Summer 7-21-2015

Quantitative Sequencing for the Determination of Kdr-type Resistance Allele (V419L,L925I, I936F) Frequencies in Common Bed Bug, Cimex lectularius L., (Hemiptera: Cimicidae) Populations Collected from Israel

Daniel J. Palencar
University of Massachusetts - Amherst

Kyle J. Gellatly
University of Massachusetts - Amherst

Kyong-Sup Yoon
Southern Illinois University Edwardsville, kyoon@siue.edu

Kosta Y. Mumcuoglu
Hebrew University-Jerusalem

Uri Shalom
Ministry of Environmental Protection, Jerusalem, Israel

OXFORD UNIVERSITY PRESS | Journal of Medical Entomology

Quantitative Sequencing for the Determination of Kdr-type Resistance Allele (V419L, L925I, I936F) Frequencies in Common Bed Bug, Cimex lectularius L., (Hemiptera: Cimicidae) Populations Collected from Israel

Journal:	*Journal of Medical Entomology*
Manuscript ID:	JME-2015-0010.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Clark, John; University of Massachusetts Amherst, Veterinary and Animal Science Palenchar, Daniel; University of Massachusetts, Amherst, Molecular Biology & Biochemistry Gellatly, Kyle; University of Massachusetts, Amherst, Molecular and Cellular Biology Sup Yoon, Kyong; Southern Illinois University, Biological Sciences and Environmental Sciences Program Mumcuoglu, Kosta Y.; Hebrew University-Hadassah Medical School, Parasitology Unit, Dept. of Microbiology Shalom, Uri; Ministry of the Environment, Division of Pest Surveillance and Control
Please choose a section from the list:	Vector Control, Pest Management, Resistance, Repellents
Field Keywords:	Resistance, Resistance Management
Organism Keywords:	Cimex

For publication in:
Journal of Medical Entomology
JME-2015-0010 revision 1
6-16-2015

Send comments and proofs to:
Dr. J. Marshall Clark [a]
Dept. of Veterinary & An. Sci.
N311B Morrill 1
637 North Pleasant St.
University of Massachusetts
Amherst, MA 01003
Tel: (413) 545-1052
Fax: (413) 577-4267
E-mail: jclark@vasci.umass.edu

Quantitative Sequencing for the Determination of Kdr-type Resistance Allele (V419L, L925I, I936F) Frequencies in Common Bed Bug, *Cimex lectularius* L., (Hemiptera: Cimicidae) Populations Collected from Israel

Daniel J. Palenchar[1], Kyle J. Gellatly[2], Kyong Sup Yoon[3], Kosta Y. Mumcuoglu[4], Uri Shalom[5], J. Marshall Clark[2,6,a]

[1]Department of Molecular Biology & Biochemistry, University of Massachusetts, Amherst, MA
[2]Molecular and Cellular Biology Program, University of Massachusetts, Amherst, MA
[3]Department of Biological Sciences and Environmental Sciences Program, Southern Illinois University, Edwardsville, IL 62026
[4]Parasitology Unit, Department of Microbiology and Molecular Genetics, the Kuvin Center for the Study of Infectious and Tropical Diseases, the Hebrew University-Hadassah Medical School, Jerusalem, Israel
[5]Divison of Pest Surveillance and Control, Ministry of Environmental Protection, Jerusalem, Israel
[6]Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA

Running head: Palenchar et al: Knockdown Resistance in Bed Bugs from Israel
Number of Figures: 4
Number of Tables: 4

ABSTRACT

Human bed bug infestations have dramatically increased worldwide since the mid-1990s. A similar phenomenon was also observed in Israel since 2005, when infestations were reported from all over the country. Two single nucleotide polymorphisms (V419L and L925I) in the bed bug voltage sensitive sodium channel confer *kdr*-type resistance to pyrethroids. Using quantitative sequencing (QS), the resistance allele frequencies of Israeli bed bug populations from across the country were determined. Genomic DNA was extracted from samples of 12 populations of bed bugs collected from Israel and DNA fragments containing the V419L or L925I and I936F mutations sites were PCR amplified. The PCR products were analyzed by QS and the nucleotide signal ratios calculated and used to predict the resistance allele frequencies of the unknown populations. Results of the genetic analysis show that resistant nucleotide signals are highly correlated to resistance allele frequencies for both mutations. Ten of the 12 tested populations had 100% of the L925I mutation and 0% of the V419L mutation. One population was heterogeneous for the L925I mutation and had 0% of the V419L mutation and another population was heterozygous for the V419L mutation and had 100% of the L925I mutation. I936F occurred only at low levels. These results indicate that bed bugs in Israel are genetically resistant to pyrethroids. Thus, pyrethroids should only be used for bed bug management with caution using effective application and careful monitoring procedures. Additionally, new and novel-acting insecticides and non-chemical means of controlling bed bugs should be explored.

Key Words: *Cimex lectularius,* common bed bug, Israel, knockdown resistance (*kdr*), pyrethroids, quantitative sequencing.

INTRODUCTION

Cimex lectularius L., the common bed bug, is an obligatorily hematophagous ectoparasitic insect that feeds on a variety of warm blooded mammals including humans (Usinger 1966, Boase 2004, Thomas et al. 2004, Reinhardt and Siva-Jothy 2007, Tawatsin et al. 2011, Zhu et al. 2010). *C. lectularius* is widely distributed throughout temperate parts of the globe, and poses serious economic, psychological, and physiological problems to humans. Effective monitoring and managing of bed bug populations and preventing or delaying development of insecticide resistance are keys to reducing and eliminating the ramifications that accompany their presence.

Human reactions to *C. lectularius* bites are highly variable (Boase 2004, Thomas et al. 2004, Wang et al. 2011). The initial bite of a bug is usually not felt. However, later the bite site itches, swells, burns, becomes inflamed or forms a weal and is very disturbing for the victim. There may be a central punctum at the site of the bite. Recurrent bites are often concentrated on the arms, legs and back, as well as on the face around the eyes. Lesions are frequently present in linear or clustered arrangements (Mumcuoglu, 2008). It is generally believed that bed bugs are not likely a vector for human disease (Blow et al. 2001, Boase 2004, Moore and Miller 2006, Reinhardt and Siva-Jothy 2007, Romero et al. 2007, Weeks et al. 2011, Doggett et al. 2012). Recently, Salazar et al. (2014) have shown that the common bed bug may be vector of *Trypanosoma cruzi* and could pose a risk for vector-borne transmission of Chagas disease. However, there is no evidence that the common bed bud actually transmits this pathogen in the field.

C. lectularius was a well-known parasite in human dwellings throughout the world until the end of WWII. The prevalence of common bed bug infestations decreased dramatically in

developed countries until the 1980s, largely due to the introduction and widespread use of insecticides, such as DDT and malathion, and probably the improvement in housing conditions and hygiene (Usinger 1966, Reinhardt and Siva-Jothy 2007). Bed bug infestations increased dramatically after the mid-1990s in the United States, the United Kingdom, various other European countries (Koganemaru and Miller 2013), Australia, Brazil, Chile (Faundez and Carvajal 2014) and China (Wang et al. 2013).

Infestations by the common bed bug were very common in what today is Israel until WWII (Kaufmann 1938, Dolev 2007). With the intensive use of DDT and lindane, the parasite became very rare and the few remaining pests were most probably those that became resistant to DDT (Levinson 1953, Cwilich et al. 1957, Gratz 1958, 1959, Busvine 1977, Mumcuoglu, 2008). During the years of 2006-2008, a 50-150% increase in the reported cases of infestations occurred in comparison to the period of 2001-2005. Bed bugs were mainly reported in hotels, residences and prisons, as well as in industrial buildings (Mumcuoglu and Shalom 2010). The most commonly used insecticides during this time were pyrethroids, followed by organophosphorous and carbamate insecticides. Banning the use of organophosphates inside houses and work places, as well as increasing numbers of foreign workers and new immigrants from developing countries, were considered additional factors for the increased number of bed bug infestations.

Bed bugs have proven to be very difficult pests to control, and insecticides have been used widely to control existing infestations (Thomas et al. 2004) and prevent reinfestations (Weeks et al. 2011). The selection of insecticide-resistant bed bugs is a significant contributing factor to their recent resurgence. Various strains of bed bugs from different geographical locations can have different resistance mechanisms (Yoon et al. 2008, Kilpinen et al. 2008, Zhu et al. 2013, Romero et al. 2009, Bai et al. 2011).

Bed bugs have demonstrated strong resistance to the pyrethroids (Seong et al. 2010), a class of insecticide that has been favored for bed bug control due to their high efficacy and low mammalian toxicity (Yoon et al. 2008). To date, bed bugs have developed multiple resistance mechanisms against pyrethroids, such as target site insensitivity due to mutations in the alpha-subunit of the voltage-sensitive sodium channel (VSSC), increased xenobiotic metabolism, and decreased cuticular penetration (Yoon et al. 2008, Zhu et al. 2013, Adelman et al. 2011, Mamidala et al. 2011, Koganemaru et al. 2013, Dang et al., 2013).

Pyrethroids target neuronal VSSCs of insects, which functions to generate and propagate action potentials in neurons (Zhu et al. 2010, Castèle and Catterall 2000). Pyrethroids selectively modify VSSC causing "repetitive discharge in motor and sensory axons and convulsive activity", which leads to paralysis and death (Morin et al. 2002). Widespread use of DDT following World War II may have inadvertently selected bed bugs for pyrethrins/pyrethroid resistance as DDT and pyrethrins/pyrethroids share the same target site (Busvine 1958, Zhu et al. 2010, Moore and Miller 2006, Boase 2004, Yoon et al. 2008). Point mutations in the alpha subunit of the VSSC in insects confer resistance to pyrethroids through target site insensitivity, resulting in knockdown resistance (*kdr)* (Yoon et al. 2008). *Kdr* and *kdr*-type mutations have been identified in many insects and result in DDT resistance and cross-resistance to the pyrethrins/pyrethroids (Clark et al. 2013).

Two mutations, a valine to leucine (V419L) and a leucine to isoleucine (L925I), have been identified by Yoon et al. (2008) in the highly deltamethrin-resistant common bed bug population (NY-BB), which conferred *kdr*-type resistance to pyrethroids. Both of these mutations result from single nucleotide polymorphisms (SNPs: **G**TC→**C**TC and **C**TT→**A**TT for V419L and L925I, respectively) in the alpha subunit gene of the common bed bug VSSC

144 (*Clvssc)*. A novel mutation I936F has also been identified in a single *d*-allethrin-resistant
145 common bed bug population from Adelaide, Australia, but it appears to give only low levels of
146 resistance and has not been validated as a *kdr*-type mutation functionally (Dang et al. 2014).
147 Using this information, a quantitative sequencing (QS) protocol was developed to
148 establish a population-based genotyping method as a molecular monitoring tool to predict the *kdr*
149 allele frequencies in common bed bug populations (Seong et al. 2010). The *kdr* allele frequencies
150 in different bed bug populations calculated by QS correlated well with filter contact vial bioassay
151 data, confirming the role of the two mutations in pyrethroid resistance (Seong et al. 2010).
152 Similar approaches based on these initial findings have been used widely to establish the role of
153 these two mutation in pyrethroid resistance in many common bed bug populations worldwide
154 (Zhu et al. 2010, Tawatsin et al. 2011, Durand et al. 2012, Tawatsin et al. 2013, Dang et al.
155 2014).
156 The purpose of this study was to predict the resistance allele frequencies of *kdr*-type
157 mutations in common bed bug populations collected from 12 locations across Israel. This
158 information is critical for those attempting to manage bed bug infestations in Israel, including
159 homeowners and pest management professionals, at a time when current approaches are failing
160 and infestations are increasing. Prior testing in other countries has shown that the frequencies of
161 *kdr*-type alleles are highly correlated to pyrethroid resistance (Yoon et al. 2008, Seong et al.
162 2010). Therefore, we hypothesize that the same two *kdr*-like mutations found in pyrethroid-
163 resistant bed bugs elsewhere (V419L and L925I) will also be found at high frequencies in the
164 Israeli populations. Additionally, previous information has identified that the L925I mutation is
165 particularly important in conferring neuronal insensitivity and pyrethroid resistance in bed bugs

(Yoon et al. 2008, Seong et al. 2010, Zhu et al. 2010). This study aims to contribute additional information towards validating or rejecting that hypothesis.

MATERIALS AND METHODS

Collection of common bed bug samples

Bed bugs were collected by pest management professionals in Israel prior to treating the infested area with insecticide. The 12 collection sites were five apartments, three one-family houses, two hotels and two industrial buildings. Each population came from a different location in Israel (Table 1). Bed bugs (8-12 per location) were individually removed from their normal habitats, including but not limited to beds, wall carpets and frames and stored in 70% ethanol prior to processing. Bed bugs were collected between February 2011 and April 2012 and sent to the Pesticide Toxicology Laboratory, University of Massachusetts-Amherst, for genotyping analysis by QS.

Sample selection for DNA extraction

All stored bed bugs from a common collection site/population were washed by removing the 70% ethanol and placing them into a 1.5 mL tube with 1 ml DNase free, double-distilled, H_2O (ddH_2O) and gently mixing the tubes by hand shaking. After 1-2 min, the wash solution was removed using a pipette. For each population, a single test sample, labeled BBM1 to BBM12, was prepared by first separating the different developmental stages. Life stages were determined by visual inspection according to Boase (2004) and Thomas et al. (2004). A single life stage (the largest and most abundant in the population) was selected as the test sample while all other bed

bugs were recombined and stored in 95% ethanol at -20°C. All test samples had multiple bed bugs of the same life stage (Table 2).

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from the 12 test samples (BBM1-12) using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Briefly, all bed bugs in a test sample were placed into 2 mL micro-centrifuge tubes containing 180 µL Buffer ATL, 0.9 µL reagent DX and five stainless steel grinding balls (0.25 g, SPEX Sample Prep, Metuchen, NJ) and homogenized in an automated homogenizer (Geno Grinder 2010, SPEX Sample Prep, Metuchen, NJ) at 1250 strokes per min for 1 min. Proteinase K (20 µL) was added to the tube and incubated for 3-24 h at 56°C. Following incubation, the lysate was transferred to a 1.5 mL tube with 100% ethanol (200 µL) and Buffer AL (200 µL) and vortexed. The sample was transferred to a DNeasy Mini spin column (Qiagen), and centrifuged for 30 s at 6000g. The flow through was discarded and the column placed into a new collection tube before washing with 500 µL buffer AW1, followed by 500 µL buffer AW2 with centrifuging for 30 s at 20,000g at each wash. The column was dried by centrifugation for 3 min at 20,000g before DNA elution. The column was placed into a new collection tube and incubated for 1 min with 50 µL buffer AE and centrifuged for 1 min at 6,000g to elute the DNA. This step was repeated to maximize total DNA yield.

Quantification of gDNA

1 µl of extracted gDNA from a single test sample was applied to a NanoDrop ND 8000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE), absorbance at 260 nm determined and used to calculate the DNA concentration in ng of DNA μL^{-1} using a modified Beer-Lambert equation. DNA purity was also determined by the ratio between absorbencies at

260 and 280 nm, with ratios between 1.5 and 1.8 indicative of pure samples. Samples were diluted to a concentration of 10 ng/µL and stored at -20°C.

PCR amplification of gDNA

15 µL of a master mix (12 µL ddH_2O, 5 µL 5X Advantage HD Buffer, 1.75 µL 2.857 mM dNTP mixture, 0.25 µL Advantage HD Polymerase and 1 µL of a 10 mM solution containing the forward and reverse primers for either V419L or L925I and I936F, Table 3) were mixed with 5 µL of template DNA (10 ng/µL) and placed onto an Eppendorf Mastercycler Pro (Hamburg, Germany) thermal cycler operated using the following parameters: 1 cycle at 95.0°C for 1 min; by 35 cycles of: 30 s at 95.0°C, 30 s at 61.0°C; and 1 min at 72.0°C. Gel electrophoresis (0.9% agarose) was used to verify quality and quantity of PCR DNA fragment amplification using ethidium bromide (1 µL of 10 mg/mL stock) staining to visualize PCR products under UV light. Where present, positive controls used an individual bed bug sample (BBS5) obtained from a laboratory-reared bed bug colony (FL-BB, Yoon et al, 2008). Negative controls for the PCR reaction used ddH_20 as template, and in all cases no band was present after PCR amplification. This confirms that all reagents were free from gDNA contamination.

PCR product purification*:*

The QIAquick PCR Purification Kit (Qiagen) was used for PCR product purification following the manufacturer's instructions. Briefly, 5 volumes of buffer PB were added to 1 volume PCR product, mixed with 10 µL of 3M sodium acetate buffer, transferred to a QIAquick spin column placed in a 2 mL collection tube and centrifuged for 30 s at 20,000g. The column was first washed with 750 µL guanidine HCl (30 s) and then with 750 µL buffer PE (30 s). After drying by centrifugation, DNA was eluted into a 1.5 mL tube by applying 50 µL and then 30 µL

of Buffer EB and centrifuging at 20,000g for 1 min. Gel electrophoresis (0.9% agarose) was used to verify quantity and quality of products as above.

Standard curve for QS analysis

The QS protocol developed by Seong et al. (2010) was followed and standard curves generated using the deltamethrin-resistant NYS-BB (Seong et al. 2010) and the insecticide-susceptible FL-BB (Yoon et al. 2008) strains of bed bugs for both mutations. Resistant and susceptible nucleotide signal intensities for each mixture were determined from sequence chromatograms and used to calculate nucleotide signal ratios (NSR) ([resistant nucleotide signal] / [resistant nucleotide signal + susceptible nucleotide signal]). Resulting nucleotide signal ratios were plotted against known resistant allele frequencies to generate standard curve equations as well as lower and upper prediction band equations (Fig. 1) using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA). The standard curves were used to predict resistance allele frequencies for the 12 unknown Israeli BBM populations at the 95% confidence level (Seong et al. 2010). A standard curve for the I936F mutation was not generated as the NSR values were consistently low.

Determination of unknown resistant allele frequencies

Triplicates for each test sample (BBM1-12), containing 5 µL of a 5 µM sequencing primer solution (3VLQS for V419L and 5LIQS for L925I and I936F, Table 3), were mixed with 10 µL of purified PCR product (Fig. 2) at a concentration of (1 ng/µL), loaded into a 96 well plate and sent to GeneWiz (South Plainfield, NJ) for sequencing. Sequence chromatograms were analyzed using Chromas lite software (Technelysium Pty Ltd., Tewantin, Australia) to determine

the nucleotide signal intensity at the respective mutation site (Fig. 3). NSR were calculated and used to predict resistance allele frequency from the Standard curves (Seong et al. 2010).

RESULTS

Standard curves for the V419L and L925I mutations were generated from known populations of deltamethrin-resistant and susceptible bed bugs (Fig. 1). The equation for the V419L and L925I standard curves were: $Y = 3.6754(1-[0.7271]^{x})$ and $Y = 2.0146(1-[0.4914]^{x})$, respectively, where Y is the resistance allele frequency. The curves show that the resistant and susceptible nucleotide signal intensities, which were used to determine the NRSs, were highly correlated to resistance allele frequencies with an R^2 value of 0.998 and 0.996 for the V419L and L925I curves, respectively. The V419L curve had smaller 95% prediction bands than the L925I curve, which may be due to different efficiencies in the dideoxy nucleotide terminator incorporation between the resistant and susceptible nucleotides in the sequencing reaction (Korch and Drabkin 1999).

After extracting gDNA from the 12 Israeli bed bug populations, absorbencies of the eluents were determined spectrophotometrically to determine the concentration and purity. For each test sample, the DNA concentrations (ng/µL) and purity (A260/A280 values) were as follows: BBM1 (123, 1.8), BBM2 (53, 1.7), BBM3 (43, 1.9), BBM4 (13, 2.9), BBM5 (76, 2.1), BBM6 (41, 1.9), BBM7 (94, 2.0), BBM8 (72, 1.9), BBM9 (120, 1.9), BBM10 (78, 2.1), BBM11 (120, 2.2), BBM12 (5.5, 2.2) All test samples yielded sufficient DNA (~10 ng/µL) of required purity for PCR amplification.

Two separate PCR reactions were performed on the 12 test sample eluents (BBM1-12) to amplify DNA fragments encompassing the V419L or L925I and I936F mutation sites within the

alpha subunit gene of *Clvssc*. The size and quality of the PCR products were determined by electrophoresis on a 0.9% agarose gel with ethidium bromide staining. The expected sizes of the V419L or L925I and I936F PCR products were 354 and 360 base pairs (bp), respectively.

The PCR products were purified and subjected to electrophoresis (Fig. 2). Bands at the expected sizes for the V419L or the L925I and I936F mutations confirmed the presence of the alleles of interest, and a band was present in the positive control, while no band was present in the negative control. Purified PCR products were sent to GeneWiz for sequencing. These sequences were analyzed with the QS protocol to determine the presence or absence of the three point mutations in the 12 test samples.

The resistance allele frequencies at the V419L mutation for all populations were 0.0 with the exception of BBM9, which was heterogeneous with a resistance allele frequency of 0.39 (Table 4). The resistance allele frequency of 0.0 corresponds to a 0% incidence of the V419L resistant allele in 11 of the 12 test samples. The 0.39 resistance allele frequency associated with one of the 12 populations samples (BBM9) corresponds to a 39% presence of the resistant allele at this site.

The resistance allele frequency for the L925I mutation was 1.0 (100% presence of the L925I-resistant allele, all bed bugs within a sample have the mutation) for 11 of the 12 populations tested (Table 4). The exception was the BBM10 sample, which had a resistance allele frequency of 0.61 (61%), indicating that this population was heterogeneous for the L925I mutation. NSRs were likewise determined for the I936F mutation for all samples but only ranged from 0.06 to 0.22 with an average NRS of 0.09.

DISCUSSION

Previously published research has shown that genetic resistance to DDT and the pyrethroids in common bed bugs is conferred, in part, by the presence of one or both of the V419l and L925I mutations. The goal of this project was to build upon these initial findings and determine the *kdr*-type resistance allele frequencies using QS in 12 bed bug populations collected from Israel in order to characterize the level and extent of these mutations, which lead to nerve insensitivity, so that monitoring and management strategies can be put into place.

Results of the DNA extraction show that the modified methods used are generally successful. For BBM1-12, the average DNA concentration was 69.8 ng/µL and only one sample (BBM12) was below the 10 ng/µL concentration prescribed for PCR. The low concentration of the BBM12 sample may partly be due to the low sample size and life stage used (n =2, third instars). BBM12 is the only sample of the original twelve that used third instars and additionally has a sample size less than or equal to all other samples. Nevertheless, the gDNA extractions, PCR amplification and purification protocols provided sufficient and suitable products for the QS analysis.

Results of the QS analysis provided useful information as to the state of *kdr*-based DDT/pyrethroid resistance in Israel bed bug populations. Eleven out of the 12 samples populations lacked the V419L mutation but all 12 populations had the L925I mutation. The only populations with differing genotypes were BBM9 and BBM10. BBM9 was heterogeneous in terms of the presence of the V419L mutation (RAF = 0.39) but was homogeneous L925I mutation (RAF = 1.0). BBM10 lacked the V419L mutation (RAF = 0.0) but was heterogeneous for the L925I mutation (RAF = 0.61). Overall, only the BBM10 population did not have a RAF of 1.0 for the L925I mutation. None of the 12 sampled populations had the I936F mutation at

either high or intermediate levels and this mutation appears to either play no role in the genetic resistance to the pyrethroids or only a minor role.

A 2010 study by Zhu et al. provides relevant information for comparison to our current results. These authors investigated *kdr*-type mutations in 93 bed bug populations from across the USA and separated the populations into the following four haplotypes based on their genotypes: A (neither mutation), B (L9251 but not V419L), C (both mutations), and D (V419L but not L9251). Of the 93 populations, haplotype B was the most common (44%), followed by C (39%), A (12%) and D (3%). These results are comparable with the current results from Israeli bed bug populations. Eleven out of the 12 populations tested in this study were haplotype B (BBM1-8 and BBM10-12) (92%), the most common haplotype. BBM9 was haplotype C (8%), the next most common haplotype. None of the samples tested in this study were haplotype A (0%) or D (0%). The most compelling piece of information comparing the current study to the Zhu et al. (2010) study is the frequencies of the L925I versus V419L mutations. Haplotype B, while the most common haplotype in both of these studies, is overwhelming the most common in the Israeli populations (Fig. 4). Interestingly, while 36 of the 93 populations tested by Zhu et al. (2010) were haplotype C (39%), only one of the bed bug populations tested in this study showed this haplotype (8%). Most recently, Dang et al. (2014) have shown that that there are high frequencies of *kdr*-type mutations associated with Australian bed bug populations, indicating the *kdr*-based pyrethroid resistance is also widespread across Australia. As found in Israel and elsewhere, haplotype B was most prevalent, haplotypes A and C were rare and haplotype D was not detected in Australia. Interestingly, two strains maintained in the laboratory, 'Moonee Ponds' and 'Darlinghurst II', appear to be losing the V419L mutation and becoming predominately haplotype B over time.

In 2008, Yoon et al. suggested that the L9251 "may play a critical role in deltamethrin resistance", after finding a cloned VSSC plasmid containing only this mutation, while all others contained both the L926I and V419L mutations. Seong et al. (2010) tested common bed bugs collected from Yongsan, Korea in 1993, 2007, 2008, and 2009 for *kdr*-type resistance allele frequencies using similar methods as the ones used in this study. Their findings showed no mutation for bed bugs collected in 1993, full saturation at both mutation sites in 2007, partial resistance for L925I (86%) and V419L (8%) in 2008, and 100% resistance for L925I with 0% resistance for V419L in 2009. The authors asserted that the L925I mutation appears to have been selected more intensively then the V419L mutation. The results from the Israeli bed bug populations presented here support his notion and further suggest that a fitness disadvantage may be associated with the V419L mutation.

Based on the trend previously shown in genetic analysis of *kdr*-based resistance in bed bugs in the USA, selective pressure appears to cause bed bugs exposed to pyrethroids to select both mutations but over time the V419L mutation is lost, leaving only the L925I mutation in heavily selected populations. If the use of pyrethroids for controlling bed bugs continues in Israel, selective pressure will likely favor the L925I haplotype. The results of this current study show that this may have already begun in Israel as 11 out of the 12 populations are homozygous for the L925I mutation with very few bed bugs maintaining the V419L mutation.

Conclusion

This paper aimed to elucidate the status of genetic resistance to pyrethroids in bed bug populations collected from Israel. Based on QS results, genetic testing showed that the majority of Israeli bed bugs examined in this study are resistant to pyrethroids by the *kdr* mechanism.

Continued use of pyrethroids on Israeli bed bugs will further select for a highly resistant populations. To effectively control these pests, alternative methods of treatments should be explored.

ACKNOWLEDGMENTS

This research was supported in part by a Commonwealth Honors College Research Academic Fellowship to D. Palenchar, University of Massachusetts Commonwealth Honors College, Amherst, MA 01003

393

394

References Cited

Alderman, Z.N., K.A. Kilcullen, R. Koganemaru, M.A.E. Anderson, T.D. Anderson, and D.M. Millar. 2011. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. PloS ONE 6:e26228.

Bai, X., P. Mamidala, S.P. Rajarapu, S.C. Jones, and O. Mittapalli. 2011. Transcriptomics of the bed bug (*Cimex lectularius*). PloS ONE 6:e16336.

Boase, C. 2004. Bed bugs – reclaiming our cities. Biologist. 51(1): 9-12.

Blow, J., M. Turell, and A. Silverman. 2001. Stercorarial shedding and transtadial transmission of hepatitis B virus by common bed bugs (Hemiptera: Cimicidae). J. Med. Entomol. 38(5): 694-700.

Busvine, J.R. 1958. Insecticide-resistance in bed-bugs. Bull. Wld. Hlth. Org. 19: 1041-1052.

Busvine J.R. 1977. Urban pests of public health importance. Royal Soc. Health J. 97: 130-134.

Castèle, S., and W. Catterall. 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochemie. 82: 883-892.

Clark, J. M, K. S. Yoon, S.H. Lee, and B. R. Pittendrigh. 2013. Human lice: past present and future control. Pestic. Biochem. Physiol. 106: 162-171.

Cwilich, R., G.G. Mer, and A.V. Meron. 1957. Bedbugs resistant to gamma-BHC (Lindane) in Israel. Nature. 179: 636-637.

Dang, K., D.G. Lilly, and S.L. Doggett. 2013. Bed bugs and insecticide resistance ; implication for pest managers. Pest (July/August): 25-27.

Dang, K., C.S. Toi, D.G. Lilly, W. Bu, and S.L. Doggett. 2014. Detection of knockdown resistance mutations in the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), in Australia. Pest Manag. Sci. doi: 10.1002/ps.3861

Doggett S.L., D.E. Dwyer, P.F. Penas, R.C. Russell. 2012. Bed bug: clinical relevance and control options. Clinical Microbio. Rev. 25:164-192.

Dolev E. 2007. Allenby's military medicine: Life and death in World War I Palestine. I.B. Tauris Publ. London, pp.86-87.

Durand, R., A. Cannet, Z. Berdjane, C. Bruel, D. Haochine, P. Delunay, and A. Izri. 2012. Infestation by pyrethroid resistant bed bugs in the suburb of Paris, France. Parasite 19: 381-387.

Faundez, E.I., and M. A. Carvajal. 2014. Bed bugs are back and also arriving in the southernmost record of *Cimex lectularius* (Heteropetra: Cimicidae) in South America. J. Med. Entomol. 51:1073-1076.

Gratz, N.G. 1958. Insecticide resistance among insects of public health importance in Israel and neighboring countries. Tavruah 4: 26-32.

Gratz, N.G. 1959. A survey of bed-bud resistance to insecticides in Israel. Bull. of the World Health Org. 20: 835-840.

Kaufmann, L. 1938. Housing in Jewish Palestine. The Jewish Agency for Palestine, Jerusalem. pp. 25-31.

Koganemaru, R., and D. Miller. 2013. The bed bug problem: past, present, and future control methods. Pestic. Biochem. and Physiol. 106: 177-189.

Koganemaru, R., D. Miller, and Z.N. Adelman. 2013. Robust cuticular penetration resistance in the common bed bug (*Cimex lectularius* L.) correlates with increase steady-state transcript levels of CPR-type cuticular protein genes. Pestic. Biochem. and Physiol. 106: 190-197.

Korch, C., and H. Drabkin. 1999. Improved DNA sequencing accuracy and detection of heterozygous alleles using manganese citrate and different fluorescent dye terminators. Genome Research 9: 588-595.

Levinson, Z.H. 1953. The control of bedbugs (*Cimex lectularius* L.) with DDT and GBH in Israel. Rivista Parassitologica. 14: 233-234.

Mamidala, P., S.C. Joned. And O. Mittapalli. 2011. Metabolic resistance in bed bugs. Insects 2: 36-48.

Masetti, M., and F. Bruschi. 2007. Bedbug infestations recorded in central Italy. Parasitology Int. 56: 81-83.

Moore, D., and D. Miller. 2006. Laboratory evaluations of insecticide product effficacy for control of *Cimex lectularius.* J. Econ. Entomol. 99: 2080-2086.

Morin, S., M. Williamson, S. Goodson, J. K. Brown, B. E. Tabashnik, and T. J. Dennehy. 2002. Mutations in the *Bemisia tabaci para* sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. Insect Biochem. Mol. Biol. 32: 1781-1791.

Mumcuoglu, K. 2008. A case of imported bed bug (*Cimex lectularius)* infestation in Israel. IMAJ. 10: 388-389.

Mumcuoglu, K., U. Shalom. 2010. Questionnaire survey of the common bed bug (*Cimex lectularius)* infestations in Israel. J. Med. Entomol. 40: 1-10.

485 **Reinhardt, K., and M. Siva-Jothy. 2007.** Biology of the bed bugs (Cimicidae). Annu. Rev.
486 Entomol. 52: 351-74.
487
488 **Romero, A., M. Potter, and K. Haynes. 2007.** Insecticide resistance in the bed bug: A factor in
489 the pest's sudden resurgence? J. Med. Entomol. 44: 175-178.
490
491 **Romero, A., M.F. Potter, and K.F. Hayes. 2009.** Evaluations of piperonyl butoxide as a
492 deltamethrin synergist for pyrethroid-resistant bed bugs. J. Econ. Entomol. 102: 2310-2315.
493
494 **Salazar, R., R. Castillo-Neyra, A.W. Tustin, K. Borrini-Mayorí, C. Náquira, and M.Z.**
495 **Levy. 2014.** Bed bugs (*Cimex lectularius*) as vectors of *Trypanosoma cruzi.* Am. J. Trop.
496 Med. Hyg. 14-0483. Published online November 17, 2014; doi:10.4269/ajtmh.14-0483.
497
498 **Seong, K., D. Lee, K. Yoon, D. Kwon, H. Kim, T. Klein, J.M. Clark, and S.H. Lee. 2010.**
499 Establishment of quantitative sequencing and filter contact vial bioassay for monitoring
500 pyrethroid resistance in the common bed bug, *Cimex lectularius.* J. Med. Entomol. 47: 592-
501 599.
502
503 **Tawatsin, A., U. Thavara, and J. Chompoosri. 2011.** Insecticide resistance in bed bugs in
504 Thailand and laboratory evaluation of insecticides for the control of *Cimex hemipterus* and
505 *Cimex lectularius* (Hemiptera: Cimicidae). J. Med. Entomol. 48: 1023-1030.
506

Tawatsin, A., K. Lorlertthum, A. Phumee, U. Thavara, J. Boon-Long, R. Boonserm, and P. Siriyasatien. 2013. Discrimination between tropical bed bug *Cimex hemipterus* and common bed bug *Cimex lectularius* (Hemiptera: Cimicidae) by PCR-RFLP. Thai J. Vet. Med. 43: 421-427.

Thomas, I., G. Kihiczak, and R. Schwartz. 2004. Bed bug bites: A review. Int. J. Dermatol. 43: 430-433.

Usinger, R.L. 1966. Monograph of Cimicidae (Hemiptera, Heteroptera). Thomas Say Foundation, University of Maryland, College Park, MD. pp. 585.

Wang, C., W. Tsai, R. Cooper, and J. White. 2011. Effectiveness of bed bug monitors for detecting and trapping bed bugs in apartments. J. Econ. Entomol. 104: 274-278.

Wang, L., Y. Xu, and L. Zeng. 2013. Resurgence of bed bugs (Hemiptera: Cimicidae) in mainland China. Florida Entomologist 96: 131-136.

Weeks, E., M. Birkett, M. Cameron, J.A. Pickett, and J.G. Logan. 2011. Semiochemicals of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), and their potential for use in monitoring and control. Pest Manag. Sci. 67: 10-20.

528 **Yoon, K., D. Kwon, J. Strycharz, C. Hollingsworth, S.H. Lee, and J.M. Clark. 2008**.
529 Biochemical and molecular analysis of deltamethrin resistance in the common bed bug
530 (Hemiptera: Cimicidae). J. Med.Entomol. 45: 1092-1101.
531
532 **Zhu, F., J. Wigginton, A. Romero, A. Moore, K. Ferguson, R. Palli, M.F. Potter, K.F.**
533 **Haynes, and S.R. Palli. 2010.** Widespread distribution of knockdown resistance mutations
534 in the bed bug *Cimex lectularius* (Hemiptera: Cimicidae), population in the United States.
535 Arch. Insect. Biochem. Physiol. 73: 242-257.
536
537 **Zhu, F., H. Gujar, J.R. Gordon, K.F. Haynes, M.F. Potter, and S.R. Palli. 2013.** Bed bugs
538 evolve unique adaptive strategies to resist pyrethroid insecticides. Sci. Rep. 3:1456.
539

540
541
542
543
544
545
546
547
548
549
550
551
552
553
554

555

556 **Table 1. Location and habitat of the 12 bed bug populations collected from Israel**

Population #	Location	Habitat
1	Jerusalem	Apartment
2	Tel Aviv	Apartment
3	Kfar Sava	Industrial Building
4	Kfar Sava	House
5	Haifa	Industrial Building
6	Hadera	Apartment
7	Eilat	Hotel
8	Tel Aviv	Hotel
9	Netanya	Apartment
10	Nazareth	House
11	Rehovot	Apartment
12	Jerusalem	House

557

558

559

560

561

562

563 **Table 2. Sample sizes and life stages of the 12 tested QS samples from bed bug**
564 **populations collected from Israel**

Population	Sample Size	Life Stage Used
BBM1	4	Adult
BBM2	2	Adult
BBM3	3	Fourth instar
BBM4	4	Fourth instar
BBM5	6	Adult
BBM6	10	Fourth instar
BBM7	2	Adult
BBM8	4	Adult
BBM9	2	Adult
BBM10	5	Fourth instar
BBM11	3	Fourth instar
BBM12	2	Third instar

565

566

567

568

569

570

571

572

573

574

575

576 **Table 3. PCR and QS primers used for the amplification of DNA fragments containing**
577 **the V419L or L929I and I936F mutations and for sequencing reactions**

Primer name	Sequence	Purpose
3'PCRVL	5'-CTGATGGAGATTTTGCCACTGATG-3'	VL sense PCR Primer
5'PCRVL	5'-GTCCGTGGCACATGTTGTTCTTCA-3'	VL antisense PCR Primer
3'PCRLI	5'-GGAGTTCGCCATCAGGGAATCTAT-3'	LI sense PCR Primer
5'PCRLI	5'-GGTCTATCAGTTTTGAGGTCATTCAG-3'	LI antisense PCR Primer
3'QSVL	5'-CCTCTTCAGCTTCTTCTTCTT-3'	VL sequencing primer
5'QSLI	5'- GTGTTTAAGCTGGCTAAGTCATGGCC-3'	LI sequencing primer

578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594

595 **Table 4. Nucleotide Signal Ratios (NSR) and Resistant Allele Frequencies (RAF) for bed**
596 **bug samples.**

Population	V419L				L925I				I936F		
	Nucleotide Signals		NSR	RAF (SD[1])	Nucleotide Signals		NSR	RAF (SD[1])	Nucleotide Signals		NSR
	Sus.	*Res.*			*Sus.*	*Res.*			*Sus.*	*Res.*	
BBM1	599	0	0.0	0.0	0	548	1.0	1.0	1049	93	0.08
BBM2	743	0	0.0	0.0	0	552	1.0	1.0	989	155	0.14
BBM3	683	0	0.0	0.0	0	582	1.0	1.0	1064	109	0.09
BBM4	516	0	0.0	0.0	0	513	1.0	1.0	979	271	0.22
BBM5	664	0	0.0	0.0	0	549	1.0	1.0	1039	81	0.07
BBM6	678	0	0.0	0.0	0	574	1.0	1.0	1082	156	0.13
BBM7	577	0	0.0	0.0	0	564	1.0	1.0	1042	106	0.09
BBM8	667	0	0.0	0.0	0	577	1.0	1.0	1046	62	0.06
BBM9	402	226	0.36	0.399 (0.032)	0	549	1.0	1.0	1093	95	0.08
BBM10	811	0	0.0	0.0	340	354	0.51	0.613 (0.002)	974	222	0.19
BBM11	757	0	0.0	0.0	0	561	1.0	1.0	1057	114	0.10
BBM12	749	0	0.0	0.0	0	555	1.0	1.0	1044	121	0.10

597 [1]SD = Standard Deviation.
598
599
600
601
602
603
604
605
606
607
608

609 **Figure Captions**

610

611 **Fig. 1.** Standard curves for V419L and L925I mutations. Resistant nucleotide signals from QS
612 were plotted against corresponding resistant allele frequencies at the V419L (A) and L925I (B)
613 mutations sites. Equations of the lines are listed above each graph. Black linear regression lines
614 are shown with 95% confidence bands in red. Nucleotide signal ratios were calculated as
615 follows: resistant nucleotide signal / [resistant nucleotide signal + susceptible nucleotide signal].

616 **Fig. 2.** Agarose gel (0.9%) electrophoresis of BBM V419L (A) and L925I purified PCR
617 products. Lane numbers correspond to BBM test samples (i.e., 1, 2, 3 for BBM1, BBM2, BBM3
618 etc.), a 100 basepair (bp) ladder (L, 100 – 1,200 bp, select marker sizes are indicated on left),
619 positive control (+, BBS5 from prior experimentation), and a negative control (-, ddH2O). An
620 asterisk (*) indicates the expected size of the respective PCR product and corresponds to the
621 experimental bands indicating successful PCR of the target DNA fragment. PCR products were
622 visualized under ultraviolet light using ethidium bromide staining.

623 **Fig. 3.** Sequencing chromatograms showing differences between susceptible, mixed, and
624 resistant nucleotide signals. On the left hand side, the VSSC amino acid substitution that results
625 in a *kdr* mutation is indicated, and the corresponding base pair change is bolded and underlined.
626 These same base pairs are indicated by an arrow in the chromatogram sequence, and its identity
627 is boxed on the chromatogram base pair readout. “N” indicates that there is no sequence
628 consensus, i.e. the population is mixed and contains both resistant and susceptible alleles.

629 **Fig. 4.** Geographic distribution of *kdr*-type mutations in common bed bug populations collected
630 from Israel. Pie charts show haplotype frequencies for the twelve bed bug populations tested.

631 Haplotypes: A (neither mutation, black), B (L925I only, white), C (both mutations, grey).
632 Haplotype D (V419L only) is not represented in any population and is omitted.

633

634

635

636

637

638

639

640

641

642

643

644

645

646

647

Fig 1.

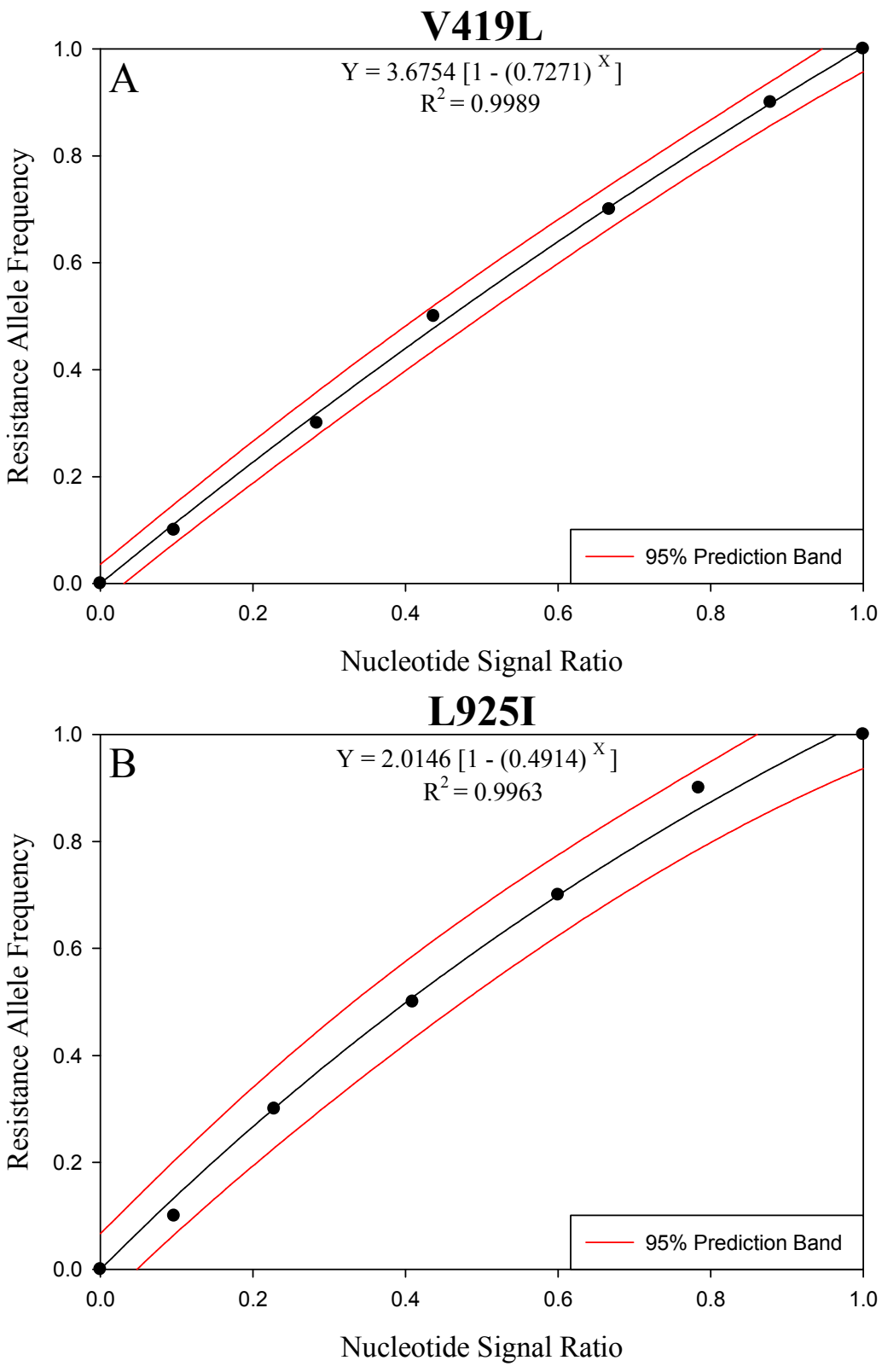
V419L
A
Y = 3.6754 [1 - (0.7271) X]
R2 = 0.9989
Resistance Allele Frequency
Nucleotide Signal Ratio
95% Prediction Band
L925I
B
Y = 2.0146 [1 - (0.4914) X]
R2 = 0.9963
Resistance Allele Frequency
Nucleotide Signal Ratio
95% Prediction Band

Fig 2.

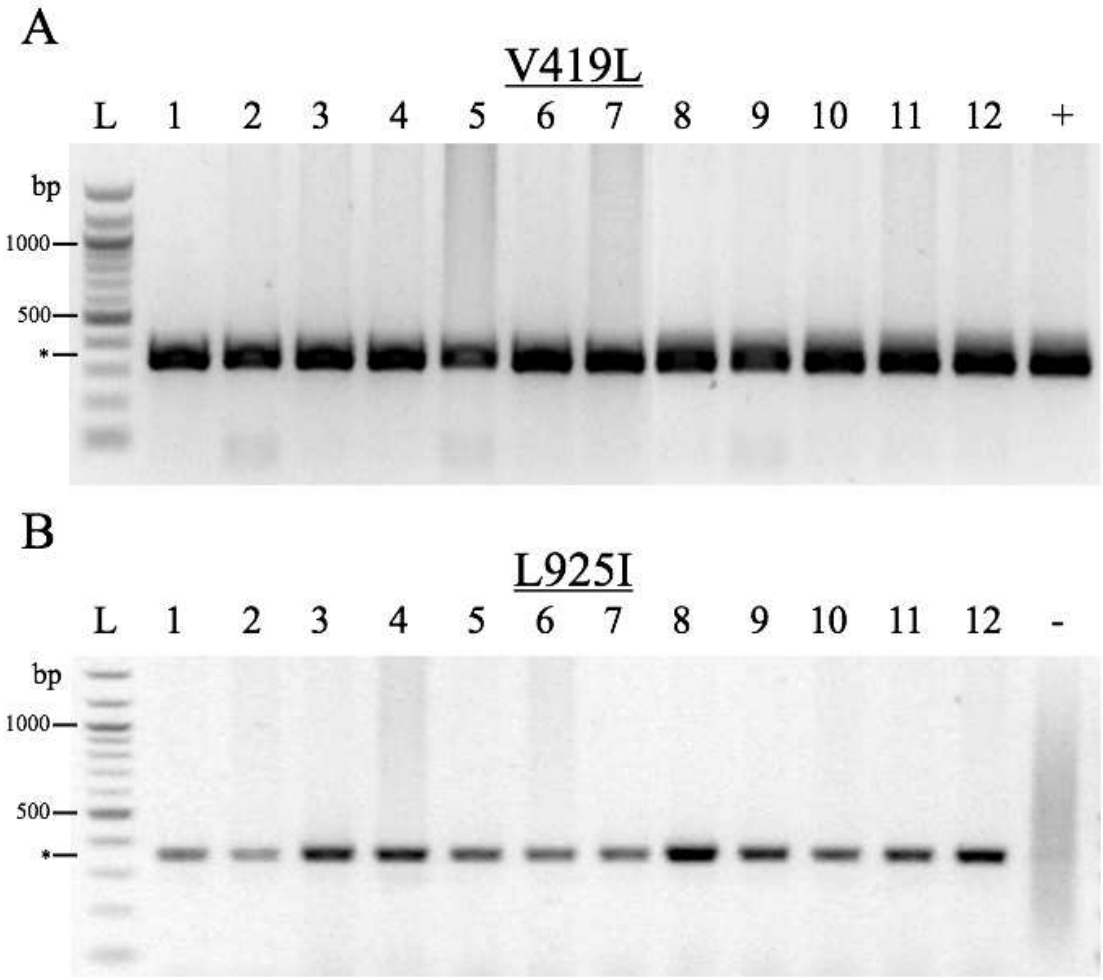
A
V419L
L 1 2 3 4 5 6 7 8 9 10 11 12 +
bp
1000
500
*
B
L925I
L 1 2 3 4 5 6 7 8 9 10 11 12 -
bp
1000
500
*

Fig 3.

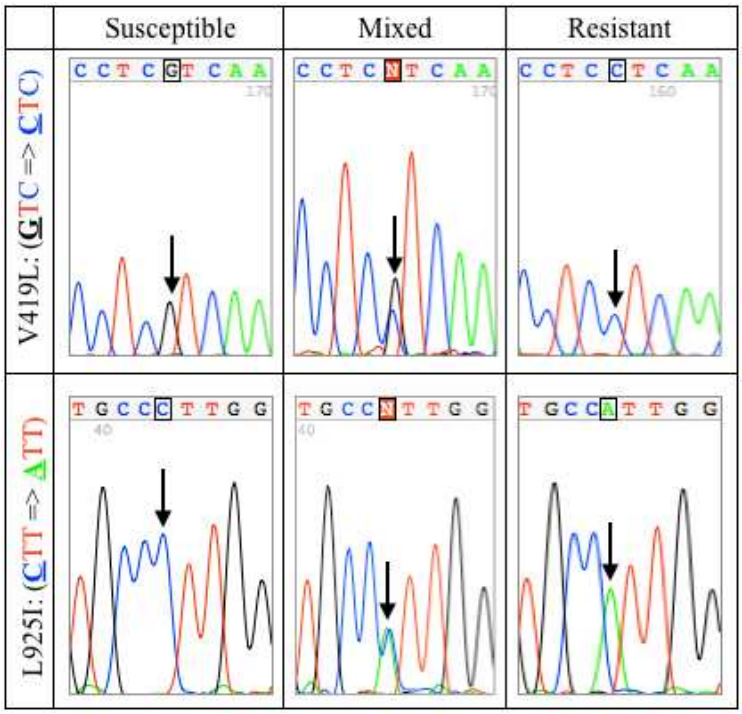

Fig 4.

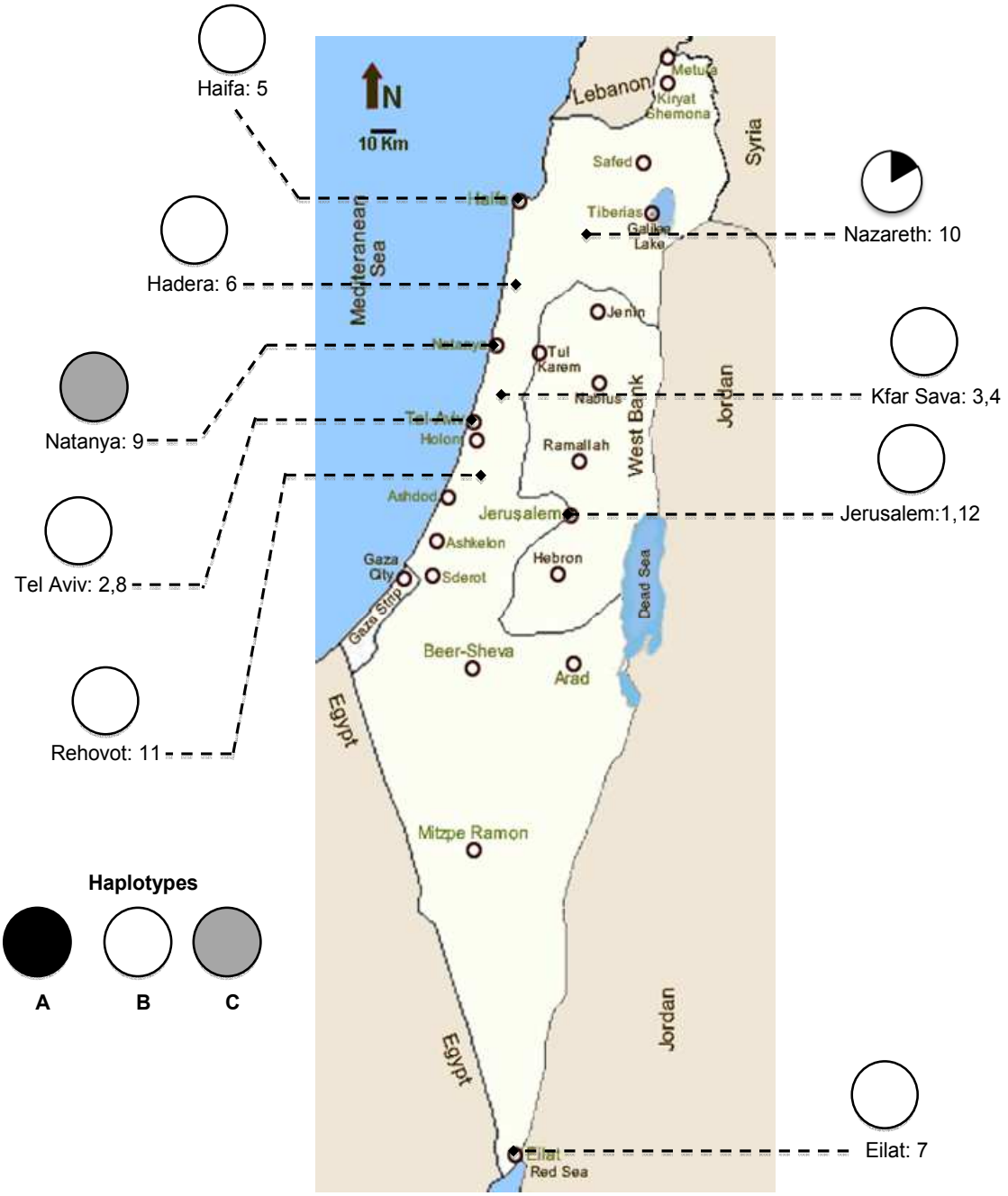
Haifa: 5
Hadera: 6
Natanya: 9
Tel Aviv: 2,8
Rehovot: 11
Nazareth: 10
Kfar Sava: 3,4
Jerusalem:1,12
Eilat: 7
Haplotypes
A
B
C
N
10 Km
Lebanon
Syria
Jordan
West Bank
Egypt
Mediterranean Sea
Red Sea
Dead Sea
Gaza Strip
Safed
Tiberias
Jenin
Tul Karem
Nablus
Ramallah
Jerusalem
Ashdod
Ashkelon
Gaza City
Sderot
Hebron
Beer-Sheva
Arad
Mitzpe Ramon
Eilat

Table 3. PCR and QS primers used for the amplification of DNA fragments containing the V419L or L929I and I936F mutations and for sequencing reactions

Primer name	Sequence	Purpose
3'PCRVL	5'-CTGATGGAGATTTTGCCACTGATG-3'	VL sense PCR Primer
5'PCRVL	5'-GTCCGTGGCACATGTTGTTCTTCA-3'	VL antisense PCR Primer
3'PCRLI	5'-GGAGTTCGCCATCAGGGAATCTAT-3'	LI sense PCR Primer
5'PCRLI	5'-GGTCTATCAGTTTTGAGGTCATTCAG-3'	LI antisense PCR Primer
3'QSVL	5'-CCTCTTCAGCTTCTTCTTCTT-3'	VL sequencing primer
5'QSLI	5'- GTGTTTAAGCTGGCTAAGTCATGGCC-3'	LI sequencing primer

Table 4. Nucleotide Signal Ratios (NSR) and Resistant Allele Frequencies (RAF) for bed bug samples.

Population	V419L				L925I				I936F		
	Nucleotide Signals		NSR	RAF (SD[1])	Nucleotide Signals		NSR	RAF (SD[1])	Nucleotide Signals		NSR
	Sus.	*Res.*			*Sus.*	*Res.*			*Sus.*	*Res.*	
BBM1	599	0	0.0	0.0	0	548	1.0	1.0	1049	93	0.08
BBM2	743	0	0.0	0.0	0	552	1.0	1.0	989	155	0.14
BBM3	683	0	0.0	0.0	0	582	1.0	1.0	1064	109	0.09
BBM4	516	0	0.0	0.0	0	513	1.0	1.0	979	271	0.22
BBM5	664	0	0.0	0.0	0	549	1.0	1.0	1039	81	0.07
BBM6	678	0	0.0	0.0	0	574	1.0	1.0	1082	156	0.13
BBM7	577	0	0.0	0.0	0	564	1.0	1.0	1042	106	0.09
BBM8	667	0	0.0	0.0	0	577	1.0	1.0	1046	62	0.06
BBM9	402	226	0.36	0.399 (0.032)	0	549	1.0	1.0	1093	95	0.08
BBM10	811	0	0.0	0.0	340	354	0.51	0.613 (0.002)	974	222	0.19
BBM11	757	0	0.0	0.0	0	561	1.0	1.0	1057	114	0.10
BBM12	749	0	0.0	0.0	0	555	1.0	1.0	1044	121	0.10

[1]SD = Standard Deviation.