Ecological and microbiological diversity of chigger mites, including vectors of scrub typhus, on small mammals across stratified habitats in Thailand

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Running title: Chigger mite ecology and microbiomes in Thailand

Abstract

Scrub typhus, caused by a bacterial pathogen (*Orientia* spp.), is a potentially life-threatening febrile illness widely distributed in the Asia-Pacific region and is emerging elsewhere. The infection is transmitted by the larval stage of trombiculid mites ("chiggers") that often exhibit low host specificity. Here, we present an analysis of chigger ecology for 38 species sampled from 11 provinces of Thailand and microbiomes for eight widespread species. In total, >16 000 individual chiggers were collected from 1 574 small mammal specimens belonging to 18 species across four horizontally-stratified habitat types. Chigger species richness was positively associated with higher latitudes, dry seasonal conditions, and host maturity; but negatively associated with increased human land use. Human scrub typhus incidence was found to be positively correlated with chigger species richness. The bacterial microbiome of chiggers was highly diverse, with *Sphingobium*, *Mycobacterium*, *Neisseriaceae* and various *Bacillales* representing the most abundant taxa. Only *Leptotrombidium deliense* was found to be infected with *Orientia*. β-diversity, but not α-diversity, was significantly different between chigger species and geographic regions, although not between habitat types. This first field survey of the chigger microbiome provides a framework for future studies on interactions between pathogens and other symbionts in these understudied vectors.

Introduction

The Trombiculoidea is a superfamily of mites (Acari: Acariformes) with a unique mode of parasitism among medically-relevant arthropod vectors. The larval stage, colloquially known as chiggers or berry bugs, is ectoparasitic on vertebrates (or occasionally invertebrates). In contrast, the deutonymph and adult stages have an edaphic lifestyle and are free-living predators of arthropods or their eggs [1]. Chiggers are the exclusive biological vectors of scrub typhus, a potentially life-threatening febrile illness of humans that historically has been associated only with the Asia-Pacific region [2]. However, recently endemic scrub typhus has been reported from the Middle East [3] and South America [4], and local transmission is suspected in sub-Saharan Africa [5]. The main aetiological agent of the disease, *Orientia tsutsugamushi* (Rickettsiales*: Rickettsiaceae*), is a vertically-transmitted chigger symbiont [6].

The epidemiology of scrub typhus remains poorly understood, largely because chiggers are minute (typically <250 µm in length) and very challenging to identify and utilise for molecular characterisation and screening [7]. In particular, interactions between climatic and physical geography, wild vertebrate hosts, and human disturbance of the environment with chigger species richness and abundance, and how these variables impact on scrub typhus incidence, are largely unexplored in most endemic regions. Moreover, our understanding of the bacterial associates of chiggers is mainly restricted to *O. tsutsugamushi* and a very small number of other potential human pathogens, such as *Bartonella* spp. [8] and *Rickettsia* spp [9]. As many cases of epidemiological-relevant interactions between human pathogens and the microbiome of arthropod vectors have been reported, our ignorance regarding the chigger microbiome is of potential concern for disease control. Indeed, this was highlighted recently by a 16S rRNA amplicon survey of a colony of the scrub typhus vector *Leptotrombidium imphalum*, which revealed a hitherto unrecognised association between a novel member of the *Amoebophilaceae* and *O. tsutsugamushi* in adult female mites [10]. The completion of the *Leptotrombidium deliense* genome project also uncovered an intimate

relationship between chiggers and soil bacteria and fungi, as genes for secondary metabolism have been acquired by lateral transfer from these microorganisms [11].

Among scrub typhus-endemic countries, Thailand has some of the highest incidence rates. The Thai Bureau of Epidemiology reported an increase in annual minimum incidence from 6.0 per 100 000 persons in 2003 to 17.1 per 100 000 in 2013 [2]. The role of the vector in this increase is unknown, but the higher prevalence of *O. tsutsugamushi* in small mammal chigger hosts from forested regions relative to areas with greater human disturbance implicates land use as a key factor in disease risk [12]. Here, we present an analysis of chigger distributions on small mammals across 11 provinces of Thailand, their associations with habitat types stratified by human disturbance, and the microbiomes of nine widely-distributed chigger species. We show that chigger species richness is influenced by mammalian host status, climatic factors and land use; whereas chigger species and geographic region, but not habitat type, significantly impact on the β-diversity of chigger microbiomes.

Materials & Methods

For a more detailed description of the Materials and Methods, see Supplemental Materials and Methods.

Trapping of small mammals and chigger collections

This study utilised chigger material collected previously for a taxonomic study in Thailand [13]. In brief, small mammals were trapped across 13 localities between 2008 – 2015, once each in the dry season and wet season. Chiggers were removed from mammal cadavers and fixed in 70 - 95% ethanol. Mites collected from each animal were counted to estimate infestation intensity and chigger abundance, as defined by Rózsa *et al*. [14]. For identification and species richness estimation, 10-20% of chiggers from each host animal were selected using size and microscopic appearance as a guide to obtain a representative sub-sample.

Ecological analysis

For ecological analysis, trapping sites were divided equally into four different types of habitats with respect to human land use (anthropization index), spanning low to high levels of disturbance [15-17]. Calculation of infestation intensity statistics of conspecific chigger species were performed using the "BiodiversityR" package. In addition, 12 chigger species that infested ≥10 individual hosts were included in an analysis of association with habitat type using the "FactoMineR" package in R.

Network analyses of host-chigger interactions

To study the community ecology of host-chigger interactions, bipartite network analyses of host-ectoparasite interactions were conducted at both community (pooled host species or pooled locations) and individual levels using "vegan" [18] and "bipartite" packages [19] implemented in R freeware. Bipartite networks were transformed to unipartite networks using the "tnet" package [20]. Unipartite network plots illustrate relative interaction patterns within a host community with respect to the co-occurrence of chigger species.

Multiple regression models of independent variables explaining chigger species richness

Generalized linear models were constructed in order to identify potential effects of host attributes (species, sex, maturity and body mass) and ecological factors (habitat, site and season) on chigger species richness at the individual host level. Poisson regression models were created for chigger species richness count data using the "lme4" package [21] in R freeware. Selection of models was based on Akaike's Information Criterion adjusted for small sample size (AICc) using the "gmulti" package [22] in R freeware. Data for scrub typhus human case numbers from the 13 studied sites were obtained from the Bureau of Epidemiology, Ministry of Public Health, Thailand (unpublished data).

DNA extraction

As clearing in Berlese's fluid destroys DNA, chiggers destined for DNA extraction were identified using autofluorescence microscopy as previously described [7]. Genomic DNA was purified using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

Library preparation and next generation sequencing of 16S rRNA amplicons

To determine the bacterial microbiome of chiggers, a dual-index nested PCR protocol for MiSeq (Illumina, San Diego, CA, USA) sequencing was applied [23-25] targeting the v4 region of the 16S rRNA gene. The second round indexing PCR was performed using the Nextera XT DNA protocol (Illumina). Each MiSeq run included three types of negative control to identify potential background contamination from sample manipulation equipment, DNA extraction kits and PCR reagents used in library preparation. Samples were submitted for sequencing with 300 bp paired-end chemistry on the Illumina MiSeq platform at the Centre for Genomic Research (University of Liverpool).

Microbiome profiling

Analyses of 16S rRNA microbiome profile were performed using the Quantitative Insights into Microbial Ecology (QIIME) software package, version 1.8.0 [26]. Operational taxonomic units (OTUs) were created using an open-reference approach using the USEARCH61 method [27] whereby reads are binned at 97% similarity [27] against the Greengene database v. 13_8 [28] followed by *de novo* OTU picking. Bacterial taxonomic assignment was performed with UCLUST. Chimeric sequences were removed using "ChimeraSlayer" [29].

Comparative analyses of the chigger microbiome

Read counts were normalized to relative abundance for graphing or rarefied to 10 000 reads for diversity calculations. Bacterial communities were categorised according to sample type (individuals and pools), selected chigger species and study sites (mixed species), as well as soil samples from Thailand and Lao PDR. For details of α- and β-diversity analyses, and principal coordinates analysis (PCoA), see Supplemental Materials and Methods.

112

113 Geobacillus *qPCR and Sanger sequencing*

114

115 A pair of PCR primers was designed to amplify a 16S rRNA gene portion for the genus *Geobacillus*
116 and related Firmicutes. Individual, 25-pooled and 50-pooled chiggers, as well as water samples from
117 the laboratory water bath (Grant Sub; Grant Instruments, Cambridge, UK) and Qiagen microbial
118 DNA-free water (negative control), were used in the qPCR assay. DNA from chiggers and 10 µl of
119 water bath samples were extracted using the DNeasy Blood & Tissue Kit (Qiagen).

120 Bacterial taxonomy was assigned using RDP Classifier Version 2.10 [30] available at
121 https://rdp.cme.msu.edu, using a >80% confidence threshold [31]. The DNA sequences were aligned
122 using ClustalW and phylogenetic tree construction was performed with the maximum likelihood
123 method using Mega software version 6.06 [32].

124 *Determination of GC content in 16S rRNA sequences*

125 We evaluated whether the influence of GC content differentially affected data obtained from
126 individual and pooled chiggers (low and high DNA concentration templates, respectively).
127 Representative sequences of the dominant bacterial OTUs from individual and pooled chiggers were
128 assessed for GC content using "Oligo Calc", an oligonucleotide properties calculator available at
129 http://biotools.nubic.northwestern.edu/OligoCalc.html [33] and their mean GC content was
130 compared by two-sample *t*-test.

131 **Results**

132 *Chigger ecology and host associations*

133 A total of 16 761 chiggers were obtained from 1 574 small mammals belonging to 18 species. The
134 overall infestation rate was 23.8%, with Bo Kleu district (Nan province) displaying the highest rate
135 recorded for a single site (95%) (Table S1). The highest mean chigger intensity (113.3) was observed

136 in *Berylmys bowersi* (Bower's white-toothed rat) (Table S2). A subsample of 2 519 chiggers
137 (approximately 15% of the total) were identified to the species level, revealing that *Rattus tanezumi*
138 (Asian house rat) and *Bandicota indica* (greater bandicoot rat) exhibited the greatest chigger species
139 richness (21 species each). Approximately half of the infested hosts (50.7%) harboured a single
140 chigger species, 33.3% harboured two, and the remainder harboured ≥3 species. *Ascoshoengastia*
141 *indica* was most prevalent (7.31%; the only species recorded from every geographic region),
142 followed by *L. deliense* (5.22%) and *Walchia micropelta* (5.16%) (Table S3).

143 A species accumulation curve plot demonstrated that the sample size of small mammals was
144 sufficient to describe the chigger species diversity accurately, since a plateau was reached at around
145 1,000 hosts (Fig. S1). Chigger species richness of sampling locations increased at higher latitudes
146 (Spearman's rank correlation = 60.81, $p = 0.0023$; Fig. S2) and varied significantly among the four
147 habitat types (in descending order) of forest, dry land, rain-fed land, and human settlement at both
148 an individual host level (Kruskal-Wallis statistic = 91.29, df = 3, $p < 0.0001$; Fig. 1b) and for the whole
149 population (Fig. 1a). Moreover, while there were no seasonal differences in chigger species richness
150 or abundance at the individual host level, chigger species richness was considerably higher in the dry
151 season than in the wet season at the whole country level (Fig. 1c). Ecological specialisation of some
152 of the most widespread chigger species (*A. indica, W. micropelta* and *Walchia pingue*) between
153 habitat types was weak (Fig. 2). However, *L. deliense* showed a preference for areas in forest or dry
154 land; whereas other species with more restricted distributions displayed predilections for human
155 settlements (*Helenicula kohlsi*), rain-fed lowland (e.g., *Walchia minuscuta*) or dry landscapes
156 (*Helenicula pilosa*) (Fig. 2).

157 Bipartite network analysis showed highly complex interactions between chigger and host species
158 (Fig. 3a). The largest chigger species assemblages at the whole host population level were found on
159 two rodent species associated with human settlements, *B. indica* and *R. tanezumi*. Interestingly, the
160 only non-rodent hosts sampled in this study, *Hylomys suillus* (Erinaceomorpha: Erinaceidae) and

Tupaia glis (Scandentia: Tupaiidae), were parasitized by several chigger species never found on rodents (Fig. 3a). Overall however, more than half of chigger species were found on more than one host species, and the species-specificity for those found on >10 individual animals was only 0.171 – 0.542. A unipartite network analysis supported the bipartite analysis, assigning *B. indica* and *R. tanezumi* with the highest Eigenvector centrality scores among all of the hosts (Fig. 3b).

Chigger-host properties and scrub typhus incidence

For each of the 13 geographic sites, bipartite network properties of host-chigger interactions were calculated at the individual host level, including the nestedness metric based on overlap and decreasing fill (NODF), network connectance, links per species, and network modularity. The highest NODF and connectance were found in the Nakhonsawan network, where chigger species richness was only four species; while the Chiangrai network exhibited an elevated chigger species richness (12 species), but with the lowest NODF and connectance (Table S4). In contrast, Chiangrai displayed the highest modularity within the network, whereas the least network modularity was found in Prachuab Kirikhan (Table S4).

We tested the effect of various independent variables on individual chigger species richness using GLMs with model selection by Akaike's Information Criterion. Host species, host maturity, site and habitat (but not host sex) were significant variables in the best 10 models (Table S5; Fig. S3a). Animals captured in forest demonstrated significantly higher chigger species richness than hosts from human settlements (estimate = -1.074, $p < 0.0001$; Table S6), and species richness was greater on mature hosts than on juveniles (estimate = -0.283, $p = 0.004$; Table S6).

We then applied the same modelling approach but included human scrub typhus cases at district level with environmental variables (elevation, annual mean temperature and latitude; Table S7), chigger species richness, and network properties (Fig. S3b). Network connectance and chigger species richness strongly influenced local scrub typhus case numbers, as the two variables appeared

in the top 10 selected models (Table S8). Finally, we performed a univariate analysis, which also showed that scrub typhus case number was positively correlated with chigger species richness (Spearman rank correlation = 45.71, p = 0.0006; Fig. 4a) and negatively correlated with host-chigger network connectance (Spearman's rank correlation = 485.45, p = 0.011; Fig. 4b). Importantly, there was no significant relationship between overall chigger abundance and scrub typhus incidence (R^2 = 0.105, *P* = 0.732; data not shown).

Microbiome of individual and pooled chigger specimens

The total number of 16S rRNA reads from the complete set of 366 samples (264 individual chiggers, 69 pooled chiggers, 18 soil samples and 15 background controls) after quality filtering, de-multiplexing and error correction was 51 896 654 (mean reads per sample = 137 657; SD = 69 522). After paired read alignment and size selection at 270 - 300 bp, the read number was 49 635 427 (mean reads per sample = 131 659; SD = 69 922), a sequence retention of 94%. The analysis of individual chigger specimens comprised nine widespread species: *A. indica, L. deliense, W. micropelta, W. minuscuta, Walchia kritochaeta, H. pilosa, H. kohlsi, Blankaartia acuscutellaris,* and *Schoengastiella ligula*. However, after removing samples with high similarity to negative controls (see Supplemental Materials and Methods), more than half (58.7%) were excluded from downstream analyses, including all of those for *W. minuscuta*.

The microbiomes of individual chiggers were dominated by several different *Geobacillus* OTUs (Fig. 5). However, *Sphingobium* (α-Proteobacteria) was also abundant, as were Comamonadaceae (especially in *Walchia* spp.) and *Brevibacillus* (particularly in *B. acuscutellaris* and *L. deliense*). Importantly, we only detected *O. tsutsugamushi* in *L. deliense* (3/39 individual specimens that passed QC), with a maximum OTU proportion of 19.58% (Fig. 5; Table 1). Other bacteria with pathogenic potential in humans were found across several chigger species, including *Mycobacterium* (11.93% of specimens), *Staphylococcus* (8.25%) and *Haemophilus parainfluenzae* (7.34%) (Table 1). However, most arthropod symbionts known to be of importance in other mite species or in insects

(*Cardinium, Pseudonocardia* and *Rickettsiella*) were rare (<2% prevalence), while *Wolbachia* remained undetected at the individual level (Table 1).

To mitigate the problem of low biomass when amplifying 16S rRNA fragments from individual chiggers, we also sequenced several pools of 50 specimens each for *A. indica, L. deliense, W. micropelta, W. minuscuta,* and *Blankaartia acuscutellaris* (Fig. 6a); as well as three mixed-species pools of 50 specimens each for all 11 Thai provinces (Fig. 6b). This strategy was successful, as fewer samples (7.2%) were removed due to high similarity with negative controls when compared with individual samples. Surprisingly, two OTUs (*Geobacillus* and *Brevibacillus*) that were highly prevalent and relatively abundant in the individual-level data were not present at a read count ≥5 in any of the pooled data (Table 1). For some of the potential pathogens, individual and pooled data showed good concordance (*Staphylococcus* and *Mycobacterium* detected in 95.38% and 73.85% of pools, respectively), whereas others that were rarely detected in individuals were robustly confirmed by the pooling strategy (*Borrelia* in 49.23% and *Corynebacterium* in 78.46% of pools, respectively) (Table 1). Pooling also provided additional evidence that three classical arthropod symbionts (*Cardinium*, *Pseudonocardia* and *Rickettsiella*) were present in chiggers (~20 – 45% of pools), while a fourth (*Wolbachia*) was present only in two (3.08%) pools (Table 1). Intriguingly, one *Neisseriaceae* OTU (933546) was detected in 95.38% of pooled samples and was particularly dominant in *L. deliense*, reaching a maximum OTU proportion of 92.48% (Table 1). In accordance with the individual chigger data, the 13 (20%) pooled samples positive for *O. tsutsugamushi* (Table 1) all contained *L. deliense*.

To investigate whether the presence of *Geobacillus* might have resulted from contamination of samples with spores in the laboratory or with bacterial DNA in the extraction kits, we first examined OTUs sequenced from negative controls, then measured levels of Firmicutes 16S rDNA by qPCR in chiggers compared with samples from the laboratory water bath. The dominant *Geobacillus* OTU observed in individual chiggers was absent in background controls (Table 1; Table S9). Despite a high

Firmicutes signal in the water bath (Fig. S4), Sanger sequencing revealed that this was derived from *Paenibacillus* spp. and related *Bacillales*, whereas *Geobacillus* spp. were only observed in individual chigger samples (Fig. S5). Finally, we calculated percent GC content for the 15 most abundant OTUs in individual specimens and the 26 most abundant OTUs in pooled samples. This showed that the GC content of the OTUs from individual specimens was significantly higher than for the pooled material ($P = 0.0094$; Fig. S6).

Factors affecting the microbial profile of chiggers

The α-diversity of bacterial OTUs determined by a richness estimator (Chao1) and the whole-tree phylogenetic diversity index (PD_whole_tree) revealed significant differences between the sample types, with pooled chigger and soil samples exhibiting higher diversity than individual chigger specimens (Kruskal-Wallis test with post-hoc Bonferroni correction, $P < 0.001$) (Table S10). The latter were not significantly more diverse than control samples. Analysis of β-diversity showed that the sample types were generally well separated from one another (ANOSIM: $R = 0.7997$, $P = 0.001$), although some background controls were nested on the periphery of the individual chigger samples (Fig. S7). Bacterial communities were significantly clustered with respect to chigger species and geographic location (study sites) in both individual and pooled chiggers ($P < 0.001$), whereas habitat (human disturbance transect) failed to show a significant effect (Fig. 7). The impact of chigger species and geographic location on β-diversity displayed similar correlation coefficients and network topology (Fig. 7).

Discussion

To the best of our knowledge, habitat type, chigger diversity and human scrub typhus incidence have never been analysed together on a countrywide scale before. The current study sampled more than one-third of known chigger species in Thailand [13] and found three significant environmental associations with species richness: a positive correlation with latitude, a negative correlation with

increased human land use, and an elevated level of chigger diversity in the dry season (in addition to significant effects of host species and maturity). Latitudinal gradients are associated with bioclimatic factors such as mean temperature, humidity and rainfall, and the species richness of animals and plants tends to increase at lower latitudes approaching the equator [34-36]. Thus, parasite diversity might also be expected to be higher at lower latitudes, and there is some evidence of this for microbial pathogens [37-39]. However, the opposite trend observed here is supported by previous studies on fleas [40]. Moreover, in Yunnan province in China, chigger diversity on small mammals was even higher than in our study and increased according to latitude up to a zenith at 25 - 26°N before decreasing further north [41], suggesting the presence of an optimum zone (our study covered 7 – 19°N). One hypothesis to explain this phenomenon is that the geographic range of individual hosts tends to be broader at higher latitudes, perhaps facilitating the accumulation of a greater diversity of ectoparasites [36].

Analysis of chigger distributions in Yunnan also concur with our findings on the impact of human disturbance of the natural environment, with a significantly greater host and chigger species richness being observed in a mountainous uncultivated landscape habitat compared with a cultivated flatland landscape [42]. We increased the resolution of the human land use analysis in our study by trapping hosts across a transect of four rather than two habitat categories, which revealed a stepwise reduction in chigger species richness as human disturbance increased, reflecting the universal process of loss of animal and plant diversity through urbanisation. Seasonality was also an apparent determinant of chigger diversity in our study, with a striking increase in species richness during the dry season compared with the wet season. However, caution is required in interpreting this finding, as our field studies were not designed to standardise sampling across the two seasons. Nevertheless, it is plausible that breeding frequency is reduced in the wet season, and/or that many chiggers emerging from underground during monsoon periods are washed into water bodies before they can attach to a host. Chigger species also have different seasonal preferences. For instance, in subtropical regions, most scrub typhus cases occur in the autumn when populations of

285 *Leptotrombidium pallidum* and *Leptotrombidium scutellare* dramatically increase (as seen in South
286 Korea [43, 44]), or the main vector may change between summer and winter (as seen with *L.*
287 *deliense* and *L. scutellare* in Taiwan, respectively [45]).

288 Here, the species richness of chiggers was identified as a positive correlate of scrub typhus incidence
289 for the first time. Since decreased human land use is associated with both increased chigger species
290 richness (this study) and higher prevalence of *O. tsutsugamushi* infection in small mammals [12],
291 increased biodiversity may be a risk factor for human scrub typhus. This contradicts a meta-analysis
292 of chigger-host relationships in Yunnan, where a lower host and chigger diversity in cultivated
293 flatland was associated with a greater abundance of chiggers, especially species that are known or
294 potential vectors of scrub typhus [42]. However, as neither human scrub typhus incidence nor *O.*
295 *tsutsugamushi* prevalence in small mammals was incorporated into the Yunnan study, and an
296 efficient scrub typhus vector (*L. scutellare*) was abundant in mountainous uncultivated land (the
297 higher biodiversity site), the impact of land use on infection risk remains an open question in that
298 region. In Taiwan, spatial modelling of land use data revealed significant positive correlations
299 between crop-vegetation mosaics and forest, as well as elevation, with scrub typhus incidence [46].
300 In contrast with our study, follow-up investigations in Taiwan found that chigger prevalence and
301 abundance on small mammals was positively associated with both human scrub typhus incidence
302 and host *O. tsutsugamushi* seropositivity [45]. Chigger species richness and host-chigger networks
303 were not explicitly incorporated in this Taiwanese study, but both the diversity of chiggers (12
304 species) and their hosts (8 species) were markedly lower than we observed in Thailand.

305 At first glance, the association between chigger species richness and scrub typhus incidence in
306 Thailand seems paradoxical as we only detected *O. tsutsugamushi* in a single species (*L. deliense*).
307 Yet it is important to emphasise that other potential vectors of scrub typhus (*e.g.*, *L. imphalum*, the
308 principal vector in northern Thailand [47]) were collected but not subjected to 16S rRNA sequencing.
309 Moreover, more than 20 species of *Leptotrombidium* have been reported from Thailand, many only

310 from the northern provinces where scrub typhus incidence is highest [13]. Although the majority of
311 these species are not known to be scrub typhus vectors, recent data on vector competence are
312 lacking, and it is plausible that transmission of *O. tsutsugamushi* by two or more vectors in the same
313 region may contribute to diversification of the pathogen and an increase in human cases, as has
314 been recently hypothesised for Taiwan [45, 48]. We also observed that human scrub typhus
315 incidence was negatively associated with host-parasite network connectance, suggesting that
316 increasing complexity of chigger-host interactions might reduce human exposure by zooprophylaxis,
317 or lead to a greater likelihood that non-vector species dominate networks.

318 With the exception of a laboratory colony of *L. imphalum* [10], the composition of the chigger
319 microbiome was largely unknown prior to our study. Our data reveal complex microbiomes that (in
320 contrast with those of many other arthropods such as certain vectors [49] or sap-feeding insects
321 [50]) are not dominated by a very small number of specialised primary and secondary symbionts.
322 Since questing chiggers emerge from underground and are associated with their host for only few
323 days before moulting into free-living nymphs, we hypothesised that they may not require symbionts
324 for dietary supplementation, and may passively accumulate soil microbes instead. Indeed, it is now
325 known that the *L. deliense* genome contains terpene synthase genes that appear to have been
326 acquired by ancient lateral gene transfer from Actinobacteria and other environmental phyla [11].
327 However, while bacterial sequences of putative soil origin were prevalent in chiggers (*e.g.*, *Bacillus*
328 *cereus* and *Mycobacterium* spp.), on the basis of the limited number of soil samples we analysed
329 here, the chigger "microbiome" is not simply a result of soil particles adhering to the mite surface.
330 The clear impact of chigger species and geographical location, but not human land disturbance, on
331 the microbial sequence profiles lends further support to the concept of an integral microbiome in
332 chiggers that may be modulated by habitat on large (several hundred km) but not small (a few km)
333 scales. This may be because their mobile hosts can travel between the human disturbance zones we
334 defined within sampling sites [16, 51]. Evidence that the host also contributes to the chigger

335 microbiome was revealed by the presence of typical mammalian-associated flora such as
336 *Staphylococcus* spp. and *Haemophilus* spp.

337 The prevalence of the classical intracellular arthropod symbionts *Cardinium*, *Rickettsiella* and
338 *Wolbachia* was quite low in individual specimens, despite their importance in other mite taxa [52].
339 While *Orientia* in some *Leptotrombidium* spp. could conceivably displace these symbionts due to
340 competition for intracellular niches, it is rare or absent in most other chigger genera [53].
341 Unfortunately, the sample size of *Orientia*-infected chiggers was too small in this study to investigate
342 the impact of the pathogen on microbiome composition. In contrast to a recent analysis of the
343 microbiome of colonised *L. imphalum*, we found no evidence for an abundant *Amoebophilaceae*
344 OTU; although this is not surprising, as it was found to be uncommon in all life stages except
345 *Orientia*-infected adult females [10], and neither this species nor life stage was included in our
346 microbiome analysis. Future studies should consider the role of *Neisseriaceae* OTU 933546 in chigger
347 biology and potential interactions with vectored pathogens. Notably, this family in the β-
348 proteobacteria exhibited a moderate prevalence in *L. deliense* individuals and contains gut
349 symbionts of bees (*Snodgrassella alvi* [54]) and termites (*Stenoxybacter acetivorans* [55]). This
350 suggests a facultative relationship in *L. deliense*, especially as OTU 933546 was also found in almost
351 30% of soil samples.

352 The high prevalence of sequences from *Geobacillus* spp. was surprising, since this is a thermophilic,
353 spore-forming genus with an optimal growth range of 45 - 70°C. *Geobacillus* spp. thrive in hot
354 composts, subterranean oilfields and hydrothermal vents, but due to their exceedingly robust spores
355 that can be transported worldwide in atmospheric currents, isolates have been obtained across a
356 vast range of temperate or cold terrestrial and marine sediments [56]. We did not surface-sterilise
357 the chiggers as there is no evidence this procedure significantly affects microbiome data obtained
358 from arthropods [57], and the risk of degradation of internal DNA when dealing with soft, minute
359 species that might exhibit small breaches in their exoskeleton was acute. In any case, the dominant

360 *Geobacillus* OTU detected in individual chiggers was absent from the soil we analysed. Despite the
361 potential ubiquity of *Geobacillus* spp. spores in the environment, it is intriguing that this genus is not
362 observed more frequently in arthropod microbiomes. In addition to aphids [58] and ants [59],
363 *Geobacillus* spp. sequences have been reported from sandflies [60], mosquitoes [61] and ticks [62].
364 In mosquitoes, *Geobacillus* spp. were identified as part of the core microbiome of dissected
365 reproductive tracts [61]; whereas in the tick *Dermacentor occidentalis*, it was associated with a
366 greater abundance of *Francisella* relative to *Rickettsia* [62]. These findings indicate that although the
367 genus *Geobacillus* is assumed to be exclusively thermophilic, it may have a potential biological role in
368 disease vectors, suggesting that some strains may actually be mesophilic.

369 The high prevalence of *Geobacillus* sequences in our individual, but not pooled, chigger data raised
370 important questions about both reagent contamination with bacterial DNA and amplification biases
371 caused by variation in GC content. Several recent studies have highlighted the pitfalls of microbiome
372 studies on low biomass samples, where bacterial DNA present in molecular biology reagents
373 competes very effectively as PCR template with bacterial DNA from the sample itself [63, 64]. Since
374 DNA from more than 180 environmental bacterial genera has been detected in commercially
375 available DNA extraction kits [64], assessing the true impact of laboratory contamination on the
376 ensuing data is extremely challenging. The conservative approach that we used here for the
377 individual chiggers was effective, but led to exclusion of more than half of these samples from
378 downstream analyses.

379 Pooling appears to be an obvious solution to the problem of low biomass samples [65], but is not
380 without its own drawbacks. Genomic GC content is now well known as a source of bias in 16S rRNA
381 datasets, with higher GC content leading to underrepresentation [66], as we observed here with
382 *Geobacillus* spp. (relatively high median GC of ~52%) in pooled samples. At lower template
383 concentrations, denaturation of DNA appears to have been more efficient, revealing OTUs that
384 would have remained hidden had we only sequenced pools.

Conclusion

This study emphasises that among human disease vectors, chiggers exhibit some of the most complex ecological relationships [67], with high species diversity and low host specificity contributing to elevated rates of coinfection on individual mammalian hosts. The diverse microbiomes of chiggers add a further layer to the network of potential interactions that *Orientia* is exposed to, and future studies should determine whether some of these commensal bacteria affect chigger vector competence. Moreover, the positive correlation we identified here between chigger species richness and scrub typhus incidence deserves further investigation in other endemic countries, especially in relation to the epidemiology of *Orientia* strain diversity [48].

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Conflicts of interest

None.

403 **Figure 1**: Effect of habitat and season on chigger species richness. (A) Chigger species accumulation
404 curves among different habitats at the host population level. (B) Mean chigger species richness per
405 host individual by habitat type. (C) Chigger species accumulation curves between the dry (red) and
406 wet (blue) season.

407 **Figure 2** Correspondence analysis showing the association between the 12 dominant chigger species
408 (Aind, *Ascoschoengastia indica*; Bacu, *Blankaartia acuscutellaris*; Hkoh, *Helenicula kohlsi*; Hpil,
409 *Helenicula pilosa*; Ldel, *Leptotrombidium deliense*; Slig, *Schoengastiella ligula*; Wdis, *Walchia*
410 *dismina*; Wkri, *Walchia kritochaeta*; Wmic, *Walchia micropelta*; Wmin, *Walchia minuscuta*; Wpin,
411 *Walchia pingue*; Wrus, *Walchia rustica*) within the four categorized habitats. The first and second
412 dimensions explain 87% of the total variance (axis 1, 59.82%; axis 2, 27.38%).

413 **Figure 3** Host-chigger associations in Thailand. (A) Bipartite graph based on presence-absence data.
414 The number of individual hosts examined is shown in parentheses. Chigger species with broad
415 host ranges are displayed in bold. (B) Unipartite network and Eigenvector centrality scores
416 illustrating the pattern of chigger sharing among 18 small mammal hosts.

417 **Figure 4** Correlation plots show the relationship between chigger ecology [(A) chigger species
418 richness; (B) host-chigger network connectance] and scrub typhus incidence in humans. Incidence
419 data are displayed as the $\log_{10}$ transformation of the number of cases per year.

420 **Figure 5** Relative abundance of bacterial OTUs in background controls and individual chiggers. (A)
421 subfamily Gahrliepiinae and subfamily Trombiculinae. (B) Tribe Schoengastiini. (C) Tribe
422 Trombiculini. The data are filtered; OTUs that represented <10% in a sample were combined in
423 "others" (light grey) to aid visualisation. Source data are included in Table S11.

424 **Figure 6** Relative abundance of bacterial OTUs in background controls and pooled samples. (A) Pools
425 by chigger species (50 individuals per sample). (B) Mixed chigger species (50 individuals per sample)

426 pooled by province. The data are filtered; OTUs that represented <10% in a sample were combined
427 in "others" (light grey) to aid visualisation. Source data are included in Table S11

428 **Figure 7** Principal coordinates analysis plots created using the unweighted UniFrac metric showing
429 bacterial community clustering of individual (left panels) and pooled chiggers (right panels) among
430 different sample categories. (A, B) chigger species; (C, D) habitat; and (E, F) study site. Control data
431 are shown for reference only and were not included in the ANOSIM.

432

433 **Table 1.** Selected bacterial taxa of public health importance, potential symbionts, and other prevalent OTUs detected in individual and pooled

434 chiggers in comparison to soils and background controls. Only the OTUs with ≥5 reads were included. Superscript "I" or "P" indicate maximum

435 OTU proportion values from individual or pooled chiggers, respectively.

Bacterial taxa (OTU identifier)	Maximum OTU Proportion (%)	Individual chigger		Pooled chigger		Soil		Control	
		Positive samples	(%)	Positive samples	(%)	Positive samples	(%)	Positive samples	(%)
Opportunistic/Potential pathogens									
Bacillus cereus (4463224)	1.63[P]	4	(3.67)	31	(47.69)	11	(61.11)	-	-
Borrelia (New.ReferenceOTU7)	34.82[P]	1	(0.92)	32	(49.23)	-	-	-	-
Campylobacter (New.CleanUp.ReferenceOTU30)	1.49[P]	-	-	3	(4.62)	-	-	-	-
Clostridium (New.ReferenceOTU2470)	3.43[P]	-	-	6	(9.23)	-	-	-	-
Corynebacterium (13485)	16.36[P]	7	(6.42)	65	(100)	-	-	2	(13.33)

Fusobacterium (2438396)	2.44[P]	1	(0.92)	6	(9.23)	-	-	-	-
Haemophilus parainfluenzae (4473129)	1.83[I]	8	(7.34)	17	(26.15)	-	-	1	(6.67)
Moraxella (1127280)	1.63[P]	-	-	4	(6.15)	-	-	-	-
Mycobacterium (4448095)	5.22[P]	13	(11.93)	48	(73.85)	18	(100)	-	-
Nocardia (102163)	17.41[P]	5	(4.58)	35	(53.84)	1	(5.55)	-	-
Orientia tsutsugamushi (301131)	19.57[I]	3	(2.75)	13	(20)	-	-	-	-
Staphylococcus (4446058)	55.99[I]	67	(61.47)	65	(100)	3	(16.66)	6	(40)
Potential arthropod symbionts									
Candidatus Cardinium (New.ReferenceOTU10)	19.06[P]	2	(1.83)	18	(27.69)	-	-	-	-
Neisseriaceae (933546)	92.48[P]	18	(16.51)	62	(95.38)	5	(27.78)	4	(26.67)
Rickettsiella (8028)	1.12[P]	2	(1.83)	12	(18.46)	-	-	-	-
Wolbachia (New.ReferenceOTU2936)	2.32[P]	-	-	2	(3.08)	-	-	-	-

Other prevalent OTUs									
Acinetobacter rhizosphaerae (4334053)	6.13[P]	4	(3.67)	33	(50.77)	-	-	-	-
Brevibacillus (3307468)	60.37[I]	61	(55.96)	-	-	-	-	-	-
Burkholderia bryophila (4320353)	1.27[I]	38	(34.86)	2	(3.07)	-	-	-	-
Geobacillus (New.ReferenceOTU5884)	10.73[I]	82	(75.23)	-	-	-	-	-	-
Nevskia (516554)	2.06[I]	27	(24.77)	3	(4.61)	7	(38.88)	-	-
Sphingobacterium multivorum (4423201)	1.16[I]	44	(40.36)	15	(23.07)	-	-	1	(6.67)
Sphingobium (4393057)	14.69[I]	82	(75.23)	11	(16.92)	1	(5.55)	-	-
Streptomyces (821185)	59.26[I]	2	(1.83)	13	(20)	1	(5.55)	-	-
Methylobacterium adhaesivum (4303249)	3.96[I]	11	(10.09)	12	(18.46)	-	-	-	-

436 References

437

438 1. Shatrov AB, Kudryashova, N.I., *Taxonomy, life cycles and the origin of parasitism in*
439 *trombiculid mites*, in *Micromammals and Macroparasites: From Evolutionary Ecology to*
440 *Management*, S. Morand, Krasnov, B.R, Poulin, R, Editor. 2006, Springer Japan: Tokyo. p.
441 119-40.

442 2. Bonell A, Lubell Y, Newton PN, Crump JA, Paris DH. Estimating the burden of scrub typhus: A
443 systematic review. PLoS Negl Trop Dis. 2017;11:e0005838. 10.1371/journal.pntd.0005838

444 3. Izzard L, Fuller A, Blacksell SD, Paris DH, Richards AL, Aukkanit N, et al. Isolation of a novel
445 Orientia species (O. chuto sp. nov.) from a patient infected in Dubai. J Clin Microbiol.
446 2010;48:4404-9. 10.1128/JCM.01526-10

447 4. Weitzel T, Dittrich S, Lopez J, Phuklia W, Martinez-Valdebenito C, Velasquez K, et al. Endemic
448 Scrub Typhus in South America. N Engl J Med. 2016;375:954-61. 10.1056/NEJMoa1603657

449 5. Masakhwe C, Linsuwanon P, Kimita G, Mutai B, Leepitakrat S, Yalwala S, et al. Identification
450 and Characterization of Orientia chuto in Trombiculid Chigger Mites Collected from Wild
451 Rodents in Kenya. J Clin Microbiol. 2018;56. 10.1128/JCM.01124-18

452 6. Takahashi M, Urakami H, Yoshida Y, Furuya Y, Misumi H, Hori E, et al. Occurrence of high
453 ratio of males after introduction of minocycline in a colony of Leptotrombidium fletcheri
454 infected with Orientia tsutsugamushi. Eur J Epidemiol. 1997;13:79-86.

455 7. Kumlert R, Chaisiri K, Anantatat T, Stekolnikov AA, Morand S, Prasartvit A, et al.
456 Autofluorescence microscopy for paired-matched morphological and molecular
457 identification of individual chigger mites (Acari: Trombiculidae), the vectors of scrub typhus.
458 PLoS One. 2018;13:e0193163. 10.1371/journal.pone.0193163

459 8. Kabeya H, Colborn JM, Bai Y, Lerdthusnee K, Richardson JH, Maruyama S, et al. Detection of
460 Bartonella tamiae DNA in Ectoparasites from Rodents in Thailand and Their Sequence
461 Similarity with Bacterial Cultures from Thai Patients. Vector-Borne and Zoonotic Diseases.
462 2010;10:429-34.

463 9. Huang Y, Zhao L, Zhang Z, Liu M, Xue Z, Ma D, et al. Detection of a Novel Rickettsia From
464 Leptotrombidium scutellare Mites (Acari: Trombiculidae) From Shandong of China. J Med
465 Entomol. 2017;54:544-9. 10.1093/jme/tjw234

466 10. Ponnusamy L, Willcox AC, Roe RM, Davidson SA, Linsuwanon P, Schuster AL, et al. Bacterial
467 microbiome of the chigger mite Leptotrombidium imphalum varies by life stage and
468 infection with the scrub typhus pathogen Orientia tsutsugamushi. PLoS One.
469 2018;13:e0208327. 10.1371/journal.pone.0208327

11. Dong X, Chaisiri K, Xia D, Armstrong SD, Fang Y, Donnelly MJ, et al. Genomes of trombidid mites reveal novel predicted allergens and laterally transferred genes associated with secondary metabolism. Gigascience. 2018;7. 10.1093/gigascience/giy127

12. Chaisiri K, Cosson JF, Morand S. Infection of Rodents by Orientia tsutsugamushi, the Agent of Scrub Typhus in Relation to Land Use in Thailand. Trop Med Infect Dis. 2017;2. 10.3390/tropicalmed2040053

13. Chaisiri K, Stekolnikov AA, Makepeace BL, Morand S. A Revised Checklist of Chigger Mites (Acari: Trombiculidae) From Thailand, with the Description of Three New Species. J Med Entomol. 2016;53:321-42. 10.1093/jme/tjv244

14. Rozsa L, Reiczigel J, Majoros G. Quantifying parasites in samples of hosts. Journal of Parasitology. 2000;86:228-32. Doi 10.2307/3284760

15. Auffray JC, Blasdell KR, Bordes F, Chabé M, Chaisiri K, Charbonnel N, et al., *Protocols for field and laboratory rodent studies*, ed. V. Herbreteau, Jittapalapong, S., Rerkamnuaychoke, W., Chaval, Y., Cosson, J.F. 2011, Bangkok: Kasetsart University Press.

16. Blasdell K, Bordes F, Chaisiri K, Chaval Y, Claude J, Cosson JF, et al. Progress on research on rodents and rodent-borne zoonoses in South-east Asia. Wildlife Research. 2015;42:98-107. doi.org/10.1071/WR14201

17. Chaisiri K, Siribat P, Ribas A, Morand S. Potentially zoonotic helminthiases of murid rodents from the Indo-Chinese peninsula: impact of habitat and the risk of human infection. Vector Borne Zoonotic Dis. 2015;15:73-85. 10.1089/vbz.2014.1619

18. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package. 2015. http://cran.r-project.org/package=vegan Accessed: 24/12/2018.

19. Dormann CF, Fründ J, Blüthgen N, Gruber B. Indices, graphs and null Models: Analyzing bipartite ecological networks. The Open Ecology Journal. 2009;2:7-24. 10.2174/1874213000902010007

20. Opsahl T. tnet: Software for analysis of weighted, two-mode, and longitudinal networks. 2015. https://cran.r-project.org/package=tnet Accessed: 24/12/2018.

21. Bates D, Machler M, Bolker BM, Walker SC. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 2015;67:1-48.

22. Calcagno V, de Mazancourt C. glmulti: An R Package for Easy Automated Model Selection with (Generalized) Linear Models. Journal of Statistical Software. 2010;34:1-29.

501 23. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global
502 patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl
503 Acad Sci U S A. 2011;108 Suppl 1:4516-22. 10.1073/pnas.1000080107

504 24. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index
505 sequencing strategy and curation pipeline for analyzing amplicon sequence data on the
506 MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013;79:5112-20.
507 10.1128/AEM.01043-13

508 25. Anonymous. 16S Metagenomic Sequencing Library Preparation. 2013, Illumina, Inc., 28 pp.
509 http://emea.support.illumina.com/downloads/16s_metagenomic_sequencing_library_prep
510 aration.html?langsel=/gb/ Accessed: 24/12/2018.

511 26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME
512 allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335-6.
513 10.1038/nmeth.f.303

514 27. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics.
515 2010;26:2460-1. 10.1093/bioinformatics/btq461

516 28. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved
517 Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of
518 bacteria and archaea. ISME J. 2012;6:610-8. 10.1038/ismej.2011.139

519 29. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA
520 sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons.
521 Genome Res. 2011;21:494-504. 10.1101/gr.112730.110

522 30. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of
523 rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261-7.
524 10.1128/AEM.00062-07

525 31. Soergel DA, Dey N, Knight R, Brenner SE. Selection of primers for optimal taxonomic
526 classification of environmental 16S rRNA gene sequences. ISME J. 2012;6:1440-4.
527 10.1038/ismej.2011.208

528 32. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary
529 Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725-9. 10.1093/molbev/mst197

530 33. Kibbe WA. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res.
531 2007;35:W43-6. 10.1093/nar/gkm234

532 34. Lindenfors P, Nunn CL, Jones KE, Cunningham AA, Sechrest W, Gittleman JL. Parasite species
533 richness in carnivores: effects of host body mass, latitude, geographical range and
534 population density. Global Ecology and Biogeography. 2007;16:496–509.

535 35. Bordes F, Morand S, Krasnov BR, Poulin R, *Parasite diversity and latitudinal gradients in*
536 *terrestrial mammals*, in *The Biogeography of Host-Parasite Interactions*, S. Morand and B.R.
537 Krasnov, Editors. 2010, Oxford University Press: New York. p. 89-98.

538 36. Morand S. (macro-) Evolutionary ecology of parasite diversity: From determinants of
539 parasite species richness to host diversification. Int J Parasitol Parasites Wildl. 2015;4:80-7.
540 10.1016/j.ijppaw.2015.01.001

541 37. Guernier V, Hochberg ME, Guegan JF. Ecology drives the worldwide distribution of human
542 diseases. PLoS Biol. 2004;2:e141. 10.1371/journal.pbio.0020141

543 38. Nunn CL, Altizer SM, Sechrest W, Cunningham AA. Latitudinal gradients of parasite species
544 richness in primates. Diversity and Distributions. 2005;11:249–56.

545 39. Bordes F, Guégan JF, Morand S. Microparasite species richness in rodents is higher at lower
546 latitudes and is associated with reduced litter size. Oikos. 2011;120:1889–96.

547 40. Krasnov BR, Shenbrot GI, Khokhlova IS, Degen AA. Flea species richness and parameters of
548 host body, host geography and host 'milieu'. Journal of Animal Ecology. 2004;73:1121–8.

549 41. Peng PY, Guo XG, Ren TG, Song WY, Dong WG, Fan R. Species diversity of ectoparasitic
550 chigger mites (Acari: Prostigmata) on small mammals in Yunnan Province, China. Parasitol
551 Res. 2016;115:3605-18. 10.1007/s00436-016-5127-x

552 42. Peng PY, Guo XG, Jin DC, Dong WG, Qian TJ, Qin F, et al. Landscapes with different
553 biodiversity influence distribution of small mammals and their ectoparasitic chigger mites: A
554 comparative study from southwest China. PLoS One. 2018;13:e0189987.
555 10.1371/journal.pone.0189987

556 43. Park GM, Shin HS. Geographical Distribution and Seasonal Indices of Chigger Mites on Small
557 Mammals Collected on the East Coast of the Republic of Korea. J Parasitol. 2016;102:193-8.
558 10.1645/15-760

559 44. Choi YJ, Lee IY, Song HJ, Kim J, Park HJ, Song D, et al. Geographical distribution of Orientia
560 tsutsugamushi strains in chiggers from three provinces in Korea. Microbiol Immunol.
561 2018;62:547-53. 10.1111/1348-0421.12639

562 45. Kuo CC, Lee PL, Chen CH, Wang HC. Surveillance of potential hosts and vectors of scrub
563 typhus in Taiwan. Parasit Vectors. 2015;8:611. 10.1186/s13071-015-1221-7

564 46. Wardrop NA, Kuo CC, Wang HC, Clements AC, Lee PF, Atkinson PM. Bayesian spatial
565 modelling and the significance of agricultural land use to scrub typhus infection in Taiwan.
566 Geospat Health. 2013;8:229-39. 10.4081/gh.2013.69

567 47. Tanskul P, Linthicum KJ. Redescription of Leptotrombidium (Leptotrombidium) imphalum
568 (Acari: Trombiculidae), with observations on bionomics and medical importance in northern
569 Thailand. J Med Entomol. 1999;36:88-91.

570 48. Kim G, Ha NY, Min CK, Kim HI, Yen NT, Lee KH, et al. Diversification of Orientia tsutsugamushi
571 genotypes by intragenic recombination and their potential expansion in endemic areas. PLoS
572 Negl Trop Dis. 2017;11:e0005408. 10.1371/journal.pntd.0005408

573 49. Rio RV, Attardo GM, Weiss BL. Grandeur Alliances: Symbiont Metabolic Integration and
574 Obligate Arthropod Hematophagy. Trends Parasitol. 2016;32:739-49.
575 10.1016/j.pt.2016.05.002

576 50. Hansen AK, Moran NA. The impact of microbial symbionts on host plant utilization by
577 herbivorous insects. Mol Ecol. 2014;23:1473-96. 10.1111/mec.12421

578 51. Morand S, Bordes F, Chen HW, Claude J, Cosson JF, Galan M, et al. Global parasite and
579 Rattus rodent invasions: The consequences for rodent-borne diseases. Integr Zool.
580 2015;10:409-23. 10.1111/1749-4877.12143

581 52. Chaisiri K, McGarry JW, Morand S, Makepeace BL. Symbiosis in an overlooked microcosm: a
582 systematic review of the bacterial flora of mites. Parasitology. 2015;142:1152-62.
583 10.1017/S0031182015000530

584 53. Traub R, Wisseman CL, Jr. Ecological considerations in scrub typhus. 2. Vector species. Bull
585 World Health Organ. 1968;39:219-30.

586 54. Kwong WK, Moran NA. Cultivation and characterization of the gut symbionts of honey bees
587 and bumble bees: description of Snodgrassella alvi gen. nov., sp. nov., a member of the
588 family Neisseriaceae of the Betaproteobacteria, and Gilliamella apicola gen. nov., sp. nov., a
589 member of Orbaceae fam. nov., Orbales ord. nov., a sister taxon to the order
590 'Enterobacteriales' of the Gammaproteobacteria. Int J Syst Evol Microbiol. 2013;63:2008-18.
591 10.1099/ijs.0.044875-0

592 55. Wertz JT, Breznak JA. Stenoxybacter acetivorans gen. nov., sp. nov., an acetate-oxidizing
593 obligate microaerophile among diverse O2-consuming bacteria from termite guts. Appl
594 Environ Microbiol. 2007;73:6819-28. 10.1128/AEM.00786-07

595 56. Zeigler DR. The Geobacillus paradox: why is a thermophilic bacterial genus so prevalent on a
596 mesophilic planet? Microbiology. 2014;160:1-11. 10.1099/mic.0.071696-0

57. Hammer TJ, Dickerson JC, Fierer N. Evidence-based recommendations on storing and handling specimens for analyses of insect microbiota. PeerJ. 2015;3:e1190. 10.7717/peerj.1190

58. Jousselin E, Clamens AL, Galan M, Bernard M, Maman S, Gschloessl B, et al. Assessment of a 16S rRNA amplicon Illumina sequencing procedure for studying the microbiome of a symbiont-rich aphid genus. Mol Ecol Resour. 2016;16:628-40. 10.1111/1755-0998.12478

59. Seipke RF, Barke J, Heavens D, Yu DW, Hutchings MI. Analysis of the bacterial communities associated with two ant-plant symbioses. Microbiologyopen. 2013;2:276-83. 10.1002/mbo3.73

60. McCarthy CB, Diambra LA, Rivera Pomar RV. Metagenomic analysis of taxa associated with Lutzomyia longipalpis, vector of visceral leishmaniasis, using an unbiased high-throughput approach. PLoS Negl Trop Dis. 2011;5:e1304. 10.1371/journal.pntd.0001304

61. Segata N, Baldini F, Pompon J, Garrett WS, Truong DT, Dabire RK, et al. The reproductive tracts of two malaria vectors are populated by a core microbiome and by gender- and swarm-enriched microbial biomarkers. Sci Rep. 2016;6:24207. 10.1038/srep24207

62. Gurfield N, Grewal S, Cua LS, Torres PJ, Kelley ST. Endosymbiont interference and microbial diversity of the Pacific coast tick, Dermacentor occidentalis, in San Diego County, California. PeerJ. 2017;5:e3202. 10.7717/peerj.3202

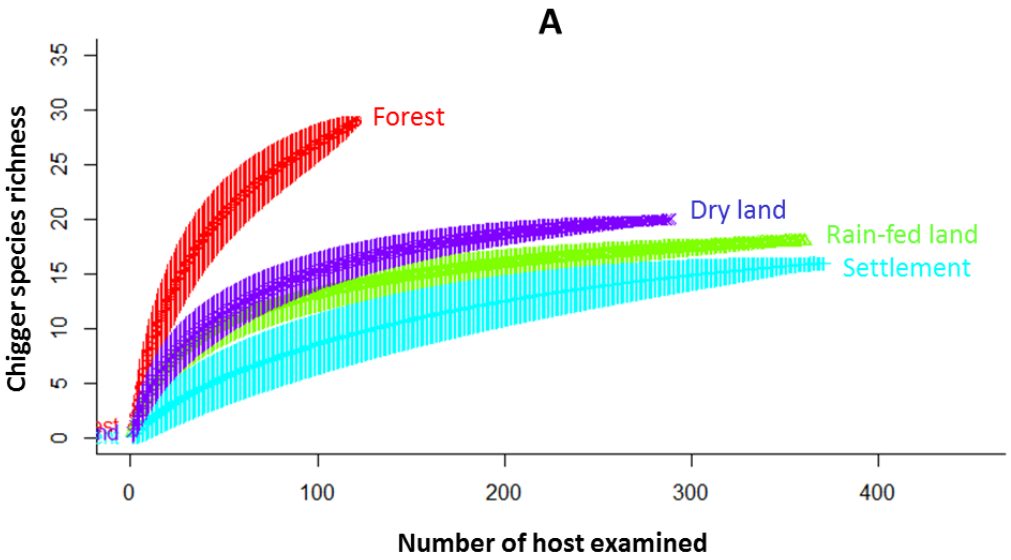
63. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014;12:87. 10.1186/s12915-014-0087-z

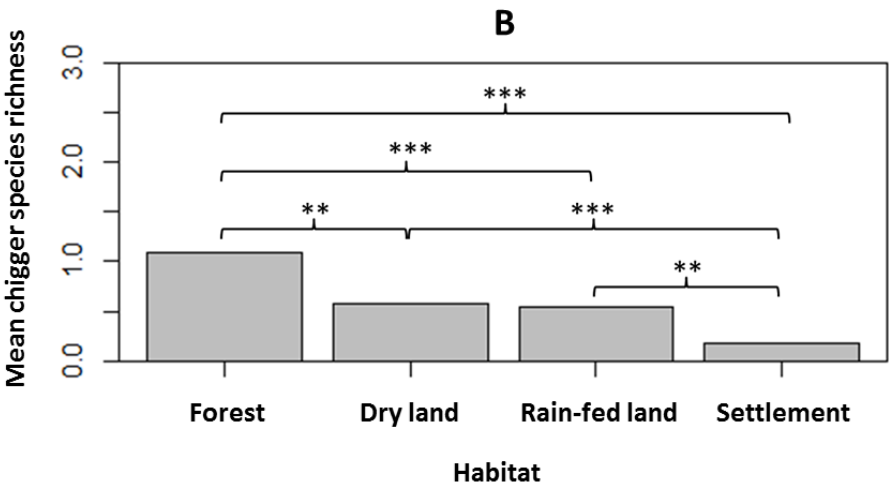
64. Glassing A, Dowd SE, Galandiuk S, Davis B, Chiodini RJ. Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. Gut Pathog. 2016;8:24. 10.1186/s13099-016-0103-7

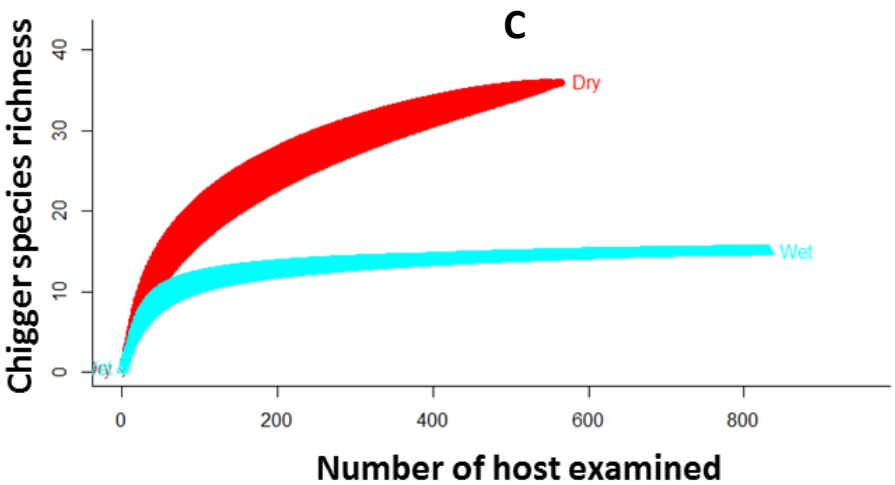
65. Kennedy K, Hall MW, Lynch MD, Moreno-Hagelsieb G, Neufeld JD. Evaluating bias of illumina-based bacterial 16S rRNA gene profiles. Appl Environ Microbiol. 2014;80:5717-22. 10.1128/AEM.01451-14

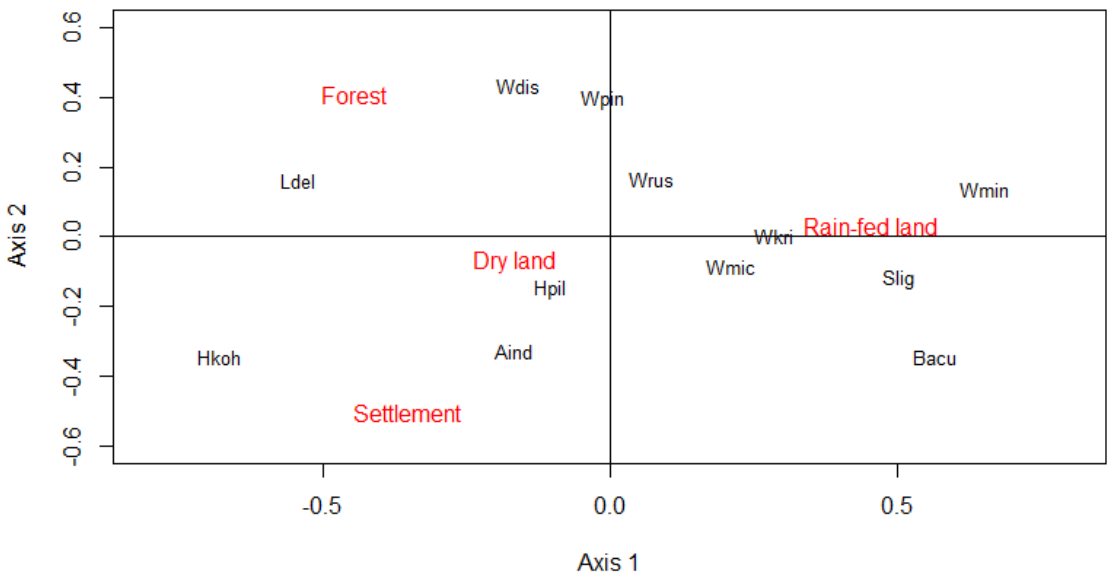
66. Laursen MF, Dalgaard MD, Bahl MI. Genomic GC-Content Affects the Accuracy of 16S rRNA Gene Sequencing Based Microbial Profiling due to PCR Bias. Front Microbiol. 2017;8:1934. 10.3389/fmicb.2017.01934

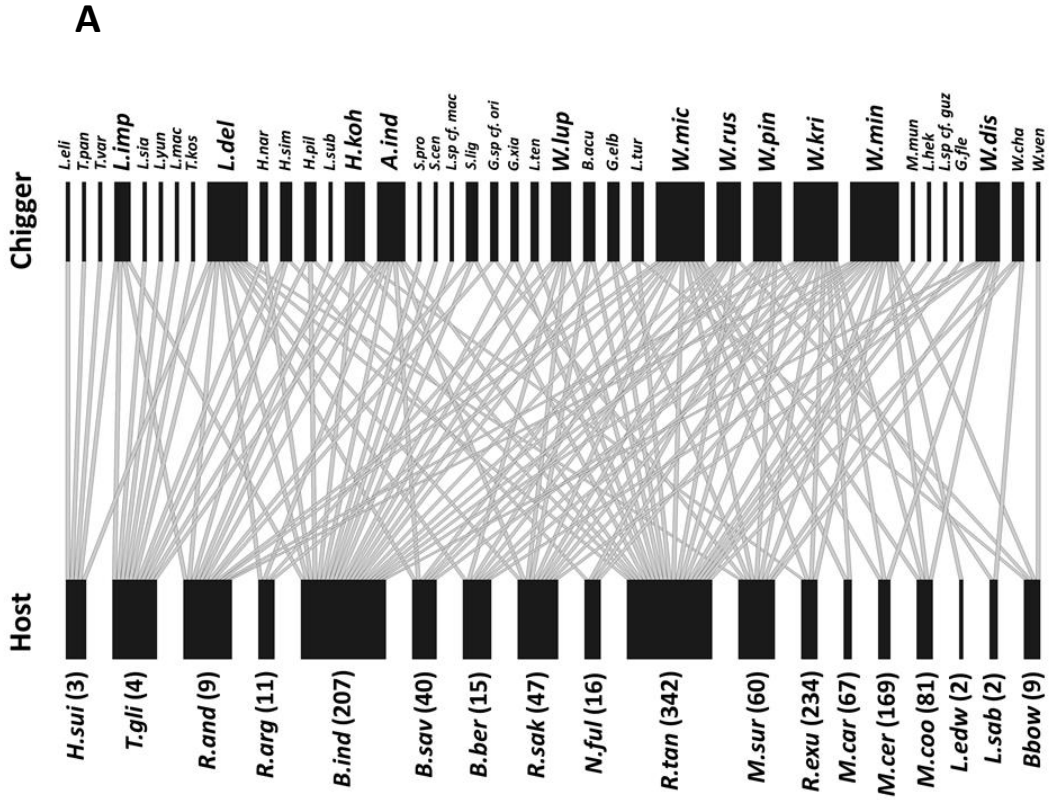
67. Traub R, Wisseman CL, Jr. The ecology of chigger-borne rickettsiosis (scrub typhus). J Med Entomol. 1974;11:237-303.

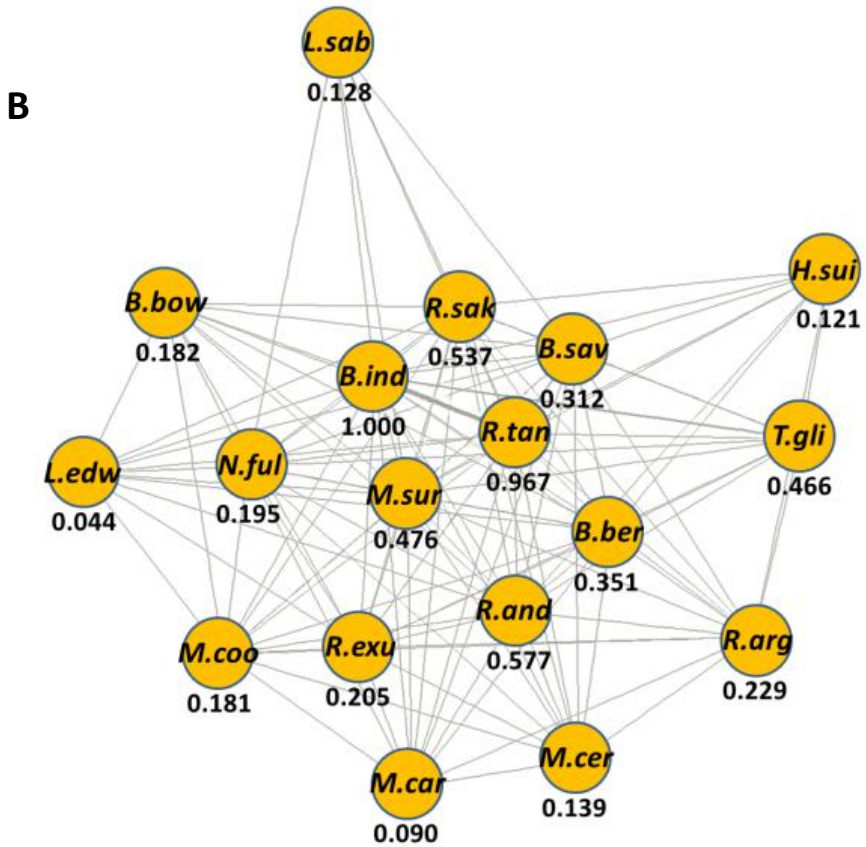


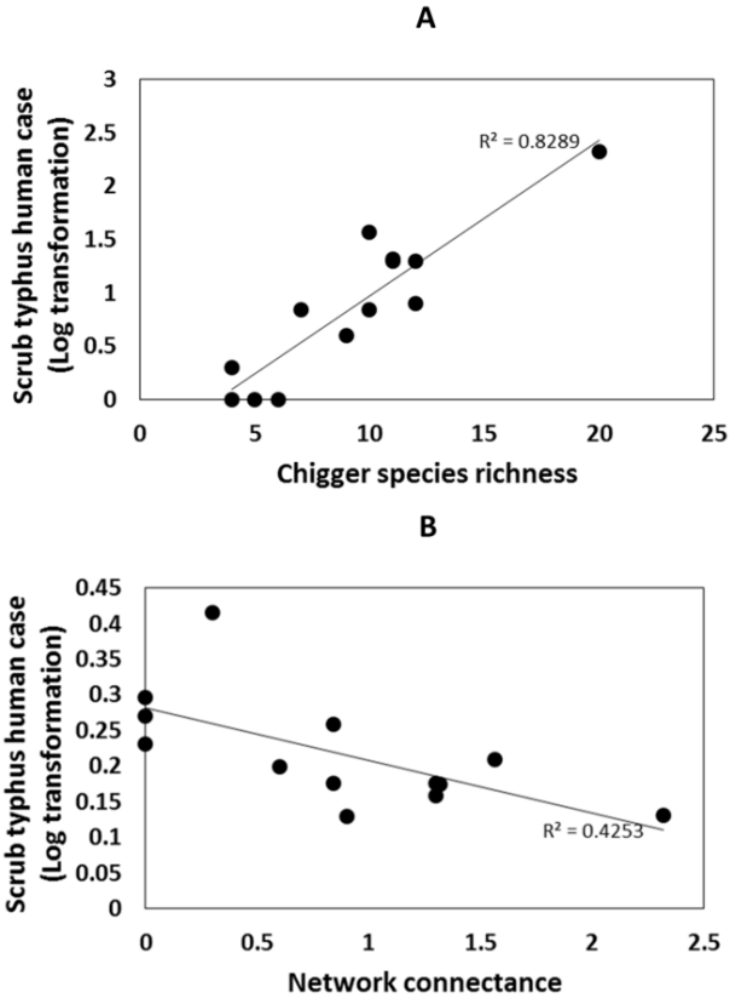


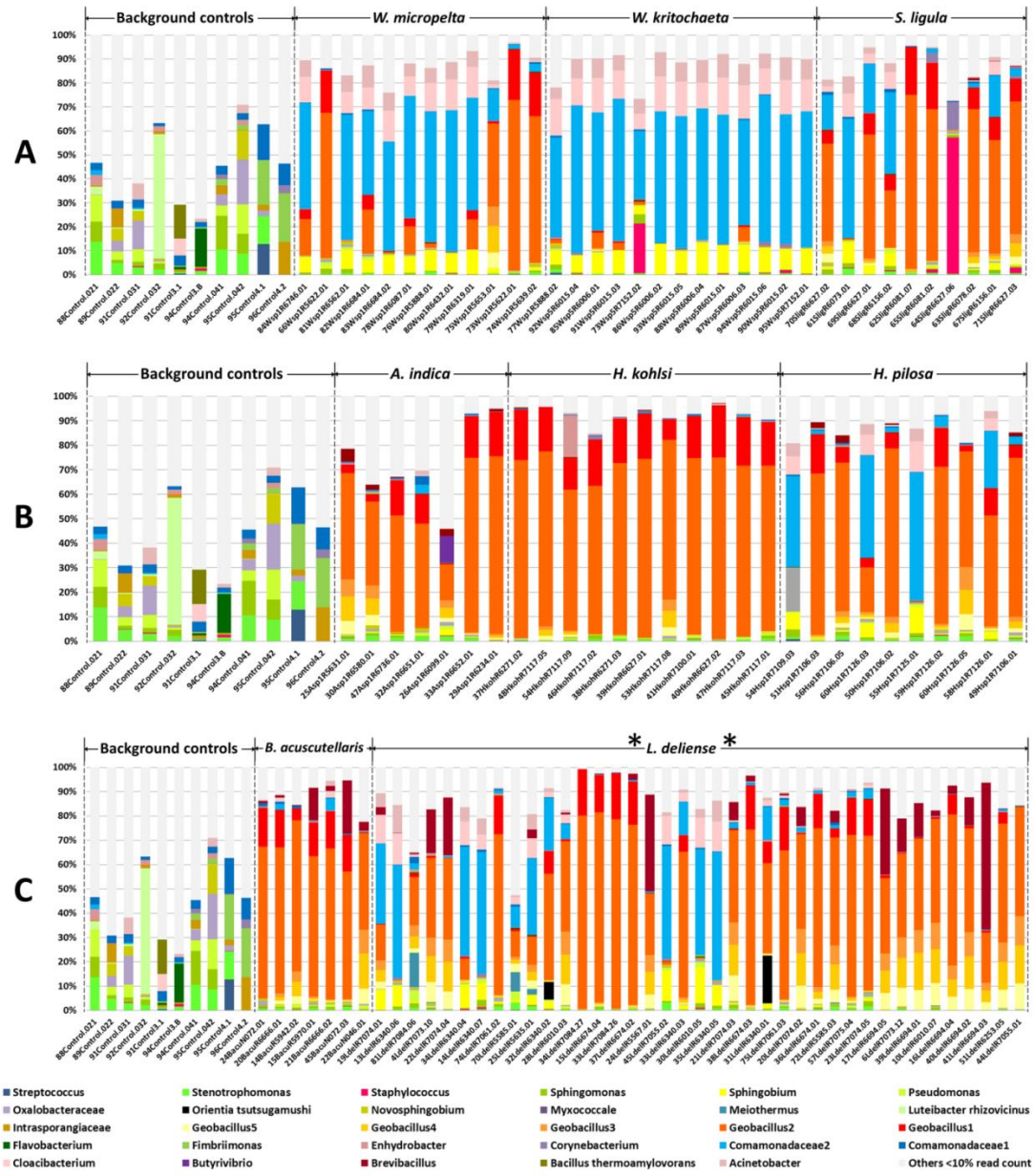












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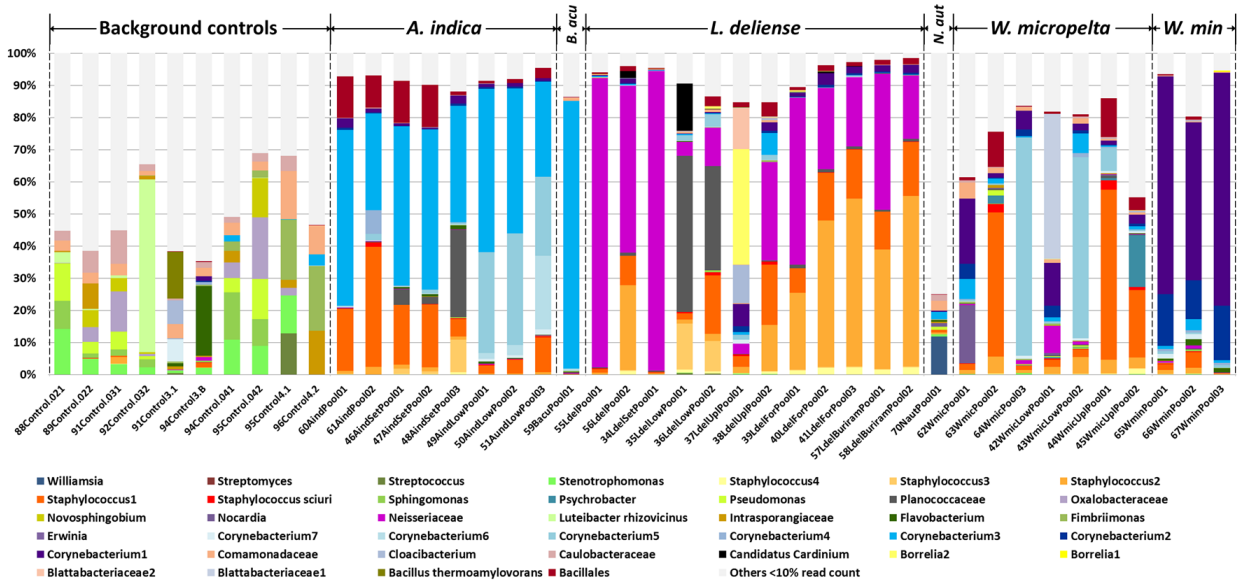

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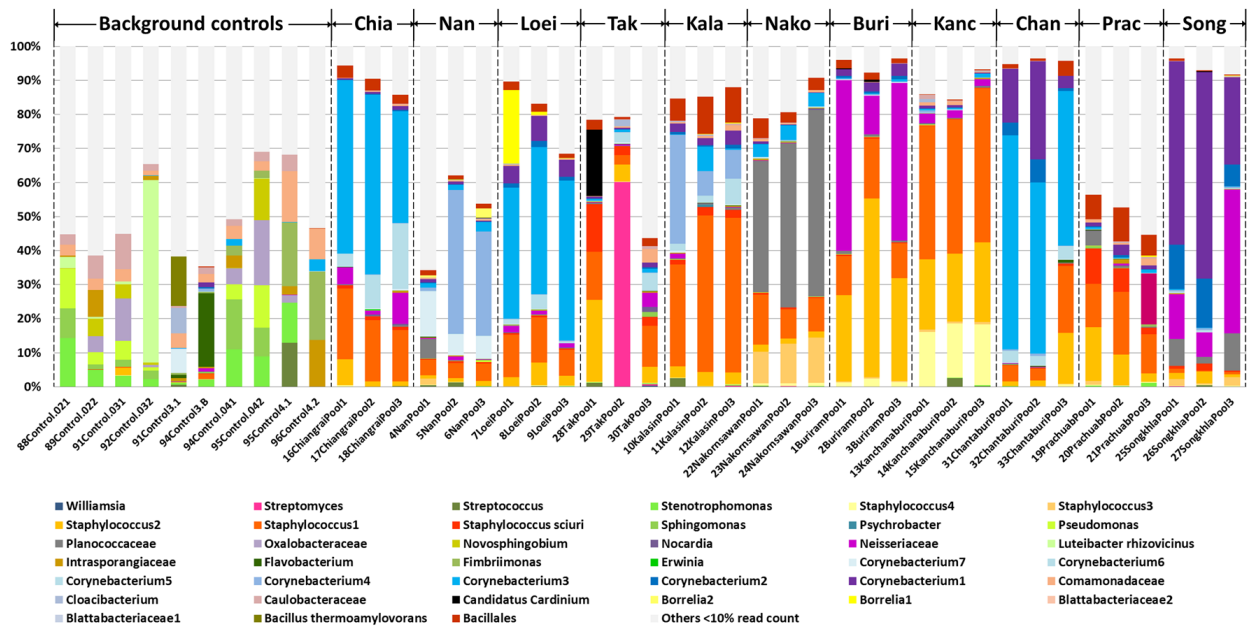
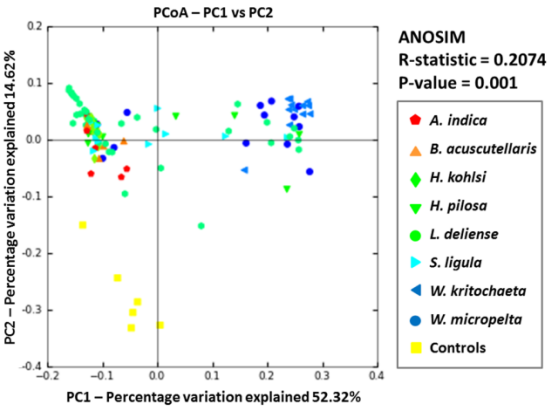


Individual

Pooled

A



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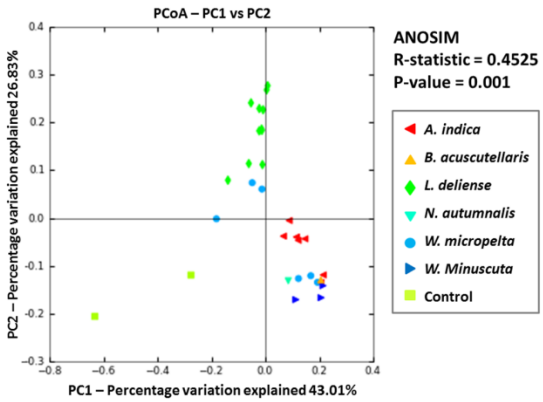

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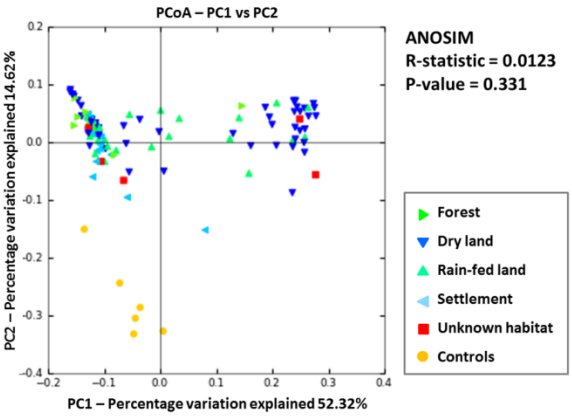

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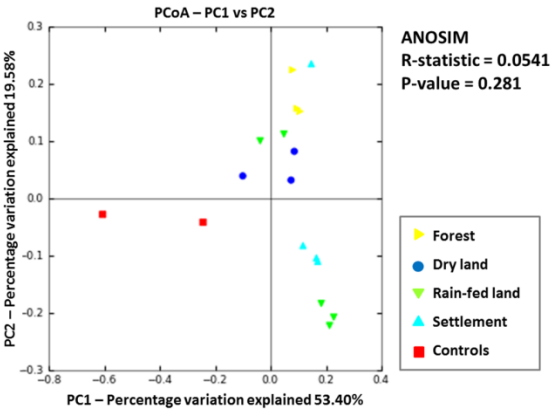

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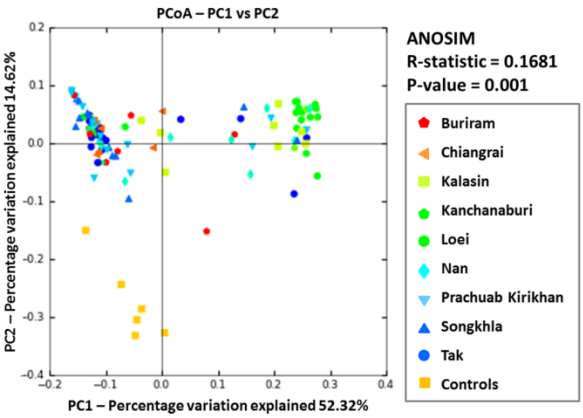

F