

## MOLECULAR AND METABOLIC INSECTICIDE RESISTANCE IN CONTINENTAL POPULATIONS OF COMMON AND TROPICAL BED BUGS

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**Abstract** The evolution of insecticide resistance in bed bug populations in recent decades is considered a significant factor contributing to their resurgence. We analyzed samples of *Cimex lectularius* and *Cimex hemipterus* from Europe (Spain 41 samples, Switzerland 2 samples, the Czech Republic 1 sample), Asia (Hong Kong 34 samples), and North America (United States of America 14 samples and Mexico 3 samples) and South America (Colombia 3 samples) to assess the prevalence and mechanisms of insecticide resistance. We identified specimens morphologically and barcoded them by sequencing the mitochondrial cytochrome c oxidase subunit I (*COI*) and the 16S ribosomal RNA (*16S* rRNA) genes. Additionally, we screened segments of the voltage-gated sodium channel (*VGSC*) and the nicotinic acetylcholine receptor (*nAChR*) genes for point mutations associated with insecticide resistance and measured the activity of detoxifying enzymes. All samples from North America and Europe were identified as *C. lectularius*, while specimens from Hong Kong were *C. hemipterus*, while the two species were found in Colombia. Out of 64 *C. lectularius* samples tested for knockdown resistance (*kdr*) mutations, 90.6% contained at least one known mutation. All 35 *C. hemipterus* samples exhibited *kdr*-mutations. A new mutation was identified in the pyrethroid target site in both common (F1524C) and tropical (F1450C) bed bugs. No resistance-associated mutations in the *nAChR* gene were found. Several populations that exhibited *kdr*-mutations also showed elevated activity of detoxifying enzymes. The high frequency of *kdr*-associated mutations in bed bug populations limits efficacy of pyrethroids.

**Key words** Bed bugs, urban pest, insecticide resistance, knockdown resistance, metabolic resistance

## INTRODUCTION

Over the last decades, *Cimex lectularius* and *Cimex hemipterus* have resurged and become a significant and difficult-to-control indoor pest (Doggett et al. 2018). Among the reasons for the later aspect, there is far-reaching evidence for the involvement of multiple mechanisms of insecticide resistance in both species of insects (Doggett et al. 2018). These mechanisms include knockdown resistance (*kdr*)-associated mutations (which can generate target-site insensitivity); increased metabolic detoxification; and reduced cuticular penetration of insecticides (Doggett et al. 2018). On the other hand, target-site mutations in nicotinic acetylcholine receptor (*nAChR*) subunits represent an important neonicotinoid resistance mechanism reported in other insects (Xu et al. 2022), however, there are no reports of mutations in the *nAChR* gene in bed bugs. In this study (Porras-Villamil et al. 2024), bed bugs collected from eight different countries were analyzed morphological and genetically barcoding the *VGSC* and *nAChR* genes. Characterization of the activity of detoxifying enzymes was also conducted in bed bugs collected from the US and Mexico.

## MATERIALS AND METHODS

The samples and methods from that study are summarized in the following paragraphs:

**Sample collection and species identification** Bed bug samples were collected by local pest management services (urban areas), preserved and stored. Some were kept in US laboratories (Mexico, the USA, and the Czech Republic) and were collected between 2008 and 2022. Samples from Spain, Hong Kong, Switzerland, and Colombia were collected in 2022 (Porras-Villamil et al. 2024). Morphological identification was based on previously published keys (Usinger 1966). For DNA barcoding, the *16S* rRNA gene (Kambhampati and Smith 1995) and the cytochrome c oxidase subunit 1 (*COI*) gene (Booth et al. 2015) were used.

**Sequence analysis of *VGSC* and *nAChR* subunit genes** A single specimen from each collection was screened for *kdr*-related mutations and mutations in *nAChR* subunit genes. Three *VGSC* gene regions were amplified using three primer pairs, two from a previous study (Ghavami et al. 2021) and a newly designed third primer; for the *nAChR*  $\alpha$  and  $\beta$  subunits several primers based on *C. lectularius* gene sequences from GenBank ( $\alpha$ : XM\_014389050.2,  $\beta$ : XM\_014400945.2) were used (Porras-Villamil et al. 2024).

**Detoxification enzyme assays and statistical analysis** To assess the detoxification activity of P450, esterases and glutathione-S-transferases the methodology outlined in Gaire et al. (2020) was followed, applying a Dunnett's test to compare the enzymatic activity of samples with the control (Fort-Dix strain). All analyses and visualizations were conducted in R (Version 4.2.3, Shortstop Beagle) (Porras-Villamil et al. 2024). The complete applied methodology can be consulted in Porras-Villamil et al. (2024).

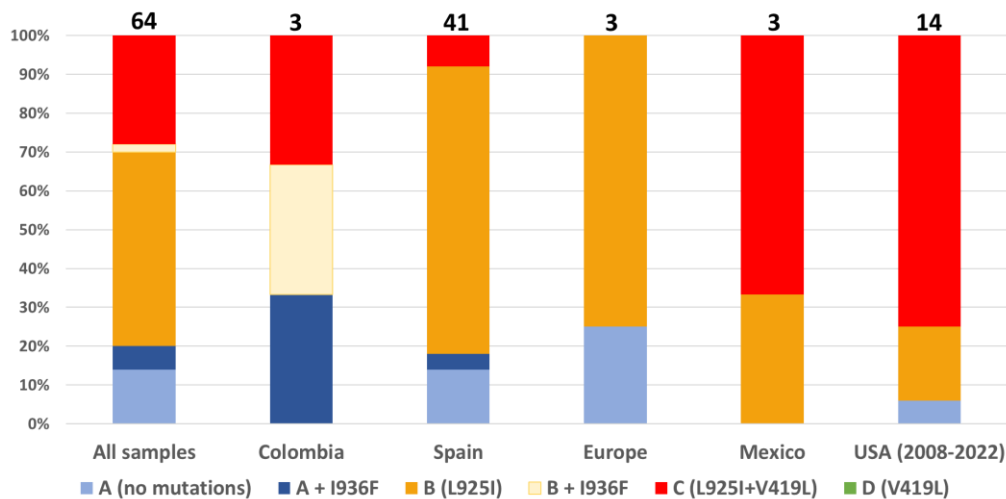
## RESULTS AND DISCUSSION

**Morphological identification** The results, described in detail in Porras-Villamil et al. (2024), provide an analysis of molecular and metabolic insecticide resistance in *C. lectularius* and *C. hemipterus* populations across eight countries (new data for Spain, Hong Kong, Mexico and Colombia) (Porras-Villamil et al. 2024). In the study, of the 99 morphologically and genetically examined samples, 66.6% were *C. lectularius* and 35.4% were *C. hemipterus* (Porras-Villamil et al. 2024). *C. lectularius* was collected in Europe and North America and *C. hemipterus* in Hong Kong. Interestingly, in Colombia, both species, *C. lectularius* (3 samples) and

*C. hemipterus* (1 sample), were found. This confirmed the presence of *C. hemipterus* in the country (Figueroa 1953) and the recent report of *C. lectularius* (Porrás-Villamil et al. 2023).

#### Identification of mutations in the VGSC in *Cimex lectularius* and *C. hemipterus*

Similar to other studies (Dogget et al. 2018), *C. lectularius* samples exhibited a high frequency of *kdr*-associated mutations, finding even the I936F mutation (Figure 1) (Porrás-Villamil et al. 2024).



**Figure 1.** Haplotypes of *kdr*-associated genotypes reported in *Cimex lectularius* (Europe and the Americas)

Furthermore, they identified a new mutation in the III-IV interlinker of the VGSC gene at the F1524C position in *C. lectularius* (Spain and Colombia samples). On the other hand, 100% of *C. hemipterus* samples presented several VGSC insecticide-resistance associated mutations (Porrás-Villamil et al. 2024) as well as a new mutation at the F1450C position (Hong Kong and Colombia samples) (Porrás-Villamil et al. 2024). The new mutations are similar to those associated with pyrethroid resistance in other insects (Yougang et al. 2020), even so, their role in resistance in bed bugs is still unclear (Porrás-Villamil et al. 2024).

**Screening of nicotinic acetylcholine receptors** In contrast, when the nAChR gene was screened, no resistance-associated mutations were found in the  $\alpha$  and  $\beta$  subunits of the nAChR gene in either bed bug species. Even so, a synonymous mutation in the  $\alpha$  subunit of *C. lectularius*, was identified in samples from the US and Spain. For *C. hemipterus*, two synonymous mutations were found in the  $\alpha$  subunit and one in the  $\beta$  subunit. Additionally, three more synonymous mutations were found in the region analyzed with the  $\beta 2$  primer (Porrás-Villamil et al. 2024).

**Characterization of detoxification enzyme activities** Finally, when they analyzed and compared the metabolic activity of metabolic enzymes, Porrás et al. (2024), found that Cytochrome P450 enzyme activity was elevated in two US samples, esterase activity was significantly higher in 5 samples (two from Mexico and three from the US), and glutathione-S-transferase activity

showed significant increases in 2 strains (one from Mexico and one from the US). This is consistent with other literature (Doggett et al. 2018)

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## CONCLUSION

In summary, the findings by Porras-Villamil et al. (2024) underscore the complexity and importance of resistance mechanisms, contributing to the current knowledge of this issue. This widespread resistance, alongside the lack of awareness among the public and governments, contributes to the ever-increasing spread of this pest.

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