

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

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Recommended Citation

Palencar, Daniel J.; Gellatly, Kyle J.; Yoon, Kyong-Sup; Mumcuoglu, Kosta Y.; Shalom, Uri; and Clark, J Marshall, "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" (2015). *SIUE Faculty Research, Scholarship, and Creative Activity*. 12.

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Cover Page Footnote

This document is an Accepted Manuscript version of an article published in the Journal of Medical Entomology published on behalf of the Entomological Society of America. The published version is available online at: <http://dx.doi.org/10.1093/jme/tjv103>

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:	<i>Journal of Medical Entomology</i>
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

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 Manuscripts

1 **For publication in:**
2 Journal of Medical Entomology
3 JME-2015-0010 revision 1
4 6-16-2015

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

15

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29

30 **Running head:** Palenchar et al: Knockdown Resistance in Bed Bugs from Israel

31 Number of Figures: 4

32 Number of Tables: 4

33

34 **ABSTRACT**

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

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

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75 INTRODUCTION

76 *Cimex lectularius* L., the common bed bug, is an obligatorily hematophagous
77 ectoparasitic insect that feeds on a variety of warm blooded mammals including humans
78 (Usinger 1966, Boase 2004, Thomas et al. 2004, Reinhardt and Siva-Jothy 2007, Tawatsin et al.
79 2011, Zhu et al. 2010). *C. lectularius* is widely distributed throughout temperate parts of the
80 globe, and poses serious economic, psychological, and physiological problems to humans.
81 Effective monitoring and managing of bed bug populations and preventing or delaying
82 development of insecticide resistance are keys to reducing and eliminating the ramifications that
83 accompany their presence.

84 Human reactions to *C. lectularius* bites are highly variable (Boase 2004, Thomas et al.
85 2004, Wang et al. 2011). The initial bite of a bug is usually not felt. However, later the bite site
86 itches, swells, burns, becomes inflamed or forms a weal and is very disturbing for the victim.
87 There may be a central punctum at the site of the bite. Recurrent bites are often concentrated on
88 the arms, legs and back, as well as on the face around the eyes. Lesions are frequently present in
89 linear or clustered arrangements (Mumcuoglu, 2008). It is generally believed that bed bugs are
90 not likely a vector for human disease (Blow et al. 2001, Boase 2004, Moore and Miller 2006,
91 Reinhardt and Siva-Jothy 2007, Romero et al. 2007, Weeks et al. 2011, Doggett et al. 2012).
92 Recently, Salazar et al. (2014) have shown that the common bed bug may be vector of
93 *Trypanosoma cruzi* and could pose a risk for vector-borne transmission of Chagas disease.
94 However, there is no evidence that the common bed bug actually transmits this pathogen in the
95 field.

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

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

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

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

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

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

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

144 (*Chvssc*). 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

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

168

169 **MATERIALS AND METHODS**

170 **Collection of common bed bug samples**

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

179

180

181 **Sample selection for DNA extraction**

182 All stored bed bugs from a common collection site/population were washed by removing
183 the 70% ethanol and placing them into a 1.5 mL tube with 1 ml DNase free, double-distilled,
184 H₂O (ddH₂O) and gently mixing the tubes by hand shaking. After 1-2 min, the wash solution was
185 removed using a pipette. For each population, a single test sample, labeled BBM1 to BBM12,
186 was prepared by first separating the different developmental stages. Life stages were determined
187 by visual inspection according to Boase (2004) and Thomas et al. (2004). A single life stage (the
188 largest and most abundant in the population) was selected as the test sample while all other bed

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

191 **Genomic DNA extraction**

192 Genomic DNA (gDNA) was extracted from the 12 test samples (BBM1-12) using the
193 DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions.
194 Briefly, all bed bugs in a test sample were placed into 2 mL micro-centrifuge tubes containing
195 180 μL Buffer ATL, 0.9 μL reagent DX and five stainless steel grinding balls (0.25 g, SPEX
196 Sample Prep, Metuchen, NJ) and homogenized in an automated homogenizer (Geno Grinder
197 2010, SPEX Sample Prep, Metuchen, NJ) at 1250 strokes per min for 1 min. Proteinase K (20
198 μL) was added to the tube and incubated for 3-24 h at 56°C. Following incubation, the lysate was
199 transferred to a 1.5 mL tube with 100% ethanol (200 μL) and Buffer AL (200 μL) and vortexed.
200 The sample was transferred to a DNeasy Mini spin column (Qiagen), and centrifuged for 30 s at
201 6000g. The flow through was discarded and the column placed into a new collection tube before
202 washing with 500 μL buffer AW1, followed by 500 μL buffer AW2 with centrifuging for 30 s at
203 20,000g at each wash. The column was dried by centrifugation for 3 min at 20,000g before DNA
204 elution. The column was placed into a new collection tube and incubated for 1 min with 50 μL
205 buffer AE and centrifuged for 1 min at 6,000g to elute the DNA. This step was repeated to
206 maximize total DNA yield.

207 **Quantification of gDNA**

208 1 μL of extracted gDNA from a single test sample was applied to a NanoDrop ND 8000
209 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE), absorbance at 260 nm
210 determined and used to calculate the DNA concentration in ng of DNA μL^{-1} using a modified
211 Beer-Lambert equation. DNA purity was also determined by the ratio between absorbencies at

212 260 and 280 nm, with ratios between 1.5 and 1.8 indicative of pure samples. Samples were
213 diluted to a concentration of 10 ng/ μ L and stored at -20°C.

214 **PCR amplification of gDNA**

215 15 μ L of a master mix (12 μ L ddH₂O, 5 μ L 5X Advantage HD Buffer, 1.75 μ L 2.857
216 mM dNTP mixture, 0.25 μ L Advantage HD Polymerase and 1 μ L of a 10 mM solution
217 containing the forward and reverse primers for either V419L or L925I and I936F, Table 3) were
218 mixed with 5 μ L of template DNA (10 ng/ μ L) and placed onto an Eppendorf Mastercycler Pro
219 (Hamburg, Germany) thermal cycler operated using the following parameters: 1 cycle at 95.0°C
220 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
221 electrophoresis (0.9% agarose) was used to verify quality and quantity of PCR DNA fragment
222 amplification using ethidium bromide (1 μ L of 10 mg/mL stock) staining to visualize PCR
223 products under UV light. Where present, positive controls used an individual bed bug sample
224 (BBS5) obtained from a laboratory-reared bed bug colony (FL-BB, Yoon et al, 2008). Negative
225 controls for the PCR reaction used ddH₂O as template, and in all cases no band was present after
226 PCR amplification. This confirms that all reagents were free from gDNA contamination.

227 **PCR product purification:**

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

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

236 **Standard curve for QS analysis**

237 The QS protocol developed by Seong et al. (2010) was followed and standard curves
238 generated using the deltamethrin-resistant NYS-BB (Seong et al. 2010) and the insecticide-
239 susceptible FL-BB (Yoon et al. 2008) strains of bed bugs for both mutations. Resistant and
240 susceptible nucleotide signal intensities for each mixture were determined from sequence
241 chromatograms and used to calculate nucleotide signal ratios (NSR) ($[\text{resistant nucleotide signal}]$
242 $/ [\text{resistant nucleotide signal} + \text{susceptible nucleotide signal}]$). Resulting nucleotide signal ratios
243 were plotted against known resistant allele frequencies to generate standard curve equations as
244 well as lower and upper prediction band equations (Fig. 1) using Sigma Plot 10.0 (Systat
245 Software Inc., San Jose, CA). The standard curves were used to predict resistance allele
246 frequencies for the 12 unknown Israeli BBM populations at the 95% confidence level (Seong et
247 al. 2010). A standard curve for the I936F mutation was not generated as the NSR values were
248 consistently low.

249

250 **Determination of unknown resistant allele frequencies**

251 Triplicates for each test sample (BBM1-12), containing 5 μL of a 5 μM sequencing
252 primer solution (3VLQS for V419L and 5LIQS for L925I and I936F, Table 3), were mixed with
253 10 μL of purified PCR product (Fig. 2) at a concentration of (1 $\text{ng}/\mu\text{L}$), loaded into a 96 well
254 plate and sent to GeneWiz (South Plainfield, NJ) for sequencing. Sequence chromatograms were
255 analyzed using Chromas lite software (Technelysium Pty Ltd., Tewantin, Australia) to determine

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

258

259 RESULTS

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

270 After extracting gDNA from the 12 Israeli bed bug populations, absorbencies of the
271 eluents were determined spectrophotometrically to determine the concentration and purity. For
272 each test sample, the DNA concentrations (ng/ μ L) and purity (A260/A280 values) were as
273 follows: BBM1 (123, 1.8), BBM2 (53, 1.7), BBM3 (43, 1.9), BBM4 (13, 2.9), BBM5 (76, 2.1),
274 BBM6 (41, 1.9), BBM7 (94, 2.0), BBM8 (72, 1.9), BBM9 (120, 1.9), BBM10 (78, 2.1), BBM11
275 (120, 2.2), BBM12 (5.5, 2.2) All test samples yielded sufficient DNA (~10 ng/ μ L) of required
276 purity for PCR amplification.

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

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

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

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

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

300

301 **DISCUSSION**

302 Previously published research has shown that genetic resistance to DDT and the
303 pyrethroids in common bed bugs is conferred, in part, by the presence of one or both of the
304 V419L and L925I mutations. The goal of this project was to build upon these initial findings and
305 determine the *kdr*-type resistance allele frequencies using QS in 12 bed bug populations
306 collected from Israel in order to characterize the level and extent of these mutations, which lead
307 to nerve insensitivity, so that monitoring and management strategies can be put into place.

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

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

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

326 A 2010 study by Zhu et al. provides relevant information for comparison to our current
327 results. These authors investigated *kdr*-type mutations in 93 bed bug populations from across the
328 USA and separated the populations into the following four haplotypes based on their genotypes:
329 A (neither mutation), B (L925I but not V419L), C (both mutations), and D (V419L but not
330 L925I). Of the 93 populations, haplotype B was the most common (44%), followed by C (39%),
331 A (12%) and D (3%). These results are comparable with the current results from Israeli bed bug
332 populations. Eleven out of the 12 populations tested in this study were haplotype B (BBM1-8
333 and BBM10-12) (92%), the most common haplotype. BBM9 was haplotype C (8%), the next
334 most common haplotype. None of the samples tested in this study were haplotype A (0%) or D
335 (0%). The most compelling piece of information comparing the current study to the Zhu et al.
336 (2010) study is the frequencies of the L925I versus V419L mutations. Haplotype B, while the
337 most common haplotype in both of these studies, is overwhelming the most common in the
338 Israeli populations (Fig. 4). Interestingly, while 36 of the 93 populations tested by Zhu et al.
339 (2010) were haplotype C (39%), only one of the bed bug populations tested in this study showed
340 this haplotype (8%). Most recently, Dang et al. (2014) have shown that there are high
341 frequencies of *kdr*-type mutations associated with Australian bed bug populations, indicating the
342 *kdr*-based pyrethroid resistance is also widespread across Australia. As found in Israel and
343 elsewhere, haplotype B was most prevalent, haplotypes A and C were rare and haplotype D was
344 not detected in Australia. Interestingly, two strains maintained in the laboratory, 'Moonee Ponds'
345 and 'Darlinghurst II', appear to be losing the V419L mutation and becoming predominately
346 haplotype B over time.

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

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

365

366 **Conclusion**

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

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

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389 **ACKNOWLEDGMENTS**

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

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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

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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

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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

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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
	<i>Sus.</i>	<i>Res.</i>			<i>Sus.</i>	<i>Res.</i>			<i>Sus.</i>	<i>Res.</i>	
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 ¹SD = Standard Deviation.
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609 **Figure Captions**

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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.

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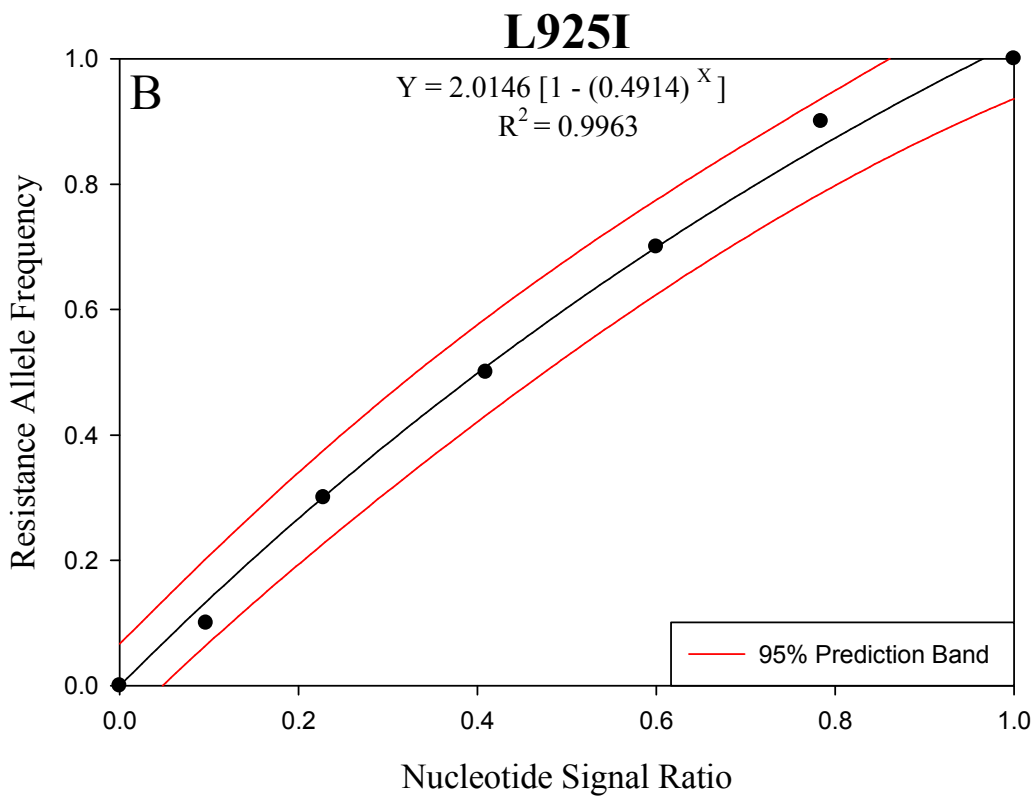
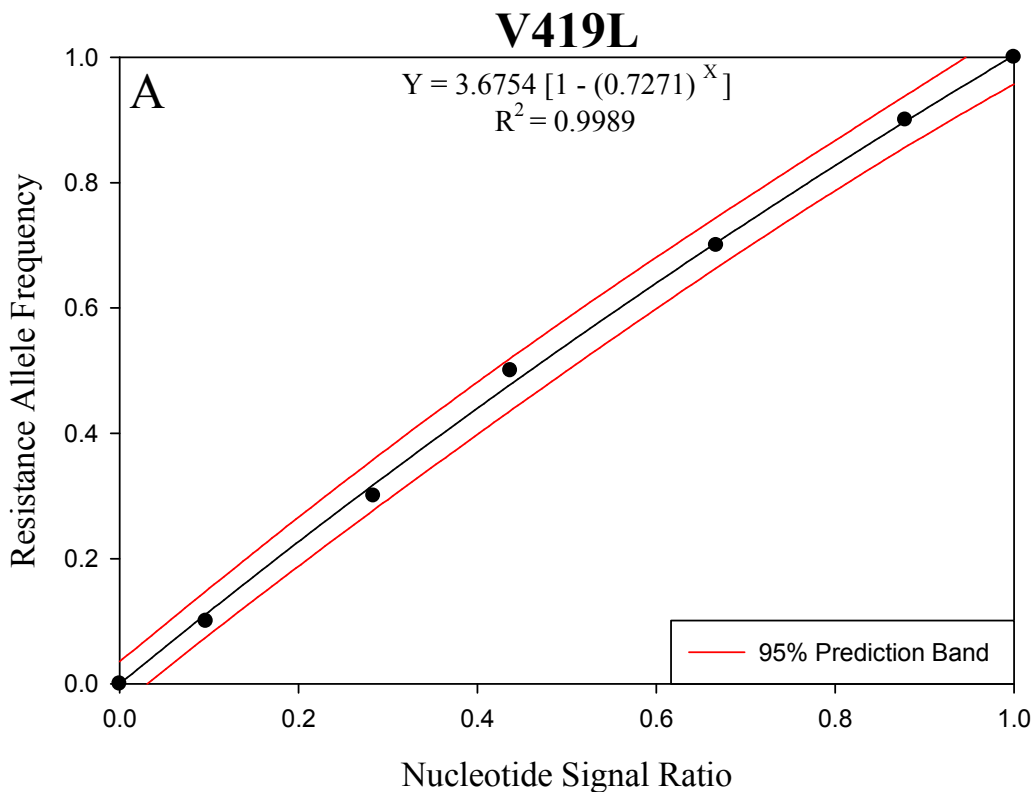
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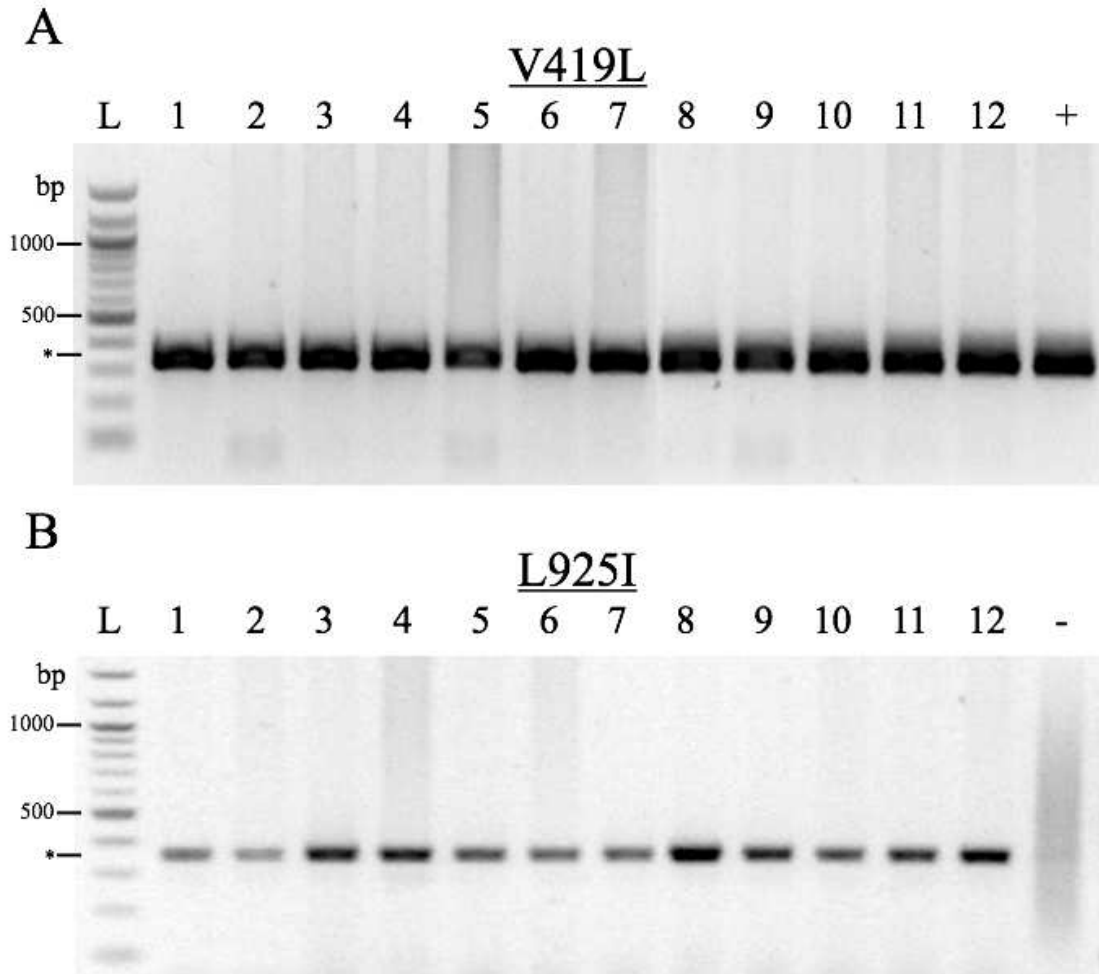
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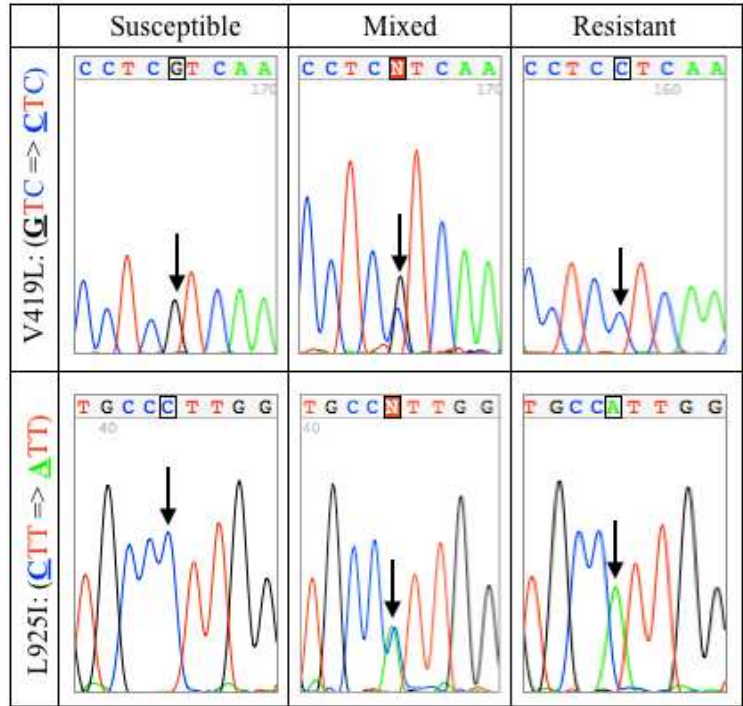
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703 Fig 4.

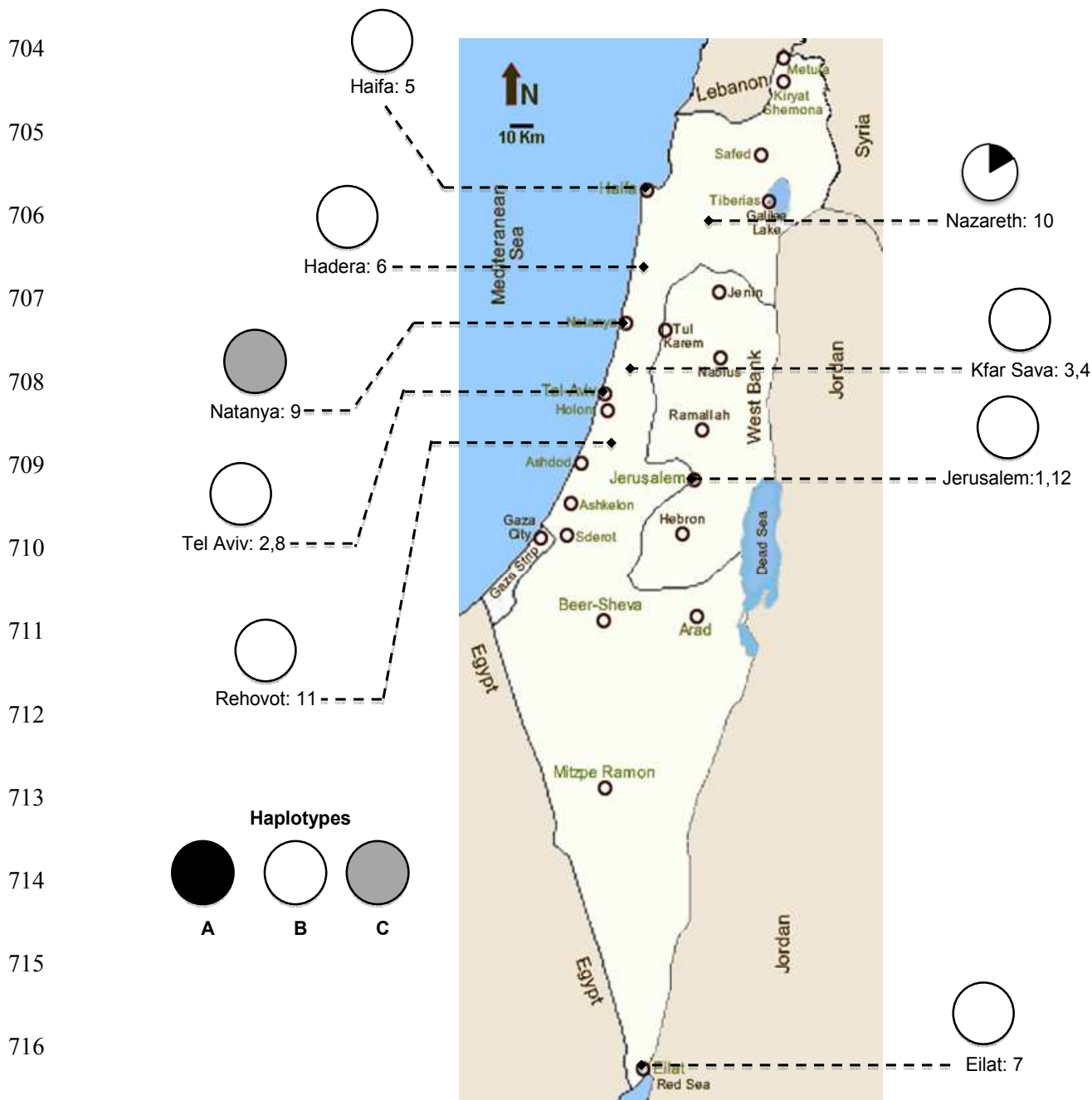


Table 3. PCR and QS primers used for the amplification of DNA fragments containing the V419L or L929I and I936F mutations and for sequencing reactions

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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		
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BBM12	749	0	0.0	0.0	0	555	1.0	1.0	1044	121	0.10

¹SD = Standard Deviation.