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APPENDIX A

The RNA codons

					Seconed	l Positi	on	\rightarrow				
		U		C A		Α		G				
Sil-		code	Amino Acid	code	Amino Acid	code	Amino Acid	code	Amino Acid			
Ø	<u>F</u>	UUU	aha S	UCU	ser	UAU	tyr	UGU		U		
J	ς, Γ	UUC	pne	UCC		UAC		UGC	Cys	С		
	U	UUA	leu	UCA		UAA	STOP	UGA	STOP	Α		
		UUG	leu	UCG		UAG	STOP	UGG	trp	G		
. 4	4	CUU		CCU		CAU	hie	CGU		U		
7	c	CUC	leu	CCC		CAC	1115	CGC		С		
1		6	6		CUA	icu	CCA	pio	CAA	ala	CGA	arg
	Y	CUG		CCG		CAG	giii	CGG		G		
		AUU		ACU	614	AAU		AGU	-	U		
		AUC	AUC ile ACC the	thr	AAC	asn		AGC				
1	A	AUA		ACA	un	AAA	her	AGA	JA AE			
		AUG	met	ACG		AAG	iys	AGG	ary	G		
		GUU		GCU		GAU	960	GGU		U		
	~	GUC	val	GCC	ala	GAC	asp	GGC	alv	С		
	G	GUA	Vell	GCA	aia	GAA	alu	GGA	עיפ	Α		
		CUIC		606		GAG	giù	000	1 1	0		

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APPENDIX B

Amino acid abbreviations

Amino acid abbreviations							
Full name of amino acid	Abbreviation (3 letters)	Abbreviation (1 letter)					
Alanine	Ala	А					
Arginine	Arg	R					
Asparagine	Asn	N					
Aspartic acid	Asp	D					
Cysteine	Cys	C					
Glutamine	Gln	Q					
Glutamic acid	Glu	E					
Glycine	Gly	G					
Histidine	His	Н					
Isoleucine	Ile	Ι					
Leucine	Leu	L					
Lysine	Lys	K					
Methionine	Met	М					
Phenylalanine	Phe	F					
Proline	Pro	P					
Serine	Ser	S					
Threonine	Thr	Т					
Tryptophan	Chiarpg Ma	Unwersity					
Tyrosine	Tyr	Y					
Valine		serve (

APPENDIX C

Chromatogram of nucleotide sequences



1. Nucleotide sequence of the IS4 - IS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD-R strain



2. Nucleotide sequence of the IS4 - IS6 domains of the Ae. aegypti voltage-gated

sodium channel gene from PMD strain



3. Nucleotide sequence of the IIS1-IIS6 domains of the Ae. aegypti voltage-gated





4. Nucleotide sequence of the IIS1-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD strain



5. Nucleotide sequence of the IIIS4-IVS2 domains of the Ae. aegypti voltage-





6. Nucleotide sequence of the IIIS4-IVS2 domains of the Ae. aegypti voltage-

gated sodium channel gene from PMD strain

Signal G:49 A:33 T:29 C:21 DT3100POP4{BDv3}v1.mob Model 3100 15-6-49_E09_RS1#IIIS4-6_F_09.ab1 Page 1 of 3 Fri, Jun 16, 2006 9:27 AM Version 3.7 ABIA Basecaller-3100APOP4_RS1#IIIS4-6_F BC 1.5.0.0 _____ Cap 9 BSU_3100 Points 1200 to 15000 Pk 1 Loc: 1200 Thu, Jun 15, 2006 8:37 PM Spacing: 15.28{15.28} CATGGT CCCG TATG CAG GG TATG AG G NGTTGTT 30 40 50 60 T T CAACG TOM CACTGCGNG GAA T GA TACG TG CONCAA GEGATACAG G CTATAC CNTG TTG ATCTTTTG G TTG ATTTT CGC TATTAT G GG TG TGCAGC TG TTTGCTG GCAAG TATTTTAAGTG CG TCG ACAAG AACAAG AC GACG 0 130 140 150 160 170 180 190 200 GCACGAG AT CATTCCGG ATGTG CCCGGG AGGTGGGAA AGCAGCCGATTCCCCGAGACCAACATCTACATGTACCTCTACTTTGTGTTCTT TCGGGTCGTTCTTCACGCTGAATCTGTTCATCGGTGTCATCATCGA 450 460 470 480 470 WM IM MΜ Signal G:49 A:33 T:29 C:21 Model 3100 15-6-49 E09 RS1#IIIS4-6 F 09.ab1 Page 2 of 3 DT3100POP4{BDv3}v1.mob BSU_3100 Fri, Jun 16, 2006 9:27 AM Thu, Jun 15, 2006 8:37 PM Version 3.7 ABI 🛕 PRISM Basecaller-3100APOP4_RS1#IIIS4-6_F BC 1.5.0.0 Cap 9 Points 1200 to 15000 Pk 1 Loc: 1200 Spacing: 15.28{15.28} ATGAAAAAGATGGGCTCGAAGAAG CAACGAGCAG AAGAAAGCC GGTG GCTCACTGGAAATGTTCATGACGGAGG ATCAG AAAAAGTAC 500 510 520 530 540 550 560 CGCTGAANGNTAT 600 610 TACAN 3GACACG AGGA GENGTOTGANGRAN NNNNNA CONNTONNONNNNNA A NNONNCOA NNONNCON NN GNOONC NO TTAGCGC GG TG CTAGACTAT CTGAACAT GATCTT CAT C

7. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltagegated sodium channel gene from field collected strain, MSR-R25. The nucleotide chromatogram of homozygous for wild type F/F1534 was shown.

MACRO Signal G: 1945 A:2372 C: 1653 T: 2919 Run Ended: 2009/7/11 20:53:21 File: Ae MSR R25 B-Ge-IIIS6 F.ab1 Sample: Ae_MSR_R25_B_Ge-IIIS6_F Lane: 31 Base spacing: 16.003092 950 bases in 11570 scans Page 1 of 2 GG NI NI NIGHT OF TO CONTROL OF A CTACK OF A MAN MAN ANA MANA MAN MANAM Manana and how many many all and a second ward and a second ward and a second and a second and a second and a second a Manufacture Manufacture and manufacture and manufacture and manual m

ลิขสิทธิ์มหาวิทยาลัยเชียงไหม Copyright[©] by Chiang Mai University All rights reserved 8. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltagegated sodium channel gene from field collected strain, Ae-LP-DelR4. The nucleotide chromatogram of heterozygous F/C1534 was shown.

File: Ae_IP_DelR4-IIIS4_F.ab1 Run Ended: 2009/577 18: 12:23 Signal G:516 A:394 C:470 T:468 M Sample: Ae_I.P_DelR4_IIIS4_F Lane: 83 Base spacing: 16:584824 769 bases in 9174 scans Page 1 of 2	ACROYGEN
NNNEN AMITHATI GETATTAT ONT T A COM CTET CTATT OCCOCTOGATE GICETT CITTATTA GET GIEGTEAAT BEAT GIEATAC GIEGTEATAC	TTCAACGTGTTAT
man was a second with the second seco	
130 TGGTGTGTTTTGATCTTTTGGTTGGATCTTCGCTATTATGGGTGIGCAGCTGTTTGCTGGCAAGTATTTTAAGGTGAGTGTACACTTCTAAAGTTGGATCCAAAAGGGCTTGATG	GAATAACCATCGG
	Unullus
AT GTT GCCTCCCTTCAGTGCGTCGACAAGAACAAGACGAACGCTGTCGCACGAGATCATTCCCGGATGTGAACGCGTCGCGCGGAGAACTACACGTGGGAGAACTCGCCGATGA	370 AACTTCG ACCACG
	MMMMMA
	ACGAATGATCTGC
AWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWW	MMM
$\begin{array}{c}510\\ TTACAATTTTACGTCCTCGATCCTTCCAGGTGGGAAAGCAGCCGATTCGCGAGGCAACATCTACATGTACCTCTACTTGTGTTCTTCATCATCTCCGCGGGTCGTTCTTCACC$	62D 63D GCTGAATCTGTT
Munimum Malina Malina Mana Mana Mana Mana Mana Mana Mana M	MMMM
File: Ae_LP_DelR4-IIIS4_F-ab.l Run Ended: 2009/5/7 18: 12:23 Signal C:516 A:394 C:470 T:468 Sample: Ae_LP_DelR4_IIIS4_F Lane: 83 Base spacing: 16.584824 769 bases in 9174 scans Page 2 of 2	
650 680 740 740 740 750 750 750 750 750 750 750 750 750 75	750 GGCTCGAAGAAGEC
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9. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltagegated sodium channel gene from field collected strain, Ae-LP-DelS2. The nucleotide chromatogram of homozygous C/C1534 was shown.

File: Ae_LP_DelS2-IIIS4_F.ab1 Run Ended: 2009/5/7 18:12:23 Signal G:5064 A:4254 C:5065 T:5203 Sample: Ae_LP_DelS2_IIIS4_F Lane: 87 Base spacing: 16.586983 807 bases in 9666 scans Page 1 of 2	
NNNATINN 10 AM TT & T GOT AT THAT ONT T COORCE OF CTATT COORCE OF COTTON COTTONT A GOT OF COT CAT COTTON CONTRACTOR	ATACCGTCCATCTTCAACGTGT
	Wand Maral Mar
	230 AAG GGCTT GATGAATAACCAT C
	CGAACTTOTACGAATGATC
Manual	Mundumundun
C/C1534	GTCGTTCTTCACGCTGAATCTG
Man Markan Mar	M. Mannahan
File: Ae_LP_DelS2-IIIS4_F.ab1 Run Ended: 2009/5/7 18:12:23 Signal G:5064 A:4254 C:5065 T:5203	
Sample: Ae_LP_DeISZ_IIIS4_F Lane: 87 Base spacing: 16.586983 807 bases in 9666 scans Page 2 of 2 630 680 680 700 710 720 TTCATCGGTGTCATCGACGACGACGACGAGAAGAAGGAAG	730 740 750 CATGAAAAAGATGGGCTCGAAGANNC
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10. Nucleotide sequence of the IIP-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, Ae-MSR-R4. The nucleotide chromatogram of homozygous for mutant V/V1016 and S/S989 were shown.

MACRO File: Ae_MSR_R4-IIP_F.ab1 Run Ended: 2009/9/16 12:38:48 Signal G:2121 A:2293 C:1918 T:2333 Sample: Ae_MSR_R4_IIP_F Base spacing: 15.754582 738 bases in 8662 scans Page 1 of 2 Lane: 67 S/S989 MAAAAAA And Mala Walk and Mar Mar MAMA Maaaamaan Maaaddaamaana Ma TCGCCAACGC GCT CAAGT TCGT GAAAAAACAAGT TAACAAGCOAGA TTTG GGTCCCT ACCCCA TGAACTCCCAAAAAGA TAG GG TA C GGT T ATTCTGAC GCC C G CGTTTCC CTGCCG TC TCTCC AATTTTAG Mm Imm m Animport

ลิขสิทธิ์มหาวิทยาลัยเชียงไหม Copyright[©] by Chiang Mai University All rights reserved 11. Nucleotide sequence of the IIP-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, Ae-MSR-R3. The nucleotide chromatogram of homozygous for mutant V/G1016 and S/P989 were shown.

MACRO File: Ae_MSR_R3-IIP_F.ab1 Run Ended: 2009/9/16 16:15:52 Signal G:3883 A:4050 C:3336 T:4074 Lane: 69 Sample: Ae_MSR_R3_IIP_F Base spacing: 14.9367695 798 bases in 9623 scans Page 1 of 2 S/P989 GACTGAAAGTAAATTGGAGCGCACAACAAGACCTGTTAT GGGAGTTCTTCTATAAGG TTCTCAGGTTGAATTGCAGTAGTTC GCTGTAAGTTC ATTCCGT CGAAATCTCCGAACTTTCATTTCATTTCGAAACGCAATACTAGACGCGCATAGAACATACAAATTTACATATAGTCAGCCTTTCATGCATTCTATCGTGCTAACCGACAAATTGTTTCCCCACCCGCCACAG V/G1016 Markan man waamma amman ACT GG AT CAAGT CGAACAT CGCCAACGC GC T CAAGT TCGT GAAAAACCAAGT TAACAAGC AGAT TGGC GT CCCAA AAA T G T CCCAT TG T T CCC G TCTTTTT TTT CTTCT TATC ACG CCC AT AT G G G

ลิขสิทธิ์มหาวิทยาลัยเชียงไหม Copyright[©] by Chiang Mai University All rights reserved 12. Nucleotide sequence of the IIP-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, Ae-MSR-R25. The nucleotide chromatogram of homozygous for mutant G/G1016 and P/P989 were shown.

MACRO File: Ae_MSR_R25_A-IIP_F.ab1 Run Ended: 2009/7/11 20:53:21 Signal G:3183 A:3505 C:2706 T:5222 Sample: Ae_MSR_R25_A_IIP_F Lane: 16 Base spacing: 16.150904 687 bases in 8056 scans Page 1 of 2 P/P989 TINNIN NETCE NG TT C G GG THTTAT GC G GG AGTG GATCGAACCCATGT GGG ATTGTAT COTT GTGGGT GACGTGTCCT GTATTCCGTTCTTTTGGCCACCGTAGTGATAGGAAATCTA AMAAAAAAA annana anna anna anna anna ann annala Malanna anna G/G1016

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APPENDIX D

Publications

 Yanola J, Somboon P, Walton C, Nachaiwieng W, Prapanthadara L. A novel F1552/C1552 point mutation in the *Aedes aegypti* voltage-gated sodium channel gene associated with permethrin resistance. Pesticide Biochemistry and Physiology. 2010; 96: 127-131.

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Pesticide Biochemistry and Physiology 96 (2010) 127-131



A novel F1552/C1552 point mutation in the Aedes aegypti voltage-gated sodium channel gene associated with permethrin resistance

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Sodium channel

ABSTRACT

Sodium channel mutations were investigated through nucleotide sequencing of three cDNA fragments amplified from permethrin resistant and susceptible Aedes aegypti from northern Thailand. There was a novel nucleotide substitution (T \rightarrow G) at the second position of codon 1552 resulting in the replacement of Phenylalanine by Cysteine in segment 6 domain III. This amino acid was indicated by another study to involve an aromatic-aromatic contact between the sodium channel protein and the first aromatic ring of the pyrethroid alcohol moiety. Reciprocal crosses between the homozygous parental susceptible and resistant strains indicated that resistance was autosomal and incompletely recessive, and highly associated with the homozygous mutation. The bioassay of the F2 progeny, formed by backcrossing the F1 with the resistant parental strain, did not show a clear plateau curve across the range of doses, suggesting that resistance to permethrin was controlled by more than one gene locus. Other possible resistance mechanisms involved are discussed.

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1. Introduction

Voltage-gated sodium channels are integral trans-membrane proteins responsible for the depolarization phase of action potentials in the membranes of neurons and most electrically excitable cells [1]. The overall organization of sodium channel proteins consists of four homologous domains (I–IV), each containing six α-helical trans-membrane segments (S1-S6) [2]. The protein is conserved among both invertebrates and vertebrates. Pyrethroid insecticides and DDT are known to deliver their insecticidal effects by disrupting the function of the insect's voltage-gated sodium channels and preventing the re-polarization phase of action potentials [3,4]. Mutations in the voltage-gated sodium channel can reduce pyrethroid binding. A substitution of Leucine to Phenylalanine (Leu to Phe) resulting from a single nucleotide mutation in domain II segment 6 of the sodium channel [5,6] has been clearly demonstrated to be associated with resistance to pyrethroid and DDT in many insect species [7–9]. In addition, more than 20 mutations in insect sodium channels have been identified that reduce channel sensitivity to insecticides or neurotoxins [10,11].

In northern Thailand, a study on insecticide susceptibility in Aedes aegypti indicated resistance to DDT and permethrin [12].

A DDT/permethrin resistant strain of Ae. aegypti, PMD-R, was established from the selection of field caught insects in 1997. Biochemical and molecular studies demonstrated that elevated DDTase activity was the major mechanism involved with DDT resistance in this strain and pyrethroid resistance was not observed to occur through metabolism [13,14]. Pyrethroid resistance appeared to mainly involve knockdown resistance. In this study, we report a novel single nucleotide mutation in the voltage-gated sodium channel gene that correlates with DDT/permethrin resistance. We also investigated the genetic inheritance of permethrin resistance and the mutant allele, based on reciprocal crosses between susceptible and resistant mosquito strains and backcrosses between F1 hybrid and resistant parental strains.

2. Materials and methods

2.1. Mosquito strains

Two strains of Ae. aegypti used in this study, the PMD and PMD-R strains, were established from field caught mosquitoes from Pang Mai Daeng, Mae Taeng District, Chiang Mai since 1997 [12] and were previously named RS and RR strains, respectively [13]. PMD is resistant to DDT whereas PMD-R is resistant to both DDT and permethrin. The colonies were maintained under insecticide selection pressure by exposure to standard WHO permethrin (0.5% and

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0.75%) papers for at least 10 generations before the resistance ratios were determined. The eggs from these mosquitoes were harvested and stored prior to use in a dry cool place. Under such conditions, the eggs can survive for a long period of time.

2.2. Bioassay for mosquito larvae and statistical analysis

The larval susceptibility test was conducted according to the WHO standard method [15]. Stock and serial dilutions of permethrin (Supelco, Belefonte, PA, USA) were prepared in ethanol. The bioassays were conducted in 400 ml beakers each containing 250 ml of distilled water with one of 5–7 different concentrations of insecticide $(0.1-500 \, \mu g \, l^{-1})$, 4 replicates per concentration. The ethanol content in each assay solution was limited to 0.4%. For both mosquito strains, batches of 25 early 4th instar larvae were tested per beaker. In the control experiments, 0.4% ethanol was included in 250 ml of water. Larval mortality was recorded after 24 h exposure. Data were analyzed by standard probit analysis [16].

2.3. Partial sequencing of Ae. aegypti voltage-gated sodium channel gene

To determine the mutations in the voltage-gated sodium channel gene in Ae. aegypti, three fragments of cDNA were amplified and sequenced. Primers to amplify these fragments were designed using sequences from the Ae. aegypti Liverpool and China susceptible strains (Liverpool cDNAs are from GenBank Accession Nos.: XM_001657308-XM_001657311 inclusive; China cDNAs are from GenBank Accession No.: AY663385). Nucleotide sequences of the 3 primer pairs, the product sizes and the regions amplified are indicated in Table 1. Total RNA was isolated from 30 mg of 4th instar larvae using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction manual. The RNA was reversed transcribed from 5 µg of total RNA using an oligo(dT)₁₂₋₁₈ primer (Invitrogen, Carlsbad, CA, USA) and Super-Script[™] III RNase H⁻ (Invitrogen, Carlsbad, CA, USA) according to the supplier's recommended protocol. First strand cDNA was then used as a template for PCR amplification of the Ae. aegypti putative knockdown resistance (kdr) region in the voltage-gated sodium channel gene.

PCR was carried out in a 50 μ l reaction volume containing 1.25 U of HotstarTaq DNA polymerase (QIAGEN, Hilden, Germany), 0.1 mM dNTPs (New England Biolabs, Ipswich, MA, USA), 1.5 mM MgCl₂ and 0.5 μ M each of the forward and reverse primers (Operon, Cologne, Germany). The amplification consisted of an initial heat activation step at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 59 °C for 1 min and 72 °C for 30 s with a final extension step at 72 °C for 7 min. The amplified fragments were analyzed by electrophoresis on a 1.0% agarose gel and visualized under UV light by ethidium bromide staining. The sodium channel gene PCR fragments were then purified using the QIAquick PCR purification Kit (QIAGEN, Hilden, Germany). The purified PCR products were sequenced using the ABI Prism Big Dye terminator cycle sequencing kit on an ABI-

Sequences of primers for amplifying sodium channel gene fragments.

PRISM 3100 automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequence data were analyzed using CLUSTAL W multiple sequence alignment program [17] and Six Frame Translation program (Sequence Launcher, BCM) [18].

2.4. Genetic inheritance of the F1552 and C1552 alleles in permethrin susceptible and resistant Ae. aegypti

A dose–response bioassay was applied to determine the genetic characteristics of resistance [19,20]. About 250 virgin adults (separated at the pupal stage) of the susceptible and the resistant strains were reciprocally crossed (PMD_{female} × PMD-R_{male}; PMD-R_{female} × PMD_{male}) to produce F₁ hybrids. F₁ progeny from both crosses were backcrossed to the resistant strain. The larval susceptibility of the parents, crosses and backcrosses was determined by exposure to various concentrations of permethrin. The dose–response curves were plotted and LC₅₀s calculated. Both the parental mosquitoes and the F₁ hybrids were examined for the presence of the F1552 and C1552 mutated alleles by DNA sequencing of the PCR products.

The degree of dominance (D) was calculated [21] on a continuous scale using the equation:

$$P = (2F_1 - P_1 - P_2)/(P_2 - P_1)$$

where F_1 is the resistance of the F_1 , P_1 and P_2 are the resistance of the susceptible and resistant parental strains, respectively (represented as log LC₅₀). Values of *D* range from -1 for completely recessive resistance to +1 for completely dominant resistance. On this scale, intermediate resistance has a dominance of 0 (zero).

3. Results

3.1. Partial sequencing of the Ae. aegypti voltage-gated sodium channel gene

Three DNA fragments encompassing 29 putative amino acid mutations [11] were amplified from cDNA templates prepared from PMD and PMD-R mosquitoes. The nucleotide sequences of these fragments cover a total of 2410 nucleotides of the voltagegated sodium channel gene. The amplified region is indicated in Table 1 and Fig. 1. These nucleotide sequences are reported in Gen-Bank (GenBank Accession Nos.: EU259807-EU259812 inclusive). Alignment of the amplified sequences show identical sequences for PMD and PMD-R except at codon 1552, which encodes Phenylalanine, where the second base "t" of PMD is substituted by "g" for PMD-R giving "tgc" which codes for Cysteine. Fig. 1 presents the deduced amino acid sequence of the sodium channel from the Liverpool strain to indicate the regions where PCR amplification was performed. In comparison with other strains, the PMD and PMD-R strains both differ from the Liverpool susceptible strain at amino acid position 436, in having an Arginine (R) rather than a Lysine (K). However a susceptible strain from China (GenBank Accession No.: AAT69681) also has an Arginine (R) at amino acid position

Primer name	Primer sequence (5'-3')	Product size (bp)	Region in sodium channel	Exon	
IS4-6_F	ATCTCGCTGCATTGAGAACA	768	IS4–IS6	1-6	
IS4-6_R	CGTAGCTGTGGCAGGAAAAG				
IIS1–6_F	GGTCCAACGTTCAAGGACAA	812	IIS1-IIS6	13-17	
IIS6_R	GAGGATGAACCGAAATTGGA				
IIIS4-6_F	TTCAAGCATTCAAAACAATG	830	IIIS5-IVS2	22-26	
IIIS4-6_R	CATCAGACACTCGCTACTGA				

^a Exon from the Aedes aegypti voltage-gated sodium channel gene. This transcript corresponds to VectorBase Transcript ID AAEL006019 and appears in supercont1.186 from nucleotide 18,685 to 163,945.

Table 1

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MTEDSDSISE	EERSLFRPFT	RESLAAIERR	IADAEAKQRE	LEKKRAE GET	GFGRKKKKKE	IRYDDEDEDE	GPOPDSTLEO	GVPIPVRNQG	SFPPELASTP	100
LEDIDSYYAN	ORTFVVVSKG	KDIFRFSATN	ALYVLD PFNP	IRRVAIVILV	HPLFSFFIIT	TILTNCILMI	MPST PTVEST	EVIFTGIYTF	ESAVKVMARG	200
FILQPFTYLR	↓ DAWNWLDFVV	IALAYVINGI	DIGNLAALRT	FRVLRALKTV	AIVPGLKTIV	GAVIESVKNL	RDVIILTMFS	LSVFALMGLQ	IYMGVL TOKC	300
IREFPMDGSW	GNLSDENWER	FNNND SNUYF	SETGDTPLCG	NSSGAGQCEE	GYICLQGYGD	NPNYGYTSFD	TFGWAFLSAF	RLMTQDYWEN	LYQLVLRSAG	400
PWHMLFFIVI	IFLGSFYLVN	LILAIVAMSY	DELQKKAEEE	E AAEEEA LRE	AEEAAAAKAA	KLEAQAAAAA	AAANPEIAKS	PSDFSCHSYE	L FVNQE KGND	500
DNNKEKMSIR	SEGLESVSEI	TRTTAPTATA	AGTAKARKVS	AGVAAFQKAS	LSLPGSPFNL	RRGSRGSHQF	TIRNGRGRFV	GWPGSDRKPL	VLSTYLDAQE	600
HLPYADDSNA	VTPMSEENGA	IIVPVYYANL	GSRHSS YTSH	QSRISYTSHG	DLLGGNTKES	RLRNRSARNT	NHSIVPPPNN	SGPNMSYVDS	NHKGQRDFDM	700
SQDCTDEAGK	IKHNDNPFIE	PSQTQTVVDN	KDVMVLNDII	E QAAGRHSRA	SDHGVSVYYF	PTEDDDEDGP	TFRDKALEFT	MRMIDVFCVU	DCCWVWLKFQ	800
EUVAFIVFD P	FVELFITLCI	VVNTL FMALD	HHDMDPDMER	ALKSGNYFFT	ATFAIEATMK	LIAMSPKYYF	QEGWNIFDFI	IVALS LLELG	LEGVQGLSVL	900
RSFRLLRVFK	LAKSWPTLNL	LISINGRING	ALGNLT FVLC	IIIFIFAVNG	NQ LFGKNY ID	NVD RFPDKD L	PRWN FTD FMH	SFMIW FRVLC	GEWIESMWDC	1000
MLVGDVSCIP	FFLATVVIGN	S6 LVVLNLFLAL	LLSNFGSSSL	SAPTADNETN	KIAEAFNRIS	RFSNWIKSNI	ANALKFVKNK	LTSQIASVQP	AGKGVCPCIS	1100
AEHGENELE L	TPDD ILADGL	LKKGWKEHNQ	LEVAIGDGME	FTIHGDLKNK	GKKNKQLMNN	SKVIGNSISN	HODNKLEHEL	NHRGMSLODD	DTASIKSYGS	1200
HKNRPFKDE S	HKGS AETMEG	EEKRD VSKED	LGIDEE LDDE	CDGEEGPLDG	ELIIHADEDE	VIEDSPADCC	PDNC YKKFPV	LAGDDDAPFU	QGWANLRLKT	1300
FQLIENKYFE	TAVITMILLS	SLALALEDVH	LPHRPILQDV	LYYMDRIFTY	S2 IFFLEMLIKU	LALGERVYFT	NAWCULDFII	VNVSLINFVA	SLCGAGGIQA	1400
FKTMRTLRAL	RPLRAMSRMO	GNRVVVNALV	QAIPSIFNVL	LVCLIFWLIF	AINGVQLFAG	KYFKCVDKNK	TTLSHEIIPD	VNACVAENYT	WENSPMNFDH	1500
VGKAYLCLFQ	VATFKGWIQI	MNDAIDSREV	GKQPIRETNI	YMYLYFVFFI PMDR:	IIS6 IFGSFF TTC -G-F1	TLNL FIGVII	DNFN EQERKA	GGSL EMFMTE	DOKK YYNAMER	MGS 160
KKPLKAIPRP	RWRPQAIVFE	IVTNKKFDMI	IVS1 IMLFIGFNML	THTLDHYKQT	DTFSAVLDYL	NMIFICIFSS	ECLMKIFALR	YHYFIEPWNL	FDFVVVILSI	1700
LGLVLSDLIE	KYFVSPTLLR	VVRVAKVGRV	LRLVKGAKGI	RTLLFALANS	LPALFNICLL	LFLVMFIFAI	FGMS FFMHVK	DKSGLDDVYN	FKTFGQSMIL	1800
LFQMSTSAGW	DGVLDGIINE	DECLP PDNDK	GYPGNC GSAT	IGITYLLAYL	VISFLIVINN	YIAVILENYS	QATEDVQEGL	TDDDYDMYYE	IWQQFD PDGT	1900
QYIRYDQLSD	FLDVLEPPLQ	IHKPNKYKII	SMDIPICRGD	MMFCVDILDA	LTKDFFARKG	NPIEETAELG	EVQARPDEVG	YEPVSSTLWR	QREEYCARVI	2000
QHAURKHKE R	OAGGGGGDDT	DADACDNDDG	DDGGGGAGDG	GSAGGGGVTS	PGVGSGSIVG	GGTTPGSGGG	GSOANLGIVV	EHNLSPKESP	DGNNDPOGRO	2100

TAVLVESDGE VTKNGHRVVI HSRSPSITSR SADV 2134

Fig. 1. The deduced amino acid sequences of the sodium channel gene in the Liverpool strain of *Ae. aegypti* obtained from manual alignment of three VectorBase ID AAEL004612, AAEL008297 and AAEL006019. The gray blocks indicated the regions amplified from cDNA of PMD and PMD-R mosquitoes by the PCR primers in Table 1. The position of the amino acid substitution, F1552C, is also shown.

436. Therefore, this difference does not seem to be related to insecticide resistance. None of the 29 putative amino acid mutations [11] were detected in the PMD-R sample.

3.2. Genetic inheritance of the F1552 and C1552 alleles in Ae. aegypti

Data from larval permethrin susceptibility tests of the parental strains, the F₁ hybrids and the F₂ backcrosses were analyzed by a plot of concentration-mortality response (Fig. 2). Statistical data from the probit analysis are presented in Table 2. The resistance ratio (RR) of the permethrin susceptible (PMD) and resistant (PMD-R) strains was about 25 as determined by LC₅₀. The concentration-mortality line of F1 hybrid mosquitoes shifts from intermediate toward, but does not reach, the susceptible line with the RR reduced to about 2.94. Sequencing of the amplified fragment from adults and larvae of F₁ hybrid progeny confirmed that they are heterozygous (F1552/ C1552), suggesting that resistance is highly associated with the homozygous point mutation. In addition, the calculated degree of dominance [21] are -0.308 for PMD (F) × PMD-R (M) and -0.347for PMD (M) \times PMD-R (F). All these results clearly suggest that permethrin resistance in PMD-R strain is partially recessive. The LC50 values of the F1 progeny obtained from both directions of crosses were similar as were the F2 backcrosses (Table 2) suggesting that there are no maternal effects or sex linkage, and thus resistance is autosomally inherited.

The offspring obtained from backcrossing the F_1 progeny with either the male or female resistant parents do not show a clear plateau curve between the concentration-mortality lines of the resistant and F_1 hybrid individuals at the 50% mortality level (Fig. 2). In addition, estimated slopes of log dose-probit mortality plots were lower for backcross progeny than for the parental strains and their F_1 hybrid progeny (Table 2). These patterns suggest an increased genetic variance in the backcross progeny compared with that of parental populations and F_1 progeny. These genotypes possess varying levels of resistance which suggests involvement of other factors or mechanisms [22].

4. Discussion

From the screening of three cDNA fragments spanning 29 putative amino acid substitutions out of the total of 33 amino acid mutations reported in correlation with insecticide resistance [10,11], we discovered a novel amino acid mutation in the permethrin resistance PMD-R strain of *Ae. aegypti*. This mutation is at amino acid position 1552 where Phenylalanine (F) was substituted by Cysteine (C). Using a PCR primer pair that can specifically amplify the mutated allele, we detected the F1552C mutation in wild populations of *Ae. aegypti* in Chiang Mai city, Ubon Rachathanee (north eastern) and Song Khla (southern) provinces, and also in Myanmar and Cambodia (unpublished data). The apparently widespread nature of this resistance allele indicates that it may play a major role in DDT/permethrin resistance in *Ae. aegypti*.

The putative binding sites in insect voltage-gated sodium channel were identified using a homology model of an available crystal structure [23]. The model predicted that insecticide resistance was associated with nine amino acid mutations located within this putative binding pocket, M918T (or V or I) on the IIS4–IIS5 linker, L925I, T929I (or V or C), L932F on the IIS5 helix and F1538I on the IIS6 helix. Position F1552 in the *Ae. aegytpi* sequence is equivalent to F1534 in the housefly Vssc1 sequence (Genbank Accession No.:

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Fig. 2. The log dose-mortality response curves and log dose-probit mortality lines (inset) of the parental strains, F1 hybrids and backcrossed individuals after exposure to permethrin.

Table 2

Toxicity of permethrin to the parental strains of Ae. aegypti, their reciprocal crosses and backcrosses

Strains	LC_{50} (95% CL) (µg l ⁻¹)	Slope (±SE)	χ^2	Р
Parental strains				
PMD	1.03 (0.92-1.17)	2.91 (+0.25)	7.53 (n = 6, df = 5)	0.11
PMD-R	25.64 (21.54-30.49)	1.95 (+0.14)	3.11 (n = 5, df = 4)	0.38
F_1 progeny				
PMD-RS1 (PMD (f) \times PMD-R (m))	3.13 (2.82-3.45)	3.46 (+0.24)	3.24 (n = 6, df = 5)	0.52
PMD-RS2 (PMD (m) \times PMD-R (f))	2.94 (2.65-3.24)	3.48 (+0.24)	2.08 (n = 6, df = 5)	0.72
Backcross progeny				
PMD-RS2 (f) \times PMD-R (m)	8.96 (7.38-10.84)	1.48 (+0.085)	9.18 (n = 7, df = 6)	0.10
PMD-R (f) \times PMD-RS1 (m)	9.36 (7.69–11.38)	1.48 (+0.088)	10.79 (n = 7, df = 6)	0.06

AAB47604) based on an alignment of the homologous sequences. Although the F1534C mutation has not been reported for resistance in housefly or other species, interpretation of the molecular model suggests that the role of F1534 is important for making aromatic–aromatic contact with the first aromatic ring of the alcohol moiety of pyrethroid [23]. Moreover, F1534 is a highly conserved residue across both arthropod and non arthropod species [23]. Therefore the F1552C mutation we observed in *Ae. aegypti* may have disrupted the ability of the channel to interact with pyrethroid.

Most studies of sodium channel mutations have focused on the M918T and L1014F changes since the kdr and super-kdr strains of housefly were found to have these point mutations in association with a resistant phenotype [9]. Although the L1014F mutation has now been identified in at least a dozen additional insect species, it has not been found in any *Ae. aegypti* pyrethroid resistant strains [24]. Four other amino acid mutations were identified in IIS6 of the sodium channel gene in pyrethroid resistant *Ae. aegypti* from various countries [25]. One of these was the V1016G mutation within the trans-membrane segment S6 of domain II (IIS6) in mosquito samples from Tak, northwestern Thailand. However, they did not examine the IIIS6 region. Recently two novel mutations in IIS6 were also discovered in Latin-American *Ae. aegypti* [26].

The genetics of resistance to permethrin have been investigated in various insect species. Resistance to permethrin was generally determined to be inherited as an autosomal and incompletely recessive trait such as in housefly [27], a predatory mite *Amblyseius fallacis* [28] and a southern cattle tick *Boophilus microplus* [29]. Our results of the inheritance analysis of permethrin resistance in *Ae. aegypti* PMD-R strain agree with these other studies.

The PMD and PMD-R strains were, respectively, permethrin susceptible and resistant strains selected from the same origin in Mae Taeng District, Chiang Mai. Both strains are resistant to DDT which our earlier studies have shown due to a glutathione transferase mechanism [13]. In this study, we found the homozygous mutations at F1552C is highly associated with permethrin resistance and probably plays a major role in the resistant characteristics of PMD-R. Both homozygous and heterozygous mutations have been detected in wild populations, and both populations survived after exposure to the discriminating dose of 0.75% permethrin (unpublished data). The heterozygous mutation in our crossing experiments showed a resistant level (LC50) of about 3 times higher than the susceptible strain (Table 2), but lower than the logarithmic average of the susceptible and resistant parental strains. A question arises whether the heterozygous mutation alone can maintain this resistance level. Our backcrossing experiment does not support monogenic control of resistance. In addition, the slopes of the concentration-mortality lines of backcross progeny (Table 2) were decreased compared with the parental strains and $F_{\rm 1}$ hybrid progeny, indicating that a number of unlinked genes contribute to resistance [22]. Our ongoing study by adding synergist piperonyl butoxide, an inhibitor of microsomal oxidase, in larval bioassays showed further decreases in permethrin resistance levels being close to the susceptible strain, suggesting that a metabolic mechanism, such as mixed function oxidases also may be involved in resistance. The results of these biochemical studies will be published elsewhere.

Therefore, in conclusion, we consider that permethrin resistance in PMD-R is possibly conferred by two major mechanisms, i.e. the homozygous mutation at F1552C in the Ae. aegypti voltage-gated sodium channel gene and a mixed function oxidase. The spread of pyrethroid resistant Ae, aegypti could have serious implications for the successful use of pyrethroid as a control measure and this problem must be closely monitored.

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High-throughput assays for detection of the FI534C mutation in the voltage-gated sodium channel gene in permethrin-resistant Aedes aegypti and the distribution of this mutation throughout Thailand

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Summary

OBJECTIVES To develop rapid monitoring tools to detect the F1534C permethrin-resistance mutation in domain IIIS6 of the *Aedes aegypti* voltage-gated sodium channel gene and determine the frequency and distribution of this mutation in Thailand.

METHODS A TaqMan SNP genotyping and an allele specific PCR (AS-PCR) assay were developed and validated by comparison with DNA sequencing of homozygous susceptible and homozygous resistant laboratory strains, their reciprocal-cross progenies, and field-caught mosquitoes. To determine the resistance phenotype of wild-caught *A. aegypti*, mosquitoes were exposed to 0.75% permethrin paper. The AS-PCR assay was used to screen 619 individuals from 20 localities throughout Thailand. RESULTS Overall, both assays gave results consistent with DNA sequencing for laboratory strains of known genotype and for wild-caught *A. aegypti*. The only slight discrepancy was for the AS-PCR method, which overestimated the mutant allele frequency by 1.8% in wild-caught samples. AS-PCR assays of permethrin-exposed samples show that the mutant C1534 allele is very closely associated with the resistant phenotype. However, 19 permethrin-resistant individuals were homozygous for the wild-type F1534 allele. DNA sequencing revealed all these individuals were homozygous for two other mutations in domain II, V1016G and S989P, which are known to confer resistance (Srisawat *et al.* 2010). The F1534C mutation is widespread in Thailand with mutant allele frequencies varying among populations from 0.20 to 1.00.

CONCLUSIONS These assays can be used for the rapid detection of the F1534C resistance mutation in *A. aegypti* populations. The F1534C, and other, mutations underlie an extremely high prevalence of pyrethroid resistance in Thailand.

keywords Aedes aegypti, pyrethroid resistance, voltage-gated sodium channel gene, mutation detection

Introduction

Aedes aegypti is a major vector of viral diseases, particularly dengue and chikungunya, which cause serious public health problems in Thailand and elsewhere. Since there is no vaccine or specific treatment, control of disease transmission is based mainly on management of breeding habitats and insecticide applications. The adverse effect of the heavy and long-term use of insecticides is the resistance of the vector throughout the world. In Thailand, *A. aegypti* is resistant to several insecticides including DDT, pyrethroids, temephos, fenitrothion and propoxur (Somboon *et al.* 2003; Paeporn *et al.* 2004; Ponlawat *et al.* 2005; Jirakanjanakit *et al.* 2007 a,b; Pethuan *et al.* 2007). This problem has hampered the control of vectors using insecticides.

There are two broad classes of resistance mechanism that play role in mosquito resistance to insecticides: targetsite insensitivity and metabolic enzyme-based resistance (Hemingway & Ranson 2000). Target-site insensitivity, known as knockdown resistance (kdr), is the important mechanism for resistance to pyrethroid and DDT and is associated with a single or multiple mutations in the voltage-gated sodium channel gene in several insect species (Soderlund & Knipple 2003; Davies *et al.* 2007). In

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A. aegypti, several mutations, e.g. G923V, L982T, I1011M, I1011V, V1016I, V1016G and D1794Y have been identified in the voltage-gated sodium channel gene and shown to confer pyrethroid resistance (Brengues *et al.* 2003; Saavedra-Rodriguez *et al.* 2007; Chang *et al.* 2009). In addition, we have recently reported a novel mutation, a phenylalanine to cysteine in *A. aegypti* at position 1552 in IIIS6 in a DDT/permethrin-resistant *A. aegypti* strain (PMD-R) from Thailand (Yanola *et al.* 2010). We refer to this mutation as F1534C in reference to the equivalent resistance mutation F1534C in the housefly Vssc1 sequence. This mutation has also been found associated with pyrethroid resistance in Vietnam and the British West Indies (Kawada *et al.* 2009; Harris *et al.* 2010).

Development of early detection and characterization of mutations associated with resistance phenotypes is essential for resistance management strategies. High-throughput molecular tools are required to screen large numbers of individuals to monitor the emergence and presence of mutations associated with resistance. There are now at least seven different methods available for detecting the mutations responsible for *kdr* in the malaria vector *Anopheles gambiae* (Bass *et al.* 2007). In *A. aegypti*, allele specific PCR (AS-PCR) has been used to screen for mutations I1011V, I1011M, V1016I and V1016G (Saavedra-Rodriguez *et al.* 2007) and F1534C (Harris *et al.* 2010), in mosquitoes from Latin America. The hot oligonucleotide ligation assay technique was also developed to detect the I1011V and V1016G mutations in Thai strains of *A. aegypti* (Rajatileka *et al.* 2008).

In this study, we developed two PCR-based assays to detect the F1534C mutation in the *A. aegypti* voltage-gated sodium channel gene, one a high-throughput allelic discrimination assay and the other a simple low-cost assay. We also used these tools to estimate the frequency of the F1534C mutation in *A. aegypti* populations in Thailand.

Materials and methods

Amplification and DNA sequencing of a fragment of the *Aedes aegypti* sodium channel gene

Genomic DNA was extracted using DNAzol Reagent (Invitrogen, Carlsbad, CA, USA). PCR primers were designed using the web-based Primer 3 program (Rozen & Skaletsky 2000) to encompass the region with the V1016G and F1534C mutations (Table 1).

PCR was carried out in a 50 μ l reaction volume containing 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen), 0.1 mM dNTPs, 1.5 mM MgCl₂ and 0.5 μ M each of the forward and reverse primers. The amplification consists of an initial heat activation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s with a final extension step at 72 °C for 2 min.

Primer name	Primer sequence (5'-3')	Product size (bp)	Region in sodium channel	Exon†	
Direct sequencing					
IIP_F	GGTGGAACTTCACCGACTTC	581	IIP-IIS6	16-17	
IIS6_R	GGACGCAATCTGGCTTGTTA				
Ge-IIIS6_F	GCTGTCGCACGAGATCATT	635	IIIS4–IIIS6	24-26	
IIIS6_R	GTTGAACCCGATGAACAACA				
TaqMan SNP genotyping					
Primers:					
F1534C SNP-F	CGAGACCAACATCTACATGTACCT	88	IIIS6	25	
F1534C SNP-R	GATGATGACACCGATGAACAGATTC				
Probes:					
F1534-PV	(VIC)-AACGACCCGAAGATGA-(MGBNFQ)				
C1534-PF	(FAM)-ACGACCCGCAGATGA-(MGBNFQ)				
AS-PCR					
F1534-f	[short GC tail]TCTACTTTGTGTTCTTCATCATATT	93	IIIS6	25	
C1534-f	[long GC tail]TCTACTTTGTGTTCTTCATCATGTG	113	IIIS6	25	
CP-r	TCTGCTCGTTGAAGTTGTCGAT				

AS-PCR, allele specific PCR.

†Exon from the *A. aegypti* voltage-gated sodium channel gene. This transcript corresponds to VectorBase Transcript ID AAEL006019 and appears in supercont1.186, nucleotides 18 685–163 945.

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The PCR fragments were purified using ExoSap-IT (USB, Columbus, OH, USA). Nucleotide sequences were determined on both strands of purified PCR products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Sequence data were analyzed using CLUSTAL W (Thompson *et al.* 1994) and the Six Frame Translation program (Sequence Launcher, BCM) (Smith *et al.* 1996).

Development of TaqMan SNP genotyping (TaqMan SNP) and AS-PCR assays

Laboratory strains of *A. aegypti* including PMD (homozygous wild type, F/F1534), PMD-R (homozygous mutant C/C1534), their reciprocal-cross progenies (PMD-RS1 and PMD-RS2, heterozygous F/C1534) (Yanola *et al.* 2010), and the New Orleans strain is a pyrethroid susceptible strain originally collected from New Orleans, USA (homozygous F/F1534, Yanola *et al.* 2010), as well as field-caught *A. aegypti* mosquitoes, were used to validate the TaqMan SNP and AS-PCR assays. Validation was done by comparison of assay genotyping with DNA sequencing as described in Yanola *et al.* (2010).

TaqMan SNP assay

Two primers and two minor groove binding probes were designed using the Custom TaqMan SNP genotyping assay service (Table 1). Primers F1534C SNP-F and F1534C SNP-R were standard oligonucleotides. The probe oligonucleotides each consist of a 5' reporter dye, a 3' non-fluorescence quencher and a minor groove binder at the 3' end. The probe F1534-PV was labelled with VIC dye fluorescence at the 5' end for the detection of the wild-type allele whereas the probe C1534-PF was labelled with 6-FAM dye fluorescence at the 5' end for the detection of the mutant allele (Figure 1a).

The TaqMan reaction contained 12.5 μl of 2X TaqMan Universal PCR Master Mix, 1.44 μM of each primer,



113 bp (Mutant allele)

Figure 1 Schematic of the (a) TaqMan SNP Genotyping and (b) the allele specific PCR assays for detection of the F1534C mutation. The primers and probes were designed based on the sequence of voltage-gated sodium channel from the PMD and PMD-R strains of *A. aegypti* (Genbank accession numbers: EU259810 and EU259811 respectively).

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0.4 μ M of each probe and 2 μ l of genomic DNA (50 ng) made up to 25 μ l with sterile water. The assay was performed using an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA), under the following thermocycling conditions: 10 min at 95 °C, 40 cycles of 92 °C for 15 min and 60 °C for 1 min. Data were analyzed by the 7500 System SDS Software version 1.3.1.

AS-PCR assay

The design of the AS-PCR assay increased specificity by following the methods of Okimoto and Dodgson (1996) and Saavedra-Rodriguez *et al.* (2007), by positioning the mismatches in each allele specific primer at the third nucleotide from the 3' terminus. To distinguish between the amplification products based on size, 5' GC tails of different lengths were attached to each allele specific primer (Germer & Higuchi 1999, Wang *et al.*, 2005).

The reaction (50 μ l) contained 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 μ M F1534-f forward primer, 0.165 μ M C1534-f forward primer and 0.5 μ M CP-r common reverse primer (Table 1). The amplification consists of a 95 °C 2 min heat activation step, 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °^C for 30 s with a 2 min final extension step at 72 °C. The amplified fragments were analyzed on a 3.0% agarose gel (Agarose 3:1; HRB, Amresco, OH, USA).

Genotyping of the F1534C mutation in wild-caught Aedes aegypti from Thailand by AS-PCR

Wild A. *aegypti* mosquitoes were collected as larvae and pupae from 20 localities around Thailand (Table S1) and reared to adulthood in the insectary. Insecticide susceptibility tests were carried out for 14 localities using one-dayold non-blood fed females using WHO 0.75% permethrin paper for 60 min exposure time and mortality recorded after 24 h. Survivors and dead individuals from this test were stored at -20 °C until tested. These mosquitoes were genotyped individually for the F1534C mutation with our developed AS-PCR method using the known genotyped laboratory strains as controls. A Pearson Chi-square test was used to compare the genotype frequency and also the allele frequency between the dead and survivor mosquito groups.

Results

Development of TaqMan SNP assay

Scatter plots of relative end point fluorescence intensities of each sample (Figure S1) show a clear clustering for each sample group of homozygous (C/C1534) mutant, homo-

zygous (F/F1534) wild type and the heterozygotes (F/C1534). The limit of detection for each genotyping was a 1:100 dilution, equivalent to 1 ng of DNA (Figure S2).

Development of AS-PCR

The wild-type homozygous samples (F/F1534) gave a single 93 bp band, the homozygous mutant samples (C/C1534) gave a single 113 bp band and the heterozy-gous (F/C1534) samples gave both of those bands (Figure S3). The sensitivity of this method was evaluated by testing with a set of DNA dilutions of each genotype. The calculated limit of detection was at a 1: 100 dilution, equivalent to 1 ng of genomic DNA.

Comparison of DNA sequencing with the TaqMan SNP and AS-PCR assays

In testing 150 known genotyped laboratory *A. aegypti* female mosquitoes, the three methods were fully consistent (Table 2). For the wild-caught mosquitoes, of 85 samples tested, the TaqMan method was as good as DNA sequencing. The AS-PCR, however, showed a discrepancy with the DNA sequencing for 2 out of 103 wild-caught mosquitoes, although these two mosquitoes were scored the same by both DNA sequencing and TaqMan SNP. Thus, the AS-PCR apparently slightly overestimated the mutant C1534 allele, by approximately 1.8%.

Distribution of the F1534C mutation in Aedes aegypti populations from Thailand

In the insecticide bioassay test of 2,154 *A. aegypti* females from 14 localities in four regions of Thailand, only 41 (1.9%) insects died (susceptible) with mortality rates ranging from 0 to 13.3%. Table 3 shows the genotyping results determined from the 41 dead mosquitoes and 465 individuals randomly selected from the 2,113 survivors (resistant). The genotype frequencies of the dead and survivors were significantly different ($\chi^2 = 80.8$, df = 2, P < 0.0001). The overall mutant allele frequency was significantly higher in the survivor group (0.84) than the dead group (0.56) ($\chi^2 = 52.1$, df = 1, P < 0.0001).

Some of the survivor group possessed a homozygous wild-type F/F1534 genotype (n = 19). We predicted that this may be due to a different mutation undetectable by our primers. Hence, we sequenced the samples and found that all were homozygous for mutations V1016G and S989P in domain II. The V1016G and S989P mutations have been reported to confer pyrethoid resistance in *A. aegypti* (Bregues *et al.* 2003; Srisawat *et al.* 2010). To determine if both mutations co-existed with F1534C, we sequenced the

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Table 2 Comparison of genotype results for the F1534C mutation obtained from the TaqMan SNP and AS-PCR assays with DNA sequencing

	TaqMan SNP genotyping/DNA sequencing (no. of samples)				AS-PCR/DNA sequencing (no. of samples)					
Strains	F/F1534	F/C1534	C/C1534	Total	F/F1534	F/C1534	C/C1534	Total		
Laboratory strains										
PMD	30/30	0/0	0/0	30/30	30/30	0/0	0/0	30/30		
PMD-R	0/0	0/0	30/30	30/30	0/0	0/0	30/30	30/30		
PMD-RS1	0/0	30/30	0/0	30/30	0/0	30/30	0/0	30/30		
PMD-RS2	0/0	30/30	0/0	30/30	0/0	30/30	0/0	30/30		
New Orleans	30/30	0/0	0/0	30/30	30/30	0/0	0/0	30/30		
Total	60/60	60/60	30/30	150/150	60/60	60/60	30/30	150/150		
Wild-caught strains										
Chiang Mai city	6/6	12/12	14/14	32/32	13/13	12/12	14/14	39/39		
Mae Taeng District	0/0	9/9	18/18	27/27	6/6	9/9	18/18	33/33		
Lampang city	2/2	7/7	10/10	19/19	5†/6	7/7	11‡/10	23/23		
Mae Sariang District	7/7	0/0	0/0	7/7	8/8	0/0	0/0	8/8		
Total	15/15	28/28	42/42	85/85	32/33	28/28	43/42	103/103		

AS-PCR, allele specific PCR.

*One sample was heterozygous F/C1534 by AS-PCR, but homozygous F/F1534 by DNA sequencing.

‡One sample was homozygous C/C1534 by AS-PCR, but heterozygous F/C1534 by DNA sequencing.

Table 3 Frequency of the F1534C mutation in the A. aegypti voltage-gated sodium channel gene within dead and survivor mosquitoes from 14 localities of Thailand determined using the AS-PCR method

				Dead				Survivors			
		Locality	N. J. J. (m. 1	No. mosquitoes			Freq	No. mosquitoes			E
Region Northern	Province		(% mortality)	F/F	F/C	C/C	C allele	F/F	F/C	C/C	C allele
Northern	Chiang Mai	Chiang Mai City	5/751 (0.7)	5	0	0	0.00	8†	20	33	0.70
		Mae Taeng District	10/116 (8.6)	6	4	0	0.20	0	7	48	0.93
	Lampang	Lampang city	0/250 (0.0)	0	0	0	0.00	5†	26	18	0.63
		Donchai, Thoen District	0/150 (0.0)	0	0	-0	0.00	0	10	22	0.84
	Mae Hong Son	Mae Sariang District	5/67 (7.5)	4	1	0	0.10	6†	8	13	0.63
	Chiang Rai	Chiang Sane District	3/28 (10.7)	0	0	3	1.00	0	2	21	0.96
	Uttraradit	Uttraradit city	2/15 (13.3)	0	0	2	1.00	0	0	13	1.00
Central	Phitsanulok	Phitsanulok City	0/70 (0.0)	0	0	0	0.00	0	11	21	0.83
	Phetchabun	Phetchabun City	0/30 (0.0)	0	0	0	0.00	0	0	29	1.00
	Nakhonsawan	Nakhonsawon city	3/134 (2.2)	0	0	3	1.00	0	10	19	0.83
Eastern	Trat	Koh Chang Subdistrict	2/40 (5.0)	0	0	2	1.00	0	9	21	0.85
Western	Tak	Tak city	8/241 (3.3)	0	0	8	1.00	0	0	24	1.00
		Mae Kasa, Mae Sot District	2/138 (1.4)	0	1	1	0.75	0	5	25	0.92
		Mae Sot, Mae Sot District	1/124 (0.8)	0	0	1	1.00	0	1	30	0.98
Total			41/2154 (1.9)	15	6	20	0.56	19	109	337	0.84

AS-PCR, allele specific PCR.

†The V1016G and \$989P mutations in domain II of the A. aegypti voltage-gated sodium channel gene were detected.

IIP–IIS6 region in 33 survivors homozygous for C/C1534 from Chiang Mai city (11), Lampang city (10) and Mae Sariang District (12). None of them possessed either the V1016G or the S989P mutation. We also sequenced the IIP–IIS6 region in nine heterozygous F/C1534 individuals

from Chiang Mai city (6) and Mae Sariang District (3) and found one of the latter was heterozygous for V/G1016 and S/P989.

Figure 2 summarizes the estimated genotype frequencies and distribution of F1534C among 2,267 mosquitoes of

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A. aegypti populations from 20 localities of Thailand. The data were derived partly from the 14 populations (n = 2,154) in Table 3 together with six other populations that were genotyped but not tested by the permethrin paper. As not all individuals in the survivor group from Table 3 were genotyped, we prevented any bias in the estimation of the population genotype frequencies (Figure 2) by estimating the absolute number of genotypes in the survivor group by multiplying the determined genotype frequencies with the total number of survivors. In a total of 2,267 mosquitoes, the homozygous wild-type F/F1534 genotype was rarely observed, with estimated frequencies ranging from 0 to 0.31. The F1534C mutation was widely distributed with the heterozygous F/C1534 genotype ranging from 0 to 0.53 and the homozygous mutant C/C1534 genotype ranging from 0.20 to 1.00. The estimated overall genotype frequencies of F/F1534, F/C1534 and C/C1534 were 9.71%, 26.36%, and 63.93%, respectively and the estimated mutant allele frequency was 0.77. Although the frequency of this resistance mutation is, overall, high in Thailand, it appears to be lower in the Provinces of Phetchaburi (sites Nongyapong and Phetchaburi city), Mae Hong Son (Mae Sariang) and Chaing Mai (Mae Taeng and Chiang Mai city), indicating some geographical variation in its distribution.

Discussion

We developed two methods, TaqMan SNP and AS-PCR assays, to detect the F1534C mutation. The TaqMan SNP method is as good as DNA sequencing, whereas the AS-PCR method showed only a very small error. A similar result was obtained in another study for the detection of the L1014F and L1014S mutations in An. gambiae s.s., in which the TaqMan SNP was the most specific method while the corresponding AS-PCR assay had a relatively low error rate (3.1%) (Bass et al. 2007). Although some reports questioned the reliability of the AS-PCR assay (Pinto et al. 2006; Verhaeghen et al. 2006), it is the most widely use method to detect the kdr mutation in malaria endemic countries because of its relatively low cost (Bass et al. 2007). Recently, Harris et al. (2010) developed their tetraplex PCR assay to detect the F1534C mutation in A. aegypti populations in Grand Cayman. Due to the greater expense of operating costs and initial outlay for special equipment for the TaqMan SNP assay, the AS-PCR may be the preferred method in disease endemic countries.

It should be noted that in some provinces small numbers of samples were tested and thus the observed frequency may not be a good estimation of genotype frequency in the area. Nonetheless, this study has provided evidence that the mutant C1534 allele is wildly distributed and significantly associated with the permethrin-resistant phenotype in *A. aegypti* populations in Thailand. The C1534 allele frequency in this study (0.77) was higher than Vietnam (0.21) (Kawada *et al.* 2009) and Grand Cayman (0.68) (Harris *et al.* 2010). We also detected the F1534C mutation by AS-PCR in *A. aegypti* in the neighbouring countries of Myanmar (Yangon city) and Cambodia (Battambang town) (7/8 alleles and 20/20 genotyped alleles, respectively; Yanola *et al.* unpublished data), indicating that this resistance mutation is widespread in Southeast Asia.

Since the 1534C allele is recessive (Harris et al. 2010; Yanola et al. 2010), only homozygous mutants were expected to be alive after a 1 h exposure to the discriminating dose (0.25% permethrin) for adult A. aegypti set by the World Health Organization (1998). However, not all homozygous mutant mosquitoes survived here (Table 3), probably due to the higher concentration of permethrin (0.75%) used in this study. In addition, 23.4% of the survivor mosquitoes were heterozygous F/C1534, probably due to additional enzyme-based resistance. Previous studies (Yaicharoen et al. 2005; Pethuan et al. 2007) and our ongoing study (Somwang et al. in press) revealed that cytochrome P450 monooxygenases and other oxidative enzymes are involved in pyrethroid resistance in A. aegypti populations in Thailand. Studies in An. gambiae s.s. also suggested that the sodium channel mutations (L1014F and L1014S) may co-operate with other unidentified mechanisms including the detoxification enzyme system (Brooke 2008; Nwane et al. 2009). As discussed by Brooke (2008), resistance could be multigenic and kdr mutations might not fully explain all the variance in the resistance phenotype. However, the strong correlation between the kdr mutation and the pyrethroid and/or DDT resistance phenotype in An. gambiae s.s. (Martinez-Torres et al. 1998; Chandre et al. 2000; Ranson et al. 2000) and A. aegypti (Harris et al. 2010) has been demonstrated previously, as well as in this study.

A small proportion of homozygous wild type F/F1534 (4.09%) individuals were able to survive the permethrin exposure (Table 3) due to the V1016G and S989P mutations in domain II. The V1016G mutation was originally discovered in a permethrin-resistant strain of *A. aegypti* from Thailand and Indonesia (Brengues *et al.* 2003) and is widely distributed in Thailand with an allele frequency of 0.23 (Rajatileka *et al.* 2008). The V1016G mutation was also found in *A. aegypti* collected from Vietnam (Kawada *et al.* 2009), Taiwan (Chang *et al.* 2009) and Bhutan (Yanola *et al.*, unpublished data). The role of the S989P mutation in resistance needs further study.



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Figure 2 Estimated frequency distributions of the homozygous wild type (F/F1534), heterozygous (F/C1534) and homozygous mutant (C/C1534) genotypes in *A. aegypti* in Thailand. For the populations from Table 3, population genotype frequencies were estimated by combining the total numbers of genotypes from the survivor and dead groups. These numbers were determined directly for the dead group and estimated for the survivor group (see main text).

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In conclusion, we have successfully developed the TaqMan SNP and AS-PCR assays for monitoring the F1534C mutation in *A. aegypti* populations. Depending on the available facilities, these assays are useful tools for the rapid detection of the F1534C resistance mutation which is essential for the development of resistance management strategies. The AS-PCR method was extremely useful to reveal the high frequency of the F1534C mutation throughout Thailand as well as indicating this may be a problem for resistance in the neighbouring countries of Myanmar and Cambodia. The development of a multiplex-PCR method to detect both the F1534C and V1016G mutations is now required to improve the surveillance of resistance alleles in wild populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Collection sites of *A. aegypti* in Thailand. Figure S1. Scatter plot of end point fluorescence intensities using the TaqMan SNP genotyping assay.

Figure S2. Effect of sample dilution on fluorescence intensity. Curves show the increase in fluorescence over time generated for mutant DNA of the following concentrations from left to right: undiluted (original 100 ng DNA template), 1:2, 1:5, 1:10, 1:20, 1: 100 and 1: 200.

Figure S3. Characteristic agarose gel and sensitivity test for the AS-PCR assay for the detection of the F1534C mutation. Lane M: 100 bp DNA ladder; lane 1: 100 ng DNA template; Lane 2-6: dilutions of 1:2, 1:10, 1:20, 1:100 and 1:200, respectively of the original 100 ng DNA template. A. Homozygous for F/F1534 allele, B. Heterozygous for F/C1534 and C. Homozygous for C/C1534.

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Oral Presentation:

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