Pesticide Biochemistry and Physiology 104 (2012) 192–200

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Multiple evolutionary origins of knockdown resistance (*kdr*) in pyrethroid-resistant Colorado potato beetle, *Leptinotarsa decemlineata*

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ARTICLE INFO

Article history:
Received 12 May 2012
Accepted 5 August 2012
Available online 31 August 2012

Keywords:
Pyrethroid resistance
Voltage sensitive sodium channel
Leptinotarsa decemlineata
Evolution
kdr

ABSTRACT

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is a serious agricultural pest that is difficult to control due to its rapid evolution of resistance to most insecticides. Knockdown resistance (*kdr*) has been identified as the chief mechanism of resistance against pyrethroid insecticides in CPB and is due to a single nucleotide polymorphism, L1014F, of the voltage-sensitive sodium channel gene, *LdVssc1*. In order to determine whether *kdr* had a single or multiple evolutionary origins in CPB, 96 larvae from 10 locations (nine from across the United States and one in Bulgaria) were collected, and partial genomic sequences for *LdVssc1* were obtained for each individual. The associated intron, three base pairs downstream from the mutation site, was used in the identification of novel resistant (F1014) and susceptible (L1014) alleles. In addition, genotyping was performed to investigate the presence of the *super-kdr* allele (L1014F + M918T). In total, 8 resistant haplotypes (all *kdr*) and 21 susceptible haplotypes were identified. While no M918T was found, a T929I mutation, which is also associated with pyrethroid resistance in other species, was present in 16 larvae from Bulgaria. A novel T929N mutation was found in conjunction with L1014F. Within the United States, collections with only susceptible haplotypes exhibited higher allelic diversity than collections with primarily resistant haplotypes. Phylogenetic analysis suggests 2–3 independent origins of *kdr*.

1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a leading pest of potato (*Solanum tuberosum* L.), but also damages tomato (*S. lycopersicum* L.), eggplant (*S. melongena* L.) and other Solanaceous plants [1]. The widespread cultivation of potatoes provided a highly suitable host plant for the beetles, allowing CPB to quickly spread from their native range in southwestern United States and Mexico across the rest of continent (and then to Europe, parts of the Middle East, Central Asia, and Western China, with the potential for further expansion into the rest of Asia, South America, Africa, and Oceania) [1–3].

CPB has been very successful at colonizing and ravaging potato fields due to a number of factors, including high feeding rates, high fecundity, and resilient dispersal strategies [4–6]. Efforts to control CPB have largely relied on insecticide use. The beetles were initially targeted by heavy use of DDT, but rapid onset of resistance reduced its effectiveness [7]. Today, CPB has developed resistance to most major classes of insecticide [8,9]. The high levels of resistance and the incidence of multiple resistance within populations [10–13] are caused by high selection pressures. CPB remains a serious pest and inflicts over $100 million annually in crop losses and chemical control costs in North America alone [14].

Pyrethroids, such as permethrin, cyfluthrin and esfenvalerate, are commonly used in CPB control. Pyrethroids exert their toxic effects by preventing the inactivation of the voltage-sensitive sodium channel (Vssc), therefore prolonging depolarization of the neuron [15].

One of the major mechanisms of resistance to pyrethroids is target-site insensitivity due to knockdown resistance (*kdr*), caused by a mutation in *Vssc* [16]. The *kdr* allele was first identified in *Musca domestica* and is due to a single nucleotide polymorphism (C to T) resulting in an amino acid change from leucine to phenylalanine (L1014F) in the transmembrane IIS6 region of the sodium channel (*Vssc1*) [17,18]. This same mutation has been observed in the orthologous *Vssc* gene of CPB (*LdVssc1*) and identified as a major factor responsible for *kdr*-like resistance in pyrethroid-resistant beetles [19–21].

Unlike in most pests, *kdr* in CPB is sex-linked [20,22,23]. Because CPB has an XO system of sex determination [24], male

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http://dx.doi.org/10.1016/j.pestbp.2012.08.001

beetles will therefore either possess or lack the *kdr* mutation while female beetles can be homozygous susceptible, homozygous resistant, or heterozygous for the trait. Bioassay results show that hemizygous resistant males and homozygous resistant females have equally high levels of resistance and that resistance in heterozygous females is incompletely recessive [20]. Therefore, X-linkage allows for pyrethroid resistance to become more easily fixed in a population since even low frequencies of resistance haplotypes could produce males with high resistance, and the effective population size with regard to the *LdVssc1* gene is reduced compared to that of autosomal genes. Furthermore, increasing selection for resistance could result in increased differences in survivorship and resistance haplotype frequencies between the sexes, due to the likelihood for males to acquire a higher resistance faster relative to females [25].

Additional *Vssc* resistance alleles have been identified, including L1014H in *M. domestica* and *Heliothis virescens* [26,27] and L1014S in *Anopheles gambiae* and *Culex pipiens* [28,29]. A second site mutation, M918T, in the *Vssc* gene is associated with the *super-kdr* trait (L1014F + M918T), which confers increased resistance to most pyrethroids. *Super-kdr* (M918T + L1014F) has only been identified in *M. domestica* and *Haematobia irritans* [30,31]. Another second site mutation, T929I, has been discovered in conjunction with L1014F in *Plutella xylostella* and has been shown to confer *super-kdr*-like resistance [32]. Incorporation of these mutations into *Drosophila* sodium channels (*para*) expressed in *Xenopus* oocytes revealed that while the M918T mutation alone resulted in a greater decrease in the sensitivity of the channel to deltamethrin when compared to the T929I mutation alone, the L1014F + T929I double mutant was much less sensitive than L1014F + M918T, with a sensitivity 10,000-fold less than the wild-type channel [33]. The T929I mutation has also been found in conjunction with a novel mutation, L932F, in pyrethroid-resistant *Pediculosis capitis* [34]. Overall, over 20 unique *Vssc* polymorphisms have been associated with pyrethroid resistance across a number of species [35–37]. Thus far, only the L1014F *kdr* mutation has been identified in CPB [21,38].

The evolutionary history of *kdr* and *kdr-type* resistance can be analyzed by comparing *Vssc* haplotypes. In *M. domestica*, results suggested multiple origins of L1014H [26] and *kdr* (Scott et al., unpublished), but a single origin of *super-kdr* [26]. Multiple origins of *kdr* and *super-kdr* were proposed in *Myzus persicae* [39]. In *A. gambiae*, analysis suggested at least 4 independent mutation events [40]. Deviations in Hardy–Weinberg equilibrium between metapopulations of *H. irritans* indicated isolated populations and multiple origins of *kdr* as well [41]. In *Bemisia tabaci*, evidence also suggested multiple origins of pyrethroid resistance, this time associated with the L925I and T929V mutations [42]. Investigation into the origins of resistance mutations and the variability among the different haplotypes can help provide information about the mobility of a pest and the evolution of insecticide resistance. Multiple origins can also provide evidence for the isolation of populations. With the persistent resistance problems found in CPB and the large impact the pest has on the potato industry, research in these areas could help in the development of better pest management strategies.

The goals of this study are to identify novel *LdVssc1* haplotypes and to determine whether *kdr* had a single or multiple origins in CPB. Larvae from different locations in the United States, as well as one site in Bulgaria, were genotyped and compared in order to build a phylogeny of *LdVssc1* haplotypes. Overall, 8 resistant (F1014) haplotypes and 21 susceptible (L1014) haplotypes were found. The T929I and T929N polymorphisms were discovered in the Bulgaria population within both samples that were independent of the L1014F mutation. Within the United States, sample groups composed of susceptible haplotypes exhibited greater diversity of alleles than those with primarily resistant haplotypes. As a whole, a large proportion of either males or homozygous females were also observed for specific haplotypes. Phylogenetic analysis suggests multiple origins of the *kdr* mutation in CPB.

2. Materials and methods

2.1. Beetle collections

Larval CPB (1st instar through 4th instar larvae) were obtained from Rodopa Mountain in Bulgaria (BU) by S. N. in June 2009. The larvae were taken from potato plants in a location where alpha-methrin had been used within the past 5 years. Although no measurements of resistance have been conducted on CPB in BU, empirical evidence suggests that control with pyrethroids has deteriorated significantly since their introduction (SN, personal communication). Following collection, larvae were kept in tubes filled with 95% ethanol for 72 h at 20–25 °C. The ethanol was then decanted prior to shipping.

New Jersey (NJ) 4th instar larvae were obtained from the NJ Department of Agriculture Beneficial Insect Rearing Lab in July 2008. The beetles had not been exposed to insecticides in over 25 years. Individuals were stored in 70% ethanol at −80 °C. Fourth instar larvae originally collected from Freeville, NY were obtained from Dr. Jennifer Thaler (Cornell University) in 2008.

Beetles from Arizona (AZ), Maine (ME), Michigan (MI), Nebraska (NE), Ohio (OH), Washington (WA), and Wisconsin (WI) were collected between July and September of 1998 off of either native host plants (if from a plains or desert location) or off of potato plants. They were frozen in liquid nitrogen and stored at −80 °C.

2.2. Bioassays

A standard neonate bioassay was conducted to characterize resistance of the AZ, NE, WA, WI, OH, MI, and ME populations to esfenvalerate. Technical grade esfenvalerate (82% purity, Dupont, Wilmington, DE) was dissolved in acetone. Each filter paper was treated with 500 μl of a discriminating concentration of esfenvalerate (100 μg/ml), allowed to dry, and moistened with 200 μl of water immediately before use [11,43]. Up to 20 neonates were placed on 5.5 cm circular filter paper within a tight fitting Petri dish. Mortality was recorded after 24 h in a growth chamber at 25 °C and 16:8 L:D. Each dish was tapped on its side and after 30 s, individuals that had righted themselves were scored as alive and those struggling and squirming were counted as dead. Because control mortality exceeded 5% only twice, once 10% (1 of 10) and once 15% (3 of 20), no corrections were made to the bioassay data.

2.3. Isolation of genomic DNA

Larvae from the BU, NJ, and NY collections were placed individually into 1.5 ml tubes with 200 μl of lysis buffer (100 mM Tris–HCl pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% w/v SDS). Each animal was homogenized with a disposable pestle (Laboratory Product Sales Inc., Rochester, NY) in an overhead stirrer (Caframo Ltd., Wiarton, Ontario) until no large pieces of tissue remained. Another 200 μl of lysis buffer was added and the sample briefly homogenized again before incubating at 65 °C for 30 min. After incubation, 800 μl of a 2.5:1 mixture of 6 M LiCl and 5 M KAc was added, and the sample was mixed by inverting before incubating on ice for 10 min. The sample was then centrifuged at 14,000*g* for 15 min, and 950 μl of the supernatant was transferred to a new 1.5 ml tube containing 570 μl of isopropanol. The sample was mixed by inverting and spun at 14,000*g* for 15 min. The supernatant was discarded, and the pellet was washed with 600 μl of 70% ethanol

and spun at 14,000*g* for 4 min. The resulting supernatant was removed by pipette, and the pellet was allowed to air dry for 15 min. DNA was resuspended in 150 μl of nuclease-free water and stored at −20 °C.

DNA of individual beetles from the AZ, NE, WA, WI, OH, MI, and ME collections was extracted by grinding frozen insect tissue in a 1.7 ml microfuge tube with disposable pestles followed by DNA extraction (QIAamp Tissue Kit, Qiagen Inc., Valencia, CA). DNA was eluted into 400 μl of the supplied buffer and stored at −20 °C.

2.4. LdVssc1 genotyping by PCR

A ~1645 bp fragment of *LdVssc1* containing the L1014F (*kdr*) codon and the associated intron was amplified in a 50 μl reaction containing 25 μl of GoTaq Green Master Mix (Promega), 22 μl of distilled H_2O, 1 μl each of the primers kdrLongF and kdrR3 (Table 1), and 1 μl of gDNA as template. A positive control reaction to amplify a 427 bp fragment of *Su(var)3–9* was amplified by PCR in a 15 μl reaction containing 7.5 μl of GoTaq Green Master Mix (Promega Corp., Madison, WI), 6.6 μl of distilled H_2O, 0.3 μl each of the primers Suvar39F and Suvar39R (Table 1), and 0.3 μl of gDNA as template. The reactions were carried out under the following conditions: 95 °C for 1 min, followed by 40 cycles of PCR (95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min 45 s), and a final extension of 72 °C for 7 min. PCR products were separated using a 1% agarose gel by electrophoresis at 150 V constant voltage for 20 min and visualized by ethidium bromide staining. The products were purified by centrifugation using the Wizard SV Gel and PCR Clean-Up System (Promega) and eluted in 50 μl nuclease-free water. The quality and concentration of the purified DNA samples were quantified by spectrophotometer. Sequencing for *LdVssc1* was performed using the primers kdrLongF, kdrR3, and kdrInternalF (Table 1). Sequencing for the *Su(var)3–9* (AJ290965) fragment used the Suvar39R primer. All sequencing was performed at Cornell's Biotechnology Resource Center.

Additionally, 2 μl of PCR product from samples with ambiguous sequences were cloned using the pGEM-T Vector System according to the manufacturer's instructions (Promega). Positive colonies were grown in liquid media, and plasmid DNA was purified by centrifugation using the PureYield Plasmid Miniprep System (Promega). DNA quality and concentration were quantified by spectrophotometer. Plasmids were sequenced in both directions using the primers T7 and SP6 (Table 1).

In order to investigate the presence of *super-kdr*, a 241 bp fragment of *LdVssc1* containing the M918T codon was amplified in a 20 μl reaction containing 10 μl of GoTaq Green Master Mix (Promega), 8.8 μl of distilled H_2O, 0.4 μl each of the primers superkdrF and superkdrR (Table 1), and 0.4 μl of gDNA as template. A positive control reaction for *Su(var)3–9* was performed in parallel as described above. PCR was conducted under the following conditions: 95 °C for 1 min, followed by 40 cycles of PCR (95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s), and a final extension of 72 °C for 10 min. PCR products were visualized, purified, and sequenced with the primers superkdrF and superkdrR as described above.

Table 1
Sequences of primers used in this study.

Primer name	Sequence (5′-3′)
Suvar39F	AAAGAAGGGCAGGCCAAAGAGCAG
Suvar39R	CTATAAGACCCCCAGGAACAGCAAACT
kdrLongF	CATGAACTACCAAGATGGAATTTCACAG
kdrR3	CCTAATCAGTTTTGCGATATCTGC
kdrR5	GCCGATAAACTTGATGAACC
kdrInternalF	TCTAGAAGGATATGTAGATC
SuperkdrF	GGCCTTGAAGGTGTTCAAGGTT
SuperkdrR	AACTGCATACCCATAACAGCAAA
T7	TAATACGACTCACTATAGGG
SP6	TATTTAGGTGACACTATAG

2.5. Analysis of LdVssc1 haplotypes

Electropherograms were inspected with Chromas Lite (Technelysium Pty. Ltd., Tewantin, Queensland). *LdVssc1* haplotypes were scored as either susceptible (L1014) or resistant (F1014). Specific haplotypes were identified by examining the associated intron that begins 3 bp downstream of the site and comparing the sequence with known haplotype sequences using the MegAlign and EditSeq applications of Lasergene 7 (DNAStar, Madison, WI). New haplotypes from heterozygous samples were identified either through plasmid DNA sequences from cloning or by comparison of known haplotypes to the undetermined sequence and recording the difference in nucleotides at heterozygous points on the electropherograms. Haplotypes were confirmed if they were observed in more than one animal or if they were identified by cloning with two or more clones containing the same sequence.

The data used for determining the phylogeny of *LdVssc1* consisted of the complete *super-kdr* associated intron sequence and 191 bp of flanking exon sequence coupled to the corresponding *kdr* associated intron sequence and 179 bp of flanking exon sequence for each resistant and susceptible haplotype. Although most of the polymorphisms exist in the intron regions, including the exon sequences made it possible to take into account the novel T929I and T929N mutations. The corresponding *Vssc* sequence in *Leptinotarsa juncta* (Accession number HQ589185) was also included as an outgroup. Genomic DNA was extracted from a single *L. juncta* 4th instar larvae collected from Ellicott City, MD in August 2010. A fragment approximately 1530 bp in length was amplified by PCR using the primers kdrLongF and kdrR5 (Table 1) following the same protocol as for the CPB samples. Column purification and sequencing of the PCR product was conducted as previously described.

Sequences were aligned using Clustal W, and relationships among sequences, including gaps, were estimated via parsimony using the dnapars v.3.52 module of PHYLIP [44]. The strict consensus tree was rooted with *L. juncta*. Each nucleotide difference in a gap was treated as a separate character state. Robustness of internal branches was estimated by calculating bootstrap values using 1000 replicates.

3. Results

3.1. Bioassays

The percent survivorship of CPB larvae to the discriminating dose of esfenvalerate is shown in Table 2. Larvae from the AZ, NE, WA, and WI collections exhibited very low levels of survivor-

Table 2
Mean percent survivorship of CPB larvae exposed to a discriminating concentration of esfenvalerate.

Population	N	Mean survivorship	SEM	Host[a]
AZ	3	0.00	–	*Sr, Se*
ME	31	0.67	0.11	*St*
MI	11	0.31	0.05	*St*
NE	7	0.00	0.00	*Sr*
OH	26	0.60	0.05	*St*
WA	9	0.01	0.01	*St*
WI	21	0.04	0.02	*St*

AZ, ME, MI, NE, OH, WA and WI represent Arizona, Maine, Michigan, Nebraska, Ohio, Washington and Wisconsin, respectively.

[a] Plants from which CPB were collected: Potato (*Solanum tuberosum (St)*), *S. rostratum* (*Sr*) and *S. elegnifolium* (*Se*).

ship, indicating the presence of susceptible *LdVssc1* haplotypes in these populations. Larvae from the ME, MI, and OH collections exhibited intermediate levels of survivorship, suggesting the presence of both susceptible and resistant haplotypes.

3.2. LdVssc1 genotyping and haplotype identification

Out of the 96 samples that were examined, 8 *kdr* haplotypes (F1014) and 21 susceptible haplotypes (L1014) were identified (Table 3, Genbank accession numbers are provided in Supplementary Table 1). Downstream of the 1014 mutation site, the intron was found to be between 1302 and 1314 bp in length (Fig. 1). The sequence alignment of the intron haplotypes and the variability among them is shown in Supplementary Table 1. The geographic distribution of the haplotypes is outlined in Fig. 2. Only 1 susceptible allele and 3 resistant alleles were found in multiple locations; the rest were each unique to only one location. With the exception of NJ, NY, and OH, each of the collections presented 3 or more haplotypes. This diversity is most prominent in the WI population, where four unique haplotypes were found after genotyping only three beetles.

Overall, 66 of 96 (0.69) individuals possessed only resistant alleles (either hemizygous males or homozygous females). Within the BU population, 16 of 25 (0.64) individuals had only resistant alleles, and 4 additional individuals were heterozygous at the *kdr* site (Table 3). Within the collections from the U.S., none of the individuals from AZ, NE, WA, and WI possessed resistant alleles, whereas all of the individuals from NJ, NY, and OH had only resistant alleles (Table 3). Both ME and MI included 13 of 14 (0.93) individuals with only resistant alleles (Table 3). These proportions correspond, for the most part, to the bioassay results. The states in which no resistant alleles were observed (AZ, NE, WA, and WI) also all had sample groups with mean percent survivorships of less than 0.05 when exposed to esfenvalerate (Table 2), supporting the notion that the populations are largely susceptible to pyrethroid insecticides. Beetles from OH had only resistant alleles and therefore showed a higher mean percent survivorship of 0.60 (SEM = 0.05). Similarly, beetles from ME had mostly resistant alleles and exhibited a mean percent survivorship of 0.67 (SEM = 0.11). However, the mean percent survivorship of a sample group from MI was lower, at 0.31 (SEM = 0.05), despite having the same proportion of individuals possessing only resistant alleles as ME (Table 3).

LdVssc1 genotyping for the *super-kdr* (M918T) region was performed on 68 of the beetles from the United States and all 25 of the beetles from BU. The *super-kdr* allele (M918T + L1014F) was not present in any of the individuals. The intron upstream of M918 was found to be 50 bp in length (Fig. 1). Two variants, defined by a single nucleotide adenine/guanine polymorphism within the intron, were detected (Fig. 3). All individuals with the *kdr1* haplotype had the adenine variant whereas individuals with all other *kdr* haplotypes possessed the guanine variant; therefore, the M918 region with the adenine SNP was incorporated into the *kdr1* haplotype as one *LdVssc1* haplotype. Genotyping of the M918 region revealed the presence of two other polymorphisms in *LdVssc1* that had not been detected in previous CPB studies (Fig. 3). The mutations T929I, characterized by a codon change from ACC to ATC, and T929N, characterized by a codon change from ACC to AAC, were present in 16 and 10 of the beetles from BU, respectively (Table 4). T929I and T929N were not present in any of the beetles from the United States that were genotyped for the M918 region of *LdVssc1*. In order to account for these mutations, the M918 regions corresponding to the unique *LdVssc1* introns were included in the haplotype sequences. T929N was only found in samples containing the *kdr5* haplotype. T929I was present in samples that contained the *v17*, *v18*, *v21*, *kdr7*, and *kdr8* haplotypes. The wild-type T929 was present in only 3 individuals from BU, as part of the *kdr4* and potentially *v16* haplotypes. The presence of *v16* in BU has yet to be confirmed, since the *kdr4* and *kdr8* haplotypes have the same *LdVssc1* intron and differ only in the presence of T929I, as do the haplotypes *v16* and *v21*, so it is un-

Table 3
LdVssc1 haplotypes present in individual CPB from ten different locations.

	Haplotypes[a]									
Sample	BU	AZ	NE	WA	WI	NJ	NY	OH	ME	MI
1	*kdr4/v21* or *kdr8/v16*[b]	*v2*	*v1*	*v9*	*v12/v16*	*kdr1*	*kdr1*	*kdr3*	*kdr2/kdr3*	*kdr6*
2	*kdr7*	*v4/v5*	*v6/v7*	*v10*	*v16/v19*	*kdr1*	*kdr1*	*kdr3*	*kdr2*	*v20*
3	*kdr5*	*v3/v5*	*v1/v8*	*v11*	*v13*	*kdr1*	*kdr1*	*kdr3*	*kdr2*	*kdr3/kdr2*
4	*kdr7*	*v5*	*v6/v16*	*v15*		*kdr1*	*kdr1*	*kdr3*	*kdr2*	*kdr3*
5	*kdr5*	*v3/v5*	*v6*	*v10*		*kdr1*	*kdr1*	*kdr1*	*kdr2*	*kdr3*
6	*v17*	*v14*				*kdr1*	*kdr1*	*kdr3*		*kdr3*
7	*kdr5/v18*							*kdr3*		*kdr3*
8	*kdr4*							*kdr3*		*kdr3*
9	*v17*							*kdr3*		*kdr3*
10	*kdr5*							*kdr3*		*kdr3*
11	*kdr4/v21* or *kdr8/v16*[b]							*kdr3*		*kdr3*
12	*kdr7*							*kdr3*		*kdr3*
13	*kdr5*									*kdr3*
14	*kdr5*									*kdr3*
15	*kdr8*									
16	*kdr5/v18*									
17	*kdr5*									
18	*v21*									
19	*kdr7*									
20	*v17*									
21	*kdr7*									
22	*kdr5*									
23	*kdr5*									
24	*kdr7*									
25	*v17*									

BU, AZ, ME, MI, NE, OH, WA and WI represent Bulgaria, Arizona, Maine, Michigan, Nebraska, Ohio, Washington and Wisconsin, respectively.

[a] Samples with two haplotypes present are known female heterozygotes. Samples with one haplotype present may be males or homozygous females.

[b] The alleles *kdr4* and *kdr8* have the same *LdVssc1* intron and differ only in the presence of T929I, as do the haplotypes *v16* and *v21*. It is unconfirmed which *LdVssc1* intron corresponds to T929 and which to I929.

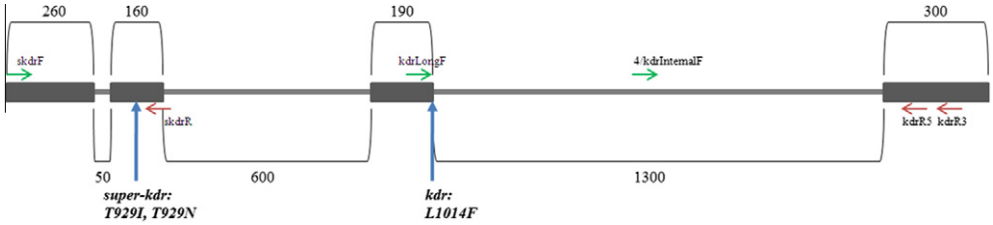

Fig. 1. Diagram of *LdVssc1* illustrating the gDNA section containing the T929I, T929N, and L1014F polymorphisms and associated introns. Locations of forward and reverse primers used for amplification are shown as arrows.

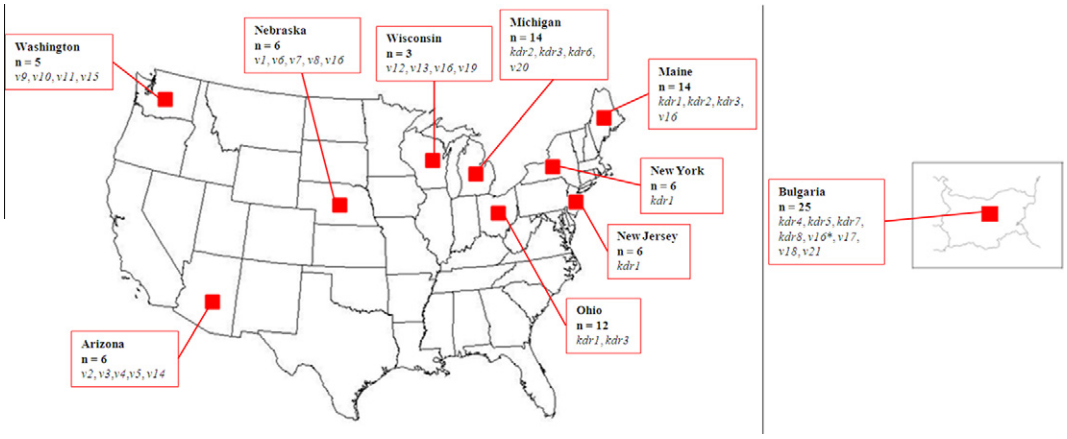

Fig. 2. *LdVssc1* haplotypes from the United States and Bulgaria.

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LdVssc1 cDNA  GGCCTTGAAGGTGTTCAAGGTTTATCTGTTTTGAGATCATTCCGACTG----------CTGAGAGTGTTCAAATTGGCCAA
CPB NJ1       GGCCTTGAAGGTGTTCAAGGTTTATCTGTTTTGAGATCATTCCGACTGGTGAGTTTCGTTTACCTAAATTTTCTTTGTATGCACATTTTTTATTGCAGCTGAGAGTGTTCAAATTGGCCAA
CPB OH1       GGCCTTGAAGGTGTTCAAGGTTTATCTGTTTTGAGATCATTCCGACTGGTGAGTTTCGTTTACCTGAATTTTCTTTGTATGCACATTTTTTATTGCAGCTGAGAGTGTTCAAATTGGCCAA
CPB Bulg2     GGCCTTGAAGGTGTTCAAGGTTTATCTGTTTTGAGATCATTCCGACTGGTGAGTTTCGTTTACCTGAATTTTCTTTGTATGCACATTTTTTATTGCAGCTGAGAGTGTTCAAATTGGCCAA
CPB Bulg3     GGCCTTGAAGGTGTTCAAGGTTTATCTGTTTTGAGATCATTCCGACTGGTGAGTTTCGTTTACCTGAATTTTCTTTGTATGCACATTTTTTATTGCAGCTGAGAGTGTTCAAATTGGCCAA
CPB Bulg8     GGCCTTGAAGGTGTTCAAGGTTTATCTGTTTTGAGATCATTCCGACTGGTGAGTTTCGTTTACCTGAATTTTCTTTGTATGCACATTTTTTATTGCAGCTGAGAGTGTTCAAATTGGCCAA

LdVssc1 cDNA  ATCGTGGCCGACTTTAAACTTACTCATTTCTATAATGGGTAGAACTATGGGTGCTCTAGGTAACCTGACCTTCGTGCTGTGCATTATTATATTTATATTTGCTGTTATGGGTATGCAGTT
CPB NJ1       ATCGTGGCCGACTTTAAACTTACTCATTTCTATAATGGGTAGAACTATGGGTGCTCTAGGTAACCTGACCTTCGTGCTGTGCATTATTATATTTATATTTGCTGTTATGGGTATGCAGTT
CPB OH1       ATCGTGGCCGACTTTAAACTTACTCATTTCTATAATGGGTAGAACTATGGGTGCTCTAGGTAACCTGACCTTCGTGCTGTGCATTATTATATTTATATTTGCTGTTATGGGTATGCAGTT
CPB Bulg2     ATCGTGGCCGACTTTAAACTTACTCATTTCTATAATGGGTAGAACTATGGGTGCTCTAGGTAACCTGATCTTCGTGCTGTGCATTATTATATTTATATTTGCTGTTATGGGTATGCAGTT
CPB Bulg3     ATCGTGGCCGACTTTAAACTTACTCATTTCTATAATGGGTAGAACTATGGGTGCTCTAGGTAACCTGAACTTCGTGCTGTGCATTATTATATTTATATTTGCTGTTATGGGTATGCAGTT
CPB Bulg8     ATCGTGGCCGACTTTAAACTTACTCATTTCTATAATGGGTAGAACTATGGGTGCTCTAGGTAACCTGACCTTCGTGCTGTGCATTATTATATTTATATTTGCTGTTATGGGTATGCAGTT
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Fig. 3. Alignment of representative *LdVssc1* sequences in the region where *super-kdr* is found. The A/G polymorphism in the intron region is shaded. The A variant was only found in individuals that possessed the *kdr1* haplotype. The M918 codon (ATG) and the T929, I929, and N929 codons (ACC, ATC, and AAC respectively) are underlined. While the *super-kdr* trait, M918T, was absent in all CPB samples, T929I and T929N were discovered in the Bulgaria population. The novel T929I and T929N mutations were not present in any samples from the United States.

Table 4
Additional polymorphisms in the *super-kdr* region of *LdVssc1*.

Polymorphism	Locations found	Number of individuals with the mutation	Associated haplotypes
M918T	None	None	n/a
T929I	BU	16	*kdr7*, *kdr8*, *v17*, *v18*, *v21*
T929N	BU	10	*kdr5*
T929	BU, AZ, ME, MI, NE, NJ, NY, OH, WA,WI	71	*kdr1*, *kdr2*, *kdr3*, *kdr4*, *kdr6*, *v1*, *v2*, *v3*, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, *v12*, *v13*, *v14*, *v15*, *v16*, *v19*, *v20*

clear which *LdVssc1* intron corresponds to T929 and which to I929 in the heterozygous samples.

3.3. Phylogeny of LdVssc1 haplotypes

Phylogenetic analysis for the *LdVssc1* haplotypes supports the hypothesis of multiple origins of *kdr* alleles (Fig. 4). The *kdr2*, *kdr3*, and *kdr7* haplotypes are within the same cluster (Group A, Fig. 4) with several of the susceptible haplotypes, indicating a shared ancestor that in all probability possessed a susceptible (L1014) allele. Similarly, the *kdr1*, *kdr5*, and *kdr6* haplotypes are within a separate cluster (Group B), as are the *kdr4* and *kdr8* haplotypes (Group C). Although the three groups are not strongly supported by the bootstrapping analysis, branches with higher bootstrap values indicate at least two origins. Within each cluster, there is also the possibility for additional separate origins of *kdr*, such as where *kdr5* diverges from *kdr1* and *kdr6*. However, the estimated genetic distances of nodes within the clusters are closer

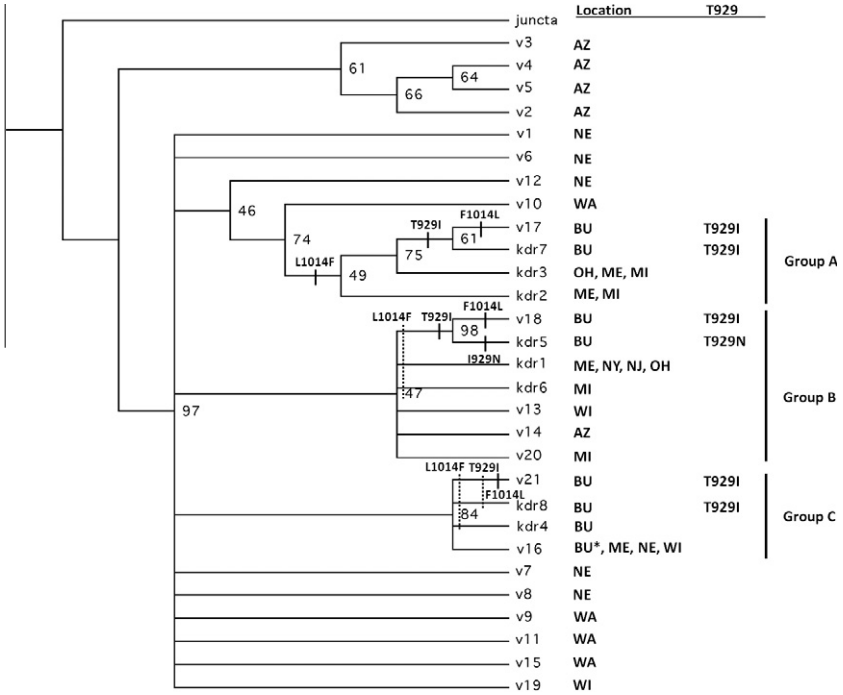

Fig. 4. Phylogenetic tree of *LdVssc1* haplotypes rooted with *L. juncta* as the outgroup showing the strict consensus of relationships among sequences, including gaps, as estimated via parsimony-based analysis. Numbers at nodes are bootstrap values. Vertical lines across branches indicate mutation events leading to specific alleles of *LdVssc1*. Mutation events marked by a solid line are highly supported, while those indicated by dashed lines indicate mutation events that are hypothetical (not yet identified).

than those of nodes between the clusters, so the notion of multiple origins in alleles separated by only one node cannot be supported with the same level of confidence. Thus, the distinct branching of the haplotypes within the phylogeny provides support for at least two, or at most three, independent origins of *kdr*.

4. Discussion

Analysis of the *LdVssc1* haplotypes reveals low allelic diversity amongst the resistant populations relative to the susceptible beetles. For locations within the United States that exhibited a high proportion of *kdr* resistant haplotypes, the majority of samples possessed the same *kdr* haplotypes within each state. For instance, in NJ and NY, all of the beetles had only the *kdr1* haplotype. Likewise, 11 of 12 individuals from OH had only the *kdr3* haplotype. MI and ME beetles had 4 different haplotypes, but three of those appeared only once in their respective state. Overall, 11 of 14 individuals from ME had only the *kdr2* haplotype, and 11 of 14 individuals from MI had only the *kdr3* haplotype. These results are not unexpected if pyrethroid insecticides have been used in these locations, since any resistant allele that had appeared in the population would have been selected for by insecticide use. Furthermore, the *kdr1*, *kdr2*, and *kdr3* haplotypes all appear in more than one population within the United States. The spread of these resistance haplotypes is probably indicative of an earlier, independent evolutionary origin and the mobility of CPB across the United States. The isolated appearances of *kdr4*, *kdr5*, *kdr7*, and *kdr8* in BU and *kdr6* in MI suggest a more recent development.

Compared to the resistant haplotypes, the susceptible alleles presented themselves in even more isolated groups among the populations. The susceptible haplotypes found in BU, AZ, ME, MI, NE, WA, and WI were all found exclusively in their respective locations, except for *v16*, which was present in NE, WI, ME, and potentially BU. This could suggest that these populations of CPB exist relatively isolated from one another so that allelic exchange is minimal or that the rate of accumulating polymorphisms is high enough for distinct haplotypes to continually emerge despite gene flow. Interestingly, despite limited sample sizes, a large number of susceptible haplotypes were identified in beetles from AZ, NE, WA, and WI. Since the haplotypes are derived from mostly intron regions of *LdVssc1*, this diversity is not unexpected.

A previous study that had sequenced *LdVssc1*, first from amplified cDNA templates prepared from pooled mRNA and then from individually amplified DNA from susceptible and resistant beetles, documented the absence of mutations at four sites other than L1014F, including M918T and T929I [21]. Therefore, the absence of *super-kdr* in 93 of the beetles in this study is consistent with previous findings, but the presence of T929I and T929N is a novel trait. L1014F + T929I had been found to confer a greater resistance to pyrethroid insecticides in *P. xylostella* than just L1014F alone, so it is possible that the same double mutation in CPB exhibits a *super-kdr-like* resistance phenotype as well.

The T929I mutation had been found in conjunction with another *Vssc* mutation in other species (T929I + L1014F in *P. xylostella* [32] and T929I + L932F in *P. capitis* [34]), which had suggested that T929I can only serve as a second site mutation, similar to M918T [33]. However, the presence of T929I in the absence of L1014F in 7 of the BU individuals introduces the possibility of T929I acting as a single mutation in conferring some level of pyrethroid resistance in CPB. In *Sitophilus zeamais*, the association of the T929I mutation and resistance has been demonstrated convincingly because weevils that survived the bioassay were homozygous for T929I, while those that died were homozygous for the wild-type allele [45]. The same T929I mutation caused high levels of resistance to both bifenthrin and DDT in *Trialeurodes vaporariorum* [46]. Heterologous expression of *para* sodium channels with the T929I mutation is sufficient to reduce the proportion of channels modified by deltamethrin, permethrin, fenfluthrin and DDT [47,48]. Therefore, the T929I mutation in CPB likely causes resistance to pyrethroids. Further genetically informative bioassays would need to be conducted in order to confirm this claim.

The changes in resistance levels at the channel level conferred by T929I and L1014F may not be the sole determinant of the origin and evolution of resistance in the field, as the fitness cost of these alleles in the absence of insecticides will also have a major influence. While channels with T929I + L1014F are extremely insensitive to deltamethrin and permethrin, these double mutant channels possess a voltage dependence of activation and inactivation that is similar to that of wild-type channels. The depolarizing shift of 3 and 5 mV in the voltage dependence of activation and inactivation, respectively, is seen in channels with L1014F. The T929I mutation causes a 5 mV depolarizing shift in the voltage dependence of inactivation [48]. Therefore, the combination of T929I + L1014F creates a channel with normal function that is insensitive to pyrethroids. As such, there may be a minimal electrophysiological fitness cost for individuals with T929I + L1014F. The major caveat to this hypothesis is that we have observed beetles with either the T929I or L1014F individual mutations in our collections. Therefore, physiological compensation by the double mutants to create channels with wild-type properties relative to each individual mutant may not be extremely important. However, fitness costs are environmentally variable [49], so while no significant changes in *Vssc* function were associated with T929I + L1014F in a heterologous expression system does not mean that they will not be observed in nature. Identification of conditions that impose a fitness cost of channels with T929I, L1014F, or a combination of both, will require further study.

T929N is a novel variant of T929I that has not been documented in other species. The only other known variant at this site is a threonine to valine substitution, T929V, found in *Ctenocephalides felis* and *B. tabaci* [50,51]. It is unclear whether T929N is associated with pyrethroid resistance and whether it confers a greater resistance when found with L1014F. The effects on resistance in the presence of T929I show that the amino acid site 929 plays a significant role in the interaction of insecticides with the sodium channel [47,48], so any amino acid change could possibly disrupt this interaction. In the case of T929I or T929V, an amino acid with a polar side chain is being substituted with an amino acid with a nonpolar side chain. With the T929N mutation a polar side chain is replaced with an amide side chain. The amide side chain of asparagine is inconsistent with the non-polar side chains of isoleucine and valine; therefore it is difficult to speculate on what effect the T929N substitution could have on resistance levels. Further electrophysiological studies would need to be conducted to determine whether T929N is associated with pyrethroid resistance.

Based on the information at hand, the absence of T929I and T929N in the United States samples suggests that these mutations originated in Europe, but have yet to evolve in North America. For instance, *kdr7*, identified in the BU population, and *kdr3*, found in the United States populations, share identical *LdVssc1* intron and flanking exon sequences, except that *kdr7* is associated with the T929I mutation whereas *kdr3* is not. This could explain why the mutations had not been found previously, since the Lee et al. (1999) study only genotyped lab strains that were originally collected in the United States. The occurrence of these new polymorphisms could be indicative of differences in selection pressures between the BU site and the sites in the United States. Sampling from additional European countries, as well as an updated collection in the United States will provide further evidence of the incidence of T929I and T929N in CPB.

It is important to note that the number of appearances of susceptible and resistant haplotypes within the populations in this study is insufficient to make generalizations about the distribution of haplotypes. The small sample sizes used in the study may not be representative of the larger populations, and further research using more beetles could reveal the presence of more novel haplotypes. For instance, many of the susceptible haplotypes could in fact be present in a number of locations, but were simply not present in the sample individuals that were genotyped. Furthermore, conclusions about the spread of pyrethroid resistance in CPB cannot be drawn from the results of this study alone. First of all, multiple factors outside of the mutations that were investigated in this study could also contribute to the development of pyrethroid resistance in a population. These include possible *LdVssc1* mutations that were outside of the region that was examined or other mechanisms of resistance such as increased cytochrome P450-mediated monooxygenase activity. Such factors could help to explain the difference in survivorship in the bioassay between the beetles from ME and those from MI despite having the same proportion of resistant alleles. Second, the collections of beetles were made at sites with different plants and insecticide use histories. For example, larvae taken off of native host plants would be expected to be less resistant to insecticides than those taken off of potato plants in agricultural fields, which would have been subjected to much higher selection pressure from insecticide use in the past. The relative proximity of potato fields to other CPB hosts could also influence the relative abundance of susceptible alleles that are found. These factors could account for the susceptibility observed in samples from the Midwest and western United States. For the purposes of identifying haplotypes to understand the evolutionary history of *kdr*, sampling from a range of populations varying in resistance levels was useful in order to incorporate both resistant and susceptible haplotypes into the phylogenetic analysis.

The distinct grouping of *kdr* haplotypes in the phylogenetic tree structure is consistent with multiple origins of *kdr* in CPB. It is most likely that each of the putative ancestral genes initially possessed the susceptible allele (L1014) and later experienced a single nucleotide substitution (CTT → TTT) to give rise to *kdr*. Following the introduction of the *kdr* mutation, further polymorphisms may have occurred within the intron to differentiate between the *kdr* haplotypes of each cluster. The occurrences of the T929N and T929I mutations also appear to be independent, since the haplotypes with the polymorphisms fall out into separate groups. For example, *v21* and *kdr8* (both T929I) are within one cluster whereas *v17* and *kdr7* (both T929I) are in another. T929N was only found in *kdr5*, suggesting that the single nucleotide substitution (ACC → AAC) for T929N occurred after that of *kdr*. However, T929I appears in both susceptible (L1014) and resistant haplotypes (F1014), so it is difficult to assess whether it was introduced before or after the introduction of *kdr*. Because *kdr7* and *kdr8* from the BU population share identical introns with that of *kdr3* and *kdr4* from the United States, respectively, it is likely that *kdr* was first introduced in the lineage in the United States while T929I was introduced later, after the migration of CPB from North America to Europe. However, the susceptible haplotypes containing T929I were only identified in the BU population as well, suggesting that either the mutation arose separately from an ancestral susceptible allele in BU or the mutation arose from the *kdr* alleles followed by a reversion of *kdr*. Since the population had been exposed to selection pressure from insecticide use in the recent years which would favor the propagation of resistance alleles, the former scenario is more probable. However, it is possible for the T929I mutation to confer pyrethroid resistance and allow reversion to L1014. In Group A (*kdr2*, *kdr3*, *kdr7* and *v17*; Fig. 4), the L1014F mutation is the ancestral character state. The appearance of T929I is then common to *kdr7* and *v17*, separating those haplotypes from *kdr2* and *kdr3*. The *v17* haplotype thus serves as an example in which the T929I mutation may have conferred enough resistance to allow 1014 to revert to L. The pattern of reversion to L1014 following the appearance of T929I is seen in Groups B and C (Fig. 4). In Group B, the L1014F mutation appeared in the *kdr1*, *kdr5*, and *kdr6* haplotypes near the base of the radiation. Shortly thereafter, the T929I mutation appears in a hypothetical haplotype that is ancestral to both *kdr5* and

v18. From this ancestral haplotype, T929I is mutated to T929N in *kdr5*, while the appearance of T929I allows the reversion of L1014F in *v18*. However, the low bootstrap value at the radiation of Group B muddles this hypothetical reconstruction, and it is just as likely that there were two independent origins of the 929 mutations (e.g. T929N in *kdr5* and T929I in *v18*).

Similarly in Group C, L1014F appears near the radiation of the group. T929I is common to *kdr8* and *v21*, which allows the reversion to L1014 in *v21*. An alternative scenario is that T929I arose at the base of the radiation of Group C that included *v21*, *kdr8*, and *kdr4*. The L1014F mutation arose in a haplotype that was common to both *kdr8* and *kdr4* where the T929I mutation was lost to give rise to the observed *kdr4* haplotype. Both of these scenarios are plausible and are supported by the high boot strap values at the radiation of Group C. However, since both Group B and C are radiations rather than the step-wise pattern of Group A, identification of additional alleles through more extensive sampling in both Groups B and C is needed to provide a more definite picture of the origin and evolution of mutations at T929.

The idea of multiple origins of *kdr* in CPB is consistent with the findings of previous studies that examined the evolutionary origins of pyrethroid resistance in other insects. Among the 8 *kdr* haplotypes identified, there exist 12 sites of single nucleotide variability and 3 sites of insertions/deletions. With the strong selection for resistance-conferring mutations in *LdVssc1* since the introduction of DDT and the quickly expanding geographic distribution of the beetle, it is unlikely that all of these changes in the haplotypes arose as independent mutations from a single resistant ancestor. Future studies with additional collections from the United States and Europe coupled to data on insecticide use at the sites could expand on these findings and provide information on the spread of the resistance alleles. Further work in genotyping known males and females in the populations could help determine allele frequencies as well. It is also of interest to define the level of resistance the T929I and T929N polymorphisms confer on their own and as second site mutations and to investigate their roles in pyrethroid resistance in CPB.

Acknowledgments

We thank Cheryl Leichter for technical assistance, and Dr. Jennifer Thaler and the New Jersey Department of Agriculture for supplying beetles. CS was supported by the Howard Hughes Medical Institute scholarship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pestbp.2012.08.001.

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