ORIGINAL ARTICLE

Molecular detection of *Rickettsia* infection in field-collected bed bugs

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Abstract

Bed bugs are now one of the most prevalent human-associated, blood-feeding pests in the urban world, but few studies of their association with human pathogens have been conducted since their resurgence. Here, we used PCR to screen samples of field-collected bed bugs (*Cimex* spp.) for the presence of *Rickettsia* bacteria and we describe the first detection of an uncharacterized *Rickettsia* in *Cimex lectularius* in nature. *Rickettsia* was detected in 5/39 (12.8%) of the bed bug samples tested. In particular, three pools from the USA and two individual insects from the UK were positive for *Rickettsia* DNA. Sequencing and analysis of a fragment of the citrate synthase gene (*gltA*) from positive samples from each country revealed that the *Rickettsia* detected in both were identical and were closely related to a *Rickettsia* previously detected in the rat flea *Nosopsyllus laeviceps*. Additional experiments indicated that the *Rickettsia* localizes to multiple tissues in the bed bug and reaches high titres. Attempts were made to infect mammalian cells in culture but these efforts were inconclusive. Our findings suggest that *Rickettsia* are secondary endosymbionts of bed bugs and have potential implications for both bed bug control and public health. However, further investigation is required to determine the pathogenicity of this *Rickettsia*, its transmission mechanisms, and its contributions to bed bug physiology.

Keywords: Bed bug, *Cimex*, detection, infection, pathogen, *Rickettsia*, symbiont
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Introduction

After nearing eradication, bed bug (*Cimex* spp.) infestations have been resurging across the globe over the past several decades. Despite sharing many close behavioural, ecological and physiological similarities to other insects that are major vectors of human pathogens, currently the prevailing dogma is that bed bugs are not significant vectors of human pathogens [1]. However, there is an argument to be made that little is still known about the ability of bed bugs to transmit infectious agents. Most work on this topic was carried out in the early twentieth century when advanced molecular techniques were not available, and is poorly described, limiting the interpretation of data. Further, because pathogen emergence is a stochastic process, many bed bug–pathogen interactions that have relevance in today's environment remain unexamined.

Bed bugs are known to harbour two primary bacterial endosymbionts: *Wolbachia* and an uncharacterized γ-proteobacterium related to the endosymbiont of the planthopper *Euscelidius variegatus*, termed BEV-like endosymbiont [2]. They also appear to acquire several species of non-pathogenic extracellular bacteria from the environment, though the stability of these associations is largely unknown [3]. Additionally, a limited number of modern studies have detected human pathogens, such as *Bartonella quintana*, in field-collected bed bugs [4,5].

In 2010, the US Centers for Disease Control and the Environmental Protection Agency released a joint statement on the public health implications of bed bugs [6]. This document included a call for new research to better understand the potential for bed bugs to transmit human pathogens following

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their resurgence. However, since the bed bug resurgence, few studies of this nature have been carried out. Here, we address this knowledge gap by screening 39 samples of field-collected bed bugs (primarily *Cimex lectularius*) from the USA and Europe for the presence of *Rickettsia*, a genus of bacteria that includes insect endosymbionts as well as important and emerging arthropod-borne human pathogens [7–9]. To do so, we employed PCR using *Rickettsia* genus-specific primers that amplify a 380-bp fragment of the citrate synthase gene (*gltA*), a common target for phylogenetic analysis of *Rickettsia* [10]. Five samples tested positive and additional sequencing was performed on a subset in an attempt to preliminarily characterize the *Rickettsia* that was detected.

Materials and methods

Bed bug collections

Bed bugs were collected from various dwellings in the USA and Europe by colleagues, placed into sterile microfuge tubes upon collection and subsequently stored at −80°C until further processing. Each sample tested represented a distinct infestation. Some collections consisted of single bed bugs only. In these cases, the insects were used only for whole-body PCR screening. However, other collections consisted of multiple bed bugs derived from the same infestation site. In these cases, some insects were pooled for whole-body PCR screening whereas others were saved at −80°C. If samples from a collection were positive for *Rickettsia* in the initial PCR, then stored insects from the same collection were used for additional experiments including: PCR of individual body segments, high-depth 16S rRNA amplicon sequencing, and inoculation into mammalian cell cultures. Information on the origin and composition of the samples tested is provided in (Table 1).

TABLE 1. Bed bug samples tested for the presence of *Rickettsia* DNA by PCR

Sample ID	Collection site	Sample composition	PCR result
South Dakota 1	Sioux Falls, SD, USA	7 insects, mixed M/F/N	+
South Dakota 2	Watertown, SD, USA	1 M/1F/1N	+
South Dakota 3	Sioux Falls, SD, USA	1M/2F	+
London 1	London, UK	1N	+
London 2	London, UK	1F	+
Czech Republic 1	Czech Republic	2M	—
Cleveland 1	Cleveland, OH, USA	1M	—
Cleveland 2	Cleveland, OH, USA	1F/5N	—
Cleveland 3	Cleveland, OH, USA	1M	—
Cleveland 4	Cleveland, OH, USA	mixed M/F/N	—
Cleveland 5	Cleveland, OH, USA	1F	—
Cleveland 6	Cleveland, OH, USA	3N	—
Cleveland 7	Cleveland, OH, USA	1M	—
Cleveland 8	Cleveland, OH, USA	1N	—
Cleveland 9	Cleveland, OH, USA	1M	—
Cleveland 10	Cleveland, OH, USA	1F	—
London 3	London, UK	1F/7N	—
Cleveland 11	Cleveland, OH, USA	1N	—
Cleveland 12	Cleveland, OH, USA	1M	—
Cleveland 13	Cleveland, OH, USA	1N	—
Cleveland 14	Cleveland, OH, USA	1N	—
Cleveland 15	Cleveland, OH, USA	1M	—
Cleveland 16	Cleveland, OH, USA	2N	—
Czech Republic 2	Czech Republic	2M	—
Cleveland 17	Cleveland, OH, USA	1F	—
Czech Republic 3	Czech Republic	2M	—
Czech Republic 4	Czech Republic	2M	—
Czech Republic 5	Czech Republic	2M	—
Cleveland 18	Cleveland, OH, USA	1N	—
Akron 1	Akron, OH, USA	2M/1F/5N	—
Czech Republic 6	Czech Republic	2M	—
Cleveland 19	Cleveland, OH, USA	1M	—
London 4	London, UK	mixed M/F/N	—
Cleveland 20	Cleveland, OH, USA	1N	—
Cleveland 21	Cleveland, OH, USA	1F	—
Cleveland 22	Cleveland, OH, USA	2F	—
Cleveland 23	Cleveland, OH, USA	1F	—
Cleveland 24	Cleveland, OH, USA	1N	—
Cleveland 25	Cleveland, OH, USA	1N	—

M indicates male insect, F indicates female insect, N indicates immature nymph insect.

PCR screening

DNA was extracted from bed bugs using the Extract-N-Amp Red Tissue kit (Sigma-Aldrich, St Louis, MO, USA). In brief, whole bed bugs were homogenized using a pestle in lysis buffer, incubated for 10 minutes at room temperature, then heated to 95°C for 3 minutes and neutralized for downstream use. Extracted DNA samples were either immediately used in PCR or stored for PCR at −20°C. The PCR were performed in 20 -μL volumes using Extract-N-Amp PCR master mix with previously described *Rickettsia* genus-specific primers Rp877p (GGGGACCTGCTCACGGCGG) and Rp1258n (ATTGCAAAAAGTACAGTGAACA) at 10-nm concentrations [10]. These primers amplify a 380-bp fragment of the *Rickettsia* citrate synthase gene (*gltA*) that is commonly used for phylogenetic analysis of *Rickettsia*. PCR consisted of an initial denaturation at 95°C for 2 minutes and 40 cycles of 30 seconds at 95°C, 30 seconds at 45°C and 55 seconds at 72°C followed by a final elongation step of 3 minutes at 72°C. PCR products were size fractionated on a 1% agarose gel containing SYBR safe dye (ThermoFisher, Waltham, MA) in Tris–acetate–EDTA buffer and gels were visualized by ultraviolet transillumination. Multiple control reactions were run in parallel. These included negative (no template) controls, positive controls using purified DNA from *Rickettsia rickettsii* as a template, and amplification controls for each sample using primers that amplify a non-specific region of the bacterial 16S rRNA gene. Samples were considered positive when both *Rickettsia*-specific primers and general 16S primers produced amplicons, and negative when general 16S primers produced an amplicon but *Rickettsia*-specific primers did not.

Sequence analysis of PCR products

Two positive PCR (South Dakota #2, London #2) were cloned into pCR 4-TOPO in *Escherichia coli* using the TOPO TA Cloning Kit according to the manufacturer's protocol

(Invitrogen, Carlsbad, CA, USA). Bacterial colonies determined to contain plasmids with inserts by X-gal selection were grown overnight in Luria–Bertani medium and plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit according to the manufacturer's protocol (ThermoFisher). Purified plasmid DNA was then used for Sanger sequencing. The resulting DNA sequences were deposited into the NCBI GenBank database (GenBank MN788122) and aligned to existing sequences from other *Rickettsia* using the NCBI nucleotide BLAST and EMBL-EBI Clustal Omega tools. A Bayesian phylogenetic tree was constructed from the multiple alignment using TOPALi v2.5 software.

Quantification of rickettsia abundance based on 16S rRNA amplicon sequencing

DNA was purified from a *Rickettsia*-positive bed bug pool (South Dakota #2) using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. Purified DNA was then used for 16S rRNA gene amplicon sequencing to determine the abundance of *Rickettsia* relative to other bacteria in the sample. In brief, sequencing was carried out at the Molecular Research DNA Lab (www.mrdnalab.com; Shallowater, TX, USA) using an Illumina MiSeq system (Illumina, San Diego, CA, USA) according to the manufacturer's guidelines. Primers for the V4 hypervariable region of the bacterial 16S rRNA gene were used to conduct PCR using the HotStarTaq Plus Master Mix Kit (Qiagen). Cycle conditions were as follows: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, products were checked in 2% agarose gels to verify successful amplification, purified using calibrated Ampure XP beads (Beckman Coulter, Brea, CA, USA) and subsequently used to prepare a DNA library according to the Illumina Truseq DNA library preparation protocol. Sequence data were processed using the MR DNA analysis pipeline. In summary, sequences were joined and depleted of barcodes. Then, sequences <150 bp and sequences with ambiguous base calls were removed. The remaining sequences were de-noised, operational taxonomic units (OTUs; 97% similarity threshold) were generated and chimeras were removed. Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). Relative abundance of *Rickettsia* was defined based on the proportion of total reads in OTUs classified to the genus.

Inoculation of cell lines with rickettsia from bed bugs

African green monkey kidney epithelial cells (Vero) were cultured in Dulbecco's modified Eagle's medium (BD Biosciences, Franklin Lake, NJ, USA) with 10% fetal bovine serum and 1% penicillin-streptomycin (MP Biomedicals, Santa Ana, CA, USA) at 37°C in a 5% CO_2 environment. Three frozen insects from a *Rickettsia*-positive pool (South Dakota #2) were surface sterilized with 70% ethanol and 10% bleach and placed into tubes of ceramic beads containing 500 µL of cell medium (lysis matrix D; MP Biomedicals). The insects were then macerated at low speed on a BeadBug homogenizer (Benchmark Scientific, Sayreville, NJ, USA) and the homogenate was inoculated into a T25 flask that was ~50% confluent. Three days after the initial inoculation, the medium was changed to antibiotic free medium and subsequent media changes occurred every 3–4 days. At multiple points following inoculation, culture supernatants and cells were checked for the presence of *Rickettsia* by PCR and Giemsa staining.

Results and discussion

Of the 39 samples that we tested, five (12.9%) contained detectable *Rickettsia* DNA (Table 1, Fig. 1a). Three of the samples were pools of mixed stage and sex collected from residences in the state of South Dakota, USA (South Dakota #1–#3), whereas the remaining two samples were individual insects collected from residences in the city of London, UK (London #1, #2). When citrate synthase gene (*gltA*) amplicons from a USA sample (South Dakota #2) and a UK sample (London #2) were sequenced, an identical sequence of 373 bp was identified in both samples. Intriguingly, BLAST analysis revealed a close match between this sequence and that of a *Rickettsia* of unknown pathogenicity that was recently detected in the rat flea *Nosopsyllus laeviceps* in China (*Rickettsia* endosymbiont of *N. laeviceps*, GenBank Sequence ID: KX457954) [11]. In particular, the sequences were 98.1% identical (366/373 bp) with no gaps (Fig. 1b).

The PCR assay used to screen field-collected samples was also performed on multiple individuals from colonies of *C. lectularius* and *Cimex hemipterus* reared in our laboratory, but no *Rickettsia* DNA was detected in these insects. Moreover, individuals from a *Rickettsia*-positive pool (South Dakota #2) that were not used in the initial PCR were dissected and the head and abdomen were tested individually by PCR. In these experiments, *Rickettsia* DNA was detected in both tissues. Purified DNA from the same *Rickettsia*-positive pool was additionally used for 16S rRNA gene amplicon sequencing to determine the abundance of *Rickettsia* relative to other bacteria present in the sample (Fig. 1c). This assay revealed that *Rickettsia* was the second most abundant genus of bacteria in the sample. Of all reads, 50.6% were taxonomically assigned to *Wolbachia*, whereas 31.6% were assigned to *Rickettsia*. In

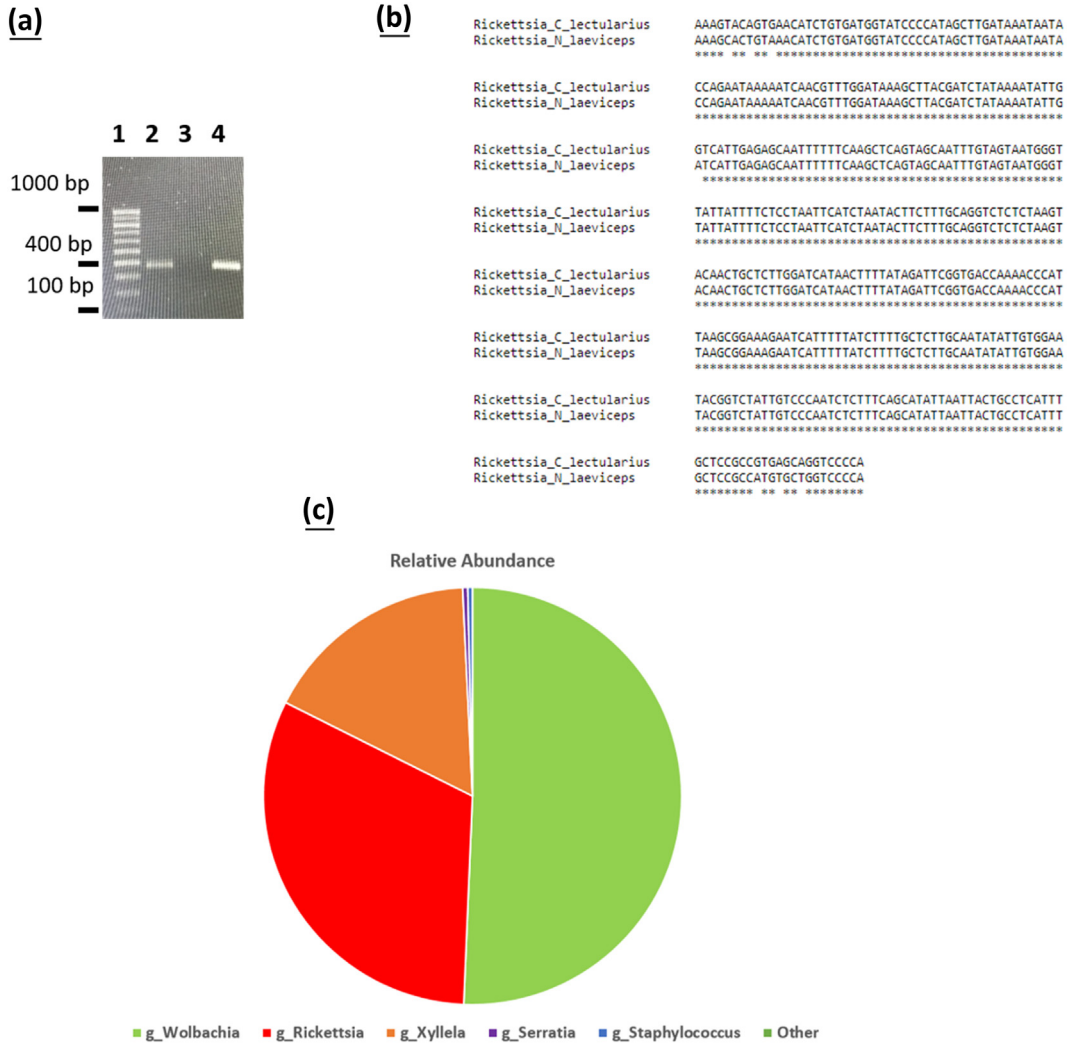

FIG. 1. Detection of *Rickettsia* in field samples of the common bed bug, *Cimex lectularius*. (a) PCR using DNA extracted from a pool of bed bugs collected in South Dakota, USA (South Dakota #2). Samples were run on a 1% agarose gel containing SYBR safe dye and visualized by ultraviolet illumination. Lane 1 (1000-bp ladder), lane 2 (*Rickettsia rickettsii* genomic DNA control), lane 3 (water control), lane 4 (bed bug DNA). (b) Sequence of a 373-bp fragment of the citrate synthase gene (*gltA*) of *Rickettsia* detected in bed bugs. The sequence was aligned to its closest match in GenBank, a sequence derived from the *Rickettsia* endosymbiont of *Nosopsyllus laeviceps*. (c) Relative abundance of *Rickettsia* in a pool of bed bugs collected in South Dakota, USA (South Dakota #2) based on 16S rRNA gene amplicon sequencing.

addition to achieving molecular detection, we attempted to infect a mammalian cell line (Vero) with *Rickettsia* from bed bugs using standard methods. Although the culture was positive for *Rickettsia* DNA by PCR 3 days after inoculation, indicating that the bed bug samples used were infected, these attempts were ultimately unsuccessful as no *Rickettsia* could be detected by PCR or Giemsa staining 15 days after the initial inoculation into the cell culture.

Together, our data provide some new insight into the biology of *Rickettsia* infection in bed bugs. The low prevalence of detection and absence of *Rickettsia* from our laboratory colonies is consistent with previous field surveys that did not find *Rickettsia* [3,12] and indicates either repeated but rare horizontal acquisitions from the environment or inefficient vertical transmission. The detection of identical sequences in bed bugs from two different continents supports the latter route of acquisition. However, a recent 16S rRNA amplicon sequencing study that focused strictly on the copulatory organs of bed bugs from a laboratory colony reported several sequence variants assigned to the genus *Rickettsia* in a proportion of the insects and indicated that these are horizontally transmitted during mating [13]. Although no additional

information on these putative *Rickettsia* reads was provided, they may be derived from the same or a similar bacterium to that reported here from wild populations, which may be sexually transmitted as well. We also stipulate that the *Rickettsia* we detected is a bed bug-adapted species. Indeed, the high relative abundance of *Rickettsia* in the pool that we examined indicates successful colonization, as does the presence of *Rickettsia* DNA in both the heads and abdomens of several individuals.

Based on a Bayesian phylogenetic tree constructed from a multiple alignment of the citrate synthase gene (*gltA*) sequence we identified (Fig. 2), the *Rickettsia* that we found in bed bugs appears to be ancestral and not a member of the typhus or spotted fever groups [11], but additional genetic information is needed to better establish its best phylogenetic placement. Although to date there is no definitive evidence that any ancestral *Rickettsia* species are pathogenic to humans and these are largely considered arthropod endosymbionts, some such as *Rickettsia canadensis* are suspected of causing febrile illness based on human serological studies. The capacity for novel *Rickettsiae* to cause disease can also be difficult to establish [7,14,15]. For example, neither *Rickettisa africae* nor *Rickettsia parkeri* were classified as pathogens until years after their discovery [14–16]. Moreover, some transitional group species such as *Rickettsia felis* exhibit properties of both insect endosymbionts and human pathogens [17,18]. While our attempts to isolate *Rickettsia* from bed bugs in mammalian cells were not successful, these experiments had multiple limitations and so should not be considered conclusive evidence that the bacterium cannot infect mammals. Primarily, it could not be determined if *Rickettsia* in the initial inoculum were viable or if low-level infections were present in Vero cultures.

Ultimately, our findings have several important implications. Bed bugs are ubiquitous, human-associated blood feeders. These insects occur not only in residential settings in the developed world, but also in many environments where vector-borne bacterial pathogens may circulate, such as shelters and camps for the homeless and refugees across the globe [19–21]. Hence, the finding of an uncharacterized *Rickettsia* in bed bugs could be a potential public health threat. In the future, imaging of tissues of infected bed bugs is needed to determine if *Rickettsia* are present in the gut or salivary glands, which could enable transmission to other hosts. Similarly, the possibility that bed bugs could be colonized by other *Rickettsia* that infect humans or animal reservoirs that are occasionally bitten should be investigated further. For instance, bed bugs can feed upon domestic cats [22], and molecular detection of the pathogen *R. felis* has been reported in *C. hemipterus* from Senegal [23]. Laboratory studies have also suggested that bed bugs could be competent vectors of *Rickettsia parkeri* [24]. Lastly, if *Rickettsia* contributes to critical aspects of the physiology of bed bugs as an endosymbiont, then this microbe could serve as a novel target to achieve control of infestations in some situations. As the work described here is the first report of an

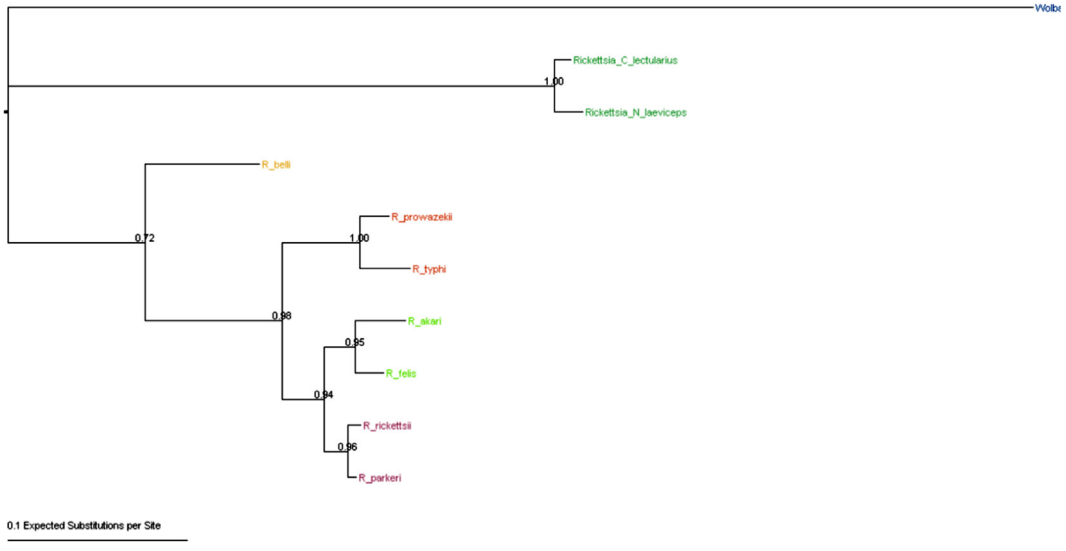

FIG. 2. Phylogeny of the *Rickettsia* sequence detected in *Cimex lectularius*. A Bayesian phylogenetic tree was constructed from multiple alignment of a fragment of the *gltA* gene. The multiple alignment included the sequence reported here from *C. lectularius* and its closest match in GenBank from *Nosopsyllus laeviceps*, as well as *Rickettsia belli*, *Rickettsia prowazekii*, *Rickettsia typhi*, *Rickettsia akari*, *Rickettsia felis*, *Rickettsia rickettsii*, *Rickettsia parkeri* and *Wolbachia*. The tree was constructed using MrBayes in TOPALi v2.5. with default settings (e.g. 100 000 generations). Letters of different colours indicate distinct clusters based on a 0.05 threshold.

uncharacterized *Rickettsia* in bed bugs from the field, additional efforts to better understand the distribution, functions and potential pathogenicity of this microbe are currently underway in our laboratory.

Conflicts of Interest

The authors declare no conflicts of interest.

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