Research Article

Received: 18 July 2017 Revised: 5 November 2017 Accepted article published: 20 November 2017 Published online in Wiley Online Library: 5 January 2018

(wileyonlinelibrary.com) DOI 10.1002/ps.4798

Novel cytochrome P450 (*CYP6D1*) and voltage sensitive sodium channel *(Vssc)* alleles of the house fly (*Musca domestica*) and their roles in pyrethroid resistance

Jing Pan,[a,b] Chan Yang,[a,b] Yan Liu,[a,b] Qi Gao,[a,b] Mei Li[a] and Xinghui Qiu[a*]

Abstract

BACKGROUND: The house fly *Musca domestica* is an important disease vector. Point mutation-mediated target-site insensitivity of the voltage sensitive sodium channel (VSSC) and increased detoxification mediated by cytochrome P450 (*CYP6D1*) overexpression have been characterized as two major mechanisms of pyrethroid resistance. In this study, genetic mutations in the *Vssc* and *CYP6D1* genes and their contribution to pyrethroid resistance were investigated.

RESULTS: Twelve lines of house flies homozygous for four genotypes were established. House flies carrying the VSSC 1014F mutation and overexpressing *CYP6D1* had higher resistance to pyrethroids than those carrying 1014F alone. The presence of the 15-bp insert in the promoter region of the *CYP6D1* gene did not necessarily result in a significant increase in *CYP6D1* mRNA and pyrethroid resistance levels. A novel *Vssc* allele carrying two mutations (G1924D and G2004S) in combination with the classic 1014F and a novel *CYP6D1* allele that is very similar to *CYP6D1v1* were identified in Chinese house flies.

CONCLUSION: This work demonstrates the effect of genetic mutations in *CYP6D1* and *Vssc* on the susceptibility of house flies to pyrethroids, and verifies that 15-bp insert-containing *CYP6D1* alleles have a single origin. These findings offer insights into the evolution of insecticide resistance and have implications for house fly control.

Supporting information may be found in the online version of this article.

Keywords: CYP6D1; evolution; *Musca domestica*; pyrethroid resistance; voltage sensitive sodium channel (*Vssc*)

1 INTRODUCTION

Insecticide resistance is a major problem in controlling medically and agriculturally important insect pests. The house fly is an important disease vector and its resistance to various classes of insecticides has been documented worldwide.[1] Two major mechanisms of pyrethroid resistance in house flies have been demonstrated: voltage sensitive sodium channel (VSSC) insensitivity and increased detoxification mediated by P450-dependent monooxygenases.[1] Both mechanisms can be present in a population-specific manner, and the relative importance of each mechanism may vary in different populations.

Three target resistance-conferring VSSC mutations including *kdr* (L1014F),[2] *kdr-his* (L1014H)[3] and *super-kdr* (M918T + L1014F)[2,4,5] have been identified in pyrethroid-resistant strains of house flies. Generally, the levels of resistance conferred by mutations in *Vssc* were in the order of *kdr-his* < *kdr* < *super-kdr*. However, there are exceptions for several pyrethroids.[6] For example, lower protection was observed for three pyrethroids with multi-halogenated benzyl groups for the *super-kdr* mutation than for *kdr*.[6] The *kdr* mutation has been commonly detected in house fly populations collected from the USA, Europe and Asia.[4,7,8] Both L1014F and L1014H were detected in Chinese field populations,[9] while M918T was identified in a deltamethrin-selected laboratory strain (BJD),[5] but not detected in field collections in 2009, probably because of low frequency.[9] Multiple origins of *kdr* mutations have been suggested.[10] Recently, several other mutations (e.g. D600N + M918T + L1014F and T929I + L1014F) have been documented in *Vssc*, with the additional D600N or T929I conferring stronger pyrethroid resistance than L1014F alone.[11]

Cytochrome P450s (CYPs) have been documented to be involved in pyrethroid resistance in house flies.[1] Over-expression of *CYP6D1* is associated with pyrethroid resistance in the LPR strain, as well as several field populations of house flies in the USA[12,13] and in China.[14] The increased transcription of *CYP6D1* in LPR is caused by both *cis* and *trans* factors on autosomes 1 and 2.[15] A resistance allele (*CYP6D1v1*) was identified in resistant LPR house

* *Correspondence to: X Qiu, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China. E-mail: qiuxh@ioz.ac.cn*

a *State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China*

b *University of Chinese Academy of Sciences, Beijing, China*

flies, and is characterized by the insertion of a 15-bp sequence in the 5′ promoter region.[13] This insert makes the *CYP6D1v1* promoter bind 5- to 10-fold less *mdGfi-1* (a transcriptional repressor) by interrupting the putative *mdGfi-1* binding site within the *CYP6D1* promoter found in susceptible strains.[16] The presence of the 15-bp insert was also detected in house flies collected across China,[5,9] and in Turkey.[8] These observations indicate that CYP6D1-mediated pyrethroid resistance is a common worldwide resistance mechanism.

Other CYPs that may be responsible for pyrethroid resistance in house flies have been suggested. For example, transcriptomic analysis identified 12 CYPs (CYP4G99, 4S24, 6A5, 6A25, 6A27, 6A36, 6A40, 6A52, 6A56, 6D10, 6GU1 and 18A1) on autosome V that were over-expressed relative to two susceptible strains in ALHF (a resistant strain displaying 23 000-fold resistance to permethrin).[1,17] Significant over-expression of more than one P450 gene in the six pyrethroid-resistant house fly populations was detected, and CYP6G4 and CYP6D1 were hypothesized to be the important P450s contributing to pyrethroid resistance of Chinese house flies.[14]

In this study, we were interested in addressing three questions regarding pyrethroid resistance in house flies. First, how much resistance may house flies gain when they carry *kdr* and/or the 15-bp insert-containing *CYP6D1* alleles? Second, are the *CYP6D1* alleles that contain the 15-bp insert identical in house flies from China and America? Third, are any other pyrethroid resistance-associated mutations in addition to the classic 1014F present in Chinese house flies?

2 EXPERIMENTAL METHODS

2.1 House flies

Five laboratory colonies (BJD, GD, SH, TJS and WHO) were used in this study. The GD and SH colonies were derived from a field collection in 2009 from Guangdong and Shanghai, respectively, in China.[9] The WHO strain is a standard insecticide-susceptible strain obtained from Dr. Michael Kristensen in 2012. TJS and BJD were two strains maintained in our laboratory.[5] These colonies were cultured at a population size of 500–1000 adults per generation in the laboratory without exposure to any insecticides.

Three independent groups (GD-A, GD-B and GD-C), with each group consisting of four genotype-specific homozygous lines (in total 12 lines), were established via procedures described below (Fig. 1). We used the GD strain as source material to create lines with specific genotypes as a result of the presence of both the 1014F mutation and the 15-bp insert-containing *CYP6D1* in the Guangdong strain at a desirable frequency.[9] First (Fig. 1, upper part), we individually genotyped virgin female flies from the GD colony using genomic DNA prepared from fly wings in order to obtain heterozygous females carrying either the *Vssc* 1014F (F) mutation or the 15-bp insert-containing *CYP6D1* allele (R); then three separate single pair crosses (A, B and C) were performed using these genotype-specific females, and males from the WHO strain; the F2 offspring were genotyped, and males and females with genotype SSFF or RRLL were selected (S represented a *CYP6D1* allele without the 15-bp insert; L represented *Vssc* 1014 L); then, six lines (three SSFFs and three RRLLs) were established and reared under standard conditions. Second (Fig. 1, lower part), three crosses (A, B and C) were conducted using RRLL males and SSFF females from the lines generated above; the individuals with SSLL, SSFF, RRLL, RRFF were identified from the F2 offspring of each cross by genotyping, and used to produce lines homozygous for the

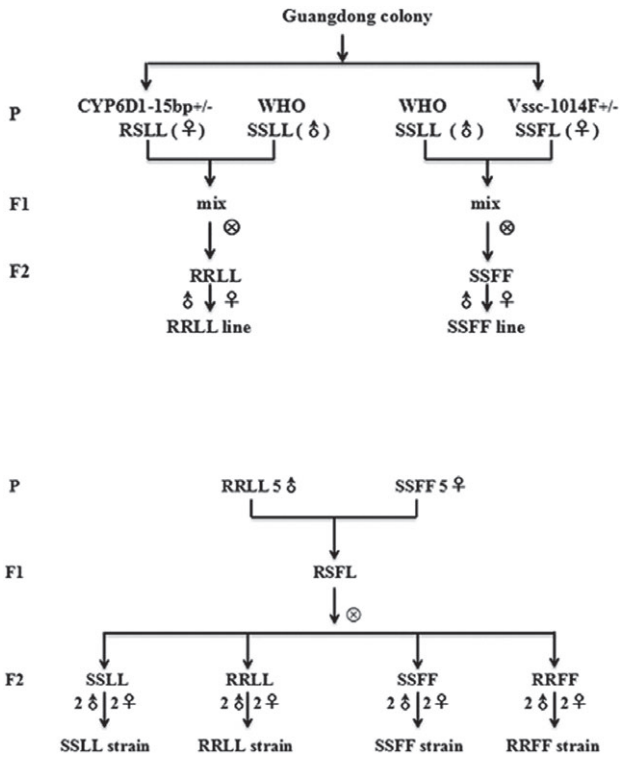

Figure 1. Procedures used to create house fly lines homozygous for different genotypes. R and S represent a *CYP6D1* allele with and without the 15-bp insert, respectively; L represents *Vssc* 1014 L; F represents *Vssc* 1014F. The final strains homozygous for the four different genotypes (bottom) were created from the RRLL and SSFF lines which were established from the crosses of allele-specific individuals from the GD colony with those from the WHO strain (upper part).

SSLL, SSFF, RRLL and RRFF genotypes, respectively. The accuracy of genotyping was confirmed by sequencing ten randomly selected individuals per line.

Flies were maintained in the laboratory at 25 °C with a photoperiod of 12:12 h (light:dark). Larvae were reared on wheat bran-based media. Adults were fed with powdered milk and sugar (1:1) and water *ad libitum*.

2.2 Toxicity bioassay

Insecticides including permethrin (95%), deltamethrin(94.62%), cyfluthrin(95%)and cypermethrin(91.16%)were dissolved in acetone and applied in a volume of 1 µL to the thoracic notum of female house flies. Each experiment was replicated at least three times. Each bioassay consisted of 15 to 30 3–5-day-old female house flies per dose and four to six doses that gave >0% and <100% mortality. Control groups received acetone alone. The treated insects were placed in 200-mL cups covered with cheesecloth, and water and food were provided. Cups with treated insects were held at 25 °C. Mortality was recorded 24 h after insecticide application. Bioassay data were pooled and analyzed based on standard probit analysis using POLO PLUS (version 2; LeOra Software, Petaluma, CA, USA), after Abbott's correction for control mortality.

2.3 RNA and DNA preparations

Total RNA was isolated from the abdomens of ten virgin female adults (3rd day) from each line using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with isolated RNA and

an oligo (dT) primer using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China). Genomic DNA was isolated from the adult flies by the method of Rinkevich *et al.* (2006).[12] Abdomens were discarded prior to the isolation of DNA.

2.4 *CYP6D1* and *Vssc* genotyping

The *CYP6D1* genotypes were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method described in Rinkevich *et al.*[12] to detect the presence or absence of the 15-bp insert. *Vssc* genotypes at the 1014 site were determined by the PCR-RFLP method described in Qiu *et al* .[18]

2.5 Cloning and sequencing of *CYP6D1* and *Vssc* genes

The genomic DNA sequences covering the 5′ promoter region and coding region of the *CYP6D1* gene were amplified using LA Taq DNA polymerase (Takara Biotechnology, Dalian, China) and primers S35 and CYP6D1-R1 (Supporting Information Table S1). PCR was carried out under the following conditions: 94 °C for 3 min,followed by 35 cycles of PCR (94 °C for 3 min, 58 °C for 30 s and 72 °C for 4 min),and an extension at 72 °C for 5 min. DNA samples from three individuals with the 15-bp insert and three without the 15-bp insert of different origins were used. The PCR products were gel-purified and directly sequenced from both directions. The sequence was further confirmed by sequencing after TA-cloning if any ambiguity occurred. The nucleotide variations detected in LPR and those in Chinese samples with the 15-bp insert were reconfirmed by PCR of three corresponding fragments using PrimeSTAR GXL DNA polymerase (Takara Biotechnology) and direct sequencing.

Full-length cDNAs of *Vssc* were amplified by PCR using PrimeSTAR GXL DNA polymerase (Takara Biotechnology) according to the protocol described in Sun *et al.*[6] PCR products were purified and directly sequenced from both directions. Ambiguities were resolved by analyzing three to five clones coupled with direct sequencing of the original PCR product.

Nucleotide sequences were aligned using CLUSTALW in MEGA7 software,[19] and the aligned sequences were analyzed using JMODELTEST[20] for evaluation of phylogenetic models. The general time reversible model incorporating invariant sites and a gamma distribution (GTR + I + G) was selected for phylogenetic analysis of *Vssc* and the Hasegawa–Kishino–Yano model with invariant sites (HKY + I) for analysis of *CYP6D1*. Phylogenetic trees were reconstructed using maximum likelihood (ML) in MEGA7 with 1000 bootstraps.

2.6 Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

qRT-PCR of *CYP6D1* expression was performed on the Mx3000P qPCR System (Stratagene, La Jolla, CA, USA) using the SYBR Premix Ex TaqII(Perfect Real Time)kit with *CYP6D1* gene-specific primer pairs (CYP6D1Q-F and CYP6D1Q-R). The ribosomal protein S3 (*RPS3*) gene (primers RPS3-F and RPS3-R; Table 1) was used as a reference gene. qRT-PCR of each cDNA sample and template-free control was performed in triplicate. Reactions were run in 20-μL volumes containing 10 μL of 2 × SYBR Premix Ex TaqII, 0.4 μL of 50 × ROX Dye II (Takara biotechnology), 0.8 μL of each forward and reverse primer (10 μM), and 1.0 μL of cDNA template with the following thermocycler parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 57 °C for 20 s and 60 °C for 30 s, and a cycle of 95 °C for 1 min and 55 °C for 30 s. The melting temperatures of amplicons were measured by taking continuous fluorescence readings whilst increasing the temperature from 55 °C to 95 °C in 0.5 °C increments, holding for 10 s after each 0.5 °C increment. The specificity of the PCR reactions was examined by melting curve analysis and product sequencing. Potential contamination was checked by including non-reverse transcriptase preparation and non-template (water) controls. The amplification efficiency was determined from a standard curve based on cycle threshold (C_t) values against serial 10× dilutions of cDNA templates. Under the conditions described in this study, the amplification efficiency was 102.7% for *RPS3* and 103.3% for *CYP6D1*, and the gene-specific PCR products were obtained. All qRT-PCR analyses were performed with three independent biological replicates and two or three technical replicates. The relative expression levels of *CYP6D1* were calculated by the comparative C_t method as described by Livak and Schmittgen.[21] One-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test was performed to determine the statistical significance of differences between means of the four strains within each group (GD-A, GD-B and GD-C, respectively) using SPSS version 13 (IBM, Armonk, NY, USA). A *P*-value of <0.05 was considered significant.

3 RESULTS

3.1 Pyrethroid susceptibilty

To evaluate precisely the effect of genetic mutations in *CYP6D1* and *Vssc* on the susceptibility of house flies to pyrethroids, we created 12 lines of house flies homozygous for four genotypes using house flies from the GD and WHO colonies as source material (Fig. 1). Bioassays showed that there was no or little difference in susceptibility to the four tested pyrethroids among the three lines (GD-A, GD-B and GD-C) with the same *CYP6D1/Vssc* genotypes, indicating that these lines have a similar genetic background (Table 1).

The median lethal dose (LD_{50}) values for the SSLL lines ranged from 0.54 ng per fly (deltamethrin) to 7.6 ng per fly (permethrin). In general, there were no significant differences in susceptibility between the SSLL lines and the WHO strain (Table 1). Compared with the susceptible WHO strain, RRLLs showed 2.7- to 3.7-fold resistance to permethrin, while no increase or a slight decline in tolerance to other three pyrethroids was observed. SSFFs exhibited 11-, 20-, 37- and 28-fold resistance to deltamethrin, permethrin, cypermethrin and cyfluthrin, respectively. Higher levels of resistance were observed in the RRFF lines, with average resistance ratios (RRs) being 28, 73, 68 and 45 for resistance to deltamethrin, permethrin, cypermethrin and cyfluthrin, respectively (Table 1).

3.2 Expression levels of *CYP6D1*

To test whether the presence of the 15-bp insert enhanced the expression of *CYP6D1*, qRT-PCR was conducted to determine the level of *CYP6D1* mRNA in the 12 lines and the WHO strain of house flies. The data showed that there was no significant difference among WHO, SSLL, RRLL and SSFF, while a 3.1- to 3.6-fold increase was observed in RRFF lines compared with corresponding SSLL lines (Fig. 2).

3.3 Full-length sequences of *CYP6D1* genes

To investigate the genetic polymorphism of *CYP6D1* alleles with or without the 15-bp insert in Chinese house flies, we sequenced genomic DNA sequences of *CYP6D1* from Chinese house flies, and compared them with those reported from LPR and aabys

Table 1. Toxicity of four pyrethroids to the WHO strain and 12 established strains of house fly

	Strain	LD_{50} (95% CI)	Slope (SE)	χ^2 (df)	*n*	RR
Deltamethrin	WHO	1.38 (0.71-2.43)	2.09 (0.18)	13.33 (3)	450	-
	SSLL-GD-A	0.78 (0.69-0.91)	2.40 (0.33)	0.29 (2)	360	0.6
	SSLL-GD-B	0.54 (0.39-0.78)	2.11 (0.20)	4.93 (3)	425	0.4
	SSLL-GD-C	0.71 (0.54-0.87)	3.39 (0.30)	6.42 (3)	450	0.5
	RRLL-GD-A	0.48 (0.40-0.57)	1.87 (0.18)	0.28 (3)	450	0.3
	RRLL-GD-B	0.62 (0.52-0.75)	1.76 (0.22)	1.89 (2)	360	0.4
	RRLL-GD-C	0.60 (0.51-0.70)	1.99 (0.18)	1.16 (3)	450	0.4
	SSFF-GD-A	14.4 (11.5-17.5)	2.98 (0.24)	4.05 (3)	450	10.4
	SSFF-GD-B	13.5 (11.3-16.2)	2.10 (0.17)	6.26 (5)	630	9.8
	SSFF-GD-C	17.4 (15.4-19.6)	2.69 (0.21)	1.58 (3)	450	13
	RRFF-GD-A	39.6 (32.8-47.2)	2.57 (0.20)	5.17 (4)	540	29
	RRFF-GD-B	36.7 (31.4-42.8)	2.66 (0.21)	7.41 (5)	630	27
	RRFF-GD-C	38.0 (33.8-42.6)	2.85 (0.22)	2.00 (3)	450	28
Permethrin	WHO	6.32 (5.42-7.31)	2.11 (0.18)	1.99 (3)	450	-
	SSLL-GD-A	6.70 (4.50-11.1)	3.00 (0.20)	12.32 (3)	450	1.1
	SSLL-GD-B	7.20 (6.20-8.20)	3.64 (0.20)	9.70 (5)	630	1.1
	SSLL-GD-C	7.63 (6.04-9.19)	4.24 (0.39)	2.05 (2)	360	1.2
	RRLL-GD-A	23.3 (20.8-26.1)	3.10 (0.20)	1.61 (3)	450	3.7
	RRLL-GD-B	17.3 (16.2-18.4)	4.99 (0.30)	1.49 (4)	540	2.7
	RRLL-GD-C	19.9 (15.4-25.9)	3.15 (0.24)	5.53 (3)	450	3.2
	SSFF-GD-A	129 (118-142)	4.30 (0.30)	0.62 (2)	360	21
	SSFF-GD-B	140 (116-165)	3.30 (0.20)	5.78 (4)	540	22
	SSFF-GD-C	109 (95.9-123)	2.71 (0.23)	1.46 (3)	450	17
	RRFF-GD-A	474 (424-530)	2.50 (0.22)	0.07 (4)	540	75
	RRFF-GD-B	473 (423-593)	2.46 (0.21)	0.79 (4)	540	75
	RRFF-GD-C	441 (395-493)	3.05 (0.23)	2.85 (3)	450	70
Cypermethrin	WHO	1.75 (1.23-2.44)	1.98 (0.17)	4.97 (3)	450	-
	SSLL-GD-A	1.60 (1.11-2.29)	1.90 (0.20)	4.97 (3)	540	0.9
	SSLL-GD-B	2.66 (1.47-4.03)	2.08 (0.26)	11.5 (4)	330	1.5
	SSLL-GD-C	1.80 (1.54-2.10)	1.99 (0.17)	2.87 (3)	450	1.0
	RRLL-GD-A	2.00 (1.79-2.30)	2.30 (0.20)	2.04 (4)	450	1.1
	RRLL-GD-B	1.38 (1.10-1.67)	1.92 (0.23)	3.90 (4)	300	0.8
	RRLL-GD-C	3.02 (2.62-3.49)	2.05 (0.15)	0.39 (4)	540	1.7
	SSFF-GD-A	52.3 (40.3-64.5)	2.30 (0.20)	5.86 (4)	540	30
	SSFF-GD-B	59.7 (50.6-68.7)	2.58 (0.32)	2.07 (4)	300	34
	SSFF-GD-C	79.9 (66.6-99.1)	1.77 (0.22)	1.65 (2)	360	46
	RRFF-GD-A	89.7 (80.4-99.2)	3.48 (0.27)	0.21 (3)	450	51
	RRFF-GD-B	111 (92.7-128)	4.27 (0.36)	3.81 (3)	419	63
	RRFF-GD-C	155 (102-246)	2.68 (0.27)	11.87 (3)	450	89
Cyfluthrin	WHO	1.84 (1.41-2.46)	1.73 (0.14)	5.45 (4)	540	-
	SSLL-GD-A	1.91 (1.44-2.50)	2.46 (0.20)	4.62 (3)	450	1.0
	SSLL-GD-B	1.95 (1.71-2.23)	2.04 (0.18)	1.91 (4)	540	1.1
	SSLL-GD-C	1.82 (1.57-2.10)	2.17 (0.18)	2.14 (3)	450	1.0
	RRLL-GD-A	1.86 (1.64-2.11)	2.58 (0.22)	2.71 (3)	450	1.0
	RRLL-GD-B	1.05 (0.93-1.18)	2.78 (0.21)	0.38 (3)	450	0.6
	RRLL-GD-C	1.80 (1.58-2.05)	2.48 (0.20)	0.94 (3)	450	1.0
	SSFF-GD-A	55.5 (48.6-62.9)	2.79 (0.26)	1.13 (2)	360	30
	SSFF-GD-B	50.2 (44.5-55.8)	2.89 (0.28)	1.99 (4)	450	27
	SSFF-GD-C	48.4 (31.5-74.4)	1.69 (0.17)	5.76 (3)	450	26
	RRFF-GD-A	75.6 (48.2-111)	2.6 (0.22)	8.52 (3)	450	41
	RRFF-GD-B	86.1 (71.6-101)	2.62 (0.20)	4.73 (4)	540	47
	RRFF-GD-C	85.1 (65.5-110)	1.95 (0.21)	3.16 (3)	450	46

SSLL, *CYP6D1*^{S}/*Vssc1014L*; RRLL, *CYP6D1*^{R}/*Vssc1014L*; SSFF, *CYP6D1*^{S}/*Vssc1014F*; RRFF, *CYP6D1*^{R}/*Vssc1014F*.
Resistance ratio (RR) = LD_{50} of a specific strain/LD_{50} of WHO.

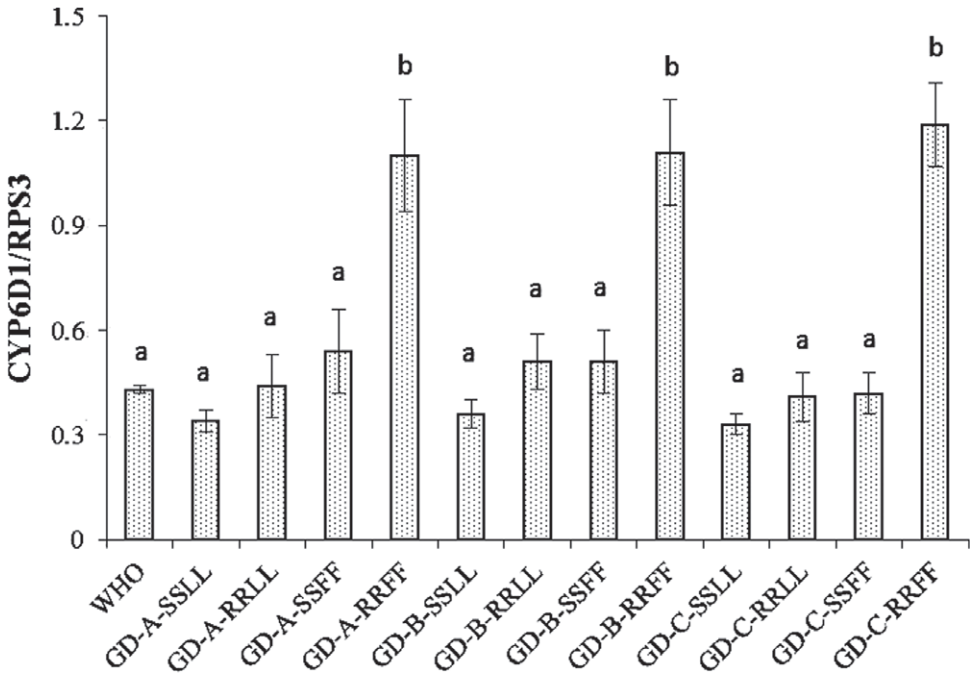

Figure 2. The mRNA ratio of *CYP6D1* to *RSP3* in the WHO strain and 12 established strains of house fly. Data represent the expression level of *CYP6D1* relative to *RPS3*. Error bars represent the standard errors, and different letters represent a significant difference at $P < 0.05$.

Table 2. Genomic sequence comparison of five *CYP6D1* alleles

	LPR[a]	BJD[b]	BJ-S	SH-S	TJ-S
Exon 1	527	527 (100%)	527 (96.96%)	527 (97.15%)	527 (96.77%)
Exon 2	162	162 (100%)	162 (95.68%)	162 (96.91%)	162 (95.06%)
Exon 3	449	449 (100%)	449 (95.54%)	449 (97.55%)	449 (95.55%)
Exon 4	249	249 (100%)	249 (96.79%)	249 (97.19%)	249 (96.79%)
Exon 5	164	164 (100%)	164 (98.78%)	164 (98.78%)	164 (98.78%)
Intron 1	74	74 (100%)	76 (85.53%)	71 (83.78%)	76 (85.53%)
Intron 2	2377	2376 (99.75%)	2872 (69.47%)	2868 (68.64%)	2871 (69.37%)
Intron 3	66	66 (98.48%)	65 (90.91%)	65 (92.42%)	65 (90.91%)
Intron 4	64	64 (98.44%)	63 (90.63%)	63 (92.19%)	63 (90.63%)

The numbers represent the length (base pairs) and percentage identity (in parentheses) calculated by optimal global alignment in BIOEDIT[22] as compared with the corresponding sequence of LPR house flies.
GD, Guangdong; SH, Shanghai; TJ, Tianjin; S, without the 15-bp insert.
Details of the BJD and TJS strains are given in Qiu *et al.*[5]
[a] Data are from Scott *et al.*[23]
[b] These sequences in BJD, SH-R and GD-R are identical; only BJD is presented in this table.

(Table 2). The accession numbers for these sequences are shown in Figure 3. The *CYP6D1* sequences having the 15-bp insert were 100% identical in the three individuals collected from different geographical locations in China; however, one indel and seven substitutions were observed when compared with the sequence from LPR (Fig. S1). These variations were present in intron 2 (five variations + one indel), intron 3 (one variation) and intron 4 (one variation), respectively. In contrast, many more differences existed in individuals lacking the 15-bp insert in both introns and exons (Figure S1 and Table 2). For example, the identity between BJ-S and SH-S was 94.04%. The phylogenetic analysis showed that the 15-bp insert-containing alleles formed one clade, while there were

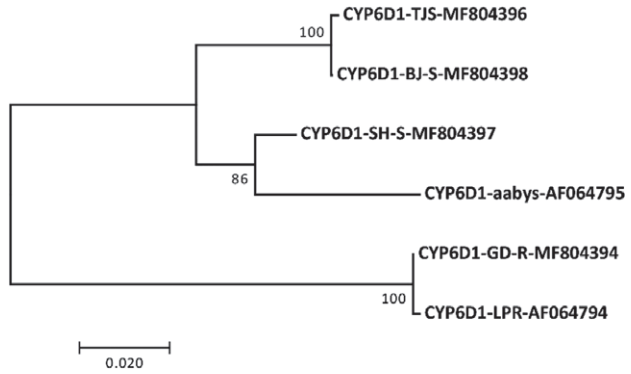

Figure 3. Maximum-likelihood phylogeny of *CYP6D1* alleles in house flies. Numbers at nodes represent the bootstrap values (%). The sequences used for tree construction are presented in Figure S1. The sequences are named using the allele or strain name followed by the accession number.

two independent clades containing the *CYP6D1* alleles without the 15-bp insert (Fig. 3).

3.4 Full-length cDNAs of *Vssc* in Chinese house flies

The full-length open reading frames of *Vssc* were obtained from the six RRLL and SSFF lines (Fig. S-2). The accession numbers for these sequences are shown in Figure 5. Alignment analysis showed that the sequences with the 1014F mutation were identical in the three SSFF lines (GD-A, B and C). RRLL-A was heterozygous at the *Vssc* locus, where two different alleles were identified by clone sequencing (GD-1014 L-1 and GD-1014 L-2; Fig. S2). The *Vssc* sequence (GD-1014 L-3) was identical in RRLL-GD-B and RRLL-GD-C house flies (Fig. S2).

The alignment of deduced amino acid sequences of *Vssc* (Fig. 4) showed a polymorphic region from amino acid residue 2021 to residue 2055. In the region from amino acid 1 to 2020, one (D2003A) and four amino acid substitutions (L1014F, G1942D, D2003A and G2004S) were observed in GD-1014Ls and GD-1014F, respectively, when compared with the allele in aabys. Notably, GD-1014F differed from KX431037 by only one conserved amino acid substitution (T2043S).

The phylogenetic tree showed that GD-1014F had a close evolutionary relationship with the other two 1014F alleles (KSkdr1A and KSkdr1B) identified in the USA, while the three 1014 L alleles identified in GD were clustered in an independent branch (Fig. 5).

4 DISCUSSION

Insects can evolve more than one resistance mechanism.[24] It is known that pyrethroid resistance can be conferred either through point mutations in *Vssc* or through CYP-mediated enhanced detoxification.[1] In our assays, the L1014F mutation caused 11- to 37- fold resistance to the four pyrethroids (Table 2). The toxicity results are consistent with those previously reported.[25]

The full-length cDNA analysis in this study identified four new *Vssc* alleles (Figs 4 and S2). These sequences confirm that *Vssc* is highly conserved in the region from amino acid 1 to 2020, but has retained a polymorphic region from amino acid 2021 to 2055.[11] Interestingly, the resistance allele GD-1014F identified in house flies from Guangdong in China has a high similarity to KSkdr1A (KX431037) and KSkdr1B (KX431038) (Figs 4 and 5) which were recently found in house flies from Kansas in the USA.[11] For example, only one conserved amino acid substitution (T2043S) was observed between GD-1014F and KSkdr1A (Fig. 4). Notably, the three alleles have G1924D and G2004S mutations in combination with the classic L1014F, and therefore the G1924D and G2004S mutations could be regarded as conserved mutations. It would be very valuable to investigate the toxicological and evolutionary significance of the addition of the two mutations to 1014F.

Although our bioassay result (Table 1) supports the notion that the addition of G1924D and G2004S to L1014F does not give higher pyrethroid resistance,[11] we could not exclude the possibility that they may confer extra resistance to other insecticides targeting VSSC. D2003A was detected in both susceptible and resistant lines, indicating that this mutation alone may play no role in pyrethroid resistance. The three mutations (D600N, M918 T and T929I) that can significantly enhance pyrethroid resistance[11] were not detected in our lines.

Previous studies have demonstrated that over-expression of *CYP6D1* in the LPR strain confers resistance to both cyano (CN) and non-CN pyrethroids.[26] Genetic studies have revealed that the factors responsible for the increased expression in the LPR strain are on autosomes 1 and 2.[15,27] To date, the *trans*-acting factor (S) on autosome 2 remains unidentified, while the 15-bp insert in the promoter of *CYP6D1* on autosome 1 has been suggested to be a *cis*-acting factor.[27] Electrophoretic mobility shift assays demonstrated that the 15-bp insert reduced the amount of mdGfi-1 (a transcriptional repressor) binding in insecticide-resistant LPR house flies by interrupting the mdGfi-1-binding site, which may lead to constitutively high levels of *CYP6D1* expression and resistance.[16] However, our bioassay results showed that the RRLL lines, which carry the 15-bp insert in the promoter region of the *CYP6D1* gene as in the LPR strain, unexpectedly exhibited no significant resistance to the three α-cyano pyrethroids, but slight (2.7–3.7-fold) resistance to the non-CN permethrin. Given that increased transcription is the underlying cause of CYP6D1-mediated insecticide resistance,[27] we examined the expression levels of *CYP6D1* in these fly lines. qRT-PCR data showed that the mRNA levels in the three RRLL lines were not significantly different from those in the SSLL or SSFF lines or the WHO strain (Fig. 2), indicating that the presence of the 15-bp insert does not result in a significant increase of *CYP6D1* mRNA in the RRLL lines. Therefore, it appears that the slight resistance to permethrin observed in the RRLL lines is not caused by enhanced detoxification catalyzed by CYP6D1. Among the 12 lines, a significant 3.1- to 3.6-fold increase of *CYP6D1* mRNA (relative to SSLL lines) was detected in RRFF lines only. The differential expression of *CYP6D1* between the RRFF and RRLL lines may indicate that the RRFF line has an undefined factor on chromosome 3 that upregulates *CYP6D1*, probably via interaction with the *mdGfi-1* repressor.

Our qRT-PCR and toxicity results indicate that the presence of the 15-bp insert in the promoter region of the *CYP6D1* gene does not necessarily mean an increase of *CYP6D1* expression and/or pyrethroid resistance. Previous genetic studies have demonstrated that autosome 1 from the LPR strain has a small role in permethrin resistance (2.4-fold resistance) in the aabys background,[28] and a factor(s) on autosome 1 by itself causes a 2–3-fold increase in *CYP6D1* mRNA and protein.[15] These observations suggest that the 15-bp insert alone is not a robust or reliable marker for *CYP6D1* over-expression and CYP6D1-mediated resistance. Further identification of the other molecular factor(s) that causes enhanced transcription of *CYP6D1* will offer new molecular tools for monitoring resistance and new target sites for insecticide development.

Figure 4. Alignment of deduced amino acid sequences of *Vssc*. Amino acids 1–960 and 1021–1920 are identical for all these sequences and are not presented in the figure. KT289928 identified in the aabys strain and KX431037 found in the KSkdr1A strain are included for comparison purposes. Asterisks (*), colons (:), and black dots (·) indicate identical, conserved and weakly conserved sites, respectively.

Genomic DNA sequencing revealed that both the 5′ flanking region and entire coding sequence of the 15-bp insert-containing *CYP6D1* allele from three individuals was exactly identical to that from the LPR strain (Table 2; Fig. S1). However, after examining the ~4500-bp full-length genomic DNA sequences, eight variations in the Chinese *CYP6D1* counterpart were detected in introns 2 to 4 when compared with *CYP6D1v1* identified in LPR. Whether or not these variations influence the transcription of *CYP6D1* is unknown. Very high similarity between the 15-bp insert-containing *CYP6D1* alleles was previously documented in a study on the NG98 strain.[13] The phylogenetic analysis revealed that 15-bp insert-containing *CYP6D1* alleles are clustered in the same clade (Fig. 3). Our data adds evidence to support the hypothesis that the 15-bp insert-containing alleles have a single origin.[5,13]

In multigenic resistance, mechanisms may interact additively, synergistically or antagonistically.[24] To examine the possible interaction between *kdr* and CYP6D1-mediated detoxification, three separate homozygous RRFF lines (GD-A, GD-B and GD-C) carrying both the 15-bp insert-containing *CYP6D1* allele and the GD-1014F mutation were generated (Fig. 1). Toxicity assays showed that, compared with the SSLL lines, the resistance levels in the RRFFs were greater than those in either RRLLs or SSFFs for three of the four pyrethroids tested. For cyfluthrin, overlapping 95% confidence intervals of LD_{50} were observed in GD-A and GD-C between RRFFs and SSFFs, although greater RR values were calculated in the RRFF lines. The increased resistance to deltamethrin, permethrin and cypermethrin in the RRFF lines compared with SSFFs can be explained by the enhanced detoxification via an increase of *CYP6D1* expression. Smaller but significant (GD-B) and insignificant (GD-A and GD-C) increases in the RR for cyfluthrin in RRFFs compared with SSFFs suggest that *CYP6D1* is ineffective in conferring resistance to the F-substituted pyrethroid (cyfluthrin), probably because the substituted phenoxybenzyl group limits cyfluthrin metabolism by CYP6D1.[25] This result is in agreement with the finding that LPR exhibits much lower resistance to cyfluthrin than to cypermethrin.[26]

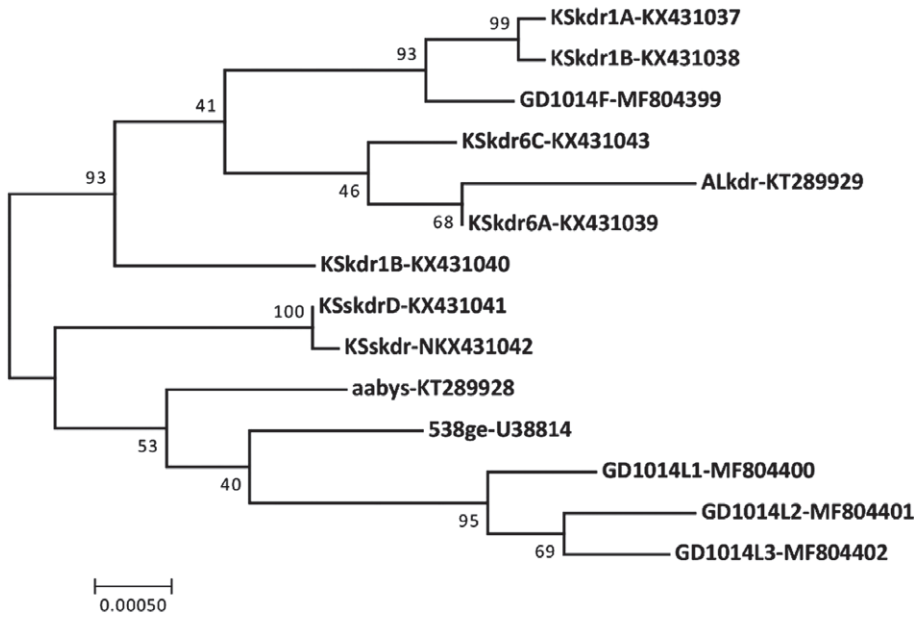

Figure 5. Maximum likelihood phylogeny of *Vssc* alleles in house flies. Numbers at nodes represent the bootstrap values (%). The sequences used for tree construction are presented in Figure S2. The sequences are named using the allele or strain name followed by the accession number.

Based on the results reported in Liu and Scott (1996),[15] in which R1 (a line exhibiting a 2- to 3-fold increase in *CYP6D1* mRNA and protein in the aabys background) was found to have 2- to 3-fold permethrin resistance, it can be deduced that the increased (3.1- to 3.6-fold) *CYP6D1* mRNA expression in the RRFF lines (compared with the SSLL lines) may contribute around 3- to 5-fold resistance to permethrin. Under this assumption, we propose that the interaction between *CYP6D1* and *kdr* is synergistic, which is in keeping with the general interactions between two or more homozygous resistance loci observed in house flies.[24]

In conclusion, we observed 11- to 37-fold resistance to pyrethroids conferred by *kdr* mutation, and synergistic effects on resistance caused by a combination of *kdr* and *CYP6D1* over-expression. Moreover, we documented two conserved 1014F-linked mutations in *Vssc*, and verified that 15-bp insert-containing *CYP6D1* alleles identified in houseflies from China and the USA had a single origin.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 31172160), Natural Science Foundation of Beijing Municipality (No.5142014) and Ministry of Science and Technology of the People's Republic of China (2012ZX10004219) to X. Qiu. We thank Dr. Michael Kristensen for kindly sending us the WHO strain of house flies. We are grateful to the respected reviewers for their critical comments and suggestions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Scott JG, Evolution of resistance to pyrethroid insecticides in *Musca domestica*. *Pest Manag Sci* **73**:716–722 (2017).
2 Williamson M, Martinez-Torres D, Hick C and Devonshire A, Identification of mutations in the house fly *para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. *Mol Gen Genet* **252**:51–60 (1996).
3 Liu N and Pridgeon JW, Metabolic detoxification and the *kdr* mutation in pyrethroid resistant house flies, *Musca domestica* (L.). *Pestic Biochem Physiol* **73**:157–163 (2002).
4 Rinkevich FD, Hamm RL, Geden CJ and Scott JG, Dynamics of insecticide resistance alleles in two different climates over an entire field season. *Insect Biochem Mol Biol* **37**:550–558 (2007).
5 Qiu X, Li M, Luo H and Fu T, Molecular analysis of resistance in a deltamethrin-resistant strain of *Musca domestica* from China. *Pestic Biochem Physiol* **89**:146–150 (2007).
6 Sun H, Tong KP, Kasai S and Scott JG, Overcoming super-knock down resistance (*super-kdr*) mediated resistance: multi-halogenated benzyl pyrethroids are more toxic to *super-kdr* than *kdr* house flies. *Insect Mol Biol* **25**:126–137 (2016).
7 Huang J, Kristensen M, Qiao C and Jespersen JB, Frequency of *kdr* gene in house fly field populations: correlation of pyrethroid resistance and *kdr* frequency. *J Econ Entomol* **97**:1036–1041 (2004).
8 Taskin V, Baskurt S, Dogac E and Taskin BG, Frequencies of pyrethroid resistance-associated mutations of *Vssc1* and *CYP6D1* in field populations of *Musca domestica* L. in Turkey. *J Vector Ecol* **36**:239–247(2011).
9 Wang Q, Li M, Pan J, Di M, Liu Q, Meng F *et al.*, Diversity and frequencies of genetic mutations involved in insecticide resistance in field populations of the house fly (*Musca domestica* L.) from China. *Pestic Biochem Physiol* **102**:153–159 (2012).
10 Rinkevich FD, Hedtke SM, Leichter CA, Harris SA, Su C, Brady SG *et al.*, Multiple origins of *kdr-type* resistance in the house fly, *Musca domestica*. *PLoS ONE* **7**:e52761 (2012).
11 Kasai S, Sun H and Scott JG, Diversity of knockdown resistance alleles in a single house fly population facilitates adaptation to pyrethroid insecticides. *Insect Mol Biol* **26**:13–24(2017).
12 Rinkevich FD, Zhang L, Hamm RL, Brady SG, Lazzaro BP and Scott JG, Frequencies of the pyrethroid resistance alleles of *Vssc1* and *CYP6D1* in house flies from the eastern United States. *Insect Mol Biol* **15**:157–167 (2006).
13 Seifert J and Scott JG, The *CYP6D1v1* allele is associated with pyrethroid resistance in the house fly, *Musca domestica*. *Pestic Biochem Physiol* **72**:40–44 (2002).
14 Gao Q, Li M, Sheng C, Scott JG and Qiu X, Multiple cytochrome P450s overexpressed in pyrethroid resistant house flies (*Musca domestica*). *Pestic Biochem Physiol* **104**:252–260 (2012).

15 Liu N and Scott JG, Genetic analysis of factors controlling elevated cytochrome P450, CYP6D1, cytochrome b5, P450 reductase and monooxygenase activities in LPR house flies, *Musca domestica*. *Biochem Genet* **34**:133–148 (1996).
16 Gao J and Scott JG, Role of the transcriptional repressor mdGfi-1 in CYP6D1v1-mediated insecticide resistance in the house fly, *Musca domestica*. *Insect Biochem Mol Biol* **36**:387–395 (2006).
17 Li M, Reid WR, Zhang L, Scott JG, Gao X, Kristensen M *et al.*, A whole transcriptomal linkage analysis of gene co-regulation in insecticide resistant house flies, *Musca domestica*. *BMC Genom* **14**:803 (2013).
18 Qiu XH, Pan J, Li M and Li Y, PCR-RFLP methods for detection of insecticide resistance- associated mutations in the house fly (*Musca domestica*). *Pestic Biochem Physiol.* **104**:201–205 (2012).
19 Kumar S, Stecher G and Tamura K, MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**:1870–1874 (2016).
20 Darriba D, Taboada GL, Doallo R and Posada D, jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* **9**:772–772 (2012).
21 Livak TD and Schmittgen KJ, Analyzing real-time PCR data by the comparative Ct method, *Nat Protoc* **3**:1101–1108(2008).
22 Hall TA, BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**:95–98 (1999).
23 Scott JG, Liu N, Wen Z, Smith FF, Kasai S and Horak CE, House-fly cytochrome P450 CYP6D1: 5′ flanking sequences and comparison of alleles. *Gene* **226**:347–353 (1996).
24 Hardstone MC and Scott JG, A review of the interactions between multiple insecticide resistance loci. *Pestic Biochem Physiol* **97**:123–128(2010).
25 Scott JG, Leichter CA, Rinkevich FD, Harris SA, Su C, Aberegg LC *et al.*, Insecticide resistance in house flies from the United States: resistance levels and frequency of pyrethroid resistance alleles. *Pestic Biochem Physiol* **107**:377–384 (2013).
26 Scott JG and Georghiou GP, Mechanisms responsible for high levels of permethrin resistance in the house fly. *Pestic Sci* **17**:195–206 (1986).
27 Liu N and Scott JG, Increased transcription of *CYP6D1* causes cytochrome P450-mediated insecticide resistance in house fly. *Insect Biochem Mol Biol* **28**:531–535 (1998).
28 Liu N and Scott JG, Genetics of resistance to pyrethroid insecticides in the house fly, *Musca domestica*. *Pestic Biochem Physiol* **52**:116–124 (1995).

