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Sequence characterization of cytochrome P450 CYP6P9 in pyrethroid resistant and susceptible *Anopheles funestus* (Diptera: Culicidae)

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ABSTRACT. *Anopheles funestus*, one of the main African malaria vectors, caused a major malaria outbreak in South Africa during 1999/2000, even though South Africa had an effective vector control program in place. The outbreak was due to pyrethroid resistant *An. funestus* invading KwaZulu/Natal. Increased activity of cytochrome P450 (monooxygenase) was responsible for the pyrethroid resistance in this species. A monooxygenase gene, CYP6P9, was highly overexpressed in the pyrethroid-resistant strain compared with a susceptible strain. Characterization of this gene as well as the redox partners involved in the catalytic cycle of P450s was investigated. The full length of the CYP6P9 sequence was isolated, sequenced and compared between the pyrethroid-resistant and -susceptible strains. Sequence identity between the two strains was 99.3%; the sequence differences were mainly outside of the conserved

regions. The functional significance is still unknown, but it is feasible that these variations are associated with differences in insecticide metabolism. A second CYP6 gene (CYP6P13) was also isolated; it shared close similarities with CYP6P9. The putative redox partners, cytochrome b_5 (cyt b_5) and NADPH-cytochrome P450 reductase (CPR), were isolated from *An. funestus* (resistant strain) and showed high levels of sequence identity to other insect cyt b_5 and CPRs. Isolation of the coding sequences CYP6P9 and its cognate redox partners enables expression of functional recombinant protein for biochemical and structural analysis.

Key words: *Anopheles funestus*; CYP6P9; CYP6P13; Cytochrome b_5; NADPH-cytochrome P450 reductase

INTRODUCTION

Africa, south of the Sahara, accounts for approximately 90% of the world's malaria mortality. This is mainly due to the predominance of the *Plasmodium falciparum* parasite, which is transmitted by malaria vectors, such as *Anopheles funestus*, one of the most efficient African malaria vectors (Gillies and De Meillon, 1968; WHO, 2003). In southern Africa, regional malaria control programs have been based on the use of various residual insecticides, including pyrethroids, carbamates and DDT for indoor spraying of houses. Some target vector populations have developed defense mechanisms against these insecticides. Pyrethroid-resistant *An. funestus* has been described from South Africa and southern Mozambique (Hargreaves et al., 2000; Brooke et al., 2001; Casimiro et al., 2006). An elevated level of detoxifying monooxygenase (cytochrome P450) was identified as the primary mode of resistance (Brooke et al., 2001). The cytochrome P450 superfamily of genes is the largest heme-thiolate gene family. P450 enzymes are involved in the metabolism of many endogenous and exogenous compounds and are found in eukaryotes as well as prokaryotes (Nelson et al., 1996). For catalysis, microsomal cytochrome P450s require electrons donated by NADPH-cytochrome P450 reductase (CPR) and sometimes cytochrome b_5 (cyt b_5).

In *An. funestus*, higher levels of CYP6P9 mRNA are found in pyrethroid-resistant strains than in pyrethroid-susceptible strains (Amenya et al., 2008; Wondji et al., 2009). Consequently, P450 may play a protective role against insecticides.

We isolated and characterized the full-length coding sequences for CYP6P9 from the resistant and susceptible strains as well as for the two redox partners, CPR and cyt b_5.

MATERIAL AND METHODS

Mosquito strains

Two strains of *An. funestus*, maintained in colonies at the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa, were used. The pyrethroid-resistant strain (FUMOZ-R) is derived from material collected in southern Mozambique and the insecticide-susceptible strain (FANG) is derived from material collected in southern Angola. Both strains are kept under standard insectary conditions. Details of selection and characteristics of pyrethroid resistance in FUMOZ-R are given in Hunt et al. (2005).

Genomic DNA isolation

Mosquito genomic DNA was extracted according to the method described by Collins et al. (1987).

cDNA synthesis

Total RNA was extracted from 1-day-old FUMOZ-R and FANG mosquitoes using TRI reagent (Sigma, USA), according to manufacturer instructions. The RNA was DNase (Roche, Germany) treated in order to remove any contaminating genomic DNA. *Superscript* III RNase H^- reverse transcriptase (Invitrogen, USA) and Oligo (dT) adaptor (5' GACTCGA GTCATCGATTTTTTTTTTTTTTVN 3') were used for first-strand cDNA synthesis from total RNA according to manufacturer instructions. cDNA templates were then used for the respective amplification of CYP6P9, cyt b_5 and CPR.

Cloning and sequencing of full-length cDNAs, CYP6P9, CYP6P13, and cyt b_5 and CPR

Amenya et al. (2005) isolated 31 partial P450 genes from *An. funestus*, these genes share more than 75% sequence identity with those of *An. gambiae.* As a result it was possible to use the *An. gambiae* sequences to design forward and reverse primers to amplify the *An. funestus*-coding regions, CYP6P9, cyt b_5 and CPR. Forward (CYPF) and reverse (CYPR) primers were used to amplify CYP6P9; forward (b5F) and reverse (b5R) primers were used to amplify cyt b_5, and forward (CPRF) and reverse (CPRR) primers were used to amplify CPR using polymerase chain reaction (PCR) (see Table 1). The amplification conditions were as follows: Stage 1, 94°C for 2 min; Stage 2, 30 cycles consisting of 94°C for 30 s, 55.1°C for 1 min and 72°C for 2.5 min; Stage 3, extension at 72°C for 10 min. The amplified fragments were cleaned using the QIAquick Gel Extraction kit (QIAGEN, Germany) and separately ligated into pGEM-T Easy vector to produce pGEM-T/CYP6P9, pGEM-T/cyt b_5 and pGEM-T/CPR. Sequencing and the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) were used to identify and determine *An. funestus* cytochrome P450 sequences. Internal forward primer (INT-CYPF) and reverse primer (INT-CYPR) were used to sequence and identify internal sequences of CYP6P9, while internal forward primer (INT-CPRF) and reverse (INT-CPRR) were used to sequence and identify internal sequence of CPR.

Table 1. Primers used to isolate the P450 genes.

Name	Sequence	Tm in °C
CYPF	5' ATG GAG CTA ATT AAC GCG GT 3'	58
CYPR	5' CTA CAA CTT TTC CAC CTT CA 3'	56
b5F	5'ATG TCG GGA AGT GAA AAC GTA 3'	56
b5R	5' TTA CTG GGT GAA GTA GAA CC 3'	58
CPRF	5' A TGG ACG CCC AGA CAG AAA CG 3'	66
CPRR	5' TTA GCT CCA CAC GTC CGC CGA GTA T 3'	69
INT-CYPF	5' CTG TGC ATT CGG GAT TGA G 3'	60
INT-CYPR	5' CCC TTG CCA ACA CTC CC 3'	62
INT-CPRF	5' AAA GTT TTG GCC AAC GGG 3'	57
INT-CPRR	5' GCA GAC GCG GTA ACA GTT 3'	59

Bioinformatics studies

Sequenced genes were submitted to the Genbank database (http://www.ncbi.nlm.nih.gov/entrez/query) using ClustalW (http://www.ebi.ac.uk/clustalw/). Multiple sequence alignment was performed between nucleotide sequences and protein sequences of interest and those of other organisms that were obtained from the nucleotide and protein databases (http://www.ncbi.nlm.nih.gov/entrez/query) using ClustalW (http://www.ebi.ac.uk/clustalw/). The positions for the start and stop codons were identified using the LASERGENE package (DNASTAR).

RESULTS

Sequence characterization of CYP6P9 in *An. funestus*

Genomic DNA and mRNA sequences from CYP6P9 (for both the resistant and susceptible strain) were used to determine the inclusion of putative positions for the start and stop codons. These were confirmed using the LASERGENE package (DNASTAR), which implies that the clones were full length copies of the cDNA. The cDNA sequence of CYP6P9 (FUMOZ-R and FANG) has an open-reading frame size of 1530 bp that encodes a protein of 509 amino acids.

Based on FUMOZ-R gDNA, one intron, which was 56 bp in length, was found situated in helix K just before the ETLR domain. When aligned with *An. gambiae* CYP6P3, *An. funestus* CYP6P9 from FUMOZ-R and FANG both shared 84.9% identity (Ranson et al., 2002; Amenya et al., 2005). When CYP6P9 (FUMOZ-R) was compared to other insect CYP6 genes involved in insecticide resistance, it revealed identities of 45.5, 34.0, 33.5, and 40.8% with CYP6A1, *Musca domestica* (Feyereisen et al., 1989); CYP6Z1, *An. gambiae* (Ranson et al., 2002); CYP6D1, *M. domestica* (Tomita et al., 1995), and CYP6F1 *Culex pipiens pallens* (Gong et al., 2005), respectively (Table 2).

Table 2. Percentage sequence identity amongst various insect CYP6 genes*.

	CYP6D1	CYP6Z1	CYP6F1	CYP6P3	FUMCYP6P9	CYP6A1	FANCYP6P9
CYP6D1	-	41.7	39.3	32.7	33.5	30.0	33.1
CYP6Z1	41.7	-	40.1	33.8	34.0	33.7	33.6
CYP6F1	39.3	40.1	-	39.6	40.8	34.0	40.8
CYP6P3	32.7	33.8	39.6	-	84.9	45.9	84.9
FUMCYP6P9	33.5	34.0	40.8	84.9	-	45.5	99.3
CYP6A1	30.0	33.7	34.0	45.9	45.5	-	45.7
FANCYP6P9	33.1	33.6	40.8	84.9	99.3	45.7	-

*Data assembled using LASERGENE package (DNASTAR, MegAlign program, Clustal W method).

A nucleotide sequence alignment performed on CYP6P9 between FUMOZ-R and FANG strains showed 99.3% identity, with only 11 nucleotide differences (Figure 1). Only four amino acid differences were noted between FUMOZ-R and FANG CYP6P9. The positions of these differences within the secondary structure were determined by identifying helices (A-L) and substrate recognition sites (SRS-1-6) using a known crystallographic CYP101A1 template (Poulos et al., 1987; Gotoh, 1992) (data not shown). Amino acid variations were noted near the NH_2 terminus (Glu^{14} to Val^{14}, based on FANG to FUMOZ-R), between helices A and B (Val^{77} to Met^{77}), F-G loop near SRS-2 (Ile^{221} to Phe^{221}) and in helix K (Ile^{361} to Met^{361}; Figure 2).

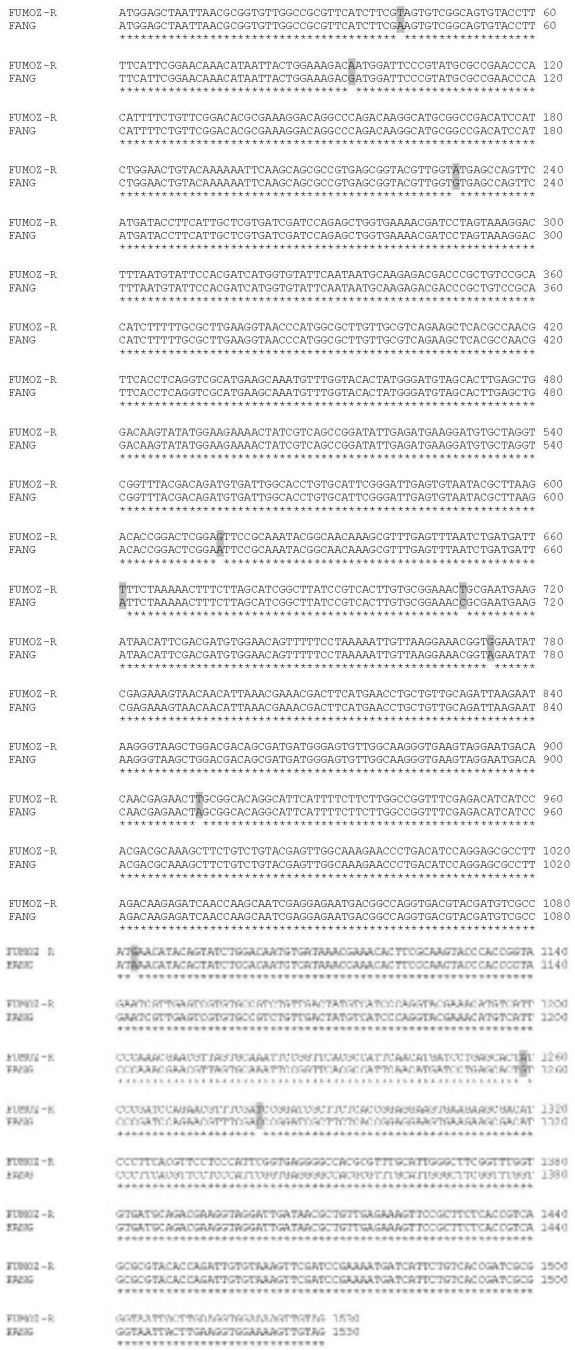

Figure 1. Nucleotide comparison of CYP6P9 cDNA from resistant and susceptible strains. Differences are noted in gray shade, and identical nucleotides are marked with an asterisk. FUMCYP6P9 FUMOZ-R (GeneBank accession number: AY729661) and FANG (GeneBank accession number: EU450763) (ClustalW, http://www.ebi.ac.uk/clustalw/).

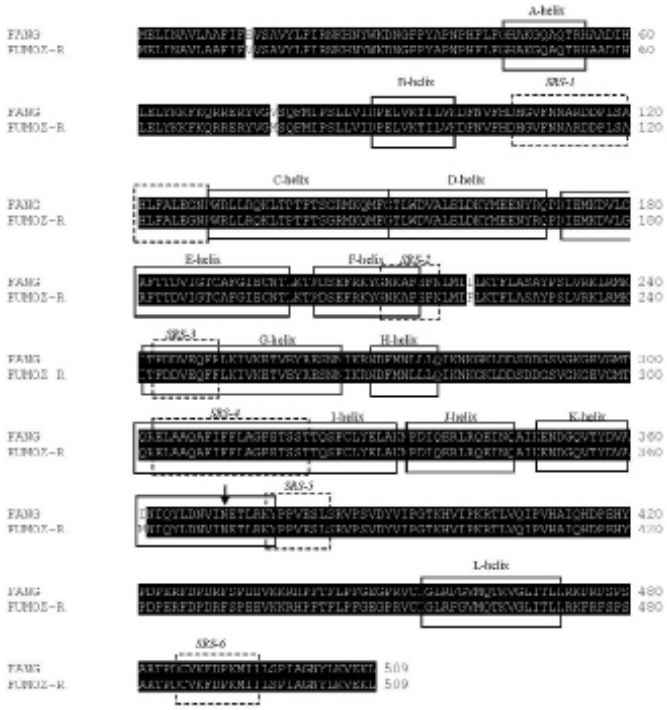

Figure 2. Amino acid comparison of CYP6P9 from resistant and susceptible strains. Helices A-L are represented by full line boxes and substrate recognition sites (SRS)-1-6 are indicated by dashed boxes. Position of intron is indicated by arrow. Identical residues are shaded in black while different residues are in white (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

Multiple sequence alignment of CYP6P9 against other insect CYP6 reveals a number of conserved regions characteristic of cytochrome P450 proteins. These include the heme-binding region (PFxxGxxxCxG, position 446-456; positions are based on FUMOZ-R/FANG sequences), which contains the highly conserved cysteine residue that holds the heme-group in place; the ExxR motif (position 372-375) situated within helix K, an oxygen-binding pocket (proton transfer groove) ((A/G)Gx(E/D) T(T/S), position 314-319), on the distal side of the heme group within helix I, also known as the P450 signature (Werck-Reichhart and Feyereisen, 2000). The YPDP motif, found in the CYP6 family (position 420-423) has an aromatic group (Tyr or Phe) and two prolines that are conserved. A microsomal cytochrome P450 motif (PERF, position 428-431) was found downstream of the YPDP motif. In the CYP6P9 and CYP6P3, Asp^{429} replaces Glu. The N-terminal region constitutes 61% hydrophobic amino acids and is involved in membrane anchoring (Rongnoparut et al., 2003).

While isolating CYP6P9 from FANG genomic DNA, a closely related CYP6 gene was isolated, named CYP6P13 (http://drnelson.utmem.edu/cytochromeP450.html). Nucleotide sequence alignment of FANG CYP6P9 and CYP6P13 showed numerous differences and had 93.7% identity (Figure 3). CYP6P13 was shown to have one intron, which was 57 bp in length. This intron was 1 bp longer than FUMOZ-R CYP6P9 gDNA.

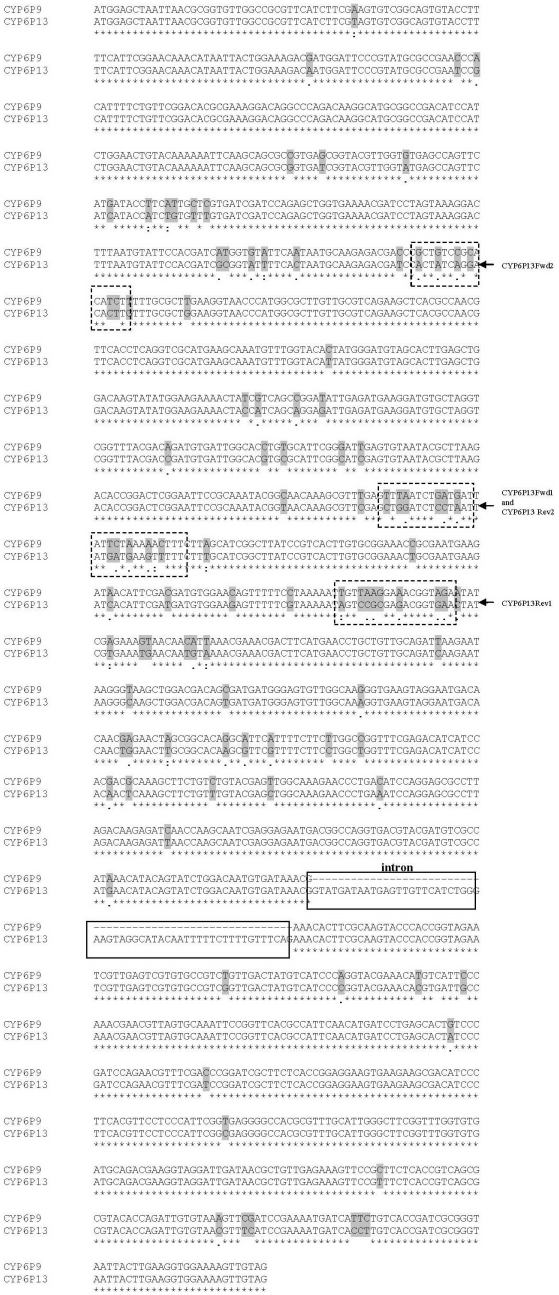

Figure 3. Nucleotide sequence alignment of FANG-CYP6P9 and FANG-CYP6P13. CYP6P9 cDNA aligned with CYP6P13 gene isolated from FANG. Intron is indicated by full line boxes. Primers were designed based on nucleotides in dashed boxes to differentiate the two genes. Differences are noted in gray shade and identical nucleotides are indicated with asterisks while similar residues are indicated with dots. CYP6P9 (GeneBank accession number: EU450763) and CYP6P13 (GeneBank accession number: EF152577) (ClustalW, http://www.ebi.ac.uk/clustalw/).

A phylogenetic tree was used to determine the relationship between CYP6P9 (FUMOZ-R and FANG) and CYP6P13. FUMOZ-R CYP6P9 and FANG CYP6P9 were on the same branch, while CYP6P13 was on a different branch (Figure 4). This indicated that CYP6P13 was from a different gene.

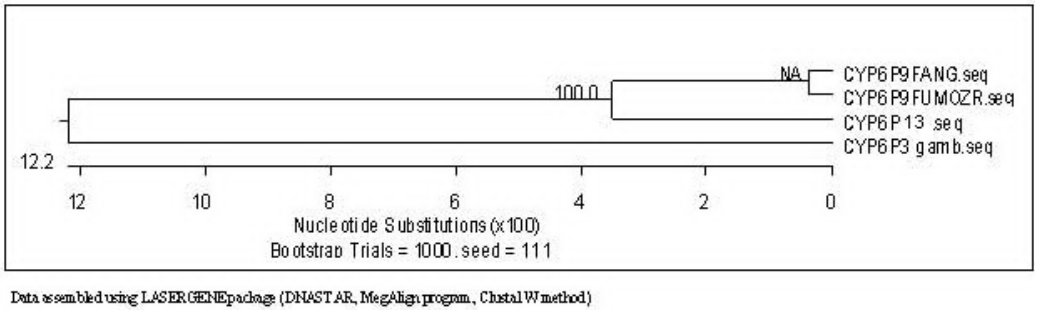

Figure 4. Phylogenetic tree of CYP6P9 and CYP6P13. CYP6P9 sequences from FUMOZ-R and FANG were closely related, while the CYP6P13 sequence was of a different gene.

Identification of *An. funestus* cytochrome b_5

Cyt b_5 from the FUMOZ-R strain was cloned and shown to be full length, with an open-reading frame of 387 bp that encodes a 128-amino acid protein. Sequence alignments revealed high conservation with its counterpart in *An. gambiae* (Nikou et al., 2003), with 93.8% identity. This was in contrast to the cyt b_5 sequences from *M. domestica* (Guzov et al., 1996) and *Drosophila melanogaster* (Kula et al., 1995), with which *An. funestus* cyt b_5 had a much lower identity of 59.4 and 64.8%, respectively. These multiple sequence alignments show that *An. funestus* cyt b_5 contains all the conserved amino acids required for the b_5-fold (Trp^{22}, Asp^{31}, His^{39}, Pro^{40}, Gly^{41}, Gly^{42}, Ala^{50}, Gly^{51}, Phe^{58}, and His^{63}; positions are based on *An. funestus* cyt b_5 sequences) and the two histidines (His^{39} and His^{63}) involved in heme-coordination (Guzov et al., 1996; Nikou et al., 2003). The amino acids involved in the "b_5-fold" are highly conserved in all sequences. The C-terminus constitutes 67% hydrophobic amino acid residues, indicative of the membrane anchor (Vergères and Waskell, 1995).

Identification of *An. funestus* NADPH-cytochrome P450 reductase

The *An. funestus* CPR sequence has an open-reading frame of 2040 bp that encodes 679 amino acids and is very closely related to that of *An. gambiae* (Nikou et al., 2003), with 96.5% identity. A comparison of *An. funestus* CPR with that of *M. domestica* (Koener et al., 1993) and *D. melanogaster* (Hovemann et al., 1997), revealed highly conserved sequences, with identities of 76.3 and 77.5%, respectively.

Anopheles funestus CPR contains all conserved regions characteristic of CPRs. It has the two conserved tyrosine residues (Tyr^{43} and Tyr^{181}; positions relative to *An. funestus* CPR) that are involved in FMN binding as well as the NADPH binding residues Ile^{531}, Phe^{542}, Lys^{625}, and Val^{641}. *Anopheles funestus* and *An. gambiae* have two substitutes at positions Arg^{82} and Gly^{188} that were

noted within the FMN domain, compared with *M. domestica* and *D. melanogaster* that have Ser and Ala at the same positions. In the FAD domain, the only substitution observed was at position Glu^{318} for both *An. funestus* and *An. gambiae*, while *M. domestica* and *D. melanogaster* had Asp at this position. A second substitution within the FAD domain was noted in *An. gambiae* at position His^{459}, while the other CPRs had Tyr at the same position. The N-terminal region is constituted of 52% hydrophobic amino acids and is involved in membrane anchoring (Wang et al., 1997).

DISCUSSION

Recently, a P450 enzyme (CYP6P9) was shown to be over-expressed in pyrethroid-resistant *An. funestus* (Amenya et al., 2008). A partial sequence of CYP6P9 was used in Northern, dot blot and real-time PCR expression studies. Preliminary microarray analysis between the pyrethroid-resistant and -susceptible colonies showed that multiple genes are differentially expressed. However, the same gene (CYP6P9) is highly overexpressed in resistant mosquitoes (Riann Christian, personal communication). Here, we report the isolation and sequence characterization of the full-length CYP6P9 gene and redox partner genes (cyt b_5 and CPR) as well as a genetic comparison of CYP6P9 between pyrethroid-resistant and -susceptible *An. funestus*. We also report the isolation of a closely related CYP6 gene (CYP6P13).

The high percentage homology between *An. funestus* and *An. gambiae* cytochrome P450s (>75%) enabled the design of degenerate primers for *An. funestus* based on *An. gambiae* sequences. These primers were used to amplify and isolate full-length genomic and cDNA sequences of CYP6P9 from the *An. funestus* FUMOZ-R and FANG strains. Sequences from these two strains showed 99.3% amino acid homology. Only four differences were noted outside the conserved regions, with one situated within the F-G loop near the SRS-2. The variations, as well as structural differences, can have an impact on the binding of substrates, affecting functionality of the enzyme.

For instance, Bergé et al. (1998) suggested that mutations in a CYP6A2 gene from a resistant *D. melanogaster* could affect the tertiary structure, thereby having an important role in the enzyme activity of the protein. Amichot et al. (2004) identified three mutations outside of the conserved regions of CYP6A2 gene that enhanced the metabolism of 1,1,1-trichloro-2,2-bis-(4'-chlorophenyl) ethane (DDT) in this DDT-resistant strain.

The presence of the heme-binding region (PFxxGxxxCxG), ExxR motif, ETLR domain and oxygen-binding-pocket ((A/G)Gx(E/D)T(T/S)) characteristic cytochrome P450 motifs were identified in the translated CYP6P9 cDNA (Werck-Reichhart and Feyereisen, 2000). Another YPDP motif present only in the CYP6 family was situated upstream of the heme-binding region. Finally, the translated CYP6P9 sequence contained a highly hydrophobic N-terminal membrane anchoring region and a PERF motif consistent with this being a microsomal cytochrome P450.

A closely related gene, CYP6P13, was only isolated using CYP6P9 forward and reverse primers. This gene was very similar to CYP6P9, with an identity of 93.7%. Phylogenetic analysis revealed that FUMOZ-R and FANG CYP6P9s were closely related. CYP6P13 also had one intron, which was 57 bp; this was only 1 bp longer than CYP6P9. CYP6P13 were most likely due to a gene-duplication event. Wondji et al. (2009) reported a duplication of CYP6P9 and called the gene CYP6P9b. Based on sequence similarities, it is highly likely that the two genes, CYP6P9b (Wondji et al., 2009) and CYP6P13, are the same gene. CYP6P13 was named in 2008 (Matambo, 2008) without prior knowledge of the publication by Wondji et

al. (2009). Wondji et al. (2009) sequenced a Bac library constructed from the unselected base colony, FUMOZ, while our reports sequence variation between the pyrethroid-selected strain, FUMOZ-R, and the pyrethroid-susceptible strain, FANG. It cannot be ruled out that the sequence differences that we observed are due to population differences; their role in pyrethroid metabolism needs to be investigated.

The cytochrome P450 redox partners, cyt b_5 and CPR, were also isolated from *An. funestus* (FUMOZ-R). *Anopheles funestus* cyt b_5 contains the "b_5-fold" and two histidines that are required for heme-coordination (Guzov et al., 1996; Nikou et al., 2003). The percentage homology of 97% with *An. gambiae* indicates that cyt b_5 function is conserved between the two species.

The deduced CPR sequences between *An. funestus* and *An. gambiae* were highly conserved, with a percentage homology of 96.5%. Nikou et al. (2003) showed that CPRs are highly conserved amongst *An. gambiae*, housefly and fruitfly sequences, with above 75% homologies. All the domains typical of CPR were present, including an N-terminal membrane anchor and the FMN and FAD/NADPH-binding domains; this suggests that the coding sequence will express a functional electron-donating enzyme.

In conclusion, we have identified two distinctive CYP6P9-coding sequences in resistant and susceptible strains (FUMOZ-R and FANG), which show differences in four amino acids. The functional significance is not yet known, but it is feasible that these variations are associated with differences in insecticide metabolism. The importance of this gene in pyrethroid resistance is supported by Wondji et al. (2007), who chromosomally mapped a quantitative trait locus (QTL) associated with pyrethroid resistance, using the same *An. funestus* FUMOZ-R and FANG strains. They found that a QTL associated with permethrin resistance is located between division 9 and 12 of chromosome arm 2R. This location coincides with a cluster of CYP6 P450's mapped by fluorescent *in situ* hybridization, suggesting that resistance is mediated by one or more CYP6 P450 genes. The isolation of the coding sequences CYP6P9 and its cognate redox partners enables the expression of functional recombinant protein for further biochemical and structural analysis. We are currently investigating the role of these mutations in pyrethroid-resistant *An. funestus* as well as conducting metabolic activity studies on CYP6P9 to determine whether this protein is able to hydrolyze pyrethroids, leading to resistance. Understanding the molecular aspects of insecticide resistance is critical in any resistance management program to ensure effective vector-control intervention.

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