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Frequencies and evolution of organophosphate insensitive acetylcholinesterase alleles in laboratory and field populations of the house fly, *Musca domestica* L.

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ABSTRACT

Resistance to organophosphate (OP) and/or carbamate insecticides can be due to mutations in the acetylcholinesterase gene (*Ace*). Genotypes of house fly, *Musca domestica* L., *Ace* were determined in twelve laboratory maintained strains (originally from North America, Europe and Asia) and two field collected populations from New York and Florida. There were 15 *Ace* alleles found and 11 of the alleles coded for a susceptible form of the enzyme (i.e., V260, A316, G342 and F407). Three of the four resistance alleles were previously described, while one is new. Phylogenetic analysis of the alleles suggests multiple origins of the F407Y mutation and multiple origins of the G342A mutation that confer OP resistance. Genotyping of field collected house flies from New York and Florida populations revealed the presence of only one resistance allele, *Acev10* (containing the non-synonymous mutations for A342 and Y407). All other alleles detected from the field-collected flies coded for a susceptible AChE. Thus, we were able to categorize individual flies as having homozygous susceptible (Ace^S/Ace^S), homozygous insensitive (Ace^I/Ace^I or *Acev10*/*Acev10*) or heterozygous AChE. The frequencies of Ace^S and Ace^I were not different between the NY2002 and FL2002 populations. Both populations were out of Castle–Hardy–Weinberg equilibrium, having an excess of Ace^S/Ace^I individuals and very few Ace^S/Ace^S individuals. Comparison of *Ace*, *Vssc* and *CYP6D1* genotypes indicates individual house flies commonly have resistance alleles at multiple loci. Comparison of genotype data with bioassays, as well as the use of genotype data in resistance studies is discussed.

1. Introduction

House flies are important vectors of numerous human and animals diseases. From the 1960s until the 1990s organophosphate and carbamate insecticides were the main tools used for control of house flies in the US and they continue to be used today. One of the major mechanisms of resistance to organophosphate and carbamates is a change in the target site (acetylcholinesterase). Insensitivity of AChE to these insecticides was first demonstrated biochemically in house flies 36 years ago [1]. Many subsequent studies indicated that patterns of cross-resistance (or the spectrum of insensitivity) to different OPs and carbamates varied, depending on the selecting agent used [2].

In contrast to several other insects, there is a single *Ace* gene in house flies (also called *Ace2* or *Drosophila melanogaster* orthologous *Ace* (*AO-AChE*)) [3–5] which is on autosome 2 [6]. Comparison of organophosphate/carbamate resistant and susceptible strains indicated five mutations (V260L, G342A, G342V, F407Y, and G445A), alone or in various combinations, (numbering of amino acids is based on the sequence of aabys *Ace* (GenBank Accession No. AF281161)) that are associated with resistance [4,7]. For example, genotyping coupled with AChE inhibition of individual house flies showed four mutations (V260L, G342A, G342V, F407Y) were associated with fenitroxon insensitivity [8]. In addition, heterologous expression of *Ace* containing one or more of these mutations confirmed their role in providing insensitivity to different organophosphates and/or carbamates [3–5].

Altered target sites are generally thought to be due to a single mutation (i.e., *Rdl* and *kdr*), or possibly the sequential accumulation of two mutations (*super-kdr*) [9]. However, house fly *Ace* alleles may have multiple resistance mutations [3–5,10] and the evolutionary pathway to this outcome is unclear. Overall, *Ace* alleles have been sequenced from relatively few strains, and none have included field-collected flies.

Little is known about the fitness cost of the *Ace* alleles that confer resistance in house flies and sequences of the *Ace* alleles present in multiple lab and field strains are needed before this question can be fully addressed. Furthermore, resistance alleles are generally thought to confer a fitness disadvantage in the absence of

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insecticides. However, it is not known if there would be epistasis between the fitness costs of multiple resistance genes in an individual. The ability to genotype multiple genes from individual flies opens the possibility to examine the relative frequency of multiple resistance alleles simultaneously in individuals in a given population, and opens the door for investigations into the interactions of fitness costs of different resistance alleles.

Herein, we describe the *Ace* alleles from 13 different laboratory strains (susceptible and insecticide resistant) of house fly. There were 15 alleles identified, and one new mutation (A316S) appears to be associated with resistance. A phylogenetic analysis of the alleles indicates multiple origins for some of the resistance mutations. The frequency of different *Ace* alleles was also determined for the house flies collected from dairies in New York and Florida (USA). Only one resistance allele was found in the field populations, but homozygous and heterozygous resistant individuals were the most common. Multiple *Vssc* alleles confer pyrethroid (and DDT) resistance [9,11–14] while overexpression of *CYP6D1v1* confers pyrethroid resistance in some strains of house fly [9,12,15–18]. Comparison of *Ace* alleles (on autosome 2), with alleles of *Vssc* (on autosome 3) and *CYP6D1* (on autosome 1) conferring pyrethroid resistance, reveals that individual field-collected flies often have resistance alleles at multiple loci. The utility of genotyping *Ace* to study insecticide resistance is discussed.

2. Materials and methods

2.1. House fly strains and populations

Twelve strains and two populations were used. The aabys (insecticide susceptible) strain has a recessive visible mutant marker on each autosome [19]. CS [20] and IsoCS [21] are insecticide susceptible strains. SRS is a standard reference susceptible strain created in 1961 [22] which was obtained from M. Kristensen (Lyngby, Denmark). Cornell-R is an organophosphate resistant strain due to insensitive AChE [1]. This strain was selected by tetrachlorvinphos (an organophosphate) from a colony collected in Tompkins Co, New York in ~1970 [1] and was maintained under biannual selection with tetrachlorvinphos. NG98 is a multi-resistant strain with pyrethroid resistance due to monooxygenase-mediated detoxification (*CYP6D1v1*) and *kdr*. This strain was established from a colony collected in Georgia in 1998 [16]. NYSPINR is a multi-resistant strain with high levels of resistance to spinosad [23], established from collections made in New York in 1999 [24]. The rspin (spinosad resistance from NYSPINR introgressed into the aabys strain) has low levels of cross-resistance to tetrachlorvinphos [23]. OCR is a cyclodiene resistant (*Rdl*) [25] and pyrethroid-susceptible strain [26] provided by Dr. F.W. Plapp Jr. in 1996 and maintained under biannual selection with dieldrin. LPR is a multi-resistant strain with high levels of pyrethroid resistance (due to increased oxidative metabolism by cytochrome P450, *CYP6D1v1* [27,28], *kdr* and decreased cuticular penetration (*pen*) [27,29]. Its parental colony was originally collected from the Learn Dairy (New York) in 1980 [30]. LPR flies used for this study were from a collection put into the ultralow freezer in 2002. YPER is a multiple resistant strain established by the selection of permethrin from a Yumenoshima population of Japan in 1997 (pyrethroid resistance due to *super-kdr* and monooxygenase-mediated detoxification [29]. NYINDR is an indoxacarb resistant strain derived from flies collected at seven different locations in New York in 1999 [31]. NY2002 was collected from a dairy in Schuyler Co. New York in the summer of 2002 and has high levels of resistance to methomyl, dimethoate and tetrachlorvinphos [32]. FL2002, was collected population from a dairy in Alachua Co., Florida in the summer of 2002 and has low levels of resistance to methomyl, dimethoate and tetrachlorvinphos [32]. The NY2002 and FL2002 were from stocks that had been placed at −80 °C after collection.

2.2. Genotyping

Individual male flies were placed in 1.5 ml tubes and frozen in liquid nitrogen. Animals were quickly pulverized with a disposable pestle (Kontes Glassware, Vineland, New Jersey, USA) and suspended in 0.5 ml of lysis buffer (100 mM Tris–Cl pH 8.0, 50 mM NaCl, 10 mM EDTA, with 1% (w/v) SDS, 0.5 mM spermidine, 0.15 mM spermine, and 0.1 mg/ml (20 U/mg) proteinase K). Samples were incubated at 60 °C for 20 min, and 75 μl of 8 M potassium acetate was added, mixed and set in an ice bath for 10 min. The samples were spun at 14,000*g* for 5 min and the supernatant was transferred to a new tube. Absolute ethanol (1 ml) was added and the samples were kept at room temperature for 10 min. The samples were spun at 14,000*g* for 10 min. Pellets were washed in 0.5 ml of 70% ethanol and spun at 14,000*g* for 5 min. The final pellet was dried in a vacuum for 10 min, suspended in 50 μl of H_2O and the DNA concentration (20 ng/μl to 2 μg/μl) was determined spectrophotometrically.

The primers for the genotyping of the *Ace* fragment were designed based on the mRNA sequence of aabys: AF281161 (Fig. 1). The *Ace* fragment was amplified by PCR in a 15 μl reaction containing 0.4 U of Taq polymerase (New England Biolabs, Beverly MA), 0.3 picomoles of S90MdAce (CATCTAAAACCGATCAGGACCATTTAATAC) and AS89MdAce (TCATCTTTAACATTTCCAATCAGAATATCG) and 0.6 μl of genomic DNA as a template. The reactions were carried out at 94 °C for 3 min, followed by 40 cycles of PCR (95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min 30 s), and a final extension at 72 °C for 10 min. The 10 μl of product was purified by incubating with 4 U Exonuclease I and 1.6 U shrimp alkaline phosphatase (USB Corporation, Cleveland OH) at 37 °C for 1 h and then at 85 °C for 15 min. The PCR products were sequenced directly (in both directions) with S88MdAce (CACATTGGACATTTACAACGCTGAGA) and AS87MdAce (GCTAAGATCTGCTGTTTTCAAAAGTGTCAT) or S86MdAce (GACAGGTTGATGCGAAAACA) and AS85MdAce (CCGGTAACAATGAGGCATTA). Sequencing was performed at Cornell Biotechnology Resource Center. Homozygous and heterozygous individuals were identified by manual inspection of the electropherograms. The deduced amino acid sequences of the *Ace* alleles were identified as sensitive or insensitive based on previous reports [3–5,7,8]. Analysis of *Vssc* and *CYP6D1* alleles were carried out by direct sequencing of PCR products as described previously [12]. Individuals homozygous for each allele in a given strain were found. All heterozygotes observed in a given strain unambiguously contained alleles identified from homozygous individuals in that strain.

2.3. Phylogenetic analysis

Alignment of sequence data using ClustalX [33] was straightforward because the only length variation among all fragments was a 3 bp insertion within the intron. The program ModelTest 3.7 [34] was used to select the best fit model under the Akaike information criterion (AIC) [35]. The model selected was the Hasegawa, Kishino, Yano plus invariant sites model (HKY + I) which was used for all maximum likelihood and Bayesian analyses.

The optimal tree and branch lengths under maximum likelihood were inferred using PAUP 4b10 [36] by conducting a heuristic search with 100 random addition replicates and TBR branch swapping. Clade robustness was assessed using the non-parametric bootstrap [37] by performing 1000 pseudoreplicates, with all parameter values re-estimated during each replicate and heuristic tree searches consisting of as-is taxon addition sequences and TBR branch swapping.

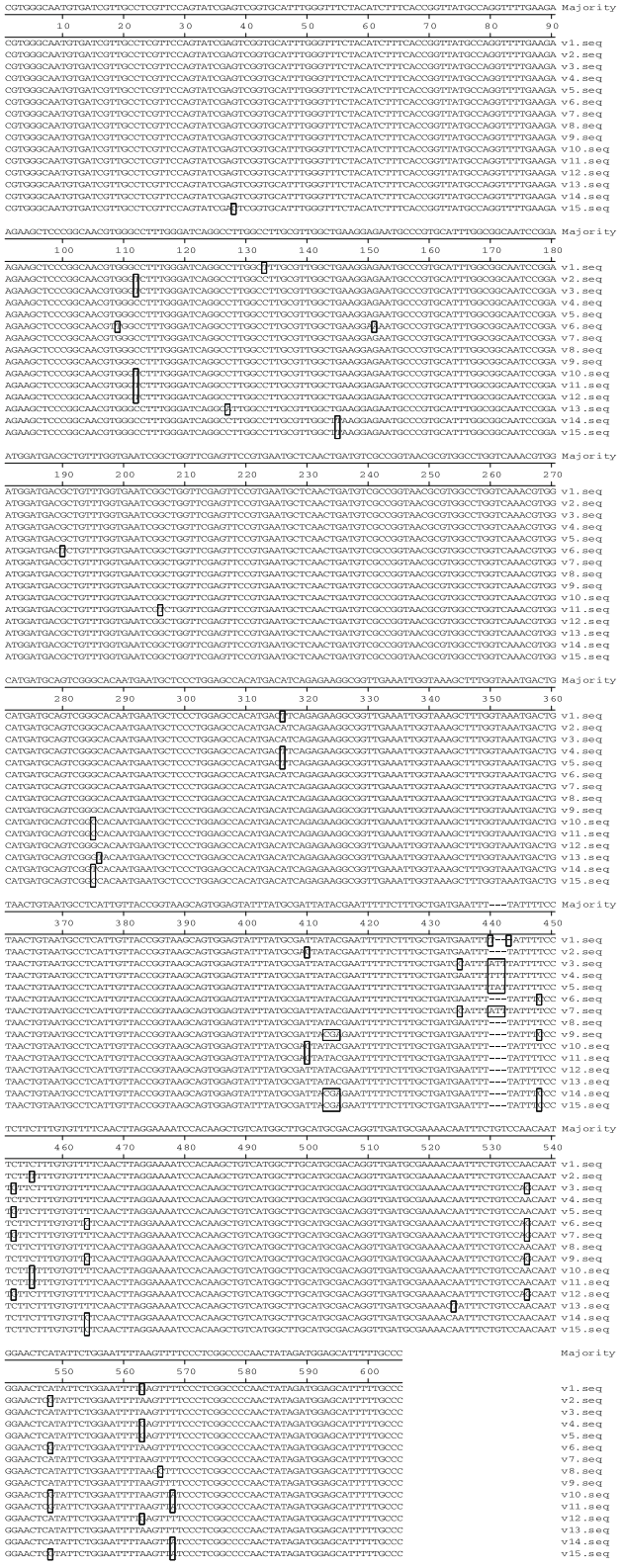

Fig. 1. Alignment of 15 *Ace* alleles from the house fly. Primer sequences have been removed. Residues differing from the consensus sequence are boxed. Accession numbers for the sequences are given in Table 1. The intron begins at position 387 and ends at 474 in the majority sequence.

Bayesian phylogenetic analysis was conducted using MrBayes 3.1.2 [38]. Two independent runs were performed, each starting from a random tree and extending for 5 million generations. The default parameters were used for the MCMC searches. Each run was sampled every 1000 generations. The first 1 million generations from each run were discarded as burn-in. The two runs quickly converged to stationarity, as indicated by several criteria, including: a low average standard deviation of split frequencies (~0.004); values of 1.0 for the potential scale reduction factor for all parameters; and the sampling of similar likelihood values at the same plateau by both runs. The post burn-in trees from each run were combined and used to calculate posterior probabilities of clades. Bootstrap values under maximum parsimony were also inferred by performing 1000 pseudoreplicates using the branch and bound algorithm in PAUP.

3. Results

3.1. Ace alleles in susceptible and insecticide resistant laboratory strains

A fragment of the house fly *Ace* gene was amplified by PCR from the genomic DNA of individual flies (Fig. 1). This fragment contained one 85–88 bp intron (Fig. 1). All the single nucleotide polymorphisms (SNPs) in the exons and intron were detected from at least three individual flies per strain. There were 15 *Ace* alleles found in the laboratory strains we examined. Most strains had two different alleles (Table 1) and 11 of the alleles (Table 2) coded for a susceptible form of the enzyme (i.e., V260, A316, G342 and F407). Four non-synonomous SNPs resulted in amino acid substitutions: V260L, A316S, G342A/V and F407Y (numbering of amino acids (and nucleotides) is based on the sequence of aabys *Ace* (GenBank Accession No. AF281161)), and four putative insensitive *Ace* alleles (Table 1) were identified. These amino acid replacements are the same as those previously reported [3,4,7], except for the A316S mutation which has not been previously reported. No other non-synonymous mutations were found in any of the fly populations. The aabys, CS, IsoCS and SRS strains contained only alleles that coded for a susceptible form of AChE (V260, A316, G342 and F407). The LPR and rspin strain were homozygous for *Acev14* (V260, A316, A342 and Y407). All other strains contained both a mixture of resistant and susceptible alleles.

3.2. Phylogeny of Ace alleles

The diverse number of alleles we found allowed for robust phylogenetic analyses of the alleles with Bayesian, maximum likelihood, and parsimony methods yielding very similar results (Fig. 2). These analyses indicate multiple origins of the F407Y mutation and multiple origins of the G342A mutation. The A316S, G342V and V260L mutations appear to have a single origin. The phylogeny also suggests a sequence of events leading to the various alleles in which F407Y and possibly G342A (*Acev10*) are

Table 1
Ace alleles from various strains of house flies.

Strain	Alleles (Genbank Accession No.)	Collection
aabys	*v1* (FJ174253), *v2* (FJ174254)	Multiple sites
CS	*v3* (FJ174255), *v4* (FJ174256)	TX, NY, MD
IsoCS	*v5* (FJ174257)	TX, NY, MD, ME
SRS	*v6* (FJ174258), *v7* (FJ174259), *v8* (FJ174260)	Italy, 1961
Cornell-R	*v9* (FJ174261), *v10* (FJ174262)	NY, ~1970
NG98	*v3*, *v11* (FJ174263)	GA, 1998
NYSPINR	*v10*, *v12* (FJ174264)	NY, 2002
rspin	*v14* (FJ174266)	Multiple sites
OCR	*v9*, *v13* (FJ174265)	OR, 1964
LPR	*v14* (FJ174266)	NY, 1980
YPER	*v3*, *v15* (FJ174267)	Japan, 1997
NYINDR	*v10*	NY, 1999

Table 2
Non-synonomous mutations in *Ace* alleles found in this study. Sequence of the four amino acids in susceptible strains (alleles 1–9, 12 and 13) are shown in the bottom line for reference.

Amino acid					
Alleles	260	316	342	407	Type[a]
v10	V	A	A	Y	Insensitive
v11	V	S	A	Y	Insensitive
v14	V	A	V	Y	Insensitive
v15	L	A	A	Y	Insensitive
v1–9, v12–13	V	A	G	F	Sensitive

[a] AChE that is insensitive or sensitive to inhibition by organophosphate and/or carbamate insecticides.

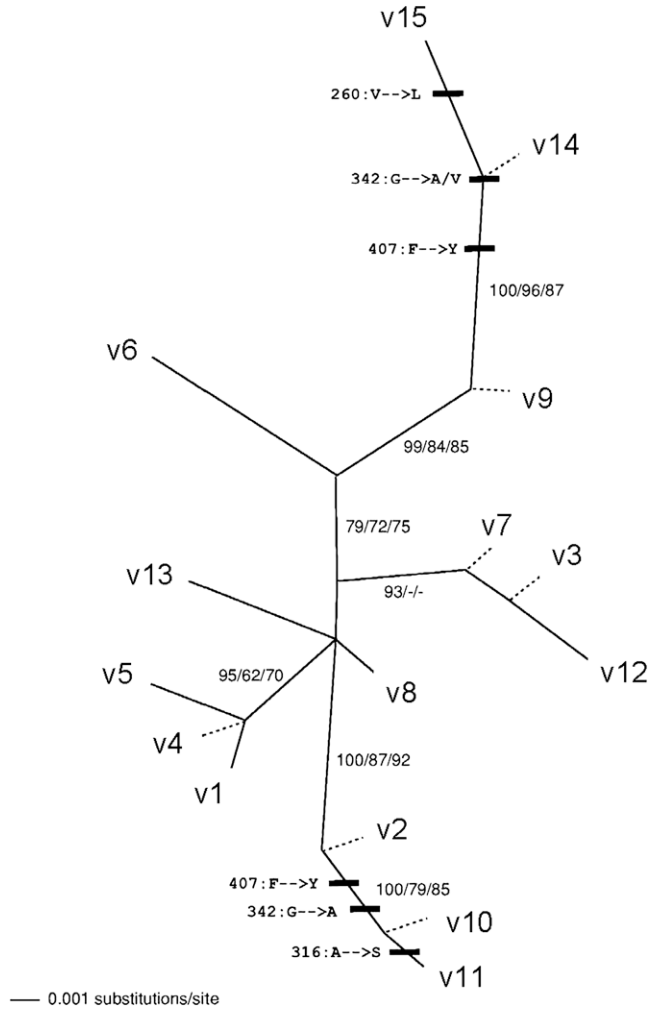

Fig. 2. Unrooted phylogeny of alleles based on maximum likelihood analysis. Numbers on branches are support values (only values >50 are shown) in the following order: Bayesian posterior probabilities/maximum likelihood bootstrap values/maximum parsimony bootstrap values.

common evolutionary precursors to both A316S (*Acev11*) and V260L (*Acev15*) in separate lineages.

3.3. Field populations of New York (NY2002) and Florida (FL2002)

Fifty-three flies from the New York population and 62 flies from the Florida population were genotyped. The only resistance allele detected in these populations was *Acev10* (containing the non-synonymous mutations for V260, A316, A342 and Y407). All other alleles detected coded for a susceptible AChE. Thus, we were able to phenotypically categorize individual flies as being homozygous susceptible (Ace^S/Ace^S), homozygous resistant (Ace^I/Ace^I or *Acev10*/*Acev10*) or heterozygous. In NY2002 there were 1, 43, and 9 individuals that were Ace^S/Ace^S, Ace^S/Ace^I and Ace^I/Ace^I, respectively. In FL2002 there were 1, 49, and 12 individuals that were Ace^S/Ace^S, Ace^S/Ace^I and Ace^I/Ace^I, respectively. The frequencies of resistant and susceptible alleles were not significantly different between the NY2002 and FL2002 populations (χ^2 (2, $N = 114$) = 0.1426, $p = 0.9048$, Fisher's exact test). Comparing the differences between the expected and observed frequencies of resistant and susceptible alleles showed that these populations were out of Castle–Hardy–Weinberg (CHW) equilibrium (NY2002; χ^2 (2, $N = 106$) = 14.54, $p = 0.0005$) (FL2002; χ^2 (2, $N = 122$) = 13.94, $p = 0.0009$) (Fisher's exact test). In both populations, the majority of flies were Ace^S/Ace^I heterozygotes and homozygous susceptible individuals were rare. Comparison of *Ace* and *CYP6D1v1* (Fig. 3a) alleles show a complete absence of individuals that are homozygous susceptible for both alleles, and that the population is clearly out of CHW equilibrium, as would be expected for loci that are under selection. A similar pattern was observed when *Ace* and *Vssc* alleles were compared (Fig. 3b). House flies in New York and Florida are composed of individuals that are heterozygous or homozygous for resistance alleles at multiple loci, and no individuals homozygous susceptible for *Vssc* or *CYP6D1* were detected.

4. Discussion

The insensitive AChE of house fly was first reported in the Cornell-R strain that was collected from a dairy in NY in about 1970 and selected by an organophosphate (tetrachlorvinphos) [1]. Thus, the *Acev10* allele found in Cornell-R was probably selected for during the period of approximately 1955-1970 when organophosphates and carbamates were routinely used for house fly control and was already in the house fly populations in New York by 1970 when this strain was collected. This allele has remained in field populations as it was detected in collections from 1999 (INDR) and 2002 (NYSPINR). The previous report of mutations responsible for organophosphate and carbamate resistance in the CH2 strain [4] agrees with our findings from the Cornell-R strain, and it appears that these may in fact be the same strains based on the description provided [39]. While it is difficult to track a geographic location for each allele, it appears that *v5* was from Maine, as this allele was found in IsoCS and not CS, and these strains vary only in the fact that IsoCS arose from backcrosses between Maine and CS house flies [21]. It was perhaps surprising that the *v11* and *v14* alleles were not found in the field-collected flies, since they were found in a strain collected from NY in 1980 and Georgia in 1998, respectively. It is possible that the organophosphates and/or carbamates that had selected for these alleles may no longer be used, leading to a decrease in these allele frequencies.

The detection of known or putative insecticide resistant *Ace* alleles varies globally by region, although there is overlap. For example, the *v10* and *v11* alleles we found in house flies from the US (Table 1), were not detected in laboratory or field collected house flies from Europe. However, putative resistant alleles were found in flies from Denmark that we did not detect [40]. One allele, with amino acid substitutions consistent with the *v14* allele (LPR strain) was also found in a strain from Denmark [40]. Alleles with amino acid substitutions consistent with the *v15* allele (detected in the YPER strain which was originally from Japan) were also reported from a strain from Japan [40] and the UK [4]. It was surprising that the Cornell-R strain was not homozygous (5 Ace^I/Ace^I and 4 Ace^S/Ace^I individuals), given that this strain is highly resistant to tetrachlorvinphos and malathion (unpublished).

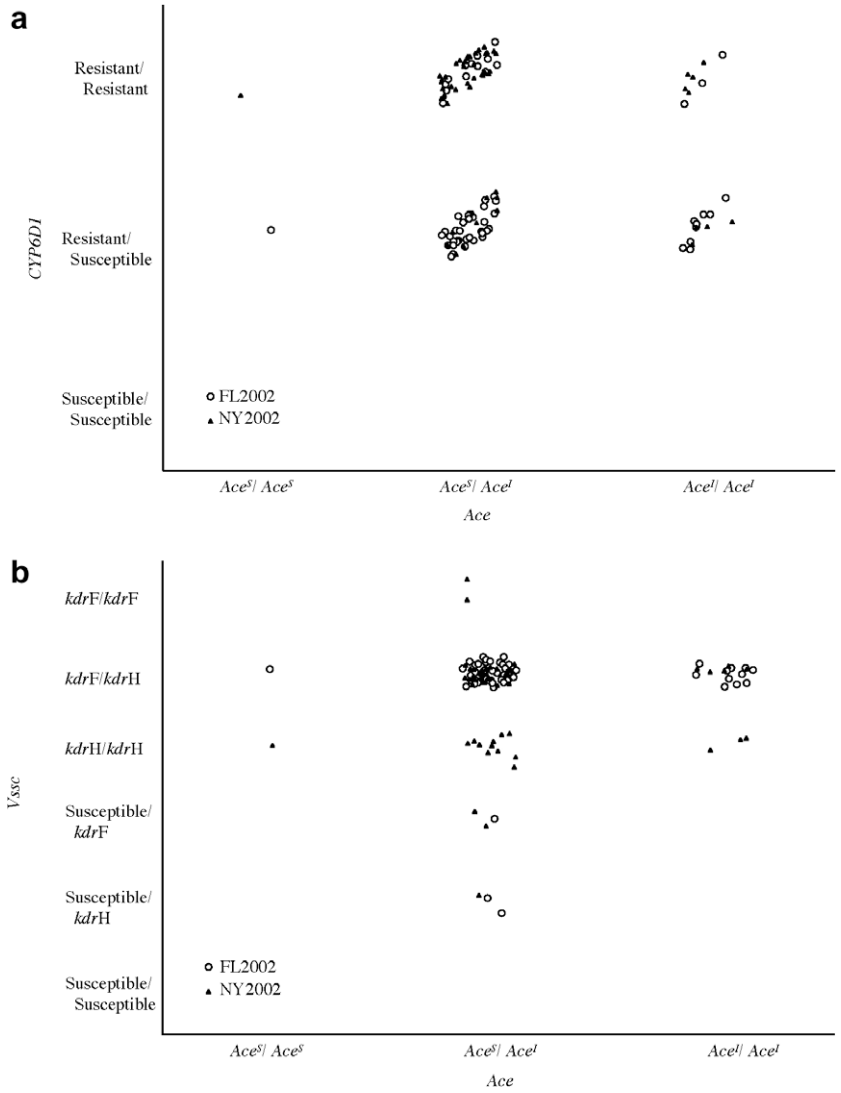

Fig. 3. Frequency plots showing genotypes of (a) *Ace* and *CYP6D1* and (b) *Ace* and *kdr* in individuals from field-collected flies (NY2002 and FL2002). The *Vssc* alleles with the L1014F and L1014H mutations are labeled as kdrF and kdrH, respectively.

The frequencies of Ace^S and Ace^I were not different between the NY2002 and FL2002 populations, although the frequency of individuals resistant to methomyl, dimethoate and tetrachlorvinphos was significantly higher in the New York flies [32]. This suggests that other mechanisms, such as detoxification are likely also involved in resistance to organophosphates and carbamates in New York flies. This appears likely as multiple mechanisms of resistance to tetrachlorvinphos [41], dimethoate [42] methomyl [43] have been found in different strains of house flies. Despite the presence of multiple resistance mechanisms, the ability to genotype individual insects remains a powerful tool for the detection of resistant individuals and for understanding the population genetics and evolution of insecticide resistance.

In both New York and Florida populations, the frequencies of Ace^S/Ace^I individuals were much higher than expected and out of the (CHW) equilibrium. The factors working on the selection of genotypes was not clear, but it was obvious that the Ace^S/Ace^I heterozygotes had an advantage in these populations. The *Acev15* allele found in some Japanese resistant strains, was not found in the field populations in the USA. This suggests that certain organophosphates, for which the *Acev15* allele confers protection (such as pyraclophos and propahophos [44], dichlorvos, malaoxon and bendiocarb [4]), might be effective for control of house flies in the USA.

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