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Frequencies of the pyrethroid resistance alleles of *Vssc1* and *CYP6D1* in house flies from the eastern United States

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Abstract

House flies were collected from four dairies in Maine, New York, North Carolina, and Florida, where high levels of resistance to permethrin have been documented. Regions of two genes, *CYP6D1* and *Vssc1*, having alleles that confer resistance to permethrin (and other pyrethroids) were analysed from individuals at each collection site. The combinations of resistance alleles for *Vssc1* and *CYP6D1* were highly variable between each state. The resistance allele *CYP6D1v1* was found at a high frequency (0.63–0.91) at all sites. Individuals homozygous susceptible for *CYP6D1* were very rare and detected only at the dairy in Maine. In addition to the typical *Vssc1* mutation responsible for resistance, *kdr* (L1014F), we also identified individuals with a L1014H mutation. Although house flies homozygous for the L1014H mutation had a lower level of resistance to permethrin, compared to L1014F, the H1014 resistance allele was frequently detected. No individuals with the *super-kdr* allele (M918T + L1014F) were detected from the field collections. The intron 3 bp downstream of the *kdr* mutation was found to be extremely variable, providing an opportunity to reconstruct a phylogeny of *Vssc1* alleles. Based on this analysis it appears the *kdr-his* mutation had multiple evolutionary origins, but that the *kdr* mutation may have had a single origin. The impacts of these findings on resistance management are discussed.

Keywords: insecticide resistance, population genetics, *Musca domestica*, *Vssc1*, *CYP6D1*, *kdr*.

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Introduction

House flies (*Musca domestica*) are capable of transmitting a wide variety of human and veterinary diseases. Thus, numerous compounds have been used to control them. Pyrethroids are currently the most widely used insecticides for house fly control, due to their effectiveness, low mammalian toxicity, and environmental safety. House flies can become resistant to pyrethroids by two major mechanisms: cytochrome P450-mediated detoxification and/or target site insensitivity (*kdr*). P450-mediated detoxification was the major resistance mechanism in the Learn Pyrethroid Resistant (LPR) strain of house flies originally collected from a dairy in New York in 1980. The LPR strain is highly resistant to unsubstituted phenoxybenzyl pyrethroids (Scott & Georghiou, 1985). Subsequent biochemical studies indicated that a single P450 isoform was responsible for pyrethroid detoxification (Wheelock & Scott, 1992b). This P450 was identified as *CYP6D1* (Tomita & Scott, 1995) and overexpression of this gene is correlated with pyrethroid resistance (Liu & Scott, 1998). Strains that overexpress *CYP6D1* possess a characteristic 15 bp insert in the 5′ flanking region that defines the *CYP6D1v1* allele. Resistant strains from New York and Georgia both overexpress *CYP6D1* (Kasai & Scott, 2000) and possess the characteristic *CYPD1v1* insert (Seifert & Scott, 2002). These results indicate that *CYP6D1v1 has* swept through house fly populations in the eastern United States although the frequency of *CYP6D1v1* in field populations remains unknown.

Use of pyrethroids (or DDT) can lead to the evolution of target site insensitivity due to a L1014F mutation (termed *kdr*) in the voltage sensitive sodium channel gene (*Vssc1*) on autosome 3 in house flies (Milani & Travaglino, 1957). Electrophysiological evidence demonstrated that *kdr* resistant strains of house fly had different sodium channel kinetics than were seen in a susceptible strain (Miller *et al*., 1979; Osborne & Hart, 1979; Salgado *et al*., 1983). House fly *Vssc1* is homologous to *Drosophila para* (Loughney *et al*., 1989) and *Vssc1* mapped to the same locus as pyrethroid and DDT resistance (Williamson *et al*., 1993). Cloning of the full-length *Vssc1* cDNA identified the L1014F mutation responsible for *kdr* resistance and the M918T + L1014F mutations that define *super-kdr* resistance (Williamson *et al*., 1996b). Electrophysiological studies of these sodium channels expressed in *Xenopus laevis* oocytes verified that

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the L1014F and M918T + I1014F mutations reduced the sensitivity of sodium channels to pyrethroids (Smith *et al*., 1997; Lee *et al*., 1999). Studies suggest that *kdr* is a major resistance mechanism in field strains of house flies in Denmark (Huang *et al*., 2004).

The *kdr* allele has been identified in house fly populations from numerous locations world wide. It is unclear if this is due to multiple evolutionary origins of the L1014F (*kdr*) mutation, or a reflection of the relatively high mobility of this pest. The observation that the M918T mutation has never been documented in flies that do not also have the L1014F mutation (Soderlund & Knipple, 2003) suggests that *super-kdr* arose from a *kdr* individual (i.e. sequential accumulation of mutations). However, a direct test for a single evolutionary origin of *kdr* in house flies has not been reported. An additional complication is the discovery of a L1014H mutation in house flies in Alabama (Liu & Pridgeon, 2002). The relative importance of this new mutation in house fly populations remains a mystery.

In the summer of 2002, house flies were collected from Androscoggin County, Maine; Schuyler County, New York; Wake County, North Carolina; and Alachua County, Florida. Flies from all four dairies showed high levels of resistance to permethrin, but lower levels of resistance to cyfluthrin and pyrethrins + piperonyl butoxide (PBO) (Hamm *et al*., 2005). Herein, we report on the frequency of resistance alleles of the two major pyrethroid resistance genes, *CYP6D1* and *Vssc1*, in the populations collected from each of these four locations. The issue of single vs. multiple evolutionary origins of *Vssc1* mutations in house flies is also discussed.

Results and Discussion

Bioassay

The levels of resistance to permethrin + PBO in *kdr* (F1014) and *kdr-his1* (H1014) house flies are shown in Table 1. Treating house flies with permethrin + PBO eliminated the contribution of *CYP6D1v1*-mediated detoxification to permethrin resistance. Therefore, we were able to study levels of resistance specifically afforded by *kdr* or *kdr-his1*. The level of resistance due to *kdr* (NC11C) was similar to that in previous reports (Farnham, 1977). Interestingly, *kdr-his1* flies were only 16-fold resistant to permethrin + PBO compared to 44-fold resistance in *kdr* flies. The *kdr*/*kdr-his1* heterozygote has an intermediate level of resistance between the two homozygotes (Table 1). The 16-fold resistance ratio of *kdr-his1* was identical to a previous report in the SeALHF strain, which also contained *kdr-his* alleles (Liu & Pridgeon, 2002).

We also attempted to isolate a strain homozygous for *kdr-his2*, a second allele containing the H1014 mutation. Three attempts resulted in strains that could not be propagated after the F_2, due to low rates of egg laying and hatching. These abnormalities were not seen with NC19His, so it is likely this is a fitness cost directly attributable to *kdr-his2*, but not to the H1014 mutation itself. Although we were unable to obtain a line homozygous for *kdr-his2* for bioassay and full-length cDNA cloning, it is likely this allele also confers resistance based on the results with the *kdr-his1* homozygous line (above) and due to electrophysiological evidence that indicates histidine at position 1014 produces pyrethroid resistant sodium channels (Zhao *et al*., 2000).

Allele frequencies of kdr, kdr-his1, super-kdr *and* CYP6D1v1

Initial studies used the PCR-RFLP method to determine *Vssc1* allele frequencies based on digestion with *Tsp* 509I, which has a recognition sequence of AATT. This can distinguish *kdr* (AAT*Ttt*, the 1014 codon is in italics) from susceptible (AAT*Ctt*) (Fig. 1A). Preliminary studies using the PCR-RFLP assay scored a high proportion of the field collected flies as susceptible, but this did not agree with bioassay data that reported resistance to cyfluthrin or pyrethrins + PBO (Hamm *et al*., 2005) (indicative of *kdr*-type resistance). These flies apparently carry the *kdr-his1* or *kdr-his2* alleles (AAT*Cat*), which lack a *Tsp* 509I cut site, explaining why we were not accurately detecting resistant genotypes with PCR-RFLP. Therefore, all *Vssc1* alleles reported herein were identified by direct sequencing.

The *kdr* and *kdr-his1* alleles were found at high frequencies in each population sampled, but the relative frequencies of the two alleles differed between the dairies in the four states ($P < 0.001$; Table 2). Together, *kdr* and *kdr-his1* composed more than 95% of all alleles found at each location. Given the lower level of resistance conferred by the *kdr-his1* allele, relative to *kdr*, the high frequency of *kdr-his1* in flies from the New York and North Carolina dairies was unexpected. Perhaps the *kdr-his1* mutation is locally

Table 1. Resistance to permethrin + PBO via topical application in house fly strains having *kdr* (NC11C), *kdr-his1* (NC19His) and their F1

Strain	*n*	LD_{50}[a] (95% CI)	Slope (SE)	RR[b]
CS	400	1.75 (1.49–2.06)	6.6 (1.4)	–
NC19His	600	28.3 (26.0–30.7)	5.0 (0.4)	16
NC11C	1020	77.2 (70.4–84.5)	2.7 (0.2)	44
F1 (NC19 × NC11C)	740	40.3 (33.5–47.5)	2.2 (0.2)	23

[a]LD_{50} in units of ng/fly.
[b]RR = LD_{50} resistant strain/LD_{50} susceptible strain.

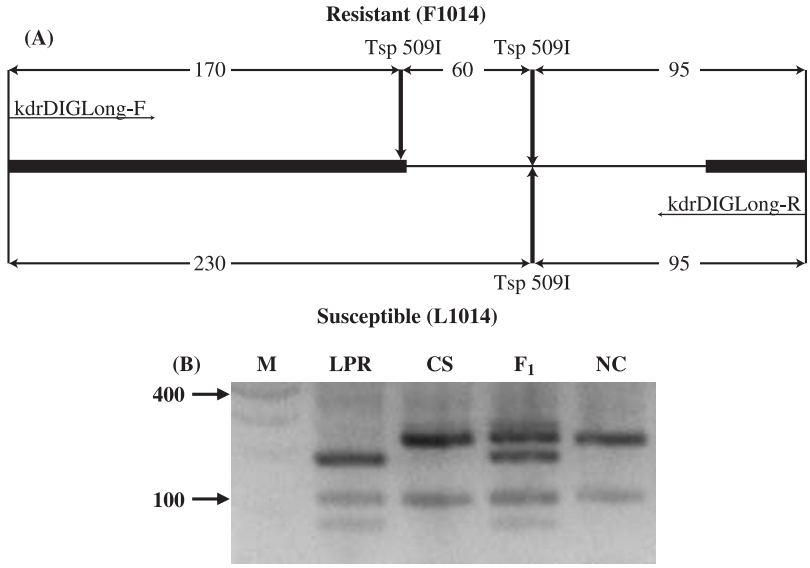

Figure 1. (A) Schematic diagram of *Tsp* 509 I restriction sites in *Vssc1* PCR products from individual 'resistant' and 'susceptible' strains of house fly. The 170 bp fragment in resistant strains is due to the *kdr* mutation (C → T) that yields the AATT restriction site of *Tsp* 509 I. The second site where both resistant and susceptible strains are cleaved lies within the intron sequence. (B) Restriction digest of amplified *Vssc1* fragments of individual flies from four strains of house flies. The bands in the LPR lane (RR) correspond to 60, 95, and 170 bp. The CS lane (SS) bands are of 95 and 240 bp. The F_1 lane bands are a combination of the LPR and CS lanes and indicate a heterozygous condition (SR) with 4 bands at 60, 95, 170, and 240 bp. The NC lane is from a field collected house fly from Wake County North Carolina having the L1014H mutation. The marker lane is Hyperladder 4 from Bio-Line.

Table 2. *Vssc1* and *CYP6D1* allele frequencies in individual house flies from four dairies in the eastern United States

		Vssc1[a]			*CYP6D1*	
Location[b]	*n*	Susceptible	*kdr*	*kdr*-his	Susceptible	Resistant
ME	49	0.05	0.84	0.11	0.20	0.80
NY	54	0.03	0.36	0.61	0.14	0.86
NC	50	0.03	0.18	0.79[c]	0.09	0.91
FL	63	0.06	0.46	0.48	0.37	0.63

[a] *Vssc1* susceptible = L1014, *Vssc1 kdr* = F1014 and *Vssc1 kdr-his* = H1014.
[b] ME = Androscoggin County Maine, NY = Schuyler County New York, NC = Wake County North Carolina and FL = Alachua County Florida.
[c] Includes both *kdr-his1* (0.75) and *kdr-his2* alleles (0.04). Only the *kdr-his1* allele was found at the other three dairies.

The null hypothesis (that allele frequency was the same at all locations) was tested using a 4×3 (*Vssc1*) or 4×2 (*CYP6D1*) contingency table. The null hypothesis was rejected in both cases with a $P < 0.001$.

adapted to environmental or insecticide pressures found at each location. House fly populations can be large at dairies, and there is evidence of gene flow between distant populations (Seifert & Scott, 2002), so it is unlikely that drift alone would cause the high frequency of *kdr-his1* seen in these local populations. The *kdr-his2* allele was only found in North Carolina and only at a low frequency (0.04). There was no occurrence of *super-kdr*, which remains restricted to Europe and Asia. Susceptible *Vssc1* alleles were at frequencies less than 6% at all locations (Table 2). This likely reflects the incompletely recessive nature of *kdr* (Milani & Travaglino, 1957; Shono, 1985), which offers RS heterozygotes limited protection.

There were no unusual patterns detected using the PCR-RFLP method for *CYP6D1v1* genotyping (Fig. 2). Sequencing of *CYP6D1* products confirmed the PCR-RFLP method was reliable. The frequency of *CYP6D1v1* ranged from 63 to 91% in the four populations (Table 2). This result indicates that *CYP6D1v1*-mediated detoxification is an important resistance factor across the eastern United States. The widespread prevalence of *CYP6D1v1* is

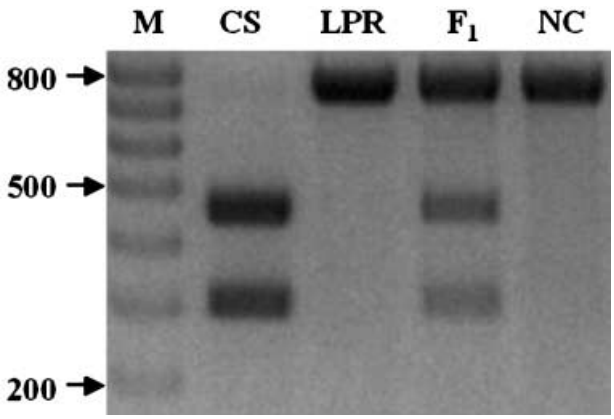

Figure 2. Restriction digest of amplified *CYP6D1* fragments of individual flies from various strains of house flies. The bands in the CS lane (SS) correspond to 279 and 432 bp. The LPR lane (RR) band is 732 bp. The F_1 lane bands are a combination of the LPR and CS lanes and indicate a heterozygous condition (RS) with 3 bands at 279, 432, and 732 bp. The NC lane is from a field collected house fly from North Carolina (RR). The marker lane is Hyperladder 4 from Bio-Line.

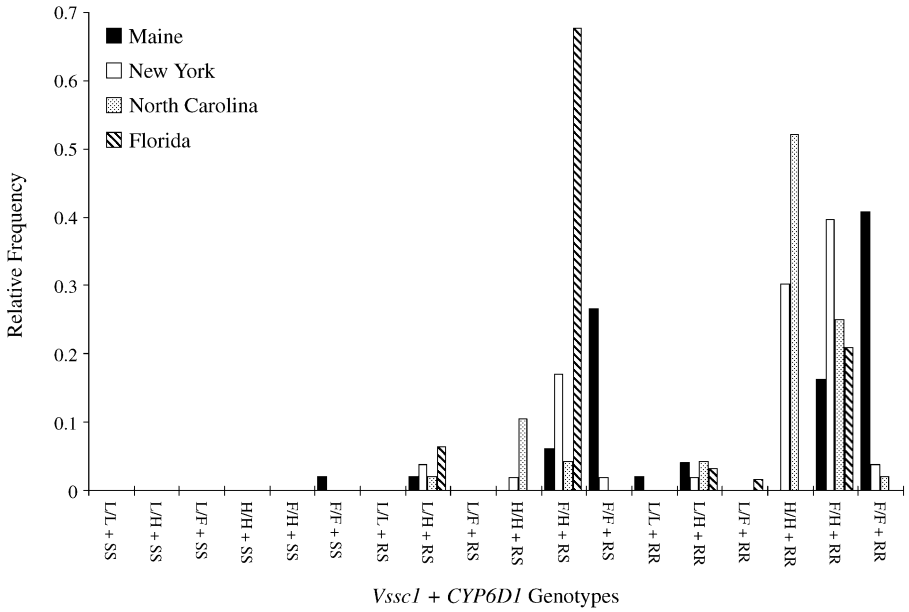

Figure 3. Frequency of *Vssc1* (homozygous or heterozygous for L, F, or H) and *CYP6D1* (resistant(R) or susceptable (s)) genotypes of individual house flies from the eastern United States. The number of individual flies genotyped is given in Table 2.

consistent with previous results suggesting *CYP6D1v1*-mediated resistance is similar between New York and Georgia (Kasai & Scott, 2000). However, there were higher levels of susceptible alleles for *CYP6D1v1* at each location compared to *kdr* and *kdr-his1*. The intermediate pattern of inheritance of *CYP6D1v1*-mediated resistance may allow susceptible alleles to be maintained in the population because the heterozygotes are resistant (Liu & Scott, 1997).

Figure 3 shows the frequency of *Vssc1* and *CYP6D1v1* genotypes from each of the four locations. If *kdr* or *kdr-his1* alone were sufficient to cause resistance under field conditions, we would expect to see equal distribution of individuals in each *kdr* or *kdr-his1* genotype independent of *CYP6D1* genotype. However, the paucity of individuals possessing *kdr* or *kdr-his1*, but lacking *CYP6D1v1* suggests that *kdr* or *kdr-his1* alone does not provide adequate resistance in the field. Similarly, if *CYP6D1v1* was the most important resistance factor under field conditions, we would expect the frequency of RR and RS individuals to be distributed independent of *Vssc1* genotype. However, most individuals also possess some combination of *kdr* or *kdr-his1*. This suggests that a combination of *kdr* or *kdr-his1* and *CYP6D1v1* is required for survival in pyrethroid treated environments. The combinations of *CYP6D1v1* and *kdr* or *kdr-his1* genotypes are representative of more than 92% of all individuals genotyped from all four locations. While this is the trend across all four locations, each site has unique combinations of each genotype. For example, Maine has more than 70% of the entire population represented by homozygous *kdr* with more than half of the individuals being homozygous for *CYP6D1v1*as well. The Florida population is mostly composed of heterozygotes for *kdr/kdr-his1* and heterozygotes for *CYP6D1v1*. The high frequency of *kdr* in Maine and the lack of *kdr* or *kdr-his1* homozygotes in Florida causes these populations to be out of Hardy–Weinberg Equilibrium (HWE) (Maine, $P = 0.042$; Florida $P = 5 \times 10^{-15}$). New York and North Carolina are represented by *kdr-his1* homozygotes or *kdr/kdr-his1* heterozygotes and *CYP6D1v1* homozygotes. Populations in New York and North Carolina are in HWE ($P >> 0.05$). The lack of uniformity in resistance genotypes indicates that these combinations may be adapted to local environmental conditions, insecticide application rates, and/or exposure methods. A high frequency of *kdr* has also been documented in house flies in Denmark (Huang *et al.*, 2004).

The observation of variable responses to similar insecticide selection pressure among populations across broad geographical areas is an interesting example of the evolutionary process. While multiple mutations can give rise to resistance, each population uses different combinations of resistance alleles to best suit their environment. For example, the H1014 mutation confers lower levels of resistance than the F1014 (*kdr*) mutation. However, in New York and North Carolina, the H1014 allele is the most common. Clearly the level of protection conferred is not the only factor determining the frequency of resistance alleles. One likely possibility is that the H1014 mutation may have a lower fitness cost (in the absence of insecticide use), at least in New York and North Carolina. This is an example of how intense selection pressure may cause populations to become geographically distinct, and may contribute to differences in populations of house flies that may drive the evolutionary

process. Given the high frequency of these alleles, it is likely that the problem of permethrin resistance along the eastern United States will not be easily solved. Other pyrethroids such as cyfluthrin, or synergism with PBO, will likely be alternatives to application of permethrin alone. It has been reported that resistance to cyfluthrin or pyrethrins + PBO is much lower than permethrin in house flies from Maine, New York, North Carolina and Florida, so these compounds may have immediate utility (Hamm *et al*., 2005). In addition to cyfluthrin, pyrethroids such as fenfluthrin and flumethrin may have increased commercial potential for house fly control because their substituents on the phenoxybenzyl group limit metabolism by *CYP6D1*, and thus prevent expression of this resistance mechanism (Scott & Georghiou, 1986). The heterogeneity of the combination of resistance alleles in populations from the eastern United States indicates that resistance management should be customized based on the frequency of major resistance alleles found at each location.

Full-length cDNA of kdr-his1

The full-length cDNA of *kdr-his1* (GenBank accession AY834743) showed 99.6% and 99.3% sequence similarity to the sodium channels from the Cooper (GenBank accession X96668) and NAIDM strains (GenBank accession U38813), respectively. Mutations unique to *kdr-his1* relative to Cooper and NAIDM sodium channels are K426R, Q465R, L1014H, D1170E, D1171I, and T1453S. The deletions of G2022-2024 and A2039, as well as the S2045T mutation are similar to those previously reported from kdr_{579} and *super-kdr*$_{530}$ in comparison to the susceptible Cooper strain (Williamson *et al*., 1996a). Another mutation at L1140M was identical to the sequence from the susceptible NAIDM strain, therefore it is unlikely to be involved in resistance. Many of these mutations are located on intracellular or extracellular domains of the sodium channel protein and are likely not involved in resistance (Soderlund & Knipple, 2003). However, the K426R mutation occurs in a position close to E435K in *Blattella germanica*, which acts as a modifier to enhance the insensitivity of L1014F (Tan *et al*., 2002). While there is uncertainty as to which mutations cause resistance, electrophysiological evidence suggests the L1014H mutation results in less sensitive sodium channels (Zhao *et al*., 2000).

Phylogeny of Vssc1 *alleles*

Sequencing of *Vssc1* products showed that the adjacent intron, three bases from the *kdr* mutation, is extremely variable (Fig. 4). There were 16 alleles identified based on sequences of this intron. Previously described susceptible alleles (Cooper and NAIDM) were given priority in designating alleles and subsequent alleles were designated by their resistance genotypes (*kdr*, *kdr-his1*, *kdr-his2*) or the order in which they were found. The 1014 codon is only 3 bp upstream of this intron, making recombination between them extremely unlikely and meaning this intron can be used to infer the mutational history of the 1014 codon. Three unique alleles were found in both the CS and Beltsville strains and nearly every strain possessed 2 or more alleles (Table 3). This diversity allowed for a phylogenetic analysis of the alleles (Fig. 5). All individuals with the F1014 or H1014 mutations (*kdr*, *kdr-his1* and *kdr-his2*) were also

Table 3. *Vssc1* alleles from various strains of house flies

Strain	Alleles	Collection	Reference
LPR	*kdr1*	NY, 1980	(Scott & Georghiou, 1985)
YPER	*super-kdr*	Japan, 1997	(Shono *et al*., 2002)
NG98	*kdr1, kdr2*	Georgia, 1998	(Kasai & Scott, 2000)
ALHF	*kdr1, kdr2*	AL, 1998	(Liu & Yue, 2001)
A2bb	*super-kdr*	Denmark, 1982	M. Kristensen (pers. comm.)
NC19	*kdr-his1*	NC, 2002	This paper
Cooper	*v1*	England (?)	I. Denholm (pers. comm.)
NAIDM	*v2*	CA (?)	(Scott & Georghiou, 1985)
aabys	*v3, v4*		Multiple sites
CS	*v2, v5, v6, v7*	TX, NY, MD	(Hamm *et al*., 2005)
Beltsville	*v8, v9, v10*	MD, 1968, &, 1969	(Pickens *et al*., 1972)
SRS	*v11*	Italy, 1961	(Keiding, 1999)
OCR	*v2, v12, v13*	Unknown	
Cornell-R	*v7, v9*	MI, ~1970	(Tripathi & O'Brien, 1973)
579_{kdr}	*kdr2*	Europe	(Williamson *et al*., 1996a)
ME	*kdr1, kdr2, kdr-his1, v10, v13*	ME 2002	(Hamm *et al*., 2005)
NY	*kdr1, kdr2, kdr-his1, v6,v 7, v8, v11, v13*	NY, 2002	(Hamm *et al*., 2005)
NC	*kdr1, kdr2, kdr-his1, kdr-his2, v13*	NC, 2002	(Hamm *et al*., 2005)
FL	*kdr1, kdr2, kdr-his1, v5, v6, v7, v12, v13*	FL, 2002	(Hamm *et al*., 2005)

ME = Androscoggin County Maine, NY = Schuyler County New York, NC = Wake County North Carolina and FL = Alachua County Florida. Accession numbers for alleles v1–13 are X96668, U38813, AY850270, AY850271, AY850268, AY851288, AY850264, AY850265, AY850266, AY850267, AY850269, AY850272 and AY850273, respectively. Accession numbers for *kdr1*, *kdr2* and *kdr-his2* are AY850260, AY850261 and AY850263, respectively. Accession numbers for *kdr-his1* are AY834743 (cDNA) and AY850262 (intron 18).

Figure 4. Sequence alignment of house fly *Vssc1* alleles. The introns are shaded and bases differing from the majority are boxed. Primer sequences are the first 30 and last 33 nucleotides.

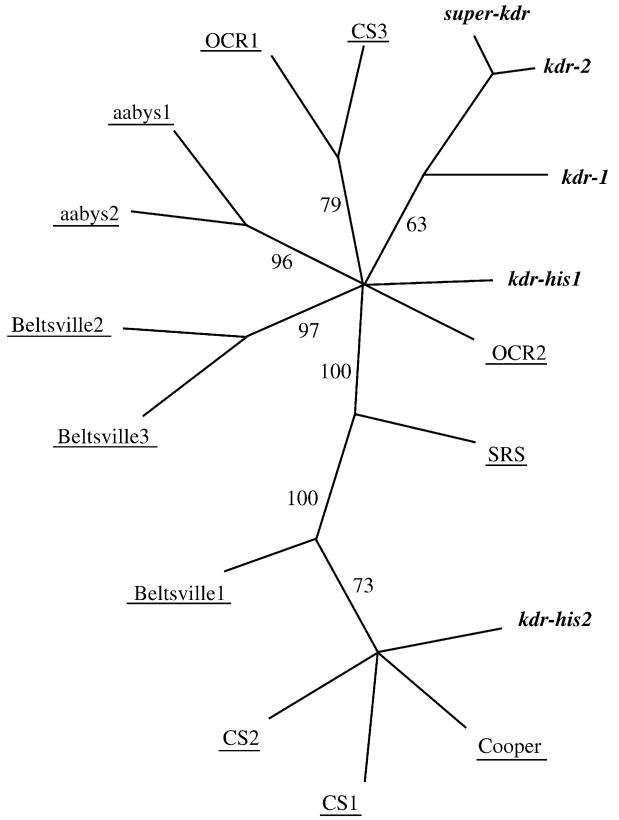

Figure 5. Unrooted phylogeny of *Vssc1* alleles from various strains of house flies. Shown is the strict consensus of the eight most parsimonious trees. Numbers above branches are bootstrap values (only those > 50% are shown). L1014 alleles are underlined. The *kdr* and *kdr-his* mutations are F1014 and H1014, respectively. Although the *super-kdr* and *kdr2* alleles had identical intron sequences, the former had both the M918T and L1014F mutations, while the later had only the L1014F mutation. The *kdr-his1* and *kdr-his2* alleles both had the H1014 mutation, but differed in their intron sequences (see Figure 4).

sequenced to determine if they had the *super-kdr* mutation. No *super-kdr* allele (i.e. M918T mutation) was found in any field collected house flies. The YPER and A2bb strains had M918T + I1014F mutations (i.e. *super-kdr*) and their intron sequences were identical to flies we designated as *kdr-2* (that had only the L1014F mutation).

The phylogenetic analysis for the *Vssc1* alleles (Fig. 5) supports the idea of multiple origins of the *kdr-his* alleles, but suggests a single origin of *kdr*. The large cluster at the top of Fig. 5 indicates that several susceptible strains, *kdr-1*, *kdr-2* and *kdr-his1* share an ancestor that in all probability possessed a susceptible (L1014) allele. This appears entirely logical, as the simplest interpretation of the data argues that the putative ancestral (L1014) gene experienced a single nucleotide substitution in one lineage (CTT → TTT) resulting in *kdr* (L1014F), and a substitution in another lineage (CTT → CAT) resulting in *kdr-his1* (L1014H). Furthermore, the exclusion of the *kdr-his2* allele from the large group containing the other resistant strains indicates an independent origin of this allele. In contrast to *kdr* in house flies, multiple evolutionary origins of *kdr* have been postulated in *Myzus persicae* (Anstead *et al*., 2005).

The use of DDT, starting in the 1940s, likely provided the initial selection for *kdr*. The use of pyrethroids, starting in the 1980s, has selected *kdr* to a high frequency. However, unlike Europe and Asia, *super-kdr* has not been detected in house flies from the USA. In the USA, *CYP6D1v1*-mediated resistance evolved in house flies (Scott & Georghiou, 1985), likely reducing the need for *super-kdr*. Increases in the amount and types of pyrethroids used over the last 20 years appears to have selected for *kdr-his*.

Intron sequences were also used to discern *Rdl* evolution in *Tribolium castaneum* (Andreev *et al*., 1999) where multiple origins of cyclodiene resistance have been proposed. As well as documenting the single vs. multiple origins of resistance, intron variability may also provide valuable information on the spread of these resistance alleles. We identified 16 alleles for this intron from house flies, and there appears to be a wide range of polymorphism within this intron in *Culex pipiens* (Martinez-Torres *et al*., 1999) and *Pediculus capitis* (Lee *et al*., 2000), suggesting it could be useful in performing analyses similar to those reported here in these medically important insects. In *Anopheles gambiae*, this intron is highly conserved within each subspecies, but differs between them (Weill *et al*., 2000). However, analysis of the intron upstream of the *kdr* mutation was used to suggest that *kdr* was introduced to the Mopti form of *A. gambiae* through introgression (Weill *et al*., 2000). It does not appear that this intron is highly variable in all species however, as identical intron sequences were found in *kdr* resistant (Ectiban-R) and susceptible (CSMA) strains of German cockroach (sequences not shown).

The combinations of resistance alleles for *CYP6D1* and *Vssc1* were highly variable between each dairy. Susceptible alleles were found at a frequency of less than 0.4 and 0.1 for *CYP6D1* and *Vssc1*, respectively. Both F1014 and H1014 mutations were observed in field collected populations, although individuals homozygous for the F1014 mutation had higher levels of resistance than individuals homozygous for the H1014 allele. Phylogenetic analysis of *Vssc1* alleles suggests multiple evolutionary origins for the *kdr-his* alleles, but not for *kdr* alleles. All of these collections were made in the middle of a spray season. In the future it will be of interest to determine the frequency of these alleles over the course of a spraying season to evaluate their relative fitness in the presence and absence of insecticide use.

Experimental procedures

House fly and cockroach strains

Thirteen laboratory strains of house fly were used. Cornell Susceptible (CS) is an insecticide-susceptible strain reared without exposure to insecticides(Hamm *et al*., 2005), SRS is an insecticide-susceptible strain (Keiding, 1999) obtained from S. Kasai (Nat.

Institute Infectious Diseases, Tokyo) in 2002, Beltsville is an insecticide-susceptible strain obtained from A. B. Broce (Kansas State University) and aabys is a susceptible strain with morphological markers on each autosome. LPR is a multiresistant strain having high levels of resistance to pyrethroid insecticides due to increased oxidative metabolism mediated by cytochrome P450 CYP6D1 (Scott & Georghiou, 1986; Wheelock & Scott, 1992a; Scott, 2001). Two other mechanisms of resistance to pyrethroid insecticides in the LPR strain are insensitivity of the nervous system (*kdr*) and decreased cuticular penetration (*pen*) (Scott & Georghiou, 1986; Shono *et al*., 2002). NG98 is a multiresistant strain (originally collected in Georgia in 1998) with 3660-fold resistance to permethrin due to monooxygenase-mediated detoxification and *kdr* (Kasai & Scott, 2000). NG98U is similar to NG98, except that the former was not selected in the laboratory (Kasai & Scott, 2000). YPER was collected in Japan and is a multiple resistant strain with more than 18,000-fold resistance to permethrin due to *super-kdr* and monooxygenase-mediated detoxification (Shono *et al*., 2002). OCR is a cyclodiene resistant strain (due to *Rdl*, Kozaki and Scott, unpublished). Cornell-R is an organophosphate resistant strain (due to altered acetylcholinesterase (Tripathi & O'Brien, 1973)). The OCR and Cornell-R strains were obtained from Dr F. W. Plapp. in 1996 and have been maintained under biannual selection with dieldrin and tetrachlorvinphos, respectively. The ALHF strain was collected in Marshall Alabama in 1998, is highly resistant to permethrin (> 1800 fold) (Liu & Yue, 2001) and was obtained from N. Liu (Auburn University) in 2004. The 579_{kdr} strain (Williamson *et al*., 1996a) was obtained from I. Denholm (Rothamsted Research, UK) The A2bb (*super-kdr*) strain was obtained from M. Kristensen (Danish Pest Infestation Laboratory, Denmark) in 2004. The A2bb strain was not homozygous for permethrin resistance (data not shown). Therefore, 3–5 d old A2bb flies were treated by residual exposure to 468 ng/cm^2 permethrin in 160 ml glass jars. Flies that were not knocked down after 30 min of exposure were collected and genotyped for *Vssc1* alleles as described below.

House flies were collected during the summer of 2002 from four dairies in Alachua County Florida, Wake County North Carolina, Schuyler County New York and Androscoggin County Maine. Adults were captured with a sweep net from inside dairy barns and around calf hutches. North Carolina flies were collected as pupae from around calf hutches. Field collected animals were used to establish laboratory colonies. Colonies were reared as previously described by Scott *et al*. (Scott *et al*., 2000). Field collected males were frozen at −80 °C. Levels of resistance to six insecticides in these strains have recently been published (Hamm *et al*., 2005).

Single pair crosses were done with Wake County (North Carolina) flies to obtain lines that are homozygous for *kdr* and *kdr-his1*. A single male house fly was placed with a single, virgin female house fly in a 270 ml paper cup filled one-quarter with media. The flies were transferred to a new cup with fresh media every day for 3 days and media was added to each previous cup. The containers were reared under standard conditions as mentioned above. Progeny (adults) from each cross were genotyped for *kdr*, *kdr-his1*, and *CYP6D1v1* as described below. The lines were propagated for homozygous *kdr* or *kdr-his1* and *CYP6D1v1* and renamed NC11C and NC19His, respectively.

Two strains of German cockroach, *Blattella germanica*, were used. CSMA is a standard susceptible strain (Scott & Wen, 1997) and Ectiban-R is a pyrethroid and DDT resistant strain having *kdr* (Scott & Matsumura, 1981; Dong & Scott, 1994; Dong, 1997).

Bioassay

House flies were bioassayed by applying a 0.5 μl drop of permethrin dissolved in acetone containing 20 mg/ml PBO to the thoracic notum of 3–5 day old adult females. Flies were provided with cotton wicks soaked in 15% sugar water and held at 25 °C. Mortality was recorded after 24 h. Files were considered dead if they were ataxic.

Isolation of genomic DNA

Individual male flies or cockroaches were placed in 1.5 ml tubes and frozen in liquid nitrogen. Animals were quickly and completely pulverized with a disposable pestle (Kontes Glassware, Vineland, New Jersey, USA) and resuspended in 0.5 ml of lysis buffer (100 mM Tris-Cl pH 8.0, 50 mM NaCl, 10 mM EDTA, with 1% (w/v) SDS, 0.5 mM spermidine, 0.15 mM spermine, and 0.1 mg/ml (20 U/mg) proteinase K. Samples were incubated at 60 °C for 20 min. After incubation, 75 μl of 8 M potassium acetate was added, mixed and set in an ice bath for 10 min. The samples were spun at 14 000 × g for 5 min and the supernatant was transferred to a new tube. One ml of absolute ethanol was added and the samples were kept at room temperature for 10 min. The samples were spun at 14 000 × g for 10 min. Pellets were washed in 0.5 ml of 70% ethanol and spun at 14 000 × g for 5 min. The final pellet was dried in a vacuum for 10 min and then resuspended in 50 μl of H_2O. A list of the primers used for the subsequent analyses is given in Table 4.

Vssc1 *genotyping by PCR-RFLP*

A 335 bp fragment of *Vssc1* was amplified by PCR in a 50 μl reaction containing 1.25 U of Taq polymerase (New England Biolabs, Beverly MA), 10 pmol of the primers *kdr*DIGLong-F and *kdr*DIGLong-R and 2 μl of genomic DNA as a template. The reactions were carried out in a Hybaid PCR Express thermal cycler (Teddington, Middlesex, UK) and using the following conditions: 94 °C for 3 min, followed by 25 cycles of PCR (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s), and a final extension at 72 °C for 10 min. PCR products were concentrated by ethanol precipitation and dried

Table 4. Sequences of primers used in this study

Primer Name	Sequence (5′–3′)
*kdr*DIGLongF	TCGCTTCAAGGACCATGAACTACCGCGCTG
*kdr*DIGLongR	CCGAAGTTGGACAAAAGCAAAGCTAAGAAAAG
Super*kdr*LongF	CTCTGCTGGAATTGGGCCTGGAGGGTGTCC
Super*kdr*LongR	AGTTGCATTCCCATCACGGCAAAGATGAAG
S*kdr*USInternal	GCCACCAGTTTTCCTTAAATATGCC
NaChan1Flong	ATGACAGAAGATTCCGACTCGATATCTGAGGAAGAA
NaChan1Rlong	CCTCGTTCACTAGCACGACTATGCCGACCAGCGGC
NaChan2Flong	GGTGGCAGTAGTACGGCCGGTGGTGGCTATCC
NaChan2Rlong	GGTATGATTTCATGGCTCAGCACAGAGTCATTACC
NaChan3Flong	GGCCACTGCGTGCTGTCTCTAGATGGGAGGG
NaChan3Rlong	TCAGACATCTGCCGTCCTGGATGTTATGCTCGGC
S35	AGCTGACGAAATTGATCAATCAGT
AS2	CATTGGATCATTTTTCTCATC
ProS1	CGTCATTTACAACGCATTAGG
roach*KDR*-F	GGATATGCCGAGATGGAACTTTAC
roach*KDR*-R	GGCTGACAGATTGGATGAACC
roachInternal-F	CTCTTTTGACGCTCTATGTAACC
roachInternal-R	GTCCTTGCCCATTCATCACAG
M13F	GTAAAACGACGGCCAGT
M13R	GTAAAACGACGGCCAGT

under a vacuum. The pellet was dissolved in 20 μl of a restriction enzyme digestion reaction mixture containing 10 U of *Tsp* 509I (New England Biolabs). The samples were incubated at 65 °C for 3–4 h. The samples were run for 25–30 min on a 3.5% agarose gel stained with ethidium bromide at a final concentration of 0.5 μg/ml and visualized under a UV lamp. Genotypes were scored as compared to the standard patterns obtained from CS, LPR and an F1 of the two strains to indicate homozygous susceptible, homozygous resistant and heterozygous susceptible-resistant, respectively (Fig. 1b).

Sequencing of Vssc1 *alleles*

PCR products from the *Vssc1* genotyping assay were also sequenced. For house fly *super-kdr* and cockroach *kdr* genotyping, the same reaction conditions were used except that the primers were Super*kdr*LongF with Super*kdr*LongR and roach*KDR*-F with roach*KDR*-R, respectively, and the extension time was increased to 1.5 min. The products were purified by incubating 8 μl of the product with 2 μl of an enzyme mix containing 4 U Exonuclease I and 1.6 U shrimp alkaline phosphatase (USB Corporation, Cleveland OH) at 37 °C for 1 h and then at 85 °C for 15 min. The *kdr*DIGLong-F primer was used to sequence house fly *kdr* fragments. Super*kdr*LongF, Super*kdr*LongR, and s*kdr*USInternal were used for sequencing house fly *super-kdr* fragments. Roach*KDR*-F, roach*KDR*-R, roachInternal-F and roachInternal-R were used to sequence the cockroach *para*^{CSMA} fragments. Sequencing was performed at Cornell's Biotechnology Resource Center. Additionally, 3 μl of PCR product from the house fly *Vssc1* amplification was cloned using the TOPO TA cloning vector according to the manufacturer's instructions (Invitrogen, Carlsbad CA). Positive colonies were grown in liquid media and plasmid isolation was performed using QIAprep Spin Miniprep kit according to the manufacturer's directions (Qiagen, Valencia CA). Purified plasmid was sequenced in each direction using M13 primers.

Analysis of Vssc1 *allele sequences*

Sequences were analysed with the Sequencher software package (Gene Codes Corp., Ann Arbor, MI) with nucleotides scored as A, T, G, C, K (G or T), R (A or G), M (A or C), S (C or G), W (A or T) or Y (C or T). A script written in C++ was used to distinguish heterozygous sequences as being combinations of alleles that were independently observed in homozygous individuals. This approach was validated using F1 individuals (i.e. heterozygotes) from known homozygous strains.

Vssc1 sequences were aligned using MegAlign software (DNA Star, Madison WI). The phylogeny of the aligned sequence data was inferred using PAUP* (Swofford, 2002) under maximum parsimony. The data for this analysis consisted of the entire intron sequence and 148 bp of the upstream exon (Fig. 4). In order to avoid the potential problem of non-independence of adjacent nucleotide differences due to multibase insertion events, we grouped together contiguous indel positions, forming a single character and scoring each unique indel motif as a separate character state. For example, the information in positions 249–257 of the alignment (Fig. 4) was reduced to a single character with six states. The most parsimonious trees were generated using the branch-and-bound algorithm with no limit to MAXTREES, a procedure guaranteed to find all optimal trees. We did not want to make any assumptions about the ancestral status of any particular susceptible or resistant allele, so the resulting trees were left unrooted. Robustness of individual groups was assessed using 1000 pseudoreplicates of the non-parametric bootstrap under the same tree search conditions as above.

CYP6D1 *genotyping*

Determination of *CYP6D1* genotype was performed using a PCR-RFLP assay. PCR reaction mixtures contained 1.25 U of Taq polymerase (New England Biolabs), 10 pmol of the primers S35 and AS2, and 2 μl genomic DNA in a 50 μl volume. PCR was conducted under the following conditions: 94 °C for 3 min, followed by 35 cycles of PCR (94 °C for 30 s, 52 °C for 30 s and 72 °C for 50 s), and a final extension at 72 °C for 10 min. Ten ul of PCR product was visualized on a 1.5% agarose gel stained with ethidium bromide. The other 40 μl was concentrated by ethanol precipitation and the pellet was dried. The DNA was resuspended and digested with 2.5 U of Hpy188 III (New England BioLabs) at 37 °C for 24 h and the restriction pattern was checked under UV on a 2% agarose gel. S35/AS2 primers produce a 732 bp band for the RR genotype and a 711 bp band for the SS genotype. A 15-bp fragment, which appears only in resistant alleles, disrupts the recognition sequence for Hpy188 III that is seen in susceptible alleles (Scott *et al*., 1999). Therefore, treatment with Hpy188 III produces 432 bp and 279 bp bands for SS genotypes, an uncut 732 bp band for the RR genotype, and bands of 732 bp, 432 bp and 279 bp for RS genotypes (Fig. 2). In every analysis, PCR products from genomic DNA of CS, LPR, and *F*1 (LPR × CS) flies were used as controls.

Additionally, PCR products were purified by mixing 8 μl of PCR product with 2 μl of an enzyme mix containing 4 U Exonuclease I and 1.6 U Shrimp Alkaline Phosphatase (USB Corporation, Cleveland OH) at 37 °C for 1 h and then at 85 °C for 15 min. Ten pmol of the primer ProS1 was added and samples were sequenced at Cornell's Biotechnology Resource Center.

Cloning full-length cDNA of kdr-his1

Total RNA was extracted from heads of 40, mixed sex, 1 day-old adults from the NC19His strain using TRIzol reagent (Invitrogen, Carlsbad CA) and resuspended in 50 μl DEPC-treated H_2O. Five ug of total RNA was reverse-transcribed using SuperScript III (Invitrogen) under the following conditions: 42 °C for 3 h and 85 °C for 15 min. The reaction was purified using a QIAquick PCR purification Kit (Qiagen) and eluted with 50 μl H_2O. The *Vssc1* gene was amplified in three separate fragments of 2.2 kb each with 100–200 bp of overlap between each fragment. These fragments were amplified in a 50 μl reaction volume using 1.25 U of Taq polymerase (New England Biolabs), 100 ng cDNA, and 10 pmol of the primers indicated below. The primer sets used were NaChanLongF1 and NaChanLongR1, NaChanLongF2 and NaChanLongR2, NaChanLongF3 and NaChanLongR3, and fragments were amplified under the following conditions: 94 °C for 5 min, followed by 35 cycles of PCR (94 °C for 30 s, 60 °C for 30 s and 72 °C for 2.5 min), and a final extension at 72 °C for 10 min. PCR products of the expected size were purified from agarose gels using QIAEX II purification kit (Qiagen) and resuspended in 30 μl H_2O. The PCR products were cloned into pGEM T-Easy vector (Promega Corp, Madison WI) according to the manufacturer's directions. Positive colonies were grown in liquid media and plasmid isolation was performed using QIAprep Spin Miniprep kit according to the manufacturers directions (Qiagen). Sequencing was performed at Cornell's Biotechnology Resource Center. Sequences from more than 6

individual clones for each fragment were aligned using MegAlign (DNA Star, Madison WI) and the full-length sequence of *kdr-his1* was determined.

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