รายงานวิจัยฉบับสมบูรณ์

โครงการ: การวิเคราะห์และจำแนกยีน Cytochrome P450 ที่แสดงความต้านทานต่อยาฆ่าแมลงชนิดไพรีทรอยด์ในยุงก้นปล่อง *Anopheles minimus* ในประเทศไทย

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สัญญาเลขที่ BRG43-8-0011

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ผู้วิจัย	สังกัด
นางสาว พรพิมล รงค์นพรัตน์	ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
นาย ธีรภาพ เจริญวิริยภาพ	ภาควิชากีฏวิทยา คณะเกษตรศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
นางสาว มธุรส พงษ์ลิขิตมงคล	ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
Dr. Michael Bangs	Medical Entomology Department, Naval Medical Research, Jakarta, Indonesia

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

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Project Title: Isolation and characterization of Cytochrome P450 genes involved in pyrethroid resistance in the malaria vector, *Anopheles minimus*, in Thailand.

Investigators: **Pornpimol Rongnoparut,** Ph.D., Principal Investigator
Department of Biochemistry, Faculty of Science, Mahidol University
E-mail: scprn@mahidol.ac.th
Theeraphap Chareonviriyaphap, Ph.D.
Department of Entomology, Faculty of Agriculture, Kasetsart University
E-mail: faasthc@ku.ac.th
Mathurose Ponglikitmongkol, Ph.D.
Department of Biochemistry, Faculty of Science, Mahidol University
E-mail: scmpl@mahidol.ac.th
Michael Bangs, Ph.D.
Medical Entomology Dept., Naval Medical Research, Jakarta, Indonesia
E-mail: bangsmj@namru2.med.navy.mil

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Abstract:

The elevated activities of the cytochrome P450 monooxygenases (P450, CYP) of insects have been implicated in many cases of insect resistance to insecticides, including DDT and pyrethroids. The goal of this study is firstly to establish a colony of *Anopheles minimus,* a malaria vector in Thailand, selected with pyrethroid as a resistant mosquito model. Second, the resistant colony is used for cloning and characterization of cytochrome P450 (P450) genes in the family 6 (*CYP6*) that are overexpressed at mRNA level.

We report the establishment of an *Anopheles minimus* mosquito colony acquiring deltamethrin resistance, by exposing subsequent mosquito generations to LD_{50} and LT_{50} values of deltamethrin. The resistant colony also presents cross-resistance to DDT during the course of deltamethrin resistance. Biochemical assays of the resistant colony indicated that cytochrome P450 acts as a primary route of insecticide detoxification in *An. minimus*.

We have primarily isolated nine isoforms of partial cDNA fragments from susceptible and resistant colonies. Among these cDNAs, five *CYP6* genes with full coding sequences were isolated from *An. minimus* using Genomic Walking and RACE methods. Two cDNA clones, CYP6AA2 and AN10, showed elevated transcription in the resistant colony upon measurement of their mRNA level, using semi-quantitative RT-PCR. The fold of mRNA increase of *CYP6AA2* and AN10 are correlated with the increasing level of resistance, during the course of insecticide selection, in *An. minimus*. The studies suggest that both *CYP6AA2* and AN10 could play and/or share role in specific resistance and/or cross-resistance against deltamethrin and DDT. Such role could further be supported by explore of the metabolic function of each gene towards insecticide substrates, including pyrethroids and DDT, using cDNA-expressed enzymes in the heterologous system. Metabolic resistance to insecticides can then be studied in this mosquito.

Keywords: Insecticide resistance, Cytochrome P450, *Anopheles minimus*, pyrethroid

บทคัดย่อ

รหัสโครงการ: BRG43-8-0011

ชื่อโครงการ: การวิเคราะห์และจำแนกยีน Cytochrome P450 ที่แสดงความต้านทานต่อสารฆ่าแมลงชนิดไพรีทรอยด์ในยุงก้นปล่อง *Anopheles minimus* ในประเทศไทย

ชื่อนักวิจัย

นางสาว พรพิมล รงค์นพรัตน์	ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
นาย ธีรภาพ เจริญวิริยภาพ	ภาควิชากีฏวิทยา คณะเกษตรศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
นางสาว มธุรส พงษ์ลิขิตมงคล	ภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
Dr. Michael Bangs	Medical Entomology Department, Naval Medical Research, Jakarta, Indonesia

ระยะเวลาโครงการ

การเพิ่มขึ้นของระดับการทำงานของเอนไซม์ cytochrome P450 monooxygenase (P450, CYP) ในแมลงเป็นสิ่งบ่งชี้ถึงสภาวะความต้านต่อสารฆ่าแมลงเช่น DDT และ pyrethroid ในแมลงเกือบทุกชนิด วัตถุประสงค์ในงานวิจัยนี้มุ่งเน้นที่ 1) การเลี้ยงยุงก้นปล่อง *Anopheles minimus* ให้เป็นกลุ่มยุงต้นแบบที่มีความต้านทานสารฆ่าแมลงไพรีทรอยด์ 2) ยุงกลุ่มที่ถูกสร้างนี้ ใช้จำลอง (cloning) และศึกษาลักษณะจำเพาะของยีน cytochrome P450 (P450) family 6 (*CYP6*) ที่มีการเพิ่มขึ้นของระดับ mRNA ได้

เรารายงานถึงการเลี้ยงยุง *Anopheles minimus* ให้มีความต้านทานสารฆ่าแมลงเดลต้าเมทริน (deltamethrin) โดยการให้ยุงสัมผัสสารเดลต้าเมทริน จนในที่สุดได้ยุงรุ่นที่มีค่า LD_{50} และ LT_{50} ต่อเดลต้าเมทริน และพบว่ายุงกลุ่มนี้สามารถเกิด cross-resistance กับ ดีดีที (DDT) ในขณะที่มีความต้านทานต่อเดลต้าเมทริน อีกด้วย ผลการศึกษาทางชีวเคมีในยุงกลุ่มนี้พบว่า cytochrome P450 ทำหน้าที่เป็นเสมือนด่านแรกที่จะกำจัดสารฆ่าแมลงในยุง *An. minimus*

ในเบื้องต้น เราสามารถแยกชิ้นส่วนๆของ *CYP6* cDNAs และจัดได้เป็น 9 กลุ่ม (isoforms) จากยุงกลุ่มที่มีความไวและกลุ่มที่มีความต้านทานต่อเดลต้าเมทริน ต่อมาในจำนวน cDNAs ที่แยกได้นี้ สามารถแยกยีน *CYP6* ในลักษณะที่เป็น full coding sequence ได้ห้ายีน โดยใช้วิธี Genome Walking และ RACE และพบว่ามี cDNA สองโคลน ได้แก่ CYP6AA2 และ AN10 ซึ่งมีระดับ transcription เพิ่มขึ้นในยุงกลุ่มที่มีความต้านทานสารฆ่าแมลง โดยการวัดระดับ mRNA ที่เพิ่มขึ้นด้วยวิธี semi-quantitative RT-PCR การเพิ่มขึ้นของระดับ mRNA ของทั้ง CYP6AA2 และ AN10 สอดคล้องกับระดับความต้านต่อสารฆ่าแมลงที่เพิ่มขึ้นในระหว่างการคัดเลือกยุงด้วยเดลต้าเมทริน จากผลการทดลองนี้น่าจะบอกได้ว่าทั้ง CYP6AA2 และAN10 อาจจะมีหน้าที่ (ร่วมกัน) ในการต้านทานอย่างจำเพาะ และ/หรือ cross-resistance ต่อเดลต้าเมทรินและดีดีที อย่างไรก็ดี ในการที่จะสรุปถึงบทบาทของยีนทั้งสองนั้น ควรจะมีข้อมูลสนับสนุนเพิ่มเติมเกี่ยวกับบทบาทของยีนแต่ละตัวที่มีต่อการสลายสารฆ่าแมลง เช่น pyrethroid และ DDT โดยการศึกษาการแสดงออกของเอนไซม์จาก cDNA ใน heterologous system และจะสามารถศึกษาถึงกลไกการเกิดสภาวะการต่อต้านสารฆ่าแมลงในยุงกลุ่มนี้ ในงานวิจัยต่อไป

คำหลัก ความต้านทานต่อสารฆ่าแมลง ไซโตโครม พี450 ยุงก้นปล่อง *Anopheles minimus* ไพรีทรอยด์

Executive Summary

The goal of this proposed study is to identify and characterize the cytochrome P450 genes in the family 6 (*CYP6*) that play role in resistance to pyrethroid insecticide in *Anopheles minimus*, a malaria vector in Thailand. Information and P450 genes obtained from the proposed study are necessary for further studies of biochemical basis of P450 enzymes and of molecular mechanisms underlying the resistance. In this study we established and used the colony of *An. minimus* selected with deltamethrin as a resistant mosquito model. The resistant colony was used to characterize CYP6 P450 cDNAs that could play role in pyrethroid resistance.

We report here the achievement of the goals we proposed in this proposal as described below.

1. Successful establishment of an *Anopheles minimus* mosquito colony acquiring deltamethrin resistance (Chareonviriyaphap T, et al, 2002, Appendix A). The F_{13}, F_{19} and F_{25} populations of the resistant colony, used in subsequent studies, showed 24%, 52% and 56% delamethrin resistance according to WHO standard susceptibility test. We have now maintained the F_{25} resistant strain. In addition, during the course of selection against deltamethrin, the F_{12} and F_{19} resistant populations were found to additionally resist to DDT with percent resistance of 20% and 40% respectively.

2. Determination of the resistance mechanisms employing biochemical assays. The F_{18} resistant mosquito showed at least 5-fold elevated level of mixed function oxidase activity comparing to the susceptible strain (Chareonviriyaphap T, et al, 2003, Appendix B). The increased activity indicated P450 as a primary route of insecticide detoxification in the resistant strain of *An. minimus*.

3. Cloning of *CYP6* genes. We isolated five *CYP6* genes containing full coding sequences from *An. minimus*.

4. Development of an approach to isolate *CYP6* genes associated with resistance (Rongnoparut et al, 2003, Appendix C). Nine isoforms of partial cDNA fragments were synthesized from F_0 susceptible and F_{13} resistant mosquitoes. Among these clones, three clones (CYP6AA2, AN10 and AN36) were found with higher frequency in F_{13} resistant colony compared to F_0 susceptible colony. However, only two of which (*CYP6AA2* and AN10) were later found overexpressed at the mRNA level in the resistant strain.

5. Isolation of 2 *CYP6* genes that could play role in insecticide resistance. One clone, *CYP6AA2*, showed elevated transciptional level in F_{13} and F_{19} resistant mosquitoes and the level of increase correlated with development of resistance. The relative amount of CYP6AA2 transcript was 2 fold greater in the F_{13} deltamethrin resistant mosquito, 4 fold in F_{19} resistant, and ~7 fold in F_{25} resistant *An. minimus* compared to F_0 susceptible strain (Rongnoparut et al, 2003, Appendix C). Another clone (AN10) has its mRNA expression level of ~2 and ~4 fold in F_{19} and F_{25} deltamethrin resistant mosquitoes, respectively, higher than F_0 susceptible strain (Manuscript in preparation). Thus, *CYP6AA2* and AN10 could play role, or possibly together, in deltamethrin resistance and perhaps DDT cross-resistance in *An. minimus*.

1. Introduction

Insecticides continue to have great importance for malaria control. In Thailand, synthetic pyrethroids are the current insecticides as the replacement for DDT in malaria vector control. However, the increased use of pyrethroids increases the selection for pyrethroid resistance in malaria vectors. Pyrethroid resistance has already been found in *Anopheles minimus* malaria vector in northern Thailand (Annual Malaria report, 1995-2000).

The cytochrome P450 monooxygenases (P450, CYP) of insects have been implicated in many cases of insect resistance to insecticides, including carbamates, DDT, organophosphates and pyrethroids (Oppenoorth, 1985). Elevated CYP activities have been detected in a number of resistant insects. For instances, increased CYP activities were present in permethrin resistant *Pseudoplusia includens*, a soybean looper (Thomas et al., 1996), in insecticide resistant *Heliothis virescens* (Zhao et al, 1996) and in diamondback moth (Yu and Nguyen, 1992). Rose et al (1995) demonstrated that elevated CYP activities in multi-resistant *H. virescens* varied from 3- to 33-fold and there was overexpression of *CYP* genes in pyrethroid resistance in *H. virescens*. However, the molecular mechanisms of resistance are poorly understood.

In insect, approximately 90 P450 genes were found in *Drosophila melanogaster* (Tijet N, 2001). Half of the 90 sequences belong to only two families, *CYP4* and *CYP6*. Insects resistant to pyrethroids are shown to possess higher levels of one or more cytochrome P450 enzymes (P450s) compared to the susceptible insects (Feyereisen, 1999). The increased level of P450 activities in resistant populations of insects results from high expression at mRNA level of P450 genes, mostly in the family 6 (*CYP6*). For example, the P450 gene such as *CYP6D1*, isolated from the pyrethroid resistant house fly *Musca domestica*, showed that increased CYP6D1 transcript and protein is an underlying cause of pyrethroid resistance (Kasai and Scott, 2000; Liu and Scott, 1998; Tomita et al, 1995). Similarly, overexpression of a number of *CYP*s that were associated with insecticide resistance was present in *Cyp6a2*, and *Cyp6a9* genes of *Drosophila melanogaster* (Brun et al, 1996; Maitra et al, 1996), and *CYP6B2* of *H. armigera* (Wang and Hobbs, 1995).

Recently, Daborn, et al (2001, 2002) have investigated the genetics of a mechanism of DDT resistance (DDT-R) in *Drosophila melanogaster* and mapped the DDT-resistant locus and identified the *Cyp6g1* in this region. Overexpression of the *Cyp6g1* gene is implicated in the enhanced detoxification of DDT and as the primary cause of DDT-R (Daborn, et al, 2001). The *Cyp6g1* gene is shown to be overexpressed in 28 DDT-resistant *D. melanogaster* strains of diverse global origin (Daborn, et al., 2002). Transgenic over-expressed *Cyp6g1* in susceptible files is sufficient to restore DDT resistance, but direct evidence of oxidative metabolism by the enzyme is not shown (Daborn, et al, 2002). *Cyp6g1* is reported to have unusally broad substrate specificity (Denholm, et al, 2002). It resists to DDT and a range of other insecticide groups, including organophosphates, neonicotinoids, and benzoylphenylureas (compounds interfering with insect development). These groups contain different modes of action. Thus such broad-spectrum substrate specificity could imply the increased risk of resistance not only to the existing agents, but also to the newly synthesized insecticide groups. A P450-based cross-resistance to DDT and permethrin has been reported in *An. albimanus* (Brogdon et al, 1999; Cuany et al, 1990).

Further study has shown that the field isolates of *D. melanogaster* resistance to DDT and other compounds is conferred by one P450 gene, *Cyp6g1*. But the DDT-selected strains in the laboratory showed upregulation of different cytochrome P450 genes, i.e. *Cyp6a8, Cyp12d1*, in addition to the *Cyp6g1* gene (Brandt, et al, 2002; Le Goff et al, 2003). Whether it is a single P450 playing roles in cross-resistance is not known. It is paramount to identify the P450-mediated pyrethroid resistance allele, and gain knowledge on the molecular mechanisms of P450-mediated resistance.

The roles of P450 genes and molecular mechanisms underlying insecticide resistance in Anopheline mosquitoes have not been reported. To date there is no known report of the role of the *CYP6* family played in pyrethroid and DDT resistance in malaria vectors. How many different P450 enzymes and genes are participated in pyrethroid resistance? What are the exact changes that alter the susceptibility of enzymes to insecticide inhibition thus conferring resistance? How might these P450 genes be regulated in insecticide-resistant and susceptible strains? Understanding the molecular basis of P450s in insecticide resistance in mosquito vectors, biochemical basis of these enzymes, the molecular structure-function relationship of P450s could lead to improved resistance diagnostics and management, and will allow us to attack existing insecticide resistance. Access to regulators of the target P450 enzyme will provide a target for synergist of current insecticides and to boost the life-span of currently available insecticides.

The goals of this study are to:

1. Establish a pyrethroid resistance colony of *An. minimus* in the laboratory and preliminarily determine whether there is an increase of mixed function oxidase enzyme activities in the pyrethroid resistance colony comparing to those of the sensitive control colony.
2. Identify and characterize P450 cDNAs belonging to the *CYP6* family.
3. Determine whether and which of the CYP6 cDNAs are associated with pyrethroid resistance.
4. Determine how many CYP6 cDNAs are associated with pyrethroid resistance.
5. Preliminarily investigate molecular basis for the *CYP6* gene-mediated resistance at the level of mRNA expression.

2. Materials and Methods

2.1 Selection for deltamethrin resistant colony of *Anopheles minimus*

We have established a deltamethrin selected mosquito strain of *Anopheles minimus* species A. The susceptible strain was originally collected from animal shelter, Rong Kwang District, Prae Province, northern Thailand in 1993. The susceptible colony is susceptible to both deltamethrin (0.05%) and DDT (4%) based on WHO bioassay (WHO, 1981; 1998).

The scheme for selection of deltamethrin resistant mosquito and the measurement of resistance in the mosquito population were performed as described (Chareonviriyaphap, et al, 2002, Appendix A). An F_0 susceptible strain of *An. minimus* females was placed under selection pressure with deltamethrin at the concentration (or time) that caused 50% mortality (LD_{50} and LT_{50}). LD_{50} dosage was used to select *An. minimus* females for F_1-F_{10}. Similarly, LT_{50} was used to select from F_{14}-F_{19}. No selection was made in F_{11}-F_{13}, F_{20}- F_{24} due to some technical problems. The progress of selection was monitored with the WHO diagnostic test (WHO 1998). A control colony was reared simultaneously in the separated room and handled in the same manner through all manipulations but was not exposed to insecticide. The control colony was tested to monitor for independent occurrence of resistance. We have now maintained the F_{25} population of which the population shows 56% deltamethrin resistance. The resistance colony was utilized throughout the studies for the isolation of the CYP6 cDNAs and for determination and comparison of their mRNA expression level.

2.2 Biochemical assays

Resistance mechanism pertaining to the deltamethrin-selected colony was examined by measurement of activity of 3 enzymes in resistant strain comparing to the sensitive strain (Chareonviriyaphap, et al, 2003, Appedix B). These enzymes include mixed function oxidases (MFO), glutathione-S-transferases (GST), and esterases. The activity levels were measured and compared between susceptible and the resistant test populations during development of deltamethrin resistance. Standard assay procedures on insects were employed. The total protein content of individual *An. minimus* mosquitoes was determined using a BioRad protein assay system.

2.3 Isolation of CYP6 cDNA fragments overexpressed in A*nopheles minimus* resistant to deltamethrin

We preliminarily isolated 4 genomic DNA fragments containing *CYP6* partial coding sequences (unpublished results). One sequence was genomic walked towards 5' region of the gene employing Genome Walker™ kit (Clontech). Thereafter 5'- and 3'-RACE were performed to identify transcriptional ends of the gene fragment following the Smart RACE cDNA amplification protocol from Clontech. The cloned sequence information allows us to further design suitable primers for screening a large number of CYP6 cDNAs from both susceptible and resistant strains as described below.

We carried out RT-PCR on total RNA isolated from both susceptible and F_{13} resistant mosquitoes in order to determine which cDNAs were overrepresented in the resistant strain. We used degenerate antisense primer that possibly targeting at heme-binding region of all cytochrome P450 sequences. The sense primer, partially degenerated, targeted at the ETLR motif and was chosen among several *An. minimus CYP6* specific primer choices. This

strategy would allow isolation of expressed cytochrome P450 genes from *An. minimus*, and probably limit the isolation to the family 6.

The primers amplified partial cDNAs from susceptible and resistant strains producing the expected product size of ~ 240 bp and the products were cloned and sequenced (Rongnoparut, et al, 2003, Appendix C). The number of clones representing identical sequences were counted and compared within and across the deltamethrin susceptible and resistant strains.

2.4 Full coding regions of selected *CYP6*s

Normally, the average size of P450 gene is ~ 1.5 kb in size. The selected and overrepresented clones (~240 bp) were genomic walked towards 5' region and 5'RACE & 3' RACE were carried out to obtain full coding sequences spanning the transcriptional most ends of the genes. The full coding sequence encompassing 5'RACE, genomic extended product, and 3' RACE fragments was generated for each of the *CYP6* corresponding gene.

2.5 Comparative expression of CYP6 mRNAs in pyrethroid resistant and susceptible strains using semi-quantitative RT-PCR technique

The semi-quantitative RT-PCR was employed to measure the expression of mRNA level of *CYP6* genes in pyrethroid resistant and susceptible strains. RT-PCR was done with RNA sample isolated from pyrethroid resistant and susceptible strains of adult mosquitoes. For comparison of expression level between susceptible and resistant strains, RT-PCR was performed with the predetermined PCR cycle at the exponential phase. For internal standard, PCR product of actin cDNA was generated with actin specific primers. The actin1D cDNA fragment was sequenced and the sequence showed that its cDNA encoded for actin protein. Band intensity of PCR products performed at the predetermined cycle number was measured by densitometer. The measured values were compared between the pyrethroid resistant and susceptible strains after normalization with the values of actin internal standard.

3. Results

We report here the selection of deltamethrin resistance in *Anopheles minimus* that later conferred cross-resistance to DDT during the course of selection. We report the isolation of *CYP6* genes, two of which were overexpressed in resistant mosquito. The fold of mRNA increase of the two *CYP6* genes is in correlation with the increase in pyrethroid resistance, suggesting that the genes play role in deltamethrin and/or DDT resistance.

3.1. Development of a deltamethrin resistant strain of *Anopheles minimus*

The ultimate goal was to establish a pyrethroid resistant colony of *Anopheles minimus*, a malaria vector in Thailand. Resistance was selected for by exposing, using the World Health Organization test protocol, sequential generations of *An. minimus* females to LD_{50} and LT_{50} values of deltamethrin insecticide. In 10 generations of exposure, resistance to deltamethrin was 22-fold higher in LD_{50} than the susceptible parent strain (Table 1). There was roughly a 3-fold increase in LT_{50} and LT_{90} when F_{19} was compared to the F_{14} generation. This continuous increase in lethal dose and lethal time values suggests that populations under selective pressure became less susceptible to deltamethrin than the parent colony. The level of deltamethrin resistance in the 13th, 19th, and 25th generations was 24% and 52%, 58% respectively (Table 2), when tested with 0.05% deltamethrin (diagnostic level) using the WHO bioassay. Detailed results for each generation of mosquitoes during selection is shown in the manuscript (Appendix A).

Table 1. Susceptibility data of Deltamethrin based on dose/mortality relationships tested against *Anopheles minimus* species A populations.

Sample	No. tested	LD_{50} (%)	LD_{90} (%)
F_0	240	0.00035	0.00137
F_{10}	360	0.00912	0.03120

Table 2. Mortality from WHO diagnostic test kit for 0.05% Deltamethrin against 2 test populations of *An. minimus* species A 24 h-post exposure.

Sample	Control colony		Selected colony	
	# Test	%Dead	#Test	%Dead
F_1	100	100	100	100
F_{13}	100	100	100	76
F_{19}	100	100	100	48
F_{25}	100	100	100	44

Anopheles minimus populations under selective pressure were also tested against diagnostic dosages of 4% DDT to check for cross-resistance. Percent mortality from DDT in the treatment colony declined in populations under deltamethrin selective pressure. Percent mortalities in tests with treatment populations against DDT were 90% in the F_8 and 60% in generation F_{19} (Table 3). The control colony remained completely susceptible to DDT (100% mortality). Detailed result is shown in Appendix A.

Table 3. Mortality from WHO diagnostic test kit for 4% DDT against *An. minimus* species A test populations 24 h-post exposure.

Test populations	Control colony			Selected colony		
	No. test	No. Dead	%Mortality	No.Test	No.Dead	%Mortality
F_0	100	100	100	100	100	100
F_8	100	100	100	100	90	90
F_{12}	100	100	100	100	80	80
F_{19}	100	100	100	100	60	60

3.2 Biochemical assays

The 52% deltamethrin resistant strain (F_{19}) showed at least 5-fold elevated level of mixed function oxidase activity comparing to the susceptible strain (Table 4, see Appendix B for detailed results), while glutathione S-transferase (GST) showed similar level of activity in both susceptible and resistant strains (Chareonviriyaphap et al, 2003). The results implicate no significant role of GST played on DDT and pyrethroid resistance. There was an increase in esterase activity in certain test populations but the conclusion can not be drawn as to its role in deltamethrin resistant *An. minimus* strain (Table 4). The results indicate P450 as a primary route of deltamethrin and/or DDT detoxification in this strain of *An. minimus*.

Table 4. Comparison of specific activities of α and β non-specific esterases and monooxygenases (MFO) from *An. minimus* susceptible control and 5 deltamethrin-exposed selected test populations.

Test populations	**Total Protein** Mean (SD) mg-protein/ml per mosquito (n)	**α Esterase** Mean (SD) m-mole α naphthol/min /mg protein (n)	**β Esterase** Mean (SD) m-mole β naphthol/min/ mg protein (n)	**MFO** Mean (SD) m-mol-products /min/mg protein (n)
F_0	0.7818±0.1836 (20)	0.0325±0.0182 (20)	0.0404±0.0216 (20)	4.2550±0.2261 (20)
F_{12}	0.7405±0.1247 (20)	0.0704±0.0160 (20)	0.0634±0.0393 (20)	4.8200±0.2239 (20)
F_{16}	0.7185±0.1083 (20)	0.0854±0.0372 (20)	0.0441±0.0245 (20)	15.24±1.8520 (20)
F_{18}	0.7385±0.1230 (30)	0.1336±0.0542 (30)	0.0484±0.0165 (30)	21.90±0.8534 (20)

(n) = sample size in parenthesis

3.3 Cloning of PCR products

Our goal was to run a preliminary screen for sequences of *CYP6* fragments from *An. minimus*. We amplified partial genomic DNA fragments of *CYP* gene. PCR amplification products of the expected size of ~ 900 bp were cloned and sequenced (unpublished results). Two clones, the AN2 and AN40, each contained a reading frame and deduced amino acids for each clone. The AN40 and AN2 sequences further showed high similarity in deduced amino acids to those of other insect *CYP6* genes. Three other clone sequences were obtained and also showed high homology to *CYP6* sequences at the deduced amino acid level, but were not selected for further studies. The complete sequence of AN2 (namely *CYP6S2*) was obtained but later showed no significant increase of its mRNA in the resistant strain and is not reported here (Thanomsing, N. MS thesis).

Alignment of the AN40 clone sequence (later identified as *CYP6P5*, Boonserbsakul, S. MS thesis) and of other *CYP6* genes provided information on conserved regions and choices for design of primers along an entire *CYP6* sequence in *An. minimus*. This information further facilitates isolation of CYP6 cDNA sequences showing elevated mRNA level in the deltamethrin resistant mosquito as described below.

3.4 Cloning of cDNAs from deltamethrin susceptible and resistant *An. minimus*

In order to isolate overexpressed CYP6 cDNAs in the pyrethroid resistant mosqutio comparing to the susceptible strain. We can not use mRNA differential display nor cDNA subtraction techniques due to the complication of gene duplication event within the organism and that our purpose is isolate the overexpressed cDNA, not a differential expressed one. We used antisense primer targeting at heme-binding region and sense primer binding at the ETLR motif sequence. The primers amplified partial cDNAs from susceptible and resistant strains producing the expected product size of ~ 240 bp and the products were cloned.

A total of 38 cDNA clones (21 from resistant strain and 17 from susceptible strain) were sequenced and scored highly with P450 genes. Deduced amino acids of the cDNA clones showed relatively high diversity and were classified into 9 isoforms (Rongnoparut et al 2003, Appendix C). The AN1 cDNA clone was found predominantly and represented 42.8% of the clones sequenced in the F_{13} resistant mosquito, while in the susceptible strain AN1 clone represented only 17.6% (Table5). The AN10 and AN36 clones were found 19% and 9.5% in the resistant strain. The number of cDNA clones found multiple times could be correlated with the level of resistance expression in each mosquito strain. Thus we selected 3 over-represented AN1, AN10 and AN36 clones and an AN40 clone found equally represented in the susceptible and resistant strains for further characterization. Interestingly the AN2 (CYP6S2) clone, previously described, showed no sequence complement to any of the cDNA clones listed in Table 5. It could be that its mRNA level is low in both susceptible and F_{13} resistant mosquitoes.

Table 5: Occurrence of cDNA clones in F_{13} deltamethrin-resistant and susceptible strains of *Anopheles minimus*.

Clone	Mosquito strain			
	Resistant (n[a])	Percent	Susceptible (n[a])	Percent
AN1	9	42.8	3	17.6
AN40	3	14.3	3	17.6
AN10	4	19.0	0	0
AN36	2	9.5	1	5.9
AN4	1	4.8	1	5.9
AN17	1	4.8	2	11.8
AN8	1	4.8	5	29.4
AN38	0	0	1	5.9
AN35	0	0	1	5.9
Total	21	100	17	100

[a] Number of cDNA clones sequenced

We have chosen to further characterize and obtain full coding sequences of AN1 (namely *CYP6AA2*), AN40 (*CYP6P5*), AN10 and AN36 (not yet assigned a *CYP6* name). The complete coding amino acid sequences deduced from all four genes showed highly similar to *CYP6* sequences. Multiple amino acid alignment of the 4 isolated *CYP* genes with other insect *CYP6* sequences revealed a high degree of homology with numerous amino acid residues conserved across other insect CYP6s. These sequences contained conserved residues representing typical features of cytochrome P450s, including a hydrophobic N-terminal membrane anchor domain, the putative heme binding site and ETLR motif. The sequences of full coding *CYP6AA2* and *CYP6P5* are reported (Rongnoparut, 2003, detailed results are shown in Appendix C). The full-length cDNA sequences of both AN10 and AN36 are completed, but the sequences are being confirmed on both strands of DNA and are not shown in this report. The sequences will be subsequently reported in a manuscript that is in preparation.

3.5 mRNA expression of *CYP* genes in deltamethrin susceptible and resistant *An. minimus* strains

a) mRNA level of *CYP6AA2* and *CYP6P5* genes

To assess the transcription expression level of *CYP6* genes in deltamethrin resistant and susceptible strains, we used semi-quantitative RT-PCR to measure expression level of each mRNA in *An. minimus*. We primarily concentrated on examining *CYP6P5* and the most over-represented *CYP6AA2* clones. Comparative mRNA expression studies, using actin1D as internal control, indicated that expression of *CYP6AA2* in deltamethrin resistant mosquitoes is correlated with development of deltamethrin resistance in *An. minimus* mosquito. The actin 1D cDNA fragment was sequenced to confirm its identity. The relative amount of CYP6AA2 transcript was 2 fold greater in the 24% deltamethrin resistant F_{13}

mosquito, while the fold of increase was a factor of 4 in 52% resistant F_{19} *An. minimus* compared to the F_0 susceptible strain (Rongnoparut, et al., 2003). The CYP6P5 transcript is equally expressed in both resistant and susceptible mosquitoes. Detailed results are shown in Appendix C.

b) The expression of mRNA level of AN10 and AN36 genes

The expression of mRNA levels was measured by comparison of semi-quantitative RT-PCR products of mRNA isolated from F_0, F_{19}, and F_{25} strains. The expression of AN10 mRNA level presented ~2 fold from F_{19} deltamethrin resistant population and ~4 fold from F_{25} deltamethrin resistant mosquitoes more than the level of F_0 susceptible population (Figure 1). Whereas, the expression of AN36 mRNA level was not significantly different between the three strains (F_0, F_{19}, and F_{25}) (Figure 2).

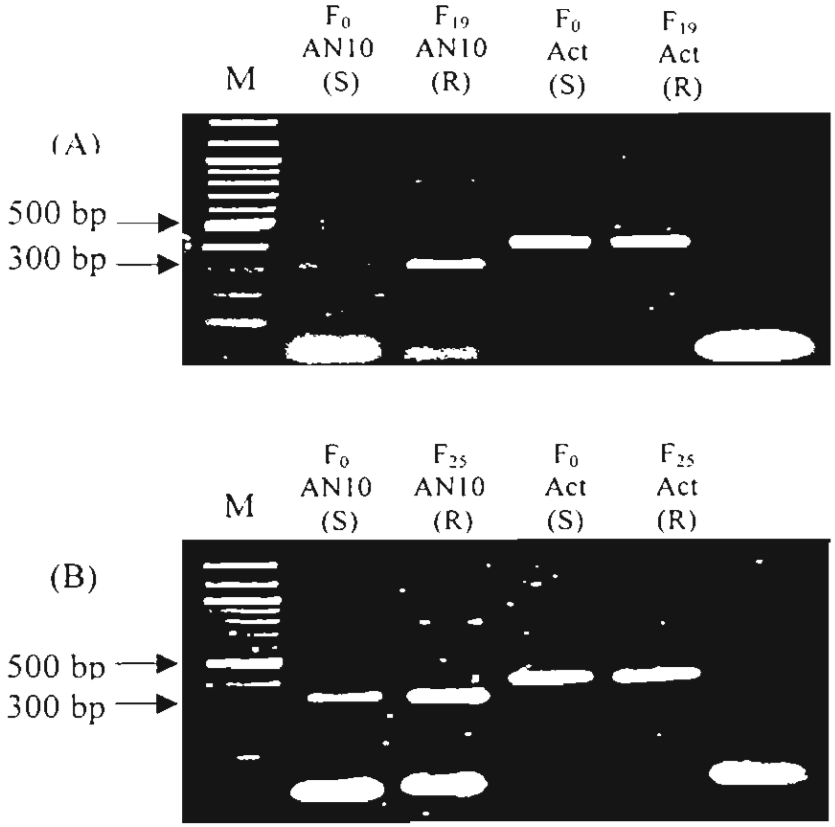

Figure 1. RT-PCR products of AN10 and actin1D (Act) internal control using Semi-Quantitative RT-PCR technique. (A) represents products of F_0 susceptible (S) and F_{19} resistant (R) strains. (B) represents products of F_0 and F_{25} resistant mosquitoes. Lane M: 100 bp ladder marker

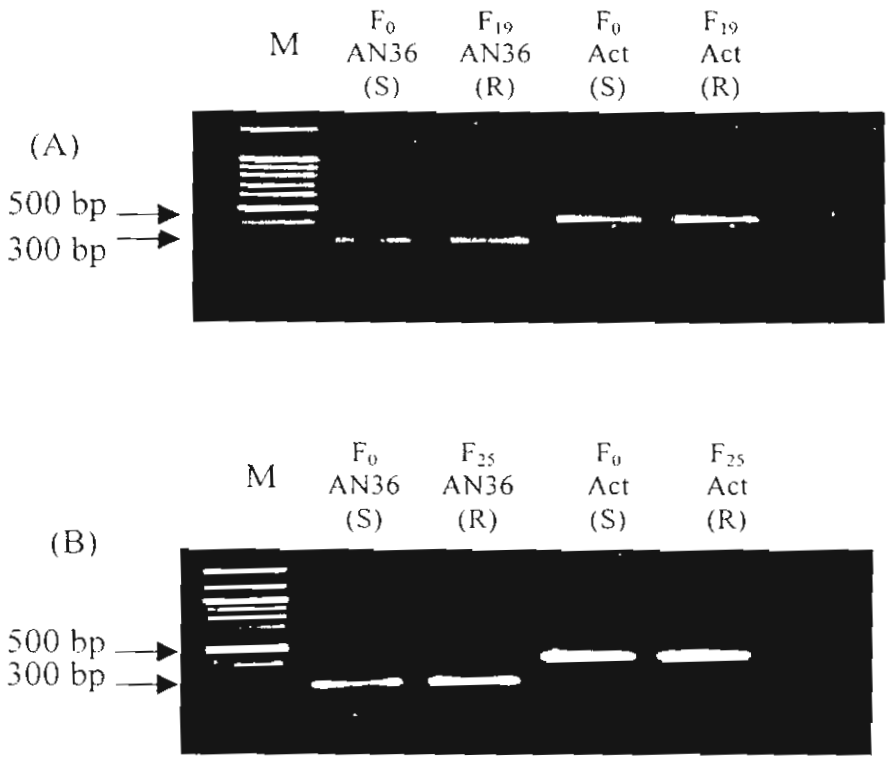

Figure 2. Semi-Quantitative RT-PCR of AN36. Lanes are as described for figure 1.

The mRNA expression of *CYP6AA2* (AN1) was investigated further in F_{25} resistant mosquito. The result showed ~7 fold mRNA expression level higher than F_0 susceptible strain as shown in Figure 3. The results demonstrated that the mRNA expression level of *CYP6AA2* and AN10 was increased as deltamethrin resistant level of *An. minimus* strains increased. However, the fold of mRNA increase in AN10 is lower than CYP6AA2 mRNA in resistant populations.

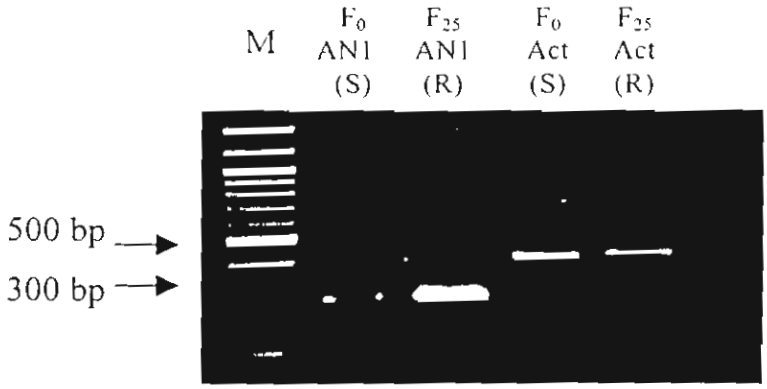

Figure 3. RT-PCR products of AN1 (*CYP6AA2*) using Semi-Quantitative technique.

4. Discussion

We have isolated at least four CYP6 cDNA fragments and obtained full coding sequences for each gene. All *CYP6* genes span approximately 1.5 kb and contain ~500 amino acid residues, typical characteristic of *CYP6* genes found in other insects. The following information indicates that all isolated *CYP6* genes are members of cytochrome P450 family six. First, their deduced amino acids contain high amino acid identity to other insect *CYP6s*. Second, the intron sequences were all found in conserved ETLR motif at ~371 glutamic (E) amino acid residue similar to the positions found for *CYP6s* in other insects, including *CYP6A1* (Feyereisen et al, 1989), and *CYP6C1* (Cohen M B, Feyereisen R, 1995). Third, They contained deduced amino acids conserved among cytochrome P450 family six, including N-terminus hydrophobic, membrane bound region of CYP protein, the heme binding (FxxGxxxCxG) *CYP* signature (Feyereisen et al, 1990) presented at ~residues 446-455, the consensus xPcxFxPE+a at positions ~420-434 residues, and the consensus WxxxR charge-paired with the heme propionate group, at 129-133 residues (Sandra et al, 1996).

Previous studies in housefly *Musca domestica*, in *Drosophila melanogaster*, and in *Helicoverpa armigera* suggested overexpression of mRNA levels of *CYP6D1*, *CYP6g1*, and *CYP6B7* respectively, in association with insecticide resistance (Feyereisen *et al.*, 1989; Tomita et al, 1995, Ranasighe et al., 1998, and Daborn et al. 2001). In addition, there is induction and the increase of CYP6B1 and CYP6B3 mRNA in *Papilio polyxenes* during feeding on furanocoumarin, indicating their involvement in plant toxin metabolism (Petersen et al, 2001). The *CYP6D1* is shown to metabolize pyrethroid (Feyereisen *et al.*, 1989; Tomita et al, 1995), while the mapping in *Drosophila* has proven the involvement of *CYP6g1* in DDT resistance (Daborn, et al. 2001). It is suggested that a single gene, *CYP6g1*, is responsible for DDT resistance (Daborn, et al. 2002). While, it is not known whether a single gene or more is participated in pyrethroid resistance.

The mRNA expression level of AN1 (*CYP6AA2*), AN10, AN36 and AN40 (*CYP6P5*) were measured and compared in F_0, F_{19}, and F_{25} deltamethrin resistant strains of *An. minimus*. The F_0, F_{19} and F_{25} showed increasing resistance against 0.05% deltamethrin. This resistant colony is shown cross-resistant to DDT with lower level of resistance than deltamethrin resistance, during the course of selection against deltamethrin. The mRNA expression level of *CYP6AA2* and AN10 was increased accordingly in F_{19} and F_{25} mosquitoes compared to F_0 susceptible strain indicating association of *CYP6AA2* and AN10 mRNA expression level with development of resistance. Whereas, those of *CYP6P5* and AN36 were not significantly different between F_0, F_{19}, and F_{25} deltamethrin resistant strains. These results suggest that *CYP6AA2* and AN10 could play role in insecticide resistance in *An. minimus*.

The overexpressed *CYP6* genes in *An. minimus* may support their functions for deltamethrin detoxification. The *CYP6AA2* and AN10 could share their roles in insecticide resistance in *An. minimus*. But the extent of mRNA increase shown in Table 6 for *CYP6AA2* is higher than for AN10. Whether only *CYP6AA2* plays role in deltamethrin resistance, while AN10 plays role in DDT resistance, or vice versa, is not known. Enzyme biochemical assays suggest an involvement of cytochrome P450 enzyme in resistant strain. Alternative explanation could include other unexplained mechanism involved in DDT resistance occurring in F_{13}, F_{19}, F_{25}, while both *CYP6AA2* and AN10 contribute to deltamethrin resistance. The third explanation could be that the occurrence of cross-resistance in *An. minimus* to pyrethroid and DDT may involve overexpression of both *CYP6AA2* (AN1) and AN10. Although, there are reports suggesting that GST enzyme is associated with DDT

resistance in insects such as *M. domestica* (Wei S.H. et al., 2001), *An. dirus* (Prapanthadara L. et al., 1998), and *An. gambiae* (Prapanthadara L. et al., 1993), the elevated GST was not detectable in *An. minimus* resistant strain used in this study (Chareonviriyaphap, et al, 2003).

Table 6. Percent mortality and mRNA expression level of F_0, F_{13}, F_{19}, and F_{25} deltamethrin and DDT resistant strains

An. minimus strain	% mortality		mRNA fold increase[a]	
	Deltamethrin	DDT	CYP6AA2 (AN1)	AN10
F_0	100	100	ND[b]	ND[b]
F_{13}	76	80	2	-
F_{19}	48	60	4	2
F_{25}	44	ND[b]	7	4

[a] The fold increase of each strains compare to F_0 mRNA level
[b] Not determined

Numerous evidences suggest that cross-resistance to DDT and pyrethroid involve knock down resistance (*kdr*) occurring by point mutation at S6II domain of the para-sodium channel gene including *M. domestica*, *D. melanogaster*, and *An. gambiae* (Liu and Pridgeon, 2002; Martinez-torres et al. 1998; Soderlund D.M. and Knippple D.C, 2003; Williamson, et al, 1993). We have cloned and sequenced the gene encoding S6II domain and found no mutation in *An. minimus* resistant strain comparing to the wide type susceptible gene (unpublished result). The result suggests that *kdr* is not involved in cross-resistance in *An. minimus*. This has left to the conclusion that the cytochrome P450 family six could be a major mechanism in the resistant strain of *An. minimus* in this study. However, cross-resistance via only *CYP6* in *An. minimus* can not be concluded. Because the resistant mosquitoes are under ongoing selection process, we have not investigated whether the addition of pyperonyl butoxide (PBO), a P450 inhibitor, would revert resistance and/or cross-resistance phenotypes. This experiment can be performed soon, however, such experiment is not included in the scope of this work. On the other hand, it is known that PBO is an inhibitor of only certain P450s, thus it might not inhibit the activity of the *CYP6s* isolated from *An. minimus* in this study.

Cytochrome P450s have been known to possess broad and overlapping substrate specificity. An example could be *CYP6g1* that play role in DDT resistance in *Drosophila melanogaster*, but has been implicated to play role cross-resistance to various insecticides (Daborn, et al 2002). The role of *CYP6AA2* and AN10 in insecticide resistance and in cross-resistance to DDT and pyrethroid could further be supported by studies of metabolic function of *CYP6AA2* and AN10. In the future study, substrate specificity could be proven for *CYP6AA2* and AN10 to each insecticide substrates including pyrethroid and DDT. The study could be accomplished by means of use of cDNA expressed protein of individual gene, CYP6AA2 or AN10, and test for the metabolic role of each gene. Their metabolic role in pyrethroid and DDT insecticides, and biochemical properties and specificity could then be clarified and may reveal the phenomenon of the specific insecticide resistance and cross-resistance.

We have recently begun to lay groundwork into a detailed understanding of the mechanisms that control the substrate and reaction selectivity of CYP6 enzymes and substrate interaction sites. The further work involves CYP6 cDNA heterologous expression in baculovirus and other system such as in *E. coli*. We have successfully expressed CYP6AA2 protein in baculovirus-infected insect cell and in *E. coli* (data not shown). The metabolic role in terms of substrate specificity towards insecticides of CYP6AA2 cDNA expressed enzyme can then be explored and inhibitors eventually screened. The analogous study on AN10 can then be followed or studied in parallel. Finally, patterns of CYP6AA2- and AN10-mediated resistance can be examined among anopheline mosquitoes.

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Output:

Internationally Peer-Reviewed Publications:

1. Chareonviriphap, T., Rongnoparut, P., and Juntarumporn, P. 2002. Selection for pyrethroid resistance in a colony of *Anopheles minimus* species A, a malaria vector in Thailand. *J. Vect. Ecol.* 27:222-229.
2. Chareonviriphap, T., Rongnoparut, P., Juntarumporn, P. and Bangs, M., 2003. Biochemical detection of pyrethroid resistance mechanisms in *Anopheles minimus* in Thailand. *J. Vect. Ecol.* 28:108-116.
3. Rongnoparut, P., Boonsuepsakul, S., Chareonviriyaphap, T., and Thanomsing, S., 2003. Molecular cloning of cytochrome P450, *CYP6P5* and *CYP6AA2* from *Anopheles minimus* resistant to deltamethrin. In press in *J. Vect. Ecol.*
4. Rodpradit, P., et al. Overexpression of a CYP6 cDNA in the pyrethroid resistance strain of *An. minimus. In preparation.*

Abstracts:

1. Sarapusit, S, Rongnoparut, P. (2003) Expression of *Anopheles minimus* CYP6AA2 cDNA in baculovirus expression system. Submitted for presentation in The 29th Congress on Science and Technology of Thailand, October, 2003
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APPENDIX A

Selection for pyrethroid resistance in a colony of *Anopheles minimus* species A, a malaria vector in Thailand

Theeraphap Chareonviriyaphap[1,4], Pornpimol Rongnoparut[2], and Piyanoot Juntarumporn[3]

[1]*Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900 Thailand*
[2]*Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400 Thailand*
[3]*Department of Pest Management, Faculty of Natural Resources, Prince of Songkhla University, Songkhla 90112 Thailand*

[4] *Corresponding Author*

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ABSTRACT: This study tested susceptibilities of *Anopheles minimus* mosquitoes to deltamethrin during each of 19 generations (although technical problems excluded selective pressure experiments during generations 11-13). The ultimate goal was to establish a pyrethroid resistant colony of this important malaria vector in Thailand. Resistance was selected for by exposing, using the World Health Organization test protocol, sequential generations of *An. minimus* females to LD_{50} and LT_{50} values of deltamethrin. The LD_{50} and LD_{90} values were determined for populations from each subsequent generation by probit analysis and significant increases (chi-square test, $P>0.01$) occurred from one generation to the next. There was approximately a 22-fold increase in the LD_{50} and a 27-fold increase in LD_{90} when the F_{10} generation was compared to the parent colony (F_1). Similarly, the LT_{50} and LT_{90} values were also increased during selection experiments during generations 14-19. There was roughly a 3-fold increase in susceptibility of F_{19} females compared to F_{14} females. In addition, deltamethrin conferred a cross-resistance to DDT in the selected colony. Baseline information from these experiments will serve as a guide for future studies on susceptibilities of wild *An. minimus* populations in Thailand. ***Journal of Vector Ecology* 27(2): 222-229. 2002.**

Keyword Index: Deltamethrin, resistance, *Anopheles minimus*, cross-selection.

INTRODUCTION

Despite years of vector control and public health activities, malaria remains a major public health concern in Thailand. Thousands of malaria cases are reported annually (Annual Malaria Reports 1995-2000). The key to preventing malaria transmission is to reduce human-vector contact using chemical insecticides (Prasittisuk 1985, Chareonviriyaphap et al. 2000). Intradomicillary spraying with DDT was employed for control after Thailand accepted the WHO plan for malaria eradication in 1950 (Prasittisuk 1985). However, the use of DDT has been decreasing over time because of changing human response to spraying coupled with environmental concerns. Furthermore, the development of physiological resistance to DDT had been detected in all 3 primary malaria vectors, *Anopheles dirus*, *An. minimus* and *Anopheles maculatus* (Chareonviriyaphap et al. 1999). Synthetic pyrethroids are the current insecticides of choice for malaria control in Thailand. The pyrethroids have shown great promise for pest control due to their low mammalian toxicity and remarkable potency at low levels that quickly immobilizes, kills and repels insects (Prasittisuk 1994, Chareonviriyaphap et al. 1997). Pyrethroids have been used for impregnation of bed nets, viz. permethrin and lambdacyhalothrin, and for intradomicillary spraying, viz. deltamethrin. Due to their effectiveness and rapid excito-repellency actions, these insecticides have been used for malaria control in many parts of the country (Chareonviriyaphap et al. 2001). *Anopheles minimus* Species A is the main malaria vector in the hill and forest fringe areas of Thailand (Baimai 1989). This species is considered sufficiently endophagous and anthropophilic and, as a consequence, is more likely to come into contact with residual insecticides used in impregnated bed-nets and intradomicilary spraying (Nutsathapana et al. 1986, Chareonviriyaphap et al. 2001).

Routine contact with chemical insecticides has led, in some cases, to high levels of insecticide resistance. Pyrethroid resistance has been documented in many species of malaria vectors, including *Anopheles gambiae* in Africa (Chandre et al. 1999); *Anopheles albimanus* in Central and South America (Penilla et al. 1999); *Anopheles stephensi* in India (Chakravorthy and Kalyanasundaram 1992); and *Anopheles funestus* from South Africa (Hargreaves et al. 2000). In addition, pyrethroid resistance was reported in a population of *An. minimus* species A from northern Thailand, approximately 1 year after pyrethroids were introduced into the vector control program (Annual Malaria Reports 1995-2000).

Recent information on pyrethroid resistance in Thailand is scarce due to few consistently and carefully designed studies. The spread of pyrethroid resistance among vector populations may result in the eventual use of less environmentally favorable insecticides. The objective of this study was to establish a deltamethrin resistant colony and to determine the level of physiological resistance in the laboratory-reared *An. minimus* species A. A selection line was established and the LD_{50} and LT_{50} values were obtained using dosage/mortality and time/mortality relationships. Mosquitoes surviving LD_{50} and LT_{50} exposures were sustained into the following generation. Susceptibility data were obtained with World Health Organization (WHO) test methods (WHO 1998).

MATERIALS AND METHODS

Mosquito test populations

A colony of *An. minimus* species A was received from the Malaria Division, Department of Communicable Disease Control (CDC), Ministry of Public Health, Nontaburi in 1997. This colony was originally collected from the animal shelter in Rong Kwang District, Prae Province, North Thailand in 1993. The colony was raised in the insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. At the time of this work, females from the colony were completely susceptible to deltamethrin (0.05%) and DDT (4%), based on a standard impregnated paper adult contact assay (WHO 1998).

Insecticide impregnated papers

All deltamethrin-impregnated papers were purchased from the WHO Vector Control Unit, Penang, Malaysia. The papers were treated at the rate of 2.75 ml of insecticide solution per 180 cm^2 and used before their specified time of expiration.

Mosquito rearing

The standard procedure for rearing *Anopheles* mosquitoes was followed, (Ford and Green 1972). All life stages were reared in an insectary (25±3°C, 80±10%RH) in the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. From the day of emergence, adult mosquitoes were provided with cotton soaked with a 10% sugar solution. Female mosquitoes were allowed to feed on a hamster on the third day postemergence. Approximately, 2-3 days later, oviposition dishes were placed in the cage containing gravid females. The eggs were placed in the center of a piece of wax paper with a hole cut in the center. The paper floated on the water surface and served to keep eggs from becoming stranded on the sides of the hatching tray. Larval food was sprinkled on the surface of the water twice daily (3 times daily after reaching the third larval stage). Pupae were collected daily, and transferred to the small bowls containing clean water. The bowls were placed in cages for adult emergence.

Selection of deltamethrin resistance

A susceptible strain of *An. minimus* females was placed under selection pressure with deltamethrin at the concentration (or time) that caused 50% mortality (LD_{50} and LT_{50}). LD_{50} dosage was used to select *An. minimus* females for F_1-F_{10}. Similarly, LT_{50} were used to select from F_{14}-F_{19}. No selection was made in the F_{11}-F_{13} generations due to technical problems. Survivals were raised as usual in the insectary at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Approximately 2,000 females were selected for each generation. The susceptibility level of deltamethrin in successive generations was monitored by determining the LD_{50} and LD_{90} values. The progress of selection was monitored with the WHO diagnostic test (WHO 1998). A control colony was reared simultaneously in the separated room and handled in the same manner through all manipulations but was not exposed to insecticide. The control colony was tested to monitor for independent occurrence of resistance.

WHO diagnostic test

Bioassays were conducted using WHO test kits for adult mosquitoes (WHO 1981a). *Anopheles minimus* females were exposed for 1 h to diagnostic dosages of deltamethrin (0.05%). For each test, 5 cylinders, 2 for the controls and 3 for the treatments, were used. Control cylinders contained filter paper impregnated with carrier whereas treatments contained paper impregnated with the diagnostic dosage of insecticide plus carrier. Twenty mosquitoes were introduced to each cylinder and maintained for 1 h in a normal vertical position. After 1

Table 1. Susceptibility data of Deltamethrin based on dose/mortality relationships tested against *Anopheles minimus* species A populations.

Sample	No. tested	LD_{50} (%)	95% CI	LD_{90} (%)	95% CI	Slope (±SE)	X^2
F0	240	0.00035	0.00031-0.00040	0.00137	0.00113-0.00173	2.20±0.15	6.06
F1	360	0.00049	0.00043-0.00057	0.00200	0.00157-0.00275	2.10±0.16	4.62
F2	240	0.00042	0.00034-0.00054	0.00500	0.00298-0.01115	1.20±0.14	1.11
F3	360	0.00027	0.00021-0.00033	0.00249	0.00169-0.00440	1.33±0.14	4.28
F4	360	0.00030	0.00015-0.00154	0.00283	0.00086-0.00401	1.32±0.26	7.27
F5	360	0.00188	0.00169-0.00210	0.00506	0.00429-0.00621	2.98±0.21	5.63
F6	239	0.00269	0.00238-0.00306	0.00828	0.00672-0.01090	2.62±0.21	3.88
F7	120	0.00292	0.00182-0.05170	0.01243	0.00656-0.05066	2.04±0.34	3.62
F8	360	0.00603	0.00472-0.00782	0.02060	0.01953-0.03078	2.00±0.15	3.69
F9	360	0.00617	0.00584-0.00741	0.02063	0.01709-0.02642	2.04±0.19	4.97
F10	360	0.00912	0.00621-0.01024	0.03120	0.02417-0.03587	1.85±0.14	2.78

h, mosquitoes were transferred to holding containers and provided with cotton pads with 10% sucrose solution. Mortality was recorded at 24 h and each test was replicated 3 times. Resistant status was evaluated according to the WHO protocol; a population is considered resistant if more than 20% of population survives the diagnostic dose compared to the susceptible colony (WHO 1981b). In addition to deltamethrin at the diagnostic dose (0.05%), susceptibility to DDT (4%) and other synthetic pyrethroids was measured in populations under selection pressure to test for cross-resistance to DDT and other synthetic pyrethroids.

Data analysis

Abbott's formula was used to correct the observed mortality. The LD_{50} and LD_{90} values were estimated from dosage-mortality regression using probit analysis (Finney 1971). Similarly, the LT_{50} and LT_{90} values were calculated from the time-mortality relationship using probit analysis (Finney 1971).

RESULTS

Anopheles minimus Species A was placed under selection pressure and each generation was tested for susceptibility to deltamethrin using dose and time mortality relationships (Tables 1 and 2). The LD_{50} and LD_{90} values for susceptibility to deltamethrin fluctuated widely during the first four generations. The reason for this variation is unknown. Under continuous selective pressure the LD_{50} and LD_{90} values continuously increased in subsequent generations. There was approximately a 22-fold increase in the LD_{50} and a 27-fold increase in the LD_{90} by the F_{10} generation (LD_{50} = 0.00912 and LD_{90}= 0.03120). The increase is based on comparisons with susceptibilities of the parent generation (F_0) (LD_{50} = 0.00035 and LD_{90}= 0.00137) (Table 1). Similarly, LT_{50} and LT_{90} values of deltamethrin also increased under selective pressure from the F_{14} to F_{19} generations. Due to technical difficulties, selective pressure was not applied in generations from F_{11} to F_{13}. There was roughly a 3-fold increase in LT_{50} and LT_{90} when F_{19} was compared to the F_{14} generation (Table 2). This continuous increase in lethal dose and lethal time values suggests that populations under selective pressure became less susceptible to deltamethrin than the parent colony.

The slopes of regression lines for test data from each generation were computed and values for slope varied 1.20 to 2.98. The highest value was obtained from the F_5 generation (2.98) and the smallest value was from the F_3 generation. Small X^2 values demonstrated that the response of mosquitoes to deltamethrin in susceptibility tests completely fit a linear model (P>0.01) (Tables 1 and 2).

Results of susceptibility tests with the single diagnostic dose of deltamethrin (0.05%) for different generations of both populations under selection pressure and populations not under selection pressure are presented in Table 3. The ability of mosquitoes to survive the diagnostic dose after 24 h is indicative of resistance in the population as defined by percent mortality in the test population. Our result showed that the parent colony was completely susceptible to deltamethrin as evidenced by 100% mortality (Table 3). Partial survival (5%) was first observed in the F_6 generation of the population under selection pressure. Percent mortality of the treatment colony continually declined toward the end of the study. The highest mortality was observed in the F_{19} generation

Table 2. Susceptibility data of Deltamethrin (0.05%) based on time/mortality relationships tested against *Anopheles minimus* species A populations.

Sample	No. tested	LT_{50} (min)	95%CI	LT_{90} (min)	95%CI	Slope (±SE)	X^2
F14	360	19.07	8.38-89.17	75.22	27.43-71.34	2.71±0.45	9.37
F15	240	29.34	18.39-58.19	73.77	43.45-96.15	2.21+0.26	5.94
F16	360	31.97	18.60-41.33	97.82	59.05-120.25	2.17±0.38	2.51
F17	360	46.32	40.32-55.43	179.90	123.57-350.67	1.69±0.32	9.52
F18	360	47.54	41.34-54.34	185.25	154.23-325.32	1.58±0.34	5.12
F19	240	55.32	45.23-78.15	204.12	189.13-398.12	1.42±0.26	4.12

Table 3. Mortality from WHO diagnostic test kit for 0.05% Deltamethrin against 2 test populations of *An. minimus* species A 24 h-post exposure.

	Control colony		Selected colony			Control colony		Selected colony	
	No. Tested	% Dead	No. Tested	% Dead		No. Tested	% Dead	No. Tested	% Dead
F1	100	100	100	100	F11	NP	NP	NP	NP
F2	100	100	100	100	F12	100	100	100	80
F3	100	100	100	100	F13	100	100	100	76
F4	100	100	100	100	F14	100	100	100	76
F5	100	100	100	100	F15	100	100	100	71
F6	100	100	100	95	F16	100	100	100	64
F7	100	100	100	90	F17	100	100	100	64
F8	100	100	NP	NP	F18	100	100	100	50
F9	100	100	NP	NP	F19	100	100	100	48
F10	100	100	100	85	F20	NP	NP	NP	NP

Table 4. Mortality from WHO diagnostic test kit for 4% DDT against *An. minimus* species A test populations 24 h-post exposure.

Test Colonies	Control colony			Selected colony		
	No.Tested	No. Dead	%Mortality	No. Tested	No. Dead	% Mortality
F0	100	100	100	100	100	100
F4	100	100	100	100	100	100
F8	100	100	100	100	90	90
F12	100	100	100	100	80	80
F16	100	100	100	100	70	70
F19	100	100	100	100	60	60

(48% mortality). The continuous decline in percent mortality of the treatment colony was indicative of increasing resistance to deltamethrin. Treatment populations were low in some generations and no diagnostic susceptibility tests could be performed. This occurred during generations F_8, F_9 and F_{11}.

Anopheles minimus populations under selective pressure were also tested against diagnostic dosages of 4% DDT to check for cross-resistance. Percent mortality from DDT in the treatment colony declined in populations under deltamethrin selective pressure. Percent mortalities in tests with treatment populations against DDT were 90% in the F_8 and 60% in generation F_{19} (Table 4). The control colony remained completely susceptible to DDT (100% mortality).

DISCUSSION

Synthetic pyrethroids were first introduced for malaria control in Thailand in 1992. Since that time they have been used to replace DDT (the DDT phase-out occurred from 1995-1999) (Annual Malaria Reports 1995-2000). Permethrin is used primarily for impregnated bed nets, whereas deltamethrin is a major insecticide for an indoor-residual house spray (Annual Malaria Reports 1995-2000). Approximately one year after bed nets impregnated with permethrin were introduced as a malaria control measure in the northern part of Thailand, evidence of physiological resistance was reported (Annual Malaria Reports 1995-2000). However, little was known about the level of physiological resistance, or the factors or mechanisms of resistance. Deltamethrin is another effective compound that should be monitored for development of resistance in vector populations. Deltamethrin is normally applied 1 or 2 times a year in malaria endemic areas of Thailand (Annual Malaria Reports 1995-2000). Continuous indoor-residual spraying of deltamethrin may select for resistance in the vector populations.

Anopheles minimus is an excellent model to study insecticide resistance in Thailand. This species is considered sufficiently endophagous and anthropophilic to come into contact with insecticide residues in houses (Nutsathapana et al. 1986). Therefore, careful and complete monitoring of malaria vectors for resistance to deltamethrin should be a primary emphasis of malaria control activities.

Resistance is defined as the acquired ability of an insect population to tolerate doses of insecticide which will kill the majority of individuals in a normal population of the same species (WHO 1975). Our results indicate that a high level of physiological resistance to deltamethrin in *An. minimus* can be developed under selective pressure in the laboratory. Resistance is shown by low mortalities of offspring from parents that survive selective pressures in preceding generations. LD_{50} and LD_{90} values of deltamethrin increased greatly over 10 generations of selective pressure. The selection experiment covered 19 generations, but due to low population numbers, no selective pressure could be applied against adults in generations 11,12 and 13. There were 22- and 27-fold increases in LD_{50} and LD_{90} values, respectively, of deltamethrin in the F_{10} compared to the parent colony (F_0). High variation characterized LD_{50} and LD_{90} values during generations F_1-F_4, i.e., immediately after selective pressure was applied. We have no explanation for unexpected levels of variation, but perhaps high variation is typical of populations in early stages of selective pressure. LT_{50} and LT_{90} values also increased during the second part of selection (F_{14}-F_{19}). There were approximately 3-fold increases in LT_{50} and LT_{90} values in F_{19} compared to the F_{14}. A similar study on rapid development of physiological resistance was reported for *Anopheles stephensi* (Verma and Rahman 1986). The authors selected for resistance to fenvalerate, another synthetic pyrethroid insecticide.

Theoretically, a susceptible colony composed of totally susceptible individuals will produce the highest slope for a regression line of dose-response data. With selective pressure from exposures to insecticides, a population will become heterozygous for resistant genotypes and as the frequency of resistant genotypes increases, the slope of the regression line for will drop off and the line will shift to the right (Brown and Pal 1971). The slope of regression lines, based on data from our selection experiments, continuously declined over time. This result is consistent with increasing physiological resistance in the test populations. However, a shift to the right without a change in slope is suggestive of increased population vigor, not physiological resistance (Brown and Pal 1971). There was no shift to the right in regression lines from our dose-response data.

Resistant status can be evaluated according to the WHO protocol; thus, a population is considered resistant if more than 20% of the population survives the diagnostic dose compared to the susceptible colony (WHO 1981b). In our tests the percent mortality continually increased from one generation to the next, hence 52% of test specimens survived after 19 generations of almost continuous selective pressure. Cross-resistance to DDT was also observed in the deltamethrin resistant colony. Cross-resistance may have occurred as a result of similar actions of DDT and pyrethroids on the voltage-dependent sodium channel of nerve axons (Brooke et al. 1999, Bloomquist 1996).

There is evidence to support a claim of cross-resistance between DDT and pyrethroids. Previous studies have shown that pyrethroid resistant populations of *Aedes aegypti* in Thailand are frequently resistant to DDT (Brealey et al. 1984, Prasittisuk and Busvine 1977). In addition, pyrethroid resistance in *An. stephensi* larvae was reported for a strain that had developed DDT resistance as a result of selection experiments in Pakistan (Omar et al. 1980). The similar mode of action of DDT and synthetic pyrethroids has led, in some cases, to a cross-resistance mechanism, commonly known as knock down resistance (kdr). Kdr is conferred by single amino acid changes in the sodium-channel insecticide-binding site in the nerve sheath. Whether the cross-resistance to DDT reported here is conferred by kdr should await further studies at the molecular level.

Several factors other than frequency of insecticide spraying serve to influence the intensity of selection and development of physiological resistance in a population. The most important factors include the frequency of the resistance gene in a population, number of genes interacting to produce the resistant character, size of population, and the dominant relationship of the gene (Ferdinand 2000). The proportion of sprayed houses with undisturbed surfaces and the extent of contamination of breeding places and outdoor resting habitats with agricultural insecticides may influence resistance development (Unpublished data).

Our study provided a baseline for susceptibility and varying levels of deltamethrin resistance in *An. minimus* in Thailand. If the level of resistance is maintained, then the resistant colony will be used to study the actions of pyrethroid insecticides and mechanisms of resistance. It has been reported that selection by toxic subtances can increase the amount of enzymes that are responsible for detoxicification (Ferrari and Georghiou 1990). Common insecticide resistance mechanisms in insect pests were reported elsewhere, including 3 possible pyrethroid resistance mechanisms, namely mixed-function oxydases (MFOs), elevated esterases, and reduced sensitivity of sodium channels (Georghiou 1986, Roberts and Andre 1994, Nelson et al. 1996, Scott et al. 1998, Feyereisen 1999). In addition, an increase in glutathion-S-transferases (GSTs) was reported in many pyrethroid resistant insects, such as *Spodoptera litturata* (Lagadic et al. 1993), *Tribolium castaneum* (Reidy et al. 1990), and *Aedes aegypti* (Grant and Matsumura 1988). Identification of elevated esterase, GSTs and MFO in this pyrethroid resistant colony will be the subject of future reports. An increase in the quantity of enzymes can be associated with gene amplification or overexpression of target genes. This appears to be the cause of protein overproduction when an organism is under environmental stress (Mouches et al. 1990). Potential genes associated with deltamethrin resistance in our selected line are under investigation.

In malaria endemic areas, there is a need for comparative studies on susceptible and refractory populations for as many known vectors as possible. This is especially true if there has been continuous intradomicillary spraying with deltamethrin and other insecticides. Additionally, such studies should be representative of different geographical conditions and be conducted with greater frequency than in the past. Detection of incipient or operationally and unacceptably high levels of physiological resistance will help public health workers take appropriate steps to counter the reductions in effectiveness of control efforts that may accompany emerging problems of insecticide resistance. Furthermore, cross-resistance or resistance as a result of agricultural uses of insecticides may evolve and adversely impact the options to switch to an alternative insecticide for disease control.

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APPENDIX B

Biochemical detection of pyrethroid resistance mechanisms in *Anopheles minimus* in Thailand

Theeraphap Chareonviriyaphap[1,5], Pornpimol Rongnoparut[2], Piyanuch Chantarumporn[3], and Michael J. Bangs[4]

[1]*Department of Entomology, Faculty of Agriculture, Kasetsart University; Bangkok 10900 Thailand*
[2]*Department of Biochemistry, Faculty of Science, Mahidol University; Bangkok 10400 Thailand*
[3]*Department of Pest Management, Faculty of Natural Resources, Prince of Songkhla University; Songkhla 90110 Thailand*
[4] *U.S. Naval Medical Research Unit No. 2, Kompleks P2M/PLP, Jl. Percetakan Negara No. 29, Jakarta 10560 Indonesia*
[5]*Corresponding author*

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ABSTRACT: Enzyme-based metabolic mechanisms of insecticide resistance were investigated, comparing a deltamethrin-susceptible parent stock and resistant colonies of *Anopheles minimus* species A using biochemical assays. The control parent colony was determined susceptible to the diagnostic lethal concentration of deltamethrin (0.05%), whereas the 6 resistant test populations at selected 4, 8, 12, 14, 16, and 18 filial generations (F_4, F_8, F_{12}, F_{14}, F_{16}, and F_{18}) demonstrated varying levels of tolerance/resistance to deltamethrin. Expression of levels of non-specific esterases, monooxygenases, and glutathione S-transferases (GSTs) were measured. Results indicated that monooxygenase activity was consistently elevated in resistant-selected test populations compared to the parent colony and increased as resistance intensified from F_8 to F_{18}. There was a 5-fold increase in monooxygenase in the F_{18} generation compared to the parental stock. Fluctuations in alpha and beta-esterase activity, measured by hydrolysis of alpha and beta-naphthylpropionate, provided no conclusive evidence of an association with pyrethroid resistance in this mosquito species. GSTs were not elevated in the 6 resistant test populations. Based on our results, it appears likely that the development of physiological resistance to deltamethrin in laboratory, resistant-selected generations of *An. minimus* is primarily associated with increased detoxification by over-expression of monooxygenases. The oxidases are the major contributors to pyrethroid resistance and the importance of *kdr* has yet to be convincingly determined. This finding represents the first report from Thailand of this metabolic mechanism of resistance in anophelines. *Journal of Vector Ecology* **28(1): 108-116. 2003.**

Keyword Index: Pyrethroids, deltamethrin, resistance, esterases, monooxygenases, glutathione s-transferases, *Anopheles minimus*, Thailand.

INTRODUCTION

Over half of the world's population resides in malarial areas, resulting in an estimated 2 to 3 million deaths annually from the disease (WHO 1996). The burden of malaria is increasing, in part, because of drug and insecticide resistance and complex social and rapid environmental changes that have intensified in the last several decades (Greenwood and Mutabingwa 2002), as well as a general breakdown of organized effective malaria control activities. In general, most countries in Southeast Asia where malaria is endemic are experiencing increased malaria problems resulting from sociological and ecological changes stemming from poorly controlled population movement and extensive exploitation of natural environments. In Thailand, malaria remains one of the most important infectious diseases affecting rural populations with over 100,000 cases reported annually during each of the last 10 years (Chareonviriyaphap et al. 2000). Recent medical surveillance indicates that malaria has expanded in the country and continues to be a serious concern along the undeveloped frontier borders with eastern Myanmar and western Cambodia (Annual Malaria Reports 1995-2001).

The prevention of malaria transmission in Thailand has relied mainly on accurate diagnosis, prompt effective

treatment of infection and reduction of the human-vector contact using residual chemical compounds (Chareonviriyaphap et al. 1999, 2000). For decades, DDT was used for malaria vector control as an interdomiciliary spray. Use of DDT in Thailand was gradually discontinued between 1995-2000 because of social and environmental concerns and replaced with synthetic pyrethroids, the current class of compounds used for vector abatement in Thailand. Pyrethroids have been used extensively for the insecticide treatment of bednets (ITN) and as indoor residual spray (IRS) in many parts of the country. The two most common compounds in use, permethrin and deltamethrin, have shown to be effective in both killing and eliciting behavioral avoidance responses (irritancy and repellency) on mosquitoes (Chareonviriyaphap et al. 2001).

Anopheles minimus (Theobald) species A is an important malaria vector throughout much of Thailand (Baimai 1989). This sibling species is considered sufficiently anthropophilic, readily entering houses and coming into contact with residual insecticides placed on bednets and interior wall surfaces (Nutsathapana et al. 1986, Chareonviriyaphap et al. 2001). Repeated contact with these insecticides has led, in some cases, to high levels of resistance in vector populations. Pyrethroid resistance has been documented in species of malaria vectors in Thailand (Chareonviriyaphap et al. 2002). Development of resistance to pyrethroids occurred in a population of *An. minimus* species A from northern Thailand within only 1 year following the introduction of pyrethroids into the vector control program (Chareonviriyaphap et al. 1999).

The increased development of mosquito resistance to pyrethroids is of particular concern for many integrated malaria control programs that utilize insecticides for vector transmission control (Brogdon and McAllister 1998). Common insecticide resistance mechanisms in insect pests against pyrethroids include P450 mediated monooxygenases, elevated non-specific esterases, and reduced sensitivity of sodium ion channels along nerve axons (Oppenoorth 1985, Georghiou 1986, Nelson et al. 1996, Roberts and Andre 1994, Scott et al. 1998, Feyereisen 1999). Moreover, increased levels of glutathione S-transferases (GSTs) have been associated with conferring pyrethroid inhibition in many insect species (Lagadic et al. 1993, Reidy et al. 1990), including *Aedes aegypti* (Grant and Matsumura et al. 1988), *Anopheles gambiae* (Ranson et al. 2001) and *Anopheles dirus* B (Prapanthadara et al. 1998). More recently, elevated GSTs have been found to bind to molecules of many pyrethroid insecticides compromising effectiveness and toxicity by a sequestering mechanism (Kostaropoulos et al. 2001).

Although the spread of pyrethroid resistance has increased in disease vectors worldwide, the actual operational impact of resistance in control of disease vectors and transmission remains limited, especially with malaria vectors in Thailand. Deltamethrin resistant *An. minimus* were established through a careful series of laboratory selection procedures. This strain conferred 52% resistance to deltamethrin with a > 25-fold increase in the LD_{50} from the parent colony (Chareonviriyaphap et al. 2002). In this study, we conducted a series of biochemical enzyme assays integrated with dose-mortality bioassays for detection of resistance and to define the underlined mechanisms involved in pyrethroid resistance in *An. minimus*.

MATERIALS AND METHODS

Test populations

A susceptible colony of *Anopheles minimus* species A was received from the Malaria Division, Department of Communicable Disease Control, Ministry of Public Health, Nonthaburi, Thailand in 1997. The origin and detailed background of this colony has been reported elsewhere (Chareonviriyaphap et al. 2002).

Deltamethrin-susceptible female mosquitoes were subjected to selection pressure against deltamethrin using dose and time mortality relationships at 50% mortality (LD_{50}/LT_{50}) cut off points per generation, as monitored by the World Health Organization (WHO) adult mosquito bioassay procedure (WHO 1998) as previously described (Chareonviriyaphap et al. 2002). One susceptible and 6 deltamethrin-selected generations were used for comparisons, F_0 (susceptible colony), F_4, F_8, F_{12}, F_{14}, F_{16}, and F_{18} to measure levels of detoxifying enzymes over the period of increasing selection pressure. The parent F0 control generation was completely susceptible to deltamethrin and DDT at recommended diagnostic dosages (0.05% and 4% respectively). This colony was maintained in the separate, deltamethrin contamination-free room and was tested repeatedly to monitor for independent occurrence of resistance. The subsequent deltamethrin tolerant/resistant generations of *An. minimus*, F_4, F_8, F_{12}, F_{14}, F_{16}, and F_{18} test populations were obtained and preserved after each selection period. Based on the WHO diagnostic test criteria (WHO 1998), the F_4 and F_8 colony were defined as "tolerant" to deltamethrin and DDT. With increasing selection, F_{12}, F_{14}, and F_{16} demonstrated approximately 20%, 24% and 36% resistance to deltamethrin and 20%, 24% and 30% to DDT, and this increased further in F_{18} to 50% and 35% resistance to deltamethrin and DDT, respectively.

Mosquito rearing

Standard procedures for rearing anophelines followed Ford and Green (1972). All life stages were reared in an environmentally controlled insectary (25 ± 3°C, 80 ± 10%RH) at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, as described previously (Charconviriyaphap et al. 2002).

Diagnostic susceptibility assay

Insecticide susceptibility bioassays were conducted using WHO test kits for adult mosquitoes (WHO 1981a). *Anopheles minimus* females were exposed to diagnostic dosages of 0.05% deltamethrin. For each test, 5 cylinders, 2 serving as controls and 3 as treatments, were used. Control cylinders contained filter paper impregnated with carrier only, while treatments contained paper impregnated with the diagnostic dosage of insecticide plus carrier. Twenty unfed female mosquitoes were allowed contact for 1 h within the cylinder placed in a vertical position. Mosquitoes were then transferred to clean holding containers and provided with cotton pads soaked with 10% sucrose solution. Mortality was recorded at 24 h post-exposure. Each test was replicated 3 times. Resistant status was determined according to WHO criteria; populations were considered resistant if more than 20% of individuals survived the diagnostic dose after 24 h compared to the susceptible control (WHO 1981b). Susceptibility to DDT (4%) was measured using the same method in generations undergoing selection against deltamethrin to assess levels of cross-resistance between insecticides.

Protein assay

The total protein content of individual *An. minimus* mosquitoes was determined using a commercial protein assay system (BioRad, Hercules, CA). Individual, freshly-killed mosquitoes were homogenized in 0.5 ml of phosphate buffer (0.2 mol, pH 7.0) with plastic microcentrifuge tube and pestle. The homogenate was frozen at –70°C until the assay was performed using 5 µl aliquots in microtiter plates, and results were compared to a derived standard curve. The plates were read after 5 min using an ELISA plate reader at 595 nm wavelength.

Enzyme determination assays

Monooxygenases

The procedure described by Vulule et al. (1999) was followed with only minor modifications. Fresh individual mosquitoes were homogenized in 50 ml distilled water in a 1 ml plastic vial. Homogenates were diluted with an additional 150 µl distilled water. Twenty µl of each homogenate was transferred to a microplate followed by addition of 80 µl 0.0625 M potassium phosphate buffer (PPB) at pH 7.0. 0.01 g of 3,3, 5',5'- Tetramethyl Benzidine (TMBZ) in 5 ml methanol was prepared and a 0.25 M sodium acetate buffer (pH 5.0) was added. Two hundred µl of TMBZ solution was then added with the 100 µl of mosquito homogenate plus PPB in each well followed by 25 µl of 3% hydrogen peroxide. The plates were read after 5 and 10 min using an ELISA plate reader at 620 nm wavelength. The quantity of monooxygenases was calculated from a standard curve using control enzyme reagents. Results were expressed as m-mole of product/min/mg protein per mosquito.

Non-specific esterases

The method of Peiris and Hemingway (1990) was used with one modification: alpha and beta-naphthyl propionate replaced alpha and beta-naphthyl acetate. The quantity of naphthol produced from the esterase reactions was calculated from standard curves of alpha and beta naphthol. The plates were read immediately after 10 min using the ELISA plate reader at 450 nm wavelength. Results were expressed as m-mole of product/min/mg protein per mosquito triturate.

Glutathione S- transferases

GST activity was assayed following the method of Brogdon and Barber (1990) with some minor technical modifications. Individual mosquitoes were homogenized in 50 µl of distilled water in 1 ml plastic vials. The homogenates were diluted with an additional 150 µl of distilled water, and 20 µl of each homogenate was transferred to a microplate well. Fifty µl of glutathione solution [0.03 g of glutathione in 50 ml PPB] and 50 µl of 1-chloro-2, 4-dinitrobenzene (CDNB) (0.01 g CDNB in 0.5 ml acetone, plus 50 ml of PPB) were added into each well. The plates were read after 30 min with the ELISA plate reader at a wavelength of 414 nm.

Data analysis

Abbott's formula was used to correct the observed mortality in adult susceptibility tests (WHO 1981a). The LC_{50} and LC_{90} values were estimated using dosage-mortality regression probit analysis (Finney 1971). Observed differences in resistance between generations were analyzed by Student's *t*-test. A one-way analysis of variance (ANOVA) was used to compare the protein content and enzyme expression levels within and between populations. All levels of statistical significance were determined at $P<0.05$.

RESULTS

Anopheles minimus species A was artificially selected for deltamethrin resistance and each generation

Table 1. Results of contact susceptibility bioassays of parent control population F_0, and F_4, F_8, F_{12}, F_{16} and F_{18} serial generations of *An. minimus* selection against deltamethrin esposure using WHO diagnostic test procedures with 0.05% deltamethrin and 24 h post-exposure mortality (Chareonviriyaphap et. al. 2002).

Sample	No.tested	LD_{50} (%)	LD_{90} (%)	Level of Resistance to Deltamethrin (%)	Level of Resistance to DDT (%)
F_0	240	0.00035	0.00137	0	0
F_4	360	0.00030	0.00283	0	0
F_8	360	0.00603	0.02060	10	10
Sample	No. tested	LT_{50} (min)	LT_{90} (min)	Level of Resistance to Deltamethrin (%)	Level of Resistance to DDT (%)
F_{12}	NA	NA	NA	20	20
F_{14}	360	19.07	75.22	24	22
F_{16}	360	31.97	97.82	36	30
F_{18}	360	47.54	185.25	52	37

NA: Not Applicable.

of adult female mosquitoes was measured independently for susceptibility to deltamethrin using contact bioassays (Table 1). Resistance to deltamethrin steadily increased with succeeding generations of exposure during the selection process (Chareonviriyaphap et al. 2002). An ANOVA found no significant differences in the total protein content among the susceptible control and the 6 generations exposed to deltamethrin ($P>0.05$) despite different levels of resistance (Table 2). All enzyme activities were calculated based on 1 mg protein levels (Table 2). Non-specific esterases, monooxygenase and GST assays were performed on the susceptible and resistant generations of *An. minimus* with sample sizes ranging from 20 to 30 mosquitoes per generation and assay (Tables 2 and 3). Alpha and beta-esterase activities fluctuated greatly between generations tested, whereas monoxygenase activity consistently increased throughout the selection period (Table 2). Alpha-esterase activity increased from the susceptible parent control to F_8 (0.1165 ± 0.0375), but decreased in F_{12} (0.0704±0.0160), and was again elevated in F_{18} (0.1336±0.0542). In all 6 exposed generations alpha-esterase was significantly elevated above the parent control (0.0325 ± 0.0182). Beta-esterase activity was found significantly reduced in F_{18} (0.0484 ± 0.0165) compared to F_4 (0.0503±0.0211), F_8 (0.1079± 0.0519), F_{12} (0.0634±0.0393), and F_{14} (0.0638±0.0280) generations despite increased insecticide selection pressure ($P<0.05$). There were no significant differences in beta-esterase levels observed between susceptible parent (0.0404± 0.0216) and the F_{18} (0.0484 ± 0.0165) resistant generation ($P>0.05$).

There was clear evidence of higher levels of monooxygenase detected in selected colonies over the parent colony. Increased specific enzyme activity was correlated well with the increased development of physiological resistance to deltamethrin (Table 2). There was an approximately 5-fold increase in monooxygenase expression in F_{18} (50% resistance level) compared to the parent susceptible (F_0), and a 4.5 fold increase in F_{18} compared to F_{12}.

The intensity of GST indicated no statistically significant differences in enzyme expression between control and selected colonies (Table 3). There were no significant differences in protein content for all 4 generations tested (Table 3). The results suggest that monooxygenases are the probable mechanism for detoxification of pyrethroids and are involved in the development of physiological resistance to deltamethrin in *An. minimus* species A in Thailand. The inconsistent levels of alpha-esterase activity among the different generations provide inconclusive evidence as to the possible role of non-specific esterases in resistant populations, but they may play a contributing role in resistance development.

DISCUSSION

Vector control in Thailand relies mainly on the reduction of human-vector contact by using chemical compounds. Indoor residual spray with deltamethrin and insecticide treated nets (ITN) using permethrin are advocated as standard tools for malaria vector control in Thailand (Annual Malaria Reports 1995-2001). *Anopheles minimus* is regarded as a major malaria vector and distributed throughout the country, closely associated with humans in the foothill rural village areas where malaria remains a serious health problem (Nutsathapana et al. 1986, Annual Malaria Reports 1995-2001). Based on field and laboratory data, this species is likely to be regularly exposed to residual insecticides used for IRS and ITN (Chareonviriyaphap et al. 2000, 2001). Selection pressure on natural vector populations exposed to frequent contacts with residual chemicals during blood locating activities and exposure to sprayed resting sites may increase the amount of detoxification enzymes produced that are responsible for insecticide resistance (Ferrari and Georghiou 1990). For these reasons, *An. minimus* was selected as a representative vector for the study of development of deltamethrin resistance in response to vector control in Thailand.

Insect populations may survive the effect of toxic chemical compounds by different physiological mechanisms including reduced target site sensitivity and elevated detoxifying enzyme production (Martinez-Torres et al. 1998, Brooke et al. 1999). Four primary insecticide resistant mechanisms have been reported associated with pyrethroid resistance, i.e., over-expression and increased production of monooxygenases (sometimes referred to as mixed function oxidases), non-specific esterases, GSTs and reduced sensitivity of sodium ion channels on the nerve membrane (*'kdr'* knock down resistance), the target site for DDT and pyrethroids (Oppenoorth 1985, Georghiou 1986, Grant and Matsumura et al. 1988, Nelson et al. 1996, Chandre et al. 1999). All three of these major groups of enzymes have been implicated in promoting detoxification of pyrethroids in resistance insects (Brogdon and McAllister 1998, Vulule et al. 1999). In general, quantitative increases in these enzymes, associated with gene amplification or over-expression of target genes, can result in protein overproduction in insects under selection pressure, thus conferring insecticide resistance (Mouches et al. 1990).

Monooxygenases have been associated with pyrethroid resistance in mosquitoes (Hemingway and Ranson, 2000), although it appears a much more common phenomenon in house flies. Elevated monooxygenases have been responsible for degradation of pyrethroids in *Anopheles pseudopunctipennis* (Ocampo et al. 2000) and *Anopheles funestus* in Africa (Brooke et al. 2001). Monooxygenases are a chain of enzymes, with the rate-limiting enzyme usually being cytochrome P450 (Nelson et al. 1996). Alterations in this rate-limiting enzyme can dictate levels of resistance to pyrethroids, organophosphates, and carbamate insecticides using this metabolic mechanism. In our study, increases in specific enzyme activity in selected generations accompanied decreased toxicity changes based on contact bioassay results on adult insects. There was a 5-fold increase in specific monooxygenase activity in the F_{18} deltamethrin-resistant generation compared to the initial parent colony. *Anopheles minimus* species A used in this study was collected from Rong Kwang District, Prae Province in 1993. This area was previously sprayed as IRS with DDT, either once or twice a year for malaria control, beginning in 1950. Additionally, DDT and other related chlorinated hydrocarbon pesticides were commonly used for crop protection against agricultural pests and termite protection of structures. In our study, *An. minimus* demonstrated susceptibility levels to DDT from 90% mortality in F_8 to 63% mortality by F_{18}, indicating possible cross-resistance between deltamethrin and DDT. Deltamethrin resistance may have been promoted from previous DDT usage in the area, wherein selection of resistance by one insecticide leads to a much broader spectrum of resistance, including insecticides an insect does not normally encounter. Cross-resistance can occur as a consequence of the similar mode of action of DDT and pyrethroids on sodium channels target sites on nerve axons, resistance resulting in a phenomenon known as "knock down resistance" (*kdr*). *Kdr* is conferred by the substitution of one amino acid in the sodium-channel insecticide-binding site in the nerve sheath. The actual mechanism of the apparent cross-resistance in *An. minimus* to DDT reported here must await further studies at the molecular level (Bloomquist 1996, Brooke et al. 1999). Nevertheless, there is good evidence to support cross-resistance between DDT and pyrethroids in various mosquito species. Previous studies have shown that pyrethroid-resistant populations of *Aedes aegypti* in Thailand are frequently resistant to DDT (Prasittisuk and Busvine 1977, Brealey et al. 1984). Pyrethroid resistance in *Anopheles stephensi* has been reported from DDT resistant strains (Omar et al. 1980, Verma and Rahman 1986), and more recently, pyrethroid resistance in *An. gambiae* in many West Africa countries has been linked, to a certain extent, by the past intensive use of DDT (Chandre et al. 2000).

Elevation of one or more broad substrate spectrum esterases is a common mechanism of insecticide resistance, especially in a number of *Culex* species, but

Table 2. Comparison of specific activities of α and β non-specific esterases and monooxygenases from *An. minimus* susceptible control and 6 deltamethrin-exposed selected test populations.

Test population Generation time deltamethrin exposure	**Total protein** Mean (+SD) mg-protein/ml per mosquito (n)	**α Esterase** Mean (+SD) m-mole α naphthol/min/mg protein (n)	**β Esterase** Mean (+SD) m-mole β naphthol/min/mg protein (n)	**Monooxygenases** Mean (+SD) m-mol-products /min/mg protein (n)
F_0	0.7818+0.1836[a](20)	0.0325+0.0182[a](20)	0.0404+0.0216[a](20)	4.2550+0.2261[a](20)
F_4	0.7490+0.0668[a](20)	0.0577+0.0143[b](20)	0.0503+0.0211[a](20)	4.1695+0.2097[a](20)
F_8	0.7476+0.1725[a](30)	0.1165+0.0375[c](30)	0.1079+0.0519[b](20)	4.74+1.1988[b](20)
F_{12}	0.7405+0.1247[a](20)	0.0704+0.0160[b](20)	0.0634+0.0393[a](20)	4.8200+0.2239[b](20)
F_{14}	0.6885+0.1438[a](20)	0.0754+0.0365[b](20)	0.0638+0.0280[a](20)	5.14+2.8156[c](20)
F_{16}	0.7185+0.1083[a](20)	0.0854+0.0372[b](20)	0.0441+0.0245[a](20)	15.24+1.8520[d](20)
F_{18}	0.7385+0.1230[a](30)	0.1336+0.0542[d](30)	0.0484+0.0165[a](30)	21.90+0.8534[e](20)

Within a column, same letter denotes no significant differences at 0.05 level of probability.
(n) = sample size in parenthesis.

Table 3. Optical density (OD) of glutathione S-transferases towards CDNB from *An. minimus* susceptible control and 3 deltamethrin-exposed test populations.

Test population Generation time deltamethrin exposure	Mean (+SD) mg protein/ml/ mosquito (n)	OD value at 414 nm
F_0 (susceptible)	0.5887+0.1977[a](20)	0.0279 ± 0.0121[a]
F_4 (tolerant)	0.6319+0.0962[a](20)	0.0156+0.0088[b]
F_{12} (20% resistance)	0.4999+0.2050[a](20)	0.0254 ± 0.0111[a]
F_{18} (50% resistance)	0.6385+0.1230[a](30)	0.0306 ± 0.0139[a]

Within a column, same letter denotes no significant difference at 0.05 level of probability.
(n) = sample size in parenthesis.

is apparently much less common in *Anopheles*. Most pyrethroid compounds, including deltamethrin, contain an ester linkage that is susceptible to hydrolysis by esterase (Oppenoorth 1985). Previous use of organophosphate and carbamate compounds may induce increased esterase production that confers cross-resistance to pyrethroids as seen in *Anopheles albimanus* from Guatemala (Brogdon and Barber 1990). Associated elevated esterase levels have been documented in many pyrethroid- resistant insects (Abdel-Aal and Soderlund 1980, Riskallah 1983, Beach et al. 1989, Rodriguez et al. 1997, Penilla et al. 1998), including *An. gambiae* from Africa (Vulule et al. 1999, Chandre et al. 1999). In our study, there was approximately a 1.8 to 4.1-fold increase in hydrolysis of alpha-naphthylpropionate to alpha-naphthol in homogenates from selected generations compared to the susceptible parent colony, but there was no observed increase in specific activity of beta esterase in F18 compared to the control. This indicates that the beta structure of the esterase enzymes does not appear to be responsible for resistance in *An. minimus*.

GSTs have been reported to play a significant role in detoxification and resistance to DDT (Ranson et al. 1997, Prapanthadara et al. 1998) and appear as a defense against pyrethroids in certain insects (Kostaropoulos et al. 2001). This enzyme appears to play an important role in many DDT-resistant insects including *Anopheles dirus* species B from Thailand and *An. gambiae* in Africa (Prapanthadara et al. 1998, Ranson et al. 2001). In contrast, GSTs were found to play only a minor role as a detoxifying enzyme in pyrethroid-resistant *An. funestus* (Brooke et al. 2001). Likewise, this enzyme was not associated with pyrethroid/DDT resistance in our *An. minimus* selected generations.

Because IRS and ITN applications depend on the use of synthetic pyrethroids as a primary malaria vector control method in Thailand, careful and routine detection and monitoring of insecticide susceptibility levels of vector populations in malaria endemic areas should be conducted over a wide geographical range to include as many known vector species as possible. Early detection of operationally unacceptable levels of resistance can prompt public health authorities to take appropriate mitigating steps to counter problems of resistance (Penilla et al. 1998). Furthermore, cross-resistance or unsusceptibility as a consequence of unintentional or extensive use of the same or related chemicals against mosquito populations and agricultural pests, remains poorly investigated in Thailand. Ongoing research will attempt to identify genes coding for deltamethrin resistance in our resistant colony. Metabolic detoxification of pyrethroids involved with increased monooxygenase production in mosquitoes will also be the subject of further investigation.

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APPENDIX C

Cloning of cytochrome P450, *CYP6P5*, and *CYP6AA2* from *Anopheles minimus* resistant to deltamethrin

Pornpimol Rongnoparut[1,3], Soamrutai Boonsuepsakul[1], Theeraphap Chareonviriyaphap[2], and Naowarat Thanomsing[1]

[1]*Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand*
[2] *Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand*
[3]*Corresponding author*

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ABSTRACT: Two new genes in the cytochrome P450 (*CYP6*) family 6 with complete coding sequences were cloned and sequenced from deltamethrin-resistant *Anopheles minimus*, a major malaria vector in Thailand. *CYP6P5* encodes a protein of 508 amino acids, while *CYP6AA2* contains 505 residues. Each encoded protein contains a hydrophobic N-terminal region and a highly conserved heme-binding region typical of P450s. Alignments of deduced amino acid sequences with other insect P450 genes indicate a high degree of identity to insect *CYP6* genes. Comparative mRNA expression studies using semi-quantitative RT-PCR analysis indicated that the relative amount of CYP6AA2 transcript was greater in the deltamethrin-resistant *An. minimus* compared to the susceptible strain. The expression of CYP6AA2 in deltamethrin-resistant mosquitoes is associated with development of deltamethrin resistance in *An. minimus* mosquito. The CYP6P5 transcript is equally expressed in both resistant and susceptible mosquitoes. ***Journal of Vector Ecology* 28(2): 2003.**

Keyword Index: Cytochrome P450 monooxygenases, cloning, insecticide resistance, deltamethrin, *Anopheles minimus*.

INTRODUCTION

The cytochrome P450 monooxygenases (P450s) or CYPs constitute a family of enzymes involved in the metabolism of a wide variety of endogenous and exogenous compounds such as steroids, fatty acids and xenobiotics (Feyereisen 1999). These divergent enzymes have multiple and overlapping substrate specificities. Examining P450 gene diversity in insects has revealed numerous P450 forms. For example, 17 genes of the *CYP4* family were identified from *Anopheles albimanus* mosquitoes (Scott et al. 1994), 14 P450 genes were identified from the Mediterreanean fruit fly *Ceratitis capitata* (Danielson et al. 1999), and 8 *CYP4* genes from *Helicoverpa armigera* (Pittendrigh et al. 1997). Recently, sequencing of *Drosophila melanogaster* genome has uncovered total P450 diversity in this species, with 90 P450 genes found (Adams et al. 2000).

Insect P450s have been implicated in insect growth, development, reproduction, insecticide resistance and tolerance to plant toxins (Feyereisen 1999, Hodgson and Kulkarni 1983, Scott et al. 1998). The cytochrome P450 enzymes confer insecticide resistance in populations of insects via an increased level of P450 activities resulting from elevated expression of P450 genes. An example is provided by CYP6D1, a P450 isolated from the house fly, *Musca domestica* (Tomita and Scott 1995). CYP6D1 was previously shown to metabolize pyrethroids at a higher level in a Learned Pyrethroid Resistant strain (Wheelock and Scott 1992), leading to increased pyrethoid detoxification and resistance. Consequently, the levels of CYP6D1 transcript and protein are elevated in the pyrethroid resistant flies compared to a susceptible strain (Kasai and Scott 2000, Tomita et al. 1995). In the multi-resistant Rutgers strain of house fly, the CYP6A1 mRNA level was higher than in the susceptible strain (Carino et al. 1994, Feyereisen et al. 1995). Other instances have shown that resistance is associated with increased expression of certain P450 mRNAs. This includes the over-expression of *Cyp6a2* in the RDDT^R insecticide-resistant strain of *Drosophila melanogaster* compared to the Canton^S sensitive strain (Brun et al. 1996), *CYP6B7* in a pyrethroid-resistant strain of *Helicoverpa armigera* (Ranasinghe and Hobbs 1998), *CYP6F1* in pyrethroid resistant *Culex quinquefasciatus* Say (Kasai et al. 2000), *CYP9A1* in a thiodicarb-selected resistant population of

Heliothis virenscens (Rose et al. 1997), and *CYP4G8* in a pyrethroid-resistant strain of *Helicoverpa armigera* (Pittendrigh et al. 1997). These broad comparative forms of P450s involved in insect detoxification have made it difficult to identify individual P450 genes that confer insecticide resistance.

Anopheles minimus is one of the most efficient malaria vectors in the Mekong region of Southeast Asia, including Thailand, Laos, Cambodia and Vietnam. This species is considered endophagic and endophilic, allowing contact with insecticide residues sprayed in houses and thus considered as an excellent model for the study of insecticide resistance in Thailand (Nutsathapana et al. 1986). In Thailand, indoor house spraying with DDT has been used in malaria control for decades[4]. Synthetic pyrethroids are promising insecticides that have replaced DDT in vector control in Thailand (phase out period, 1995-1999). The intensive and sometime indiscriminate use of pyrethroids in agriculture and for the control of disease vectors can lead to development of pyrethroid resistance (Miller, 1988). Pyrethroid resistance was recently reported in a population of *An. minimus* from northern Thailand[4].

We have recently established a deltamethrin-resistant mosquito strain of *An. minimus* species A (Chareonviriyaphap et al. 2002). The resistant strain showed an elevated level of mixed function oxidases compared to a susceptible strain, implicating P450 as a primary route of detoxification in this strain of *An. minimus*. Although insecticide resistance mechanisms mediated by cytochrome P450s in insects have been extensively studied, there is no published report of the role that the *CYP6* family plays in pyrethroid resistance in malaria vectors. To identify resistance-associated cytochrome P450 in *An. minimus*, we focused on isolation of *CYP6* gene members and investigated whether there is increased expression at the transcription level in the pyrethroid-resistant mosquito. In this article we report the isolation and sequence analysis of 2 genes encoding cytochrome P450s in *An. minimus*. Semi-quantitative RT-PCR analysis indicated an increased expression of CYP6AA2 mRNA in the *An. minimus* resistant to deltamethrin, while CYP6P5 mRNA are equally expressed in both resistant and susceptible mosquitoes.

MATERIALS AND METHODS

Mosquito test populations

Three test populations of *An. minimus*, one susceptible (F_0) and 2 deltamethrin-resistant (F_{13} and F_{19}), were used for this study. At the time of selection, the parent colony remained susceptible to both deltamethrin (0.05%) and DDT (4%) based on WHO bioassay[5,6]. The resistant colonies (F_{13} and F_{19}) are established from the susceptible strain (F_0) by sequential exposure of females to increasing doses of deltamthrin and measuring the progress of selection by the standard WHO diagnostic test[6]. The original and detailed backgrounds of *An. minimus* from this study were recently published (Chareonviriyaphap et al. 2002).

Genomic DNA amplification and cloning

Genomic DNA was isolated from the F_{13} adult mosquitoes. The degenerate primers used were the antisense primer 5' CG (G/A/T/C) (T/G) G (G/T/C) CC(T/C) TC (A/G/T) CC (G/A) AACGG 3' (CYPR) corresponding to conserved amino acids FGDGPR surrounding the heme binding site, and the sense primer 5' GATGTGAT (T/C) GG (A/C/T) AG (C/T) GT (G/A/C/T) GC (C/G) TT (G/T/C) GG 3' (CYPFL) corresponding to VIGXCAFG (see Figure 2 for locations of primers). PCR reaction was performed in a DNA thermal cycler (PE Applied Biosystems, Boston, Maryland, USA) using one denaturation step at 94°C for 10 min, followed by 30 cycles of 1 min each at 94°C, 58°C, and 72°C. The last elongation step was lengthened to 10 min at 72°C. PCR product with the expected size was subjected to cloning in the pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). Clone products were sequenced with dye terminator sequencing using an ABI 377 automated DNA sequencer at Bioservice unit, National Science and Technology Development Agency, Bangkok, Thailand.

RT-PCR and cloning of partial cDNA

Completely F0 susceptible or the F_{13} deltamethrin selected adult mosquitoes were homogenized and total RNA was prepared using NucleoSpin RNA II kit (Macherey-Nagel, rue Gutenberg, France) following manufacturer's instructions. RNA was reverse transcribed to single-stranded cDNA using Superscript RNaseH- reverse transcriptase kit (Gibco/BRL, Rockville,

[4]Annual Malaria Reports 1980-2001. Malaria Division, Department of Communicable Disease Control, Ministry of Public Health, Thailand.

[5]WHO 1981. Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorine, organophosphate and carbamated insecticide-diagnostic test. WHO/VBC/81.806, Geneva, Switzerland, 6 pp.

[6]WHO 1998. Test procedure for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces.

Maryland, USA) and CYPR primer. PCR was performed on the resulting cDNAs with CYPFS, 5 'A (G/A) AC (T/G) CTTCG (C/T) AAGTA (T/C) CC 3', corresponding to the amino acid residues ETLRKYP in the ETLR motif and a CYPR primer (Figure 2). PCR products from susceptible and resistant strains were cloned into pGEM-T Easy vector and clone products were sequenced as previously described.

Genomic DNA walking, 5' RACE, 3'RACE and full coding regions of *CYP6P5* and *CYP6AA2*

The corresponding genomic DNA for AN1 and AN40 clones were walked towards the 5' region of the genes employing Genome WalkerTM kit (Clontech, Hampshire, UK). Thereafter, 5'- and 3'-RACE were performed to identify transcriptional ends of the AN1 and AN40 gene fragments. 5' and 3' RACE followed the Smart RACE cDNA amplification protocol (Clontech). Genome walking, 5' and 3' RACE by PCR were conducted with the primers provided with the kits. Typical PCR cycle parameters on the PE 2400 Thermal Cycler (PE Applied Biosystems) followed the conditions recommended by the supplier. To obtain intact *CYP6P5* genomic DNA containing full coding region, primers synthesized spanning start and stop codon sequences (derived from the sequence information of 5'RACE and 3'RACE products) of *CYP6P5* were used for PCR amplification. PCR amplification on *CYP6P5* on genomic DNA used AN40F (5' ATGGAGCTCATTAACCT AGT 3') and INTAN40R (5' CTACACCTTTTCCACCTTCA 3') primers. To obtain full coding CYP6AA2 cDNA, RT-PCR was performed following the Smart RACE cDNA amplification protocol. The full coding CYP6AA2 cDNA was synthesized using antisense primers provided with the kit for first strand cDNA synthesis and PCR amplified with AN1F (5' GTCGAGCGCAGTTGTATG 3') sense primer and the kit's antisense primer. All PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced. All sequence information in this study was derived from sequencing on both strands of DNA.

Semi-quantitative RT-PCR analysis

RT-PCR was performed with RNA samples isolated from F_{13} and F_{19} deltamethrin-resistant and susceptible adult mosquitoes. A comparative study of the expression of *CYP6P5* and *CYP6AA2* in resistant and susceptible strains was carried out with specific oligonucleotide primer pairs for the two genes. Two pairs of primers were designed according to *CYP6AA2* sequence. One pair, QAN1-1 (5' AACGGAATGCGATAGTACGGC 3') and QAN1-2 (5' TTTCCAACACTTCGGCACGCA3') and another pair, QAN1-3 (5' CTGGAGGCATCATTCCGGTT 3') and QAN1-4 (5' CGATCTCACAAATCGTGGTAAA CATC 3') were used for RT-PCR in F_{13} and F_{19} deltamethrin-resistant mosquitoes, respectively. The primer pair INTAN40F: (5' GTTGAGGAGAATGATGGACA 3') and INTAN40R: (5' CTACACCTTTTCCACCTTCA 3') was used in RT-PCR of *CYP6P5*. For RT-PCR of internal actin gene control, actin primer pair sequences (ACTF, 5'-AGCAGGA GATGGCCACC-3' and ACTR, 5'-TCCACATCTGCTGGAAGG-3') were designed following previously published primer sequences (Kasai et al. 1998). Upon obtaining actin cDNA product, we sequenced to confirm its identity as actin 1 D cDNA (data available upon request). Antisense primers were used for first strand cDNA synthesis using SuperscriptTM II RNaseH reverse transcriptase (Gibco/BRL). RT-PCR performed

Table 1. Occurrence of cDNA clones in F_{13} deltamethrin-resistant and F_0 susceptible populations of *Anopheles minimus*.

Clone	Mosquito strain			
	Resistant (n[a])	Percent	Susceptible (n[a])	Percent
AN1	9	42.8	3	17.6
AN40	3	14.3	3	17.6
AN10	4	19.0	0	0
AN36	2	9.5	1	5.9
AN4	1	4.8	1	5.9
AN17	1	4.8	2	11.8
AN8	1	4.8	5	29.4
AN38	0	0	1	5.9
AN35	0	0	1	5.9
Total	21	100	17	100

[a]Number of cDNA clones sequenced

Table 2. Percentage identity of the deduced amino acid sequences of the P450 genes[a]

P450	Percent deduced amino acid identity	
	CYP6P5	CYP6AA2
CYP6AA2	41	—
CYP6P5	—	41
CYP6A1	43	40
CYP6B1	31	33
CYP6D1	32	33
CYP6E1	39	40
CYP6F1	38	40

[a]*CYP6A1* and *CYP6D1* from *M. domestica* (Feyereisen et al. 1989, Tomita and Scott 1995), CYP6B1 from *Papilio polyxenes* (Cohen et al 1994); *CYP6E1* and *CYP6F1* from *Cx. quinquefasciatus* (Kasai et al. 1998, 2000).

with the predetermined PCR cycle at the exponential phase was compared between susceptible and resistant strains. Each cycle included denaturation at 94°C for 1 min, primer annealing at 58°C for 30 sec, and extension at 72°C for 1 min. PCR band intensities were compared between those of resistant and susceptible strains after normalization with actin standard band intensities. Band intensities were measured on a densitometer (Biorad's Image analysis model) using Molecular AnalystR TM software version 1.4.

RESULTS

Cloning of PCR and RT-PCR products

Our goal was to run a preliminary screen for sequences of *CYP6* fragments from *An. minimus*. We amplified a partial genomic DNA fragment of cytochrome P450 gene using a pair of degenerate primers in the PCR reaction. The primer sequences were based on amino acid sequences of previously isolated *CYP6* P450s from the mosquito *Culex quinquefasciatus* Say (Kasai et al. 1998, 2000), *Drosophila melanogaster* (Brun et al. 1996) and *Musca domestica* (Tomita and Scott 1995). An antisense CYPR primer was designed corresponding to amino acids of the most conserved heme-binding region among all P450 enzymes. The sense CYPFL primer specified the VIGXCAFG peptide sequences among CYP6 amino acid sequences. PCR amplification products of the expected size of ~900 bp were cloned and sequenced. One clone, the AN40, showed high amino acid sequence identity in deduced amino acids compared to those of other insect *CYP6* genes. Deduced amino acid sequence of AN40 fragment showed 49% identity to *CYP6A1* from *M. domestica* (Feyereisen et al. 1989) and 43 % identity to *CYP6F1* from *Cx. quinquefasciatus*, (Kasai et al. 2000). Alignment of AN40 sequence and of other *CYP6* genes provided information on conserved regions of a *CYP6* sequence in *An. minimus*, further facilitating the isolation of CYP6 cDNA sequences in this mosquito.

In an attempt to isolate a CYP6 cDNA associated with pyrethroid resistance in *An. minimus*, we carried out RT-PCR on total RNA isolated from completely susceptible and F13 deltamethrin-resistant strains to determine which cDNA(s) was over-represented in the resistant strain. Although several primer choices based on sequence information of the AN40 clone were selected, we found the primer pair CYPFS and CYPR the

Figure 1. Alignment of amino acid sequences deduced from the nucleotide sequences of cloned RT-PCR products, excluding priming sites.

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AN1  1 PVPQLIRVSTQPYTVEATNVTLDRDTMLMVPIYAIHHDANIYPEPERFDPDRFAPDAVHSRHTHAFL 67
AN5  1 PLESLTRVPVRDYTIPGTKHVIPKDTVIQIPVYALQHDPEFYPDPDQFNPDRFLPEEVKQRHPYVFL 67
AN8  1 PIEALSRVPNCDVTMPGTNHVIPKNTLIQIPVYAIQRDPEFYPDPDQFNPDRFLPEEVKQRHPYVFL 67
AN10 1 PLESITRAPEQDYTIPGTKHVIPKAHDGQIPIYALHHDPEYYPEPERFDPERSSRSGKRTSPYVYM 66
AN35 1 GLPILNRECTIDYPVPDSDIVIRKGTQVIIPLLSISMNEKYFPNPELYSPERF-DEATKNYDPDAYY 66
AN36 1 PLETTVRVTSQDYTIPGTEHVIPRKVGVQIPVFAIHRDPELYPDPECFDPDRFTKEESKKRPAYTFL 67
AN38 1 ALAVLNRECTIDYPVPDSDVVIRKGTQVIIPLLGISMNEKYFPNPELYSPERF-DEATKNYDPDAYY 66
AN40 1 PVESLNRVPSVDYLIPGTKHVIPKRTLVQIPVHAIQNDPDHYPDPERFDPDRFNPEEVKKRHPFTFI 67
AN41 1 PVETLTRKPARDYVIPGTKHIIPEGTIVQIPIYAIQRDPDIFPDPEHFDPDRFMPEEVK-RHPYVFL 66
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Figure 2. Comparison of deduced amino acid sequences of *CYP6P5* and *CYP6AA2* from *An. minimus* to those of other insect P450s: *CYP6E1* and *CYP6F1* from *Cx. quinquefasciatus* (Kasai *et al.*, 1998, 2000); *CYP6A1* and *CYP6D1* from *M. domestica* (Feyereisen *et al.*, 1989; Tomita and Scott, 1995). Gaps in the alignment are indicated by —. Conserved amino acids across all compared sequences are bold letters. Degenerate primers synthesized corresponding to amino acids are shown as bars.

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CYP6A1    MDFGSFLLYALGVLASLALYFVRWNFGYWKRRGIPHE-EPHLVMGNVKGLRS-KYHIGEI   58
CYP6B1    MLYLLALVTVLAGLLHYYFTRTFNYWKKRNVAG-PKPVPFFGNLKDSVLRRKPQVMV    56
CYP6D1    MLLLLLLIVVTTLYIFAKLIIYTKWERLGFESD-KATIPLGSMAKVFHKERPFGLV     54
CYP6E1    MLLYLVTIVTWLVYVWIKRRYSYWKDRGVPSL-RVSFPAGNLQGIG—HRHLGLI      52
CYP6F1    MFAWIICAAAAVPLVYFLIVYQFSYWKRRGITQL-TPSFPFGDLGPFFRQRSSLGVV   56
CYP6P5    MELINLVLAAFIFVWSVVYLFIRNKHNYWKDNGFPYAPNPIIFLFGHAKGQTL-TKHAADI 59
CYP6AA2   MGYVNVVFYLVLPALGLLYYYVKQHYRYWANRNIPQL-EASFPVGNMKGVGS-KLHFNDV  58

CYP6A1    IADYYRKFK-GSDPLPGIFLGHKPAAVVLDKELRKRVLIKDFSNFANRGLYYNEKDDPLT  117
CYP6B1    YKSIYDEFPN—EKVVGIYRMTTPSVLLRDLDIIKHVLIKDFESFADRGVEFS—LDGLG   112
CYP6D1    MSDIYDKCHEK—VVGIYLFFKPALLVRDAELARQILTTDFNSFHDRGLYVDEKNDPMS   111
CYP6E1    MQDLYGKLKGSGAKFGGIYSFLKPMVMVLDLDFAKDVLVREFQYFHDRGMYYNERDDPLS 112
CYP6F1    YADVYRLCKRLP—FVGIYLSLRPMLVVNDPELIKNVLVRDFDHFHDRGLYVNEEKDPLS  114
CYP6P5    HLELYKQFKQRGDRYVGMSQFIIPSVFVIDPELVKTIMVKDFNVFHDRGVFTNAKDDPLS 119
CYP6AA2   LGEAYDKGKSKTAPLVGLYFMLKPVLIVTDLDMVKRILVKDFNSFHDRGLYVNERDDPLS 118

CYP6A1    GHLVMVEGEKWRSLRTKLSPTFTAGKMKYMYNTVLEVGQRLLEVMYEKLEVSS—ELDMR  175
CYP6B1    ANIFHADGDRWRSLRNRFTPLFTSGKLKSMLPLMSQVGDRFINSIDEVSQTQP—EQSIH  170
CYP6D1    ANLFVMEGQSWRTLRMKLAPSFSSGKLKGMFETVDDVADKLINHLNERLKDGQTHVLEIK 171
CYP6E1    AHLVSLEGDKWKSLRTKLTPTFTSGKMKMMFGTIEEVVDRLEGCIRVRVESGE—CIEIR  170
CYP6F1    GHLFALGGEQWRHHRSKLTPTFTSGRLKEMFTNLVQIGRVLQDHVAKRAGED——IEIR   170
CYP6P5    GHLFALEGNPWRLLRQNVTPTFTSGRMKQMFGTLWDVALELDKYMEENYRQP—DMEMK  176
CYP6AA2   GHLFALDGERWRYLRNKLSPTFTSGKIKLMFTTICEIGDEFLASVTRYVDREA—PIDVK  176

CYP6A1    DILARFNTDVIGSVAFGIECNSLRNPHDRFLAMGRKSIEVPRHNALIMA—FIDSFPEL   232
CYP6B1    NLVQKFTMTNIAACVFGLNLDEG—MLKTLEDLDKHIFTVNYSAELDMM———YPGI     221
CYP6D1     SILTTYAVDIIGSVIFGLEIDSFTHPDNEFRVLSDRLFNPKKSTMLERIRNLSTFMCPPL 231
CYP6E1    DIISRFAMDVIGSCAFGLDCNSLVLSDPPFWKMSLKASTSTKLQFLISL—FATTYRKF   227
CYP6F1    DVMARYTTDIIASVGFGIENDSINEKGNIFREMGTKVFSPDLKTILRLT—STFFTPKL   227
CYP6P5    DVLGRFTTDVIGTCAFGIECNTLKTPDSEFRKYGNKAFEFNLSIMIKIF—LASSYPEL   233
CYP6AA2   LLSQCFTCDVVGSVAFGLKCNSLKNEGSKLLEIGDKVFKPPAWRNMLTF—MLISCKKM  233
                    CYPFL ——→

CYP6A1    SRKLGMRVLPEDVHQFFMSSIKETVDYREKNNIRRNDFLDLVLDLKN————————N    280
CYP6B1    LKKLNGSLFPKVVSKFFDNLTKNVLEMRKGTPSYQKDMIDLIQELREKKTLELSRKHE—  279
CYP6D1    AKLLSRLGAKDPITYRLRDIVKRTIEFREEKGVVRKDLLQLFIQLRNTGKISDDNDKLWH 291
CYP6E1    SNQIGICVLPNDVSDFYLGAVRDTIKFRMDNQASRKDFMDLLIKLED————————     274
CYP6F1    NALFGFKFIAQEIEDFIMNVVRETLEYRESNKVVRKDMMQLLMQLRNSGTVSIDDR—W  284
CYP6P5    VRALKMKITFDDVERFFLKIVRETVDYREQNNVKRNDFMNLLLQIKNKGKLD———D    286
CYP6AA2   AKRLHLPALPSEVGSFFMPLVSETVHDRERNAIVRPDFLNLLIQLKNKG————T     283

CYP6A1    PESISKLG—GLTFNELAAQVFVFFLGGFETSSSTMGFALYELAQNQQLQDRLREEVNEV  338
CYP6B1    —NEDVKALELTDGVISAQMFIFYMAGYETSATTMTYLFYELAKNPDIQDKLIAEIDEV  336
CYP6D1    DVESTAENLKAMSIDMIASNSFLFYIAGSETTAATTSFTIYELAMYPEILKKAQSEVDEC 351
CYP6E1    ————————NFTFNEIAAQAFVFFQAGYETSSITMTFCLYELALNQELQERARKSVEDV  324
CYP6F1    DIEVSTN-KKKLSLEQVTAHAFVFFIAAYETSSTTISFCLFELARNPEIQKKVQQEIDQV 343
CYP6P5    SEDIVGKGEVGMTQLELAAQAFVFFLAGFETSSTTQSFCLYELAKNPEIQERLRQEINQA 346
CYP6AA2   VEDESSEGLEKLTLDEVAAQAFVFFFAGFETSSTTLSFALFELANNPAIQERVRAEVLEK 343

CYP6A1    FDQFK—EDNISYDALMNIPYLDQVLNETLRKYPVGVGSALTRQTLNDYVVPHNPKYVL  395
CYP6B1    LSRH——DGNITYECLSEMTYLSKVFDETLRKYPV—ADFTQRNAKTDYVFPG-TDITI   389
CYP6D1    LQRHGLKPQGRLTYEAIQDMKYLDLCVMETTRKYPG—LPFLNRKCTQDFQVPD-TKLTI 408
CYP6E1    LKRH——GSFSYETIQDMEFLNCCVKETLRKYPP—VANLFREITKNYKVPE-TDITL   376
CYP6F1    LASH——NGEITYDNINEMKYLENCIDETLRKYPA—VPFLNRECSKDYKIPG-TDTTI  396
CYP6P5    VEEN——DGQVTYDVAMNIQYLDNVINETLRKYPP—VESLNRVPSVDYLIPG-TKHVI  399
CYP6AA2   LKLH——DGQITYDALKEMTYLDQVINETLRMYPP—VPQLIRVSTQPYTVEA-TNVTL  396
                                  CYPFS ——→
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Continued

Figure 2. Continued.

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CYP6A1    PKGTLVFIPVLGIHYDPELYPNPEEFDPERFSPEMVKQRDSVDWLGFGDGPRNCIGMRFG   455
CYP6B1    KKGQTIIVSTWGIQNDPKYYPNPEKFDPERFNPENVKDRHPCAYLPFSAGPRNCLGMRFA   449
CYP6D1    PKETGIIISLLGIHRDPQYFPQPEDYRPERFADE-SKDYDPAAYMPFGEGPRHCIAQRMG   467
CYP6E1    EKGYRVVIPVYGIHHDPDIYPNPEVFNPERFIPELSTNRHPMAYLPFGEGPRTCIGERFA   436
CYP6F1    EKGTSLVIPVLGLHRDPDHYPEPDRFIPERFSN—FEDISTKPYLPFGAGPRNCIGLRLG   454
CYP6P5    PKRTLVQIPVHAIQNDPDHYPDPERFDPDRFNPEEVKKRHPFTFIPFGEGPRICIGLRFG   459
CYP6AA2   DRDTMLMVPIYAIHHDANIYPEPERFDPDRFAPDAVHSRHTHAFLPFGDGPRNCIGMRFG   456
                                                  ←——— CYPR

CYP6A1    KMQSRLGLALVIRHFRFTVCSR—TDIPMQINPESLAWTPKNNLYLNVQAIRKKIK      509
CYP6B1    KWQSEVCIMKVLSKYRVEPSMK—SSGPFKFDPMRLFALPKGGIYVNLVRR           498
CYP6D1    VINSKVALAKILANFNIQPMPR—QEVEFKFHSAPVLVPVNGLNVGLSKRW           516
CYP6E1    LMETKIGLSRLLQKFRFKLAPQTSTRIELNKTGVFLSIQGNLWMKVKKTCHNLTVVTEPAAEN   499
CYP6F1    KLQTKAGLVMMLSKFNVRLADETYASKELALDARSVVLMPVGGIKVSISERRAS       508
CYP6P5    VMQTKVGLITLLRKFRFSPSAR—TPDRVTFEPKMITLSPNAGNYLKVEKV           508
CYP6AA2   LLEVKFGIVQMLSKLRFTVNSR—MQLPIKLSKAAAMLEVEGGIWLNATKL          505
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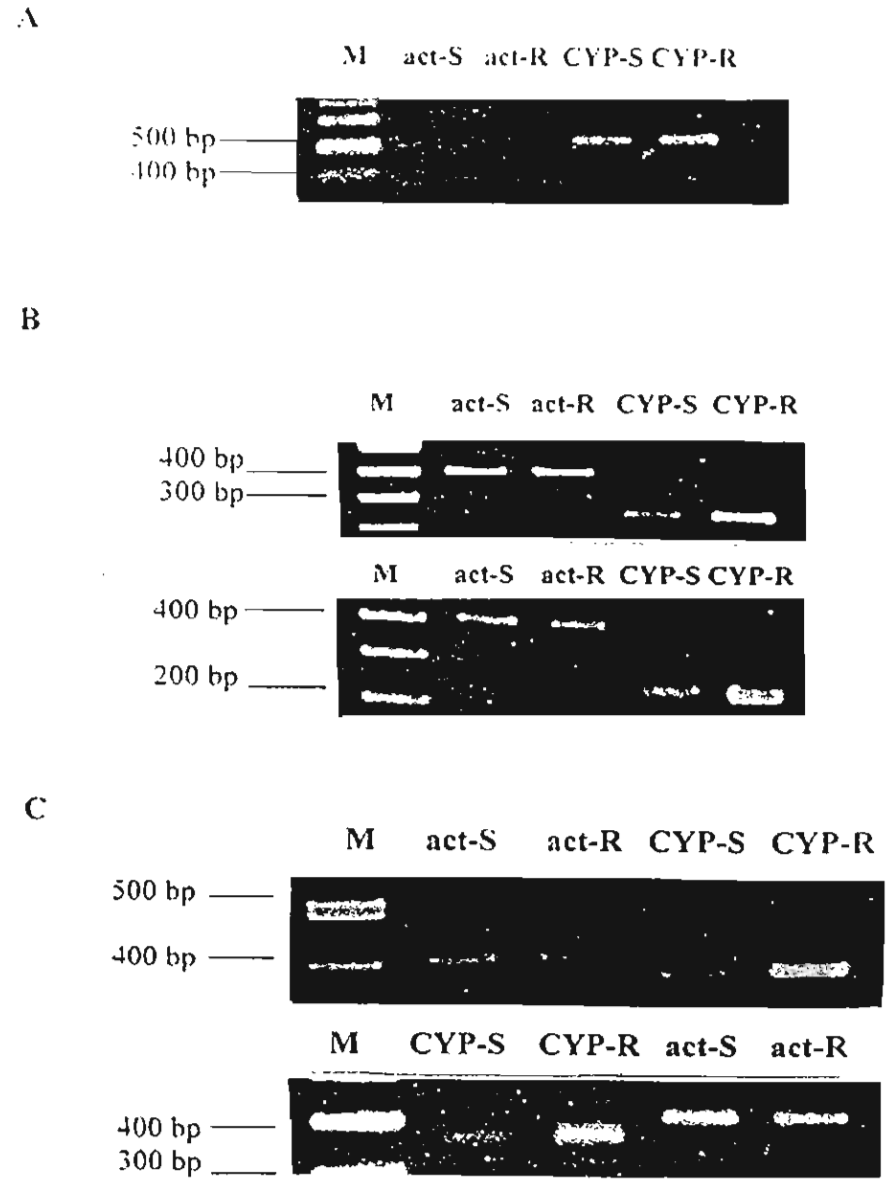

Figure 3. Expression of *CYP6P5*, *CYP6AA2* and actin mRNAs. Total RNA template isolated from F_0 deltamethrin susceptible (lane S) and F_{13} & F_{19} resistant (lane R) strains of *An. minimus* were RT-PCR amplified with *CYP6P5*, *CYP6AA2* and actin specific primers. **A:** RT-PCR product of CYP6P5 and actin mRNAs in susceptible and F_{19} pyrethroid resistant mosquitoes. **B:** RT-PCR product of CYP6AA2 amplified with QAN1-1 and QAN1-2 primers and actin mRNAs in susceptible and pyrethroid resistant strains. **C:** RT-PCR product of CYP6AA2 amplified with QAN1-3 and QAN1-4 primers and actin mRNAs in susceptible and pyrethroid resistant strains. **Top panels,** F13 deltamethrin resistance; **Bottom panels,** F_{19} deltamethrin resistance. Lanes M: Marker; Act-S and CYP-S: actin and CYP products from deltamethrin sensitive strain; Act-R and CYP-R: actin and CYP products from deltamethrin resistant strain.

best choice (based on sequence information of the derived clones thereafter) for the purpose of this study. We used the degenerate CYPR antisense primer targeting at heme-binding region and the low degenerated sense CYPFS primer binding at the ETLR motif sequence. The primers amplified partial cDNAs from susceptible and resistant strains producing the expected product size of ~240 bp and the products were cloned.

A total of 40 cDNA clones was screened and sequenced. Two clones showed sequences unrelated to P450 genes. The remaining 38 clones (21 from resistant strains and 17 from susceptible strains) were scored highly with P450 genes. Deduced amino acids of the cDNA clones showed relatively high diversity and were classified into 9 isoforms (Table 1) with alignment excluding the primer targeting sites (Figure 1). The AN1 cDNA clone was found predominantly and represented 42.8% of the clones sequenced in the F13 resistant strain, while in the F0 susceptible strain the AN1 clone represented only 17.6% (Table 1). The number of AN1 cDNA clones found multiple times could be correlated with the level of resistance expression in each mosquito strain. Thus we selected the AN1 clone for further characterization. All 9 isoforms showed deduced amino acid sequence homology to *CYP6* family.

Isolation of complete coding sequence of *CYP6P5* and *CYP6AA2* genes

To attain complete coding sequences for the AN1 and AN40 clones, genome walked, 5' and 3' RACE products of the two corresponding genes were obtained. Thereafter, intact full coding CYP6AA2 cDNA and CYP6P5 full coding genomic DNA was obtained. Upon alignment of DNA sequences from genomic and cDNA PCR products, the *CYP6P5* and *CYP6AA2* genes each had a short intron in the same amino acid position as that of *CYP6* gene family from the house fly (Cohen et al. 1994). The genes containing complete coding regions for clones AN1 and AN40 were named *CYP6P5* and *CYP6AA2*. The complete *CYP6P5* (Genbank Accession no. AY128947) and *CYP6AA2* (Genbank Accession no. AY129952) combined sequences each contained an open reading frame coding for 508 and 505 amino acid residues, respectively (Figure 2). Multiple amino acid alignment of both *CYP6AA2* and *CYP6P5* with other insect CYP6 sequences revealed a high degree of identity with numerous amino acid residues conserved across all insect CYP6 enzymes (Figure 2, Table 2). These sequences contained conserved residues representing typical features of cytochrome P450s, including a hydrophobic N-terminal membrane anchor domain, the putative heme binding site and ETLR motif.

CYP6P5 and CYP6AA2 mRNA expression in deltamethrin-susceptible and resistant *An. minimus* strains

To assess the transcription expression level of *CYP6P5* and *CYP6AA2* genes in deltamethrin-resistant and susceptible strains, we used semi-quantitative RT-PCR to measure the expression level of each mRNA in *An. minimus*. PCR products of *CYP6P5* using INTAN40F and INTAN40R primers and of *CYP6AA2* using QAN1-1 and QAN1-2 primers were 500 bp and 240 bp, respectively (Figure 3). The internal standard, PCR product of actin 1D cDNA, with the size of 400 bp, was generated with actin-specific primers. Band intensity for *CYP6P5* amplified from F_{19} resistant mosquitoes was similar to that amplified from the susceptible strain (Figure 3A). A similar result was obtained when F_{13} resistant mosquitoes were used (unpublished data). In contrast, RT-PCR band intensities for *CYP6AA2* amplified from F_{13} resistant mosquitoes were approximately twice those from the F_0 susceptible strain after normalization with actin 1D (Figure 3B), and were approximately 3-4 fold higher when amplified from F_{19} resistant mosquitoes. A different pair of primers, QAN1-3 and QAN1-4, based on a different region of CYP6AA2 cDNA sequence was used for semi-quantitative RT-PCR in susceptible and resistant mosquitoes. This was to ensure that the mRNA level increase was the resultant product of CYP6AA2 mRNA and not due to a high level of sequence identity between members of P450 gene family as has been reported high sequence similarities among *CYP6B* genes in *Helicoverpa* species and *Papilio* species (Li et al. 2001; 2002). The resulting 360 bp band intensity was reproducible when QAN1-3 and QAN1-4 primers were used demonstrating approximately 2 and 3-4 fold increases in band intensity in the RT-PCR amplification of F_{13} and F_{19} resistant mosquitoes, respectively, compared to the F_0 susceptible mosquito (Figure 3C). Thus, the level of CYP6AA2 mRNA increase is correlated well with the increased level of deltamethrin resistance in *An. minimus*.

DISCUSSION

We used the deltamethrin-resistant colony (F_{13} and F_{19}) established from a pyrethroid susceptible *An. minimus* mosquito strain (F_0) by systematic selection against deltamethrin (Chareonviriyaphap et al. 2002). The F_{13} and F_{19} resistant mosquitoes showed approximately 1.2 and 5 fold increase in specific activity of mixed function oxidases (MFOs) compared to the F_0 susceptible strain (Chareonviriyaphap et al., unpublished data). The higher MFOs activity was increased in the test populations of mosquitoes as the selection against

deltamethrin continued and the increase was found associated with changes in bioassay analysis (Chareonviriyaphap et al. 2002, Chareonviriyaphap et al. unpublished data). The results implicated the involvement of P450 in deltamethrin resistance in the *An. minimus*. We have therefore placed an emphasis on the study of P450 and cloned P450 genes as a first step towards an understanding of pyrethroid resistance mechanisms in *An. minimus*.

A pair of primers used to amplify partial P450 cDNAs and cloned sequences from both F_{13} resistant and F_0 susceptible strains were compared. The first primer was a degenerate primer targeted to the P450 heme-binding site, which is conserved in all P450 proteins. The second was based on the ETLR motif of the AN40 sequence and those that shared high homology among *CYP6* sequences. This was similar to previously published approaches for isolation of *CYP4* and *CYP6F1* genes from *An. albimanus* and *Cx. quinquefasciatus* Say mosquitoes (Kasai et al. 2000, Scott et al. 1994). This strategy allowed isolation of expressed cytochrome P450 genes from *An. minimus*, probably limiting the isolation coverage to the family six. The sequences of partial cDNA clones, although not full complements of the P450 genes, nevertheless revealed P450 isoforms expressed in *An. minimus* (Figure 1). Among these, seven isoforms were detected in the resistant strain and eight in the susceptible strain, of which six were commonly found in both strains (Table 1). The cDNAs showed > 40% deduced amino acid identity to known *CYP6* genes indicating that they belong to family six The nine isoforms exhibited a wide range of variation, as measured by deduced amino acid sequences. The level of *CYP6* diversity sampled in this study is comparative to that sampled in *Cx. quinquefasciatus* Say using a similar strategy (Kasai et al. 2000) and that sampled in the adult Mediterranean fruit fly, *Ceratitis capitata* (Danielson et al. 1999). However, this level is much less than that previously reported in *Drosophila melanogaster*, where *22 CYP* genes were found belonging in the *CYP6* family (Tijet et al. 2001). Thus, the degree of heterogeneity observed in this study does not reflect total *CYP6* diversity in *An. minimus*.

The sequenced partial cDNA clones may not correlate with the relative abundance of product expressed by *CYP6* genes in the resistant strain. To some extent, the abundance of the PCR products could depend on the binding affinity of the priming sites. However, we can only roughly associate the transcript level and relative abundance of a particular cDNA sequence in the PCR products by the multiple number of cDNA clones found. One predominant cDNA clone (AN1), accounted for ~43% among the clones of the resistant strain (Table 1), could be the most highly expressed gene in the F_{13} deltamethrin resistant strain. Moreover there were increases of ~2 and ~3-4 in expression of the CYP6AA2 transcript (the AN1 full complement) in the F_{13} resistant compared to the F_0 susceptible strain. However, the AN40 (identified as *CYP6P5*), representing ~14% and ~17% in the resistant and susceptible strains, respectively, was shown to have equal levels of mRNA in both strains. These data support the congruence of the occurrence of the multiple cDNA clones with the relative amount of particular cDNAs in PCR products in relation to the transcription level.

We compared mRNA expression level of CYP6P5 and CYP6AA2 in the F_{13} and F_{19} deltamethrin-resistant and the F_0 susceptible strains. The CYP6P5 mRNA level was unchanged in the resistant strain compared to the sensitive strain indicating that CYP6P5 is not associated with deltamethrin resistance in this strain of *An. minimus*. In contrast, a 3-4 fold higher CYP6AA2 mRNA level in the F_{19} resistant strain over the F_0 susceptible strain is comparable to that observed for MFOs activity. These results suggest that there is an association between increased CYP6AA2 mRNA level and deltamethrin resistance. Although at present there is no direct evidence showing the involvement of CYP6AA2 in deltamethrin resistance, the CYP6AA2 mRNA increase was stepwise in association with elevated resistance suggesting that CYP6AA2 could play a role in pyrethroid resistance in *An. minimus*. Other unidentified isoforms of cytochrome P450 that might play a role in resistance could not be ruled out. Further proof is required to confirm that CYP6AA2 could play a role in deltamethrin resistance. This could involve analysis of pyrethroid metabolism *in vitro* by virtue of heterologous expression of the CYP6AA2 gene and exploitation of its enzyme activity against pyrethroids. Its metabolic role in pyrethroid insecticide, biochemical properties and specificity, the regulatory processes and the genetic mechanisms remain to be clarified.

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