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Evolutionary plasticity of monooxygenase-mediated resistance

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Abstract

The cytochrome P450 monooxygenases are an important metabolic system involved in the detoxification of xenobiotics, and are thus one of the major mechanisms by which insects evolve insecticide resistance. However, comparatively little is known about the evolutionary constraints of this insecticide resistance mechanism. We investigated the genetic basis of resistance in a strain of house fly (NG98) from Georgia, USA that had evolved 3700-fold resistance to the pyrethroid insecticide permethrin, and compared this to other permethrin resistant strains of house flies from the US and Japan. Resistance in NG98 was due to *kdr* on autosome 3 and monooxygenase-mediated resistance on autosomes 1, 2, and 5. These results indicate that the genes which evolve to produce monooxygenase-mediated resistance to permethrin are different between different populations, and that the P450 monooxygenases have some degree of plasticity in response to selection. Monooxygenase-mediated resistance appears to evolve using different P450s, and possibly different regulatory signals controlling P450 expression, even in strains selected with the same insecticide.

Keywords: Cytochrome P450 monooxygenases; *kdr*; CYP6D1; Evolution of resistance; House fly

1. Introduction

Insecticide resistance is a valuable phenomenon for investigating Darwinian processes in natural populations [1,2] because the selection pressure is strong, the selective agent is known, the evolution of resistance is rapid and because experimental populations can be readily manipulated. In the last decade, identification of the genes responsible for insecticide resistance has led to novel insights about the evolution and population genetics of resistance, the fitness cost (in the absence of insecticides) of resistance genes, monogenic vs. polygenic basis of resistance, and coadaptation [1–3].

Although resistance almost invariably occurs following use of insecticides, the number of possible mutations responsible for resistance can be quite limited. For example, resistance to cyclodiene insecticides is due exclusively to the same single mutation in a GABA gated chloride channel subunit in multiple species worldwide [4]. However, most of our current understanding of the evolution of insecticide resistance comes from mutations of the target sites (i.e., *Rdl*, *kdr*, *super-kdr*, etc.) or from overexpression of esterases that act to sequester the insecticide (and in some cases to slowly detoxify it) [5,6]. In contrast, even

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though P450 monooxygenases are one of the major mechanisms of insecticide resistance [7], comparatively little is known about the evolutionary constraints of this insecticide resistance mechanism.

The cytochrome P450 monooxygenases are an important metabolic system involved in the detoxification/activation of xenobiotics (as well as in the anabolism and catabolism of endogenous compounds including hormones and pheromones). As such, P450 monooxygenases play an important role in insecticide resistance. P450 monooxygenases have such a phenomenal array of metabolizable substrates because of the presence of numerous P450s (60–111) in each species, as well as the broad substrate specificity of some P450s [8]. Studies of monooxygenase-mediated resistance (reviewed in [7]) have indicated that resistance can be due to increased expression of one P450 (via increased transcription [9]) involved in detoxification of the insecticide and might also be due to a change in the structural gene itself [10]. However, it is not clear how much plasticity exists within the P450 monooxygenases in terms of the development of insecticide resistance to populations selected with the same insecticide. Would all populations utilize the same P450(s) or different ones?

Pyrethroid insecticides have been widely used for the control of insects, especially Diptera, for over a decade. The first pyrethroid to be used was permethrin, and this was (almost exclusively) the only pyrethroid used at dairy and poultry facilities in the USA from the mid-1980s to the mid-1990s. For house fly control on dairies and poultry facilities, pyrethroids were initially highly effective. Unfortunately, in some populations of house flies this class of insecticides has been rendered largely ineffective due to the development of very high levels of resistance. Decades of research have shown that there are only two major mechanisms of resistance to pyrethroids in house flies: increased monooxygenase-mediated detoxification and insensitivity of the voltage sensitive sodium channel (VSSC). There are two alleles associated with insensitivity of the VSSC: *kdr* and *super-kdr* [11]. House fly populations throughout the world have been found with *kdr*, while *super-kdr* has been found in Europe and Japan, but not in the USA.

How mobile are house flies? A recent study has found very little detectable gene flow between North America and sub-Saharan Africa [12,13]. However, another recent study found identical *CYP6D1v1* alleles in the resistant NG98 and LPR strains from Georgia (GA, USA) and New York (NY), respectively, indicating that CYP6D1 resistance must have evolved once and then spread [10]. Mark-release-recapture studies of house fly movement indicate this insect is very mobile [14] and can move at least 6 miles in a 24 h period [15]. Yet this is still far short of the several hundred miles from GA to NY. Thus, it was suggested that humans may act as vehicles for the movement of house flies (in cars, trains, planes, etc.), or flies may be able to take advantage of seasonal trade winds to accomplish long distance dispersal as in the case of potato leaf hopper [16]. Whether or not flies are as mobile as mosquitoes, where at least one gene for resistance appears to have evolved once and then spread throughout the world [17], remains to be determined.

Herein, we examined the genetic basis for monooxygenase-mediated pyrethroid resistance in a strain of house fly collected from GA. These results are compared to those with other populations from NY, Alabama (AL), and Japan in an effort to understand how much variability exists in monooxygenase-mediated resistance in populations that have been selected with the same pyrethroid insecticide (permethrin).

2. Materials and methods

2.1. Chemicals and house fly strains

Permethrin was from Chem Service (West Chester, PA). Two parental strains were used: aabys, a susceptible strain with the recessive morphological markers *ali-curve* (*ac*), *aristapedia* (*ar*), *brown body* (*bwb*), *yellow eyes* (*ye*), and *snipped wings* (*snp*) on autosomes 1, 2, 3, 4, and 5, respectively, and NG98, a strain that has 3700-fold resistance to permethrin which was collected from a poultry facility in 1998 [18]. House flies were

reared as described previously [19]. Adult flies were fed powdered milk:granulated sugar (1:1) and water ad libitum.

2.2. Bioassay and genetic analysis

Bioassays were carried out by topical application of a 0.5-μl drop of insecticide in acetone solution to the thoracic notum of 3–5-day-old female flies. Each replicate consisted of 20 flies per dose and at least three doses, giving greater than 0 and less than 100% kill. All tests were run at 25 °C and were replicated four times. Mortality was assessed 24 h after treatment. Bioassay data were pooled and analyzed by standard probit analysis [20], as adapted to personal computer use by Raymond [21] using Abbott's [22] correction for control mortality. Resistance ratios (RRs) were calculated by dividing the resistant strain LD_{50} by the susceptible strain LD_{50}.

2.3. Linkage analysis

The chromosomes involved in resistance to permethrin in the NG98 strain were evaluated (four independent replications) using the F_1 male backcross method of Tsukamoto [23] using a total of 9013 male and female flies at a diagnostic dose of 20 ng permethrin per fly applied as described above. This method involves crossing susceptible marker strain females with resistant (R) males, backcrossing the F_1 males to susceptible marker females and testing separately each phenotypic class of the progeny with a diagnostic dose (expected to kill 100% of the susceptible strain) of the insecticide (Fig. 1). Since crossing over is very rare in male house flies, this method permits the detection and measurement of the effect of each chromosome (i.e., the resistance contributed by individual R chromosomes). In this type of analysis, the percent survival is converted to an arc-sin unit, the 'effect' of each chromosome, or combination of chromosomes, is calculated, and subjected to an analysis of variance with significance being determined by an *F* test [23]. By this type of analysis, the chromosomes with genes contributing to the resistance can be detected (significant *F* value). Additionally, greater- or less-than-additive effects between chromosomes can also be detected.

2.4. Amplification of the para-homologous VSSC α-subunit cDNA and sequencing

To determine if the NG98 strain contained either the *kdr* or *super-kdr* mutations found in house flies, this region of the *para*-homologous VSSC α-subunit gene was sequenced. Total RNA was isolated from individual abdomens of four house fly adults (two males and two females) using the acid guanidine–phenol–chloroform method [24] with Isogen (Nippon Gene, Tokyo, Japan). First strand cDNA was synthesized with isolated RNA (equivalent of 1/10

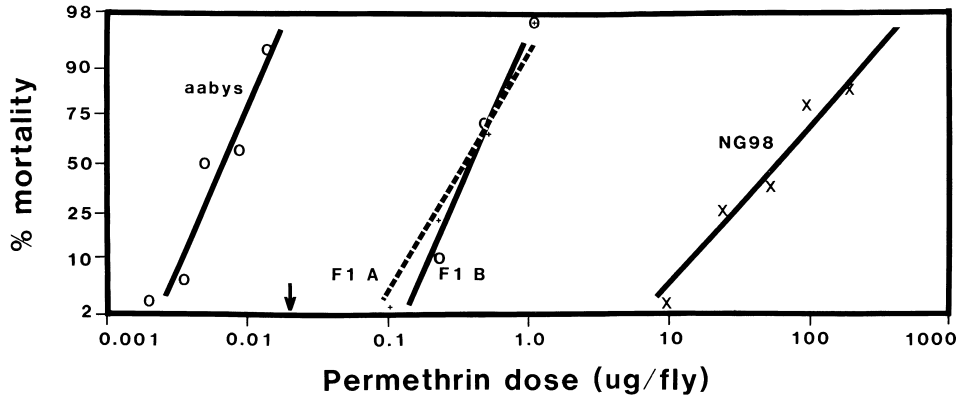

Fig. 1. Log dose-probit lines for susceptible (aabys), resistant (NG98), and F_1 (aabys × NG98) house flies. The arrow indicates the diagnostic dose (20 ng/fly) used for the factorial analysis of resistance (Table 1). F_1 A (+) was NG98 male × aabys female. F_1 B (○) was NG98 female × aabys male. Percent mortality is on a probit scale.

abdomen) and an Oligo(dT) primer. A partial sequence of the *para*-homologous sodium channel α-subunit cDNA (a part of segment 5 and 6 of the domain II) was amplified by reverse transcription-mediated polymerase chain reaction (RT-PCR) using primers (mdsc-1: 5′-CGAAGTACTACTTC CAGGAAG-3′ and mdsc-3: 5′-TCATGACCCAA CTCCAGTTC-3′) designed based on the house fly *para*-homologous sodium channel α-subunit gene sequence [11] with ExTaq polymerase (Takara, Tokyo, Japan) under the following conditions: the reaction was kept at 94 °C for 3 min, then 35 cycles of PCR (94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min) were done, and the samples were finally kept at 68 °C for 5 min. The PCR products were purified using a QIAquick PCR purification Kit (Qiagen) were used for the sequencing reactions as templates and were analyzed on a Genetic Analyzer 310 system (PE Applied Biosystems, Foster, CA). Sequences were determined from both directions with internal primers (mdsc-2: 5′-GCCTGTCGGT GTTGAGAAG-3′ and mdsc-4: 5′-CATGTTCTG ATGGTTGGTC-3′) designed based on the house fly *para*-homologous sodium channel α-subunit gene [11].

3. Results

Permethrin resistance in the NG98 strain was inherited as a slightly recessive trait with a degree of dominance [25] of −0.14 (Fig. 1). This value is intermediate within the *D* values for the high levels of permethrin resistance reported in other strains: YPER ($D = -0.49$ [19]), LPR ($D = -0.21$ [26], $D = -0.28$ calculated from [27]) and ALHF ($D = 0.13$, calculated from [28]). Reciprocal crosses between NG98 and aabys gave F_1 progeny with similar log dose-probit lines (Fig. 1) indicating all resistance genes were autosomal.

The factorial analysis of resistance in the NG98 strain indicated the relative contribution to resistance by the different autosomes was $5 > 3 > 1 > 2$ (Table 1). There was a greater-than-additive interaction between autosomes 3 and 5.

To determine if *kdr* or *super-kdr* was present in NG98 we sequenced a portion (putative transmembrane segments S4-S6 of domain II) of the *para*-homologous sodium channel α-subunit in NG98 (accession # AF521558 and AF521559). The deduced amino acid sequence from the NG98 strain (Fig. 2) indicates this strain has the *kdr* mutation (homologous to L1014F), but not the *super-kdr* mutation [29]. The *kdr* mutation has previously been detected in the USA in flies from Florida [11] and New York [19]. Given the abundant toxicological and electrophysiolgical evidence linking the *kdr* gene to insensitivity (and resistance) to pyrethroids it appears clear that pyrethroid resistance in NG98 is due in part to *kdr*. Although *super-kdr* should provide a selective advantage to flies exposed to

Table 1
Factorial analysis of permethrin resistance in the NG98 strain of house fly

Autosome(s)	Effect	Mean square	*F* value
5	546	18660	56.6*
4	62	238	0.7
4 + 5	−9	5	0.0
3	408	10383	31.5*
3 + 5	199	2476	7.5*
3 + 4	−38	90	0.3
3 + 4 + 5	4	1	0.0
2	248	3831	11.6*
2 + 5	33	69	0.2
2 + 4	15	14	0.0
2 + 4 + 5	−14	12	0.0
2 + 3	59	216	0.7
2 + 3 + 5	−63	245	0.7
2 + 3 + 4	−59	219	0.7
2 + 3 + 4 + 5	−7	3	0.0
1	277	4798	14.5*
1 + 5	122	934	2.8
1 + 4	67	280	0.8
1 + 4 + 5	61	232	0.7
1 + 3	−47	136	0.4
1 + 3 + 5	−80	398	0.4
1 + 3 + 4	−12	10	0.0
1 + 3 + 4 + 5	−11	7	0.0
1 + 2	−7	3	0.0
1 + 2 + 5	−56	199	0.6
1 + 2 + 4	63	250	0.8
1 + 2 + 4 + 5	28	47	0.1
1 + 2 + 3	−83	435	1.3
1 + 2 + 3 + 5	−63	248	0.8
1 + 2 + 3 + 4	0	0	0.0
1 + 2 + 3 + 4 + 5	7	3	0.0
Error		330	

* Statistically significant at the 0.01 level.

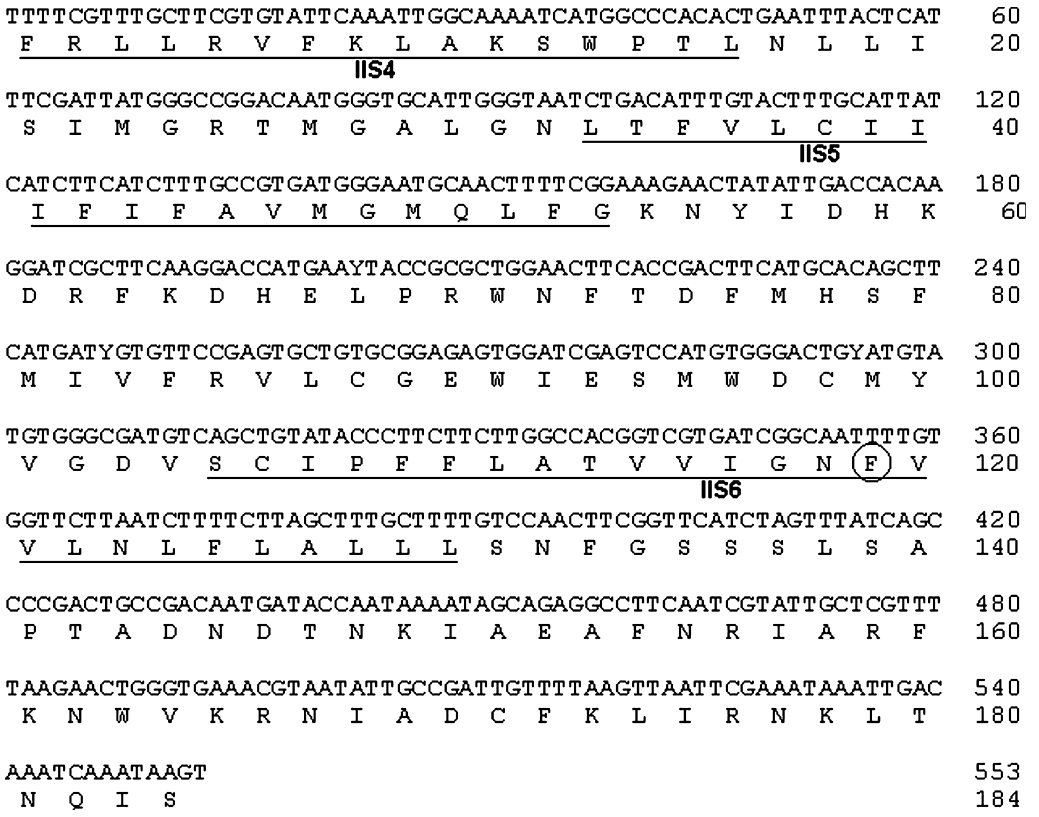

Fig. 2. Nucleotide and deduced amino acid sequence of the *para*-homologous sodium channel gene from the NG98 strain of house fly (Accession No. AF521558 and AF521559). Putative transmembrane segments (S4, S5, and S6) of domain II are underlined. The amino acid corresponding to the *kdr* mutation in NG98 is circled (L119F). The L119F mutation shown here is homologous to the L1014F mutation described by Williamson et al. [11]. Two alleles were detected and the sites of the polymorphism are indicated by a "Y."

pyrethroid insecticides (i.e., they have higher levels of permethrin resistance in laboratory bioassays) it has not been detected in the USA. Thus, it appears that either there is very limited movement of flies (i.e., transport) from other continents into the US or that there is no selective advantage for *super-kdr* flies (beyond the other resistance genes they possess) in the USA. Two *VSSC* alleles were detected in the NG98 strain and they differed at three nucleotide positions (C203T, T247C, and T295C, Fig. 2). However, both alleles (*kdr*) coded for the same protein sequence. The most frequent nucleotide at positions 203, 247, and 295 appeared to be C, T, and T, respectively, as flies homozygous for this allele were found, but only heterozygotes for the other allele were detected.

4. Discussion

Detection of the autosomes involved in insecticide resistance in the house fly has been accomplished via factorial analyses of backcross progeny (as in this paper), or by the isolation of isochromosomal strains. The LPR strain of house fly has been analyzed by both of these methods and nearly identical results were obtained (compare [27] and [30]). This indicates that comparing strains that were analyzed by these two methods is feasible.

In house flies, autosomes 1, 2, 3, and 5 have been historically associated with pyrethroid resistance, with the major mechanisms of resistance being monooxygenase-mediated resistance on autosomes 1, 2, and 5 [7], and *kdr*/*super-kdr* (and *pen*) on autosome 3 [31,32]. Pyrethroid resistance

in house flies has been detected throughout the world, and investigations into the mechanisms and genetics responsible for the very high levels of resistance (>1000-fold) have been carried out with strains from the US and Japan: LPR (from Horseheads, NY), ALHF (AL), YPER (Japan), and NG98 (GA) [19,28,33,34]. Permethrin resistance (6000-fold) in LPR is due to monooxygenase-mediated resistance on autosomes 1 and 2, plus target site insensitivity (*kdr*) and decreased penetration on autosome 3 [19,26,30,35–38]. The monooxygenase-mediated resistance is due to increased transcription of *CYP6D1v1* due to factors on autosomes 1 and 2 [9] and possibly to a unique allele (*CYP6D1v1*) on autosome 1 [10]. ALHF (collected in AL) has 1800–9200-fold permethrin resistance [28,34], due to *kdr* (Liu and Pridgeon, personal communication) and a factor on autosome 5 that can be overcome by piperonyl butoxide [28,34]. YPER (from Yumenoshima Island, Japan) has >18,400-fold resistance due to *super-kdr* (and perhaps *pen*) on autosome 3 and monooxygenase-mediated resistance on autosomes 1, 2, and 5 based on synergist, and biochemical studies [19]. The number of alleles responsible for monooxygenase-mediated pyrethroid resistance in other strains of house flies is not known (although the trait is polygenic in ALHF and YPER as described above). Although it is not possible to determine the precise number of applications or the exact insecticides used at each of these locations over the past 60 years, there are some generalizations that can be made. First, it is likely that all strains are from populations that have been treated with organochlorine, cyclodiene, organophosphates, and a pyrethroid (permethrin). Second, the strains from the USA come from populations that have had more similar insecticide treatments (both intervals of use and specific insecticides) than the strain from Japan. Further, YPER was from an area that was treated with organophosphates for a longer period of time, and with pyrethroids for a shorter period of time, relative to the strains from the USA [19]. Another important consideration is that although the selection pressures may be similar at the different sites, the relative fitness cost associated with a given resistance allele may be different in dissimilar climates [39].

Previous work has shown that permethrin resistance in NG98 is due to monooxygenase-mediated detoxification, based on synergist, biochemical, immunological and Northern blot analyses (due in part to *CYP6D1* [10,18]), and *kdr* (and perhaps decreased penetration) [18]. Results from this study are consistent with those results and indicate that monooxygenase-mediated resistance is associated with autosomes $5 \gg 2 > 1$ (Table 1) (given that *kdr* is on autosome 3 and that monooxygenase-mediated resistance has never been associated with autosome 3).

Comparison of the results with NG98 and the three other cases of high level permethrin resistance (YPER, LPR, and ALHF) indicates the genetic basis of this trait is surprisingly complex (Table 2), even though all three strains of house fly were homozygous (i.e., similar slope of dose–response lines compared to homozygous susceptible strains) and selected with the same pyrethroid (permethrin). While the strain from Japan (YPER) had *super-kdr* and the strains from the US (ALHF, LPR, and NG98) had only *kdr*, a more notable difference between the strains was that they did not evolve an identical molecular basis of monooxygenase-mediated resistance. The most important autosome in terms of monooxygenase-mediated permethrin resistance varied between strains, being

Table 2
Relative importance of each autosome to permethrin resistance in the house fly

Strain	Collection site	Ranking of autosomes involved in resistance[a]	References
NG98	Georgia, USA	$5 > 3 > 1 > 2$	This paper
ALHF	Alabama, USA	$3 \geqslant 5 \gg 1 \sim 2$	[28]
LPR	New York, USA	$3 > 1 > 2$	[27,30,35]
YPER	Yumenoshima, Japan	$2 > 3 > 5 \sim 1$	[19]

[a] Autosome 3: *kdr* in NG98, ALHF, and LPR, *super-kdr* in YPER. Autosomes 1, 2, and 5 represent monooxygenase-mediated resistance genes.

autosome 1 for LPR, autosome 2 for YPER, and autosome 5 for ALHF and NG98. One of the most dramatic differences is between ALHF, in which autosome 5 is responsible for nearly all of the monooxygenase-mediated resistance, and LPR, in which autosome 5 is of no importance. In the LPR strain monooxygenase-mediated permethrin resistance is due to *CYP6D1* and is associated with autosomes 1 and 2. In YPER, *CYP6D1* expression is only slightly increased and YPER does not have the *CYP6D1v1* allele (on autosome 1) that is associated with resistance. Therefore, it appears clear that YPER and LPR derive their monooxygenase-mediated resistance to pyrethroids from different P450 isoforms.

Although autosome 2 is important in the LPR and YPER strains (and to a lesser degree in NG98), it is of nearly no consequence in the ALHF strain indicating that the proposed "master gene" on autosome 2 [40] is not involved in all cases of monooxygenase-mediated pyrethroid resistance. While the differing genetic basis underlying pyrethroid resistance is likely due to the different selection histories of each strain (prior to collection and selection with permethrin), these results portend great difficulty for the development of diagnostic tools that could be used reliably over large areas to monitor monooxygenase-mediated pyrethroid resistance. The involvement of different P450s in resistance in different strains suggests that the patterns of cross-resistance may potentially vary between strains. In addition, the relative plasticity with which the P450 monooxygenases are able to respond to produce resistance helps to explain another reason why this mechanism is so widespread.

In conclusion, the genetic basis of resistance mediated by the cytochrome P450 monooxygenases is variable in different populations. It appears that pyrethroid resistance can evolve using different P450s (and possibly different regulatory signals controlling P450 expression) even in strains selected with the same insecticide.

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