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NAME: Mr. Chatchai Tananchai

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR
(Professor Theeraphap Chareonviriyaphap, Ph.D.)

THESIS CO-ADVISOR
(Assistant Professor John Paul Grieco, Ph.D.)

DEPARTMENT HEAD
(Professor Theeraphap Chareonviriyaphap, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON

DEAN
(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

BITING PATTERNS OF *ANOPHELES DIRUS* AND *ANOPHELES BAIMAII* AND RESPONSE TO INSECTICIDES BY *ANOPHELES DIRUS*

CHATCHAI TANANCHAI

A Thesis Submitted in Partial Fulfillment of
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Chatchai Tananchai 2012: Biting Patterns of *Anopheles dirus* and *Anopheles baimaii* and Response to Insecticides by *Anopheles dirus*. Master of Science (Entomology), Major Field: Entomology, Department of Entomology. Thesis Advisor: Professor Theeraphap Chareonviriphap, Ph.D. 85 pages.

Two species from the Dirus complex, *Anopheles dirus* and *Anopheles baimaii,* were surveyed from Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand between September 2009 and August 2010. A total of 598 *An. dirus* (91.2%) and 58 *An. baimaii* (8.8%) was identified. The trophic behavior, host preference and seasonal abundance were determined for both *An. dirus* and *An. baimaii.* Both species demonstrated exophagic and zoophilic activities. Outdoor landing catches by *An. dirus* occurred between 2300 and 2400 hr, and indoor landing showed activity between 1900 and 2000 hr. whereas *An. baimaii* presented two peaks in indoor human landing, between 1900 and 2000 hr and between 0200 and 0300 hr, outdoor human landing activity peaks showed between midnight and 0100 hr. Significantly greater numbers of *An. dirus* were collected from cattle compared to humans ($P < 0.05$). Both species were also more abundant during the wet season rather than the dry and hot seasons.

In addition, the behavioral response of wild-caught populations of *An. dirus* to the operational field dose of three synthetic pyrethroids (0.025 g a.i./m^2 of bifenthrin, 0.03 g a.i./m^2 of alpha-cypermethrin and 0.02 g a.i./m^2 of lambda-cyhalothrin) was evaluated for contact and noncontact actions using an exito-repellency escape chamber. DEET was used as the repellent standard for comparison with the other three synthetic pyrethroids. Result showed that test populations rapidly escaped from direct contact with treated surfaces for each of the three synthetic pyrethroids and DEET. Alpha-cypermethrin (88.70%) demonstrated the strongest irritant action, followed by DEET (80.05%) and lambda-cyhalothrin (72.49%). Fewer mosquitoes escaped from noncontact treatment chambers as compared contact trials but a significant escape response compared to the matched controls ($P<0.05$) was achieved. I conclude that a better understanding of the behavioral responses of vectors to various chemicals will allow for greater efficiency in program design for targeting appropriate vectors.

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Chatchai Tananchai
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LIST OF ABBREVIATIONS

An. = Anopheles
KD = Knockdown
MOPH = Ministry of Public Health
P = Probability Value
χ^2 = Chi-Square
WHO = World Health Organization
% = Percent
RH = Relative Humidity
°C = Degree(S) Celsius
cm = Centimeter
ml = Millilitre
mM = Millimolar
min = Minute
μl = Microlitre
hr = Hour
sec = Second
α = Alpha
λ = Lambda

BITING PATTERNS OF *ANOPHELES DIRUS* AND *ANOPHELES BAIMAII* AND RESPONSE TO INSECTICIDES BY *ANOPHELES DIRUS*

INTRODUCTION

Many people live in tropical and subtropical areas of the world are at risk of infection from a wide variety of vector-borne diseases, most notably malaria. Globally, between 100-300 million people live in malaria endemic areas (World Health Organization (WHO), 2009). Southeast Asia accounts for 30% of the global malaria morbidity and about 8% of the global mortality, with approximately 26,000 deaths reported per year (WHO, 2007b), where the four human malaria parasites (*Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale and Plasmodium malariae*) are transmitted by mosquitoes in the genus *Anopheles* (Service and Townson, 2002). From a total of 76 *Anopheles* species, three species complex are major vectors of malaria in Thailand, including *Anopheles dirus* complex, *Anopheles minimus* complex and *Anopheles maculatus* complex (Chareonviriyaphap *et al*., 2000). Members of the *Anopheles dirus* complex occur primarily in forested foothills and deep forest areas. The *An. minimus* complex is widespread in the hilly forested regions and *An. maculatus* complex be found at the margins of hilly forest zones and in rubber-plantation areas. The members of the *An. dirus* complex, however, serve as the most efficient malaria vectors (Rattanarithikul *et al.,* 2006; Manguin *et al.,* 2008; Singha and Chandra, 2011).

Anopheles dirus is comprised of a complex of mosquito species known as important vectors of malaria throughout the Southeast Asia. This vector taxon contains at least seven morphologically similar species in the Oriental Region (Peyton, 1989; Sawadipanich *et al.,* 1990), including *Anopheles dirus* sensu stricto (former *An. dirus* A), *Anopheles cracens* Sallum (species B), *Anopheles scanloni* Sallum (species C), *Anopheles baimaii* (species D), *Anopheles nemophilous* (species F), *Anopheles elegans* (species E) and *Anopheles takasagoensis* Morishita. Within this complex, two species, *An. baimaii* and *An. dirus* are major vectors for human malaria transmission in Thailand (Manguin *et al.,* 2008; Sungvornyothin *et al.,* 2009; Singha and Chandra, 2011). *Anopheles dirus* is a common species found throughout Thailand, while *An. baimaii* is found along the western regions of Thailand (Sallum *et al.,* 2005). These two species are closely related and are strongly associated with forested and hilly environments along western areas of the Thai–Myanmar border (Baimai *et al.,* 1988; Rattanarithikul *et al.*, 1995, 2006; Manguin *et al.,* 2008). Both *An. baimaii* and *An. dirus* have been proven extremely difficult to control due to a diverse array of host seeking behaviors and preferences, biting patterns and larval breeding habitats (Pates and Curtis, 2005; Sinka *et al.,* 2011).

Both insecticide residual spray (IRS) and insecticide-treated nets (ITN) are common control methods for malaria vectors in Thailand. However, one of the most effective IRS chemicals (DDT), has been banned from further use in Thailand. DDT has gradually been replaced by other more toxic pyrethroids (Chareonviriyaphap *et al.,* 2000). From the beginning, synthetic pyrethroids have been used primarily for IRS as well as for treated fabric material used in bed-nets and curtains (Chareonviriyaphap *et al.,* 2004). Synthetic pyrethroids elicit a repellent response for many insect species and cause mosquitoes to move away from sprayed surfaces (Lockwood *et al.,* 1984; Miller, 1990; Lindsay *et al.,* 1991). The extensive and continued use of pyrethroids should act as a stimulus to increase attention on the impact these chemicals have on the behavior of the mosquito vectors. The use of IRS in homes has severed as a major means of controlling malaria transmission, yet little is known of the precise role irritant and repellent actions have on specific malaria vectors. Behavioral responses can be separated into two types of actions, contact irritancy and noncontact repellency (Roberts *et al.,* 1997). An irritant response occurs after physical contact with a chemically treated surface, while repellency is an avoidance response without physical contact with the chemical (Chareonviriyaphap *et al.,* 1997; Roberts *et al.,* 1997; Pothikasikorn *et al.,* 2005). Although behavioral responses have been recorded with various species of *Anopheles* from Thailand using the excito-repellency test box, none have observed these responses in wild caught *An.*

dirus when exposed to recommended field doses of bifenthrin, alpha-cypermethrin, lambda-cyhalothrin and DEET.

A better understanding of the biology, ecology and behavior of sibling species is critically important to help identify their respective roles in disease transmission and to help the vector control personnel to design the appropriate steps for vector management. Various studies on biting cycle and host preference of these complexes have been described in Thailand (Scanlon and Sandhinand, 1965; Wilkinson *et al.,* 1970; Baimai *et al.,* 1988c; Rosenberg *et al.,* 1990; Rattanarithikul *et al.,* 1996b; Sungvornyothin *et al.,* 2009). Only the phenotypic wing patterns are used to identify between species yet these features are not enough. Until recently, use of morphological features in combination with molecular techniques have served as the standard for separating members of this complex (Jaichapor *et al.*, 2005; Sungvornyothin *et al.,* 2006; Manguin *et al.,* 2008). Currently, several molecular identification using polymerase-chain reaction (PCR) based methods for identifying members in the Dirus complex have been developed (Walton *et al.,* 1999, 1999a; Huong *et al.,* 2001; Manguin *et al.,* 2002). This method is a simple and rapid multiplex allele-specific PCR technique (ASPCR) and has been used to identify species in this taxon (Walton *et al.,* 1999, 2000). The AS-PCR method can easily distinguish members of the Dirus complex, as well as closely related species that occur in tropical and subtropical areas.

OBJECTIVES

1. To define the biting patterns and seasonal abundance of *Anopheles dirus* and *Anopheles baimaii* in western Thailand.

2. To characterize the behavioral responses of *Anopheles dirus* to the recommended field application rate of three synthetic pyrethroids (bifenthrin, alpha-cypermethrin, and lambda-cyhalothrin) as compared with DEET.

LITERATURE REVIEW

1. Malaria in Thailand

Malaria represents a substantial health and development problem in the Southeast Asia (SEA). In this region, approximately 1.3 billion people (77% of the total population) are at risk for transmission with an estimated 90-160 million infections and around 100-120 thousand deaths occurring each year. Around 33% are at moderate to high risk of malaria and 67% are at low risk of malaria. During 2000-2008 in the SEA, the annual malaria incidence remained between the range of 2.16 million and 2.83 million.

In Thailand, the incidence of malaria has been significantly reduced. The disease is prevalent along the undeveloped borders of eastern Myanmar, western Cambodia and northern Malaysia (Chareonviriyaphap *et al.,* 2000; Ministry of Public Health (MOPH), 2009). The current status of malaria in Thailand is represent in Figure 1 and Table 1. More than 70% of the malaria cases occur along the Thai-Myanmar border. The reason malaria remains high in some locations is a consequence of the low economic conditions, uncontrolled population movements and unstable political climate found along international boundaries (MOPH, 2009). Based on the malaria surveillance activities in Thailand from 2005-2011 (Table 1), reported cases of malaria peaked during 2007 with 25,193 and 5,696 cases in Thai and non-Thai populations, respectively. Since this time, the number of cases has declined to 9,306 and 5,536 cases in 2011, in Thai and non-Thai populations, respectively. In addition, more malaria cases occur in Thailand during the rainy season (especially from May to June) (Figure 2). Malaria is transmitted by the bite of an infective anopheline mosquito with the primary malaria vectors being found in both the Minimus and Dirus complexes as well as the *An. maculatus* group (Rattanarithikul *et al*., 1996).

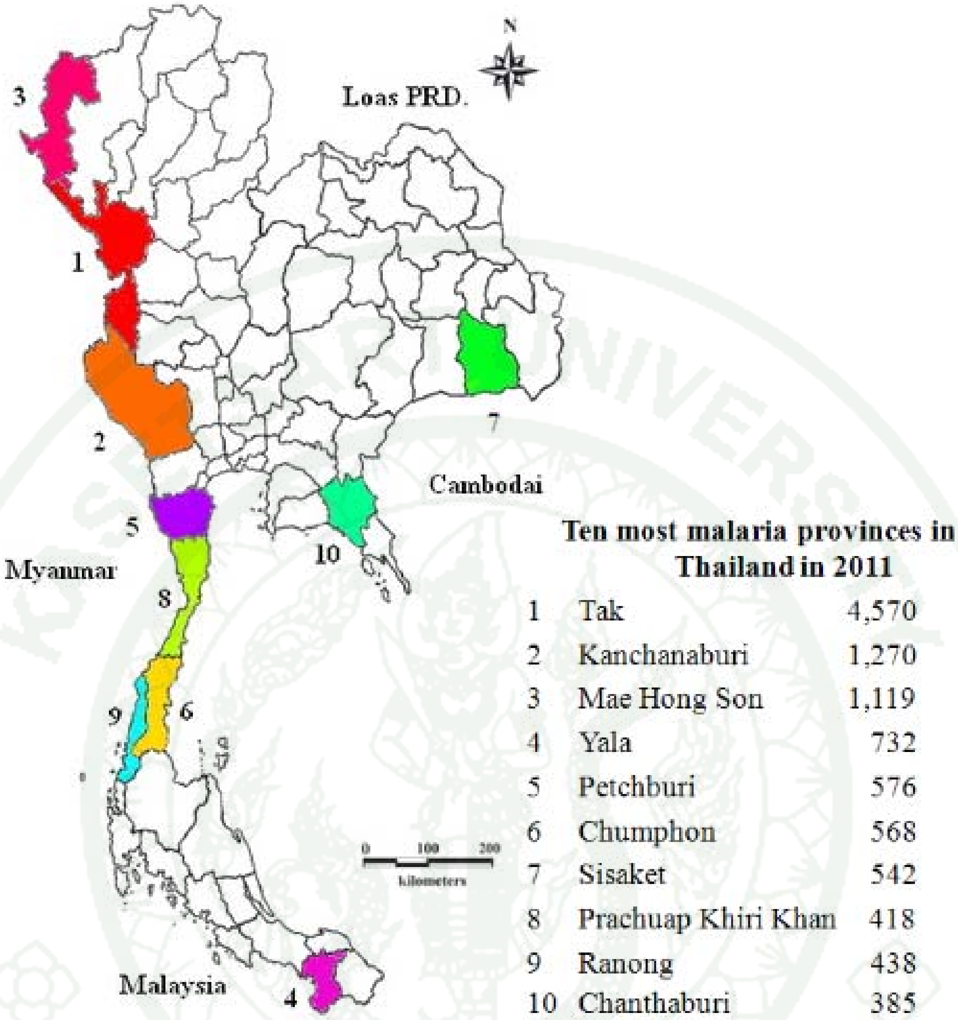
Table 1 Malaria surveillance in Thailand from 2005-2011

Year	Total of population	No. of malaria cases		Morbidity rate	Mortality rate	CFR[2] (%)
		Thai	Non-Thai			
2005	62,195,878	21334	6797	45.23	0.11	0.25
2006	62,623,416	22151	6811	46.25	0.08	0.18
2007	62,933,515	25193	5696	49.08	0.06	0.12
2008	63,214,022	21729	7173	45.72	0.06	0.12
2009	63,457,439	16390	6839	36.61	0.03	0.08
2010	63,701,703	18202	7437	40.25	0.05	0.13
2011[1]	63,525,062	9306	5536	23.36	0.01	0.05

Source : Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health 2011

[1] Preliminary report (January – September 2011) (MOPH 2011)

[2] Case fatality rate



Ten most malaria provinces in Thailand in 2011

1	Tak	4,570
2	Kanchanaburi	1,270
3	Mae Hong Son	1,119
4	Yala	732
5	Petchburi	576
6	Chumphon	568
7	Sisaket	542
8	Prachuap Khiri Khan	418
9	Ranong	438
10	Chanthaburi	385

Figure 1 Map of Thailand, depicting the general distribution of the ten most malarious provinces in Thailand, 2011 (Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health)

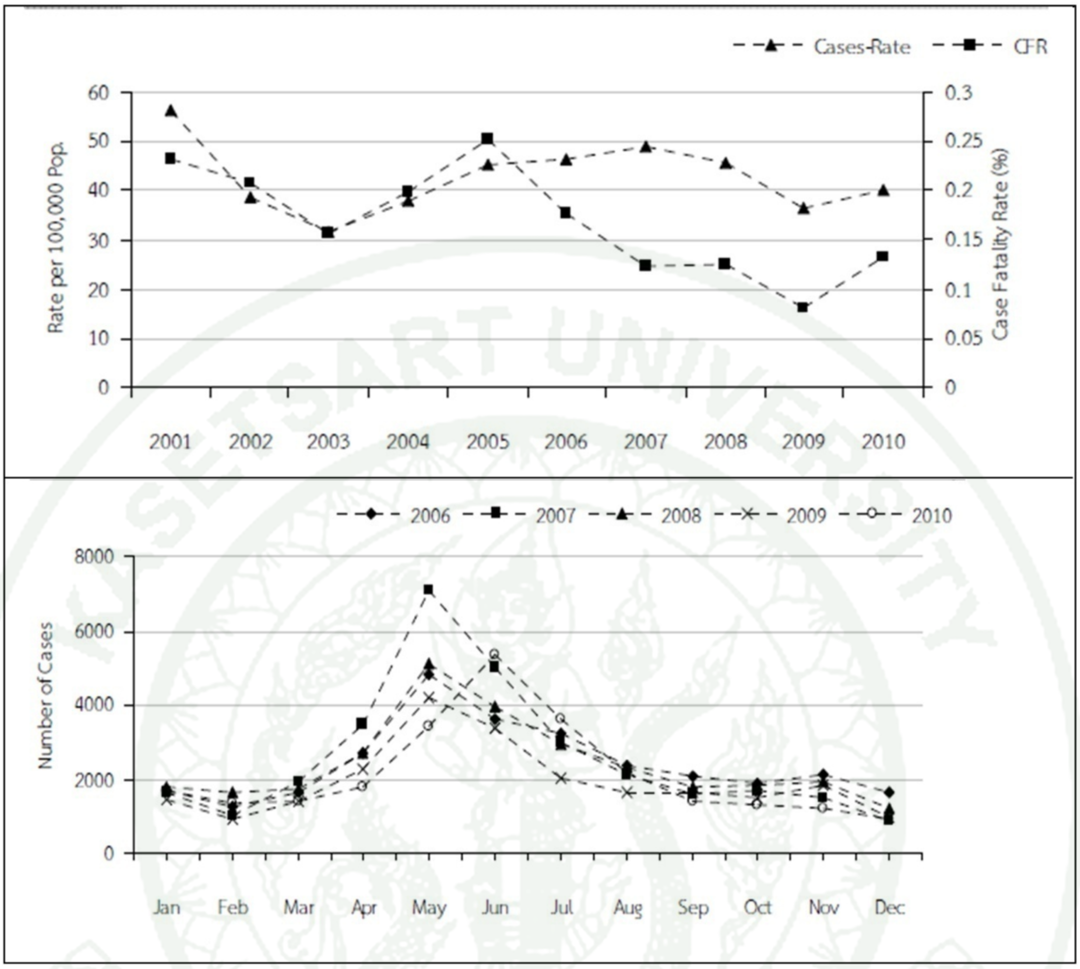


Figure 2 Malaria cases per 100,000 population and case fatality rates for Thailand from 2001-2010 (upper) and cases of malaria by month for Thailand from 2006-2010 (lower) (MOPH 2011)

2. Malaria vectors in Thailand

Seventy-six *Anopheles* species are recognized in Thailand and represent two groups (the Leucosphyrus group (Neomyzomyia series) and the Maculatus group (Neocellia series)) and one subgroup (the Minimus subgroup (Mysomyia series)). The primary malaria vectors are comprised of *An. baimaii* (Green *et al.,* 1991) and *An. dirus* (Rosenberg *et al.,* 1990) in the *An. dirus* complex, *An. minimus* (Rattanarithikul*et al.,* 1996a) in the *An. minimus* complex and *An. maculatus* and *An. pseudowillmori* (Green *et al.,* 1991) in the *An. maculatus* complex.

The *Anopheles minimus* Theobald complex is made up of at least three sibling species, *Anopheles minimus* (species A), *Anopheles harrisoni* (species C) and *Anopheles yaeyamaensis* (species E). Two of these species, *An. minimus* and *An. harrisoni,* are present in Thailand but only *An. minimus* is considered to be major malaria vector in Thailand (Rattanarithikul *et al.,* 2006; Manguin *et al.,* 2008). *Anopheles minimus* can be found throughout Thailand. *Anopheles harrisoni,* however, appears restricted to the western Thai-Myanmar border and occurs in sympatry with *An. minimus*. *Anopheles yaeyamaensis* is found up to the Ishigaki Island of the Ryukyu Archipelago, Japan (Harbach, 2004; Somboom *et al*., 2005; Garros *et al*., 2006; Sungvornyothin *et al*., 2006b).

The *Anopheles maculatus* complex contains at least eight closely related species; *Anopheles maculatus* s.s. Theobald, *Anopheles sawadwongporni* Rattanarithikul and Green, *Anopheles dravidicus* Christophers, *Anopheles notanandai* Rattanarithikul and Green, *Anopheles willmori* (James), *Anopheles psuedowillmori* (Theobald) (Green *et al.,* 1985; Rattanarithikul and Green, 1986; Rattanarithikul and Harbach, 1990; Kittayapong *et al.,* 1990; Green *et al.,* 1992) have been reported from Thailand and *Anopheles dispar* and *Anopheles greeni* (Rattanarithikul and Harbach, 1990) have been reported from the Philippines. Two of these species*, An. pseudowillmori* and *An. maculatus,* have been incriminated as either primary or secondary malaria vectors in Thailand (Green *et al.,* 1991, 1992; Rattanarithikul *et al.,* 1996a). *Anopheles sawadwongporni* and *An. maculatus* are relatively common species often found in high density throughout Thailand, especially along the border regions with Myanmar and Malaysia, whereas *An. pseudowillmori* and *An. willmori* are considered secondary vectors and are found in northwestern Thailand along the Myanmar border (Green *et al*., 1991, 1992b).

The *Anopheles dirus* complex contains at least seven species; *An. dirus* Peyton & Harrison sensu stricto (former *An. dirus* A), *An. cracens* Sallum and Peyton (species B), *An. scanloni* Sallum and Peyton (species C), *An. baimaii* Sallum and Peyton (species D), *An. nemophilous* Peyton & Ramalingam (species F), *An. elegans* James (species E) and *An. takasagoensis* Morishita. Five species within the Dirus complex are present in Thailand. Of these, four species, *An. dirus* s.s., *An. cracens*, *An. scanloni* and *An. baimaii,* are regarded as malaria vectors with infectivity rate ranging between 0.3% and 13% (Rosenberg, 1982; Peyton, 1989; Sallum *et al.,* 2005). Only *An. baimaii* and *An. dirus* are considered to be important malaria vectors in Thailand (Rattanarithikul *et al.,* 2006; Manguin *et al.,* 2008). *Anopheles dirus* has a wide distribution in Eastern Asia and it is known to occur along the Thai-Myanmar border extending eastward across Thailand to Laos, Vietnam, Cambodia and Hainan Island (China) (Rosenberg and Maheswary, 1982; Tun-Lin *et al.,* 1985; Baimai *et al.,* 1988a). *Anopheles cracens* is known from southern (peninsular) Thailand, peninsular Malaysia, and Sumatra (Indonesia) (Manguin *et al.,* 2008). *Anopheles scanloni* occurs in a small foci along the borders of southern Myanmar and western and southern Thailand. *Anopheles baimaii* is found across southwestern China (Yunnan Province), western Thailand, Myanmar, and extends from Bangladesh to northeastern India and the Andaman Islands (India) (Sallum *et al.,* 2005). *Anopheles elegans* is widespread in the hilly forests of southwestern India (Sawadipanich *et al.,* 1990). *Anopheles nemophilous* has a patchy distribution along the Thai–Malay peninsula and Thai border areas with Myanmar and Cambodia. Finally, *An. takasagoensis* is restricted to Taiwan (Peyton & Harrison, 1980). Published field data have indicated that *An. dirus* and *An. baimaii* are closely related species and strongly associated with forested, forest fringe, and hilly environments along western areas of the Thai–Myanmar border, especially in Kanchanaburi and Tak Provinces (Baimai *et al.,* 1988; Rattanarithikul *et al.,* 1995, 2006; Manguin *et al.,* 2008).

3. Behavioral response to insecticides and vector control

Malaria vector control is intended to protect individuals against infective mosquito bites and, at the community level, to reduce the intensity of local malaria transmission. The two most powerful and most broadly applied interventions are ITN and IRS to control malaria transmission (Chareonviriyaphap *et al.,* 2000; WHO, 2009). IRS with DDT proved to be successful in field trials prompting DDT to become the insecticide of choice for malaria control programs from1950 to 1970 (WHO, 2002). In Thailand, the use of DDT was supported by various international donor agencies and was first used in Chiang Mai from 1949-1951. Although, DDT is a good insecticide for vector control, Thailand gradually discontinued its use in the mid 90's because of the changing human perception to spraying and the implied adverse long-term impact on the environment. The use of DDT for vector control has slowly been replaced with synthetic pyrethroids (Chareonviriyaphap *et al.,* 2000).

Synthetic pyrethoids are currently the insecticide of choice for malaria control programs in Thailand. Pyrethroids have shown great promise for controlling pest populations due to their relatively low mammalian toxicity, broad spectrum of efficacy in controlling indoor mosquito populations, and afford ability (Elliott *et al.,* 1978; Roberts and Andre, 1994; Hemingway and Ranson, 2000). Synthetic pyrethroids elicit a repellent response from many insect species and cause mosquitoes to move away from sprayed areas (Lockwood *et al.,* 1984; Miller, 1990; Lindsay *et al.,* 1991). The extensive and continued use of pyrethroids should act as a stimulus to increased attention on the impact these chemicals have on the behavior of the mosquito vectors. The use of IRS and ITNs in homes has severed as a major means of controlling malaria transmission.

Behavioral responses or "insecticide avoidance" can be separated into two types of behavioral actions, contact irritancy and noncontact repellency (Roberts *et al.,* 1997). Irritant responses occurs when an insect moves away from an insecticide after direct physical contact with the chemical residue, whereas repellent actions result from an insect detecting chemicals from a distance and avoiding the treated surfaces before making physical contact (Roberts *et al.,* 1997; Potikasikorn *et al.,* 2005; Tanasinchayakul *et al*., 2006).

Bifenthrin

Bifenthrin (IUPAC 2-methylbiphenyl-3-ylmethyl (*Z*)-(1*RS*, 3*RS*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate] is a third-generation synthetic pyrethroid insecticide. This chemical is characterized by greater photostability and insecticidal activity as compared with previous pyrethroids (Mokry and Hoagland, 1989). Bifenthrin affects the central peripheral nervous system of insects causing paralysis (Miller and Salgado, 1985) and acts on sodium channels at the nerve cell endings to depolarize the pre-synaptic terminals. It has also been shown to affect cellular ATP-ase production. Bifenthrin is a non-alpha cyano pyrethroid insecticide and has been used as an acaricide in orchards, nurseries, and homes. Because it exhibits low mammalian toxicity and does not cause irritation after dermal application, bifenthrin is considered a good potential candidate for treatment of mosquito nets.

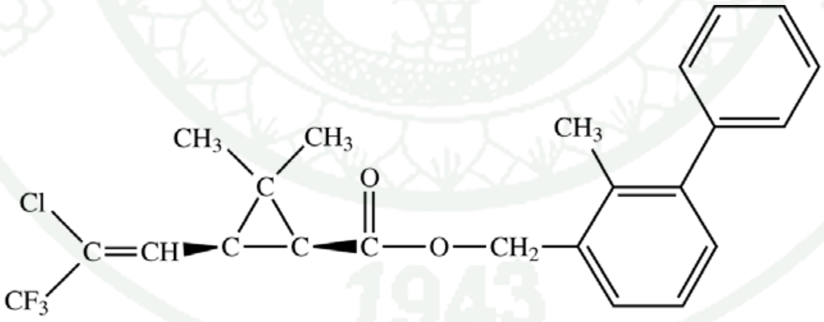


Figure 3 Chemical structure of bifenthrin

Source : Department of Health and Ageing (2007)

Alpha-cypermethrin

Alpha-cypermethrin ((*R*)-cyano(3-phenoxyphenyl) methyl (1*S*,3*S*)-*rel*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate) is a non-systemic, contact toxicant. It consists of the active isomer of the synthetic phyrethroid insecticide cypermethrin and is highly effective against a broad spectrum of insects. It acts by preventing transmission of nerve impulses, by blocking the passage of sodium ions through channels in nerve membranes, thus preventing the axon from transmitting signals. Typically this intoxication results in a rapid "knockdown" and mortality. Alpha-cypermethrin has been used to control mosquitoes, flies and other insect pests in both human and animal dwellings (WHO, 2007).

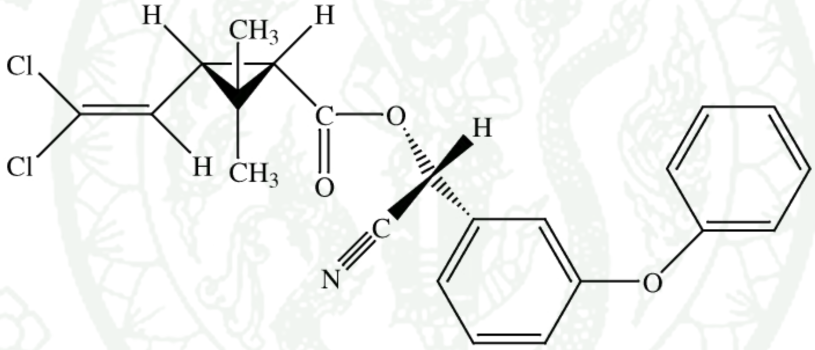


Figure 4 Chemical structure of alpha-cypermethrin

Source : Department of Health and Ageing (2007)

Lambda-cyhalothrin

Lambda-cyhalothrin (Cyano-3-phenoxybenzyl (1s+1r)-cis-3-(z-2-chloro-3, 3, 3-trifluoroprop-1-enyl)-2, 2-dimethyl cyclopropanecarboxylate) is a synthetic pyrethroid insecticide and acaricide used to control a wide range of pests in a variety of applications. Cyhalothrin act on ion channels within the nerve cells (neurons) to disrupt proper function of the cells of both the peripheral and central nervous systems. At lower doses, this may take the form of stable, repetitive firing of the neuron, but at high doses may result in depolarization of the nerve cell and blockage of signal conduction. These effects may result in observable signs such as: tingling, burning or numbness sensations (particularly at the point of skin contact); tremors, in coordination of movement, paralysis or other disrupted motor function; and confusion or loss of consciousness.

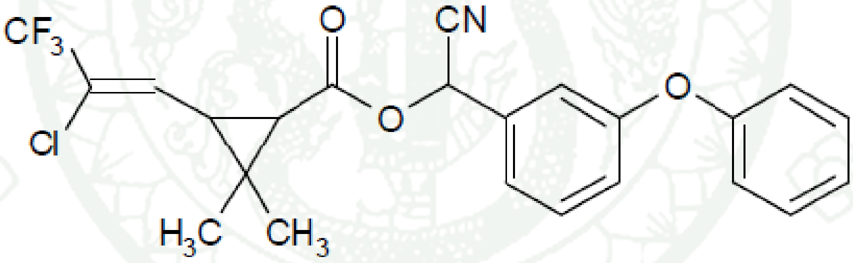


Figure 5 Chemical structure of lambda-cyhalothrin

Source : WHO (2003)

DEET

DEET (*N,N*-diethyl-m-toluamide or *N,N*- diethyl-3-methylbenzamide) is the most common active ingredient in commercially available insect repellents. DEET was first registered in the U.S. in 1957 after first being developed by the U.S. Army in 1946 for use by military personnel in insect-infested areas. DEET has been shown to inhibit odor-evoked currents (activation) mediated by the insect odorant receptor complex (Ditzen *et al.,* 2008), effectively inhibiting perception of host odors and chemo-attractant cues. DEET has shown to prevent bites from mosquitoes, ticks, fleas, biting flies and chigger mites.

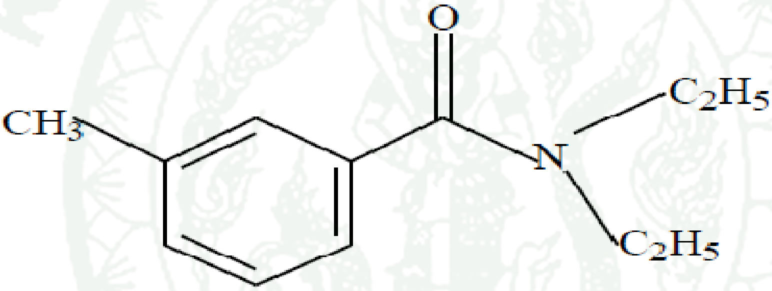


Figure 6 Chemical structure of DEET

Source : Zeiger (1999)

4. Identification of species complex

The term species complex covers morphologically similar sibling species which can only be differentiated using infertility, cytogenetic, biochemical or molecular criteria. Each sibling species within a given complex will have its own ecology and behavior patterns and degree of efficiency as a vector. Most vector species are considered as complexes. In Southeast Asia, *An. balabacensis* was first distinguished from *An. leucosphyrus*, and then divided into three different species, comprised of *An. balabacensis*, *An. dirus* and *An. takasagoensis*. The following are also considered as species complexes in the Oriental region; *An. minimus*, *An. fluviatitis*, *An. calicifacies*, *An. philippinensis*, *An. maculatus* and *An. sundicus*.

Morphological identifications of *Anopheles dirus*

In the *An. dirus* complex, both of *An. baimaii* and *An. dirus* are considered to be important malaria vectors and they are also sympatric species. One morphological characteristic can be used to differentiate between two species. *Anopheles dirus* presents with a presector dark spot (PSD) on the radius vein that extends basally beyond the PSD spot on the costa vein and a PSD spot that reaches the humeral dark (HD) spot of the costa vein or at least surpasses the middle of the presector pale spot (PSP) of the costa vein of at least one wing. *Anopheles baimaii* presents with a presector dark spot (PSD) on the radius vein usually at level of PSD spot on costa vein or that extends only slingtly basally and usually no more than to the middle of presector pale spot (PSP) of costa vein (Figure 12).

Molecular identifications of *Anopheles dirus*

Several previous techniques have been used for the identification of members of the *Anopheles dirus* complex. These include by specific differences of the polytene and mitotic chromosomes (Baimai, 1988; Baimai *et al.,* 1988a,c,d; Sawadipanich *et al.,* 1990). Four of these isomorphic species (*An. dirus*, *An. cracens*, *An. scanloni* and *An. baimaii*) can be separated by using isozyme electrophoresis (Green *et al.,* 1992), DNA probes (Panyim *et al.,* 1988), non-radio- active DNA hybridization (Audtho *et al.,* 1995), rDNA- PCR of ITS2 (Xu *et al.,* 1998) and by PCR-RFLP (Yasothornsrikul *et al.,* 1988). However, all of these methods have major disadvantages that preclude their large-scale application (Walton *et al.,* 1999a), because they require specific technical skills (cytotaxonomy), frozen materials (isozymes), a large amount of DNA (three to five mosquitoes per well for PCR-RFLP), or they allow the identification of only one, two or three species (DNA probes, non-radio- active DNA hybridization, rDNA- PCR of ITS2). Currently, several molecular methods, such as Allele-specific polymerase chain reaction (ASPCR), sequence characterized amplified region (SCAR) and rapid polymerase chain reaction have been developed to detect fixed differences in the ITS2 of the rDNA and are considered useful tools to help identify the species within the Dirus complex (Walton *et al.,* 1999; Huong *et al.,* 2001; Manguin *et al.,* 2002).

MATERIALS AND METHODS

1. Blood feeding activity and seasonal abundance of *Anopheles dirus* and *Anopheles baimaii* (Diptera: Culicidae) in western Thailand

1.1 Study Area

This study was conducted in Pu Teuy Village, a village located in Sai Yok District, Kanchanaburi Province, western Thailand (14° 17'N, 99° 11'E). The collection site is in a small valley surrounded by deep forest and lies approximately 400 m above sea level. The occupations of local residents are linked closely with the forest. There is a narrow, slow running stream (2 m in width at an average 0.5 m depth) that is bordered by native vegetation. Surrounding vegetation is primarily agricultural land and dense secondary forest (Sungvornyothin *et al.,* 2009) (Figure 7). This area is known as a potential site for *An. dirus* and other malaria vectors.

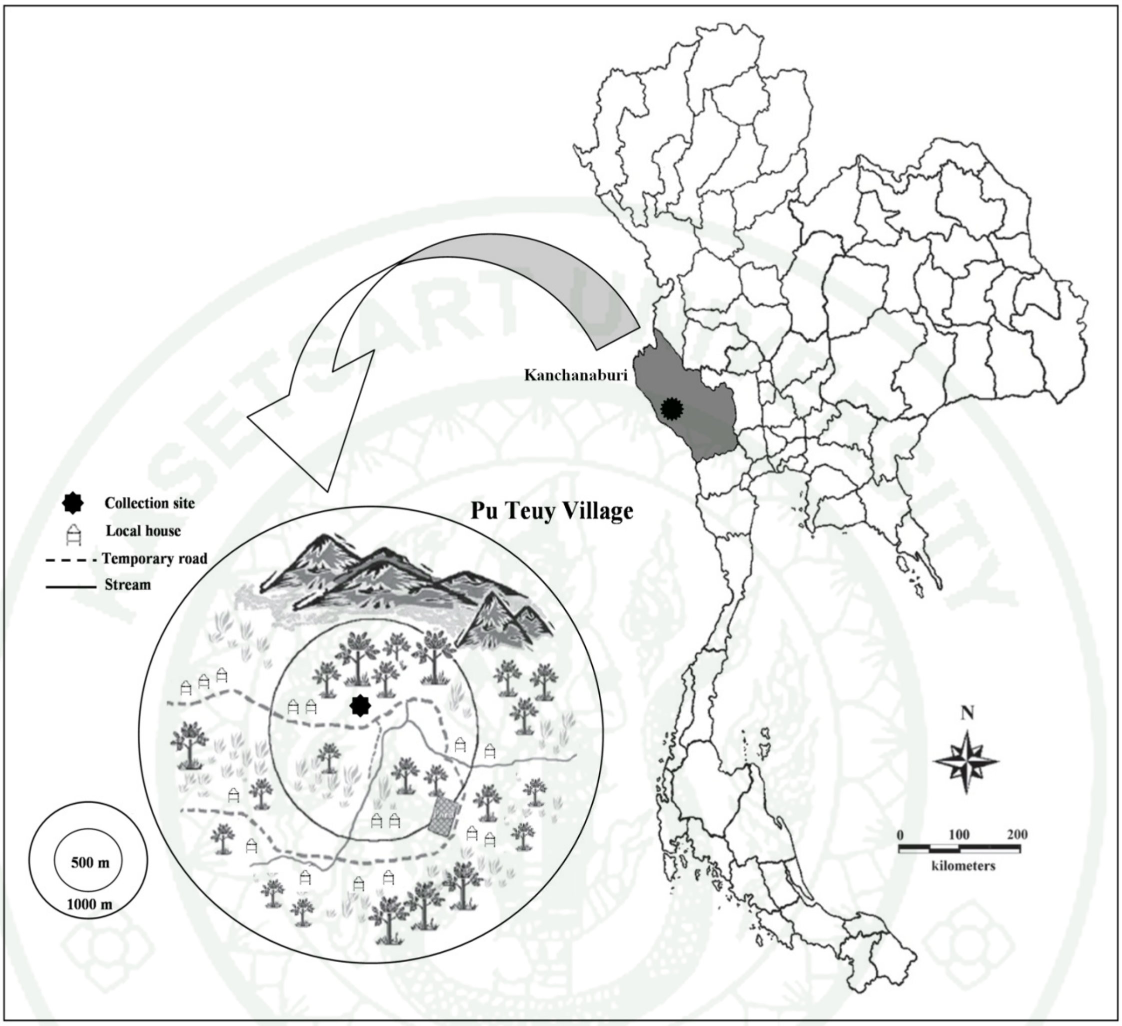


Figure 7 Collection site of Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand

1.2 Adult Mosquito Collections

Adult female mosquitoes were collected from human landing collections and cattle bait for two consecutive nights per month beginning from 1800 to 0600 hr, from September 2009 to August 2010.

Each night, three collection methods were apply, to include human landing indoor (HLI), human landing outdoor (HLO), and cattle bait collections (CBC) from 1800 to 0600 hr (Figures 9-11). Eight human hosts were divided into two groups, each group comprised of four persons, two person teams indoors and two person teams outdoors. The first team working from 1800 to 2400 hr and the second team from 2400 to 0600 hr. Indoor collectors were positioned in the center of the house and exposed their legs while outdoor collectors were positioned at least 50 m away from house. Human landing collections were performed for 45 min every hour with a 15 min break. One cattle was used as animal bait and was positioned 50 m away from the human collection sites (Figure 8). The collector used an aspirator to collect the mosquitoes resting on the cattle net for 15 min every hour. All collected adult mosquitoes were kept in mosquito cups and were provided a sugar pad soaked with a 10% sugar solution. Collected mosquitoes were labeled by sites and hour of collection and each cup was returned to local laboratory in the field for morphological identification. Formal human-use approval was granted by the Ethical Research Committee convened by the Research and Development Institute, Kasetsart University, Thailand (KURDI-1/2543-1421457).

Hourly ambient temperature and relative humidity were recorded from 1800 to 0600 hrs, using manual thermometers and hygrometers (BARICO GmbH, Villingen-Schwenningen, Germany). Records of daily rainfall were also obtained from a manual rain gauge (RAIN GAUGE AUGE KIT, England) positioned at the study site.

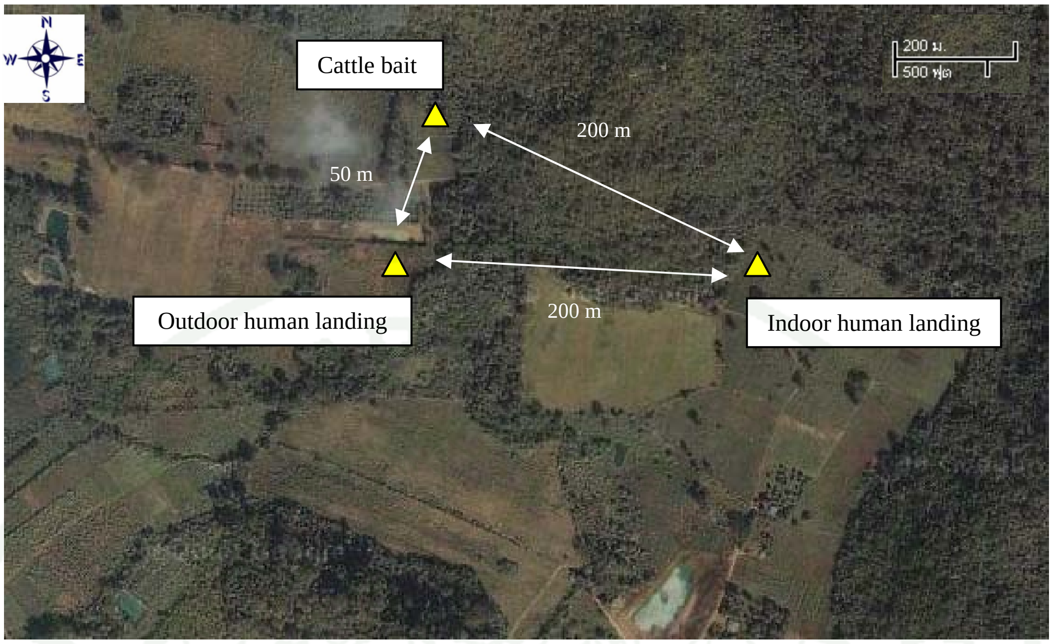


Figure 8 Collection points of Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand

Figure 9 Human landing indoor collections

Figure 10 Human landing outdoor collections

Figure 11 Cattle bait collections

1.3 Morphological identification

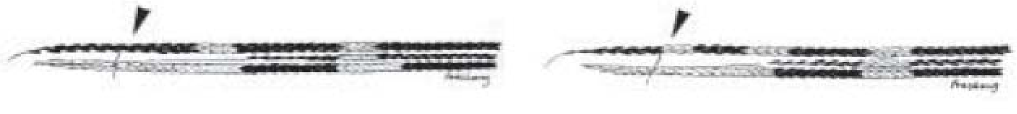
All female anopheline mosquitoes were identified to species using the illustrated morphological key of Rattanarithikul and Panthusiri (1994) and Rattanarithikul *et al*. (2006).

Members of the Dirus complex were identified as *An. dirus* if the exhibited a presector dark spot (PSD) on the radius vein that extends basally beyond the PSD spot on the costa vein and a PSD spot that reaches the humeral dark (HD) spot of the costa vein or at least surpasses the middle of the presector pale spot (PSP) of the costa vein of at least one wing (Figure 12).

phenotype of *An. dirus* phenotype of *An. baimaii*

Figure 12 Different morphological characteristics of *Anopheles dirus* and *Anopheles baimaii*

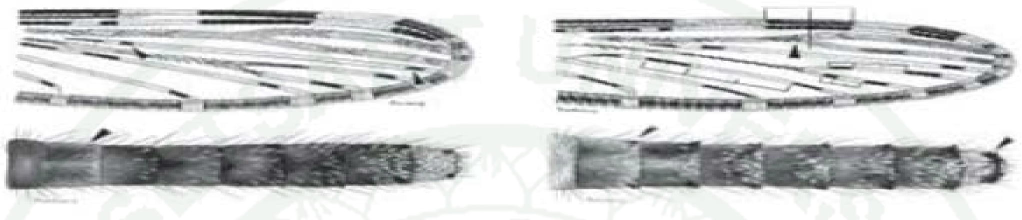
The *An. minimus* is identified if a presector pale spot (PSP) is present on costal vein of both wings, whereas *An. harrisoni* is obtained if both presector pale and humeral pale spots (HP) are present at least on one wing (Sungvornyothin *et al*., 2006a) (Figure 13).



phenotype of *An. minimus* phenotype of *An. harrisoni*

Figure 13 Different morphological characteristics of *Anopheles minimus* and *Anopheles harrisoni*

The *An. maculatus* has R_{2+3} with one dark spot on both wings and presector dark (PSD) spot on R is usually shorter than PSD spots on sub costa and costa. R_{4+5} have 2 dark spots and abdominal terga VII, VIII cover with dark scales on posterolateral corners and sometimes on VI (Figure 14).



phenotype of *An. maculatus* phenotype of *An. sawadwongporni*

Figure 14 Different morphological characteristics of *Anopheles maculatus* and *Anopheles sawadwongporni*.

1.4 Molecular identification

Female mosquito specimens of *Anopheles* mosquitoes were extracted genomic DNA from single adult mosquitoes according to procedures of Linton *et al.* (2001) and Manguin *et al.* (2002).

PCR conditions for the identification method

Anopheles dirus

Molecular analysis was performed using an Allele Specific Polymerase Chain Reaction (AS-PCR) assay (Walton *et al*., 1999a). The rDNA ITS2 was amplified using primers ITS2A and ITS2B. In the final volume of 25 μl PCR reaction mixture contained 2.5 μl of 10X reaction buffer, 10 mM of each dNTP, 10 μM of primer, 10% dimethyl sulfoxide (DMSO), 0.2 units of *Taq* DNA polymerase and 2 μl of DNA template. The conditions of PCR cycles were as follows by an initial denaturation step at 96°C for five min, 32 amplification cycles at 94°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec each, and a final extension step at 72°C for 10 min. The PCR products were visualized using electrophoresis on a 2% agarose gel at 100 V for 30 min. Primer names, sequences, and sizes of the PCR products are shown in Table 2.

Anopheles minimus

The AS-PCR assay developed by Garros *et al*. (2004a), amplification by PCR and sequencing of the Internal Transcribed Spacer 2 (ITS2 and ITS2B) region. The PCR mixture contained 17.75 μl ultrapure distilled water, 2.5 μl of 10X reaction buffer (Qiagen, Valencia, CA), 10 mM of each dNTP, 10 µM of primer, 0.5 units of *Taq* DNA polymerase and 0.5 μl of DNA template. After an initial denaturation step at 94°C for two min, 40 amplification cycles were programmed as follows: 94°C for 30 sec, 54°C for 30 sec, 72°C for 40 sec, and a final extension step at 72°C for five min. One negative control was included per test run. Products were visualized by electrophoresis on a 2% agarose gel. Primer names, sequences, and sizes of the PCR products are shown in Table 3.

Anopheles maculatus

The PCR assay developed by Walton *et al.* (2007). The rDNA ITS2 was amplified using primers 5.8F and 28R (Collins and Paskewitz, 1996). The concentrations of the reactants were 2.5 μl of 10x reaction buffer, 10 mM of each dNTP, 10 μM of primer, 0.2 units of *Taq* DNA polymerase and 2 μl of DNA template. The conditions of PCR cycles were as follows by an initial denaturation step at 94°C for five min, 35 amplification cycles at 94°C for one min, 61°C for 30 sec and 72°C for 30 sec each, and a final extension step at 72°C for five min. The PCR products were visualized to electrophoresis on 2% agarose gel. Primer names, sequences, and sizes of the PCR products are shown in Table 4.

Table 2 List of primers used for identification of five species of the *Anopheles dirus* complex

Species	Primer name	Sequence (5’ to 3’)	Size of the product (bp[2])
Universal forward primer	D-U	CGC CGG GGC CGA GGT GG	
An. dirus (A) and *An. scanloni* (C)	D-AC[1]	CAC AGC GAC TCC ACA CG	526(A), 349(C)
*An. cracen*s	D-B	CGG GAT ATG GGT CGG CC	514
An. baimaii	D-D	GCG CGG GAC CGT CCG TT	306
An. nemophilous	D-F	AAC GGC GGT CCC CTT TG	223

[1] D-AC binds at different places to the ITS2 DNA of *An. dirus* and *An. scanloni*

[2] bp = number base pairs

Table 3 List of primers, product size and Tm designed for *Anopheles minimus* complex

Species	Primer name	Sequence (5’ to 3’)	Size of the product (bp)	Tm (^{o}C)
Universal forward primer	ITS2	TGT GAA CTG CAG GAC ACA T		54.5
Anopheles pampanai	PAM	TGT ACA TCG GCC GGG GTA	90	56.0
Anopheles aconitus	ACO	ACA GCG TGT ACG TCC AGT	200	58.2
Anopheles harrisoni	MIC	GTT CAT TCA GCA ACA TCA GT	180	53.2
Anopheles varuna	VAR	TTG ACC ACT TTC GAC GCA	260	53.7
Anopheles minimus	MIA	CCC GTG CGA CTT GAC GA	310	57.6

*The internal transcribed spacer 2 (ITS2A) is the universal primer that binds to the same position on the ITS2 DNA for all five species, while the specific primers (PAM to MIA) bind at different places on the ITS2 DNA of the corresponding species

Tm = melting temperature

Table 4 List of primers designed and product size for *Anopheles maculatus* complex

Species	Primer name	Sequence (5' to 3')	Size of the product (bp)
Universal forward primer	ITS2	ATC ACT CGG CTC GTG GAT CG	
Anopheles maculatus	MAC	GAC GGT CAG TCT GGT AAA GT	180
Anopheles pseudowillmori	PSEU	GCC CCC GGG TGT CAA ACA G	203
Anopheles sawadwongporni	SAW	ACG GTC CCG CAT CAG GTG C	242
Anopheles maculatus K	K	TTC ATC GCT CGC CCT TAC AA	301
Anopheles dravidicus	DRAV	GCC TAC TTT GAG CGA GAC CA	477

1.5 Data analysis

Three key important factors were chosen for analysis, including climatic seasons, time periods and categories of collection. Seasonality included three period: wet (June to October), dry (November to February), and hot (March to May). Biting activity was divided into four time period representative of evening (1800-2100 hr), late night (2100-2400 hr), pre dawn (2400-0300 hr) and dawn (0300-0600 hr). Collection methods were identified as either indoor, outdoor human landing or cattle bait.

The nocturnal biting cycle of *An. dirus*, and *An. baimaii* were tabulated by averaging number landing per human by hour for indoor and outdoor collections and by averaging the number of mosquitoes captured per cow per hour. Comparisons of landing data were analyzed by nonparametric Kruskal-Wallis tests. The accepted level of significance was determined at 0.05% (P-valve < 0.05), followed by multiple linear regression and correlation coefficient (r^2) analysis using variables with the interaction between mosquitoes captured and environmental. All data were analyzed using the SPSS program package (version 17.0, SPSS, Chicago, IL).

2. Insecticide induced behaviors of *Anopheles dirus* in Thailand

2.1 Mosquito populations

Anopheles dirus was captured from Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand (14° 17'N, 99° 11'E). The collection site is located 400 m away from the base of a steep mountainous ridge and is surrounded by natural dense forest (Sungvornyothin *et al.,* 2009) (Figure 7). Female mosquitoes were collected from cattle using mouth aspirators during 15 min intervals per hour from 1800 and 0600 hr. Collected mosquitoes were then identified, placed in clean plastic cups covered with netting, and provided a cotton pad that was soaked with a 10% sugar solution.

2.2 Morphological identification

Adult mosquitoes were identified using the morphological keys of Sallum *et al.* (2005) and Rattanarithikul *et al.* (2006). Female mosquitoes showing a presector dark spot (PSD) on the radius vein that extends basally beyond the PSD spot on the costa vein and a PSD spot that reaches the humeral dark (HD) spot of the costa vein or at least surpasses the middle of the presector pale spot (PSP) of the costa vein of at least one wing were identified as *An. dirus* (Figure 12).

2.3 Insecticide impregnated papers

Standard doses of each test compound (0.025 g a.i./m^2 of bifenthrin, 0.03 g a.i./m^2 of alpha-cypermethrin,0.02 g a.i./m^2 of lambda-cyhalothrin and 2 g a.i./m^2 of DEET) were impregnated onto 15 x 17.5 cm filter paper sheets. The filter papers were treated according to WHO protocol using acetone solutions of insecticide and silicone oil (WHO, 1998). Drops of the insecticide were applied to the filter paper at a rate of 2.75 ml of insecticide solution per 180 cm^2. Control papers (without chemical) were prepared using only a diluent (i.e. acetone or ethanol). The papers were dried for 24 hr prior to testing.

2.4 Behavioral tests

Excito-repellency system

The test system was developed and described by Chareonviriyaphap *et al*. (2002) and modification being a reduction in size (Tanasinchaykul *et al.,* 2006). The chamber (external dimensions size 23.5 cm x 24.5 cm x 23.5 cm) is made up of several components numbered from 1 to 7; (1) a rear door cover, (2) a Plexiglas panel with rubber latex-sealed door, (3) a Plexiglas holding frame, (4) an outer chamber, (5) a screened inner chamber, (6) a front door, (7) an exit portal (Figures 15 and 16). To assemble chamber, all four side walls are connected by sliding the aluminum tongue and groove elements. A screened inner chamber is prepared in the same manner, consisting of a four sided frame that can be inserted easily inside the outer chamber. A Plexiglas holding frame and panel are attached to one end and an exit portal slot is attached to the opposite end of the box. A receiving cage (21.5 cm x 24.5 cm x 21 cm paper box) is connected to the exit portal for collecting any escape mosquitoes. The complete test consisted of two treated test chambers and two paired control chambers.

Tests were conducted to compare two types of behavioral responses (irritancy and repellency) of *An. dirus* in contact and noncontact exposure chambers using four different chemicals (0.025% bifenthrin, 0.03% alpha-cypermethrin, 0.02% lambda-cyhalothrin, and 5% DEET). In each test, 15 female mosquitoes were carefully introduced into each of four chambers by using a mouth aspirator after which the rear door was closed (Figure 16). Mosquitoes were allowed a 3-min adjustment period before the escape funnel was opened to initiate the observation period. Mosquitoes escaping from the chamber into the receiving cage were recorded at 1-min intervals for a period of 30 min, ambient temperatures (25±5 ºC) and relative humidity (80±10% RH) were recorded during the experiment. All tests were performed during the day between 0800 and 1630 hr and each test was replicated four times. After each test period, the numbers of dead or knockdown specimens were recorded separately from each exposure chamber. Live escaped specimens and those remaining inside the treatment and control chambers were collected and held separately in clean containers topped with cotton pads soaked in a 10% sugar solution. After 24 hr, mosquito mortality was recorded.

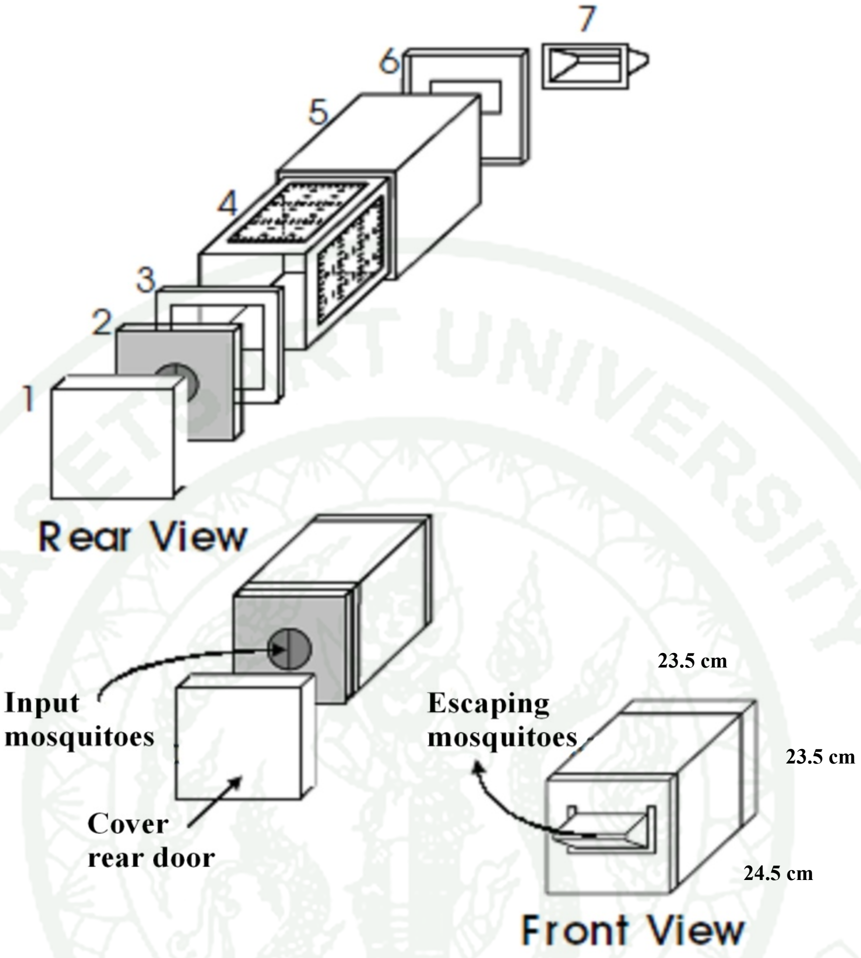


Figure 15 Excito-repellency test chamber for observe insecticide behavioral responses

1. = Rear door
2. = Plexiglass panel with rubbered door
3. = Plexiglass holding frame
4. = Screend inner chamber
5. = Outer chamber
6. = Front door
7. = Exit window

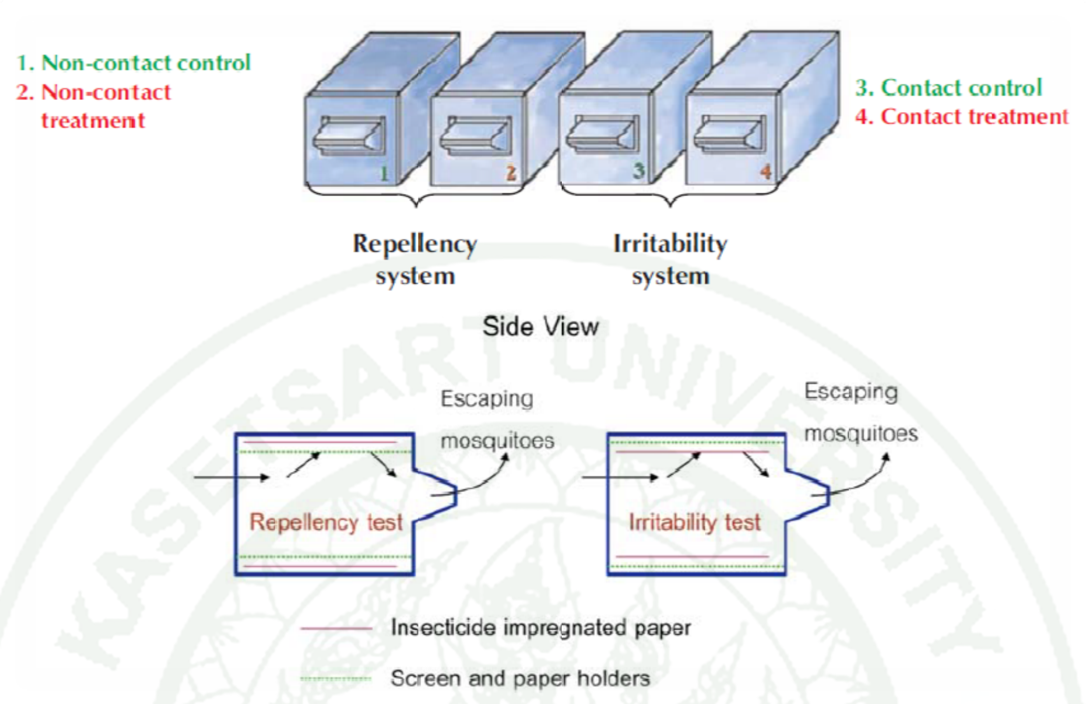


Figure 16 Excito-repellency test chamber used to study insecticide behavioral responses

1. = Control noncontact chamber
2. = Treatment noncontact chamber
3. = Control contact chamber
4. = Treatment contact chamber

2.5 Data analysis

Percentage escape obtained from the treated exposure chambers was adjusted based on paired control escape responses using Abbott’s formula (Abbott, 1925). The Kaplan-Meier survival analysis method was used to analyze and evaluate the behavioral response data (Kleinbaum, 1995, Roberts *et al.,* 1997). Survival analysis was also used to estimate the probability of escape time (ET) at which 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of the test populations vacated the chamber. Patterns of escape were evaluated within the test cohorts and between different treatment groups using the log-rank method (Mantel and Haenzel, 1959). Statistical significance was determined using SAS 9.0 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Results

1. Blood feeding activity and seasonal abundance of *Anopheles dirus* and *Anopheles baimaii* (Diptera: Culicidae) in western Thailand

A survey on adult Anopheline mosquito diversity, collected from September 2009 to August 2010 at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, western Thailand, the results are presented in Table 3. From a total of 9,824 specimens, 656 belong to the Dirus complex, 8,802 were the Minimus complex and 366 were the Maculatus complex. Six important species were molecularly identified to include *An. dirus* (6.08%), *An. baimaii* (0.59%), *An. minimus* (4.94%), *An. harrisoni (*84.64%), *An. maculatus* (2.43%) and *An. sawadwongporni* (1.29%) (Table 5 and Figures 15-17). Among these, four species, *An. dirus*, *An. baimaii*, *An. minimus* and *An. maculatus* are considered to be the important malaria vectors in Thailand (Manguin *et al.,* 2010; MOPH, 2010).

A total of 656 *Anopheles dirus* complex were captured throughout the study period to include 598 (91.2%) *An. dirus* and 58 (8.8%) *An. baimaii*. Both species were found to be more abundant in the wet season (especially from July to August) and both were positively correlated with increased rainfall throughout the year (Table 6 and Figure 20). For *An. dirus*, 378 (63.2%) were captured on cattle, 168 (28.1%) were collected from outdoor human landing collection, and 52 (8.7%) were obtained from indoor human landing collection. In contrast, a total of 58 specimens of *An. baimaii* were captured from all three types of collections. Twenty-nine (50%) were captured on cattle, 23 (39.7%) were obtained from outdoor human landing collection, and 6 (10.3%) were from indoor human landing collection (Table 6). In general, both species of the Dirus complex were found more attractive to cattle than to humans and, in the latter case, more outdoors than indoors, regardless of the time periods and seasons.

Landing patterns of *An. dirus* and *An. baimaii* were observed for both indoor and outdoor human collections and for cattle baited collection during the study period (Figures 17-19). More *An. dirus* were collected than *An. baimaii* from all collection methods employed in the study. The indoor biting activity of *An. dirus* demonstrated two peaks, the largest peak around 1900 and 2000 hr and a smaller one at 0200 and 0300 hr (Figure 17). The outdoor human landing showed one peak of activity and was elevated at 2300 and 2400 hr (Figure 18). Conversely, cattle bait catches showed one prominent peak for *An. dirus* in the early evening (1900 and 2000 hr) followed by a decline throughout the remainder of the night (Figure 19). Although low numbers of *An. baimaii* were obtained, both outdoor activity peaks were clearly defined with a distinct outdoor peak between 2400 and 0100 hr, whereas indoor peak presented between at 1900 to 2000 hr and at 0200 and 0300 hr (Figures 17 and 18). Cattle bait catches showed two clear peaks in the early evening (1900 and 2100 hr) and midnight (2400 and 0100 hr) (Figure 19). Both *An. dirus* and *An. baimaii* were more attractive to cattle bait than to human bait, indicating they are more zoophilic than anthropophilic.

Total number of landing mosquitoes per hour was subjected to a Kruskal-Wallis tests, with seasons (dry, hot, and wet), collection methods (indoor and outdoor human bait and cattle bait) and time intervals (early evening, late evening, predawn, and dawn) as discriminating factors. A strong significant differences in number of *An. dirus* and *An. baimaii* were found between seasons ($\chi^2 = 70.55$; df = 2; $P < 0.0001$ *An. dirus* and $\chi^2 = 27.34$; df = 2; $P < 0.0001$ *An. baimaii*), and between indoor, outdoor human landing and cattle bait ($\chi^2 = 11.59$; df = 2; $P = 0.003$ *An. dirus* and $\chi^2 = 6.07$; df = 2; $P = 0.048$ *An. baimaii*), There was no significant differences in the number of both species collected between the four quarterly evening time intervals ($\chi^2 = 2.21$; df = 3; $P = 0.529$ *An. dirus* and $\chi^2 = 3.68$; df = 3; $P = 0.298$ *An. baimaii*), were observed.

Data from all collection methods were pooled to determine the correlation between mosquito abundance and environmental variables. *Anopheles dirus* and *An. baimaii* densities were strongly associated well with the total rainfall ($r^2 = 0.454$; $P = 0.016$ *An. dirus* and $r^2 = 0.609$; $P= 0.003$ *An. baimaii*) but not related with relative humidity and minimum and maximum ambient air temperatures ($P > 0.05$) (Table 7 and Figure 20).

Table 5 Monthly numbers of *Anopheles* mosquitoes collected at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, during one year study period (September 2009-August 2010)

Month	*Anopheles* mosquitoes						
	Dirus		Minimus		Maculatus		Total
	DIR	BAI	MIN	HAR	MAC	SAW	
September	30	3	9	372	-	-	414
October	54	9	7	234	-	-	304
November	23	4	35	939	-	-	1001
December	0	1	21	676	-	-	698
January	0	0	65	824	-	-	889
February	0	0	64	1115	-	-	1179
March	0	0	34	713	4	0	751
April	4	0	31	554	26	8	623
May	17	4	43	873	39	12	988
June	51	5	89	783	43	30	1001
July	124	13	74	994	77	42	1324
August	295	19	14	239	50	35	652
Total	598	58	486	8316	239	127	9824
	(6.08%)	(0.59%)	(4.94%)	(84.64%)	(2.43%)	(1.29%)	

DIR ; *An. dirus*
BAI ; *An. baimaii*
MIN ; *An. minimus*
HAR ; *An. harrisoni*
MAC ; *An. maculatus*
SAW ; *An. sawadwongporni*

Table 6 Monthly capture totals of *Anopheles dirus* and *Anopheles baimaii* from three collection methods from Pu Teuy Village, Sai Yok District, Kanchanaburi Province

Month	*An. dirus*				*An. baimaii*				T[4]	H[5]	R[6]
	In[1]	Out[2]	Cattle[3]	total	In	Out	Cattle	total			
Sep	7	9	14	30	0	0	3	3	28.97	78.38	168.70
Oct	8	4	42	54	1	3	5	9	28.20	79.44	156.90
Nov	0	5	18	23	1	3	0	4	20.08	85.44	156.90
Dec	0	0	0	0	1	0	0	1	22.47	78.37	7.00
Jan	0	0	0	0	0	0	0	0	20.37	83.47	12.10
Feb	0	0	0	0	0	0	0	0	26.11	77.63	0.00
Mar	0	0	0	0	0	0	0	0	25.63	83.06	76.50
Apr	0	0	4	4	0	0	0	0	27.33	84.01	26.00
May	2	3	12	17	1	2	1	4	26.36	83.11	63.00
Jun	1	14	36	51	0	0	5	5	26.06	92.08	183.00
Jul	12	50	62	124	1	6	6	13	24.86	88.83	291.00
Aug	22	83	190	295	1	9	9	19	25.80	87.25	208.00
Total	52	168	378	598	6	23	29	58			

[1] Indoor collection

[2] Outdoor collection

[3] Cattle bait collection

[4] T = Temperature (°C)

[5] H = Humidity (%)

[6] R= Rainfall (mm)

Table 7 Correlation between total numbers of *Anopheles dirus* and *Anopheles baimaii* and rainfall, temperatures and humidity in Pu Teuy Village, Sai Yok District, Kanchanaburi Province

Variables	*An. dirus*				*An. baimaii*			
	r	r^2	*F*	Sig.(*P*)	r	r^2	*F*	Sig.(*P*)
Rain fall	0.674	0.454	8.327	0.016	0.781	0.609	15.593	0.003
Temperature	0.140	0.020	0.200	0.664	0.170	0.029	0.296	0.598
Humidity	0.454	0.206	2.601	0.138	0.460	0.212	2.683	0.132

$P < 0.05$

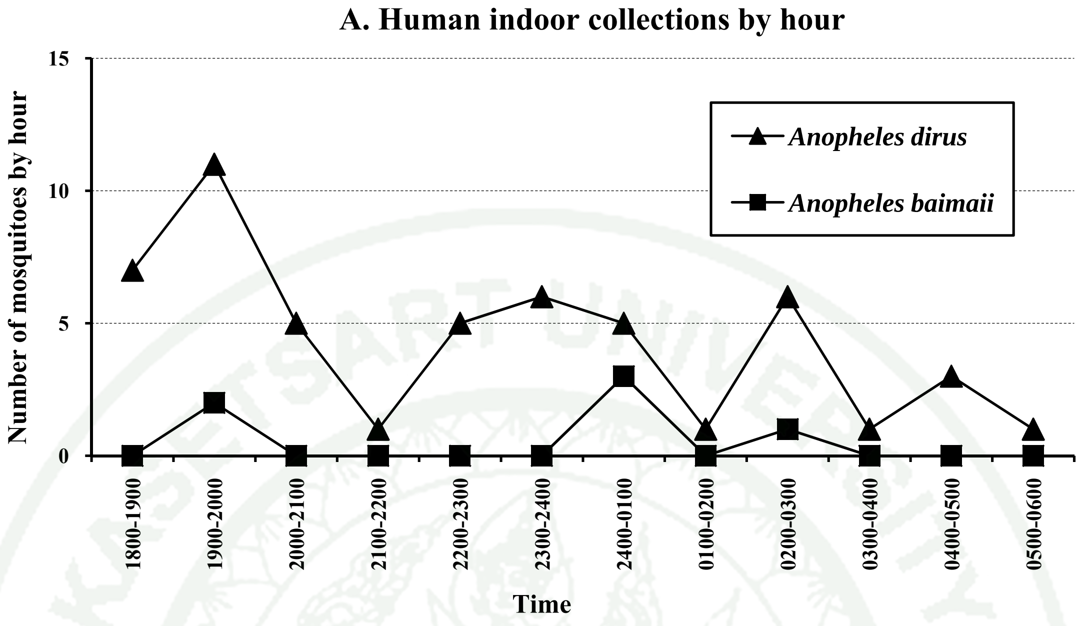


Figure 17 Landing patterns of *Anopheles dirus* and *Anopheles baimaii* for indoor human landing collections by hour

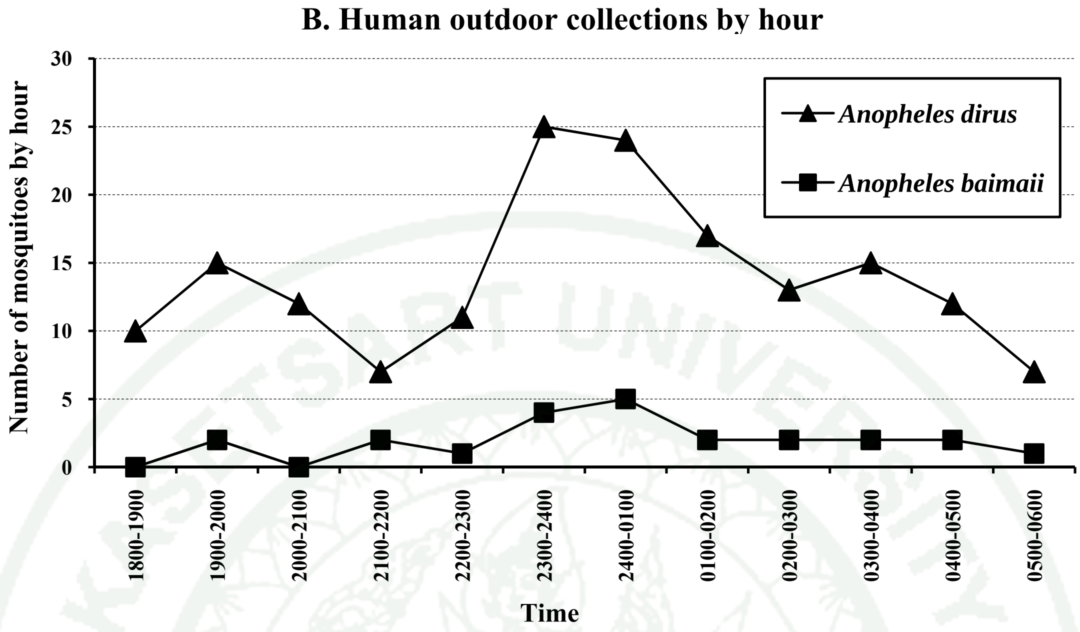


Figure 18 Landing patterns of *Anopheles dirus* and *Anopheles baimaii* for outdoor human landing collections by hour

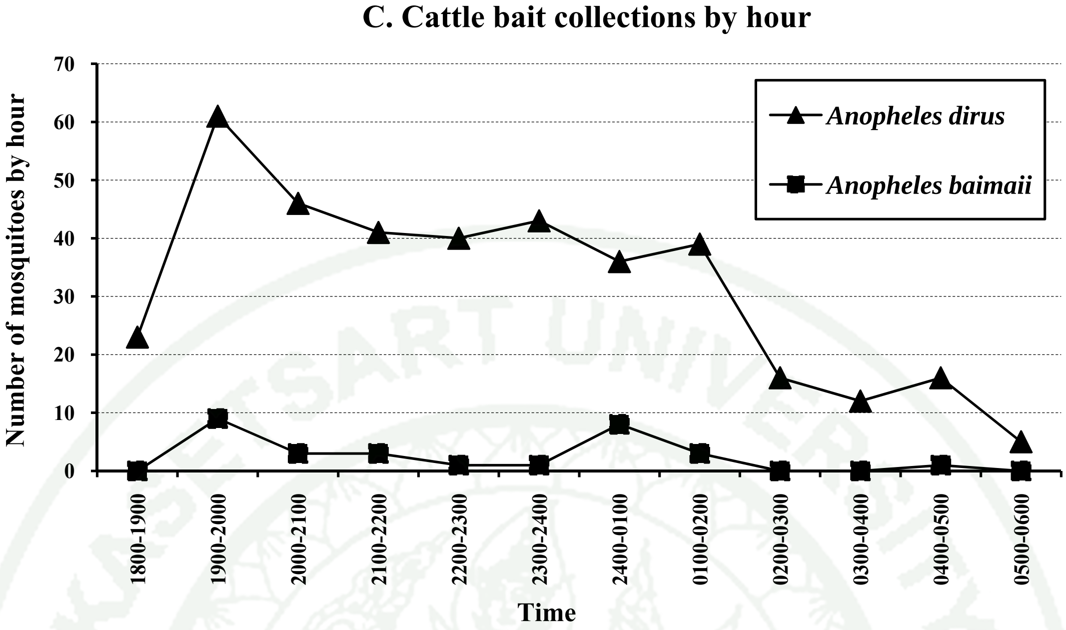


Figure 19 Landing patterns of *Anopheles dirus* and *Anopheles baimaii* for cattle bait collections by hour

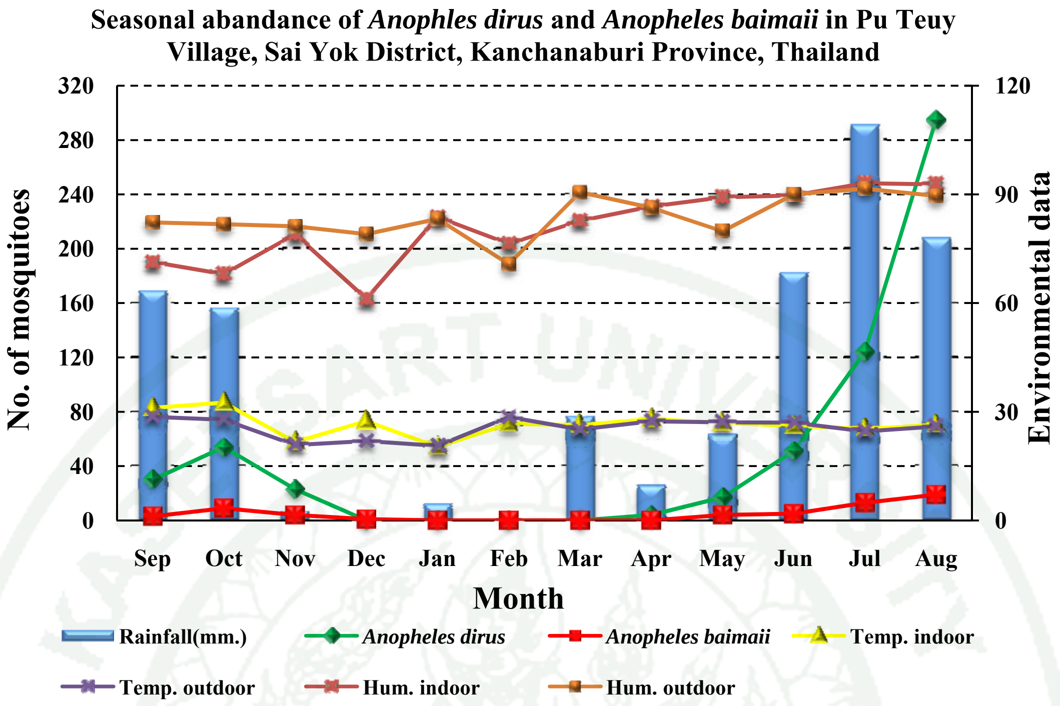


Figure 20 Monthly collections of *Anopheles dirus* and *Anopheles baimaii* in relation to average ambient air temperature, humidity and rainfall in Pu Teuy Village, Sai Yok District, Kanchanaburi Province

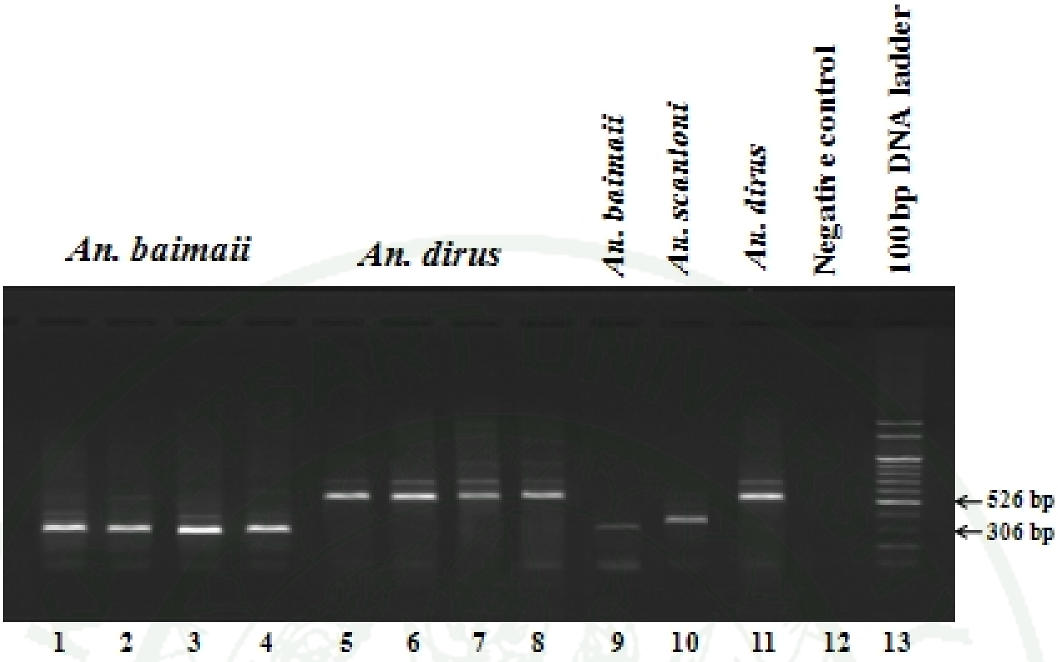


Figure 21 Multiplex Allele-Specific PCR assay. Lanes 1-4 = *An. baimaii*; lanes 5-8 = *An. dirus*; lanes 9-11 = positives of *An. baimaii*, *An. scanloni* and *An. dirus*; lane 12 = negative control; lane 13 = 100 bp DNA ladder

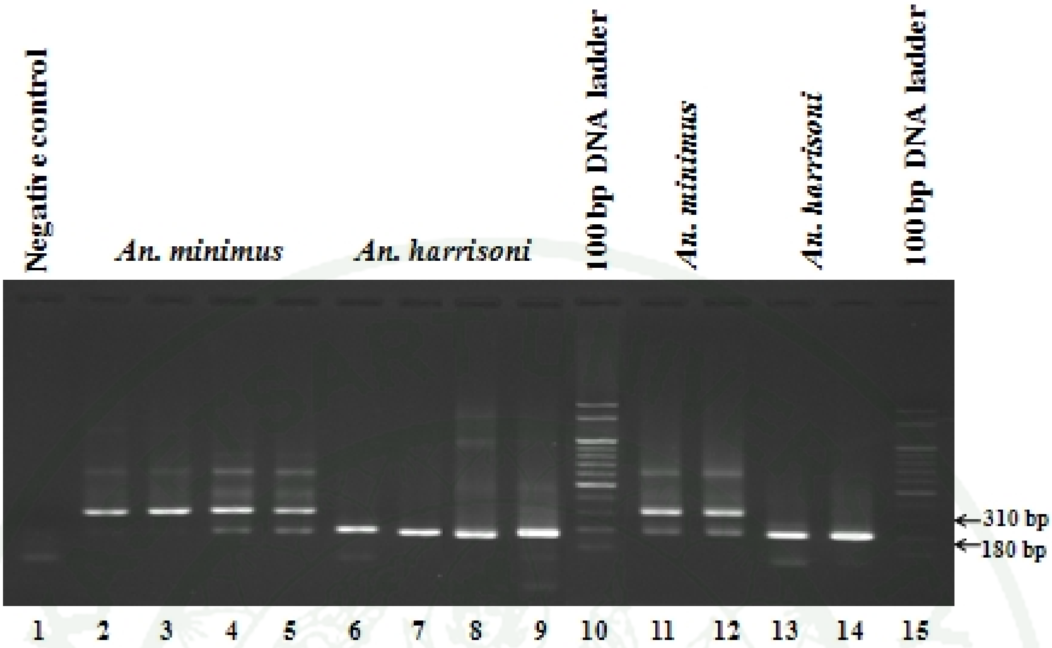


Figure 22 Multiplex Allele-Specific PCR assay. Lanes 1 = negative control; lanes 2-5 = *An. minimus*; lanes 6-9 = *An. harrisoni*; lanes 10 and 15 = 100 bp DNA ladder; lanes 11-14 = positives of *An. minimus* and *An. harrisoni*

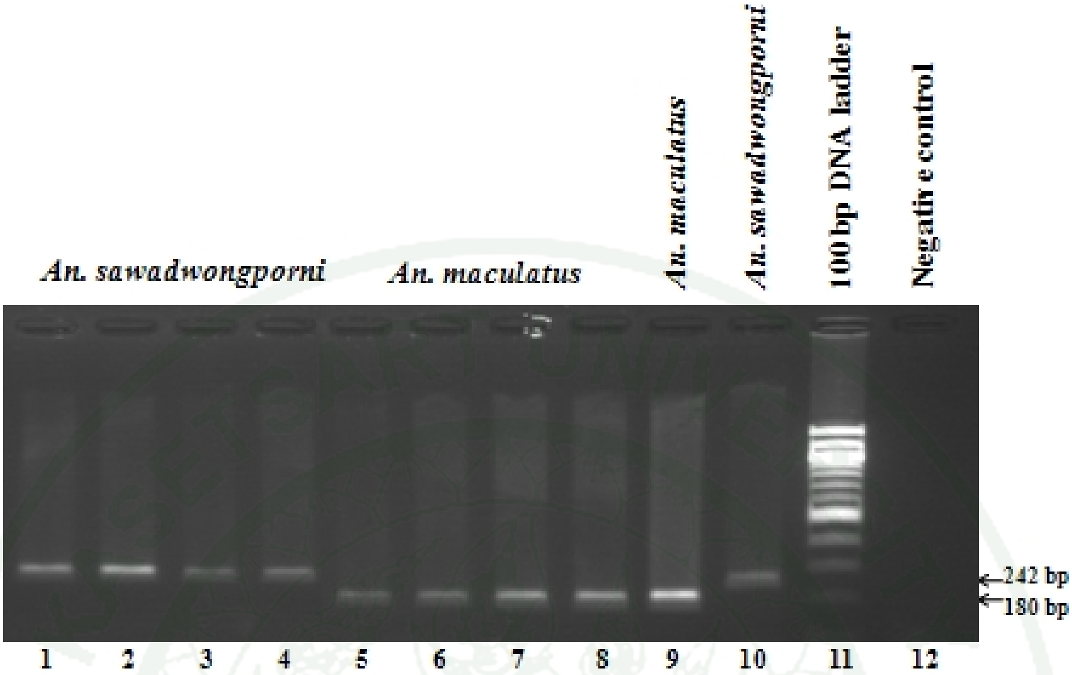


Figure 23 Multiplex Allele-Specific PCR assay. Lanes 1-4 = *An. sawadwongporni*; lanes 5-8 = *An. maculatus*; lanes 9-10 = positives of *An. maculatus* and *An. sawadwongporni*; lane 11 = 100 bp DNA ladder; lane 12 = negative control

2. Insecticide induced behaviors of *Anopheles dirus* in Thailand

The behavioral responses of *Anopheles dirus* wild-caught population were exposed to three synthetic pyrethroids (operation field concentrations) and DEET using an excito-repellency test system. Two types of behavioral responses, contact irritancy (move away from the treated area after making physical contact with test compound) and noncontact repellency (move away from the treated area before making physical contact with test compound). Percent mortality for escaped and non-escaped mosquitoes from both the control and treatment chambers are presented in Table 8. In general, patterns and rates of escape were much stronger in contact trials (68.32-84.91%) as compared to noncontact trials (0-52.52%) for all four compounds. In contact trials, percent escape of *An. dirus* for alpha-cypermethrin (84.91%) was higher than for bifenthrin (68.32%), lambda-cyhalothrin (68.60%) and DEET (77.01%). Relatively low numbers of mosquitoes escaped from the control chambers (5-20%) in both the contact and noncontact configuration (8.47-17.24%). In noncontact trials, DEET (52.52%) produced the greatest repellent response, followed by bifenthrin (21.88%), alpha-cypermethrin (11.54%), and lambda-cyhalothrin (0%).

Percent mortality of escaped and non-escaped specimens after a 24 hr holding period from both the contact and noncontact assays are presented in Table 8. In contact trials, mortality rates of escaped mosquitoes ranged between 6.25 and 25% whereas those remaining in the test chamber showed mortality rates, ranging from 6.12 to 73.33%. The highest mortality rate for escaped mosquitoes from the control chambers was observed in assays matched with bifenthrin (18.18%). In noncontact trials, the percent mortality of non-escaped specimens was generally low, varying between 0 and 7.50%. No mortality of escaped mosquitoes was observed from noncontact trials for all four compounds (Table 8).

The escape patterns generated for the insecticide-treated chambers are expressed in 1-min intervals for 25%, 50%, and 75% (ET_{25}, ET_{50}, and ET_{75}) of the test population to exit the test chambers (Table 9). ET values are generally low for contact trials compared to noncontact trails, regardless of test chemical. For contact trails, the ET_{25} values for bifenthrin, alpha-cypermethrin, lambda-cyhalothrin and DEET were 11, 3, 4 and 3 min, respectively. The ET_{50} values for bifenthrin, alpha-cypermethrin, lambda-cyhalothrin and DEET were 26, 5, 10 and 9 min, respectively. The ET_{75} values for bifenthrin and lambda-cyhalothrin could not be calculated because of insufficient numbers of mosquitoes exiting during the 30-min test. For the noncontact trails, the ET_{25} and ET_{50} could only be estimated for DEET whereas the exit times for the three synthetic pyrethroids could not be estimated, except bifenthrin (ET_{25} = 29) (Table 9). The remaining noncontact trials produced insufficient numbers of escaped mosquitoes to calculate exit times.

Differences in the patterns of escape behavior were analyzed using the log-rank method and statistical significance was set at 0.05 (Tables 10 and 11). Significant differences in escape behavior were observed when comparing paired control versus contact trials and contact versus noncontact trials for all four compounds ($P < 0.05$). Significant differences were also observed when noncontact trials was compared to paired control trials for bifenthrin ($P = 0.0227$) and DEET ($P < 0.0001$). A comparison of the escape response of mosquitoes between pairs of each of the four test chemicals in contact and noncontact trials are presented in Table 11. Significant differences in contact trials were observed when alpha-cypermethrin was compared to bifenthrin ($P < 0.0001$), DEET ($P = 0.0887$) and lambda-cyhalothrin ($P = 0.0076$). In noncontact trials, significant differences were observed for all chemical pairs ($P < 0.05$), except when bifenthrin was compared to alpha-cypermethrin ($P = 0.4818$) (Table 11).

The proportions of *An. dirus* mosquitoes remaining in the exposure chamber treated with bifenthrin, alpha-cypermethrin, lambda-cyhalothrin and DEET. These proportions were used to develop escape rate patterns representing the probability of escaping from the exposure chamber in contact and paired control trials (Figure 24), and noncontact and paired control trials (Figure 25). In contact trials, alpha-cypermethrin produced the greatest irritant response as compared with the other chemicals ($P<0.05$). In noncontact trials, significantly stronger escape responses were observed in DEET compared to the other compounds ($P<0.05$).

Table 8 Percentage escape and 24 hr mortality of *Anopheles dirus* exposed to bifenthrin, alpha-cypermethrin, lambda-cyhalothrin and DEET in contact and noncontact trials

Condition	Test Population	Mosquitoes (No. of test)	% escaped*	% mortality	
				Escaped	Remain
Contact	BIF[1]	Treatment(58)	68.32	6.97(3/43)	73.33(11/15)
		Control(60)	18.33	18.18(2/11)	6.12(3/49)
	ALP[2]	Treatment(58)	84.91	15.68(8/51)	14.28(1/7)
		Control(60)	20	0	8.33(4/48)
	LAM[3]	Treatment(57)	68.60	25(10/40)	29.41(5/17)
		Control(60)	5	0	0
	DEET	Treatment(58)	77.01	6.52(3/46)	66.66(8/12)
		Control(60)	10	0	12.96(7/54)
Noncontact	BIF	Treatment(57)	21.88	0	7.50(3/40)
		Control(59)	10.17	0	3.77(2/53)
	ALP	Treatment(60)	11.54	0	4.34(2/46)
		Control(60)	13.33	0	1.92(1/52)
	LAM	Treatment(57)	0	0	0
		Control(59)	8.47	0	1.85(1/54)
	DEET	Treatment(56)	52.52	0	4.54(1/22)
		Control(58)	17.24	0	0

[1]Bifenthrin, [2]Alpha-cypermethrin , [3]Lambda-cyhalothrin

* Adjusted rate based on control response

Table 9 Escape time (ET) in minutes for 25% (ET_{25}), 50% (ET_{50}) and 75% (ET_{75}) of *Anopheles dirus* to escape from the excite repellency test chambers containing one of four chemicals using a 30 minute of exposure period

Test condition	BIF[1]			ALP[2]			LAM[3]			DEET		
	ET_{25}[4]	ET_{50}[5]	ET_{75}[6]	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Contact	11	26	+	3	5	12	4	10	+	3	9	30
Noncontact	29	+	+	+	+	+	+	+	+	8	22	+

[1] Bifenthrin, [2] Alpha-cypermethrin, [3] Lambda-cyhalothrin

+ Insufficient number of mosquitoes escapes from test chamber

ET: Escape Time

[4] ET_{25} = Escape time = Time in minutes for 25% of female mosquitoes to escape from excito-repellency test chambers

[5] ET_{50} = Escape time = Time in minutes for 50% of female mosquitoes to escape from excito-repellency test chambers

[6] ET_{75} = Escape time = Time in minutes for 75% of female mosquitoes to escape from excito-repellency test chambers

Table 10 Comparison of escape responses between contact and control, contact and noncontact and noncontact and control trials for *Anopheles dirus*

Chemical	Treatment pairs		
	Control vs. Contact	Contact vs. Noncontact	Noncontact vs. Control
BIF[1]	<0.0001	<0.0001	0.0227
ALP[2]	<0.0001	<0.0001	0.1450
LAM [3]	<0.0001	<0.0001	0.4618
DEET	<0.0001	0.0227	<0.0001

[1]BIF = Bifenthrin, [2]ALP = Alpha-cypermethrin, [3]LAM = Lambda-cyhalothrin

Significance set at $P < 0.05$

Table 11 Comparison of escape responses of *Anopheles dirus* between pairs of four test chemicals in contact and noncontact trial

Chemical	Treatment pairs	
	Contact	Noncontact
BIF[1] vs. ALP[2]	<0.0001	0.4818
BIF vs. LAM[3]	0.3465	0.0011
BIF vs. DEET	0.0869	0.0010
ALP vs. LAM	0.0076	0.0046
ALP vs. DEET	0.0887	<0.0001
LAM vs. DEET	0.3408	<0.0001

[1]BIF = Bifenthrin, [2]ALP = Alpha-cypermethrin, [3]LAM = Lambda-cyhalothrin

Significance set at $P < 0.05$

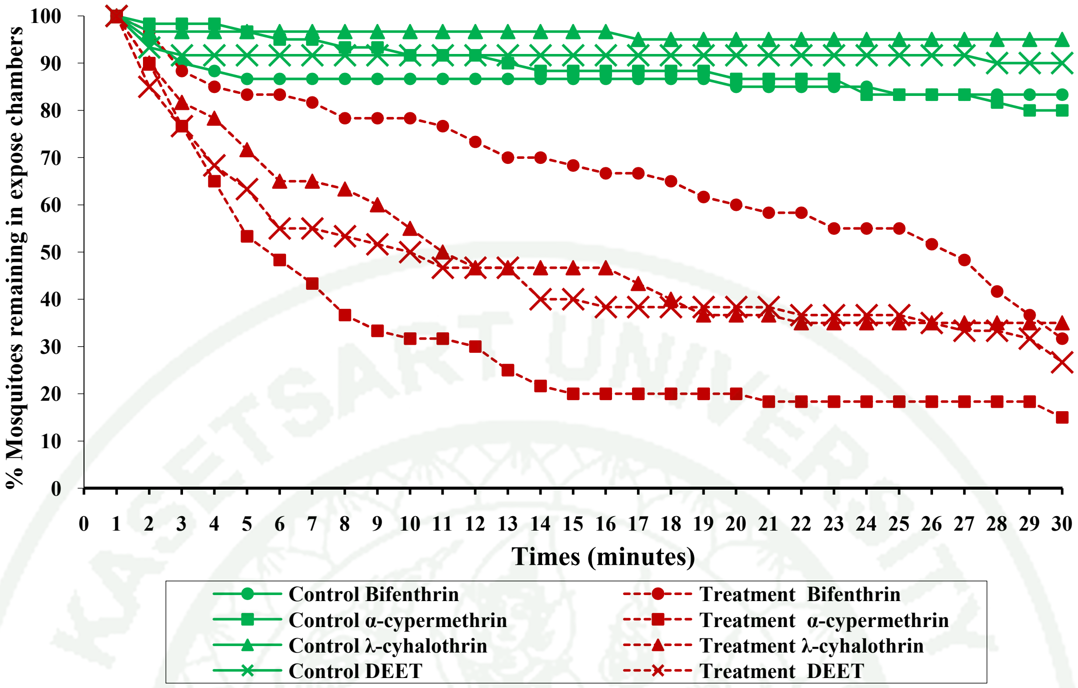


Figure 24 Escape probability of *Anopheles dirus* exposed to bifenthrin = 0.025 g/m², alpha-cypermethrin = 0.03 g/m², lambda-cyhalothrin = 0.02 g/m² and DEET = 2 g/m^2 for treatment and control contact trials

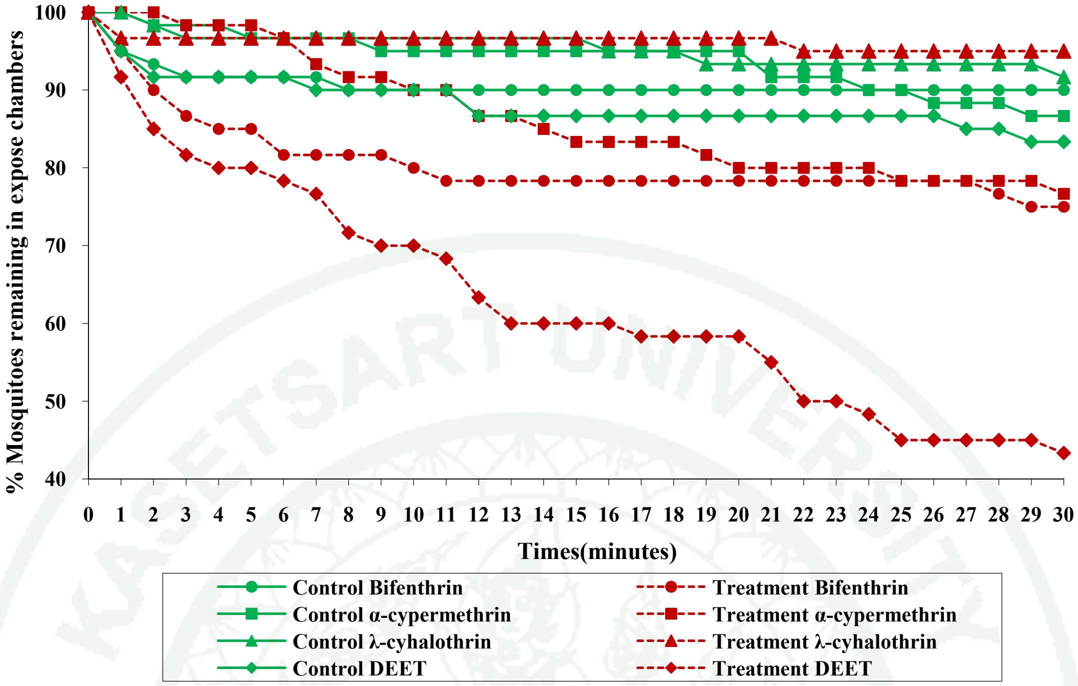


Figure 25 Escape probability of *Anopheles dirus* exposed to bifenthrin = 0.025 g/m², alpha-cypermethrin = 0.03 g/m², lambda-cyhalothrin = 0.02 g/m² and DEET = 2 g/m^2 for treatment and control noncontact trials

Discussion

1. Blood feeding activity and seasonal abundance of *Anopheles dirus* and *Anopheles baimaii* (Diptera: Culicidae) in western Thailand

From a total of 76 known *Anopheles* species found in Thailand, some members of Leucosphyrus and Maculatus groups and Minimus subgroup are recognized as important malaria vectors (Rattanarithikul *et al.,* 2006). In this study, six species are found belong to the two groups and one subgroup, including two species in the Leucosphyrus group, *An. dirus* and *An. baimaii*, two species in the Maculatus group, *An. maculatus* and *An. sawadwongporni* and both species in the Minimus subgroup, *An. minimus* and *An. harrisoni*. Four identified species, *An. dirus*, *An. baimaii*, *An. maculatus*, *An. minimus*, are designated as potential vectors of malaria. Previous studies have successfully described the trophic behavior, biting activity, and seasonal abundance in the Maculatus group and Minimus subgroup (Sungvornyothin *et al.,* 2006b; Meunworn *et al.,* 2006) whereas none has been observed such activities in the most two important vectors of malaria within the Leucosphylus group, *An. dirus* and *An. baimaii*.

Anopheles baimaii and *An. dirus* are considered to be primary malaria vectors in Thailand (Rattanarithikul *et al.,* 2006). These two closely related species cannot differentiated from each other by morphological characters (Walton *et al.,* 1999; Sungvornyothin *et al.,* 2006a). Both are forest and forest-fringe inhabiting mosquitoes that are considered highly anthropophilic (Baimai *et al.,* 1984; Rattanarithikul *et al.,* 2006). The preferred breeding habitats of these two species are animal footprints, wheel-tracks and temporary ground pools. In addition, larval breeding sites can be found in water jars, cut tree stumps, and root holes. *Anopheles dirus* is the only species that is found throughout Thailand and often occurs in sympatry with *An. baimaii* (Rattanarithikul *et al.,* 1995). Both species are considered very anthropophilic in blood-feeding preference and demonstrate both exophagic and endophagic behavior (Sinka *et al.,* 2011), and in some cases a generally greater tendency toward exophily (Baimai *et al.,* 1988; Manguin *et al.,* 2008). Previous findings showed complications using morphology in the identification of these sibling species complex (Manguin *et al.,* 2008; Sungvornyothin *et al.,* 2009). In this study, specimen of mosquitoes reported were subjected to a multiplex AS-PCR, to generate accurate species identification and describe with reliability the trophic behavior, seasonal abundance, and host preference of *An. dirus* and *An. baimaii* in Pu Teuy Village in Kanchanaburi Province.

Both species of *An. dirus* and *An. baimaii* demonstrated strong zoophilic behavior. More mosquitoes were caught on cattle bait compared to human landing from both inside and outside of houses (63.2% of *An. dirus* and 50% of *An. baimaii*). Results of previous studies on biting activity and host preference of *An. dirus* complex in central Thailand demonstrated a delayed and more prolonged peak feeding activity between 2000 and 2400 hr (Baimai *et al.,* 1988c; Wilkinson *et al.,* 1970). In southern Thailand, Scanlon and Sandhinand (1965) reported that peak biting activity of occurred between 2400 and 0300 hr. Rosenberg *et al.* (1990) observed peak biting occurring between 2200 and 0100 hr, and Rattanarithikul *et al.* (1996b) reported a single early-evening biting peak between 2000 and 2200 hr. In western Thailand, Sungvornyothin *et al.* (2009) observed the biting activity between 2000 to 2100 hr in both human baited and 2000 and 2300 hr in cattle baited. All of those biological and behavioral studies on *An. dirus* used morphological characters in the identification. Our study took advantage of PCR technology to identify the sibling species of the *An. dirus* complex and described individual biting cycles and blood feeding activities. The indoor biting activity of *An. dirus* occurred throughout the night long as than undistinct peak between 1900 and 2000 hr. Outdoor human landing activity presented a prominent peak between 2300 and 2400 hr. On the other hand, cattle baited collections showed one clear peak in the early evening between 1900 and 2000 hr, followed by a declining throughout the night long. *Anopheles baimaii* was collected in small proportion compared to *An. dirus* and showed an ambiguous is pattern of peak activity. Outdoor activity was peaked between 2400 and 0100 hr. Cattle bait catches showed two clear peaks in the early evening at 1900 and 2100 hr and at 2400 and 0100 hr. Accurate identification of the species with the use multiplex PCR technology could explain the differences in feeding patterns of these sibling species complex when compared to earlier published reports.

The seasonal abundance of *Anopheles* mosquitoes appeared to be influenced by several factors. In this study, adult densities were found to be positively associated with increased rainfall (July to August). This is shown by the greatest numbers found during the wetter period of the year with rainfall dependent abundance pattern was reported previously in Thailand and Bangladesh (Rosenberg and Maheswary, 1982; Baimai *et al.,* 1988; Sungvornyothin *et al.,* 2009), the reason that the larval habitats of *An. dirus* preferring temporary breeding habitats such as animal footprints, wheel tracks, and temporary ground pools common during the wet season. Contradictory finding was observed with *An. minimus* (Sungvornyothin *et al.,* 2006) and *An. maculatus* in the same locality (Muenworn *et al.,* 2008), wherein these two species was shown to prefer breeding at the edges of slow-running streams (Chareonviriyaphap *et al.,* 2003; Sungvornyothin *et al.,* 2006). In contrast, negative association was found with higher mean ambient temperatures and relative humidity.

Although a cases of malaria is low in Pu Teuy Village (Sungvornyothin *et al.,* 2009), the area remains at risk to increased transmission due to great human movement (parasite introduction) from more malarial areas. Also efficient malaria vectors such as, *An. dirus*, *An. minimus* and *An. maculatus* have been commonly found in abundance particularly during the rainy periods of the year.

2. Insecticide induced behaviors of *Anopheles dirus* in Thailand

Although studies have been carried in Thailand out to evaluate the behavioral responses of anopheline mosquitoes to various cyano-pyrethroid insecticides using the excito-repellency test chamber (Chareonviriyaphap *et al.,* 2001, 2004; Muenworn *et al.,* 2006; Thanispong *et al.,* 2009; Mongkalangoon *et al.,* 2009), none have evaluated the active properties of bifenthrin, alpha-cypermethrin and lambda-cyhalothrin, three commonly used synthetic pyrethroids, on field-collected *An. dirus*. In this study, both contact excitatory (*irritancy*) and noncontact spatial repellency responses of *An. dirus* were quantitatively evaluated using the excito-repellency test system to provide a more complete understanding of vector behavior when exposed to insecticides. All three synthetic pyrethroids produced strong excitation responses among exposed female *An. dirus* without resulting in mortality following contact. Noncontact repellency was also evident but had much less an influence on escape (*avoidance*) behavior than direct contact irritancy. Following the indoor application of ‘*excito-repellent*’ compounds may result to aversion in normal indoor biting behavior. This in effect may reduce disease transmission risk inside the house but may increase the probability for transmission outside the home by promoting greater exophagic behavior. This information should be incorporated in designing more effective strategies targeting specific vector species.

For decades, several groups of chemical compounds have been widely used in the national public health vector control program in Thailand (Jirakanjanakit *et al.,* 2007; Thanispong *et al.,* 2008; Chuaycharoensuk *et al.,* 2011). Synthetic pyrethroids represent one of the most commonly used insecticides for controlling indoor and outdoor blood feeding mosquitoes, particularly *Anopheles* malaria vectors. This compound has been found to exhibit a certain degree of excito-repellency in many agricultural and medically important insects (Lockwood *et al.,* 1984; Miller, 1990; Lindsay *et al.,* 1991; Roberts and Andre, 1994). The continued use of pyrethroids demonstrate their effectiveness as vector control tool and signifies the need for further evaluations of the avoidance behavior elicited by these compounds across mosquito species and in other arthropod pests. With pyrethroids playing a major role in most indoor residual spray campaigns and as the main active ingredient in most insecticide treated bed nets, the role of the irritant and repellent actions of pyrethriods should be thoroughly defined for specific malaria vectors before beginning any large scale control campaign.

After an excito-repellency test system was introduced and used to separate two types of behavioral actions (Roberts *et al.,* 1997), several studies to evaluate the excito-repellent behavior in *Anopheles* mosquitoes have subsequently been reported (Chareonviriyaphap *et al.,* 1997, 2001, 2004; Pothikasikorn *et al.,* 2005; Muenworn *et* *al.,* 2006; Pothikasikorn *et al.,* 2007). Overall, synthetic pyrethroids produce much stronger irritant responses in *Anopheles* compared to repellent actions (Chareonviriyaphap *et al.,* 2001, 2004; Muenworn *et al.,* 2006; Pothikasikorn *et al.,* 2007). In this study, a greater avoidance response for *An. dirus* was also observed in contact trials, compared to noncontact and paired control trials. There were also significant differences in the escape responses documented between noncontact trials and their matched controls.

All the three pyrethroids produced a rapid and pronounced irritant response in *An. dirus*. The most dramatic avoidance response after physical contact was observed when *An. dirus* were exposed to the operational dose of alpha-cypermethrin. Similar findings of the irritant actions of pyrethroids were previously observed in both laboratory and field test populations of *An. dirus* (Chareonviriyaphap *et al.,* 2004). Additional laboratory studies also demonstrated a relatively strong irritant effect of alpha-cypermethrin in three test populations of *Aedes aegypti* from Thailand (Thanispong *et al.,* 2009). These studies also showed that most mosquitoes exited from the treated chamber before receiving a lethal dose of alpha-cypermethrin. Among the three test pyrethriods used in the current study, bifenthrin elicited the weakest escape response. Bifenthrin is a broad spectrum synthetic pyrethroids used for the control of a variety of agricultural pests and exhibits a very low vapor pressure (Hougard *et al.,* 2002). Bifenthrin may exhibit a weaker excito-repellent effect compared to the others due to this low vapor pressure. A low irritant and knockdown response was also found for bifenthrin as compared with permethrin and deltamethrin (WHO, 2001). These same studies concluded that although bifenthrin demonstrated an excito-repellent effect, it was a weak enough response to produce consistently high mortality. It was postulated that the weak behavioral response allowed mosquitoes to rest on the treated surface for a sufficient time to acquire a lethal dose of insecticide as compared to other chemicals such as deltamethrin or DEET (Tisgratog *et al.,* 2011). Although noncontact repellency to various synthetic pyrethroids has been recognized in several species of *Anopheles* (Lien, 1991; Chareonviriyaphap *et al.,* 1999, 2001; Sungvornyothin *et al.,* 2001), this type of chemical action was not significantly pronounced in our study.

Apart from synthetic pyrethroids, DEET is the most commonly used chemical for disrupting human-vector contact and exhibits strong behavioral responses in mosquitoes (Surgeoner, 1995; Cox, 2005). Although DEET has long been classified as a repellent, more recent studies have concluded that it may act as an inhibitor to mask host cues rather than as a true repellent (Dogan *et al.,* 1999; Dogan and Rossignol, 1999). Based on electrophysiological responses, DEET was shown to inhibit odor-evoked currents mediated by the insect odorant receptor complex (Ditzen *et al.,* 2008), effectively inhibiting perception of host odors and chemo-attractant cues. There is no clear conclusion as to whether DEET performs as a true repellent or an inhibitor based on the findings of this study. However, the differences in escape response between contact and noncontact trials did allow us to separate and prioritize the order of action for the irritant or repellent actions of the compounds tested. The intensity of these actions were also determined based on the escape response from matched control and noncontact tests to arrive at an adjusted percent repellency. It must be stated, however, that neither test configuration used a known attractant inside the DEET-treated chamber, a requirement to evaluate if inhibition was a mode of action. Those mosquitoes that escaped from the noncontact test configuration seem to have done so as the result of repellency alone. Regardless, the endpoint obtained from DEET was that it continued to function as a deterrent even in the absence of host cues. Based on our findings from the excito-repellency test system, DEET appears to act as a contact irritant and a moderate spatial repellent.

Significant differences in the escape responses were documented for all paired contact and noncontact trials. In addition, the escape responses for all paired noncontact tests were found to be significantly different as compared to their matched controls. Mortality was low for mosquitoes escaping the treated chambers in both of the contact and noncontact trials, an indication that behavioral avoidance greatly reduces the opportunity for residual insecticides to impact survival through toxicity. Pothikasikorn *et al.* (2005) confirmed that *An. minimus* and *An. harrisoni* are rapidly irritated by lambda-cyhalothrin and deltamethrin. Chareonviriyaphap *et al.* (2004) reported on an extensive study that defined the excito-repellent action of deltamethrin on four anopheline species, all representing important malaria vectors in Thailand. Although repellency was less profound than contact irritancy, the escape responses were statistically significant compared to the matched controls.

Pu Teuy Village is considered nearly malaria-free with only a few cases reported. Even then, the area remains vulnerable to increased transmission due to human movement from highly endemic areas (Chareonviriyaphap *et al.,* 2003; Muenworn *et al.,* 2006; Pothikasikorn *et al.,* 2005; Sungvornyothin *et al.,* 2006b). In addition, Pu Teuy Village is located in close proximity to intact forest and forest-fringe zones that are ideal habitats for *An. dirus* s.l., one of the efficient vectors of malaria in Thailand (Rattanarithikul *et al.,* 2006). I suggest that further evaluations be made to characterize the behavioral responses elicited by vector control insecticides for each species in order to help define the chemicals impact as part of the control programs in Thailand. This will help define the relative risk for malaria transmission associated with particular species that can assist in prioritization and design of appropriate vector prevention and control strategies.

A better understanding of the irritant and repellent actions of chemicals in malaria vectors is essential when evaluating the full impact these compounds may have on both mosquitoes and disease transmission. A standardize approach and quantification of a chemical's primary and secondary mode of action will help optimize our currently available public health tools and hopefully promote the development of newer chemicals and other innovative control methodologies. I believe excito-repellent assays should be an integral component of any insecticide evaluation to determine its full capabilities and potential to mitigate disease transmission.

CONCLUSION

1. Blood feeding activity and seasonal abundance of *Anopheles dirus* and *Anopheles baimaii* (Diptera: Culicidae) in western Thailand

Two sibling species of *An. dirus* complex present in Thailand, *An. dirus* and *An. baimaii*, which are known for their sympatry in Kanchanaburi Province were evaluated in this study. Of 656 *An. dirus* complex collected between September 2009 to August 2010 at Pu Teuy Village, Sai Yok District, Kanchanaburi Province, 598 (91.2%) were identified as *An. dirus* and 58 (8.8%) were identified as *An. baimaii*. Both *An. dirus* and *An. baimaii* were collected in higher numbers by cattle baited collection (63.2% *An. dirus* and 50% *An. baimaii*) as compared with human landing collection. Outdoor human collections (28.1% *An. dirus*, 39.7% *An. baimaii*) exceeded indoor human collections (8.7% *An. dirus*, 10.3% *An. baimaii*). From the landing patterns, the indoor landing activity of *An. dirus* presented two peaks, the largest peak 1900 and 2000 hr and a smaller one at 0200 and 0300 hr while from the outdoor human landing pattern there was elevated activity at 2300 and 2400 hr. From the cattle baited collections, one prominent peak was observed for *An. dirus* in the early evening (1900-2000 hr) followed by a decline throughout the remainder of the night. whereas *An. baimaii* presented two peaks in indoor human landing, at 1900 and 2000 hr and at 0200 and 0300 hr, outdoor human landing catches showed between midnight to 0100 hr and during early evening (1900-2000 hr) and midnight (2400-0100 hr) in cattle bait collections. In general, both *An. dirus* and *An. baimaii* were more abundant during the wet season (especially from July to August) compared to dry or hot season.

2. Insecticide induced behaviors of *Anopheles dirus* in Thailand

Behavioral response of *An. dirus* wild-caught population exposed to an operational field dose of three synthetic pyrethroids (bifenthrin, alpha-cypermethrin and lambda-cyhalothrin) and DEETwere evaluated using an exito-repellency test chamber. The escape response to three pyrethroids and DEET were significantly stronger in contact trials (irritancy) than in noncontact trials (repellency) ($P<0.05$). All test specimens rapidly escaped from the test chamber when exposed todirect contact with a surface treated with any of the three synthetic pyrethroids or DEET. Alpha-cypermethrin demonstrated the strongest irritant action (88.70% escape), followed by DEET (80.05%), lambda-cyhalothrin (72.49%) and bifenthrin (71.46%). In noncontact trials, fewer mosquitoes escaped from the test chambers as compared with contact trials, although a significant escape response was still observed as compared to the controls ($P<0.05$). This demonstrated that the pyrethroids act primarily as irritants and only secondarily as repellents.

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APPENDIX

Appendix Table 1 Kruskal-Wallis tests of total number landing mosquitoes/h, seasons (dry, hot, and wet), collection methods (indoor and outdoor human bait, and cattle bait) and time intervals (early evening, late evening, predawn, and dawn) as discriminating factors of *Anopheles dirus*

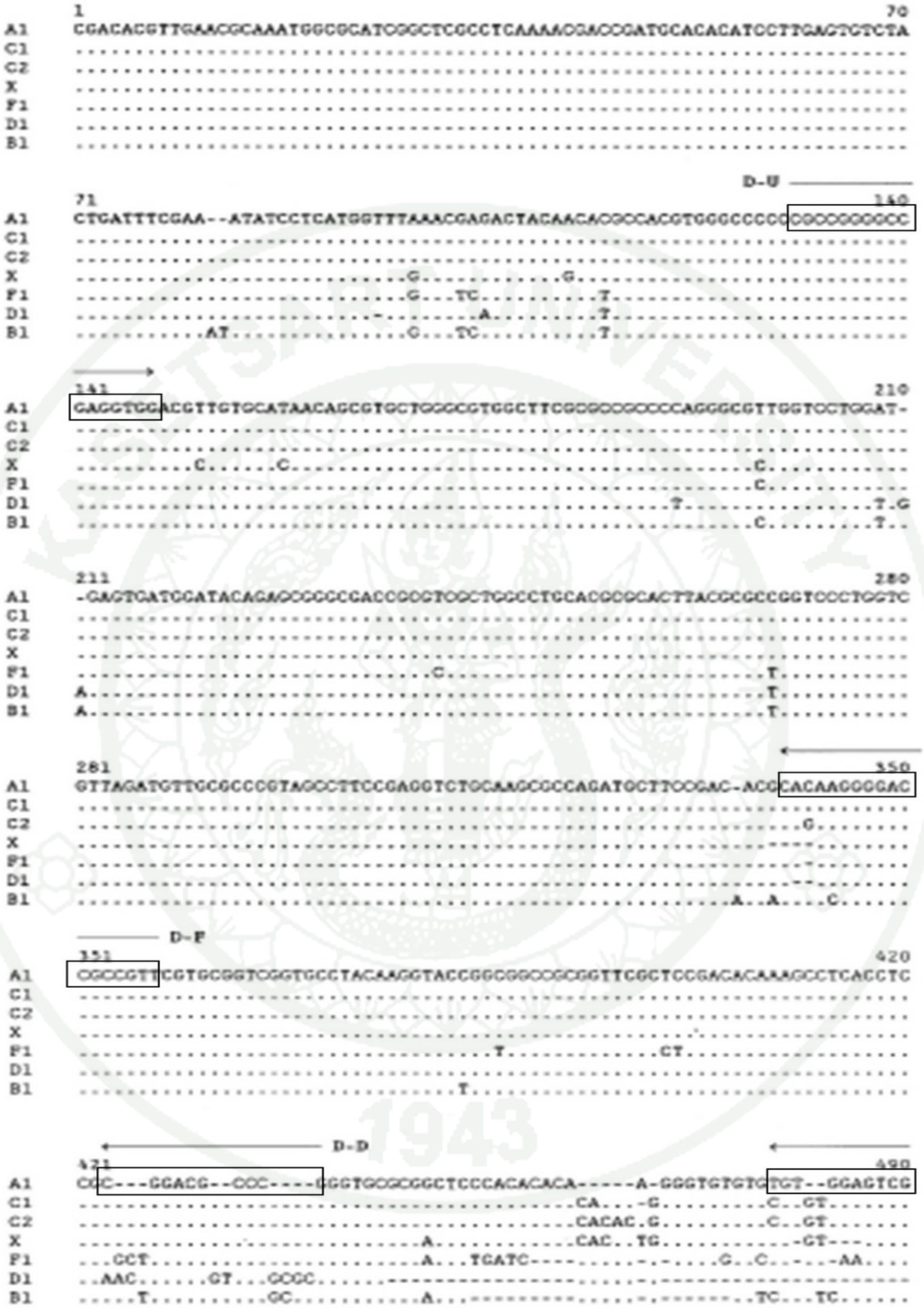
Factor		N	Mean Rank	Chi-Square (χ^2)	df	Sig. (*P*)
Season	Dry	36	35.95	70.552	2	<0.0001
	Hot	36	39.13			
	Wet	36	88.44			
	Total	108				
Type of Collection	Indoor	36	42.97	11.596	2	0.003
	Outdoor	36	53.72			
	Cattle	36	66.81			
	Total	108				
Time period	18-21	27	57.65	2.213	3	0.529
	21-24	27	59.50			
	24-03	27	51.91			
	03-06	27	48.94			
	Total	108				

($P < 0.05$)

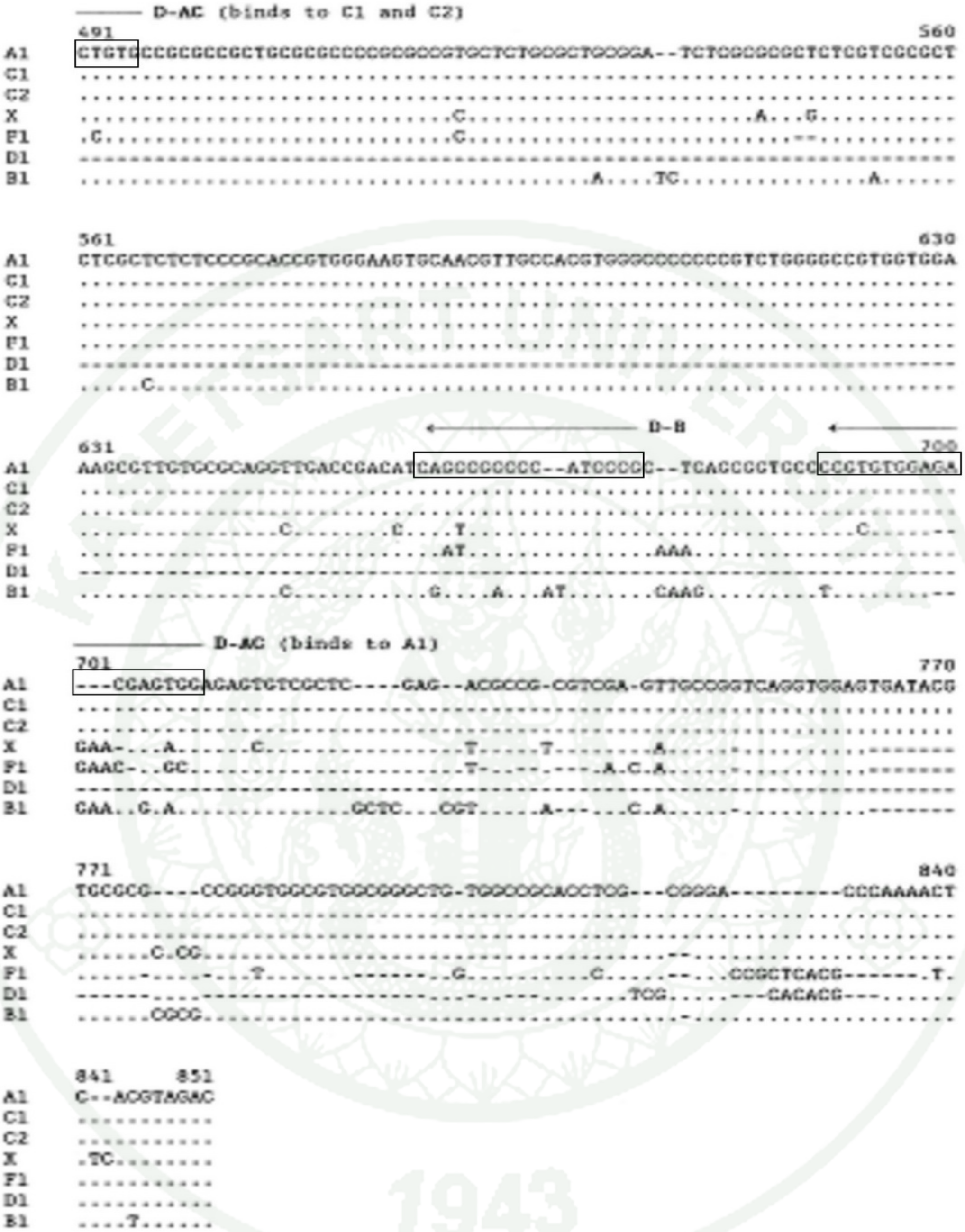
Appendix Table 2 Kruskal-Wallis tests of total number landing mosquitoes/h, seasons (dry, hot, and wet), collection methods (indoor and outdoor human bait, and cattle bait) and time intervals (early evening, late evening, predawn, and dawn) as discriminating factors of *Anopheles baimaii*

Factor		N	Mean Rank	Chi-Square (χ^2)	df	Sig. (*P*)
Season	Dry	36	46.10	27.340	2	<0.0001
	Hot	36	45.78			
	Wet	36	71.63			
	Total	108				
Type of Collection	Indoor	36	47.42	6.070	2	0.048
	Outdoor	36	61.39			
	Cattle	36	54.69			
	Total	108				
Time period	18-21	27	51.07	3.683	3	0.298
	21-24	27	56.28			
	24-03	27	60.93			
	03-06	27	49.72			
	Total	108				

($P < 0.05$)



Appendix Figure 1 Alignment of the nucleotide sequence of the internal transcribed spacer 2 (ITS2) from five species of the *Anopheles* species, shaded boxes indicate primer selection sites



Appendix Figure 1 (Continued)

CURRICULUM VITAE

NAME : Mr. Chatchai Tananchai

BIRTH DATE : May 27, 1984

BIRTH PLACE : Phrae, Thailand

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE**
2003-2007	Kasetsart University	B.Sc.

WORK PLACE : Student research assistance at the Department of Entomology, Kasetsart University