Genomic and Bioinformatic Analysis of NADPH-Cytochrome P450 Reductase in Anopheles stephensi (Diptera: Culicidae)

C. Suwanchaichinda1,2 and L. B. Brattsten1

1Department of Entomology, Rutgers University, New Brunswick, NJ 08901
2Corresponding author; e-mail: csuanch@hotmail.com

ABSTRACT. The cytochrome P450 monooxygenase (P450) enzyme system is a major mechanism of xenobiotic biotransformation. The nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR) is required for transfer of electrons from NADPH to P450. One CPR gene was identified in the genome of the malaria-transmitting mosquito Anopheles stephensi Liston (Diptera: Culicidae). The gene encodes a polypeptide containing highly conserved flavin mononucleotide-, flavin adenine dinucleotide-, and NADPH-binding domains, a unique characteristic of the reductase. Phylogenetic analysis revealed that the A. stephensi and other known mosquito CPRs belong to a monophyletic group distinctly separated from other insects in the same order, Diptera. Amino acid residues of CPRs involved in binding of P450 and cytochrome c are conserved between A. stephensi and the Norway rat Rattus norvegicus Berkenhout (Rodentia: Muridae). However, gene structure particularly within the coding region is evidently different between the two organisms. Such difference might arise during the evolution process as also seen in the difference of P450 families and isoforms found in these organisms. CPR in the mosquito A. stephensi is expected to be active and serve as an essential component of the P450 system.

Key Words: binding domain, gene structure, phylogenetic tree, sequence analysis

The mosquito Anopheles stephensi Liston (Diptera: Culicidae) is one of the major vectors that transmit the human malaria parasite Plasmodium falciparum Welch. Geographical distribution of the mosquito ranges from the Middle East to the Indian subcontinent (Kiszewski et al. 2004). Various types of insecticides have been used in controlling populations of mosquito vectors. Insecticide resistance or changes in insecticide susceptibility have been documented in A. stephensi (Ganesh et al. 2003, Enayati and Ladonni 2006, Tiwari et al. 2010, Tikar et al. 2011, Shetty et al. 2012).

The cytochrome P450 monoxygenase (P450) system is well known as a major mechanism of xenobiotic biotransformation. Involvement of specific P450 isoforms in insecticide resistance has been characterized in several insects, including mosquitoes (Nikou et al. 2003, Shen et al. 2003, Djouaka et al. 2008, Yang and Liu 2011, Bariami et al. 2012). As part of the redox reaction, a nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR) is needed in transferring electrons to P450s. CPR is a flavoprotein containing binding domains for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH, as determined by X-ray crystallography of rat CPR (Wang et al. 1997). NADPH serves as electron donor, whereas FAD and FMN serve as cofactors in the pathway of electron transfer. In eukaryotic microsomes, both P450s and CPRs are integral membrane proteins. Although there is such an extensive diversity of P450 isoforms identified in animals (Nelson 2011), each animal’s genome generally carries only one CPR gene (Porter et al. 1990). Thus, interaction between the two enzymes in the microsomal environment is probably in the ratio of multiple P450s to one CPR. Feyereisen (2005, 2012) suggests a ratio of 6–18 P450s to 1 reductase in insects. Lycett et al. (2006) showed that silencing the CPR gene could impact insecticide susceptibility in the mosquito Aedes aegypti.

To date, only a few mosquito CPRs have been identified and deposited in the NCBI GenBank database: Aedes sollicitans (D. Sun and L.B.B., unpublished data), Aedes aegypti (Nene et al. 2007), Anopheles funestus (Matambo et al. 2010), A. gambiae (Nikou et al. 2003), Anopheles minimus (Kaewpa et al. 2007), and Culex quinquefasciatus (Arensburger et al. 2010). By using A. gambiae microarray platforms, a few possible P450 transcripts were identified in A. stephensi (Vontas et al. 2007). A small nucleotide sequence, possibly representing a fragment of a CPR gene, was obtained from A. stephensi by multilocus DNA sequencing as part of a phylogenetic study among different anopheline mosquitoes (Dixit et al. 2010). However, a complete sequence of the A. stephensi CPR gene has not, to date, been determined.

In this study, a complete coding sequence of a CPR gene was identified by comparative genomic analysis of the A. stephensi genome; the whole genome shotgun sequence of A. stephensi was originally deposited in the NCBI GenBank database by Hall and Jiang, Virginia Tech University, in 2012. The deduced amino acid sequence was analyzed by using bioinformatic and phylogenetic analyses. Conserved residues were further characterized in relation to rat and known mosquito CPRs. This study was undertaken because of our long-standing interest in the role of the P450 enzyme system in the responses of insects, especially mosquitoes, to insecticides applied in an attempt to control their populations, and also in their responses to allelochemical substances in their ecological habitats (Suwanchaichinda and Brattsten 2002). (Note: the nucleotide sequence of the A. stephensi CPR transcript is available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK008720.)

Materials and Methods

Genomic Analysis. To screen the NCBI nonredundant nucleotide database for any possible CPR gene identified in A. stephensi, the nucleotide sequence of the A. gambiae CPR gene was used as a query in a BlastN search. The A. gambiae CPR nucleotide and amino acid sequences were then used in BlastN and TBlasN searches, respectively, against the genome sequences in the NCBI database. An identified genome contig was compared with the A. funestus CPR gene to reconfirm its identity. Additionally, the Ae. sollicitans CPR cDNA, which was cloned and sequenced in our laboratory, was used as an inferential evidence for the annotation of the A. stephensi CPR gene.
Sequences obtained as results of both nucleotide and genome sequence database searches were subsequently used for further analysis.

**Sequence Characterization.** An identified genome contig containing a possible *A. stephensi* CPR gene was manually analyzed by comparing it with previously identified mosquito CPR genes to obtain a complete coding region. The intron–exon organization (from initiation codon to stop codon) and the sizes of exons of the *A. stephensi* CPR gene were determined according to CPR genes in the genome releases of *A. gambiae*, *Ae. aegypti*, and *C. quinquefasciatus* available in VectorBase (http://www.vectorbase.org). The CPR gene of the Norway rat (*Rattus norvegicus*) was used as a representative of vertebrates for comparison. Information on the intron–exon organization of the rat CPR gene was obtained from Ensemble (http://www.ensembl.org).

A hypothetical translation of the complete open reading frame of the *A. stephensi* CPR transcript was performed to obtain a deduced amino acid sequence. The translated protein was then used in a BlastP search against the NCBI nonredundant protein database to confirm the *A. stephensi* CPR identity compared with other known CPRs in invertebrates and vertebrates. Amino acid residues involved in binding to cytochrome P450s and cytochrome c were predicted based on the rat CPR previously identified by Shen et al. (1989) and Shen and Kasper (1995). ClustalW was also used to construct a multiple sequence alignment between mosquito and rat CPRs.

**Phylogenetic Analysis.** The deduced amino acid sequence of *A. stephensi* CPR was used in a BlastP search for known homologous CPRs deposited in the GenBank. Subsequently, CPRs representing vertebrates and arthropods with complete open reading frames were selected for phylogenetic analysis. The CPR amino acid sequences were first analyzed using T-Coffee multiple sequence alignment with default parameters (Notredame et al. 2000) to determine variable and conserved regions. A region of low homology at the N-termini was manually removed, and the remaining portion of the sequences was realigned using ClustalW embedded in the MEGA 4.0 software (Tamura et al. 2007). The created alignment file was then used for construction of a neighbor-joining phylogenetic tree. Gaps were eliminated from the algorithmic analysis, and 1,000 replications were performed as a bootstrap test of phylogeny. A cut-off value of 50% was set for the bootstrap consensus tree.

**Results**

**Genomic and Phylogenetic Analysis.** The BlastN search, using the nucleotide sequence of the *A. gambiae* CPR gene against the genome sequences in the NCBI GenBank database, identified a specific contig (GenBank ALPR01027092) of the *A. stephensi* genome. This contig was confirmed to contain one CPR gene by comparative genomic analysis. A small fragment of nucleotide sequence (GenBank HM171646) was acquired as a result of a BlastN search against the nonredundant nucleotide sequence. This partial sequence was incorporated in this study to correct a genomic sequencing error. The corrected genomic sequence was then compared with CPR genes previously identified in other mosquito species, which yielded a complete coding region of the *A. stephensi* CPR gene from the initiation codon (ATG) to the stop codon (TAA). The existence and expression of the gene were confirmed by the presence of an expressed sequence tag (EST) (Patil et al. 2009).

The identified *A. stephensi* CPR gene contained an open reading frame of 2,040 bp, coding for a polypeptide of 679 amino acids (Fig. 1).
mosquitoes and other selected arthropods. A rat CPR was used as an outgroup. As expected, all mosquito CPRs formed a cluster distinctly separated from other organisms (Fig. 2). In particular, CPRs of *A. stephensi* and other *Anopheles* mosquitoes were grouped together within a monophyletic group. The branch representing mosquito CPRs bifurcated from the same node as did the branch that represented other dipteran CPRs. This indicated that they possibly descended from a common ancestor.

The genomic DNA sequences of mosquito CPRs were analyzed for intron–exon organizations. Only species with publicly released genomes were included in this analysis. Based on the region of nucleotides from the initiation codon to the stop codon, seven exons were identified in the mosquito CPR genes (Fig. 3). The sizes of the exons of the *A. stephensi* CPR gene are generally similar to those of *A. gambiae*, *Ae. aegypti*, and *C. quinquefasciatus*. On the other hand, 15 exons were identified in the rat CPR within the coding region. The sizes of exons 3, 4, and 7 of the *A. stephensi* CPR were identical to exons 4, 5, and 15 of the rat CPR, respectively. There was only a slight difference in exon 1 between the two groups. The rat CPR exons 2 and 3 were comparable with the mosquito CPR exon 2, whereas the rat CPR exons 6–14 corresponded to the mosquito CPR exons 5 and 6. The last exon of the *A. stephensi* CPR appeared to be conserved, when compared with the last exon of the rat CPR (Fig. 4). CPRs in insects representing Diptera, Coleoptera, and Lepidoptera also contained some amino acid residues identical to those in *A. stephensi* and rat within this particular region.

**Table 1. Amino acid sequence identity of CPRs between *A. stephensi* and other selected organisms**

| GenBank accession no. | Species    | Common name                  | Total score | Max identity (%) |
|-----------------------|------------|------------------------------|-------------|-----------------|
| AB077954              | *A. funestus* | African malaria mosquito     | 1,392       | 97              |
| AA024765              | *A. gambiae*  | African malaria mosquito     | 1,386       | 97              |
| ABL75156              | *A. minimus*    | Southeast Asian malaria mosquito | 1,378     | 97              |
| XP_001656715          | *Ae. aegypti* | Yellow fever mosquito        | 1,273       | 88              |
| ACIO02091             | *Ae. sollicitans*  | Eastern saltmarsh mosquito   | 1,264       | 87              |
| XP_001865801          | *C. quinquefasciatus* | Southern house mosquito    | 1,243       | 87              |
| NP_477158             | *Drosophila melanogaster* | Fruit fly                    | 1,120       | 78              |
| ADD19306              | *Glossina morsitans morsitans* | Tsetse fly                   | 1,102       | 77              |
| Q07994                | *Musca domestica*  | House fly                    | 1,094       | 77              |
| XP_971174             | *Tribolium castaneum*  | Red flour beetle           | 998         | 70              |
| AOX95746              | *Spodoptera exigua*   | Beet armyworm               | 991         | 69              |
| AAR26515              | *Mamestra brassicae*  | Cabbage moth                | 985         | 68              |
| NP_001104834          | *Bombyx mori*       | Silkworm                    | 978         | 68              |
| EHJ63867              | *Danaus plexippus*   | Monarch butterfly           | 976         | 69              |
| XP_002423980          | *Pediculus humanus corporis* | Human body louse           | 972         | 67              |
| AFS05057              | *Cimex lectularius*  | Bed bug                     | 970         | 66              |
| XP_002400171          | *Ixodes scapularis*  | Black-legged tick           | 816         | 59              |
| AAA41683              | *R. norvegicus*      | Norway rat                  | 792         | 55              |

*E* value = 0.0.
Amino Acid Sequence Analysis. A multiple sequence alignment among mosquito CPRs was carried out to determine conserved regions involved in ligand bindings. The *A. stephensi* CPR clearly contained all conserved FMN-, FAD-, and NADPH-binding domains (Fig. 5). Catalytic residues (Ser460, Cys631, Asp676, and Trp678) were identified in the mosquito CPR. These residues may be essential in the hydride transfer reaction as previously characterized in the rat CPR (Shen et al. 1999, Hubbard et al. 2001). The FMN-binding domain of the mosquito CPR contained two conserved tyrosine residues (Tyr143 and Tyr181) that might be critical in FMN binding (Shen et al. 1989; Fig. 5). In addition, Phe residues at positions 86 and 219 were conserved in all mosquitoes except *A. minimus*, which instead contained Leu at both positions. Phe\(^{86}\) and Phe\(^{219}\) are involved in FMN binding (Sarapusit et al. 2008, 2010).

The FAD- and NADPH-binding domains carry conserved sequence motifs recognized within the ferredoxin reductase structural family. One of the most conserved motifs is Arg-x-Tyr-Ser(Thr), which is found in all members of the family (Dym and Eisenberg 2001). Indeed, Arg-Tyr-Tyr-Ser\(^{460}\) was detected within the FAD-binding domain of the *A. stephensi* CPR. This motif was also conserved in *A. funestus*, *Ae. aegypti*, *Ae. sollicitans*, and *C. quinquefasciatus*. However, the *A. minimus* and *A. gambiae* CPRs contained Ser and His, respectively, instead of Tyr at the position 459. Another conserved sequence motif within this particular protein family is Met-x-x-x-Gly-Thr(Ser)-Gly(Ala)-Ile-x-Pro, identified in the NADPH-binding domain of all mosquito CPRs (Fig. 5). With one exception, the mosquito CPRs carried Leu instead of Ile in the motif.

One major function of microsomal CPRs is the electron transfer from electron donors to P450s. The interaction between the two
Fig. 5. Conserved regions for ligand bindings in the *A. stephensi* CPR. The multiple sequence alignment illustrates FMN-, FAD-, and NADPH-binding domains of mosquito CPRs according to Pfam. The binding domains are boxed (green, FMN; blue, FAD; purple, NADPH). Triangles above amino acids indicate catalytic residues involved in hydride transfer. Essential residues involved in ligand bindings are highlighted.
enzymes requires specific bindings that involve certain residues within the molecules. Acidic residue clusters 207-Asp-Asp-Asp 209 and 213-Glu-Glu-Asp 217 in rat CPR interact with cytochrome c or P450 (Shen and Kasper 1995). The A. stephensi CPR contained these conserved binding clusters as shown in the multiple sequence alignment in Fig. 6. Residues 210-Asp-Asp-Asp 212 in the A. stephensi CPR appeared to be highly conserved in comparison with rat and other mosquitoes. However, the residue Glu at position 214 in the rat CPR was substituted with Asp in all mosquitoes. Substitution of Asp at position 215 in the rat CPR with Tyr was observed only in Anopheles but not in Aedes and Culex mosquitoes.

Discussion

Genome, proteome, and transcriptome have become valuable resources of information for studying mosquito vectors in combination with the availability of bioinformatic tools. Genomic analysis in this study identifies one CPR gene in the mosquito A. stephensi. The gene encodes a polypeptide containing 679 amino acid residues. The length of the translated protein is exactly equal to that of CPRs in other known mosquitoes previously characterized. The amino acid sequence of A. stephensi CPR shows the highest identity with the CPRs of A. funestus (Matambo et al. 2010), A. gambiae (Nikou et al. 2003), and A. minimus (Kaewp et al. 2007). The identity of these mosquito CPRs is consistent with the results of phylogenetic analysis, showing that they belong to the same monophyletic group. It is likely that they perform similar physiological functions. CPRs of the other few known dipteran species are grouped together as another cluster. These two phylogenetic groups apparently bifurcate from the same node. Lepidoperan CPRs clearly form a separate cluster. CPRs of the bed bug (Cimex lectularius L.) and human body louse (Pediculus humanus corporis L.) fall into the same clade, which is consistent with a previous study (Zhu et al. 2012).

Since the completion of human and rat genome sequences, the number of genome sequence projects in insects has increased and promises to accelerate with the 5,000 insect genome project (Levine 2011). With the mosquito genomes available to date, genomic sequences of CPR genes of mosquitoes and vertebrates were analyzed for intron–exon organization. From the initiation codon to the stop codon, 7 and 15 coding exons were found in the mosquito and rat CPRs, respectively. This comparison reveals unique differences and similarities between the two groups. The sizes of exons 3, 4, and 7 of the A. stephensi and other mosquito CPRs are similar to exons 4, 5, and 15 of the rat CPR. However, there is an intron between exons 2 and 3 in the rat CPR, which does not exist in the region where the mosquito CPR exon 2 is located. There is an intron between exons 5 and 6 in the mosquito CPRs, which is not found in the region where the rat CPR exon 13 is located. Exons 3 and 4 of the mosquito CPRs and exons 4 and 5 of the rat CPR are highly conserved, likely because they represent the FMN-binding domain. Codons corresponding to the two Tyr residues involved in FMN binding (Shen et al. 1989) obviously reside within these two exons. In the case of the last exon of the mosquito and rat CPRs (exons 7 and 15, respectively), their similarity is consistent with their protein structure homology, particularly the sequence conservation at the C-terminal region (Fig. 4). Therefore, the similarities between the mosquito and rat CPRs are not restricted to their gene structures but extend to their protein structures as well. In addition, some amino acid residues in other insect CPRs as shown in Fig. 4 are also identical to those in A. stephensi and rat CPRs within this region. These findings provide an insight into the evolutionary relationship of gene organization and domain formation in insect and vertebrate CPRs. Although the completion of genome assembly of A. stephensi is being generated, chromosomal location of the CPR gene in the mosquito is still unknown without gene mapping. However, the CPR gene was mapped on chromosome X in A. gambiae (Holt et al. 2002). It is possible that the CPR gene in A. stephensi may also be located on the same chromosome.

Based on the study of rat CPR, Porter and Kasper (1986) propose that CPRs originated from the fusion of two ancestral genes coding for a flavodoxin and a ferredoxin reductase. CPRs are unique proteins in the way they contain FMN-, FAD-, and NADPH-binding domains, in addition to binding sites for cytochrome c and P450. There is also a connecting domain between the FMN- and FAD-binding domains (Wang et al. 1997). The A. stephensi CPR has ligand-binding sites similar to those of other mosquitoes, and the locations of these sites are consistent with the binding domains as previously described in other species (Porter and Kasper 1986, Koener et al. 1993). As shown in the multiple sequence alignment among mosquito CPRs (Fig. 5), the A. minimus CPR contains Leu instead of Phe residues at both 86 and 219 positions. Sarapusit et al. (2008, 2010) conducted kinetic studies of the wild-type and mutant A. minimus CPRs and found that substitutions of the Leu residues with Phe increased retention of the FMN cofactor and stability of the enzyme. In comparison with the wild-type CPR, single mutation (L86F or L219F) and double mutation (L86F/L219F) of the enzymes also enhanced deltamethrin degradation when the mutants were reconstituted with the A. minimus P450 CYP6AA3 (Sarapusit et al. 2010). Both Phe 86 and Phe 219 may be necessary for FMN binding and the FMN domain stabilization and thus aid the P450 in insecticide metabolism.

The highly conserved sequence motifs, Arg-x-Tyr-Ser(Thr) and Met-x-x-Gly-Thr(Ser)-Gly(Ala)-Ile-x-Pro, as part of the FAD- and NADPH-binding domains, respectively, are present in the A. stephensi CPR. These sequence motifs are also conserved in other mosquito species. However, the residue at position 459 of the former motif in A. minimus and A. gambiae CPRs differs from that in other mosquito CPRs. In addition, Sarapusit et al. (2013) recently identified Cys 427 of the A. minimus CPR as another residue possibly involved in FAD binding. This residue is conserved in A. stephensi, A. gambiae, Ae. aegypti, and Ae. sollicitans but not in A. funestus and C. quinquefasciatus. Further experiments will be required to elucidate the effects, if any, of these substitutions on the functions of the enzyme particularly in different organisms.

Interaction between the two protein components is required to transfer electrons to an electron acceptor by a CPR. Nisimoto (1986) originally specified two neighboring clusters consisting of acidic residues

**Fig. 5. Continued**
(207)Asp-Asp-Asp(209) and (213)Glu-Glu-Asp(215) that are important in the interaction with cytochrome c. In comparison with the rat enzyme, two acidic clusters (210)Asp-Asp-Asp(212) and (216)Glu-Asp-Tyr(218) are found in the A. stephensi CPR. The first cluster is entirely identical in the two species, but there are two differences in the second cluster. Specifically, one of the two (Asp(217)) is a conserved substitution that is found in the bacteria Desulfovibrio vulgaris Hildenborough flavodoxin (Dubourdieu and Fox 1977). A study based on site-directed mutagenesis in a rat CPR suggests that the first acidic cluster interacts primarily with P450s, whereas the second cluster interacts primarily with cytochrome c (Shen and Kasper 1995). It is likely that the A. stephensi CPR can perform electron transfers to P450s in the mosquito. Whether the A. stephensi enzyme can efficiently transfer electrons to cytochrome c will require further investigation. The assumption is supported by activity studies of recombinant CPRs in A. minimus (Kaewpa et al. 2007), A. gambiae (Lian et al. 2011), and A. sollicitans (C.S., unpublished data). Although there are similarities between mosquito and vertebrate CPRs, variations in terms of ligand-binding capacities and enzyme kinetics between the two groups are likely (Lian et al. 2011).

Similar to P450s, microsomal CPRs are typically present as integral proteins anchored on the membrane of the endoplasmic reticulum. The anchoring segment of CPRs is a type I signal-anchor sequence with N'-lumen and C'-cytoplasm topology (Kida et al. 1998). The membrane anchoring is also critical for the reductase function. Solubilized reductase without the N'-hydrophobic segment seems to lose its ability to interact with P450s (Black and Coon 1982). As expected, in the case of the A. stephensi CPR, the protein does not have a secretion signal peptide but does carry an N'-hydrophobic portion predicted to be a transmembrane region. This suggests that the mosquito CPR can function as part of the electron transfer system to terminal electron acceptors like P450s, which also reside in the microsomal membrane.

In conclusion, analysis of the A. stephensi genome reveals a gene encoding for a CPR. The enzyme is highly conserved among mosquitoes and also comparatively similar to CPR enzymes in other organisms. These findings facilitate the understanding of CPR evolution and the role the enzyme plays in electron transfer to its electron acceptor including the P450 family of enzymes, which are often of critical importance in the metabolism of xenobiotic compounds. The results of this study facilitate further elucidation of the mosquito A. stephensi CPR functions, including their expected involvement in xenobiotic metabolism.

Acknowledgments

This study was supported in part by Hatch funds from the New Jersey Agricultural Experiment Station.

References Cited

Arensburger, P., K. Megy, R. M. Waterhouse, J. Abrudan, P. Amedeo, B. Antelo, L. Bartholomay, S. Bidwell, E. Caler, F. Camara, et al. 2010. Sequencing of Culex quinquefasciatus establishes a platform for mosquito comparative genomics. Science 330: 86–88.

Bariani, V., C. M. Jones, R. Poupardin, J. Vontas, and H. Ranson. 2012. Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, Aedes aegypti. PLoS Negl. Trop. Dis. 6: e1692.

Black, S. D., and M. J. Coon. 1982. Structural features of liver microsomal NADPH-cytochrome P450 reductase: hydrophobic domain, hydrophilic domain, and connecting region. J. Biol. Chem. 257: 5929–5938.

Dixit, J., H. Srivastava, M. Sharma, M. K. Das, O. P. Singh, K. Raghavendra, N. Nanda, A. P. Dash, D. N. Saksena, and A. Das. 2010. Phylogenetic inference of Indian malaria vectors from multilocus DNA sequences. Infect. Genet. Evol. 10: 755–763.

Djouaka, R., F. A. A. Bakare, O. N. Coulibaly, M. C. Akogbeto, H. Ranson, J. Hemingway, and C. Strode. 2008. Expression of the cytochrome P450s, CYP6P3 and CYP6F2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. BMC Genomics 9: 538.

Dubourdieu, M., and J. L. Fox. 1977. Amino acid sequence of Desulfovibrio vulgaris flavodoxin. J. Biol. Chem. 252: 1453–1463.

Dym, O., and D. Eisenberg. 2001. Sequence-structure analysis of FAD-containing proteins. Protein Sci. 10: 1712–1728.

Enayati, A. A., and H. Ladouni. 2006. Biochemical assay baseline data of permethrin resistance in Anopheles stephensi (Diptera, Culicidae) from Iran. Pakistan J. Biol. Sci. 9: 1265–1270.

Feyerisen, R. 2005. Insect cytochrome P450, pp. 1–77. In L. I. Gilbert, K. Iatrou, and S. S. Gill (eds.), Comprehensive molecular insect science. Elsevier, Oxford, United Kingdom.

Feyerisen, R. 2012. Insect CYP genes and P450 enzymes, pp. 236–316. In L. I. Gilbert (ed.), Insect molecular biology and biochemistry. Academic Press, London, United Kingdom.

Ganesh, K. N., J. Urmila, and V. A. Vijayan. 2003. Pyrethroid susceptibility & enzyme activity in two malaria vectors, Anopheles stephensi (Liston) & A. culicifacies (Giles) from Mysore, India. Indian J. Med. Res. 117: 30–38.

Gasteiger, E., C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel, and A. Bairoch. 2005. Protein identification and analysis tools on the ExPaSy server, pp. 571–607. In J. M. Walker (ed.), The proteomics protocols handbook. Humana Press, Totowa, NJ.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. Ribeiro, R. Wides, et al. 2002. The genome sequence of the malaria mosquito Anopheles gambiae. Science 298: 129–149.

Hubbard, P. A., A. L. Shen, R. Paschke, C. B. Kasper, and J. J. Kim. 2001. NADPH-cytochrome P450 oxidoreductase. Structural basis for hydride and electron transfer. J. Biol. Chem. 276: 29163–29170.

Kaewpa, D., S. Boonsuepsakul, and P. Rongnoparut. 2007. Amino acid sequence of cucumber mosaic virus 3D polypeptide of bipartite RNA virus. Arch. Virol. 152: 1571–1579.

Kiszewski, A., M. Melling, A. Spielman, P. Malaney, S. E. Sachs, and J. Sachs. 2004. A global index representing the stability of malaria transmission. Am. J. Trop. Med. Hyg. 70: 486–498.

Koener, J. F., F. A. Carinlio, and R. Feyerisen. 1993. The cDNA and deduced protein sequence of house fly NADPH-cytochrome P450 reductase. Insect Biochem. Mol. Biol. 23: 439–447.

Larkin, M. A., G. Blackshields, N. P. Brown, D. N. McGettigan, H. McWilliam, F. Valencia, I. M. Wallace, A. Wilm, R. Lopez, et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

Levine, R. 2011. ISk: the 5,000 insect genome project. Am. Entomol. 57: 110–113.

Lian, L. Y., P. Widdowson, L. M. McLaughlin, and M. J. Paine. 2011. Biochemical comparison of Anopheles gambiae and human NADPH P450 reductases reveals different 2'-5'ADP and FMN binding traits. PLoS One 6: e20574.
Shen, A. L., and C. B. Kasper. 1995. Role of acidic residues in the interaction of NADPH-cytochrome P450 reductase with cytochrome P450 and cytochrome c. J. Biol. Chem. 270: 27475–27480.

Shen, A. L., D. S. Sem, and C. B. Kasper. 1999. Mechanistic studies on the reductive half-reaction of NADPH-cytochrome P450 reductase. J. Biol. Chem. 274: 5391–5398.

Shen, A. L., T. D. Porter, T. F. Wilson, and C. B. Kasper. 1989. Structural analysis of the FMN binding domain of NADPH-cytochrome P450 reductase by site-directed mutagenesis. J. Biol. Chem. 264: 7584–7589.

Shen, B., H. Dong, H. Tian, L. Ma, X. Li, G. Wu, and C. Zhu. 2003. Cytochrome P450 genes expressed in the deltamethrin-susceptible and -resistant strains of Culex pipiens pallens. Pestic. Biochem. Physiol. 75: 19–26.

Shetty, V., D. Sanil, and N. J. Shetty. 2012. Insecticide susceptibility status in three medically important species of mosquitoes, Anopheles stephensi, Aedes aegypti and Culex quinquefasciatus, from Bruhat Bengaluru Mahanagara Palike, Karnataka, India. Pest Manag. Sci. 69: 257–267.

Suwanachaihind, C., and L. B. Brattsten. 2002. Induction of microsomal cytochrome P450s by tire-leachate compounds, habitat components of Aedes albopictus mosquito larvae. Arch. Insect Biochem. Physiol. 49: 71–79.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.

Tikar, S. N., M. J. Mendki, A. K. Sharma, D. Sukumaran, V. Veer, S. Prakash, and B. D. Parashar. 2011. Resistance status of the malaria vector mosquitoes, Anopheles stephensi and Anopheles subpictus towards adulticides and larvicides in arid and semi-arid areas of India. J. Insect Sci. 11: 85.

Tiwari, S., S. K. Ghosh, V. P. Olja, A. P. Dash, and K. Raghavendra. 2010. Reduced susceptibility to selected synthetic pyrethroids in urban malaria vector Anopheles stephensi: a case study in Mangalore city, South India. Malar. J. 9: 179.

Vontas, J., J. P. David, D. Nikol, J. Hemingway, G. K. Christophides, C. Louis, and H. Ranson. 2007. Transcriptional analysis of insecticide resistance in Anopheles stephensi using cross-species microarray hybridization. Insect Mol. Biol. 16: 315–324.

Wang, M., D. L. Roberts, R. Paschke, T. M. Shea, B. S. Masters, and J. J. Kim. 1997. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc. Natl Acad. Sci. U S A 94: 8411–8416.

Yang, T., and N. Liu. 2011. Genome analysis of cytochrome P450s and their expression profiles in insecticide resistant mosquitoes, Culex quinquefasciatus. PLoS One 6: e29418.

Zhu, F., S. Sams, T. Moural, K. F. Haynes, M. F. Potter, and S. R. Palli. 2012. RNA interference of NADPH-cytochrome P450 reductase results in reduced insecticide resistance in the bed bug, Cimex lectularius. PLoS One 7: e31037.

Received 1 February 2013; accepted 18 March 2013.