CHAPTER IV

DISCUSSION

From the screening of three cDNA fragments of the *Ae. aegypti* voltage-gated sodium channel gene spanning 29 putative amino acid substitutions out of the total of 33 amino acid mutations reported in correlation with insecticide resistance (Davies *et al.*, 2007, Soderlund and Knipple, 2003), we discovered a novel amino acid mutation in the permethrin resistance PMD-R strain of *Ae. aegypti.* This mutation is at amino acid position 1534 where Phenylalanine (F) was substituted by Cysteine (C). The numbering of this amino acid position is based on the housefly *Vssc1* sodium channel sequences (Ingle *et al.*, 1996). Later the F1534C mutation in IIIS6 was also found in pyrethroids-resistant *Ae. aegypti* populations from Vietnam (Kawada *et al.*, 2009) and Grand Cayman (Harris *et al.*, 2010).

The putative binding sites in insect voltage-gated sodium channel were identified using homology modeling based on crystal structure of the open potassium channel Kv1.2 (Long *et al.*, 2005). The model predicted that the pyrethroids-binding site in the sodium channel is located within a hydrophobic cavity formed by the transmembrane helices IIS5 and IIIS6, as well as the linker IIS4-IIS5 (O'Reilly *et al.*, 2006). There are nine amino acid mutations located within this putative binding pocket, M918T (or V or I) in the IIS4-S5 linker, L925I, T929I (or V or C), L932F in the IIS5

helix and F1538I in the IIIS6 helix (Davies *et al.*, 2007, Soderlund and Knipple, 2003). The F1534C mutation in IIIS6 is one of amino acid mutation located in the pyrethroids-binding pocket. The interpretation of the molecular model suggests that the role of the amino acid F1534 is important for making aromatic-aromatic contact with the first aromatic ring of the alcohol moiety of pyrethroids (O'Reilly *et al.*, 2006). Moreover, the amino acid F1534 is a highly conserved residue across both arthropod and non arthropod species (Figure 3.12). Therefore the F1534C mutation we observed in *Ae. aegypti* may have disrupted the ability of the channel to interact with pyrethroids.

The involvement of the *kdr* associated mutation in pyrethroids resistance is confirmed by using the *Xenopus* oocyte expression system to examine the sensitivity of the mutant sodium channel to pyrethroids. Ten sodium channel mutation have been confirmed to reduce channel sensitivity to pyrethroids (Dong, 2007). Recently, the F1534C mutation was demonstrated to reduce the sensitivity of cockroach sodium channel to type I pyrethroids (i.e. permethrin, bioresemthrin and NRDC157) but not to type II pyrethroids (i.e. cypermethrin, deltamethrin and cyhalothrin) (Hu *et al.*, 2011). Therefore, this study confirmed the involvement of the F1534C mutation in permethrin resistance. Both type I and type II pyrethroids prolong the opening of the sodium channels resulting in a slow decaying sodium channel tail current associated with repolarization (Lund and Narahashi, 1981, Vijverberg *et al.*, 1982). However, type II pyrethroids alter gating kinetics more drastically than type I pyrethroids and cause much more slowly decaying tail current upon repolarization.

Most studies of sodium channel mutations have focused on the M918T and L1014F mutations in domain II of the sodium channel gene since the *kdr* and *super-kdr* strains of housefly were found to have these point mutations in association with a resistant phenotype (Williamson *et al.*, 1996). 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* pyrethroids resistant strains (Davies *et al.*, 2007). Several sodium channel mutations have been identified in pyrethroids resistant *Ae. aegypti* from various countries (Brengues *et al.*, 2003, Chang *et al.*, 2009, Saavedra-Rodriguez *et al.*, 2007). Two of these were the S989P and V1016G mutations within IIP and IIS6 regions of sodium channel gene in mosquito samples from Thailand (Brengues *et al.*, 2003, Srisawat *et al.*, 2010). However, they did not examine the IIIS6 region.

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 (Shono *et al.*, 2002), a predatory mite *Amblyseius fallacis* (Thistlewood *et al.*, 1995) and a southern cattle tick *Boophilus microplus* (Li *et al.*, 2008). 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 (Prapanthadara *et al.*, 2002). In this study, we found the homozygous mutations at F1534C is highly associated with permethrin resistance and probably plays a major role in the resistant characteristics of PMD-R.

The heterozygous mutation in our crossing experiments showed a resistant level (LC_{50}) of about 3 times higher than the susceptible strain (Table 3.2), but lower than the logalithmic average of the susceptible and resistant parental strains. A question arises whether the heterozygous mutation alone can maintain this resistance level. The study by adding synergist piperonyl butoxide (PBO), an inhibitor of oxidase, in larval bioassays showed the F1 hybrid strains with heterozygous mutation (F/C1534) was decrease in permethrin resistance levels being close to the susceptible strain with homozygous wild type (F/F1534) (Figure 3.15), suggesting that a metabolic mechanism, such as mixed function oxidases also may be involved in resistance. Our backcrossing experiment does not support monogenic control of resistance. In addition, the slopes of the concentration-mortality lines of backcross progeny (Table 3.1) were decreased compared with the parental strains and F1 hybrid progeny, indicating that a number of unlinked genes contribute to resistance (Georghiou, 1969).

Recently, the larvae bioassay by adding the PBO and BNPP (bis(4-nitrophenyl)-phosphate) showed that the LC_{50} of PMD and PMD-R strains were reduce about twofold and threefold, respectively for the oxidase inhibitor (PBO) but little or no effect of esterase inhibitor (BNPP) was observed in both strains (Somwang *et al.*, 2011). Somwang *et al.* (2011) also demonstrated the enzymatic involvement of ADH, ALDH, and particularly, P450s in permethrin resistance pathway by using enzymes extracted from the PMD and PMD-R strains.

We have successfully 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 have 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) has developed their tetraplex PCR assay to detect the F1534C mutation in *Ae. 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 *Ae. 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 *Ae. aegypti* in the neighboring countries of Myanmar (Yangon city) and Cambodia (Battambang town) (7/8 alleles and 20/20 genotyped alleles, respectively), indicating that this resistance mutation is widespread in Southeast Asia.

Since the 1534C allele is recessive (Harris *et al.*, 2010 and this study), only homozygous mutants were expected to be alive after a 1 hour exposure to the discriminating dose (0.25% permethrin) for adult *Ae. aegypti* set by the WHO (1998).

However, not all homozygous mutant mosquitoes survived throughout the test (Table 3.4), 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 (Pethuan *et al.*, 2007, Somwang *et al.*, 2011, Yaicharoen *et al.*, 2005) has revealed that cytochrome P450 monooxygenases and other oxidative enzymes are involved in pyrethroids resistance in *Ae. aegypti* populations in Thailand. Studies in *An. gambiae s.s.* have 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 pyrethroids and/or DDT resistance phenotype in *An. gambiae s.s.* (Chandre *et al.*, 2000, Martinez-Torres *et al.*, 1998, Ranson *et al.*, 2000) and *Ae. 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 3.4) due to the V1016G and S989P mutations in domain II. The V1016G mutation was originally discovered in a permethrin resistant strain of *Ae. 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 *Ae. aegypti* collected from Vietnam (Kawada *et al.*, 2009) and Taiwan (Chang *et al.*, 2009). The combination of the V1016G and S989P mutations was demonstrated in

deltalmethrin-resistant Khu Bua strain of *Ae. aegypti* (Srisawat *et al.*, 2010). We also obseved this combination in permethrin resistance of *Ae. aegypti* from field populations (Table 3.5). Moreover, we identified the co-existence of both mutations in domain II and the F1534C mutation in domain III. However, the correlation between the mutations in domain II and the F1534C mutation in resistance need further study.