Evolutionary Changes in Gene Expression, Coding Sequence and Copy-Number at the Cyp6g1 Locus Contribute to Resistance to Multiple Insecticides in Drosophila

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Abstract

Widespread use of insecticides has led to insecticide resistance in many populations of insects. In some populations, resistance has evolved to multiple pesticides. In Drosophila melanogaster, resistance to multiple classes of insecticide is due to the overexpression of a single cytochrome P450 gene, Cyp6g1. Overexpression of Cyp6g1 appears to have evolved in parallel in Drosophila simulans, a sibling species of D. melanogaster, where it is also associated with insecticide resistance. However, it is not known whether the ability of the CYP6G1 enzyme to provide resistance to multiple insecticides evolved recently in D. melanogaster or if this function is present in all Drosophila species. Here we show that duplication of the Cyp6g1 gene occurred at least four times during the evolution of different Drosophila species, and the ability of CYP6G1 to confer resistance to multiple insecticides exists in D. melanogaster and D. simulans but not in Drosophila willistoni or Drosophila virilis. In D. virilis, which has multiple copies of Cyp6g1, one copy confers resistance to DDT and another to nitenpyram, suggesting that the divergence of protein sequence between copies subsequent to the duplication affected the activity of the enzyme. All orthologs tested conferred resistance to one or more insecticides, suggesting that CYP6G1 had the capacity to provide resistance to anthropogenic chemicals before they existed. Finally, we show that expression of Cyp6g1 in the Malpighian tubules, which contributes to DDT resistance in D. melanogaster, is specific to the D. melanogaster–D. simulans lineage. Our results suggest that a combination of gene duplication, regulatory changes and protein coding changes has taken place at the Cyp6g1 locus during evolution and this locus may play a role in providing resistance to different environmental toxins in different Drosophila species.

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Introduction

Different mechanisms have evolved that allow organisms to avoid or detoxify harmful chemicals found in their environment or food source. One possible mechanism is a change in a detoxification enzyme that confers a novel metabolic activity, such as the glycine to aspartic acid replacement in the active site of the detoxification enzyme in Drosophila melanogaster, which lowers the activity of the enzyme. All orthologs tested conferred resistance to one or more insecticides, suggesting that CYP6G1 had the capacity to provide resistance to anthropogenic chemicals before they existed. Finally, we show that expression of Cyp6g1 in the Malpighian tubules, which contributes to DDT resistance in D. melanogaster, is specific to the D. melanogaster–D. simulans lineage. Our results suggest that a combination of gene duplication, regulatory changes and protein coding changes has taken place at the Cyp6g1 locus during evolution and this locus may play a role in providing resistance to different environmental toxins in different Drosophila species.

Rst2/DDT, a locus in D. melanogaster that is associated with resistance to multiple classes of insecticides in some strains, was mapped to a small region on chromosome 2R containing several P450s. Of these, a single P450 gene, Cyp6g1, is overexpressed in resistant strains. There are no amino acid differences in the proteins encoded by the resistant and susceptible alleles of this gene, consistent with evidence that suggests resistance evolved very recently [9,10]. The insertion of the long terminal repeat (LTR) of an Ac/Ds transposable element into the 5’ region of Cyp6g1 is associated with resistance to the insecticide permethrin [6], and gain of Cyp6g1 expression in the brain of Tribolium castaneum is responsible for deltamethrin resistance [7], while in the aphid Myzus persicae, an increase in copy number of the Cyp6g3 gene is associated with resistance to neonicotinoid insecticides [8].
A complex, adaptive allelic series exists at this locus, including gene copy-number variation and the insertion of various transposable elements, and each step towards the most derived allele is associated with higher transcription of \textit{Cyp6g1} and increased resistance to dichlorodiphenyltrichloroethane (DDT) \cite{12}. The importance of this gene in adaptation is further highlighted by parallel evolution in the closely related species \textit{Drosophila simulans}. A \textit{Doc} transposable element insertion 5' to the \textit{Cyp6g1} gene has been identified in \textit{D. simulans} populations. Similar to the \textit{Accord LTR} in \textit{D. melanogaster}, the \textit{Doc} element is associated with a selective sweep, overexpression of \textit{Cyp6g1} and DDT resistance \cite{13}. The constitutive overexpression of \textit{Cyp6g1} is a significant adaptive response, as exposure to insecticides results in very little induction of transcription of \textit{Cyp} genes \cite{14}. 

Overexpression of \textit{Cyp6g1} in the metabolic tissues provides an example of how increased expression of a single gene can confer resistance to a wide variety of xenobiotics with different chemical structures \cite{11,15}. Because this resistance occurs without changes to the protein sequence, this suggests that the capability of the CYP6G1 protein to provide resistance to various classes of chemicals is present in all \textit{D. melanogaster} strains, and may be an ancestral feature of the protein. If so, then many other insects

\textbf{Figure 1. Cyp6g1 and Cyp6g2 copy number in twelve Drosophila species.} Cyp6g1 is duplicated in \textit{D. willistoni}, \textit{D. grimshawi} and some strains of \textit{D. melanogaster}, and triplicated in \textit{D. virilis}. The third copy of \textit{Cyp6g1} in the strain of \textit{D. virilis} used for this study has an inactivating mutation, but this mutation is not present in the sequenced strain, so it is not formally a pseudogene. Comparison with the phylogeny of the species suggests that multiple independent duplication events occurred (cladogram inferred from Stark \textit{et al.} \cite{21}). In contrast, \textit{Cyp6g2} has 1:1 orthologs in all twelve \textit{Drosophila} species analyzed.

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might also have the potential to become resistant to the same classes of chemicals by overexpression of Cyp6g1. In contrast, if the ability to confer resistance to chemicals is only encoded by D. melanogaster Cyp6g1 and not by Cyp6g1 orthologs in closely related species, this would suggest that the potential for broad-spectrum resistance is a derived capacity of CYP6G1 originating from amino acid changes in the D. melanogaster lineage. Distinguishing these two possibilities may lead to a better understanding of how resistance to multiple classes of insecticides evolves, as well as contribute to pest management strategies.

Here, we address this question by examining the Cyp6g1 gene in four Drosophila species that last had a common ancestor approximately 40 million years ago [16]. The contribution of regulatory and coding sequence adaptation to the evolution of insecticide resistance was studied by comparing the expression and resistance phenotypes associated with Cyp6g1 orthologs. Transgenic expression of the Cyp6g1 orthologs in a consistent genetic background was used to determine whether changes in the coding sequences result in different abilities to provide resistance to xenobiotics, and whether the resistance potential of Cyp6g1 only exists in D. melanogaster or is found in other members of the genus.

**Results**

**Gene duplication of Cyp6g1 has occurred multiple times in the Drosophila genus**

As the number of P450s varies between genomes [17,18], and the copy number of Cyp6g1 is polymorphic in D. melanogaster [12], the presence or absence and copy number of Cyp6g1 in 12 Drosophila species with complete reference genomes was investigated [19]. This was accomplished using searches in FlyBase [20] and manual annotation. Orthologs were found in other sequenced Drosophila species using the predicted amino acid sequence of CYP6G1 from the sequenced y, cn bw sp strain of D. melanogaster as a query in BLASTp searches. In the genomes of D. simulans, Drosophila sechellia, Drosophila yakuba, Drosophila erecta, Drosophila ananassae, Drosophila pseudoobscura, Drosophila persimilis and Drosophila mojavensis, Cyp6g1 was found as a single copy, as in the D. melanogaster y, cn bw sp genome. In Drosophila willistoni and Drosophila grimshawi, Cyp6g1 duplications were found, although one copy in D. willistoni is truncated and presumably a pseudogene. In Drosophila virilis, three copies of Cyp6g1 were found [Fig. 1].

There are two possible explanations for the multiple copies of Cyp6g1 in different lineages: either there were independent gene duplication events that occurred in each species that has more than one copy of Cyp6g1, or the common ancestor of all twelve species had multiple copies of Cyp6g1, but the species with only one copy have independently lost one or more of the ancestral copies. To distinguish between these possibilities, an unrooted neighbour-joining tree of the Cyp6g1 orthologs from the 12 Drosophila genomes was plotted [Fig. 2]. In the species where there are multiple copies of Cyp6g1, the paralogs cluster with each other rather than with Cyp6g1 orthologs from other species. Considered with the phylogeny of the species [21], this indicates that gene amplification events in each lineage occurred independently, suggesting that some selective advantage to multiple copies of Cyp6g1 may have existed during the evolution of these species.

**Transgenic overexpression of Cyp6g1 orthlogs from different Drosophila species does not produce similar resistance profiles**

Transgenic overexpression of D. melanogaster Cyp6g1 (Dmel-Cyp6g1) in the midgut, Malpighian tubules and fat body confers resistance to four different insecticides (DDT, lufenuron, nitenpyram and diazinon) with very different chemical structures [11,15]. When expressed in tobacco cell culture, Dmel-CYP6G1 is able to perform hydroxylation of imidacloprid and dechlorination of DDT—one different chemical reactions on two chemically different insecticides [22]. This indicates that Dmel-CYP6G1 is a P450 with broad substrate specificity.

In order to investigate the functional divergence of CYP6G1, and to determine whether the capacity of Dmel-CYP6G1 to provide resistance to a range of chemicals was ancestral to the different Drosophila species or arose in the D. melanogaster lineage, orthologs from D. melanogaster (Dmel-Cyp6g1, cloned from the single-copy y, cn bw sp strain), D. simulans (Dsim-Cyp6g1), D. willistoni (Dwil-Cyp6g1) and the two expressed paralogs from D. virilis (Dvir-Cyp6g1a and Dvir-Cyp6g1b) were cloned into a specific transgene insertion site in a consistent genetic background (the 86Fb strain) in D. melanogaster. The third copy of Cyp6g1 (Dvir-Cyp6g1c) from D. virilis was not detected by RT-PCR in several life stages and had an inactivating mutation in the strain used for this study, which is not present in the sequenced strain (data not shown). The use of a defined transgene insertion site allowed us to compare the capability of each ortholog to confer resistance directly, without confounding position effects [23]. The 5′Cyp6g1HR-3a-GAL4 driver was used to overexpress the orthologs in the midgut, Malpighian tubules and fat body, an approach which has been validated for testing the resistance potential of genes from distantly related insects [24].

Transgenic flies expressing each ortholog were exposed to three different insecticides (DDT, nitenpyram and dicyclanil) to determine whether overexpression conferred resistance (i.e. increased survival) compared to the background strain [Fig. 3A]. Overexpression of Dmel-Cyp6g1 or Dsim-Cyp6g1 resulted in increased survival on DDT, nitenpyram and dicyclanil. Cyp6g1 from D. simulans and D. melanogaster produce qualitatively identical responses under these conditions, but the functions of all the other orthologs were divergent. Dwil-Cyp6g1 conferred resistance only to dicyclanil. Of the two orthologs from D. virilis, Cyp6g1a conferred resistance to DDT, and Cyp6g1b conferred resistance to nitenpyram [Fig. 3A]. Although the survival of flies expressing Dvir-Cyp6g1a on dicyclanil was statistically higher than control flies, the increase was only 1.01–1.10 fold, suggesting that the resistance is not biologically relevant. The functions of the two copies of Cyp6g1 from D. virilis were qualitatively different despite the 94% identity of their amino acid sequences [Fig. S1].

**Tissue-specific expression of Cyp6g1 has diverged in the Drosophila genus**

The differences in the range of chemicals to which the Cyp6g1 orthologs from different Drosophila species provide resistance when overexpressed raise other questions. Are the Cyp6g1 orthologs expressed in the same tissues as Dmel-Cyp6g1 or do they have restricted expression patterns in specialized tissues, as is the case for Cyp6g2, which is specifically expressed in the corpus allatum [17]? To investigate this, RNA in situ hybridization with probes directed against Dsim-Cyp6g1, Dwil-Cyp6g1 and Dvir-Cyp6g1 was performed on third instar larvae of these species. Because Dvir-Cyp6g1a and Dvir-Cyp6g1b are very similar at the nucleotide level, it
was not possible to design probes that discriminate between the two copies.

The expression of Dmel-Cyp6g1 has been previously described. Expression in the midgut, Malpighian tubules and fat body is controlled by two distinct tissue-specific enhancers, one that drives expression in the Malpighian tubules and another that drives expression in the midgut and fat body \[11,25\]. mRNA for the Cyp6g1 orthologs from all four species was detected in the midgut. However, Malpighian tubule expression as reported in D. melanogaster was detected in D. simulans but not in D. willistoni or D. virilis. Fat body expression was detected in all species except D. willistoni (Fig. 4). These results indicate that gene expression of Cyp6g1 has evolved in the Drosophila genus, and expression of Cyp6g1 in the Malpighian tubules is restricted to the D. melanogaster–D. simulans lineage.

**Discussion**

In this paper, evidence that Cyp6g1 is duplicated at least four times in the Drosophila genus is presented (Fig. 1). Based on phylogenetic analysis of the protein sequences, it can be concluded that the duplications were independent events (Fig. 2). There is strong evidence that the duplication in D. melanogaster occurred very recently \[12\]. In contrast, Cyp6g2, which is expressed in the corpus allatum in D. melanogaster \[17\], has a 1:1 ortholog in all 12 Drosophila species. This supports the inference that across the different Drosophila species, there may be selection for increased Cyp6g1 copy number, presumably via selection for an increased amount of gene product.

In order to determine whether the potential for Dmel-CYP6G1 to provide resistance to insecticides is ancestral, the resistance conferred by overexpressing Cyp6g1 orthologs from four different species was tested. Similar to Dmel-Cyp6g1, overexpression of Dsim-Cyp6g1 conferred resistance to the three insecticides tested (DDT, nitenpyram and dicyclanil), which corroborates the report of parallel evolution in D. melanogaster and D. simulans at the Cyp6g1 locus \[13\]. Although metabolism of the insecticides was not tested and the metabolic detoxification of insecticides by Cyp6g1 is not well understood \[22\], and some orthologs may have the ability to confer resistance to insecticides that were not tested, these results suggest that Dsim-CYP6G1 has metabolic ability and broad substrate specificity similar to Dmel-CYP6G1. In contrast, overexpression of Dwil-Cyp6g1 only conferred resistance to one of the three insecticides tested (dicyclanil), and the two expressed copies of Cyp6g1 in D. virilis, Dwir-Cyp6g1a and Dwir-Cyp6g1b,
Evolution at Cyp6g1 on Insecticide Resistance

Figure 3. Overexpression of different Cyp6g1 orthologs confers resistance to different insecticides. A) Changes in survival following exposure to three classes of insecticide by expression of Cyp6g1 orthologs from D. melanogaster, D. simulans, D. willistoni and D. virilis in a consistent genetic background. Resistance ratio (RR) was calculated by comparing the concentration of insecticide that killed 50% of insects (the LC50) between the line expressing the ortholog and the background strain, 86Fb, which was genetically identical except for the absence of the Cyp6g1 construct. Results marked with an asterisk were statistically significant (p<0.05). Orthologs from D. melanogaster and D. simulans were functionally identical at a qualitative level, both providing resistance to all three chemicals, but the resistance profile varied between the other three orthologs. These results suggest that adaptation of the protein has occurred repeatedly in the species. Notably, the CYP6G1 orthologs from the two species where resistance to a range of insecticides or expression in the Malpighian tubules alone results in insecticide resistance, and tissue-specific RNA knockdown of Cyp6g1 in the Malpighian tubules results in lower survival on DDT, whilst knockdown in the fat body and central nervous system do not affect survival [26].

In D. melanogaster, transgenic overexpression of Cyp6g1 in the Malpighian tubules alone results in insecticide resistance, and tissue-specific RNA knockdown of Cyp6g1 in the Malpighian tubules results in lower survival on DDT, whilst knockdown in the fat body and central nervous system do not affect survival [26]. Ubiquitous RNA knockdown of Cyp6g1 does not cause lethality or observable developmental effects, suggesting that Cyp6g1 is not involved in development, unlike other P450s tested including Cyp6g2 [17]. The expression studies indicate that the tissue-specific expression of Cyp6g1 has evolved in the four species tested. Cyp6g1 is not expressed in the Malpighian tubules of D. willistoni or D. virilis. Given that expression of Cyp6g1 in D. melanogaster is controlled by two different enhancers, one for the Malpighian tubules and one for the midgut and fat body, this suggests modularity in the evolution of Cyp6g1 expression in the different species. Notably, the Cyp6g1 orthologs from the two species where Cyp6g1 is expressed in the Malpighian tubules, D. melanogaster and D. simulans, provide resistance to a range of insecticides. The results presented here do not distinguish whether capacity to provide resistance to a range of insecticides or expression in the Malpighian tubules evolved first in these two species. However, a combination of Malpighian tubule expression and the capacity to provide resistance to a range of insecticides, as observed in D. melanogaster and D. simulans strains without transposable element insertions, may have conferred a low level of resistance, even before Cyp6g1 expression was boosted by transposable element insertion, leading to high level resistance to a broad range of insecticides.

The extent to which an enzyme metabolises an insecticide may vary over a continuum. The comparisons between orthologs were based on a threshold of resistance, which is expected to be related to the amount of metabolic activity the enzyme has towards the insecticide, but this was not measured. When genes that are associated with metabolism-based insecticide resistance in pest species are expressed in D. melanogaster, the levels of resistance are generally lower than in the pest species [24]. In this study, the amount of transcript produced for each ortholog was not measured and antibodies for quantification of the proteins are not available. Previous studies have indicated that a high level of transcript is produced using this system, but even if the amount of transcript was controlled across the different transgenic lines, effects such as codon bias and translational efficiency might still result in differing amounts of functional protein, and other factors may be lacking in D. melanogaster that are required for the enzyme to function optimally [15]. Nevertheless, the fact that resistance was conferred to at least one of the insecticides by each ortholog indicates that a significant amount of functional protein was produced, and supports the hypothesis that the potential for the Cyp6g1 gene to provide resistance existed in the last common ancestor of the four Drosophila species, approximately 40 million years ago. This implies that the ability of Cyp6g1 to provide resistance to anthropogenic insecticides when overexpressed predates the existence of the compounds, so this property may be a side effect of adaptation to detoxify allelochemicals present in the food source. Previous evidence indicates that selection with plant allelochemicals affects tolerance to insecticides in moths, and P450 induction by allelochemicals is correlated with insecticide resistance [27,28].

The different resistance profiles of the orthologs suggest that the chemicals to which the CYP6G1 protein has the potential to cause...
resistance are not the same throughout the Drosophila lineage, and it may have evolved to detoxify allelochemicals specific to the habitat of each species. This possibility is highlighted by the duplication of Cyp6g1 in D. virilis, which gave rise to two enzymes with different capacities for resistance. Comparison of the predicted amino acid sequences of the orthologs did not reveal differences that correlate with the differences in resistance when overexpressed (Table S2, Fig. S1). Although a homology model for CYP6G1 has been produced [29], there is not enough information about the relationship between P450 structure and function to allow prediction of metabolic capacity based on primary sequence [30,31], and the lack of functional validation of the model means that these results must be interpreted with caution.

The data presented here suggest that the evolution of resistance to different insecticides involves adaptive changes in both tissue-specific gene expression and coding sequence. These findings may have implications for strategies to combat metabolic insecticide resistance in the field, and contribute to the understanding of the mechanisms by which different evolutionary changes lead to resistance to multiple insecticides. If the ability of P450s to provide resistance to anthropogenic insecticides is a side effect of their evolution to detoxify host xenobiotics, then supposedly naïve insect populations may possess an enzyme activity that can provide resistance to novel insecticides, despite not being optimised for their metabolism. In this case, a regulatory change to increase P450 expression in the appropriate tissues, such as a transposable element insertion [10,13], would be enough to confer resistance to an insect population.

**Materials and Methods**

**Annotation of orthologs and phylogenetic analyses**

The genomic region surrounding Cyp6g1 in each of the 12 Drosophila species was manually annotated using the Artemis software package [32]. The predicted amino acid sequences of the orthologs were aligned and an unrooted, neighbour-joining tree with 1000 bootstrapping iterations was produced with ClustalX 2.1 [33].
RNA in situ hybridization

RNA in situ hybridization was performed as per published protocols [11]. The D. melanogaster probe was used for in situ hybridization for D. simulans. The primers used to design D. willistoni and D. virilis probes are listed in the Table S1.

Overexpression of Cyp6g1 orthologs and insecticide resistance assays

The Cyp6g1 orthologs were cloned using multiple steps into pUASattB using the EcoRI sites and the primers listed in the Table S1 from cDNA synthesized from a Caribbean isolate of D. willistoni Quechua (UCSD stock number 14030-0814.10), a Californian isolate of D. virilis (UCSD stock number 15010-1051.00) and a population of D. simulans. The UAS-Cyp6g1 constructs were transformed into the y\(^\text{+}\) M[vas-int.Dm]ZH-2A w\(^{\text{+}}\); M(3)PS-RFP.attP2/ZH-86Fb recipient strain (herein referred to as 86Fb), which has a defined integration site on Chromosome III, using the attP-attB system and \(\Phi\)C31 integrase [23]. Expression of the Cyp6g1 orthologs was achieved using the Gal4-UAS system by crossing males carrying the UAS-Cyp6g1 constructs to virgin 5\(^{\text{th}}\) Cyp6g1/HR-3a females, which express Gal4 in the midgut, Malpighian tubules and fat body [11]. The background of the progeny was controlled by repeating the cross using 86Fb males instead of UAS-Cyp6g1 males. 3–8 replicates of twenty 4-day-old, mated, adult female progeny were exposed to concentrations of 1–5 \(\mu\)g/vial \(^{-1}\) of DDT (Sigma) using 24 hour contact assays in glass scintillation vials [9]. The number of replicates for each cross at each dose was determined by the abundance of adult flies produced by the cross, and no data were excluded from the analysis. Ten replicates of twenty-five first instar larvae were reared on food containing 0.8–4.5 \% w/v nitenpyram (Novartis) and five replicates on 0.9–1.6 \% w/v dicyclan (Novartis) and emergence was counted after 15 days. Dosage mortality curves were constructed and LC\(_{50}\) values estimated using PriProbIt [34] and resistance ratios and confidence intervals were calculated using the method described by Robertson et al. [35].

Supporting Information

Figure S1 Multiple alignment of the predicted amino acid sequences of the CYP6G1 orthologs that were transgenically expressed in D. melanogaster. There were no changes observed in any of the putative P450 functional domains [36], except a serine to phenylalanine substitution at residue 317 in Dsim-CYP6G1, which did not result in any functional differences between Dsim-CYP6G1 and Dmel-CYP6G1 in our experiments.

Table S1 Primers used to amplify probes for RNA in situ hybridisation and Cyp6g1 orthologs for overexpression studies.

Table S2 Pairwise amino acid identity (%) between CYP6G1 orthologs.

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Author Contributions

Conceived and designed the experiments: TWRH PJD PB HC. Performed the experiments: TWRH TS CL RTG PJD HC. Analyzed the data: TWRH TS PJD PB HC. Wrote the paper: TWRH TS PB HC.

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