

Molecular Cloning and Characterization a Novel Gene Encoding CYP4H28v2 from the Mosquito, *Aedes aegypti*

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Abstract—Cytochrome P450 is a group of heme-containing enzymes. They have been detected in virtually all organisms examined from bacteria to mammals. In insects, P450s are involved in the oxidative metabolism of a large number of exogenous compounds (xenobiotics) from natural or anthropogenic origins (plant allelochemicals and insecticides) and endogenous compounds such as steroid hormones ecdysteroid hormones and pheromones. Thus the P450s have an important role in controlling insect growth, development, and evolutionary adaptation to environments containing potentially toxic compounds. A novel cDNA clone encoding a cytochrome P450 gene has been isolated from the mosquito, *Ae. aegypti* strain VCRU. The cDNA is 1731 bp in length and has an open reading frame from 277 bp to 1647 bp encoding a protein of 529 aa. The classic heme-binding characteristic motifs of P450s (FXXGXRXCXG, RxxR and EVLR) are present and conserved in this gene. The EVDTFMFEGHDTT motif characteristic of CYP4 family is also found in this new gene. The full length cDNA clone for CYP4H28v2 is 98% identical to CYP4H28 of The Liverpool strain *Ae. aegypti*. So, we propose that this is an allele of that gene.

Keywords— Cytochrome P450, CYP4 family, *Aedes aegypti*, RACE.

I. INTRODUCTION

AEGYPTI is the most important mosquito vector of Dengue fever in the world and in Malaysia. The mosquito is closely related to human population and as a result they breed near human dwellings in rural and urban areas. Cytochrome P450 is a group of heme-containing enzymes. P450 superfamily have been identified more than three hundred P450s especially in insect, were divided 27 P450 families and more than 70 subfamilies, in *Drosophila melanogaster* and *Anopheles gambiae* are present (90, 111) P450s respectively [1]. Cytochrome P450 are involved in the oxidative metabolism of a large number of exogenous compounds (xenobiotics) from natural or anthropogenic origins (plant allelochemicals and insecticides) and endogenous compounds [2], [3]. Insect cytochrome P450s participate in the biosynthesis of juvenile hormone, ecdysteroids, cuticular hydrocarbons and pheromone [2].

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Some of these enzymes are involved in the detoxication and activation of insecticides [4], [5], [6], [3]. Insect cytochrome P450s also have important function in the metabolism of plant allelochemicals in herbivorous insects as they adapt to their plant hosts [7].

CYP450 superfamily is extensive in insects specially CYP4 family. For instance, CYP6A1 in *Musca domestica* was 1st insect P450 gene [8]. 2 subfamilies of CYP4 in *Manduca sexta* were identified (CYP4L, CYP4M) [9]. About 21 genes of CYP4 family have been identified in *Drosophila* and in *A. aibimanus* about 17 partial CYP4 family fragments have been isolated belong to several CYP4 subfamilies [10]. Expression and down-regulation of Four CYP4 in *D. melanogaster* [11]. Additionally, previous studies have measured the cytochrome P450-dependent monooxygenase activity by effect of atrazine in *Chironomus tentans*, increased activity larvae of CYP4G33 in *C. tentans* as a result to atrazine exposure [12]. In the present study, we cloned and sequence CYP4 genes of *Ae. aegypti*.

II. METHODS

A. Total Of RNA Isolation And 1st-Strand CDNA Synthesis

Instar *Ae. aegypti*, was provided by the Vector Control and Research Unit (VCRU), Universiti Sains Malaysia. They were kept at 25 ± 2 °C and fed on powdered bovine liver Total RNA was isolated using (RNeasy Mini Kit, Qiagen) according to the manufacturer's instruction. First-strand cDNA synthesis was performed using 5µg of total RNA with oligo (dT)₁₈ primer and M-MuLV Reverse Transcriptase from First Strand cDNA Synthesis Kit (Fermentas). The total volume of 1st-strand cDNA Synthesis products were 20 µl of reaction solution according to the manufacturer's instruction and stored at -20 °C.

B. PCR, Cloning And Sequencing

cDNA template was used for PCR using specific primers designed from a previously cloned DNA sequence (NCBI accession: AY205085.1) coding for a putative CYP4 gene in *Ae. aegypti*. As F001 primer and R459 primer (Table I). 25µl of PCR reaction using *Taq* polymerase (Kapa Biosystems) and the temperature cycles consisting an initial denaturation step 94 °C for 2 min, 7 cycles of 94 °C for 30 s, 42 °C for 30 s, and

72 °C for 1 min, followed by 27 cycles 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, then a final extension 72 °C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis in 0.5 x TBE buffer. This revealed a single DNA band of approximately 450 bp. The PCR product was purified using PCR clean up kit (Promega).

Cloning of fragment (450bp) was carried out according to the method described by Zhou et al (2006). pGEM-T vector was used to clone the 450 bp DNA fragment, cells were grown overnight at 37 °C on LB-agar plates with ampicillin (100mg/ml), IPTG and X-gal. White colonies were incubated overnight at 37 °C with shaking on LB broth containing 100mg/ml ampicillin. Plasmid extraction was done using minipreps DNA kit (Promega).

DNA sequencing was performed by 1st base laboratory. Sequencing analysis was done using BLASTX and BLASTN in (NCBI).

C. Pcr 3' And 5' Ends (Race)

Specific gene primer SGP1 and SGP2 (table I), designed from a previously cloned sequence result. The amplification of the 3' end was performed with anchor primer (table 1). 25µl of PCR reactions 12.5 µl of Taq Hot start 2X Master mix with standard buffer, 2 µg of 1st – strand cDNA, (0.5 µl) 10 pmol each primers (SGP1 and anchor primer). Thermal cycling was carried out as follows: 94 °C for 5 min, followed by 7 cycles of 94 °C for 30 s, 43 °C for 30 s, 72 °C for 1 min and 27

cycles of 94 °C for 30 s, 51.5 °C for 30 s, 72 °C for 1 min, final extension step at 72 °C for 10 min.

Nested PCR was performed by using PCR product with SGP2 and anchor primer. Conditions for PCR were identical to those described above after optimization of the annealing temperature. Nested PCR product was analyzed by electrophoresis on 1% agarose gel. And subsequently cloned and sequenced.

The 5' end RACE was performed using SMARTer™ RACE cDNA Amplification Kit (Clontech). The sequence of the gene specific primer is given in table I. PCR was performed with Advantage™ 2 GC PCR Kit (Clontech). Thermal cycling was carried out as follows: 5 cycles of 94 °C for 30 s 72 °C for 3 min; followed by 5 cycles of 94 °C for 30 s 68 °C for 30 s, 72 °C for 3 min, then another 30 cycles of 94 °C for 30 s, 66 °C for 30 s 72 °C for 3 min. PCR product was cloned and sequenced.

D. Confirmation Of The Cloned Gene

To confirm the sequences of 3 and 5 ends were from the same gene by using gene specific primers (Forward and Reverse in table I). Using 1st-strand Cdna.

TABLE I

Primer name	Sequence of primer
Primer F00	5' - GATACGTTTCATGTTTGAGGGGCA-3'
Primer R459	5' -GCGATCTTTTGGCCTATGC-3'
SGP ₁	5' -CATGTTTGAAGGACACGATACGACC-3'
SGP ₂	5' - TGTACGGTGAGATTCGGCAGGT-3'
Anchor primer (T) ₂₅ V	5' -TTTTTTTTTTTTTTTTTTTTTTTTTTTGCA- 3'
Primer 5' RACE	5' -ACCGATGCAATTTCCGCGTCCCACACTG-3'
Forward Primer	5' -TCCACTCCCTTAGGGCAACCGGGGT-3'
Reverse primer	5' - TTATCTGCTCTCGATTCTTAACGCCATATT-3'

LIST OF PRIMER NAME, PRIMER SEQUENCE (5'-3')

III. RESULTS

The full length of cDNA sequence of this CYP4 family gene has been named CYP4H28v2 by submission to the P450 Nomenclature Committee (Dr. David Nelson) (GenBank accession number, KC481237). The nucleotide sequence and the deduced amino acid sequences of the full length cDNA are shown in Fig 1. The gene consists of 1731 with an open reading frame of 1587 bp coding for a protein of 529 amino acids. The alignment of CYP4H28v2 with several genes from the CYP4 family reveals a high degree of sequence similarity with CYP4H28 (98%) from *Ae. aegypti*. The latter is an allele from the Liverpool strain. In addition homology comparison with genes from the CYP4 family from several different insect show between 50-50% identity (Table II). The amino acid sequence of CYP4H28v2 contains conserved regions of P450

typically found in genes from this superfamily. For example, the heme binding region (FXXGXRXCXG) [2], [13] is present at amino acids residues 469 to 478. Furthermore, other P450 motifs of P450 such as ExxR and PxxxxP are present at amino acid residues 396 to 399, 445 to 450 respectively.

In addition, specific conserved region of CYP4 family containing a 13-residues motif EVDTFMFEGHDTT [12], [14] is present at amino acid residues 328 to 340 (Fig 1).

TABLE II
PERCENTAGE OF AMINO ACID IDENTITY AMONG INSECT P450 GENES
WITH CYP4H28V2 FROM AE. AEGYPTI STRAIN VCRU

Insect species	family	% identity	Accession
<i>Aedes aegypti</i>		98%	EAT45359.2
<i>Aedes aegypti</i>	CYP4H28	98%	XP 001656766.1
<i>Anopheles gambiae</i>		55%	XP 317096.4
<i>Culex quinquefasciatus</i>	P450 4C1	52%	XP 001843663.1
<i>Anopheles gambiae</i>		54%	XP 317098.4
<i>Aedes aegypti</i>	P450	53%	XP 001654558.1
<i>Anopheles gambiae</i>		52%	XP 321208.5
<i>Culex quinquefasciatus</i>	P450 4d1	50%	XP 001843622.1

IV. DISCUSSION.

Degenerate primers designed from conserved amino acid sequences of gene using PCR have proven to be an effective method to obtain full length of gene were successfully cloned, even though cytochrome P450s have a very different sequence particularly in insects [15]. The sequence features of P450 genes can be listed as follows, first, the most common and

easily identified P450 sequence motif is the heme-binding FxxGxxxCxG [2]. Secondly, the sequence motif (A/G) GxxT corresponding to an I-helix which is a conserved alpha helical region. Thirdly, the motif WxxxR corresponding to a C-helix sequence, conserved in most eukaryotic P450s [16]. The K-helix (EXXR) is the best conserved [16], [17]. In addition, there are specific conserved region for some families of P450, such as motif (EVDTFMFEGHDTT) [12].

Molecular cloning of CYP4H28v2 is the first step study in the study of family IV P450 in *Ae. aegypti*, This family has not been very well studied and their role in the detoxification of insecticides. In future research, we will study the expression of CYP4H28v2 and identify the biological role of CYP4H28v2 in *Ae. Aegypti*.

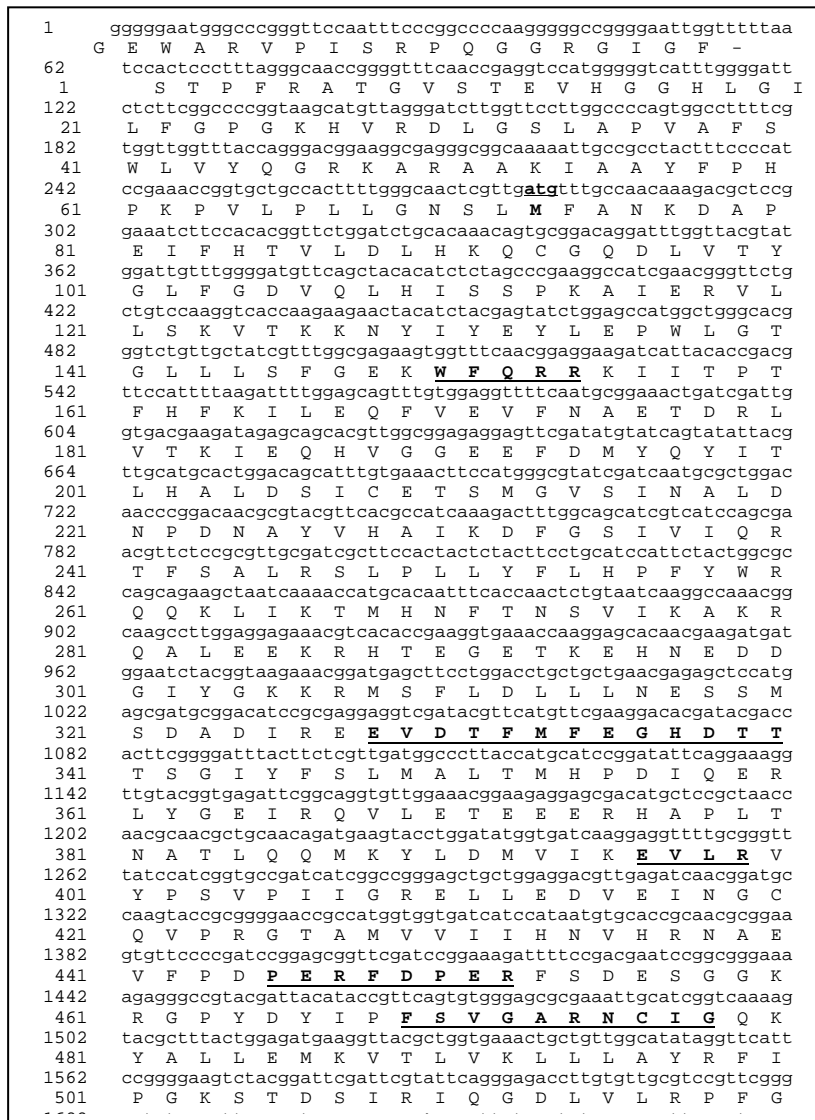


Fig. 1 Both Nucleotide and deduced amino acid sequence of CYP4H28v2 are numbered on the left. Amino acids in conserved regions within P450 and CYP4 family are underlined. The putative start codon ATG and the stop codon TGA are double underlined. The canonical polydenylation signal AATAAA are in bold and is found 26 nucleotides upstream of the beginning of the poly-A tail.

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