DEVELOPMENT OF A MULTIPLEX PCR METHOD TO DETECT THE V1016G AND F1534C MUTATIONS IN THE VOLTAGE-GATED SODIUM CHANNEL GENE, AND THEIR EFFECTS ON PYRETHROID RESISTANCE IN THE MOSQUITO *Aedes aegypti*

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DOCTOR OF PHILOSOPHY

IN PARASITOLOGY

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หัวข้อดุษฎีนิพนธ์	การพัฒนาวิธี Multiplex PCR เพื่อตรวจหาการกลายพันธุ์ V1016G และ F1534C ใน Voltage-gated Sodium Channel Gene และผลของการกลายพันธุ์ ต่อการดื้อไพรีทรอยด์ในยุงลาย *Aedes aegypti*
ผู้เขียน	นายเจษฎา ไทรงามสุข
ปริญญา	ปรัชญาดุษฎีบัณฑิต (ปรสิตวิทยา)
คณะกรรมการที่ปรึกษา	รศ.ดร. ปรัชญา สมบูรณ์ อาจารย์ที่ปรึกษาหลัก ผศ.ดร. อติพร แซ่อึ้ง อาจารย์ที่ปรึกษาร่วม ผศ.ดร. จินตนา ยาโนละ อาจารย์ที่ปรึกษาร่วม

บทคัดย่อ

การกลายพันธุ์ที่ voltage-gated sodium channel (VGSC) gene หรือที่รู้จักกันในนาม knockdown resistance (*kdr*) เป็นกลไกการดื้อสารเคมีฆ่าแมลงไพรีทรอยด์ที่สำคัญในยุงลาย *Aedes aegypti* ซึ่งเป็นพาหะของโรคไข้เดงกี่ โดยการกลายพันธุ์จาก valine ไปเป็น glycine (V1016G) และการกลายพันธุ์จาก phenylalanine ไปเป็น cysteine (F1534C) นั้นพบได้ทั่วไปในกลุ่มประชากรยุงลาย *Ae. aegypti* ในประเทศไทยและอีกหลายประเทศในทวีปเอเชีย ยิ่งไปกว่านั้นความถี่ของอัลลีลกลายพันธุ์ G1016 และ C1534 ยังคงสูงขึ้นอย่างต่อเนื่องในช่วงหลายปีที่ผ่านมา ด้วยเหตุนี้การที่จะตรวจติดตามความถี่อัลลีลการกลายพันธุ์ทั้งสองในวงกว้าง จึงจำเป็นที่จะต้องมีวิธีการที่ง่ายและราคาไม่แพง นอกจากนี้ อัลลีลที่ควบคุมลักษณะการดื้อสารเคมีฆ่าแมลงยังมีความเกี่ยวข้องกับความแข็งแรงของยุงในสภาวะที่ปราศจากสารเคมีฆ่าแมลง ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้คือ 1) พัฒนาและตรวจสอบประสิทธิภาพวิธี multiplex PCR เพื่อตรวจหาการกลายพันธุ์ V1016G และ F1534C ที่ voltage-gated sodium channel (VGSC) gene ในยุงลาย *Ae. aegypti* และ 2) ศึกษาความแข็งแรงสัมพัทธ์ในยุง *Ae. aegypti* ที่ดื้อต่อสารเคมีฆ่าแมลง 3 สายพันธุ์คือ PMD, PMD-R, UPK-R และลูกผสม F_1 (PMD-R x UPK-R)

วิธี multiplex PCR เพื่อตรวจหาการกลายพันธุ์ V1016G และ F1534C ได้ถูกพัฒนาขึ้นมาในการศึกษาครั้งนี้ โดยมีการนำชุด Primer เพื่อตรวจหาการกลายพันธุ์ที่ตำแหน่ง 1016 จากการศึกษาครั้งก่อนมาใช้ร่วมกับชุด Primer ที่ออกแบบใหม่ เพื่อตรวจหาการกลายพันธุ์ที่ตำแหน่ง 1534 ในการศึกษาครั้งนี้ สภาวะที่เหมาะสมของการทำ PCR ได้ถูกทดสอบ และเปรียบเทียบผลที่ได้จากวิธี multiplex PCR กับผลจากการทำ DNA sequencing โดยใช้ตัวอย่างยุงจากห้องปฏิบัติการที่ทราบลักษณะการกลายพันธุ์ก่อนแล้วและยุงเก็บมาจากภาคสนาม และยังได้ทำการเปรียบเทียบประสิทธิภาพของวิธีนี้กับวิธี allele specific PCR (AS-PCR) เพิ่มเติม ผลปรากฏว่า ผลที่ได้จากวิธี multiplex PCR ตรงกับผลของวิธี DNA sequencing ทั้งหมด และยังให้ผลที่ถูกต้องแม่นยำมากกว่าวิธี AS-PCR นอกจากนี้ได้มีการทดสอบประสิทธิภาพของสีย้อมดีเอ็นเอที่ไม่เป็นพิษ 2 สี คือ Ultrapower™ และ RedSafe™ เปรียบเทียบกับ Ethidium Bromide (EtBr) ซึ่งผลออกมาคือสีย้อมที่ไม่เป็นพิษทั้งสองสีให้ผลที่ดีและสามารถใช้ทดแทน EtBr ซึ่งมีความเป็นพิษได้ ดังนั้นวิธี multiplex PCR ที่ได้พัฒนาขึ้นในการศึกษานี้จึงเป็นวิธีที่น่าเชื่อถือและเป็นประโยชน์ในการศึกษาตรวจติดตาม *Ae. aegypti* ในพื้นที่ที่มีการกลายพันธุ์ทั้งสองตำแหน่งนี้ปรากฏอยู่ร่วมกัน

จากการศึกษาความแข็งแรงสัมพัทธ์ในด้านการเจริญเติบโตและการสืบพันธุ์ของยุงลาย *Ae. aegypti* ที่ดื้อต่อสารเคมีฆ่าแมลง 3 สายพันธุ์คือ PMD, PMD-R, UPK-R และยุงลูกผสม F_1 (PMD-R x UPK-R) ภายใต้สภาวะแวดล้อมมาตรฐานเดียวกันในห้องปฏิบัติการและปราศจากสารเคมีฆ่าแมลง; PMD เป็นสายพันธุ์ที่ดื้อต่อดีดีที แต่ไม่มีอัลลีล *kdr*; PMD-R เป็นสายพันธุ์ที่ดื้อต่อดีดีทีและเพอเมททริน มีอัลลีล C1534 แบบโฮโมซัยกัส; UPK-R เป็นสายพันธุ์ที่ดื้อต่อดีดีที เพอเมททรินและเดลต้าเมททริน มีอัลลีล P989 และ G1016 แบบโฮโมซัยกัส; ยุงลูกผสม F_1 (PMD-R x UPK-R) นั้นดื้อต่อดีดีทีและดื้อในระดับปานกลางต่อเพอเมททรินและเดลต้าเมททริน มีอัลลีลทั้งสามตำแหน่งแบบเฮเทอโรซัยกัส (S/P989, V/G1016 และ F/C1534)) ผลการศึกษาพบว่าสายพันธุ์ที่ดื้อต่อดีดีที (PMD) มีความแข็งแรงมากที่สุดเมื่อเทียบกับสายพันธุ์ที่ดื้อต่อทั้งดีดีทีและไพรีทรอยด์ (PMD-R และ UPK-R) และยุงลูกผสมความเสื่อมถอยของสุขภาพที่พบได้เหมือนกันในยุงสายพันธุ์ดื้อต่อดีดีทีและไพรีทรอยด์และยุงลูกผสม ประกอบด้วย ความยาวของปีกลดลง, อัตราการฟักเป็นลูกน้ำลดลง อายุขัยของยุงตัวเต็มวัยเพศเมียสั้นลงและอายุขัยของไข่ยุงในการเก็บรักษาสั้นลง แต่ทว่าไม่พบความเสื่อมถอยด้านอัตราการกินเลือด นอกจากนี้จำนวนไข่ที่ผลิตลดลงพบแต่ในยุงสายพันธุ์ PMD-R ในขณะที่ยุงสายพันธุ์ UPK-R ใช้ระยะเวลาในการเจริญเติบโตมากกว่าสายพันธุ์อื่น ยุงลูกผสมซึ่งมีอัลลีลการกลายพันธุ์แบบเฮเทอโรซัยกัสนั้นมีความแข็งแรงมากกว่ายุงที่มีอัลลีลการกลายพันธุ์แบบโฮโมซัยกัส ซึ่งผลที่ได้จากการศึกษาครั้งนี้สอดคล้องกับการพบความถี่การกลายพันธุ์แบบเฮเทอโรซัยกัสที่สูงในประชากร *Ae. aegypti*

ตามธรรมชาติในจังหวัดเชียงใหม่ การเข้าใจความสัมพันธ์ระหว่างความแข็งแรงกับการดื้อต่อสารเคมีฆ่าแมลงสามารถนำไปใช้ในการคาดคะเนแนวโน้มการดื้อต่อสารเคมีฆ่าแมลงในอนาคต และยังสามารถนำไปใช้ปรับปรุงมาตรการควบคุมประชากรยุงพาหะได้

Dissertation Title	Development of a Multiplex PCR Method to Detect the V1016G and F1534C Mutations in the Voltage-gated Sodium Channel Gene, and Their Effects on Pyrethroid Resistance in the Mosquito *Aedes aegypti*
Author	Mr. Jassada Saingamsook
Degree	Doctor of Philosophy (Parasitology)
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ABSTRACT

Mutation of the voltage-gated sodium channel (VGSC) gene, or knockdown resistance (*kdr*) gene, is an important resistance mechanism of the dengue vector *Aedes aegypti* mosquitoes against pyrethroids. In Thailand and many countries in Asia, a valine to glycine substitution (V1016G) and a phenylalanine to cysteine substitution (F1534C) are common in *Ae. aegypti* populations. The G1016 and C1534 allele frequencies have been increasing in recent years, and hence there is a need to have a simple and inexpensive tool to monitor the alleles in large scale. Moreover, the persistence of the resistance phenotype is associated with high fitness of resistance alleles in the absence of insecticide pressure. Therefore, the objectives of this study are to 1) develop and validate a multiplex PCR method for detection of the V1016G and F1534C mutations in the voltage-gated sodium channel gene of *Ae. aegypti* and 2) investigate the relative fitness cost in three insecticide-resistant strains of *Ae. aegypti*, PMD, PMD-R and UPK-R, and F_1 (PMD-R x UPK-R) hybrid.

A multiplex PCR to detect V1016G and F1534C mutations has been developed in the current study. This study utilized primers from previous studies for detecting the mutation at position 1016 and newly designed primers to detect variants at position 1534. The PCR conditions were validated and compared with DNA sequencing using known *kdr* mutant laboratory strains and field collected mosquitoes. The efficacy of this method was also compared with allele-specific PCR (AS-PCR). The results of our multiplex PCR were in complete agreement with sequencing data and better than the AS-PCR. In addition, the efficiency of two non-toxic DNA staining dyes, Ultrapower™ and RedSafe™, were evaluated by comparing with ethidium bromide (EtBr) and the results were satisfactory. Our multiplex PCR method is highly reliable and useful for implementing vector surveillance in locations where the two alleles co-occur.

The relative development and reproductivity fitness cost were determined in three insecticide-resistant strains of *Ae. aegypti*, PMD, PMD-R and UPK-R, and F_1 (PMD-R x UPK-R) hybrid under similar laboratory conditions in the absence of insecticide; the PMD strain is resistant to DDT with no *kdr* alleles; the PMD-R is resistant to DDT and permethrin with C1534 homozygous allele; the UPK-R is resistant to DDT, permethrin and deltamethrin with P989+G1016 homozygous alleles; and the hybrid is resistant to DDT and intermediate resistant to permethrin and deltamethrin with S/P989, V/G1016 and F/C1534 heterozygous alleles. The DDT-resistant PMD strain had the highest fitness compared with the two DDT/pyrethroid-resistant strains (PMD-R and UPK-R) and hybrid. Consistent fitness costs were observed in the DDT/pyrethroid-resistant strains and hybrid, including shorter wing length, reduced egg hatchability, shorter female lifespan and shorter viability of eggs after storage, whereas no effect was observed on blood feeding rate. In addition, reduced egg production was detected in the PMD-R strain and prolonged developmental time was seen in the UPK-R strain. The corresponding hybrid that is heterozygous for *kdr* alleles was fitter than either of the homozygous mutant genotypes. This is in accordance with the high frequency of heterozygous genotypes observed in nature populations of *Ae. aegypti* in Chiang Mai city. Understanding fitness-resistance association information helps predict resistance trends in the future and improving the integrated vector control programs.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
AS-PCR	Allele-Specific PCR
bp	Base pair
cm	Centimeter
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue virus
df	Degree of freedom
DF	Dengue fever
DNA	Deoxyribonucleic Acid
e.g.	For example
et al.	And others
EtBr	Ethidium bromide
F_1	First generation of progeny
g	Gram
GABA	Gamma-aminobutyric acid
GST	Glutathione-S Transferases
h	Hour

LIST OF ABBREVIATIONS (continued)

HOLA	Heated Oligonucleotide Ligation
i.e.	That is
kdr	Knockdown resistance
MFO	Mixed Function Oxidases P450
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
ng	Nanogram
no.	Number
oz.	Ounce
P	Probability
PCR	Polymerase Chain Reaction
P450s	Cytochrome P450 monooxygenases
RH	Relative humidity
s	Second
SE	Standard error
SNP	Single Nucleotide Polymorphism

LIST OF ABBREVIATIONS (continued)

TBE	Tris-borate-EDTA buffer
µg/ml	Microgram per milliliter
µl	Microliter
ULV	Ultra-low volume
µM	Micromolar
UV	Ultraviolet
V	Voltage
VGSC	Voltage-gated sodium channels
VSD	Voltage sensing domains
vs	Versus
v/v	Volume per volume
WHO	World Health Organization

LIST OF SYMBOLS

+	Plus
±	Plus minus
<	Less than
>	More than
=	Equals
%	Percentage
°C	Degree Celsius
:	Per

ข้อความแห่งการริเริ่ม

1) วิทยานิพนธ์นี้เป็นการศึกษาแรกที่ทำการพัฒนาวิธี Multiplex PCR เพื่อตรวจหาการกลายพันธุ์ V1016G และ F1534C ใน voltage-gated sodium channel gene ในยุงลาย *Ae. aegypti*

2) วิทยานิพนธ์นี้เป็นการศึกษาแรกที่ทำการเปรียบเทียบความแข็งแรงในด้านการเจริญเติบโตและการสืบพันธุ์ระหว่างยุงลาย *Ae. aegypti* ที่ดื้อต่อสารเคมีฆ่าแมลงสามสายพันธุ์และหนึ่งสายพันธุ์ลูกผสม โดยแต่ละสายพันธุ์มีระดับการดื้อต่อสารเคมีฆ่าแมลงและการกลายพันธุ์ที่ voltage-gated sodium channel ที่แตกต่างกัน ประกอบด้วย 1) สายพันธุ์ที่ไม่มีการกลายพันธุ์ (PMD) 2)สายพันธุ์ที่มีการกลายพันธุ์ F1534C แบบโฮโมซัยกัส (PMD-R) 3) สายพันธุ์มีการกลายพันธุ์ S989P+V1016G แบบโฮโมซัยกัส (UPK-R) และ 4) ลูกผสม F_1 (PMD-R x UPK-R) ที่มีการกลายพันธุ์แบบเฮเทอโรซัยกัสทั้งสามตำแหน่ง 989 1016 และ 1534

STATEMENT OF ORIGINALITY

1) This was the first study to develop a multiplex PCR method for detection of the V1016G and F1534C mutations in the voltage-gated sodium channel gene of *Ae. aegypti*.

2) This was the first study to investigate the relative development and reproductive fitness cost in three insecticide-resistance strains and one hybrid of *Ae. aegypti*. Each strain confers different resistance level and *kdr* mutation including 1) without *kdr* mutation (PMD) 2) homozygous F1534C mutation (PMD-R) 3) homozygous S989P+V1016G mutations (UPK-R) and 4) heterozygous mutations at all three positions 989, 1016, and 1534 (F1 (PMD-R x UPK-R) Hybrid).

CHAPTER 1

Introduction

1.1 Statement and significant of the problem

Aedes aegypti is the primary vector of viral diseases, including dengue fever (DF), yellow fever, chikungunya and zika. DF has been a worldwide epidemic in tropical and subtropical areas, including Thailand. At present, DF causes more illness and death to humans than any other arbovirus diseases. Since several human candidate vaccines against dengue are still under development and there is no specific treatment. Besides, the strategies like releasing the strain carrying *Wolbachia* symbiont that decreases the mosquito vectorial capacity, or of the genetically modified mosquitoes that suppress the natural populations, are currently being evaluated in the field and not available for large-scale applications. The control of disease transmission is, therefore based mainly on the management of breeding sites and insecticide applications.

In Thailand, synthetic pyrethroids have been widely used for controlling mosquitoes due to their fast action, high insecticidal activity, and low mammalian toxicity. However, intensive use of pyrethroid insecticides has resulted in the development of resistance in *Ae. aegypti*. Pyrethroid insecticides act on the nervous system, modifying the gating kinetics of voltage-gated sodium channels (VGSC). There are two major mechanisms of pyrethroid resistance in resistant insects: (1) accelerate the rate of pyrethroid detoxification by increasing metabolic enzyme activity (cytochrome P450) or level (esterases and glutathione S-transferases); (2) reduce sensitivity of the target site via a point mutation in the VGSC gene that leads to amino acid substitution. The latter mechanism is called knockdown resistance (*kdr*) and is the main mechanism of resistance in *Ae. aegypti* in Thailand.

Ten amino acid substitutions have been detected in *Ae. aegypti* VGSC: G923V, L982W, S989P, I1011M, I1011V, V1016G, V1016I, T1520I, F1534C, and D1763Y. Of these, only three substitutions, I1011M, V1016G, and F1534C, are shown to associate with pyrethroid resistance. Meanwhile, S989P and V1016I are associated with pyrethroid resistance when in combination with other mutations. In Thailand, V1016G (always accompany by S989P) is found to be associated with deltamethrin and permethrin resistance in *Ae. aegypti* populations, whereas F1534C confers resistance to permethrin. Determination of the allele frequency of the mutations in order to monitor the resistant status of the vector is important for vector control strategy. Detection of these mutations is currently based on molecular methods including DNA sequencing, Taqman assay, Heated Oligonucleotide Ligation assay (*HOLA*) and Allele-specific PCR assay (AS-PCR). Among these, AS-PCR is the cheapest and most convenient method, whereas the other methods require high initial equipment costs, expensive reagents and/or well-trained technical personnel, meaning that they can be difficult to implement in many laboratories. At present, detection of V1016G and F1534C by AS-PCR needs at least two separate reactions that consume time and reagents. This study aims to develop a simple, rapid, highly sensitive and specific multiplex PCR method that can detect both V1016G and F1534C mutations in single reaction, then visualizes the result by gel electrophoresis. This method will require approximately half the cost and time when compared to existing methods, making it very useful for laboratories with limited resources.

Although resistance mechanisms help mosquitoes to survive under the continuous insecticide pressure, these actions are costly and may negatively affect mosquito's fitness (e.g., adult longevity, larval development time, fecundity, fertility, mating competitiveness and blood feeding capability) when the insecticides are withdrawn. The fitness reduction may be caused by pleiotropy in the resistance genes themselves or as a consequence of hitch-hiking effect. It is difficult to associate fitness disadvantages specifically with resistance in field populations. Evaluation of relative fitness of resistant and susceptible genotypes obtained from laboratory studies are useful when considering the impact of resistance alone on biological fitness. Our previous study revealed that the presence of homozygous F1534C mutation reduced the size of mutant

Ae. aegypti (Plernsub et al., 2013), the fitness cost affecting by V1016G alone and F1534C+V1016G combination have not yet investigated. This study aimed to determine whether pyrethroid resistance in laboratory *Ae. aegypti* strains each conferring different *kdr* mutations affects specific fitness components when compared to the pyrethroid susceptible strain. Understanding these aspects is vital for improving the management in vector control programmes and predicting the resistance trend in the future.

1.2 Literature review

1.2.1 Biology of *Aedes aegypti*

The mosquito *Ae. aegypti* was first recorded in Thailand in 1907, as well as being noted in Singapore and Malaysia (Theobald, 1907). Initially, it was limited in city areas or along the trade routes. Nevertheless, it has now become commonplace in urban areas of Thailand and has spread widely to rural areas in the last 60 years (Bosio et al., 2005). *Ae. aegypti* is the primary vector of flaviviruses such as dengue virus (DENV), yellow fever virus, chikungunya virus and Zika virus. These are the most crucial mosquito-borne viruses that cause significant public health concerns in tropical and sub-tropical countries throughout the world (WHO, 2016a, b, 2017). *Ae. aegypti* is an invasive black and white striped day-biting mosquito which mainly feed on human blood. It can also feed on animal blood including bovine, swine, cat, rat, and chicken which represents <1% of blood meals (Ponlawat and Harrington, 2005). The remarkable feature of this species is the white scales on the dorsal surface of the thorax that form a lyre or violin shape (Figure 1.1). The adult is a small to medium-sized mosquito, approximately 4 to 7 mm. Figure 1.2 demonstrates the life cycle of *Ae. aegypti*. During the adult stage, the lifespan can range from 2 weeks to a month, depending on environmental conditions. After taking a complete blood meal, females produce on average 100 to 200 eggs per batch, however, the number of eggs produced is dependent on the size of the blood meal. Females can produce up to 5 batches of eggs during a lifetime. Regularly, eggs will be placed at varying distances above the waterline, and a female will not lay the entire clutch at a single site, but rather spread out the eggs over two or more sites. Eggs of *Ae. aegypti* are elongate, smooth, ovoid-shaped, and about

one millimeter long. When first laid, eggs appear white but within minutes turn a shiny black. In warm climates, such as the tropics, eggs may develop in as little as two days, whereas in colder temperate climates, development can take up to a week. *Ae. aegypti* eggs can survive desiccation for months and hatch once submerged in water resulting in the difficulty of controlling *Ae. aegypti*. Larvae of *Ae. aegypti* can be distinguished from other genera by the unaided eye due to their short siphon which is used to breathe oxygen and it is held above the water surface while the rest of the body hangs vertically. Larvae feed on organic particulate matter in the water. There are four larval instars. Males develop faster than females, so males generally pupate earlier. If temperatures are cold, *Ae*. *aegypti* can remain in the larval stage for months so long as the water supply is sufficient. After the larval stage, it enters the pupal stage. Pupae do not feed, and approximately after 3-4 days, the adults emerge head first by ingesting air to expand the abdomen thus breaking the pupal case (Foster and Walker, 2002; Hopp and Foley, 2001; Nelson, 1986; Yimer et al., 2016).

Figure 1.1 Adult female *Aedes aegypti* (Yimer et al., 2016)

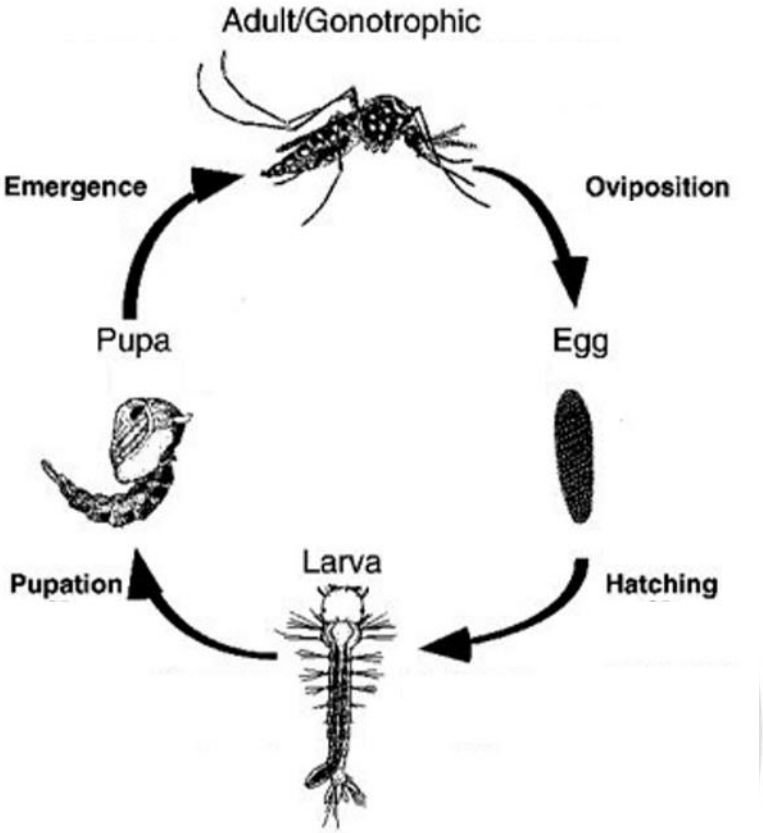


Figure 1.2 *Aedes aegypti* mosquito life cycle. (Yimer et al., 2016)

1.2.2 Dengue situation

DF is a prime public-health concern throughout tropical and sub-tropical regions of the world, including Thailand. In general, dengue infection causes a flu-like illness by presenting only mild symptoms and usually not fatal. However, it occasionally develops into a potentially lethal complication called severe dengue (dengue hemorrhagic fever and dengue shock syndrome), particularly among children. Although, a vast majority of cases are asymptomatic and hence the actual numbers of dengue cases are underreported, and many cases are misclassified. The number of dengue cases has grown dramatically around the world in recent decades. Since the effective vaccine is not available and a lack of specific antiviral treatments, the distribution of this disease is leading to significant health, economic and social problems within the populations of the endemic areas.

Dengue is recognized as the most rapidly spreading mosquito-borne viral disease, with a 30-fold increase in global incidence over the past 50 years (Mackenzie et al., 2004). The World Health Organization (WHO) estimates that 50–100 million dengue infections occur each year and that almost half the world's population lives in countries where dengue is endemic (WHO, 2012). The prevalence of dengue has been estimated that 3900 million people living in 128 countries are at risk of infection with dengue viruses (Brady et al., 2012). Another study, 390 million dengue infections per year have been reported, of which 96 million (67–136 million) manifest clinically (Bhatt et al., 2013).

In Thailand, the first dengue hemorrhagic fever outbreak happened in Bangkok in 1958 (Hammon et al., 1960). Since then, much of urban is regarded as hyper-endemic for dengue infection with all four serotypes (DEN-1, DEN-2, DEN-3, DEN-4) regularly circulating and now occurring more commonly in rural areas. In 2018 alone, there were 85,849 dengue cases and 111 deaths reported in the country. Cases were reported in all 77 Thai provinces. Overall, the Central region has the highest rate of infection. Meanwhile, the provinces with the highest rates of infection include Nakhon Pathom, Nakhon Sawan, Samut Sakhon, Nakhon Si Thammarat and Phichit (Ministry of Health, 2019).

1.2.3 Vector control

The control of disease transmission is, therefore based mainly on the management of breeding sites and insecticide applications (WHO, 2012). Several insecticide groups, including organochlorines (DDT), organophosphates (e.g., malathion, fenitrothion and temephos), and carbamates (e.g., propoxur) were extensively used for controlling the mosquitoes over 50 years in Thailand until pyrethroids have been introduced in the early 1990s (Chareonviriyaphap et al., 2013).

1.2.4 Pyrethroids

Pyrethroid insecticides are a major class of neurotoxic insecticides. They are synthetic analogues of the naturally occurring insecticidal esters of chrysanthemic acid (pyrethrins I) and pyrethric acid (pyrethrin II), originally found in the flowers of

Chrysanthemum cinerafolis. Pyrethrin and pyrethroid insecticides affect both the peripheral and central nervous systems of insects. They initially bind to a sodium channel protein on nerve cells, and then stimulate them to produce repetitive discharges, eventually causing paralysis. This effect is similar to, but more pronounced than, that of DDT. In insects, this produces an incapacitating, and also known as 'knockdown'. The amplitude of the sodium current continues undiminished until the level of hyperexcitability overwhelms the capacity of the cell to maintain the activity of the sodium pump. Higher lipophilicity of the insecticide chemical gives better knockdown rates as pyrethroid penetrates to the target more quickly. However, such compounds may not give good 'kill-rate' due to a tendency to dissociate from the target. Type I pyrethroids (e.g., permethrin (Figure 1.3)) are generally good knockdown agents due to their ability to induce repetitive firing in axons; resulting in restlessness, un-coordination and hyperactivity followed by prostration and paralysis. Type II compounds, as typified by deltamethrin (Figure 1.3), have a cyano group at the α-benzylic position (the α-carbon of the 3-phenoxybenzyl alcohol) and cause a pronounced convulsive phase. This results in a better kill-rate because depolarization of the nerve axons and terminals is irreversible (Bloomquist, 1996). The differing physiological effects are thus explained by the fact that the duration of modified sodium currents by Type I compounds lasts only tens or hundreds of milliseconds, while those of Type II compounds last for several seconds or longer.

Pyrethroid insecticides have become the most popular insecticide because of their fast action, low cost with high insecticidal activity, low mammalian toxicity and less harmful to the environment (van den Berg et al., 2012). To conclude, they act on the nervous system by modifying the gating kinetics of voltage-gated sodium channels (VGSC), resulting in rapid immobilization (knockdown) and death (Davies et al., 2007). Several formulations of pyrethroids have been widely used for controlling adult *Ae. aegypti*, for both of domestic (i.e., aerosol cans, mosquito coils and impregnated materials) and public uses (i.e., thermal fogging and ultra-low volume (ULV) space spraying).

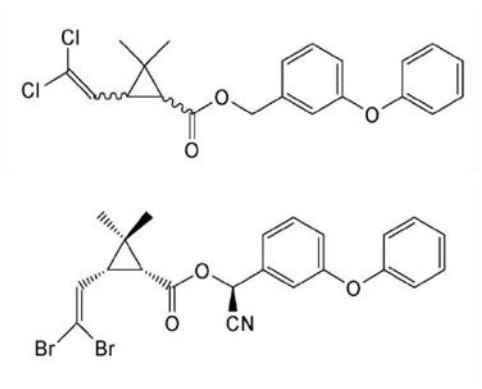


Figure 1.3 Chemical structure of permethrin and deltamethrin

1.2.5 Insecticide resistance status in Thailand

The intensive and extensive use of insecticides has generated high selective pressure on mosquito populations and led to the development of insecticide resistance (WHO, 2012). This problem is the principal factor reducing the efficacy of disease vector control programs. In Thailand, DDT resistant *Ae. aegypti* was first evidenced in the mid-1960s (Neely, 1966). Temephos is the main larvicide used in the control of *Ae. aegypti*. Temephos resistance of *Ae. aegypti* was reported from several provinces including, Bangkok, Nakhon Sawan, Tak, Phatthalung and Surat Thani (Ponlawat et al., 2013). Resistance to pyrethroids and other classes of insecticides in *Ae. aegypti* has been reported throughout Thailand (Jirakanjanakit et al., 2007a; Jirakanjanakit et al., 2007b; Paeporn et al., 2004; Pethuan et al., 2007; Ponlawat et al., 2005; Somboon et al., 2003).

1.2.6 Insecticide resistance mechanism

Insecticide resistance in *Ae. aegypti* consists of four mechanisms: increased metabolic activity (David et al., 2013; Hemingway et al., 2004), target site insensitivity (Hemingway and Ranson, 2000; Soderlund and Knipple, 2003), cuticle thickening (Kasai et al., 2014; Wood et al., 2010), and behavioral avoidance (Chareonviriyaphap et al., 2013). The first two mechanisms are considered to be of particular importance and well documented.

1.2.6.1 Metabolic resistance

The detoxifying enzymes become more effective in metabolizing the insecticide, preventing it from reaching its target in the nervous system. The processes include 1) increase in the number of available detoxifying molecules (by gene expression activation or amplification) or 2) mutation in the coding portion of the detoxifying enzyme gene, resulting in enhancing its product metabolizes the insecticide. There are three major enzyme superfamilies involving these processes: Mixed Function Oxidases P450 (MFO), Glutathione-S Transferases (GST) and Esterases (Hemingway and Ranson, 2000; Montella et al., 2007). Studies have shown that P450s are the most strongly associated with pyrethroid resistance (Jeffrey and Shinji, 2001; Kasai et al., 2014 Li et al., 2007). These superfamilies are produced by various genes (Ranson et al., 2002), and it is not unusual for different enzymes to produce the same metabolites. Additionally, an alteration in one type of enzyme may lead to cross-resistance among multiple classes of insecticides (Ranson et al., 2011).

1.2.6.2 Target site resistance

This mechanism is characterized by modification of the insect molecule where the insecticide binds, inhibiting its effects. Neurotoxic insecticides have their ultimate target in insect central nervous system: the nicotinic acetylcholine receptors for spinosyns and neonicotinoids, the enzyme acetylcholinesterase (AChE) for organophosphates and carbamates, the gamma-aminobutyric acid receptor (GABA) for cyclodienes, and the voltage-gated sodium channel for DDT and pyrethroids. Although the mutated target molecule reduces or even abolishes, its affinity for the insecticide, this alteration must not result in loss of function regarding the insect physiological processes (Ffrench-Constant et al., 1998; Raymond et al., 2001).

Since pyrethroids are the most common insecticide nowadays, pyrethroid resistance has an immense impact on mosquito control programs. The resistance of *Ae. aegypti* in Thailand to pyrethroids is associated with elevated activity of MFO enzymes, whereas esterases and GST play a little role (Lumjuan et al., 2014; Plernsub et al., 2016a; Prapanthadara et al., 2002; Somwang et al., 2011). However,

little has been done to characterize metabolic resistance mechanisms using genome-wide transcriptional analyses and functional validations. Also, this resistance is strongly linked to *kdr* mutations which have been proved in our previous studies (Stenhouse et al., 2013, Yanola et al., 2010).

1.2.7 *Kdr* mutation

Kdr mutation refers to the mutation in the voltage-gated sodium channel (VGSC). The insect VGSC consists of 4 internally homologous domains (I-IV) connected by intracellular linkers, with each domain consisting of 6 membrane-spanning segments (S1-S6) joined by intracellular or extracellular loops. Within the axon membrane, the S5 and S6 segments come together to form a central ion-conducting pore, with the S1-S4 segments forming the voltage-sensing part of the channel. Re-entrant ion hairpin loops (P-loops) between S5 and S6 form the narrow ion-selective filter at the extracellular end of the pore (Figure 1.4) (Davies et al., 2007).

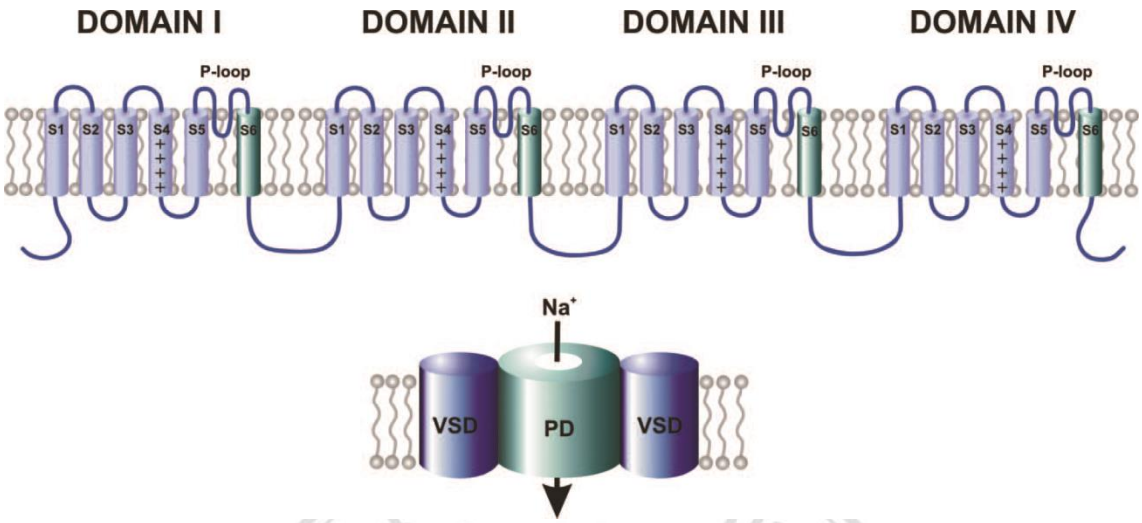


Figure 1.4 The transmembrane topology of the voltage-gated sodium channel. The pore-forming unit consists of a single polypeptide chain with four internally homologous domains (I–IV), each having six transmembrane helices (S1–S6). The domains assemble to form a central aqueous pore (PD), lined by the S5, S6 helices and S5 – S6 linkers (P-loops). The S1–S4 helices are responsible for the voltage sensitivity of the channel and assemble to form four independent voltage sensing domains (VSD) (Note: only two of the four VSD's are shown in the figure). The voltage-dependence of channel activation is thought to derive from the movement of the four positively charged (+) S4 segments (Davies et al., 2007).

The first *kdr* trait was found in the house fly (*Musca domestica*). It is a single nucleotide polymorphism resulted in leucine to phenylalanine substitution at position 1014 (L1014F), in the IIS6 segment. Following then, this mutation has been investigated in a large number of insects, and it is termed classic *kdr* (Ingles et al., 1996; Knipple et al., 1994; Miyazaki et al., 1996; Williamson et al., 1996; Williamson et al., 1993). The second mutation, M918T in conjunction with L1014F (M918T+L1014F); this genotype leads to a higher resistance level to pyrethroids, called super *kdr* phenotype (Ingles et al., 1996; Williamson et al., 1996). The *kdr* mutation has been studied globally in numerous arthropod vectors. Besides, some *kdr* mutations have been found in multiple insect species, while others have been found only in one species

(Figures 1.5) (Davies et al., 2007; Dong, 2007; Rinkevich et al., 2013; Soderlund, 2008; Soderlund and Knipple, 2003).

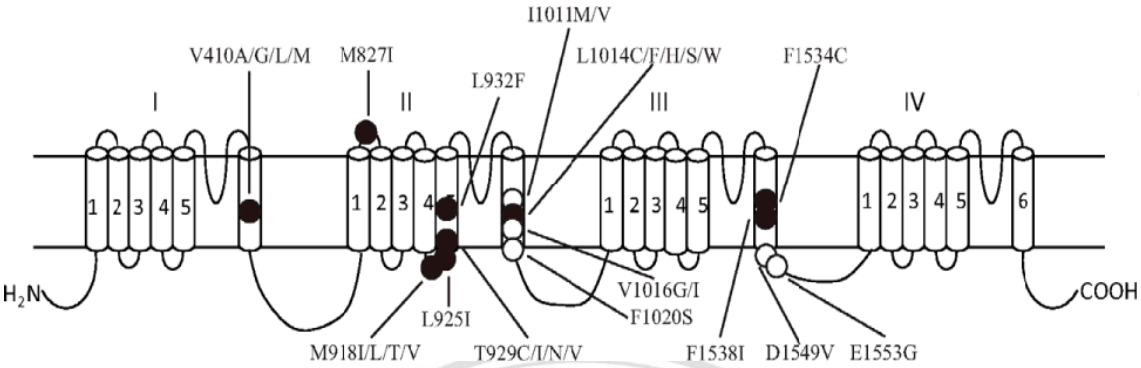
In *Ae. aegypti*, ten mutations at eight codons positions in VGSC gene (domains II-IV), comprising 15 haplotypes have been demonstrated (Table 1.1). These mutations vary in frequency, geographical spread and effects on resistance (Figure 1.6). In Thailand, three *kdr* mutations, i.e., V1016G (co-found with S989P) and F1534C, based on the mutation positions of house fly VGSC, have been reported (Plernsub et al., 2016a; Stenhouse et al., 2013; Yanola et al., 2010).

The first *kdr* mutation, V1016G, a valine to glycine substitution at position 1016 within domain II of VGSC is associated with varying degrees of resistance to type I and II pyrethroids, such as permethrin and deltamethrin, respectively (Brengues et al., 2003; Stenhouse et al., 2013). The G1016 allele has been found throughout Thailand, with an average frequency of 0.23 (Stenhouse et al., 2013). This allele has also been reported elsewhere in Asia, i.e., Indonesia, Vietnam, Taiwan, Bhutan, Myanmar, Singapore, Malaysia, China and Saudi Arabia (Al Nazawi et al., 2017; Brengues et al., 2003; Chang et al., 2009; Ishak et al., 2015; Kasai et al., 2014; Kawada et al., 2009; Li et al., 2015; Yanola et al., 2011). In addition, another *kdr* mutation at the same position, a valine to isoleucine substitution (V1016I) occurs among *Ae. aegypti* population in Latin America (Alvarez et al., 2014; Deming et al., 2016; Harris et al., 2010; Linss et al., 2014; Vera-Maloof et al., 2015), Vietnam (Bingham et al., 2011), and recently found in Ghana (Kawada et al., 2016).

The second *kdr* mutation, phenylalanine to cysteine substitution at position 1534 within domain III (F1534C), is associated with resistance to type I pyrethroids (Yanola et al., 2010). Similar to the G1016 allele, the C1534 allele has been found throughout Thailand with higher average frequency (0.77) (Yanola et al., 2011). Furthermore, the F1534C mutation is the most widespread *kdr* mutation and occurs across three continents: Asia, the Americas and Africa (Al Nazawi et al., 2017; Alvarez et al., 2014; Deming et al., 2016; Harris et al., 2010; Ishak et al., 2015; Kasai et al., 2014; Kawada et al., 2016; Kawada et al., 2009; Kawada et al., 2014; Kushwah et al., 2015; Li et al., 2015; Linss et al., 2014; Muthusamy and Shivakumar, 2015; Sayono et al., 2016; Stenhouse et al., 2013; Vera-Maloof et al., 2015; Wuliandari et al., 2015).

The third *kdr* mutation, a serine to proline substitution at position 989 in domain II (S989P), this mutation is likely to co-occur with V1016G mutation in many countries (Al Nazawi et al., 2017; Kasai et al., 2014; Kawada et al., 2014; Li et al., 2015; Srisawat et al., 2010; Stenhouse et al., 2013). Even though the S989P mutation was not detected in Malaysia (Ishak et al., 2015) and Taiwan (Chang et al., 2009). In the *Xenopus* oocyte expression system, the S989P mutation alone did not affect permethrin or deltamethrin sensitivity, but in these S989P+V1016G or S989P+V1016G+F1534C mutation combination significantly reduced the VGSC sensitivity to deltamethrin (Hirata et al., 2014). Generally, P989+G1016 and C1534 mostly occur on opposite chromosomes. However, the triple mutant haplotype has been detected at low frequency in at least three discrete areas including Myanmar, Indonesia and Singapore (Kasai et al., 2014; Moyes et al., 2017; Sayono et al., 2016), possibly generated by independent recombination events. Although P989+G1016 and C1534 recessive alleles, there is an additive effect of the combination of these alleles; the heterozygous form of S989P+V1016G+F1534C confers high resistance to pyrethroids (Plernsub et al., 2016a; Plernsub et al., 2016b).

A



B

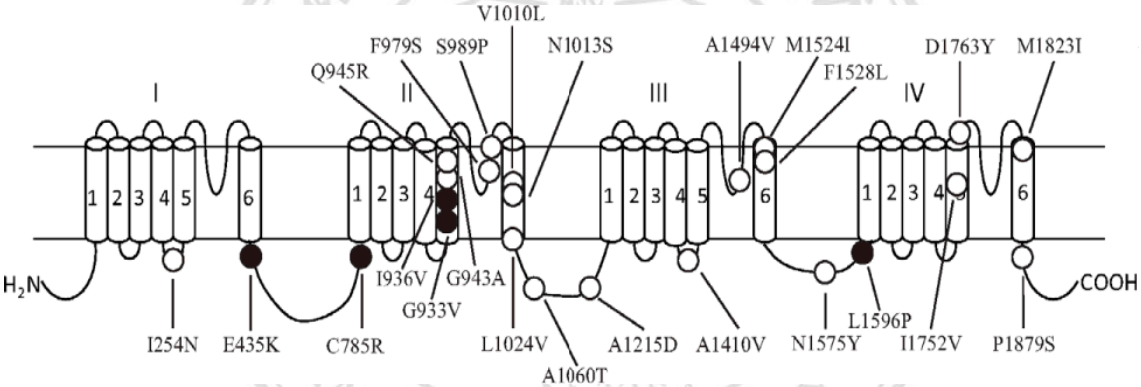


Figure 1.5. Position of pyrethroid resistance-associated sodium channel mutations that are detected in more than one species (A) and only in one species (B). The mutations with solid circles have been confirmed to reduce sodium channel sensitivity to pyrethroids in *Xenopus* oocytes. The mutations with open circles have not been examined in *Xenopus* oocytes yet. Mutations positions are designated based on house fly sodium channel numbering (Genbank accession number: X96668) (Rinkevich et al., 2013).

Table 1.1. Geographical distribution of known VGSC mutations and haplotypes in *Aedes aegypti*. Codons (para numbering) give wild type amino acid (reviewed by Moyes et al., 2017)

IIS4-5	IIS5-6	IIS5-6	IIS6	IIIS6	IIIS6	IIIS6	IVS5	Mutation(s)	Americas	Africa	Middle East	Asia
			V1016					G	.	.		Thailand, Indonesia, Vietnam, Malaysia, Taiwan
		S989						P	.	.		Indonesia
		S989	V1016					P+G	.	.	Saudi Arabia	Thailand, Singapore, Myanmar, China, Indonesia
		S989				F1534		P+C	.	.		Indonesia
		S989	V1016			F1534		P+G+C	.	.		Myanmar, Indonesia, Singapore
			V1016			F1534		G+C	.	.		Singapore, Indonesia
						F1534		C	Venezuela, Mexico, French Guiana, Guadeloupe	Ghana	Saudi Arabia	Vietnam, Thailand, Singapore, Myanmar, Indonesia, Malaysia, India
			V1016					I	Multiple Latin American countries, Columbia, Mexico, Grand Cayman, Brazil, Venezuela, French Guiana, Guadeloupe	Ghana		.
			V1016			F1534		I+C	Grand Cayman, Brazil, Venezuela, Mexico, French Guiana, Guadeloupe	.		.
			I1011					M	Brazil, French Guiana, Guadeloupe	.		.
				T1520		F1534		I+C	.	.		India
			I1011					V	Multiple Latin American countries	.		Thailand
G923			I1011					M+V	French Guiana, Brazil, Martinique	.		.
	L982							W	.	.		Vietnam
			V1016				D1763	G+Y	.	.		Taiwan

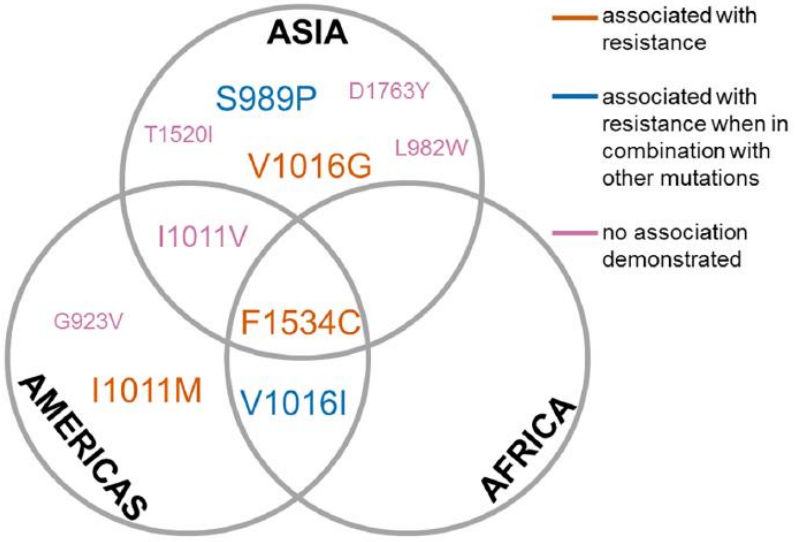


Figure 1.6 The geographical distribution of the 10 known voltage-gated sodium channel (VGSC) mutations in *Aedes aegypti* across the 3 continents in which they have been detected. Association of each mutation with pyrethroid resistance is shown in the key. Font size gives an indication of relative frequency (Moyes et al., 2017).

1.2.8 Detection of *kdr* mutations

Several methods can perform detection of *kdr* alleles in mosquitoes. Nucleotide sequencing is considered to be the most accurate method as a gold standard. The advantages of DNA sequencing over the other methods: (1) allowing direct visualization of the nucleotide allele sequences. (2) eliminating the problem of unspecific amplification or hybridization of PCR based protocols. (3) enabling visualizing potential novel variations that would never be identified by PCR diagnostic SNP techniques. However, this method is expensive and not suitable for examining a large number of mosquitoes. A number of PCR-based techniques for detecting *kdr* alleles have been reported. An assay was optimized for use in a real time PCR machine, although the amplified products could also be detected *via* agarose gel electrophoresis (Saavedra-Rodriguez et al., 2007). An alternative technique using a heated oligonucleotide ligation assay (HOLA) was developed (Rajatileka et al., 2008). Although this assay does not involve the use of radioisotope or any specialized

machine, it requires additional reagents that can contribute to increased costs. We recently developed allele-specific PCR-based assays (AS-PCR) to detect the F1534C (Yanola et al., 2011) and V1016G mutations (Stenhouse et al., 2013). Although techniques are simpler and genotyping results can only be determined by gel electrophoresis, testing one sample requires two separate reactions, i.e., one for F1534C and the other V1016G. In addition, AS-PCR often shows a discrepancy with DNA sequencing (Yanola et al., 2011). Therefore, a multiplex PCR to detect both F1534C and V1016G mutations in a single reaction is needed to develop. This technique can reduce the cost and time consumed in monitoring the mutant allele frequencies in many countries where both V1016G and F1534C mutations co-exist.

1.2.9 Fitness costs associated with insecticide resistance

Although resistance mechanisms help mosquitoes survive under continuous insecticide pressure, these actions are costly and may negatively affect mosquito's fitness (e.g., size of body, adult longevity, larval development time, fecundity, fertility, mating competitiveness and blood feeding capability) when the insecticides are withdrawn. The fitness reduction may be caused by pleiotropy in the resistance genes themselves or a consequence of hitch-hiking effects.

According to each resistance mechanism, overexpression of a resistance-conferring gene is thought to result in a fitness cost because of a resource and energy reallocation at the expense of metabolic and developmental processes. A constitutive low-expression and target-site modification can result in a fitness cost when the target-site substitution or the low-expressed gene are essential for the viability of the insect, or when this change results in a near loss of function. Any molecular alteration may also have pleiotropic effects on other traits, causing a morphological and possibly a behavioral change impairing the resistant individual's survival and/or reproductive success in the natural population (Kliot and Ghanim, 2012).

Several studies have shown that insecticide resistance reduces the reproductive fitness of *Ae. aegypti* (Belinato et al., 2012; Brito et al., 2013; Diniz et al., 2015; Kumar et al., 2009; Martins et al., 2012; Mebrahtu et al., 1997) as well as some *Anopheles* and *Culex* species (Amin and White, 2009; Hardstone et al., 2009; Kumar

and Pillai, 2011; Kumar et al., 2009; Li et al., 2002; Otali et al., 2014; Rowland, 1991). For example, decreased longevity and increased larval development time have been reported in pyrethroid-resistant *Ae. aegypti* and also *Culex pipiens* (Belinato and Valle, 2015; Jaramillo et al., 2014; Li et al., 2002; Martins et al., 2012). In competition analyses, *Ae. aegypti* with *kdr* mutations had longer larval development times when reared alongside the susceptible strain under stringent conditions (Brito et al., 2013). By contrast, pyrethroid-resistant *Anopheles funestus* in southern Africa did not show any loss of fitness under laboratory conditions (Okoye et al., 2007). These studies have suggested that fitness cost may vary depending on mosquito species and resistance mechanisms.

While the investigation of fitness cost in field populations is difficult and complicated, evaluation of relative fitness obtained from laboratory studies is useful when considering the impact of resistance alone on biological fitness (Brito et al., 2013; Martin et al., 2012). Over 20 years of our studies regarding insecticide resistance mechanisms in *Ae. aegypti* populations in Thailand, we have detected four major genotypic forms of *kdr* mutant mosquitoes, no *kdr* alleles (wild type), C1534 homozygote, P989 + G1016 homozygote and S/P989 + V/G1016 + F/C1534 heterozygote (Plernsub et al., 2016a; Stenhouse et al., 2013; Yanola et al., 2010). We also succeeded in establishing three strains of *Ae. aegypti* harboring no *kdr* alleles (PMD strain), C1534 homozygous allele (PMD-R strain) and P989 + G1016 homozygous allele (UPK-R strain). These strains also showed differences in levels of detoxifying enzyme activities and response to insecticide susceptibility (Choovattanapakorn et al., 2017; Lumjuan et al., 2011; Lumjuan et al., 2014; Plernsub et al., 2016a; Plernsub et al., 2016b; Prapanthadara et al., 2002; Prapanthadara et al., 2005; Somwang et al., 2011; Son-un et al., 2018). It is known that maintenance of resistance, including *kdr* genes, often involves a fitness cost in the absence of insecticide (Brito et al., 2013). Recent studies revealed that the heterozygous form was most common (about 46% of the population) in Chiang Mai Province, and showed a high level of pyrethroid resistance (Plernsub et al., 2016a; Plernsub et al., 2016b). It was also postulated that the heterozygote might be fitter than homozygous mutant genotypes and plays a vital role in maintaining C1534 and G1016 alleles in wild populations (Plernsub et al., 2016a). However, the maintenance of polymorphism for heterozygous

alleles is not clearly understood. Studying the fitness cost in resistant heterozygotes is imperative because the heterozygotes will be more common at the early stages of insecticide selection. Also, the dominance of any possible pleiotropic effects of the resistance can be better studied in these heterozygous individuals (Argentine et al., 1989; McCarroll and Hemingway, 2002). Identifying fitness costs as a result of all patterns of resistance alleles can be an advantage in designing the integrated vector control programs and predicting resistance trends in the future.

1.3 Purposes of this study

1.3.1 To develop and validate a multiplex PCR method for detection of the V1016G and F1534C mutations in the voltage-gated sodium channel gene of *Ae. aegypti*.

1.3.2 To investigate the relative fitness cost of three insecticide-resistant strains of *Ae. aegypti*, PMD, PMD-R and UPK-R, and F_1 (PMD-R x UPK-R) hybrid on various life-history parameters.

1.4 Usefulness of this study

The multiplex PCR method developed during this study will provide another means for assessing pyrethroid insecticide resistance in this medically important mosquito species. Due to its simplicity, and lack of expensive equipment or reagents, this method will be especially useful for laboratories with limited resources. Together with the understanding of fitness cost associated with pyrethroid resistance, the knowledge of this study can improve in designing integrated vector management and predicting resistance trends in the future.

CHAPTER 2

Materials and Methods

2.1 Development of a multiplex-PCR for detection of knockdown resistance mutations, V1016G and F1534C, in pyrethroid resistant *Aedes aegypti*

2.1.1 Mosquito samples

Five laboratory strains, two F_1 hybrids, DNA samples and field-collected *Ae. aegypti* mosquitoes were used to develop, optimize and validate the multiplex PCR method. The three laboratory strains included a pyrethroid susceptible strain, PMD (homozygous wild type for both V1016 and F1534 alleles, V/V1016+F/F1534), and two pyrethroid resistant strains, PMD-R (homozygous wild type for the V1016 allele but homozygous mutant for the C1534 allele, V/V1016+C/C1534) (Somboon et al., 2003; Yanola et al., 2010), and UPK-R (homozygous mutant for G1016 allele but homozygous wild type for the F1534 allele, G/G1016+F/F1534) (Plernsub et al., 2016a). The PMD and PMD-R strains originated from a rural area of Chiang Mai Province and have been maintained in our laboratory since 1997. The UPK-R strain was established from mosquitoes collected from Chiang Mai city and maintained since 2006. An F_1 hybrid was derived from the cross between the PMD (male) and UPK-R (female) strains to produce heterozygous mutant for the G1016 allele but homozygous wild type for the F1534 allele, V/G1016+F/F1534. Another F_1 hybrid was derived from the cross between the PMD-R (male) and UPK-R (female) strains to produce heterozygous mutant for both G1016 and F1534 allele, V/G1016+F/C1534.

The other two laboratory strains were Dagon Myothit North Yangon (YG) and Than Bya Zayet Monstate (MS) strains. These two strains originated from Myanmar, however, the *kdr* genotypes of each strain were not determined until the present study.

The DNA samples of *Ae. aegypti* collected from wild populations in Thailand, Myanmar, Cambodia, Bhutan and Pakistan were obtained from previous studies (Stenhouse et al., 2013; Yanola et al., 2011).

Field-collected mosquito samples were obtained from larval surveys in various temples around Chiang Mai city, Thailand, as temples are numerous and readily accessible throughout Chiang Mai, and from households from several rural villages in Mae Taeng district. We also collected the immature stages from Myanmar (Yangon city) and Indonesia (Ternate Island and Soppeng Regency, South Sulawesi). The samples collected were reared to adulthood, identified morphologically (Rattanarithikul et al., 2010) and preserved in absolute ethanol until the multiplex PCR was performed.

2.1.2 Development of multiplex PCR method

This assay was designed by combining two sets of primers. The first set was previously designed to genotype the V1016G mutation (Saavedra-Rodriguez et al., 2007) and was used to develop an AS-PCR assay (Stenhouse et al., 2013). This set consists of three primers: a common forward primer (Gly1016f) and two specific reverse primers, Val1016r and Gly1016r, that differ in length and distinguish the V1016 and G1016 alleles, respectively (Figure 2.1). In the case of a heterozygote, both products would be amplified.

The second set of primers was newly designed using the web-based Primer3Plus software (Untergasser et al., 2012). The cDNA nucleotide sequences of VGSC domains IIIS4-IVS2 of the *Ae. aegypti* PMD strain (GenBank: EU259810.1) and PMD-R strain (GenBank: EU259811.1), which were submitted by Yanola et al. (Yanola et al., 2010), were used as reference sequences. This set of primers were designed to genotype the F1534C mutation (Figure 2.2, 2.3) and consisted of four primers (tetra primer PCR assay). In this assay, the outermost primers (c1534-f and c1534-r) amplified a control band of 368 bp. Two internal allele-specific primers, Ae1534F-r and Ae1534C-f, were designed to work in conjunction with the external primers to give amplified products of either 232 bp for the F1534 allele or 180 bp for the C1534 allele, respectively. In the case of a heterozygote, all three products would be amplified. All primer sequences used in this study are shown in Table 2.1.

For optimization, DNA samples extracted from the laboratory strains, PMD, PMD-R, UPK-R and (PMD-R × UPK-R) F_1 hybrids were used. Our preliminary study revealed that the PCR conditions from previous reports (Saavedra-Rodriguez et al., 2007; Stenhouse et al., 2013) were not suitable for our multiplex technique. Therefore, several PCR conditions were optimized for a total reaction volume of 10 µl: primer annealing temperature (50-65 °C), concentration of each primer (0.1–0.5 µM), *Taq* DNA polymerase concentration (0.05–0.5 unit), $MgCl_2$ concentration (0.5–3.0 mM) and dNTP concentration (50–200 µM). Furthermore, the lowest amount of genomic DNA (DNA template) that still gave a clear result on agarose gel, i.e., the detection limit of this method, was also determined.

2.1.3 V1016G and F1534C genotyping by multiplex PCR

Genomic DNA from each alcohol-preserved mosquito was extracted using DNAzol® reagent (Invitrogen, Carlsbad, California, USA). After PCR reactions, the amplified products were analyzed on 3% agarose gel with a low molecular weight DNA ladder (New England Biolab, Ipswich, Massachusetts, USA) used to estimate the band size. The electrophoresis was run for 50 min at 100 V in TBE buffer. The gel was then submerged in 0.5 µg/ml ethidium bromide (EtBr) (Invitrogen) solution for 15 min, de-stained for 5 min in distilled water, and visualized in a UV transilluminator.

Since EtBr is known to be a strong mutagen and is treated as hazardous waste, as alternatives we tried using Ultrapower™ (BioTeke, Beijing, China) and RedSafe™ (iNtRON Biotechnology, Gyeonggi-do, Korea) dyes, which are advertised as non-toxic and have no hazard waste. For Ultrapower™ staining, the dye solution (10,000×) was diluted 100-fold in 6× loading dye (New England Biolab), then 1 µl of diluted dye was mixed with 5 µl of PCR product. 1 µl of diluted dye was also added to 5 µl of the DNA ladder before loading on the gel. For RedSafe™ staining, 5 µl of this dye (20,000×) was mixed in with 100 ml molten agarose gel prior to gel pouring. Visualization was done immediately after gel electrophoresis.

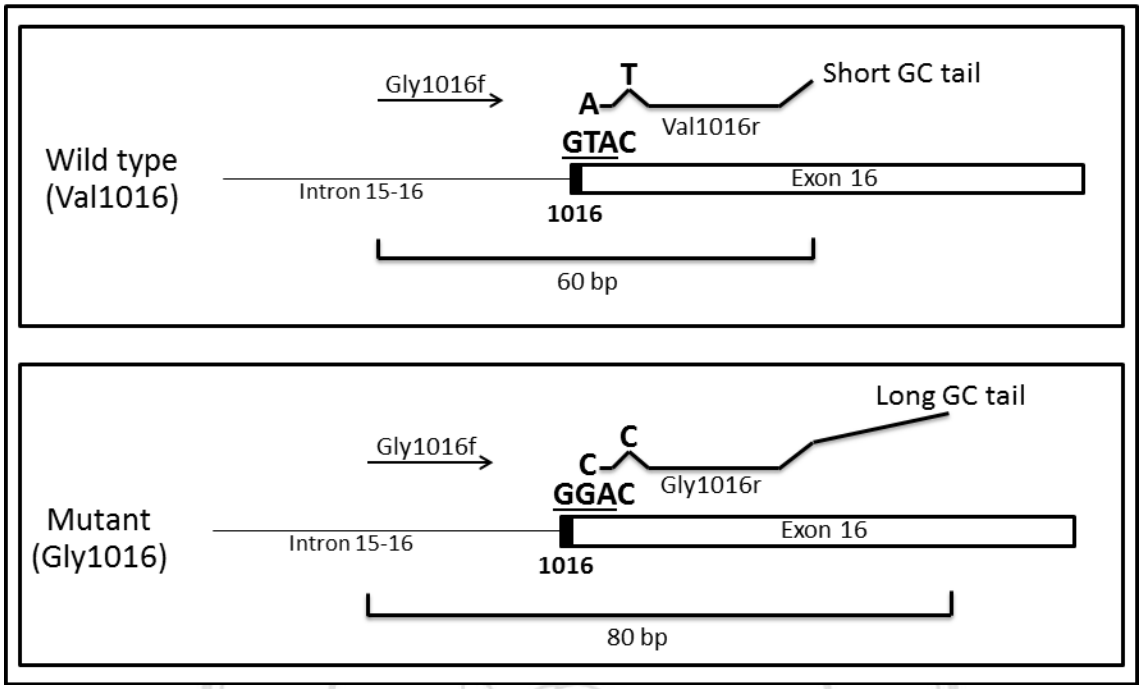


Figure 2.1 Schematic of the AS-PCR assay for detection of the V1016G mutation as described in Stenhouse et al. (Stenhouse et al., 2013).

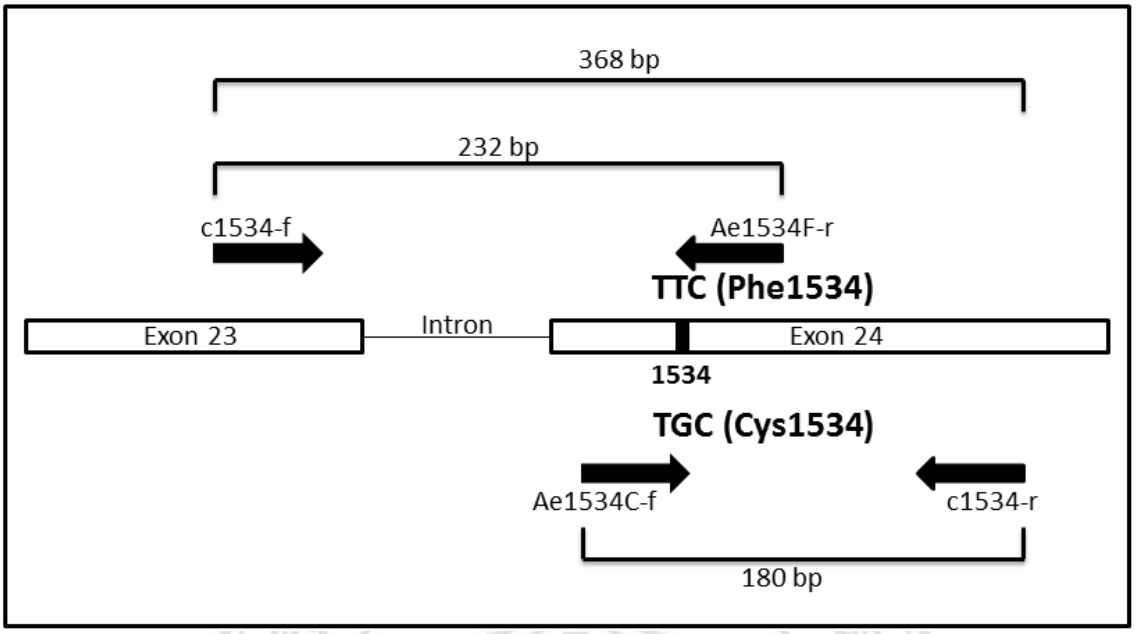


Figure 2.2 Schematic of the tetra primer AS-PCR assay developed herein for detection of the F1534C mutation

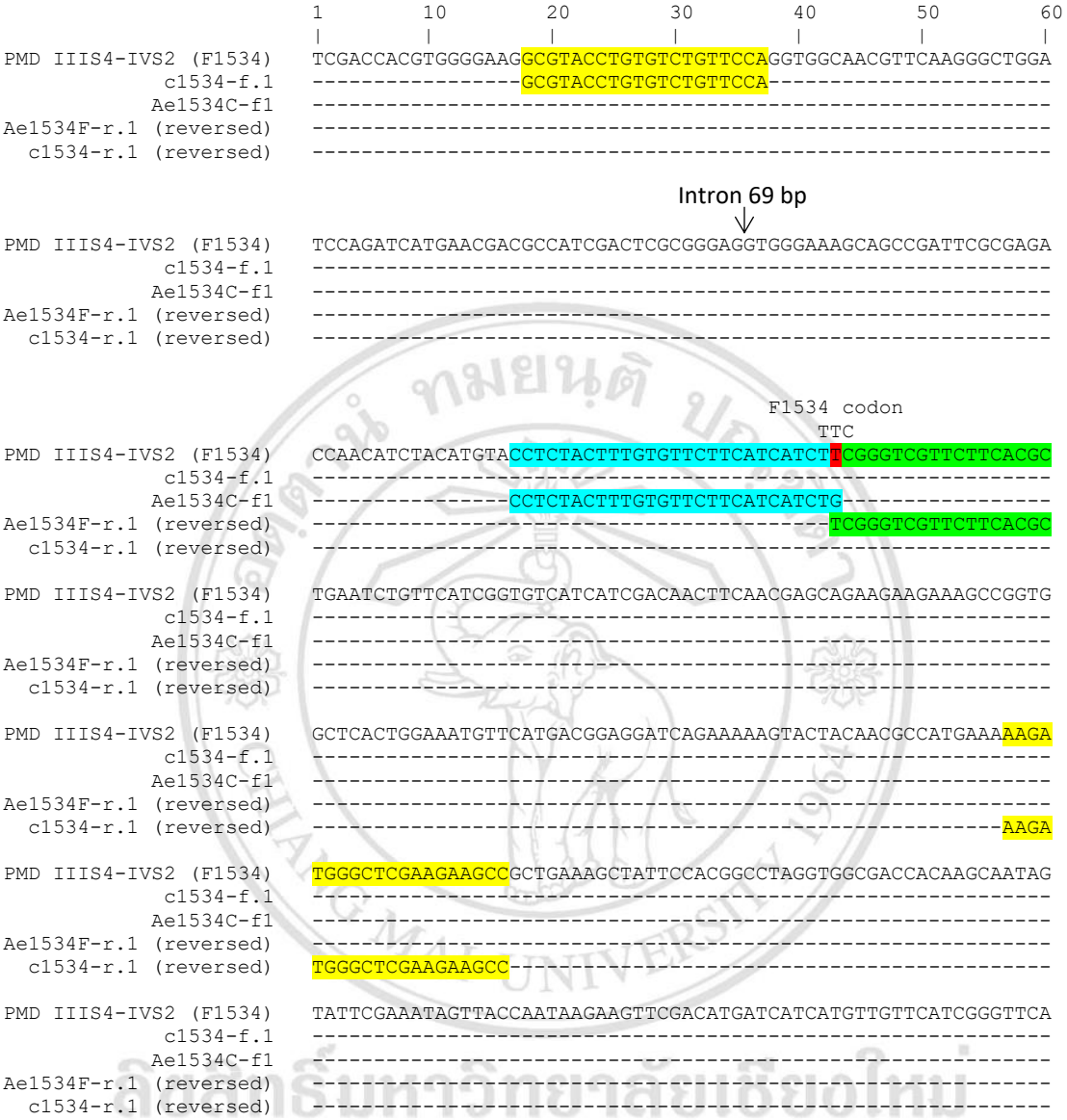


Figure 2.3 The alignment illustrates the location of F1534 codon on the cDNA nucleotide sequences of VGSC domains IIIS4-IVS2 of the *Ae. aegypti* PMD strain (GenBank: EU259810.1), and primer binding locations. The sequence of backward primers is reversed for the alignment.

Table 2.1 Sequences of primers used in this study

Primer name	Primer sequence (5'-3')	Product size (bp)	Exon[a]
Direct sequencing			
IIP_F	GGTGGAACTTCACCGACTTC	581	15
IIS6_R	GGACGCAATCTGGCTTGTTA		16
Ge-IIIS6_F	GCTGTCGCACGAGATCATT	635	23
IIIS6_R	GTTGAACCCGATGAACAACA		25
Multiplex PCR			
1016 genotyping			
Gly1016f	ACCGACAAATTGTTTCCC		15–16[b]
Val1016r	[short GC tail][c] AGCAAGGCTAAGAAAAGGTTAATTA	60	16
Gly1016r	[long GC tail][d] AGCAAGGCTAAGAAAAGGTTAACTC	80	16
1534 genotyping			
c1534-f	GCGTACCTGTGTCTGTTCCA	368	23
c1534-r	GGCTTCTTCGAGCCCATCTT		24
Ae1534F-r	GCGTGAAGAACGACCCGA	232	24
Ae1534C-f	CCTCTACTTTGTGTTCTTCATCATCTG	180	24

[a]Exon from the *Ae. aegypti* VGSC gene. This transcript corresponds to VectorBase Transcript ID AAEL006019

[b]Intron between exon 15 and 16

[c]Short GC tail sequence: 5'-GCG GGC-3'

[d]Long GC tail sequence: 5'-GCG GGC AGG GCG GCG GGG GCG GGG CC-3'

2.1.4 DNA sequencing

In order to validate the multiplex PCR method, the results of samples tested by this assay were compared by using DNA sequencing data obtained from previous studies (Stenhouse et al., 2013; Yanola et al., 2011) as well as the present study. The IIS6 and IIIS6 regions of the VGSC gene, which encompass the V1016G and F1534C mutations, respectively, were amplified and purified. This method has been described previously (Yanola et al., 2011). For domain IIS6 amplification, each PCR was carried out in a 20 µl reaction volume, containing: 2 µl of DNA sample (50 ng), 0.4 units of Platinum *Taq* DNA polymerase (Invitrogen), 1.6 µl of 2.5 mM dNTPs mix (200 µM) (New England Biolabs), 0.6 µl of 50 mM $MgCl_2$ (1.5 mM), 2 µl of 10× PCR buffer (1×) (Invitrogen), and 2 µl of 5 µM each of IIP-F (0.5 µM) and IIS6_R primers (0.5 µM) (Table 2.1), and made up to 20 µl with sterile water. The amplification consisted of 95 °C for a 2 min heat activation step, followed by 35 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s with a 2 min final extension step at 72 °C. Amplifying domain IIIS6 used the same conditions, but the primers were changed to Ge-IIIS6_F and IIIS6_R.

The amplified products were purified using Illustra™ ExoProStar™ 1-Step DNA purification reagent (GE Healthcare Life Sciences, Buckinghamshire, UK) and sent to Macrogen, Inc. (Seoul, Korea) for direct sequencing in both the forward and reverse directions. Sequence data were analyzed using Geneious software, version 5.3.6 (Biomatters Ltd, UK). Finally, for each mosquito sample, sequencing results were compared against the genotype previously obtained by the multiplex PCR method.

2.1.5 Allele-specific PCR (AS-PCR)

The efficacy of multiplex PCR was compared with the AS-PCR methods for detecting F1534C and V1016G mutations as described in previous studies (Stenhouse et al., 2013; Yanola et al., 2011). The tested materials included DNA samples from the previous studies and newly extracted field samples.

2.2 Investigation of relative development and reproductivity fitness cost in three insecticide-resistant strains of *Aedes aegypti* from Thailand

2.2.1 Mosquito strains

The three strains of *Ae. aegypti*, PMD, PMD-R and UPK-R, as mentioned in 2.1.1 were used in this study. Initially, exposure of natural *Ae. aegypti* adults reared from wild larvae in northern Thailand to WHO DDT papers (4%) for 30 min revealed low or no mortality, indicating that DDT resistance was very common (Somboon et al., 2003). Attempts have been made to establish a DDT-susceptible strain but not successful. The PMD and PMD-R strains (formally called R^dS^p and R^dR^p, respectively) were established from isofemale lines reared from wild larvae from Ban Pang Mai Daeng, a rural village of Chiang Mai Province since 1997 (Prapanthadara et al., 2002). Briefly, half of each isoline was exposed to WHO permethrin papers (0.25%) for 1 h Isolines that showed 100% mortality were checked for *kdr* alleles by DNA sequencing and those without *kdr* mutation alleles were pooled and colonized as the DDT-resistant PMD strain. Exposure of female and male progeny to DDT papers continued for at least 10 generations. This strain is susceptible to permethrin and deltamethrin, but resistant to DDT (<5% mortality), although no further exposure to DDT (<5% mortality) (Choovattanapakorn et al., 2017).

The permethrin resistant PMD-R strain was established from several C1534 homozygous isofemale lines that had survived permethrin exposure bioassays. Exposure of female and male progeny to permethrin papers continued for at least 10 generations. This strain is resistant to permethrin and DDT determined by adult bioassays (0-5% mortality), conferred by *kdr* and metabolic enzymes. DDT resistance in the PMD and PMD-R strains is associated with increased DDTase and MFO activities which were about 10-fold and four-fold, respectively, higher than the susceptible Rockefeller strain (Prapanthadara et al., 2002). Later, Prapanthadara et al. 2005 revealed that DDTase activity of GST isoenzymes in the PMD-R was about 10-fold higher than the PMD strain. Although the amount of MFO in the PMD and PMD-R strains was not significantly different (Somwang et al., 2011), there are, however, differences in another oxidative enzyme system between the two strains; two aldehyde dehydrogenase

(ALDH) genes, ALDH9948 and ALDH14080 were upregulated in the PMD-R strain and involved in permethrin metabolism (Lumjuan et al., 2014). Mosquitoes of the 82nd generation of both strains were used in this study.

The deltamethrin resistant UPK-R strain was established from isofemale lines reared from wild larvae from Chiang Mai city since 2006 (Plernsub et al., 2016a)**.** Several homozygous P989 + G1016 isofemale lines that had survived deltamethrin (0.05%) exposure bioassays were pooled and colonized. Exposure of female and male progeny to deltamethrin papers continued for at least 10 generations. This strain is resistant to not only deltamethrin (3-8% mortality), but also DDT and permethrin (0-5% mortality), conferred by *kdr* and metabolic enzymes. The UPK-R strain showed a greater level of MFO, esterase and GST activities (1-2 fold) than the PMD strain (Choovattanapakorn et al., 2017), but DDTase activity has not yet been investigated. Mosquitoes of the 41st generation of this strain was used in this study**.**

Our previous studies revealed that permethrin resistance levels of UPK-R and PMD-R were higher than the susceptible PMD strain by 325-fold and 25-fold, respectively, as determined by larval bioassays, while their deltamethrin resistance levels were higher than PMD by 53-fold and 13-fold, respectively. The adults of PMD-R and UPK-R colonies were occasionally (1-2 times a year) exposed to the standard WHO permethrin (0.75%) and deltamethrin (0.05%) papers, respectively, to confirm maintenance of resistance status. No serious deteriorative signs of inbreeding depression, e.g., high mortality of immature stages and reduced longevity of adults, were observed in the three strains.

We created a hybrid, harboring S/P989, V/G1016 and F/C1534 alleles, by crossing UPK-R females with PMD-R males. The F_1 hybrid showed intermediate resistance to permethrin and deltamethrin (Plernsub et al., 2016a). The genotypes of the three strains and the hybrid were confirmed by multiplex PCR and DNA sequencing (Saingamsook et al., 2017).

2.2.2 Mosquito rearing

All laboratory *Ae. aegypti* strains were reared and maintained in the insectary at 25-27 ^{o}C, ~70% relative humidity with a 12-h day/night cycle under our standard rearing conditions (Stenhouse et al., 2013). Dried mosquito eggs were placed into 25x35x6 cm-plastic trays filled with 3 liters of distilled water for hatching. After hatching, the first instar larvae (approximately 300 per tray) were fed on finely ground dog-biscuit (Tesco, Thailand). The water in the trays was changed two to three times a week to avoid stagnation. The pupae were transferred to plastic cups containing distilled water and placed into a 30x30x30 cm mosquito cage. After emergence, the adults were provided with 10% sucrose and 10% v/v multivitamin syrup (Seven Seas, Thailand) soaked onto cotton wool which was changed daily. One week after emergence, adult female mosquitoes were fed by an artificial membrane feeding method with heparinized cow blood (Finlayson et al., 2015). Three days post feeding, an oviposition cup lined with filter paper and filled with distilled water was placed in the cage and females were allowed to lay eggs for a few days. The filter papers with eggs were air dried for 4-5 days and stored in a sealed plastic bag.

2.2.3 Effect of fitness cost on biological parameters

2.2.3.1 Developmental time and viability of mosquitoes

This experiment was to compare the development time and viability of larvae until adult emergence in different strains under the same conditions. Eggs were submerged in water and the larvae hatched within 24 h were used. Four replicates of 50 larvae per strain were gently transferred to plastic cups (26 oz.) containing 500 mL of distilled water. Thereafter, 50 mg of finely ground dog biscuit was offered daily until pupation. The numbers of pupae were counted daily until the last larva pupated. The numbers of adults emerged were counted and sexed.

2.2.3.2 Size of mosquitoes

This experiment was to determine if there was a significant difference in the size of mosquitoes from different strains. The size of individual adult females was

estimated by the average length of both right and left wings. Thirty females per strain obtained from the same rearing conditions from 2.2.3.1 were randomly selected. The wings were cut and placed on a glass slide with normal saline and covered by a coverslip. Wing length was measured as the distance from the axillary incision to the wing tip (the end of vein R_3 (Rattanarithikul et al., 2010)), using a digital camera attached to a compound microscope (BX53, Olympus, Japan) and cellSens standard software version 1.18 (Olympus, Japan).

2.2.3.3 Blood feeding capacity

This experiment was to compare the amount of blood feeding by females of different strains. Seven-day-old females were deprived of sugar solution 24 h prior to blood feeding. After fasting, female mosquitoes were divided at random into two groups. The first group, triplicates of 100 females each, was transferred to a cage containing the artificial membrane feeder as above and allowed to feed for 30 min. The number of fully engorged females was counted. Shortly after feeding, they were killed by freezing in -20 ^{o}C for five min and weighed in pools (five each). The second group, without blood feeding, was killed and weighed in the same manner. The amount of blood taken was calculated from the ratio of female groups with and without a blood feeding.

2.2.3.4 Fecundity

This experiment aimed to determine if there was a significant difference in fecundity of females of different strains. Two hundred pairs of one-day-old males and females were confined in a cage for a week before a blood meal was offered as described above. Forty fully engorged females were randomly aspirated and kept individually in oviposition cups (8 oz.) lined inside with filter paper and provided with a 10% sugar solution. Five days post feeding, 25 mL of distilled water was poured into the cup, and the females were allowed to lay eggs for three days. Deposited eggs were counted and air dried for a week.

2.2.3.5 Egg viability after storage

This experiment was to determine the viability of eggs after different storage durations. About 160 blood-fed females from 2.2.3.4 were allowed to lay eggs for three days in the cage with an oviposition cup lined with filter paper. The papers with eggs were air dried for a week for embryo development. Afterward, the papers were cut into small pieces (each piece containing at least 100 eggs, estimated visually) and stored in a tight plastic food container. Hatchability of eggs was determined on week 0, as the control, and every two weeks for 38 weeks. To determine egg hatchability, three pieces of papers were randomly picked and checked for the presence of lids before they were submerged for 24 h in 300 mL of distilled water mixed with 0.3 g of finely ground dog food to induce hatching. The egg hatching rate was determined by examining 100 eggs/paper under a stereomicroscope. Eggs with opened lids and no larva inside were counted as viable (successful hatching).

2.2.3.6 Adult longevity

This experiment was to determine if there was a significant difference in the longevity of adult mosquitoes among the different strains. Fifty pairs of males and females, one-day-old, were released in duplicate into 30x30x30 cm cages and provided with a 10% sugar solution which was changed daily. The bottom of the cages was lined with white paper so that dead mosquitoes were easily seen. The dead mosquitoes were counted and removed every day. Comparisons of survival curves were analyzed with both Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

2.2.3.7 Larval competition

This experiment aimed to determine the competitive ability between larvae of the PMD-R and UPK-R strains when they were reared together under conditions of limited room and food. Duplicates of 50 one-day-old larvae of each strain were put together in a small cup (8 oz.) containing 100 mL distilled water. 20 mg of dog food was added to each cup every other day. Pupae were separated daily and transferred

to a new cup containing distilled water for the emergence of adults. The emerging adults were recorded daily and genotyped for G1016 and C1534 alleles by multiplex PCR (Saingamsook et al., 2017).

2.2.3.8 Colony competition

This experiment was to determine the reproductivity of adults of the PMD-R and UPK-R strains when they were mixed together in the cages in the absence of insecticide pressure. To ensure that the females were virgin before releasing into the cages, pupae of each strain were sexed and separated. Forty one-day-old females of each strain were released, followed by forty males of both strains (80 pairs per cage in total). After one week, a blood meal was offered. Four days after blood feeding, an oviposition cup was introduced in the cage and allowed to stand for three days for egg laying. The F_1 progeny eggs were air dried, induced to hatch and reared to the adult stage under the standard laboratory conditions as above. Thirty adult females and males in each cage were randomly selected and genotyped. Since our previous study revealed that S/P989 allele was found in all the PMD-R x UPK-R F_1 hybrids, we determined only the frequency of G1016 and C1534 alleles by multiplex PCR (Saingamsook et al., 2017).

2.2.3.9 Statistical analysis

Comparisons of mean values of life-trait parameters among PMD, PMD-R, UPK-R and F_1 (PMD-R x UPK-R) hybrid were conducted using analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison Test. Qualitative data were analyzed by Fisher's exact test. Others were indicated in experimental methods or results. Analyses were made using Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

CHAPTER 3

Results

3.1 Development of a multiplex-PCR for detection of knockdown resistance mutations, V1016G and F1534C, in pyrethroid resistant *Aedes aegypti*

3.1.1 Development of multiplex PCR method

Tests for the optimization of PCR conditions resulted in the following multiplex PCR protocol. Each PCR reaction was performed in a 10 µl volume containing: 1 µl of DNA sample (25 ng), 0.4 units of Platinum *Taq* DNA polymerase (Invitrogen), 0.8 µl of 2.5 mM dNTPs mix (200 µM) (New England Biolabs), 0.3 µl of 50 mM $MgCl_2$ (1.5 mM), 1 µl of 10× PCR buffer (1×) (Invitrogen), and primer concentrations: Gly1016f (0.5 µM), Val1016r (0.25 µM), Gly1016r (0.5 µM), c1534-f (0.25 µM), c1534-r (0.25 µM), Ae1534F-r (0.1 µM) and Ae1534C-f (0.5 µM), and made up to 10 µl with sterile water. The amplification consisted of 95 °C for a 2 min heat activation step, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with a 2 min final extension step at 72 °C.

For these two polymorphic sites, there are nine possible genotypes, all of which were present in the laboratory and field-collected strains (Table 3.1, Figure 3.1). The detection limit of this method was also evaluated by testing with a set of DNA dilutions of each genotype. The double heterozygous patterned sample (V/G1016+F/C1534) needed the highest amount of DNA template (2 ng) to get a reliable result (Figure 3.2). Thus, the detection limit of this method was 2 ng of genomic DNA.

Table 3.1 Comparison of genotyping results for V1016G and F1534C mutations from multiplex PCR and DNA sequencing

Strain	Year of collection	Multiplex PCR genotyping/DNA sequencing (no. of samples)									
		VV/FF	VV/FC	VV/CC	VG/FF	VG/FC	VG/CC	GG/FF	GG/FC	GG/CC	Total
Laboratory strains											
PMD		10/10	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	10/10
PMD-R		0/0	0/0	10/10	0/0	0/0	0/0	0/0	0/0	0/0	10/10
UPK-R		0/0	0/0	0/0	0/0	0/0	0/0	10/10	0/0	0/0	10/10
(PMDxUPK-R) F_1 hybrid		0/0	0/0	0/0	10/10	0/0	0/0	0/0	0/0	0/0	10/10
(PMD-RxUPK-R) F_1 hybrid		0/0	0/0	0/0	0/0	10/10	0/0	0/0	0/0	0/0	10/10
YG[a]		0/0	0/0	1/1	0/0	1/1	0/0	5/5	3/3	0/0	10/10
MS[b]		0/0	0/0	0/0	0/0	2/2	2/2	0/0	5/5	1/1	10/10
Field-collected strains											
Thailand											
Chiang Mai city, Chiang Mai	2016	0/0	0/0	15/15	0/0	20/20	0/0	12/12	0/0	0/0	47/47
Mae Taeng district, Chiang Mai	2016	0/0	0/0	12/12	0/0	5/5	0/0	1/1	0/0	0/0	18/18
Mae Sariang district, Mae Hong Son[c]	2010	0/0	2/2	6/6	0/0	6/6	0/0	2/2	0/0	0/0	16/16
Total		10/10	2/2	44/44	10/10	44/44	2/2	30/30	8/8	1/1	151/151

[a] Dagon Myothit North Yangon strain

[b] Than Bya Zayet Monstate strain

[c] Mosquito DNA samples were obtained from Yanola et al., 2011.

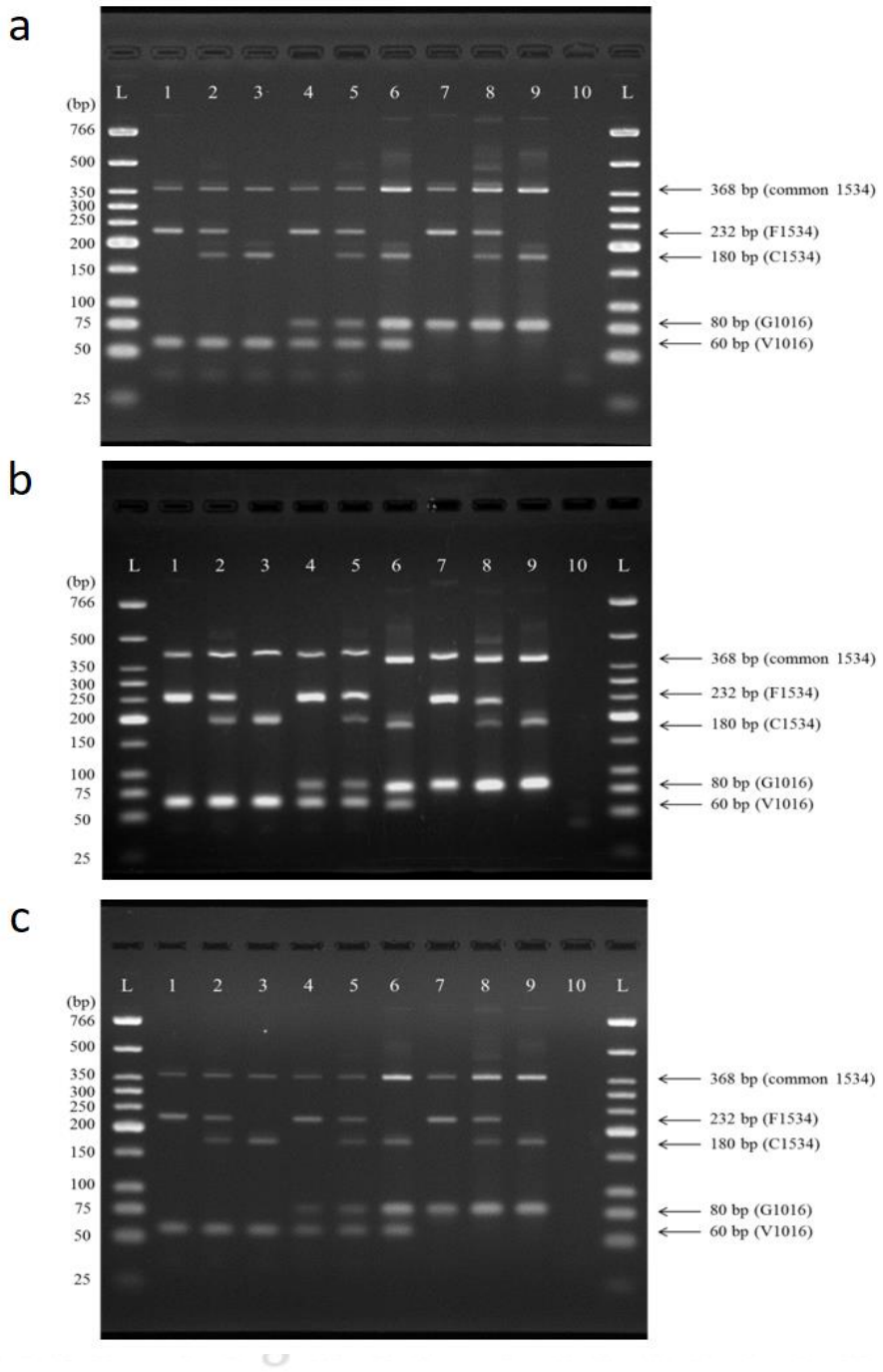


Figure 3.1 Gel electrophoresis results. a Stained with ethidium bromide. b Stained with Ultrapower^{TM} dye. c Stained with RedSafe^{TM} dye. All panels represent each of the nine possible genotypes. Lanes L: contains low molecular weight DNA ladder. Lane 1–9: contain PCR products by using a single mosquito DNA sample as template. Lane 1: VV/FF; Lane 2: VV/FC; Lane 3: VV/CC; Lane 4: VG/FF; Lane 5: VG/FC; Lane 6: VG/CC; Lane 7: GG/FF; Lane 8: GG/FC; Lane 9: GG/CC; Lane 10: negative control in which distilled water was used as the template in the PCR reaction

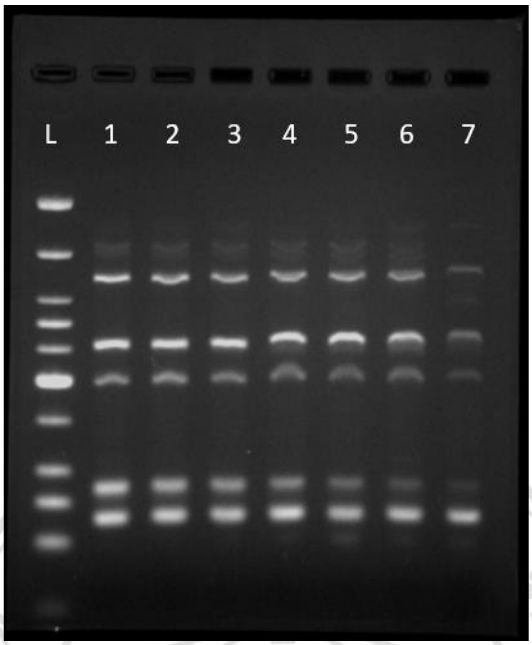


Figure 3.2 Gel electrophoresis results of the double heterozygous patterned sample (V/G1016+F/C1534). The PCR products were stained with Ultrapower^{TM} dye. All panels represent the PCR products from each of DNA amount used as template. Lanes L: contains low molecular weight DNA ladder. Lane 1: 25 ng; Lane 2: 12.5 ng; Lane 3: 6.25 ng; Lane 4: 3.12 ng; Lane 5: 1.56 ng; Lane 6: 0.78 ng; Lane 7: 0.39 ng.

To compare the efficiency of three DNA staining dyes on this multiplex PCR, the PCR products from the same set of DNA templates were stained by EtBr (Figure 3.1a), Ultrapower^{TM} (Figure 3.1b) and RedSafe^{TM} (Figure 3.1c), and the gels were visualized (with the best adjustment) under UV light. The EtBr and RedSafe^{TM} staining methods gave clear and accurate results and the PCR product band sizes were correct, when determined by DNA Ladder. The RedSafe^{TM} staining dye had a more faded result than EtBr; however, it had fewer non-specific bands. Ultrapower^{TM} staining gave the brightest result, but bands were oversized when determined by DNA ladder. The C1534 (180 bp), F1534 (232 bp) and common 1534 (368 bp) bands were estimated as 200, 250 and 400 bp, respectively. The results were still interpretable by using these oversized bands, instead of original sizes.

3.1.2 Comparison of DNA sequencing with the multiplex PCR method

A total of 151 samples from laboratory and field materials covering the nine genotypes were tested; all were successfully amplified by the multiplex PCR and sequenced. The sequences of all samples were in agreement with the multiplex PCR (Table 3.1). Thus, this multiplex PCR method has 100% specificity. However, there were limited numbers in a few genotypes (VV/FC, VG/CC and GG/CC) because they were rare in populations. The sequences of representative genotypes have been deposited in GenBank with accession numbers as follows: MF794972-3 (VV/FF), MF794974-5 (VV/FC), MF794976-7 (VV/CC), MF794978-9 (VG/FF), MF794980-1 (VG/FC), MF794982-3 (VG/CC), MF794984-5 (GG/FF), MF794986-7 (GG/FC) and MF794988-9 (GG/CC).

3.1.3 Comparison of AS-PCR with the multiplex PCR method

A total of 169 field samples tested for F1534, C1534, V1016 and G1016 alleles by the AS-PCR were in agreement with the multiplex PCR, except one sample from Mae Sariang district which showed homozygous C/C1534 by AS-PCR, but heterozygous F/C1534 by the multiplex PCR (Table 3.2); the sequence of this sample (accession number MF794990) agrees with the multiplex PCR.

Table 3.2 Comparison of genotyping results for V1016G and F1534C mutations from multiplex PCR and AS-PCR

Strain	Year of collection	Total	Multiplex PCR genotyping/AS-PCR genotyping (No. of samples)					
			1534			1016		
			F/F	F/C	C/C	V/V	V/G	G/G
Thailand								
Mae Sariang district, Mae Hong Son[a]	2010	15	4/4	5/4	6/7[c]	9/9	2/2	4/4
Ranot district, Song Khla[a]	2008	10	0/0	3/3	7/7	10/10	0/0	0/0
Ubon Ratchathani city, Ubon Ratchathani[a]	2008	9	0/0	1/1	8/8	8/8	1/1	0/0
Myanmar								
Yangon city[a]	2008	10	4/4	6/6	0/0	10/10	0/0	0/0
Yangon city	2016	32	15/15	16/16	1/1	0/0	0/0	32/32
Cambodia								
Battambang city[a]	2008	10	0/0	0/0	10/10	10/10	0/0	0/0
Bhutan								
Phuntsholing city[a]	2006	12	12/12	0/0	0/0	0/0	0/0	12/12
Pakistan								
Lahore city[b]	2012	39	0/0	0/0	39/39	39/39	0/0	0/0
Indonesia								
Ternate Island	2017	20	19/19	1/1	0/0	0/0	1/1	19/19
Soppeng Regency, South Sulawesi	2017	12	12/12	0/0	0/0	0/0	0/0	12/12
Total		169	66/66	32/31	71/72	86/86	4/4	79/79

[a]Mosquito DNA samples were obtained from Yanola et al., 2011

[b]Mosquito DNA samples were obtained from Stenhouse et al., 2013

[c]One sample was homozygous C/C1534 by AS-PCR, but heterozygous F/C1534 by multiplex PCR and DNA sequencing

3.2 Investigation of relative development and reproductivity fitness cost in three insecticide-resistant strains of *Aedes aegypti* from Thailand

3.2.1 Development and fecundity

The mean duration of larvae developing to pupae in the UPK-R strain was about one day longer than the other strains and hybrid, while the pupation and adult emergence rates were not significantly different. Consequently, the adult emergence time in the UPK-R strain was about one day longer than the others (Table 3.3, Figure 3.3). The observed sex ratio was 1:1 for all (Table 3.4). Significant differences in mean wing length were observed; specimens from the pyrethroid-susceptible PMD strain had the longest wing lengths, followed by UPK-R and hybrid mosquitoes, whereas those from the PMD-R strain were the shortest. Mean numbers of eggs per female were not significantly different, except for PMD-R females which laid about 15-20% fewer eggs than the others. The mean egg hatching rate of the PMD strain was about 12-16% higher than the other strains and hybrid. The hatching rate of hybrid eggs was about 5% lower than the two parental resistant strains. Therefore, the reproductive fitness of the pyrethroid-susceptible PMD strain was greatest, followed by the hybrid which appeared superior to the pyrethroid-resistant PMD-R and UPK-R strains.

3.2.2 Blood feeding capability

Blood feeding rates among the PMD, PMD-R, UPK-R and hybrid strains were not significantly different (Table 3.5). The average weights of PMD and hybrid specimens, measured in pools, were greater than those from the PMD-R and UPK-R strains. However, the amount of blood ingested by PMD specimens was significantly greater than the others. The increasing weight ratios after blood feeding were not significantly different in all groups, indicating that their capacity of blood ingestion was similar regardless of body size.

Table 3.3 Comparison of life-trait parameters of PMD, PMD-R and UPK-R strains and F_1 (PMD-R x UPK-R) hybrid.

Parameter	Strain (mean±SE)*			
	PMD	**PMD-R**	**UPK-R**	**Hybrid**
Pupation time (day) [1]	11.6±0.1 [a]	11.5±0.1 [a]	12.6±0.1 [b]	11.8±0.1 [a]
Pupation rate (%) [2]	89.5±4.0 [a]	85.0±2.5 [a]	89.0±1.3 [a]	95.0±1.7 [a]
Adult emergence time (day) [3]	14.9±0.1 [a]	14.6±0.1 [a]	15.8±0.1 [b]	14.9±0.1 [a]
Adult emergence rate (%) [4]	89.0±3.8 [a]	85.0±2.5 [a]	87.5±1.0 [a]	95.0±1.7 [a]
Wing length (mm)	3.29±0.02 [a]	2.98±0.03 [b]	3.14±0.02 [c]	3.08±0.01 [c]
Eggs/female (egg)	94.8±2.6 [a]	74.7±3.8 [b]	91.9±1.7 [a]	87.4±1.6 [a]
Eggs hatching rate (%)	96.0±0.6 [a]	84.7±0.3 [b]	84.7±0.3 [b]	80.7±0.9 [c]

*The same superscript letters in each row indicate no significant difference ($p>0.05$, ANOVA, followed by Bonferroni's Multiple Comparison Test).

[1] Duration of L_1 larvae developing to pupae.

[2] Percent of larvae developing to pupae.

[3] Duration of L_1 larvae developing to adults.

[4] Percent of larvae developing to adults.

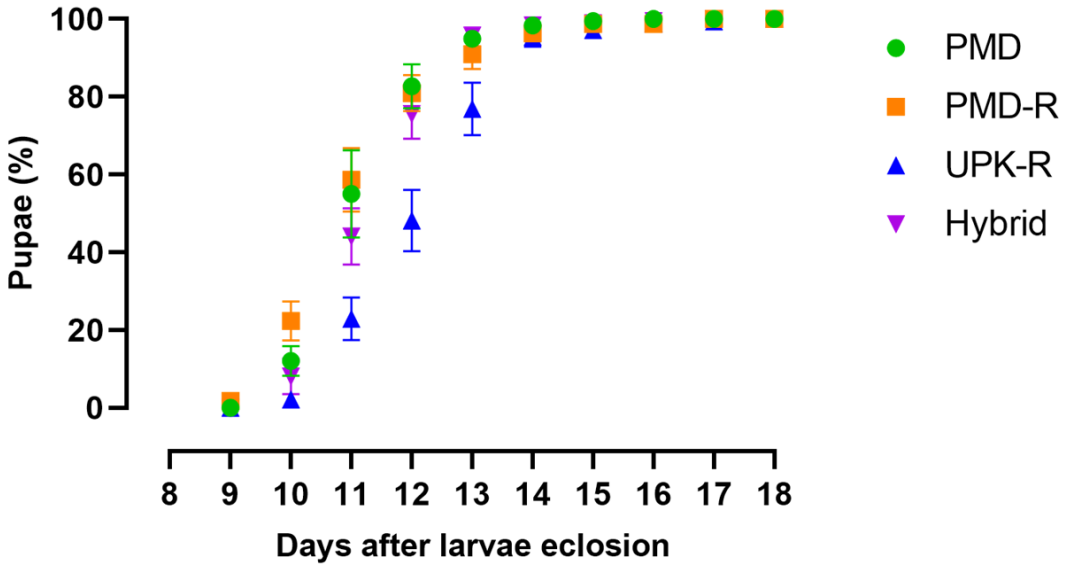


Figure 3.3 Development time of larvae to pupae of PMD, PMD-R, UPK-R strains and F_1 (PMD-R x UPK-R) hybrid. The cumulative mean percentage of pupae formation and standard error are indicated.

Table 3.4 *Aedes aegypti* sex ratio of each strain.

Strain	Male	Female	P
PMD	102	76	0.2021
PMD-R	93	77	0.4472
UPK-R	84	91	0.7484
Hybrid	109	81	0.1810

Numbers of males and females obtained from PMD, PMD-R, UPK-R and hybrid strains. P = probability for Fisher's exact test.

Table 3.5 Blood feeding capacity of the PMD, PMD-R, UPK-R and F_1 (PMD-R x UPK-R) hybrid.

Parameter	PMD	PMD-R	UPK-R	Hybrid
Blood feeding rate (%)	94.0±0.6 [a]	93.0±0.6 [a]	95.0±1.2 [a]	95.7±0.3 [a]
No. of pool [1]	6	6	6	7
Before blood meal (mg) [2]	12.58±0.23 [a]	9.90±0.61 [b]	10.75±0.12 [b]	11.54±0.31 [a]
After blood meal (mg) [2]	27.55±0.45 [a]	20.37±0.73 [b]	22.18±0.28 [b,c]	23.83±0.43 [c]
Weight of blood ingested (mg) [2]	14.97±0.25 [a]	10.47±0.76 [b]	11.43±0.31 [b]	12.29±0.43 [b]
Weight increase ratio	2.19±0.02 [a]	2.09±0.13 [a]	2.07±0.03 [a]	2.07±0.06 [a]

Values indicate mean and standard error. The same superscript letters in each row indicate no significant difference ($p>0.05$, ANOVA, followed by Bonferroni's Multiple Comparison Test).

[1] Five females in each pool.

[2] Average per pool

3.2.3 Viability of eggs after storage

The hatching rate of PMD eggs was significantly higher than the others (Figure 3.4), with about 50% hatchability after storage for 24 weeks. By contrast, the hatching rate of UPK-R eggs was reduced by 50% after 9 weeks. The hatching rates of PMD-R and hybrid eggs were not significantly different, with about 50% hatchability after 11 weeks.

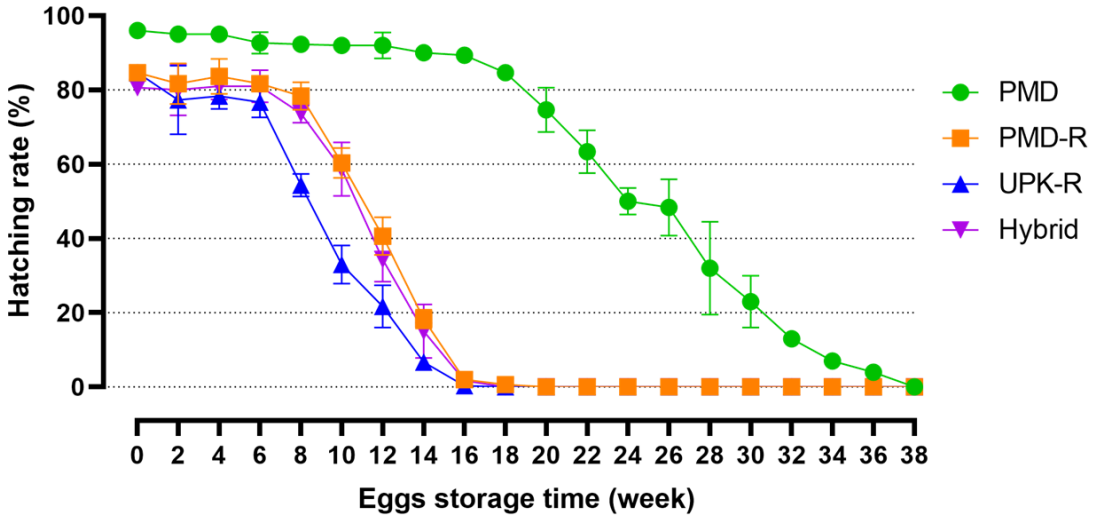


Figure 3.4 Egg hatching rates of PMD, PMD-R, UPK-R and F_1 (PMD-R x UPK-R) hybrid. Mean and standard deviation are indicated.

3.2.4 Adult longevity

Longevity of males was generally less than the females (Figure 3.5a, 3.5b). The longevity of males from the PMD-R strain was the shortest (medium survival time, MST 30 days) whereas the rest were not significantly different (MST 40 days) (Table 3.6). The longevity of PMD females was longest (MST 90 days), whereas the PMD-R was shortest (MST 50 days). No significant differences were observed between the female UPK-R and F_1 (PMD-R x UPK-R) hybrid (MST 60 days) (Table 3.6).

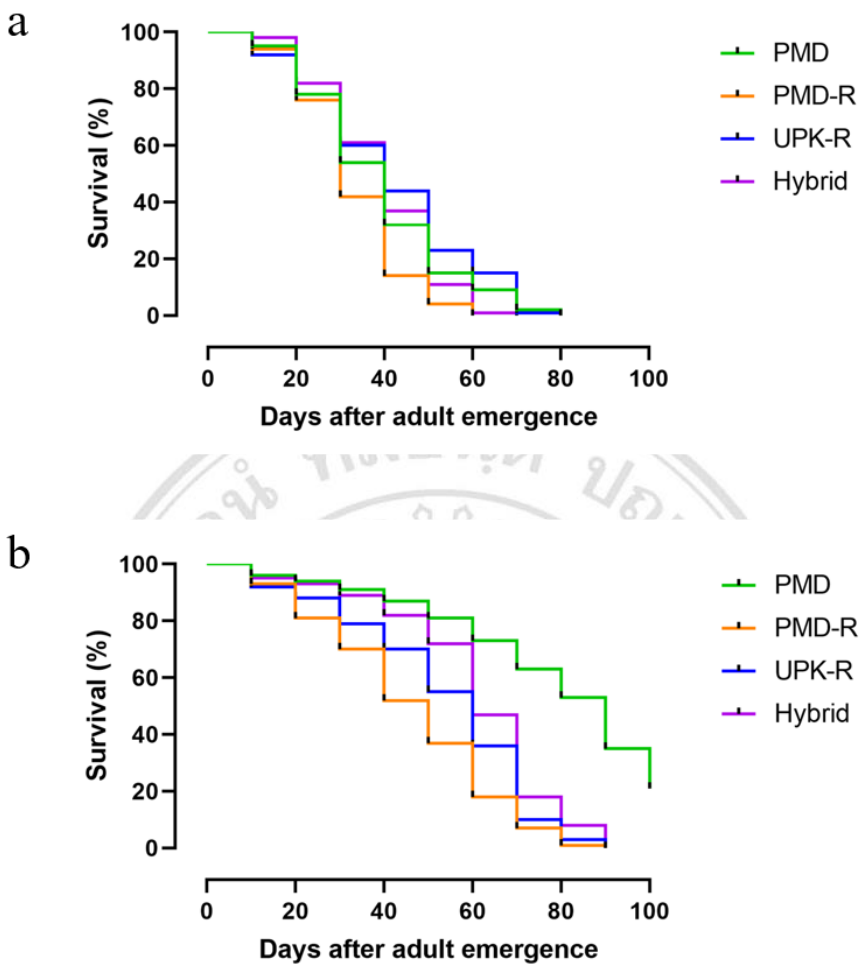


Figure 3.5 Survival curves of males (a) and females (b) of PMD, PMD-R and UPK-R strains, and F_1 (PMD-R x UPK-R) hybrid under laboratory conditions.

Table 3.6 P value of pair comparisons of survival curves of each strain

Comparison	sex	Log-rank (Mantel-Cox) test			Gehan-Breslow-Wilcoxon test		
		χ^2	df	P	χ^2	df	P
PMD vs PMD-R	Male	9.43	1	**0.0021**	4.79	1	0.0286
	Female	91.26	1	**<0.0001**	71.42	1	**<0.0001**
PMD vs UPK-R	Male	1.60	1	0.2054	1.11	1	0.2927
	Female	69.09	1	**<0.0001**	50.89	1	**<0.0001**
PMD vs Hybrid	Male	0.09	1	0.7605	0.44	1	0.5067
	Female	51.56	1	**<0.0001**	34.48	1	**<0.0001**
PMD-R vs UPK-R	Male	19.36	1	**<0.0001**	10.02	1	**0.0015**
	Female	7.55	1	**0.0060**	7.52	1	**0.0061**
PMD-R vs Hybrid	Male	12.29	1	**0.0005**	10.36	1	**0.0013**
	Female	26.03	1	**<0.0001**	27.08	1	**<0.0001**
UPK-R vs Hybrid	Male	3.98	1	0.0461	0.49	1	0.4851
	Female	5.87	1	0.0154	5.95	1	0.0147

Family-wise significance level P value = 0.05

Bonferroni corrected threshold P value = 0.0083

P value less than 0.0083 was considered a significant difference (Bold letter).

The relative reproductive fitness cost on life-trait parameters of the pyrethroid-resistant strains and the hybrid, compared with the pyrethroid-susceptible PMD strain, is summarized in Table 3.7. The resistant strains showed deteriorative effects on six out of eight life-trait parameters, whereas the hybrid showed only four, indicating that the hybrid had higher fitness than its parents.

Table 3.7 Summary of relative fitness cost on life-trait parameters of pyrethroid-resistant strains PMD-R and UPK-R and F_1 (PMD-R x UPK-R) hybrid compared with the pyrethroid-susceptible PMD strain.

Life-trait parameter	PMD-R	UPK-R	Hybrid
Developmental time (larval to adult)	equal	longer	equal
Wing length	shorter	shorter	shorter
Blood feeding rate	equal	equal	equal
Blood ingestion volume	smaller	smaller	equal
Eggs/female	lower	equal	equal
Egg hatchability	lower	lower	lower
Egg viability duration	shorter	shorter	shorter
Adult longevity	shorter	shorter	shorter

3.2.5 PMD-R and UPK-R competition

3.2.5.1 Larval competition

When larvae from the PMD-R and UPK-R strains were reared together under limited space and food, larval development was prolonged (Figure 3.6). About 50% cumulative adult emergence of PMD-R took place on day 25 which was about 5 days faster than UPK-R. However, the final emergence rate of UPK-R adults was higher than PMD-R by 7%.

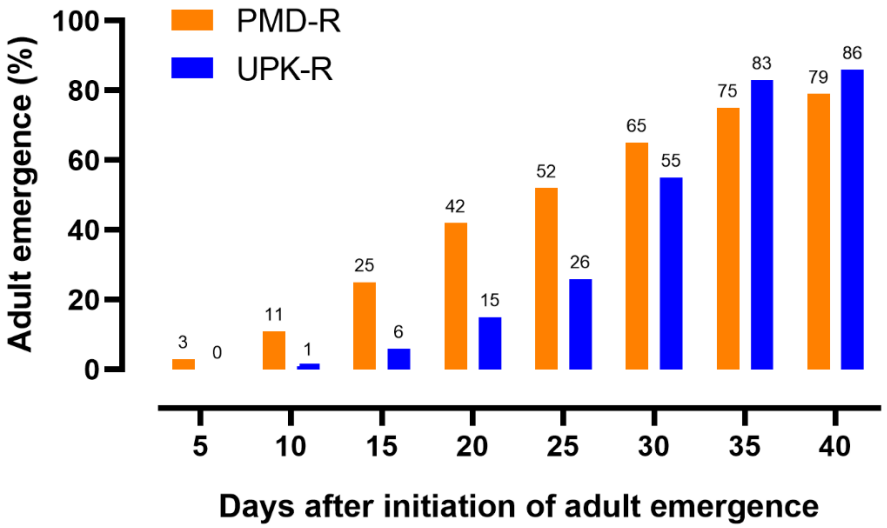


Figure 3.6 Cumulative adult emergence from the first appearance until the last emergence of PMD-R and UPK-R strains when they were reared together under limited space and food. The percents of emerging adults are indicated above each bar.

3.2.5.2 Colony competition

When equal pairs of males and females of PMD-R and UPK-R strains were confined in the same cages, the F_1 adult progeny reared from oviposited eggs showed differences in genotype frequency (Table 3.8). The heterozygous (V/G1016 + F/C1534) genotype predominated (46.7%), followed by homozygous G/G1016 + F/F1534 (38.3%) and homozygous V/V1016 + C/C1534 (15.0%). The calculated G allele and C allele were 0.63 and 0.37, respectively.

Table 3.8 Frequencies of V1016G and F1534C alleles in F_1 adult progeny reared from eggs obtained from cages containing equal pairs of males and females of PMD-R and UPK-R strains.

***Kdr* alleles**	**No. of mosquito (%)**		**Total**
	Cage		
	A	**B**	
V/V1016 + C/C1534	5 (16.7)	4 (13.4)	9 (15.0)
G/G1016 + F/F1534	10 (33.3)	13 (43.3)	23 (38.3)
V/G1016 + F/C1534	15 (50.0)	13 (43.3)	28 (46.7)
Total	30 (100.0)	30 (100.0)	60 (100.0)

CHAPTER 4

Discussion

4.1 Development of a multiplex-PCR for detection of knockdown resistance mutations, V1016G and F1534C, in pyrethroid resistant *Aedes aegypti*

We have successfully developed the multiplex PCR method to detect both V1016G and F1534C *kdr* mutations in *Ae. aegypti* in a single reaction. The results of samples tested by our multiplex PCR method and DNA sequencing were in complete agreement to detect all nine possible *kdr* genotypic patterns derived from V1016G and F1534C mutations: VV/FF, VV/FC, VV/CC, VG/FF, VG/FC, VG/CC, GG/FF, GG/FC and GG/CC. According to previous studies, only three patterns, VV/CC, VG/FC and GG/FF, were found in Chiang Mai city (Plernsub et al., 2016b) and several provinces throughout Thailand (Stenhouse et al., 2013), which is in agreement with the current study. While VV/FC can be found in other provinces i.e., Mae Hong Son, Song Khla and Ubon Ratchathani, the wild type (VV/FF) and double homozygous mutant (GG/CC) were rarely detected, and only found in Myanmar. For VG/CC and GG/FC genotypes, these patterns have never been reported in Thailand, but have been found in Myanmar and elsewhere (Kawada et al., 2014; Sayono et al., 2016). Hence, we found these genotypic patterns in YG, MS and field-collected Yangon strains from Myanmar. The samples from Cambodia and Pakistan had only C1534 mutant allele (with VV/CC pattern), while Bhutan had only G1016 mutant allele (with GG/FF pattern).

In a previous study, we developed an AS-PCR assay to detect the F1534C mutation, but there were slight discrepancies between the AS-PCR results and those from sequencing (Yanola et al., 2011). A similar situation was found in the current study when we performed the AS-PCR. However, our multiplex PCR method showed no discrepancy with the sequence data; hence, this method is as good as DNA sequencing for both V1016G and F1534C mutations. Our multiplex PCR method is

simple, has a lower cost and needs less special equipment compared to other molecular techniques, e.g. DNA sequencing, Taqman assay and Heated Oligonucleotide Ligation assay.

In this study, we used two alternative safe nucleic acid stains to compare with the traditional EtBr stain when determining the multiplex PCR results. EtBr has high sensitivity for DNA staining, provides accurate band sizes, and is cheaper than the safe dyes used. However, due to its carcinogenic property, it is preferable to use alternative safe stains, such as Ultrapower™ and RedSafe™, which are non-toxic, non-mutagenic, non-carcinogenic, and leave no hazardous waste. However, both have some disadvantages. Although the prestain safe dye Ultrapower™ staining had higher sensitivity than EtBr and RedSafe™, it gave oversized bands when determined by the DNA ladder. This may be because the dye bound to the double strand DNA PCR product before gel electrophoresis and thus reduced product mobility. This problem has been investigated with other prestain safe dyes, SYBR® Gold and SYBR® Green I (Huang and Fu, 2005). Nonetheless, for this multiplex PCR method, the results were still interpretable by using these oversized bands, instead of true sizes. For RedSafe™ staining, which was mixed into the gel, the band sizes were accurate and had less non-specific bands than EtBr, but this had the lowest sensitivity. Following the manufacturer protocols, 1 µl RedSafe™ can stain 10 samples, while 1µl Ultrapower™ can stain 100 samples. Because the price per volume is similar, Ultrapower™ is more cost effective by 10-fold. We therefore prefer to use Ultrapower™, particularly when testing a large number of samples.

Further development of multiplex PCR to include the serine to proline mutation (S989P) in domain II of VGSC is challenging, since P989 allele has a synergistic effect with G and C alleles in reducing the sensitivity of VGSC (Hirata et al., 2014; Plernsub et al., 2016a). However, adding an additional mutation would increase the number of possible genotypes and exponentially increase the complexity of banding patterns, potentially making the method unreliable due to difficulty in interpretation of the banding patterns. At present, detection of S989P may be less important in some countries where this mutation is known to co-occur or be highly associated with V1016G, such as Thailand, Singapore, Myanmar and China (Kasai et al., 2014; Kawada

et al., 2014; Li et al., 2015; Stenhouse et al., 2013). However, in countries where P989 allele has not been detected or has a low frequency (Chang et al., 2009; Ishak et al., 2015; Sayono et al., 2016; Wuliandari et al., 2015; Yanola et al., 2010), monitoring this mutation in wild populations by an AS-PCR (Li et al., 2015) may be a necessary component of the surveillance system.

4.2 Investigation of relative development and reproductivity fitness cost in three insecticide-resistant strains of *Aedes aegypti* from Thailand

The current study determined relative fitness of a pyrethroid-susceptible strain (PMD) and two pyrethroid-resistant strains (PMD-R and UPK-R), including the F_1 hybrid (PMD-R x UPK-R) of *Ae. aegypti* under similar laboratory conditions. Unfortunately, *Ae. aegypti* populations in Thailand are widely resistant to DDT (Somboon et al., 2003) and hence, a fully susceptible strain was unavailable for comparison. Pyrethroid resistance in our resistant strains is conferred by *kdr* mutations and, to a lesser extent, metabolic enzymes (MFO) based on a synergist assay (Plernsub et al., 2016a). In addition, since all strains originated from Chiang Mai province, and were resistant to DDT, their genetic backgrounds are expected to be genetically similar to each other and to the natural populations on which they were based. This allows us to reasonably infer that the major contributor to relative fitness differences in this study depends on *kdr* genotype and that the responses found are likely to be relevant to the natural populations.

The pyrethroid-susceptible PMD strain had the highest fitness compared with the two pyrethroid-resistant strains (PMD-R and UPK-R) and the hybrid. The resistant strains showed fitness costs on seven out of eight parameters (Table 3.7), whereas there were only four affected parameters observed in the hybrid. The resistance ability of the hybrid is intermediate between the two resistant strains (Plernsub et al., 2016a), implying that the fitness cost is positively related to the level of resistance. Consistent fitness costs on the life-trait parameters occurring in all resistant strains and hybrid included shorter wing length, reduced egg hatchability, shorter female lifespan and decreased viability of eggs after storage, whereas no effect was observed on blood feeding rate. The effects on developmental time, egg production and blood ingestion were variable among *kdr* genotypes.

The UPK-R strain, which displayed the highest pyrethroid resistance level, had significantly prolonged larval development times, consequently resulting in increased adult emergence times than the others (PMD, PMD-R and hybrid). This implies that not all resistance alleles caused a delayed larval development time. It also suggests that double homozygous *kdr* mutations (P989 + G1016 alleles) in UPK-R strain might have more effects than single homozygous mutations (C1534 allele) in PMD-R strain. In *An. gambiae*, Platt et al. (Platt et al., 2015) reported an additive fitness cost in male homozygous mutations for both sodium-gated (*kdr*) and GABA-gated chloride channels.

The prolonged larval development time in the UPK-R strain was confirmed when the larvae of this strain were reared together with PMD-R larvae under limited space and food. This result agrees with other previous studies showing that increased development time occurred in *Ae. aegypti* and *Cx. quinquefasciatus* selected for pyrethroid resistance in the laboratory (Brito et al., 2013; Li et al., 2002; Martins et al., 2012) and also in field collected *Ae. aegypti* with high resistance to organophosphates (Martins et al., 2012). Prolonged larval development is disadvantageous and increases the risk of exposure to extrinsic factors such as container habitat elimination, predation, disease or xenobiotics, all of which can reduce larval survival (Bourguet et al., 2004; van Uitregt et al., 2012). Avoiding to those risks, shorter development time can boost the chance of adult emergences, increasing the vector density which one aspect of vectorial capacity. Development thresholds could explain the delay in developmental time. Larvae require a certain amount of accumulated nutrients to trigger metamorphosis to the next stage. Resistant larvae may need more resources to sustain resistance mechanisms, leading to increased development thresholds (Diniz et al., 2015). However, Diniz et al. (2015) suggested that prolonged development times might benefit temephos-resistant *Ae. aegypti*, allowing for better use of nutrients available in the rearing container which could ease or compensate for the fitness disadvantage of maintaining resistance mechanisms.

Wing length indirectly represents the body size of adult mosquitoes. Specimens from the pyrethroid-susceptible PMD strain were bigger than those of the resistant strains and hybrid, suggesting that resistance alleles had an adverse effect on the size of

mosquitoes, as found in our previous study (Plernsub et al., 2013). Furthermore, among the resistant strains (PMD-R and UPK-R), their body sizes were significantly different from each other, suggesting that the degree of size reduction was dependent on the level of resistance or different *kdr* alleles. The relationship between temephos resistance and short wing length in *Ae. aegypti* has also been reported elsewhere (Diniz et al., 2015). However, Jaramillo et al. (2014) found that the resistance affected only the wing shape but not the wing size in *Ae. aegypti* resistant to lambda-cyhalothrin. In *Culex* mosquitoes, shorter wing lengths were seen in *Cx. pipiens* that overproduced acetylcholinesterase (Bourguet et al., 2004). Additionally, pyrethroid-resistant *Cx. quinquefasciatus* mosquitoes with elevated levels of P450 enzymes were smaller in size than those of the susceptible strain (Hardstone et al., 2009).

Blood feeding capacity is an essential parameter for determining vectorial capacity since parasite or pathogen loads and number of eggs produced are directly proportional to the volume of ingested blood. Blood feeding rates in all groups were similar, ranging from 93.0 to 95.7%, and indicated that the resistance alleles did not affect the ability to respond to blood meal stimuli. In contrast, a substantial reduction of feeding rate was observed in deltamethrin and temephos-resistant *Ae. aegypti* females under laboratory conditions (Belinato et al., 2012). Resistant female mosquitoes are likely to ingest less blood volume compared to susceptible individuals (Belinato et al., 2012; Li et al., 2002; Martins et al., 2012; Otali et al., 2014). Due to smaller body size, the volume of blood ingested by the two resistant strains was less than the susceptible PMD strain. Mosquito weights taken before a blood meal can be used to infer body size and wing length, and the results for both parameters are correlated as we expected. However, the weight increase ratios were not different, indicating no fitness cost on blood feeding capacity in the resistant strains. This implies that reduced blood ingestion was related to the difference in mosquito body size, not to the reduced willingness of blood sucking.

Reduced egg production and egg hatchability in insecticide-resistant *Ae. aegypti* has been reported elsewhere (Kumar et al., 2009; Martins et al., 2012; Mebrahtu et al., 1997). In *An. funestus*, however, egg production rates between the pyrethroid-resistant and susceptible strains did not vary by much (Okoye et al., 2007). In the current study,

only the PMD-R females laid fewer eggs, suggesting that not all resistance alleles affect egg production. The resistance alleles are correlated with reduced egg hatchability (by 12-16%). Thus, reduced larval production would lead to a reduction of adult density and a decrease in vectorial capacity among resistant mosquitoes.

The eggs of *Ae. aegypti* can tolerate dry conditions for a very long time (Christopher, 1960). They are ready to hatch once submerged in water. This property is an advantage for their survival under periods of desiccation. Moreover, the eggs of this species are the main form of passive dispersal, making it widespread and difficult to control. Under continuous insecticide exposure, this ability is very crucial for eggs of resistant mosquitoes because it helps to maintain resistant individuals (as well as resistant genes or mechanisms) in nature. In the current study, PMD eggs showed longer viability than the others after storage in dry conditions, whereas the UPK-R showed the most rapid loss of viability. About 90% of PMD eggs survived after 18 weeks of storage, but none of the eggs from the resistant strains or hybrid survived. This suggests that the survival of eggs under desiccation is shorter when the resistance level is increased. This fitness cost has an impact on the maintenance of resistant alleles during periods of prolonged desiccation. The viability rate of PMD, which is resistant to DDT, at week 21 was about 70% which is similar to the egg viability rate of all temephos-resistant groups of *Ae. aegypti* at day 150 (80%) reported by Diniz et al. (2015). However, results under laboratory conditions may not reflect what would happen in nature. Therefore, the viability of eggs of resistant mosquitoes under natural conditions needs further investigation.

Our results clearly demonstrated that the longevity of *Ae. aegypti* adults was shorter in the resistant strains. Over 80% of the PMD females survived up to 60 days which was slightly longer than other studies (Belinato et al., 2012; Martins et al., 2012). The longevity of PMD-R mosquitoes, despite having lesser resistance, was shorter than UPK-R mosquitoes of both sexes. Large variations in longevity of resistant mosquitoes have been observed in previous studies. Reduced longevity has been observed in pyrethroid-resistant *Ae. aegypti* and *Cx. pipiens pallens* (Jaramillo et al., 2014; Li et al., 2002; Martins et al., 2012), and also temephos-resistant *Ae. aegypti* (Belinato et al., 2012; Diniz et al., 2015). By contrast, Hardstone et al. (2009) reported that *Cx.*

quinquefasciatus females resistant to permethrin survived longer than those of susceptible strain when provided with sugar. However, no differences in longevity were observed in some studies, for example, between pyrethroid-resistant (Rock-*kdr*) and susceptible (Rock) *Ae. aegypti* strains (Brito et al., 2013), and also between some strains of *An. gambiae* and *An. stephensi* which were resistant to gamma-HCH and dieldrin (Rowland, 1991). This implies that the longevity of resistant adults in the laboratory may be related not only to resistance levels but also some other factors, e.g., genetics, adaptation and selection. Longevity reduction in females is important since this would affect the extrinsic incubation period of pathogens as well as blood feeding frequency, both of which are major aspects of vectorial capacity. Survival in the laboratory, however, cannot reflect the longevity of wild *Ae. aegypti* mosquitoes which usually live for a few weeks (Scott et al., 1997). Additionally, lab conditions may not reflect their reproductivity in nature since male and female mosquitoes are usually only reproductively active in the first few weeks after emergence (Clements, 1992). Therefore, the longevity and reproductivity of resistant mosquitoes in natural conditions should be further investigated.

Previous studies among laboratory-reared *Ae. aegypti* showed that large males had greater mating capacities than small males (Helinski and Harrington, 2011; Ponlawat and Harrington, 2007, 2009). Berticat et al. (2002) performed a mating competition in *Cx. pipiens* mosquitoes which revealed that susceptible males had more mating competitiveness (with both susceptible and resistant females) than those resistant to organophosphate. In *An. gambiae*, *kdr* heterozygote males were more likely to mate than homozygote resistant males and were more competitive than homozygote susceptible males (Platt et al., 2015). However, there was no fitness cost on mating and insemination capability in a mating competition experiment between a susceptible *Ae. aegypti* strain (Rock) and a pyrethroid-resistant (Rock-*kdr*) strain (Brito et al., 2013). In the current study, when adults of UPK-R and PMD-R were confined in the same cage, the heterozygous form predominated in the F_1 progeny, with a higher frequency of the G1016 allele over the C1534 allele. This may be attributed by the result of the present study showed that the UPK-R strain produced more eggs than the PMD-R strain (Table 3.3). In addition, the body size of UPK-R mosquitoes was larger than those of the PMD-R strain, and likely gave them an advantage in mating competition. Under natural

conditions, however, it is not known if resistant *Ae. aegypti* mosquitoes harboring homozygous P989 + G1016 alleles have more mating capability than those with the homozygous C1534 allele.

In Chiang Mai city, Plernsub et al. (2016b) reported that the majority (46%) of *kdr* genotypes in *Ae. aegypti* population consisted of the heterozygous form, followed by the homozygous C1534 (39%) and homozygous P989 + G1016 (15%) forms. The frequency of these *kdr* alleles appears to have increased only very slowly over the last decade of monitoring (Stenhouse et al., 2013; Yanola et al., 2011). This may be due to balancing selection under insecticide pressure in relation to the fitness cost of resistance alleles. Although the fitness costs of resistance among natural populations of mosquitoes remains to be determined, the results of the current study suggest that the fitness of life-trait parameters in the heterozygous form may be higher than the homozygous P989 + G1016 mutant (UPK-R), which had increased larval development times as well as decreased viability of eggs under desiccation. Fitness was also higher than that seen in the homozygous C1534 mutants (PMD-R), which had smaller body sizes, lower fecundity and decreased longevity. Similarly, the fitness of heterozygote resistant males was greater than that of homozygotes in *An. gambiae* (Platt et al., 2015). Since the heterozygous mutants expressed a high pyrethroid-resistant phenotype, this has a significant impact on *Ae. aegypti* control programs using pyrethroid-based approaches (Plernsub et al., 2016a; Plernsub et al., 2016b). Therefore, there is a need for both alternative methods or chemicals that overcome *kdr* in the long term (such as insect growth regulators), as well as continued monitoring of resistance genotypes in *Ae. aegypti* populations.

CHAPTER 5

Conclusion

Insecticide resistance in mosquito vectors is one of the most important factors that hampers the effectiveness of insecticide-based control methods, causing difficulties to stop transmission of mosquito-borne diseases. *Kdr* mutations, V1016G and F1534C, have been proved as an essential mechanism of pyrethroid resistance in *Ae. aegypti* in Thailand. The multiplex PCR developed during this study has high specificity and sensitivity to detect V1016G and F1534C *kdr* mutations in *Ae. aegypti* that allows all possible genotypes to be identified in a single step. Due to this method was proved to be highly reliable, cost-effective and simple. It will be useful for monitoring mutant allele and genotype frequencies in wild populations throughout Thailand, and many other disease endemic countries in Asia, where these two alleles are prevalent.

Although insecticide resistance helps mosquitoes to survive insecticide exposure. Collectively the data presented here on Chiang Mai derived laboratory strains indicate that in the absence of pyrethroid insecticides *Ae. aegypti* mosquitoes in Chiang Mai that carry *kdr* alleles, particularly in the homozygous form, are likely to have reduced fitness compared to non-*kdr* genotypes. This fitness cost has a broad basis in multiple life history, development and reproductive traits including prolonged development time, smaller body size, decreased blood ingestion amount, lower fecundity, reduced egg hatching rate, shorter lifespan, decreased viability of eggs under desiccation. The high use of pyrethroid insecticides in Chiang Mai coupled with the high fitness of the *kdr* allele hybrid in both the absence and presence of insecticide are likely maintaining both *kdr* alleles in Chiang Mai. Despite this, the much higher fitness of non-*kdr* genotypes in the absence of insecticide means that it is theoretically possible to restore pyrethroid susceptibility to Chiang Mai populations by the periodic withdrawal of pyrethroid insecticides that would favour the spread of non-*kdr* alleles. Given the primary

importance of pyrethroids for disease control and the continuing threat from arboviral diseases vectored by *Ae. aegypti*, the plausibility of this approach should be given consideration.

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LIST OF PUBLICATIONS

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Publications

2015 Finlayson, C., **Saingamsook, J.**, & Somboon, P. (2015). A simple and affordable membrane-feeding method for *Aedes aegpyti* and *Anopheles minimus* (Diptera: Culicidae). *Acta Trop, 152*, 245-251. doi:10.1016/j.actatropica.2015.09.026

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2018 Son-un, P., Choovattanapakorn, N., **Saingamsook, J.**, Somboon, P., Yanola, J., Lumjuan, N., & Walton, C. (2018). Effect of relaxation of deltamethrin pressure on metabolic resistance in a pyrethroid-resistant *Aedes aegypti* (Diptera: Culicidae) strain harboring fixed P989P and G1016G *kdr* alleles. *J Med Entomol, 55*(4), 975-981. doi:10.1093/jme/tjy037

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Training experience

Workshop attendance on "The care and practice techniques for Laboratory animals" at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand in 2013.

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Conference attendance on the TRF Seminar Series in Basic Research CII: From molecular to market at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand in 2014.

Certificate of completion for successfully completing the "Scientific Writing Workshop" at the Graduate School, Chiang Mai University, Chiang Mai, Thailand in 2014.

Seminar attendance on “Next generation sequencing (NGS) Technology” by Illumina Technology at Kantary Hill Hotel, Chiang Mai, Thailand in 2014.

Certificate for “License to Use Animals for scientific purposes” license number U1-00999-2558 by Institute of Animals for Scientific Purpose Development (IAD) and National Research Council of Thailand (NRCT), Chiang Mai, Thailand in 2015.

Workshop attendance and certificate on “Thalassemia diagnosis testing and evaluation” by Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai Thailand in 2016.

Workshop attendance and certificate on “Basic Real time PCR technique for gene quantification and detection” at Gibthai Co.,Ltd, Chiang Mai, Thailand in 2017.

Certificate of “Biosafety in Laboratory 2017” by Chiang Mai University, Chiang Mai, Thailand in 2017.

Certificate of participation on “Training Programs in Epidemiology and Public Health Interventions Network (TEPHINET)” in partnership with Ministry of Public Health of Thailand. At Chiang Mai, Thailand 2017.

Symposium attendance on “Manchester Molecular and Genome Evolution Symposium (MAGE2018)” at Michael Smith Building, The University of Manchester, England in 2018.

Teaching experience

Inform knowledge in collecting of stool for stool examination and medical parasites: morphology of parasites, transmission, pathogenesis, diagnosis, treatment and prevention for high school students at

- Debsirin Chiang Mai School, Chiang Mai, Thailand (2012)
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Teaching assistance; helping foreign students working at Chiang Mai University, on their year in industry, from The University of Manchester, England. Involved in helping with their projects and general wellbeing (2014-2019).

Experiences

Poster presentation. **Saingamsook J**, Yanola J, Lumjuan N, Somboon P. First detection of knockdown resistance gene mutation in *Musca domestica* field populations in Chiang Mai Province, Thailand. TRF seminar series in basic research CII: From molecular to market, 2 May 2014, at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Poster presentation. **Saingamsook J**, Plernsub S, Yanola J, Lumjuan N, Somboon P. Development of a Multiplex PCR Method to Detect the V1016G and F1534C Mutations in the Voltage-Gated Sodium Channel Gene of the Mosquito *Aedes aegypti* from Chiang Mai Province, Thailand. RGJ – Ph.D. Congress 17, 8-11 June 2016, at Jomtien Palm Beach Hotel & Resort, Pattaya, Chonburi, Thailand.

Poster presentation. Saingamsook J, Yanola J, Lumjuan N, Walton C, Somboon P. Relative fitness cost on development and reproductivity of pyrethroid susceptible and resistant strains conferred by knockdown resistance mutations, S989P, V1016G and F1534C, in *Aedes aegypti* in Thailand, The 18th Awaji international forum on infection and immunity, at Awaji Yumebutai International Conference Center, Awaji City, Hyogo, Japan.