

# Polymorphism and Distribution of *Ace* Gene Involved in the Resistance of *Musca Domestica* to Organophosphates in Guizhou Province of China

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## Research

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## Abstract

Acetylcholinesterase is the primary target of organophosphates (OPs) and carbamates in insects. As gene mutation has been verified as an important mechanism of insecticide resistance in insects, in this study, we investigated the status of OPs resistance and the polymorphism of *ace* gene (that encodes acetylcholinesterase) in housefly (*Musca domestica* L) field populations in Guizhou Province, China. Bioassays showed that the houseflies had 142.16–303.54-fold resistance to dichlorvos (DDVP) and 122.13–363.98-fold resistance to temephos. Molecular analysis revealed resistance-causing mutations of *ace* gene at loci of 260, 342 and 407 in the housefly populations, with a total frequency of 55%, 100% and 94%, respectively. In addition, 11 combinations of *ace* mutation were observed across the detected populations. The most frequently detected combination was L/V+A/V+Y, followed by L+A+Y and L/V+A+Y. No significant relationship was found between single mutation/combination mutations and DDVP resistance. These results indicate that the OPs resistance is prevalent among the housefly populations in Guizhou Province, with a similar pattern of allele mutation of *ace* across China. The target resistance can not fully account for the resistance of houseflies to OPs in Guizhou.

## Introduction

Houseflies, *Musca domestica*, are a transmission vector of more than 100 human and animal pathogens, including bacteria, parasites, viruses, and rickettsia[1]. Until now, chemicals with strong insect lethality have been widely used to control houseflies. Currently, the use of harmful insecticides has amounted to 1.8 billion kilograms in China and the amount of hazardous insecticide use in the USA has reached 31.75 million kilograms in urban settings[2, 3]. Although the chemical insecticides can effectively control houseflies and the diseases they carry, the long-term, extensive use of insecticides can lead to the development of insecticide resistance in houseflies[4, 5]. A study in 48 Chinese cities has suggested that the houseflies have developed a strong resistance to several common insecticides, such as dichlorvos (DDVP), temephos and deltamethrin[6]. This indicates that the resistance to organophosphate (OP) insecticides including DDVP and temephos has become a serious concern in the control of local houseflies in China.

It has been revealed that the resistance of houseflies to OPs is mainly attributed to the insensitive target-site acetylcholinesterase (AChE)[3, 7, 8]. AChE (EC 3.1.1.7), encoded by the *ace* gene, is the key enzyme of the cholinergic system in insects[9]. OPs and carbamates (CBs) can irreversibly bind with AChE and cause phosphorylation or carbamylation of the enzyme at the active serine site, leading to the accumulation of acetylcholine at synapses[10, 11]. This in turn leaves the acetylcholine receptor permanently open, resulting in the death of the insect[10]. However, modification or mutation of the *ace* gene can change the structure of AChE, thereby reducing or eliminating the binding affinity of insecticides at the target-site and inducing the resistance to OPs and CBs[8, 12, 13]. For houseflies, there is only a single *ace* gene in the whole genome[14]. Currently, six mutations (V260L, A316S, G342A/V, F407Y and G445A) have been verified to be present separately or in combination within a particular active site of AChE that can change the current of the catalytic triad and restrict the binding ability to insecticides, thus being responsible for the development of resistance [8, 15, 16].

In Guizhou Province located in the southwest part of China, the insecticide resistance of houseflies has been reported only by one study (1999) [17]. With the campaign of establishing the national sanitary cities across China, the resistance of houseflies to propoxur and DDVP has been reported in some major cities of Guizhou, such as Anshun, Guiyang and Xingyi, in recently years[18–20]. However, little is known about the underlying genetic variability of AChE that confers the resistance to OPs and CBs. In this study, we set out to explore the resistance status of houseflies to OPs and investigate the genetic mutations of *ace* in the housefly (*Musca domestica* L) field populations across Guizhou Province.

## Materials And Methods

### Collection and rearing of houseflies

The housefly observation, adult housefly collection, and field studies in Guizhou Province were conducted from April 2018 to December 2019. Adult *M. domestica* houseflies were collected in urban or suburban areas distributed in 7 different regions in Guizhou Province (Fig. 1). In the current study, about 100 houseflies were collected by the sweep net mainly in waste transfer stations and refused dump of the farmer markets or old residential buildings, and then mixed to represent a local population. All of the field-collected populations were routinely reared with a mixture of milk powder and granulated sugar at a ratio of 1:1 in an appropriate amount of water, at a constant indoor temperature of 25±1 °C, with humidity of 70±10% under a 12 h light/12 h dark cycle. Housefly eggs were laid in wheat bran (100 g), with milk powder (5 g), granulated sugar (5 g) and water (130 mL), as the random mating of houseflies occurred in the breeding cage. The eggs were hatched to pupate on the dry surface of the feed within 7 days. The adult susceptibility bioassay and molecular tests were conducted using the first-generation, field-collected houseflies of 3–5 days old with a similar body weight of 18–22 mg.

### Bioassays

Bioassays were performed in accordance with the Bioassay Methods for *Musca domestica* (GB/T 26350 (2010)) released by the Standardization Administration of the People's Republic of China[21].

The 97.6% DDVP and 87.4% temephos solutions provided by the Chinese Center for Disease Control and Prevention (CDC) were first dissolved in acetone and then half-diluted to a series of concentrations. The customized 0.35 µL pipettes purchased from Nanjing Agricultural University (Nanjing, China) were used to conduct bioassays for each housefly population. The assays at each concentration of DDVP and temephos were performed in three replicates, with acetone being used as a negative control. The regression equations were obtained using the mortality 24 h after drug exposure in each test recorded after. The LD<sub>50</sub> (lethal dose, 50%) value was calculated based on the corresponding insecticide concentrations. The mortality of the control group was below 5%. The resistance ratio (RR) was obtained from dividing the LD<sub>50</sub> of different populations by the LD<sub>50</sub> of the susceptible housefly.

## Extraction of genomic DNA

The genomic DNA of houseflies was extracted according to the description by Yang et al.[20]. A whole adult housefly was homogenized in 300  $\mu$ L extraction buffer in a 1.5 mL Eppendorf tube, and proteinase K (50  $\mu$ g) was added. The homogenates were incubated at 56 °C overnight. Then a solution (300  $\mu$ L) of chloroform and isoamyl alcohol (24:1) was added. After shaking violently for several times, the samples were centrifuged at 10000 rpm/min at 4 °C for 10 min. The supernatant was transferred to a new tube, and a 0.1-fold volume of 3 M sodium acetate (4 °C) and a 2-fold volume of pure ethanol were added to precipitate DNA for 2 h. Afterwards, the supernatant was discarded after centrifugation at 12000 rpm/min for 5 min at 4 °C, and the DNA was washed twice with 70% ethanol (1 mL). Finally, the DNA was resolved with ddH<sub>2</sub>O and stored at -20 °C until use.

## Amplification and sequencing of *ace* gene

The *ace* gene fragment was amplified by PCR in a 25- $\mu$ L reaction system containing 12.5 $\mu$ L of Premix Taq™ (TAKARA Bio Inc., Shiga, Japan), 8.5  $\mu$ L of ddH<sub>2</sub>O, 2  $\mu$ L of DNA template, and 1  $\mu$ L of each of 10  $\mu$ M forward primer S90MdAce (5'-CATCT AAAAC CGATC AGGAC CATT T AATAC-3') and 10  $\mu$ M reverse primer AS89MdAce (1 $\mu$ L) (5'-TCATC TTAA CATT CCAAT CAGAA TATCG-3') [22]. PCR reactions were run in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) under the following conditions: 94 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 10 min [16]. PCR products (5  $\mu$ L) were identified and bi-directionally sequenced by Majorbio Bio Tech Co., Ltd. (Shanghai, China). Homozygous and heterozygous individual houseflies were identified by manual inspection of the sequencing and chromatograms. .

## Statistical analysis

The LD<sub>50</sub> of insecticides in the tested populations was calculated with a probit analysis based on the recorded concentration-mortality data. Association between *ace* genotype and phenotypical resistance was analyzed with the Spearman's correlation coefficient. All analyses were conducted using SPSS 22.0 software (IBM, Armonk, NY, USA).

## Results

### Resistance of housefly populations to insecticides

The resistance of houseflies to DDVP and temephos was analyzed in 5 and 4 populations, respectively. The LD<sub>50</sub> values of DDVP and temephos ranged 0.56865-1.21415  $\mu$ g/housefly and 13.8005-41.12605  $\mu$ g/housefly, respectively. The two OPs showed a high resistance ratio, 122.13~363.95-fold for temephos and 142.16~303.54-fold for DDVP (Table 1).

### The distribution and frequency of *ace* mutations

A total of 237 PCR products of *ace* gene were obtained and sequenced. Nonsynonymous mutations were detected only at the loci of V260, G342, and F407 among the field-collected populations (Fig. 2). The frequencies of these mutant alleles were 55%, 100%, and 94% respectively. In addition, 23.63% and 4.24% of the houseflies were homozygous for L260 and V260 respectively, whereas 72.13% were heterozygous. Only 2 mutation genotypes (A/V or A/A) were detected at the locus 342, and the frequencies were 63.94% and 36.06%, respectively. At the locus 407, three genotypes (F/F, F/Y and Y/Y) were found and 91.7% of the houseflies were homozygotes for Y/Y (Table 2). The KL and GY were two top populations for the simultaneous mutation frequency of the three loci, while the CH and ZY populations had the lowest simultaneous mutation frequency of the three loci.

Eleven combinations of *ace* mutation were detected among the populations (Fig. 3). The combination of L/V+A/V+Y had the highest frequency (59.9%), followed by the L+A+Y (22.8%) and L/V+A+Y (8%). The ZY population had seven combinations, the AS population had five combinations, the LPS, HS and GY populations each had four combinations, and the CS population had the least 2 combinations. No distinct differences among the populations had been found.

### Relationship between *ace* mutation and insecticide resistance

According to the bioassay results of DDVP in 5 field populations, we conducted the correlation analysis to explore the relationship between single or combined mutations of *ace* gene and DDVP resistance phenotype (Fig.4). However, no significant correlation was detected between them.

## Discussion

In China, OPs and CBs have been widely used in the public health field since 1950s, because of the low costs and high efficiency[23]. The development of insecticide resistance during housefly control has been widely reported in China. Previous studies on houseflies collected from Xingyi City and Anshun City in Guizhou have also shown a high resistance to OPs and CBs[18-20]. The current bioassay results further revealed the presence of a high resistance to OPs in houseflies in Guizhou Province.

The *ace* gene of housefly has been demonstrated as an indicator for the response to OPs and CBs stress[22]. In this study, 6 alleles at loci 260, 342, and 407 were detected in all 7 housefly populations in Guizhou Province. It is noteworthy that the frequency of allele mutation was 100% at locus 342 and 94% at locus 407. This is consistent with previous report on *ace* gene in houseflies in Guangdong and Shanghai, China[24]. Previous studies have shown that both G342A/V and F407Y mutations, which are located close to the active-site triad of AChE at the base of the gorge, can cause resistance to OPs and CBs, with G342A/V mutation likely to affect the orientation of the catalytic serine and F407Y decreasing the available space within the acyl-binding pocket[8].

Meanwhile, the 342V mutation has a much more significant effect on the properties of AChE than 342A, which causes a >10-fold decrease in substrate affinity compared to the wild-type enzyme[8,25]. In the present study, the frequency of the 342A/V genotype (61.9%) was higher than that of the 342A/A genotype (36.1%), which might suggest that the 342A/V genotype confers higher resistance to OPs. Interestingly, 342V homozygotes have not been detected in the present or previous studies, either in China or in other countries outside of China[16,22,24]. It is further suggested that houseflies with the 342V mutation carry higher fitness cost[24,26]. For mutations at the locus 407, 407Y conferred 1.8-fold resistance to DDVP[8], and the genotype 407Y/Y (91.7%) ranked top in frequency among three genotypes in all detected populations. Similar results have been found in other places in China[24].

It has been found that combined mutations of *ace* often confer higher resistance than single mutations[8,25]. Here, 11 combinations of three mutations (V260L, G342A/V, and F407Y) were observed in the field populations. Houseflies with any of these combinations showed resistance to OPs and CBs. V/L+A/V+Y and L+A+Y were the top 2 combinations in frequency, which caused 48-fold resistance to DDVP compared to the wild-type[8]. This mutation pattern of *ace* has also been found in other regions of China [24], and in Japan, Turkey[22] and Europe[25], but not in the USA[16], which indicates that the resistance alleles vary globally in different regions. The combination V/L + A + Y has been detected in Guangdong Province in south of China, but not in Shanghai, Shandong, Beijing, or Jilin, which are located in east and north of China[24]. In the present study, the combination V/L + A + Y was found in the housefly populations, and also ranked third in frequency. This suggests that this pattern of mutation is common in south China.

There was no distinct distribution division of *ace* resistance alleles either for single mutation or combined mutations, among the different field populations in Guizhou. This might be the result of two evolutionary forces (mutation and migration). The allelic diversity in field populations might enable them survive against the different insecticides[27]. Correspondingly, application of a multiplicity of treatments on field populations might result in the population heterogeneity, composed of a mixture of different alleles.

Although the current study showed a high resistance phenotype, no significant relationship was found between either single mutation or combined mutations and DDVP resistance. This indicates that target resistance is not the only mechanism involved in the housefly resistance to OPs in Guizhou. Further studies are needed to clarify the resistance mechanism.

## Conclusion

In conclusion, the resistance to OPs exists widely in houseflies in Guizhou Province. A similar pattern of allele mutation of *ace* has been found across China. The findings imply a need for monitoring on insecticide use to avoid the abuse of insecticides. It also demonstrates that target resistance is not enough to account for housefly resistance to OPs in Guizhou.

## Declarations

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### Authors` Contribution

WJH and TWL designed the whole study, genetic analysis and wrote the manuscript. MR and YX performed housefly collection, bioassay and genotyping. LQG and WYM performed housefly collection and data processing. CJZ performed data processing and genetic analysis. WG provided technical support in equipment use. All authors read and approved the final manuscript.

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### Ethics approval and participant consent

All the field studies on housefly were approved by the Institutional Animal Care and Use Committee of Guizhou Medical University (China).

### Consent for publication

No applicable.

### Availability of data and material

No.

## Competing interests

The authors declare that they have no competing interests.

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## Tables

**Tab. 1** Organophosphates resistance of the field-collected houseflies in Guizhou Province

Insecticide	Population	n	LD <sub>50</sub> & 95% CI (µg/housefly)	Slope	RR
DDVP	AS	450	0.7231 (0.6468~0.8071)	2.56	180.78
	GY	450	1.21415 (0.90335~1.6856)	2.621	303.54
	HS	450	0.56865 (0.52115~0.69685)	3.538	142.16
	KL	450	0.72205 (0.6538~0.79625)	2.58	180.51
	ZY	450	0.63315 (0.55825~0.721)	3.712	158.29
	Susceptible	-	0.004 (0.003~0.005)	1.052	1
Temephos	AS	450	24.01385 (11.1251~35.161)	2.16	212.51
	GY	450	17.26095 (11.1251~26.95385)	2.356	152.75
	HS	450	13.8005 (11.64275~17.2522)	1.858	122.13
	KL	450	41.12605 (33.2248~54.005)	1.326	363.95
	Susceptible	-	0.113 (0.054~0.178)	0.894	1

**Tab. 2** Distribution, polymorphisms and mutation frequencies of *ace* alleles and genotypes at locus 260, 342 and 402 in houseflies in Guizhou province, China

Population	n	Loci and Genotype Frequency %									Mutation Allele Frequency %				Simultaneous Mutation Frequency of the Three Loci %
		260			342			407			260	342	407		
		L/V	L/L	V/V	A/V	A/A	F/Y	Y/Y	F/F	L	A	V	Y		
AS	31	77.4	12.9	9.7	71.0	29.0	0	90.3	9.7	51.6	64.5	35.5	90.3	46.6	
CS	53	54.7	45.3	0	54.7	45.3	0	100.0	0	27.4	72.7	27.3	100.0	27.4	
GY	30	77.7	22.3	0	46.7	53.3	6.7	93.3	0	72.7	76.7	23.7	96.7	70.3	
HS	33	81.8	18.2	0	81.8	18.2	15.2	81.8	3	59.1	59.1	40.9	89.4	52.8	
KL	30	53.3	46.7	0	56.7	43.3	3.3	96.7	0	73.8	71.7	28.3	98.4	72.6	
LPS	30	93.3	6.7	0	70.0	30.0	0	100.0	0	53.4	65.0	35.0	100.0	53.4	
ZY	30	66.7	13.3	20	66.7	33.3	6.7	80.0	13.3	46.7	66.7	33.3	83.4	38.9	
total	237	72.13	23.63	4.24	63.94	36.06	4.56	91.69	3.71	55	68	32	94	51.7	

## Figures



**Figure 1**

Sampling sites of field houseflies in Guizhou Province, China. Anshun (AS), 26°16'N 105°59'E; Chishui (CS), 28°34'N 105°42'E; Guiyang (GY), 26°38'N 106°47'E; Huishui (HS), 26°07'N 106°39'E; Kaili (KL), 26°35'N 107°58'E; Liupanshui (LPS), 26°35'N 104°49'E; Zunyi (ZY), 27°43'N 106°54'E. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

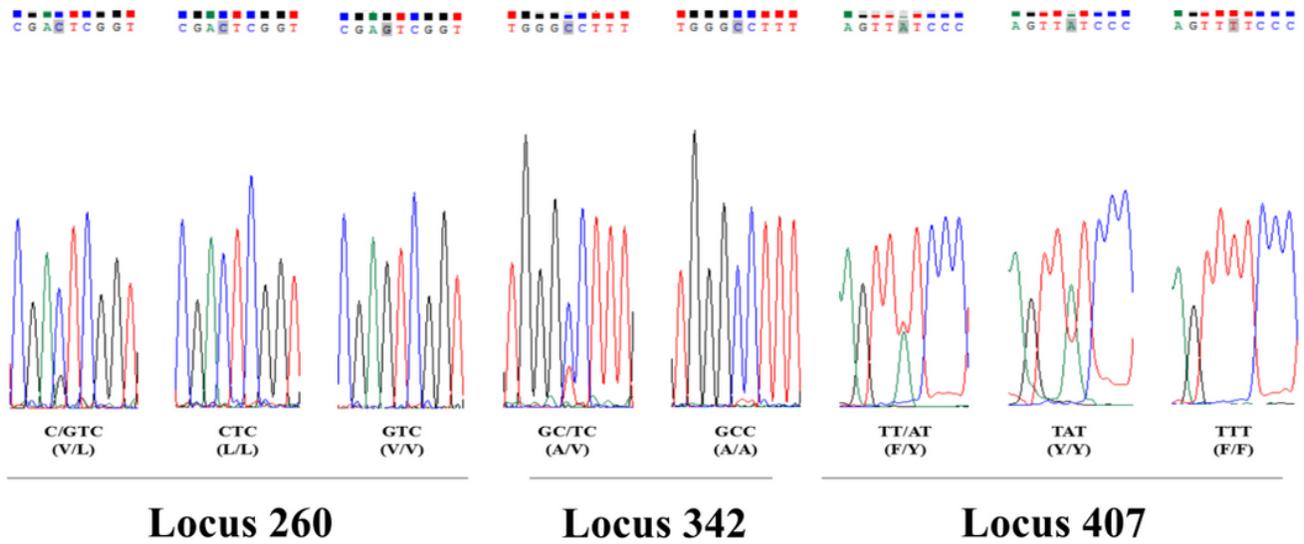


Figure 2

Chromatograms of eight ace genotypes detected at locus 260, 342 and 407 in *Musca domestica* from Guizhou province. AFLVY in brackets are abbreviations for alanine, phenylalanine, leucine, valine and tyrosine respectively.

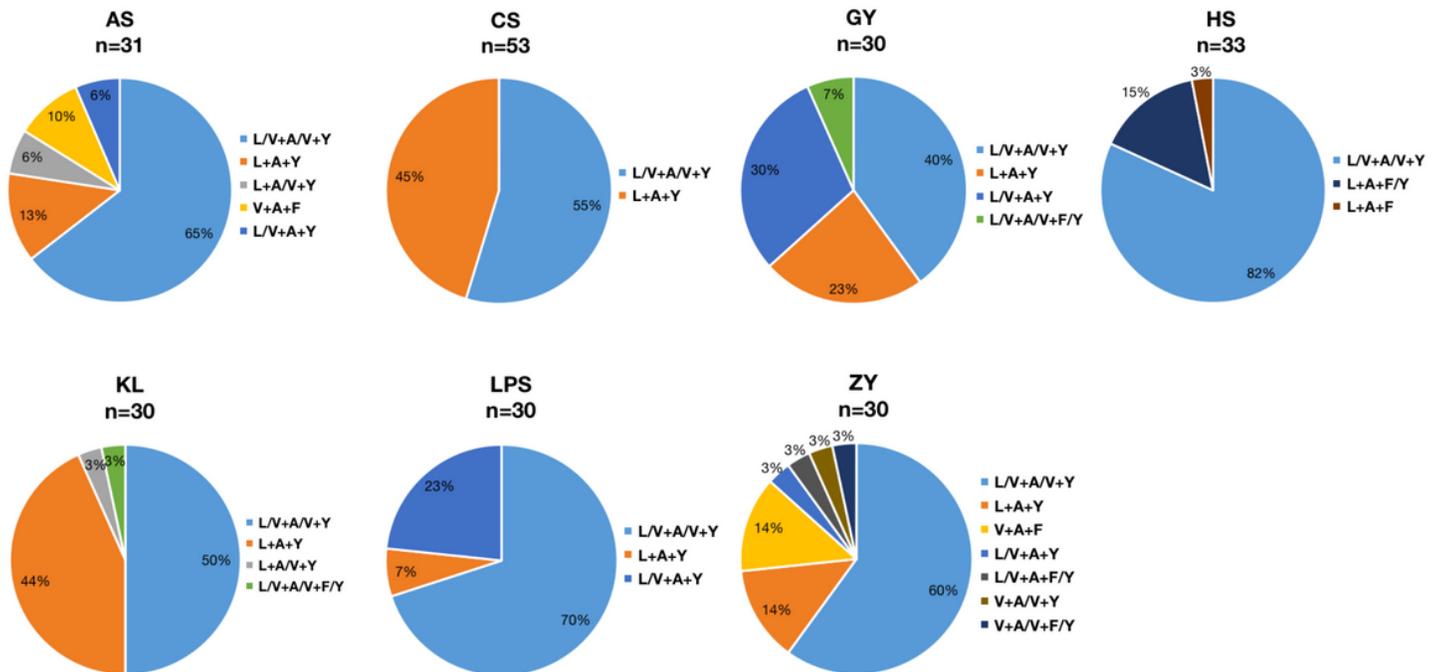


Figure 3

The percentage of 10 ace mutation combinations at loci 260, 342 and 407 in 7 housefly populations. The predominant amino acid substitution type was L/V+A/V+Y (blue), followed by L+A+Y (orange).

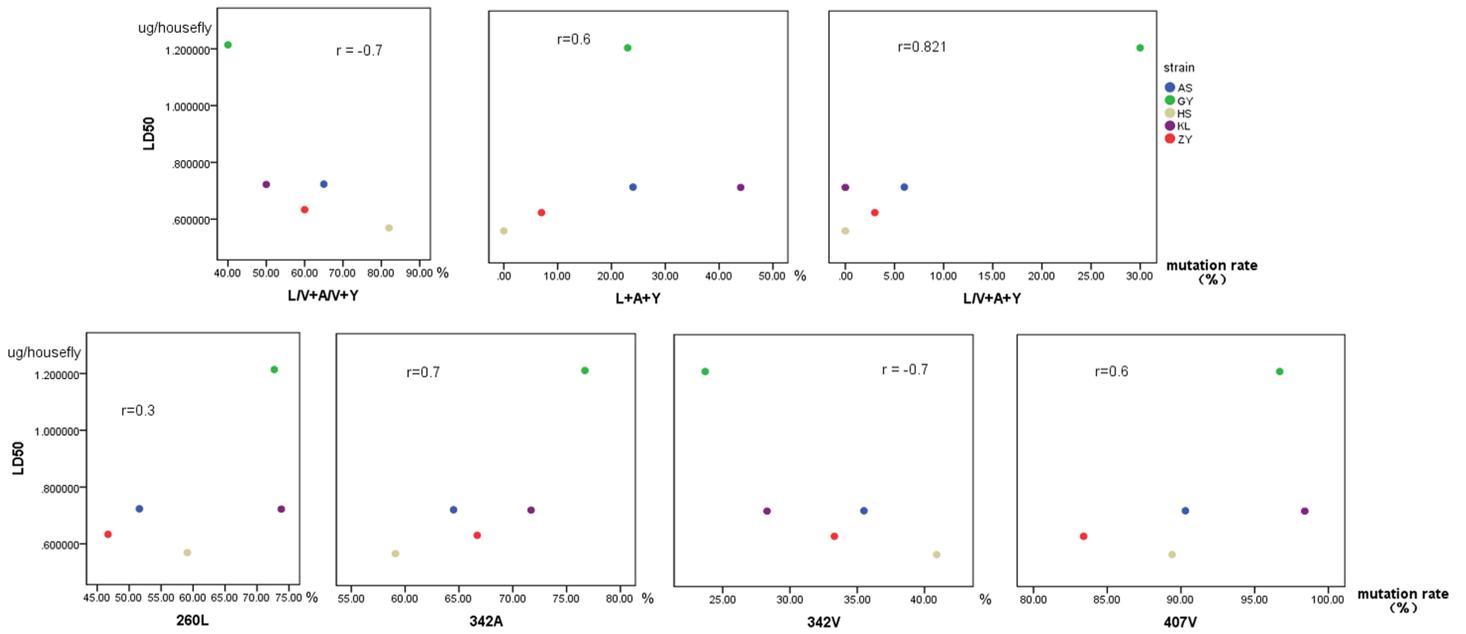


Figure 4

The correlation between single mutation/ combined mutations of ace gene and DDVP resistance to field populations of the housefly in Guizhou province.

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