

Recent Detection of Multiple Populations of the Tropical Bed Bug (Hemiptera: Cimicidae) Exhibiting *kdr*-Associated Mutations in Hawaii

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Abstract

In recent years, bed bugs have experienced a remarkable resurgence on a near global scale. While reports have primarily focused on the common bed bug, *Cimex lectularius* (L.), which has resurged largely in temperate regions, in tropical regions the tropical bed bug, *Cimex hemipterus* (F.) (Hemiptera: Cimicidae), has reemerged as well. Recent reports of *C. hemipterus* introductions to subtropical and temperate regions, outside of the species' natural distribution, suggest the potential for establishment and further spread. Establishment may be aided by insecticide resistance mechanisms, such as the presence of knockdown resistance (*kdr*)-associated mutations, which potentially confer resistance to pyrethroid, pyrethrin, and organochloride insecticides. Here, we present the first report of the detection and likely establishment of *C. hemipterus* in Honolulu, Hawaii, from samples collected in 2009 and 2019. Furthermore, through partial sequencing of the voltage-gated sodium channel, we report the presence of *kdr*-associated mutations in all samples. These findings have implications for the implementation of control strategies aimed at eradicating infestations.

Key words: *Cimex hemipterus*, voltage-gated sodium channel, knockdown resistance, insecticide resistance, introduced species

The tropical bed bug, *Cimex hemipterus* (F.) (Hemiptera: Cimicidae), is an obligate hematophagous ectoparasite that primarily feeds on humans (Usinger 1966). Like its close relative, the common bed bug, *Cimex lectularius* (L.), it has undergone a resurgence in recent years (Doggett and Cains 2018, Fourie and Cafford 2018, Lee et al. 2018, Miller 2018, Naylor et al. 2018, Rao and Rao 2018). However, unlike *C. lectularius*, which primarily occurs in temperate regions, *C. hemipterus* is most commonly found within the 30° north and south latitudes (Usinger 1966, Zorrilla-Vaca et al. 2015), hence the common name. The reasons for the resurgence are comparable for both species, and are believed to include international trade and travel, ineffective control, and the evolution of mechanisms conferring insecticide resistance (Doggett et al. 2003, Dang et al. 2017). Due to its physical resemblance to *C. lectularius*, accurate identification requires examination of the ratio of the length and width of the pronotum and the pronotum shape (Usinger 1966, Ghauri 1973), PCR-RFLP analysis (Tawatsin et al. 2013), or DNA sequencing (Balvin et al. 2015). Note that morphological identification is only reliable from the third instar onwards. Given these complexities, mistaken identification of *C. hemipterus* as *C. lectularius* may be common (Naylor et al. 2018), which may in turn lead to

an underestimation of infestation rates and spread outside of the natural range. *Cimex hemipterus* infestations outside of the tropics/subtropics have recently been reported, including records in Russia (Gapon 2016), Sweden (Naylor et al. 2018), France (Bérenger and Pluot-Sigwalt 2017), Italy (Masini et al. 2020), Japan (Komatsu et al. 2018), southern Australia (Doggett and Russell 2008), and the continental USA (Campbell et al. 2016). However, while infestations are reported, the establishment of populations capable of local or regional spread may be dependent on the climatic conditions. Tropical climates are characterized by temperatures at or exceeding 17.8°C, thus suboptimal winter temperatures may limit or prevent population establishment of *C. hemipterus* beyond a given infestation. As such, infestations reported in temperate regions may be ephemeral, and incapable of establishment.

While not investigated to the same extent, multiple verified or candidate insecticide resistance mechanisms previously reported in *C. lectularius* likely share mechanisms with *C. hemipterus* (Dang et al. 2017). These include metabolic-associated resistance mechanisms, such as P450s (How and Lee 2011), esterases (Lilly et al. 2016), and glutathione *S*-transferases (Karunaratne et al. 2007), in addition to those that may confer target site insensitivity, which include

altered acetylcholinesterase (Karunaratne et al. 2007) and knock-down resistance (*kdr*) (Dang et al. 2015, Punchihewa et al. 2019). As in *C. lectularius*, *kdr*-associated mutations in the voltage-gated sodium channel (VGSC) of *C. hemipterus* have been reported, and are considered a factor responsible for the resurgence. These point mutations reduce target-site sensitivity to pyrethrin, pyrethroids, and some organochlorides. Dang et al. (2015) reported four *kdr*-associated mutations in *C. hemipterus* sampled from Africa, Asia, and Australia. Punchihewa et al. (2019) later identified five additional *kdr*-associated point mutations in *C. hemipterus* collected in Sri Lanka. However, unlike *C. lectularius*, little is known regarding the distribution and frequency of *kdr*-associated mutations across *C. hemipterus* populations (Booth et al. 2018).

Understanding the patterns of spread and establishment of invasive species outside of their natural range is of fundamental importance, particularly for species that may be economically or medically detrimental. Knowledge of the factors promoting establishment in the introduced areas, such as insecticide resistance, may be of significance in the formulation of management strategies aimed at eradication. Here, we report the first records of *C. hemipterus* on the Hawaiian Island of Oahu. The species was first detected in a collection made in Honolulu in 2009, and subsequent infestations were detected in follow-up collections in 2019. Furthermore, we report the presence of *kdr*-associated mutations within these infestations.

Materials and Methods

Sample Collection and DNA Extraction

Specimens presumed to be *C. lectularius* were collected from eight distinct infestations of residential homes or apartments in Honolulu, Oahu Island, Hawaii (Fig. 1). Collections were made by local pest management companies (see Acknowledgments) over two periods: one made 8 October 2009, and the remaining seven between 6 June 2019 and 1 July 2019. Specimens were immediately preserved in 100% ethanol, and later stored at -20°C until use. Prior to species

identification, genomic DNA was extracted from longitudinally bisected adults (seven infestations) or nymphs (one infestation), using a Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD). DNA was subsequently eluted three times in 25 μl of AE buffer (QIAGEN) in order to maximize yield. DNA was then stored at -20°C until use.

Species Identification

Species identification was undertaken using two independent methods performed on each sample analyzed.

Morphology

The morphology of the pronotum lateral margin of adult specimens ($n = 7$) was examined, following the method of Ghauri (1973), as outlined in Campbell et al. (2016). Specifically, the lateral margin of *C. lectularius* is more ‘upturned’, compared to *C. hemipterus* (Fig. 2). Comparisons were made to known samples of *C. lectularius*. Both adult males and females were examined.

Mitochondrial DNA

For all specimens, a 334 bp fragment of the 16S rRNA gene, proven to be discriminatory for *Cimex* species, was amplified using primers LR-J-13017 (5'-TTACGCTGTTATCCTAA-3'; Kambhampati and Smith [1995]) and LR-N-13398 (5'-CACCTGTTTAACAAAA ACAT-3'; Simon et al. [1994]). Amplification was performed using the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 46°C for 45 s and 72°C for 1 min, and a final extension period at 72°C for 10 min. Polymerase chain reaction (PCR) products were purified using Exo-SAP-IT (Affymetrix Inc., Santa Clara, CA), and bi-directionally amplified using the BigDye Terminator v3.1 cycle kit (Thermo Fisher Scientific, Waltham, MA). Samples were sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA), and the resulting chromatograms visualized and edited using the CLC Genomics Workbench (QIAGEN). Sequence homology was compared to *Cimex* species, whose sequences were publically available on GenBank, using the

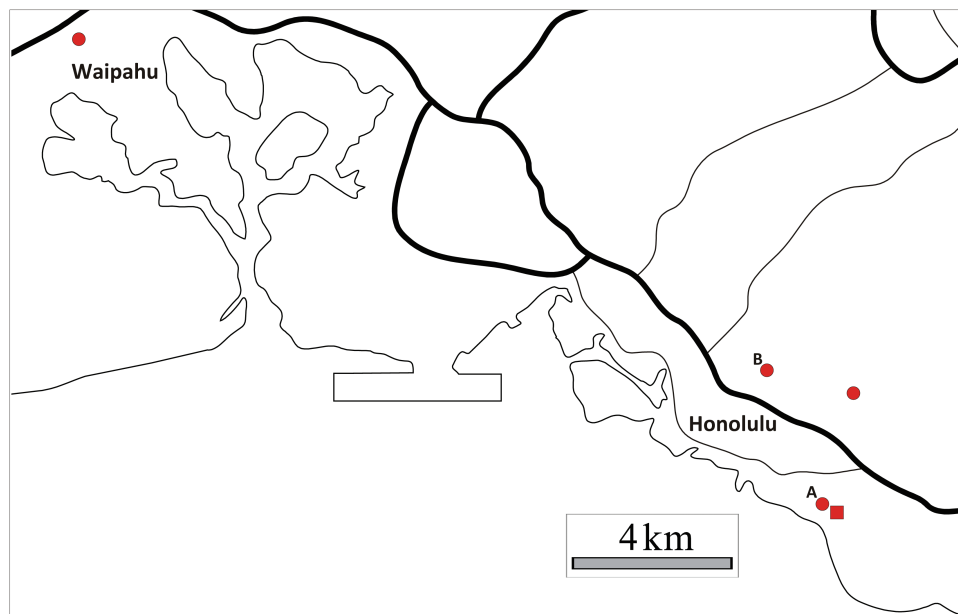


Fig. 1. Map of collection sites of *Cimex hemipterus* from Honolulu, Hawaii. Locations are approximate due to maintaining homeowner anonymity. The 2009 collection is represented by a square, whereas 2019 collections are depicted as circles. From site A, samples were collected from three unique apartments, whereas from site B, samples were collected from two unique apartments.

National Center for Biotechnology Information (NCBI) Nucleotide BLAST (v2.10.0) function.

Voltage-Gated Sodium Channel Sequencing

One individual from each infestation was screened for the presence of nine *kdr*-associated mutations (L899V, M918I, D953G, and L1014F, Dang et al. 2015; Y/I995H, V1010L, I1011F, V1016E, and L1017F/S, Punchihewa et al. 2019). PCR amplification was performed using primer combinations BBParaF3/BBParaR3; following methodology outlined in Zhu et al. (2010). PCR products were purified as above, and sequenced using the BBparaR3 primer. The presence or absence of mutations were scored by eye and recorded.

Results

Morphology

All adult specimens examined exhibited a smooth curvature of the pronotum lateral margin, corresponding to *C. hemipterus*.



Fig. 2. *Cimex hemipterus* male (left) from Honolulu, Hawaii, and *C. lectularius* male (right) from Portland, Oregon. The lines indicate the margin of the pronotum, which indicates the deep excavation of *C. lectularius* and lack thereof in *C. hemipterus*.

In contrast, known *C. lectularius* were found to have a domed pronotum that is deeply excavated, producing a thin, exaggerated margin (Fig. 2). Given that half of each specimen was used for DNA extraction (prior to the initial identification as *C. hemipterus*), we were unable to use pronotum width and height ratio as described by Usinger (1966) as a species identifier.

Molecular Species Identification

Molecular identification was confirmed for all specimens through sequencing of a 334 bp fragment of the 16S rRNA gene. The sample collected in 2009 (GenBank Accession No. MN944418) shared a sequence similarity of 98.89% with *C. hemipterus* (GenBank Accession No. MK908977). All samples from the 2019 collections exhibited an identical sequence (GenBank Accession No. MN946488), differing from the 2009 sample by a single mutation. These shared a similarity of 100% to *C. hemipterus* (GenBank Accession No. MK908977). As expected given the phylogenetic structure of the Cimicidae (Roth et al. 2019), *Cimex pipistrelli* and *Cimex vicarius* were the next most closely related species, with sequence similarity of ~92% and ~91%, respectively (*C. pipistrelli* GenBank Accession No. MG596872, *C. vicarius* GenBank Accession No. KF018722). Sequence similarity to *C. lectularius* was considerably lower: 2009 = 88.95%, 2019 = 89.35% similarity (GenBank accession no. KJ937978).

Voltage-Gated Sodium Channel Sequencing

Knockdown resistance-associated mutations were found in all specimens (Table 1). The specimen collected in 2009 was found to possess three of the nine previously reported mutations; namely D953G, Y/I995H, and L1014F. For those collected in 2019, all exhibited the M918I, D953G, Y/I995H, and L1014F mutations. V1016E and L1017F/S were not detected in the protein translations of the BBParaF3/BBParaR3 amplified fragments. Where present, mutations were always in the homozygous state.

Discussion

Specimens were identified as *C. hemipterus* independently through morphological and/or molecular methods, and this represents the first report of this species in Hawaii. Given both the temporal and spatial sampling, it is likely that the species has been present on the island for at least 10 yr, with establishment and spread aided by insecticide resistance mechanisms. The identification of multiple independent residential infestations, each exhibiting *kdr*-associated mutations, has significant implications for this species' establishment, spread and control in Hawaii.

It is unknown when *C. hemipterus* was first introduced to Oahu, since distinguishing the species from *C. lectularius* requires methods unlikely to be part of a regular pest management inspection. However, no prior records of *C. hemipterus* presence within the Hawaiian islands were discovered in the literature. Additionally,

Table 1. Knockdown resistance-associated mutation profiles for *Cimex hemipterus* collected in Honolulu, Hawaii during 2009 and 2019

Populations	Knockdown resistance mutations						
	L899V	M918I	D953G	Y/I995H	L1014F	V1010L	I1011F
2009	L (S)	M (S)	G (R)	H (R)	F (R)	V (S)	I (S)
2019*	L (S)	I (R)	G (R)	H (R)	F (R)	V (S)	I (S)

S = susceptible, R = resistant.

*As all populations from 2019 shared the same profile, they are reported as a group.

a search of the entomology collection held at the Bernice Pauahi Bishop Museum, Honolulu, Hawaii, failed to yield any *Cimex* spp. that may be indicative of presence prior to the recent resurgence. Of note, no samples within our collections from Hawaii have been found to be *C. lectularius*. The samples investigated here were all derived from residential properties; therefore, which species may exist within tourist-associated properties is at present unknown. The samples presented here fall into two temporally different groupings; one haplotype present in 2009, and another present in 2019. Understanding whether the later samples represent a separate introduction or mtDNA mutation would require additional genomic sequencing. Likewise, estimating propagule number and geographic origin is at this point impossible. While understanding the origin of introduced species is often aided by DNA sequencing (e.g., [Ascunce et al. 2011](#)) given that many species exhibit patterns of phylogeographic structure ([Avisé 2000](#)), for species which rely solely on human-mediated dispersal, such as bed bugs, phylogeographic structure is often lost or absent, thus accurate identification of origin is impossible ([Fitzpatrick et al. 2012](#)). With *C. hemipterus*, this is further complicated by the low level of mitochondrial diversity reported for the species ([Masran and Majid 2017](#)). While few 16S sequences exist at present in GenBank for *C. hemipterus*, they group primarily into one haplotype (similar to MK908977), with two additional variants recorded; one relating to the 2009 sample presented here. As such, understanding the origin of invasion will require more extensive population sampling and genomic sequencing (e.g., using SNPs).

Regardless of origin or propagule number, bed bug infestations appear resilient to the potentially negative effects of inbreeding ([Booth et al. 2018](#)), likely due to the purge of mildly deleterious alleles. As a result, infestations can establish from the introduction of only a pair of individuals or even a gravid female and can subsequently spread rapidly among units within multiunit apartment buildings ([Booth et al. 2012](#)). [Masran and Majid \(2019\)](#) reported microsatellite allele diversity within infestations of *C. hemipterus* as being higher than previously reported for *C. lectularius* ([Booth et al. 2012, 2015](#); [Saenz et al. 2012](#); [Fountain et al. 2014](#); [Akhoundi et al. 2015](#); [Narain et al. 2015](#)). However, due to a similar biology and life history, *C. hemipterus* likely has the same potential to establish from genetically depauperate propagules.

Once established, control of *C. hemipterus* may be more difficult than for *C. lectularius*. While effective control methods implemented by pest management professionals may be comparable to those that show success in controlling *C. lectularius* (e.g., heat treatments, broad spectrum insecticides, etc.), the use of smooth-surface pitfall traps either as part of homeowner-implemented *do-it-yourself* methods or pest management professional treatment, may prove ineffective. Using scanning electron microscopy, [Kim et al. \(2017\)](#) observed a greater number of tenant hairs on the tibial pads of *C. hemipterus*, when compared to *C. lectularius*. These hairs are thought to impart a higher vertical friction force, permitting a greater climbing ability in *C. hemipterus*, enabling adults to escape from traps. In contrast, nymphal instars lack the tibial pad, thus, pitfall traps may be an effective monitoring and control method for juvenile *C. hemipterus* ([Kim et al. 2017](#)).

Similarly, the presence of multiple *kdr*-associated mutations likely makes control through the use of over-the-counter pyrethroid-based insecticides ineffective. We found four of the nine previously identified SNPs in the VGSC—M918I, D953G, Y1995H, and L1014F. We did not detect L899V, V1010L, or I1011F mutations in the Hawaiian *C. hemipterus* samples and the role of D953G in pyrethroid resistance has not been investigated in functional studies. The

L1014F and M918I mutations (and related substitutions at these sites), on the other hand, have been associated with *kdr* or *super-kdr* resistance in many insect species, and expression studies confirmed that they confer site insensitivity to pyrethroids ([Dong et al. 2014](#)). Moreover, co-occurrence of these two resistance-associated mutations further reduces or abolishes the VGSC sensitivity to pyrethroids. Therefore, these two mutations likely confer resistance to pyrethroids in *C. hemipterus* as well.

The discovery of *C. hemipterus* in Hawaii is evidence of this species' spread and potential to be introduced into new areas, such as southern states in the United States ([Campbell et al. 2016](#)). Further, with the discovery of infestations in temperate regions ([Doggett and Russell 2008](#), [Gapon 2016](#), [Komatsu et al. 2018](#), [Béranger and Pluot-Sigwalt 2017](#), [Naylor et al. 2018](#), [Masini et al. 2020](#)), the species may be able to persist in areas outside the typical tropical and subtropical range previously reported. Thus, presence of *C. hemipterus* within temperate regions may be more common than previously considered, however whether populations can truly establish is debatable. As such, future research should focus on monitoring the introduction of *C. hemipterus* in high traffic areas of the world to identify range expansions. Furthermore, population studies of *C. hemipterus* could be conducted with high-resolution, genome-wide markers, such as SNPs, that would aid in the inference of population origin, gene flow, and diversity across its range.

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