IDENTIFICATION AND EXPRESSION OF THE CYTOCHROME P450 REDUCTASE GENE IN *Aedes sollicitans* (Walker) (DIPTERA: CULICIDAE)

by

DEBIN SUN

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ABSTRACT OF THE DISSERTATION

Identification and Expression of the Cytochrome P450 Reductase Gene in *Aedes Sollicitans* (Walker) (Diptera: Culicidae)

By DEBIN SUN

Dissertation Director:
Professor Lena B. Brattsten, Ph.D

Aedes sollicitans (Walker) is an important vector of mosquito-borne diseases. Cytochrome P450 system is one of the most important enzyme families involved in the metabolic responses of living organisms to foreign chemicals. To explore the specific functions of individual cytochrome P450s, it is important to identify the cytochrome P450 reductase gene.

The cytochrome P450 reductase genes were identified in *Ae. sollicitans* from Cape May, Middlesex and Ocean County, New Jersey. The cytochrome P450 reductase gene has an open reading frame of 2040 bp encoding 679 amino acid residues. The comparison among 679 amino acid residues deduced from the cytochrome P450 reductase gene obtained from three mosquito populations showed that mutations had occurred at the nucleotide level but did not produce amino acid changes except in one case. In Ocean County mosquito population, the amino acid serine was changed to glycine. The amino acid change was not considered to cause any functional change of cytochrome P450 reductase since the mutation was not located in any functional region.

Quantitative real-time expression analysis showed that the expressions of CYP6BB1 and cytochrome P450 reductase mRNAs were not significantly different in fourth instar larvae or in different body parts of adult female mosquitoes in populations

from three locations. The study implied that CYP6BB1 was ubiquitously expressed in female mosquitoes and its expression occurred in both larvae and adults.

The cytochrome P450 reductase gene was expressed in *E. coli* cells and molecular mass of the protein was estimated to be 78 kDa. Its activity was assayed in both the cytosolic and cell membrane fractions. The activity in the cytosol was much higher than that in the membrane fractions. The cytochrome P450 reductase activities in microsomes of *Ae. sollicitans* collected from the three locations were assayed. The results showed no statistically significant differences.

This is the first study of cytochrome P450 reductase in *Ae. sollicitans*. The identification and expression of the cytochrome P450 reductase gene provide further insights on how cytochrome P450s metabolize substrates and make it possible to explore the specific functions of each cytochrome P450 gene. The study is not only meaningful for *Ae. sollicitans* but also for other mosquito species.

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IDENTIFICATION OF

CYTOCHROME P450 REDUCTASE GENE IN *Ae. sollicitans*

INTRODUCTION

The Mosquito *Aedes sollicitans*

Aedes sollicitans (Walker), a salt marsh mosquito species, is an aggressive biter that can obtain in large numbers in salt marsh habitats. It is a medium-sized mosquito species with a bright scale pattern. Prominent features include black-and-white banded legs, a conspicuous band of white scales around the center of the proboscis, a pale-scaled, longitudinal stripe on the dorsum of the abdomen, and a thorax with dense patches of white scales on the sides and brown, yellow, golden and white scales on the dorsum. It mainly distributes in the eastern seaboard of the United States and Canada.

It is a vector of several diseases, including eastern equine encephalitis (Crans et al. 1986; Crans et al. 1996), Venezuelan equine encephalitis (Turell et al. 1992), the dog heartworm *Dirofilaria immitis* Leidy (Parker 1986; Parker 1993) in addition to being a major nuisance. *Ae. sollicitans* is also a competent vector of the recently (1999) arrived West Nile Virus (Turell et al. 2001a; Turell et al. 2001b).

Aedes sollicitans females require a blood meal to produce eggs. Females obtain blood from both mammals and birds. They make feeding flights mainly during dusk and after dark. However, females are ready to feed at any time during the day. Eggs are laid

individually on moist substrate in salt marshes, especially in areas with salt meadow grass or salt grass.

In New Jersey, *Ae. sollicitans* is a medically important mosquito species. It has been ranked as the second most important pest species statewide by mosquito control agencies and was ranked number one in the southern half of the state (Crans et al. 1996).

Ae. sollicitans females are easy to capture because they are aggressive biters, even during the day, if disturbed. Their mating behavior is not described although it is presumed to take place in large swarms above the expanse of salt marshes. It may resemble the dance of *Culex* mosquitoes above rising sticks or poles (Spielman 2001). However, caged mating in the laboratory is not successful.

Generally there are three ways to obtain mosquitoes or their eggs: soaking sod samples dug from the salt marshes (Elmore and Fay 1958), forced mating of *Ae. sollicitans* (McCuiston and White 1976), or raising larvae from field-collected females (Brattsten et al. submitted). Although it is time and labor intensive to collect female mosquitoes in the field, feed them blood in the lab and hatch their eggs for research use, raising larvae from field-collected females is still the most efficient way to obtain enough mosquito larvae for research.

The Cytochrome P450 Reductase

Cytochrome P450s are one of the most important families of proteins involved in the metabolic responses of living organism to foreign chemicals (Gutierrez et al. 2003). The cytochrome P450 system is thought starting to evolve about 3.5 billion years ago from a single gene (Loomis 1988). The system can catalyze at least twelve different

types of oxidation reactions on thousands of substrates (Black 1993). Most of the eukaryotic P450 enzymes are microsomal. However, several are known to be mitochondrial (Alzahrani 2009). The membrane-bound cytochrome P450 enzymes with the catalytic function are difficult to purify because of interactions with hydrophobic membrane compeonents, hydrophilic substrates, and the hydrophobic domain of cytochrome P450 reductase.

These systems include a cytochrome P450 reductase that supplies electrons to metabolize endogenous and xenobiotic compounds. The cytochrome P450 system can also interact with cytochrome *b5* and NADPH-cytochrome *b5* reductase (Hildebrandt and Estabrook 1971). This interaction can influence the rate of catalysis (Bernhardt 1996) at least in some vertebrates. The cytochrome P450 reductase plays an important role in the transfer of electrons from NADPH to the ferric heme of cytochrome P450. This enzymatic process has the unique characteristic of exhibiting biphasic kinetics (Gigon et al. 1969). Unlike the cytochrome P450 genes, of which there are on average 100 in any insect species, there is usually only one cytochrome P450 reductase gene (Feyereisen 1999). Since the cytochrome P450 has been shown to form a 1:1 functional reductase-P450 complex (Miwa et al. 1979; Miwa and Lu 1984), the reductase must be capable of supplying electrons to each of the different P450 enzymes (Backes and Kelley 2003). So far, the cytochrome P450 reductase gene has been indentified in *Ae. aegypti* (Nene et al. 2007), *Culex quinquefasciatus* (Atkinson et al. unpublished), *Anopheles funestus* (Matambo et al. unpublished), *Anopheles gambiae* (Nikou et al. 2003), *Anopheles minimus* (Kaewpa et al. 2007), *Drosophila mettleri* (Dec et al. 1997), *Mamestra brassicae* (Maibeche-Coisne et al. 2005), *Bombyx mori* (Horike et al. 2000), *Bombyx*

mandarina (Wang et al. unpublished), *Drosophila melanogaster* (Hovemann et al. 1997), *Musca domestica* (Koener et al. 1993), *Tribolium castaneum* (NCBI. unpublished) and *Apis mellifera* (NCBI. unpublished). *Ae. sollicitans*, despite its importance, is not among the mosquito species in which the cytochrome P450 reductase gene has been identified until now.

Unlike the amino acid sequences of cytochrome P450s, which are highly variable, sometimes sharing as little as 20% of sequence identities, the amino acid sequences of cytochrome P450 reductase are relatively conserved between closely related species. The cytochrome P450 reductase in *Ae. aegypti* shares 90% of its amino acids with that of *Cx. quinquefasciatus*, and 87% with that of *An. funestus*. The amino acid residues of cytochrome P450 reductase in *Ae. aegypti*, although phylogenetically distant from *Homo sapiens*, share a 56% identity with humans. Considering their genetic distance, this is a surprisingly high number. All these cytochrome P450 reductases contain two major highly conserved domains, an FAD domain with an NAD(P)H and an FMN domain (Feyereisen 1999). The high similarity between the cytochrome P450 reductases in different insect species not only shows the functional identity and evolutionary relationship between them, but provides a useful tool to study new cytochrome P450 reductase genes in other species with the aid of the polymerase chain reaction (PCR).

The coexpression of a specific cytochrome P450 gene with the reductase gene allows testing of what substrate(s) the cytochrome P450 gene can react with and specific activities of the reaction. Thus, to obtain the specific functions of a particular cytochrome P450 gene, this gene should be expressed simultaneously with the cytochrome P450

reductase gene in *E. coli* or other expression systems. This also tests whether the cytochrome P450 reductase gene is functionally expressed.

In this study, the conserved domains of the cytochrome P450 reductase of several insect species including *D. melanogaster* (CAA63639), *M. domestica* (AAA29295), *An. gambiae* (AAO24765), *M. brassicae* (AAR26515) and *B. mori* (BAA95684) were used to design a pair of degenerate primers. Reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) were used to obtain the full-length cytochrome P450 reductase gene in *Ae. sollicitans* and it was verified by analyzing its conserved domains and similarity to the cytochrome P450 reductase genes in other insect species.

MATERIALS AND METHODS

Mosquito Collecting and Rearing

Female mosquitoes were collected at salt marsh sites in Middlesex County, Ocean County and Cape May County, New Jersey. Females were caged and transported back to the Center for Vector Biology in New Brunswick, New Jersey. A 10% sugar water solution was given immediately to the mosquitoes after collecting.

Bovine blood meals were supplied with a Hemotek® apparatus (Discovery Workshops, Accrington, United Kingdom) to females the day after the correction. Cages with females were placed in large plastic bags until they were transferred to glass shell vials. The plastic-bagged cages were kept at room temperature. Paper towel or gauze saturated with 10% sugar water was placed on top of the cage (Brattsten et al. submitted).

After being fed three to four times, the mosquitoes were identified and transferred to glass shell vials with moist cotton balls covered by screen to lay eggs. Only *Ae. sollicitans* females and eggs were saved. The eggs were first dried for about 3 weeks at room temperature and then stored in a Percival environmental incubator set at 24℃, 16/8h of L:D and about 80% relative humidity with wet paper towels on the top to keep moisture level high. When needed, the eggs were hatched by immersion in deoxygenated water. The larvae were fed pulverized Purina® rat chow every other day. The larvae were raised in pure fresh water, kept clean with paper towels and changed to clean water as needed.

RNA Extraction and cDNA Synthesis

Fifty fourth-instar larvae hatched from Middlesex County mosquito eggs were homogenized in 1 mL TRIzol® Reagent (Invitrogen, Carlsbad, CA) with a motoried Brinkmann homogenizer (Brinkmann Instruments Inc, Riverview, FL). The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform (Fisher Scientific, Pittsburgh, PA), 0.2 mL per mL of TRIzol® reagent, was added to the sample tubes. The tubes were capped securely and shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2 - 3 minutes. The samples were centrifuged at 12000×g for 15 minutes at 4℃ in a Beckman L8-M ultracentrifuge. The aqueous phase was transferred to a fresh tube and the RNA from the aqueous layer was precipitated by mixing with 0.5 mL isopropanol (Sigma, St. Louis, MO) per mL TRIzol® reagent. The samples were incubated at room temperature for 10 minutes and then centrifuged at 12000×g for 15

minutes at 4 ℃. The supernatant was removed. The RNA pellet was washed with 75% ethanol, adding at least 1 mL per mL of TRIzol® reagent. The samples were mixed by vortexing and centrifuged at 7500 ×g for 5 minutes at 4 ℃. The RNA pellet was dried for 10 minutes and then dissolved in nuclease-free water. The RNA solution was incubated at 60 ℃ for 10 minutes. Reverse transcription PCR was used to obtain the cDNA of cytochrome P450 reductase using iScript™ cDNA Synthesis Kit (Bio-rad Laboratories, Hercules, CA). The 20 μL reverse transcription PCR system contained 1 μg total RNA, 4 μL 5×iScript™ Reaction mix, 1 μL iScript Reverse Transcriptase and was filled with nuclease-free water to 20 μL. The reaction was performed at 25°C for 5 min, 42°C for 30 min and 85 ℃ for 5 min. The reverse-transcribed cDNA was kept at -20 ℃ for future use.

The same methods were used to extract the total RNA from 50 fourth-instar larvae hatched from Ocean County mosquito eggs and 50 fourth-instar larvae hatched from Cape May County mosquito eggs, separately. All cDNAs were kept at -20 ℃ for future use.

Degenerate Primer Design, Amplification and Cloning of Cytochrome P450 Reductase cDNA Fragment

Cytochrome P450 reductases in the house fly (*M. domestica*), malaria mosquito (*An. gambiae*), fruit fly (*D. melanogaster*), cabbage moth (*M. brassicae*), and domestic silkworm (*B. mori*) were downloaded from GeneBank of the National Center for Biotechnology Information (NCBI) and aligned by Megalign in LASERGENE® sequence software suite, V 7.0 (DNASTAR, Madison, WI). Based on two conserved regions flanking a sequence segment of about 250 amino acids of NADPH-cytochrome

P450 reductase, one pair of degenerate primers was designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA). The forward primer CPR F 5' GAAGGG HATGGTBGCBGAYCC 3' (Table 1) was based on amino acid region KGMVADP (from AA119 to AA125) and the reverse primer CPR R 5' GGGAAYGGRTGYTTYT TRCTGC 3' (Table 1) was based on the amino acid region SSKKHPFP (from AA368 to AA375) (Figure 1).

To obtain the partial sequences of the cytochrome P450 reductase gene, a 50 µL PCR reaction was set up containing 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.15 µM of each dNTP, 0.6 µM of each degenerate primer, 1 µL of cDNA template from Middlesex County mosquito larvae diluted 20 times, 1.25 U of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and an appropriate amount of Milli-Q ultra pure water to make up to 50 µL.

The PCR reaction mixture was denatured at 94 ℃ for 2 minutes and amplified by 40 cycles at 94 ℃ for 30 seconds, 58 ℃ for 30 seconds and 72 ℃ for 1 min. The reaction was finally extended at 72 ℃ for 10 min and held at 4 ℃ (Table2). After the amplification, the obtained PCR product solution was electrophorezed on a 1.2% agarose gel with a 100bp DNA ladder (Promega, Madison, WI). The target DNA fragment with a size of about 730 bp was cut, retrieved and purified by QIAquick® Gel Extraction Kit according to the user guide (Qiagen, Valencia, CA) (Figure 2).

The purified DNA was cloned into the pCR®4-TOPO® vector (Invitrogen, Carlsbad, CA) and subsequently transformed into the One Shot® TOP 10 (Invitrogen, Carlsbad, CA) chemically competent *E. coli* cells by the TOPO TA Cloning® Kit

(Invitrogen, Carlsbad, CA) following the manufacturer's manual for sequencing. The transformed *E. coli* cells were spread onto Luria-Bertani (LB) agar plates containing 75 μg/mL ampicillin and cultured in an incubator overnight at 37 ℃. Then, 5 well isolated colonies were picked up and cultured in LB medium containing 75 μL/mL ampicillin at 37 ℃ and shaken for 16 hours. The *E. coli* cells were harvested and plasmids were isolated from the LB medium with QIAprep® Minipep Kit (Qiagen, Valencia, CA). The plasmids were digested with the restriction enzyme EcoR I before sequencing and the inserted DNA fragment was confirmed by 1.2% agarose gel electrophoresis. The plasmid DNA was then sent to Genewiz (Piscataway, NJ) to be sequenced with two universal sequencing primers, M13F (5'-d(TGTAAAACGACGGCCAGT)-3') and M13R (5'-d(CAGGAAACAGCTATGAC)-3'). After the nucleotide sequences were obtained, the protein sequences were deduced. Both the nucleotide sequences and protein sequences were blasted in the nucleotide database and protein database in the NCBI databse and were confirmed to be a cDNA fragment of cytochrome P450 reductase gene.

The cDNA fragments of the cytochrome P450 reductase gene from Ocean County and Cape May County were also obtained and identified using the methods described above.

Cloning and Sequencing of 5'- and 3'-ends of Cytochrome P450 Reductase Gene

After the DNA fragment sequences were identified, gene-specific primers for RACE (Rapid Amplification of cDNA Ends) were designed based on the known sequences. Total RNA was extracted from 50 fourth instar mosquito larvae from Middlesex County using TRIzol® reagent with the same methods as described above and

run on a RNA formaldehyde denaturing agarose gel (Figure 3) to test its quality in order to obtain the intact, full-length mRNA. Following GeneRacer™ Kit's User Manual (Invitrogen, Carlsbad, CA), 5 µg of total RNA from Middlesex County mosquito larvae was used to remove the 5' phosphates, eliminating truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA oligo. The dephosphorated RNA was treated with tobacco acid pyrophosphate (TAP) to remove the 5' cap structure from the intact, full-length mRNA, leaving only 5' phosphate that is required for ligation to the GeneRacer™ RNA oligo. The 5' end of the treated mRNA was ligated with GeneRacer™ RNA oligo (5′ CGACUGGAGCACGAGGACACUGACAUGGACUGAA GGAGUAGAAA 3′) by T_4 RNA ligase (Invitrogen, Carlsbad, CA) in order to create ligated full-length mRNA. The ligated full-length mRNA was reverse transcribed by the GeneRacer™ Oligo dT Primer (5′ GCTGTCAACGATACGCTACGTAACGGCATGAA GTG$(T)_{24}$ 3′) (Table 1) to create RACE-ready first-strand cDNA using GeneRacer™ kit with SuperScript™ III RT Module (Invitrogen, Carlsbad, CA).

To obtain the 5' end, the 5'-RACE was performed with GeneRacer™ 5' primer and reverse primer CPR FR (5' AGGGCTCGCCATTCAGAGGGTGCTT 3') (Table 1). A nested 5'-RACE was also performed with GeneRacer™ 5' Nested primer (Table 1) and CPR FR (Table 1). To obtain the 3' end, the 3'-RACE was performed with GeneRacer™ 3' primer (Table 1) and forward primer CPR FF (5' ACAGCCCCGCTAC TACTCTATTTCCTC 3') (Table 1). A nested 3'-RACE was also performed with GeneRacer™ 3' Nested primer (Table 1) and CPR FF. The 50 µL PCR reactions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.15 mM of each dNTP, 0.3 µM of each RACE primer, 1 µL of cDNA template diluted 20 times, 1 Unit of

Platinum® *Taq* polymerase and an appropriate amount of Milli-Q ultra pure H_2O to make up to 50 µL. The PCR reaction mixture for 5'-RACE was denatured at 94 ℃ for 2 minutes and amplified by 40 cycles at 94 ℃ for 30 seconds, 60 ℃ for 30 seconds and 72 ℃ for 2 min. The reaction was finally extended at 72 ℃ for 10 min and held at 4 ℃ (Table 2). The PCR reaction mixture for 3'-RACE was denatured at 94 ℃ for 2 minutes and amplified by 40 cycles of 94 ℃ for 30 seconds, 60 ℃ for 30 seconds and 72 ℃ for 2 min 30 seconds. The reaction was finally extended at 72 ℃ for 10 min and held at 4 ℃ (Table 2). All the RACE products were electrophorezed on 1.2% agarose gel and target amplicons were retrieved and purified by QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA).

The purified DNA was cloned into pCR®4-TOPO® vector (Invitrogen, Carlsbad, CA) and subsequently transformed into One Shot® TOP 10 (Invitrogen, Carlsbad, CA) chemically competent *E. coli* cells using TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) following the manual for sequencing. The transformed *E. coli* cells were spread onto LB agar plates containing 75 µg/mL ampicillin and cultured in the incubator overnight at 37 ℃. Then, 5 well isolated colonies were picked up and cultured in LB medium containing 75 µL/mL ampicillin at 37 ℃ and shaken for 16 hours. The *E. coli* cells were harvested and plasmid DNA was isolated from LB medium by QIAprep® Minipep Kit (Qiagen, Valencia, CA). The plasmid DNA was digested with restriction enzyme EcoR I before sequencing and the inserted DNA fragment was confirmed by 1.2% agarose gel electrophoresis. The plasmid DNA was then sent to Genewiz (Piscataway, NJ) to be sequenced with two universal sequencing primers M13F and M13R. After the nucleotide sequences were obtained, the sequences were analyzed and assembled to obtain putative

full-length P450 reductase sequences using SeqMan program in LASERGENE® sequence software suite, V 7.0 (DNASTAR, Madison, WI). The protein sequences were deduced from the putative full-length P450 sequences.

Verification of Full-length P450 Reductase gene-like cDNA Sequences

To verify the putative sequences, a pair of primers, CPRVF (5' CTCGGTTCAG ACTGGTCGTGTTT 3') and CPRVR (5' TGCGTGAGTGAGTGAATGAGTG 3') , flanking the coding region of the cytochrome P450 reductase gene was designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA) and used in the verification PCR reaction. To eliminate the possible errors produced by Platinum® *Taq* DNA polymerase, a thermostable high-fidelity proof-reading polymerase Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) was used. The 50 µL PCR reaction included 60 mM Tris-SO_4 (pH 8.9), 2 mM MgSO4, 0.2 mM each of dNTP, 0.3 µM each of primers, 1 µL of cDNA template diluted 20 times, 1 unit of Platinum® *Taq* High Fidelity and an appropriate volume of Milli-Q ultra pure water. The PCR reaction mixture was denatured at 94 ℃ for 2 minutes and amplified by 40 cycles at 94 ℃ for 30 seconds, 58 ℃ for 30 seconds and 68 ℃ for 2 min 30 seconds. The reaction was extended at 68 ℃ for 10 min and held at 4 ℃ (Table 2). The PCR amplicons were electrophorezed on a 1.2% agarose gel and the target PCR product was purified by QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). In order to perform TA cloning, a 3'-A overhang was added to the 3' end of purified amplicon. Then the PCR product with 3'-A was TA cloned into pCR®4-TOPO® vector (Invitrogen, Carlsbad, CA) and subsequently transfected into One Shot® TOP 10 (Invitrogen, Carlsbad, CA) chemically competent *E.*

coli cells using TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) for sequencing following the manual. Because the PCR product was 2400 bp long and the sequencing itself was limited, there was a 300 bp gap that could not be sequenced. Two more sets of verification primers CPRVF1 (5' TATCCTGTCAACGATCAGGACTT 3'), CPRVF2 (5' GAATTCGTTCATCAAGAAGCTG 3'), CPRVR1 (5' GATGTACTGCACAGCTT CACTT 3') and CPRVR2 (5' AAGTCCTGATAAGTCCTGATCGTTGACAGGATA 3') were designed and combined with CPR VF and CPR VR to cover the gap to verify the coding region (Table 1). Three PCR products were amplified by CPRVF/CPRVR2, CPRVF1/CPRVR1 and CPRVF2/CPRVR, respectively, using 50 μL PCR reaction system as described above (Table 2). The target PCR products were retrieved and purified. A 3'-A overhang was added to the amplicons and they were TA cloned by TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA) as described above and sequenced by Genewiz (Piscataway, NJ). The putative full-length cytochrome P450 reductase gene was obtained through the overlapped regions of each sequence.

The cytochrome P450 reductase genes in Ocean County mosquito population and Cape May County mosquito population were obtained and verified using the same methods described for obtaining cytochrome P450 reductase gene from Middlesex County mosquito population.

Sequence Analysis

The sequence analyses for the cytochrome P450 reductase gene obtained from Middlesex County mosquitoes, Ocean County mosquitoes and CapeMay County mosquitoes were performed with SeqMan and MegAlign in Lasergene® sequence

software suite, V 7.0 (DNASTAR, Madison, WI). The BLASTN program was used to search the sequence database of the National Center for Biotechnology Information for nucleotide sequences similar to the cytochrome P450 reductase gene. The BLASTP program was also used to search the NCBI for amino acid sequence similar to cytochrome P450 reductase. Phylogenetic analysis was performed in MrBayes 3.1.2 and GeneDoc V 2.7.000 was used to annotate the alignments.

RESULTS

The Cytochrome P450 Reductase Fragment Identified By Degenerate Primers

Using the reverse transcribed-PCR with the degenerate primers, two bands were obtained, one of which was very strong and the other much weaker. The strong band was the target band with an approximate size of 730 bp. The target band was retrieved, purified, cloned and sequenced. The sequence was blasted in the NCBI genebank and was identified to be a cDNA fragment of the cytochrome P450 reductase gene (Figure 4).

The deduced amino acid sequence from the DNA fragment had 237 amino acid residues. The 237 amino acid residue sequences were also blasted using BLASTP in the NCBI database. It had two conserved regions characteristic of the cytochrome P450 reductase gene, one is the flavodoxin-1 superfamily and the other is the FNR-like superfamily (Figure 5). Thus the partial amino acid sequences were confirmed to be a part of cytochrome P450 reductase protein. The verified cDNA fragment of cytochrome P450 reductase gene was used to design new primers to amplify the full-length gene.

Cytochrome P450 Reductase cDNA Sequences Identified by RACE

The full-length cDNA of cytochrome P450 reductase gene from Middlesex County mosquitoes was obtained using the methods described above. It had 2870 bp with an open reading frame of 2040 bp encoding 679 amino acid residues (Figure 6). A start codon ATG and the most common stop codon TAA were present. The 5'-untranslated region (5'-UTR) of cytochrome P450 reductase cDNA was short with a slightly over 100 bp and the 3'-UTR was 730 bp long. The cytochrome P450 reductase gene had a 23 bp poly(A) tail.

The cytochrome P450 reductase gene in Middlesex County mosquitoes, Ocean County mosquitoes and Cape May County mosquitoes was identified using Platinum® *Taq* polymerase and verified separately using Platinum® *Taq* DNA Polymerase High Fidelity with three pairs of primer combinations CPRVF/CPRVR2, CPRVF1/CPRVR1 and CPRVF2/CPRVR. The putative ORF of the full-length cDNA sequence of cytochrome P450 reductase gene was blasted in the NCBI database. The blast results showed that it had all the conserved domains characteristic of cytochrome P450 reductase gene and were highly similar to those of other insect cytochrome P450 reductases (Figure 4). The deduced amino acid residue sequences were blasted in the NCBI database as well. The blast results showed that it had all the conserved domains characteristic of other insect cytochrome P450 reductases (Figure 5) and the deduced amino acid residues were highly similar to other insect cytochrome P450 reductases (Figure 7). The cytochrome P450 reductase gene sequence in Middlesex County mosquitoes was submitted to the National Center for Information Biotechnology (NCBI) with the accession number FJ487605.1. Its deduced protein sequence was also submitted to NCBI with the accession

number ACL01092. According to the blasting results of the nucleotide sequences and protein sequences from the nucleotide database and protein database in NCBI, the putative full-length cDNA sequence of the cytochrome P450 reductase gene were confirmed.

The full-length sequences of cytochrome P450 reductase gene from Ocean and Cape May County mosquitoes had the same length of open reading frame with 2040 bp encoding 679 amino acid residues (Figure 8, Figure 9). They were also confirmed as the cytochrome P450 reductase genes following the same procedure.

Comparison of cDNA Sequences of Cytochrome P450 Reductase Gene

The coding area of three cDNA sequences of cytochrome P450 reductase gene from Middlesex, Ocean and Cape May County mosquitoes was compared using Genedoc V2.7.000. The alignment results showed that there were a few nucleotide mutations in all three coding areas despite the fact that the cytochrome P450 reductase gene was relatively conserved (Figure 10). There were point mutations at 9 nucleotide sites.

Comparison of Amino Acid Sequences of Cytochrome P450 Reductase

The 679 amino acids deduced from the cytochrome P450 reductase gene obtained from Middlesex County mosquitoes, Ocean County mosquitoes and Cape May County mosquitoes were compared using Genedoc V 2.7.000. The comparison showed that the mutations occurred at the nucleotide level and did not lead to any amino acid changes except in one case. In Ocean County mosquitoes (Figure 11), the amino acid serine was changed to glycine at nucleotide position 1951 and amino acid position 651. NCBI

analysis using the BlastP suite and the conserved domains one by one showed that the mutation was not located in any functional region of cytochrome P450 reductase. Consequently it is unlikely that the function of cytochrome P450 reductase would be affected by this change.

The cytochrome P450 reductase was relatively conserved. Unlike the cytochrome P450s, the cytochrome P450 reductase does not experience a high selection pressure. A phylogenetic tree was constructed from the MrBayes 3.1.2 with 100 bootstrap trails using 14 published insect cytochrome P450 reductases (Figure 12).

DISCUSSION

The mosquito *Ae. sollicitans* and its Classification

I chose to use *Aedes sollicitans* rather than *Ochlerotatus sollicitans* because of remaining issues with systematics of the genus (Reinert 2000).

The Cytochrome P450 Reductase in *Ae. sollicitans*

The full-length cytochrome P450 reductase gene showed high similarity to other full-length insect cytochrome P450 reductase genes. It showed the highest similarity to the reductase gene of *Ae. aegypti* with 86% percent identity. Next in similarity was the reductase gene in *Cx. quinquefasciatus* 82% and then those in *Anopheles* mosquitoes 78%. It had a 74% similarity to the cytochrome P450 reductase gene in the fruit fly *D. melanogaster*.

The amino acid sequences of cytochrome P450 reductase in *Ae. sollicitans* showed a 95% identity with its counterpart in *Ae. aegypti*. It showed a 90% identity to that in *Cx. quinquefasciatus,* 87% identity to that in *An. funestus* and *An. minimus,* 86% identity to that in *An. gambiae,* 78% identity to that in *D. melanogaster* and *M. domestica*, 73% identity to that in *Dr. mettleri,* and 68% identity to that in *B. mori*, *B. mandarina* and *A. mellifera*.

The comparison among the 679 amino acid residues deduced from cytochrome P450 reductase gene obtained from Middlesex County, Ocean County and Cape May County mosquitoes showed that mutations occurred at the nucleotide level but did not lead to amino acid changes except in one case. In Ocean County mosquito population, the amino acid serine was changed to glycine. This change, however, was not considered to cause any functional change of cytochrome P450 reductase since the mutation was not located in any functional region as confirmed by NCBI analysis using the Blastp suite and conserved domains.

The cytochrome P450 reductase was not highly selected so it could be used to construct a phylogenetic tree. The tree revealed that the cytochrome P450 reductase of *Ae. sollicitans* was most closely related to that of *Ae. aegypti*. The two formed a single branch with 100 bootstrap supports. The tree also revealed the evolutionary relationship between the selected 14 insect species reductases. Mosquitoes, including *Aedes*, *Culex* and *Anopheles* appeared as one group related to other Dipterans, *M. domestica* and *Drosophila*. Insect reductase sequences from other phyla, Lepidoptera, Hymenoptera, and Coleoptera, were less related to the mosquito reductase sequences.

An Amino Acid Mutation in Cytochrome P450 Reductase From Ocean County Mosquito Population

The cytochrome P450 reductases were obtained from Middlesex County, Ocean County and Cape May County mosquito populations. The comparison between reductases from the three populations showed that all three had 679 amino acids and that no differences occurred between them except one amino acid in the reductase from Ocean County mosquito population. The amino acid glycine at position 651 in Ocean County mosquito population was different from the amino acid serine at position 651 in the Middlesex and Cape May County mosquito populations. The amino acid serine has a polar side chain (-OH) whereas the amino acid glycine is the smallest amino acid with a nonpolar side chain (H). Amino acids vary in their hydrophilic or hydrophobic physical properties depending on the polarity of the side chain. These properties are important in protein structure and functions and also in protein-protein interactions. The importance of the physical properties of the side chains comes from the influence this has on the interactions of the amino acid residues with other structures, both within a single protein and between proteins. Hydrophilic and hydrophobic interactions of the proteins do not have to rely only on the side chains of amino acids themselves. Posttranslational modifications can also change the proteins, forming hydrophobic or hydrophilic, for instance. In the cytochrome P450 reductase, putative conserved domain analysis in NCBI showed that amino acid at position 651 is not involved in any conserved domain. Thus, we believe that the change in the amino acid at the position 651 probably will not change the function or activity of the protein. However, the exact effect of the amino acid change

at position 651 of the cytochrome P450 reductase needs to be determined by further experiments.

CHAPTER 2

RELATIVE mRNA EXPRESSION OF CYP6BB1 AND CYTOCHROME P450 REDUCTASE GENE IN *Ae. sollicitans*

INTRODUCTION

Metabolism catalyzed by cytochrome P450s affects resistance to insecticides, physiological regulations and adaptation to environments in insects (Feyereisen 1999; Scott 1999). These effects have been evidenced in many insect species.

P450 over-expressions are not uncommon (Scott 1999) and result in increased insecticide detoxification rates, that could lead to insecticide resistance (Brattsten 1992). For instance, CYP6A1 mRNA levels of larva and adults in the Rutgers strain of house fly, *M. domestica*, were at least 10 times higher than in susceptible strains (Carino et al. 1994). CYP12A1 of the house fly is phenobarbital-inducible, and is constitutively overexpressed in an insecticide-resistant strain (Feyereisen 2006). CYP6D1 mRNA was also over-expressed in the Learn pyrethroid-resistant house fly *M. domestica* (Kasai et al. 2000). Other P450s over-expressed in insecticide-resistant strains of insects include CYP6A2 (Waters et al. 1992; Brun et al. 1996; Dunkov et al. 1997), CYP4E2 (Amichot et al., 1994), CYP6B2 (Wang and Hobbs 1995), CYP6A9 (Maitra et al. 1996), CYP9A1 (Rose et al. 1997), CYP12A1 (Grieneisen et al. 1993), CYP4G8 (Pittendrigh et al. 1997), CYP6G1 (Daborn et al. 2002), CYP6BG1 and CYP6BG2 (Bautista et al. 2009), P450 MA (Scharf et al. 1999), CYP6P3 and CYP6M2 (Djouaka et al. 2008; Muller et al. 2008), etc. Over-expression of these P450s associated with insecticide resistance is usually due to increased transcription rate of the gene, a common mechanism of P450 regulation in

mammals, or a stabilization of the mRNA or both (Feyereisen 1999), and does not result from gene amplification.

A characteristic of many P450s is their relatively low constitutive expression in the absence of substrate and highly elevated expression in the presence of their own substrates or many other xenobiotic compounds, such as insecticides, plant allelo-chemicals, fungal metabolites, hormones, growth regulators, barbiturates and other drugs, pollutants and xenobiotics present in habitats (Terriere 1984; Fuchs et al. 1994; Amichot et al. 1998; Scott et al. 1998; Stevens et al. 2000). The induction of P450s causes overproduction of the enzymes leading to increased catalytic activities and insecticide detoxification rates (Suwanchaichinda and Brattsten 2002). This induction is usually tissue-specific, rapid, dose-dependent, and reversible upon removal of the inducer. In the fruit fly *D. melanogaster* and the house fly *M. domestica*, P450s of subfamilies CYP6A and CYP6D have been isolated and shown to be responsive to phenobarbital (Feyereisen 1999). Insect P450s display characteristic expression behaviors depending on their specific functions. An interesting example is that the *CYP6B1* gene, initially cloned because of its xanthotoxin responsiveness, was shown to be selectively induced by linear, but not angular, furanocoumarins (Prapaipong et al. 1994; Hung et al. 1995). Instead, another P450 gene, *CYP6B3* (88% identity at the amino acid level to CYP6B1), was inducible by linear as well as angular furanocoumarins (Hung et al. 1995). It has been proposed that induction and over-expression in insecticide resistance should not be considered exclusive of each other because insecticides can be inducers and induction can lead to increased tolerance to insecticides (Brattsten 1988).

Insecticide resistance has been connected to a fitness cost (Davies et al. 1996). Resistance to organophosphates in the mosquito *Cx. pipiens* is a convenient model for investigating the fitness cost of resistance. Two main loci, *Ace 1* and *Ester*, were responsible for organophosphate resistance. Resistance alleles at these two esterase loci induced an overproduction of esterase, due to either gene amplification or gene regulation (Rooker et al. 1996), apparently affecting the mosquito development time and wing length (Bourguet et al. 2004). Fitness cost also occurred in insect resistance to Bt toxins. Fitness cost of Bt resistance occurred when, in the absence of Bt toxins, fitness was lower for resistant insects than for susceptible insects (Gassmann et al. 2009). The pattern of expression of individual P450s can vary within and/or between life stages and can be tissue specific depending on specific functions. For example, CYP4D1 in *Drosophila* and CYP6A1 in *M. domestica* were expressed in all developmental stages (Gandhi et al. 1992; Cohen and Feyereisen 1995), whereas CYP6Z1 in *An. gambiae* and CYP6D1 in *M. domestica* were adult specific (Scott et al. 1996; Nikou et al. 2003), and CYP6B2 was larva specific in *H. armigera* (Ranasinghe and Hobbs 1999). CYP6D1 was found in all tissues of adult house fly (Scott et al. 1998), while CYP6L1 was sex-specifically expressed, only in the testis of adult *Blattella germanica* (Wen and Scott 2001). A male-specific P450s, Cyp312a1, was also found in *D. melanogaster* (Kasai and Tomita 2003). Male-specific P450s were also found in *Ips paraconfusus* (Huber et al. 2007). Of which, the expression patterns of Cyp4AY1, Cyp4BG1, and, especially, Cyp9T1 in males suggested roles for these genes in male-specific aggregation pheromone production. Other P450s, such as P450s, *sad* (CYP315A1) and *dib* (CYP302A1) (Warren et al. 2002), CYP4C7 (Sutherland et al. 1998) and CYP15A1 (Helvig et al. 2004), were involved in

the hormone metabolism. The pheromone-metabolizing P450s such as CYP4S4 were expressed in the antenna of insects (Reed et al. 1994; Wojtasek and Leal 1999; Maibeche-Coisne et al. 2002). This differential expression may influence the fitness cost.

In this study, adult female mosquitoes were dissected and thoraxes, heads, Malphigian tubules and guts were used to extract RNA to perform real-time PCR. The aim of this study was to observe the different mRNA expression patterns of CYP6BB1 and expression level of the cytochrome P450 reductase gene in mosquito populations from three field sites in New Jersey. The CYP6BB1 mRNA expression was tested in different body parts to determine its expression level. The expression of the cytochrome P450 reductase gene was also compared in different body parts in three mosquito populations.

MATERIALS AND METHODS

Total RNA was isolated from fourth instar larvae from Cape May County, Middlesex County and Ocean County using TRIzol® reagent (Invitrogen, Carlsbad, CA) separately and treated with DNase I (Ambion, Austin, TX) to eliminate genomic DNA contamination. Malphigian tubules, heads, guts and thoraxes were dissected from newly emerged female adult mosquitoes from Cape May County, Middlesex County and Ocean County. Total RNA was isolated from them separately using TRIzol® reagent and treated with DNase I. First-strand cDNAs were synthesized from 1 μg of total RNA using iScript™ cDNA synthesis Kit (Bio-Rad, Hercules, CA) according to the user guide.

In this study, SYBR green was used to monitor DNA synthesis. SYBR green is a dye that binds to double-stranded DNA but not to single-stranded DNA and is frequently used in real-time PCR.

Real-Time PCR Primer Design and Standard Curve Construction

In this experiment, quantitative real-time PCR was used to quantify the differences in CYP6BB1 and cytochrome P450 reductase mRNA expression. Real-time PCR monitors the progress of PCR in "real time", i.e., the actual number of double-stranded DNA copies during the extension step at each PCR cycle, by detecting a fluorescent marker (such as SYBR green) which binds only to double-stranded DNA. The two most common methods to analyze real-time PCR are relative quantification and absolute quantification. Absolute real-time PCR estimates the starting copy number by relating the PCR signal to a standard curve, while relative real-time PCR estimates the change in mRNA expression levels by comparing the PCR signal of the target transcript to that of a control (a so-called housekeeping or reference gene). It is easier to use than absolute real-time PCR because it does not use a calibration curve, and is useful for most situations in which physiological changes in gene expression are studied (http://www.biocompare.com/articles/technologySpotlight/933/TechnologySpotlightArticle.html).

Designing optimal primer pairs is critical to have a successful quantitative real time PCR. The primer pairs should be able to yield a highly specific PCR product and have minimal or nonspecific annealings. The primers for CYP6BB1 were 5' GCGGA

AAGATGGGTTTACTG 3’ and 5’ CGGTCAAGATGTGCGTATTC 3’ with a 141bp amplicon (Table 1). The primer pair CPR RTF and CPR RTR for the cytochrome P450 reductase gene was 5’ CTACGAAGAGGAATTGGAGGATT 3’ and 5’ TCTTCCTCCA GCAGATGAGTAAC 3’with a 110bp amplicon (Table 1). Due to possible RNA integrity, different PCR efficiency, different amount of starting cDNA and operational errors, it is important to control errors internally so a target gene expression against a reference gene expression is routinely normalized. The reference gene should not be regulated or influenced by the experimental conditions. The most commonly used reference genes include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HRRT) and 18S ribosomal RNA (Huggett et al. 2005). A pair of primers for 18S rRNA was designed in this study. The forward primer and reverse primer were 5’ AGATACCGCCCTAGTTCTAACC 3’ and 5’ GTGCCCT TCCGTCAATTCC 3’, repectively. All the real time PCR primers except CPR RTF and CPR RTR were designed in the software Beacon Design 4.0 (PREMIER Biosoft International, Palo Alto, CA). The CPR RTF and CPR RTR were designed using the online tools—http://frodo.wi.mit.edu/primers/. The quality of the primers was evaluated by melting curve analysis and the primer amplification efficacy was calculated by standard curves constructed from a dilution series of PCR products for each pair of primers.

Real-time Quantitative RT_PCR Reaction

Real-time quantifications of CYP6BB1 and CYP6P10 mRNAs were normalized to 18S rRNA using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules,

CA). The 25 µl real-time PCR reaction for CYP6BB1 was made up of 200 ng of template cDNA, 2 µl of each 10 µM gene specific primers, 12.5 µl 2 ×iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR GreenI, 20 nM fluorescent and stabilizers. The reactions were triplicated and amplified using the following amplification conditions: 95 ℃ for 3 minutes followed by 40 cycles of 95 ℃ for 10 seconds and 60 ℃ for 1 minute. A melting curve analysis was performed after the amplification. It was performed by increasing temperature in 0.5 ℃ increments from 55 ℃ to 95 ℃ for 80 cycles of 10 seconds each and analyzed by the accompanied software in the machine. Two negative controls, one without primers and the other without cDNA, were performed at the same time by replacing primers and cDNA with sterile distilled water. Both the real-time PCR reaction system and reaction conditions for the cytochrome P450 reductase were the same as those for CYP6BB1. The specificity of the primers was verified by the presence of one single peak on the melting curve graph.

In this study, the expression of each of CYP6BB1 gene and cytochrome P450 reductase gene was compared in different body parts. So the relative quantification method was selected. A mathematical model for relative quantification in real-time PCR was set up (Pfaffl 2001). In this model, the expression ratio is calculated from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control. Control levels were included in the model to standardize each reaction run with respect to RNA integrity, sample loading and inter-PCR variations (Pfaffl 2001). The following Pfaffl model

$$\text{Expression Ration} = (E_{target})\Delta Ct_{target}(control\text{-}sample) \div (E_{reference})\Delta Ct_{reference}(control\text{-}sample)$$

was used to calculate the relative expression levels using the corresponding software REST 2005 BETA V1.9.12 with a Pair Wise Fixed Reallocation Randomisation Test (Pfaffl et al. 2002). In the equation, E_{target} used the real-time PCR efficiency of the target gene transcript while $E_{reference}$ was the real-time PCR efficiency of the reference gene transcript; ΔCt was the threshold cycle at which a significant increase in fluorescence intensity was first detected. ΔCt_{target} and $\Delta Ct_{reference}$ were the differences between the threshold cycles of control-sample of target gene and reference gene. The PCR efficiencies were calculated according to E=10[-1/slope] (Rasmussen 2001).

The Middlesex County mosquito population was used as control while the Ocean County and Cape May County mosquito populations were used as sample populations. When it came to body parts comparison, the body parts in the Middlesex County mosquitoes were used as control while the corresponding body parts in the Ocean County mosquitoes and Cape May County mosquitoes were used as samples. Both CYP6BB1 and cytochrome P450 reductase gene were tested.

RESULTS

Primer Quality

Double-stranded DNA melts at a temperature which is mainly decided by its base content and length. As the temperature increases, the double-stranded DNA denatures and single-strand DNA is released; as the temperature drops, the opposite occurs.

When the double-stranded DNA was denatured, the SYBR green was released and fluorescence levels declined sharply. The negative first derivative values of the fluorescence intensity, which was the result of temperature, were used to plot the melting curve. The melting curve analysis was performed for each pair of primers which resulted in single products respectively (Figure 13), showing that the real-time PCR product was amplified specifically by the primers and no primer dimers were formed in the reactions.

Standard Curves

Each pair of primers was diluted to perform the real-time PCR and the corresponding melting curve again produced a single peak showing the specific amplification of each pair of primers (Figure 13). The standard curves calculated from the C_t of each dilution series showed that the slopes of the real-time PCR amplified by CYP6BB1, CPR and 18S rRNA primers were -3.209, -3.3857, and -3.836. The amplification efficiencies of CYP6BB1, CPR and 18s rRNA primers were 2.049, 1.974 and 1.823 respectively. The relative correlation coefficient r^2 for all three primer pairs was >0.99, suggesting the standard curves were good.

Relative mRNA Expression of CYP6BB1 and CPR

The real-time PCR showed varied threshold cycles, indicating that the expressions of CYP6BB1 and CPR mRNA were different in whole larvae and different body parts of adult mosquito populations from three different counties. The single peak in the melting curve in the real-time PCR confirmed that the amplifications of CYP6BB1 and CPR mRNA were specific.

When the mRNA expression levels between the three mosquito populations were compared, CYP6BB1 expression levels were not significantly different in fourth instar larvae. The body parts in the three populations were compared and showed that there were no significant differences between them.

The mRNA expression levels of the cytochrome P450 reductase between whole fourth-instar larvae and between different body parts of non-blood-fed mosquitoes showed slight differences but none of these differences were significant.

DISCUSSION

Cytochrome P450s are considered "remarkable enzymes because there are so many of them and they do so many things" (Estabrook 1996). In insects, there are at least 59 known insect P450 families. Each insect species has about 60-120 P450 genes according to Nelson (2009). Insect P450s are important in insect physiology and development as well as adaptation to hosts and environments. They may also have other critical functions that we do not yet know (Feyereisen 1999).

The insect P450s' role in insecticide metabolism has been widely studied. P450-mediated metabolism is an extremely important metabolic system involved in the catabolism and anabolism of xenobiotics and endogenous compounds (Scott 1999). The molecular mechanism behind P450-mediated resistance is mainly due to increased transcription rate or stabilization of mRNA which results in over-expressed levels of P450 enzymes (Scott 1999) or less importantly, mutations (Lamb et al. 1997; Delye et al. 1998; Amichot et al. 2004).

Some of the insect P450s, such as CYP4D1 in *Drosophila* and CYP6A1 in *M. domestica,* can be expressed at all life stages. Others, such as CYP6Z1 in *An. gambiae,* CYP6B2 in *H. armigera* and CYP6D1 in *M. domestica,* are not. Some of the insect P450s can be expressed ubiquitously in different tissues, such as CYP6D1 in adult house fly, while some others can either be adult-specific, larva-specific, sex-specific or tissue-specific, such as CYP6L1 in adult *B. germanica*. Some of the insect P450s are expressed sex-specifically, such as CYP6L1 in male *B. germanica,* Cyp312a1 in male *D. melanogaster* and Cyp4AY1, Cyp4BG1, Cyp9T1 in male *I. paraconfusus,* while some others are expressed in both sexes, such as CYP6Z1 in *An. gambiae* and Cyp4G27 in *I. paraconfusus* (Huber et al. 2007), Cyp6a2, Cyp6a8, Cyp6d5 and Cyp12d1 in *D. melanogaster* (Le Goff et al. 2006), etc. CYP6BB1 was expressed in larvae, adult males and adult females, suggesting its expression was neither life stage specific nor sex specific, however, it is unclear whether CYP6BB1 is ubiquitous or tissue specific (Huang et al. 2008). The results in this study showed that CYP6BB1 was expressed in all four tissues examined, suggesting that its expression was ubiquitous, rather than tissue specific.

The CYP6BB1 allele was relatively over-expressed in adults from Ocean County mosquito population, but not in larvae (Huang et al. 2008). In those studies, the CYP6BB1 was over-expressed in blood-fed Ocean County mosquitoes. In this study, we did not find any significant expression differences between the four different body parts in non-blood-fed adult mosquitoes, indicating an endogenous function for CYP6BB1.

CHAPTER 3

EXPRESSION OF CYTOCHROME P450 REDUCTASE GENE IN *Escherichia coli*

INTRODUCTION

The production of proteins and active enzymes in model expression systems has been critical to several areas of molecular biology, including biotechnology, gene regulation, and enzyme structure and function. Knowing the cDNA of a particular protein means that we can study it in molecular detail but not without an appropriate expression system. So far a handful of systems have been used either to express cytochrome P450 or cytochrome P450 reductase, such as yeast, bacteria, mammalian cells, insect cells and stable expression systems such as B lymphoblastoid Cells and V79 cells, etc. (Gonzalez and Korzekwa 1995). Several factors should be considered when deciding which cDNA expression system to use for a particular enzyme. Yield and expense are of primary concern. Bacterial systems and insect cells (Baculovirus system) are usually selected as the expression system. *E. coli* has an advantage in terms of low cost, ease of use and the high yield of protein with a relatively short culture period. According to our experimental objective, we used *E. coli* as the expression system.

Cytochrome P450 monooxygenases are responsible for metabolism of endogenous compounds and xenobiotics. The activity requires the NADPH-cytochrome P450 reductase to transfer electrons from NADPH to cytochrome P450, using flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Wang et al. 1997). The cytochrome P450 reductase, like cytochrome P450, is capable of reducing other electron

acceptors, including cytochrome b5 (Schenkman and Jansson 1999), squalene oxidocyclase and cytochrome c, important in sterol biosynthesis (Ono et al. 1977), haem and cytochrome c biosynthesis (Wang and de Montellano 2003). The genes coding for the cytochrome P450 reductase have been isolated from *Homo sapiens, Rattus norvegicus* and several insect species, including members of the orders Coleoptera, Lepidoptera, Hymenoptera and Diptera. In mosquitoes, it has been isolated in *An. gambiae* (Nikou et al. 2003), *An. funestus* (http://www.ncbi.nlm.nih.gov/sites/ entrez?cmd= Retrieve&db= nucleotide&dopt= GenBank&RID=5MVZPX6801S&log%24=nucltop&blast_rank= 4&list_uids=139538809), *An. minimus* (Kaewpa et al. 2007), *Cx. quinquefasciatus* (http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db= nucleotide&dopt= GenBank&RID=5MVZPX6801S&log%24=nucltop&blast_rank=3&list_uids= 170060431) and *Ae. aegypti* (Nene et al. 2007). Some of the sequences are not published but appear as part of annotated genome sequences of the insects. The cytochrome P450 reductase genes from *An. minimus* (Kaewpa et al. 2007), *B. mori* (Horike et al. 2000), *M. domestica* (Andersen et al. 1994) and *D. melanogaster* CPR expression (Helvig et al. 2004) were expressed in *E. coli cells*.

MATERIALS AND METHODS

Total RNA Extraction and Full-length cDNA Synthesis

Total RNA from fifty fourth-instar larvae hatched from Middlesex County mosquito eggs was isolated using the TRIzol® Reagent (Invitrogen, Carlsbad, CA). The quality of total RNA was checked using RNA formaldehyde denaturing agarose gel electrophoresis (Figure 2.). The total RNA was treated with calf intestinal phosphatase

(CIP) to remove the 5’ phosphates; the dephosphorylated RNA was treated with tobacco acid pyrophosphatase (TAP) to remove the 5’ cap structure from intact, full-length mRNA; the GeneRacer RNA Oligo was ligated to the 5’ end of the mRNA using T_4 RNA ligase; the ligated mRNA using SuperScript™ III RT and the GeneRacer™ Oligo dT Primer was reverse transcribed to create RACE-ready first-strand cDNA with known priming sites at the 5’ and 3’ ends. The RACE-ready first-strand cDNA was used to amplify the coding region of the cytochrome P450 reductase gene and cloned into the expression vector. All these steps strictly followed the manual and used the following materials:

Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), Qiaprep® Spin Miniprep Kit and Qiaquick® Gel Extraction Kit (Qiagen, Valencia, CA), pGEMT easy vector (Promega, Madison, WI), expression vector pTricHis (Invitrogen, Carlsbad, CA), *E. coli* strain XL-1 Blue cells (Stratagene, La Jolla, CA), restriction enzymes BamHI and XhoI (New England Biolabs Inc, Ipswich, MA), T_4 DNA ligase and isopropyl-β-D thiogalactopyranoside (IPTG) (Promega, Madison, WI), oligonucleotide primers (Eurofins MWG Operon, Huntsville, AL) and cytochrome c and NADPH (Sigma-Aldrich, St. Louis, MO).

The microsomes from fourth instar mosquito larvae were prepared in 0.1M sodium phosphate buffer with 1mM EDTA and pH 7.5. First, it was centrifuged at a low speed (10,000 × g) for 10 minutes to precipitate the nuclei, mitochondria, cell debris, and bacteria, followed by a high speed spin (240, 000 × g) for 20 minutes resulting in a supernatant and microsomal pellet. The pellet was gently resuspended in Tris-HCl buffer at pH 7.7 and stored at -80 ℃ until use.

Amplification of the Coding Region of the Cytochrome P450 Reductase Gene

A pair of gene-specific primers flanking the coding area is designed based on the full-length cytochrome P450 reductase gene. In order to create two restriction digestion sites, two adapters containing the digestion sites of BamHI and XhoI were attached added to the primers. The forward primer was CPR5P (5' CTGGGATCCATGGACGCACAAA CGGAACCGG 3') and reverse primer was CPR3P (5' CTGCTCGAGTTAGCTCCACA CATCGGCCGAG 3'). The 50 μL PCR reaction system contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5% dimethyl sulfoxide (DMSO), 2mM $MgSO_4$, 0.3 mM each of dNTP, 0.4 mM of each primer, 1 μL of cDNA template diluted 20 times, 1 unit of Platinum® *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA), and an appropriate volume of Milli-Q ultra pure H_2O. The PCR reaction mixture was denatured at 94℃ for 2 minutes, and subsequently amplified by 40 cycles of 94℃ for 15 seconds, 55℃ for 30 seconds, and 68℃ for 2 minutes and 15 seconds. The PCR reaction was then extended at 68℃ for 10 minutes and held at 4℃. The PCR products were run on a 1.5% agarose gel with a 2kb DNA ladder (Invitrogen, Carlsbad, CA). The band corresponding to approximately 2040bp was cut out and purified by the Qiaquick® Gel Extraction kit (Qiagen, Valencia, CA). The Platinum Pfx DNA polymerase has proofreading 3' to 5' exonuclease activity and can not be used directly for TA cloning. In order to perform the subsequent TA cloning, a 3'-A overhang was added to the purified PCR product. The 25 μL reaction included 1 unit of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2mM $MgCl_2$, 0.6 mM dATP (Qiagen, Valencia, CA), and an appropriate amount of PCR product purified from the gel. It was incubated at 70°C for 1 hour. The purified DNA with 3'-A overhang was then cloned into the pGEMT

easy vector. The ligation reaction included 5 μL of 2×Rapid Ligation Buffer, T_4 DNA ligase, 1 μL of pGEMT Easy Vector (50ng), 3 μL of incubation solution and 1 μL of T_4 DNA Ligase (3 Weiss units/μL). The ligation reaction was incubated overnight at 4℃ in order to obtain the maximum number of transformants. Ligation reaction of 2 μL was mixed with 50 μL of JM109 competent cells (Promega, Fitchburg, WI) and placed on ice for 20 minutes. The mixture was heat-shocked for 45 seconds at 42℃ before being placed on ice for 2 minutes. SOC medium (Invitrogen, Carlsbad, CA) of 950 μL at room temperature was added to the tube and it was incubated for 1.5 hours at 37℃ with shaking at 150rpm. 100 μL of transformation culture was plated onto each LB/ampicillin/IPTG/X-Gal plate. The plates were then incubated 18 hours at 37℃. A well separated and white colony grown on the plate was selected and cultured in LB medium containing 75 μg/mL ampicillin for 16 hours at 37℃ with shaking. The overnight culture of JM109 competent cells in 1.5 mL of LB medium was transferred to 1.5 mL of Eppendorf tubes and centrifuged at 4000 rpm to collect the cells. The pelleted bacterial cells were resuspended and plasmid DNA was eluted according to QIAprep® Miniprep kit manual (Qiagen, Valencia, CA). The plasmid DNA was sent to Genewiz (Piscataway, NJ) for sequencing to verify the insertion of the cytochrome P450 reductase gene. After the verification process, the plasmid DNA was digested with BamHI and XhoI and electrophoresed on a 1.2% agarose gel. The 20 μL digestion reaction was set up as follows: 10 μL of purified plasmid DNA, 2ng/μL of BSA, 2 μL of 10×NEBuffer 3, 1 μL each of restriction enzymes BamHI and XhoI and an appropriate amount of ddH_2O. The digestion reaction was incubated at 37℃ for 3 hours. The released target DNA was cut out and purified using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA).

Recombinant Expression DNA

The pTrcHis vectors are pBR322-derived expression vectors which can express recombinant protein efficiently. The pTrcHis vector has a multiple cloning site that has eight unique restriction sites including BamH I and Xho I. The pTrcHis vector was digested using restriction enzymes BamH I and Xho I. The 20 μL digestion reaction was set up as follows: 2 μL of expression vector pTrcHis A, 0.2 μL of 100×BSA, 2 μL of 10×NEBuffer 3, 1 μL each of restriction enzymes BamHI and XhoI and an appropriate amount of ultrapure water The digestion reaction was incubated at 37 ℃ for 4 hours. The digestion reaction was electrophoresed on 1.2% agrarose gel and the target band was cut out and purified using QIAquick® Gel Extraction Kit. The ligation reaction for digested cytochrome P450 reductase and digested expression vector pTrcHis was set up as follows: 6 μL of digested cytochrome P450 reductase gene, 2 μL of digested vector pTrcHis, 3 Weiss units of T_4 DNA ligase and 1 μL of 10×T_4 DNA ligase buffer. The ligation reaction was incubated at room temperature for 2 hours. The different sticky restriction digestion ends could insure that the cytochrome P450 reductase gene, digested by enzymes BamH I and Xho I, easily cloned into the multiple cloning sites in frame with the starting codon ATG so the insert gene could be expressed successfully.

Expression of Cytochrome P450 Reductase Gene in XL-1 Blue Competent Cells

The XL1-Blue competent cells often serve as host for cloning using plasmid vectors. They are tetracycline resistant and ampicillin resistant. The XL1-Blue cells have two other very important characters. First, they are endonuclease (*endA*) deficient, which

greatly improves the quality of miniprep DNA. Second, they are recombination (*recA*) deficient, which greatly increases the insert stability.

The XL1-Blue competent cells were thawed on ice, gently mixed and 100 µL of the cell suspension was added to the pre-chilled 14-mL BD Falcon polypropylene round-bottom tubes (BD Biosciences, San Jones, CA). A 1.7 µL portion of β-mercaptoethanol was added to the incubation to increase the transformation efficiency and the cells were incubated on ice for 10 minutes. The cells were swirled gently every 2 minutes. Ligation reaction of 2 µL was added to the cells and the tube was swirled gently to mix them. The tube was heat-shocked at 42 ℃ in a water bath for 45 seconds after 30 minutes incubation on ice. After the heat-shocking, the tube was cooled down on ice for at least 2 minutes and 900 µL of preheated SOC medium was added to the tube. Then the tube was incubated at 37 ℃ for 1 hour with shaking at 225rpm. A sample of 100 µL of the transformation mixture was streaked onto LB agar plates containing 75 µg/mL ampicillin. The LB agar plates were incubated at 37 ℃ overnight for the colonies to grow. Five well separated single colonies were selected and each was cultured in 5 mL of LB medium at 37 ℃ for 16 hours with shaking at 225rpm separately. The XL1-Blue cells grown in the tubes were harvested by centrifugation at 5000 rpm for 10 minutes in a Beckman L8-M ultracentrifuge. The pelleted bacterial cells were resuspended and the plasmid DNA was purified by QIAprep Miniprep kit according to the manual.

The purified plasmid DNA was sent to Genewiz for sequencing to confirm the integrity of the open reading frame of the cytochrome P450 reductase gene. A pair of sequencing primers pTrcHis-Forward (GAGGTATATATTAATGTATCG) and pTrcHis -Reverse (CGCTTCTGCGTTCTGATTTA) based on the sequence of the expression

vector pTrcHis was designed for the sequencing. The sequencing result confirmed that the cytochrome P450 reductase gene was inserted into the multiple cloning sites of the expression vector pTrcHis in frame with the ATG. The XL1-Blue cells corresponding to the tube which has the correct insert were streaked onto LB plates with 50 μg/mL of ampicillin and incubated at 37 ℃ overnight. Next day, a well separated single recombinant *E. coli* colony was picked and grown in 5 mL of LB medium with shaking overnight again. The next day 50 mL of LB medium with 50 μg/mL of ampicillin was inoculated with 0.2 mL of the overnight culture. The 50 mL of culture was grown at 37 ℃ with shaking at 250 rpm to an optical density at 600 nm of 0.6. Cells in 1 mL were collected prior to IPTG induction. The sample was centrifuged and the supernatant was aspirated and kept at -20 ℃. This was the time zero sample. IPTG was added to the culture mmedium to give a final concentration of 1mM and the cells were incubated at 37 ℃ with shaking at 250 rpm. Samples were taken at one hour intervals for 5 consecutive hours. Each sample was centrifuged and the cell pellet was stored at -20 ℃.

SDS-PAGE of the Expressed Cytochrome P450 Reductase

After all the time points had been collected, the pellets were resuspended in 100 μL of 20 mM phosphate buffer at neutral pH and frozen on dry ice. The frozen lysates were thawed at 42 ℃. The freeze and thaw process was repeated for 3 additional times and centrifuged at 10,000 rpm at 4 ℃ for 10 minutes to pellet the insoluble protein. The supernatant was removed to a fresh tube and the pellet was resuspended in 100 μL of Laemmli Buffer (Bio-Rad, Hercules, CA). The 100 μL of supernatant sample was mixed

with 100 μL of 2 × Laemmli Buffer. The pellet samples and supernatant samples were analyzed on 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). After the protein electrophoresis, the gel was stained with Coomassie Brilliant Blue and a band with increasing intensity in the expected size range was detected. The gel showed that 4h after the IPTG addition was the optimal time to havest the XL1-Blue competent cells wash (Figure 15).

A well separated single recombinant *E. coli* colony was picked and grown in 5 mL of LB medium (75 μg/mL of ampicillin) with shaking overnight. The next day 50 mL of LB medium with 50 μg/mL of ampicillin was inoculated with 0.2 mL of the overnight culture. The 50 mL of culture was grown at 37 ℃ with shaking at 250 rpm to an optical density at 600 nm of 0.6. A final concentration of 1 mM IPTG was added to the culture solution and the cells were incubated at 37 ℃ with shaking at 250 rpm. The samples were taken 4 hours after IPTG was added.

The XL1-Blue competent cells were centrifuged at 4 ℃ at 10,000 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended with 50 mL of lysis buffer. The lysis buffer (pH 8.0) was made up of 10 mM Tris-Hcl (pH 8.0), 1 mM EDTA (pH 8.0) and 1 mM PMSF. The cells were broken by a sonicator for a total of 20 minutes, with 30 seconds on and 30 seconds off. The broken cells were centrifuged at 4 ℃ at 55,000 rpm for 20 minutes. The supernatant was collected and the pellets were resuspended using 100 mL of Tris-Hcl (pH 7.7). Both the supernatant and the pellet solution were stored at -80 ℃ until use.

ASSAY OF CYTOCHROME P450 REDUCTASE

BCA standard protein assay

The protein concentration in the samples was determined with the BCA assay procedure (Bio-Rad, Hercules, CA).

Reductase Assay

A portion of 0.8 mL 0.1 M Tris-HCl buffer (pH 7.7), 0.2 mL of cytochrome c (4.4 mg/mL) in Tris-HCl buffer and enzyme suspension was added to each of 2 matched glass cuvettes and a zero baseline was obtained. The specific activity of the cytochrome P450 reductase was calculated as product per minutes and mg protein using the molar extinction coefficient for reduced cytochrome c as 21,000 $M^{-1}cm^{-1}$ product. One of these two cuvettes was used as reaction blank. The reaction in a total volume of 1 mL was started by adding NADPH to the sample cuvette. The time-dependent absorption change at 550nm was monitored up to 2 minutes by using Unicam spectrophotometer model UV1 (West Yorkshine, UK).

The microsomes in *Ae. sollicitans* from Middlesex County, Ocean County and Cape May County were assayed using the same method as for the expressed cytochrome P450 reductase in *E. coli*.

RESULTS

The condition for the production of the 6×His-tagged CPR fusion protein was the induction by addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at A_{600} of 0.6 and cultivation at 37 ℃ for an additional 4 hours. The cytochrome P450 reductase

activity was detected in the cell lysate by showing cytochrome c reduction. The molecular weight of the CPR recombinant protein estimated on SDS-PAGE was 84kDa, in accordance with the expected size of the sum of 6×His-tagged (6.2 kDa) and cytochrome P450 reductase protein (78 kDa) (Figure 15).

The CPR protein was present in both cytosolic and membrane fractions. Both cytosolic and membrane fractions contained NADPH-cytochrome P450 reductase activity, with the activity in cytosol much higher than in the membrane fraction (Table 4).

The cytochrome P450 reductase activities in microsomes of *Ae. sollicitans* collected from three different locations in New Jersey were assayed and the results in all three locations are listed in Table 4.

DISCUSSION

The mosquito *Ae. sollicitans* NADPH-cytochrome P450 reductase enzyme was successfully heterologously expressed in XL-1 Blue *E. coli* expression system. Both the cytosolic fraction and membrane fraction of the cytochrome P450 reductase contained cytochrome c reducing activity. This was attested by measurement of the cytochrome c reduction activity spectrophotometrically. The same results were found for *D. melanogaster* and *An. minimus* cytochrome P450 reductase expression (Kaewpa et al. 2007). The specific activities assayed are listed in Table 4.

The cytochrome P450 reductase was expressed in XL-1 Blue *E. coli* cells, cultured at 37 ℃ for 48 hours after IPTG was added and then harvested by centrifugation. Table 4 shows that the specific activity of the cytochrome P450 reductase gene expressed in *E. coli* was 5.6-fold higher in the cytosolic fraction than that in the membrane fraction.

This was also observed in the *D. melanogaster* cytochrome P450 reductase expressed in *E. coli*, in which the cytochrome c reductase activity was present in both cytoplasmic and membrane fractions, with the specific activity in the soluble fraction 5-10-fold higher than in the membrane fraction (Helvig et al. 2004).

However, when compared to the specific activity of the cytochrome P450 reductase gene in *An. minimus* expressed in *E. coli*, the specific activity of the cytochrome P450 reductase gene in *Ae. sollicitans* expressed in *E. coli* was much higher, in both the cytosolic fraction and membrane fraction. The specific activity of the expressed cytochrome P450 reductase gene from *Ae. sollicitans* in the membrane fraction harvested 48 hours after IPTG addition was 2.3-fold higher than that from *An. minimus* while the specific activity of the cytochrome P450 reductase gene expressed in the cytosolic fraction harvested 48 hours after IPTG addition from *Ae. sollicitans* was 6.65-fold higher than that from *An. miminus.* When comparing the content of the expressed recombinant cytochrome P450 reductase in the membrane fraction to that in abdominal microsomes of *Ae. sollicitans*, the microsomal cytochrome P450 reductase content was low, only about 15% of that expressed in IPTG-treated *E. coli*. The result was comparable to house fly microsomal CPR and *An. minimus* microsomal cytochrome P450 reductase, both of which were about 19% of activity expressed in *E.coli*.

Murataliev (1999) reported that 0.1 M K-phosphate buffer pH 7.6 interfered with the binding of NADPH and cytochrome c increasing K_m for the substrate 3-5 fold. The same effect of phosphate was found on the mammalian P450 reductase (Phillip and Langdon. 1962). In our experiment, to avoid the inhibitory effect, all assays and incubations were carried out in 0.1 M Tris-HCl pH 7.7 buffer instead of phosphate buffer.

This may explain the differences between the specific activity of the cytochrome P450 reductase gene from *Ae. sollicitans* expressed in *E. coli* and that from *An. minimus* expressed in *E. coli*.

We also reported the specific activities of the cytochrome P450 reductase in microsomes of *Ae. sollicitans* collected from three different locations in New Jersey (Table 4). The activity in the Cape May County mosquito population was higher than that in the Middlesex County and Ocean County mosquito populations. It was much lower when compared to the activity in midgut microsomes in southern army worm, Sp*odoptera eridania* (Cramer), which has an activity of 168.94 nmol/min/mg (STDEV 16.80 nmol/min/mg) (Brattsten et al. 1986). In the tobacco budworm, *Heliothis virescens*, the reductase activity in gut and fatbody microsomes was 42.35 nmol/min/mg (Brattsten 1987). However, our data is comparable to the microsomal reductase activity in the boll weevil, *Anthonomus grandis grandis* Boheman, which has an activity of 2.87 nmol/min/mg (Brattsten 1987).

CHAPTER 4

CONCLUSIONS AND OUTLOOK

The salt marsh mosquito *Ae. sollicitans* is an important nuisance species in New Jersey and a vector of several viral diseases. The microsomal cytochrome P450 system, which entirely depends on cytochrome P450 reductase activity, is one of the most important families of enzymes involved in the metabolic responses of living organism to foreign chemicals. The cytochrome P450s in *Ae. sollicitans* were studied in our lab and partial and full-length sequences were obtained for 13 cytochrome P450 genes. These sequences were submitted to NCBI and named by the P450 nomenclature committee. As an important part of the enzyme system, it is imperative to study the cytochrome P450 reductase in *Ae. sollicitans*.

The cytochrome P450 reductase gene in *Ae. sollicitans* collected from Middlesex County was identified and verified by analyzing its conserved domains and similarity to the cytochrome P450 reductase genes in 13 other insect species. The base sequence and its deduced protein were submitted to NCBI. The cytochrome P450 reductase genes in Ocean County and Cape May County *Ae. sollicitans* were also identified and the amino acid sequences were deduced.

Our results showed that the Open Reading Frame (ORF) in the cytochrome P450 reducase gene is 2040 bp, encoding 679 amino acids. It has a very high similarity to the cytochrome P450 reductase in other insect species, with a 95% identity with its counterpart in *Ae. aegypti,* 90% identity to that in *Cx. quinquefasciatus,* 87% identity to that in *An. funestus* and *An. minimus,* an 86% identity to that in *An. gambiae*, a 78% identity to that in *D. melanogaster* and *M. domestica*, a 73% identity to that in *Dr.*

mettleri, a 68% identity to that in *B. mori*, *B. mandarina* and *A. mellifera*. Interestingly, a relatively large genetic distance allows considerable gene sequence identity. The identity of cytochrome P450 reductase from *Ae. sollicitans* and *Homo sapiens* is 55%; 54% identity to that in *Mus musculus*; 55% identity to that in *Oryctolagus cuniculus*, and 55% identity to that in *Sus scrofa*. This implies that cytochrome P450 reductase is highly conserved.

The phylogenetic tree revealed that the cytochrome P450 reductase of *Ae. sollicitans* was most closely related to that of *Ae. aegypti*. The tree also revealed the evolutionary relationship between the published 14 reductase sequences from insects. Mosquitoes, including *Aedes*, *Culex* and *Anopheles* appeared as one group related to other Dipterans, *M. domestica* and *Drosophila*. Insect reductase sequences from other phyla, Lepidoptera, Hymenoptera, and Coleoptera, were less related to the mosquito reductase sequences.

The function of an individual cytochrome P450 isozyme can only be identified when its sequence is coexpressed with cytochrome P450 reductase gene. We now have a good start for this. Another possibility is to blend the purified cytochrome P450 protein with the purified reductase protein. This can be done practically in insects only by way of DNA.

Comparing the 679 amino acid residues deduced from cytochrome P450 reductase gene obtained from Middlesex, Ocean and Cape May County *Ae. sollicitans* populations showed that mutations occurred at the nucleotide level but did not lead to amino acid changes except in one case. The Ocean County mosquito population has a serine at position 651 whereas Middlesex and Cape May have a glycine in this position. This

amino acid change was not located in any functional region as confirmed by NCBI analysis using the Blastp suite and conserved domains, and hence was not considered to cause any functional change of cytochrome P450 reductase although one amino acid is lipophilic and the other has a hydrophobic side chain making it generally more reactive than the glycine. However, the exact effect of the amino acid change at position 651 of the cytochrome P450 reductase needs to be further studied.

Relative real-time expression analysis showed that the expressions of CYP6BB1 were not significantly different in different body parts of adult *Ae. sollicitans* from the three locations in New Jersey. In previous studies in our lab, CYP6BB1 was found significantly expressed in Ocean County mosquitoes when compared to Middlesex County mosquitoes after blood-feeding, indicating that CYP6BB1 may be involved in the metabolism of endogenous compounds. The expression of CYP6BB1 was found in four body parts of female adult *Ae. sollicitans,* indicating that CYP6BB1 is ubiquitously expressed in female adults, rather than locally expressed. The mRNA expression levels of the cytochrome P450 reductase between the whole fourth-instar larvae and different body parts of non-blood-fed mosquitoes showed no significant differences.

The cytochrome P450 reductase gene was expressed in XL-1 Blue *E. coli* cells and the molecular mass of the protein was estimated to be 78 kDa according to SDS-PAGE. The reductase activity was assayed in both the cytosolic and cell membrane fractions. The activity was much higher in the soluble fraction of the cells than that in the membrane fraction. The cytochrome P450 reductase activities in microsomes of *Ae. sollicitans* collected from three different locations in New Jersey were assayed. The results showed differences that were not statistically significant.

To better explore the functions of vast cytochrome P450s, a number of different methods has been used, including sequencing the whole genome, cloning and expressing the gene with cytochrome P450 reductase, and using known P450 as a probe. However, considering the huge cost of sequencing the whole genome and vast diversity of cytochrome P450s, many chose *in vitro* coexpression of cytochrome P450s with cytochrome P450 reductase. Several systems have been used to express cytochrome P450 reductase and cytochrome P450s together. Among them, the *E. coli* expression system is the most widely used due to its advantages over other systems, including rapid growth in a very short time, easy to manipulate, inexpensive, high yields of the heterologously expressed protein and broad variety of expression vectors. In this study, an *E. coli* expression system to express cytochrome P450 reductase was set up and successfully expressed cytochrome P450 reductase. Using this system to coexpress with cytochrome P450s in the future work, the functions of the identified cytochrome P450s in *Ae. sollicitans* are plausible.

Identification of the cytochrome P450 reductase gene and the availability of its sequence may provide a way for us to design a RNAi to block the expression of cytochrome P450 reductase. The inhibition of the expression of cytochrome P450 reductase will hinder the availability of electrons for cytochrome P450s to function appropriately, thus reducing in the efficiency of mosquitoes to detoxify insecticides and improving the efficiency of insecticides application at the same time.

This is the first study of cytochrome P450 reductase in *Ae. sollicitans,* including its relative expression and DNA sequence. The identification and expression of the cytochrome P450 reductase gene will provide further insights on how cytochrome P450s

metabolize substrates and make it possible to explore the specific functions of each cytochrome P450 gene in *Ae. sollicitans* and in other mosquito species.

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TABLES

Table 1. Primers used in degenerate PCR, RACE and CPR-verification PCR reactions

	Primer Sequence (5'-3')
CPR F	GAA GGG HAT GGT BGC BGA YCC
CPR R	GGG AAY GGR TGY TTY TTR CTG C
GeneRacer™ Oligo dT Primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$
CPR FF	ACAGCCCCGCTACTACTCTATTTCCTC
CPR FR	AGGGCTCGCCATTCAGAGGGTGCTT
GeneRacer™ 5' primer	CGACTGGAGCACGAGGACACTGA
GeneRacer™ 3' primer	GCTGTCAACGATACGCTACGTAACG
GeneRacer™ 5' Nested Primer	GGACACTGACATGGACTGAAGGAGTA
GeneRacer™ 3' Nested Primer	CGCTACGTAACGGCATGACAGTG
CPRVF	CTCGGTTCAGACTGGTCGTGTTT
CPRVR	GTGCGTGAGTGAGTGAATGAGTG
CPRVF1	TATCCTGTCAACGATCAGGACTT
CPRVF2	AGAATTCGTTCATCAAGAAGCTG
CPRVR1	GATGTACTGCACAGCTTCACTT
CPRVR2	AAGTCCTGATAAGTCCTGATCGTTGACAGGATA

Table 2. PCR conditions used in degenerate PCR, RACE and CPR-verification reactions.

Reactions	Conditions
Degenerate PCR	Initial denature at 94 ℃ for 2 minutes 40 cycles of Denature at 94 ℃ for 30 sec Anneal at 58 ℃ for 30 sec Extend at 72 ℃ for 1 min Final extension at 72 ℃ for 10 min
5'-RACE and Nested 5'-RACE	Initial denature at 94 ℃ for 2 minutes 40 cycles of Denature at 94 ℃ for 30 sec Anneal at 60 ℃ for 30 sec Extend at 72 ℃ for 2 min Final extension at 72 ℃ for 10 min
3'-RACE and Nested 3'-RACE	Initial denature at 94 ℃ for 2 minutes 40 cycles of Denature at 94 ℃ for 30 sec Anneal at 60 ℃ for 30 sec Extend at 72 ℃ for 2 min Final extension at 72 ℃ for 10 min
Verification PCR	Initial denature at 94 ℃ for 2 minutes 40 cycles of Denature at 94 ℃ for 30 sec Anneal at 58 ℃ for 30 sec Extend at 68 ℃ for 2 min 30 sec Final extension at 68 ℃ for 10 min

Table 3. Primers used for real-time PCR of CYP6BB1 and CPR.

Primer Sequence (5’-3’)	
18S F	AGATACCGCCCTAGTTCTAACC
18S R	GTGCCCTTCCGTCAATTCC
CYP6BB1 F	GCGGAAAGATGGGTTTACTG
CYP6BB1 R	CGGTCAAGATGTGCGTATTC
CPR RTF	CTACGAAGAGGAATTGGAGGATT
CPR RTR	TCTTCCTCCAGCAGATGAGTAAC

Table 4. Specific activities of cytochrome P450 reductase expressed in *E. coli* in cytosolic and cell membrane fractions and in microsomes of *Ae. sollicitans* larvae representing three populations in New Jersey.

	Membrane	Cytosol
Average Specific activity in Membrane (nmol/min/mg) (SE) (N)	130.4 (24.4) (3)	727.6 (80.5) (3)

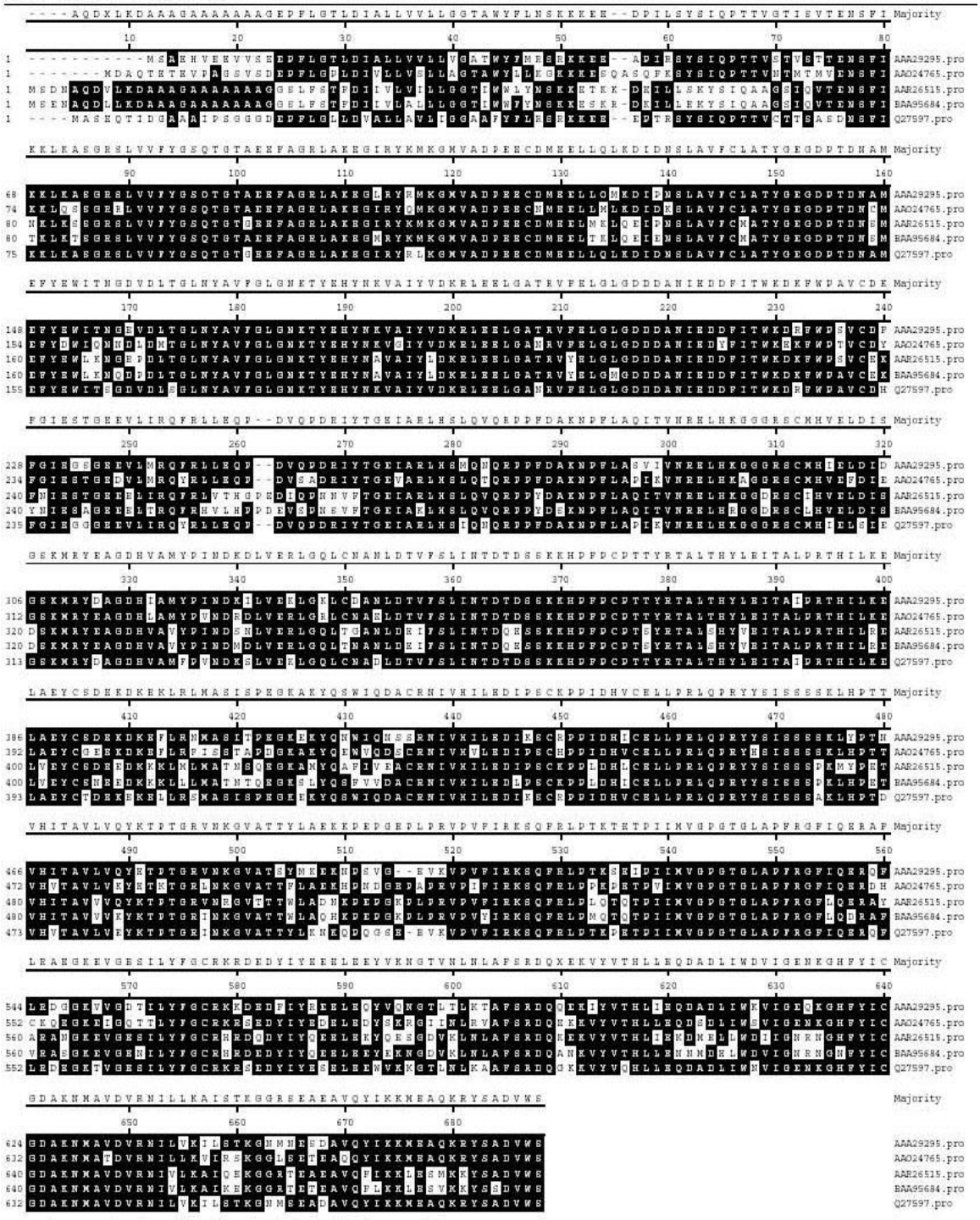
4A Specific activities in *E. coli* in cytosolic and cell membrane fractions

Location	Specific activity in microsomes (nmol/min/mg) (SE) (N)
Middlesex County	1.71 (0.35) (3)
Ocean County	1.26 (0.30) (3)
Cape May County	2.13 (1)

4B Specific activities in microsomes of *Ae. sollicitans* larvae representing three populations in New Jersey.

FIGURES

Figure 1. Alignment of amino acid sequences from five insect species produced by Megalign (Lasergene V 7.0).



The shaded regions are the conserved regions shared by *M. domestica* (Genbank: AAA29295), *An. gambiae* (Genbank: AAO24765), *M. brassicae* (Genbank: AAR26515), *B. mori* (Genbank: BAA95684), *D. melanogaster* (Genbank: CAA63639). Two highly conserved regions, KGMVADP (from amino acid AA 119 to AA 125) and SSKKHPFP (from AA 368 to AA 375), were selected to design the degenerate primers using Primer Premier 5.0.

Figure 2. Agarose gel electrophoresis of PCR product amplified by a pair of degenerate primers.

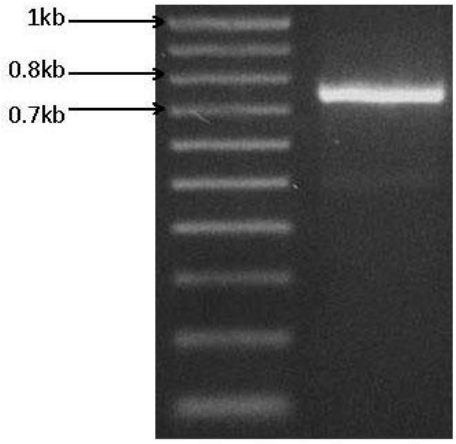


Figure 3. RNA formaldehyde denaturing agarose gel electrophoresis of total RNA from fourth instar mosquito larvae.

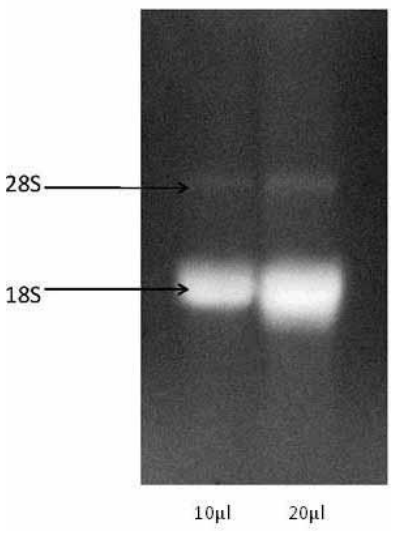


Figure 4. BlastN results for partial and full-length sequence of cytochrome P450 reductase gene from *Ae. sollicitans* in the NCBI database.

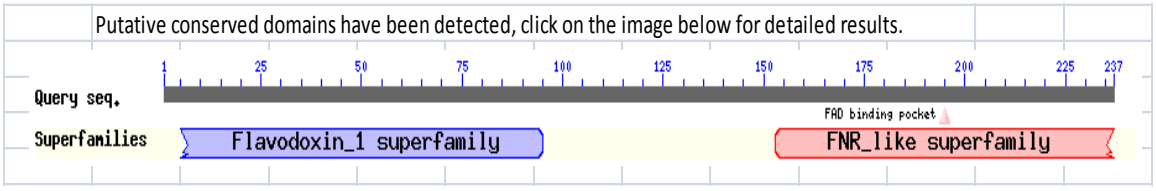
Accession	Description	Max identity
XM_001656665.1	*Aedes aegypti* NADPH-cytochrome P450 reductase mRNA	86%
XM_001865766.1	*Culex quinquefasciatus* NADPH-cytochrome P450 reductase mRNA	85%
EF057735.1	*Anopheles minimus* NADPH-cytochrome P450 reductase mRNA	81%
AY183375.1	*Anopheles gambiae* NADPH-cytochrome P450 reductase mRNA	81%
EF152578.1	*Anopheles funestus* NADPH-cytochrome P450 reductase mRNA	80%

4A Partial nucleotide sequence

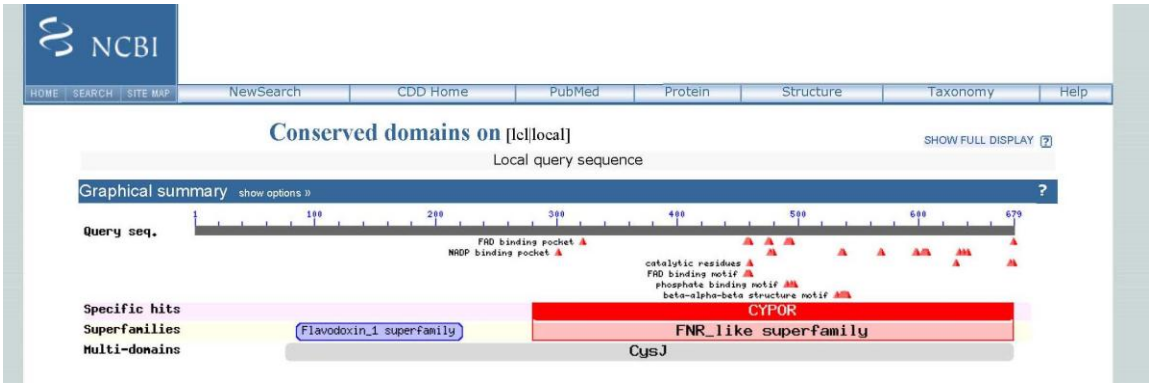
Accession	Description	Max identity
XM_001656665.1	*Aedes aegypti* NADPH-cytochrome P450 mRNA	86%
XM_001865766.1	*Culex quinquefasciatus* NADPH-cytochrome P450 reductase mRNA	82%
EF152578.1	*Anopheles funestus* NADPH-cytochrome P450 reductase mRNA	78%
EF057735.1	*Anopheles minimus* NADPH-cytochrome P450 reductase mRNA	78%
AY183375.1	*Anopheles gambiae* NADPH-cytochrome P450 reductase mRNA	78%
X93090.1	*Drosophila melanogaster* NADPH-cytochrome P450 reductase mRNA	74%

4B Full-length nucleotide sequence

Figure 5. BlastP results for amino acid residues of the cytochrome P450 reductase from *Ae. sollicitans* in the NCBI database.



5A Partial amino acid residue sequence



5B Full-length amino acid residue sequence

Figure 6. Sequences of cytochrome P450 reductase gene and its deduced amino acid residue sequence from *Ae. sollicitans* collected from Middlesex County, New Jersey.

TCAAGTTTTGCTCTCGGTTCAGACTGGTCGTGTTTCCTTCTAGGCGCTTTTCCCCGGTAATTTTCGTGTGCGTTTTCCGTGTCGTCGAATATCAGCATC
AGCAGCAGCAGTGACGACCCTAGAGCACCTTTCCGGTAACATACCACGATGGACGCACAAACGGAACCGGAAGTACCTCCGGCACCGGTCAGCGA
GGAGCCATTCCTGGGCCCGCTAGACATTATCCTGCTGACAGTGCTGATCGGCGGAGCCGCCTGGTATTTCCTCAAAAACAAAAAGAAGGACACACA
GGCTAGTCAGTTCAAATCGTACTCGATACAGCCAACCACCGTCAACACGATGACCATGGCTGAGAATTCGTTCATCAAGAAGCTGAAATCGTCCGG
TCGTCGGTTAGTGGTATTCTACGGTTCGCAGACCGGAACGGCCGAAGAATTTGCCGGTCGTTTGGCCAAGGAAGGTCTCCGTTACCAGATGAAGG
GTATGGTAGCCGATCCGGAAGAATGTGATATGGAGGAGCTACTTTCACTGAAGGACATCGACAAGTCGTTGGCCGTGTTCTGTTTAGCTACGTACG
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TTGGTAACAAAACCTATGAACACTACAATAAGGTTGGAATCTACGTCGACAAGCGACTGGAGGAACTGGGAGCTAACAGGGTGTTCGAGCTGGGT
CTCGGAGATGATGATGCCAACATTGAAGATGACTTCATCACCTGGAAGGACAAGTTCTGGCCAGCAGTTTGTGATCACTTTGGCATTGAAAGCTCT
GGCGAGGATGTCCTGATGCGCCAGTACCGTTTGCTTGAGCAGCCGGAAGCTCCAGCGGAGCGTTTGTATACCGGCGAAGTGGCTCGTCTGCATTCT
CTGCAAACGCAACGTCCACCATTCGATGCCAAGAATCCCTTCCTGGCCCCGATTAAGGTCAACCGGGAACTGCACAAGGCCGGTGATCGGTCCTGC
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GTTAGCTAAAATAACGTCTAAGGATGTGAATGATGCAATATACATTTTTGGAACCAAAAAAAAAAAAAAAAAAAAAA

6A Nucleotide sequence of cytochrome P450 reductase gene

MDAQTEPEVPPAPVSEEPFLGPLDIILLTVLIGGAAWYFLKNKKKDTQASQFKSYSIQPTTVNTMTMAENSFIK
KLKSSGRRLVVFYGSQTGTAEEFAGRLAKEGLRYQMKGMVADPEECDMEELLSLKDIDKSLAVFCLATYGEGD
PTDNCMEFYDWIQNNDVDFSGLNYAVFGLGNKTYEHYNKVGIYVDKRLEELGANRVFELGLGDDDANIEDD
FITWKDKFWPAVCDHFGIESSGEDVLMRQYRLLEQPETPAERLYTGEVARLHSLQTQRPPFDAKNPFLAPIKV
NRELHKAGDRSCMHIEFDIEGSKMRYEAGDHLAMYPVNDQDLVLRLGKLCNADLDTIFSLINTDTDSSKKHPF
PCPTTYRTALTHYLEITALPRTHILKELAEYCSDEKDKEFLRFMCSTNPEGKAKYQEWVQDSCRNIVHVLEDLPS
CRPPVDHICELLPRLQPRYYSISSSSKLYPTTVHVTAVLVKYQTKTGRVNNGVATTFLSMKHPLNGEPLPRVPIFI
RKSQFRLPAKTETPVIMVGPGTGLAPFRGFIQERDFHKKDGKDIGQTILYFGCRKRAEDYIYEEELEDYAQSGTI
KLRTAFSRDQPQKVYVTHLLEEDMDLLWDVIGEKKGHFYICGDAKNMATDVRNILLKVLQSKGNMSESEAV
QYIKKMEAQKRYSADVWS

6B Deduced amino acid sequence of cytochrome P450 reductase protein

Figure 7. The first 50 sequence hits of BlastP search of cytochrome P450 reductase in NCBI protein database.

XP_001656715.1	NADPH- cytochrome P450 [Aedes aegypti] >gb|EAT45397.1|
XP_001865801.1	NADPH-cytochrome P450 reductase [Culex quinquefasciatus]
ABO77954.1	NADPH-cytochrome P450 reductase [Anopheles funestus]
AAO24765.1	NADPH cytochrome P450 reductase [Anopheles gambiae]
ABL75156.1	NADPH-cytochrome P450 reductase [Anopheles minimus]
XP_002065280.1	GK14755 [Drosophila willistoni]
XP_002002669.1	GI11531 [Drosophila mojavensis]
XP_001962292.1	GF14516 [Drosophila ananassae]
NP_477158.1	cytochrome P450 reductase, isoform A [Drosophila melanogaster]
XP_002051335.1	GJ12806 [Drosophila virilis]
XP_002088202.1	GE13802 [Drosophila yakuba]
XP_002038080.1	GM18621 [Drosophila sechellia]
XP_001970000.1	GG10405 [Drosophila erecta]
CAA63639.1	NADPH-cytochrome P450 reductase [Drosophila melanogaster]
ADD19306.1	NADPH-cytochrome P450 reductase [Glossina morsitans morsitans]
XP_001355866.1	GA11069 [Drosophila pseudoobscura pseudoobscura]
XP_002078355.1	GD23403 [Drosophila simulans]
Q07994.1	gb|AAA29295.1| NADPH cytochrome P450 reductase [Musca domestica]
XP_001988124.1	GH10739 [Drosophila grimshawi]
AAB48964.1	NADPH-cytochrome P450 reductase [Drosophila mettleri]
XP_971174.1	PREDICTED: similar to nadph cytochrome P450 [Tribolium castaneum]
XP_310593.4	AGAP000500-PA [Anopheles gambiae str. PEST]
AAR26515.1	antennal oxidoreductase [Mamestra brassicae]
XP_002423980.1	NADPH--cytochrome P450, putative [Pediculus humanus corporis]
NP_001104834.1	NADPH cytochrome P450 reductase [Bombyx mori]
ABJ97709.1	NADPH cytochrome P450 reductase [Bombyx mandarina]
XP_001119949.1	PREDICTED: similar to NADPH--cytochrome P450 reductase [Apis mellifera]
XP_001945312.1	PREDICTED: similar to NADPH cytochrome P450 reductase [Acyrthosiphon pisum]
XP_001649352.1	NADPH cytochrome P450 [Aedes aegypti]
ACX61585.1	GH28443p [Drosophila melanogaster]
NP_723173.1	cytochrome P450 reductase, isoform B [Drosophila melanogaster]
BAF49701.1	NADPH-P450 reductase [Xenopus laevis]
XP_002400171.1	cytochrome P450, putative [Ixodes scapularis]
NP_001078953.1	P450 (cytochrome) oxidoreductase [Xenopus laevis]
ACN10454.1	NADPH--cytochrome P450 reductase [Salmo salar]
AAX37331.1	NADPH-cytochrome P450 oxidoreductase [Danio rerio]
NP_001030154.1	cytochrome P450 reductase [Danio rerio]
XP_002130153.1	PREDICTED: similar to cytochrome P450 reductase isoform 1 [Ciona intestinalis]
XP_415768.2	PREDICTED: similar to NADPH-cytochrome P450 oxidoreductase [Gallus gallus]
XP_002130184.1	PREDICTED: similar to cytochrome P450 reductase isoform 2 [Ciona intestinalis]
NP_001166473.1	NADPH--cytochrome P450 reductase [Cavia porcellus]
XP_849143.1	PREDICTED: similar to NADPH--cytochrome P450 reductase isoform 3 [Canis familiaris]

XP_002194936.1	PREDICTED: similar to P450 (cytochrome) oxidoreductase [Taeniopygia guttata]
CAF91751.1	unnamed protein product [Tetraodon nigroviridis]
XP_001631497.1	predicted protein [Nematostella vectensis]
NP_001153762.1	P450 (cytochrome) oxidoreductase [Oryctolagus cuniculus]
NP_001116127.1	P450 (cytochrome) oxidoreductase [Equus caballus]
NP_001030467.1	cytochrome P450 reductase [Bos taurus]
Q3SYT8.3	RecName: Full=NADPH--cytochrome P450 reductase
XP_858049.1	PREDICTED: similar to NADPH--cytochrome P450 reductase isoform 4 [Canis familiaris]
XP_001109664.1	PREDICTED: P450 (cytochrome) oxidoreductase isoform 2 [Macaca mulatta]
GenBank No.	Description

Figure 8. Sequences of cytochrome P450 reductase gene and its deduced amino acid residue sequence from *Ae. sollicitans* collected from Ocean County, New Jersey

ATGGACGCACAAACGGAACCGGAAGTACCTCCGGCACCGGTCAGCGAGGAGCCATTCCTGGGCCCGCTA
GACATTATCCTGCTGACAGTGCTGATCGGCGGAGCCGCCTGGTATTTCCTCAAAAACAAAAAGAAGGAC
ACACAGGCTAGTCAGTTCAAATCGTACTCGATACAGCCAACCACCGTCAACACGATGACCATGGCTGAGA
ATTCGTTCATCAAGAAGCTGAAATCGTCCGGTCGTCGGTTAGTGGTATTCTACGGTTCGCAGACCGGAAC
GGCCGAAGAATTTGCCGGTCGTTTGGCCAAGGAAGGTCTCCGTTACCAGATGAAGGGTATGGTAGCCGA
TCCGGAAGAATGTGATATGGAGGAGCTACTTTCACTGAAGGACATCGACAAGTCGTTGGCCGTGTTCTG
TTTAGCTACGTACGGTGAGGGTGATCCCACCGATAATTGCATGGAATTCTACGATTGGATTCAGAACAAC
GATGTCGACTTCTCGGGGCTGAATTATGCGGTGTTTGGACTTGGTAACAAAACCTATGAACACTACAATA
AGGTTGGAATCTACGTCGACAAGCGTCTGGAGGAACTGGGAGCTAACAGGGTGTTCGAGCTGGGTCTC
GGAGATGATGATGCCAACATTGAAGATGACTTCATCACCTGGAAGGACAAGTTCTGGCCAGCAGTTTGT
GATCACTTTGGCATTGAAAGCTCTGGCGAGGATGTCCTGATGCGCCAGTACCGTTTGCTTGAGCAGCCGG
AAACTCCAGCGGAGCGTTTGTATACCGGCGAAGTGGCTCGTCTGCATTCTCTGCAAACGCAACGTCCACC
ATTCGATGCCAAGAATCCCTTCCTGGCCCCGATTAAGGTCAATCGGGAACTGCACAAGGCCGGTGATCG
GTCCTGCATGCATATTGAATTTGATATTGAGGGTTCCAAAATGCGCTACGAAGCCGGCGATCATTTGGCC
ATGTATCCTGTCAACGATCAGGACTTGGTGTTGAGATTAGGAAAGCTGTGCAATGCCGATTTGGATACGA
TTTTCTCGTTGATCAATACCGATACCGATAGTAGCAAGAAGCATCCCTTCCCTTGCCCGACGACTTACAGA
ACCGCTTTGACGCACTACCTGGAAATCACTGCCTTGCCTAGAACTCATATTCTCAAGGAACTGGCCGAATA
CTGCAGCGACGAGAAGGATAAGGAATTCCTACGGTTCATGTGTTCGACCAACCCGGAAGGCAAGGCCAA
GTACCAGGAATGGGTTCAGGACAGCTGTCGAAACATTGTTCACGTGCTGGAGGACCTTCCATCGTGTCGT
CCACCGGTTGATCACATTTGCGAACTGTTGCCTCGTCTACAGCCCCGCTACTACTCCATTTCCTCATCATCC
AAGCTGTATCCCACTACGGTCCACGTCACAGCGGTGCTGGTCAAATACCAAACCAAAACCGGACGCGTCA
ACAATGGCGTTGCAACGACATTCCTTTCGATGAAGCACCCTCTGAATGGTGAACCGTTGCCACGCGTGCC
GATCTTCATTCGCAAGAGCCAGTTCCGGTTGCCAGCGAAAACGGAAACGCCCGTGATCATGGTTGGCCC
GGGAACCGGTCTGGCACCGTTCCGAGGCTTCATCCAGGAGCGAGACTTCCACAAGAAGGATGGCAAGG
ACATTGGCCAGACGATCCTGTACTTTGGCTGCCGCAAGCGGGCTGAGGACTACATCTACGAAGAGGAGT
TGGAGGATTACGCTCAAAGCGGTACGATCAAACTGCGGACAGCATTCTCCCGGGATCAACCGCAAAAGG
TGTACGTTACTCATCTGCTGGAGGAAGACATGGACCTGCTCTGGGATGTGATAGGGGAAAAGAAGGGTC
ACTTCTACATTTGCGGTGATGCCAAAAACATGGCCACCGATGTGCGAAACATTCTGCTCAAGGTGCTACA
GGGCAAGGGCAACATGAGCGAAAGTGAAGCTGTGCAGTACATCAAAAAGATGGAAGCCCAAAAGCGGT
ACTCGGCCGATGTGTGGAGCTAA

7A. Nucleotide sequence of cytochrome P450 reductase gene

MDAQTEPEVPPAPVSEEPFLGPLDIILLTVLIGGAAWYFLKNKKKDTQASQFKSYSIQPTTVNTMTMAENSFIK
KLKSSGRRLVVFYGSQTGTAEEFAGRLAKEGLRYQMKGMVADPEECDMEELLSLKDIDKSLAVFCLATYGEGD
PTDNCMEFYDWIQNNDVDFSGLNYAVFGLGNKTYEHYNKVGIYVDKRLEELGANRVFELGLGDDDANIEDD
FITWKDKFWPAVCDHFGIESSGEDVLMRQYRLLEQPETPAERLYTGEVARLHSLQTQRPPFDAKNPFLAPIKV
NRELHKAGDRSCMHIEFDIEGSKMRYEAGDHLAMYPVNDQDLVLRLGKLCNADLDTIFSLINTDTDSSKKHPF
PCPTTYRTALTHYLEITALPRTHILKELAEYCSDEKDKEFLRFMCSTNPEGKAKYQEWVQDSCRNIVHVLEDLPS
CRPPVDHICELLPRLQPRYYSISSSSKLYPTTVHVTAVLVKYQTKTGRVNNGVATTFLSMKHPLNGEPLPRVPIFI
RKSQFRLPAKTETPVIMVGPGTGLAPFRGFIQERDFHKKDGKDIGQTILYFGCRKRAEDYIYEEELEDYAQSGTI
KLRTAFSRDQPQKVYVTHLLEEDMDLLWDVIGEKKGHFYICGDAKNMATDVRNILLKVLQGKGNMSESEAV
QYIKKMEAQKRYSADVWS

7B. Deduced amino acid sequence of cytochrome P450 reductase protein

Figure 9. Sequences of cytochrome P450 reductase gene and its deduced amino acid residue sequence from *Ae. sollicitans* collected from Cape May County, New Jersey.

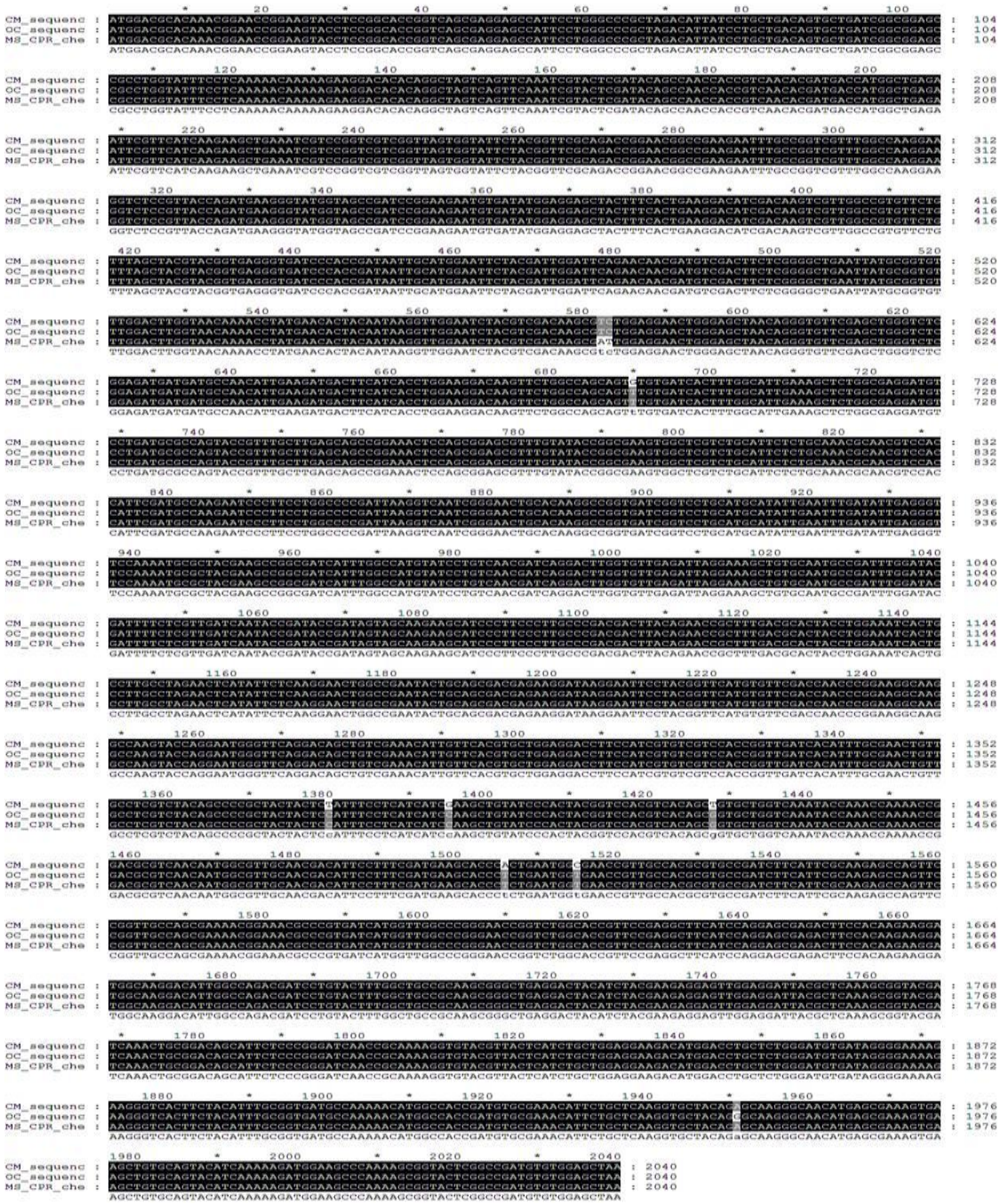
ATGGACGCACAAACGGAACCGGAAGTACCTCCGGCACCGGTCAGCGAGGAGCCATTCCTGGGCCCGCTA
GACATTATCCTGCTGACAGTGCTGATCGGCGGAGCCGCCTGGTATTTCCTCAAAAACAAAAAGAAGGAC
ACACAGGCTAGTCAGTTCAAATCGTACTCGATACAGCCAACCACCGTCAACACGATGACCATGGCTGAGA
ATTCGTTCATCAAGAAGCTGAAATCGTCCGGTCGTCGGTTAGTGGTATTCTACGGTTCGCAGACCGGAAC
GGCCGAAGAATTTGCCGGTCGTTTGGCCAAGGAAGGTCTCCGTTACCAGATGAAGGGTATGGTAGCCGA
TCCGGAAGAATGTGATATGGAGGAGCTACTTTCACTGAAGGACATCGACAAGTCGTTGGCCGTGTTCTG
TTTAGCTACGTACGGTGAGGGTGATCCCACCGATAATTGCATGGAATTCTACGATTGGATTCAGAACAAC
GATGTCGACTTCTCGGGGCTGAATTATGCGGTGTTTGGACTTGGTAACAAAACCTATGAACACTACAATA
AGGTTGGAATCTACGTCGACAAGCGTCTGGAGGAACTGGGAGCTAACAGGGTGTTCGAGCTGGGTCTC
GGAGATGATGATGCCAACATTGAAGATGACTTCATCACCTGGAAGGACAAGTTCTGGCCAGCAGTGTGT
GATCACTTTGGCATTGAAAGCTCTGGCGAGGATGTCCTGATGCGCCAGTACCGTTTGCTTGAGCAGCCGG
AAACTCCAGCGGAGCGTTTGTATACCGGCGAAGTGGCTCGTCTGCATTCTCTGCAAACGCAACGTCCACC
ATTCGATGCCAAGAATCCCTTCCTGGCCCCGATTAAGGTCAATCGGGAACTGCACAAGGCCGGTGATCG
GTCCTGCATGCATATTGAATTTGATATTGAGGGTTCCAAAATGCGCTACGAAGCCGGCGATCATTTGGCC
ATGTATCCTGTCAACGATCAGGACTTGGTGTTGAGATTAGGAAAGCTGTGCAATGCCGATTTGGATACGA
TTTTCTCGTTGATCAATACCGATACCGATAGTAGCAAGAAGCATCCCTTCCCTTGCCCGACGACTTACAGA
ACCGCTTTGACGCACTACCTGGAAATCACTGCCTTGCCTAGAACTCATATTCTCAAGGAACTGGCCGAATA
CTGCAGCGACGAGAAGGATAAGGAATTCCTACGGTTCATGTGTTCGACCAACCCGGAAGGCAAGGCCAA
GTACCAGGAATGGGTTCAGGACAGCTGTCGAAACATTGTTCACGTGCTGGAGGACCTTCCATCGTGTCGT
CCACCGGTTGATCACATTTGCGAACTGTTGCCTCGTCTACAGCCCCGCTACTACTCTATTTCCTCATCATCG
AAGCTGTATCCCACTACGGTCCACGTCACAGCTGTGCTGGTCAAATACCAAACCAAAACCGGACGCGTCA
ACAATGGCGTTGCAACGACATTCCTTTCGATGAAGCACCCACTGAATGGCGAACCGTTGCCACGCGTGCC
GATCTTCATTCGCAAGAGCCAGTTCCGGTTGCCAGCGAAAACGGAAACGCCCGTGATCATGGTTGGCCC
GGGAACCGGTCTGGCACCGTTCCGAGGCTTCATCCAGGAGCGAGACTTCCACAAGAAGGATGGCAAGG
ACATTGGCCAGACGATCCTGTACTTTGGCTGCCGCAAGCGGGCTGAGGACTACATCTACGAAGAGGAGT
TGGAGGATTACGCTCAAAGCGGTACGATCAAACTGCGGACAGCATTCTCCCGGGATCAACCGCAAAAGG
TGTACGTTACTCATCTGCTGGAGGAAGACATGGACCTGCTCTGGGATGTGATAGGGGAAAAGAAGGGTC
ACTTCTACATTTGCGGTGATGCCAAAAACATGGCCACCGATGTGCGAAACATTCTGCTCAAGGTGCTACA
GAGCAAGGGCAACATGAGCGAAAGTGAAGCTGTGCAGTACATCAAAAAGATGGAAGCCCAAAAGCGGT
ACTCGGCCGATGTGTGGAGCTAA

8A. Nucleotide sequence of cytochrome P450 reductase gene

MDAQTEPEVPPAPVSEEPFLGPLDIILLTVLIGGAAWYFLKNKKKDTQASQFKSYSIQPTTVNTMTMAENSFIK
KLKSSGRRLVVFYGSQTGTAEEFAGRLAKEGLRYQMKGMVADPEECDMEELLSLKDIDKSLAVFCLATYGEGD
PTDNCMEFYDWIQNNDVDFSGLNYAVFGLGNKTYEHYNKVGIYVDKRLEELGANRVFELGLGDDDANIEDD
FITWKDKFWPAVCDHFGIESSGEDVLMRQYRLLEQPETPAERLYTGEVARLHSLQTQRPPFDAKNPFLAPIKV
NRELHKAGDRSCMHIEFDIEGSKMRYEAGDHLAMYPVNDQDLVLRLGKLCNADLDTIFSLINTDTDSSKKHPF
PCPTTYRTALTHYLEITALPRTHILKELAEYCSDEKDKEFLRFMCSTNPEGKAKYQEWVQDSCRNIVHVLEDLPS
CRPPVDHICELLPRLQPRYYSISSSSKLYPTTVHVTAVLVKYQTKTGRVNNGVATTFLSMKHPLNGEPLPRVPIFI
RKSQFRLPAKTETPVIMVGPGTGLAPFRGFIQERDFHKKDGKDIGQTILYFGCRKRAEDYIYEEELEDYAQSGTI
KLRTAFSRDQPQKVYVTHLLEEDMDLLWDVIGEKKGHFYICGDAKNMATDVRNILLKVLQSKGNMSESEAV
QYIKKMEAQKRYSADVWS

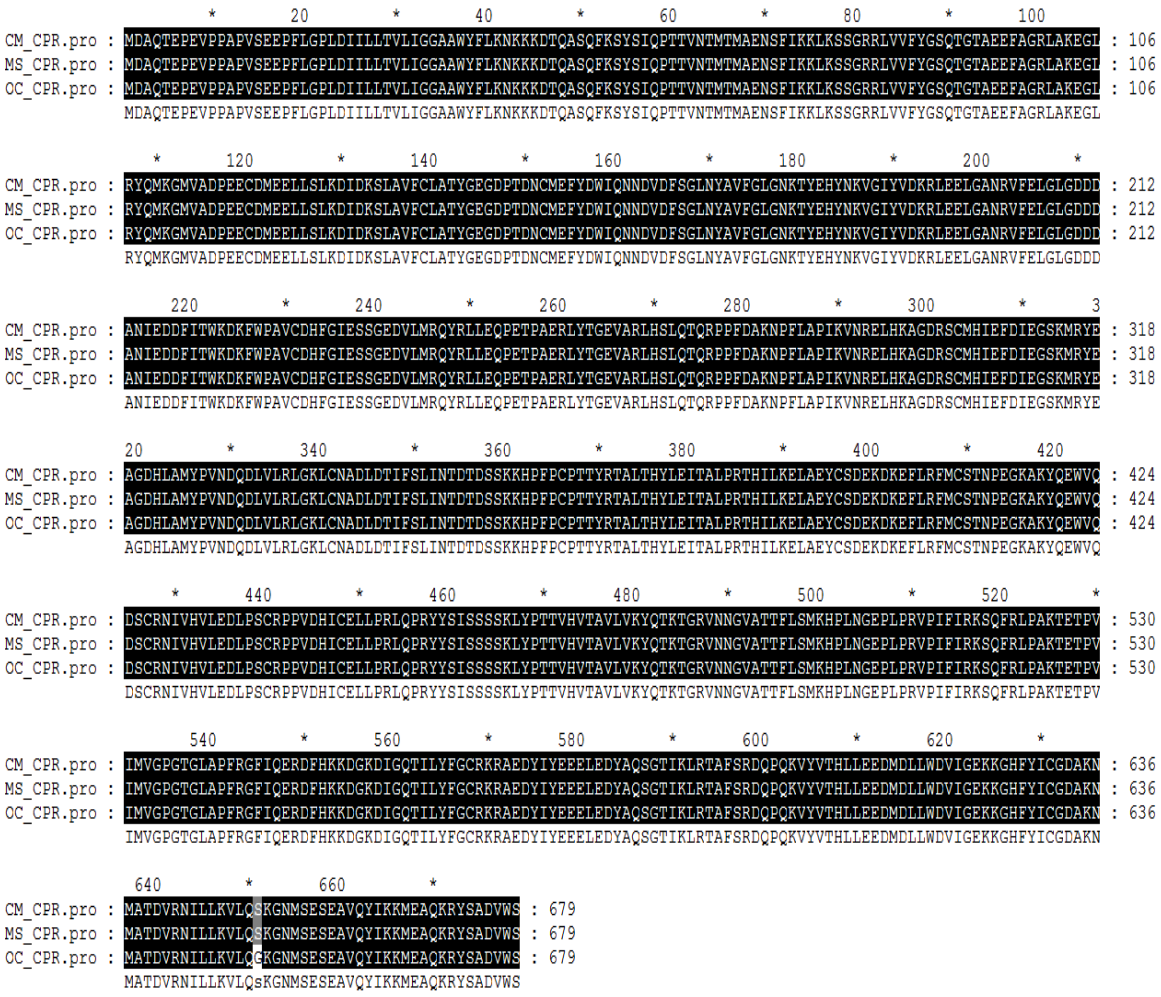
8B. Deduced amino acid sequence of cytochrome P450 reductase protein

Figure 10. Alignment of nucleotide sequence of cytochrome P450 reductase gene from Cape May (CM_Sequence), Middlesex (MS_Sequence), and Ocean (OC_Sequence) County mosquitoes.



The conserved regions are indicated in black and are listed in upper case at the bottom of the alignment. Nucleotides with substitutions are marked in gray and listed at the bottom with lower case. The alignment positions are listed on top of the alignment. The numbers on the right of the alignment are positions of each cytochrome P450 reductase gene.

Figure 11. Alignment of amino acid residues of the cytochrome P450 reductase from Cape May (CM_CPR), Middlesex (MS_CPR), and Ocean (OC_CPR) County mosquitoes.



The conserved regions are indicated in black and are listed in upper case at the bottom of the alignment. Amino acid with substitutions is marked in gray and listed at the bottom with lower case. The alignment positions are listed on top of the alignment. The numbers on the right of the alignment are positions of each cytochrome P450 reductase.

Figure 12. Neighbor-joining phylogenetic tree of published cytochrome P450 reductases from 14 insect species. The bootstrap value for 1000 trials is indicated for each branch in percent.

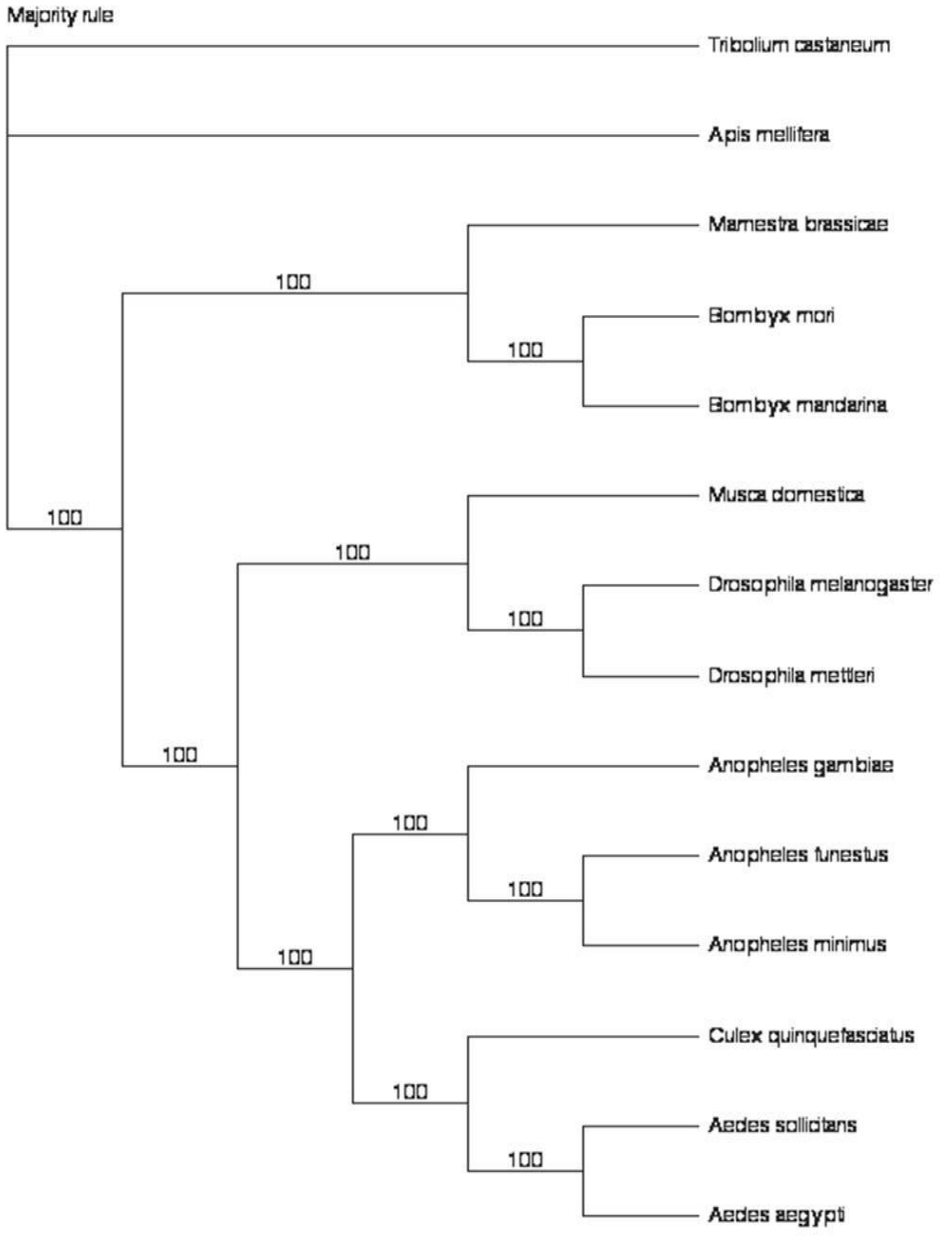
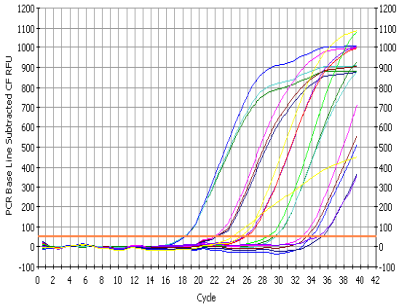
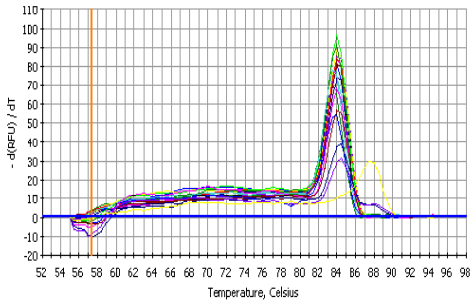


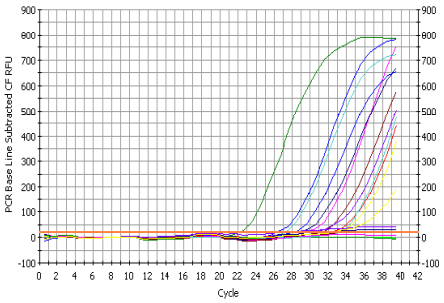
Figure 13. Standard curves for PCR product dilutions for each primer pair.



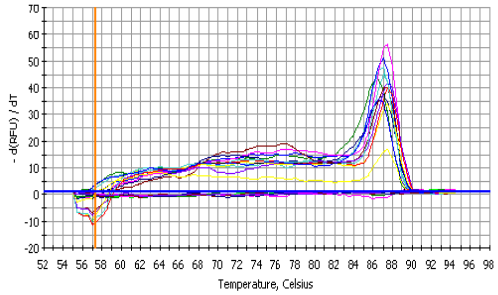
(A1)



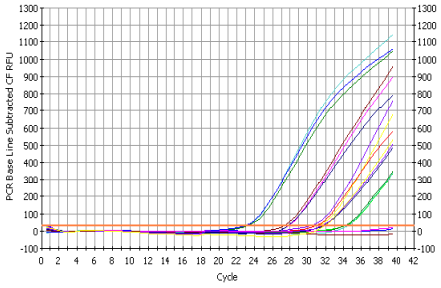
(B1)



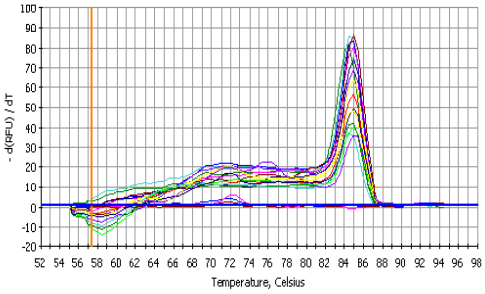
(A2)



(B2)



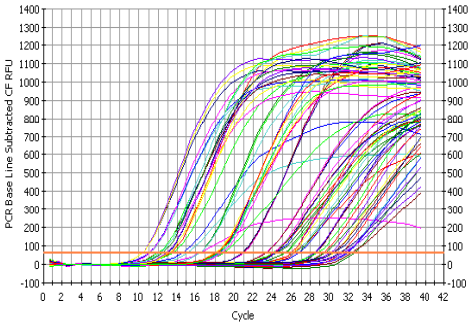
(A3)



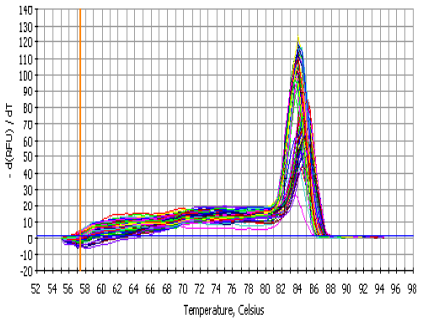
(B3)

(A1), (A2), (A3): the real-time output display detecting the increased fluorescence of SYBR green bound to dsDNA from each series of 1/10 dilutions of PCR products of 18S, CPR and CYP6BB1. (B1), (B2), (B3): melting curve for 18S, CPR and CYP6BB1.

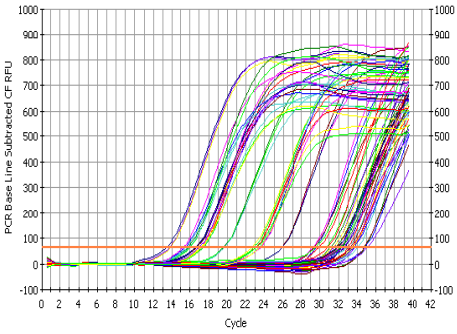
Figure 14. Melting curves of the cytochrome P450 reductase and CYP6BB1 in different body parts of female adult mosquitoes from Cape May, Ocean and Middlesex County.



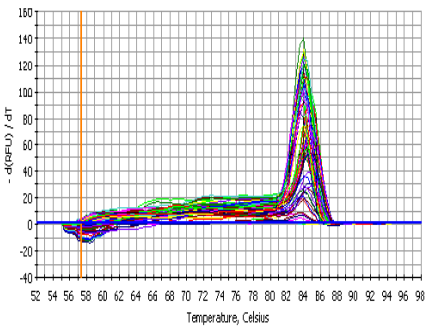
(A1)



(B1)



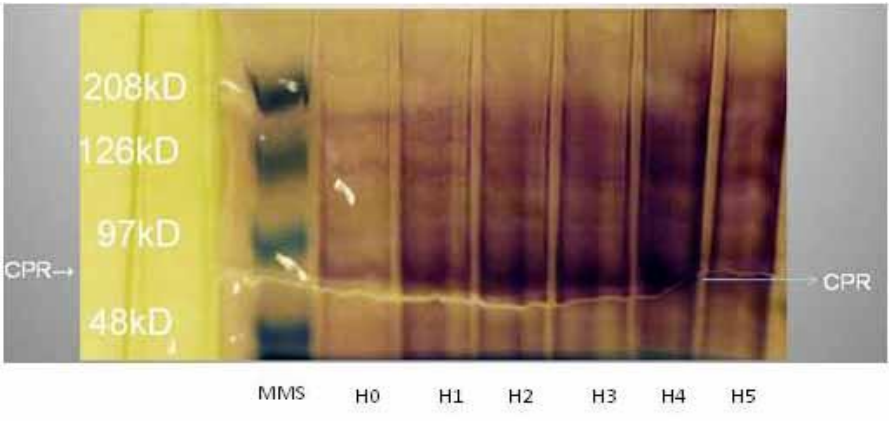
(A2)



(B2)

A1, A2: the real-time PCR output for the reductase. B1, B2: the real-time PCR output for CYP6BB1.

Figure 15. SDS-PAGE of the cytochrome P450 reductase pellets. From left to right: Molecular mass standards, CPR at hour 0, 1, 2, 3, 4 and 5.



CURRICULUM VITAE

Debin Sun

Education

2010 Ph.D., Entomology

Rutgers University, New Brunswick, New Jersey, US

2001 M.S., Breeding of Economically Important Animals

Southwest Agricultural University, Chongqing, China

1996 B.S., Sericulture

Southwest Agricultural University, Chongqing, China

Working Experience

06/09-present Biologist, Division of Environmental Services, County of Essex, NJ

09/03-06/09 Graduate Assistant, Department of Entomology, Rutgers University, NJ

07/01-08/03 Technologist, China University of Mining and Technology

09/96-08/98 Technician, Suyu Institute of Sericulture, Jiangsu, P. R. China

Publications

Sun De-Bin, Lu Cheng. 2000. QTL Research and Its Application Prospect in Sericulture. *Acta Sericologica Sinica* 26(Z1).

Chen Da-Xia, Sima Yang-Hu, Sun De-Bin, Lu Cheng and Xiang Zhong-Huai. 2003. Preliminary AFLP Analysis of Segregation of Springs in Silkworm *Bombyx mori* Cross Combinations. *Journal of Southwest Agricultural University* 25(2): 101-104.

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Sima Yang-Hu, Li Bin, Xu Hai-ming, Chen Da-Xia,Sun De-Bin, Zhao Ai-Chun, Lu Cheng and Xiang Zhong-Huai. 2005. Studies on Location of QTLs Controlling Cocoon Traits in Silkworm. *Journal of Genetics and Genomics* 32(6): 625-632.

Sima Yang-Hu, Chen Da-Xia, Sun De-Bin, He Si-Mei, Qian He-Ying, Li Bin, Lu Cheng, Xiang Zhong-Huai. 2005. The Character of Molecular Markers Between Segregation Lines of Hybridization in Silkworm *Bombyx mori*. *Acta Sericologica Sinica* 31(1):36-41.

Sima Yang-Hu, Li Bin, Chen Da-Xia, Sun De-Bin, Zhao Ai-Chun, Zhang Lie, Lu Cheng, He Si-Mei and Xiang Zhong-Huai. 2006. Construction and Analysis of an AFLP Molecular Linkage Map of the silkworm (*Bombyx mori*). *Chinese Journal of Agricultural Biotechnology* 3(1):25-32.

S. Huang, D. Sun, L. B. Brattsten, 2008. Novel cytochrome P450 CYP6BB1 and CYP6P10 alleles in the salt marsh mosquito *Aedes sollicitans* (Walker) (Diptera: Culicidae). *Arch. Insect Biochem. Physiol*., 67:139-154.