


The double-mutation (M918I + L1014F) *kdr* allele is fixed in *Cimex hemipterus* populations in Guangxi, China

Yuhan Zhao¹, Xiangyang Feng², Mei Li¹ and Xinghui Qiu¹ 

Research Paper

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Abbreviations:

COI: cytochrome c oxidase subunit I; *kdr*: knock down resistance; PCR: polymerase chain reaction; VGSC: voltage-gated sodium channel

Author for correspondence:

Xinghui Qiu,
Email: qiuxh@ioz.ac.cn

¹State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China and ²Guangxi Zhuang Autonomous Region Centre for Diseases Control and Prevention, Nanning 530028, China

Abstract

Four putative knockdown resistance (*kdr*) mutations have been documented in the voltage-gated sodium channel (VGSC) gene of *Cimex hemipterus* from several countries. However, no information regarding *kdr* mutations in any Chinese tropical bed bug population is available to date. In this study, a double-mutation (M918I + L1014F) *kdr* allele was identified in six *C. hemipterus* populations across Guangxi Zhuang Autonomous Region of China. The frequency of this allele was 100% in all the six examined populations. In addition, only two cytochrome c oxidase I (COI) gene haplotypes, with one synonymous nucleotide variation, were identified in a total of 48 individuals from six locations. The fixation and broad geographic distribution of this resistant allele questions the continued use of pyrethroids in the treatment of tropical bed bug infestations. The very low genetic diversity within and among these populations indicates that these bed bugs may have a single origin.

Introduction

The tropical bed bug *Cimex hemipterus* (Hemiptera: Cimicidae) is distributed mainly in tropical and subtropical regions (Doggett *et al.*, 2018a). This bloodsucking pest of significant health importance feeds on humans and birds. Bites by bed bugs may cause skin reactions including the formation of papular lesions with associated itch (Doggett *et al.*, 2018b), and the resulting scratching may further lead to secondary infections including cellulitis, ecthyma, impetigo and lymphangitis (Lai *et al.*, 2016; Doggett *et al.*, 2012, 2018a, 2018b). In addition to the direct effects of bites and secondary infections, bed bug infestations impose substantial social, mental, and financial distresses (Yoon *et al.*, 2008).

Over the last 15–20 years, bed bugs have been undergoing a dramatic global resurgence with many infestation reports around the world (Davies *et al.*, 2012; Dang *et al.*, 2017; Naylor *et al.*, 2018). The reappearance or introduction of the tropical bed bug has been documented in tropical and subtropical regions (Davies *et al.*, 2012; Campbell *et al.*, 2016; Naylor *et al.*, 2018). Although several factors, such as an increase in global travel and trade, have been suspected to be attributable to the resurgence of bed bugs, the evolution of insecticide resistance has largely been incriminated as the main factor for the resurgence of this pest (Romero *et al.*, 2007; Lai *et al.*, 2016; Dang *et al.*, 2017).

With the extensive use of pyrethroids, resistance to this chemical group has been documented in *C. hemipterus* (Myamba *et al.*, 2002; Karunaratne *et al.*, 2007; Tawatsin *et al.*, 2011; Dang *et al.*, 2015). Knockdown resistance, which is caused by non-synonymous mutations within the voltage-gated sodium channel (VGSC) gene, is an important mechanism of pyrethroid and DDT in the bed bugs. So far, four mutations (L899V, M918I, D953G, and L1014F) were identified in the VGSC gene of *C. hemipterus* collected from multiple countries (Dang *et al.*, 2015). Similar to observations from *Musca domestica*, the M918I mutation was always found together with the L1014F mutation (Dang *et al.*, 2015). Strains of *C. hemipterus* carrying the two mutations (M918I and L1014F) were reported to be associated with higher resistance to d-allethrin than that those with just the L1014F mutation (Dang *et al.*, 2015). A causal link between one or both of the mutations at sites 918 and 1014 with pyrethroid resistance was reported in several other insects (Williamson *et al.*, 1996; Vais *et al.*, 2003; Usherwood *et al.*, 2007; Sonoda, 2010; Sonoda *et al.*, 2012; Dong *et al.*, 2014). However, the role of the conserved mutations L899V and D953G within the VGSC gene in insecticide resistance has yet to be evaluated (Dang *et al.*, 2015).

Although many studies addressing *kdr* mechanisms have been conducted on common bed bugs (*C. lectularius*) (Zhu *et al.*, 2010; Dang *et al.*, 2017), few reports on the tropical bed bug are available. The presence of possible *kdr* mutations has been investigated in tropical bed bugs collected from Australia, India, Malaysia, Kenya, and Thailand (Dang *et al.*, 2015). However, to

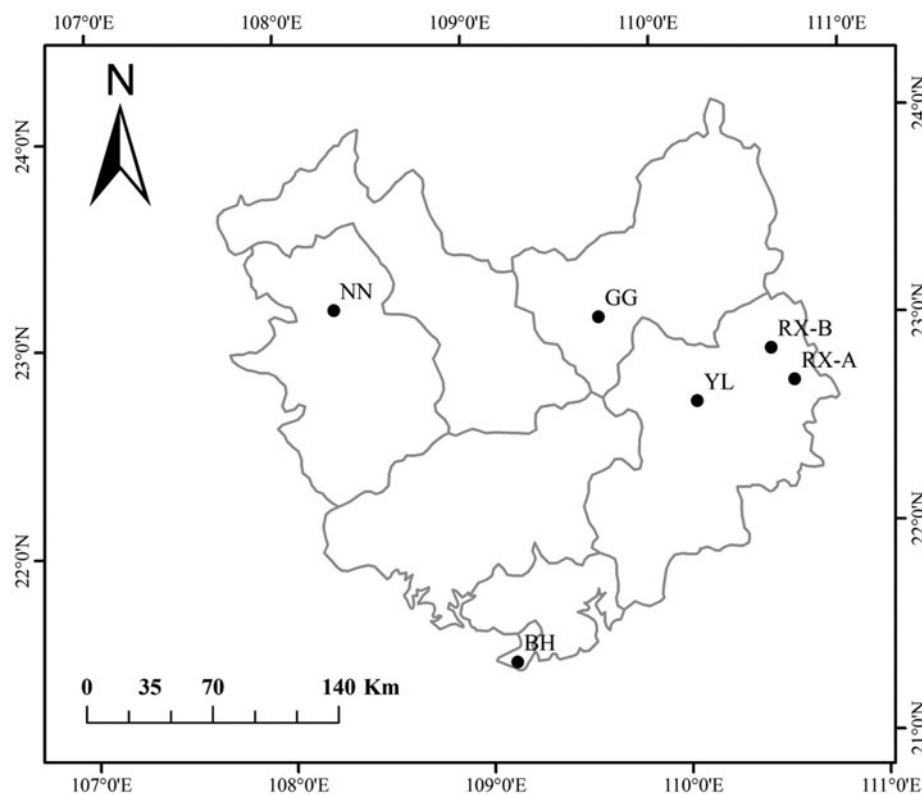


Figure 1. Map of the sampling locations for *Cimex hemipterus*, Guangxi Zhuang Autonomous Region, China. NN, Nanning; GG, Guigang; YL, Yulin; RX, Rongxian; BH, Beihai.

Table 1. *COI* haplotypes identified in the tropical bed bugs from Guangxi, China

Populations	Locations	Structure	Individuals examined	Frequency (%)	
				H1	H2
RX-A	Yangmei, Rongxian	School dormitory	10	100	0
RX-B	Rongzhou, Rongxian	School dormitory	10	20	80
YL	Yulin	Hotel	6	0	100
GG	Guigang	School dormitory	10	0	100
BH	Beihai	Residency	6	0	100
NN	Nanning	Hospital	6	0	100

Note: The GenBank accession numbers are MK883766 and MK883767 for H1 and H2, respectively

our knowledge, no study on *kdr* mutation in Chinese tropical bed bug populations has been reported.

Guangxi is located in a subtropical monsoon climate zone with high average annual temperatures, which is suitable for bed bug occurrence. Moreover, control failure of bed bugs using pyrethroids in the field was reported by local pest control operators. In this context, we attempted to investigate the presence, distribution, and frequency of *kdr* mutations of tropical bed bugs in Guangxi of China, through the application of DNA sequencing. Such information will be useful for monitoring and managing insecticide resistance.

Materials and methods

Sample collection

The bed bugs were collected from six different locations in Guangxi, China (fig. 1, table 1). RXA and RXB samples were

caught in 2014, and the others were collected in 2018. The bed bugs were placed in 95% ethanol and stored at -20°C until use.

DNA extraction and gene sequencing

The genomic DNA from individual bed bugs was extracted by the method of Rinkevich *et al.* (2006), and stored at -18°C until use.

A fragment (~550 bp) of the cytochrome c oxidase I (COI) gene was amplified using primers CHCOI-F (5'-TATTCGG-AATGTGGGCAGGG-3') and CHCOI-R (5'-CACCCGCTAA TACAGGTAGGA-3'). The PCR mixture in a total volume of 25 μl , containing 12.5 μl of 2 \times Taq MASTER MIX (Tiangen Co., Beijing), 0.2 μM each primer, and 100–200 ng of DNA as a template. The thermal cycling profile was: an initial step of denaturation at 95°C for 2 min, followed by 34 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 50 s, and a final extension step

at 72°C for 5 min. The PCR products were gel-purified and directly sequenced using the forward primer CHCOI-F.

Similarly, a fragment of the *VGSC* gene, encoding for the domain IIS4–IIS6 region and encompassing five putative *kdr* mutation sites (M918, L925, T929, L932, and L1014), was amplified using primers CHVG-SC-F and CHVG-SC-R (Dang *et al.*, 2015). The PCR products were sequenced using the reverse primer CHVG-SC-R.

Bioinformatics analysis

Data from DNA sequencing were checked and cleaned manually. All confirmed DNA sequences were aligned by using Muscle 3.8 (Edgar, 2004), and variable sites were identified. NCBI resources (<https://www.ncbi.nlm.nih.gov/>) including databases and software indicated in the following text were used for bioinformatics analysis.

Results

COI haplotypes

PCR products of the *COI* gene fragment were amplified from 48 individuals collected from six locations. Sequence alignment (472 bp in length) identified two haplotypes (H1 and H2, table 1). Chromatograms revealed only one haplotype within an individual, and only one synonymous nucleotide variation was observed between the two haplotypes (fig. 2). H1 was detected in two populations, while H2 was widely distributed in five of the six populations (table 1). Blast search revealed that H2 was shorter than, but 100% identical to the corresponding region of several *COI* sequences of tropical bed bugs from other Asian countries, such as MH607404 (Bangladesh), MG770889 (Iran), and KF018754 (Malaysia.).

kdr mutations

PCR products of the *VGSC* gene from each bed bug with a size of around 800 bp were sequenced. The DNA sequences excluding ambiguous terminal regions (with a length of 776 bp) were processed for further analysis. Sequence alignment indicated that all the *VGSC* sequences were identical and occurred in the homozygous form in the 44 examined individuals. This fragment (GenBank accession: MK883768) included three introns and four exons, with a sequence identity of 95.26% to the corresponding region of the common bed bug *VGSC* gene (GenBank No. JRLE01000129.1) (fig. 3). We notice that the length of exon 19 was 188 bp, rather than 190 bp as described in Dang *et al.* (2015). The result was confirmed by re-sequencing two randomly selected samples using the forward primer CHVG-SC-F.

To identify possible mutations within these exon regions, the deduced protein sequence was compared with those from the common bed bug *C. lectularius*, housefly *M. domestica*, and German cockroach *Blattella germanica* (fig. 4). Two non-synonymous variations, respectively, at sites 918 (ATA) and 1014 (TTC) were observed, resulting in M918I (ATG to ATA) and L1014F (CTG to TTC) substitutions. The previously reported L899V and D953G (Dang *et al.*, 2015) were not detected in our samples.

Discussion

This study is the first detection to date of the putative *kdr* mutations in Chinese tropical bed bug populations. We found that all the investigated bed bugs carried both M918I and L1014F mutations (table 2). Based on the findings of Dang *et al.* and others (Williamson *et al.*, 1996; Vais *et al.*, 2003; Usherwood *et al.*, 2007; Sonoda, 2010; Sonoda *et al.*, 2012; Dong *et al.*, 2014; Dang *et al.*, 2015), we predict that these bed bug populations in Guangxi exhibit a high level of resistance to pyrethroids. This result provides an explanation for the control failure of bed bugs reported by local pest control operators in the field. This situation also calls for a decrease in reliance on pyrethroids, and highlights the need of alternative insecticides with different modes of action or alternative approaches such as heat treatments or fumigation in order to make bed bug control more effective.

Notably, all these examined bed bugs that were collected in different locations share an identical *VGSC* haplotype. Moreover, *VGSC* exists in homozygous forms in these samples. In addition, while two *COI* haplotypes exist, they differ by only one synonymous nucleotide variation. These data reveal a very low genetic diversity within and among these populations. The limited diversity within each population may be explained by observations that bed bug populations are often highly inbred. The low genetic diversity among these six populations, which were collected from different locations (fig. 3), probably is a consequence of strong anthropogenic selection by synthetic insecticides and followed by a founder effect. It is very likely that these populations in this study have a single origin, then establish through the introduction of singly mated female or a small group of extremely related individuals carrying the M918I + L1014F allele, and spread due to human-mediated movement. Although the M918I + L1014F alleles were found in populations from Australia, India, and Thailand (Dang *et al.*, 2015), we are not sure if these alleles have a common origin, because the corresponding genomic DNA sequences of the *VGSC* gene in these samples used by Dang *et al.* (2015) are not publicly available.

The two haplotypes (H1 and H2) of *COI* identified from a total of 48 individuals differ from each other at a single nucleotide (fig. 2). Interestingly, H2 is distributed in five of the six locations, while only sites RX-A and RX-B contain individuals of H1, and RX-B is the only location sampled which contains both H1 and H2 (table 1). A Blast search using H2 against the GenBank nucleotide collection (non-redundant) database (on 14 November 2019) yielded 12 entries with 100% identity and 100% coverage from *C. hemipterus* individuals sampled from Bangladesh, Indonesia, Iran, and Malaysia, demonstrating a wide distribution of H2. By contrast, no hit that is 100% identical to H1 was retrieved. This observation makes us propose that H1 may be a newly evolved haplotype. Further studies are required in order to clarify this hypothesis *via* investigating more samples from different locations. The coexistence of both H1 and H2 in RX-B implies that the bed bugs carrying H1 are spreading from RX-A to nearby RX-B. Taking action to limit the potential migration of bed bugs across locations is recommended.

This work focused on the investigation of *kdr* mutations. Based on lessons from studies on the related common bed bug *C. lectularius*, in which multiple resistance mechanisms have been identified (Yoon *et al.*, 2008; Mamidala *et al.*, 2012; Zhu *et al.*, 2013), it is possible that other resistance mechanisms such as increased

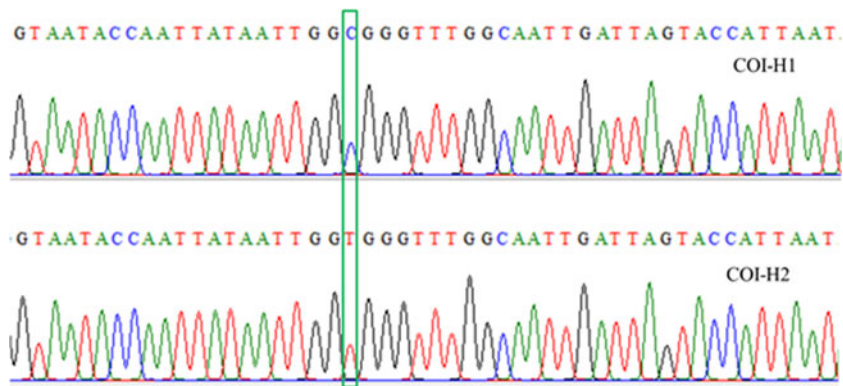


Figure 2. Chromatograms showing the nucleotide variation in the COI gene detected in the tropical bed bugs from Guangxi, China. The base variation is boxed.

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Chem-vgsc-GX      ATGAAGTTGATTGCAATGAGTCCAAAATATTACTTTCAAGAAGGCTGGAATATATTTGAT
Clec-vgsc-ref    ATGAAGTTGATAGCAATGAGTCCAAAATATTACTTTCAAGAAGGCTGGAATATATTTGAT
*****
Chem-vgsc-GX      TTTATCATAGTCGCTCTATCGTTATTAGAAGTCTAGTCTTGAAGGAATCAAGGTCTATCA
Clec-vgsc-ref    TTTATCATAGTCGCTCTATCGTTATTAGAAGTCTAGTCTTGAAGGAATCAAGGTCTATCA
*****
Chem-vgsc-GX      GTTTGGAGTCATTCAGATTGGTATGTTTATAACTAAAATTAATCCAACCTACCTTCT
Clec-vgsc-ref    GTTTGGAGTCATTCAGATTGGTATGTTTATAACTGAAAATTAATCCAACCTACCTTCT
*****
Chem-vgsc-GX      TATTAATAATTAATAAACTAATAATAATTCAGCTCAGGGTGTTAAGCTGGCTAAGTCA
Clec-vgsc-ref    TACTAATAACTAATAAACTAATAATAATTCAGCTCAGGGTGTTAAGCTGGCTAAGTCA
**
Chem-vgsc-GX      TGGCCAACACTCAATCTTCTCATTCCATTATAGGCAGAACAGTGGGTGCCCTCGGTAAT
Clec-vgsc-ref    TGGCCAACACTCAATCTTCTCATTCCATTATGGGCAGAACAGTGGGTGCCCTCGGTAAT
*****
Chem-vgsc-GX      TTAACCTTTGTGTGTCATTATTATATTCATCTTTGCTGTGATGGGAATGCAGTTGTT
Clec-vgsc-ref    TTAACCTTTGTGTGTCATTATTATTTTCATCTTTGCTGTGATGGGAATGCAGTTGTT
*****
Chem-vgsc-GX      GGAAAAAATTATATCGGTAATTTTTACAATGAATGTATAATTTTGACACGTGTACAA
Clec-vgsc-ref    GGAAAGAACTATATCGGTAATTTTTG-AATGAATGTCTAATTTTGACATGTGTACAA
****
Chem-vgsc-GX      ATTGATTATATACTTACAGATAACATGGATAGATCCCTGATGGAGAATCCCGAGGTG
Clec-vgsc-ref    ATTGATTATATTCT---AGATAATATGGATAGATCCCTGATGGCGAACTCCCGAGGTG
*****
Chem-vgsc-GX      GAATTTTACAGATTCATGCATTTCGTTTATGATAGTGTTAGGGTCCTGTGTGGAGAGTG
Clec-vgsc-ref    GAATTTTACAGATTCATGCATTTCGTTTATGATAGTGTTAGGGTCCTGTGTGGAGAGTG
*****
Chem-vgsc-GX      GATAGAATCGATGTGGATTGTATGCACGTGGGAGATGTTTCTTGCAATCCATTTTTTCT
Clec-vgsc-ref    GATAGAATCGATGTGGATTGTATGCATGTGGGAGATGTTTCTTGCAATCCATTTTTTCT
*****
Chem-vgsc-GX      AGCGACAGTAGTAATTGGGAATTCGTTGTAAGTGCATTTTTTAATTAATAAAAATCAGT
Clec-vgsc-ref    AGCGACAGTAGTAATTGGGAATCGGTTGTAAGTGCATTTTTACTAATAAAAATCAGT
*****
Chem-vgsc-GX      TGT---TTAAACAGTGTGTTTGTAACTGTTTCGATGATTTTTTTAGGTTCTCAA
Clec-vgsc-ref    TGTTTTATTAACCTTTTTGGGTTAACTGTTTGGATA---TTTTTTAGGTTCTCAA
****
Chem-vgsc-GX      CCTTTTCTAGCCCTGTTGCTCAGTAATTTGGCTCGTCGAGTCTTTCAGCTCCGACAG
Clec-vgsc-ref    CCTTTTCTAGCCCTGTTGCTCAGTAATTTGGCTCGTCGAGTCTTTCAGCTCCGACAG
*****
    
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Figure 3. Exon-intron structure of the amplified genomic fragment of the VGSC genes from *C. hemipterus*, aligned with the corresponding region from the common bed bug *C. lectularius* (JRLE01000129.1). The two codons for M918I (ATA) and L1014F (TTC) mutations were indicated in bold. The introns are underlined.

Author contribution. Conceived and designed the experiments: XHQ and XYF. Performed the experiments: YHZ and ML. Analyzed the data: XHQ and YHZ. Contributed reagents/materials/analysis tool: ML and XYF. Wrote the paper: XHQ and YHZ. All authors read and approved the final manuscript.

Conflict of interest. The author(s) declare that they have no competing interests.

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