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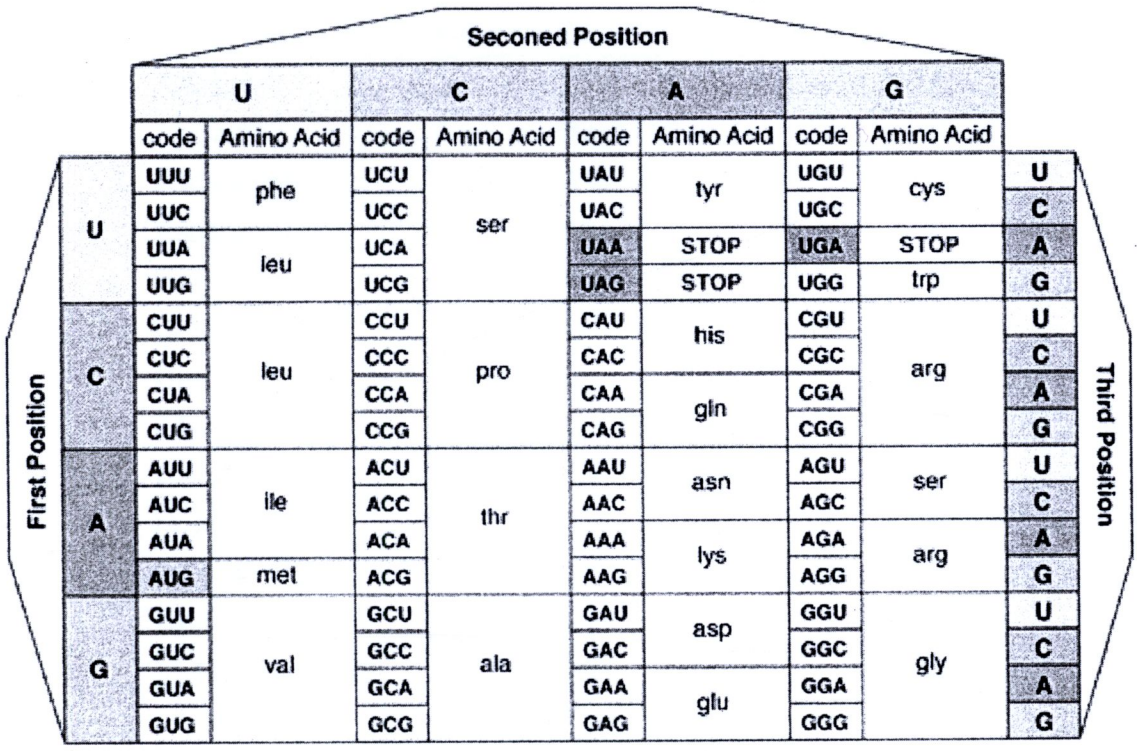
APPENDICES

APPENDIX A

The RNA codons



First Position	Seconed Position: U								Third Position
	code	Amino Acid	code	Amino Acid	code	Amino Acid	code	Amino Acid	
U	UUU	phe	UCU	ser	UAU	tyr	UGU	cys	U
	UUC		UCC		UAC		UGC		C
	UUA	leu	UCA		UAA	STOP	UGA	STOP	A
	UUG		UCG		UAG	STOP	UGG	trp	G
C	CUU	leu	CCU	pro	CAU	his	CGU	arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	ile	ACU	thr	AAU	asn	AGU	ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	lys	AGA	arg	A
	AUG	met	ACG		AAG		AGG		G
G	GUU	val	GCU	ala	GAU	asp	GGU	gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	glu	GGA		A
	GUG		GCG		GAG		GGG		G

APPENDIX B

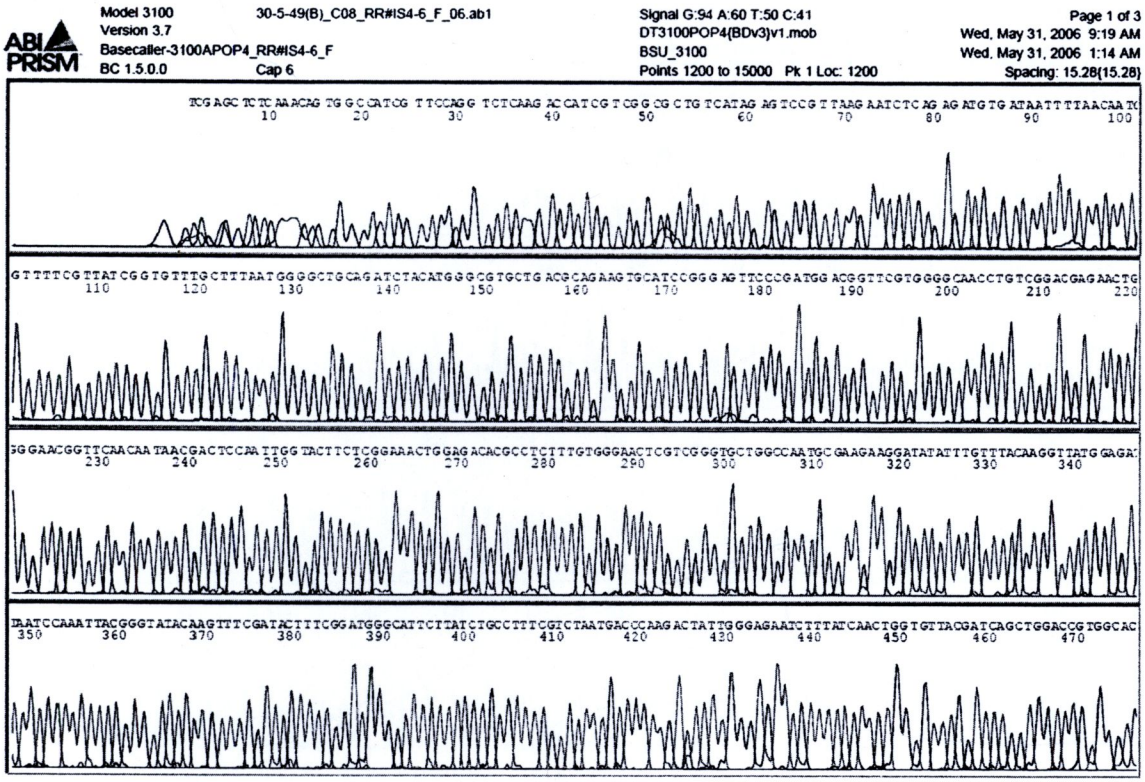
Amino acid abbreviations

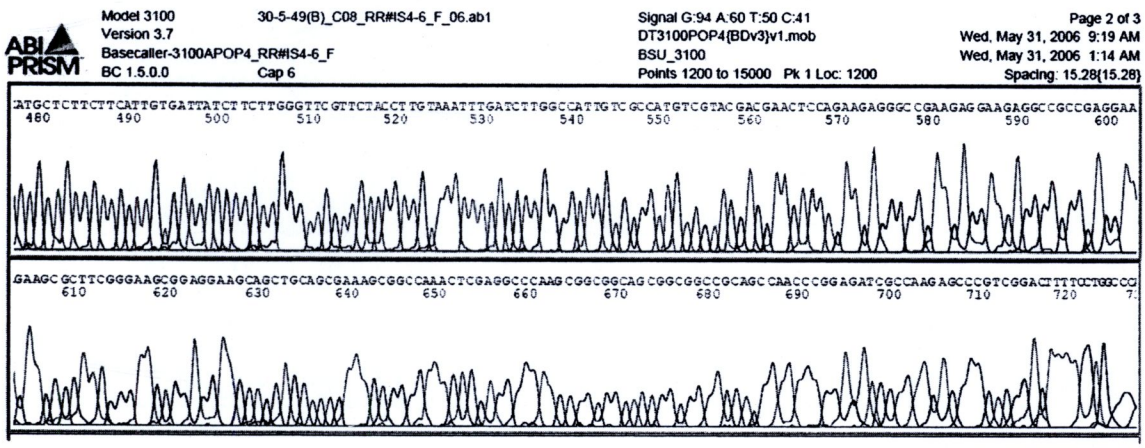
Full name of amino acid	Abbreviation (3 letters)	Abbreviation (1 letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX C

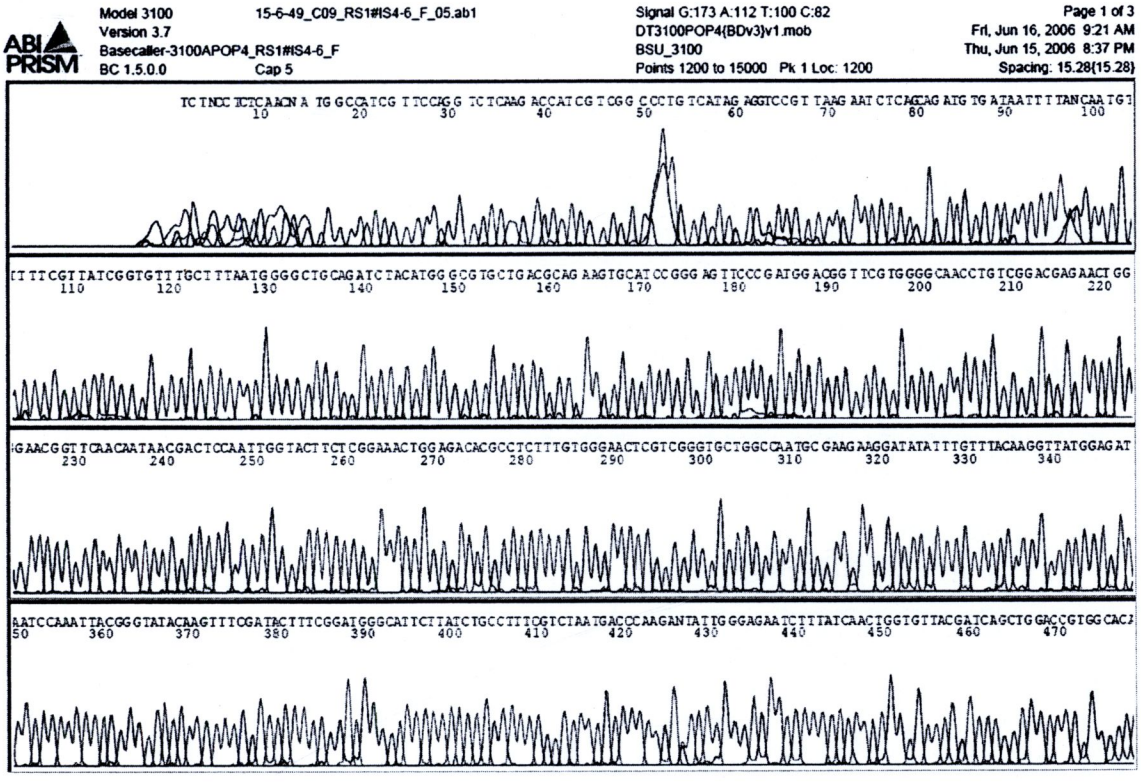
Chromatogram of nucleotide sequences

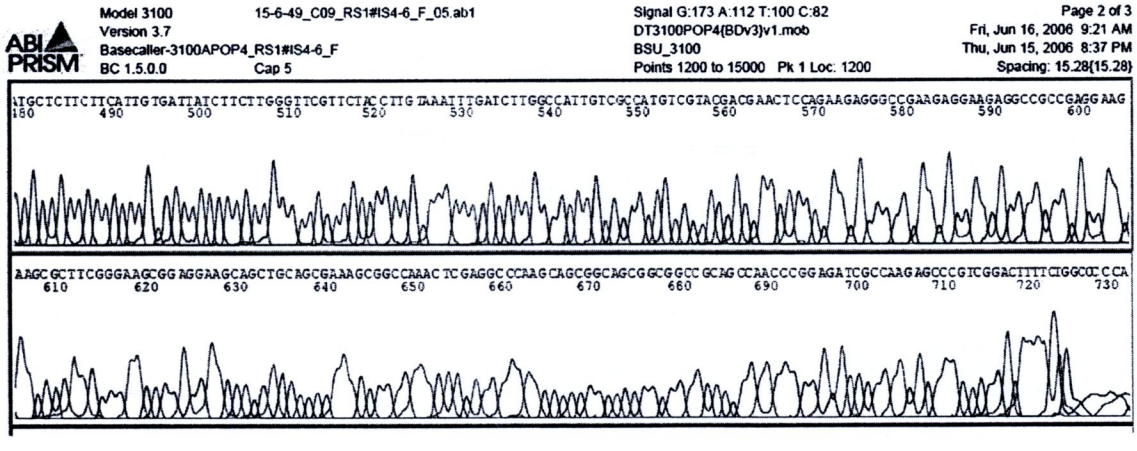
1. Nucleotide sequence of the IS4 - IS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD-R strain



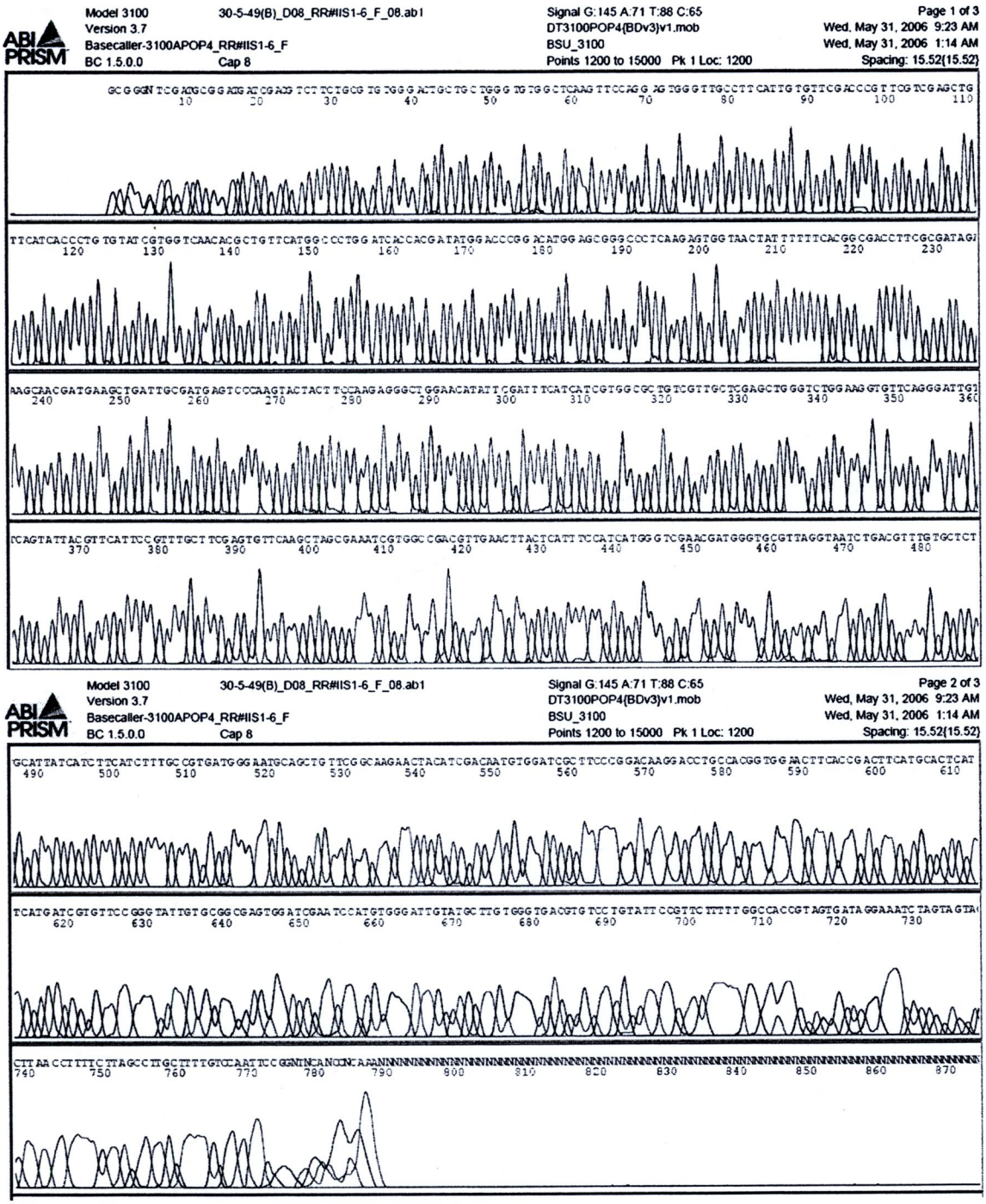


2. Nucleotide sequence of the IS4 - IS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD strain

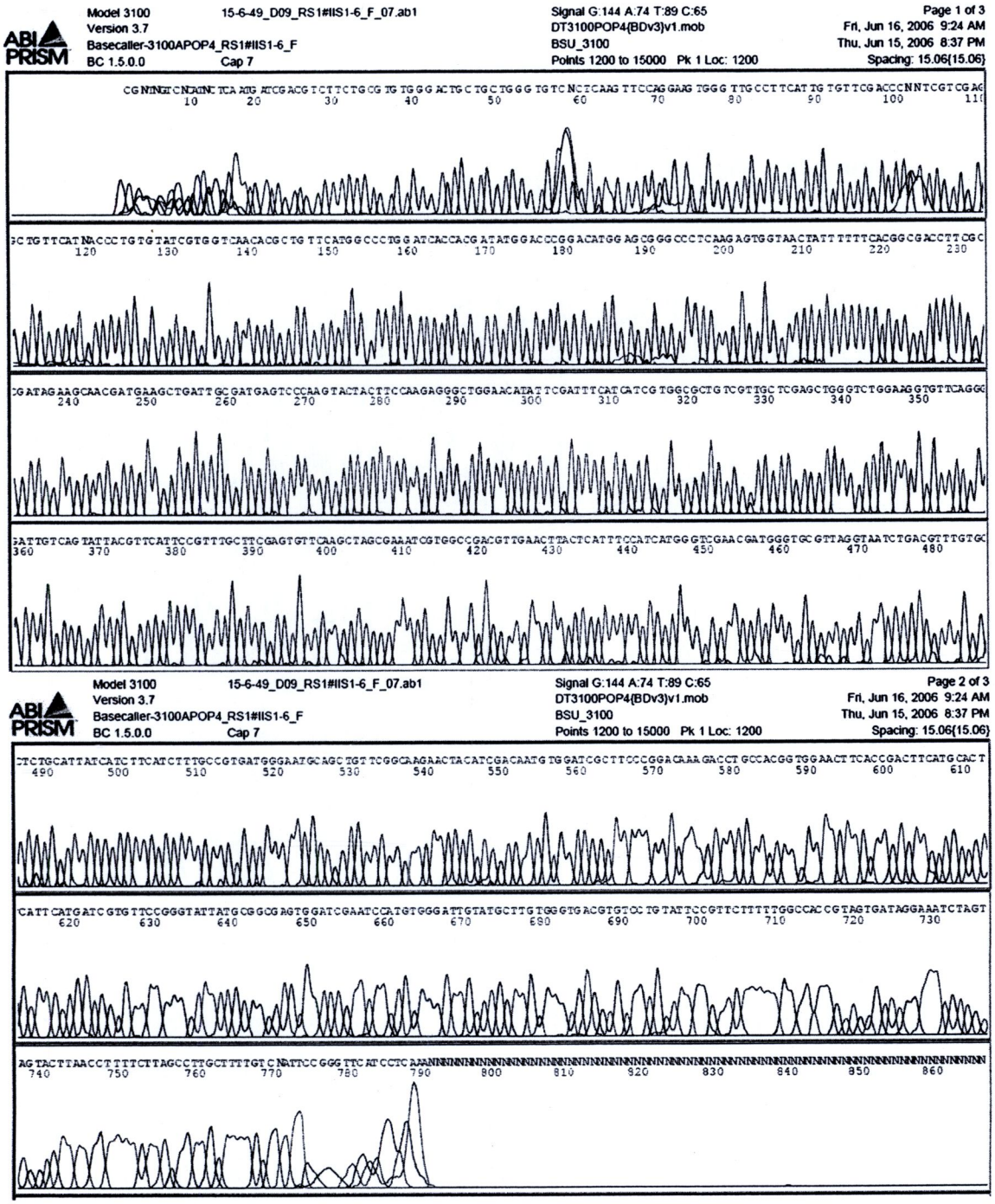




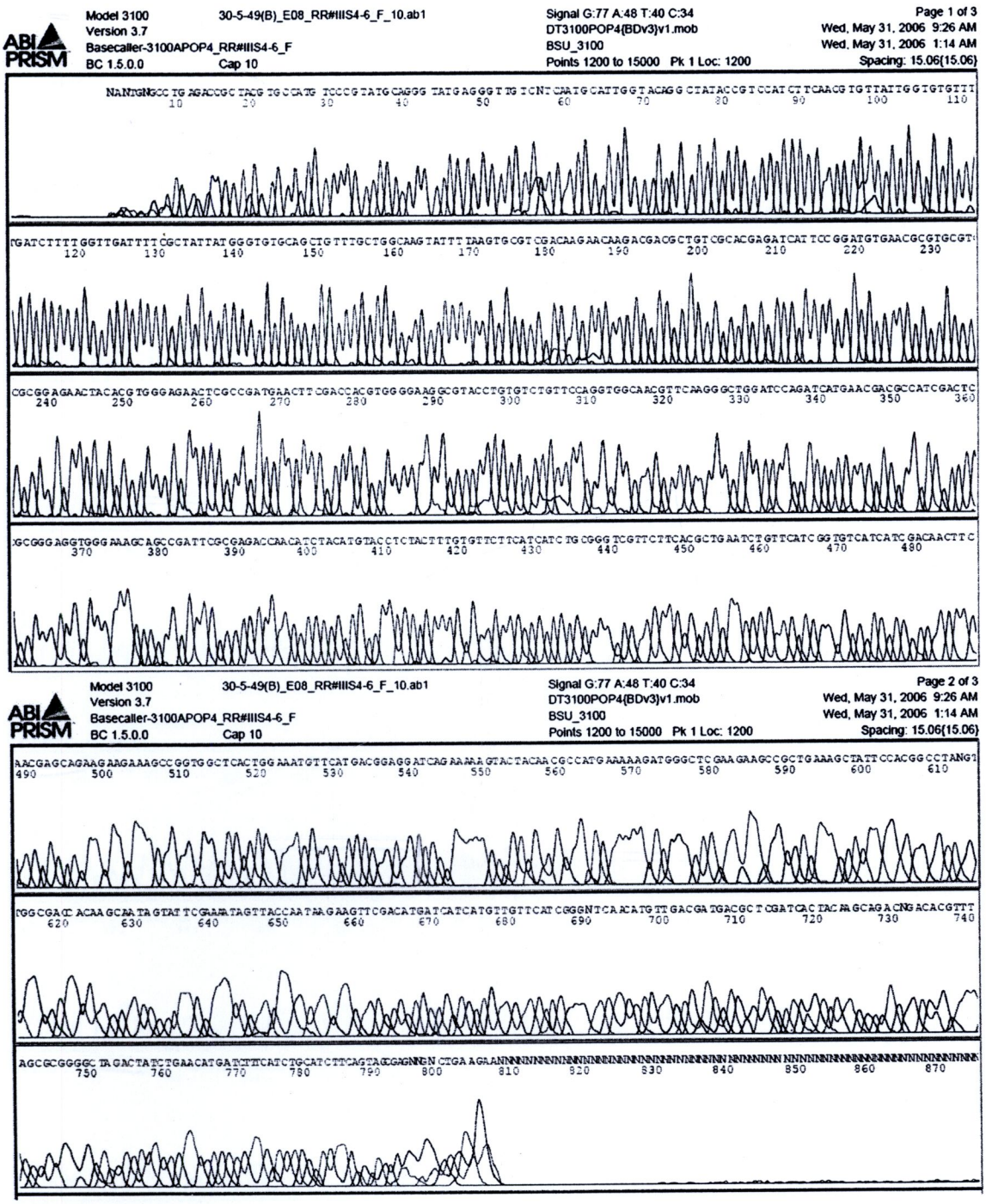
3. Nucleotide sequence of the IIS1-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD-R strain



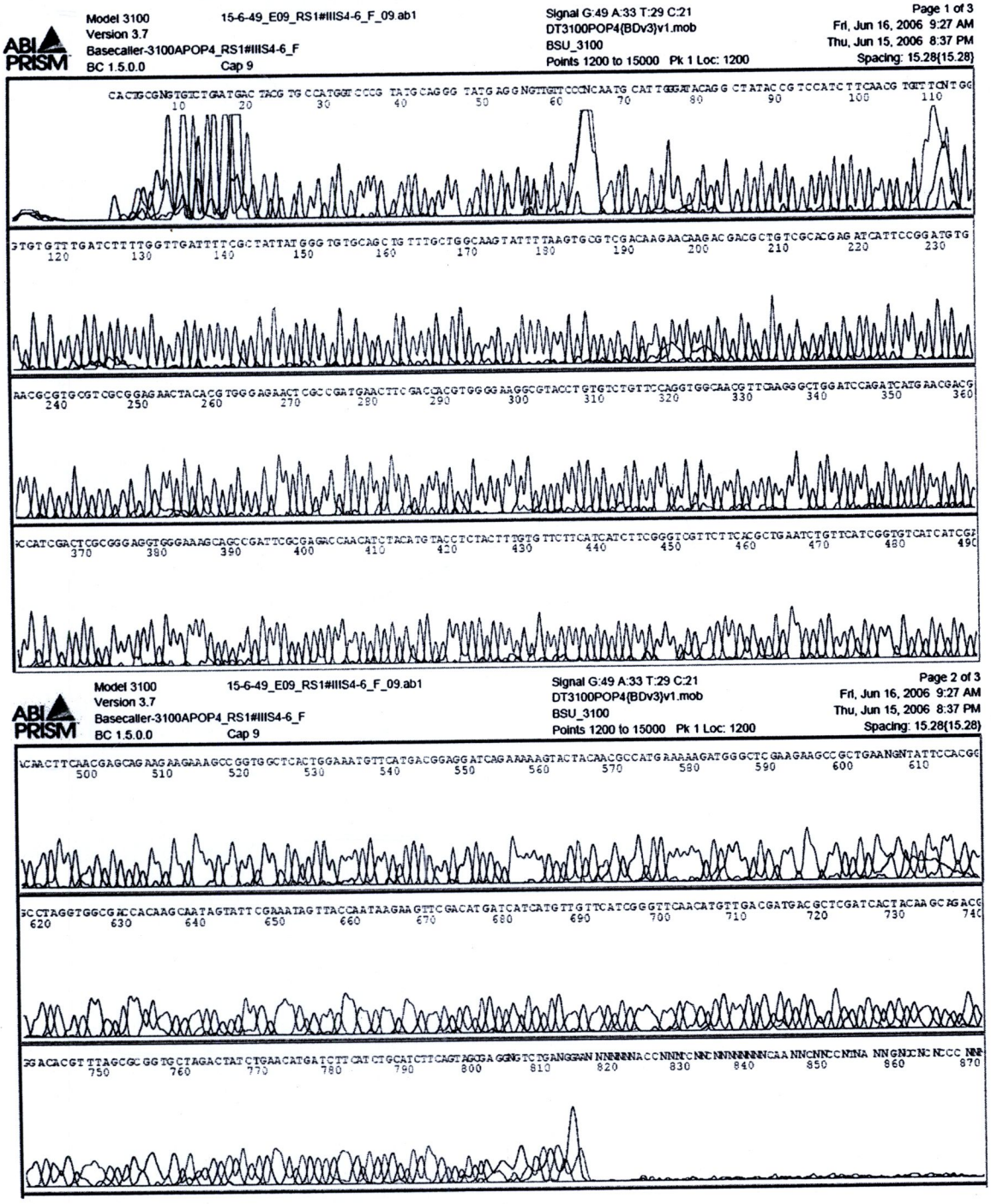
4. Nucleotide sequence of the IIS1-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD strain



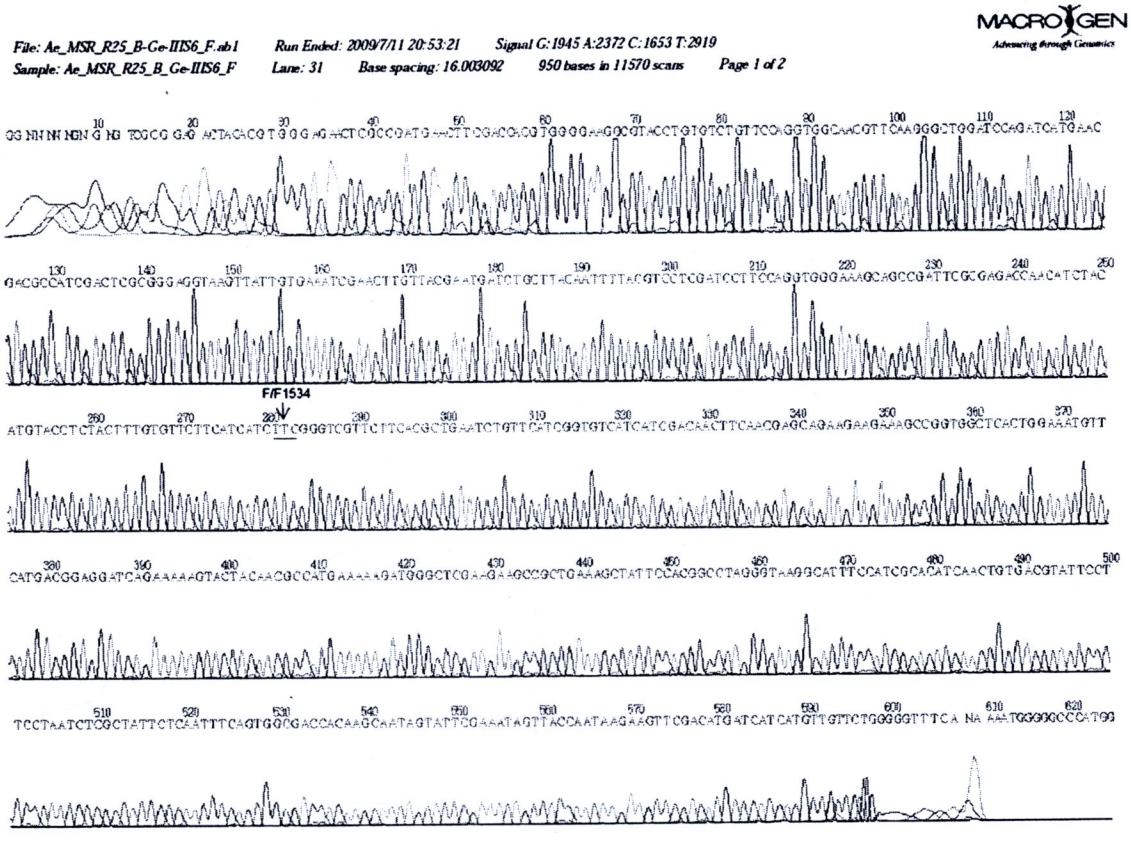
5. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD-R strain



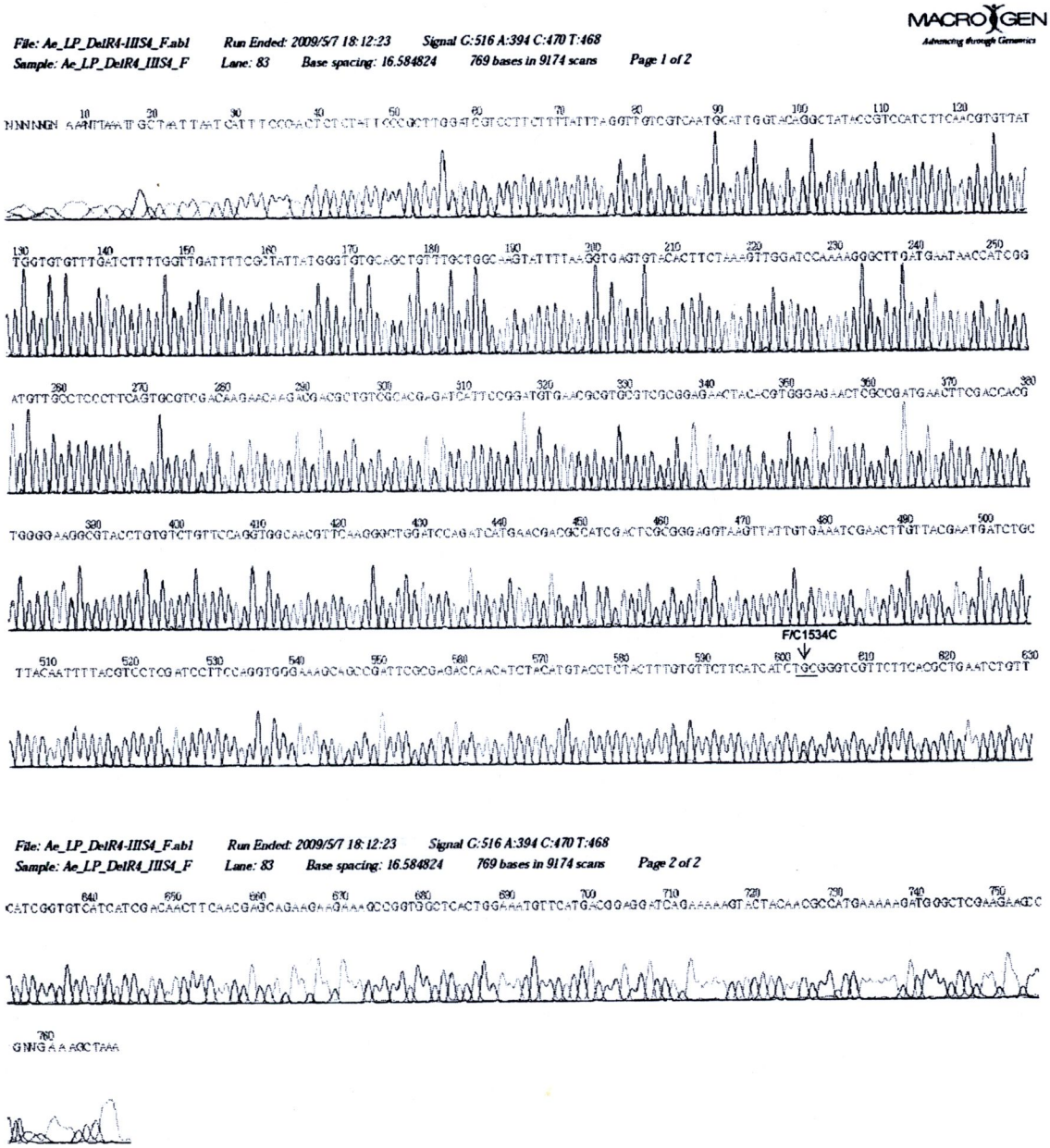
6. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltage-gated sodium channel gene from PMD strain



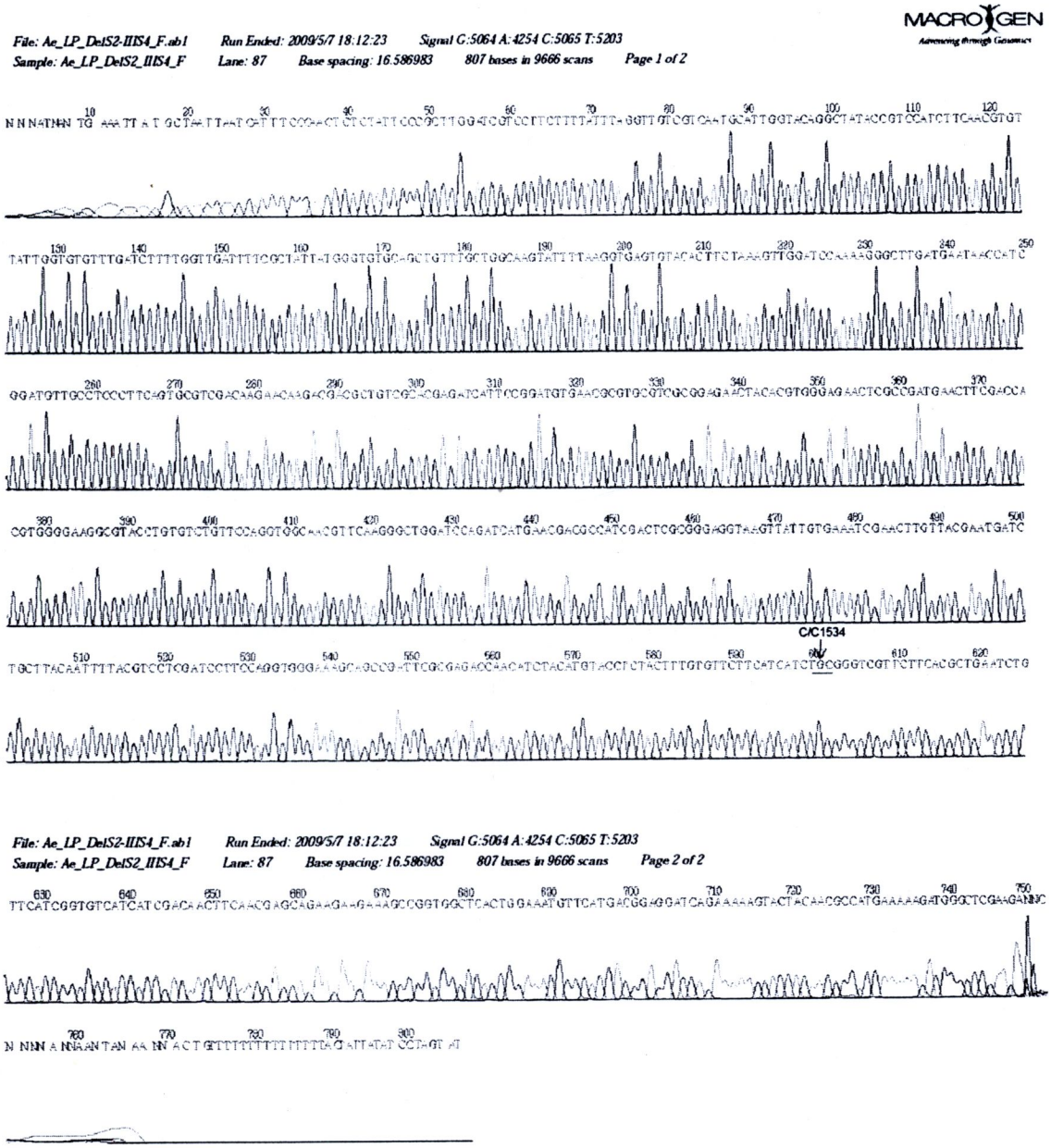
7. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, MSR-R25. The nucleotide chromatogram of homozygous for wild type F/F1534 was shown.



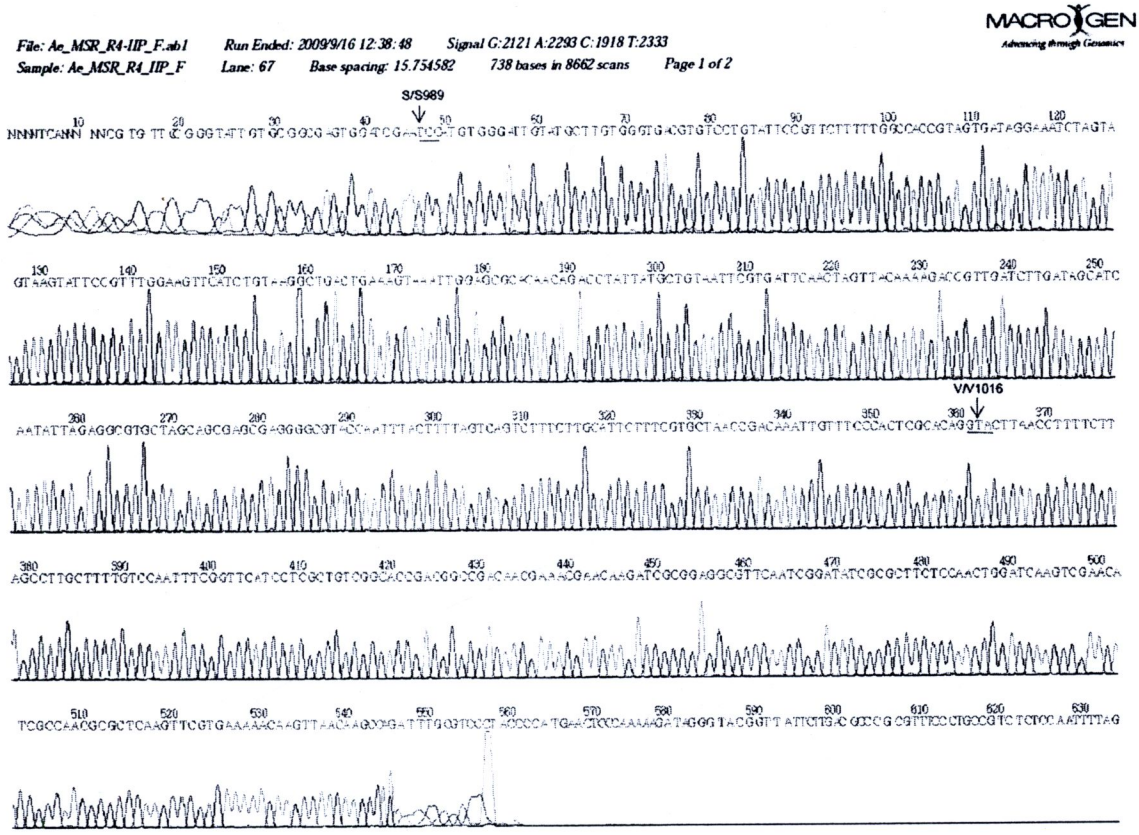
8. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, Ae-LP-DelR4. The nucleotide chromatogram of heterozygous F/C1534 was shown.



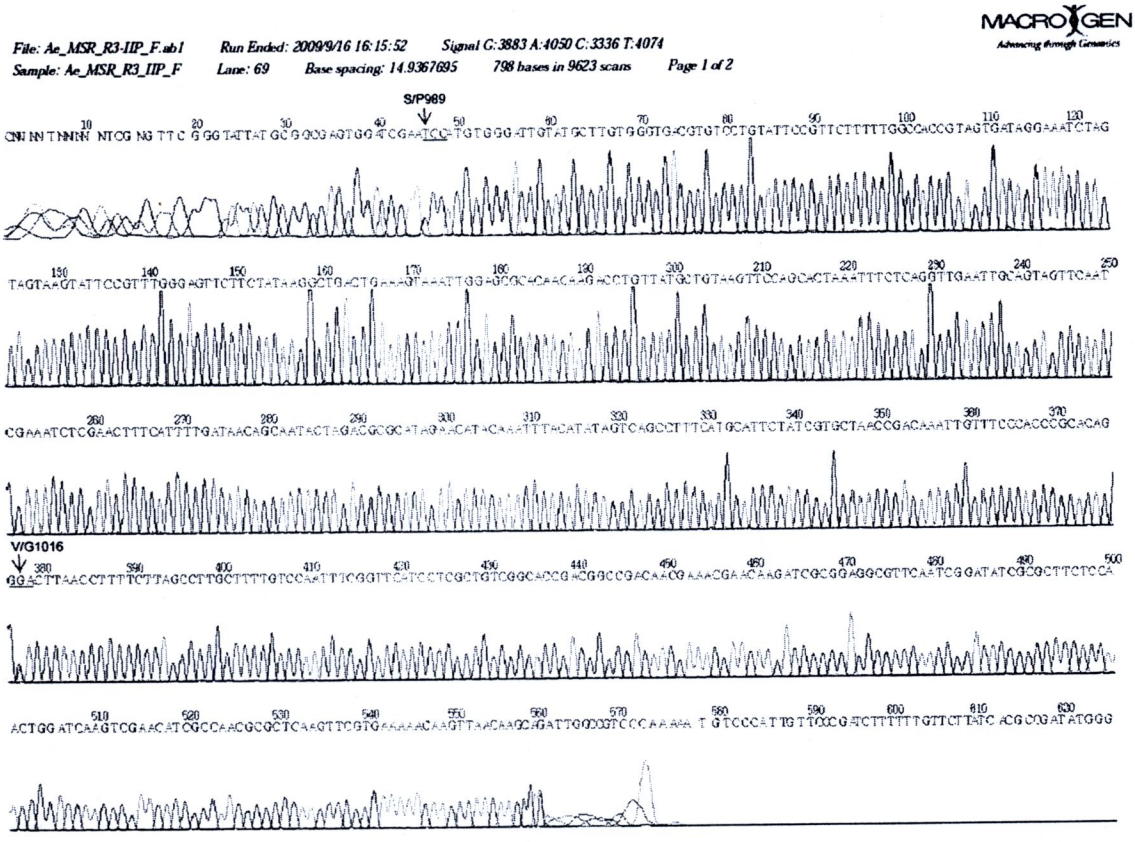
9. Nucleotide sequence of the IIIS4-IVS2 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, Ae-LP-DelS2. The nucleotide chromatogram of homozygous C/C1534 was shown.



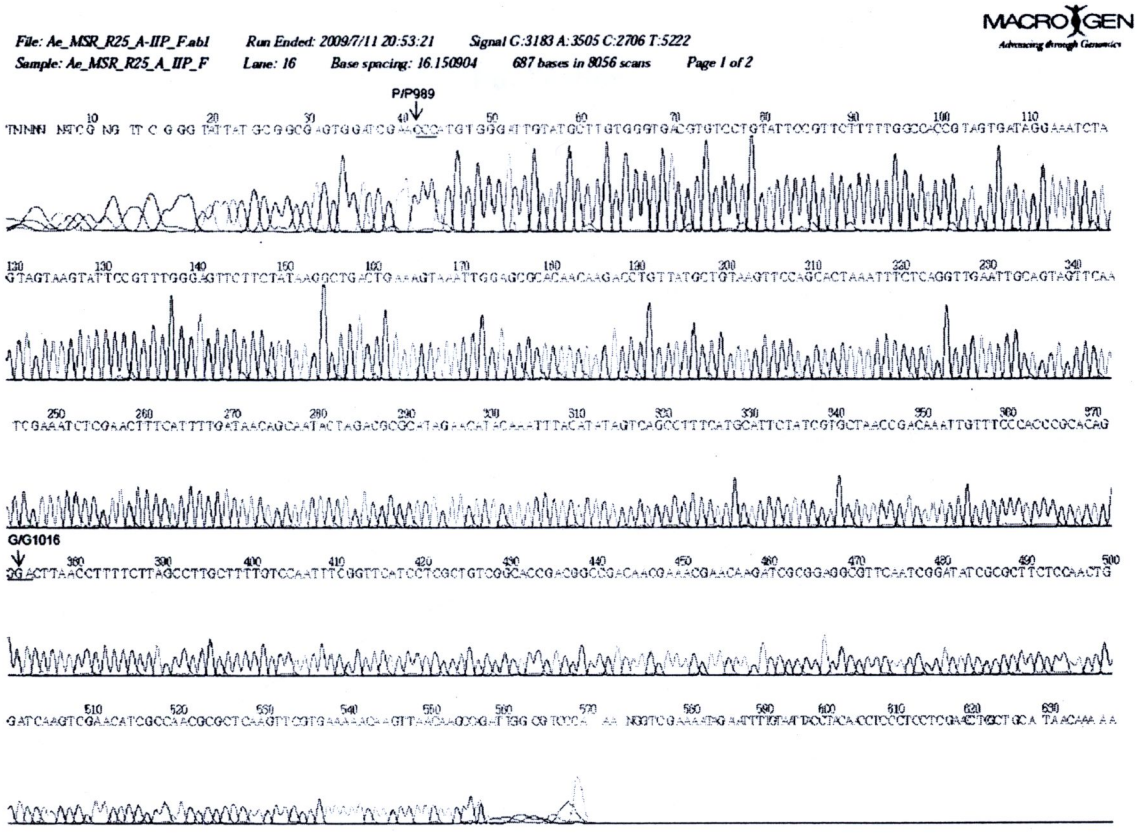
10. Nucleotide sequence of the IIP-IIS6 domains of the *Ae. aegypti* voltage-gated sodium channel gene from field collected strain, Ae-MSR-R4. The nucleotide chromatogram of homozygous for mutant V/V1016 and S/S989 were shown.



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.



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.



APPENDIX D

Publications

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

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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 → 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 F_2 progeny, formed by backcrossing the F_1 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.

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 F_1 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 SuperScript™ 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 μl 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 $MgCl_2$ and 0.5 μM 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-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 ($PMD_{female} \times PMD\text{-}R_{male}$; $PMD\text{-}R_{female} \times PMD_{male}$) to produce F_1 hybrids. F_1 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 LC_{50}s calculated. Both the parental mosquitoes and the F_1 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:

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

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 LC_{50}). 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 voltage-gated sodium channel gene. The amplified region is indicated in Table 1 and Fig. 1. These nucleotide sequences are reported in GenBank (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

Table 1
Sequences of primers for amplifying sodium channel gene fragments.

Primer name	Primer sequence (5′–3′)	Product size (bp)	Region in sodium channel	Exon[a]
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.

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 F_1 hybrids and the F_2 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 LC_{50}. The concentration–mortality line of F_1 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 F_1 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 −0.347 for PMD (M) × PMD-R (F). All these results clearly suggest that permethrin resistance in PMD-R strain is partially recessive. The LC_{50} values of the F_1 progeny obtained from both directions of crosses were similar as were the F_2 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 IIIS6 helix. Position F1552 in the *Ae. aegytpi* sequence is equivalent to F1534 in the housefly *Vssc1* sequence (Genbank Accession No.:

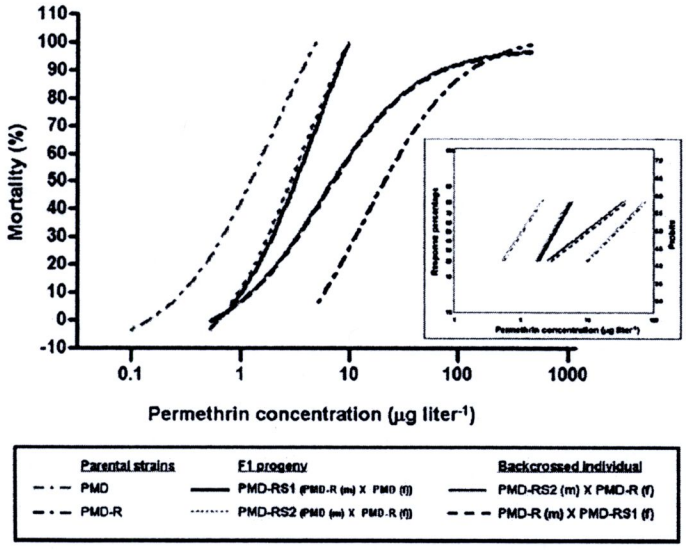

Fig. 2. The log dose–mortality response curves and log dose–probit mortality lines (inset) of the parental strains, F_1 hybrids and backcrossed individuals after exposure to permethrin.

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	*P*
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) × 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) × 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) × PMD-R (m)	8.96 (7.38–10.84)	1.48 (+0.085)	9.18 (n = 7, df = 6)	0.10
PMD-R (f) × 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 (LC_{50}) 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_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.

Acknowledgments

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High-throughput assays for detection of the F1534C 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: target-site 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 μl reaction volume containing 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen), 0.1 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 μM 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.

Table 1 Sequences of oligonucleotides used to amplify fragments of the voltage-gated sodium channel gene, for the TaqMan SNP genotyping assay and for the AS-PCR assay. The long 26-bp GC tail has the sequence 5′-GCGGGCAGGGCGGCGGGGGCGGGGCC-3′ and the short 6-bp GC tail has the sequence 5′-GCGGGC-3′

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.

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,

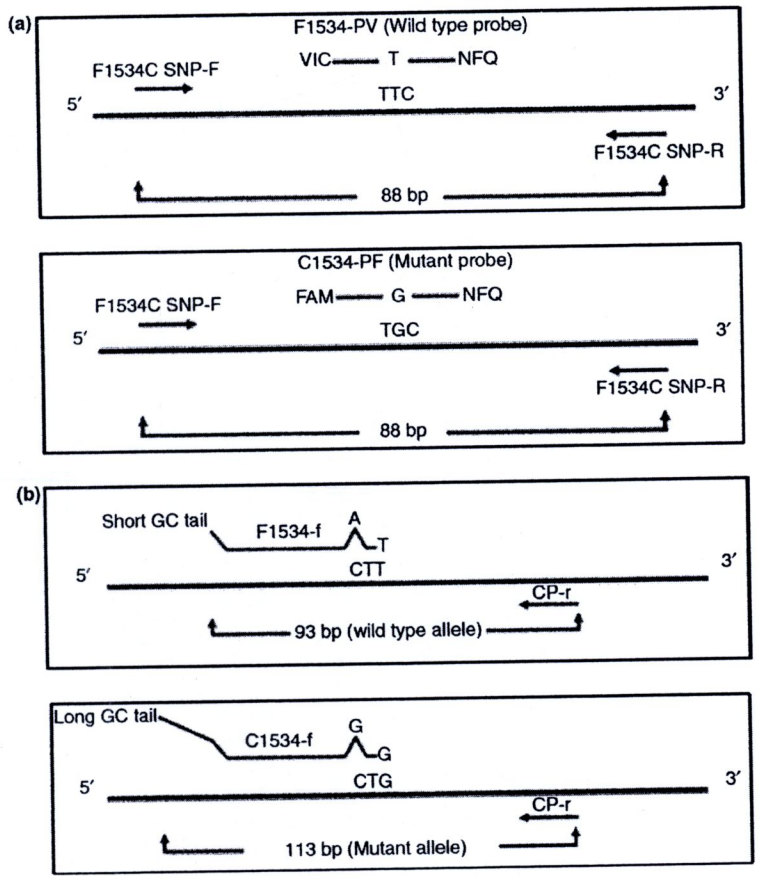

Figure 1 Schematic of the (a) TaqMan SNP Genotyping and (b) the allele specific PCR assays for detection of the F1534C mutation. The primers and probes were designed based on the sequence of voltage-gated sodium channel from the PMD and PMD-R strains of *A. aegypti* (Genbank accession numbers: EU259810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

0.4 μM of each probe and 2 μl of genomic DNA (50 ng) made up to 25 μl 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 μl) contained 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM F1534-f forward primer, 0.165 μM C1534-f forward primer and 0.5 μM 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-day-old 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, homozygous (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 heterozygous (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

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

Strains	TaqMan SNP genotyping/DNA sequencing (no. of samples)				AS-PCR/DNA sequencing (no. of samples)			
	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

Region	Province	Locality	No dead/total (% mortality)	Dead				Survivors			
				No. mosquitoes			Freq. C allele	No. mosquitoes			Freq. C allele
				F/F	F/C	C/C		F/F	F/C	C/C	
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 S989P 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

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.

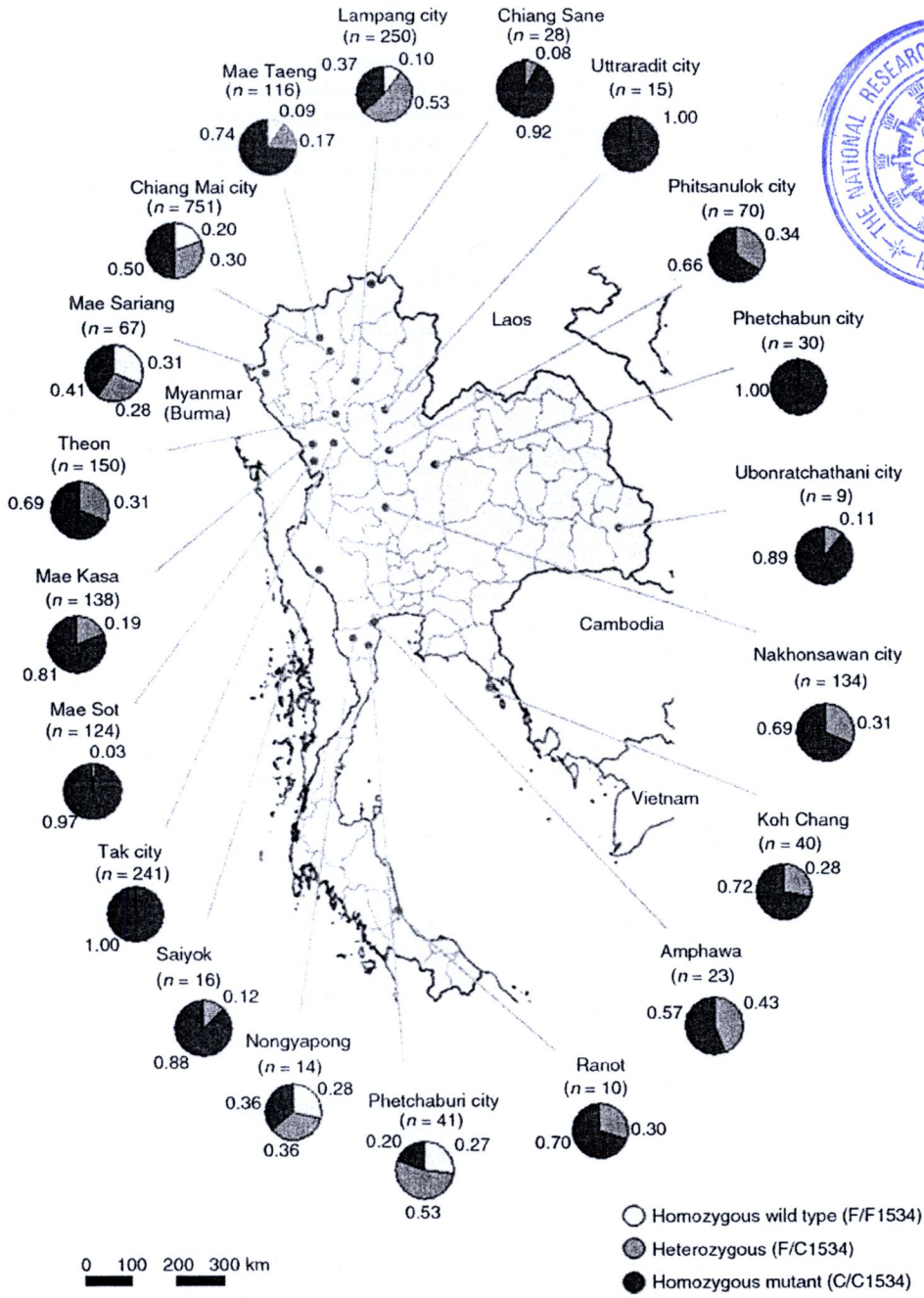

Figure 2 Estimated frequency distributions of the homozygous wild type (F/F1534), heterozygous (F/C1534) and homozygous mutant (C/C1534) genotypes in *A. aegypti* in Thailand. For the populations from Table 3, population genotype frequencies were estimated by combining the total numbers of genotypes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

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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