200Pool3	*B. indica*	*L. imphalum & W. kritochaeta*	Thoet Thai, Chiang Rai
R207Pool2	*B. indica*	*L. imphalum*	Thoet Thai, Chiang Rai
R209Pool5	*R. tanezumi*	*L. imphalum & W. kritochaeta*	Thoet Thai, Chiang Rai
R211Pool4	*R. tanezumi*	*L. imphalum & W. kritochaeta*	Thoet Thai, Chiang Rai
R240Pool3,5,6&7	*T. glis*	*L. turdicola & H. naresuani*	Mae Mon, Chiang Rai
T14	*R. losea*	*L. deliense*	Penghu Islands, Taiwan

An analysis of the proportion of the core genes of *O. tsutsugamushi* covered by at least 1 read was performed for the 17 best-sequenced samples and is displayed in Figure 76. The results show that for most samples around 10/657 core genes were not covered by at least 1 read. Several genes were repeatedly incompletely covered among these samples. The R240 pools from Ban Mae Mon showed higher numbers of incompletely covered core genes ranging from 30 to 40.

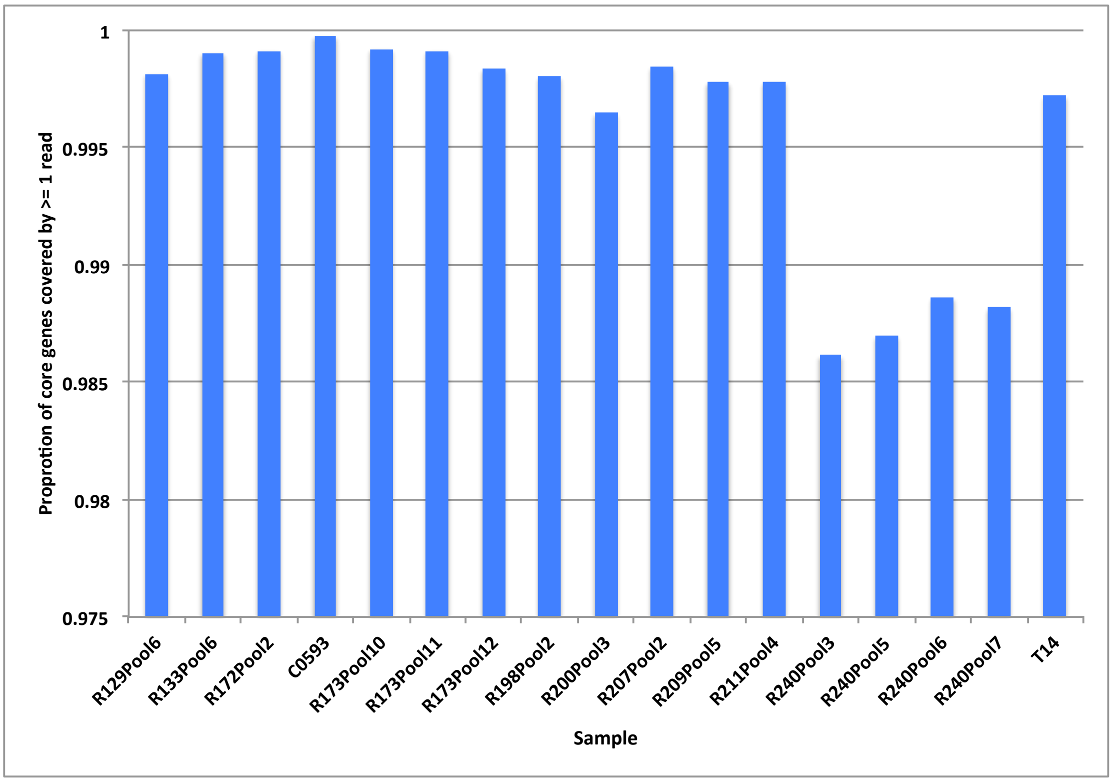


Figure 76 Proportion of *O. tsutsugamushi* core genes covered by at least one read for the 17 best-sequenced samples.

A phylogenetic tree of multiple sequence alignments for all samples with >50,000 bases called was created using the ML method. These consisted of 17 samples: 11 chigger pools and 1 individual chigger from Ban Thoet Thai, 4 pools from Ban Mae Mon and 1 individual from the Penghu Islands, Taiwan (Table 26).

In an initial attempt to understand the phylogenetic relationship between these 17 samples, 6920 positions definitively called as homozygous in all these samples were used to generate the ML tree (Figure 77). The number of sequence positions called across the genome for samples used in the phylogenetic analysis is shown in Appendix C, Table 3. The number of positions called in each sample and pairwise distance matrix and number of differences between sites are shown in Appendix C, Table 4. The GTR+R4 model was selected as the best-fit.

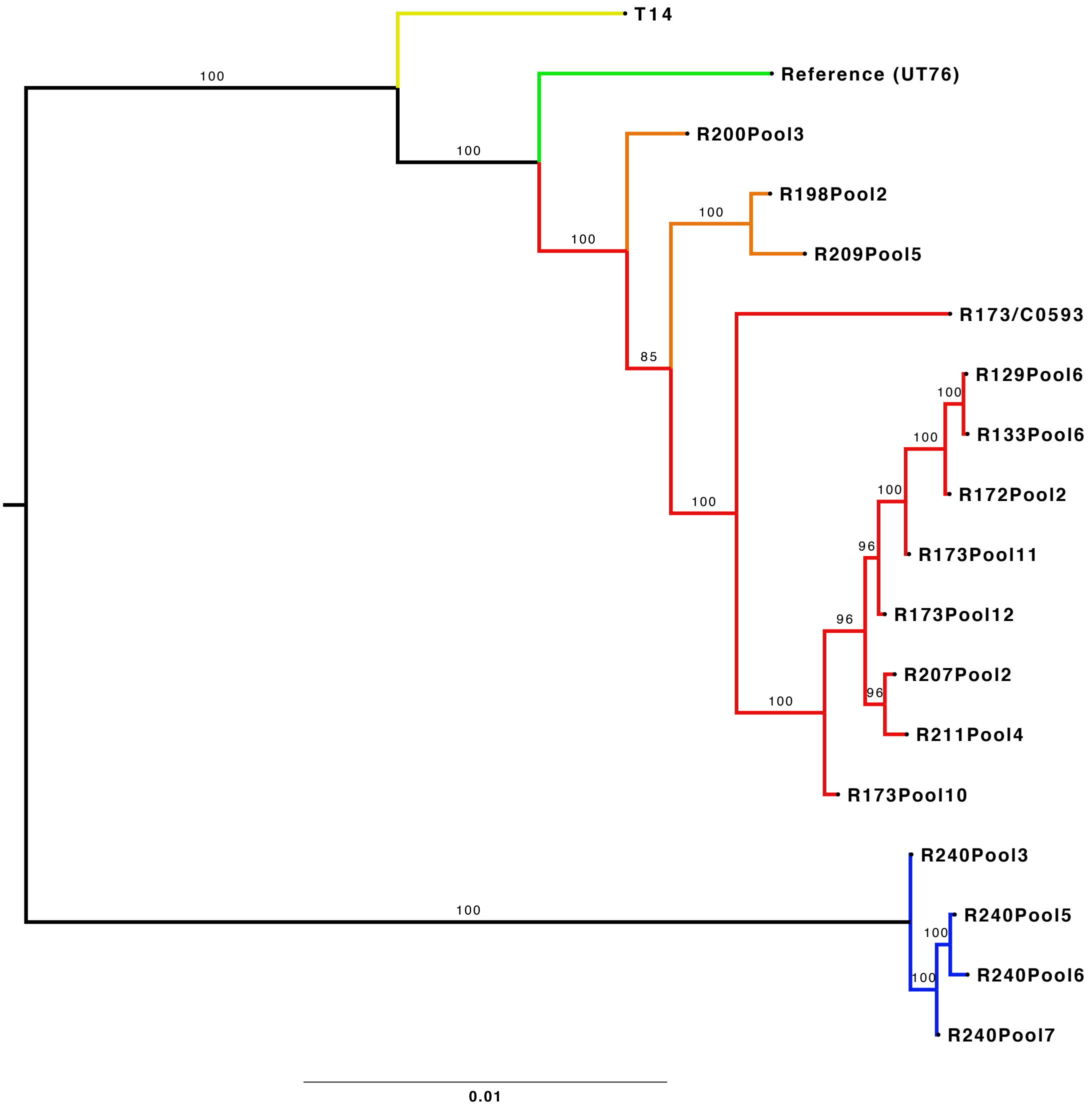


Figure 77 Maximum-likelihood phylogenetic tree of 17 samples compared to the reference. Blue = Ban Mae Mon, Orange = Ban Thoet Thai Group 1, Red = Ban Thoet Thai Group 2, Green = reference genome UT76 and yellow = Penghu Islands, Taiwan

Branch bootstrap values, which can be interpreted as the relative (%) support of the data for the tree topology represented by the pairings of isolates or groups of isolates on either side of the labelled branch, were all in the range 96 to 100, except for 1 branch at 85. This latter, lower bootstrap value appears to reflect uncertainty in the splitting order of sequences from a single location. Samples from Ban Mae Mon (R240 pools in blue) are clearly distinct from samples from Ban Thoet Thai (red/orange) which group together. Even at a scale of ~3km^2 in Ban Thoet Thai there appears to be some local sequence structure. The Taiwan chigger and the reference genome are grouped separately.

To evaluate the ML tree, with a different method based on a different proportion of the data, a pairwise distance matrix was calculated, based for each pair on the sites at which high-confidence ("homozygous") calls could be made for both samples (~330,000 to ~1,100,100) and the number of sites at which those calls differed (183 to ~36,000) was created (Appendix C, Table 5). The UPGMA tree generated was very close to the ML tree. A comparison of the 2 phylogenies is shown in Figure 78.

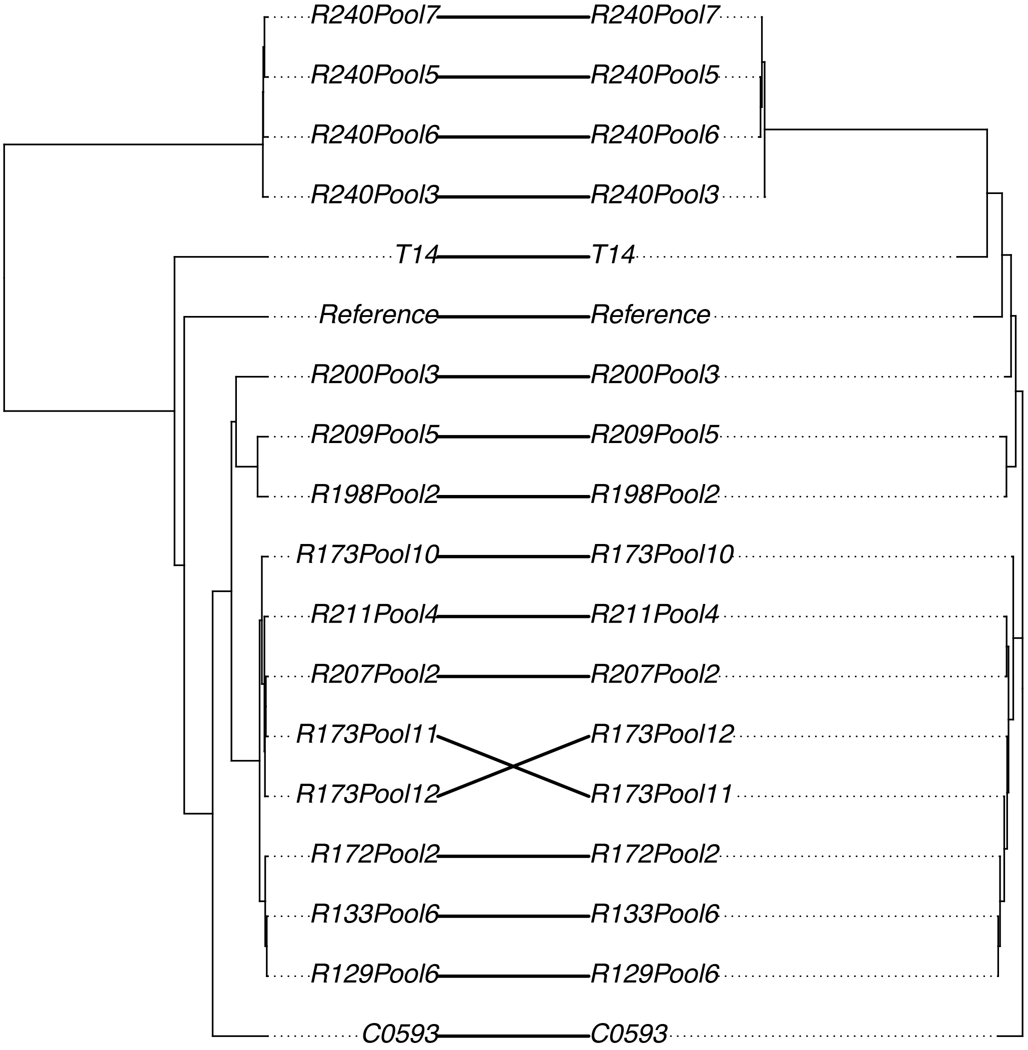


Figure 78 Comparison of phylogenies generated using the ML method for positions definitively called as homozygous in all samples (left) and UPGMA tree (right) for the difference between the alignments scaled by the number of positions called.

5.6 Discussion

Novel methods for the targeted enrichment of *O. tsutsugamushi* DNA were developed and successfully tested on a set of human, chigger and rodent DNA samples. The sequence data generated for some samples provides an opportunity to compare *O. tsutsugamushi* strains with greater resolution than previously possible. The study included strains sequenced from chiggers collected from a single host animal, strains from chiggers from several animals at single study site of <10 km^2 and from two sites about 45km apart. A wider comparison of chigger and human strains from Chiang Rai, Laos and Taiwan was also made. Samples were collected over an 18-month period.

Evidence that, from a limited number of samples with sufficient genomic scale data, relatively close genomic relationships existed at small spatial scales such as those of a single study site or individual host animal, support my hypothesis, firstly that novel methods could improve the collection of genomic scale data and that secondly such data would be useful in understanding the structure of *O. tsutsugamushi* genomic variation. My data also revealed the challenges of directly sequencing natural samples and of the importance in refining methods used.

The extremely low quantity of DNA present in many samples, the low proportion of *O. tsutsugamushi* DNA and the difficulties of assembling the *O. tsutsugamushi* genome have presented considerable challenges. Only 17 of the 183 samples sequenced provided sufficient sequence coverage to easily allow robust comparisons between strains. Unfortunately, many of the human samples were sequenced using the first method, before the introduction of the WGA step. Had these samples been available for the revised method, they may have provided more on-target sequence for analysis. We plan to analyse this data in more detail, using for example pairwise methods, and also possibly, exploiting the ability of ML methods to account for missing data, in order to add more samples with lower coverage to the analysed dataset.

Concern remains over the possibility of false-positive 47 kDa *O. tsutsugamushi* PCR on samples due to non-specific primer binding. This was investigated and discussed in Chapter 3. Two low-positive samples (Ct values of 35 and 37.8) were sequenced without a WGA step and subjected to read classification using Centrifuge and visualization with Pavian [370,371]. One showed a small number (4,200) of reads classified as *O. tsutsugamushi* and the other showed none. A recent study from Uttar Pradesh, India reported more PCR positives using the GroEL gene compared to the 56kDa gene, suggesting diverse *O. tsutsugamushi* strains may not be captured by single-gene methods and that a nested or multiplex gene approach may be more suitable [381]. An additional limitation lies with the quantitative PCR used to generate Ct values. Over the course of the 18 months of laboratory work, batches of standards used for quantitation were changed twice and this may have introduced some variability between qPCR results.

Some refinement to the sequence capture probe set would be expected to improve sequence capture of targeted DNA. In the spike-in experiment it was noted that off-target vero cell DNA sequences were being captured. Some rodent sequences were also captured, despite masking performed by the probe set manufacturer. Recently the first full genome of *L. deliense* has been published and this could be used to check for any capture of off-target chigger DNA [140]. The more distantly related *O. chuto* genome has not been well assembled to date, however improvements to the assembly with long read technology could allow its sequences to be incorporated in the probe set together with other recently sequenced genomes [350].

The first set of human samples and colony chiggers sequenced revealed the variation in sequence coverage among the samples and high duplication rates. This sequencing was performed using the low-input Nextera XT library preparation method. Just 2 of the human buffy coat samples produced coverage of >90%. Buffy coat and eschar samples performed better than whole blood as the latter sample type contains the lowest quantity of DNA. Perhaps surprisingly, the colony chigger samples performed well with 5/6 having >70% coverage at 10x. The quantity of DNA in the colony chiggers appears higher than that seen in almost all individual wild chiggers, perhaps because of different conditions in the laboratory setting or different handling of samples, but further investigation would be needed to resolve this.

The spike-in experiment demonstrated some inconsistencies in results between copy-number dilutions. The total reads per sample is driven by both pooling and relative copy number of on-target sequences. At quality control, the mass of different samples was noted to vary. Equal amounts of libraries were pooled for enrichment and sequencing in this project, which tends to obscure the relative yields of on-target sequence. Variability may also be created at the library preparation stage. The size distribution of library inserts among Nextera XT spike-in samples was not uniform: samples with less DNA altogether had a sub-optimal broad peak between 550-600bp. This suggests that some fragments may have been too long to sequence or amplified less efficiently. It is unclear why the fragment sizes ended up larger than expected, although there is a known relationship between more DNA and longer fragments in Nextera libraries. The kit blocking mix is usually optimized for capturing from human background DNA, so rodent repetitive DNA may have been less efficiently blocked.

The yield of unique on-target reads, particularly at the low copy number dilutions (5,000 and 10,000 copies) was higher for WGA followed by standard library preparation than for Nextera XT. Copy-number standards were designed to correspond to real-life chiggers, and comparisons suggested that they did match some such samples. Duplication rates were also reduced with WGA. This method was therefore selected for subsequent library preparation.

A general trend towards higher percentage mapped was seen for lower Ct values on an exponentiated scale (Figure 75). However, there was considerable inconsistency likely due to a combination of the factors described above. Rodent samples performed particularly poorly. Despite some having Ct values as low as 32, none produced coverage of more than 4.4% at 1x. This may be due to the high quantity of DNA likely to be present in extracted rodent samples, with the proportion of *O. tsutsugamushi* DNA being low leading to poor sequencing. Any metagenomic sequencing, including probe-enrichment sequencing, suffers from lower sensitivity when there is a relatively high content of competing nucleic acid in the sample.

Among the 17 best-sequenced samples, >98.5% of the core genes of the reference sequence were covered by at least one read. For most samples, the regions of no coverage were confined to a very few genes, some of which were present in all samples. Intriguingly, for chigger pools from Ban Mae Mon (R240), more genes (30-40 per sample) were incompletely covered, and most of these were present in all samples, even though the total volume of on-target reads (equivalently, the average coverage of the core genome) was similar in these samples as in other high-performing samples. Possible explanations include extreme genomic sequence divergence in a subset of core genes (in spite of the robustness of the methodology to sequence divergence [366]) or variation in gene structure, such as truncation caused by genome re-arrangement in a group of related strains, or insertions that dirupt read mapping. Across the core gene set as a whole, the R240 samples formed a distinct phylogenetic group, which could be consistent with of the suggested explanations.

Based on the preliminary methods used, only a small number of samples that were sequenced were suitable for phylogenetic analysis, the results reveal clear phylogeographic clustering. At a larger scale the samples from Ban Mae Mon (R240), Ban Thoet Thai (red and orange in Figure 77), the Taiwan chigger (T14) and the Thai reference genome (UT76) were clearly grouped separately. Interestingly, there seemed to be clustering even at the scale of a single study site of ~3km^2. Ban Thoet Thai rodents R198, R200 & R209 were trapped within 25m of each other in Jan 2018 and carried closely related *O. tsutsugamushi* sequences, although the bootstrap value of 85 implies a close relationship in which the branching order of the 3 samples is somewhat uncertain. The other samples from the same village were trapped up to 230m apart, over a 12-month period. R173/C0593 occupy branches separated by several branches on the phylogenetic tree. C0593 was an individual chigger collected from rodent R173, from which pools were also collected. The genetic distance between C0593 and R173 pools suggests significantly different strains. R173 was a large (800g) male bandicoot rat, perhaps with a bigger territorial/foraging range and thus able to acquire chiggers from a larger area with more strain diversity (Figure 79).

The addition of recently fully sequenced samples may improve the resolution of the phylogenetic analysis [350]. In future analyses, the use of imputation as an option in the maximum likelihood tree construction will allow the inclusion of sites in the sequence alignment that are not determined in every sample. This should allow less-well sequenced samples to be included in the analysis, increasing the number of potential relationships that can be evaluated.

Figure 79 Location of samples collected in Ban Thoet Thai, with reference (colour coding) to Figure 77, showing phylogeographic differences at the scale of a single study site

R240 pools are a considerable genetic distance from the other samples, perhaps differing by 4-5%. This level of sequence variation is high and raises the intriguing question of whether there could be more than one *Orientia* species and if perhaps these others species are not or weakly pathogenic to man. As can be seen in Table 26 all the chigger pools and individuals from Ban Thoet Thai consisted of the known vector *L. imphalum* (with or without some *Walchia* species). The Taiwanese chigger was the known human vector *L. deliense*. The R240 pools were collected from the scansorial tree shrew *T. glis* and consisted of *L. turdicola* and *H. naresuani* – neither known to be human vectors or previously reported as being infected with *O. tsutsugamushi*. This could explain the surprising genetic difference from the other samples.

A single recent study has reported phylogenetic comparisons of *O. tsutsugamushi* strains from chiggers collected from the same host animal, based on sequencing of a single gene (encoding the 56 kDa antigen) with the Roche 454 platform generating average read lengths of 633 nucleotides and a median number of reads per sample of 10,198 [321]. Results revealed mixed infections; with some chiggers containing a single genotype and others mixed genotypes. In some cases these mixed genotypes were combinations of those found in chiggers with single genotype infections on the same animal. This lends weight to the possibility of transfer of strains through co-feeding on the same host, a key pre-requisite of horizontal gene transfer between *O. tsutsugamushi* strains that potentially marks them out as unique among rickettsial intra-cellular bacterial parasites.

Important questions remain about the role of recombination between strains in infected chiggers and to what extent the accessory genome of *Orientia* is open or closed. The sequence capture approach used in this study recovers sequences that are previously known, but does not assess the number of new genes. Thus this method does not result in a complete genome assembly and so is unsuitable for identifying the core or accessory genome. With improvements to the sequencing methods and the accumulation of more high-quality sequences it may be possible to characterise recombination at the population level by comparing clustering of mutations between different samples collected from, for example, the same host animal or animals trapped in the same area. However, *O. tsutsugamushi* genomes are known to have poorly conserved synteny, which is likely to complicate analysis.

The maximum sequence divergence (~4%) at core genome positions between pairs of samples revealed during this experiment provides evidence that our enrichment method with the existing sequence probe panel is reasonably robust to variation and almost certainly more robust than a large-scale PCR-based sequence survey would be. The methods developed in this project have, for the first time in scrub typhus research, demonstrated phylogeographic clustering of *O. tsutsugamushi* strains at the international, provincial and highly localised scales. This shows that both closely related strains and more distantly related strains may co-exist at a very localised site. As methods improve and can be applied to a greater range of samples, particularly sympatric rodents and exposed humans, further insights into this fascinating phylogeographic variation will be revealed with important consequences for diagnostic tests and vaccine development strategies.

CHAPTER 6

Conclusions and future work

6 Chapter 6: Conclusions and future work

6.1 Introduction

Scrub typhus is an important and neglected tropical disease in South and East Asia with an expanding geographical distribution. The burden of disease is high in both Thailand and Laos. In Thailand the provinces with the highest incidence are located in the northwest. In the districts of Chiang Rai Province investigated in this study, mean annual incidence ranged from 121 to 218/100,000 population over the last two decades. Reported seroprevalence in Thailand ranges from 2.7% to 59.5% depending on the population selected [39]. In Laos, 15% of fever presenting to the main tertiary referral hospital in Vientiane is due to scrub typhus [41]. With natural immunity probably not lasting beyond 2 to 3 years [62,63], repeated infections in exposed populations is likely, thus contributing to the high burden and morbidity of the disease. In many regions the disease is probably overlooked with infection going unreported. Although treatment with antibiotics is simple and effective, appropriate therapy is frequently not initiated due to lack of diagnostics and awareness. Poor understanding of the ecology of scrub typhus, including disease transmission, has prevented the promotion of potentially simple public health interventions that could reduce the burden of disease.

Much of the research on the natural history of scrub typhus took place prior to the 1970s. The last detailed review of the ecology of the disease was performed in 1974 [14]. In this project I aimed to develop and utilise modern techniques to revisit fundamental aspects of the epidemiology and ecology of the disease. A systematic review of the literature going back to the 1920s provides data on testing of *Orientia* in vectors and non-human hosts from 30 countries. A summary of the key themes in scrub typhus ecology provides a framework for our current understanding, highlights the many gaps in knowledge and proposes minimum reporting criteria for future studies.

This study undertook field investigations to identify a site of high scrub typhus disease transmission in Thailand and Laos. The difficulty in identifying such a site illustrates the low density of *O. tsutsugamushi* in vector chiggers and small mammal hosts and that the intensity of infection is probably patchy across endemic regions. A low-input targeted enrichment sequencing method was developed and successfully applied to PCR positive samples to begin to understand the population genetics and interaction of *O. tsutsugamushi* strains between vectors and hosts, including humans. Geographical information systems tools were used to map infected cases to the environment and statistical methods were applied to investigate the complex ecological interactions of infected and uninfected vectors and hosts with habitat and climate at the scale of a single study site.

6.1.1 Vectors and hosts

Approximately 5% of the 17,000 chiggers collected in this study were identified to species level, with 46 species identified from 15 genera in 2 subfamilies. A possible further 7 species were recorded for which reliable identification to species level could not be made. Of individual chiggers tested, 8.6% were *O. tsutsugamushi* PCR positive and 25.9% of chigger pools tested positive. Six new chigger species, including one new genus, *Trombiculindus*, tested positive for *O. tsutsugamushi* for the first time to the best of our knowledge (Appendix A-3). In total 244 small mammals of 19 species were trapped from 10 sites in Northern Thailand and Laos. At least one organ tested PCR positive from 16% of small mammals, of which 72% were from the genus *Rattus* and 11% *Bandicota indica*. Laboratory analysis highlighted the importance of testing multiple organs, particularly lung and spleen, to identify a positive individual.

Seventy-five small mammals (31%) tested positive from at least one of the following: chigger pool, individual chigger or animal tissue. In only 23/39 organ PCR positive small mammals did either an individual chigger or pool of chiggers from that mammal test positive. This could be explained by assuming that the transmitting chigger(s) had already detached from the host. There is both laboratory and field evidence that many species of chiggers and even Macronyssidae and ticks can acquire *O. tsutsugamushi* from a rickettsaemic host [102,104,112,129-131]. At least one positive chigger pool or individual was detected in 51 PCR negative small mammals. In the laboratory setting transmission rates of *O. tsutsugamushi* from infected chiggers to uninfected small mammals is high [150]. The number of small mammals testing negative despite the presence of infected chiggers was surprising. This may be because infected chiggers had not yet fully attached, or rickettsaemia was yet to develop in the host or possibly that host immunity prevents rickettsaemia detectable by PCR.

6.1.2 Habitat

The habitats at all 3 main study sites were described in detail to determine whether habitat may play a role in the presence or absence of *O. tsutsugamushi* infected vectors and hosts. All sites consisted of farmed land including plantations, fallow areas and patches of mixed secondary forest to varying extents. Two sites also contained small perennial streams. Among the most common plants identified were a number of invasive grasses and weeds.

In an analysis of habitat types and infected vectors and hosts, paddy field/riverbank, cornfields and mixed secondary forest were most central in the unipartite network analysis. A key vector species, *L. deliense* and the frequent host species *R. tanezumi* were identified as true habitat generalists with Paired-differences indices (PDI) of 0.33 and 0.42 respectively. This ability to inhabit many ecological niches probably contributes to these species' success. In the GLM, fixed effects of habitat types on the best-fit model for *O. tsutsugamushi* positivity showed rice paddy field, mixed secondary forest and fallow areas to have the strongest positive effects on the model.

The challenges of classifying habitats accurately and the likely exposure of some host species to several habitats (particularly at habitats edges) make these analyses challenging. Moreover, that important vector chigger species are habitat generalists suggest habitat type is not critical in influencing *O. tsutsugamushi* positivity. However, any requirement for presence of water and moist ground requires further evaluation.

6.1.3 Seasonality

The seasonality of scrub typhus disease in humans appears to be associated with rainfall and temperature. In more northern latitudes, temperature probably plays the greater role, whereas closer to the equator rainfall is more important. Indeed, Thai national scrub typhus data shows much lower seasonal variation in central and southern provinces compared to northern and northeastern. In northern latitudes, including the Penghu islands, temperature is probably more important, as chiggers are relative inactive at lower temperatures [193-195]. Chigger species involved in transmission are also thought to vary in different regions (and through different seasons) of South Korea and Japan and may be responsible for the varying patterns of scrub typhus in these countries [170-172,179,180]. In this study, chigger indices certainly rose after the onset of the rains and fell during the dry season. However, overall chigger numbers remained relatively high throughout the year and the proportion of PCR positive chigger pools were actually higher during the lower-incidence scrub typhus season. On the Penghu Islands, chigger indices drop to nearly zero during winter. GLM identified the end of the dry season as most strongly associated with *O. tsutsugamushi* PCR positive samples.

These findings suggest that human behaviour and exposure to chiggers is probably more important than previously considered. Farming activity rises sharply at the end of the dry season. A high proportion of people in the highest incidence areas are involved in farming and during these periods spend most of the day in the field, typically eating and resting on the ground during the day. This provides the necessary opportunity for chiggers to attach. Studies in South

Korea and Japan have identified certain farming activities to be associated with increased risk of acquiring scrub typhus [268,269].

6.1.4 High and low risk sites

Despite considerable effort, no focus of high transmission was identified in Laos. Epidemiological investigation into possible exposure sites may increase the likelihood of identifying infected vectors and hosts. Three sites of high transmission were eventually identified in Chiang Rai Province and investigated 4 times each over an 18-month period. Sites of high human incidence would be expected to contain greater numbers of infected vector chigger species and have higher small mammal densities as maintaining hosts. Investigating such sites, as was apparent in some areas, can be challenging, as small mammals are not always readily trapped.

Scrub typhus has been described as a disease of patchy distribution at both the large scale (endemic region), small scale and even at the micro-scale of less than a few metres. In the 1940s Audy coined the phrase “mite island” which has been repeated in the literature ever since. Such islands have been reported in an enormous range of habitats, but not from very arid areas or from deep forest. If the disease is indeed patchy in its distribution, what size are the high-risk areas? What factors determine high and low risk sites and what influences the dynamics of such a site over time?

During the year preceding this study, several human cases were diagnosed from the 3 villages investigated. One village had higher rates of positive vectors and hosts. GIS mapping of positives and negatives over 18 months at each village did not identify any clear tendency for a particular area at any study site to be persistently positive or negative. Based on free-living chigger collections at the micro-scale, chiggers did appear to be patchily distributed, however this may be due to the suitability of microenvironments needed for chiggers to thrive.

At the site with the highest *O. tsutsugamushi* infection rates, chigger indices were consistently highest of all sites with a mean of 151, compared to 86 and 62 at the other main sites and 27 and 13 at Lao sites. Chigger diversity was lowest with an *H'* of 1.39, 95% CIs [0.99, 1.71] compared to 1.57 [1.28, 1.86] and 1.66 [1.37, 1.92] at the other main sites. Ban Thoet Thai, had the highest proportion of recognised vector species *L. deliense* and *L. imphalum* at 71%, compared to 44 and 27% at the other main sites and 2 and 4% at the Lao sites. The factors that determine the presence and density of these key vector species are unknown. The findings in this study at the scale of a single village differ markedly from those found in a study of 13 sites in 11 provinces in Thailand, which suggested scrub typhus was more associated with higher chigger diversity [201]. It may be that at this larger scale the findings reflect the fact that areas of human disease are more associated with communities involved in agriculture at the interface of forest/fallow areas and farmland. On the Penghu Islands in Taiwan, chigger diversity is very low, with just the key vector *L. deliense* predominating. Small mammal density at the main study sites is difficult to measure. Based on trap success rates, Ban Song Kwair was highest, however this site had consistent trapping by hunters. The density of rodents in Ban Thoet Thai was certainly high in the small area under investigation.

The findings of Audy and colleagues on the Indo-Burma border 70 years ago and their description of mite islands reflected an unusual relationship between humans and the ecology of *O. tsutsugamushi* and this may have led to an overemphasis on the importance of mite islands in the subsequent literature. In WW2 young soldiers with zero previous exposure to scrub typhus (except those in the allied armies with origins in India and Nepal and perhaps those with Japanese and Chinese origins) were suddenly exposed in very large numbers at high human density to small areas of territory in extremely basic living conditions – a form of 'rural mass gathering'. In today's world such mass short-term naïve human invasions of small areas of rural tropical environments are rare. In more recent years the high incidence of human disease (particularly outbreaks) on, for example the Penghu Islands, probably reflects the introduction of immunologically naïve military personnel engaged in activities with high exposure risk.

If military tactical decisions led to deposition of these high densities of naïve humans in areas of high *O. tsutsugamushi*-infected chigger density, the heterogenous distribution of *O. tsutsugamushi* would have highlighted the importance of mite islands that may be less important in stable dispersed rural communities. Hence, mite islands may be of less public health importance today, even though they may still exist in some biotypes, as large numbers of people are not deposited in them and people may pass through mite islands relatively infrequently. The lack of rural mass gatherings such as occurred in WW2 would also make it much harder to detect putative mite islands today as relatively few people would be exposed and their ranging patterns are likely to be large. This does not mean that mite islands are not important as greater understanding may help prevent the severe public health issues that occurred in the 1940s, in case of future ‘rural mass gathering’ arising from military action and training, civilian camping and orienteering, and refugee crises with urban people seeking shelter in rural environments.

6.1.5 *O. tsutsugamushi* sequencing

Novel low-input targeted enrichment sequencing methods were successfully developed to allow the sequencing of *O. tsutsugamushi* DNA extracted directly from human, chigger and small mammal samples. Improvements were made to the methods by introducing a WGA step prior to library preparation with reduction in duplication rates. Samples varied widely in the quantity of DNA present. Less than 20 out of nearly 350 samples contained more than 10,000 copies of *O. tustsugamushi*, while ~50% of samples contained less than 100 copies (equivalent to a Ct value of ~36). Chigger pools and a small number of buffy coat and eschar samples and individual chiggers contained the highest quantity of *O. tsutsugamushi* DNA. Sequence coverage was thus very low in the majority of samples. Improvements to the sample DNA extraction methods, capture probe set design and downstream analysis would improve sequence coverage for a greater proportion of samples. However it is likely that detailed phylogenetic comparisons for samples well below 100 copies of *O. tsutsugamushi* will remain challenging.

6.1.6 Phylogenetics

Comparison of a set of 17 of the most completely sequenced samples with between 50,000 and 1,150,000 bases called, revealed clear evidence of phylogeographic clustering at the international, provincial and even very localised scales. Chiggers collected from the same village showed close genetic similarity. Even within one village, there appeared to be differences between samples collected 25m apart compared to those collected >200m away. However, in one case larger genetic diversity existed between an individual chigger and chigger pools collected from the same host, suggesting higher diversity can co-exist. A considerable genetic distance of ~4-5% was observed between the *O. tsutsugamushi* sequenced from chigger pools in Ban Mae Mon (R240), compared to all other samples. These pools consisted of unusual chigger species and host, perhaps explaining the divergence. This degree of difference raises the question of taxonomic classification and whether other "species" (like *O. chuto*) may exist. This diversity could also explain aspects of human disease virulence, geographic differences and suitability to infect humans, others hosts and chiggers. The degree of both genetic and geographic resolution revealed by the methods developed in this study should pave the way for much greater understanding *of Orientia* phylogeography.

6.1.7 Future work

Many of the fundamental aspects of the life cycle and transmission of *O. tsutsugamushi* remain poorly understood with little recent research using advanced laboratory techniques and analytic tools. A single laboratory chigger colony currently exists worldwide. Establishing further colonies to repeat and expand on previous studies would be extremely useful. Investigating aspects such as transovarial transmission, particularly after the acquisition of *O. tsutsugamushi* from a host, and whether chiggers may feed on more than one host would be valuable. Performing these studies with other species and genera of trombiculid mites would help to better understand the role of different genera in disease transmission.

Field studies in this project identified a range of factors associated with a high transmission site. Repeating this work at both sites of low transmission such as some districts of central Thailand and other high-risk sites in other regions could provide useful insights into the key determinants of risk. Furthermore, investigation of atypical habitats such as deep secondary forest, marshland or dry habitats may develop understanding of the role of different habitats.

The scale of patchiness of disease risk across endemic regions (mite islands) remains poorly understood. Are *O. tsutsugamushi* infected vectors completely absent in some areas or are they still present but at very low density? A single positive chigger pool was found at 1 site in Laos and 1 site in the lower-risk area to the east of Chiang Rai city.

Finding free-living chiggers even in areas with high small mammal chigger indices proved difficult. Work into understanding the habitats occupied by free-living chiggers would be beneficial. Recent advances in technology for tracking small animals and birds could be applied to common hosts to understand their home ranges and potential sites for chiggers to attach and detach. These methods could also be used to study ground-dwelling and long-distance migrant birds, potentially provide fascinating insights into the movement and dispersal of infected chiggers over wider areas and the possibility for setting up new infected areas.

The role of humans in disease acquisition has been identified as a critical area for study by this project. Study of high and low risk sites (ideally with associated collection of vectors and hosts) would begin to reveal the aspects of behaviour most associated with acquiring scrub typhus. Tracking technology could also be applied to humans to determine time spent in different habitats at different times of the year and how this relates to infection risk. Additional study into land use, environmental change and climate may be useful in understanding future risk for differing populations. With scrub typhus now being identified at sites far from the traditional region, much work is needed into how the ecology differs in these areas.

This project provides the first detailed whole-genome sequences for DNA samples collected directly from humans, chiggers and rodents. Some preliminary insights into the population genetics of this pathogen are presented. The method has many potential advantages over existing methods, but challenges remain. However improvements are likely to increase resolution much beyond existing single gene or limited MLST approaches. More phylogenetic analysis could be attempted by further assessment of the pairwise differences between samples. Genetic distances between samples could also be calculated.

The application of these methods to samples collected in South America and Africa should provide exciting new insights into the taxonomy of *Orientia*. Studies are now urgently needed in these regions to understand the extent and relevance of human infection with *O. tsutsugamushi*-like species.

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APPENDIX

PRISMA 2009 Checklist

8.1 APPENDIX A-1

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3-4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	2-3
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	3
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	3-4
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	3-4
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	3
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	3
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	3-4

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	3-4
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	5-6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	5-6
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	7-21
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7-21
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	8-9
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	40-41
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	40-41
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	40-44
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

8.2 APPENDIX A-2

8.2.1 Table 1 Summary of laboratory test categories and list of tests for each category

Molecular	**Serological**	**Combination serological & molecular**	**Culture +- microscopy**	**Combination culture & serological**	**Combination culture & molecular**	**Microscopy**
56 kDa PCR	Direct immunofluorescence (DIF)	ELISA & PCR	Xenodiagnosis	Xenodiagnosis & complement fixation	Xenodignosis & PCR	Organ impression smears
47 kDa PCR	Indirect immunofluorescence (IIF)	56 kDa, nested PCR & sequencing	Xenodiagnosis & microscopy	Xenodiagnosis & OXK/Weil Felix	Xenodiagnosis & L929 cell culture & PCR	Giemsa stain or unspecified
GroEL PCR	ELISA	IF & PCR		Xenodiagnosis & DIF/IIF	L929 cell culture & PCR	
Nested PCR	OXK/Weil Felix test			Xenodiagnosis & L929 cell culture & IIF		
16s rRNA sequencing	Complement fixation			Xenodiagnosis & unspecified serological method		
Unspecified molecular method	Passive haemagglutination assay					
	Indirect Immunoperoxidase					
	Unspecified serological (IgM/IgG) method					
	Unspecified antigen-based method					

8.2.2 Table 2 Vector genera and species tested for *O. tsutsugamushi*, combining all laboratory tests

Group	Chigger mites & other Acari	Total tested	Total positive	Percentage positive
Trombiculidae	*Actomatacrus* sp.	?	3	-
Ixodida	*Amblyomma auricularium*	4	0	0.0
Ixodida	*Amblyomma ovale*	32	0	0.0
Ixodida	*Amblyomma parvum*	241	0	0.0
Ixodida	*Amblyomma sculptum*	1033	0	0.0
Ixodida	*Amblyomma* sp.	269	0	0.0
Ixodida	*Amblyomma tigrinum*	1	0	0.0
Trombiculidae	*Ascoschoengastia indica*	1273	8	0.6
Trombiculidae	*Ascoschoengastia* sp.	1448	14	1.0
Trombiculidae	*Blankaartia acuscutellaris*	919	66	7.2
Trombiculidae	*Blankaartia* sp.	61	1	1.6
Trombiculidae	*Cheladonta ikaoensis*	42	2	4.8
Laelapidae	*Echinolaelaps echidninus*	?	1	-
Trombiculidae	*Eltonella ichikawa*	6	0	0.0
Trombiculidae	*Euschoengastia koreaensis*	1127	5	0.4
Trombiculidae	*Euschoengastia miyagawai*	?	1	-
Trombiculidae	*Euschoengastia* sp.	?	1	-
Trombiculidae	*Eutrombicula* sp.	9	0	0.0
Trombiculidae	*Eutrombicula wichmanni*	161	14	8.7
Trombiculidae	*Gahrliepia octosetosa*	3	0	0.0
Trombiculidae	*Gahrliepia saduski*	1617	5	0.3
Trombiculidae	*Gahrliepia* sp.	1058	7	0.7
Ixodida	*Haemaphysalis flava*	?	1	-
Ixodida	*Haemaphysalis humerosa*	6	0	0.0
Ixodida	*Haemaphysalis hystricis*	?	1	-
Ixodida	*Haemaphysalis* sp.	?	2	-
Trombiculidae	*Helenicula* sp.	?	2	-
Ixodida	*Ixodes* sp.	12	2	16.7
Laelapidae	*Laelaps turkestanicus*	?	1	-
Trombiculidae	*Leptotrombidium akamushi*	26	28	107.7
Trombiculidae	*Leptotrombidium arenicola*	16	6	37.5
Trombiculidae	*Leptotrombidium arvinum*	898	45	5.0
Trombiculidae	*Leptotrombidium chiangraiensis*	211	49	23.2
Trombiculidae	*Leptotrombidium deliense*	31749	16387	51.6
Trombiculidae	*Leptotrombidium dicum*	1	0	0.0
Trombiculidae	*Leptotrombidium fletcheri*	30	6	20.0
Trombiculidae	*Leptotrombidium fuji*	1595	3	0.2
Trombiculidae	*Leptotrombidium hsui*	1	0	0.0
Trombiculidae	*Leptotrombidium imphalum*	458	69	15.1
Trombiculidae	*Leptotrombidium intermedium*	9486	12	0.1
Trombiculidae	*Leptotrombidium kawamurai*	34	7	20.6
Trombiculidae	*Leptotrombidium kitasatoi*	421	1	0.2
Trombiculidae	*Leptotrombidium laxoscutum*	46	0	0.0

Trombiculidae	*Leptotrombidium linhuaikongense*	87	17	19.5
Trombiculidae	*Leptotrombidium miyajima*	8	0	0.0
Trombiculidae	*Leptotrombidium murotoense*	?	1	-
Trombiculidae	*Leptotrombidium nangii*	?	1	-
Trombiculidae	*Leptotrombidium orientale*	1786	33	1.8
Trombiculidae	*Leptotrombidium pallidum*	16153	1732	10.7
Trombiculidae	*Leptotrombidium palpale*	6385	67	1.0
Trombiculidae	*Leptotrombidium pavlovskyi*	3564	161	4.5
Trombiculidae	*Leptotrombidium peniculatum*	469	7	1.5
Trombiculidae	*Leptotrombidium scutellare*	12403	329	2.7
Trombiculidae	*Leptotrombidium* sp.	2837	122	4.3
Trombiculidae	*Leptotrombidium tachensis*	?	1	-
Trombiculidae	*Leptotrombidium taishanicum*	34	2	5.9
Trombiculidae	*Leptotrombidium waiganmensis*	?	1	-
Trombiculidae	*Leptotrombidium yui*	5	0	0.0
Trombiculidae	*Leptotrombidium zetum*	430	2	0.5
Trombiculidae	*Lorillatum* sp.	159	1	0.6
Trombiculidae	*Microtrombicula chamlongi*	35	7	20.0
Trombiculidae	*Neoschoengastia* sp.	?	1	-
Trombiculidae	*Neotrombicula gardellai*	138	0	0.0
Trombiculidae	*Neotrombicula japonica*	1907	39	2.0
Trombiculidae	*Neotrombicula microti*	4	6	150.0
Trombiculidae	*Neotrombicula mitamurai*	39	7	17.9
Trombiculidae	*Neotrombicula nagayoi*	4	3	75.0
Trombiculidae	*Neotrombicula pomeranzevi*	7	5	71.4
Trombiculidae	*Neotrombicula tamiyai*	9552	0	0.0
Trombiculidae	*Neotromicula shiraii*	?	1	-
Trombiculidae	*Odontacarus majesticus*	185	0	0.0
Trombiculidae	*Odontocarus* sp.	567	21	3.7
Macronyssidae	*Ornithonyssus bacoti*	7	1	14.3
Ixodida	*Rhipicephalus microplus*	1	0	0.0
Ixodida	*Rhipicephalus sanguineus*	1	0	0.0
Trombiculidae	*Schoengastia* sp.	35	3	8.6
Trombiculidae	*Trombicula sadoensis* n. sp.	?	1	-
Trombiculidae	*Trombicula tosa*	?	1	-
Trombiculidae	*Walchia chinensis*	257	19	7.4
Trombiculidae	*Walchia disparunguis pingue*	1	0	0.0
Trombiculidae	*Walchia fragilis*	18	0	0.0
Trombiculidae	*Walchia pacifica*	136	10	7.4
Trombiculidae	*Walchia parapacifica*	10	0	0.0
Trombiculidae	*Walchia* sp.	?	53	-
	Multiple species	328	169	51.5
	Not identified	19284	1029	5.3

8.2.3 Table 3 Non-human "host" species tested for *O. tsutsugamushi* (all laboratory tests combined)

Group	Host species	Total Tested	Total Positive	Percentage positive
Artiodactyla	*Bos taurus* (cow)	706	12	1.7
Artiodactyla	*Capra aegagrus hircus* (goat)	292	25	8.6
Artiodactyla	*Sus scrofa* (domestic pig)	60	1	1.7
Artiodactyla	*Ovis aries* (sheep)	510	16	3.1
Aves	Birds	150	0	0.0
Aves	*Carpodacus sibircus*	?	1	-
Aves	*Gallus gallus domesticus* (chicken)	13	4	30.8
Aves	*Climacteris* sp.	1	0	0.0
Aves	*Cracticus nigrogularis*	1	0	0.0
Aves	*Cracticus torquatus*	1	0	0.0
Aves	*Dacelo gigas*	1	0	0.0
Aves	*Emberiza spodocephala*	?	2	-
Aves	*Eopsaltria chrysorrhoa*	4	0	0.0
Aves	*Macropygia phasianella*	1	0	0.0
Aves	*Motacilla cinerea*	16	3	18.8
Aves	*Myzantha melanocephala*	4	0	0.0
Aves	*Philemon corniculatus*	2	0	0.0
Aves	*Passer domesticus*	91	5	5.5
Aves	*Strepera graculina*	1	1	100.0
Aves	*Trichoglossus chlorolepidoptus*	7	0	0.0
Canidae	*Cerdocyon thous*	78	0	0.0
Canidae	*Canus lupus familiaris* (dog)	1711	325	19.0
Canidae	*Nasua nasua*	31	0	0.0
Chiroptera	*Eptesicus serotinus*	308	38	12.3
Chiroptera	*Miniopterus schreibersii*	47	1	2.1
Chiroptera	*Murina leucogaster*	2	0	0.0
Chiroptera	*Myotis formosus*	1	0	0.0
Chiroptera	*Myotis macrodactylus*	9	0	0.0
Chiroptera	*Plecotus auritus*	1	0	0.0
Chiroptera	*Rhinolophus ferrumequinum*	385	58	15.1
Chiroptera	*Vespertilio superans*	44	2	4.5
Cricetidae	*Alticola roylei*	21	4	19.0
Cricetidae	*Arvicola scherman*	64	2	3.1
Cricetidae	*Clethrionomys rufocanus*	81	10	12.3
Cricetidae	*Clethrionomys rutilus*	13	0	0.0
Cricetidae	*Cricetulus barabensis*	27	2	7.4
Cricetidae	*Cricetulus migratorius*	303	7	2.3
Cricetidae	*Cricetulus triton*	655	77	11.8
Cricetidae	*Cricetus* sp.	4	0	0.0
Cricetidae	*Hyperacrius fertilis*	31	5	16.1
Cricetidae	*Hyperacrius wynnei*	11	2	18.2
Cricetidae	*Lagurus lagurus*	84	8	9.5
Cricetidae	*Microtus agrestis*	7	0	0.0

Cricetidae	*Microtus arvalis*	49	6	12.2
Cricetidae	*Microtus fortis*	375	33	8.8
Cricetidae	*Microtus maximowiczii*	33	1	3.0
Cricetidae	*Microtus montebelli*	252	81	32.1
Cricetidae	*Microtus pennsylvanicus*	?	0	-
Cricetidae	*Microtus subterraneus*	4	0	0.0
Cricetidae	*Myodes andersoni*	1	0	0.0
Cricetidae	*Myodes glareolus*	302	44	14.6
Cricetidae	*Myodes regulus*	160	16	10.0
Cricetidae	*Myodes smithii*	14	10	71.4
Cricetidae	*Oecomys mamorae*	25	0	0.0
Didelphidae	*Gracilianus agilis*	11	0	0.0
Didelphidae	*Monodelphis domestica*	4	0	0.0
Didelphidae	*Thylamys macrurus*	14	0	0.0
Echimyidae	*Clyomys laticeps*	8	0	0.0
Echimyidae	*Thrichomys fosteri*	77	0	0.0
Erinaceidae	*Echinosorex gymnura*	4	0	0.0
Erinaceidae	*Erinaceus amurensis*	4	0	0.0
Felidae	*Leopardus pardalis*	7	0	0.0
Herpestidae	*Herpestes javanicus*	1	0	0.0
Lagomorpha	*Ochotona roylei*	6	0	0.0
Marsupialia	*Didelphis albiventris*	1	0	0.0
Marsupialia	*Echymipera kalubu*	30	4	13.3
Marsupialia	*Isoodon macrourus*	196	33	16.8
Marsupialia	*Perameles nasuta*	29	0	0.0
Muridae	*Acomys wilsoni*	21	0	0.0
Muridae	*Apodemus agrarius*	11656	3704	31.8
Muridae	*Apodemus argenteus*	41	6	14.6
Muridae	*Apodemus flavicollis*	95	15	15.8
Muridae	*Apodemus peninsulae*	290	26	9.0
Muridae	*Apodemus speciosus*	2435	866	35.6
Muridae	*Apodemus sylvaticus*	82	2	2.4
Muridae	*Apomys datae*	?	0	-
Muridae	*Arvicanthis niloticus*	6	0	0.0
Muridae	*Bandicota bengalensis*	233	43	18.5
Muridae	*Bandicota indica*	2176	301	13.8
Muridae	*Bandicota savilei*	54	12	22.2
Muridae	*Bandicota* sp.	17	1	5.9
Muridae	*Berylmys berdmorei*	33	3	9.1
Muridae	*Berylmys bowersi*	204	99	48.5
Muridae	*Chiropodomys gliroides*	6	1	16.7
Muridae	*Chrotomys whiteheadi*	?	0	-
Muridae	*Gerbillus gleadowi*	27	0	0.0
Muridae	*Golunda ellioti*	4	0	0.0
Muridae	*Hapalomys delacouri*	1	0	0.0
Muridae	*Hydromys chrysogaster*	8	0	0.0

Muridae	*Lenothrix canus*	63	3	4.8
Muridae	*Leopoldamys edwardsi*	20	5	25.0
Muridae	*Lophuromys sikapusi*	1	0	0.0
Muridae	*Mastomys erythroleucus*	147	0	0.0
Muridae	*Mastomys natalensis*	8	1	12.5
Muridae	*Maxomys inas*	15	0	0.0
Muridae	*Maxomys moi*	4	1	25.0
Muridae	*Maxomys rajah*	277	97	35.0
Muridae	*Maxomys sp.*	5	4	80.0
Muridae	*Maxomys surifer*	306	130	42.5
Muridae	*Maxomys whiteheadi*	372	101	27.2
Muridae	*Melomys cervinipes*	173	16	9.2
Muridae	*Melomys littoralis*	37	5	13.5
Muridae	*Melomys lutillus*	180	12	6.7
Muridae	*Melomys sp.*	3	0	0.0
Muridae	*Meriones erythrourus*	30	0	0.0
Muridae	*Meriones libycus*	4	0	0.0
Muridae	*Meriones tamariscinus*	1	1	100.0
Muridae	*Micromys minutus*	30	8	26.7
Muridae	*Milardia kondana*	8	0	0.0
Muridae	*Milardia meltada*	33	1	3.0
Muridae	“Mouse”	455	10	2.2
Muridae	*Mus booduga*	17	2	11.8
Muridae	*Mus caroli*	310	53	17.1
Muridae	*Mus cervicolor*	31	0	0.0
Muridae	*Mus commisarius*	?	0	-
Muridae	*Mus cookii*	11	0	0.0
Muridae	*Mus fragilicauda*	1	0	0.0
Muridae	*Mus musculus*	1333	198	14.9
Muridae	*Mus platythrix*	42	0	0.0
Muridae	*Mus saxicola*	16	0	0.0
Muridae	*Mus* sp.	68	5	7.4
Muridae	*Nesokia indica*	69	8	11.6
Muridae	*Niviventer confucians*	41	5	12.2
Muridae	*Niviventer coninga*	3	1	33.3
Muridae	*Niviventer cremoriventer*	122	12	9.8
Muridae	*Niviventer fulvescens*	67	4	6.0
Muridae	*Niviventer niviventer*	12	2	16.7
Muridae	*Rattus andamanensis*	40	10	25.0
Muridae	*Rattus annandalei*	499	282	56.5
Muridae	*Rattus argentiventer*	1074	397	37.0
Muridae	*Rattus blandfordi*	33	2	6.1
Muridae	*Rattus bukit*	12	0	0.0
Muridae	*Rattus canus malaisia*	?	0	-
Muridae	*Rattus conatus*	58	6	10.3
Muridae	*Rattus concolor browni*	51	2	3.9

Muridae	*Rattus coxingi*	13	7	53.8
Muridae	*Rattus diardi*	2	0	0.0
Muridae	*Rattus everetti*	7	7	100.0
Muridae	*Rattus exulans*	1034	175	16.9
Muridae	*Rattus flavipectus*	1516	1025	67.6
Muridae	*Rattus fuscipes*	84	4	4.8
Muridae	*Rattus gestri*	4	0	0.0
Muridae	*Rattus huang*	1	0	0.0
Muridae	*Rattus leucopus*	14	2	14.3
Muridae	*Rattus losea*	2614	742	28.4
Muridae	*Rattus luzonicus*	40	0	0.0
Muridae	*Rattus mackenziei*	1	0	0.0
Muridae	*Rattus mindanensis*	291	68	23.4
Muridae	*Rattus mordax*	30	0	0.0
Muridae	*Rattus muelleri*	605	235	38.8
Muridae	*Rattus nitidus*	1	0	0.0
Muridae	*Rattus norvegicus*	6050	1049	17.3
Muridae	*Rattus panglima*	2	2	100.0
Muridae	*Rattus praetor*	1	0	0.0
Muridae	*Rattus rattoides*	48	8	16.7
Muridae	*Rattus rattus*	7228	1487	20.6
Muridae	*Rattus rattus diardii*	226	18	8.0
Muridae	*Rattus rattus frugivorus*	5	2	40.0
Muridae	*Rattus rattus rufescens*	16	1	6.3
Muridae	*Rattus rattus satarae*	23	2	8.7
Muridae	*Rattus rattus septicus*	9	1	11.1
Muridae	*Rattus rattus sladeni*	?	0	-
Muridae	*Rattus rattus thai*	201	44	21.9
Muridae	*Rattus remotus*	?	0	-
Muridae	*Rattus ruber*	1	0	0.0
Muridae	*Rattus sabanus*	2474	896	36.2
Muridae	*Rattus* sp.	394	17	4.3
Muridae	*Rattus tanezumi*	359	65	18.1
Muridae	*Rattus tiomanicus*	4117	984	23.9
Muridae	*Rattus turkestanicus*	3	3	100.0
Muridae	*Rhombomys opimus*	305	4	1.3
Muridae	“Rodent”	1270	93	7.3
Muridae	*Sicista concolor*	7	0	0.0
Muridae	Swiss albino mouse	?	0	-
Muridae	*Tatera indica*	98	8	8.2
Muridae	*Uromys caudimaculatus*	47	7	14.9
Muridae	*Zapus hudsonius*	58	0	0.0
Mustelidae	*Melogale personata*	7	1	14.3
Reptilia	Lizards	51	0	0.0
Reptilia	*Physignathus lesueurii*	8	0	0.0
Reptilia	*Varanus* sp.	1	0	0.0

Reptilia	*Varanus varius*	1	0	0.0
Sciuridae	*Callosciurus caniceps*	46	4	8.7
Sciuridae	*Callosciurus erythraeus*	1	0	0.0
Sciuridae	*Callosciurus flavimanus*	1	0	0.0
Sciuridae	*Callosciurus nigrovittatus*	241	17	7.1
Sciuridae	*Callosciurus notatus*	791	14	1.8
Sciuridae	*Callosciurus prevostii*	18	0	0.0
Sciuridae	*Callosciurus* sp.	4	0	0.0
Sciuridae	*Dremomys rufigens*	1	0	0.0
Sciuridae	*Funambulus pennantii*	9	0	0.0
Sciuridae	*Funambulus tristriatus*	5	0	0.0
Sciuridae	*Hylopetes spadiceus*	125	0	0.0
Sciuridae	*Iomys horsfieldii*	2	0	0.0
Sciuridae	*Lariscus insignis*	2	0	0.0
Sciuridae	*Marmota baibacina*	58	4	6.9
Sciuridae	*Marmota bobak*	26	0	0.0
Sciuridae	*Menetes berdmorei*	21	2	9.5
Sciuridae	*Menetes* sp.	30	0	0.0
Sciuridae	*Petinomys setosus*	2	0	0.0
Sciuridae	*Pteromyscus pulverulentus*	7	0	0.0
Sciuridae	*Spermophilopsis leptodactylus*	2	0	0.0
Sciuridae	*Sundasciurus brookei*	2	0	0.0
Sciuridae	*Sundasciurus hippurus*	1	0	0.0
Sciuridae	*Sundasciurus lowii*	22	0	0.0
Sciuridae	*Sundasciurus tenuis*	26	0	0.0
Sciuridae	*Tamias sibiricus*	249	64	25.7
Simiformes	*Macaca fascicularis*	27	12	44.4
Soricidae	*Crocidura attenuata*	11	0	0.0
Soricidae	*Crocidura dsinezumi*	2	0	0.0
Soricidae	*Crocidura horsfieldii*	1	0	0.0
Soricidae	*Crocidura lasiura*	279	6	2.2
Soricidae	*Crocidura olivieri*	2	0	0.0
Soricidae	*Crocidura* sp.	25	0	0.0
Soricidae	*Crocidura suaveolens*	25	1	4.0
Soricidae	*Neomys fodiens*	2	0	0.0
Soricidae	"Shrews"	62	0	0.0
Soricidae	*Sorex caecutiens*	?	0	-
Soricidae	*Sorex minutus*	2	0	0.0
Soricidae	*Suncus murinus*	836	158	18.9
Talpidae	*Talpa mogra*	3	0	0.0
Talpidae	*Urotrichus taipoides*	10	3	30.0
Tupaiidae	*Tupaia belangeri*	11	6	54.5
Tupaiidae	*Tupaia glis*	200	43	21.5
Tupaiidae	*Tupaia gracilis*	8	0	0.0
Tupaiidae	*Tupaia minor*	17	0	0.0
Tupaiidae	*Tupaia* sp.	11	0	0.0

Tupaiidae	*Tupaia tana*	86	0	0.0
Viverridae	*Paradoxurus hermaphroditus*	5	0	0.0
Viverridae	*Viverricula indica*	1	0	0.0
	Multiple listed	4643	1480	31.9
	Not Identified	15413	3169	20.6

8.2.4 Table 4 Median chigger index (mean number of chiggers per host species) for species where reported.

Species	**Median chigger index**
Apodemus agrarius	78
Apodemus argenteus	89
Apodemus peninsulae	8
Apodemus speciosus	53
Bandicota bengalensis	22
Bandicota indica	54
Bandicota savilei	58
Berylmys berdmorei	28
Callosciurus erythraeus	0
Gallus gallus domesticus (chicken)	2
Clethrionomys rufocanus	29
Clethrionomys rutilus	16
Cricetulus triton	76
Crocidura lasiura	1
Funambulus tristriatus	17
Golunda ellioti	11
Micromys minutus	15
Microtus fortis	0
Milardia kondana	240
Milardia meltada	131
Mus booduga	5
Mus caroli	2
Mus musculus	1
Mus platythrix	7
Myodes smithii	180
Niviventer confucians	26
Niviventer coninga	0
Niviventer fulvescens	17
Rattus argentiventer	170
Rattus blandfordi	18
Rattus coxingi	14
Rattus exulans	8
Rattus flavipectus	85
Rattus losea	51
Rattus mindanensis	4
Rattus norvegicus	50
Rattus rattus	45
Rattus rattus diardii	400
Rattus rattus rufescens	47
Rattus rattus satarae	130
Rattus remotus	8
Rattus tanezumi	394

Rattus tiomanicus	78
Sorex caecutiens	0
Sorex minutus	0
Suncus murinus	29
Multiple listed	92
Not identified	74

8.2.5 Table 5 Median percentage infestation rate (per cent hosts with at least 1 chigger attached) for species where reported.

Species	**Median percentage infestation**
Apodemus agrarius	61
Apodemus speciosus	14
Bandicota bengalensis	25
Bandicota indica	95
Bandicota savilei	100
Berylmys berdmorei	67
Callosciurus erythraeus	0
Clethrionomys rufocanus	74
Clethrionomys rutilus	77
Cricetulus triton	89
Crocidura lasiura	4
Echymipera kalubu	100
Funambulus tristriatus	42
Golunda ellioti	56
Isoodon macrourus	47
Melomys cervinipes	61
Melomys lutillus	45
Menetes berdmorei	88
Microtus fortis	0
Milardia kondana	96
Milardia meltada	100
Mus booduga	38
Mus caroli	26
Mus musculus	15
Mus platythrix	28
Myodes regulus	100
Neomys fodiens	0
Niviventer confucians	48
Niviventer coninga	50
Niviventer fulvescens	41
Rattus argentiventer	99
Rattus coxingi	27
Rattus exulans	33
Rattus flavipectus	89
Rattus leucopus	47
Rattus losea	93
Rattus mindanensis	25
Rattus norvegicus	46
Rattus rattus	56
Rattus rattus diardii	41
Rattus rattus rufescens	64
Rattus rattus satarae	92

Rattus remotus	50
Rattus sp.	62
Rattus tanezumi	100
Rattus tiomanicus	96
Sorex caecutiens	0
Sorex minutus	0
Suncus murinus	59
Tatera indica	0
Tupaia glis	100
Uromys caudimaculatus	47
Not Identified	17

8.2.6 Table 6 List of all study sites and administrative level (accuracy score)

Site id	Province name	Latitude	Longitude	Country	Site name	Administrative level§
1	Addu Atoll	-0.6333333	73.1666667	Maldives	Port X	2
2	Unknown	14.058324	108.277199	Vietnam	Unknown	0
3	Akita	39.210589	140.514679	Japan	Omono River, Jumonji	2
4	Shizuoka	35.294381	138.853603	Japan	East Fuji Maneuvre Area, Kitago 5km NE Higashi-Fuji, Oshima 6km NE Higashi-Fuji	2
5	Quebec	47.032	-70.628	Canada	Grosse Isle	3
6	Anhui	32.574618	118.126657	China	Sanjie	4
7	Fujian	26.6628	118.1926	China	Ningde, Nanping, Sanming, Pucheng	1
8	Guangdong	21.663457	110.924191	China	Maoming	2
9	Henan	32.146984	114.091023	China	Xinyang City	2
10	Shandong	36.727	117.1502	China	Jinan Suburbs	2
11	Hebei	36.9941667	113.9277778	China	Taihang Mountains	3
12	Shandong	35.89396	117.9249	China	Unknown	1
13	Fujian	26.665617	119.547933	China	Ningde	2
14	Inner Mongolia	49.2009	119.7646	China	Hulun Buir area	2
15	Hebei	38.037057	114.468665	China	Unknown	1
16	Xinjiang	44.85387	82.051005	China	Bole	3
17	Xinjiang	43.432939	83.380331	China	Nalati Grassland	4
18	Jiangsu and Shandong	35.048	118.654	China	Multiple	0
19	Guangdong	23.257277	117.287954	China	Nanpeng	4
20	Shandong	35.265961	117.977325	China	Fei County	3
21	Jilin	42.862821	130.366036	China	Hunchun	3
22	Shandong	36.255833	117.105556	China	Mount Tai	4
23	Fujian	25.544055	118.75951	China	Unknown	1

24	Zhejiang	30.583333	119.833333	China	Hangzhou	2
25	Shandong	36.6686	117.0204	China	Fei County	3
26	Beijing	39.90403	116.407526	China	Beijing	1
27	Xinjiang	44.022064	89.593964	China	Changji	3
28	Fujian	24.44	118.33	Taiwan	Kinmen County	3
29	Guangdong	20.916667	110.6	China	Naozhou	4
30	Oro	-8.762016	148.366596	Papua New Guinea	Dobadura	4
31	Queensland	-16.383	145.335	Australia	Whyanbeel Parish, west of Dayman Point state school	4
32	Queensland	-16.419	145.39	Australia	Portion 252 east of road to Daintree	4
33	Queensland	-16.361	145.356	Australia	Portion R100 northern boundary of Portion 14v	4
34	Maharastra	18.7546171	73.4062342	India	Lonavala	4
35	Maharastra	18.4454128	73.7801447	India	Khadakvasla	4
36	Maharastra	18.3838904	73.6035782	India	Panchet	4
37	Maharastra	18.366304	73.755876	India	Singhgadh	4
38	Maharastra	18.5699361	73.8506427	India	Khadki	4
39	Maharastra	18.4770906	73.8906869	India	Kondhwa (Khurd)	4
40	Maharastra	18.5911292	73.91882	India	Lohegaon	4
41	Maharastra	18.558007	73.8075201	India	Aundh	4
42	Maharastra	18.5023996	73.8784824	India	Pune Cantt	4
43	Maharastra	18.4923382	73.870286	India	Ram Tekri	4
44	Fujian	25.479	119.7429	China	Beicuo, Pingtan Island	4
45	Anhui	32.777055	117.989199	China	Mingguang	3
46	Unknown	35.86166	104.19539	China	Unknown	0
47	Udon Thani	17.3647	102.8159	Thailand	Unknown	1
48	Chiang Mai	18.70606	98.98172	Thailand	Unknown	1
49	Chiang Rai	19.91048	99.84058	Thailand	Unknown	1
50	Nonthaburi	13.85911	100.5217	Thailand	Unknown	1

51	Khon Kaen	16.43219	102.8236	Thailand	Unknown	1
52	Prachaup Kirikhan	11.81237	99.79733	Thailand	Unknown	1
53	Sa Kaeo	13.82404	102.0646	Thailand	Unknown	1
54	Tak	16.88399	99.12585	Thailand	Unknown	1
55	Ubon Ratchathani	15.1264018	105.4302383	Thailand	Chong Mek	4
56	Petchaburi	12.96492	99.64259	Thailand	Unknown	1
57	Guangdong	22.037097	113.917236	China	Wanshan Archipelago	4
58	Guangdong	23.430771	117.092538	China	Nan'ao	3
59	Guangdong	20.914179	110.096586	China	Leizhou	3
60	Xinjiang	43.834689	90.286028	China	Mori Kazakh Autonomous County	3
61	Fujian	25.221115	119.47541	China	Nanri	4
62	Yunnan	25.63911	101.0065	China	NE of Xundian Hui & Yi counties, NW of Yulong Naxi county & south of Simao district	1
63	Queensland	-16.45	145.373333	Australia	Mossman	4
64	Taiwan strait	24.658084	119.697944	Taiwan	Pescadores Islands	1
65	Ardennes region	49.6807359	4.7961215	France	Unknown	2
66	Unknown	16.1533563	-14.920809	Senegal	Senegal River	1
67	Loei, Nan, Buriram	17.143	101.87	Thailand	Multiple	0
68	Chiang Rai	20.4414	99.9289	Thailand	Viang Hom	4
69	Chiang Rai	20.4166	99.9911	Thailand	Rong	4
70	Chiang Mai	19.737001	98.973428	Thailand	Aruno Tai	4
71	Chiang Mai	19.742155	98.936953	Thailand	Muang Na	4
72	Mae Hong Son	18.170158	97.930459	Thailand	Mae Sa Riang	4
73	Mae Hong Son	18.80928	98.03255	Thailand	Mae Ja Ton	1
74	Tak	16.68982	98.51774	Thailand	Rim Mei	4
75	Tak	16.709573	98.505422	Thailand	Wang Ta Kian	4
76	Ratchaburi	13.53341	99.58085	Thailand	Bo Moo	1
77	Ratchaburi	13.65447	99.210315	Thailand	Tago Lang	4
78	Phetchaburi	12.94651	99.6194	Thailand	Teng Nuea	1

79	Phetchaburi	12.94651	99.6194	Thailand	Suan Yai Pattana	1
80	Prachuap Khiri Khan	11.77577	99.69108	Thailand	Dan Sing Khon	4
81	Prachuap Khiri Khan	11.29382	99.37646	Thailand	Klong Loi	4
82	Kanchanaburi	14.5822	99.0501	Thailand	Tai Mhuang	1
83	Kanchanburi	14.5822	99.0501	Thailand	Pra Chum Mai	1
84	Chumphon	10.919176	99.221389	Thailand	Ran Tad Phom	4
85	Chumphon	10.841477	99.038575	Thailand	Santi Nimitr	4
86	Ranong	9.9666	98.7005	Thailand	Nai Krung	1
87	Ranong	10.59796	98.81012	Thailand	Had Tun	4
88	Chiang Rai	19.847971	99.707009	Thailand	Ban Mae Sad	4
89	Xinjiang	42.52464	87.53959	China	Unknown	1
90	Jiangsu	32.86843	120.3203	China	Dongtai	3
91	Fujian	24.445567	118.082658	China	The Wan Shi Botanical Gardens, Siming, Xiamen	4
92	Samut Prakan	13.5991	100.5998	Thailand	Unknown	1
93	Bangkok	13.75633	100.5018	Thailand	Unknown	1
94	Nonthaburi	13.8136	100.4227	Thailand	Amphoe Bang Kruai	2
95	Samar	12.24455	125.0388	Philippines	Unknown	1
96	Phit Sanulok	16.7512	100.3114	Thailand	31st Border Patrol Police Base	4
97	Nonthaburi	13.833333	100.516667	Thailand	Tambon Tha Khanun, Bangkruai	4
98	Shizuoka Prefecture	35.32186	138.8817	Japan	Takigahara Army Base, Gotemba	4
99	Pahang	3.170556	102.3192	Malaysia	Bukit Mendi	4
100	Jakarta	-6.13298	106.8267	Indonesia	Ancol	4
101	Guangdong	23.681774	113.056042	China	Qingyuan	2
102	Sonsorol	5.326812	132.2239	Palau	Unknown	1
103	Koror Island	7.341063	134.4772	Palau	Unknown	1
104	Fengping	23.584856	121.502581	Taiwan	Unknown	3

105	Fujian	25.461402	119.720433	China	Pingtan Island	4
106	Biak Island	-1.2	136.06	Indonesia	Sorido	4
107	Baik Island	-1.15	136.03	Indonesia	Insrom	4
108	Biak Island	-1.15	136.216	Indonesia	Adibat	4
109	Biak Island	-0.933	136	Indonesia	Sarwom	4
110	Biak Island	-0.9166	136.05	Indonesia	Korem	4
111	Owi Island	-1.25	136.21	Indonesia	Unknown	4
112	Unspecified	31.91622	59.62805	Iran	Multiple	0.5
113	Shizuoka	35.29409	138.8537	Japan	Mt. Fuji	2
114	Queensland	-16.926	145.728	Australia	Cairns	3
115	Queensland	-17.086	145.829	Australia	Unknown (Near Cairns)	2
116	Hanoi	20.99378	105.8467	Vietnam	Truong Dinh	4
117	Hanoi	21.0039	105.8403	Vietnam	Dong Da	4
118	Hanoi	21.02204	105.8486	Vietnam	Ha Dong	4
119	Hanoi	20.99087	105.865	Vietnam	Mai Dong	4
120	Hanoi	20.98361	105.8422	Vietnam	Giap Bat	4
121	Hanoi	21.01325	105.8657	Vietnam	Huu Nghi	4
122	Selangor	3.183288	101.600004	Malaysia	Bukit Lanjan	4
123	Selangor	2.9411111	101.5263889	Malaysia	Bukit Kemandul	4
124	Selangor	3.151666	101.909713	Malaysia	Ulu Lui	4
125	Selangor	3.363928	101.6028641	Malaysia	Serendah	4
126	Selangor	2.8469444	101.6725	Malaysia	Bukit Tampoi	4
127	Selangor	2.889606	101.744002	Malaysia	Bukit Tunggal	4
128	Selangor	2.904825	101.77317	Malaysia	Kampung Bahagia	4
129	Selangor	2.8091667	101.6266667	Malaysia	Bukit Tadom	4
130	Selangor	3.2106	101.5052	Malaysia	Elmina Estate	4
131	Selangor	3.0741786	101.5702813	Malaysia	Kuala lumpur metroplitan	4
132	Selangor	3.1724827	101.7084615	Malaysia	Jalan Fletcher	4
133	Selangor	3.1278871	101.5944885	Malaysia	Petaling Jaya	4

134	Selangor	3.1383679	101.7667557	Malaysia	Ampang New Village	4
135	Selangor	3.0886261	101.7082528	Malaysia	Salak South New Village	4
136	Selangor	3.1980496	101.5682343	Malaysia	Jinjang North New Village	4
137	Selangor	3.245244	101.658614	Malaysia	Selayang Baharu	4
138	Toyama	36.817727	137.59804	Japan	A (Kurobe River)	4
139	Toyama	36.837732	137.58565	Japan	B (Kurobe River)	4
140	Toyama	36.957075	137.540425	Japan	C (Kurobe River)	4
141	Toyama	36.894245	137.485187	Japan	D (Kurobe River)	4
142	Toyama	36.915438	137.423517	Japan	E (Kurobe River)	4
143	Toyama	36.863686	137.604588	Japan	F (Kurobe River)	4
144	Toyama	36.665615	137.198899	Japan	G (Zinzu River)	4
145	Toyama	36.649384	137.141583	Japan	H (Yamada River)	4
146	Toyama	36.781445	137.05695	Japan	I (Oyabe River)	4
147	Toyama	36.749331	137.110054	Japan	J (Gejo River)	4
148	Gifu	35.438655	137.044642	Japan	Kani City to Sakahogi (Kiso river)	3
149	Gifu	35.555756	137.044642	Japan	Kuze town (Ibi river)	4
150	Gyeonggi-do	35.555756	136.50708	South Korea	Chipo-ri	4
151	Gyeonggyi-do	38.249585	127.419701	South Korea	Kumhwa	4
152	Gyeonggyi-do	37.8321286	127.1389957	South Korea	Songu-ri	4
153	Gyeonggyi-do	38.181725	127.352273	South Korea	Monkey-ri	4
154	Gyeonggyi-do	38.09644381	127.0748335	South Korea	Yonchon	3
155	Gyeonggyi-do	38.098781	126.90699	South Korea	Ko-wang ni	4
156	Gyeonggyi-do	38.101428	126.984203	South Korea	Unspecified near 38th parallel	3
157	Pahang	4.443021	102.38481	Malaysia	Lata Berkoh, Taman Negara National Park	4
158	Suraburi	14.528915	100.910142	Thailand	Unknown	1
159	Chaiyaphum	15.806817	102.031503	Thailand	Unknown	1
160	Bangkok Metropolitan	13.7663606	100.548603	Thailand	Unknown	1
161	Chantaburi	12.6112485	102.103781	Thailand	Unknown	1

162	Assam	27.328385	95.835226	India	Stilwell Road, 9 sites between 4.6 and 22 miles	3
163	Kachin	25.442275	97.351685	Myanmar	Camp Landis	3
164	Kachin	25.394812	97.203095	Myanmar	Myitkyina, 15mi West	3
165	Manipur	24.663954	93.906346	India	Unknown	1
166	Uttarakhand	29.68167	79.85884	India	Kumaon	2
167	Jammu & Kashmir	34.391564	73.77279081	India	Teetwal	4
168	Shizuoka	35.32175	138.89711	Japan	Gotenba-Oyama district (11 sites rodents, 17 sites free-living chiggers)	1
169	Chiang Rai	20.2244	99.9481	Thailand	Ban Pa Gook (4 sites around village)	4
170	Chiang Rai	19.847971	99.707009	Thailand	Ban Mae Sad(4 sites around village)	4
171	Seoul	37.5333	126.9833	South Korea	Yongsan Garrison	4
172	Unknown	-2.5489	118.0149	Indonesia	Unknown	0
173	Unknown	4.4106	109.6181	Malaysia	Unknown	0
174	Gyeonggi-do	38.078055	127.07694	South Korea	Firing Point 10, Yeoncheon-gun	3
175	Akita	39.437034	140.49696	Japan	Omagari area	2
176	Hokkaido	43.0583491	141.4763946	Japan	Nopporo area	4
177	Akita	39.214785	140.52125	Japan	Jumonji area along Omono river	3
178	Selangor	2.94111111	101.5263889	Malaysia	Bukit Kemandul	4
179	Niigata	37.74453571	139.1825675	Japan	Gosen	3
180	Kanagawa	35.14417061	139.6207704	Japan	Miura	3
181	Shizuoka	35.3605555	138.7277777	Japan	Mt Fuji foothills	3
182	Shizuoka	34.65109151	138.8585273	Japan	Minami-Izu	4
183	Oita	33.303591	131.267837	Japan	Hijudai	3
184	Oita	33.5633274	131.7323015	Japan	Kunisaki	3
185	Mpumalanga	-24.65	31.333	South Africa	Bushbuckridge Municipal Area	3
186	Bengal	22.76742781	88.38834551	India	Barrackpore	4
187	Selangor	3.18908	101.5585	Malaysia	Oil Palm Estate	1
188	Chiang Rai	22.22448	99.94814	Thailand	Ban Pa Gook	4
189	Chiang Rai	20.2106498	99.930182	Thailand	Ban Nongkroung	4

190	Hua-lien	23.808491	121.4848195	Taiwan	Shou-feng & Fong-lin lowlands	2
191	Unknown	23.6958	121.094	Taiwan	9 sites around Taiwan	0
192	Yilan	24.56563799	121.6425298	Taiwan	Unknown	1
193	Hualien	23.74786975	121.3812831	Taiwan	Unknown	1
194	Lanyu Island	22.0435616	121.548418	Taiwan	Unknown	3
195	Taitung	22.85665323	121.0407154	Taiwan	Unknown	1
196	Taoyuan	24.900437	121.2627249	Taiwan	Unknown	1
197	Taichung	24.23354757	120.897715	Taiwan	Unknown	1
198	Kaoping	22.57786	120.453434	Taiwan	Unknown	1
199	Matsu	26.18463571	120.054454	Taiwan	Unknown	3
200	Kinmen	24.45180955	118.3680931	Taiwan	Unknown	3
201	Hualien	23.923	121.566	Taiwan	Ji-an & Shou-feng villages	2
202	Singapore	1.35688321	103.9721258	Singapore	Changi camp	2
203	Gyeonggi	37.234	127.707	South Korea	Songsan, Hwaseong	4
204	Gyeonggi	37.28768071	127.0180568	South Korea	Jangan, Hwaseong	4
205	Gyeonggi	37.29802371	127.6371628	South Korea	Yeoju	2
206	Gyeonggi	37.75986881	126.7801781	South Korea	Paju	2
207	Gangwon	38.1466091	127.3132256	South Korea	Cheorwon	1
208	Gangwon	37.88131531	127.7299707	South Korea	Chuncheon	1
209	Chungbuk	36.99101131	127.9259497	South Korea	Chungju	1
210	Jeollanam	35.20249471	127.4626534	South Korea	Gurye	1
211	Gyeongnam	35.5665758	128.1657995	South Korea	Hapcheon	1
212	Jeollanam-do	35.601917	127.502133	South Korea	Hwangjeon-myeon, Suncheon-si	3
213	Gaafu Dhaalu atoll	0.28999361	73.45706051	Maldives	Gadhdhoo island	3
214	Selangor	3.17137	101.61882	Malaysia	Village, Bukit Lanjan forest reserve	4
215	Selangor	3.17516	101.61638	Malaysia	Lalang, Bukit Lanjan forest reserve	4
216	Selangor	3.17381	101.61515	Malaysia	Scrub/edge, Bukit Lanjan forest reserve	4
217	Selangor	3.17162	101.61165	Malaysia	Forest, Bukit Lanjan forest reserve	4

218	Tainan	23.14866	120.33236	Taiwan	Unknown	1
219	Shandong	35.286128	118.184602	China	Feixian County, Fangcheng town, along Jun river	4
220	Queensland	-17.1816467	145.4551226	Australia	Rocky Creek	4
221	Queensland	-17.3632312	146.0183888	Australia	Bramston Beach	4
222	Queensland	-17.5226261	146.0285223	Australia	Innisfail	4
223	Queensland	-17.6074357	145.7812177	Australia	Palmerston	4
224	Queensland	-17.5144717	145.6044448	Australia	Millaa Millaa	4
225	Queensland	-17.609167	145.483611	Australia	Ravenshoe	4
226	Queensland	-18.1923688	145.5991846	Australia	Kirrama	4
227	Queensland	-18.6494046	146.1624851	Australia	Ingham	4
228	Queensland	-21.1424956	149.1821469	Australia	Mackay	4
229	Himachal Pradesh	30.900879	76.965108	India	Kasauli	4
230	Himachal Pradesh	30.907903	76.962862	India	Pasteur Institute Estate	4
231	Himachal Pradesh	30.902052	76.981129	India	Garkhal	4
232	Himachal Pradesh	39.976158	76.989798	India	Subathu	4
233	Guangdong	23.3306	116.55566	China	Shantou	2
234	Unknown	15.04277	100.77886	Thailand	18 Provinces	0
235	Gyeonggi	37.891975	126.722283	South Korea	North Carolina Range: Jangdan-myeon, Paju-si	4
236	Gyeonggi	37.9208389	126.745844	South Korea	Warrior Base: Gunnae-myeon, Paju-si	4
237	Gyeonggi	37.8956389	126.802391	South Korea	Monkey range: Jindong-myeon, Paju-si	4
238	Gyeonggi	37.954125	126.804755	South Korea	Story range: Jindong-myeon, Paju-si	4
239	Gyeonggi	37.967311	126.84083	South Korea	Dagmar North: Jeokseong-myeon, Paju-si	4
240	Gyeonggi	38.0487056	127.105049	South Korea	Firing Point 60: Yeoncheon-gun	4
241	Gyeonggi	38.0178583	126.719477	South Korea	Rodriguez Range: Youngjung-myeon, Pocheon-gun	4
242	Unknown	23.738737	120.948901	Taiwan	Unknown	0

243	Puducherry & Tamil Nadu	12.021736	79.6672	India	12 villages	3
244	Khasan	42.429882	130.677629	Russia	River valleys & lakes	2
245	Taitung	22.026205	121.55264	Taiwan	Iratai village, Lanyu Island	4
246	Taitung	22.050337	121.513253	Taiwan	Ya-yu village, Lanyu	4
247	Taitung	22.037456	121.566448	Taiwan	Ivarinu village, Lanyu	4
248	Taitung	22.027832	121.543047	Taiwan	Imurud village, Lanyu	4
249	Taitung	22.057958	121.564367	Taiwan	Iraramuruk village, Lanyu	4
250	Taitung	22.078773	121.527319	Taiwan	Iraralai village, Lanyu	4
251	Primorye	42.87204	131.365381	Russia	Khasan & Slavyan districts	1
252	Khatlon	37.470684	69.342326	Tajikistan	Parhar, Kyzylzu river bank	2
253	Khatlon	37.217971	69.106235	Tajikistan	Panj, beside Panj river	2
254	Vahdat	38.747004	69.300416	Tajikistan	Romit Reserve along Sardai Miyona river	2
255	Heilongjiang	47.05712	127.82671	China	Qing'an county	3
256	Fujian	24.4028	117.78984	China	Longhai county	3
257	Guangdong	23.23216	113.36582	China	Guangzhou suburbs	2
258	Hainan	16.329777	112.025511	China	Xisha Islands	3
259	Da Nang	16.124599	108.268078	Vietnam	Tien Sha peninsula	3
260	Quang Nam	15.755481	108.194261	Vietnam	Hill 244	2
261	Manus	-2.848308	146.231647	Papua New Guinea	South Bat Island	4
262	Zhejiang	29.17951	120.08884	China	Unknown	1
263	Yunnan	24.976	101.4894	China	Unknown	1
264	Tibet	29.320762	95.325017	China	Medog county	3
265	Hunan	28.60033	109.99545	China	Guzhang county	3
266	Maharashtra	21.145578	79.085529	India	Nagpur	3
267	Maharashtra	21.077943	78.967216	India	Raipur	4
268	Shandong	35.5933556	119.533363	China	Huangdao, Qingdao County	3
269	Shandong	35.361947	118.089868	China	4 villages in NE Fei County	3

270	Shandong	35.9424	117.912	China	4 counties in Shandong Province	2
271	Selangor	2.89678	101.729	Malaysia	Jendaram	4
272	Selangor	3.133659	101.55279	Malaysia	Subang	4
273	Shandong	37.16681	117.83393	China	Zouping County	3
274	Shandong	35.87939	117.92819	China	Fei & Zouping counties	2
275	Chiba	34.907436	139.89914	Japan	Shirahama	4
276	Tokyo	33.103021	139.80355	Japan	Hachijo Island	3
277	Niigata	37.446265	138.85127	Japan	Nagaoka	3
278	Kochi	33.72	133.55	Japan	Ida	1
279	Kochi	33.72	133.55	Japan	Okata	1
280	Hokkaido	43.221236	142.863405	Japan	Unknown	1
281	Aomori	40.79319	141.23618	Japan	Tohoku	4
282	Unknown	36.16211	139.6951	Japan	Kanto Region	0.5
283	Unknown	36.13862	137.82993	Japan	Chubu Region	0.5
284	Unknown	34.73723	135.59412	Japan	Kinki Region	0.5
285	Unknown	34.77413	132.86211	Japan	Chugoku Region	0.5
286	Unknown	33.752548	133.642631	Japan	Shikoku Island	0.5
287	Unknown	32.644429	130.955909	Japan	Kyushu Island	0.5
288	Kyoto	35.55	135.13	Japan	Nodagawa river	3
289	Sabah	6.049497	116.686206	Malaysia	Poring, Primary Forest	4
290	Sabah	6.049772	116.700785	Malaysia	Poring, Secondary Forest	4
291	Sabah	6.049378	116.703475	Malaysia	Poring, Edge Habitat	4
292	Selangor	3.250108	101.600025	Malaysia	Bukit Lagong	4
293	Selangor	3.354802	101.839087	Malaysia	Janda Baik	4
294	Anhui	33.03227	117.03227	China	Huaiyuan county	3
295	Anhui	32.78804	116.56753	China	Fengtai county	3
296	Anhui	30.59842	117.89465	China	Qingyang county	3
297	Oro	-9.1313	148.6919	Papua New Guinea	Buna	3

298	Torres Islands	-13.249749	166.616667	Vanuatu	Unknown	2
299	Banks Islands	-13.5257102	167.3266991	Vanuatu	Ureparapara	4
300	Banks Islands	-13.6794304	167.6707923	Vanuatu	Mota Lava	4
301	Banks Islands	-13.8443151	167.4472495	Vanuatu	Vureas Bay, Vanua Lava	4
302	Gaua	-14.28096	167.5160788	Vanuatu	Unknown	4
303	Espiritu Santo	-15.3003549	166.9182097	Vanuatu	Unknown	1
304	Guadalcanal	-9.577328399	160.1455805	Solomon Islands	Unknown	1
305	Makira	-10.5737447	161.8096941	Solomon Islands	Unknown	2
306	Ndende (Nendo Island)	-10.7685	165.82109	Solomon Islands	Head of Graciosa Bay	4
307	Ndende (Nendo Island)	-10.723837	165.798697	Solomon Islands	Government Station	4
308	Ndende (Nedno Island)	-10.798664	165.8395	Solomon Islands	Lueselemba Bay	4
309	Ndende (Nendo Island)	-10.723404	165.833585	Solomon Islands	East side of Graciosa Bay	4
310	Banks Islands	-13.832484	167.58326	Vanuatu	Grouped	3
311	Ndende (Nendo Island)	-10.722391	165.923375	Solomon Islands	Grouped	3
312	Delhi	28.484121	77.18785	India	Unknown	3
313	Unknown	36.206	138.253	Japan	Unknown	0
314	Mato Grosso do Sul	-18.9833	-56.65	Brazil	Nhumirim ranch, Pantal region	4
315	Maharashtra	18.456039	73.55018	India	6 sites	1
316	Maharashtra	18.371551	73.760739	India	Sinhgarh	3
317	Maharashtra	17.9278	73.6493	India	Mahabaleshwar	3
318	Oita	32.9666	131.4	Japan	Taketa	3
319	Oita	33.199	131.517	Japan	Hasama	3
320	Oita	32.233	131.606	Japan	Oita City	3

321	Fujian	26.64025	118.68218	China	Fu zhou, ping tan, putian and jian yang	1
322	Unknown	15.4992608	102.6419295	Thailand	Various sites	0
323	Da Nang	16.078238	108.071177	Vietnam	Unknown, Da Nang	1
324	Chittoor District	13.4566	79.00394	India	Chittoor district, Andhra Pradesh	2
325	North Andaman	13.264254	92.982727	India	Diglipur	4
326	South Andaman	11.7549735	92.655838	India	Port Blair	3
327	Gyeonggi Province	37.979839	126.851165	South Korea	Dagmar North Training Area	4
328	Chonnam Province	35.19316	126.829055	South Korea	Gwangsangu and Bukgu, Gwangju Metropolitan area	1
329	Seoul	37.461516	126.965171	South Korea	Mt Gwanak	4
330	Selangor	3.087569	101.614615	Malaysia	Sungei Way Rubber plantation	3
331	Selangor	3.080095	101.56258	Malaysia	Subang at 7mile mark of Batu Tiga-Subang Road	3
332	Pampanga	15.1038	120.445	Philippines	SW of Clarke Air Base	2
333	East Java	-7.985764	112.594287	Indonesia	Mulyorejo, Malang	4
334	East Java	-7.999479	112.615125	Indonesia	Bandungrejosari, Malang	4
335	East Java	-7.974543	112.626762	Indonesia	Klojen, Malang	4
336	Chiang Mai	18.936643	98.82291	Thailand	Ban Ponyeang	4
337	Chonburi	13.244467	101.504483	Thailand	6 sites in Bothong district	2
338	Chonburi	13.18688	101.54355	Thailand	Ban Vangri	4
339	Chonburi	13.2323	101.456	Thailand	Ban Thaprang	4
340	Chonburi	13.23561	101.45922	Thailand	Ban Thapsung	4
341	Gifu	35.283101	136.547815	Japan	Yoro foothills	3
342	Phangnga	8.67104	98.42208	Thailand	Unknown	1
343	Cheju Island	33.480461	126.383598	South Korea	Kumdogri, Aiwol-ub	4
344	Cheju Island	33.487571	126.416373	South Korea	Tonggui- ri	4
345	Cheju Island	33.476973	126.551965	South Korea	Ara-i-dong	3
346	Cheju Island	33.447672	126.539559	South Korea	Odong-dong	3
347	Cheju Island	33.390445	126.485429	South Korea	Eorimog	4

348	Cheju Island	33.415582	126.433616	South Korea	Kosong-ri	4
349	Cheju Island	33.501547	126.497393	South Korea	Unknown	1
350	Unknown	4.04475	102.16336	Malaysia	Peninsular Malaysia, site unknown	0.5
351	Khon Kaen	16.21271	102.52007	Thailand	Mancha Khiri	4
352	Khon Kaen	16.61639	102.0962	Thailand	Chum Phae	4
353	Khon Kaen	16.71909	101.87154	Thailand	Phu Pha Man	4
354	Khon Kaen	16.20253	102.76643	Thailand	Ban Haet	4
355	Khon Kaen	15.92382	102.45806	Thailand	Waeng Yai	4
356	Khon Kaen	16.49189	102.61814	Thailand	Ban Fang	4
357	Goyang-gun	37.6805	126.8391	South Korea	Dorai 5-ri	2
358	Goyang-gun	37.67037	126.841525	South Korea	Goyang-ri	4
359	Goyang-gun	37.649568	126.870506	South Korea	Wonhung 2-ri	4
360	Goyang-gun	37.62775	126.839046	South Korea	Haingsin 1-ri	4
361	Paju-gun	37.779311	126.85017	South Korea	Kwangtan 3-ri	4
362	Gyonggi-do	37.50935	126.76656	South Korea	Bucheon-si	4
363	Yesan-gun	36.701752	126.6699	South Korea	Deogsan-ri	4
364	Cheongwon-gun	36.489743	127.411	South Korea	Simog-ri	4
365	Chollanam-do	34.99721	127.04353	South Korea	Hwasun	2
366	Chollanam-do	35.205265	126.713759	South Korea	Won-dong, Kwangju	3
367	Chollanam-do	35.190435	126.910139	South Korea	Wuchi-dong, Kwangju	3
368	Chollanam-do	35.205265	126.713759	South Korea	Bia-dong, Kwangju	3
369	Chungchongnam-do	36.611094	129.298	South Korea	Yongi	2
370	Chungchongnam-do	36.4645	127.1212	South Korea	Kongju	1
371	Chungchongnam-do	36.396788	127.347911	South Korea	Tan-dong, Taejon	3
372	Chon-nam	35.173231	126.859211	South Korea	Kwangju	2
373	Chon-nam	34.6725	126.92524	South Korea	Changhung	2
374	Chon-nam	34.772462	127.662137	South Korea	Yocheon	2

375	Chon-bug	35.959727	126.995427	South Korea	Iksan	2
376	Chon-bug	36.02865	126.92917	South Korea	Iri	2
377	Kyong-nam	34.85083	128.428692	South Korea	Tongyong	2
378	Kyong-bug	36.453657	129.37976	South Korea	Yongdeok	2
379	Kangwon	37.927158	127.741511	South Korea	Chunseong	2
380	Kangwon	37.448628	129.164628	South Korea	Samcheok	2
381	Kangwon	37.536359	129.114714	South Korea	Tonghae	2
382	Kyonggi	37.682933	126.839626	South Korea	Koyang	2
383	Niigata	37.9153	139.1468	Japan	Kawamura Nakasu	4
384	South	35.213654	128.581433	South Korea	Masan	2
385	Gyonggi-do	37.905642	127.028694	South Korea	Jeonggam-dong, Dongducheon-si	3
386	Gyonggi-do	37.50931	126.766866	South Korea	Bugog-dong, Bucheon-si	3
387	Kyonggi	37.912304	127.206533	South Korea	Pochon	3
388	Kyonggi	37.012596	127.114585	South Korea	Pyongtaek	2
389	Kyonggi	37.806544	127.048385	South Korea	Yangju	2
390	Kyonggi	37.880701	127.206048	South Korea	Yongjongdo	3
391	Kyong-buk	35.903954	129.226886	South Korea	Kyongju	2
392	Kyong-buk	36.682275	128.870409	South Korea	Kumrung	1
393	Kyong-buk	36.174892	128.111518	South Korea	Kimchon	2
394	Kyong-buk	36.454851	128.171682	South Korea	Sangju	2
395	Kyong-buk	37.023292	129.388257	South Korea	Uljin	3
396	Kyong-buk	36.673097	128.458475	South Korea	Yechon	3
397	Kyong-buk	36.613559	128.193713	South Korea	Munkyong	2
398	Kyong-buk	36.618027	128.737959	South Korea	Andong	3
399	Kyong-nam	35.560394	128.502171	South Korea	Changnyong	3
400	Kangwon	38.668636	128.318547	South Korea	Kosong	4
401	Kangwon	38.454237	128.454594	South Korea	Keojin	4
402	Choong-nam	36.211354	127.102691	South Korea	Nonsan	2
403	Choong-nam	36.92053	126.633677	South Korea	Dangjin	2

404	Choong-nam	36.608839	126.663444	South Korea	Hongseong	3
405	Choong-buk	36.492037	126.553709	South Korea	Cheongwon	3
406	Chollanam-do	35.26867	127.02371	South Korea	Damyang	2
407	Chon-nam	34.641702	126.773775	South Korea	Kangjin	2
408	Cheju	33.462337	126.327607	South Korea	Aewol	3
409	Cheju	33.475995	126.54499	South Korea	Ara	3
410	Niigata	38.112	138.393	Japan	Sado Island, Umezu district	2
411	Chon-buk	35.936666	127.257499	South Korea	Wanju	2
412	Kyongsangbuk-do	36.296655	128.097201	South Korea	Cho-o 1, Sangju-si	4
413	Kyongsangbuk-do	36.305582	128.097692	South Korea	Cho-o 2, Shangju-si	4
414	Kyongsangbuk-do	35.952037	128.015493	South Korea	Yobae, Kumrung-gun	4
415	Kyongsangbuk-do	36.024429	128.045654	South Korea	Mipyong, Kumrung-gun	4
416	Kyongsangbuk-do	36.112158	127.994602	South Korea	Unsu, Kimchon-si	4
417	Kyongsangbuk-do	36.548172	128.729605	South Korea	Jeokha, Andong-si	4
418	Kyongsangbuk-do	36.518598	128.86518	South Korea	Imha, Andong-si	4
419	Kyongsangbuk-do	36.738957	128.085797	South Korea	Jinan, Munkyong-si	4
420	Kyongsangbuk-do	36.73354	128.1557	South Korea	Koyo, Munkyong-si	4
421	Chungchongbuk-do	36.948664	127.910086	South Korea	Tanwol, Chungju-si	4
422	Chungju-si	36.912518	127.961803	South Korea	Sesong, Chungju-si	4
423	Kyongsangbuk-do	36.617717	128.500393	South Korea	Jiksan, Yechon-gun	4
424	Unknown	12.889	121.77	Philippines	Unknown Cameron	0
425	Pahang	4.502	101.39	Malaysia	Highlands Johor	2
426	Johor	1.668	103.785	Malaysia	several sites	1
427	Negri Sembilan	2.516	102.239	Malaysia	Unknown	1
428	Selangor	3.264	101.588	Malaysia	6 sites	1
429	Nakhon Ratchasima	14.95728	102.11057	Thailand	Unknown	1
430	Ubon Ratchathani	15.18319	105.11287	Thailand	Unknown	1
431	Sialkot	32.497222	74.536111	Pakistan	Sialkot	2

432	Sakhalin Oblast	49.94017	143.6239	Russia	Sakhalin & Shikotan (Kuril Islds.)	1
433	Maharashtra	19.12752	72.8811	India	Bombay City	3
434	Sakhalin Oblast	43.7948	146.747	Russia	Shikotan Island	3
435	Sakhalin Oblast	44.14993	145.875	Russia	Kunashir Island	3
436	Choallanam-do	35.349	126.403	South Korea	Yongkwang	2
437	Chollanam-do	35.295	127.294	South Korea	Koksung	2
438	Queensland	-17.4151	145.4365	South Korea	Wondecla	4
439	Chollanam-do	34.80974	127.18264	South Korea	Posong	2
440	Chollanam-do	35.10168	126.54436	South Korea	Hampyong	2
441	Hong Kong	22.322421	114.13531	Hong Kong	Stonecutters Island	3
442	Osaka	34.717239	135.505627	Japan	Osaka City	2
443	Kerala	8.662156	76.80928	India	Khizuvalam	4
444	Kerala	8.67988	76.82401	India	Mamom	4
445	Kerala	8.659932	76.8217	India	Kochalummoodu	4
446	Kerala	8.49389	76.95364	India	Rajaji Nagar	4
447	Chollanam-do	35.0033	127.3139	South Korea	Cheogye	3
448	Gyeongnam-do	35.4541	128.5805	South Korea	Gangjeon	2
449	Chon-nam	35.2001	126.5018	South Korea	Yongwang (Chonbul)	3
450	Chon-nam	35.2178	126.3232	South Korea	Yongkwang (Wolya)	3
451	Chon-nam	35.264264	126.476361	South Korea	Yongkwang (Gunso)	3
452	Gyenoggi	37.8353	126.8894	South Korea	North, Twin Bridges Training Area, Paju county	3
453	Gyeonggi	37.8101	126.8793	South Korea	Twin Bridges Training Area, South, Paju county	3
454	Xinjiang	45.673	84.582	China	Altai & Yili areas	1
455	Shandong	35.74572	117.9245	China	Feixian, Junan & Jinan counties	2
456	Yamagata Prefecture	38.308987	140.14949	Japan	Asahi-Machi	4
457	Saitama	35.9846	139.0898	Japan	Hitsujiyama Park	4
458	Kagoshima	31.583	130.466	Japan	Uwatoko, Matsumoto Town	4

459	Kagoshima	31.7	130.616	Japan	Kamihazeyama, Aira Town	4
460	Kagoshima	31.5	130.433	Japan	Imakoba, Fukiage Town	4
461	Buri Ram	15.1024	103.097	Thailand	Unknown	1
462	Chachoengsao	13.786	101.092	Thailand	Unknown	1
463	Chiang Rai	20.25	99.9386	Thailand	Changchawa sub-district, Mae Chan district	3
464	Phitsanulok	16.7597	100.2713	Thailand	Ekatosort Army Base	4
465	Phitsanulok	16.7554	100.2913	Thailand	Combined data, Ekatorot & 31st Border police bases	3
466	Aichi Prefecture	35.2275	137.3027	Japan	Okura area, Obara Village	4
467	Aichi Prefecture	35.22519	137.3285	Japan	Okura and Hirahata area, Obara Village	3
468	Saitama Prefecture	36.1894	139.5183	Japan	Site number 1	4
469	Saitama Prefecture	35.8731	139.5749	Japan	Site number 2	4
470	Saitama Prefecture	36.0594	139.4739	Japan	Site number 3	4
471	Saitama Prefecture	35.9618	138.9787	Japan	Site number 4	4
472	Saitama Prefecture	35.9713	139.5303	Japan	Site number 5	4
473	Lan-Yu Island	22.025421	121.539354	Taiwan	Hungtou	4
474	Lan-Yu island	22.07883	121.529329	Taiwan	Langtao	4
475	Lan-Yu Island	22.037774	121.566534	Taiwan	Yehin	4
476	Penghu Island group	23.606179	119.513551	Taiwan	Hsi-Yu, Yuweng island	4
477	Taitung Prefecture	23.157553	121.366029	Taiwan	Chengkung	3
478	Sarawak	2.529346	112.328754	Malaysia	Selangau Health Center	4
479	Sarawak	2.53254	112.324787	Malaysia	Selangau	4
480	Muang Lampaya	13.81215	100.024784	Thailand	Nakhon Pathom	4
481	Selangor	3.29794	101.77925	Malaysia	Ulu Gombak Forest Reserve	4
482	West Bengal	26.8309	88.2353	India	3 villages in Kurseong subdistrict	3
483	Chieng Mai	18.803763	98.996269	Thailand	Muang	4
484	Chieng Mai	18.874536	99.048919	Thailand	Sansai	4

485	Chieng Mai	18.73896	99.17482	Thailand	Sankumpang	4
486	Chieng Mai	18.948755	98.885656	Thailand	Maerim	4
487	Chieng Mai	18.720558	99.037689	Thailand	Sarapee	4
488	Rajburi and Nakorn- pathom provinces	13.64855	99.73485	Thailand	Unknown	0.5
489	Perak	4.22829	100.5578	Malaysia	Pangkor Island	3
490	Punjab	32.48218	74.85751	Pakistan	Charwa, Sialkot	4
491	Punjab	31.20649	73.83183	Pakistan	Lahore area	2
492	Punjab	30.183419	71.527876	Pakistan	Multan, lower Indus	2
493	Khyber Pakhtnkhwa	34.14434	73.23312	Pakistan	Abbottabad	2
494	Khyber Pakhtunkhwa	33.91301	73.400014	Pakistan	Murree foothills	3
495	Gilgit-Baltistan	34.907035	73.654373	Pakistan	Kaghan valley forest & glacial till	3
496	Gilgit-Baltistan	34.94003	73.7932	Pakistan	Kaghan valley alpine meadow	3
497	Gilgit-Baltistan	35.653121	74.279269	Pakistan	Upper Indus	3
498	Gilgit-Baltistan	36.1974	73.18514	Pakistan	Gilgit Agency mountain desert	3
499	Gilgit-Baltistan	36.16775	74.17751	Pakistan	Nalta, isolated coniferous forest	3
500	Chiba	35.1349	139.8657	Japan	Awa district	3
501	Tokyo	35.55511	139.4428	Japan	Machida district	3
502	Tokyo	35.7408	139.3278	Japan	Fussa district	3
503	Tokyo	35.72903	139.2223	Japan	Itsukaichi district	3
504	Toyama	36.57051	137.12244	Japan	mountain village	1
505	Yamagata	38.19171	140.10008	Japan	Shirataka town	3
506	Yamagata	38.8062	140.2228	Japan	Sakegawa	3
507	Yamagata	38.75745	140.1464	Japan	Tozawa	3
508	Yamagata	38.75526	139.8305	Japan	Tsuruoka	3
509	Yamagata	38.29519	140.2627	Japan	Yamanobe	3
510	Kyoto Prefecture	35.566924	135.152635	Japan	Iwataki	4

511	Niigata Prefecture	37.820019	139.115024	Japan	Kawaguchi	4
512	Kraskino	42.71347	130.7845	Russia	Field	3
513	Taumi	42.5566	130.6947	Russia	Lake side	3
514	Hasan	42.4419	130.6446	Russia	Lake side	3
515	Fatashi	42.865	130.9196	Russia	River side	3
516	Fadeevka	43.0571	131.1918	Russia	Lake side	3
517	Novo Georgievka	44.0316	131.4012	Russia	River side	3
518	Cherniytino	43.5532	131.86248	Russia	River side	3
519	Karnataka	13.137758	78.133217	India	Kolar, 13 sites in & around	2
520	Chonburi	13.27715	101.47932	Thailand	Bo Thong & Si Racha districts	1
521	Central Luzon	15.15	120.45	Philippines	Unknown	1
522	Keelung	25.1457056	121.7904019	Taiwan	Badouzih Fishing Harbor	4
523	Keelung	25.1388806	121.7508906	Taiwan	Keelung Harbor	4
524	Taoyuan	25.0796514	121.234217	Taiwan	Taoyuan Airport	4
525	Taoyuan	24.988267	121.014516	Taiwan	Yongan Fishing Harbor	4
526	Hsinchu	24.9271532	120.9713198	Taiwan	Potou Fishing Harbor	4
527	Hsinchu	24.83833	120.92	Taiwan	Nangliao Fishing Harbor	4
528	Hsinchu	24.849406	120.918782	Taiwan	Hsinchu Fishing Harbor	4
529	Hsinchu	24.7652733	120.903358	Taiwan	Haishan Fishing Harbor	4
530	Yunlin	24.941328	121.901466	Taiwan	Dashi Fishing Harbor	4
531	Yunlin	24.597159	121.857558	Taiwan	Suao Harbor	4
532	Taichung	24.261151	120.522772	Taiwan	Taichung Wuchi Harbor	4
533	Yunlin	23.800252	120.177637	Taiwan	Mailiao Harbor	4
534	Tainan	22.9968813	120.155553	Taiwan	Anping Fishing Harbor	4
535	Tainan	22.98133	120.222423	Taiwan	East District, Tainan city	4
536	Kaohsiung	22.867754	120.193199	Taiwan	Hsinda Fishing Harbor	4
537	Kaohsiung	22.814995	120.209705	Taiwan	Hsingang Fishing Harbor	4
538	Kaohsiung	22.762183	120.236169	Taiwan	Mito Fishing Harbor	4
539	Kaohsiung	22.727105	120.254688	Taiwan	Uhliao Fishing Harbor	4

540	Kaohsiung	22.699588	120.272685	Taiwan	Zhoying Military Area	4
541	Kaohsiung	22.5970794	120.314721	Taiwan	Qianzhen District, Kaohsiung City	4
542	Kaohsiung	22.6483444	120.326254	Taiwan	Sanmin District, Kaohsiung City	4
543	Kaohsiung	22.7823697	120.378138	Taiwan	Yanchao District, Kaohsiung City	4
544	Kaohsiung	22.5991796	120.288768	Taiwan	Kaohsiung International Harbor	4
545	Kaohsiung	22.4832774	120.39975	Taiwan	Chungyun Fishing Harbor	4
546	Kaohsiung	22.574629	120.344807	Taiwan	Kaohsiung International Airport	4
547	Pingtung	22.4715189	120.436001	Taiwan	Yanpu Fishing Harbor	4
548	Kaohsiung	22.4793396	120.39773	Taiwan	Linyuan Fishing Harbor	4
549	Pingtung	22.464693	120.443651	Taiwan	Donggang Fishing Harbor	4
550	Pingtung	22.373024	120.584896	Taiwan	Shueidiliao Fishing Harbor	4
551	Pingtung	22.3630694	120.59266	Taiwan	Fangliao Fishing Harbor	4
552	Hualien	24.294636	121.754889	Taiwan	Hoping Fishing Harbor	4
553	Hualien	23.979627	121.624168	Taiwan	Hualien Harbor	4
554	Taitung	22.7916741	121.192057	Taiwan	Fugang Fishing Harbor	4
555	Shandong	35.25421	118.3479	China	Linyi	2
556	Shandong	36.25936	117.10212	China	Tai'an	2
557	Shandong	36.1032	117.4163	China	Xintai & Daiyue, Tai'an	2
558	Gifu	35.4146	137.0237	Japan	Kani river	2
559	Gifu	35.428563	137.00241	Japan	Kiso river	2
560	Shandong	35.9237	117.7644	China	Xintai, Daiyue & Yinan	2
561	Shandong	35.8386	118.319	China	6 districts	1
562	Gifu	35.473	137.412	Japan	Ena	2
563	Gifu	36.1484	137.2525	Japan	Takayama	2
564	Okayama	35.0829	133.692	Japan	Katsuyama	3
565	Okayama	34.9408	133.9614	Japan	Kumenan	3
566	Okayama	34.69	133.748	Japan	Souja	3
567	Okayama	34.8676	134.0989	Japan	Wake	3
568	Tokyo	34.0916	139.5248	Japan	Miyake Island, Izu Shichito	4

569	Saga	33.32585	130.2863	Japan	Saga	2
570	Saga	33.3666	130.3535	Japan	Kanzaki	2
571	Saga	33.3579	130.4193	Japan	Kamimine	3
572	Saga	33.429	130.5123	Japan	Kiyama	3
573	Okayama	34.8814	133.8122	Japan	Kamogawa	3
574	Akita	39.4028	140.4719	Japan	Onakajima	4
575	Akita	39.5092	140.4947	Japan	Nagatoro	4
576	Aomori	40.8677	141.129	Japan	Noheji	4
577	Aomori	40.743	141.265	Japan	Tohoku	4
578	Aomori	40.389	141.262	Japan	Sannohe	4
579	Aomori	41.152	140.825	Japan	Wakinosawa	4
580	Aomori	40.7101	140.578	Japan	Namioka	4
581	Aomori	40.6639	140.504	Japan	Fujisaki	4
582	Aomori	40.635	140.55	Japan	Inakadate	4
583	Aomori	40.611	140.465	Japan	Hirosaki	4
584	Aomori	40.4808	140.6214	Japan	Ikarigaseki	4
585	Aomori	40.585	139.925	Japan	Iwasaki	4
586	Fukui	36.066	136.5	Japan	Nomuki, Katsuyama	3
587	Ishikawa	36.593	136.583	Japan	Kenmin Park, Kanazawa	4
588	Gunma	36.410197	139.058349	Japan	Miyagi	3
589	Gunma	36.2611	139.31054	Japan	Kasukawa	3
590	Gunma	36.49336	139.00512	Japan	Fujimi	3
591	Gunma	36.47067	138.84795	Japan	Haruna	3
592	Gunma	36.404404	138.951319	Japan	Misato	3
593	Gunma	36.43195	138.783251	Japan	Kurabuchi	3
594	Gunma	36.40549	139.33069	Japan	Kiryu	3
595	Gunma	36.43109	139.27391	Japan	Omama	3
596	Gunma	36.52145	139.238186	Japan	Kurohone	3
597	Gunma	36.4224	139.278	Japan	Seta Azuma	3

598	Mandalay	21.966	96.087	Myanmar	Mandalay	2
599	Manipur	24.452	94.026	India	Palel and surroundings	3
600	Bago	18.497	95.506	Myanmar	Paungde	4
601	Sagaing	23.207	94.298	Myanmar	Kalewa	4
602	Sagaing	24.226	94.311	Myanmar	Tamu	4
603	Uttarakhand	29.592	79.646	India	Almora	2
604	Manipur	24.97	93.88	India	Kanglatongbi	4
605	Manipur	25.092	94.36	India	Ukhrul Road	3
606	Manipur	24.83	93.94	India	Imphal	2
607	Addu Atoll	-0.6958	73.156	Maldives	Gan Island	4
608	Uttarakhand	29.348	79.551	India	Bhimtal	4
609	Uttarakhand	29.322	79.581	India	Naukuchiatal	4
610	Uttarakhand	29.349	79.531	India	Sattal	4
611	Gunma	36.4186	139.23771	Japan	Niisato	4
612	Gunma	36.30443	139.11491	Japan	Tamamura	4
613	Gunma	36.29112	139.37547	Japan	Ota	4
614	Gunma	36.35882	139.31564	Japan	Yabuzuka honmachi	4
615	Gunma	36.48947	139.00045	Japan	Shibukawa	4
616	Gunma	36.4475	139.00977	Japan	Yoshioka	4
617	Gunma	36.55	139.18333	Japan	Akagi	4
618	Gunma	36.55251	138.92216	Japan	Onogami	4
619	Gunma	36.37063	138.66945	Japan	Komochi	4
620	Gunma	36.4775	139.0443	Japan	Kitatachibana	4
621	Kagoshima	31.586	130.989	Japan	Ohsumi	4
622	Gunma	36.25848	139.07444	Japan	Fujioka	4
623	Gunma	36.23507	138.97648	Japan	Yoshii	4
624	Gunma	36.29286	138.79836	Japan	Nakazato	4
625	Gunma	36.08314	138.77738	Japan	Ueno	4
626	Gunma	36.25984	138.88957	Japan	Tomioka	4

627	Gunma	36.30075	138.7404	Japan	Myougi	4
628	Gunma	36.1585477	138.7114062	Japan	Nanmoku	4
629	Gunma	36.2429538	138.9216824	Japan	Kanra	4
630	Gunma	36.3263676	138.8873239	Japan	Annaka	4
631	Gunma	36.3131395	138.8020768	Japan	Matsuida	4
632	Gunma	36.5898925	138.8409863	Japan	Nakanojo	4
633	Gunma	36.6208865	138.9434494	Japan	Takayama	4
634	Gunma	36.5314431	138.6388879	Japan	Agatsuma	4
635	Gunma	36.5709	138.8255	Japan	Agatsuma Azuma	4
636	Gunma	36.6460769	139.0441608	Japan	Numata	4
637	Gunma	36.7862665	139.1625989	Japan	Tone	4
638	Gunma	36.7724689	139.2252431	Japan	Katashina	4
639	Gunma	36.6822686	138.9928865	Japan	Tsukiyono	4
640	Gunma	36.695	138.914	Japan	Niiharu	4
641	Gunma	36.6946691	139.1065343	Japan	Kawaba	4
642	Gunma	36.664609	139.128654	Japan	Shirasawa	4
643	Gunma	36.2448485	139.5419678	Japan	Tatebayashi	4
644	West Java	-7.049	106.576	Indonesia	Loji	4
645	West Java	-6.79	106.576	Indonesia	Cihamerang	4
646	West Sumatra	-0.98	100.36	Indonesia	Air Manis	4
647	West Sumatra	-0.202	100.341	Indonesia	Koto Rantang	4
648	North Sulawesi	1.447	124.813	Indonesia	Malalayang	4
649	North Sulawesi	1.368	124.832	Indonesia	Kinilow	4
650	East Kalimantan	-1.203	116.965	Indonesia	Manggar Baru	4
651	East Kalimantan	-1.023	116.865	Indonesia	Bukit Bangkirai	4
652	Shandong	35.757	117.946	China	Mengyin County	3
653	Gifu	35.492	136.728	Japan	Unknown	1
654	Kalimantan	-1.113	116.915	Indonesia	East Kalimantan (2 sites)	1
655	Gwangju	35.19318	126.8291	South Korea	2 sites	1

656	Banten	-6.233	106.5321	Indonesia	Duku	2
657	Jakarta	-6.1564	106.8612	Indonesia	Jakarta	1
658	Riau	0.772	101.796	Indonesia	Unknown	1
659	Hualien	24.05	121.58	Taiwan	Fallow land	1
660	Hualien	24.1	121.57	Taiwan	Ploughed land	1
661	Gyeonggyi-do	37.0249	127.2845	South Korea	Anseong	2
662	Gyeonggyi-do	37.7469	127.0329	South Korea	Uijongbu	2
663	Gangwon	37.4161	128.1533	South Korea	Anheung-myeon, Pyeongchang	3
664	Gyeonggyi-do	36.9508	127.0432	South Korea	Songhwa-ri, Pyeongtaek	4
665	Gyeonggyi-do	38.0902	127.274	South Korea	Uncheon, Pocheon	3
666	Gyeonggyi-do	37.0331	127.5742	South Korea	Neungseo-myeon, Yeoju	3
667	Gangwon	37.9385	127.7465	South Korea	Sinbuk-eup, Chuncheon	3
668	Chungbuk	36.4894	127.718	South Korea	Boeun-eup	3
669	Chungnam	36.8417	127.1905	South Korea	Anseo-ri, Cheonan	4
670	Kyongbuk	35.9172	128.2267	South Korea	Daega myeon, Seongju-gun	3
671	Kyongnam	35.298	128.407	South Korea	Haman	2
672	Chon-buk	35.8649	127.1659	South Korea	Wongeuk, Wangju	3
673	Chungnam	36.7117	126.8496	South Korea	Yesan	3
674	Gyeonggi-do	37.74	126.45	South Korea	Gangwha	3
675	Gyeonggi-do	38.1206	127.0813	South Korea	Yeoncheon	2
676	Gyeonggi-do	37.338	126.8353	South Korea	Ansan	3
677	Chungnam	36.8256	127.0078	South Korea	Asan-si	2
678	Gyeongbuk	36.9387	128.7349	South Korea	Bongwha-gun	2
679	Gyeonggi-do	37.6311	126.9087	South Korea	Ungbong Mt.	3
680	Gyeonggi-do	37.8835	127.6087	South Korea	Kekwan Mt.	3
681	Gyeonggi-do	37.9406	127.6122	South Korea	Gaduk Mt.	3
682	Gyeonggi-do	38.107	127.337	South Korea	Mungsung Mt.	3
683	Gangwon	37.995	127.503	South Korea	Whaak Mt.	3
684	Gangwon	37.805	127.598	South Korea	Jumbong Mt.	3

685	Gangwon	37.866	127.979	South Korea	Gali Mt.	3
686	Gangwon	37.728	128.465	South Korea	Gyebang Mt.	3
687	Gangwon	37.732	128.593	South Korea	Odae Mt.	3
688	Gangwon	38.053	128.256	South Korea	Hansok Mt.	3
689	Gangwon	37.879	128.458	South Korea	Gachil Mt.	3
690	Gangwon	37.835	127.621	South Korea	Samak Mt.	3
691	Chungnam	36.714	126.631	South Korea	Kaya Mt.	3
692	Chungnam	36.673	127.037	South Korea	Kwangduk Mt.	3
693	Chungnam	36.357	127.232	South Korea	Gyeryong Mt.	3
694	Gyeongbuk	36.813	128.044	South Korea	Choryong Mt.	3
695	Gyeongnam	35.337	127.73	South Korea	Jiri Mt.	3
696	Jeonbuk	35.816	128.567	South Korea	Taeduk Mt.	3
697	Jeonbuk	35.973	127.776	South Korea	Togyu Mt.	3
698	Chungnam	36.839	127.104	South Korea	Cheonan-si	2
699	Seoul	37.594	126.98	South Korea	Seoul	1
700	Gyeongnam	35.522	128.746	South Korea	Miryang-si	2
701	Gyeongnam	35.047	128.068	South Korea	Uiryeong	2
702	Gyeongnam	34.994	128.32	South Korea	Goseong	2
703	Gyeongnam	35.554	127.729	South Korea	Hamyang	2
704	Gyeonggyi-do	38.096	127.019	South Korea	Gunnam-myeon, Yeoncheon	3
705	Gyeonggyi-do	37.971	126.92	South Korea	Chuksungmyeon	3
706	Gyeonggyi-do	38.083	127.281	South Korea	Wuncheonmyeon	3
707	Jeollabuk-do	35.98	126.72	South Korea	Kunsan city	2
708	Gyeongbuk	35.59	129.316	South Korea	Ulsan city	2
709	Gyeongnam	35.238	129.083	South Korea	Pusan city	2
710	Gangwon	38.207	128.586	South Korea	Sokcho city	2
711	Jeollanam-do	34.817	126.392	South Korea	Mokpo city	2
712	Jeollanam-do	34.792	127.677	South Korea	Yeosu city	2
713	Gyeonggyi-do	38.01	127.3	South Korea	US Army camp	2

714	Gyeonggyi-do	38.03	127.5	South Korea	ROC Army camp	2
715	Gyeonggyi-do	38.078	127.53	South Korea	Sachang-ri	4
716	Gyeonggyi-do	37.6	127.5	South Korea	Unknown	1
717	Gangwon	38.05	128.2	South Korea	Unknown	1
718	Jeolla	35.3	127	South Korea	Unknown	1
719	Gyeongsang	35.6	128.2	South Korea	Unknown	1
720	Chungcheongbuk-do	36.9	127.7	South Korea	Unknown	1
721	Chungcheongnam-do	36.7	126.8	South Korea	Unknown	1
722	Gyeongsangbuk-do	36.78	128.9	South Korea	Unknown	1
723	jeju	33.4	126.5	South Korea	Unknown	1
724	Gyeonggyi-do	37.872	127.522	South Korea	Gapyeong-gun	2
725	Gangwon	38.112	128.16	South Korea	Inje-gun	2
726	Gangwon	37.768	127.891	South Korea	Hongcheon-gun	2
727	Gangwon	38.129	127.715	South Korea	Hwacheon-gun	2
728	Primorye	46.7608	143.3334	Russia	Lake Tunaicha	3
729	Primorye	46.4586	143.4247	Russia	Igrivaya river	3
730	Primorye	46.4679	142.3293	Russia	Uryum river	3
731	Primorye	46.5624	143.0291	Russia	3 sites combined	1
732	Primorye	42.459	130.655	Russia	Lake Doretsni	3
733	Primorye	42.429	130.633	Russia	Tumyn'tszyan river	3
734	Primorye	42.683	130.7596	Russia	Yanchikhe river	3
735	Primorye	42.685	130.663	Russia	Cherukhe river	3
736	Primorye	42.936	131.3749	Russia	Brus'ya river	3
737	Primorye	43.559	131.901	Russia	Suifun river Russkii	3
738	Primorye	43.0224	131.8601	Russia	island Vladivostok	3
739	Primorye	43.1265	131.9565	Russia	Khasan district	2
740	Primorye	42.9337	131.1612	Russia	Shkotovsk district	2
741	Primorye	43.3172	132.6183	Russia	Shkotovsk district	2

742	Primorye	43.2802	133.3041	Russia	Partizansk district	2
743	Primorye	43.1389	133.1247	Russia	Suchansk district	2
744	Primorye	43.4977	131.8417	Russia	Nadezhdinski district	2
745	Primorye	43.9805	133.14079	Russia	Anuchinski district	2
746	Primorye	44.3817	132.1783	Russia	Khorolski district	2
747	Primorye	44.3142	134.2674	Russia	Chuguevski district	2
748	Primorye	43.9725	133.0452	Russia	Ivanovski district	2
749	Primorye	46.6584	135.9187	Russia	Pozharski district	2
750	Primorye	43.105	131.928	Russia	Ob'yaseniya river	3
751	Primorye	43.0675	131.951	Russia	Patrokl bay	3
752	Primorye	43.188	132.105	Russia	Shamora bay	3
753	Primorye	42.965	131.7302	Russia	Popova island	3
754	Primorye	42.8774	131.659	Russia	Rikorda island	3
755	Primorye	43.2165	132.3772	Russia	Kangauz river	3
756	Primorye	42.9903	132.4065	Russia	Promyslovka town	3
757	Primorye	42.8617	132.4156	Russia	Putyatina island	3
758	Primorye	42.7557	132.3414	Russia	Askol'd island	3
759	Primorye	42.8559	133.0149	Russia	Suchan river, Suchanskii	3
760	Primorye	43.2802	133.3041	Russia	Suchan river, Partizanski	3
761	Primorye	44.5472	132.3785	Russia	Luzanova hill (sopka)	3
762	Primorye	42.7138	130.784	Russia	Kraskino	3
763	Primorye	43.8183	131.8421	Russia	Slavyanka river	3
764	Primorye	44.4088	131.3809	Russia	Pogranichny	3
765	Sakhalin Oblast	43.8138	146.7495	Russia	Krabozavodsk	3
766	Sakhalin Oblast	43.8676	146.8366	Russia	Bezymyannyi stream	3
767	Sakhalin Oblast	45.0709	147.8348	Russia	Iturup island	3
768	Primorye	42.5059	130.6987	Russia	Lake Tal'mi	3
769	Jiangsu	32.6	119.94	Russia	Taizhou	3
770	Chiloe	-41.89	-73.83	Chile	Ancud area	4

771	Chiloe	-42.49	-73.79	Chile	Castro Area	4
772	Gyeonggi-do	37.0403	126.8697	South Korea	Jangan-myeon, Hwaseong-si	4
773	Chungcheongnam-do	36.762	126.8678	South Korea	Seonjang-myeon, Asan-si	4
774	Jeollanam-do	35.3036	126.7858	South Korea	Bugil-myeon, Jangseong-gun	4
775	Khon Kaen	16.65	102.96	Thailand	Khon Kaen military base	3
776	Chaiyaphum	16.2	101.9	Thailand	Chaiyaphum military base	3
777	Buriram	14.3	102.8	Thailand	Buriram military base	3
778	Chantaburi	12.68	102.15	Thailand	Chantaburi military base	3
779	Rayong	12.85	101.1	Thailand	Rayong military base	3
780	Multi	16.2	102.2	Thailand	Chaiyaphum & Khon Kaen	0.5
781	Multi	14.6	101.9	Thailand	Chaiyaphum, Buriram, Chantaburi, Lopburi, Rayong, Chachoengsao	0.5
782	Multi	15.2	102.1	Thailand	Chaiyaphum, Chantaburi, Kohn Kaen, Rayong	0.5
783	Baringo	0.48	36.01	Kenya	Perkerra, Marigat	4
784	Uttar Pradesh	26.799	83.359	India	Gorakhpur, 12 villages	2
785	Tak	16.410034	98.749251	Thailand	Unknown	1
786	Loei	17.351113	101.15821	Thailand	Unknown	1
787	Sisaket	14.477182	104.489909	Thailand	Unknown	1
788	Chumphon	10.578439	99.212795	Thailand	Unknown	1
789	Phang Nga	8.664258	98.452195	Thailand	Unknown	1
790	Central	7.26	80.6	Sri Lanka	Rajawatta	4
791	Central	7.18	80.52	Sri Lanka	Thambavita	4
792	Central	7.32	80.5	Sri Lanka	Western Slopes	3
793	Southern	6.02	80.25	Sri Lanka	Unawatuna	4

§ 0 = unknown location in country, 0.5 = recognized subregion of a country, 1 to 4 following administrative levels listed in the International Organization for Standardization codes ISO 3166-1 and ISO 3166-2.

8.3 APPENDIX A-3

Complete list of tromiculid mites and other Acari reported to be positive for *O. tsutsugamushi*. Location, laboratory test used and reference provided.

SPECIES	COUNTRY	LOCATION	LATITUDE	LONGITUDE	DATE OF COLLECTION	LABORATORY TEST	1st AUTHOR	2nd AUTHOR	TITLE	JOURNAL	YEAR	NOTES
Actomatacrus sp.	China	Fujian	26.640	118.682	1953-54	Xenodiagnosis & serology	Yu, E.S.	Lin, S.H.	Study on the condition of natural infection with R. tsutsugamushi among mites and dometic animals in Fukien	Acta Microbiologica Sinica	1957	
Actomatacrus sp.	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Ascoschoengastia indica	Thailand	Nonthaburi	13.833	100.517	1992	Direct immunofluorescence	Frances, S.P.	Watcharapichat, P.	Occurrence of Orienta tsutsugamushi in Chiggers (Acari:Trombiculidae) and small animals in an Orchard near Bangkok, Thailand	J. Med. Entomol.	1999	
Ascoschoengastia indica	Malaysia	Selangor	3.298	101.779	1949	Xenodiagnosis	Traub, R	Frick, LP	Observations on the occurence of Rickettsia tsutsugamushi in rats and mites in the Malayan Jungle	Am. J. Hyg.	1950	
Ascoschoengastia indica	China	Fujian	26.640	118.682	1953-54	Xenodiagnosis & serology	Yu, E.S.	Lin, S.H.	Study on the condition of natural infection with R. tsutsugamushi among mites and dometic animals in Fukien	Acta Med. Biol. (Niigata)	1957	
Ascoschoengastia indica	Thailand	Nan	19.144	100.714	Nov-16	47 kDa RT PCR	This project					
Ascoschoengastia sp.	Thailand	Nonthaburi	13.833	100.517	1992	Direct immunofluorescence	Frances, S.P.	Watcharapichat, P.	Occurrence of Orienta tsutsugamushi in Chiggers (Acari:Trombiculidae) and small animals in an Orchard near Bangkok, Thailand	J. Med. Entomol.	1999	
Ascoschoengastia sp.	Thailand	Phitsanulok	16.751	100.311	1988-90	Direct immunofluorescence	Tanskul, P.	Strickman, D.	Rickettsia tsutsugamushi in chiggers (Acari: Trombiculidae) associated	J. Med. Entomol.	1994	

									with rodents in central Thailand			
Ascoschoengastia sp.	Thailand	Sisaket	14.477	104.490	2015	PCR (56kDa + 47kDa)	Takhampunya, R.	Korkusol, A.	Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand	Plos Negl. Trop. Dis.	2018	
Ascoschoengastia sp.	Thailand	Multiple sites	16.200	102.200	2017-18	PCR (56kDa + 47kDa)	Linsuwananon, P.	Krairojananan, P.	Surveillance for scrub typhus, Rickettsial diseases, and Leptospirosis in US and multinational military training exercise Cobra Gold Sites in Thailand	US Army Med. Dep. J.	2018	
Ascoschoengastia sp.	Thailand	Chiang Rai	20.256	99.652	Nov-16	47 kDa RT PCR	This project					
Blankaartia acuscutellaris	Thailand	Phitsanulok	16.751	100.311	1989-90	Direct immunofluorescence	Tanskul, P.	Strickman, D.	Rickettsia tsutsugamushi in chiggers (Acari: Trombiculidae) associated with rodents in central Thailand	J. Med. Entomol.	1994	
Blankaartia acuscutellaris	Thailand	Phitsanulok	16.751	100.311	1993	Xenodiagnosis & serology	Frances, S.P.	Watcharapichat, P.	Investigation of the role of Blankaartia acuscutellaris (Acari: Trombiculidae) as a vector of scrub typhus in central Thailand	J. Med. Entomol.	2001	
Blankaartia acuscutellaris	Thailand	Phitsanulok	16.760	100.271	1989-90	Direct immunofluorescence	Tanskul, P.	Strickman, D.	Rickettsia tsutsugamushi in chiggers (Acari: Trombiculidae) associated with rodents in central Thailand	J. Med. Entomol.	1994	
Blankaartia sp.	Thailand	Phang Nga	8.664	98.452	2015	PCR (56kDa + 47kDa)	Takhampunya, R.	Korkusol, A.	Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand	Plos Negl. Trop. Dis.	2018	
Blankaartia sp.	Thailand	Chumphon	10.578	99.213	2015	PCR (56kDa + 47kDa)	Takhampunya, R.	Korkusol, A.	Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from	Plos Negl. Trop. Dis.	2019	

									wild-caught small mammals in Thailand			
Cheladonta ikaoensis	Japan	Shizuoka	35.294	138.854	1974	Xenodiagnosis	Asanuma, K.	Kitaoka, M.	Leptotrombidium scutellare as a vector of scrub typhus at the endemic area of the foothills of Mt. Fuji, Japan	J. Hyg. Epidemiol. Microbiol. Immunol.	1974	
Cheladonta ikaoensis	Japan	Shizuoka	35.361	138.728	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Echinolaelaps echidninus	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Euschoengastia koreaensis	South Korea	Jeollanam-do	35.304	126.786	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Euschoengastia koreaensis	South Korea	Gangwon	38.147	127.313	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Euschoengastia miyagawai	Japan	Niigata	38.112	138.393	1957-58	Xenodiagnosis & microscopy	Saito, Y.	Otsuru, M.	Notes on Trombiculid mites collected in Sadi Island of Japan and isolation of rickettsia, with a description of Trombicula (Noetrombicula) sadoensis n. sp.	Acta Med. Biol. (Niigata)	1959	
Euschoengastia sp.	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Eutrombicula wichmanni	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living

Gahrliepia elbeli	Thailand	Nan	19.138	100.718	Nov-16	47 kDa RT PCR	This project					
Gahrliepia saduski	Japan	Oita	33.304	131.268	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Gahrliepia saduski	Japan	Yamagata	38.309	140.149	2012	PCR (56kDa)	Seto, J.	Suzuki, Y	Poposed vector candidate: Leptotrombidium palpale for Shikokoshi type Orientia tsutsugamushi	Microbiol. Immunol.	2013	
Gahrliepia saduski	Japan	Oita	32.233	131.606	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction	J. Med. Entomol.	2001	Free living
Gahrliepia sp.	Thailand	Phang Nga	8.664	98.452	2015	PCR (56kDa + 47kDa)	Takhampunya, R.	Korkusol, A.	Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand	Plos Negl. Trop. Dis.	2018	
Gahrliepia sp.	Thailand	Multiple sites	14.600	101.900	2017-18	PCR (56kDa + 47kDa)	Linsuwanonon, P.	Krairojananan, P.	Surveillance for scrub typhus, Rickettsial diseases, and Leptospirosis in US and multinational military training exercise Cobra Gold Sites in Thailand	US Army Med. Dep. J.	2018	
Gahrliepia sp.	Thailand	Chiang Rai	19.859	99.609	Apr-17	47 kDa RT PCR	This project					Free living
Gahrliepia xiaowoi	Thailand	Chiang Rai	19.859	99.609	Apr-17	47 kDa RT PCR	This project					
Haemaphysalis flava	Japan	Unknown	36.206	138.253	2014	PCR (56kDa)	Namikawa K.	Tanabe A.	Canine Orientia tsutsugamushi infection: report of a case and its epidemicity	Southeast Asian J. Trop. Med. Public Health	2014	
Haemaphysalis hystricis	Japan	Unknown	36.206	138.253	2014	PCR (56kDa)	Namikawa K.	Tanabe A.	Canine Orientia tsutsugamushi infection: report of a case and its	Southeast Asian J. Trop. Med. Public	2014	

									epidemicity	Health		
Haemaphysalis sp.	Japan	Unknown	36.206	138.253	2014	PCR (56kDa)	Namikawa K.	Tanabe A.	Canine Orientia tsutsugamushi infection: report of a case and its epidemicity	Southeast Asian J. Trop. Med. Public Health	2014	
Haemaphysalis sp.	India	Manipur	24.452	94.026	1945	Xenodiagnosis	Multiple		Scrub typhus investigations in South East Asia. A report on investigations on scrub typhus by the G.H.O. (India) Field Typhus Research Team, and the Medical Research Council Field Typhus Team, based on the Scrub Typhus Research Laboratory, South East Asia Command	Scrub Typhus Research Laboratory, South East Asia Command	1947	
Helenicula naresuani	Thailand	Nan	19.138	100.718	Nov-16	47 kDa RT PCR	This project					
Helenicula sp.	Thailand	Khon Kaen	16.650	102.960	2017-18	PCR (56kDa + 47kDa)	Linsuwananon, P.	Krairojananan, P.	Surveillance for scrub typhus, Rickettsial diseases, and Leptospirosis in US and multinational military training exercise Cobra Gold Sites in Thailand	US Army Med. Dep. J.	2018	
Ixodes sp.	China	Shandong	35.924	117.764	2010-12	PCR (56kDa)	Zhang, M.	Zhao, Z.T.	Molecular epidemiology of Orientia tsutsugamushi in chiggers and ticks from domestic rodents in Shandong, northern China	Parasit. Vectors	2013	
Laelaps turkestanicus	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Leptotrombidium akamushi	Solomon Islands	Ndende (Nendo Island)	-10.799	165.840	1975	Xenodiagnosis & microscopy	Miles, J.A.R.	Austin, F.J.	Scrub typhus in the Eastern Solomon Islands and Northern Vanuatu (New Hebrides)	Am. J. Trop. Med. Hyg.	1981	
Leptotrombidium akamushi	Solomon Islands	Ndende (Nendo Island)	-10.769	165.821	1975	Xenodiagnosis & microscopy	Miles, J.A.R.	Austin, F.J.	Scrub typhus in the Eastern Solomon Islands and Northern Vanuatu	Am. J. Trop. Med. Hyg.	1982	

									(New Hebrides)			
Leptotrombidium akamushi	Solomon Islands	Ndende (Nendo Island)	-10.724	165.799	1975	Xenodiagnosis & microscopy	Miles, J.A.R.	Austin, F.J.	Scrub typhus in the Eastern Solomon Islands and Northern Vanuatu (New Hebrides)	Am. J. Trop. Med. Hyg.	1983	
Leptotrombidium akamushi	Solomon Islands	Ndende (Nendo Island)	-10.723	165.834	1975	Xenodiagnosis & microscopy	Miles, J.A.R.	Austin, F.J.	Scrub typhus in the Eastern Solomon Islands and Northern Vanuatu (New Hebrides)	Am. J. Trop. Med. Hyg.	1984	
Leptotrombidium akamushi	Japan	Niigata	37.745	139.183	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium akamushi	Japan	Niigata	37.915	139.147	1966	Xenodiagnosis & microscopy	Saito, Y.		Parasitism of chiggers on the meadow mouse on the islet 'Kawamura nakasu' lying in the lower regions of river Agano, Niigata prefecture, Japan, in 1966, with isolation of Rickettsia from the host and parasites	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium akamushi	Japan	Akita	39.437	140.497	1965	Xenodiagnosis & serology	Kitaoka, M.	Asanuma, K.	Transmission of Rickettsia orientalis to man by Leptotrombidium akamushi at a scrub typhus endemic area in Akita Prefecture, Japan	Am. J. Trop. Med. Hyg.	1974	
Leptotrombidium akamushi	Japan	Akita	39.437	140.497	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium akamushi	Japan	Akita	39.437	140.497	1965	Xenodiagnosis & serology	Kitaoka, M.	Asanuma, K.	Monthly observations on rickettsia and complement fixing antibody response in Microtus montebelli placed once on the ground endemic of scrub typhus or inoculated experimentally with Rickettsia orientalis.	J. Hyg. Epidemiol. Microbiol. Immunol.	1968	
Leptotrombidium akamushi	Malaysia	Selangor	2.897	101.729	1969	?	Rapmund, G.	Upham, R.W.	Transovarial development of scrub typhus in a colony	Trans. R. Soc. Trop. Med.	1969	Free living

									of vector mites	Hyg.		
Leptotrombidium arenicola	Indonesia	Jakarta	-6.133	106.827	1977	Xenodiagnosis	Dennis, D.T.	Hadi, T.R.	A survey of scrub and murine typhus in the Ancol section of Jakarta, Indonesia	Southeast Asian J. Trop. Med. Public Health	1981	
Leptotrombidium arenicola	Malaysia	Perak	4.228	100.558	1960	Xenodiagnosis	Upham, R.W.	Hubert, A.A.	Distribution of Leptotrombidium (Leptotrombidium) arenicola (Acarina: Trombiculidae) on the ground in West Malaysia	J. Med. Entomol.	1971	
Leptotrombidium arvinum	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	
Leptotrombidium arvinum	Thailand	Chiang Mai	18.706	98.982	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	
Leptotrombidium chiangraiensis	Thailand	Chiang Rai	20.211	99.930	1994-01	Xenodiagnosis & serology	Lerdthusnee, K.	Khunthirat, B.	Vector competence of Leptotrombidium chiangraiensis chiggers and transmission efficacy and isolation of Orientia tsutsugamushi	J. Med. Entomol.	2003	
Leptotrombidium chiangraiensis	Thailand	Chiang Rai	20.250	99.939	1993-96	Xenodiagnosis & serology	Tanskul, P.	Linthicum, K.J.	A new ecology for scrub typhus associated with a focus of antibiotic resistance in rice farmers in Thailand	J. Med. Entomol.	1998	
Leptotrombidium deliense	Australia	Queensland	-16.383	145.335	1970-71	Xenodiagnosis	Campbell, R.W.	Domrow, R.	Rickettsioses in Australia: isolation of Rickettsia tsutsugamushi and R. australis from naturally infected arthropods	Trans. R. Soc. Trop. Med. Hyg.	1974	
Leptotrombidium deliense	Papua New Guinea	Manus	-2.848	146.232	1945	Xenodiagnosis & microscopy	Philip, C.B.	Kohls, G.M.	Studies on Tsutsugamushi disease (scrub typhus, mite-borne typhus) in New Guinea and adjacent islands. Tsutsugamushi disease with high endemicity on a small	Am. J. Hyg.	1945	

									South Sea island.			
Leptotrombidium deliense	Indonesia	Riau	0.772	101.796	?	Xenodiagnosis	Walch, E.	Keukenschrijver, N.C.	Eenige opmerkingen aangaande de epidemiologie van de pseudotyphus	Nederlands Tijdschrift voor Geneeskunde	1924	
Leptotrombidium deliense	Singapore	Singapore	1.357	103.972	1953	Xenodiagnosis	Lawley, B.J.		The discovery, investigation and control of scrub typhus in Singapore	Trans. R. Soc. Trop. Med. Hyg.	1957	
Leptotrombidium deliense	Malaysia	Selangor	3.080	101.563	1948	Xenodiagnosis	Philip, C.B.	Traub, R.	Chloramphenicol (chloromycetin) in the chemoprophylaxis of scrub typhus (Tsutsugamushi disease). 1. Epidemiological observations on hyperendemic areas of scrub typhus in Malaya	Am. J. Hyg.	1949	
Leptotrombidium deliense	Malaysia	Selangor	3.088	101.615	1948	Xenodiagnosis	Philip, C.B.	Traub, R.	Chloramphenicol (chloromycetin) in the chemoprophylaxis of scrub typhus (Tsutsugamushi disease). 1. Epidemiological observations on hyperendemic areas of scrub typhus in Malaya	Am. J. Hyg.	1949	
Leptotrombidium deliense	Thailand	Rajburi and Nakhon Pathom	13.649	99.735	1963	Xenodiagnosis	Trishnananda, M	Harinasuta, C	Studies on the vector of Rickettsia tsutsugamushi infection in Thailand	Ann. Trop. Med. Parasitol.	1966	
Leptotrombidium deliense	Thailand	Nonthaburi	13.833	100.517	1993-95	Direct immunofluorescence	Frances, S.P.	Watcharapichat, P.	Seasonal Occurrence of Leptotrombidium deliense (Acari: Trombiculidae) attached to sentinel rodents in an orchard near Bangkok, Thailand	J. Med. Entomol.	1999	
Leptotrombidium deliense	Thailand	Nonthaburi	13.833	100.517	1992	Direct immunofluorescence	Frances, S.P.	Watcharapichat, P.	Occurrence of Orienta tsutsugamushi in Chiggers (Acari:Trombiculidae) and small animals in an Orchard near Bangkok, Thailand	J. Med. Entomol.	1999	

Leptotrombidium deliense	Thailand	Phitsanulok	16.751	100.311	1988-89	Direct immunofluorescence	Tanskul, P.	Strickman, D.	Rickettsia tsutsugamushi in chiggers (Acari: Trombiculidae) associated with rodents in central Thailand	J. Med. Entomol.	1994	
Leptotrombidium deliense	Thailand	Phitsanulok	16.760	100.271	1988-89	Direct immunofluorescence	Tanskul, P.	Strickman, D.	Rickettsia tsutsugamushi in chiggers (Acari: Trombiculidae) associated with rodents in central Thailand	J. Med. Entomol.	1994	
Leptotrombidium deliense	China	Guangdong	20.914	110.097	1998-04	PCR (56kDa)	Wang, S.S.	Huang, J.L.	Study on the characterisitics of tsutsugamushi disease in the epidemic areas of south islands in China	Chinese Journal of Epidemiology	2007	
Leptotrombidium deliense	China	Guangdong	20.917	110.600	1998-04	PCR (56kDa)	Wang, S.S.	Huang, J.L.	Study on the characterisitics of tsutsugamushi disease in the epidemic areas of south islands in China	Chinese Journal of Epidemiology	2007	
Leptotrombidium deliense	China	Guangdong	22.037	113.917	1998-04	PCR (56kDa)	Wang, S.S.	Huang, J.L.	Study on the characterisitics of tsutsugamushi disease in the epidemic areas of south islands in China	Chinese Journal of Epidemiology	2007	
Leptotrombidium deliense	Taiwan	Taitung	22.050	121.513	1990	xenodiagnosis/cell culture & indirect IFA & PCR	Tamura, A.	Ohashi, N.	Characterization of Orientia tsutsugamushi isolated in Taiwan by immunofluorescence and restriction fragment length polymorphism analyses	FEMS Microbiol. Lett.	1997	
Leptotrombidium deliense	Taiwan	Lan-Yu island	22.079	121.529	1990	xenodiagnosis/cell culture & indirect IFA & PCR	Tamura, A.	Ohashi, N.	Characterization of Orientia tsutsugamushi isolated in Taiwan by immunofluorescence and restriction fragment length polymorphism analyses	FEMS Microbiol. Lett.	1997	
Leptotrombidium deliense	China	Guangdong	23.257	117.288	1998-99	Xendiagnosis & PCR	Wang, S.	Jiang, P.	Demonstration of the natural focus of Tsutsugamushi disease in the Nan Peng Lie Islands in China	Southeast Asian J. Trop. Med. Public Health	2001	

Leptotrombidium deliense	China	Guangdong	23.257	117.288	1998-04	PCR (56kDa)	Wang, S.S.	Huang, J.L.	Study on the characterisitics of tsutsugamushi disease in the epidemic areas of south islands in China	Chinese Journal of Epidemiology	2007	
Leptotrombidium deliense	China	Guangdong	23.431	117.093	1998-04	PCR (56kDa)	Wang, S.S.	Huang, J.L.	Study on the characterisitics of tsutsugamushi disease in the epidemic areas of south islands in China	Chinese Journal of Epidemiology	2007	
Leptotrombidium deliense	Taiwan	Fengping	23.585	121.503	1970	Xenodiagnosis & microscopy	Gale, J.L.	Irving, G.S.	Scrub typhus in Eastern Taiwan, 1970	Am. J. Trop. Med. Hyg.	1974	
Leptotrombidium deliense	Myanmar	Sagaing	24.226	94.311	?	Xenodiagnosis	Multiple		Scrub typhus investigations in South East Asia. A report on investigations on scrub typhus by the G.H.O. (India) Field Typhus Research Team, and the Medical Research Council Field Typhus Team, based on the Scrub Typhus Research Laboratory, South East Asia Command	Scrub Typhus Research Laboratory, South East Asia Command	1947	
Leptotrombidium deliense	Taiwan	Fujian	24.440	118.330	1999-00	Nested PCR	Wang, H.C.	Chung, C.L.	Studies on the vectors and pathogens of scrub typhus on murine-like animals in Kinmen Country, Taiwan	Formosan Entomol.	2004	
Leptotrombidium deliense	China	Fujian	24.446	118.083	1992	Xenodiagnosis	Cheng, G.H.	Zhang, C.X.	First isolation of Rickettsia tsutsugamushi from Leptotrombidium deliensis in Xiamen City with scanning electron microscope observation	Journal of Xiamen University Natural Science	1995	
Leptotrombidium deliense	India	Manipur	24.452	94.026	1945	Xenodiagnosis	Multiple		Scrub typhus investigations in South East Asia. A report on investigations on scrub typhus by the G.H.O. (India) Field Typhus Research Team, and the Medical Research Council	Scrub Typhus Research Laboratory, South East Asia Command	1947	

									Field Typhus Team, based on the Scrub Typhus Research Laboratory, South East Asia Command			
Leptotrombidium deliense	Taiwan	Taiwan strait	24.658	119.698	1962	Xenodiagnosis	Cooper, W.C.	Lien, J.C.	Scrub typhus in the Pescadores Islands: An epidemiologic and clinical study	Am. J. trop. Med. Hyg.	1964	
Leptotrombidium deliense	Taiwan	Taiwan strait	24.658	119.698	2006-10	Immunofluorescence + PCR	Kuo, C.C.	Lee, P.L.	Surveillance of potential hosts and vectors of scrub typhus in Taiwan	Parasit. Vectors	2015	
Leptotrombidium deliense	Taiwan	Taiwan strait	24.658	119.698	1975	Xenodiagnosis & microscopy	Olson, J.G	Ho, C.M	Isolation of Rickettsia tsutsugamushi from mammals and chiggers (Fam. Trombiculidae) in the Pescadores Islands, Taiwan.	Trans. R. Soc. Trop. Med. Hyg.	1978	
Leptotrombidium deliense	China	Fujian	25.221	119.475	1997	Nested PCR	Huang, Z.S.	Guo, H.B.	Epidemiologic study of scrub typhus [Orienta tsutsugamushi] on Nanri Island	Chinese Journal of Zoonoses	1998	
Leptotrombidium deliense	Myanmar	Kachin	25.395	97.203	1945	Xenodiagnosis	Mackie, T.T.		Observations on Tsutsugamushi disease (scrub typhus) in Assam and Burma; Preliminary report.	Trans. R. Soc. Trop. Med. Hyg.	1946	
Leptotrombidium deliense	China	Fujian	25.479	119.743	2002	Xendiagnosis & PCR	Cao, M.	Guo, H.	Spring scrub typhus, People's Republic of China	Emerg. Infect. Dis.	2006	
Leptotrombidium deliense	China	Fujian	25.544	118.760	1997-98	Nested PCR	Yan, Y.S.	Zheng, J.	Detection of Orienta tsutsugamushi in chigger mites collected in Fujian coastal and mountain areas in recent years.	Chinese Journal of Zoonoses	1999	
Leptotrombidium deliense	China	Fujian	26.640	118.682	1953-54	Xenodiagnosis & serology	Yu, E.S.	Lin, S.H.	Study on the condition of natural infection with R. tsutsugamushi among mites and dometic animals in Fukien	Acta Microbiologica Sinica	1957	
Leptotrombidium deliense	India	Uttar Pradesh	26.799	83.359	2015	PCR (GroEL)	Sadanandane, C.	Jambulingam, P.	Occurrence of Orientia tsutsugamushi, the Etiological Agent of	Vector Borne Zoonotic Dis.	2018	

									Scrub Typhus in Animal Hosts and Mite Vectors in Areas Reporting Human Cases of Acute Encephalitis Syndrome in the Gorakhpur Region of Uttar Pradesh, India.			
Leptotrombidium deliense	India	Assam	27.328	95.835	1945	Xenodiagnosis	Traub, R.		Observations on Tsutsugamushi disease (scrub typhus) in Assam and Burma. The mite Trombicula deliensis Walch, and its relation to scrub typhus in Assam	Am. J. Hyg.	1949	
Leptotrombidium deliense	India	Assam	27.328	95.835	1945	Xenodiagnosis	Mackie, T.T.		Observations on Tsutsugamushi disease (scrub typhus) in Assam and Burma; Preliminary report.	Trans. R. Soc. Trop. Med. Hyg.	1946	
Leptotrombidium deliense	China	Tibet	29.321	95.325	1973	Xenodiagnosis	Fan, R.S.	Zhang, Y.	Baseline survey on taxonomy and population fluctuation of murine animals and their ectoparasites: forcast research on the related diseases of natural focus in Maoming Port.	Chinese Journal of Vector Biology and Control	2003	
Leptotrombidium deliense	India	Uttarakhand	29.592	79.646	?	Xenodiagnosis	Multiple		Scrub typhus investigations in South East Asia. A report on investigations on scrub typhus by the G.H.O. (India) Field Typhus Research Team, and the Medical Research Council Field Typhus Team, based on the Scrub Typhus Research Laboratory, South East Asia Command	Scrub Typhus Research Laboratory, South East Asia Command	1947	
Leptotrombidium deliense	Pakistan	Punjab	32.482	74.858	1962-65	Xenodiagnosis	Traub, R.	Wisseman, C.L.	The occurrence of scrub typhus in unusual habitats in West Pakistan	Trans. R. Soc. Trop. Med. Hyg.	1967	

Leptotrombidium deliense	China	Unknown	35.862	104.195	1957	Serology (OXK)	Ch'iu, F-H.	Chung, H-L.	Isolation of Rickettsia tsutsugamushi from patients, rats and mites	Chinese Med. J.	1962	
Leptotrombidium deliense	Malaysia	Johor	1.668	103.785	?	Direct immunofluorescence	Shirai, A.	Dohany, A.L.	Serological classification of Rickettsia tsutsugamushi organisms found in chiggers (Acarina: Trombiculidae) collected in Peninsular Malaysia	Trans. R. Soc. Trop. Med. Hyg.	1981	Free living
Leptotrombidium deliense	Malaysia	Pahang	3.171	102.319	1975-77	Direct immunofluorescence	Dohany, A.L.	Shirai, A.	Variation in populations of chigger vectors of scrub typhus in developing oil palm areas of different ages	Jap. J. Med. Sci. Biol.	1980	Free living
Leptotrombidium deliense	Malaysia	Selangor	3.183	101.600	1973-74	Direct immunofluorescence	Roberts, L.W.	Muul, I.	Numbers of Leptotrombidium (Leptotrombidium) deliense (Acarina: Trombiculidae) and prevalence of Rickettsia tsutsugamushi in adjacent habitats of peninsular Malaysia	Southeast Asian J. Trop. Med. Public Health	1977	Free living
Leptotrombidium deliense	Malaysia	Selangor	3.211	101.505	1976-77	Xenodiagnosis & direct IF	Shirai, A.	Robinson, D.M.	Rickettsia tsutsugamushi infections in chiggers and small mammals on a mature oil palm estate	Southeast Asian J. Trop. Med. Public Health	1978	Free living
Leptotrombidium deliense	Thailand	Rajburi and Nakhon Pathom	13.649	99.735	1963	Xenodiagnosis	Trishnananda, M	Harinasuta, C	Studies on the vector of Rickettsia tsutsugamushi infection in Thailand	Ann. Trop. Med. Parasitol.	1966	Free living
Leptotrombidium deliense	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Leptotrombidium deliense	Thailand	Ubon Ratchathani	15.183	105.113	1977-78	Indirect immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Leptotrombidium deliense	China	Hainan	16.330	112.026	2000	PCR (56kDa)	Wang, S.S.	Zhan, D.C.	Sequence analysis of Orientia tsutsugamushi DNA from mites collected in Xisa archipelago, China	Southeast Asian J. Trop. Med. Public Health	2002	Free living

Leptotrombidium deliense	Thailand	Chiang Mai	18.706	98.982	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Leptotrombidium deliense	Taiwan	Taiwan strait	24.658	119.698	1962	Xenodiagnosis	Cooper, W.C.	Lien, J.C.	Scrub typhus in the Pescadores Islands: An epidemiologic and clinical study	Am. J. trop. Med. Hyg.	1964	Free living
Leptotrombidium deliense	India	Assam	27.328	95.835	?	Xenodiagnosis	Davis, G.E.	Austrian, R.C.	Observations on Tsutsugamushi disease (scrub typhus) in Assam and Burma	Am. J. Hyg.	1947	Free living
Leptotrombidium deliense	Thailand	Chiang Rai	20.026	99.758	2016-17	47 kDa RT PCR	This project					
Leptotrombidium deliense	Thailand	Chiang Rai	20.247	99.646	2016-17	47 kDa RT PCR	This project					
Leptotrombidium deliense	Thailand	Nan	19.147	100.721	2016-17	47 kDa RT PCR	This project					
Leptotrombidium deliense	Thailand	Nan	20.026	99.758	Apr-17	47 kDa RT PCR	This project					Free living
Leptotrombidium fletcheri	Papua New Guinea	Oro	-9.131	148.692	1945	Xenodiagnosis	Manwaring, W. H.		Vectors of Tsutsugamushi disease (scrub typhus)	Cal. West. Med.	1945	
Leptotrombidium fletcheri	Papua New Guinea	Oro	-8.762	148.367	1943	Xenodiagnosis & serology	Blake, F.G.	Maxcy, K.F.	Studies on Tsutsugamushi disease (scrub typhus, mite-borne typhus) in New Guinea and adjacent islands: epidemiology, clinical observations, and etiology in the Dobadura area	Am. J. Hyg.	1945	
Leptotrombidium fletcheri	Malaysia	Johor	1.668	103.785	?	Direct immunofluorescence	Shirai, A.	Dohany, A.L.	Serological classification of Rickettsia tsutsugamushi organisms found in chiggers (Acarina: Trombiculidae) collected in Peninsular Malaysia	Trans. R. Soc. Trop. Med. Hyg.	1981	Free living
Leptotrombidium fletcheri	Malaysia	Selangor	3.264	101.588	?	Direct immunofluorescence	Shirai, A.	Dohany, A.L.	Serological classification of Rickettsia tsutsugamushi organisms found in chiggers (Acarina:	Trans. R. Soc. Trop. Med. Hyg.	1981	Free living

									Trombiculidae) collected in Peninsular Malaysia			
Leptotrombidium fletcheri	Malaysia	Selangor	3.211	101.505	1976-77	Xenodiagnosis & serology	Shirai, A.	Robinson, D.M.	Rickettsia tsutsugamushi infections in chiggers and small mammals on a mature oil palm estate	Southeast Asian J. Trop. Med. Public Health	1978	Free living
Leptotrombidium fuji	Japan	Aichi	35.228	137.303	1998	Cell culture & 56 kDa PCR	Tamura, A.	Makisaka, Y.	Isolation of Orientia tsutsugamushi from Leptotrombidium fuji and its characterization	Microbiol. Immunol.	2000	
Leptotrombidium fuji	Japan	Yamagata	38.309	140.149	2012	PCR (56kDa)	Seto, J.	Suzuki, Y	Poposed vector candidate: Leptotrombidium palpale for Shikokoshi type Orientia tsutsugamushi	Microbiol. Immunol.	2013	
Leptotrombidium fuji	Japan	Oita	32.233	131.606	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction	J. Med. Entomol.	2001	Free living
Leptotrombidium fuji	Japan	Oita	33.199	131.517	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction	J. Med. Entomol.	2001	Free living
Leptotrombidium imphalum	Thailand	Chiang Rai	20.250	99.939	1993-96	Xenodiagnosis & direct IF	Tanskul, P.	Linthicum, K.J.	A new ecology for scrub typhus associated with a focus of antibiotic resistance in rice farmers in Thailand	J. Med. Entomol.	1998	
Leptotrombidium imphalum	Thailand	Chiang Rai	20.246	99.644	2016-17	47 kDa RT PCR	This project					
Leptotrombidium imphalum	Thailand	Chiang Rai	20.247	99.646	Aug-17	47 kDa RT PCR	This project					Free living

Leptotrombidium intermedium	China	Shandong	35.924	117.764	2010-12	PCR (56kDa)	Zhang, M.	Zhao, Z.T.	Molecular epidemiology of Orientia tsutsugamushi in chiggers and ticks from domestic rodents in Shandong, northern China	Parasit. Vectors	2013	
Leptotrombidium intermedium	Japan	Niigata	38.112	138.393	1957-58	Xenodiagnosis & microscopy	Saito, Y.	Otsuru, M.	Notes on Trombiculid mites collected in Sadi Island of Japan and isolation of rickettsia, with a description of Trombicula (Noetrombicula) sadoensis n. sp.	Acta Med. Biol. (Niigata)	1959	
Leptotrombidium intermedium	Japan	Yamagata	38.309	140.149	2012	PCR (56kDa) & indirect IF	Seto, J.	Suzuki, Y	Poposed vector candidate: Leptotrombidium palpale for Shikokoshi type Orientia tsutsugamushi	Microbiol. Immunol.	2013	
Leptotrombidium intermedium	Japan	Kyoto	35.550	135.130	1996-99	Indirect immunofluorescence	Takahashi, M.	Misumi, H.	Mite Vectors (Acari: Trombiculidae) of Scrub Typhus in a New Endemic Area in Northern Kyoto, Japan	J. Med. Entomol.	2004	Free living
Leptotrombidium intermedium	Japan	Kyoto	35.550	135.130	1997-01	xenodiagnosis/cell culture & indirect IFA	Misumi, H.	Takahashi, M.	Distribution of infective spots composed of unfed larvae infected with Orientia tsutsugamushi in Leptotrombidium mites and their annual fluctuations on the soil surface in an endemic area of tsutsugamushi disease (Acari: Trombiculidae)	Med. Entomol. Zool.	2002	Free living
Leptotrombidium intermedium	Japan	Kyoto	35.567	135.153	1997-98	Indirect immunofluorescence	Urakami, H	Takahashi, M	Detection, isolation and characterization of Orientia tsutsugamushi in Leptotrombidium intermedium	Med. Entomol. Zool.	2000	Free living
Leptotrombidium intermedium	Japan	Niigata	37.820	139.115	1996	Xenodiagnosis & indirect IF	Urakami, H	Takahashi, M	Detection, isolation and characterization of Orientia tsutsugamushi in Leptotrombidium intermedium	Med. Entomol. Zool.	2000	Free living

Leptotrombidium kawamurai	Japan	Hokkaido	43.058	141.476	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium kawamurai	Japan	Hokkaido	43.058	141.476	1959-68	Xenodiagnosis & complement fixation	Kitaoka, M.	Asanuma, K,	Seasonal occurrence of trombiculid mites species and Leptotrombidium kawamurai (Acarina, Trombiculidae) as a carrier of Rickettsia orientalis in the Nopporo area, Hokkaido, Japan.	J. Hyg. Epidemiol. Microbiol. Immunol.	1973	
Leptotrombidium kitasatoi	Japan	Yamagata	38.309	140.149	2012	PCR (56kDa) & indirect IF	Seto, J.	Suzuki, Y	Poposed vector candidate: Leptotrombidium palpale for Shikokoshi type Orientia tsutsugamushi	Microbiol. Immunol.	2013	
Leptotrombidium kitasatoi	Japan	Oita	32.233	131.606	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction	J. Med. Entomol.	2001	Free living
Leptotrombidium linhuaikongense	China	Anhui	32.777	117.989	2009-12	Xenodiagnosis	Cao, M.	Che, L.	Determination of scrub typhus suggests a new epidemic focus in Anhui Province, China	Sci. Rep.	2016	
Leptotrombidium linhuaikongense	China	Shandong	35.266	117.977	1995-03	Xendiagnosis & PCR	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern China	Cell Biochem. Biophys.	2013	
Leptotrombidium linhuaikongense	China	Shandong	35.266	117.977	1995-03	PCR (56kDa)	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern	Cell Biochem. Biophys.	2013	

									China			
Leptotrombidium linhuaikongense	China	Shandong	35.266	117.977	1995-99	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	First isolation of Orientia (O.) tsutsugamushi from larvae and reared nymphs of Leptotrombidium (L.) linhuaikongense collected from wild rodents in Fei County, Shandong Province, China	Syst. Appl. Acarol.	2002	
Leptotrombidium linhuaikongense	China	Shandong	35.286	118.185	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Isolation of Rickettsia tsutsugamushi from Trombiculid mites (Acari: Trombiculidae) in Feixian county, Shandong province, China	Entomologia Sinica	1999	
Leptotrombidium linhuaikongense	China	Shandong	35.362	118.090	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Epidemiological study of autumn-winter type scrub typhus in a new endemic focus of Fei County, Shandong Province, China	Syst. Appl. Acarol.	2000	
Leptotrombidium linhuaikongense	China	Shandong	35.879	117.928	1995-02	Xendiagnosis & PCR	Liu, Y.	Zhao, Z.	Characterization of Orientia tsutsugamushi strains isolated in Shandong Province, China by immunofluorescence and restriction fragment length polymorphism (RFLP) analyses	Southeast Asian J. Trop. Med. Public Health	2004	
Leptotrombidium linhuaikongense	China	Shandong	35.924	117.764	2010-12	PCR (56kDa)	Zhang, M.	Zhao, Z.T.	Molecular epidemiology of Orientia tsutsugamushi in chiggers and ticks from domestic rodents in Shandong, northern China	Parasit. Vectors	2013	
Leptotrombidium murotoense	Japan	Oita	33.304	131.268	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium nangii	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Leptotrombidium	South	Jeollanam-do	34.673	126.925	1992-93	Immunofluorescence	Ree, H. I	Chang, W. H	Detection of Orientia	Med. Entomol.	1997	

orientale	Korea					+ PCR			tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Zool.		
Leptotrombidium orientale	South Korea	Gwangju	35.193	126.829	2014-15	Immunofluorescence + PCR	Park, J. W	Chung, J.K	Seroepidemiological Survey of Zoonotic Diseases in Small Mammals with PCR Detection of Orientia tsutsugamushi in Chiggers, Gwangju, Korea	Korean J. Parasitol.	2016	
Leptotrombidium orientale	South Korea	Gwangju	35.193	126.829	2014-16	PCR (56kDa)	Park, J.W.	Kim, S.H.	Molecular epidemiology of an Orientia tsutsugamushi gene encoding 56-kDa type-specific antigen in chiggers, small mammals and patients from Southwest region of Korea	Am. J. Trop. Med. Hyg.	2018	
Leptotrombidium orientale	South Korea	Jeollanam-do	35.218	126.323	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium orientale	South Korea	Jeollanam-do	35.304	126.786	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium orientale	South Korea	Gyeongsangnam-do	35.567	128.166	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Leptotrombidium orientale	South Korea	Jeollabuk-do	35.960	126.995	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium orientale	South Korea	Chungcheongnam-do	36.762	126.868	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	

Leptotrombidium orientale	South Korea	Chungcheongbuk-do	36.991	127.926	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Leptotrombidium orientale	South Korea	Gangwon-do	37.449	129.165	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium orientale	Russia	Primorsky Krai	42.430	130.678	1963-65	Xenodiagnosis & Indirect IF	Kulagin, S.M.	Tarasevich, I.V.	On the natural focus of scrub typhus in the south of the Primorie area of the USSR	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium orientale	Russia	Primorsky Krai	42.459	130.655	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Leptotrombidium pallidum	Japan	Oita	33.304	131.268	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	Japan	Shizuoka	34.651	138.859	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	Japan	Shizuoka	34.651	138.859	1952-60	Xenodiagnosis	Asanuma, K.	Okubo, K.	Determination of the vector mites of scrub typhus in Japan	Jap. J. Med. Sci. Biol.	1962	
Leptotrombidium pallidum	South Korea	Jeollanam-do	34.673	126.925	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	South Korea	Jeollanam-do	34.997	127.044	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	

Leptotrombidium pallidum	Japan	Kanagawa	35.144	139.621	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	Japan	Kanagawa	35.144	139.621	1952-60	Xenodiagnosis	Asanuma, K.	Okubo, K.	Determination of the vector mites of scrub typhus in Japan	Jap. J. Med. Sci. Biol.	1962	
Leptotrombidium pallidum	South Korea	Jeollanam-do	35.173	126.859	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	South Korea	Jeollanam-do	35.200	126.502	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	Japan	Aichi	35.225	137.329	1989-90	xenodiagnosis/cell culture & 56 kDa PCR	Tamura, A.	Makisaka, Y.	Isolation of Orientia tsutsugamushi from Leptotrombidium fuji and its characterization	Microbiol. Immunol.	2000	
Leptotrombidium pallidum	South Korea	Jeollanam-do	35.264	126.476	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	South Korea	Jeollanam-do	35.304	126.786	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium pallidum	Japan	Gifu	35.429	137.002	1984	Xenodiagnosis & indirect IF	Kasuya, S.	Hioki, A.	Studies on Tsutsugamushi disease in Gifu Prefecture. 2. A speculation on the vector	Kansenshogaku Zasshi	1985	
Leptotrombidium pallidum	Japan	Gifu	35.439	137.045	1984-86	Xenodiagnosis & indirect IF	Iwasa, M.	Kasuya, S.	Trombiculid Mites (Acari: Trombiculidae) and Rickettsia tsutsugamushi Isolated from Wild Rodents in a New Endemic Area of Japan	J. Med. Entomol.	1990	
Leptotrombidium	Japan	Gifu	35.492	136.728	1994	Xenodiagnosis &	Yamashita, T.	Kasuya, S.	Transmission of Rickettsia	J. Clin.	1994	

pallidum						indirect IF			tsutsugamushi strains among humans, wild rodents, and trombiculid mites in an area of Japan in which Tsutsugamushi disease is newly endemic	Microbiol.		
Leptotrombidium pallidum	Japan	Kyoto	35.550	135.130	1996-99	xenodiagnosis/cell culture & indirect IFA	Takahashi, M.	Misumi, H.	Mite Vectors (Acari: Trombiculidae) of Scrub Typhus in a New Endemic Area in Northern Kyoto, Japan	J. Med. Entomol.	2004	
Leptotrombidium pallidum	Japan	Kyoto	35.550	135.130	1996-99	Indirect immunofluorescence	Takahashi, M.	Misumi, H.	Mite Vectors (Acari: Trombiculidae) of Scrub Typhus in a New Endemic Area in Northern Kyoto, Japan	J. Med. Entomol.	2004	
Leptotrombidium pallidum	Japan	Kyoto	35.550	135.130	1997-01	xenodiagnosis/cell culture & indirect IFA	Misumi, H.	Takahashi, M.	Distribution of infective spots composed of unfed larvae infected with Orientia tsutsugamushi in Leptotrombidium mites and their annual fluctuations on the soil surface in an endemic area of tsutsugamushi disease (Acari: Trombiculidae)	Med. Entomol. Zool.	2002	
Leptotrombidium pallidum	Japan	Saitama	35.985	139.090	1985-86	Xenodiagnosis & indirect IF	Takahashi, M.	Murata, M.	Aggregated distribution of infective spots composed of Leptotrombidium pallidum, highly prevalent with Rickettsia tsutsugamushi, demonstrated by sentinel voles, Microtus montebelli, on the ground	Jpn. J. Exp. Med.	1990	
Leptotrombidium pallidum	Japan	Saitama	35.985	139.090	1985	Direct immunofluorescence	Takahashi, M.	Murata, M.	Vector mite of Tsutsugamushi disease in the area of Hitsujiyama, Chichibu City, Saitama Prefecture: accumulation of Rickettsia tsutsugamushi in the	Journal of Saitama Medical School	1992	

									endemic spot			
Leptotrombidium pallidum	Japan	Saitama	35.985	139.090	1985	Xenodiagnosis & indirect IF	Takahashi, M.	Murata, M.	Vector mite of Tsutsugamushi disease in the area of Hitsujiyama, Chichibu City, Saitama Prefecture: accumulation of Rickettsia tsutsugamushi in the endemic spot	Journal of Saitama Medical School	1992	
Leptotrombidium pallidum	Japan	Saitama	35.985	139.090	1984-85	Xenodiagnosis & indirect IF	Takahashi, M.	Murata, M.	Trombiculid mites and Rickettsia tsutsugamushi isolated from wild rodents and mites in an endemic area of Saitama Prefecture, Japan	Japanese Journal of Sanitary Zoology	1993	
Leptotrombidium pallidum	South Korea	Chungcheongnam-do	36.762	126.868	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium pallidum	Japan	Toyama	36.818	137.598	1979-84	Xenodiagnosis & indirect IF	Ishikura, M.	Watanabe, M.	Epidemiological Studies on the Background of the Endemic Occurrence of Tsutsugamushi Disease in Toyama Prefecture. I. Epidemiology of Infection with Rickettsia tsutsugamuchi among Field Rodents in Endemic and Nonendemic Areas	Microbiol. Immunol.	1985	
Leptotrombidium pallidum	South Korea	Gyeonggi-do	37.040	126.870	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium pallidum	South Korea	Gyeonggi-do	37.234	127.707	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Leptotrombidium pallidum	South Korea	Gyeonggi-do	37.298	127.637	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub	Vector Borne Zoonotic Dis.	2011	

									typhus, in a novel mite species, Euschengastia koreaensis, in Korea			
Leptotrombidium pallidum	Japan	Niigata	37.446	138.851	1952-60	Xenodiagnosis	Asanuma, K.	Okubo, K.	Determination of the vector mites of scrub typhus in Japan	Jap. J. Med. Sci. Biol.	1962	
Leptotrombidium pallidum	South Korea	Gangwon-do	37.536	129.115	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	South Korea	Gyeonggi-do	37.681	126.839	1989	Indirect immunofluorescence	Ree, H.I	Lee, I. Y	Determination of the vector species of tsutsugamushi disease in Korea	Korean J. Parasitol.	1991	
Leptotrombidium pallidum	South Korea	Gyeonggi-do	37.683	126.840	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	Japan	Niigata	37.745	139.183	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	Japan	Niigata	37.915	139.147	1966	Xenodiagnosis & microscopy	Saito, Y.		Parasitism of chiggers on the meadow mouse on the islet 'Kawamura nakasu' lying in the lower regions of river Agano, Niigata prefecture, Japan, in 1966, with isolation of Rickettsia from the host and parasites	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	South Korea	Gangwon-do	37.927	127.742	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium pallidum	South Korea	Gangwon-do	38.147	127.313	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite	Vector Borne Zoonotic Dis.	2011	

									species, Euschengastia koreaensis, in Korea			
Leptotrombidium pallidum	Japan	Yamagata	38.309	140.149	2012	PCR (56kDa) & indirect IF	Seto, J.	Suzuki, Y	Poposed vector candidate: Leptotrombidium palpale for Shikokoshi type Orientia tsutsugamushi	Microbiol. Immunol.	2013	
Leptotrombidium pallidum	Japan	Akita	39.211	140.515	1969-70	Xenodiagnosis	Asanuma, K.	Kitaoka, M.	Occurrence of Scrub Typhus in clinical endemic area in Japan but not transmitted by Leptotrombidium akamushi	J. Med. Entomol.	1972	
Leptotrombidium pallidum	Japan	Akita	39.215	140.521	1970-71	Xenodiagnosis & complement fixation	Kitaoka, M.	Asanuma, K.	Experiments on chickens placed on ground in endemic of classical scrub typhus in Akita prefecture, Japan	J. Hyg. Epidemiol. Microbiol. Immunol.	1976	
Leptotrombidium pallidum	Japan	Akita	39.437	140.497	1964-66	Complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	Japan	Aomori	40.710	140.578	1975-76	Xenodiagnosis	Takada, N.		Epidemiology of Tsutsugamushi disease in Aomori Prefecture	Japanese Journal of Sanitary Zoology	1982	
Leptotrombidium pallidum	Russia	Primorsky Krai	42.430	130.678	1963-65	Xenodiagnosis & indirect IF	Kulagin, S.M.	Tarasevich, I.V.	On the natural focus of scrub typhus in the south of the Primorie area of the USSR	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pallidum	Russia	Primorsky Krai	42.459	130.655	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Leptotrombidium pallidum	Russia	Primorsky Krai	42.459	130.655	1963	Xenodiagnosis	Kudryashova, N.I.	Tarasevich, I.V.	Trombiculids in a natural focus of tsutsugamushi disease in the south of the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1964	
Leptotrombidium pallidum	Japan	Oita	32.233	131.606	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales:	J. Med. Entomol.	2001	Free living

									Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction			
Leptotrombidium pallidum	Japan	Oita	33.199	131.517	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction	J. Med. Entomol.	2001	Free living
Leptotrombidium pallidum	Japan	Shizuoka	35.322	138.897	1986-90	Xenodiagnosis & indirect IF	Kawamori, F.	Akiyama, M.	Epidemiology of Tsutsugamushi Disease in Relation to the Serotypes of Rickettsia tsutsugamushi Isolated from Patients, Field Mice, and Unfed Chiggers on the Eastern Slope of Mount Fuji, Shizuoka Prefecture, Japan	J. Clin. Microbiol.	1992	Free living
Leptotrombidium pallidum	Japan	Kyoto	35.550	135.130	1996-99	Indirect immunofluorescence	Takahashi, M.	Misumi, H.	Mite Vectors (Acari: Trombiculidae) of Scrub Typhus in a New Endemic Area in Northern Kyoto, Japan	J. Med. Entomol.	2004	Free living
Leptotrombidium pallidum	Japan	Kyoto	35.550	135.130	1997-01	xenodiagnosis/cell culture & indirect IFA	Misumi, H.	Takahashi, M.	Distribution of infective spots composed of unfed larvae infected with Orientia tsutsugamushi in Leptotrombidium mites and their annual fluctuations on the soil surface in an endemic area of tsutsugamushi disease (Acari: Trombiculidae)	Med. Entomol. Zool.	2002	Free living
Leptotrombidium pallidum	Japan	Kyoto	35.567	135.153	1997-98	Indirect immunofluorescence	Urakami, H	Takahashi, M	Detection, isolation and characterization of	Med. Entomol. Zool.	2000	Free living

									Orientia tsutsugamushi in Leptotrombidium intermedium			
Leptotrombidium palpale	Japan	Oita	33.304	131.268	1964-66	complement fixation	Kitaoka, M.	Okubo, K.	Epidemiological survey by means of complement fixation test on scrub typhus in Japan	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium palpale	South Korea	Jeollanam-do	34.997	127.044	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	South Korea	Jeollanam-do	35.173	126.859	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	South Korea	Jeollanam-do	35.200	126.502	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	South Korea	Jeollanam-do	35.218	126.323	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	South Korea	Jeollanam-do	35.264	126.476	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	China	Shandong	35.266	117.977	1995-03	Xendiagnosis & PCR	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern China	Cell Biochem. Biophys.	2013	
Leptotrombidium palpale	China	Shandong	35.266	117.977	1995-03	PCR (56kDa)	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub	Cell Biochem. Biophys.	2013	

									typhus patients, rodents, and chiggers from a New Endemic focus of Northern China			
Leptotrombidium palpale	China	Shandong	35.266	117.977	1995-96	Direct immunofluorescence	Liu, Y.X.	Wu, Q.Y.	First isolation of Rickettsia Tsutsugamushi from Leptotrombidium palpalis	Chinese Journal of Vector Biology and Control	1998	
Leptotrombidium palpale	China	Shandong	35.286	118.185	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Isolation of Rickettsia tsutsugamushi from Trombiculid mites (Acari: Trombiculidae) in Feixian county, Shandong province, China	Entomologia Sinica	1999	
Leptotrombidium palpale	South Korea	Jeollanam-do	35.304	126.786	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium palpale	China	Shandong	35.362	118.090	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Epidemiological study of autumn-winter type scrub typhus in a new endemic focus of Fei County, Shandong Province, China	Syst. Appl. Acarol.	2000	
Leptotrombidium palpale	South Korea	Gyeongnam-do	35.567	128.166	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Leptotrombidium palpale	China	Shandong	35.879	117.928	1995-02	Xendiagnosis & PCR	Liu, Y.	Zhao, Z.	Characterization of Orientia tsutsugamushi strains isolated in Shandong Province, China by immunofluorescence and restriction fragment length polymorphism (RFLP) analyses	Southeast Asian J. Trop. Med. Public Health	2004	
Leptotrombidium palpale	China	Shandong	35.924	117.764	2010-12	PCR (56kDa)	Zhang, M.	Zhao, Z.T.	Molecular epidemiology of Orientia tsutsugamushi in chiggers and ticks from domestic rodents in Shandong, northern China	Parasit. Vectors	2013	

Leptotrombidium palpale	South Korea	Jeollabuk-do	35.960	126.995	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	South Korea	Jeollabuk-do	36.029	126.929	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium palpale	Japan	Yamagata	38.309	140.149	2012	56kDa & nested PCR, sequencing, indirect immunofluorescence	Seto, J.	Suzuki, Y	Poposed vector candidate: Leptotrombidium palpale for Shikokoshi type Orientia tsutsugamushi	Microbiol. Immunol.	2013	
Leptotrombidium palpale	Russia	Primorsky Krai	42.872	131.365	1963-65	Xenodiagnosis & microscopy	Kulagin, S.M.	Tarasevich, I.V.	The investigation of scrub typhus in the USSR	J. Hyg. Epidemiol. Microbiol.	1968	
Leptotrombidium palpale	Russia	Primorsky Krai	42.936	131.375	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Leptotrombidium pavlovskyi	Russia	Primorsky Krai	42.429	130.633	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Leptotrombidium pavlovskyi	Russia	Primorsky Krai	42.430	130.678	1963-65	Xenodiagnosis & indirect IF	Kulagin, S.M.	Tarasevich, I.V.	On the natural focus of scrub typhus in the south of the Primorie area of the USSR	Acta Med. Biol. (Niigata)	1967	
Leptotrombidium pavlovskyi	Russia	Primorsky Krai	42.683	130.760	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Leptotrombidium pavlovskyi	Russia	Primorsky Krai	42.683	130.760	1963	Xenodiagnosis	Kudryashova, N.I.	Tarasevich, I.V.	Trombiculids in a natural focus of tsutsugamushi disease in the south of the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1964	
Leptotrombidium	Russia	Primorsky Krai	42.685	130.663	1964-65	Direct	Kudryashova,	Mirolyubova,	Natural infection of	Meditsinskaya	1968	

pavlovskyi						immunofluorescence	N.I.	L.N.	Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Parazitologiya i Parazitarnye Bolezni		
Leptotrombidium pavlovskyi	Russia	Primorsky Krai	42.872	131.365	1963-65	Xenodiagnosis & microscopy	Kulagin, S.M.	Tarasevich, I.V.	The investigation of scrub typhus in the USSR	J. Hyg. Epidemiol. Microbiol.	1968	
Leptotrombidium pavlovskyi	Russia	Primorsky Krai	42.429	130.633	1963	Xenodiagnosis	Kudryashova, N.I.	Tarasevich, I.V.	Trombiculids in a natural focus of tsutsugamushi disease in the south of the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1964	Free living
Leptotrombidium peniculatum	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Leptotrombidium scutellare	China	Fujian	24.440	118.330	1999-00	Nested PCR	Wang, H.C.	Chung, C.L.	Studies on the vectors and pathogens of scrub typhus on murine-like animals in Kinmen Country, Taiwan	Formosan Entomol.	2004	
Leptotrombidium scutellare	China	Fujian	25.544	118.760	1997-98	Nested PCR	Yan, Y.S.	Zheng, J.	Detection of Orienta tsutsugamushi in chigger mites collected in Fujian coastal and mountain areas in recent years.	Chinese Journal of Zoonoses	1999	
Leptotrombidium scutellare	China	Jiangsu	32.868	120.320	1994	PCR (56kDa)	Guo, H.B.	Wu, G.H.	Studies on detection of Rickettsia tsutsugamushi DNA in the single larva of Leptotrombidium (L.) scutellare collected from the endemic areas by PCR	Chinese Journal of Zoonoses	1996	
Leptotrombidium scutellare	South Korea	Cheju Island	33.480	126.384	1991-92	Indirect immunofluorescence	Ree, H.I.	Lee, I.Y	Study on vector mites of tsutsugamushi disease in Cheju Island, Korea	Korean J. Parasitol.	1992	
Leptotrombidium scutellare	South Korea	Cheju Island	33.488	126.416	1991-92	Indirect immunofluorescence	Ree, H.I.	Lee, I.Y	Study on vector mites of tsutsugamushi disease in Cheju Island, Korea	Korean J. Parasitol.	1992	
Leptotrombidium scutellare	South Korea	Jeollanam-do	34.673	126.925	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids	Med. Entomol. Zool.	1997	

									using polymerase chain reaction in Korea			
Leptotrombidium scutellare	South Korea	Jeollanam-do	34.772	127.662	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	Japan	Chiba	34.907	139.899	1952-61	Xenodiagnosis	Asanuma, K.	Okubo, K.	Determination of the vector mites of scrub typhus in Japan	Jap. J. Med. Sci. Biol.	1962	
Leptotrombidium scutellare	South Korea	Jeollanam-do	34.997	127.044	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	Japan	Chiba	35.135	139.866	1952	Xenodiagnosis	Asanuma, K.	Kitaoka, M.	Evidences for Trombicula scutellaris to be a vector of scrub typhus in Chiba prefecture, Japan	Japanese Journal of Sanitary Zoology	1959	
Leptotrombidium scutellare	South Korea	Jeollanam-do	35.173	126.859	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	South Korea	Jeollanam-do	35.193	126.829	2014-15	Immunofluorescence + PCR	Park, J. W	Chung, J.K	Seroepidemiological Survey of Zoonotic Diseases in Small Mammals with PCR Detection of Orientia tsutsugamushi in Chiggers, Gwangju, Korea	Korean J. Parasitol.	2016	
Leptotrombidium scutellare	South Korea	Gwangju	35.193	126.829	2014-16	PCR (56kDa)	Park, J.W.	Kim, S.H.	Molecular epidemiology of an Orientia tsutsugamushi gene encoding 56-kDa type-specific antigen in chiggers, small mammals and patients from Southwest region of Korea	Am. J. Trop. Med. Hyg.	2018	
Leptotrombidium	South	Jeollanam-do	35.200	126.502	1995	Immunofluorescence +	Ree, H. I	Chang, W. H	Detection of Orientia	Med. Entomol.	1997	

scutellare	Korea					PCR			tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Zool.		
Leptotrombidium scutellare	South Korea	Jeollanam-do	35.218	126.323	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	South Korea	Jeollanam-do	35.264	126.476	1995	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	China	Shandong	35.266	117.977	1995-03	Xendiagnosis & PCR	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern China	Cell Biochem. Biophys.	2013	
Leptotrombidium scutellare	China	Shandong	35.266	117.977	1995-03	PCR (56kDa)	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern China	Cell Biochem. Biophys.	2013	
Leptotrombidium scutellare	China	Shandong	35.286	118.185	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Isolation of Rickettsia tsutsugamushi from Trombiculid mites (Acari: Trombiculidae) in Feixian county, Shandong province, China	Entomologia Sinica	1999	
Leptotrombidium scutellare	Japan	Shizuoka	35.294	138.854	1952-60	Xenodiagnosis	Asanuma, K.	Okubo, K.	Determination of the vector mites of scrub typhus in Japan	Jap. J. Med. Sci. Biol.	1962	
Leptotrombidium scutellare	Japan	Shizuoka	35.294	138.854	1954-62	Xenodiagnosis	Asanuma, K.	Kitaoka, M.	Leptotrombidium scutellare as a vector of scrub typhus at the endemic area of the foothills of Mt. Fuji,	J. Hyg. Epidemiol. Microbiol. Immunol.	1974	

									Japan			
Leptotrombidium scutellare	South Korea	Jeollanam-do	35.304	126.786	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium scutellare	China	Shandong	35.362	118.090	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Epidemiological study of autumn-winter type scrub typhus in a new endemic focus of Fei County, Shandong Province, China	Syst. Appl. Acarol.	2000	
Leptotrombidium scutellare	South Korea	Gyeongnam-do	35.567	128.166	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Leptotrombidium scutellare	China	Shandong	35.757	117.946	1986	Xenodiagnosis & indirect IF	Xiangrui, C.	Jinju, W.	Recent studies on scrub typhus and Rickettsia tsutsugamushi in Shandong Province - China	Eur. J. Epidemiol.	1991	
Leptotrombidium scutellare	China	Shandong	35.879	117.928	?	Xendiagnosis & PCR	Liu, Y.	Zhao, Z.	Characterization of Orientia tsutsugamushi strains isolated in Shandong Province, China by immunofluorescence and restriction fragment length polymorphism (RFLP) analyses	Southeast Asian J. Trop. Med. Public Health	2004	
Leptotrombidium scutellare	China	Shandong	35.924	117.764	2010-12	PCR (56kDa)	Zhang, M.	Zhao, Z.T.	Molecular epidemiology of Orientia tsutsugamushi in chiggers and ticks from domestic rodents in Shandong, northern China	Parasit. Vectors	2013	
Leptotrombidium scutellare	South Korea	Jeollabuk-do	35.960	126.995	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids	Med. Entomol. Zool.	1997	

									using polymerase chain reaction in Korea			
Leptotrombidium scutellare	South Korea	Jeollabuk-do	36.029	126.929	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	South Korea	Gyeongsangbuk-do	36.454	129.380	1992-93	Immunofluorescence + PCR	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium scutellare	South Korea	Chungcheongnam-do	36.762	126.868	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium scutellare	South Korea	Chungcheongnam-do	37.040	126.870	2009	Nested PCR	Choi, Y.J.	Lee, I.Y.	Geographical distribution of Orientia tsutsugamushi strains in chiggers from three provinces of Korea	Microbiol. Immunol.	2018	
Leptotrombidium scutellare	China	Shandong	37.167	117.834	1995-03	PCR (56kDa)	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern China	Cell Biochem. Biophys.	2013	
Leptotrombidium scutellare	Malaysia	Pahang	4.502	101.390	?	Indirect immunofluorescence	Shirai, A.	Dohany, A.L.	Serological classification of Rickettsia tsutsugamushi organisms found in chiggers (Acarina: Trombiculidae) collected in Peninsular Malaysia	Trans. R. Soc. Trop. Med. Hyg.	1981	Free living
Leptotrombidium scutellare	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Leptotrombidium scutellare	Thailand	Chiang Mai	18.706	98.982	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers	Southeast Asian J. Trop.	1981	Free living

									collected in Thailand	Med. Public Health		
Leptotrombidium scutellare	Japan	Kagoshima	31.700	130.616	2000-01	Direct immunofluorescence	Takahashi, M.	Urakami, H.	Detection and serotyping of Orientia tsutsugamushi from the unfed larval trombiculid mite Leptotrombidium scutellare (Nagayo, Miyagawa, Mitamura, Tamiya, et Tenjin, 1921) (Acari: Trombiculidae)	Med. Entomol. Zool.	2002	Free living
Leptotrombidium scutellare	China	Jiangsu	32.600	119.940	2013	Nested PCR	He, Y.L.	Yang, H.Y.	Study of epidemic area on Tsutsugamushi disease in Taizhou from 2013 to 2014	Chinese Journal of Preventive Medicine	2017	Free living
Leptotrombidium scutellare	Japan	Oita	32.967	131.400	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture, Japan, by nested polymerase chain reaction	J. Med. Entomol.	2001	Free living
Leptotrombidium scutellare	Japan	Shizuoka	35.322	138.897	1986-90	Xenodiagnosis & indirect IF	Kawamori, F.	Akiyama, M.	Epidemiology of Tsutsugamushi Disease in Relation to the Serotypes of Rickettsia tsutsugamushi Isolated from Patients, Field Mice, and Unfed Chiggers on the Eastern Slope of Mount Fuji, Shizuoka Prefecture, Japan	J. Clin. Microbiol.	1992	Free living
Leptotrombidium sp.	Indonesia	East Kalimantan	-1.023	116.865	2007-08	PCR & ELISA	Widjaja, S.	Williams, M.	Geographical assessment of Rickettsioses in Indonesia	Vector Borne Zoonotic Dis.	2016	
Leptotrombidium sp.	Indonesia	North Sulawesi	1.447	124.813	2007-08	ELISA	Widjaja, S.	Williams, M.	Geographical assessment of Rickettsioses in Indonesia	Vector Borne Zoonotic Dis.	2016	

Leptotrombidium sp.	Thailand	Phang Nga	8.664	98.452	2015	PCR (56kDa)	Takhampunya, R.	Korkusol, A.	Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand	Plos Negl.	2018	
Leptotrombidium sp.	Thailand	Chachoengsao	13.786	101.092	2010	Xendiagnosis & PCR	Takhampunya, R.	Tippayachai, B.	Characterization based on the 56kDa type- specific antigen gene of Orientia tsutsugamushi genotypes isolated from Leptotrombidium mites and the rodent host post-infection	Am. J. trop.	2014	
Leptotrombidium sp.	Thailand	Multiple sites	15.200	102.100	2017-18	PCR (56kDa)	Linsuwananon, P.	Krairojananan, P.	Surveillance for scrub typhus, Rickettsial diseases, and Leptospirosis in US and multinational military training exercise Cobra Gold Sites in Thailand	US Army Med. Dep. J.	2018	
Leptotrombidium sp.	Taiwan	Hualien	23.923	121.566	2007-08	PCR (56kDa)	Kuo, C.C.	Wang, H.C.	The potential effect of exotic Pacific rats Rattus exulans on vectors of scrub typhus	J. Appl. Ecol.	2011	
Leptotrombidium sp.	Taiwan	Hualien	24.100	121.570	2007-08	PCR (56kDa)	Kuo, C.C.	Huang, J.L.	Cascading effect of economic globalization on human risks of scrub typhus and tick-borne rickettsial diseases.	Ecol. Appl.	2012	
Leptotrombidium sp.	Pakistan	Gilgit-Baltistan	34.907	73.654	1962-65	Xenodiagnosis	Traub, R.	Wisseman, C.L.	The occurrence of scrub typhus in unusual habitats in West Pakistan	Trans. R. Soc. Trop. Med. Hyg.	1967	
Leptotrombidium sp.	Thailand	Chiang Mai	18.706	98.982	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers	Southeast Asian J. Trop. Med. Public	1981	Free living
Leptotrombidium tachensis	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	

Leptotrombidium taishanicum	China	Shandong	35.924	117.764	2010-12	PCR (56kDa)	Zhang, M.	Zhao, Z.T.	Molecular epidemiology of Orientia tsutsugamushi in chiggers and ticks from domestic rodents in Shandong, northern China	Parasit. Vectors	2013	
Leptotrombidium waiganmensis	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Leptotrombidium zetum	South Korea	Gyeongnam-do	34.851	128.429	1992-93	PCR (56kDa) & indirect IF	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Leptotrombidium zetum	South Korea	Jeollabuk-do	35.960	126.995	1992-93	PCR (56kDa) & indirect IF	Ree, H. I	Chang, W. H	Detection of Orientia tsutsugamushi DNA in individual trombiculids using polymerase chain reaction in Korea	Med. Entomol. Zool.	1997	
Lorillatum sp.	Thailand	Sisaket	14.477	104.490	2015	PCR (56kDa)	Takhampunya, R.	Korkusol, A.	Heterogeneity of Orientia tsutsugamushi genotypes in field-collected trombiculid mites from wild-caught small mammals in Thailand	Plos Negl. Trop. Dis.	2018	
Microtrombicula chamlongi	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Neoschoengastia sp.	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Neotrombicula japonica	South Korea	Gyeonggi-do	37.760	126.780	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub typhus, in a novel mite species, Euschengastia koreaensis, in Korea	Vector Borne Zoonotic Dis.	2011	
Neotrombicula japonica	South Korea	Gangwon-do	38.147	127.313	2005	PCR (56kDa)	Lee, H.I.	Shim, S.K.	Detection of Orientia tsutsugamushi, the causative agent of scrub	Vector Borne Zoonotic Dis.	2011	

									typhus, in a novel mite species, Euschengastia koreaensis, in Korea			
Neotrombicula japonica	Russia	Primorsky Krai	42.429	130.633	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula japonica	Russia	Primorsky Krai	42.430	130.678	1963-65	Xenodiagnosis & indirect IF	Kulagin, S.M.	Tarasevich, I.V.	On the natural focus of scrub typhus in the south of the Primorie area of the USSR	Acta Med. Biol. (Niigata)	1967	
Neotrombicula japonica	Russia	Primorsky Krai	42.459	130.655	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula japonica	Russia	Primorsky Krai	42.685	130.663	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula japonica	Russia	Primorsky Krai	42.872	131.365	1963-65	Xenodiagnosis & microscopy	Kulagin, S.M.	Tarasevich, I.V.	The investigation of scrub typhus in the USSR	J. Hyg. Epidemiol. Microbiol.	1968	
Neotrombicula japonica	Russia	Primorsky Krai	42.936	131.375	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula japonica	Russia	Primorsky Krai	43.022	131.860	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula japonica	Japan	Oita	32.967	131.400	1997-99	Nested PCR	Pham, X.D.	Otsuka, Y.	Detection of Orientia tsutsugamushi (Rickettsiales: Rickettsiaceae) in unengorged chiggers (Acari: Ttombiculidae) from Oita Prefecture,	J. Med. Entomol.	2001	Free living

									Japan, by nested polymerase chain reaction			
Neotrombicula microti	Russia	Sakhalin Oblast	43.795	146.747	1968-73	Xenodiagnosis	Somov, G.P.	Shubin, F.N.	Tsutsugamushi fever in the Kuril Islands	Zh. Mikrobiol. Epidemiol. Immunobiol.	1976	
Neotrombicula microti	Russia	Sakhalin Oblast	43.795	146.747	?	Xenodiagnosis	Somov, G.P.	Shubin, F.N.	A major result of studying Tsutsugamushi fever in the Soviet Far East	Folia Microbiol.	1976	
Neotrombicula mitamurai	Russia	Primorsky Krai	42.430	130.678	1963-65	Xenodiagnosis & indirect IF	Kulagin, S.M.	Tarasevich, I.V.	On the natural focus of scrub typhus in the south of the Primorie area of the USSR	Acta Med. Biol. (Niigata)	1967	
Neotrombicula mitamurai	Russia	Primorsky Krai	42.683	130.760	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula mitamurai	Russia	Primorsky Krai	42.685	130.663	1963	Xenodiagnosis	Kudryashova, N.I.	Tarasevich, I.V.	Trombiculids in a natural focus of tsutsugamushi disease in the south of the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1964	
Neotrombicula mitamurai	Russia	Primorsky Krai	42.872	131.365	1963-65	Xenodiagnosis & microscopy	Kulagin, S.M.	Tarasevich, I.V.	The investigation of scrub typhus in the USSR	J. Hyg. Epidemiol. Microbiol.	1968	
Neotrombicula mitamurai	Russia	Primorsky Krai	42.936	131.375	1964-65	Direct immunofluorescence	Kudryashova, N.I.	Mirolyubova, L.N.	Natural infection of Trombiculid mites with the rickettsiae of tsutsugamushi disease in the Maritime Province	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	1968	
Neotrombicula nagayoi	Russia	Sakhalin Oblast	44.150	145.875	1973	Xenodiagnosis	Somov, G.P.	Shubin, F.N.	Tsutsugamushi fever in the Kuril Islands	Zh. Mikrobiol. Epidemiol. Immunobiol.	1976	
Neotrombicula nagayoi	Russia	Sakhalin Oblast	44.150	145.875	?	Xenodiagnosis	Somov, G.P.	Shubin, F.N.	A major result of studying Tsutsugamushi fever in the Soviet Far East	Folia Microbiol.	1976	
Neotrombicula pomeranzevi	Japan	Hokkaido	43.058	141.476	1959-68	Xenodiagnosis & complement fixation	Kitaoka, M.	Asanuma, K,	Seasonal occurrence of trombiculid mites species and Leptotrombidium kawamurai (Acarina, Trombiculidae) as a carrier of Rickettsia orientalis in	J. Hyg. Epidemiol. Microbiol. Immunol.	1973	

									the Nopporo area, Hokkaido, Japan.			
Neotrombicula pomeranzevi	Russia	Sakhalin Oblast	43.795	146.747	1968-73	Xenodiagnosis	Somov, G.P.	Shubin, F.N.	Tsutsugamushi fever in the Kuril Islands	Zh. Mikrobiol. Epidemiol. Immunobiol.	1976	
Neotrombicula pomeranzevi	Russia	Sakhalin Oblast	43.795	146.747	?	Xenodiagnosis	Somov, G.P.	Shubin, F.N.	A major result of studying Tsutsugamushi fever in the Soviet Far East	Folia Microbiol.	1976	
Neotrombicula shiraii	Japan	Niigata	38.112	138.393	1957-58	Xenodiagnosis & microscopy	Saito, Y.	Otsuru, M.	Notes on Trombiculid mites collected in Sadi Island of Japan and isolation of rickettsia, with a description of Trombicula (Noetrombicula) sadoensis n. sp.	Acta Med. Biol. (Niigata)	1959	
Odontacarus sp.	Thailand	Nakhon Ratchasima	14.957	102.111	1977-78	Direct immunofluorescence	Shirai, A.	Tanskul, P.L.	Rickettsia tsutsugamushi strains found in chiggers collected in Thailand	Southeast Asian J. Trop. Med. Public Health	1981	Free living
Ornithonyssus bacoti	India	Maharashtra	21.146	79.086	2016	PCR (56kDa)	Bhate, R.	Pansare,N.	Prevalence and phylogenetic analysis of Orientia tsutsugamushi in rodents and mites from Central India	Vector Borne Zoonotic Dis.	2017	
Schoengastia sp.	Thailand	Buriram	14.300	102.800	2017-18	PCR (56kDa)	Linsuwananon, P.	Krairojananan, P.	Surveillance for scrub typhus, Rickettsial diseases, and Leptospirosis in US and multinational military training exercise Cobra Gold Sites in Thailand	US Army Med. Dep. J.	2018	
Schoutedenichia sp.	Thailand	Chiang Rai	20.246	99.647	Apr-17	47 kDa RT PCR	This project					
Schoutedenichia sp.	Thailand	Chiang Rai	20.026	99.758	Apr-17	47 kDa RT PCR	This project					
Trombicula sadoensis n. sp.	Japan	Niigata	38.112	138.393	1957-58	Xenodiagnosis & microscopy	Saito, Y.	Otsuru, M.	Notes on Trombiculid mites collected in Sadi Island of Japan and isolation of rickettsia, with a description of	Acta Med. Biol. (Niigata)	1959	

									Trombicula (Noetrombicula) sadoensis n. sp.			
Trombicula tosa	Japan	Kochi	33.720	133.550	1952-60	Xenodiagnosis	Asanuma, K.	Okubo, K.	Determination of the vector mites of scrub typhus in Japan	Jap. J. Med. Sci. Biol.	1962	
Trombiculindus variaculum	Thailand	Chiang Rai	20.246	99.647	Nov-16	47 kDa RT PCR	This project					
Walchia chinensis	China	Fujian	24.440	118.330	1999-00	Nested PCR	Wang, H.C.	Chung, C.L.	Studies on the vectors and pathogens of scrub typhus on murine-like animals in Kinmen Country, Taiwan	Formosan Entomol.	2004	
Walchia chinensis	China	Fujian	25.544	118.760	1997-98	Nested PCR	Yan, Y.S.	Zheng, J.	Detection of Orienta tsutsugamushi in chigger mites collected in Fujian coastal and mountain areas in recent years.	Chinese Journal of Zoonoses	1999	
Walchia chinensis	China	Zhejiang	29.180	120.089	?	?	Fan, M.Y.	Walker, D.H.	Epidemiology and ecology of rickettsial diseases in the People's Republic of China	Rev. Infect. Dis.	1987	
Walchia chinensis	China	Hainan	16.330	112.026	2000	PCR (56kDa)	Wang, S.S.	Zhan, D.C.	Sequence analysis of Orientia tsutsugamushi DNA from mites collected in Xisa archipelago, China	Southeast Asian J. Trop. Med. Public Health	2002	
Walchia kritochaeta	Thailand	Chiang Rai	20.246	99.647	2016-17	47 kDa RT PCR	This project					
Walchia kritochaeta	Thailand	Chiang Rai	20.026	99.760	2016-17	47 kDa RT PCR	This project					
Walchia kritochaeta	Thailand	Nan	19.143	100.714	Nov-16	47 kDa RT PCR	This project					
Walchia micropelta	Thailand	Chiang Rai	20.045	99.952	Nov-16	47 kDa RT PCR	This project					
Walchia micropelta	Thailand	Nan	19.143	100.714	Nov-16	47 kDa RT PCR	This project					
Walchia minuscuta	Thailand	Nan	19.132	100.722	Apr-17	47 kDa RT PCR	This project					
Walchia pacifica	China	Shandong	35.266	117.977	1995-03	Xendiagnosis & PCR	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents,	Cell Biochem. Biophys.	2013	

									and chiggers from a New Endemic focus of Northern China			
Walchia pacifica	China	Shandong	35.266	117.977	1995-03	PCR (56kDa)	Liu, Y.	Jia, N.	Consistency of the key genotypes of Orientia tsutsugamushi in scrub typhus patients, rodents, and chiggers from a New Endemic focus of Northern China	Cell Biochem. Biophys.	2013	
Walchia pacifica	China	Shandong	35.286	118.185	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Isolation of Rickettsia tsutsugamushi from Trombiculid mites (Acari: Trombiculidae) in Feixian county, Shandong province, China	Entomologia Sinica	1999	
Walchia pacifica	China	Shandong	35.362	118.090	1995-96	Xenodiagnosis & indirect IF	Liu, Y.	Yang, Z.	Epidemiological study of autumn-winter type scrub typhus in a new endemic focus of Fei County, Shandong Province, China	Syst. Appl. Acarol.	2000	
Walchia pacifica	China	Shandong	35.879	117.928	?	Xendiagnosis & PCR	Liu, Y.	Zhao, Z.	Characterization of Orientia tsutsugamushi strains isolated in Shandong Province, China by immunofluorescence and restriction fragment length polymorphism (RFLP) analyses	Southeast Asian J. Trop. Med. Public Health	2004	
Walchia sp.	Thailand	Chiang Rai	20.044	99.959	Nov-16	47 kDa RT PCR	This project					

8.4 APPENDIX B-1

Guide to small mammals trapped during fieldwork in Northern Thailand and Laos, 2015-18

Bandicota indica (Bechstein, 1800)

Figure 1: Greater Bandicoot Rat

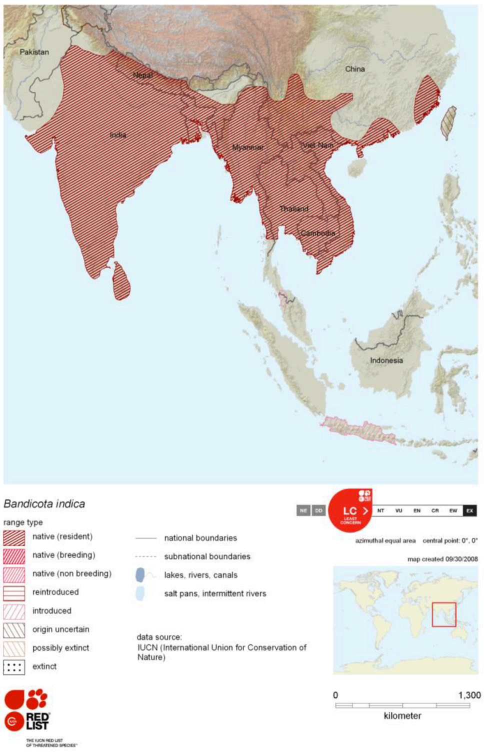
46 individuals trapped from 5 sites in Chiang Rai & Nan Provinces, Thailand & Vientiane Province, Laos.

Identification

- Large size (mean total length 482mm & weight 476g)
- Typical dorsal guard hairs
- Tail shorter than head & body
- Hind foot very large

Ecology

- Lowland rice paddies and cultivated areas, typically near water
- Terrestrial, burrowing, good swimmer, aggressive nature
- Feeds on plant material and invertebrates
- Frequent human food source



Berylmys berdmorei (Blyth, 1851)

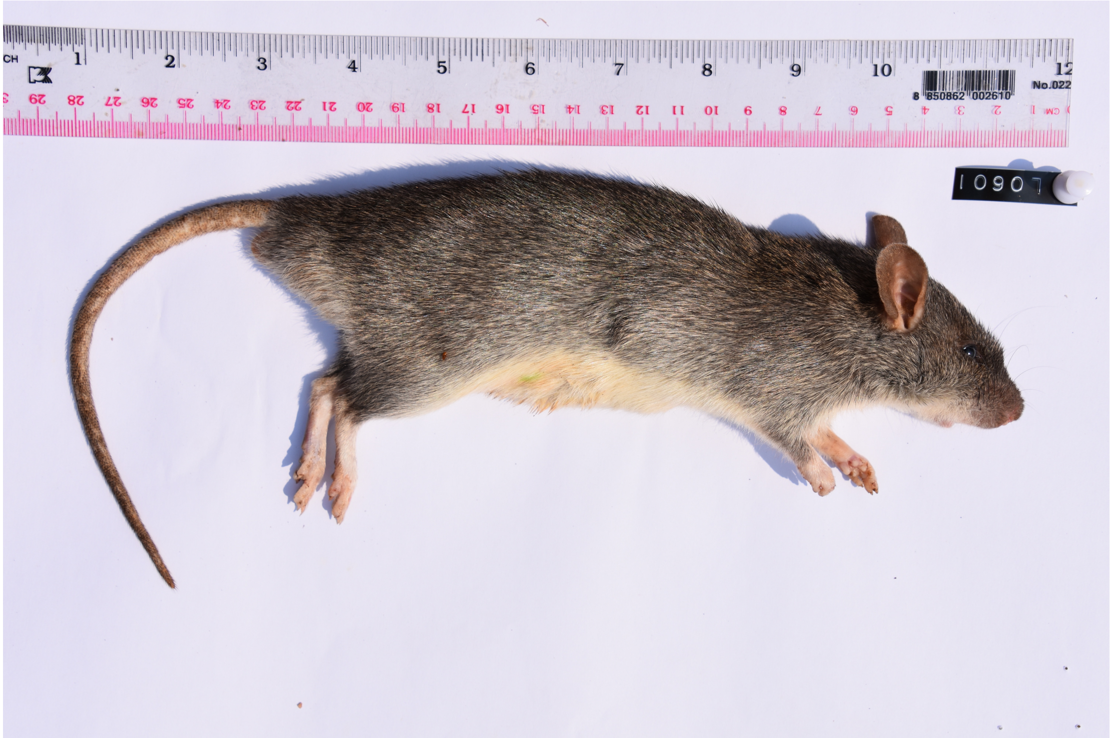


Figure 2: Berdmore's Berylmys or Small White-toothed Rat

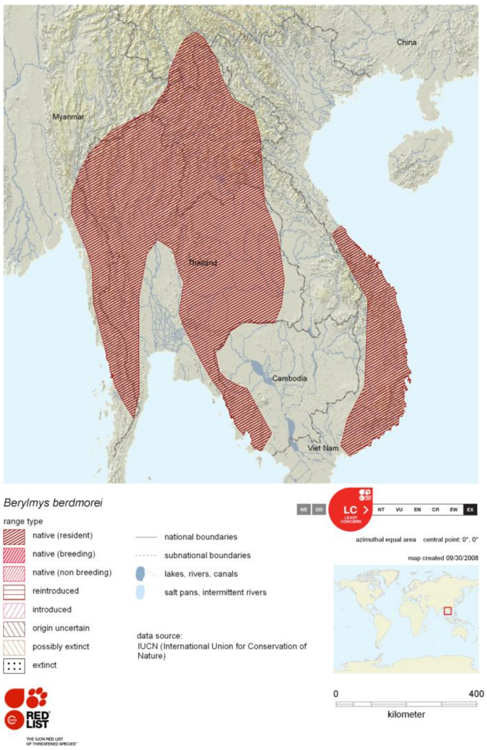
7 individuals trapped from 3 sites in Chiang Rai & Nan Provinces, Thailand & Vientiane Province, Laos.

Identification

- Medium size (mean total length 367mm & weight 215g)
- Silver grey dorsum with pure white ventrum
- Tail 20% shorter than head & body
- Hind foot whitish-grey

Ecology

- Upland forest, bamboo groves, plantations and cultivated areas
- Terrestrial, burrowing
- Quiet, passive nature
- Feeds on plant material and invertebrates



Berylmys bowersi (Anderson, 1879)

Figure 3: Bower's berylmys or Large white-toothed rat

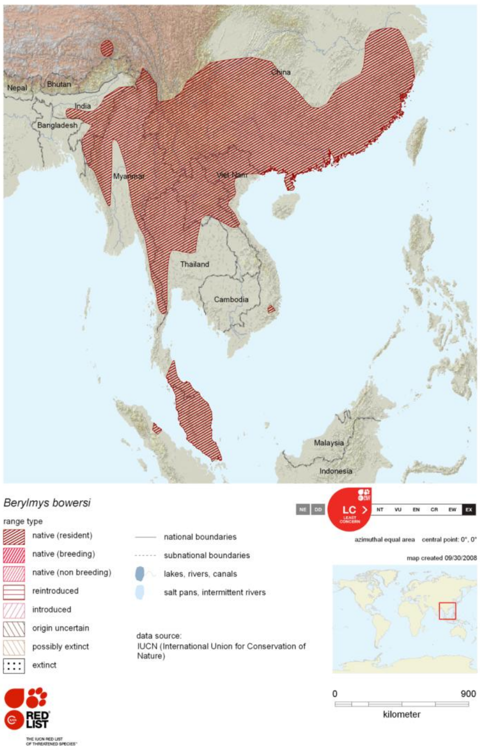
5 individuals trapped from 3 sites in Chiang Rai & Nan Provinces, Thailand.

Identification

- Large size (mean total length 518mm & weight 366g)
- Grey dorsum, flat spiney hairs & pure white ventrum
- 5% longer tail than head & body and paler beneath
- Hind foot white with grey stripe

Ecology

- Disturbed scrub habitat, cultivated land and various types of forest
- Quiet, passive nature
- Feeds on plant matter, fruit etc.
- Human food source



Leopoldamys edwardsi (Thomas, 1882)

Figure 4: Edward's Long-tailed Giant Rat

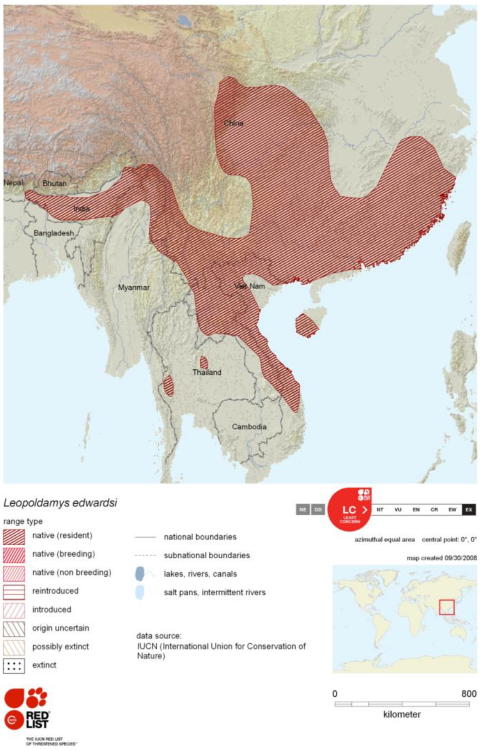
5 individuals trapped from 2 sites in Nan Province, Thailand & Vientiane Province, Laos.

Identification

- Large size (mean total length 572mm & weight 317g)
- Reddish-brown dorsum, creamy white ventrum
- Very long tail, darker above and white beneath
- Hind foot long with brown stripe

Ecology

- Montane, evergreen, mixed deciduous and disturbed forest
- Semi-terrestrial, lives in burrows
- Human food source



Maxomys surifer (Miller, 1900)

Figure 5: Indomalayan Maxomys or Red Spiny Rat

15 individuals trapped from 3 sites in Chiang Rai & Nan Provinces, Thailand & Vientiane Province, Laos.

Identification

- Medium size (mean total length 359mm & weight 144g)
- Reddish-brown dorsum, creamy white ventrum, spiny fur
- Tail similar to head & body, darker above and white beneath
- Hind foot long and narrow

Ecology

- Bamboo, evergreen and deciduous forest, edge habitat
- Mostly terrestrial
- Fruit, roots, insects, small vertebrates

Mus caroli (Bonhote, 1902)

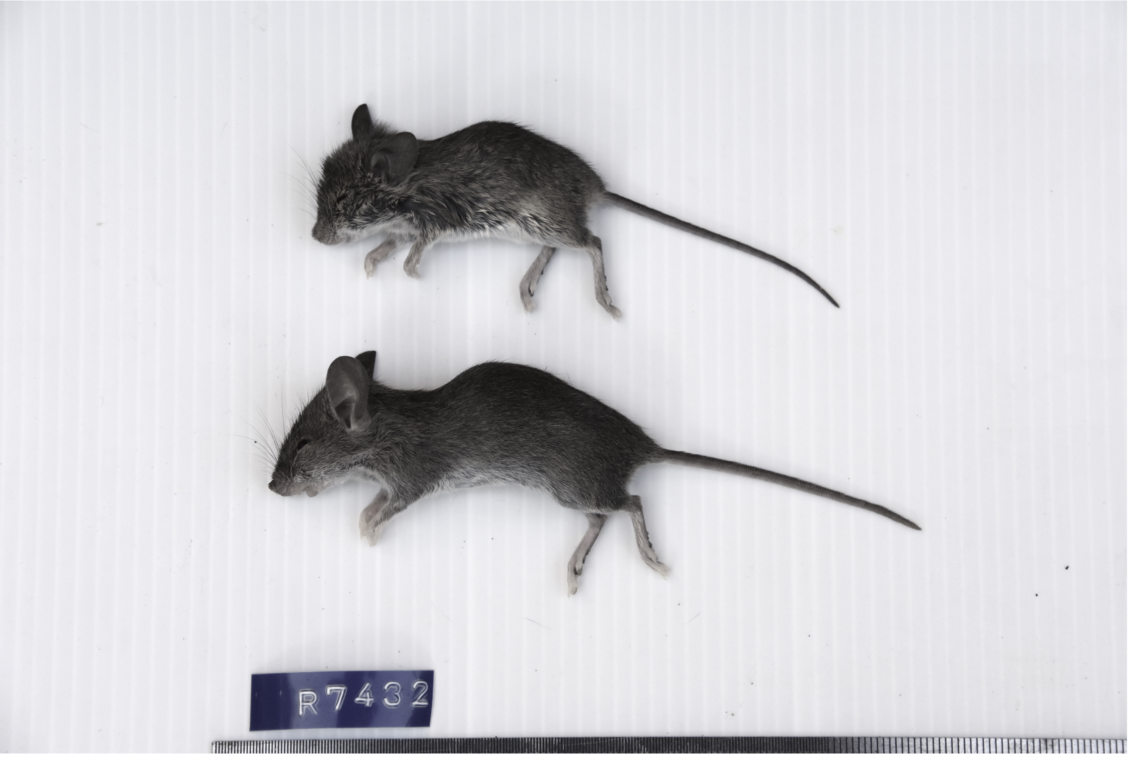


Figure 6: Ryukyu Mouse or Ricefield Mouse

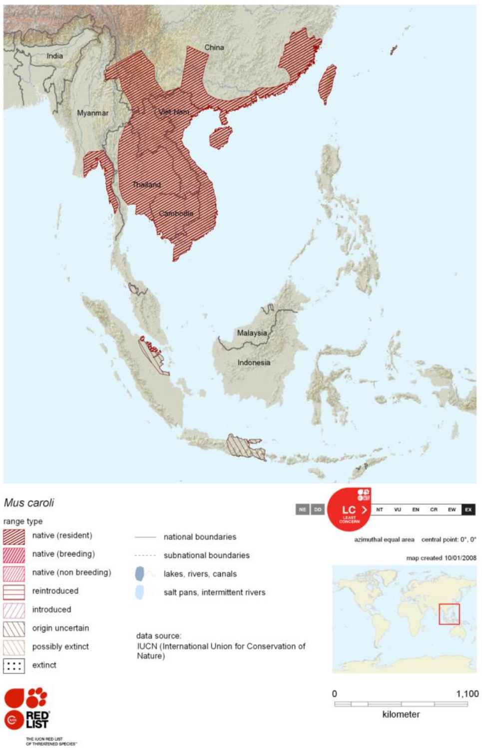
6 individuals trapped from 3 sites in Chiang Rai Province, Thailand & Vientiane Province, Laos.

Identification

- Small size (mean total length 159mm & weight 14g)
- Grey-brown dorsum & grey-white ventrum, large ears
- Tail similar to head & body, darker above and white beneath
- Hind foot large

Ecology

- Scrub & grassland, secondary growth, cultivation & paddy fields
- Terrestrial
- Seeds & invertebrates



Mus cookii (Ryley, 1914)

Figure 7: Cook's Mouse

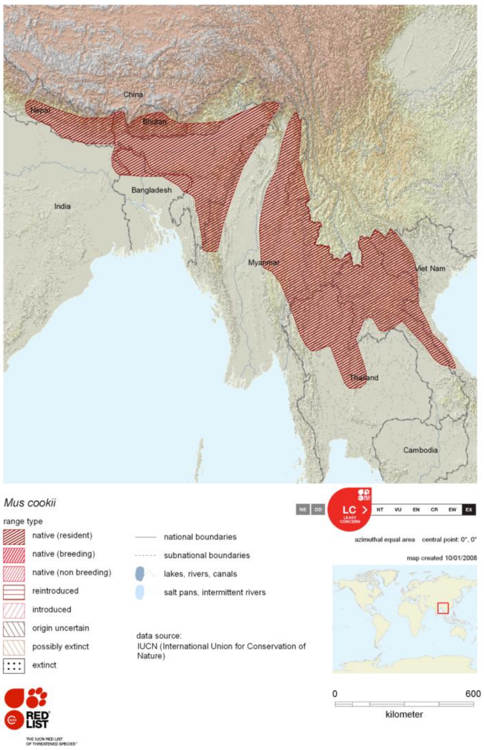
10 individuals trapped from 3 sites in Chiang Rai Province, Thailand.

Identification

- Medium size mouse (mean total length 172mm & weight 22g)
- Grey-brown dorsum & grey-white ventrum, large ears
- Tail slightly shorter than body length
- Hind foot large, light brown

Ecology

- Dry cultivation including rice, grasslands & various forest types
- Terrestrial



Mus cervicolor (Hodgson, 1845)

Figure 8: Fawn-coloured Mouse

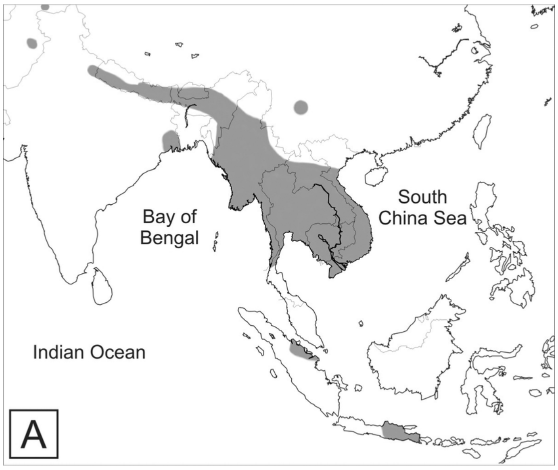
2 individuals trapped from 1 site in Vientiane Province, Laos.

Identification

- Small size (mean total length 147mm & weight 17g)
- Brown dorsum & grey-white ventrum, large ears
- Tail shorter than head & body, darker above and white beneath
- Hind foot white above

Ecology

- Very wide range of habitats including cultivated areas
- Terrestrial
- Seeds & insects



Niviventer langbianis (Robinson & Kloss, 1922)

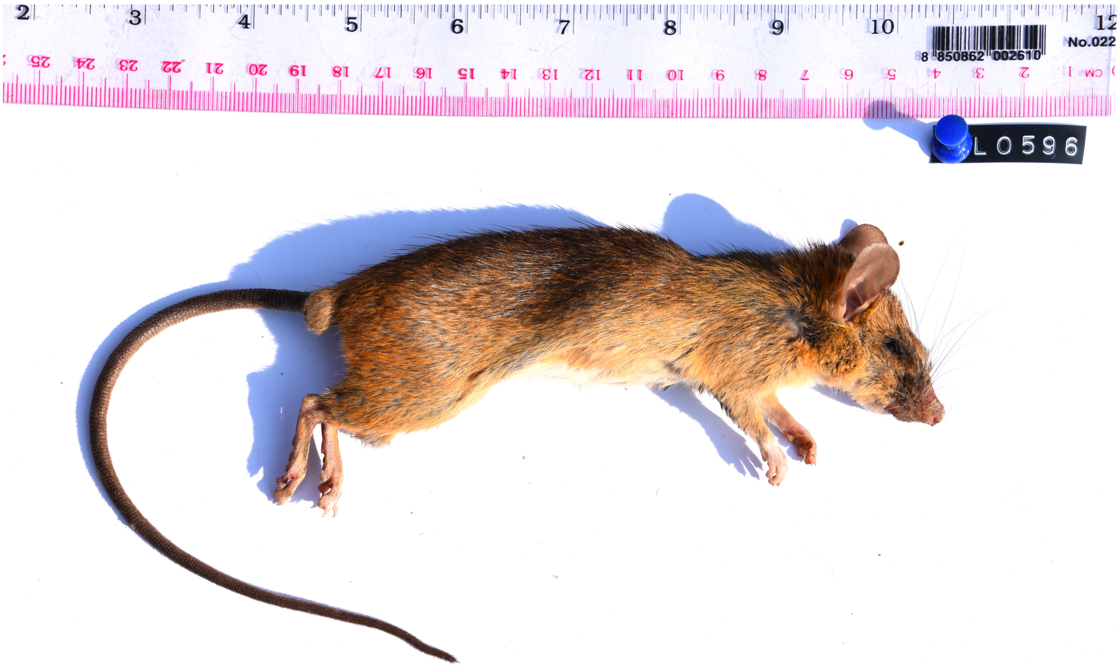


Figure 9: Indochinese arboreal Niviventer or Lang Bian White-bellied Rat

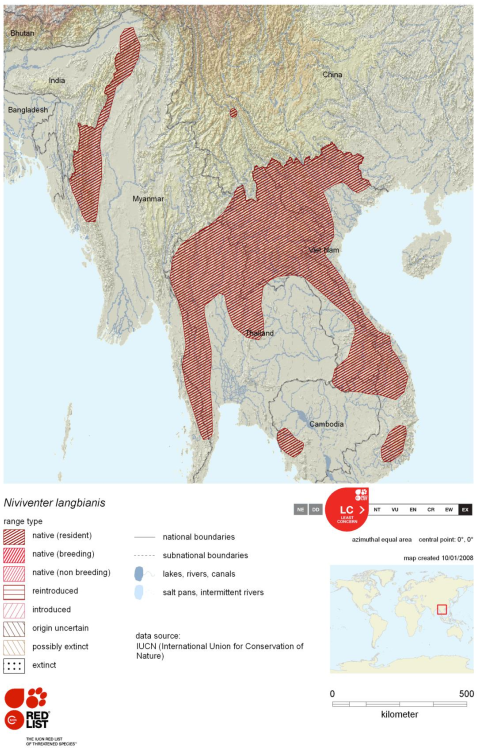
1 individual trapped from 1 site in Vientiane Province, Laos.

Identification

- Medium size (mean total length 310mm & weight 75g)
- Brownish grey with yellow-orange tinge, pale ventrum, larger ears than other Niviventer
- Tail longer than head & body, uniformly dark
- Hind foot short and broad

Ecology

- Tropical evergreen, mixed deciduous forest and lightly disturbed forest
- Mainly arboreal



Niviventer fulvescens (Gray, 1867)

Figure 10: Indomalayn Niviventer or Chestnut White-bellied Rat

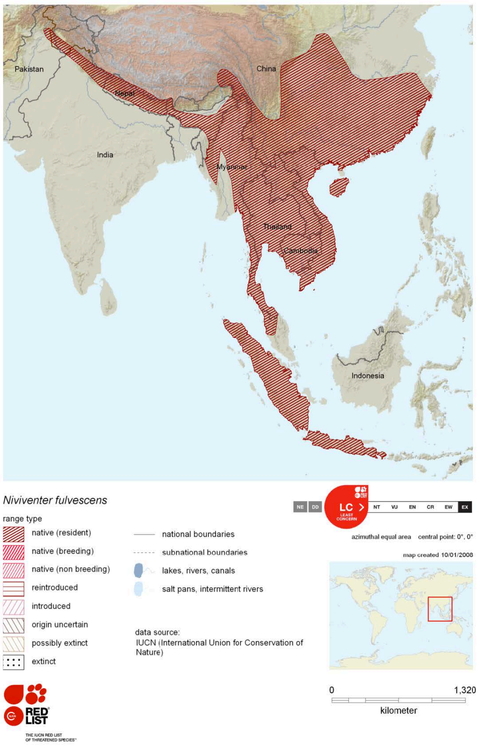
13 individuals trapped from 4 sites in Chiang Rai & Nan Provinces, Thailand & Vientiane Province, Laos.

Identification

- Medium size (mean total length 300mm & weight 74g)
- Red-brown with yellow tinge, white-cream ventrum, spiny hairs
- Tail longer than head & body, dark above, pale below
- Hind foot long & slender

Ecology

- Wide variety of forest habitats, & forest edge near cultivation
- Semi-arboreal



Rattus nitidus (Hodgson, 1845)

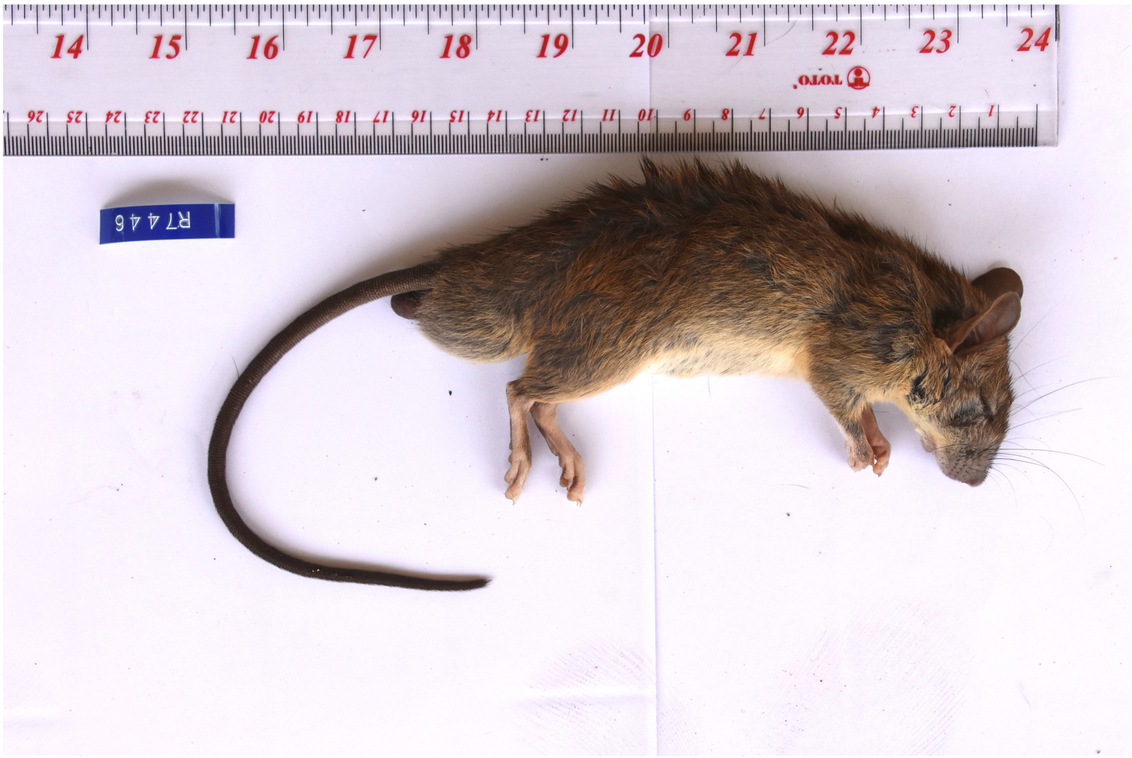


Figure 11: Himalayan Field Rat

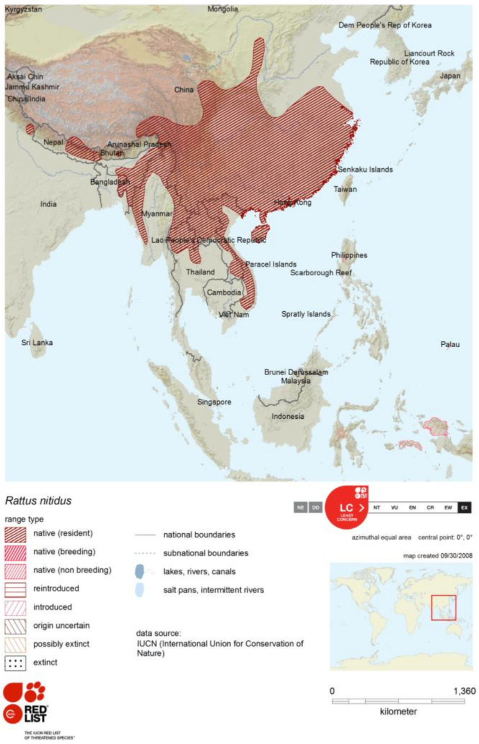
10 individuals trapped from 3 sites in Chiang Rai Province, Thailand.

Identification

- Medium size (mean total length 296mm & weight 106g)
- Bromnish grey dorsum & dull grey ventrum
- Tail equal to head & body, uniformly dark
- Hind foot pearly white

Ecology

- Various forest habitats, cropland and human settlements
- Terrestrial
- Can be agricultural pest



Rattus exulans (Peale, 1848)

Figure 12: Polynesian or Pacific Rat

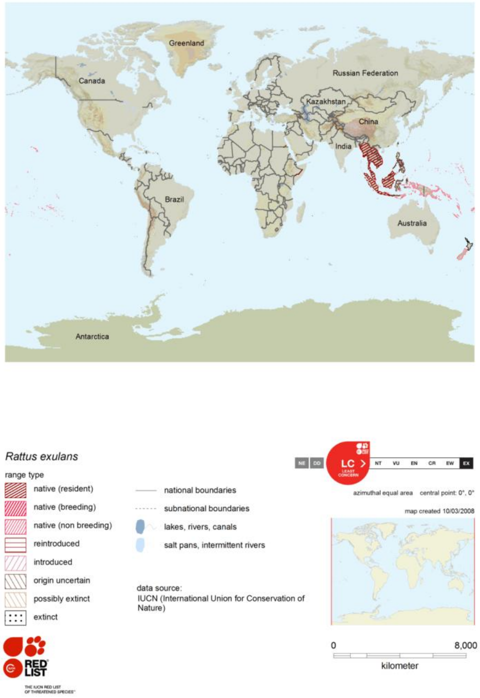
25 individuals trapped from 6 sites in Chiang Rai & Nan Provinces, Thailand & Vientiane Province, Laos.

Identification

- Smallest Rattus (mean total length 241mm & weight 36g)
- Grey-olive-brown dorsum, pale grey ventrum, spiny fur
- Tail 10% longer than head & body, uniformly dark
- Hind foot pale above

Ecology

- Close associated with human settlements and nearby cultivation
- Semi-terrestrial
- Eats plant & animal material



Rattus tanezumi (Temminck, 1844)

Figure 13: Oriental House Rat

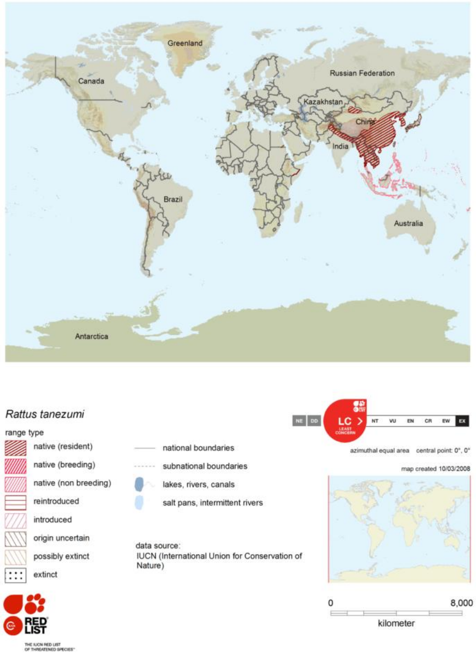
57 individuals trapped from 9 sites in Chiang Rai & Nan Provinces, Thailand.

Identification

- Medium size (mean total length 347mm & weight 118g)
- Grey-brown with reddish tinge, cream ventrum, large ears
- Tail equal to head & body, uniformly dark
- Hind foot grey-white

Ecology

- Ubiquitous and highly adaptable
- Terrestrial
- Eats plant & animal material



Rattus losea (Swinhoe, 1871)

Figure 14: Losea Rat or Lesser Rice-field Rat

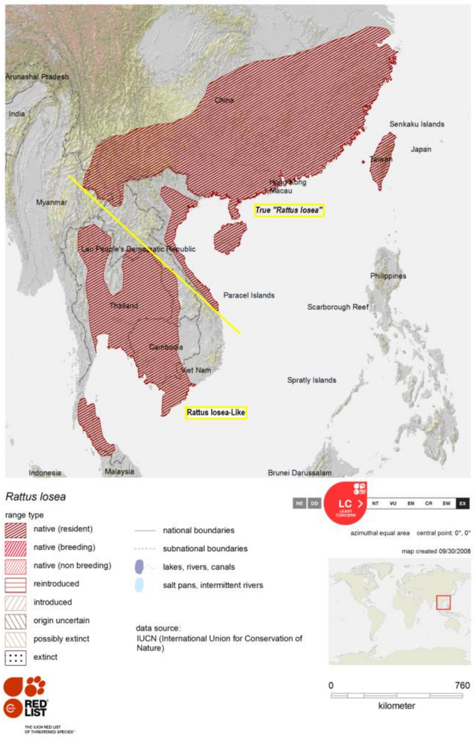
5 individuals trapped from 1 sites in Vientiane Province, Laos.

Identification

- Medium size (mean total length 284mm & weight 87g)
- Brownish-grey dorsum, white ventrum with grey under fur, small ears
- Tail shorter than head & body, uniformly dark
- Hind foot flesh coloured

Ecology

- Scrub, grass, plantations and forest edge, often close to paddy fields
- Terrestrial
- Can be agricultural pest



Rattus andamanensis (Blyth, 1860)

Figure 15: Indochinese Forest Rat or Sikkim Rat

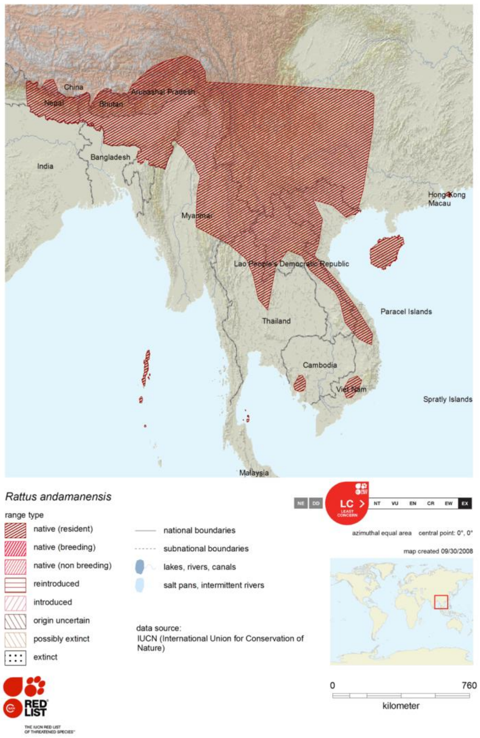
23 individuals trapped from 2 sites in Chiang Rai Province, Thailand & Vientiane Province, Laos.

Identification

- Medium size (mean total length 375mm & weight 115g)
- Dark brown dorsum, cream ventrum, long guard hairs
- Tail longer than head & body, uniformly dark
- Hind foot whitish above

Ecology

- Dry evergreen, deciduous and bamboo forest, cultivation and close to human habitation
- Arboreal



Menetes berdmorei (Blyth, 1849)

Figure 16: Indochinese Ground Squirrel

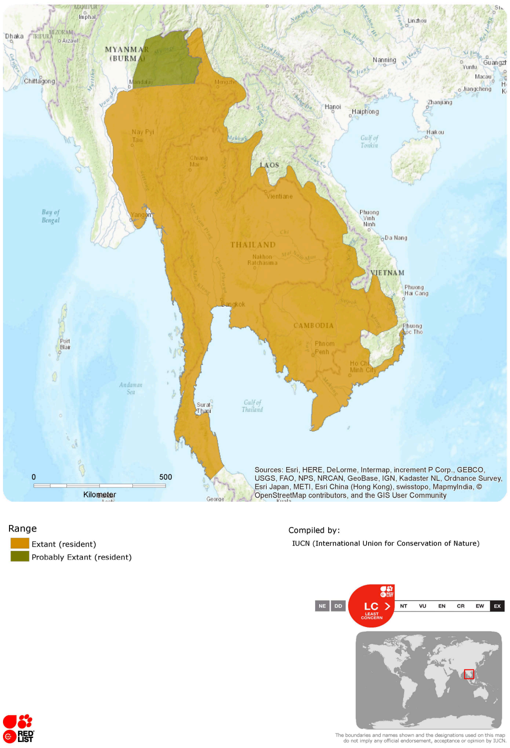
4 individuals trapped from 3 sites in Chiang Rai Province.

Identification

- Medium size (mean total length 350mm & weight 190g)
- Grey-brown dorsum, white ventrum and distinctive beige and black stripes along flanks
- Tail shorter than head & body
- Hind foot whitish above

Ecology

- Various forest types, forest edge and cultivation
- Mainly terrestrial



Tupaia glis/belangeri (Diard, 1820)

Figure 17: Common Tree Shrew [Species complex with *T. belangeri*]

7 individuals trapped from 4 sites in Chiang Rai Province & Vientiane Province, Laos.

Identification

- Medium size (mean total length 360mm & weight 142g)
- Dark brown dorsum, small, pale shoulder stripe, orange-rufous ventrum, small ears, long snout
- Tail shorter than head & body
- Hind foot large, brown above, sharp claws

Ecology

- Primary & secondary forest, tolerates habitat disturbance, cultivation & near settlements
- Semi-terrestrial
- Insects, eggs, fruit & leaves

Suncus murinus (Linnaeus, 1766)

Figure 18: Asian House Shrew

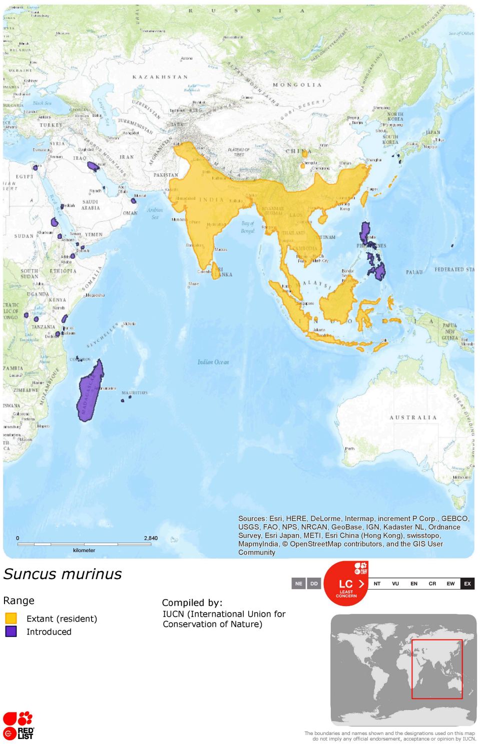
2 individuals trapped from 1 site in Chiang Rai Province.

Identification

- Medium size (mean total length ~180mm with large variation)
- Grey-black, short, velvety fur, long pointy snout, typical strong musky odour
- Tail shorter than head & body
- Hind foot grey-black

Ecology

- Wide range of habitats from forest to scrubland, cultivation and urban settlements
- Terrestrial
- Invertebrates



Callosciurus erythraeus (Pallas, 1779)

Figure 19: Pallas's Squirrel

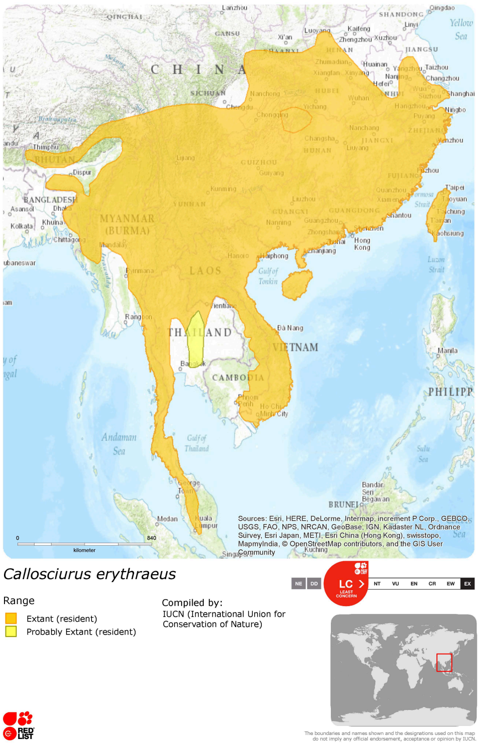
1 individual trapped from 1 site in Chiang Rai Province.

Identification

- Medium size (mean total length 450mm)
- Speckled olive-brown dorsum, ventrum reddish-brown. Highly variable.
- Tail longer than head & body
- Hind foot large, strong claws

Ecology

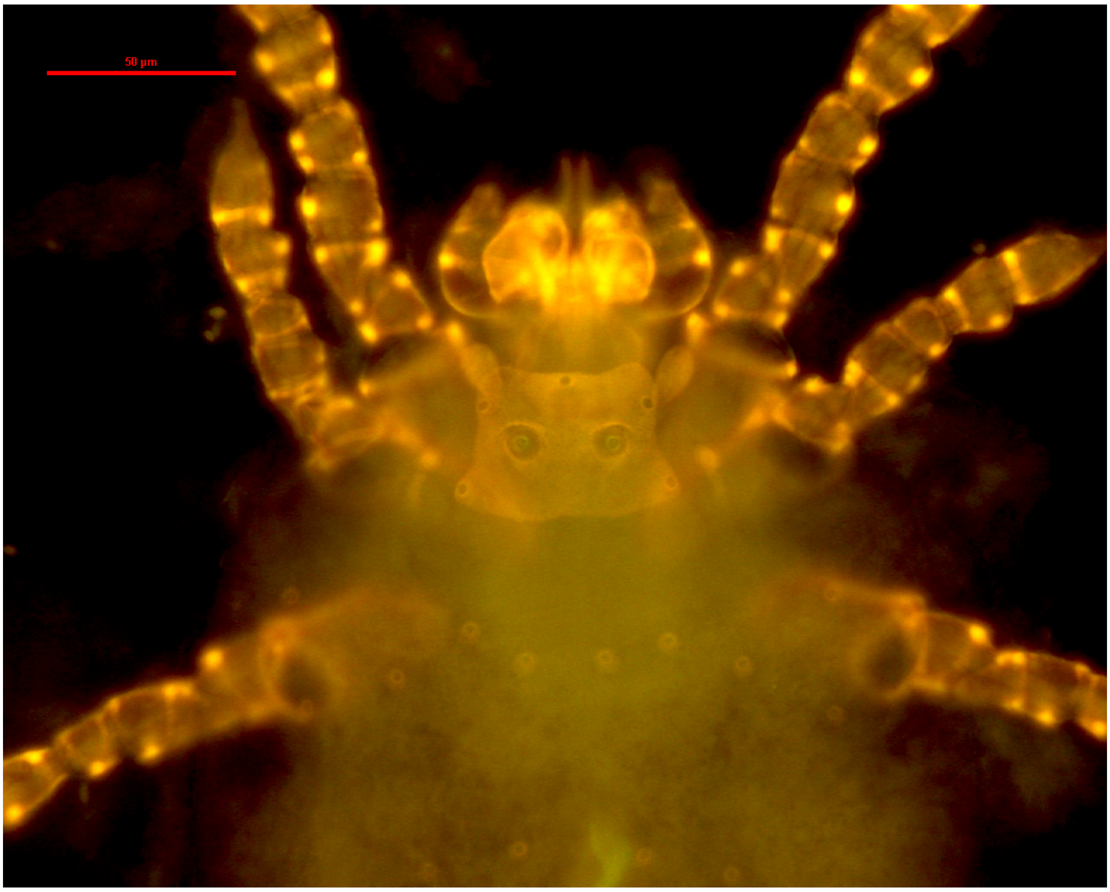
- Wide range of forest types and degraded habitats
- Arboreal
- Leaves, fruit, insects, seeds and nuts



8.5 APPENDIX B-2

Guide to chigger species collected during fieldwork in Northern Thailand and Laos, 2015-18

Ascoschoengastia indica (Hirst, 1915)



Taxonomy
Trombiculinae > Schoengastiini > Ascoschoengastia

Synonyms
Schoengastia indica, Euschoengastia indica, Neoschoengastia indica, Laurentella indica, Trombicula indica

Distribution

- India & Sri Lanka to Thailand, Taiwan, Australia and Japan.
- Collected from wide range of habitats at 8/12 sites

Hosts (no. of chiggers/positives)
Leopoldamys edwardsi (2/1), Maxomys surifer (1), Rattus andamanensis (26), R. exulans (17), R. nitidus (3), Rattus sp. (2), R. tanezumi (13/1), Tupaia glis (3)

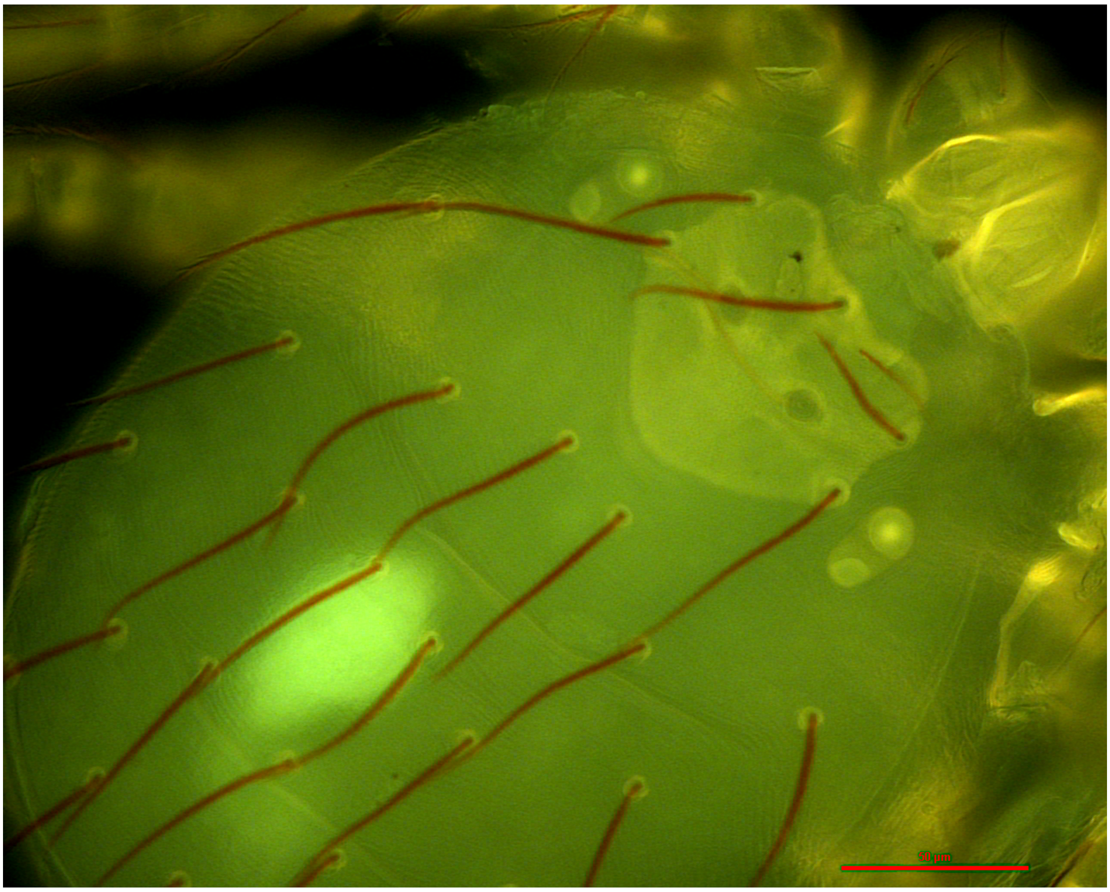
Key identification features

- Small-medium size
- Trapezoidal scutum, lightly punctate
- Anterolateral shoulders
- Sensilla setae claviform
- Cheliceral with sub-apical tooth
- Coxae conspicuously punctate
- Leg 1 with 3 genualae

Reference keys

- Audy, 1956
- Vercammen-Grandjean, 1968
- Chung, 2015

Blankaartia acuscutellaris (Walch, 1922)



Taxonomy
Trombiculinae > Trombiculini > Blankaartia

Synonyms
Trombicula acuscutellaris, Tragardhula acuscutellaris

Distribution

- India & Sri Lanka to Thailand, Taiwan, Australia and Japan.
- Collected only from rice fields, Phonhong, Laos

Hosts (no. of chiggers/positives)
Rattus losea (2)

Key identification features

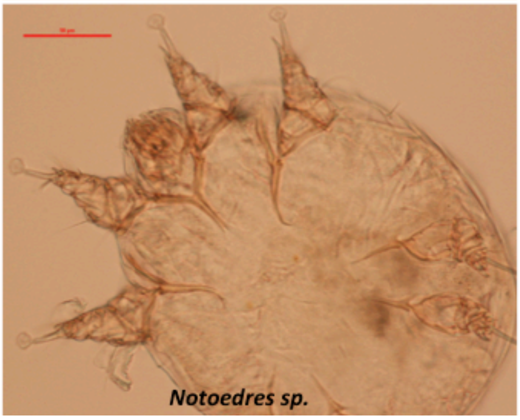
- Red colour
- Pentagonal scutum
- Anterolateral shoulders
- Sensilla setae flagelliform
- Distinct eyes
- Coxae striate punctate
- Ip ~1000 μm

Reference keys

- Nadchatram, 1974
- Vercammen-Grandjean, 1968
- Fernandes, 2003

Acariformes (non-chiggers)


Cheyletus sp.


Notoedres sp.


Laelapidae sp.

Taxonomy

Trombidiformes >Cheyletidae
Sarcoptidae > Notoedres (mange mite)
Dermanyssoidae >Laelapidae

Distribution

- Not known
- Collected from forest and cultivation areas at Ban Song Kwair, Ban Mae Mon & Ban Thoet Thai

Hosts (no. of chiggers/positives)

Menetes berdmorei (Cheyletus) (3)
Bandicota indica (2), Berylmys bowersi (2), Callosciurus erythraeus (2), M. berdmorei (Laelapidae) (1)
Rattus tanezumi (Notoedres) (1)

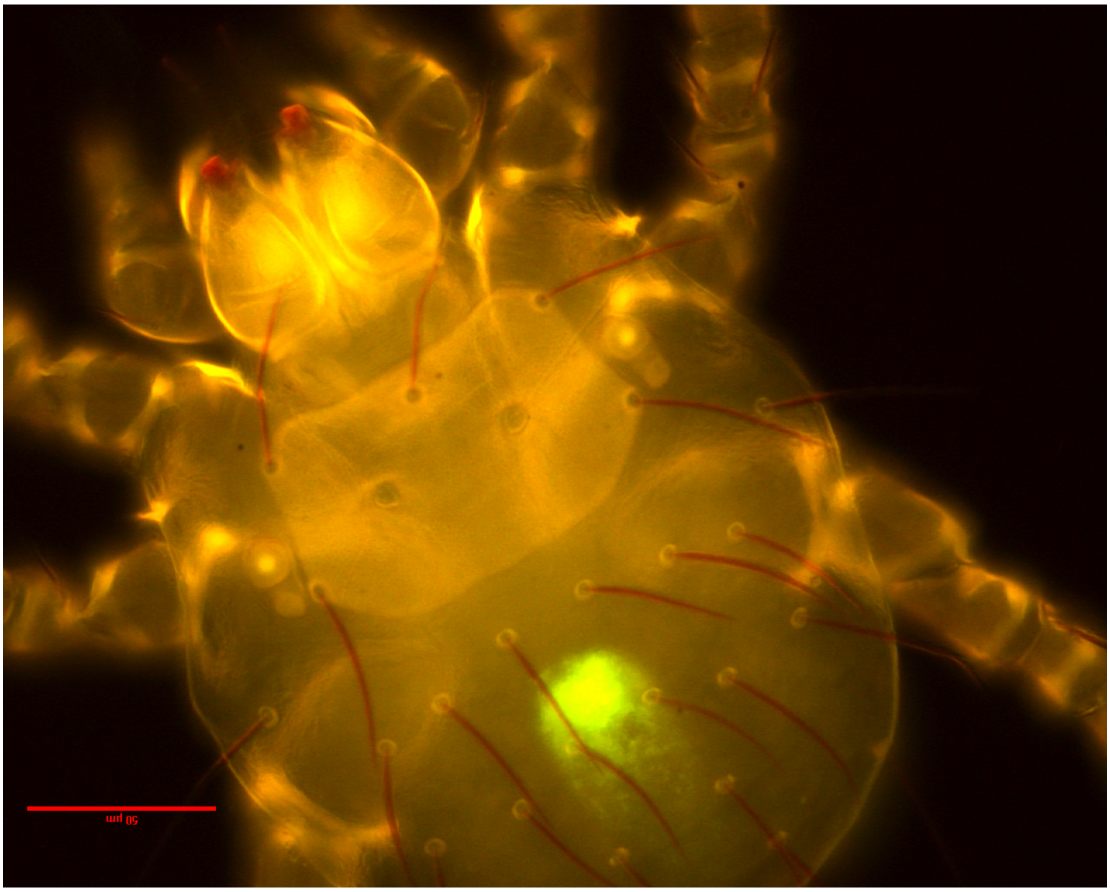
Key identification features

- Cheyletus species have large claws for hunting other mites
- More than 1 Laelapidae species found

Reference keys

- Krantz & Walter, 2009
- Klompen, 1992

Eutrombicula wichmanni (Oudemans, 1905)



Taxonomy
Trombiculinae > Trombiculini > Eutrombicula

Synonyms
Trombicula wichmanni

Distribution

- China, Indonesia, Japan, Malaysia, Papua New Guinea, Philippines
- Collected from edge of rice fields (7) (Feuang, Laos), Palm oil plantation, Laos (1), overgrown weedy areas, Huay Mae Sai (1) & Ban Song Kwair (1), Chiang Rai.

Hosts (no. of chiggers/positives)
All collected by black plate (10)

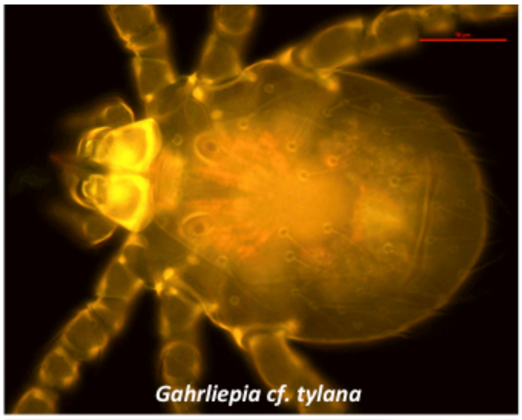
Key identification features

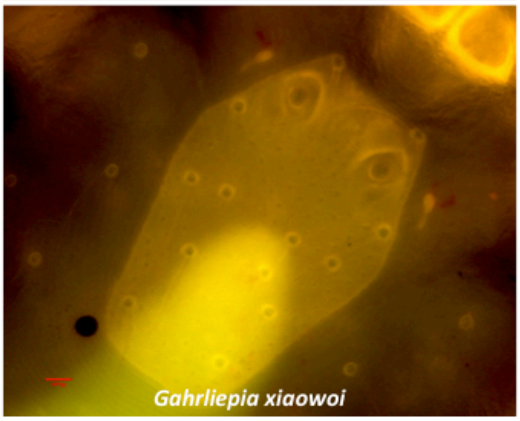
- Medium-large chigger
- Reddish colour
- Large rectangular scutum
- Indistinct anterolateral shoulders
- Distinct eyes
- Sensilla setae flagelliform
- Palpal claw 2-pronged
- NDV 32

Reference keys

- Fuller, 1952
- Vercammen-Grandjean, 1968

Garhrliepia elbeli, G. tenella, G. tylana (Traub & Morrow, 1955), *G. xiaowoi* (Wen & Xiang, 1984)





Taxonomy

Trombiculidae > Gahrliepiini > Gahrliepia sp.

Synonyms
Typhlothrombidium sp., Gateria sp.

Distribution

- China, Myanmar, Thailand
- Collected from 4 sites in Chiang Rai & Nan Provinces

Hosts (no. of chiggers/positives)
Berylmys berdmorei (1 G. tenella)
Berylmys bowersi (1/1) G. elbeli)
Rattus tanezumi, Tupaia glis (2 G. cf. xiaowoi)
Bandicota indica (1 G. cf. tylana)

Black plate: *G. (cf.) xiaowoi* (14/1)

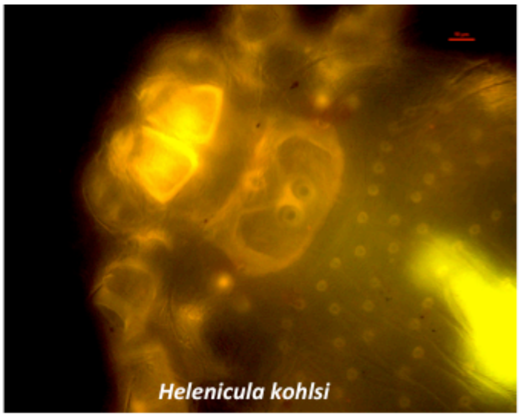
Key identification features

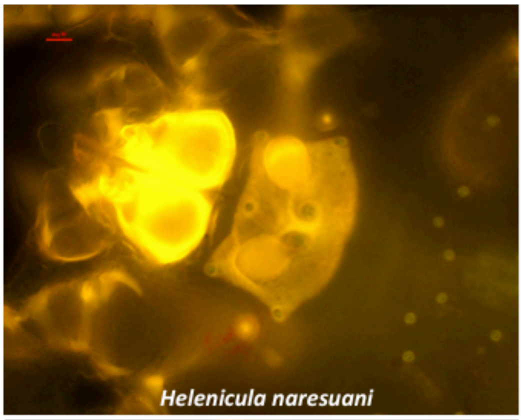
- Medium to large size
- Markedly elongate scutum, widened and punctate or scrobiculate
- 2 or more post-posterolateral setae
- Sensillae clavate
- Eyes usually present

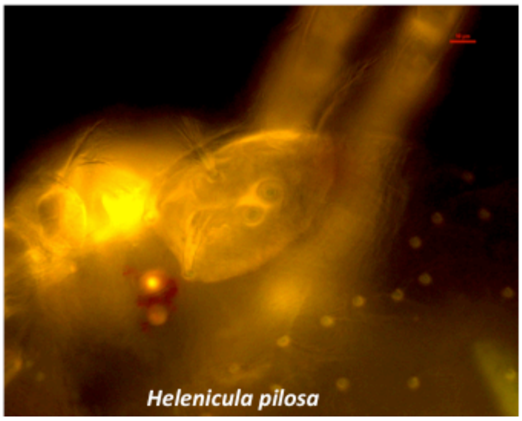
Reference keys

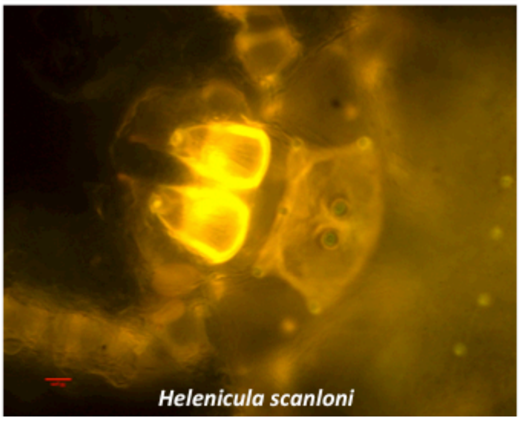
- Traub & Morrow, 1955
- Wen & Xiang, 1984

Helenicula kohlsi (Philip & Woodward, 1946), *H. naresuani* n. sp. (Stekolnikov, 2016), *H. pilosa* (Abonnenc & Taufflieb, 1957), *H. scanloni* (Domrow & Nadchatram, 1964)









Taxonomy

Trombiculinae > Schoengastiini > Helenicula

Synonyms

Euschoengastia sp., Trombidium sp. Globuraloschoengastia sp., Neoschoengastia sp., Schoengastia sp.

Distribution

- Nepal, Thailand, China & Vietna
- Collected from forest & forest edge at 4 sites

Hosts (no. of chiggers/positives)

Rattus exulans, R. nitidus, R. tanezumi (***H. kohlsi*** *3*)
Berylmys bermorei, B. bowersi, R. andamanensis, Rattus sp., Suncus murinus, Tupaia glis (***H. naresuani*** *12/1 ex B. bowersi*)
Bandicota indica (***H. pilosa*** *1*)
Menetes berdmorei, Niviventer fulvescens, R. tanezumi, T. glis (***H. scanloni*** *11*)

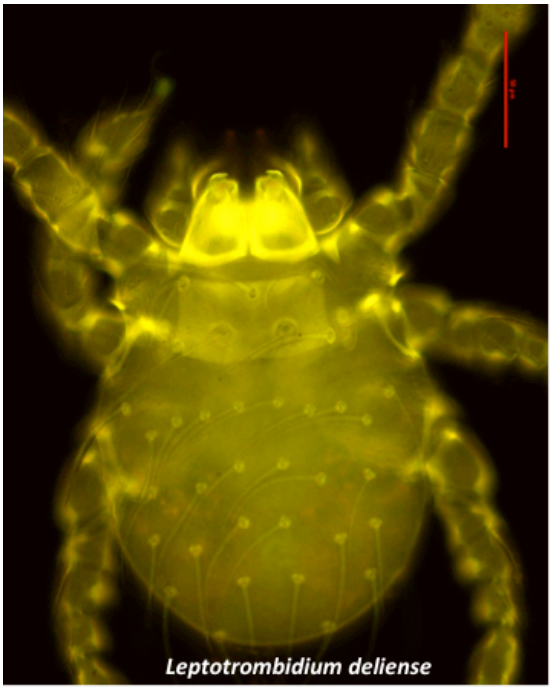
Key identification features

- Medium size
- Rectangular or subquadrate scutum
- Scutum sensillar bases close together – gap less than diameter of either base
- Sensilla setae globose
- Claw 3-pronged

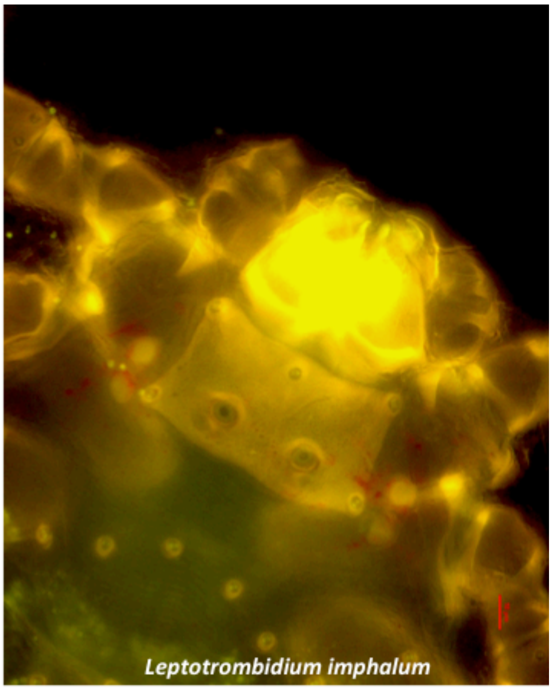
Reference keys

- Nadchatram & Traub, 1971
- Domrow & Nadchatram, 1964

Leptotrombidium deliense (Walch, 1922), *L. imphalum* (Vercammen-Grandjean & Langston, 1976)

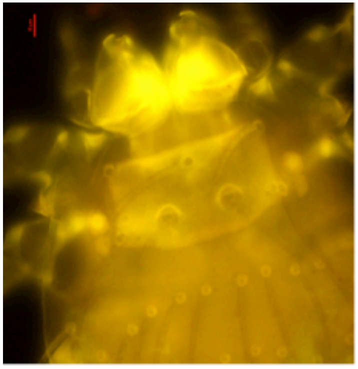


Leptotrombidium deliense

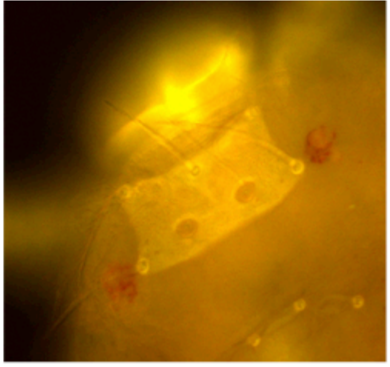


Leptotrombidium imphalum

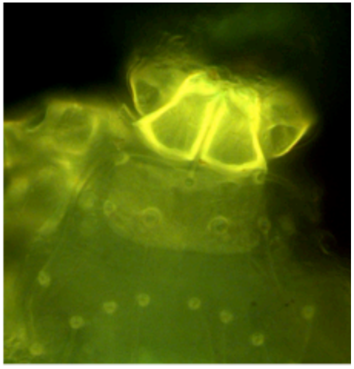
L. turdicola (V-G. & Langston, 1976), *L. harrisoni* (Tanskul & Gingrich, 1986), *L. arvinum* (Schluger, 1960), *L. fletcheri* (Womersley & Heaslip, 1943), *L. dendrium* (Tanskul, 1991), *L. elisbergi* (Traub & Lakshana, 1966)



Leptotrombidium turdicola



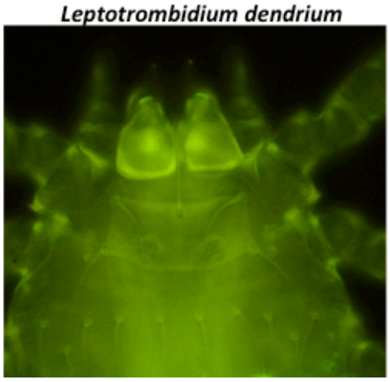
Leptotrombidium harrisoni



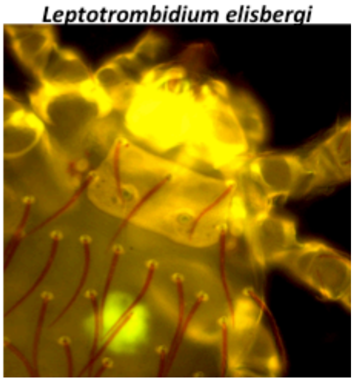
Leptotrombidium arvinum

Leptotrombidium cf. fletcheri

Leptotrombidium dendrium



Leptotrombidium elisbergi



Leptotrombidium spp.

Hosts (no. of chiggers/positives)

*Bandicota indica, Berylmys berdmorei, B. bowersi, Maxomys surifer, Menetes berdmorei, Mus cookii, Niviventer sp., Rattus andamanensis, R. exulans, R. nitidus, R. tanezumi, Suncus murinus (**L. deliense** 117/3: ex B. indica, 6 ex B. berdmorei, 1 ex R. andamanensis, 6 ex R. exulans, 1 ex R. tanezumi)*

*B. indica, Niviventer sp., R. nitidus, R. tanezumi (**L. imphalum** 83/2 ex B. indica, 4 ex R. tanezumi)*

*M. berdmorei, R. tanezumi, T. glis (**L. turdicola** 10)*

*R. tanezumi (**L. harrisoni** 4)*

*S. murinus (**L. arvinum** 1)*

*R. andamanensis (**L. cf. fletcheri** 2)*

*R. tanezumi, S. murinus (**L. elisbergi** 2)*

Blackplate

L. deliense (67/2)
L. dendrium (23)
L. imphalum (7/1)
L. turdicola (4)

Taxonomy

Trombiculinae > Trombiculini > Leptotrombidium

Synonyms

Trombicula sp., Trombidium sp., Mehracula sp., Montivagnum sp., Hsuella sp.

Distribution

- China, India, Myanmar, Malaysia, Maldives, Taiwan, Philippines (ranges vary by species)
- Collected from most habitats at 8/11 sites
- *L. deliense* present at all 8 sites, *L. imphalum* from 3 sites

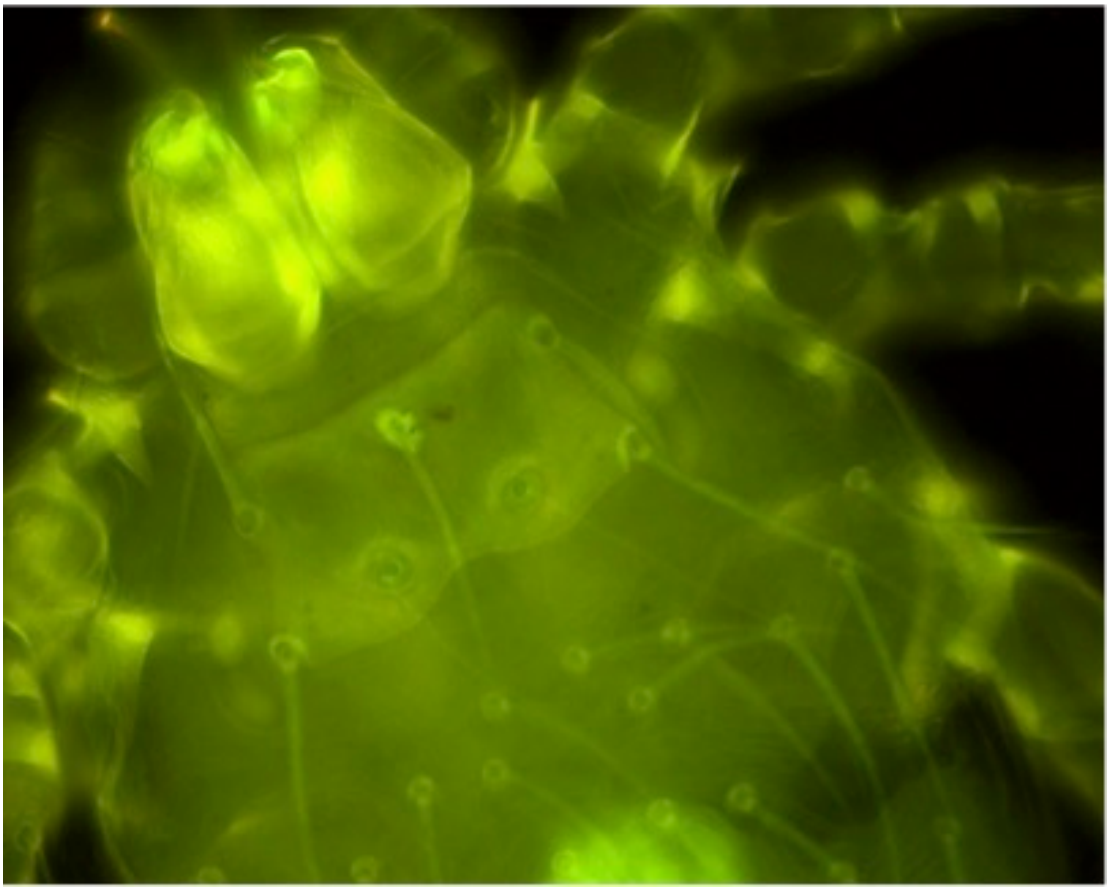
Key identification features

- Small to medium sized
- Subrectangular scutum
- Posterior margin bilobate or straight
- Sensilla flagelliform and branched
- 8-10 setae in 1st dorsal row
- Leg coxae unisetose

Reference keys

- Vercammen-Grandjean & Langston, 1976
- Stekolnikov, 2013

Lorillatum kianjoei (Nadchatram & Traub, 1947)



Taxonomy
Trombiculinae > Trombiculini > Lorillatum

Synonyms
Leptotrombidium kianjoei

Distribution

- Thailand, Laos & Vietnam.
- Collected only from Thoulakhom District, Laos

Hosts (no. of chiggers/positives)
Leopoldamys edwardsi (2), Maxomys surifer (2), Rattus andamanensis (1)

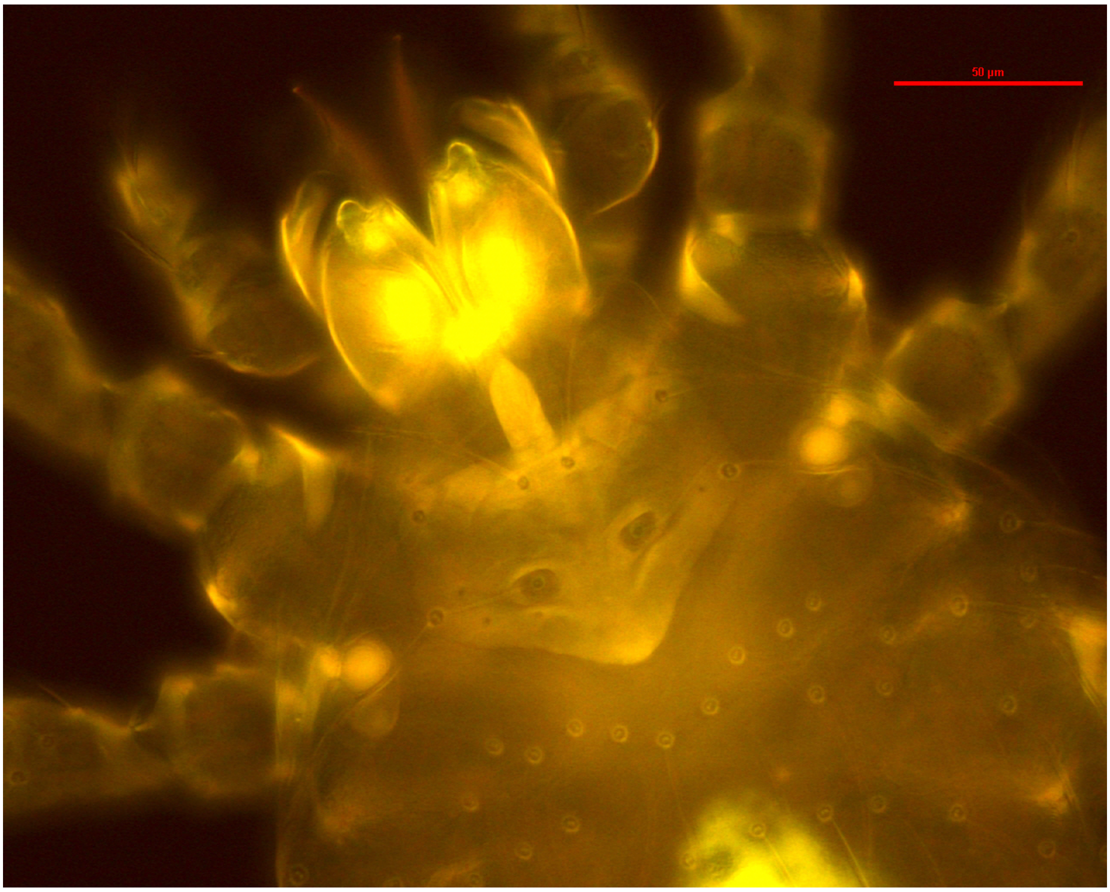
Key identification features

- Rectangular scutum
- Anterolateral shoulders
- Sensilla setae flagelliform
- Posterolateral setae long
- Long whip-like setae on legs
- Barbed palpal femoral setae
- fD = 42-45 (2H.8.8.8.8.4.4.2)
- Ip <900

Reference keys

- Nadchatram & Traub, 1964
- Vercammen-Grandjean & Langston, 1976

Odontacarus audyi (Radford, 1946)



Taxonomy
Leuwenhoekiinae > Leuwenhoekiini > Odontacarus

Synonyms
Actomatacrus audyi, Leogonius audyi

Distribution

- India, Malaysia, Thailand
- Collected only by black plate from Ban Song Kwair, Chiang Rai Province

Hosts (no. of chiggers/positives)
Collected from black plates only. Parasitizes birds.

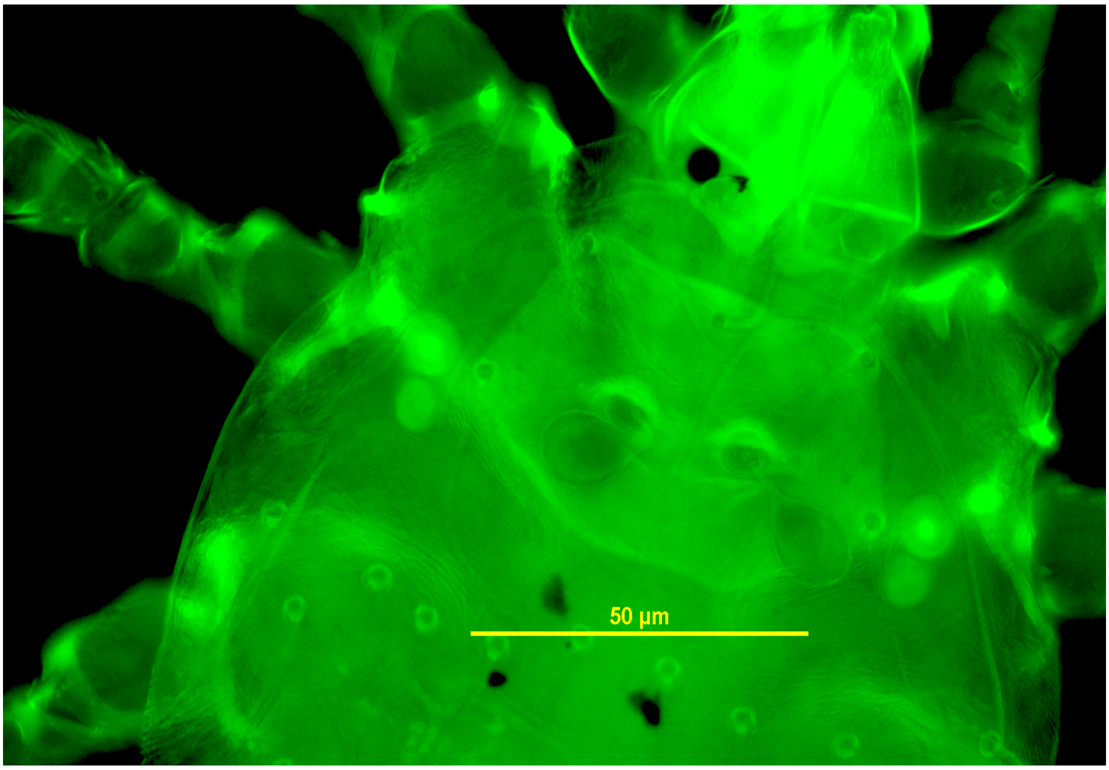
Key identification features

- Orange colour
- Anterior scutal projection "nasus"
- Subpentagonal scutum, with 2 AM setae
- Legs 6-segmented
- fPp = B/B/Bbb. 7B. Ga=B

Reference keys
No full key for genus available

- Nadchatram, 1963
- Fernandes & Kulkarni, 2003

Schoengastia kanhaensis (Mitchell and Nadchatram, 1966)



Taxonomy
Trombiculinae > Schoengastiini > Schoengastia

Synonyms
Radfordiana species

Distribution

- India, Thailand
- Collected from Phonhong District, Laos only

Hosts (no. of chiggers/positives)
Bandicota indica (2), Rattus losea (1)

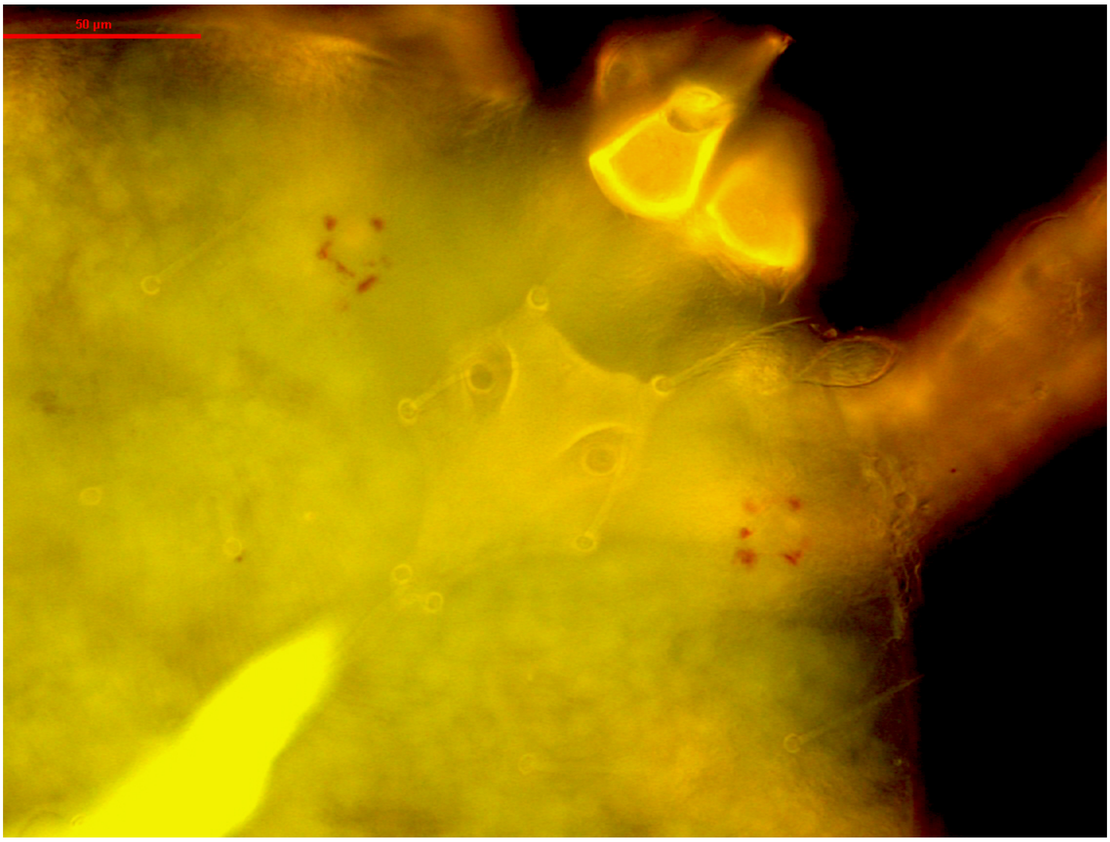
Key identification features

- Subpentagonal scutum with posterior margin deeply convex
- Sensilla setae globose
- Cheliceral blade long, dorsal row of teeth and 3-pronged cap
- fPp = N/N/NNB. 7BS. G=N
- AL>PL>>AM
- Ip900-1100

Reference keys

- Nadchatram & Goff, 1980
- Goff, 1982

Schoengastiella ligula (Radford, 1946)



Taxonomy
Trombiculinae > Gahrliepiini > Schoengastiella

Synonyms
Gahrliepia ligula

Distribution

- China, India, Myanmar, Thailand, Malaysia.
- Collected from Ban Song Kwair, Thailand only.

Hosts (no. of chiggers/positives)
Rattus andamanensis (1), Rattus nitidus (2), Rattus tanezumi (5)

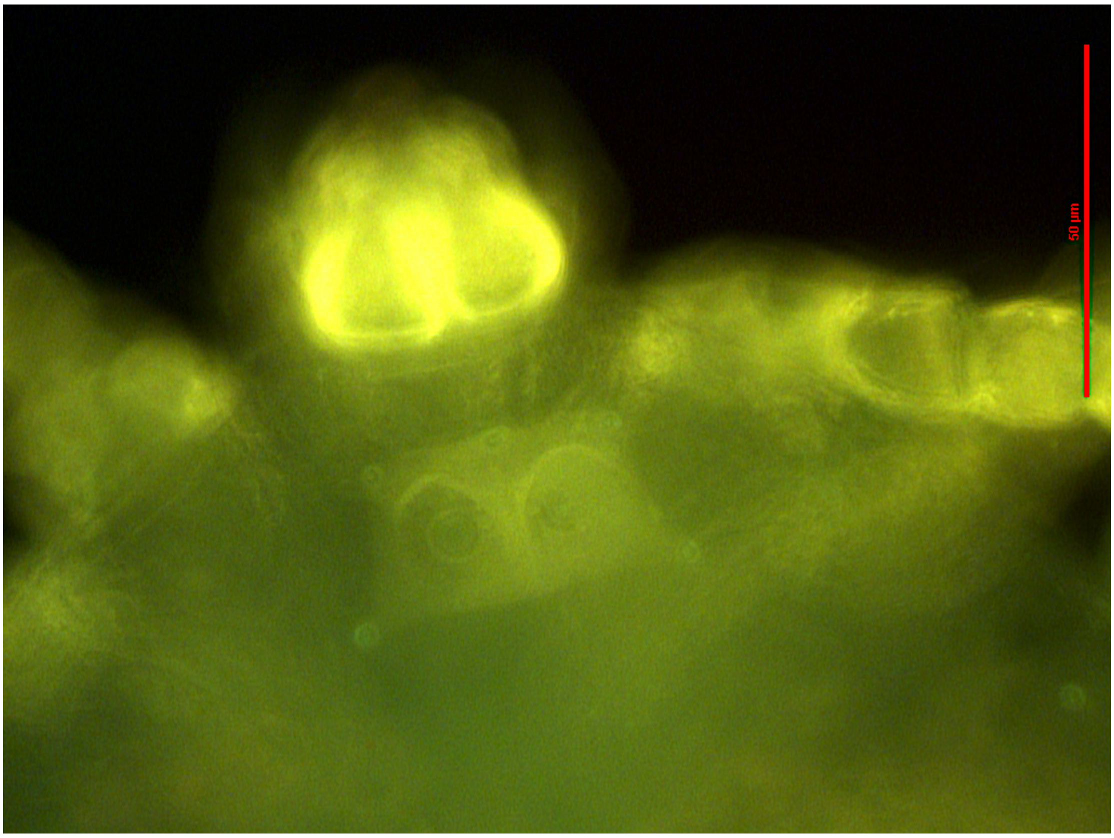
Key identification features

- Scutum narrowing posterior to PL, tongue-like
- 2 psuedo-PL setae close together near apex
- Sensilla setae globose
- Cheliceral with sub-apical tooth
- fD 38-44 and fV ~50

Reference keys

- Traub & Evans, 1954
- Womersley, 1952

Susa traubi (Nadchatram & Lakshana, 1965)



Taxonomy
Trombiculinae > Schoengastiini > Susa

Synonyms
Cheladonta traubi, Euschoengastia traubi

Distribution
- Thailand, Malaysia, Indonesia
- Collected from Ban Song Kwair only

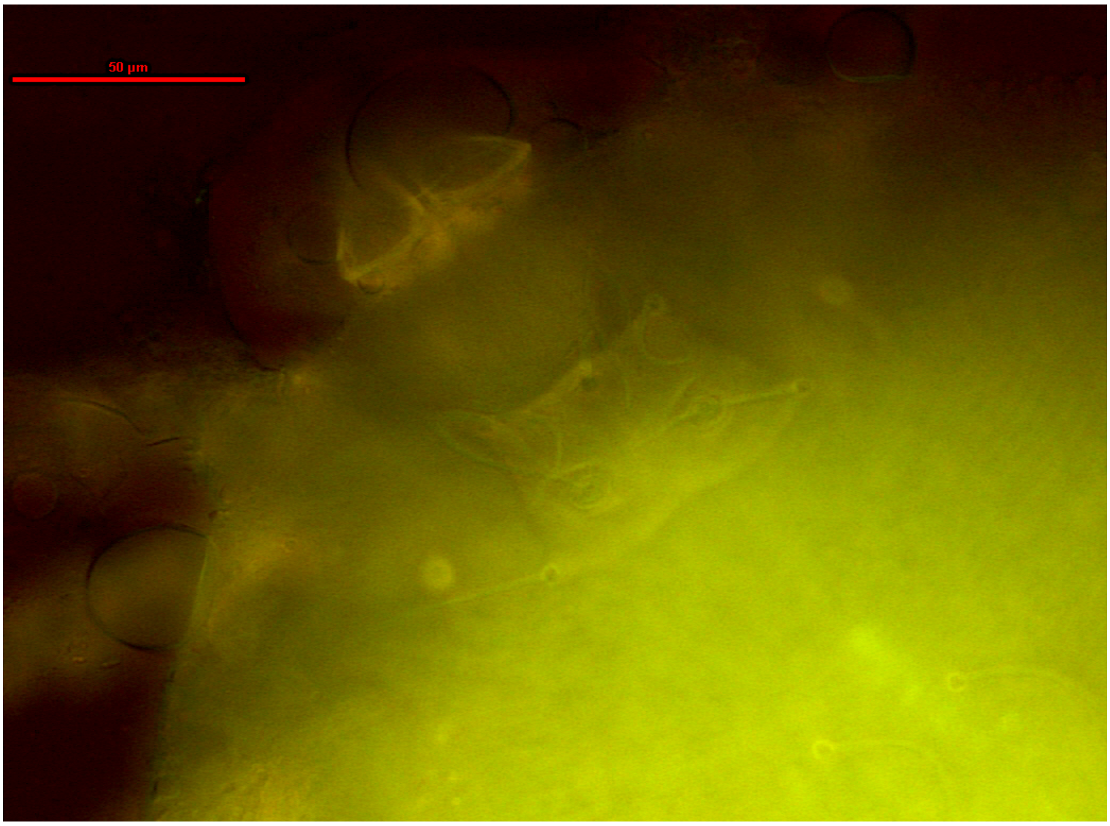
Hosts (no. of chiggers/positives)
Rattus tanezumi (1)

Key identification features

- Pale colour
- Subquadrate scutum
- Sensilla setae globose
- fPp 4B, 4BS or 5B
- Cheliceral with sub-apical tooth & row of minute teeth
- fD ~40
- Ip short (~) 650

Reference keys
No full key for genus available
- Nadchatram & Lakshana, 1965
- Lakshana, 1969

Schoutedenichia species (Jadin and Vercammen-Grandjean, 1954)



Taxonomy
Trombiculinae > Schoengastiini > Schoutedenichia

Synonyms
Ascoschoengastia, Schoengastia, Euschoengastia

Distribution

- Africa and Asia
- Collected from Ban Thoet Thai and Ban Song Kwair only.

Hosts (no. of chiggers/positives)
Rattus tanezumi (1/1), Rattus sp. (1/1)

Key identification features

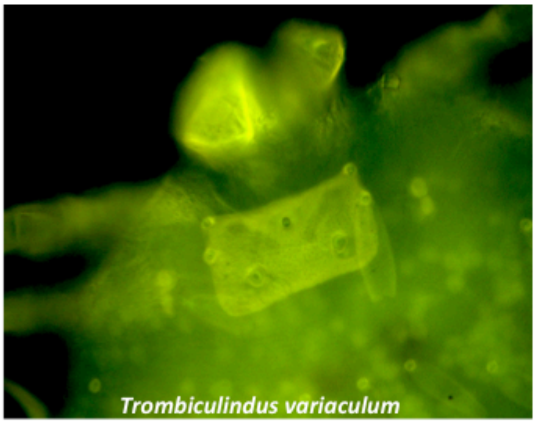
- Trapezoidal scutum, concave between PLs
- SB widely set
- Sensilla setae globose
- Tibia III absent tibiala
- Legs 7-segmented

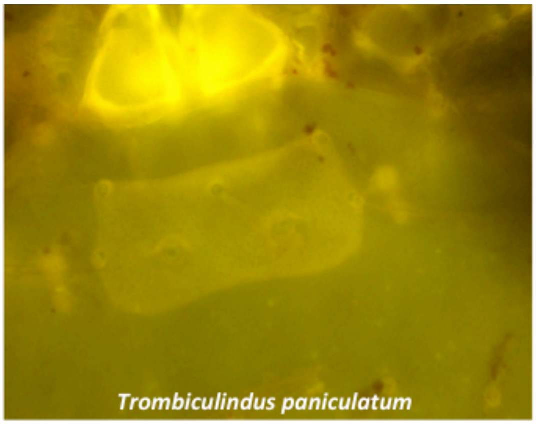
Reference keys
No full key for genus available

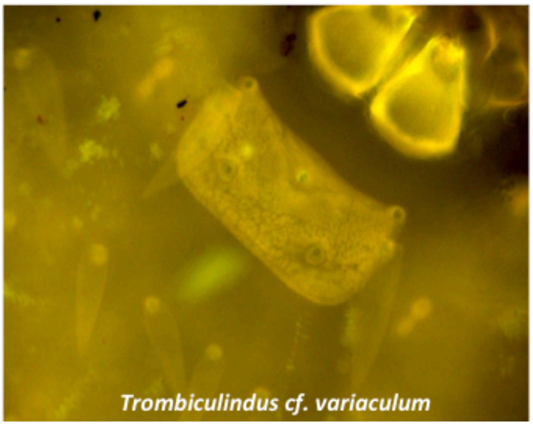
- Domrow, 1962
- Nadchatram & Dohany, 1974
- Fernandes & Kulkarni, 2003

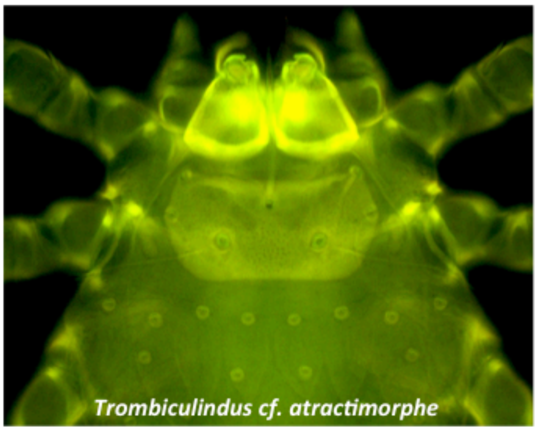
N.B. Unfortunately both specimens damaged and full morphometry for species identification not possible.

Trombiculindus cf. atractimorphe, Trombiculindus paniculatum, Trombiculindus (cf.) variaculum (Traub, Nadchatram & Lakshana, 1968)


Trombiculindus variaculum


Trombiculindus paniculatum


Trombiculindus cf. variaculum


Trombiculindus cf. atractimorphe

Taxonomy
Trombiculinae > Trombiculini > Trombiculindus

Synonyms
Trombicula, Neotrombicula, Leptotrombidium

Distribution

- India & Sri Lanka to Thailand, Indonesia and Philippines.
- Collected from 3 sites in Chiang Rai Province

Hosts (no. of chiggers/positives)
Suncus murinus (1 T. cf. atractimorphe), Maxomys surifer (1 T. cf. variaculum), Rattus exulans (1/1 T. variaculum).

15 T. cf. atractimorphe & 1 T. cf. variaculum from black plate

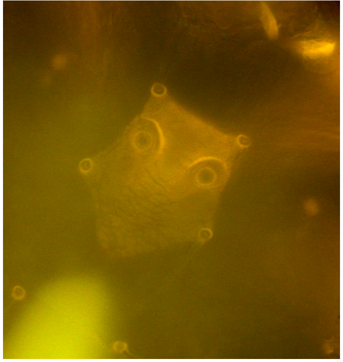
Key identification features

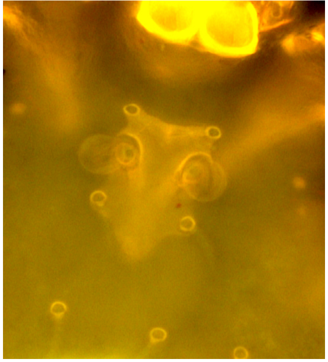
- SRectangular scutum, densely punctate
- AM submarginal
- Differs to Leptrombidium species in:
- Expanded leaf-like PL and dorsal setae
- PLs closer to AL margin than PL margin
- Cheliceral with sub-apical tooth

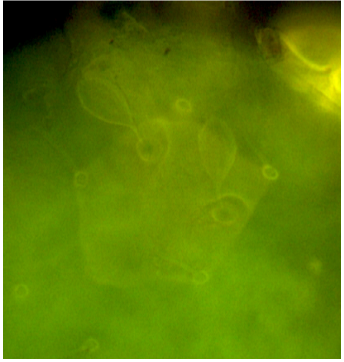
Reference keys

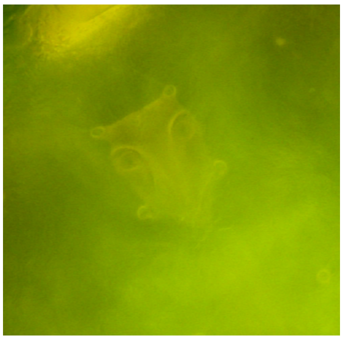
- Vercammen-Grandjean, 1976

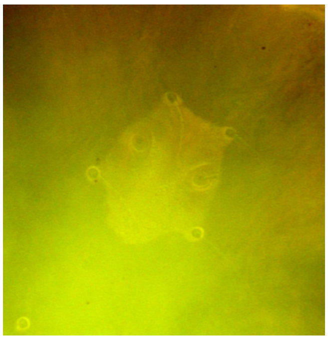
***Walchia kritochaeta, W. micropelta, W. alpestris* (Traub & Evans, 1957), *W. ewingi lupella* (Fuller, 1949), *W. minuscuta* (Chen, 1978), *W. rustica* (Gater, 1932)**

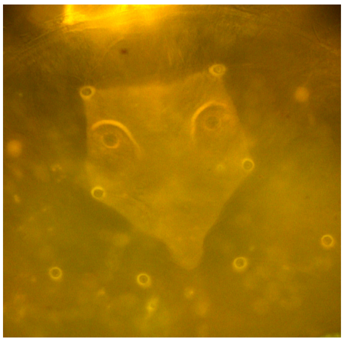

Walchia kritochaeta


Walchia micropelta

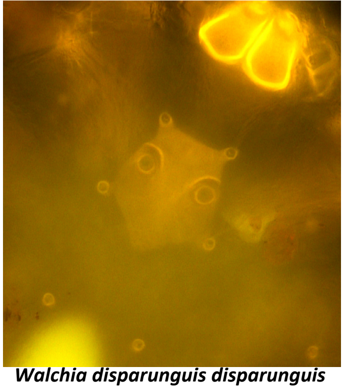

Walchia ewingi lupella

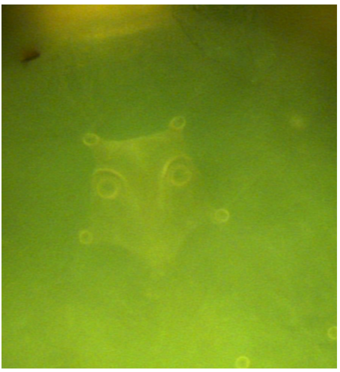
Walchia minuscuta


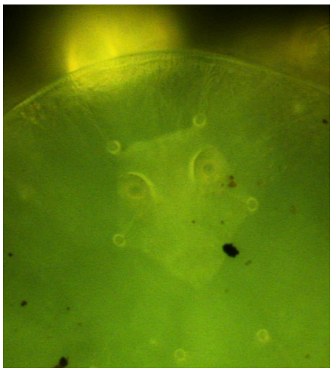
Walchia alpestris


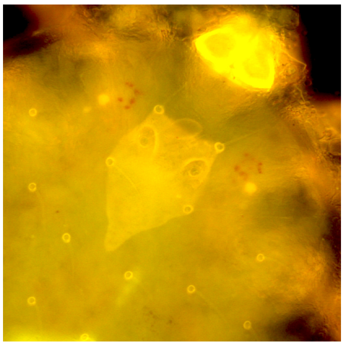
Walchia rustica


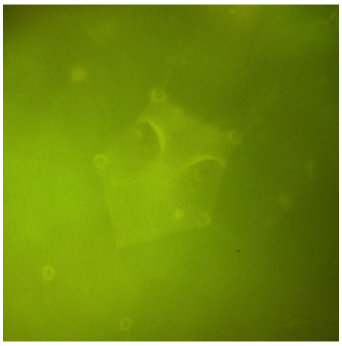
***Walchia disparuguis, W. disparunguis disparunguis* (Oudemans, 1929), *W. ewingi ewingi* (Fuller, 1949), *W. turmalis, W. rustica* (Gater, 1932), *W. isonychia* (Nadchatram & Traub, 1964)**

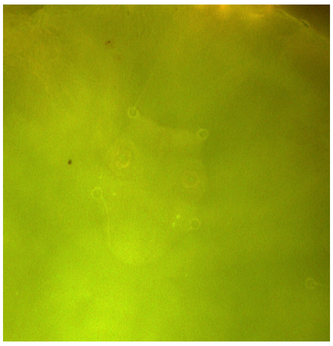

Walchia disparunguis disparunguis


Walchia disparunguis

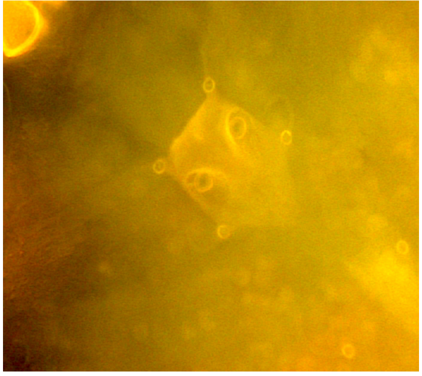

Walchia ewingi ewingi

Walchia turmalis


Walchia isonychia


Walchia ventralis


Walchia dismina (Schluger et al., 1960)



Hosts (no. of chiggers/positives)

B. berdmorei, B. bowersi ***W. alpestris*** *(7)*

B. indica, Maxomys sp., M. cervicolor, M. cookii, Niviventer sp., R. andamanensis, R. exulans, R. nitidus, R. tanezumi ***W. micropelta*** *(70/7: 1 ex B. indica & 6 ex Maxomys)*

B. indica, B. berdmorei, L. edwardsi, M. surifer, M. cookii, R. tanezumi ***W. minuscuta*** *(14/3: ex M. surifer)*

M. cookii (2) ***W. dismina***

M. cookii (2), R. losea (1) ***W. disparunguis***

M. surifer (4), R. tanezumi (2) ***W. disparu. Disparunguis***

B. indica (5) ***W. ewingi ewingi***

B. indica (31), M. caroli (1), R. exulans (1) ***W. ewingi lupella***

B. indica (1), M. surifer (2) ***W. isonychia***

B. indica, B. berdmorei, L. edwardsi, Maxomys sp., M. cookii, Niviventer sp., R. exulans, R. nitidus, R. tanezumi ***W. kritochaeta*** *(70/13: 2 ex B.indica, 1 ex B. berdmorei, 2 ex M. surifer, 6 ex R exulans, 2 ex r. tanezumi.*

R. andamanensis (5) ***W. rustica***

R. tanezumi (3) ***W. turmalis***

B. bowersi (2) ***W. ventralis***

Blackplate: ***W. turmalis (1)***

Taxonomy
Trombiculinae > Gahrliepiini > Walchia

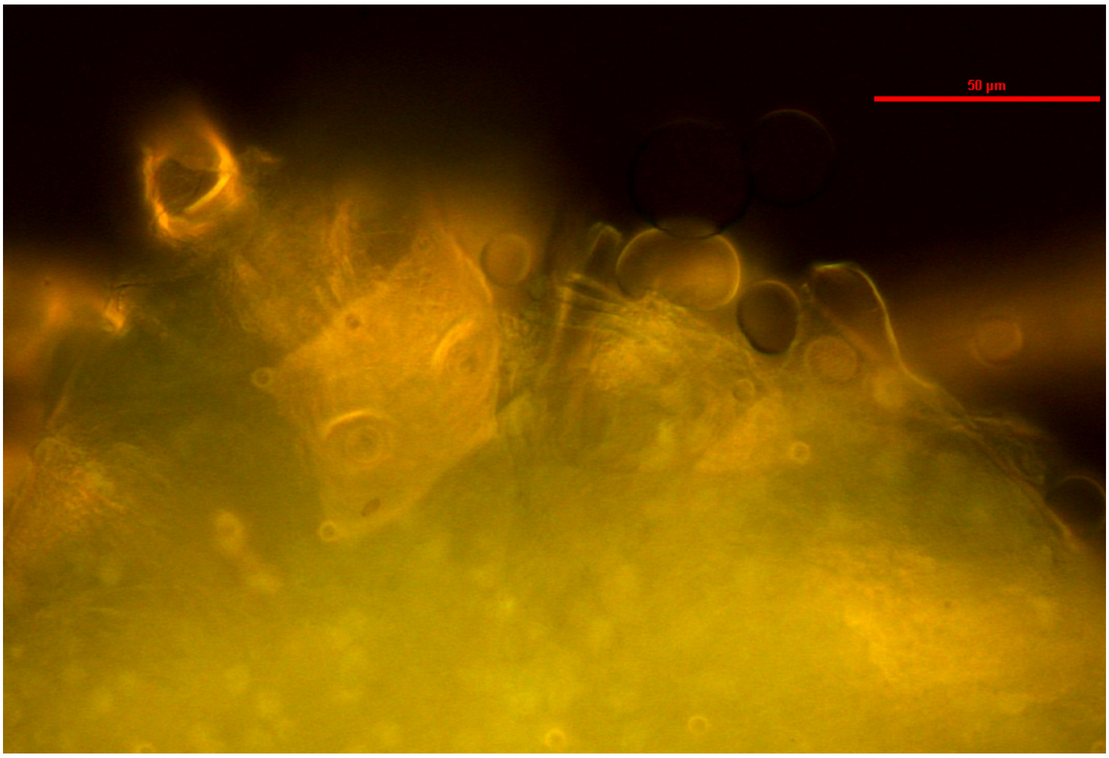
Synonyms
Trombidium, Gahrliepia

Distribution
- China, India, Myanmar, Malaysia, Indonesia, Thailand, Laos, Japan, Korea, Africa & New World
- Collected from all small mammal trapping sites

Key identification features
- White to yellow colour, some very small
- Shield shaped, elongated beyond PLs, with 4 scutal setae
- Ga and Palpal femoral setae nude
- Chelicerae simple
- Dorsal setae usually 2H+6…, short
- Number of Cox III setae & difference in width of claws may be useful

Reference keys
- Traub & Evans, 1957
- Nadchatram & Traub, 1964

Walchiella species (Fuller, 1952)



Taxonomy
Trombiculinae > Schoengastiini > Walchiella

Synonyms
Schoengastia, Euschoengastia, Trombicula

Distribution

- Thailand, Asiatic-Pacific Region, India
- Collected from Ban Song Kwair only

Hosts (no. of chiggers/positives)
Rattus andamanensis (1)

Key identification features

- White to yellow colour
- Squarish scutum
- PLs shorter than ALs
- Sensilla setae lanceolate and barbed
- SBs closer to lateral margins than each other
- Cheliceral with tricuspid cap
- No mastitarsala

Reference keys
No full key available for genus

- Domrow, 1962
- Nadchatram & Dohany, 1974

NB Unfortunately this specimen was damaged and identification to species was not possible.

Glossary of abbreviations

Abbreviation	Description
AL	Anterolateral seta length
AM	Anteromedian seta length
PL	Posterolateral seta length
AP	Distance between anterolateral & posterolateral setae
ASB	Distance from line connecting sensillary bases to anterior-most margin of scutum
PSB	Distance from line connecting sensillary bases to posterior-most margin of scutum
SD	Scutal depth (ASB + PSB)
AW	Distance between bases of anterolateral setae
PW	Distance between bases of posterolateral setae
SB	Distance between sensillary bases
PPW1 & PPW2	Distance between 1st or 2nd pair of usurped setae (Gahrliepiinae)
PPP1 & PPP2	Distance from 1st or 2nd pair of usurped setae to posterior scutal margin
Pa	Length of anterior leg (leg 1)
Pm	Length of median leg (leg 2)
Pp	Length of posterior leg (leg 3)
Ip	Total leg length (Pa + Pm + Pp)
fD	Number of dorsal setae
fV	Number of ventral setae
NDV	Total number of setae (fD + fV)
fPp	Palpal formula
fSc	Scutal formula
fCx	Coxal setae formula
B or N	Barbed or Nude
Sens	Sensilla

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8.6 APPENDIX C

8.6.1 Table 1 Sequencing statistics for human samples from Thailand and Laos, colony chiggers of 3 species, 2 rodent lung samples and negative controls. Sequencing followed the Nextera XT library preparation method.

Sample id	Sample type	Ct value	Total reads	Mean coverage of core genome	On-target unique reads	% reads mapped to reference genome	% duplicates	% of core genome covered at 1x	% of core genome covered at 5x	% of core genome covered at 10x
UI30239	Neg control - whole blood	n/a	12985138	0.47	1389	0.53	98.67	0.8	0.1	0.1
UI30243	Neg control - whole blood	n/a	151258	0	205	0.35	68.09	0	0	0
UI35856E	Eschar crust	32.9	21100410	341.7	93036	34.56	99.39	51.2	50.4	50.2
UI35934E	Eschar crust	38.5	19128164	9.2	8115	3.60	99.37	2.9	1.7	1.7
UI36672E	Eschar crust	38.4	8234892	2.3	2210	1.69	99.15	1.1	0.6	0.6
UI37494E	Eschar crust	32.9	6868070	48.2	14927	16.93	99.36	10	9.6	9.5
UI37719E	Eschar crust	32.7	18732338	10.39	4985	1.74	99.17	3.5	2.4	2.4
UI37880	Neg control - buffy coat	n/a	1231644	0.06	1286	0.48	81.15	1.1	0.1	0.1
UI37899	Neg control - buffy coat	n/a	9770162	0	160	0.00	0.00	0.4	0	0
C0066	Negative chigger	n/a	1625016	0	78	0.01	58.82	0.1	0	0
C0067	Negative chigger	n/a	5910538	0.08	772	0.23	96.63	0.8	0	0
Lc-1	Lc - colony chigger	30.2	1559774	42.17	146934	98.41	93.30	40.2	39.2	39
Lc-2	Lc - colony chigger	28.6	4009618	166.17	229515	77.14	94.85	94.1	93.8	93.4
Ld-1	Ld - colony chigger	29.0	4877270	205.74	302293	78.85	94.47	95.8	95.5	95.2
Ld-2	Ld – colony chigger	29.2	1672222	65.37	110081	70.97	93.40	78.7	77.9	76.7
Li-4	Li – colony chigger	30.2	2879596	78.29	104138	51.56	95.10	73.8	73.1	72.5
Li-5	Li – colony chigger	29.1	2692954	88.82	122966	59.92	94.68	82.5	82	81.3
MIL6240	Buffy coat	36.4	8379252	39.61	15848	9.96	99.08	11.1	10.1	10.1
R0002P	Neg control - rodent lung	n/a	22082604	0.36	5337	0.66	97.60	1.6	0.1	0.1
R0013P	Rodent lung	35.1	3607416	0.43	8188	0.88	77.41	1.9	1	0.9

R0025P	Rodent lung	38.7	3227358	0.11	2334	0.43	85.89	0.7	0.2	0.2
R0028P	Neg control – rodent lung	n/a	4068932	0.09	7581	0.62	74.30	0.9	0.1	0.1
STP-001-W00-ES	Eschar swab	36.6	3743122	1.35	790	1.12	98.93	0.5	0.2	0.2
STP-001-W00-WB	Whole blood	35.6	4446618	0.66	963	0.69	97.91	1	0.2	0.2
STP-002-W00-WB	Whole blood	37.1	10475610	0.41	948	0.28	97.82	0.7	0.1	0.1
STP-003-W00-WB	Whole blood	38.6	10485908	0.86	1267	0.42	98.31	0.7	0.2	0.2
STP-004-W00-EC	Eschar crust	33.7	8083684	12.44	4210	3.51	99.23	3.1	2.5	2.5
STP-009-W00-EC	Eschar crust	38.1	3599342	3.7	1694	3.03	99.20	1.1	0.8	0.8
STP-009-W00-WB	Whole blood	37.1	10019496	2.65	1602	0.76	98.79	1.4	0.8	0.8
STP-010-W00-ES	Eschar swab	38.3	2678868	2.63	2264	2.80	98.47	1.5	1.3	1.3
STP-010-W00-WB	Whole blood	31.2	6116682	42.94	13912	15.24	99.25	10.5	10.2	10.1
STP-011-W00-EC	Eschar crust	33.8	11733992	111.39	37195	22.03	99.25	22.2	21.6	21.5
STP-011-W00-WB	Whole blood	36.4	4892654	1.9	2318	1.47	98.37	1.4	1.3	1.2
STP-019-W00-EC	Eschar crust	37.5	7670142	33.71	25645	29.96	99.50	3.9	2.9	2.9
STP-019-W00-WB	Whole blood	37.1	12453124	0.48	1308	0.39	98.16	0.9	0.1	0.1
STP-024-W00-WB	Whole blood	37.1	15065644	3.59	2434	0.83	98.85	1.6	0.9	0.9
STP-027-W00-WB	Whole blood	35.5	9428944	3.56	2708	1.21	98.68	1.6	1	1
STP-030-W00-WB	Whole blood	36.6	5194134	0.86	1418	0.71	97.97	0.7	0.4	0.4
STP-034-W00-EC	Eschar crust	33.7	12105100	163.69	63077	29.25	99.14	37.7	36.6	36.4
STP-034-W00-WB	Whole blood	30.3	9909622	49.97	21111	11.00	99.05	15	14.2	14.1
STP-044-W00-ES	Eschar swab	38.6	16292366	8.44	11184	6.85	99.48	3.5	1.4	1.4
STP-044-W00-WB	Whole blood	38.6	15306758	0.82	1292	0.15	96.53	1	0.3	0.3
STP-045-W00-WB	Whole blood	36.9	9593406	1.7	1464	0.58	98.39	1.4	0.4	0.4
STP-048-W00-BU-C	Buffy coat	27.3	1385312	21.82	57172	35.84	91.76	53.4	52.3	49.8
STP-048-W00-ES	Eschar swab	41.4	19352482	3.89	3115	1.00	99.07	1.4	0.6	0.6
STP-050-W00-EC	Eschar crust	29.5	211120	1.5	4912	17.98	90.61	6.1	5.9	5.4
STP-050-W00-WB	Whole blood	36.9	13893000	1.8	1599	0.51	98.66	1	0.4	0.4
STP-051-W00-EC	Eschar crust	33.1	9914482	152.65	53448	33.87	99.24	33.1	32.4	32.2
STP-051-W00-WB	Whole blood	33.1	9828360	25.84	8942	5.22	99.12	7.8	7.1	7.1
STP-052-W00-EC	Eschar crust	33.9	6318962	11.34	5082	5.36	99.25	3.5	3	3
STP-054-W00-WB	Whole blood	34.8	11437002	12.34	4583	2.60	99.18	3.9	3.1	3.1
STP-055-W00-ES	Eschar swab	37.1	6881756	8.76	3595	3.58	99.25	2.3	1.9	1.9

STP-055-W00-WB	Whole blood	33.5	9946052	7.63	3201	1.94	99.05	3.2	1.6	1.6
STP-057-W00-WB	Whole blood	33.9	15384190	4.76	3825	1.20	98.84	2	1	1
STP-059-W00-BU-C	Buffy coat	32.5	19241616	12.01	5108	1.61	99.09	4.4	3.1	3.1
UI29931	Buffy coat	31.0	9359670	53.93	23011	15.61	99.21	14	13.4	13.3
UI30029	Buffy coat	35.9	8853516	0.31	1108	0.52	98.59	0.5	0	0
UI30104	Buffy coat	35.2	14221666	5.25	2500	0.97	99.02	1.8	1.2	1.1
UI30862	Buffy coat	35.9	7613298	2.77	1501	0.84	98.62	1.3	0.9	0.9
UI31120	Buffy coat	33.4	9403130	4.84	2637	1.46	98.91	2.1	1.2	1.2
UI31199	Buffy coat	30.4	8844724	13.51	6290	4.52	99.18	4.3	3.5	3.4
UI31209	Buffy coat	32.4	6350924	7.16	4667	3.78	98.95	2.5	2.2	2.2
UI31230	Buffy coat	36.1	11197860	3.95	2967	0.96	98.53	1.7	0.9	0.9
UI31231	Buffy coat	31.9	11288206	2.42	2107	0.84	98.54	1.9	0.6	0.6
UI31248	Buffy coat	35.0	11185654	0.71	1285	0.47	98.44	0.9	0.1	0.1
UI31331	Buffy coat	32.9	10135836	6.34	3500	1.27	98.45	2.5	1.7	1.7
UI31445	Buffy coat	35.7	8015022	2.87	850	0.57	98.83	1.1	0.6	0.6
UI31467	Buffy coat	28.2	2561736	91.63	172179	75.31	93.71	92.9	92.3	91.4
UI32074	Buffy coat	31.9	3489236	7.15	2481	3.96	99.18	2.5	2.2	2.2
UI32426	Buffy coat	32.3	15611978	268.17	106409	45.87	99.29	40.6	39.8	39.7
UI32456	Buffy coat	36.1	5581602	2.38	1662	1.43	98.83	1.1	0.7	0.7
UI32574	Buffy coat	32.9	8641438	0.01	180	0.00	1.11	0.6	0	0
UI32883	Buffy coat	31.1	43138246	1727.35	479765	75.18	99.28	97.3	97.1	97
UI32955	Buffy coat	34.7	10206396	7.51	4596	2.17	98.88	2.3	1.8	1.8
UI32990	Buffy coat	35.3	9129466	9.99	3393	2.49	99.20	3.5	2.4	2.4
UI33288	Buffy coat	33.2	8492882	23.58	9725	6.92	99.13	6.4	5.8	5.8
UI33303	Buffy coat	34.7	3486026	3.07	1258	1.73	98.80	1.5	1	1
UI33771	Buffy coat	35.9	9065028	10.8	5091	3.50	99.12	3.9	3.2	3.1
UI34384	Buffy coat	34.7	16624612	41.51	14517	5.83	99.23	11.3	9.9	9.7
UI35934	Buffy coat	38.2	4487444	1.87	1204	1.12	98.76	0.6	0.4	0.4
UI35942	Buffy coat	38.5	8672428	1.65	1727	0.83	98.61	0.8	0.4	0.4
UI36627	Buffy coat	35.7	9826732	75.16	30418	18.50	99.19	19.1	18.4	18.3
UI36672	Buffy coat	40.0	7093002	0.85	816	0.55	98.74	0.9	0.2	0.2
UI39238E	Eschar crust	28.1	552540	0.66	36191	16.59	71.26	44.9	0.4	0

T01E	Eschar crust	n/a	568480	2.01	63543	29.77	73.08	77.5	9.7	0.9

Lc = Leptotrombidium chiagnraiensis, Ld = L. deliense, Li = L. imphalum.
Human samples in *italics* are from Lao patients and the rest from Chiang Rai, Thailand. T01E was from a patient probably infected on Green Island, Taiwan.
Two samples in **bold** were sequenced later using the WGA and standard library preparation method.

8.6.2 Table 2 Sequencing statistics for individual chiggers, pooled chiggers and rodent tissues. Sequencing performed using WGA followed by standard library preparation. Two rodent lung tissues were included with the human samples and colony chiggers below.

Sample id	Sample type	Ct value	Total reads	Mean coverage of core genome	On-target unique reads	% reads mapped to reference genome	% duplicates	% of core genome covered at 1x	% of core genome covered at 5x	% of core genome covered at 10x
C0325	C	35.0	282958	0.08	4414	3.66	67	0.2	0	0
C0339	C	30.4	68675066	765.43	27419146	96.47	70	99.8	99.8	99.8
C0438	C	30.3	3343152	11.06	515886	91.78	89	97.7	76.3	45.7
C0456	C	32.0	239512	0.23	9335	15.09	81	19	0	0
C0546	FC	34.1	1333440	7.22	381218	71.14	70	36.7	17.8	11.7
C0593	C	32.5	219953112	2308.82	88459194	96.61	70	99.9	99.9	99.9
R0054L	Li	33.6	5090032	0.02	7599	0.25	49	2	0	0
R0054P	Lu	34.2	707768	0.02	9415	2.33	54	2.2	0	0
R0130L	Li	34.8	987714	0.04	3418	0.64	55	3.2	0	0
R0130P	Lu	32.1	757196	0.03	3443	0.86	55	2.4	0	0
R0131P	Lu	34.2	615874	0.01	2712	0.83	55	0.6	0	0
R0209P	Lu	32.5	700210	0.05	2795	0.82	61	4.4	0	0
R125Pool3		32.9	962612	1.02	36015	8.00	65	58.5	1.1	0
R125Pool4		32.8	1511498	0.66	27719	4.33	69	44.4	0.6	0
R125Pool6		32.7	890232	0.88	31195	7.57	65	54.2	0.6	0
R129Pool6		25.4	4373758	41.09	1725115	84.77	66	99.8	99.5	99
R130Pool4		28.8	930332	4.18	141922	34.20	67	94.6	36.8	5.2
R130Pool5		32.6	809996	0.1	13515	4.30	71	3.5	0.3	0.2
R130Pool6		29.7	723794	0.34	15634	4.52	63	24.5	0.3	0
R131Pool1		32.6	960018	2.2	90068	24.14	72	85.1	9.5	0.2
R131Pool3		29.3	760600	2.24	65462	20.02	69	85	10.8	0.3
R131Pool4		28.9	1019384	3.48	131308	35.90	74	94.4	27.7	1.5
R131Pool5		28.5	693280	1.89	57463	17.36	64	77.3	7.1	0.3
R132Pool2		32.2	1242130	6.33	224386	36.59	63	98.4	65.8	16

R132Pool5		29.6	857336	3.74	127445	34.58	68	95	31	2.1
R132Pool9		31.7	724510	1.35	56171	17.35	67	69.9	2.7	0.1
R133Pool2		29.8	1003790	2.16	79830	18.97	70	84.6	8.9	0.3
R133Pool4		30.7	792234	1.29	45950	18.75	78	68.7	2	0
R133Pool6		28.8	8070940	85.73	3208783	89.93	68	99.8	99.6	99.6
R133Pool7		27.7	1147944	5.24	200152	41.91	70	98	54.4	8.1
R133Pool8		29.1	794828	0.77	30001	9.29	71	49	0.5	0
R144Pool3		33.1	730082	0.01	62467	19.42	66	0.2	0	0
R151Pool6		31.1	734180	0.9	34499	12.06	72	55	0.9	0.1
R162Pool7		29.3	615918	1.56	57267	21.03	67	71.9	4.9	0.1
R166Pool3		31.6	678176	0.52	21328	7.32	68	38.3	0.2	0
R167Pool2		30.3	1244050	3.8	151704	31.61	72	95.6	33.2	2.1
R167Pool5		30.3	1410268	4.13	171832	30.79	72	96.1	38.2	3.2
R167Pool6		32.5	759462	0.77	30628	8.61	65	49.9	0.5	0
R167Pool7		33.0	907790	1.29	50178	10.84	61	67.3	2.1	0.1
R167Pool8		30.1	953584	1.44	63737	17.40	73	71.9	3	0.1
R172Pool1		29.5	1784766	9.78	414276	61.31	73	99.5	89.3	46.9
R172Pool2		26.2	4398598	34.56	1335424	80.48	73	99.9	99.6	99.2
R172Pool6		31.6	1015934	2.46	103276	28.12	74	87.8	12.9	0.4
R173Pool10		25.9	4699038	42.6	1475854	82.38	73	100	99.7	99.4
R173Pool11		27.3	5909356	48.77	1848464	85.43	74	99.9	99.8	99.6
R173Pool12		29.0	3989040	33.55	1229597	78.63	72	99.9	99.6	99
R173Pool8		28.7	1331508	6.52	229238	45.69	73	99.1	69	16.5
R173Pool9		26.8	1646068	9.82	382327	62.39	74	99.7	91.1	48.5
R174Pool1		26.1	1550288	7.75	315125	43.71	66	99.4	80.6	27.7
R174Pool10		29.1	1103952	3.36	131960	27.64	68	94.3	26	1
R174Pool2		24.6	1139468	5.21	188252	41.87	72	98.4	55.2	6.9
R174Pool3		28.8	805536	0.78	36229	10.54	68	50.9	0.5	0
R174Pool4		30.2	986522	2.8	119541	31.42	72	89.6	17	0.8
R174Pool5		28.3	875090	1.15	51636	16.15	74	64.1	1.8	0
R174Pool6		30.2	778528	1.99	86452	29.33	72	78.6	8.7	0.4
R174Pool7		32.9	954202	1.08	43904	10.36	67	61.8	1.4	0.1

R174Pool8		29.3	1208790	3.29	129804	28.48	73	92.3	25.2	1.4
R174Pool9		30.3	743342	0.3	18333	6.94	74	24.6	0	0
R175Pool9		28.9	620870	0.58	16941	6.29	68	39.9	0.3	0
R178Pool2		31.9	648212	0.33	11772	3.86	64	25.7	0.1	0
R198Pool2		32.0	1914614	17.43	652854	78.96	68	99.7	94.3	73.7
R198Pool4		30.9	1054082	2.77	106552	24.70	70	89.3	17.4	0.9
R200Pool3		32.7	1620102	12.99	450129	59.91	65	99.7	92	63.8
R204Pool2		32.8	897472	1.41	53655	15.72	72	70.6	3.2	0.1
R206Pool1		31.8	890574	0.33	14322	3.74	68	26.8	0	0
R206Pool4		31.6	847976	1.34	48613	12.24	65	70.7	2.1	0
R206Pool5		32.8	925464	0.76	32097	7.88	68	49.8	0.4	0
R206Pool6		25.3	678096	0.5	18415	7.02	72	36.7	0.1	0
R207Pool2		31.5	2455480	18.96	669138	66.03	70	99.8	98.6	89
R208Pool2		30.1	1016990	1.81	66574	20.65	78	79.1	5.8	0.1
R209Pool5		28.7	3637180	31.93	1127597	75.18	70	99.8	99.6	98.5
R211Pool4		32.9	2564534	19.05	714751	72.79	73	99.8	99.2	92.4
R211Pool6		31.5	1159424	5	194098	37.31	67	97.8	51.1	7.2
R234Pool10		31.2	836014	3.08	97546	26.35	67	90	20	0.8
R234Pool3		32.4	867794	0.39	13825	3.92	69	28.8	0.2	0.1
R234Pool4		32.5	747178	0.19	7906	2.23	63	15.9	0.1	0
R234Pool7		30.0	9538	0	87	1.43	40	0.1	0	0
R234Pool8		30.4	959694	2.6	80516	20.02	69	86.4	13.7	0.4
R234Pool9		31.6	836364	2.14	65071	16.39	64	81.6	8.5	0.2
R235Pool3		33.2	409726	1.31	40043	20.36	64	66.1	3	0.1
R240Pool3		28.7	1404350	14.31	420685	71.23	69	98.4	95.2	73.3
R240Pool5		27.7	3829890	43.44	1299918	88.60	72	98.5	98.2	97.8
R240Pool6		26.2	6772906	94.21	2728724	92.72	68	98.6	98.3	98.2
R240Pool7		29.9	3782834	42.3	1245348	79.72	69	98.5	98.2	97.3
R240Pool8		30.6	678078	4.52	123062	44.83	70	93.9	38.9	4.1
R240Pool9		31.5	538562	0.58	13663	5.30	63	38.4	0.3	0.1
R242Pool1		32.7	686386	0.79	25760	7.52	62	48.7	0.8	0
R44Pool4		28.1	1228576	6.26	178935	33.71	69	98.8	67.3	15.3

R44Pool7		33.0	963168	0.22	10804	2.35	63	18.5	0	0
R45Pool7		32.9	875000	2.31	71507	16.81	63	84.6	11.7	0.4
R45Pool8		30.9	600548	0.08	2634	0.94	62	7.2	0	0
T14	T	34.2	184980396	1708.03	76601246	96.22	68	99.7	99.6	99.6

C = chigger from rodent (individual); FC = Free-living chigger; Li = Rodent liver; Lu = Rodent lung; Pool = pool of between 5 and 50 individuals; T = Individual chigger from Taiwan.
Samples in **bold** were used for phylogenetic comparisons in Chapter 5, including in Figures 9, 10 & 11

8.6.3 Table 3 Number of sequence positions called across the genome out of ~2.1 million total sites for the 17 most completely sequenced samples included in the phylogenetic analysis

Sample ID	**Number of positions called**
C0593	1158668
T14	908598
R129Pool6	1169615
R133Pool6	1220731
R172Pool2	1156026
R173Pool10	1137561
R173Pool11	1178425
R173Pool12	1132425
R198Pool2	677581
R200Pool3	642554
R207Pool2	922829
R209Pool5	957303
R211Pool4	941089
R240Pool3	531817
R240Pool5	812343
R240Pool6	856697
R240Pool7	799635

8.6.4 Table 4 Pairwise matrix with number of positions differing between pairs in the subset of positions called across all strains (above the diagonal), which were used to generate the maximum-likelihood tree, and the equivalent pairwise distance (proportion of differences) between pairs (below the diagonal), that were used to generate the UPGMA tree.

	C0593	R129 Pool6	R133 Pool6	R172 Pool2	R173 Pool10	R173 Pool11	R173 Pool12	R198 Pool2	R200 Pool3	R207 Pool2	R209 Pool5	R211 Pool4	R240 Pool3	R240 Pool5	R240 Pool6	R240 Pool7	Ref	T14
C0593	0	285	285	278	282	278	278	314	315	278	323	279	5616	5674	5674	5674	1115	1211
R129Pool6	0.0099	0	0	7	17	7	7	67	93	7	102	8	5501	5559	5559	5559	899	1049
R133Pool6	0.0101	0.0002	0	7	17	7	7	67	93	7	102	8	5501	5559	5559	5559	899	1049
R172Pool2	0.0079	0.0004	0.0004	0	10	0	0	60	86	0	95	1	5494	5552	5552	5552	892	1042
R173Pool10	0.0054	0.0014	0.0017	0.0010	0	10	10	66	90	10	103	11	5500	5558	5558	5558	896	1046
R173Pool11	0.0062	0.0004	0.0006	0.0003	0.0007	0	0	60	86	0	95	1	5494	5552	5552	5552	892	1042
R173Pool12	0.0061	0.0008	0.0009	0.0005	0.0007	0.0003	0	60	86	0	95	1	5494	5552	5552	5552	892	1042
R198Pool2	0.0082	0.0071	0.0072	0.0048	0.0029	0.0033	0.0030	0	123	60	53	61	5516	5574	5574	5574	894	1058
R200Pool3	0.0074	0.0077	0.0078	0.0054	0.0026	0.0033	0.0034	0.0043	0	86	134	87	5528	5586	5586	5586	901	1078
R207Pool2	0.0078	0.0014	0.0014	0.0010	0.0009	0.0003	0.0005	0.0043	0.0047	0	95	1	5494	5552	5552	5552	892	1042
R209Pool5	0.0074	0.0081	0.0084	0.0060	0.0037	0.0044	0.0044	0.0014	0.0045	0.0053	0	96	5536	5592	5592	5592	927	1086
R211Pool4	0.0076	0.0021	0.0021	0.0015	0.0011	0.0004	0.0004	0.0040	0.0042	0.0006	0.0050	0	5493	5551	5551	5551	891	1041
R240Pool3	0.0355	0.0357	0.0357	0.0336	0.0295	0.0301	0.0306	0.0321	0.0323	0.0324	0.0312	0.0318	0	61	61	61	5792	5837
R240Pool5	0.0404	0.0404	0.0406	0.0383	0.0341	0.0348	0.0353	0.0365	0.0368	0.0366	0.0355	0.0361	0.0007	0	5850	5894	5850	5894
R240Pool6	0.0422	0.0420	0.0424	0.0400	0.0358	0.0366	0.0370	0.0378	0.0384	0.0381	0.0371	0.0374	0.0007	0.0006	0	5894	5850	5894
R240Pool7	0.0400	0.0400	0.0402	0.0380	0.0339	0.0345	0.0349	0.0362	0.0367	0.0365	0.0353	0.0358	0.0007	0.0005	0.0007	0	5850	5894
Ref	0.0160	0.0156	0.0162	0.0127	0.0090	0.0100	0.0091	0.0095	0.0093	0.0106	0.0110	0.0097	0.0350	0.0403	0.0421	0.0398	0	1473
T14	0.0162	0.0158	0.0159	0.0137	0.0103	0.0111	0.0112	0.0111	0.0122	0.0124	0.0108	0.0117	0.0316	0.0362	0.0377	0.0360	0.0153	0

8.6.5 Table 5 Matrix of pairwise comparisons between isolates used in phylogenetic analysis, showing the number of differences x_{ij} between pairs (above the diagonal) and the number of positions called (y_{ji}) in both isolates of a pair (below the diagonal). These pairwise distances (calculated as x_{ij}/y_{ji}) were used to construct a UPGMA tree.

	C0593	**R129 Pool6**	**R133 Pool6**	**R172 Pool2**	**R173 Pool10**	**R173 Pool11**	**R173 Pool12**	**R198 Pool2**	**R200 Pool3**	**R207 Pool2**	**R209 Pool5**	**R211 Pool4**	**R240 Pool3**	**R240 Pool5**	**R240 Pool6**	**R240 Pool7**	**Ref**	**T14**
C0593	0	5976	6243	8368	12898	12898	13281	11160	9065	9040	8569	8318	8394	14743	24837	26804	24494	13861
R129Pool6	489644	0	1923	2360	4529	4715	4809	3320	2009	2072	2616	2342	1564	10876	16869	18158	16762	5975
R133Pool6	562296	444843	0	908	5200	4602	4719	3124	2148	1947	2473	2342	1869	12115	19098	20374	18855	6459
R172Pool2	773524	527355	637566	0	6363	7293	7673	5371	3942	3867	3978	3876	3314	15641	26521	28686	26134	10490
R173Pool10	794464	608854	636591	863386	0	10315	10790	8234	6448	6228	6689	6576	5542	17794	30179	32834	29542	18507
R173Pool11	816510	614120	651557	900977	1047203	0	183	431	469	827	1304	1936	1546	18414	31455	34075	30778	18285
R173Pool12	833531	617373	655580	913552	1071247	1164220	0	413	706	1010	1320	1979	1883	18625	32099	35025	31323	19724
R198Pool2	812830	613058	649876	898276	1039713	1130647	1147845	0	355	580	884	1390	1054	17378	29890	32452	29280	14727
R200Pool3	820330	612639	648518	902605	1043098	1130862	1154394	1127747	0	363	257	380	752	15493	27099	29731	26512	11822
R207Pool2	805533	606678	642852	887762	1016943	1100282	1115522	1099144	1112859	0	439	393	770	15727	27238	29659	26620	10340
R209Pool5	693453	558263	581618	750841	857136	913786	916100	913315	911685	907119	0	494	767	15121	24332	26077	24115	9826
R211Pool4	708340	552425	581055	777151	862647	926308	929807	925999	928828	924620	811572	0	988	15236	25044	26760	24588	9156
R240Pool3	812188	605354	643350	891680	1016983	1087486	1103176	1085243	1100207	1077797	895403	914636	0	15185	26397	28851	25897	10183
R240Pool5	465971	336563	377376	502074	500644	516493	521001	517897	515164	513711	466512	478391	513981	0	354	397	352	18636
R240Pool6	685418	458504	523877	746828	746628	779160	790585	780152	778800	771982	664559	693929	774029	531055	0	447	374	32755
R240Pool7	711818	473461	538962	773563	778176	810599	825996	810710	812480	802481	685293	714663	805922	531278	811268	0	577	36091
Ref	679599	456806	520632	739958	738102	769582	779792	770424	768893	762426	660261	687737	764444	530129	786874	798470	0	31861
T14	908598	642554	677581	957303	1158668	1169615	1220731	1156026	1178425	1132425	922829	941089	1137561	531817	812343	856697	799635	0