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of breeding places and outdoor resting habitats with agricultural insecticides may influence resistance development (Unpublished data).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

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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

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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.

```
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.

```
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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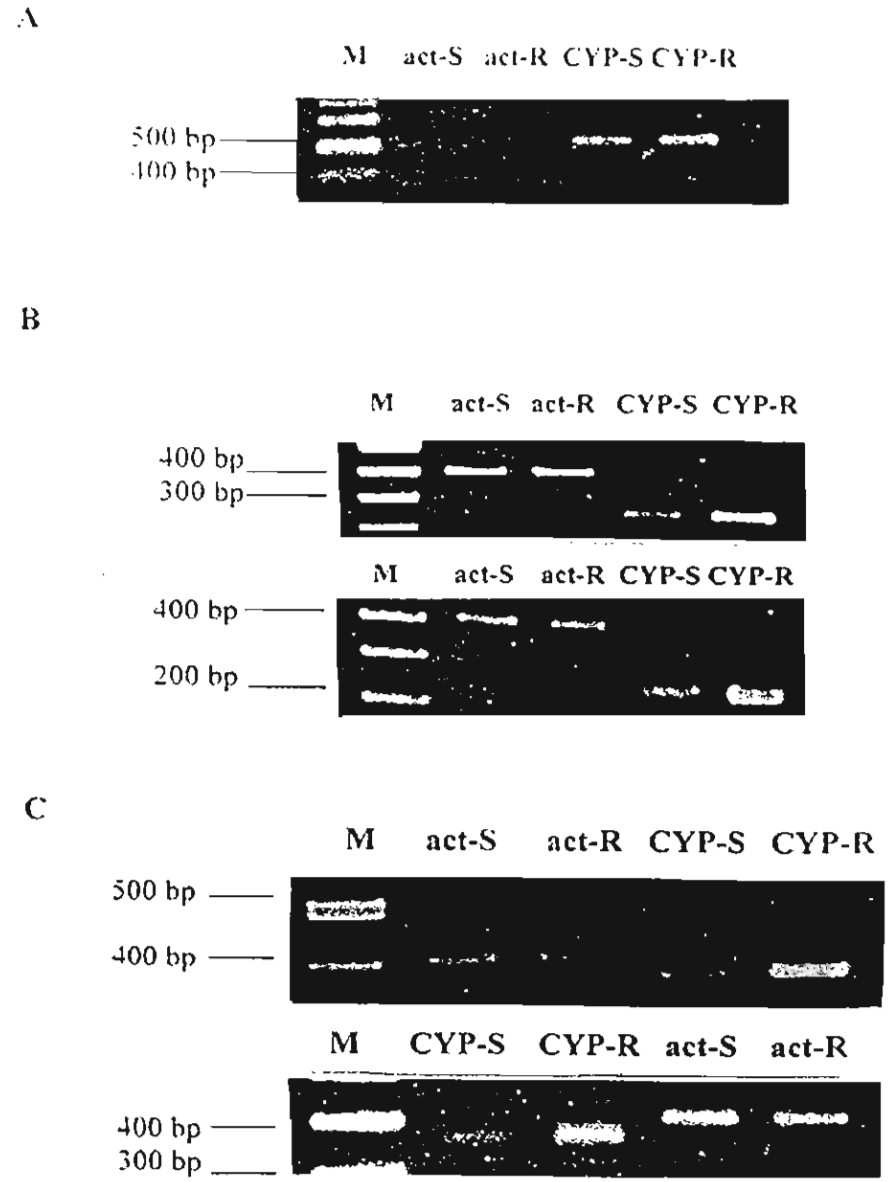

Figure 3. Expression of *CYP6P5*, *CYP6AA2* and actin mRNAs. Total RNA template isolated from F_0 deltamethrin susceptible (lane S) and F_{13} & F_{19} resistant (lane R) strains of *An. minimus* were RT-PCR amplified with *CYP6P5*, *CYP6AA2* and actin specific primers. **A:** RT-PCR product of CYP6P5 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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