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Diversity and frequencies of genetic mutations involved in insecticide resistance in field populations of the house fly (*Musca domestica* L.) from China

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ABSTRACT

Insecticides have been extensively used for house fly control in China, with dichlorvos and deltamethrin being widely used. Knowledge about the current status of insecticide resistance and the underlying genetic changes is crucial for developing effective fly control strategies. The susceptibility to dichlorvos and deltamethrin, and the frequencies of genetic mutations involved in insecticide resistance were studied in five field populations of the house fly collected across China. Bioassay results show that flies exhibit 14- to 28-fold resistance to dichlorvos and 41- to 94-fold resistance to deltamethrin, indicating that dichlorvos and deltamethrin resistance are common in house fly populations in China. Molecular analysis reveals that flies from the five various locations carry resistance alleles at multiple loci and have diverse allelic types, different relative frequencies and combinations of each allele. Four non-synonymous single nucleotide polymorphisms (SNPs) (i.e. V260L, G342A/V, F407Y) in acetylcholinesterase (*Ace*) and two mutations (W251L/S) in a carboxylesterase (*MdαE7*) were commonly present in the field house flies. The L1014H rather than L1014F mutation in the voltage sensitive sodium channel gene (*Vssc*) was widely distributed in Chinese house flies. *CYP6D1v1*, which confers pyrethroid resistance, was found in all the five tested populations in China, although its frequency in house fly from Shandong province was very low. Our results suggest that resistance monitoring and management of house flies should be customized for a given location.

1. Introduction

The house fly (*Musca domestica*) is a cosmopolitan species and important sanitary pest of humans and animals. It is a mechanical carrier of more than 100 human and animal pathogens [1], including those resistant to antibiotics [2]. In addition, house flies are one of the most serious pests at dairy, horse, sheep, and poultry facilities [3]. House fly activity results in lowered levels of egg and milk production and reduced feed conversion [3]. The control of house flies often depends on insecticides. Organophosphates (OP) and pyrethroids have been used widely as insecticides for house fly control in many countries, including China [4,5], and they continue to be the most frequently used insecticides for house fly control in China.

Extensive use of insecticides has led to resistance in house flies worldwide [6,7]. Resistance is usually associated with a few genes and a limited number of mutations in each gene. One major type of resistance to OPs is target site (acetylcholinesterase, AChE) insensitivity [8]. Six AChE mutations (V260L, A316S, G342A/V, F407Y, and G445A, numbering based on the sequence of aabys AChE, accession number AF281161, corresponding to positions 180, 236, 262, 327 and 365, respectively, if numbering is based on the mature AChE protein sequence [9]) alone or in various combinations play a role in conferring resistance [8–11]. The other major type of resistance (metabolic resistance) to OP insecticides is due to insecticide detoxification by P450 monoxygenases or carboxylesterases. OP resistance in certain strains of *M. domestica* (such as the Rutgers Diazinon-R) is associated with reduction in the carboxyesterase activity of a particular esterase enzyme [12]. Resistance to some organophosphates has been associated with lower levels of carboxylesterase in the resistant strains, and this was named the "mutant aliesterase" mechanism of resistance [13]. In the blow fly, *Lucilia cuprina*, a mutation in a carboxylesterase (*αE7*) was found to lower the activity of general esterase and increase the hydrolysis of the OP insecticide diazinon [14]. The orthologous gene has been identified in house fly and mutations in this gene may be responsible for the "mutant aliesterase" resistance to some OPs in house flies [12].

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Two major forms of pyrethroid resistance in house flies have been observed: target-site (voltage sensitive sodium channel) insensitivity and increased detoxification mediated by P450 dependent monooxygenases [15]. The molecular basis of target insensitivity is well-characterized [16]. Cloning of the full-length voltage sensitive sodium channel gene (*Vssc*) cDNA identified *kdr* (L1014F) [17], *kdr-his* (L1014H) [18] and *super-kdr* (M918T + L1014F) mutations [17]. Over-expression of *CYP6D1* confers pyrethroid resistance in the LPR strain, as well as several populations of house flies in the USA [4,19,20] and China [7].

In a previous study [7], combinations of resistance alleles for *CYP6D1* (*CYP6D1v1*) and *Vssc* (*kdr* and *super-kdr*) were detected to be associated with deltamethrin resistance in a strain of house fly (BJD) collected in Beijing, China and selected with deltamethrin. Although there are many field surveys about the status of insecticide resistance in house flies in China to various types of insecticides [21,22], little is known of the underlying genetic changes that confer resistance in field populations. In this study, we conducted a survey of resistance to the commonly recommended organophosphate (dichlorvos) and pyrethroid (deltamethrin), and investigated the genetic mutations involved in insecticide resistance in Chinese field populations of *M. domestica* across a broad geographical area.

2. Materials and methods

2.1. House flies

House flies were collected from a pig farm or municipal dumps at five different provinces (Table 1) in China during September and October in 2009. Adults (>100 individuals, designated as the parental generation) were caught by sweep net. Collections from each site were transported to the laboratory and allowed to randomly mate in order to establish corresponding laboratory colonies. Field-collected flies were stored in 100% ethanol after laying eggs and kept in −20 °C for molecular analysis. The F2 or F3 progeny flies were used for toxicity bioassays. An insecticide susceptible strain (S-lab) [23], which has been maintained in our lab without exposure to any insecticide, was used as a control strain. Flies were maintained at 25 °C and photoperiod of 12:12 h (L:D). Larvae were reared on wheat bran-based media. Adults were fed powered milk: sugar (1:1) and water *ad libitum*. According to personal communications with the employees at these sampling sites, OPs such as dichlorvos, and pyrethroids such as deltamethrin have been used in fly control.

2.2. Bioassays

Deltamethrin (99%, Roussel UCLAF) or dichlorvos (DDVP, 86.7%, Shandong Dacheng Pesticide Limited Company, China) were dissolved in acetone and applied in 0.928 μL to the thoracic notum of female house flies. Each experiment was replicated at least three times. Each bioassay consisted of twenty-five 3–5 days old house flies per dose and five or six doses that gave >0% and <100% mortality. Control groups received acetone alone. The treated insects were put in 200 mL cups covered with cheese cloth and water and food was 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 the POLO program, after Abbott's correction for control mortality.

2.3. Isolation of genomic DNA and analysis of alleles

Genomic DNA was isolated from the adult flies which had been stored in 100% ethanol and frozen at −20 °C by the method of Rinkevich et al. [4]. Abdomens were discarded prior to the isolation of DNA.

The *Ace* fragment (~800 bp) was amplified by PCR according to Kozaki et al. [8] The PCR products of *Ace* were directly sequenced with S90mdace [8] by BGI Company (Beijing, China).

A region (~750 bp) of the *MdαE7* gene was amplified by PCR in a 20 μL reaction containing 1U Taq polymerase (Takara LA, Takara), 4 pmol of primers (Md_aliest_F and Md_aliest_R [24],) and 2 μL of genomic DNA as a template. The reaction were performed at 95 °C for 3 min, followed by 35 cycles of PCR (95 °C for 30 s, 50 °C, 30 s, 72 °C 1 min) and then extension at 72 °C for 5 min. Pilot experiments showed noisy signals in electropherogram when the PCR products were sequenced directly. Therefore, five individual flies were randomly selected for sequence analysis through TA cloning. The PCR products were ligated into pGEM and sequenced. Three to five clones were sequenced for each individual.

A fragment of *Vssc* (~350 bp) was amplified by PCR using primers of kdr-Diglong F and kdr-Diglong R, and PCR products were directly sequenced with kdr-Diglong F according to the method of Rinkevich et al. [4].

CYP6D1 5′ flanking fragments (~730 bp) were generated by PCR using primers S35 and AS2. Genotypes were determined using a PCR-RFLP method [4].

3. Results and discussion

3.1. Organophosphate and pyrethroid resistance

Insecticide resistance in the house fly has been reported by a number of surveys worldwide [23,25–30]. The susceptibility of house flies in China to two commonly recommended insecticides was determined in this study. LD_{50} values (Table 2) revealed that all five field populations collected from various locations of China differed significantly from the laboratory susceptible strain (S-Lab), showing that field populations of house flies developed resistance to both dichlorvos (14- to 28-fold) and deltamethrin (41- to 94-fold) (Table 2). In China, OPs (commonly DDVP and malathion, both are dimethyl OPs) and pyrethroids (commonly deltamethrin) have been used extensively for public health for more than 20 years (OPs were first introduced in 1952, and pyrethroids by the end of 1970's). Thus, it is not surprising that widespread resistance was found in this study. While it is uncertain whether or not

Table 1
Sampling sites of field collected house flies in 2009.

Populations	Guangdong (GD)	Shanghai (SH)	Shandong (SD)	Beijing (BJ)	Jilin (JL)
Location	Guangzhou, Guangdong Province	Feng Xian, Shanghai Municipality	Jinan, Shandong Province	Chaoyang, Beijing Municipality	Changchun, Jilin Province
Longitude	113°18′E	121°26′E	117°59′E	116°22′E	125°16′E
Latitude	23°06′N	30°54′N	36°39′N	39°59′N	43°53′N
Type of breeding sites	Dump	Pig farm	Dump	Dump	Dump

Table 2
Toxicity of dichlorvos and deltamethirin to the Chinese field collected strains of house flies.

Insecticides	Populations	Generation	*n*	LD_{50} (ng per fly) (95% CL)	Slope (SE)	RR
Dichlorvos	S-lab	Laboratory		6.37+		1
	Guangdong	F2	200	138 (123–154)	4.7 (0.6)	22
	Shanghai	F2	314	181 (164–202)	3.8 (0.4)	28
	Shandong	F3	252	105 (92.2–118)	3.5 (0.4)	16
	Beijing	F3	300	101 (86.4–120)	2.7 (0.3)	16
	Jilin	F2	301	88.8 (78.1–101)	4.8 (0.5)	14
Deltamethrin	S-lab	Laboratory	540	0.84 (0.73–1.02)	2.8 (0.3)	1
	Guangdong	F2	300	78.8 (64.9–98.9)	2.2 (0.2)	94
	Shanghai	F2	237	35.0 (30.1–40.4)	3.7 (0.5)	42
	Shandong	F3	300	43.5 (37.0–51.2)	2.7 (0.3)	52
	Beijing	F2	301	42.0 (34.7–50.6)	3.2 (0.3)	50
	Jilin	F2	304	34.5 (28.1–42.3)	1.8 (0.2)	41

RR: LD_{50} field strain/LD_{50} susceptible strain. +: Data from Ref. [23].

the levels of resistance we found here have led to control failures, there is unquestionably a loss of efficacy associated with resistance to dichlorvos and deltamethrin at these sites.

3.2. AChE mutations

AChE (EC 3.1.1.7) is the target of two of the most commonly used types of insecticides (organophosphate and carbamate insecticides). Five mutations in *Ace* have been identified in resistant house fly stains [8,9,31–36]. To see if any of these mutations were present in Chinese populations of house flies, a fragment of *Ace* was sequenced (Table 3). In all five field fly populations, the V260L, G342A/V and F407Y mutations were identified. However, the A316S mutation that was found in the NG98 strain of house fly [8] collected in Georgia, USA was not observed. At position 260, two genotypes (heterozygous 260 V/L and homozygous 260 L/L) with different frequencies, but no 260 V/V homozygotes, were identified in these field populations. Three genotypes (342G/A, 342A/A, and 342A/V) were observed in individuals from Shandong, Beijing and Jilin, whereas susceptible *Ace* (342G) allele was not detected in Guangdong or Shanghai flies. Notably, the frequency of the F407Y mutation was 1.0 in four populations (Guangdong, Shanghai, Shandong and Jilin) and was 0.98 in the Beijing population (Table 3). Three of these mutations (V260L, G342A and F407Y) were previously identified from the carbamate-resistant strain (SH-CBR) which was collected from Shanghai and selected with propoxur followed by methomyl [33].

The relative roles of different mutations in causing insensitivity to different OPs or/and carbamates have been characterized by functional expression of *Ace* mutants with the baculovirus [9] or *Pichia pastoris* expression systems [31]. Generally, different mutations give varying spectra or levels of insecticide resistance [9,11]. For example, sensitivity to dichlorvos was illustrated to decrease in the order of G342V (58-fold) >G342A (4.3-fold) >F407Y (1.8-fold) >V260L (1.3-fold) [9]. G326V decreased sensitivity by 58-fold to dichlorvos, but 85-fold to bendiocarb [9]. G342 and F407 were suggested to be the key residues controlling insensitivity to trichlorphon [31]. G342A/V and F407Y are highly conserved mutations across a wide range of species, reinforcing the importance of these changes in conferring resistance [9].

Table 3
Frequency of *Ace* genotype (in percentage) in field collected house flies from China.

Population	*n*	Residue Site						
		260		342			407	
		C/GTC (V/L)	CTC (L)	GC/GC (G/A)	GCC (A)	GC/TC (A/V)	TTT (F)	TAT (Y)
Guangdong	34	58.8	41.2	0	55.9	44.1	0	100
Shanghai	69	95.6	4.4	0	4.4	95.6	0	100
Shandong	66	72.7	27.3	4.5	22.7	72.7	0	100
Beijing	58	32.2	67.8	15.6	53.4	31.0	1.7	98.3
Jilin	40	22.5	77.5	22.5	60.0	17.5	0	100

The previously reported A316S mutation was not found. A = alanine, F = phenylalanine, L = leucine, V = valine, Y = tyrosine.

Biochemical studies showed that the 342V mutation gave greater insensitivity to dichlorvos and bendiocarb, compared to other mutations [9]. Thus, it can be speculated that the 342V mutation should provide better protection than 342A for house flies in an environment where methylcarbamates or dimethyl OPs are used. However, 342 V homozygotes were not observed in any of the house fly populations across China, while individuals homozygous for 342A were found at frequencies of 4.4–60%. One explanation for 342 V/V being rare could be that this mutation has a fitness cost in the absence of OP use. Acetylcholinesterase is a key enzyme in the cholinergic synapses and it plays an important role in nerve transmission. To maintain its physiological function, this enzyme can not afford any alterations that reduce its catalytic efficiency. *In vitro* analysis showed that the 342V mutant had a much more marked effect on the properties of the AChE relative to 342A and the other known mutations, with a greater than 10-fold decrease in substrate affinity compared with the wild type enzyme [9]. Therefore, it is logical to believe that house flies with the 342V mutation carry fitness cost, although this hypothesis needs further testing.

It has been elucidated that combined mutations of *Ace* often give higher resistance than single mutations [9,32]. For example, combination of G342V + F407Y causes enhanced (240-fold) insensitivity to dichlorvos [9]. In this study, we found that all flies analyzed carried more than one *Ace* mutation. Six combinations of the four mutations (V260L, G342A, G342V, F407Y) were observed (Table 4). All combinations appear to be an OP/carbamate insensitive type. Among the five house fly populations, the majority of flies could be categorized into combination 1 (260L-342A-407Y) and combination 5 (260V/L-342A/V-407Y). Combination 1 was detected in the 77 M strain, which was established from flies collected in the UK in 1991, and these mutations combined caused 48-fold insensitivity to dichlorvos compared to the wild type [9]. Similarly, a higher level and wider spectrum of resistance was achieved by combining three or four point mutations in *Drosophila melanogaster* [35], with triple mutant (I161V-G265A-F330Y) the most frequent allele in analyzed natural populations [36]. Wheter the different combinations of *Ace* mutations are the result of selection with different insecticides, or are compensatory mutations that evolve to offset the fitness cost associated with resistance mutations is an interesting question that will require further study.

Taken together, we found that mutations causing insensitivity to OPs and carbamates were diverse and at a high frequency.

Table 4
Frequency (in percentage) of combinations of mutations in *Ace* in field collected house flies from China.

	Residue Site			Population				
	260	342	407	Guangdong	Shanghai	Shandong	Beijing	Jilin
Combination 1	L	A	Y	38.2	4.3	22.7	55.2	60.0
Combination 2	L	G/A	Y	0	0	4.5	13.8	17.5
Combination 3	L	G/A	F	0	0	0	1.7	0
Combination 4	V/L	A	Y	17.6	0	0	0	0
Combination 5	V/L	A/V	Y	44.1	95.7	72.7	31.0	17.5
Combination 6	V/L	G/A	Y	0	0	0	0	5.0
n				34	69	66	58	40

A = alanine, F = phenylalanine, L = leucine, V = valine, Y = tyrosine.

Overall, 407Y-carrying haplotypes are nearly fixed, but among them several haplotypes with amino acid variations at 260 and 342 positions are still segregating in the natural populations. It is noteworthy that no individual homozygous susceptible for *Ace* was found in and of the five populations. The detected multiple mutations and high frequency of resistant alleles are consistent with dichlorvos resistance in these strains (Table 2). Future studies on the relative insensitivity and associated fitness caused by different *Ace* point mutation (alone and in combination) will shed light on the relative role and evolutionary dynamics of each mutation or combination of mutations in insecticide resistance.

3.3. MdαE7 mutations

Mutations of *LcαE7* (homology of *MdαE7*) have been documented to confer two forms of resistance in Australian *L. cuprina*, which can be explained by biochemical changes caused by two amino acid substitutions (Gly137Asp and Trp251Leu) [37]. To see if common mutations selected in field-collected Chinese house flies were present, a fragment (~750 bp) of the *MdαE7* gene, including two introns (II and III), was amplified from individual flies from the five populations. Clone sequencing revealed varying (0–57) nucleotide differences among clones from each DNA sample. The presence of many polymorphic sites in certain individuals indicated that these flies might have highly different alleles or contain duplicated copies of *MdαE7*. Gene duplication of the *αE7* gene was identified in *L. cuprina* [38,39]. The presence of gene duplication of *MdαE7* in these field house flies is unknown.

By sequencing clones, we observed two synonymous variations at position 137 encoding Gly. GGT was detected in samples from all the five provinces, whereas GGA was found only in Guangdong and Shanghai. Three non-synonymous substitutions (Trp251Leu, Trp251Ser, Trp251Cys) in *MdαE7* have been documented in the literature [37,40–43], and all except 251Cys were found in this study. The 251Leu mutation was not found in Guangdong. Co-occurrence of 251Ser and 251Leu was detected in Shanghai, Shandong, Beijing and Jilin. The six possible genotypes at this site were observed in the tested 25 individuals (Fig. 1). Because only five individuals from each location were analyzed in this study, the possible differences of the number and frequencies of each genotype among different populations are not clear. No 137Asp mutation, which had been characterized to be diagnostic for diazinon-type resistance in the house fly [12], was detected in any of the 25 individuals sequenced.

It has been previously reported that the Trp251Leu substitution can enhance OP hydrolysis and confer the malathion type of OP resistance in *L. cuprina* [37,38]. Biochemical analyses elucidated that a substitution of the bulky Trp to any of five small amino acids, including Ser and Leu, produced an enzyme with some OP hydrolase and malathion carboxyesterase activities [44]. We observed only one homozygote for 251Trp in the 25 tested flies, indicating that homozygous susceptible individuals for residue 251 were rare. Our results suggest that the malathion-type (dimethyl OP) resistance-conferring mutations have been selected and are widespread in Chinese field house flies.

3.4. Vssc mutations

The results of *Vssc* genotyping are summarized in Table 5. We identified SNPs encoding Leu, Phe or His at position 1014, respectively. Four genotypes (1014 L/L, 1014 L/F, 1014 L/H, 1014 H/H) were found in individual flies (Table 5). The 1014 F/F and H/F genotypes were not detected in this study, which had been observed in house flies from the USA [4].

The susceptible allele (1014L) was found at high frequencies (>65%) in all five populations in this study, similar to the results from Florida, USA during 2003–2004 [45]. In contrast, lower 1014L frequencies were observed in house flies from Danish livestock farms in 1997 (1–54%) [46], and from the eastern United States in 2002 (3–6%) [4]. The frequencies of individuals homozygous for 1014L from the five populations ranged from 44% to 92%. Field collected flies from northern China were 0% to 84% homozygous susceptible [5].

Notably, we found for the first time that *kdr-his* was widely distributed in China. The *kdr-his* allele occurred at a frequency ranging from 4.1% to 32.8%. This mutation was detected in heterozygotes in all the five populations, and *kdr-his* homozygotes were observed in populations from Guangdong and Shandong at a frequency of 2.8% and 9.8% respectively. Interestingly, the *kdr* allele (1014F) which had been observed worldwide in house flies, was detected only in Guangdong and its frequency (4.9%) was lower than *kdr-his* (11.8%). *kdr-his* was also previously detected in the eastern United States, at a frequency ranging from 11% to 79% [4,45].

The 1014F allele has a worldwide distribution and was detected before 1014H [18,47]. A strain (BJD) of house fly collected from a poultry farm in Beijing in 1983 that was selected in the laboratory with deltamethrin was found to have only the *kdr* allele [7]. In addition, the frequency of *kdr* was 25–56% in several field populations from Beijing in 2004 [5]. Thus, the geographically limited and lower frequency of the classic *kdr* than *kdr-his* in Chinese house flies found in this study was unexpected. The factors which result in a likely decreased frequency of 1014F are yet to be explored, but changes in allele frequencies over time have been reported [45]. The 1014H mutation confers lower levels of permethrin resistance than the 1014F substitution [4], although the relative level of protection *kdr-his* provides to deltamethrin is not known. Electrophysiological characteristics of wild-type and 1014L modified *D. melanogaster para* channels co-expressed with tipE in *Xenopus oocytes* demonstrated that 1014F provided a high level of resistance against all permethrin, deltamethrin and DDT, while 1014H most effectively combated deltamethrin [48]. Therefore, it is possible that the 1014H mutation may be better adapted to specific insecticide pressure than the 1014F mutation. We would hypothesize that local selection would make 1014H prevalent, and perhaps gradually replace 1014F to provide a specific protection coupled with a lower fitness cost. The prevalence of the 1014H in Chinese

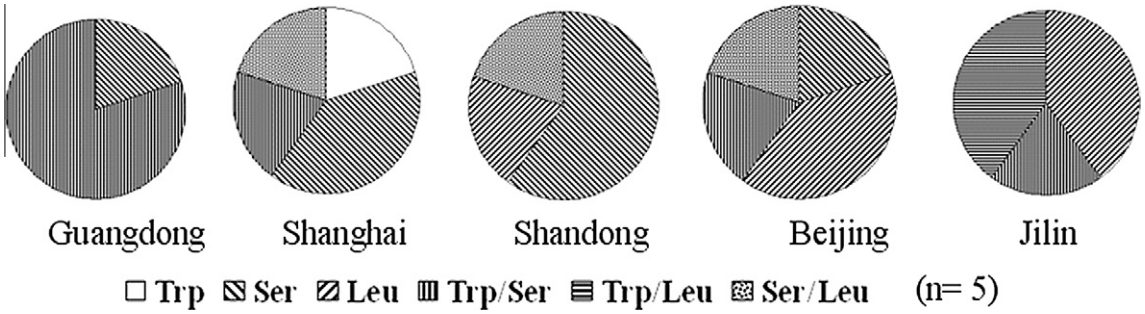

Fig. 1. The percentage of flies with different genotypes at site 251 of MdaE7.

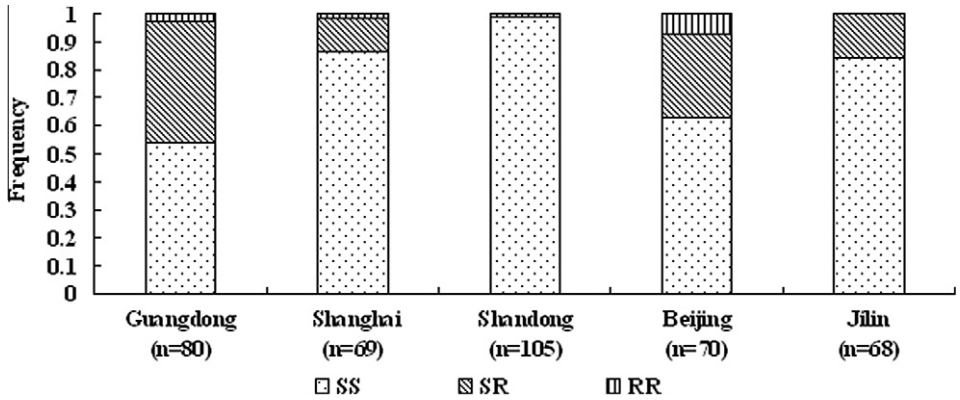

Fig. 2. CYP6D1 genotype and allele frequencies in five field populations of house flies collected across China. RR = resistant homozygote, SS = susceptible homozygote, SR = heterozygote.

Table 5
Vssc genotype (homozygous or heterozygous for L, F, or H) and allele frequencies in five field populations of house flies collected across China.

Population	*n*	L/L (%)	L/F (%)	L/H (%)	H/H (%)	Susceptible (%)	*kdr* (%)	*kdr-his* (%)
Guangdong	72	69.4	9.7	18.1	2.8	83.3	4.9	11.8
Shanghai	61	91.8	0	8.2	0	95.9	0	4.1
Shandong	61	44.3	0	45.9	9.8	67.2	0	32.8
Beijing	56	89.3	0	10.7	0	94.6	0	5.4
Jilin	63	76.2	0	23.8	0	88.1	0	11.9

L = leucine, F = phenylalanine, H = histidine at amino acid residue 1014 in the voltage sensitive sodium channel protein. *kdr* = 1014F, *kdr-his* = 1014H.

populations of house fly presents another case supportive of the hypothesis that the level of protection conferred is not the only factor determining the frequency of resistance alleles [4]. It would be interesting and important to track the evolutionary process, and to evaluate the relative fitness and resistance spectrum of these alleles.

The *super-kdr* allele was found in strains from Asia, Europe [4,7,17] and in field populations from New York [45]. No individuals with the M918T mutation were detected from the house flies sampled in this study ($n = 95$, data not shown), consistent with a survey in China in 2003–2004 [5]. It has been reported that higher levels of resistance are conferred by *super-kdr* than *kdr* or *kdr-his* to most pyrethroids [4]. Thus the lack of *super-kdr* in Chinese populations is good news for control efforts.

3.5. Distribution and frequencies of CYP6D1v1

Enhanced detoxification of insecticides by cytochrome P450 monoxygenases has been reported to confer resistance. Strains of house flies that over-expressed CYP6D1 posses a characteristic 15-bp insert in the 5′ flanking region and this allele was named as *CYP6D1v1* [49–51]. It has been elucidated that this 15 bp insert makes the CYP6D1 promoter in resistant strains bind 5- to 10-fold less *mdGfi*-1 (a transcriptional repressor) through interrupting the putative *mdGfi*-1 binding site within the CYP6D1 promoter found in susceptible strains [52]. The *CYP6D1v1* allele has been detected in the USA, Europe and was also found in the BJD strain collected from Beijing, China [7]. Thus, we explored whether *CYP6D1vl* was selected in the field house fly populations across China. Our results showed that *CYP6D1v1* was discovered (0.5–24.4%, Fig. 2) in all five populations collected in China, although the frequency of *CYP6D1v1* in house flies from Shandong was very low (0.5%). The frequencies of *CYP6D1* susceptible homozygotes ranged from 54% to 99%. Individuals homozygous for *CYP6D1v1* were found in Guangdong, Shanghai and Beijing at low frequencies (1–8%). While CYP6D1 confers resistance to phenoxybenzyl pyrethroids in house flies from many populations in the USA, there is evolutionary plasticity in the evolution of P450 mediated resistance [15], with one strain of permethrin resistant house fly from Japan having P450 monooxygenase mediated pyrethroid resistance that is not due to CYP6D1 [53]. Thus, it is possible that there could be a P450, in addition to CYP6D1, that is involved in deltamethrin resistance in house flies from Asia.

3.6. Combinations of CYP6D1 and Vssc resistance allele

CYP6D1v1 and multiple *Vssc* alleles confer resistance to pyrethroid insecticides in house flies [4,7]. *Vssc* and *CYP6D1* resistance alleles may be present in individual house flies either independently or in various combinations. In this study, ten combinations of *Vssc* and *CYP6D1* genotypes were identified in the five populations (Table 6). Most individuals (44–77%) possessed a combination of susceptible alleles of both *Vssc* and *CYP6D1*. *CYP6D1v1*

Table 6
Frequencies of *VSSC* and *CYP6D1* haplotypes of individual house flies from five populations across China.

Combination	Guangdong (n = 72)	Shanghai (n = 60)	Shandong (n = 61)	Beijing (n = 57)	Jilin (n = 60)
L/L + 6D1S/S	32 (44.4%)	46 (76.7%)	27 (44.3%)	31 (54.3%)	41 (68.3%)
L/L + 6D1S/R	16 (22.2%)	8 (13.3%)		16 (28.1%)	5 (8.3%)
L/L + 6D1R/R	2 (2.8%)	1 (1.7%)		4 (7.0%)	
L/F + 6D1S/S	4 (5.6%)				
L/F + 6D1S/R	3 (4.2%)				
L/H + 6D1S/S	1 (1.4%)	5 (8.3%)	28 (45.9%)	4 (7.0%)	11 (18.3%)
L/H + 6D1S/R	12 (16.7%)			1 (1.8%)	3 (5.0%)
L/H + 6D1R/R				1 (1.8%)	
H/H + 6D1S/S			6 (9.8%)		
H/H + 6D1S/R	2 (2.8%)				

Vssc haplotypes are named based on the amino acid at position 1014 (L, F or H). *CYP6D1* haplotypes are named R (*CYP6D1v1*) or S (susceptible alleles). The possible combinations of 1014L/F + 6D1R/R, 1014F/F + 6D1S/S, 1014F/F + 6D1S/R, 1014F/F + 6D1R/R, 1014L/H + 6D1R/R, 1014F/H + 6D1S/S, 1014F/H + 6D1S/R, 1014F/H + 6D1R/R, 1014H/H + 6D1R/R were not found in this study.

could be present independent of *kdr or kdr-his*, with 25%, 22.6% and 32.4% of individuals carrying *CYP6D1v1* alone in Guangdong, Shanghai, Shandong population, respectively. Similarly, *Vssc* resistance mutations occurred independently of *CYP6D1v1*, with 3.2–57.4% of individuals carrying *kdr-his* or *kdr*, but having a susceptible *CYP6D1* allele. Rinkevich et al. found that most individuals possess some combination of *CYP6D1v1* and a *Vssc* resistance allele (*kdr* or *kdr-his*), hence it was suggested that a combination of *CYP6D1v1* and *kdr* was needed for survival of house flies from the eastern United States [4]. Due to the differences in bioassay methods used in their study compared to our study, it is impossible to compare resistance levels in the two papers, making it difficult to understand if the higher frequencies of *Vssc* and *CYP6D1* resistance alleles is due to higher resistance in the US populations or not. However, the relatively high frequencies of susceptible *Vssc* and *CYP6D1* alleles detected in the Chinese populations (for example Shandong population) reported here seems potentially inconsistent with their levels of deltamethrin resistance (41- to 94-fold), especially given that *kdr* (and *kdr-his*) is inherited as an incompletely recessive trait and *CYP6D1v1* resistance is intermediate [54]. Without an understanding of the level of resistance conferred by *kdr-his* (homozygotes and heterozygotes) to deltamethrin it is difficult to predict if another mechanism may contribute to the relatively high deltamethrin resistance observed in these house fly populations (Table 2). Data in Table 6 also revealed that house flies from different areas possessed distinct patterns of combination of *Vssc* and *CYP6D1* genotypes, the most diverse in Guangdong and the least in Shandong. These combinations of alleles likely reflect the differences in local environments and insecticide application history at each location. The lack of uniformity in resistance genotypes has been documented in house flies from the eastern United States [4].

4. Conclusion

Our results reveal widespread and multiple resistance patterns in field populations of the house fly in China. Molecular investigations demonstrate that individual field flies often have resistance alleles at multiple loci and exhibit diverse combinations of each allele. Multiple mechanisms and mutations render the organisms well adapted to varying environments and insecticide pressure, implying a potential problem in house fly control practice with insecticide use. Common mutations, which existed in all examined resistance-conferring genes (*Ace*, *MdaE7*, *Vssc* and *CYP6D1*) of house flies collected from different geographical locations, calls for further detailed studies addressing the number of the evolutionary origins for different resistance alleles. The heterogeneity of resistance genotypes and the differences in relative frequency of multiple resistance alleles in different populations in China suggest that resistance monitoring and management should be customized for each location. The relatively high frequencies of susceptible *Vssc* and *CYP6D1* alleles detected in Chinese populations provide the possibility of developing control strategies designed to maintain susceptibility through the use of different insecticides in a mosaic or rotation. Sequencing of the house fly genome would greatly facilitate our understanding of the evolution of insecticide resistance [3] and would help to identify what other genes (if any) carry mutations responsible for insecticide resistance.

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References

[1] B. Greenber, Flies and Disease, Sci. Amer. 213 (1965) 92–99.
[2] L. Macovei, B. Miles, L. Zureki, Potential of houseflies to contaminate ready-to-eat food with anti biotic-resistant enterococci, J. Food. Prot. 71 (2008) 435–439.
[3] G. Scott, N. Liu, M. Kristensen, A.G. Clark, A Case for Sequencing the Genome of *Musca domestica* (Diptera: Muscidae), J. Med. Entomol. 46 (2009) 175–182.
[4] F.D. Rinkevich, L. Zhang, R.L. Hamm, S.G. Brady, BP. Lazzaro, J.G. Scott, Frequencies of the pyrethroid resistance alleles of Vssc1 and CYP6D1 in house flies from the eastern United States, Insect. Molec. Biol. 15 (2006) 157–167.
[5] X.M. Cao, F.L. Song, T.Y. Zhao, Y.D. Dong, C.X. Sun, B.L. Lu, Survey of deltamethrin resistance in house flies (*Musca domestica*) from urban garbage dumps in northern China, Environ. Entomol. 35 (2006) 1–9.
[6] J. Keiding, Review of the global status and recent development of insecticide resistance in field populations of the housefly *Musca domestica* (Diptera: Muscidae), B. Entomol. Res. 89 (1999) S9–S67.
[7] X. Qiu, M. Li, H. Luo, T. Fu, Molecular analysis of resistance in a deltamethrin-resistant strain of *Musca domestica* from China, Pestic. Biochem. Physiol. 89 (2007) 146–150.
[8] T. Kozaki, S.G. Brady, J.G. Scott, Frequencies and evolution of organophosphate insensitive acetylcholinesterase alleles in laboratory and field populations of the house fly *Musca domestica* L. Pestic, Biochem. Physiol. 95 (2009) 6–11.
[9] S.B. Walsh, T.A. Dolden, G.D. Moores, M. Kristensen, T.D. Lewis, A.L. Devonshire, M.S. Williamson, Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance, Biochem. J. 359 (2001) 175–181.
[10] T. Kozaki, T. Shono, T. Tomita, Y. Kono, Polymorphism in the acetylcholinesterase gene of the housefly, *Musca domestica* L. (Diptera: Muscidae), Appl Entomol Zool 36 (2001) 377–380.
[11] T. Kozaki, T. Shono, T. Tomita, Y. Kono, Amino acid substitutions conferring insecticide insensitivity in *Ace*-paralogous acetylcholinesterase, Pestic. Biochem. Physiol. 85 (2006) 123–132.

[12] C. Claudianos, R.J. Russell, J.G. Oakeshott, The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly, Insect Biochem. Mol. Biol 29 (1999) 675–686.
[13] F.J. Oppenoorth, Biochemistry and genetics of insecticide resistance. In: Kerkut GA, Gilbert LI, (Ed.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Pergamon Press, Oxford. (1985) pp. 731–774.
[14] R.D. Newcomb, P.M. Campbell, R.J. Russell, J.G. Oakeshott, cDNA cloning, baculovirus expression and kinetic properties of the esterase, E3, involved in organophosphorus resistance in *Lucilia cuprina*, Insect Biochem. Mol. Biol. 27 (1997) 15–25.
[15] J.G. Scott, S. Kasai, Evolutionary plasticity of monooxygenase-mediated resistance, Pestic. Biochem. Physiol. 78 (2004) 171–178.
[16] K. Dong, Insect sodium channels and insecticide resistance, Invert. Neurosci. 7 (2007) 17–30.
[17] M.S. Williamson, D. MartinezTorres, C.A. Hick, A.L. Devonshire, Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Molec. Gen. Genet. 252(1996) 51-60.
[18] N.N. Liu, J.W. Pridgeon, Metabolic detoxification and the *kdr* mutation in pyrethroid resistant house flies, *Musca domestica* (L.), Pestic. Biochem. Physiol. 73 (2002) 157–163.
[19] S. Kasai, J.G. Scott, Overexpression of cytochrome P450CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia, Pestic. Biochem. Physiol. 68 (2000) 34–41.
[20] J. Seifert, J.G. Scott, The *CYP6D1v1* allele is associated with pyrethroid resistance in the house fly, *Musca domestica*, Pestic. Biochem. Physiol. 72 (2002) 40–44.
[21] J. Yin, Y. Song , H. Wu, X. Yu, J. Yu, Investigation on the resistance of housefly in Huangshi city from 1996 to 2007, Chin. J. Vector Biol. Con. 19 (2008) 429–431.
[22] J. Wu, H. Chu, W. Yang, Z. Chen, Resistance of *Musca domestica* to insecticides and control measures in Jiangsu province from 2004 to 2006, Chi. J. Hyg. Insect. Equip. 113 (2007) 424-426.
[23] Y. Jin, G. Feng, Toxicity of organophosphate insecticides to pyrethroid resistant house flies *Musca domestica vinica*, Acta Entomologia Sinica 44 (2001) 182–186.
[24] J.G. Scott, L. Zhang, The house fly aliesterase gene (MdalphaE7) is not associated with insecticide resistance or P450 expression in three strains of house fly, Insect Biochem. Mol. Biol. 33 (2003) 139–144.
[25] M. Kristensen, A.G. Spencer, J.B. Jespersen, The status and development of insecticide resistance in Danish populations of the housefly *Musca domestica* L, Pest Manag. Sci. 57 (2001) 82–89.
[26] P.E. Kaufman, J.G. Scott, D.A. Rutz, Monitoring insecticide resistance in house flies (Diptera: Muscidae) from New York dairies, Pest Manag. Sci. 57 (2001) 514–521.
[27] L. Pap, R. Farkas, Monitoring of resistance of insecticides in-house fly (*Musca domestica*) populations in hungary, Pestic. Sci. 40 (1994) 245–258.
[28] J.G. Scott, T.G. Alefantis, P.E. Kaufman, D.A. Rutz, Insecticide resistance in house flies from caged-layer poultry facilities, Pest Mana. Sci. 56 (2000) 147–153.
[29] P.A. Chapman, C.P. Morgan, Insecticide Resistance in *Musca domestica* L from Eastern England, Pestic. Sci. 36 (1992) 35–45.
[30] G.R. Acevedo, M. Zapater, A.C. Toloza, Insecticide resistance of house fly, *Musca domestica* (L.) from Argentina, Parasitol. Res. 105 (2009) 489–493.
[31] C.S. Kim, W.T. Kim, K.S. Boo, S. Kim, Mutagenesis and expression of the acetylcholinesterase gene from a strain of *Musca domestica*: The change from a drug-resistant to a sensitive enzyme, Mol. Cells 15 (2003) 208–215.
[32] M. Kristensen, J. Huang, C.L. Qiao, J.B. Jespersen, Variation of *Musca domestica* L. acetylicholinesterase in Danish housefly populations, Pest Manag. Sci. 62 (2006) 738–745.
[33] L.M. Tao, M.G. Shi, J.Z. Yuang, P.J. Zhuang, C.X. Zhang, Z.H. Tan, Resistance pattern and point mutations of insensitive acetylcholine sterase in a carbamate-resistant strain of housefly (*Musca domestica*), Pestic. Biochem. Physiol. 86 (2006) 1–6.
[34] T. Hotelier, V. Negre, P. Marchot, A. Chatonnet, Insecticide resistance through mutations in cholinesterases or carboxylesterases: data mining in the ESTHER database, J. Pestic. Sci. 35 (2010) 315–320.
[35] M. Shi, A. Lougarre, C. Alies, I. Fremaux, Z. Tang, J. Stojan, D. Fournier, Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance, BMC Evolutionary Biology 4 (2004) 5.
[36] P. Menozzi, M. Shi, A. Lougarre, Z. Tang, D. Fournier, Mutations of acetylcholinesterase which confer insecticide resistance in *Drosophila melanogaster* populations, BMC Evol. biol. (4) (2004) 4.
[37] P.M. Campbell, J.L. Yen, A. Masoumi, R.J. Russell, P. Batterham, J.A. McKenzie, Cross-resistance patterns among *Lucilia cuprina* (Diptera: Calliphoridae) resistant to organophosphorus insecticides, J. Econ. Entomol. 91 (1998) 367–375.
[38] K.A. Smyth, T.M. Boyce, R.J. Russell, J.G. Oakeshott, MCE activities and malathion resistances in field populations of the Australian sheep blowfly (*Lucilia cuprina*), Heredity 84 (2000) 63–72.
[39] R.D. Newcomb, D.M. Gleeson, C.G. Yong, R.J. Russell, J.G. Oakeshott, Multiple mutations and gene duplications conferring organophosphorus insecticide resistance have been selected at the Rop-1 locus of the sheep blowfly, *Lucilia cuprina*, J. Mol. Evol. 60 (2005) 207–220.
[40] V. Taskin, M. Kence, B. Gocmen, Determination of malathion and diazinon resistance by sequencing the *Md* alpha E7 gene from Guatemala, Colombia, Manhattan, and Thailand housefly (*Musca domestica* L.) strains, Russ. J. Genet. 40 (2004) 377–380.
[41] V. Taskin, M. Kence, The genetic basis of malathion resistance in housefly (*Musca domestica* L.) strains from Turkey, Russ. J. Genet. 40 (2004) 1215–1222.
[42] C.J. Hartley, R.D. Newcomb, R.J. Russell, Yong CG, Stevens JR, Yeates DK, J.G. Oakeshott, Amplification of DNA from preserved specimens shows blowflies were preadapted for the rapid evolution of insecticide resistance, Proc. Natl. Acad. Sci. USA 103 (2006) 8757–8762.
[43] F. Gacar, V. Taskin, Partial base sequence analysis of Md alpha E7 gene and ali-esterase enzyme activities in field collected populations of house fly (*Musca domestica* L.) from Mediterranean and Aegean Regions of Turkey, Pestic. Biochem. Physiol. 94 (2009) 86–92.
[44] R.J. Russell, R. Heidari, A.L. Devonshire, B.E. Campbell, K.L. Bell, S.J. Dorrian, J.G. Oakeshott, Hydrolysis of organophosphorus insecticides by in vitro modified carboxylesterase E3 from *Lucilia cuprina*, Insect Biochem. Mol. Biol. 34 (2004) 353–363.
[45] F.D. Rinkevich, R.L. Hamm, C.J. Geden, J.G. Scott, Dynamics of insecticide resistance alleles in house fly populations from New York and Florida, Insect Biochem. Mol. Biol. 37 (2007) 550–558.
[46] J. Huang, M. Kristensen, C.L. Qiao, J.B. Jespersen, Frequency of *kdr* gene in house fly field populations: Correlation of pyrethroid resistance and *kdr* frequency, J. Econ. Entomol. 97 (2004) 1036–1041.
[47] M.S. Williamson, I. Denholm, C.A. Bell, A.L. Devonshire, Knockdown resistance (*kdr*) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*), Molec. Gen. Genet. 240 (1993) 7–22.
[48] M.J. Burton, I.R. Mellor, I.R. Duce, T.G.E. Davies, L.M. Field, M.S. Williamson, Differential resistance of insect sodium channels with *kdr* mutations to deltamethrin permethrin and DDT, Insect Biochem. Mol. Biol. 41 (2011) 723–732.
[49] F.F. Smith, J.G. Scott, Functional expression of house fly (*Musca domestica*) cytochrome P450 CYP6D1 in yeast (*Saccharomyces cerevisiae*), Insect Biochem. Mol. Biol. 27 (1997) 999–1006.
[50] N. Liu, J.G. Scott, Increased transcription of CYP6D1 causes cytochrome P450 mediated insecticide resistance in house fly, Insect Biochem. Mol. Biol. 28 (1998) 531–535.
[51] J.G. Scott, N. Liu, Z.M. Wen, F.F. Smith, S. Kasai, C.E. Horak, House-fly cytochrome P450 CYP6D1: 5 'flanking sequences and comparison of alleles, Gene 226 (1999) 347–353.
[52] J.W. Gao, J.G. Scott, Role of the transcriptional repressor mdGfi-1 in CYP6D1v1-mediated insecticide resistance in the house fly, *Musca domestica*., Insect Biochem. Mol. Biol. 36 (2006) 387–395.
[53] T. Shono, S. Kasai, E. Kamiya, Y. Kono, J.G. Scott, Genetics and mechanisms of permethrin resistance in the YPER strain of house fly, Pestic. Biochem. Physiol. 73 (2002) 27–36.
[54] N. Liu, J.G. Scott, Inheritance of CYP6D1-mediated pyrethroid resistance in house fly (Diptera: Muscidae), J. Econ. Entomol. 90 (1997) 1478–1481.