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The *CYP6D1v1* Allele Is Associated with Pyrethroid Resistance in the House Fly, *Musca domestica*

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CYP6D1 is the cytochrome P450 responsible for pyrethroid resistance in the LPR strain of house fly, which was originally collected from New York and was recently implicated as a mechanism of resistance in house flies from Georgia. We sequenced *CYP6D1* from the NG98 strain of house fly from Georgia and found that the *CYP6D1* allele in this strain is identical to that found in the LPR strain (*CYP6D1v1*). This is in contrast to the other five alleles of *CYP6D1* from pyrethroid-susceptible strains which were all unique (i.e., different from all other strains). These results indicate that CYP6D1-mediated resistance may have evolved once and then spread between these two states. This is unexpected as house flies are not documented to disperse over long distances. The finding of identical alleles in the pyrethroid-resistant NG98 and LPR strains supports the hypothesis that the different CYP6D1 protein in resistant strains contributes to their resistance.

Key Words: Insecta; *Musca domestica*; cytochrome P450 monooxygenases; pyrethroid resistance.

INTRODUCTION

CYP6D1 is the cytochrome P450 responsible for pyrethroid resistance in the Learn-PyR (LPR) strain of house fly (1). The increased level of detoxification of pyrethroids by the LPR strain is a result of the overexpression of CYP6D1 (2, 3). The mechanism underlying this phenomenon is increased transcription of the *CYP6D1* gene (4). Alleles of *CYP6D1* have been sequenced from LPR and five pyrethroid-susceptible strains of house flies, and several differences in the nucleotide sequence have been documented (5). One of the most interesting differences is that the LPR strain open reading frame (ORF) codes for a protein that differs from all susceptible strains by five amino acids ($Asp_{150} \rightarrow$ Ala, $Ile_{153} \rightarrow$ Leu, $Thr_{165} \rightarrow$ Ser, $Glu_{218} \rightarrow$ Gln and $Met_{227} \rightarrow$ Ile). This is in contrast to a related house fly P450 (*CYP6D3*) in which LPR is not uniquely different from susceptible strains (6). This suggests that there is a functional consequence for LPR having this particular *CYP6D1* allele. Another notable difference in *CYP6D1* sequences was the presence of a 15-bp insert in the 5′ flanking region found only in the LPR strain. This 15-bp insert has been suggested to play a role in the increased transcription of *CYP6D1* in LPR (5, 7). A limitation of the previous studies was that there was only one resistant strain (LPR) with which to make correlations.

The NG98 strain of house fly was collected from a poultry facility in Georgia in 1998. Previous studies revealed that it was highly resistant to permethrin, the resistance was suppressible with piperonyl butoxide, and CYP6D1 levels were elevated relative to susceptible strains (8). This strain offers a unique opportunity to test the hypothesis that *CYP6D1* alleles are involved in resistance.

Herein we report the sequence of *CYP6D1* from two strains (NG98 and Bryant) of house fly from Georgia and the development of an allele-specific PCR for detection of *CYP6D1* alleles carrying the 15-bp insert. Implications for the evolution of CYP6D1-mediated resistance are discussed.

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MATERIALS AND METHODS

House Flies

Six strains of house flies were used in this study. Cornell susceptible (CS) is an insecticide-susceptible strain reared in our laboratory without exposure to insecticides (9), and aabys is a susceptible strain with morphological markers on each autosome (obtained from Dr. T. Hiroyoshi, Osaka University). LPR is a multiresistant strain having high levels of resistance to pyrethroid insecticides due to increased oxidative metabolism mediated by cytochrome P450 CYP6D1 (1, 10, 11). Two other mechanisms of resistance to pyrethroid insecticides in the LPR strain are insensitivity of the nervous system (*kdr*) and decreased cuticular penetration (*pen*) (10). The NG98-U strain was established from flies collected at a poultry farm in Georgia in 1998 (8). NG98-U was selected for two generations in the laboratory to produce the NG98 strain which was highly resistant to permethrin due to monooxygenase-mediated detoxification and *kdr* (8). Bryant was a strain of house fly collected from the same poultry farm as NG98-U, but was maintained without selection pressure in Georgia by Craig Sheppard (University of Georgia). House flies were reared as described previously (12).

Sequencing of CYP6D1 from NG98

We used a strategy of PCR amplification and cloning to determine the sequence of the *CYP6D1* allele from the NG98 strain using methods reported previously (5) and described briefly below. PCR was used to amplify the 5′ flanking region of *CYP6D1* from the NG98 strain using DNA (13) as template, with S34 (5′-CTCCATAGTATCGTGGAGGGT-3′) and A28 (5′-CCCCTTGAGTTTACCCGACG-3′) primers. The PCR product was then purified (5) and clones were constructed by inserting the product into pCR 2.1 vector using the Original TA Cloning Kit (Invitrogen) as described by the manufacturer. The 3′ end of the gene was sequenced in a similar manner except that primers S18 (5′-AAAAGGCCCAATCTGAGGTG-3′) and A20 (5′-CTTAACCACTTGCTCTTCAC-3′) were used. PCR was used to amplify a portion of the *CYP6D1* ORF from the NG98 strain using mRNA (6) as template with primers S26 (5′-TCGTCGGGTAAACTCAAGGGG-3′) and AS27 (5′-TGCCTTTGCAGACACTCATC-CAC-3′). With this set of clones nearly the entire sequence of *CYP6D1* could be determined (except for introns 1 and 2).

Sequencing

Plasmid DNA was sequenced by the Cornell Biotechnology Center. The sequence of a given clone was determined a minimum of two times or until all ambiguities could be resolved. A minimum of three clones were sequenced for each region of the gene (see above).

Allele-Specific PCR

An allele-specific PCR was developed to determine whether individual flies contained the 15-bp insert characteristic of the LPR *CYP6D1v1* allele. DNA was isolated from individual flies (13) by homogenization in 500 μl lysis buffer (100 mM Tris, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, and 0.5 mM spermidine) and then 5 μl of proteinase K (233 U/ml) was added. The mixture was extracted three times with equal volumes of phenol, phenol:chloroform (1:1), and chloroform, respectively. A 0.1 volume of 2.5 M sodium acetate was added and gently mixed. Two volumes of ethanol were added and the tube was held at −80°C for 1 h. The DNA was pelleted by centrifugation. *CYP6D1* was amplified by PCR under the following conditions. The reaction mixtures (20 μl) contained 2.0 μl 10× PCR buffer (Gibco), 0.4 μl dNTP (10 mM), 0.6 μl $MgCl_2$ (50 mM), 0.2 μl *Taq* (5 U/μl; Gibco), 0.4 μl of a primer (slpr) specific for the 15-bp insert found in pyrethroid resistant strains (100 pmol/μl) (5′-GCATTCGAATCATTCTG-TTTCAC-3′), 0.4 μl of primer AS2 (100 pmol/μl) (5′-CATTGGATCATTTTTCTCATC-3′), 14.0 μl H_2O, and 2.0 μl genomic DNA (~500 ng). Reaction mixtures were held at 94°C for 5 min, then 30 cycles of PCR (94°C for 45 s, 55°C for 45 s, and 72°C for 50 s) were done, and the samples were finally kept at 72°C for 5 min.

TABLE 1
Percentage Identity (Based on Nucleotide Sequence) of Seven *CYP6D1* Alleles with Each Other and with *CYP6D3*

	LPR	NG98[a]	CS	ISK	aabys	Rutgers	Bryant Minus[b]
CYP6D3	74.9	74.9	74.0	73.5	74.4	74.9	69.8
LPR		100	96.5	96.0	97.8	98.2	94.8
NG98[a]			96.5	96.0	97.8	98.2	94.8
CS				99.6	97.8	98.2	98.4
ISK					97.3	97.8	98.4
aabys						99.6	95.8
Rutgers							96.4

[a] NG98 sequence was identical to the sequence of the Bryant flies, which had the 5′ flanking sequence 15-bp insert.
[b] Bryant Minus are flies from the Bryant strain lacking the 5′ flanking sequence 15-bp insert.

PCR products were visualized with ethidium bromide on a 1.2% agarose gel.

RESULTS

CYP6D1 was sequenced from the NG98 strain and it is nearly identical to the sequence from LPR. There were only two nucleotide differences, one each in introns 3 and 4. Thus, the ORF and 5′ flanking sequences (up 658 nt before the start codon) were identical in NG98 and LPR. This represents the first *CYP6D1* allele with such a high degree of similarity to another strain (Table 1, Fig. 1).

Given that resistant and susceptible strains differed by the presence or absence of the 15-bp insert in the *CYP6D1* 5′ flanking region, we developed a method for genotyping flies based on this feature. Using individual flies we compared the frequency of this insert and the results are shown in Table 2. These results are consistent with what we know about pyrethroid resistance in these strains; the 15-bp insert was found only in the homozygous resistant strains (LPR and NG98) and not in the homozygous susceptible strains (CS and aabys). The heterozygous resistant strains (NG98-U and Bryant) have individuals with or without the insert. The 15-bp insert was detected in F_1 individuals, indicating that this PCR technique detects the insert whether the individual is heterozygous or homozygous for the *CYP6D1v1* allele (Table 2). This technique appears to be well suited to resistance monitoring in house flies, at least in North America where CYP6D1-mediated resistance has been documented, although for this purpose modification of the procedure to be able to distinguish heterozygous individuals is clearly desirable.

Due to the tremendous similarity of NG98 to

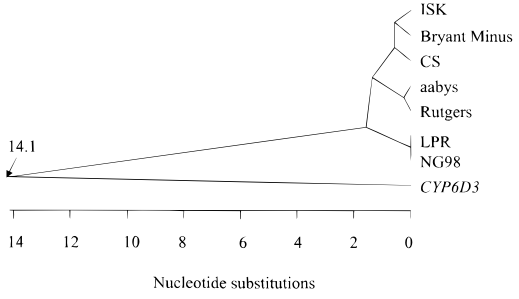

FIG. 1. Diagrammatic representation of the relatedness between *CYP6D3* and the seven alleles of *CYP6D1* (indicated by strain name).

TABLE 2
Relative Frequency of the 15-bp Insert (Characteristic of the *CYP6D1v1* Allele) in Different House Flies

Strain	Number	
	With 15-bp insert	Total tested
aabys	0	20
CS	0	20
LPR	20	20
F_1 (LPR × CS)	20	20
NG98-U	12	20
NG98	20	20
Bryant	23	25

LPR it was necessary to confirm that this was not due to contamination of the NG98 strain by LPR. We again obtained flies from Georgia (Bryant strain). These pupae were allowed to emerge, the adults were frozen, and DNA was extracted without rearing of the strain. Given that this strain was from a field collection and had not been selected with pyrethroids in the laboratory, we expected that it would not be homozygous for resistance and this was confirmed by our allele-specific PCR (Table 2). Using primers S1 (5′-ACCCTCTATATCTTTGCCAA-3′) and AS14 (5′-ACATTGTCGACTTCTTTGGG-3′) we sequenced the *CYP6D1* allele from three individuals of the Bryant strain having the 15-bp insert and from two individuals lacking the insert. The three individuals having the 15-bp insert had sequences that were 100% identical to NG98 and LPR (i.e., *CYP6D1v1*). Those lacking the insert (Bryant Minus; Table 1, Fig. 1) had a unique sequence different from the other alleles that have been sequenced and which appears to be the allele originally present in this population before the genetic sweep of the resistance allele (*CYP6D1v1*) occurred.

DISCUSSION

Given the identical alleles found in the NG98 and LPR resistant strains from Georgia and New York it becomes a question as to whether these resistance-associated alleles arose independently or whether there is actually gene flow between these two regions. The phenomenal nucleotide identity between the NG98 and the LPR strains strongly suggests that CYP6D1 resistance must have evolved once and then spread. Studies of house fly movement indicate that 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 Georgia to New York. Could house flies actually disperse this far? Given the relatively short life span of a house fly the answer to this question appears to be no (at least in one generation), unless the flies were aided in some way. Here there are two possibilities. First, humans may act as vehicles for the movement of house flies (in cars, trains, planes, etc.). Second, 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). Furthermore, it is possible that the spread of CYP6D1-mediated resistance occurred over many years and generations.

One important aspect for the development of effective resistance management tactics is to understand the geographic area over which a strategy must be implemented. For example, there is apparently widespread migration of house flies among New York dairies as the pattern of resistance to insecticides is similar at geographically widespread dairies independent of their insecticide use (17, 18). If there is actually mixing of populations from places as distant as New York and Georgia, this suggests that resistance management would have to be undertaken over an enormous area to have the greatest chance of success. While resistance management has been undertaken in some large areas (e.g., against cotton pests in cotton-growing regions of Australia), the larger the size of the area the more difficult it becomes to successfully implement resistance management strategies.

Another interesting finding is that the flies from Georgia had both the altered ORF and the 15-bp insert in the 5′ flanking region that had previously been found only in LPR. Thus, these traits do not seem to segregate. While one could envision CYP6D1-mediated resistance occurring in different steps (change in the ORF, change in the autosome 1 transcription factor, and change in the autosome 2 transcription factor), it appears that at least the changes in the ORF and in the 15-bp insert may have arisen together (i.e., from a individual common ancestor). The strong correlation that now exists between the resistance and the *CYP6D1v1* allele provides good evidence, albeit indirect, that the *CYP6D1v1* allele plays a role in resistance. Future studies with heterologous expression systems will be necessary to determine quantitatively the selective advantage of this allele (i.e., in terms of pyrethroid metabolism).

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REFERENCES

1. G. D. Wheelock and J. G. Scott, The role of cytochrome $P450_{lpr}$ in deltamethrin metabolism by pyrethroid resistant and susceptible strains of house flies, *Pestic. Biochem. Physiol.* **43,** 67 (1992).
2. G. D. Wheelock and J. G. Scott, Immunological detection of cytochrome P450 from insecticide resistant and susceptible house flies (*Musca domestica*), *Pestic. Biochem. Physiol.* **38,** 130 (1990).
3. T. Tomita, N. Liu, F. F. Smith, P. Sridhar, and J. G. Scott, Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica, Insect Mol. Biol.* **4,** 135 (1995).
4. N. Liu and J. G. Scott, Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly, *Insect Biochem. Mol. Biol.* **28,** 531 (1998).
5. J. G. Scott, N. Liu, Z. Wen, F. F. Smith, S. Kasai, and C. E. Horak, House fly cytochrome P450 *CYP6D1*: 5 prime flanking sequences and comparison of alleles, *Gene* **226,** 347 (1999).
6. S. Kasai and J. G. Scott, Expression and regulation of CYP6D3 in the house fly, *Musca domestica* (L), *Insect Biochem. Mol. Biol.* **32,** 1 (2002).
7. S. Kasai and J. G. Scott, Cytochrome P450s CYP6D3 and CYP6D1 are part of a P450 gene cluster on autosome 1 in the house fly, *Insect Mol. Biol.* **10,** 191 (2001).
8. S. Kasai and J. G. Scott, Overexpression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia, *Pestic. Biochem. Physiol.* **68,** 34 (2000).
9. J. G. Scott, P. Sridhar, and N. Liu, Adult specific expression and induction of cytochrome $P450_{lpr}$ in house flies, *Arch. Insect Biochem. Physiol.* **31,** 313 (1996).
10. J. G. Scott and G. P. Georghiou, Mechanisms responsible for high levels of permethrin resistance in the house fly, *Pestic. Sci.* **17,** 195 (1986).
11. J. G. Scott, Molecular basis of insecticide resistance: Cytochromes P450, *Insect Biochem. Mol. Biol.* **29,** 757 (1999).
12. P. J. Korytko and J. G. Scott, CYP6D1 protects thoracic ganglia of house flies from the neurotoxic insecticide cypermethrin, *Arch. Insect Biochem. Physiol.* **37,** 57 (1998).
13. N. Liu, T. Tomita, and J. G. Scott, Allele-specific PCR reveals that the cytochrome $P450_{lpr}$ gene is on chromosome 1 in the house fly, *Musca domestica, Experientia* **51,** 164 (1995).
14. K. D. Quarterman, M. Willis, and J. W. Kilpatrick, Urban fly dispersal in the area of Savannah, Georgia, *J. Econ. Entomol.* **47,** 405 (1954).
15. F. C. Bishop, Dispersion of flies by flight, *J. Agric. Res.* **21,** 729 (1921).
16. E. J. Shields and A. M. Testa, Fall migratory flight initiation of the potato leafhopper, *Empoasca fabae* (Homoptera: Cicadellidae): Observations in the lower atmosphere using remote piloted vehicles, *Agric. Forest Meterol.* **97,** 317 (1999).
17. J. G. Scott, R. T. Roush, and D. A. Rutz, Resistance of house flies to five insecticides at dairies across New York, *J. Agric. Entomol.* **6,** 53 (1989).
18. P. E. Kaufman, J. G. Scott, and D. A. Rutz, Monitoring insecticide resistance in house flies (Diptera: Muscidae) from New York dairies, *Pest Manag. Sci.* **57,** 514 (2001).