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Research Article

Distribution and Frequency of Pyrethroid Resistance-Associated Mutations in Host Lineages of the Bed Bug (Hemiptera: Cimicidae) Across Europe

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Abstract

For over two decades, the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae) has been undergoing a dramatic global resurgence, likely in part to the evolution of mechanisms conferring resistance to insecticides. One such mechanism is knock-down resistance (*kdr*), resulting from nonsynonymous mutations within the voltage-gated sodium channel (VGSC) gene. To date, three mutations have been identified in *C. lectularius*, V419L, L925I, and I936F. Using Sanger sequencing, the frequency and distribution of these VGSC mutations across 131 populations collected from the bat-associated and human-associated lineages of *C. lectularius* found in Europe are documented. All populations from the bat-associated lineage lacked mutations at the three sites. In contrast, the majority of populations associated with humans (93.5%) possessed the mutation at the L925I site. The I936F mutation, previously only reported in Israel and Australia, was found in nine populations spread across several European countries, including the Czech Republic and Switzerland. The high frequency of *kdr*-associated resistance already reported in *C. lectularius* and the occurrence and broad geographic distribution of this additional VGSC mutation, questions the continued use of pyrethroids in the treatment of infestations.

Key words: *Cimex lectularius*, voltage-gated sodium channel, insecticide, *kdr*, knockdown resistance

Originally an ectoparasite associated with bats (Horváth 1913), the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), became associated with humans likely prior to the Neolithic period (Balvín et al. 2012), and has remained so with no evidence of genetic exchange with the ancestral bat-associated lineage since (Booth et al. 2015). By the late 1960s, the human-associated lineage experienced a significant population decline in developed countries, assisted by the widespread application of DDT and pyrethroids (Usinger 1966). However, over the past two decades, a dramatic population resurgence has been observed globally (Davies et al. 2012, and references therein). Within temperate regions, this resurgence has comprised mainly *C. lectularius*, whereas tropical and subtropical regions have witnessed the reappearance or introduction of the tropical bed bug, *C. hemipterus* F. (Hemiptera: Cimicidae) (Davies et al. 2012, Doggett et al 2012, Campbell et al. 2016). More recently, periodical reports of *C. hemipterus* in Europe have been made (Naylor et al. 2018). While the re-emergence of these species has been linked to factors including an increase in flight traffic, a lack of awareness among the public and pest controllers, and ineffective pest control (Doggett et al. 2004, Reinhardt et al. 2008), the evolution of insecticide resistance has likely played a role in the resurgence and spread (Reinhardt and Sive-Jothy 2007; Dang et al. 2017).

Cimicid insects have evolved a number of diverse mechanisms that confer resistance to a variety of insecticides (Adelman et al. 2011, Mamidala et al. 2012, Koganemaru et al. 2013, Zhu et al. 2013, Dang et al. 2015b, Romero and Anderson 2016, Runjaic et al. 2017). Of these, knock-down resistance (*kdr*) has received significant attention (Yoon et al. 2008; Zhu et al. 2010; Durand et al. 2012; Tomita et al. 2012; Booth et al. 2015; Dang et al. 2015a, b; Palenchar et al. 2015; Raab et al. 2016). This mechanism functions to reduce an individual's sensitivity to pyrethrin, pyrethroids, and organochlorides. This is driven by nonsynonymous mutations in the voltage-gated sodium channel (VGSC) α-subunit gene; a number of which have been inferred to confer resistance in multiple species of pest insect (e.g. Dong, 1997, 2007; Lee et al. 2000; Soderlund 2005; Dang et al. 2015b). In *C. lectularius*, three such mutations have been identified: V419L and L925I (Yoon et al. 2008), and I936F (Dang et al. 2015a). Note that the former amino acid is the wild type and is associated with susceptibility, whereas the latter is the mutation associated with putative resistance. The presence of two of these mutations, V419L and L925I, has been found to be highly

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correlated with pyrethroid resistance in *C. lectularius* (Seong et al. 2010). Insecticide resistance conferred by the I936F mutation has yet to be fully evaluated. Previous studies denote these mutations by haplotype: A = susceptible at both 419 and 925; B = 419 susceptible, 925 resistant; C = 419 and 925 resistant; and D = 419 resistant, 925 susceptible (Zhu et al. 2010). When the I936F mutation has been investigated, these haplotypes are given a superscripted b (e.g., A^b = 419 and 925 susceptible, 936 resistant) (Dang et al. 2015a).

The distribution and frequency of the V419L and L925I mutations in *C. lectularius* has been investigated to varying degrees within the United States (Zhu et al. 2010, Raab et al. 2016), Europe (Durand et al. 2012, Booth et al. 2015), Japan (Tomita et al. 2012), Australia (Dang et al. 2015a), and Israel (Palenchar et al. 2015) (see Table 1). These studies have revealed an alarming pattern of geographically widespread insecticide resistance, with most populations exhibiting one or both mutations, and few exhibiting the susceptible haplotype. For example, across the United States, out of 110 sampled locations, ~41% were haplotype B, ~41% were haplotype C, and ~3% exhibited haplotype D (Zhu et al. 2010). In Europe, VGSC mutations were absent in all populations of the bat-associated lineage of *C. lectularius*, whereas the human-associated populations primarily exhibited the haplotype B (90%), and only 4% lacked the mutations (Booth et al. 2015). The distribution and frequency of the I936F mutation is still largely unknown outside of Australia and Israel (Dang et al. 2015a, Palenchar et al. 2015). This is due primarily to the method by which the V419L and L925I mutations have been identified, i.e., allele-specific amplification (e.g., Zhu et al. 2010). Following this approach, primers bind specifically based on the presence or absence of a mutation, and as such only amplify when the primer corresponding to the specific base (susceptible or mutation) is present. This PCR produced is then visualized on an agarose gel, thus any mutations present in other locations in the amplified fragments are missed. As allele-specific primers have not been developed for the I936F site, information regarding the presence or absence of the mutation at this site has largely remained unaddressed. While more costly, Sanger sequencing provides a more effective approach for both the identification of known resistance alleles, and the identification of new mutations which may be informative when considering the evolutionary relationships among populations. It should be noted that novel mutations should subsequently be profiled for resistance through bioassay when the mutations are found to be nonsynonymous.

An extensive VGSC Sanger sequence-based population screening of *C. lectularius* incorporating the three currently known *kdr*-associated mutations is missing for most countries. Here, data are presented for host-associated lineages of *C. lectularius* collected across a substantial geographic area in Europe; specifically those associated with bats, and those associated with humans. This study significantly expands on a previous study by Booth et al. (2015), through greatly expanding the number of populations screened and increasing geographic spread. Furthermore, frequency and distribution data for the I936F mutation are presented. As previous studies have suggested an uneven distribution of VGSC-associated haplotypes between the United States, and Europe, Australia, Japan, and Israel, this new study attempts to bridge that gap in order track the rise and spread of VGSC-associated mutations globally and to provide information that may help understand the relationships of European populations to others around the world.

Materials and Methods

In total, 393 *C. lectularius*, representing 131 unique collection sites, were sampled from bat roosts (n = 78 specimens from 26 locations, in 6 countries: Czech Republic = 13, Germany = 3, Hungary = 3, Serbia = 2, Slovakia = 4, Switzerland = 1) and human dwellings (n = 315 specimens from 105 locations, in 14 countries: Czech Republic = 52, Germany = 3, Slovakia = 5, Switzerland = 12, Austria = 1, Bulgaria = 1, France = 4, Finland = 1, Great Britain = 2, Italy = 7, Netherlands = 1, Norway = 8, Poland = 5, Sweden = 3) (Supp. Table S1 [online only]). All bat roosts sampled were within the attics of human-built structures. Upon collection, specimens were preserved in 96% ethanol. Genomic DNA was extracted from three individual insects per location using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and subsequently stored at −18°C until genetic analyses were performed.

Partial (see Results) or complete VGSC-mutation haplotypes were generated following the methodology outlined by Zhu et al. (2010). Two genomic fragments were amplified, each capturing either the 419 or 925 sites linked to the resistance to pyrethroids in *C. lectularius*. The latter fragment also spanned the 936 site recently identified by Dang et al. (2015a). Primer combinations BBParaF1/BBParaR1 (V419L) and BBParaF3/BBParaR3 (L925I) were used, as previously described by Zhu et al. (2010). Fragments were amplified independently for three individuals from each collection site and PCR products mixed in equal volumes according to the strength of

Table 1. Review of frequencies of *kdr*-associated haplotypes reported in studies of human-associated populations of *C. lectularius*

				Haplotype						
				A	B	C	D	A^b	B^b	C^b
Study	Region	No. of pops. screened	No. of het. pops.	None	L925I	V419L L925I	V419L	I936F	L925I I936F	V419L L925I I936F
Zhu et al. 2010[a]	United States	93	0	12 (12.9)	42 (45.2)	36 (38.7)	3 (3.2)	0	0	0
Dang et al. 2015a	Australia	32	4	3 (9.4)	25 (78.1)	2 (6.3)	0	5 (15.6)	1 (3.1)	0
Palenchar et al. 2015	Israel	12	12	0	11 (91.6)	1 (8.3)	0	0	11[b] (91.6)	1 (8.3)
Durand et al. 2012[a]	France	198[c]	0	0	198 (100)	0	0	0	0	0
Booth et al. 2015[a]	Europe	49	2	4 (8.1)	46 (93.9)	1 (2.0)	0	0	0	0
This study	Europe	105	9	4 (4.2)	98 (93.3)	2 (1.9)	0	9 (8.6)	0	0

Numbers for each haplotype include heterozygous locations. Representation by percentage is in parentheses (note that due to the presence of heterozygous populations, these values may exceed 100 % for a given study).

[a]Studies not reporting the I936F site.

[b]I936F mutation reported to occur at only low levels.

[c]Two high-rise multi-apartment buildings sampled, totaling 198 apartments (102 and 96, respectively).

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bands on 2% agarose electrophoresis gels (1× TBE). The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and Sanger sequencing was performed bi-directionally using a commercial sequencing service (Macrogen Inc., Seoul, South Korea).

Chromatograms were aligned using CodonCode Aligner 3.0 (CodonCode Corporation, Centerville, MA) and inspected by eye. Heterozygotes were identified through the presence of overlapping peaks at each specific site and must exhibit both nucleotides linked to pyrethroid/organochloride resistance.

Results

All bat-associated samples exhibited the susceptible nucleotide at both the 925 and 936 sites. Despite repeated attempts, eleven samples failed to produce an unambiguous sequence at the 419 site. As a result, this prevented the allocation of a haplotype at this site for these specimens (see Supp Table 1 [online only]). The remaining fifteen locations exhibited the susceptible form at this site (Supp Table 1 [online only], Fig. 1). Eight populations from the human-associated samples were found to be heterozygous in their *kdr* profile, while the rest were homozygous (Table 2; Supp Table 1 [online only], Fig. 1.). The vast majority (98 locations, 93.3%) of the human-associated samples exhibited haplotype B. Only two (1.9%) locations were found to possess both 419 and 925 mutations (haplotype C): one in Finland, and one in north Poland. Likewise, haplotype A was found in only a small number of populations (n = four, 4.3%): one homozygous in Germany, one in Poland, one in Great Britain and one heterozygous (together with haplotype B) in Switzerland. The haplotype A^b containing the I936F mutation was found in nine (8.6%) populations. Two of these were homozygous: one population from Switzerland and one from Bulgaria, while seven populations were heterozygous containing both haplotypes A^b and B: three populations from Czech Republic, two from Switzerland, one from Germany, and one from Norway.

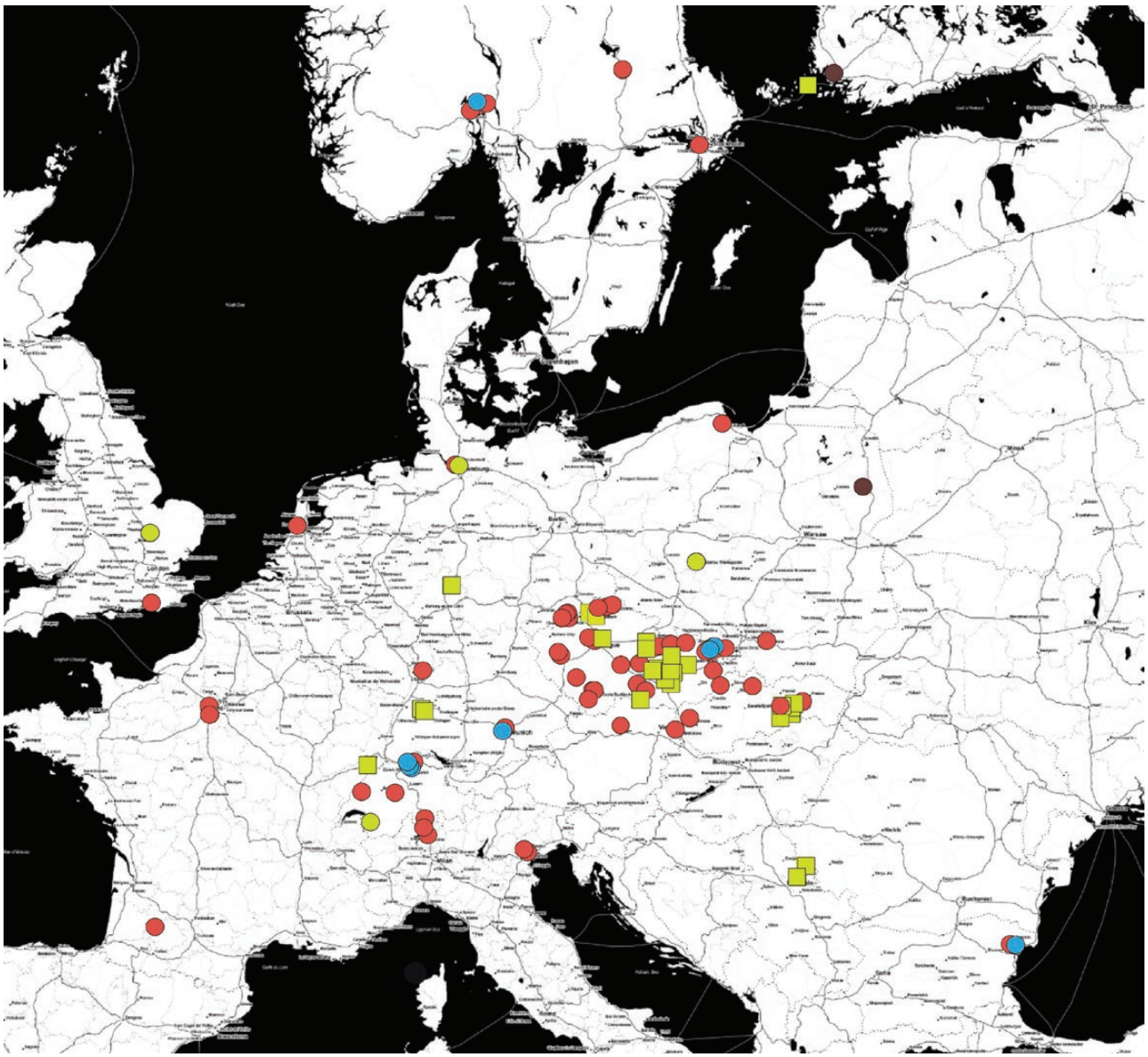

Fig. 1. Map of Europe identifying sampling locations for *C. lectularius*. Square icons represent bat-associated samples whereas circles represent human-associated samples. Haplotypes are differentiated by color: A (no mutations) = yellow, A^b (I936F) = blue, B (L925I) = red, and C (V419L and L925I) = brown.

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Table 2. Number of human-associated populations of *C. lectularius* per country found to exhibit susceptible or resistant *kdr*-associated haplotypes

			Haplotype			
			A	B	C	A[b]
Country	No. of pops. screened	No. of het. pops.	None	L925I	V419L L925I	I936F
Austria	1	0	0	1	0	0
Bulgaria	1	0	0	0	0	1
Czech Republic	52	3	0	53	0	3
Finland	1	0	0	0	1	0
France	4	0	0	4	0	0
Germany	3	1	1	2	0	1
Great Britain	2	0	1	1	0	0
Italy	7	0	0	7	0	0
Netherlands	1	0	0	1	0	0
Norway	8	1	0	8	0	1
Poland	5	0	1	3	1	0
Slovakia	5	0	0	5	0	0
Sweden	3	0	0	3	0	0
Switzerland	12	3	1	11	0	3
Total populations	105	8	4	98	2	9

Numbers for each haplotype include heterozygous populations.

Discussion

This study represents the most extensive screening to date of the three previously identified VGSC-associated mutations in *C. lectularius*, sampled across European populations of both bat- and human-associated lineages. The findings significantly increase the sample size for the human-associated lineage in Europe when compared to a previous study (Booth et al. 2015) and provide new information on both frequency and distribution of the I936F mutation; a site for which data have previously been unavailable outside of Australia and a small number of populations in Israel.

In relation to the bat-associated populations, the findings complement those of Booth et al. (2015), with all populations exhibiting susceptible nucleotides at all sites. This absence of resistance haplotypes in concert with the lack of evidence for contemporary gene-flow between bat- and human-associated populations (Balvín et al. 2012, Booth et al. 2015), supports the susceptible form (i.e., haplotype A—V419, L925, I936) as representing the ancestral amino acid composition at the VGSC. Given that the I936F mutation was absent in the bat-associated lineage, it may be surmised that this mutation arose within the human-associated lineage, similar to the mutations at sites 419 and 925; however, this will need to be evaluated through additional population screenings of both bat- and human-associated lineages. It should be noted here that while the human-associated lineage split from the ancestral bat-associated lineage ~225,000 year ago (Balvin et al. 2012), the bat-associated samples analyzed here were all collected within human-built structures (e.g., churches, factories, castles, etc.). We cannot comment as to whether these were ever exposed to insecticide application.

This sample of 105 human-associated European populations further supports the findings of previous studies which report high frequencies of resistance-associated haplotypes in the VGSC of European populations (Durand et al. 2012, Booth et al. 2015); specifically haplotype B. While resistance levels of the screened populations were not determined through insecticide assays in this study, these mutations have previously been shown to be associated with *kdr* (Zhu et al. 2010, Dang et al. 2015a). The prevalence of haplotype B across Europe is congruent with rates previously reported in Europe, Australia, Japan, and Israel (Durand et al. 2012, Tomita et al. 2012, Booth et al. 2015, Dang et al. 2015a, Palenchar et al. 2015). Comparable to those studies, haplotypes A and C were found in very few locations (Table 2, Supp. Table S1 [online only], Fig. 1). This contrasts with the pattern reported in United States, where B and C are common and largely in comparable frequencies (Zhu et al. 2010, Booth et al. 2018), and haplotype A is not uncommon (~13%) (Zhu et al. 2010). The distribution of the susceptible form (haplotype A) within the European human-associated population is scarce, with no evident geographic pattern of localization. It is noteworthy that no population sampled within the Czech Republic was found to exhibit this susceptible haplotype; a country represented by almost half of the total samples. Together with the relatively late resurgence of *C. lectularius* resurgence in the Czech Republic (Naylor et al. 2018), and assuming that the VGSC mutations have only one or a few independent origins, it is unlikely that any local refugia contributed to the resurgence of *C. lectularius* in the Czech Republic. It is, therefore, likely that introduction occurred through tourism or immigration.

Within the human-associated samples, the mutation I936F was observed infrequently, but at levels comparable to previous studies reported from Australia and Israel (Dang et al. 2015a, Palenchar et al. 2015). Its distribution covers several countries across Europe, with multiple instances within both the Czech Republic and Switzerland. Within the former, all instances occurred in or within 20km of Ostrava, the third largest city in the country. This region is affected by a declining economy, with a large proportion of the population living in government-assisted housing. There, the risk of bed bug infestation appears high and, due to the living conditions, local spread is likely. Recent studies have emphasized the potential for *C. lectularius* to establish and spread within multiapartment buildings upon introduction, often from what might be a single pregnant female (Doggett and Russell, 2008, Booth et al. 2012, Raab et al. 2016). In Switzerland, while samples were collected within multiple cities, all instances of the I936F mutation were recorded in samples collected within apartment buildings in Zurich. Single instances were then recorded in Norway, Germany, and Bulgaria, with the latter two being collected from hotels or student lodgings.

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No evidence was found for temporal variation in the presence of the I936F mutation, as suggested by Dang et al. (2015a).

To date the I936F mutation has not been reported within the United States, either in the original paper identifying the 419 and 925 mutations (Yoon et al. 2008), or in subsequent population level screenings (Zhu et al. 2010, Raab et al. 2016). In regards to the latter, this likely resulted from the use an allele-specific PCR approach. However, a recent unpublished screening (Booth, unpublished data) suggests that the I936F mutation within the south central United States is extremely rare (~2.06% of populations screened). This, in concert with the contrasting frequencies of both V419L and L925I mutations between the examined regions of the United States and the Old World reveals a highly contrasting pattern of VGSC profiles and suggests that the exchange of *C. lectularius* between the European and United States populations screened may be rare. Unfortunately little is known about the geographic origin of the VGSC mutations and a population genetic comparison of the respective regions using high-resolution markers is currently missing.

This study presents two contrasting patterns of the distribution and frequency of the VGSC mutations among host-associated lineages of *C. lectularius* in Europe. First, bat-associated lineages appear to lack mutations associated with knock-down resistance, further supporting a lack of movement from this source into the human-associated population, and vice versa. Second, across the human-associated population of Europe haplotype B is widespread. As the production and application of organophosphate-based insecticides is heavily regulated across the European Union (e.g., the Biocidal Products Regulation 528/2012), control of *C. lectularius* has largely had to rely on carbamate, pyrethroid insecticides, and more recently, desiccant dusts. Likely, the lack of substantial control due to knock-down resistance has aided the species establishment and spread across Europe and other regions of the Old World. Comparison of the United States and Europe VGSC profiles suggests that the global human-associated population of *C. lectularius* is not homogenous, but instead dispersal may be highly biased to within the New World or Old World respectively. This has significant implications for the spread of novel mutations conferring insecticide resistance, *kdr*-associated or otherwise, and thus, warranting a global assessment of populations using high-resolution genetic markers.

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Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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