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TITLE: Characterization of the *Anopheles minimus* Complex in Thailand: Seasonal Population Surveys, Morphological-Molecular Identifications, and Behavioral Responses to Insecticides

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THESIS

CHARACTERIZATION OF THE *ANOPHELES MINIMUS* COMPLEX IN THAILAND: SEASONAL POPULATION SURVEYS, MORPHOLOGICAL-MOLECULAR IDENTIFICATIONS, AND BEHAVIORAL RESPONSES TO INSECTICIDES

MONTHATHIP KONGMEE

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Monthathip Kongmee 2012: Characterization of the *Anopheles minimus* Complex in Thailand: Seasonal Population Surveys, Morphological-Molecular Identifications, and Behavioral Responses to Insecticides. Doctor of Philosophy (Entomology), Major Field: Entomology, Department of Enotomolgy. Thesis Advisor: Professor Theeraphap Chareonviriyaphap, Ph.D. 115 pages.

Two different ecological breeding habitats of the Minimus Complex from Bong Ti Noy (BTN) and Pu Teuy (PT) were characterized using the Geographical information system (GIS) and the remote sensing technology in combination with field data. Differences in species diversity existed between the two study sites, indicating that surrounding land cover is associated very well with species-specific productive larval breeding habitats. Fluctuations of larval population densities in BTN were strongly affected by rainfall patterns. Changing environmental habitats associated with human activity also influenced larval population densities from PT. This information on environmental modifications of larval habitats is a potentially important strategy for anopheline larval control.

The characteristics of specific species within the Minimus Complex using molecular technique indicated that no morphological characters are completely reliable for distinguishing the adults of Minimus Complex species. Using molecular markers, nine species were obtained from BTN and eight species were identified from PT. Such correct identification is absolutely essential and mandatory for any relevant for the additional application of successful control strategies.

In addition, behavioral responses of these two species within the Minimus Complex, *Anophles harrisoni* and *Anopheles minimus* were evaluated following exposure to two pyrethroid insecticides, bifenthrin or deltamethrin, using an excito-repellency test system in the presence and absence of live host cues. The result demonstrated that deltamethrin elicited stronger irritant chemical effects than bifenthrin but that behavioral responses in vector populations are dampened in the presence of an available host. This information is useful for estimating probability of pathogen transmission when using irritant chemicals in proximity to a blood-meal source.

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April 2012

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INTRODUCTION

Malaria is currently one of the most important mosquito-borne infectious diseases in the tropics (World Health Organization [WHO], 2010), and kills over a million people annually and cause clinical cases between 300-500 million each year. The phenomenal malaria transmission trend as well as the geographical of disease transmission has been changing over time in several countries in Asia and Southeast Asia. In Thailand, malaria is most prevalent along the international borders of eastern Myanmar, northern Malaysia, and western Cambodia (Bureau of Epidemiology, 2010). These areas are frequently and strongly associated with tribal populations that are highly migratory because of transient employment opportunities for example logging, mining and road construction, hunting, gem mining and illegal activities. The nomadic nature of these populations, along with occupational movement and uncontrolled migration along the borders, confounds the problems associated with cross-border transmission and control (Chareonviriyaphap *et al*., 2000; Chaveepojnkamjorn and Pichainarong, 2004, 2005; Pichainarong and Chaveepojnkamjorn, 2004; Cui *et al*., 2011).

To better assess the risk of malaria transmission in a given area, surveillance studies are conducted to characterize the bionomics of target vector speies. Different *Anopheles* species have shown to have a vast diversity in breeding habitats such as marshes, rice fields, plantations, forests, forest fringes, and foothills. Changes in qualitative and quantitative environmental variables of mosquito larval habitats generally result in changes in larval mosquito abundance and related adult mosquito populations. Despite this understanding, relatively limited research has been conducted to examine the biology and habitats of *An. minimus* complex larval

mosquitoes in Thailand (Rattanarithikul *et al.*, 1995; Overgaard *et al.*, 2002), including the seasonal abundance of this two sympatric species in the Minimus Complex (*An. harrisoni* and *An. minimus*) and other closely related species (*An. aconitus*, *An. pampani*, and *An. varuna*) from a riparian, freshwater habitat.

Also important is an understanding of the spatial and temporal changes in anopheline mosquito abundance. Geographical information systems (GIS) and remote sensing technologies are useful tools to describe such changes. These technologies, in combination with field data, allow researchers to identify drivers of potential vector breeding sites and target control intervention methods. In Thailand, at least one or two species within the *Anopheles minimus* complex play a major role in malaria transmission. Previous research characterizing the habitats of *An. minimus* Theobald (former species A) and *An. harrisoni* Harbach and Manguin (former species C) have shown differences in larval habitat distribution based on land cover. However, the satellite imagery used did not include multi-temporal data. In addition, *An. minimus* has recently been reported where previously it was not collected (Kengluecha *et al.*, 2005; Rongnoparut *et al.*, 2005). This indicates potential changes in the surrounding environment that may be useful in predicting the distribution of *An. minimus* and *An. harrisoni* larval habitats for positive and negative locations. This requires establishing a database of environmental and mosquito attributes as well as the acquisition and processing of satellite imagery.

Although closely related, species within sibling complexes can differ in vector competency required for pathogen transmission. The accurate identification of sympatric sibling species of important vectors directly contributes to more beneficial studies and effective control (Curtis and Townson, 1998; Chen *et al.*, 2002; Oyewole *et al.*, 2007; Sinka *et al.*, 2011). Moreover, the understanding of behavior of each single vector species is critically important to help identify their respective role in disease transmission. The presence or absence of the humeral pale spot and the presector pale spot on the costa of the wing have been widely used to recognize *An. minimus* from *An. harrisoni* (Sharpe *et al.*, 1999; Van Bortel *et al.*, 1999; Rwegoshora *et al.*, 2002; Garros *et al.*, 2005b). However, using morphological characters of wing pattern alone often

leads to species misidentification. Green *et al*. (1990) obtained 37% misidentification, Van Bortel *et al*. (1999) otained 33% misidentification, and Sungvornyothin *et al.* (2006a) a lower percentage (13.2%). Several molecular-based tools have been developed that reliably identify individual species in the complex (Green *et al.*, 1990; Garros *et al.*, 2004a, b; Garros *et al.*, 2006; Sungvornyothin *et al.*, 2006a). Allozyme electrophoresis was first used to identify species within the *An. minimus* complex and related species, including *An. aconitus* (Green *et al.*, 1990; Van Bortel *et al.*, 1999). Although this technique remains the gold standard to identify the individual species within the Minimus Complex, meticulous and careful handling of specimens in which need to be frozen, is absolutely essential (Manguin *et al.*, 2008a). Recently, PCR-based methods examining DNA isolated from mosquitoes have been developed to identify members in this complex and other related species. (Sucharit and Komalamisra, 1997; Sharpe *et al*., 1999; Van Bortel *et al.*, 2000; Kengne *et al.*, 2001; Garros *et al.*, 2004a, b).

Even with correct identification and knowledge of where breeding sites for important vectors exist, decisions must be made regarding how to control target arthropods. Behavioral responses of the two important species within the Minimius Complex in response to insecticides in the presence of host stimuli are unclear. A few studies on the movement pattern of Anopheline mosquitoes have been performed in the field (Polsomboon *et al.* 2008; Malaithong *et al*., 2010) but none have been tested under laboratory-controlled conditions. The characterization of Anopheline mosquito response to insecticides includes both repellency and irritancy behaviors. Irritancy results from physical contact with chemical-treated surfaces. In contrast, repellency occurs when an insect detects the chemical in the vaport phase and avoids the treated surface without making physical tarsal contact (Chareonviriyaphap *et al*., 1997; Roberts *et al*., 1997; Potikasikorn *et al*., 2005). An excito-repellency test system developed by Chareonviriyaphap *et al*. (2002) has been used to quantitatively measure behavioral responses of female *An. harrisoni* and *An. minimus* mosquitoes following exposure to pyrethroid compounds. Specifically, comparisons of escape rates and time were made under contact (irritancy) and noncontact (repellency) test conditions with and without host cues.

OBJECTIVES

The objectives of this study were

1. To characterize the environmental variables that are associated with high-density breeding sites of *Anopheles harrisoni* and *Anopheles minimus* in order to identify predictors of species-specific habitat distribution.

2. To describe the larval density of *Anopheles harrisoni* and *Anopheles minimus* in two different ecological breeding habitats during different seasons.

3. To compare the reliability of a potential diagnostic character of *Anopheles harrisoni* and *Anopheles minimus* collected from two different ecological breeding habitats.

4. To quantify the behavioral responses of female *Anopheles harrisoni* and *Anopheles minimus* upon exposure to residual pyrethroid insecticides using an excito-repellency system in the presence and absence of live host.

LITERATURE REVIEW

1. Malaria

Malaria is one of the most important mosquito-borne infectious diseases caused by four different species of *Plasmodium* namely *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. These pathogens are transmitted to human by the bites of infective mosquitoes (Bruce-Chwatt, 1985). There are more than 3,500 known species of mosquitoes worldwide (Harbach, 2011). Out of that, only around 70 to 80 species of *Anopheles* mosquitoes are capable of transmitting the infection (Manguin *et al*., 2008b). Even though malaria caused by *P.vivax* is the most common, *P. falciparum* infection is most lethal (WHO, 2011). Moreover, the fifth *Plasmodium* species, *P. knowlesi*, that capable affects humans, a primate malaria parasite commonly found in Southeast Asia. This species causes malaria in long-tailed (*Macaca fascicularis*) and pig-tailed (*M. nemestrina*) macaques but reflect in humans, is either minor naturally or artificially tested in by *An. latens* mosquitoes, in the Kapit Division of Malaysian Borneo (Singh *et al*., 2004; Tan *et al*., 2008).

Malaria is widespread throughout the tropics, even though previously occurred in many temperate regions. Malaria is endemic in 106 countries with an estimated at 3.3 billion people living in malaria risk areas (WHO, 2011). These, 2.1 billion have been at low risk (< 1 reported case per 1000 population), 94% of whom are living in geographic regions other than the WHO African Region. The 1.2 billion who are at high risk (> 1 case per 1000 population) are living mostly in the WHO African (47%) and South-East Asia Regions (37%). There were an estimated 216 million episodes of malaria in 2010, with a wide uncertainty interval from 149 million to 274 million cases. Approximately 81%, or 174 million (113 - 239 million) cases, were in the African Region, with the South-East Asian Region accounting for another 13%. There were an estimated 655,000 (537,000 - 907,000) malaria deaths in 2010, of which 91% (596,000, range 468,000 - 837,000) were in the African Region. Approximately 86% of malaria deaths globally were of children under five years old (WHO, 2011).

Besides the chidren and pregnant women, malaria appears to have a great risk to traveler amd immigrants, with imported cases increasing in non-endemic areas. Treatment and control appear extremely difficult with the spread of drug-resistant strains of parasites and insecticide-resistant strains of mosquito vectors. Health education, better case management, better control tools and concerted action are needed to limit the burden of the disease. During the 1950s and 1960s, a vigorous campaign to eradicate malaria was waged through out the world with great success. The disease was in the process of being eliminated in some regions. But over the past few decades, resurgence is being witnessed. The dream of the global eradication of malaria is beginning to fade with the growing number of cases, rapid spread of drug resistance in people and increasing insecticide resistance in mosquitoes.

2. Malaria in Thailand

Malaria has significantly reduced over the past three decade (Bureau of Epidemiology, 2000-2011). The morbidity rate (per 100,000 population) of malaria has fallen from 83.54 cases in 2000 to 32.62 cases in 2011 (Table 1). Top ten provinces with high reported malaria cases in Thailand include Tak, Mae Hong Son, Ranong, Kanchanaburi, Yala, Chumphon, Trat, Phangnga, Chanthaburi, and Prachuap Khiri Khan (Figure 1). Four recognized species of human malaria parasite *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are found prevalent in Thailand (Chareonviriyaphap *et al*., 2000). *Plasmodium falciparum* and *P. vivax* are relatively common compared to *P. malariae* and *P. ovale*. The proportion of *P. vivax* has been increasing over the last forty years while *P. falciparum* seems to be reducing (Figure 2). The first case that infected in 2000 report on the fifth species of malaria, *P. knowlesi* in Thailand was published by Jongwutiwes *et al*. (2004). Additionally, three malaria cases infected with *P. knowlesi* have been reported in 2009, 2 cases in Yala and 1 case in Chanthaburi Provinces (Bureau of Epidemiology, 2009).

From recent studies, multidrug resistance of *P. falciparum* malaria has been reported along the Thailand-Cambodia border (Prasittisuk, 1985; Tan-ariya *et al*., 1995), and resistance has been confirmed against artemisinin (WHO, 2011). In 2009

and 2010, therapeutic drug efficacy studies in Mekong region countries including Thailand also have shown suspected artemisinin resistance in western Thailand along the Myanmar border as evidenced by ≥ 10% of cases with parasites detectable on day 3 after treatment with an artemisinin-based combination therapy (ACT). Day 3 parasite detection is one of earliest signs of potential artemisinin drug resistance (WHO, 2010). Besides drug treatment, one of the most effective methods for malaria prevention and control has been by vector control, mainly by use of routine residual insecticide spray inside houses and distribution of pyrethroid-impregnated bed nets.

For decades, DDT was used for malaria control as an indoor residual spray (IRS) in Thailand. DDT use was completely stopped for public health use in 2001 although a phase out period occured from 1995 to 1999 (Chareonviriyaphap *et al*., 2000). The reasons for the removal of DDT from malaria control in Thailand were due mainly to a perceived adverse impact on environment and a public negative attitude towards DDT. However, the true impact of DDT on mosquito vectors in terms of behavioral responses and disease transmission remains poorly understood and needs further clarification. In 2000, the mathematical framework for understanding the repellent, irritant and toxic properties of insecticides on mosquito populations and how they function in control of malaria has been proposed (Roberts *et al*., 2000). In addition, a new classification system for the actions of IRS chemicals used for malaria control has been suggested by Roberts *et al*. and found that DDT primarily functioned as the most repellent compound that keep mosquitoes outside of huts.

DDT was replaced by synthetic pyrethroids, deltamethrin and permethrin. The first has been primarily used in IRS whereas the latter has been applied as insecticide-treated netting (ITN). An alternative pyrethriod, bifenthrin is used to treat mosquito nets (Hougard *et al*., 2002, Batra *et al*., 2005, Chouaibou *et al*., 2006). The increased use of pyrethroids in IRS and ITN are generally more accepted by human populations. However, several countries in Africa have introduced, or are planning to reintroduce, DDT in IRS operations (WHO, 2007).

Table 1 Number of malaria cases, morbidity rate, deaths, case fatality rate, and mortality rate in Thailand 2000-2011

Year	Cases	Morbidity rate (per 100,000 population)	Deaths	CFR* (%)	Mortality rate (per 100,000 population)
2000	51,849	83.54	102	0.20	0.17
2001	34,925	56.25	81	0.23	0.13
2002	24,100	38.53	50	0.21	0.08
2003	19,910	31.63	31	0.16	0.05
2004	23,656	37.83	47	0.20	0.08
2005	28,131	45.23	71	0.25	0.11
2006	28,962	46.25	51	0.18	0.08
2007	30,889	49.08	38	0.12	0.06
2008	28,902	45.72	36	0.12	0.06
2009	23,229	36.61	19	0.08	0.03
2010	25,639	40.25	34	0.13	0.05
2011	20,835	32.62	11	0.05	0.02

*CFR (Case fatality rate) = the proportion of cases that is fatal within a specified time

Source: Bureau of Epidemiology (2000-2011)

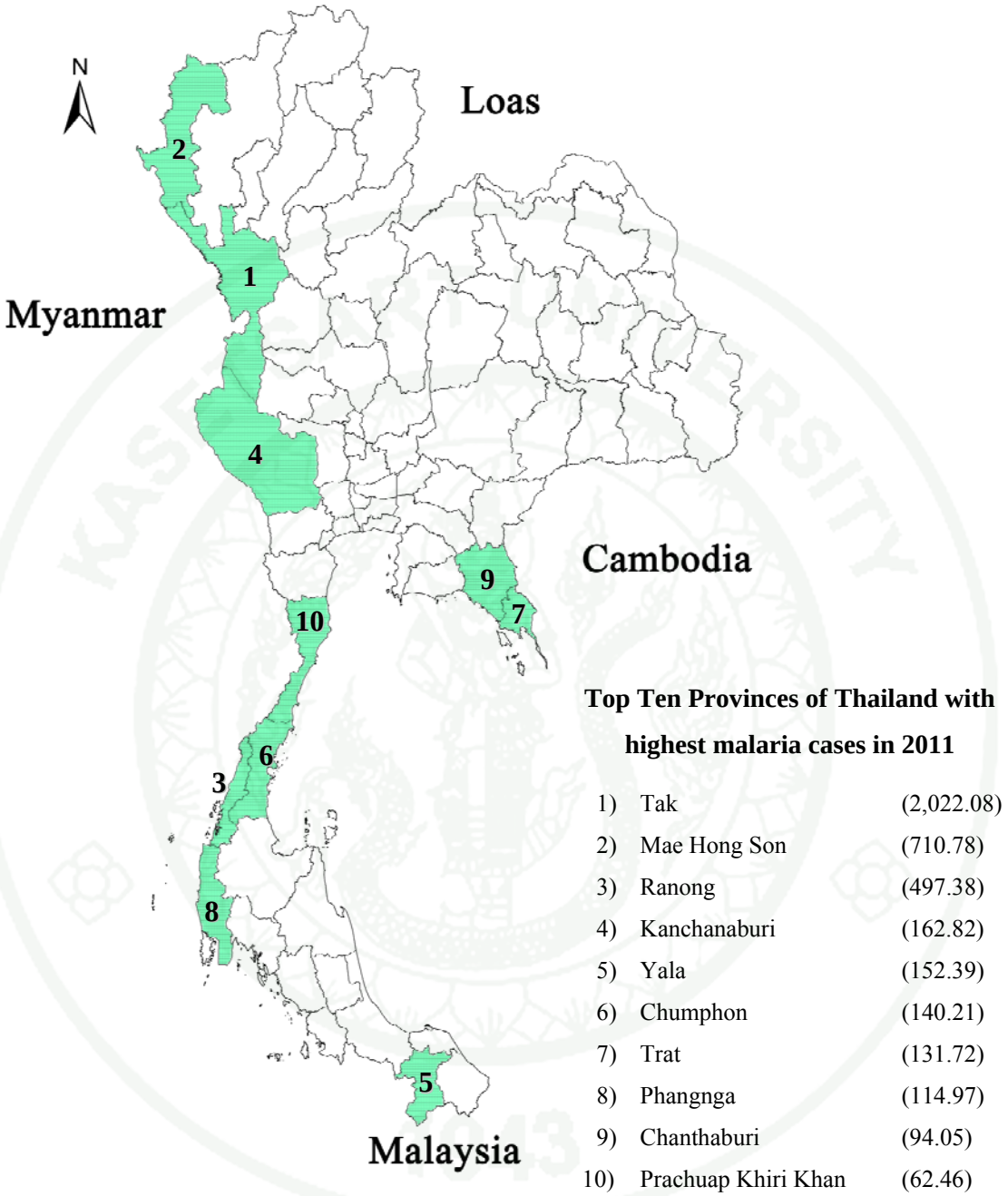

Figure 1 Map of Thailand, top ten provinces have had high reported malaria cases (mobidity rate, per 100,000 population) in Thailand, 2011

Source: Bureau of Epidemiology (2011)

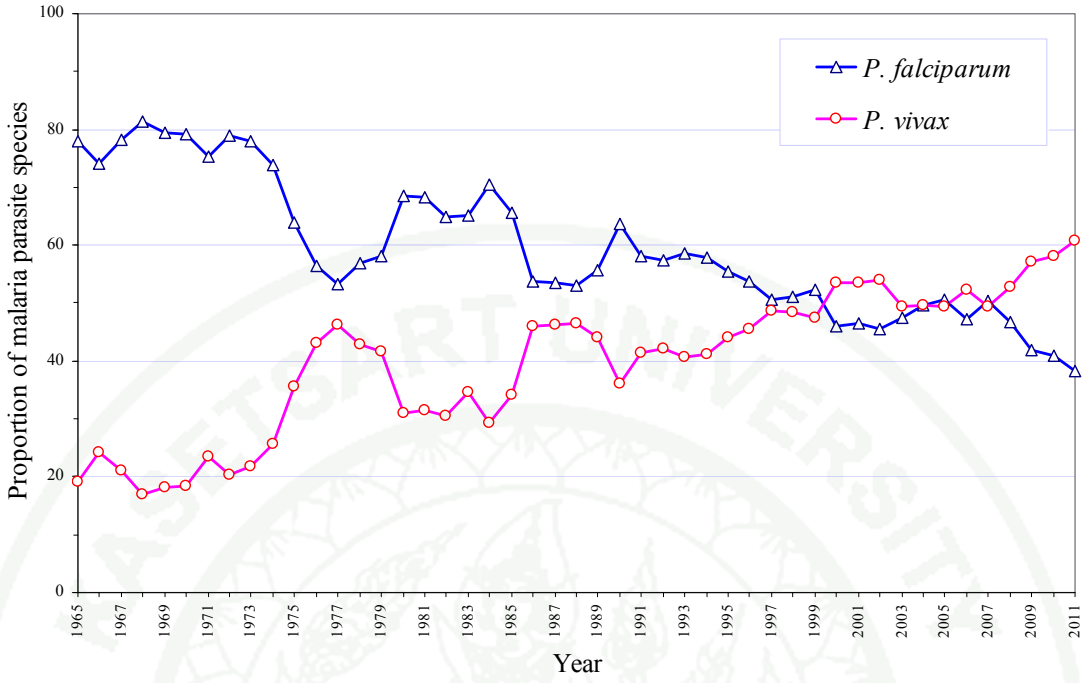

Figure 2 Proportion of malaria parasite species, *Plasmodium falciparum* and *Plasmodium vivax* in Thailand 1965-2011

Source: Bureau of Vector Borne Disease (2011**)**

3. Malaria vectors in Thailand

A well-described publication containing references to the Anophelines of Thailand has been published by Theobald (1910) whereas the first papers describing the genus *Anopheles* and the role of Anopheline species in the transmission of malaria in Thailand was produced by Barnes (1923a, b). He listed 17 species of *Anopheles* and included important notes on their biology and vector relationships. Thurman (1959) documented 47 identified species of anophelines in Thailand. Subsequently, 52 species of *Anopheles* known to occur in Thailand was recorded (Scanlon *et al*., 1968). Harrison *et al*. (1990) listed a total of 72 species of *Anopheles*, including four unnamed species that had been confirmed using cytogenetic and molecular techniques. Recently, a total of 73 species of *Anopheles*, including 71 named species, a new species near *An. gigas*, and an informally designated species, *An. minimus* C (formally named as *An. harrisoni* in 2007) was reported (Rattanarithikul *et al*., 2006).

In Thailand, primary malaria vectors are members of Leucosphyrus group (Neomyzomyia series), Maculatus group (Neocellia series) and Funestus group (Myzomyia series). Two species within the Neomyzomyia series, *An. baimaii* (former *An. dirus* species D*)* (Green *et al*., 1991) and *An. dirus* (Rosenberg *et al*., 1990; Green *et al*., 1991); one species within the Neocellia series, *An. pseudowillmori* (Green *et al*., 1991); and two species within the Myzomyia series, *An. minimus* (former *An. minimus* species A) (Rattanarithikul *et al*., 1996a), and *An. aconitus* (Gould *et al*., 1967; Green *et al*., 1991; Maheswary *et al*., 1992) are discriminated as a malaria vectors in Thailand.

4. Minimus Complex

The Minimus Complex belongs to Minimus Subgroup. It is a part of the Funestus Group which includes four other subgroups; Aconitus, Culicifacies, Funestus, and Rivulorum (Harbach, 2011). This complex comprises three genetically related species: (1) *An. harrisoni* Harbach & Manguin (former species C), (2) *An. minimus* Theobald (former species A) and (3) *An. yaeyamaensis* Somboon & Harbach (former species E).

The genetic evidence for the Minimus Complex was first provided for populations in Thailand (Green *et al*., 1990), then confirmed in Vietnam (Van Bortel *et al*., 1999), where sympatric homozygotes at the Odh (Octanol deshydrogenase) locus occurred in the absence of heterozygotes. The species were informally named *An. minimus* species A and C (Green *et al*., 1990). The Minimus Complex was confirmed using molecular markers. The single-strand conformational polymorphism-polymerase chain reaction (SSCP-PCR) assay (Sharpe *et al*., 1999). The study by Chen *et al*. (2002) showed that *An. minimus* forms A and B in China (previously described by Yu and Li, 1984; Yu, 1987) are morphological variants of *An. minimus*. In Japan, Somboon *et al*. (2001, 2005) provided morphological, cytogenetic, and molecular and hybridization evidence for the recognition of another sibling species, designated species E, on the Ishigaki Island of the Ryukyu Archipelago. Crossing experiments between species E and either species A or C showed that F1 crosses were sterile (Somboon *et al*., 2001, 2005). Both studies showed hybrid male sterility, which is generally accepted as very clear evidence of specific status. There is now no reason to cast doubt on the specific status of species E (Van Bortel and Coosemans, 2003). However, the complex may include two other species, *An. minimus* species D (Baimai, 1989) and specimen no. 157 (Sharpe *et al*., 1999) in Thailand. The specific status of these two entities is uncertain and needs further study. It seems that species D is a chromosomal variant of *An. minimus* species A (V. Baimai, unpublished data). Today, the taxonomy of the Minimus Complex is complete. Harbach *et al*. (2006) designated a neotype to fix the identity of *An. minimus s.s*., *An. harrisoni* was recently described and named by Harbach *et al*. (2007). Based on available genetic, molecular

and morphological distinctions, species E is formally described and named as *An. yaeyamaensis* (Somboon *et al*., 2010). Despite with the formal taxonomy, the three species cannot be distinguished based on morphology (Jaichapor *et al*., 2005; Sungvornyothin *et al*., 2006a) and their separation from closely related sympatric species is problematic due to overlapping characters. The situation is complicated by the morphological variability of *An. minimus* (Jaichapor *et al*., 2005).

Anopheles harrisoni is confined to a particular site of China (Chen *et al*., 2002), Laos (Phuc *et al*., 2003), Thailand (Sharpe *et al*., 2000; Kengne *et al*., 2001; Garros *et al*., 2006; Manguin *et al.*, 2008a), and Vietnam (Van Bortel *et al*., 1999; Phuc *et al*., 2003; Garros *et al*., 2008), whereas *An. minimus* is distributed primarily in the India-Indochina peninsular regions (Harrison, 1980). *Anopheles harrisoni* occurs in sympathy with *An.minimus* in several locations in southern China (Chen *et al*., 2002), northern Laos (Phuc *et al*., 2003), northern and western Thailand (Sucharit, 1988; Sungvornyothin *et al*., 2006a), and northern and southern Vietnam (Van Bortel *et al*., 1999; Phuc *et al*., 2003; Garros *et al*., 2008). *Anopheles yaeyamaensis* has a restricted distribution in the Yaeyama and Miyako Island groups, Ryukyu Archipelago of Japan (Somboon *et al*., 2001; Somboon *et al*., 2010). In Thailand there are two species in the Minimus Complex, *An. minimus* is widespread throughout the country (Sucharit *et al*., 1988; Baimai, 1989; Green *et al*., 1990) and *An. harrisoni* was previously collected from Mae Sot in Tak Province and Mae Rim in Chiangmai Province, North of Thailand in 2006 but no clear confirmation was made at the time (R. Rattarithikul, unpublished data). A previous recently study by Sungvornyothin *et al*. (2006) reported that *An. harrisoni* was collected and described by using a molecular identification assay from Ban Nam Dip, Mae Sot District, Tak Province. Furthermore, *An. harrisoni* is found predominantly in western Thailand, particularly Kanchanaburi Province (Sucharit *et al.*, 1988; Green *et al.*, 1990; Sharpe *et al.*, 1999), especially in two villages of Sai Yok District, Pu Ong Ka Village (Kengluecha *et al.*, 2005; Rongnoparut *et al.*, 2005) and Pu Teuy Village (Sucharit *et al.*, 1988; Sungvornyothin *et al.*, 2006a, b) where both sibling species can be found in sympatry. Additionally, other closely related species in the Aconitus Subgroup (*An. aconitus, An. pampani, An. varuna*), the Jeyporiensis Subgroup (*An. jeyporiensis*), and the

Culicifacies Subgroup (*An. culicifacies*) can occupy similar habitats as that of *An. minimus* Subgroup (Rattanarithikul *et al*, 1995; Rattanarithikul *et al.*, 2006).

Members of the Minimus Complex occur is the forested foothill and hilly regions with clear-water canals and streams and slow moving current (Harrison, 1980). However, larvae of *An. minimus* are also found in water tanks in the suburban of Hanoi, Vietnam (Van Bortel *et al*., 1999, 2003). In western Thailand, *An. harrisoni* was found in fewer types of habitats than *An. minimus*, which occurs in a variety of habitats ranging from agricultural fields to forests with a closed canopy (Rongnoparut *et al*., 2005).

Genus *Anopheles*

Subgenus *Cellia*

Myzomyia Series

Funestus Group (Garros *et al*., 2005a)

jeyporiensis James

Aconitus Subgroup (Chen *et al*., 2003)

aconitus Dönitz

filipinae Manalang

mangyanus (Banks)

pampanai Büttiker & Beales

varuna Iyengar

Culicifacies Subgroup (Garros *et al*., 2005a)

culicifacies Giles (species A, B, C, D and E) (Kar *et al*., 1999)

Funestus Subgroup (Garros *et al*., 2005a)

aruni Sobti

confusus Evans & Leeson

funestus Giles

funestus-like species (Spillings *et al*., 2009)

longipalpis (Theobald) (Type C) (Koekemoer *et al*., 2009)

parensis Gillies

vaneedeni Gillies & Coetzee

Minimus Subgroup (Chen *et al*., 2003)

flavirostris (Ludlow)

leesoni Evans

longipalpis (Theobald) (Type A) (Koekemoer *et al*., 2009)

Fluviatilis Complex (Sarala *et al*., 1994)

fluviatilis James (species S, T and U)

Minimus Complex (Green *et al*., 1990)

harrisoni Harbach & Manguin (species C)

minimus Theobald (species A)

yaeyamaensis Somboon & Harbach (species E)

Rivulorum Subgroup (Garros *et al*., 2005a)

brucei Service

fuscivenosus Leeson

rivulorum Leeson

rivulorum-like species (Cohuet *et al*., 2003)

Figure 3 Taxonomic hierarchy of *Anopheles minimus* complex

Source: Harbach (2011)

6. Geographic information system (GIS) and remote sensing

Geographic information system (GIS) is a powerful tool for studying and mapping the spatial relationships of objects. A GIS is a computer program designed to collect, retrieve, transform, display, and analyze spatial data. GIS can incorporate georeferenced data to produce maps or layers (Gilbert, 1997). The spatial data managed by a GIS program may include items such as localities where mosquitoes have been collected, densities of mosquitoes, and distribution of mosquitoes relative to environmental parameters, such as elevation, precipitation, and vegetation, including human artifacts, such as political boundaries or roads, and crop field boundaries. Similar data are typically grouped into individual layers or themes. The display of these layers can be turned on or off and modified to display various data. An important strength of the GIS is that it can simultaneously relate layers of data at the same points in space and can analyze and map out the results (Noonan, 2003).

Remote sensing is the acquisition of information about an object or phenomenon, without making physical contact with the object. Remote sensing data, such as aerial photos and satellite imageries, is usually done with the help of mechanical devices known as remote sensors. Often, these sensors are positioned away from the object of interest by using helicopters, planes, and satellites. Most sensing devices record information about an object by measuring an object's transmission of electromagnetic energy from reflecting and radiating surfaces (Pidwirny, 2006). As remote sensing techniques provide valuable information on such environmental conditions (Hay *et al*., 1997; Beck *et al*., 2000), several studies have used remote sensing imagery to map the distribution of vector species at different spatial scales (Srivastava *et al*., 2001; Benedict *et al*., 2007; Vanwambeke *et al*., 2007). Remote sensing techniques are becoming increasingly important for identifying mosquito habitats, investigating malaria epidemiology and assisting malaria control.

Success of the approach to select for a particular vector control intervention requires a good stratification of control areas in time and space, accurate information

on vector biology and ecology, detailed information on vectorial capacity, and malaria transmission and epidemiology. Therefore, both correct species identification and real-time monitoring of the geographical distribution are needed to provide accurate information on elucidating the nature of the malaria vector species complex. In Thailand, the potential applications of remote sensing or GIS technologies have been demonstrated in studying the epidemiology of dengue hemorrhagic fever (Sithiprasasna *et al*., 1997), to identify the breeding habitats of major malaria vectors and their distribution (Sithiprasasna *et al*., 2003), to survey for dengue virus-infected *Aedes* mosquitoes (Sithiprasasna *et al*., 2004), and to predict malaria transmission risk (Sithiprasasna *et al*., 2005). In addition, Rongnoparut *et al*. (2005) was used these techniques to characterize the breeding habitats of *Anopheles minimus* complex in five different districts of Kanchanaburi Province in western Thailand.

Most larval habitats are highly localized and can be described in terms of environmental parameters such as elevation, precipitation, temperature, vegetation and water quality. Larval habitat quality may, however, be inferred by using vegetation as an indicator of environmental conditions necessary for successful oviposition and larval/pupal survival (Gabinaud, 1987). Remote sensing data can be used at a regional scale to identify and monitor temporal and spatial vegetation characteristics which can then be used to infer attributes of larval habitat (Jovanovic, 1987). Successful larval control is, in part, dependent on the ability to identify larval habitat and distinguish between high and low-producing sites in a timely manner.

8. Behavioral responses to insecticides

Behavioral responses can be objectively and quantitatively assessed by using an excito-repellency test system (Roberts *et al*., 1997). It was first developed in 1970 in an attempt to access the behavioral responses of mosquitoes to insecticides (WHO, 1970). Through several studies, the test system has been further modified and improved to evaluate the behavioral responses of various mosquito species (Quinones and Suarez, 1989; Ree and Loong, 1989; Evans, 1993). In 1997, the improved test system distinguished between two distinct types of behavioral responses

(Chareonviriyaphap *et al*., 1997; Roberts *et al*., 1997): contact irritancy (defined as insects leaving an insecticide-treated surface after tarsal contact with the residual chemical) and noncontact repellency (an insecticide to act from an area effect, diverting insects away from treated surfaces without actual physical contact with the chemical). Later, a portable version was developed that allowed direct assessment of mosquito behavior at field sites (Chareonviriyaphap *et al*., 2002).

Mosquito behavioral responses to insecticides have been previously documented by many investigators using experimental huts, semi-field and field studies to describe the movement patterns, host-seeking and resting behavior of female mosquitoes in the presence of hosts with or without exposure to insecticide (Cullen and De Zulueta, 1964; Grieco *et al*., 2000; Chareonviriyaphap *et al*., 2005; Suwonkerd *et al*., 2006; Malaithong *et al*., 2010). Other studies include determination of the influence of environmental factors (temperature, humidity, precipitation, wind, etc.) on seasonal mosquito biting activity and periodicities (Rowley and Graham, 1968; Clements, 1999; Suwannachote *et al*., 2009; Paaijmans and Thomas, 2011). Previous studies have been reported on behavioral responses on the synthetic chemicals (Grieco *et al*., 2000; Potikasikorn *et al*., 2005; Chareonviriyaphap *et al*., 2006; Pothikasikorn *et al*., 2007). Recently works on the behavioral response to insecticides (DEET and bifenthrin) of *An. minimus* and *An. harrisoni* was studied by Tisgratog *et al*. (2011) reported that field-collected *An. minimus* demonstrated a more rapid escape response to DEET than to bifenthrin, whereas *An. harrisoni* showed a converse response. A study by Boonyuan *et al*. (2011) to access the escape responses of mated and unmated nulliparous *Ae. aegypti* mosquitoes to deltamethrin in the presence or absence of a live animal host using an excito-repellency test system and revealed that deltamethrin concentrations decrease to sublethal levels, mating status and host cues play a more significant role in escape behavior of *Ae. aegypti* mosquitoes.

MATERIALS AND METHODS

Part 1 Survey of two species in the Minimius Complex, *Anopheles harrisoni* and *Anopheles minimus* from two different potential breeding sites

1.1 Study sites

The study was conducted in two villages of Pu Teuy (PT) and Bong Ti Noy (BTN), Sai Yok District, Kanchanaburi Province, Thailand (Figure 4). Pu Teuy is considered nearly malaria-free, and only a few cases are documented each year (Vector Borne Diseases Control Unit, 2006, 2007, 2008). The site is the potential site for *An. harrisoni* (Sungvornyothin *et al*., 2006b) and located in a hilly zone and mostly surrounded by primary and secondary forest and with agricultural area. The larval collection site in PT (14^{o} 20′ N, 98^{o} 59 ′E) is a flowing small stream running through the village (~300 m above sea level). The PT stream covered with native vegetation along its margin.

In BTN, the occurrence of malaria cases was higher than in PT (Table 2). This study site is the potential site for *An. minimus* (Phothikasikorn, 2006). The site is also located in a hilly zone and surrounded by forest and agricultural area. The larval collection site (14^{o} 17′ N, 99^{o} 11′ E) was a seasonal running stream (~100 m above sea level) bordered by grasses along its margins in dry season. This stream becomes a river in wet season.

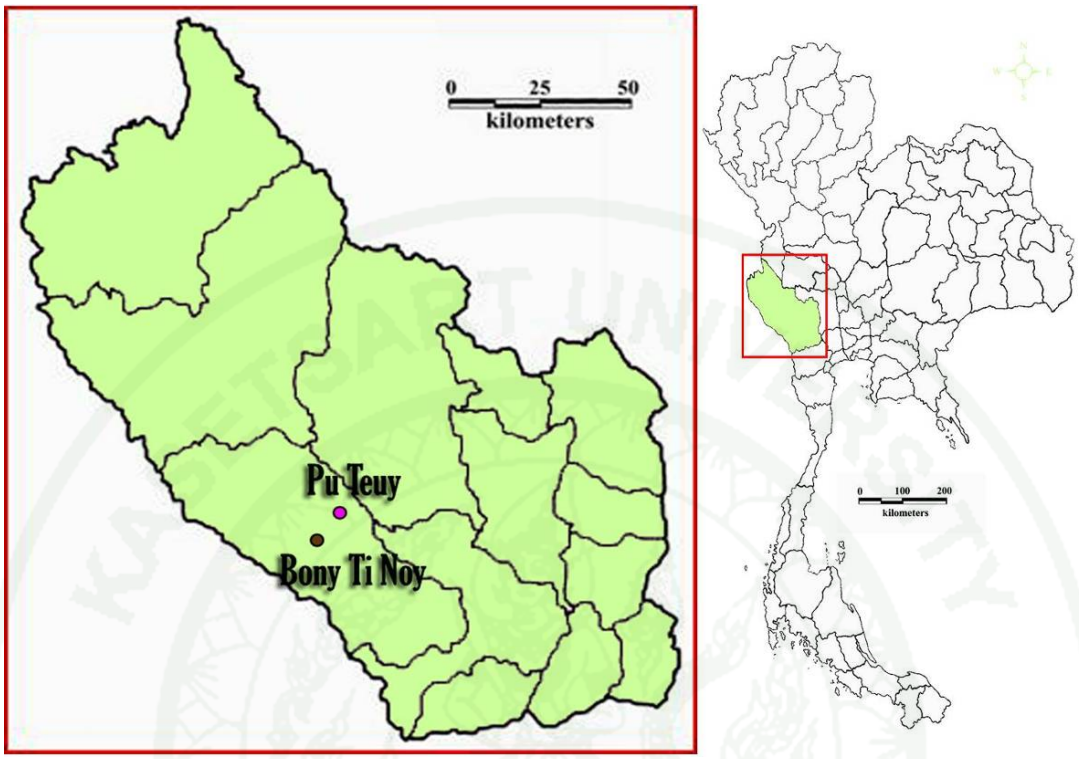

Figure 4 Location of study sites, Bong ti Noy and Pu Teuy Villages, Sai Yok District, Kanchanaburi Province

Table 2 Characters of the two study sites, Bong ti Noy and Pu Teuy Villages, Sai Yok District, Kanchanaburi Province

Characters	Bong Ti Noy	Pu Teuy
1. Population[1]	524, 462, 475	496, 476, 378
2. Malaria cases[1]	36, 39, 39	3, 3, 0
• Thai	21, 27, 28	0, 0, 0
• Non-Thai	15, 12, 11	3, 3, 0
3. Common species	*An. minimus*	*An. harrisoni*
4. Altitude	~100 m above sea level	~300 m above sea level
5. Collection sites	Stream becomes a river in wet season	Small stream

[1] Data during 2006 to 2008 from Vector Borne Diseases Control Unit 4.1.5 (Tha Sao Subdistrict, Sai Yok District, Kanchanaburi Province), Bureau of Vector Borne Disease, Ministry of Public Health

1.2 Larval collections

Anopheline larvae were sampled along the same stretch of stream during a two year period at December 2006 - November 2008, every month at PT and once every two months at BTN. Three teams of two collectors each performed the larval sampling in the morning (0800-1200 h) and afternoon hours (1300-1600 h). Ten dips per collector were performed along the stream margins at each sentinel point (~30 m distance between sentinel points) with 20 dips total taken at each location per collection period. A total of 73 different points at PT and 58 points at BTN were sampled along a designated area of the stream. Coordinates of sampling points were recorded using a hand-held Global Positioning System device (Garmin GPS Map76s).

All larval mosquitoes were kept alive in 200 ml plastic bags at 20-25 ºC in a styrofoam box during transport from Kanchanaburi to the insectary of Kasetsart University, Bangkok, for processing and species identification. Larvae were

categorized into different instar stages, followed by counting and recording, and then reared in 3.5 inches diameter plastic bowl (15-20 larvae per bowl) under identical physical and nutritional conditions throughout the study period.

1.3 Environmental variables

The physical and chemical characteristics of larval habitat, depth and width of water body, temperature, velocity, turbidity and the density of vegetation (cover water surface, emergent, and submerge vegetation) as well as debris at each sampling point were recorded for each sampling period and throughout the study. Water pH and conductivity were measured in the second year of the study.

Water temperature was measured in the middle of the stream and 10 cm below the water surface or at a half of the water depth if the depth was less than 10 cm. The velocity was measured by timing a float of a table tennis ball at 1 m distance to calculate the velocity (m/s). Water turbidity was measured using a secchi disc. Turbidity measurements were conducted by lowering the secchi disc until it disappeared and this depth noted. The secchi disk was then raised until it reappeared and this depth noted. The secchi depth is the midpoint between these two depths (Figure 5). If the two depths differ by more than 10 cm, the measurement was repeated. Water samples from each sampling point (at 10 cm depth of the stream) consisted of using a plastic bottle to obtain 120 ml of water and each sample maintained at 4 ºC until measuring water pH and conductivity using a Consort C831 multi-channel analyzer.

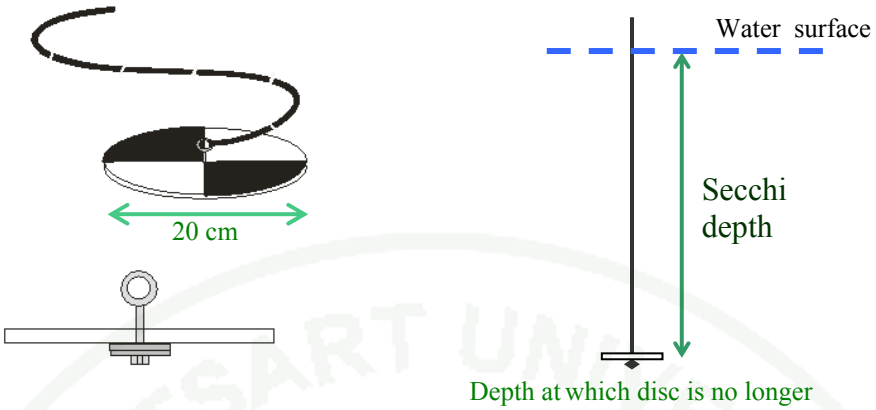

Figure 5 Using secchi disc for turbidity measurement

The density of vegetation (riparian canopy coverage, emergent, and submerge vegetation) as well as debris surrounding each sampling point in a 1 m^2 area (0.5 m from the bank and 1 m long on both sides) was classified (Figure 6). The density of vegetation was scored as 0 to 3, 0 (< 1%), 1 (1-20%), 2 (21-50%) and 3 (> 50%).

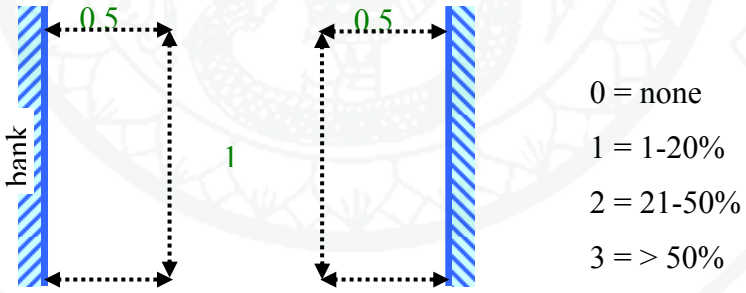

Figure 6 Scoring the vegetation and debris density in a 1 m^2 area

Other attributes such as houses, roads, major intersection, and boundaries of crop fields was marked as separate GPS points for image processing. Precipitation data was obtained from the Sai Yok District Meteorological Station, Thai Meteorological Department, located near the village.

1.4 Morphological identification

Larval anophelines were carefully reared to adults and morphologically identified (Rattanarithikul *et al.*, 2006). All larvae were reared under the insectary condition at 25 ± 5 ºC and 70 ± 10% relative humidity, with a 12:12 h light:dark photoperiod. For the adult specimens belonging to the Minimus Complex were initially identified as either *An. minimus* if the presector pale (PSP) spot was present on the wing costa or as *An. harrisoni* if the humeral pale (HP) spot phenotype was present (Sungvornyothin *et al.*, 2006a) and on at least one of the wings for this study (Figure 7).

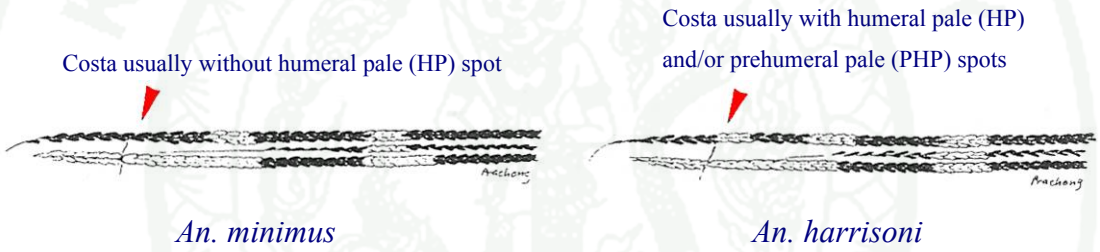

Figure 7 Morphological characters of mosquito wing use to identify adult mosquitoes, *Anopheles minimus* and *Anopheles harrisoni*

Source: Rattanarithikul *et al.* (2006)

1.5 Molecular identification

Only specimens of the Minimus Subgroup and related species in the Aconitus Subgroup were subjected to molecular identification using an allele-specific multiplex assay examining the ITS-2 regions of DNA (Garros *et al.*, 2004b). Mosquitoes were individually processed and genomic DNA extracted from the whole body. Following amplification of DNA using a PCR method, species-specific primers were used in the one-step reaction to differentiate *An. minimus*, *An. harrisoni*, *An.*

aconitus, *An. pampani*, and *An. varuna*. In a final volume of 25 μL, PCR amplification conditions are as follows: 2.5 μL of 10x reaction buffer, 2.5 mM of each dNTP, 10 μM of each primer, 0.5 units of Taq polymerase, and 0.5 μL of DNA template. The PCR cycles are as follows: one cycle at 94 ºC for 2 minutes, following by 40 cycles at 94 ºC for 30 seconds, 50 ºC for 30 seconds, and 72 ºC for 40 seconds each. An additional auto extension cycle at 72 ºC for 10 minutes was included at the end. The PCR products were subjected to electrophoresis on a 2% agarose gel at 100 v for 25 minutes and stained with GelStar® (Lonza Rockland, Inc., Maine, USA).

1.6 Land use mapping and analysis

Land cover was assessed using aerial photographs of both study areas (Figures 8A and 8B) which was taken in 2006. Land cover was manually digitized with ArcGIS 9.2 program (ESRI, Inc., USA) using the following classes: forest, shrub, plantation, and pasture within a 1 km buffer zone (Figure 8) around each sampling point (i.e., probable flight range of *Anopheles* mosquito from breeding habitat). Percentage of each land cover class was then quantified within 5, 10, 20 and 30 m individual buffer zones surrounding high and low density habitats at both sites. Whereas in dry and hot seasons at BTN, in 5-10 m radius around a sampling point was surrounded by a dry stream bed that contains water only during times of heavy rain, then percentage of each land cover class was quantified within 40 m buffer zones as well.

The physical and chemical variables between high and low density habitats were compared using an independent t-test. Statistical significance for all tests of probability was set at 5% ($P < 0.05$).

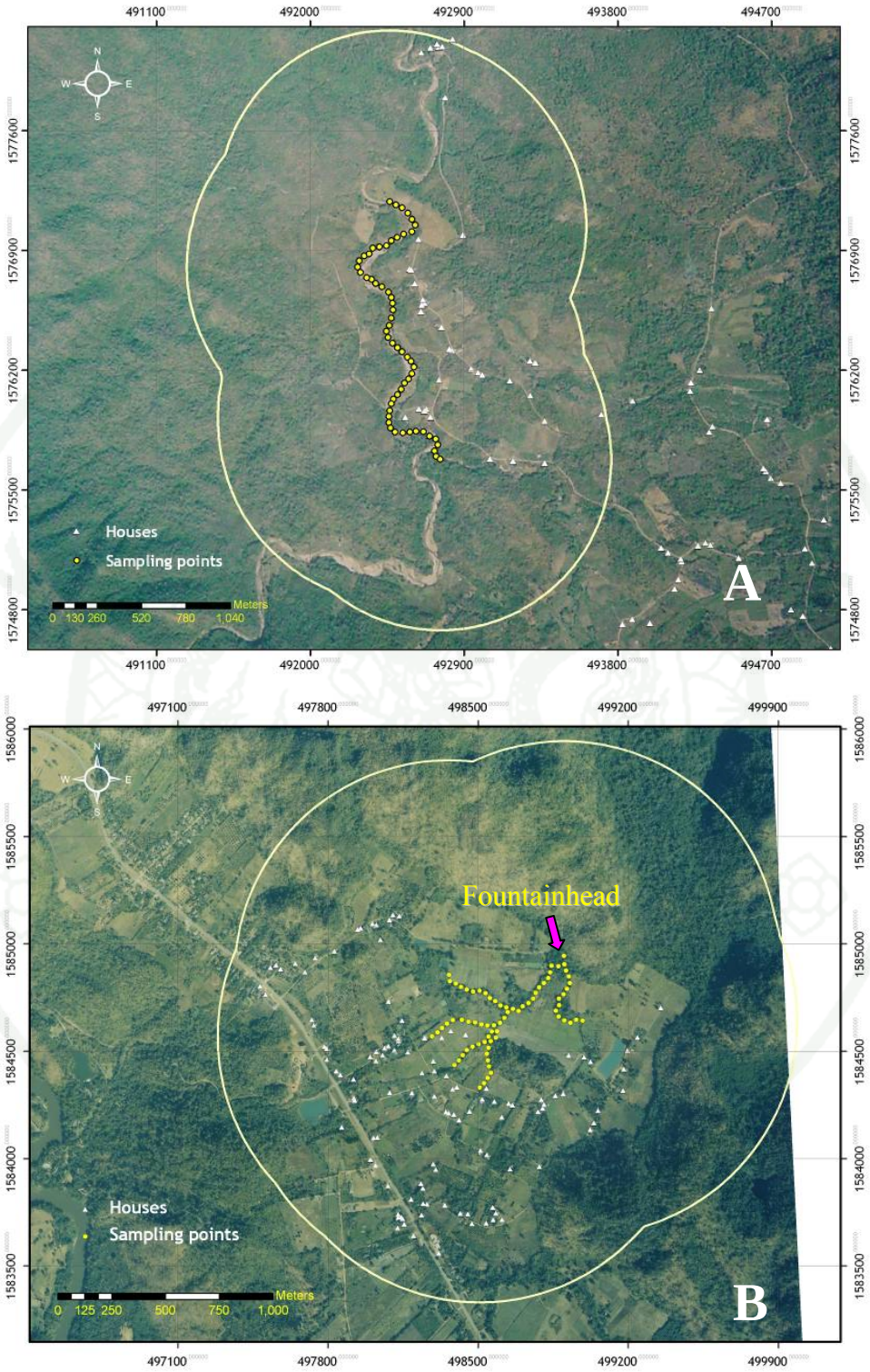

Figure 8 Buffer zone of 1 kilometer around all sampling points (yellow circle) in study areas (A) Bong Ti Noy and (B) Pu Teuy of Sai Yok district, Kanchanaburi province, Thailand

Part 2 Seasonal abundance of larvae of *Anopheles harrisoni* and *Anopheles minimus* in two different potential breeding sites

2.1 Study sites

The sampling survey was conducted in PT and BTN villages in Sai Yok District, Kanchanaburi Province. Collection site is the main stream of both villages as previously described in Part 1.

2.2 Larval collections

Anopheline larvae were sampled along the same stretch of stream during December 2006-November 2008, every month at PT and once every two months at BTN. Three teams of two collectors each performed the larval sampling. Ten dips per collector were performed along the stream margins at each sentinel point with 20 dips total taken at each location per collection period. A total of 1,460 dips (73 sampling points at PT) and 1,160 dips (58 points at BTN) of larval sampling each month. The numbers of collected larvae were recorded for each life stage and were reared to adult and identified to species by morphological and molecular identification as previously described in Part 1.

2.3 Environmental variables

The physical and chemical characteristics of water body, depth and width of water body, temperature, velocity, and turbidity were recorded each sampling period and throughout the study. For water quality, pH and conductivity was measured in the second year of the study. The detail of measure methods as previously described in Part 1.

2.4 Data analysis

Statistical analyses of data (SPSS Version 16.0 for Windows, SPSS Inc., Chicago, IL) included the Pearson Chi-square test to determine the homogeneity of the proportion of mosquitoes, by species, collected each period. The relationship between larval mosquito density and rainfall was determined by simple regression analysis. Variation in larval density of targeted species between seasons and year was compared by a generalized linear model (GLM) univariate analysis followed by a least significant difference test. The seasonal differences, based on the rainfall patterns, were classified accordingly as, “dry” (December to February), “hot” (March to May) and “wet” (June to November). The number of each mosquito species was recorded and compared by season. The physical and chemical attributes of water between seasons and year was compared using a paired t-test. Statistical significance for all tests of probability was set at 5% ($P < 0.05$).

Part 3 Identification of the two species of the Minimus Complex, *Anopheles harrisoni* and *Anopheles minimus* collected from two different ecological breeding habitats by morphological and molecular identification

3.1 Study sites

The study was conducted in two villages of PT and BTN in Sai Yok District, Kanchanaburi Province. PT is the potential site for *An. harrisoni* and BTN is the potential site for *An. minimus.*

3.2 Larval collection

Anopheline larvae were sampled during December 2006-November 2008, every month at PT and once every two months during the same time period at BTN. The methodology of larval collection was explained in Part 1. All *Anopheles* larvae sampling each month were reared to adult and identified to species by morphological and molecular identification.

3.3 Morphological identification

All adults were identified by using the morphological characters follow standard key (Rattanarithikul *et al.*, 2006). Proboscis morphology (the presence of pale scaling on the distal half of the proboscis) was used to separate *An. aconitus* from Minimus Subgroup and other related species (*An. varuna*). The absence of PSP spot was used to separate *An. varuna* from Minimus Complex. For Minimus Complex specimens identified as either *An. harrisoni* if the HP spot phenotype was present and on at least one of the wings for this study or as *An. minimus* if the PSP spot was present on the wing costa (Appendix Figure 1)

3.4 Molecular identification

Following morphological identification, only specimens of the Minimus Subgroup and related species in the Aconitus Subgroup were subjected to molecular identification using an allele-specific multiplex assay examining the ITS-2 regions of DNA (Garros *et al.*, 2004b) as previously described in Part 1. Lengths of amplified species-specific products were 180 bp for *An. harrisoni*, 200 bp for *An. aconitus*, 260 bp for *An. varuna*, and 310 bp for *An. minimus* (Figure 9).

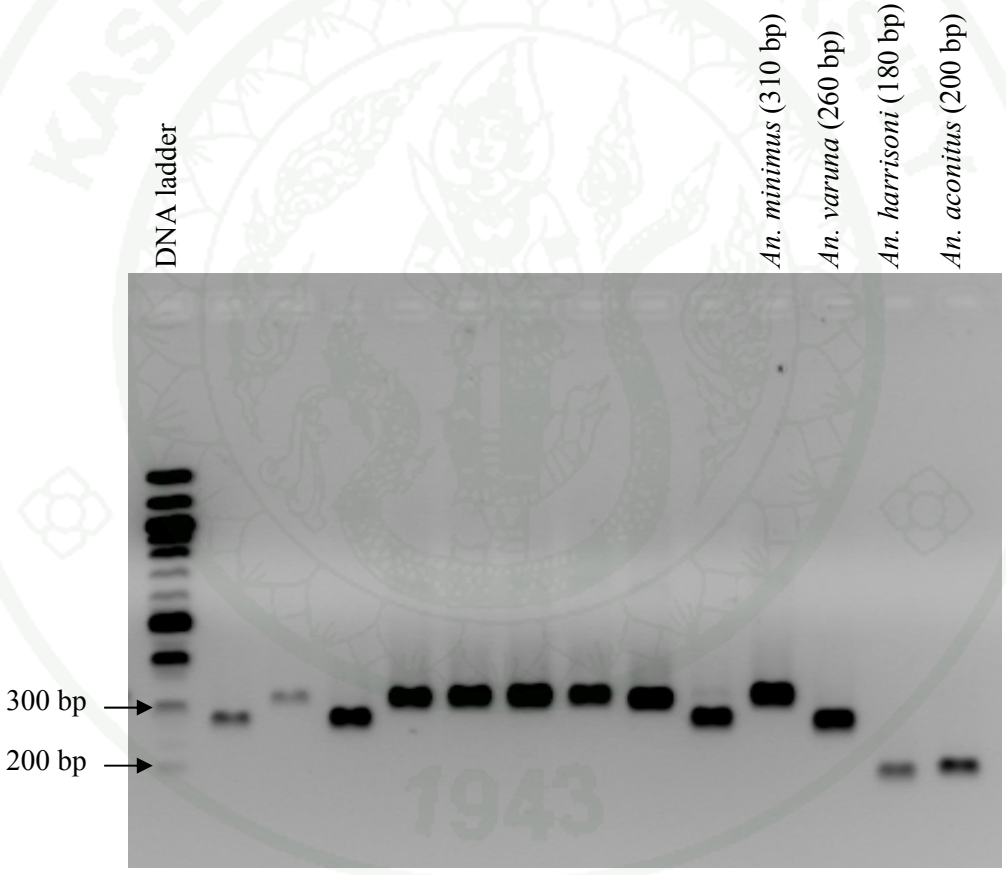

Figure 9 Amplified fragments using an allele-specific multiplex assay for identifying members of the Minimus Subgroup and Aconitus Subgroup

3.5 Data analysis

To test the reliability of the presence/absence of the specific characteristics to identify the species in a group of closely related species, biomedical tests will be used (Altman, 1991) to evaluate diagnostic power of the morphological character. Several values provide insights on the reliability of the test (Appendix Figure 2). The sensitivity and specificity will be calculated, by comparing the observed test outcome with the produce of the golden standard, i.e. the molecular identification. Another way to characterize a diagnostic test was to calculate the proportion of correctly classified individuals as an index of validity (Iv). The index of validity is the probability of agreement between the molecular and the morphological identifications. The three paired of reliability test, (1) *An. aconitus* vs Minimus Complex (*An. harrisoni* and *An. minimus*) and other related species (*An. varuna*), (2) *An. varuna* vs. Minimus Complex, and (3) *An. harrisoni* vs *An. minimus*. The positive predictive value (PPV) provides the probability of having *An. aconitus* if the proboscis with distal pale area on dorsum and venter was present, as *An. varuna* if the costa without PSP spot was present, and as *An. harrisoni* if the HP phenotype is present. There is a corresponding negative predictive value (NPV) predicting the probability of rightly identifying Minimus Complex or *An. varuna* if the proboscis entirely dark or with ventral pale patch, as Minimus Complex if the costa with PSP spot was present, and as *An. minimus* if the PSP phenotype is present (Appendix Figure 1).

Part 4 Behavioral responses of *Anopheles harrisoni* and *Anopheles minimus* to bifenthrin and deltamethrin using an excito-repellency system and a live host

4.1 Mosquito populations

Two anopheline mosquito species were used in this study: *An. harrisoni* and *An. minimus*. *Anopheles harrisoni* egg batches (F_{18} - F_{20} generations), originally collected from PT, Kanchanaburi Province, Thailand, were received from the insectary at Chiangmai University. *Anopheles minim*us was originally collected from Prae Province, northern Thailand, in 1993 and maintained in insectary-controlled conditions at Department of Communicable Disease Control (CDC), Ministry of Public Health, Nonthaburi, Thailand. Egg batches (F_{87} - F_{90} generations) were subsequently received from CDC and both species were raised in the insectary at Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Female mosquitoes were permitted to imbibe human blood on the third day after emergence for egg development. Larval stages were reared in enameled pans under identical physical and nutritional conditions throughout the study period. Four- to 7-day-old female mosquitoes were used for all tests. All adult test mosquitoes were denied blood feeding and provided with only 10% sugar solution for nutritional energy. The sugar solution was removed and mosquitoes starved for 24 h before the test.

4.2 Insecticide-impregnated papers

Test paper (Whatman® No. 1) 27.5 cm × 35.5 cm was impregnated with standard field dose: 0.025 g/m^2 of bifenthrin and 0.02 g/m^2 of deltamethrin. All treated papers were prepared according to World Health Organization specification (WHO, 2006); a filter-paper impregnated at 1% contains 6.6 mg of technical insecticide, or 367 mg/m². Control paper was impregnated with solvent (acetone and mixed with silicon oil) only.

4.3 Excito-repellency test system

The test system has been previously described by Chareonviriyaphap *et al.* (2002) with recent design changes presented in Boonyuan *et al.* (2011) (Figure 10A). The system consists of four chambers for contact trials and four chambers for noncontact trials. For contact trials, the impregnated papers are lined on the inside of the screened inner chamber, allowing mosquitoes to rest directly on the treated inner surface making tarsal contact. For noncontact trials, the impregnated papers are lined on the outside of screened inner chamber, which prevents mosquitoes resting directly on the surface of the impregnated paper and thereby without making tarsal contact. Each trial comprised two paired control (untreated) chambers (with and without host) and two paired test (treated) chambers (with and without host) as shown in Figures 10C and 10D.

Twenty-five female mosquitoes were introduced into each test chamber (control and treated) using a mouth aspirator. Mosquitoes were allowed a 3-min period for adjustment to chamber conditions after which the escape funnels were opened to begin the observation period. The numbers of mosquitoes escaping from the chamber containing the control and treated papers and into the receiving cage were recorded at 1-min intervals for a total 30-min observation period. Immediately after the 30-min exposure time expired, the number of dead or knocked-down mosquitoes that remained inside the test chamber and those that escaped was recorded separately. All mosquitoes that either escaped or remained inside the chamber were collected and held separately by category of response in small holding containers with cotton pads soaked in 10% sugar solution to monitor 24-h mortality rates. All assays were replicated four times per treatment condition and conducted between 0800 and 1600 h under laboratory conditions with temperatures ranging from 25 ^{o}C to 28 ^{o}C and the relative humidity ranging from 45% to 65%.

Host cues were provided by the addition of live adult guinea pigs (*Cavia cobaya* Pallas). One guinea pig was positioned inside the chamber containing the

impregnated papers and protected from mosquito biting by placing the host inside a cylindrical cage covered by nontreated netting material (Figure 10B).

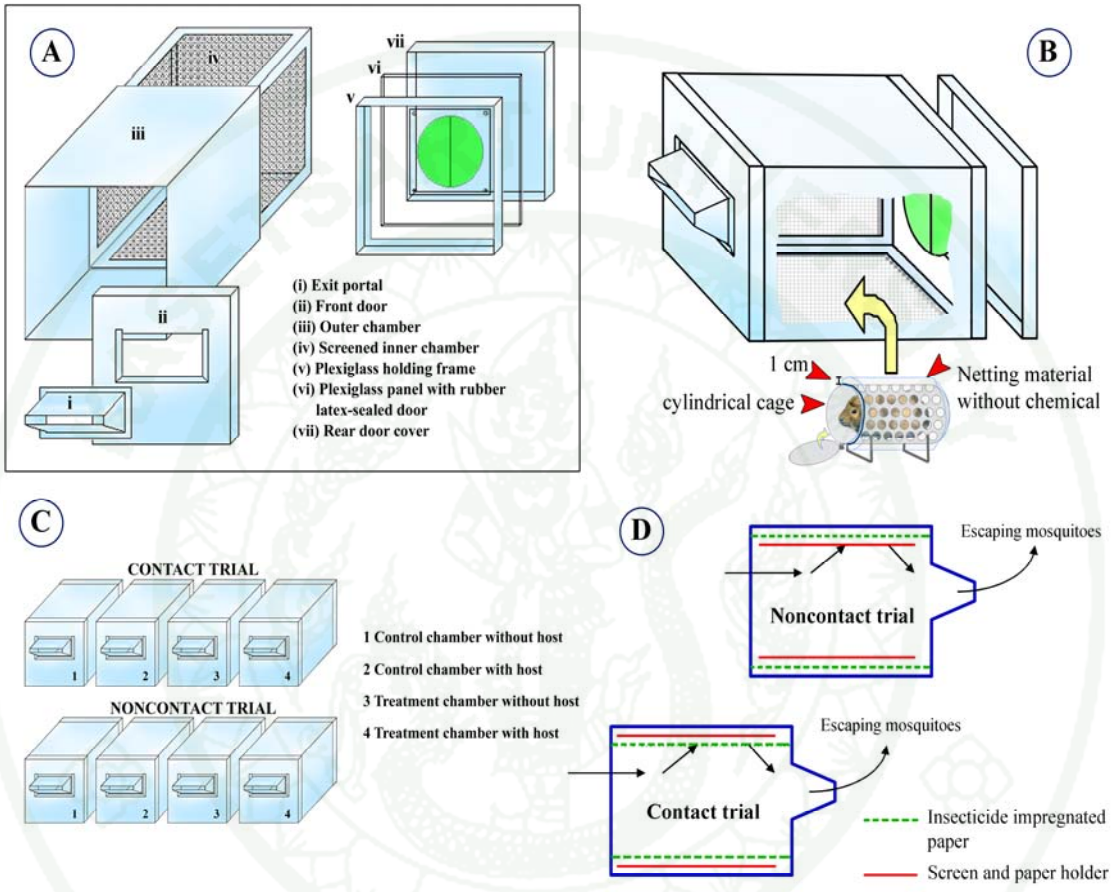

Figure 10 (A) Excito-repellency test chamber, (B) the chamber with live host (guinea pig), (C) composition of excito-repellency test chamber for behavioral studies, (D) side view of test chamber for contact and noncontact trials

4.4 Data analysis

Kaplan-Meier survival analysis was used to analyze and interpret the behavioral response data (Kleinbaum, 1995). For excito-repellency data, a life table survival analysis approach was used to estimate mosquito escape rates and compare differences between test populations and insecticides (Roberts *et al.,* 1997). For this study, the probability of mosquito response to insecticides was estimated in relation to differences of mosquito escape in the present or absence of live host, in either contact or noncontact trials, including the time in minutes for 25%, 50%, and 75% of the test population to escape into the receiving chamber (ET_{25}, ET_{50}, and $ET_{75)}$. The escape patterns (the time and density of escape from the treated and control chambers) of both mosquito species against bifenthrin or deltamethrin in two host conditions (presence or absence) between paired control and treatment in contact trials, paired control and treatment in noncontact trials, and paired contact and noncontact trials was compared.

A log-rank test (Mantel and Haenzel, 1959) was used to compare the curves or patterns of escape behavior between different treatments. Statistical significance for all tests was set at $P < 0.05$.

RESULTS

Part 1 Survey of two species in the Minimius Complex, *Anopheles harrisoni* and *Anopheles minimus* from two different potential breeding sites

A total of 7,012 identified mosquito larvae were collected at PT and a total of 3,513 at BTN. *Anopheles harrisoni* (93.91%) was the most common species at PT while *An. minimus* (69.83%) was the most common species identified from BTN. The mean number of larval mosquitoes was used for determining cut-off point of high and low density habitats.

1.1 Environmental data

Summarized environmental data from high and low density habitats of target mosquito species (*An. harrisoni* and *An. minimus*) and other important vectors, such as *An. maculatus*, at BTN and PT are shown in Table 3. The most obvious difference in environmental data between BTN and PT was conductivity levels with higher values in PT. Data from PT indicate high density habitats of *An. harrisoni* were located near the lower branches of PT stream (close to the fountainhead of the stream) while the opposite was true for habitats within the higher branch area of the stream (Figure 11B). Excluding the water pH and temperature with significantly lower values, the value of conductivity, stream width, and depth were all at higher levels in high density habitats than low density habitats ($P < 0.05$). Flow rate between the two habitat classes were not significantly different. High density habitats of *An. harrisoni* were associated with mid-range coverage of riparian canopy and debris (scored 2, 21-50%), the opposite was true for the low density habitats. In addition, there was less than 1% of submergent vegetation for high density habitats of *An. harrisoni*. There were only two *An. harrisoni* found in BTN and the habitat was associated with low-range of riparian canopy coverage, submergent vegetation, and debris.

There were no significant differences among all environmental data (except the water pH) between high and low density habitats of *An. minimus* in both

sites. However, the high density habitats of *An. minimus* in PT had a higher range of riparian canopy coverage and emergent vegetation and lower range of submergent vegetation than BTN. Likewise in *An. maculatus* at BTN, there were no significant differences of environmental data (except the water pH and vegetation data) between high and low density habitats. The pH of water in high density habitats was lower than in low density habitats. The higher number of *An. maculatus* larvae was found in BTN associated with the riparian canopy coverage and submergent vegetation (score of 2) while low density habitats of this species scored a 1 (1-20%). Contrary in PT, the lower number of *An. maculatus* larvae were present in the mid-range of riparian canopy coverage and low-range of submergent vegetation (scored 0). In addition, the pH, width, depth and temperature of high density habitats were significantly different from low density habitats with higher values of pH and temperature, and lower values of width and depth.

The shortest distance from sampling points to houses located around the sampling area is shown in Figure 11. Mean shortest distance from high and low density habitats to houses is shown in Table 3. In PT, the shortest distance to houses was significant different between high and low density habitat of *An. harrisoni* and *An. maculatus* ($P < 0.01$) and no significant different in *An. minimus* ($P = 0.288$). The average distance from *An. harrisoni* high density habitats to houses was ~323 m. On the other hand, the average distance from low density habitats of *An. harrisoni* to houses were ~71 m. Contrary for *An. maculatus* in PT, the average distance from high density habitats to houses was ~108 m while the average distance from low density houses was further (~207 m). In BTN, the shortest distance to houses was not significantly different between high and low density habitats of *An. minimus* and *An. maculatus* ($P = 0.892$ and 0.066, respectively) with each resulting ~200 m from homes. For *An. harrisoni* in BTN, the two sampling points positive for this species occurred at the shortest distance to houses of 62 and 196 m.

Table 3 Environmental data in high and low density area of three mosquito species at Pu Teuy and Bong Ti Noy (data collected from December 2006 to November 2008)

Variable data	Bong Ti Noy		Pu Teuy	
	High density	Low density	High density	Low density
An. harrisoni[1]	Two mosquitoes[5]		> 5.90	< 1.50
pH	7.84±0.12		6.87±0.07	7.39±0.10*
Conductivity (mS/cm)	285.75±45.57		759.29±3.39	737.74±34.29*
Width (m)[2]	4.35±2.14		1.95±0.52	1.36±0.52*
Depth (m)	0.22±0.11		0.38±0.06	0.11±0.07*
Temp (^{o}C)	26.87±3.38		25.05±0.20	25.67±0.15*
Flow rate (m/s)	0.45±0.32		0.28±0.05	0.32±0.07
Shortest dist. (m)[3]	128.93±94.43		323.54±73.69	70.79±48.63*
Coverage[4]	1		2	1
Emergent[4]	0		1	1
Submergent[4]	1		0	1
Debris[3]	1		2	1
An. minimus[1]	> 4.50	< 2.50	> 0.16	< 0.05
pH	7.87±0.06	7.81±0.02*	7.02±0.18	7.18±0.24*
Conductivity (mS/cm)	259.60±2.03	263.97±14.71	754.91±11.96	748.43±27.12
Width (m)[2]	5.33±1.39	4.81±1.15	1.57±0.43	1.71±0.64
Depth (m)	0.28±0.04	0.28±0.05	0.28±0.13	0.24±0.13
Temp (^{o}C)	28.53±1.07	27.04±1.23*	25.22±0.26	25.34±0.35
Flow rate (m/s)	0.42±0.06	0.45±0.05	0.30±0.04	0.30±0.09
Shortest dist. (m)[3]	176.30±64.03	179.85±76.65	212.54±120.04	173.23±126.48
Coverage[4]	1	1	2	2
Emergent[4]	0	0	1	1
Submergent[4]	1	1	0	0
Debris[3]	1	1	1	1

Table 3 (Continued)

Variable data	Bong Ti Noy		Pu Teuy	
	High density	Low density	High density	Low density
An. maculatus group[1]	> 1.20	< 0.51	> 0.12	< 0.01
pH	7.80±0.05*	7.86±0.05	7.26±0.23	7.04±0.17*
Conductivity (mS/cm)	260.56±0.95	271.56±26.34	739.21±33.64	756.80±3.41
Width (m)[2]	5.56±1.67	4.78±0.77	1.30±0.40	1.69±0.48*
Depth (m)	0.29±0.04	0.27±0.05	0.17±0.13	0.28±0.11*
Temp (^{o}C)	27.26±1.22	27.71±1.65	25.57±0.26	25.16±0.26*
Flow rate (m/s)	0.43±0.06	0.42±0.06	0.30±0.05	0.27±0.09
Shortest dist. (m)[3]	200.82±52.78	164.02±57.67	108.17±84.27	207.09±115.31*
Coverage[4]	2	1	1	2
Emergent[4]	0	0	1	1
Submergent[4]	2	1	0	0
Debris[3]	1	1	1	1

[1] The cut-off point of high and low density habitats using mean number of mosquitoes

[2] Once measurement when beginning of collecting at Pu Teuy

[3] Mean distances from sampling point to the nearest houses

[4] Mode value (the value that occurs the most frequently in data set or a probability distribution)

[5] Only two individuals of *An. harrisoni* were found in study period at Bong Ti Noy

* Identifies results of t-tests with statistically significant ($P < 0.05$) differences between high and low density bahitats

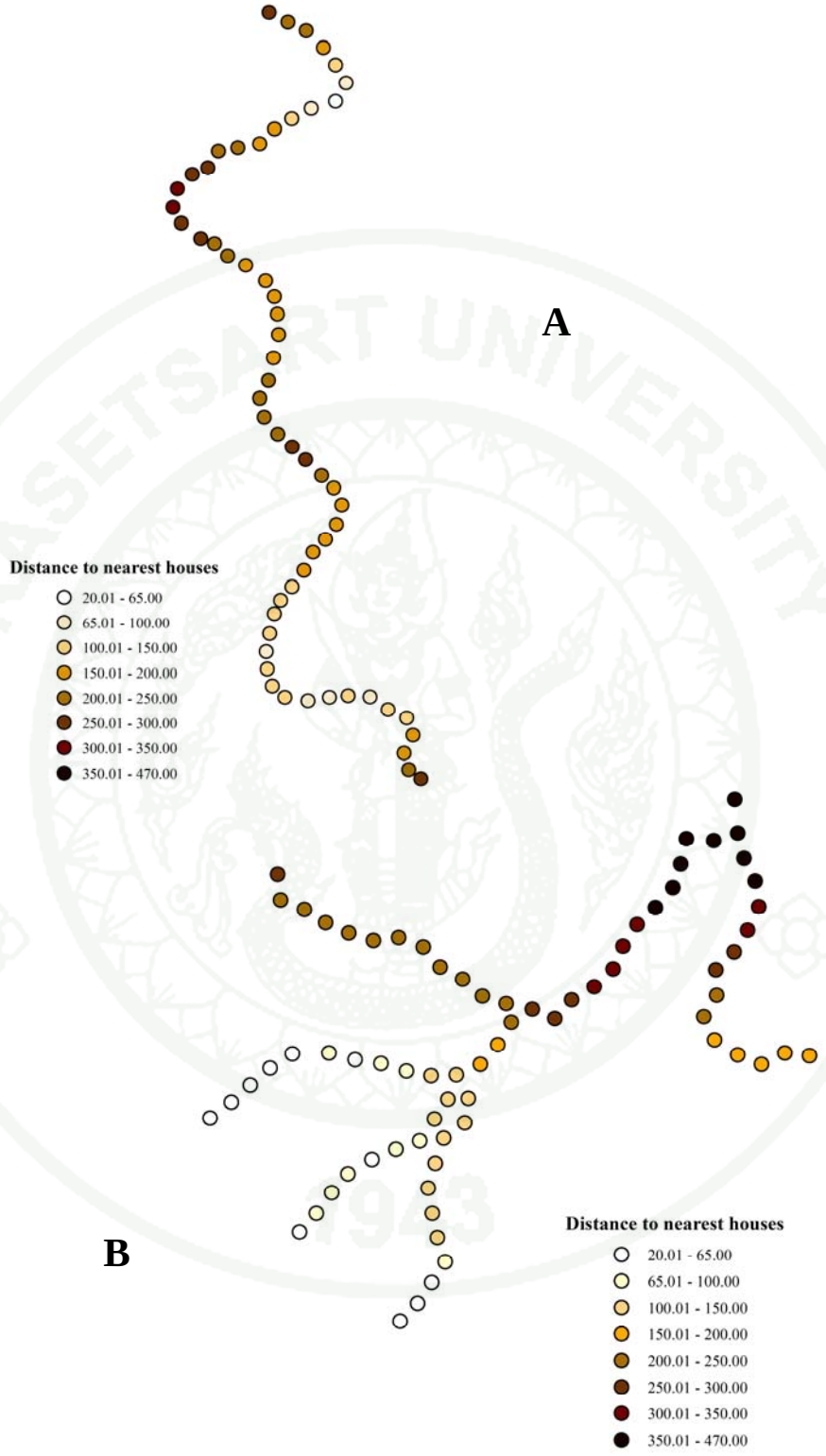

Figure 11 The shortest distance from sampling point to houses of larval habitats in (A) Bong Ti Noy and (B) Pu Teuy

1.2 Land cover

A 1 km buffer zone around sampling points was classified to four land cover classes (% pixel): plantation, forest, pasture, and shrub (Figure 12). In BTN, the largest area was forest (49.2%), followed by pasture (22.9%), shrub (18%), and plantation (9.9%). For PT, the largest area was forest (57.4%), followed by plantation (20.3%), pasture (14.6%), and shrub (7.7%). Percentage of land cover area in 5, 10, 20, 30, and 40 m individual buffer zones of high and low density of target species in both study sites was shown in Figures 13 and 14.

In BTN, the majority of land cover within 5-40 m radius of sampling points was riverbed and area that used to be riverbed more than 50% pixels. The habitat was found *An. harrisoni* associated with forest and shrub land cover within 30 m and 40 m buffer zones more than other land use types in the same buffer zones. Whereas, high density habitats of *An. minimus* was associated with all land cover (excluding forest land cover) with highest proportion of pasture land cover, while low density habitats was associated with all land cover including forest land cover. High density habitat within 5-30 m radius of *An. maculatus* was associated with shrub land cover while low density habitat were associated with forest and pasture land cover.

In PT, high density habitats within 5-30 m radius of *An. harrisoni* was greatly associated with forest land cover (66-92% pixels) while low density habitats were associated with higher proportion of plantation, pasture, and shrub than forest. This trend remained similar for all buffer zones analyzed. Contrary in *An. maculatus*, low density habitats within 5-30 m radius was associated with forest land cover (45-54% pixels) while high density habitats were associated with all land cover class especially plantation land cover. On the other hand, the high and low density habitats of *An. minimus* was associated with all land cover classes but associated with forest land cover more than other land cover class. Forest land cover of high density habitat of *An. minimus* was higher (39-55 % pixels) than low density habitat (37-39 % pixels) for all buffer zones.

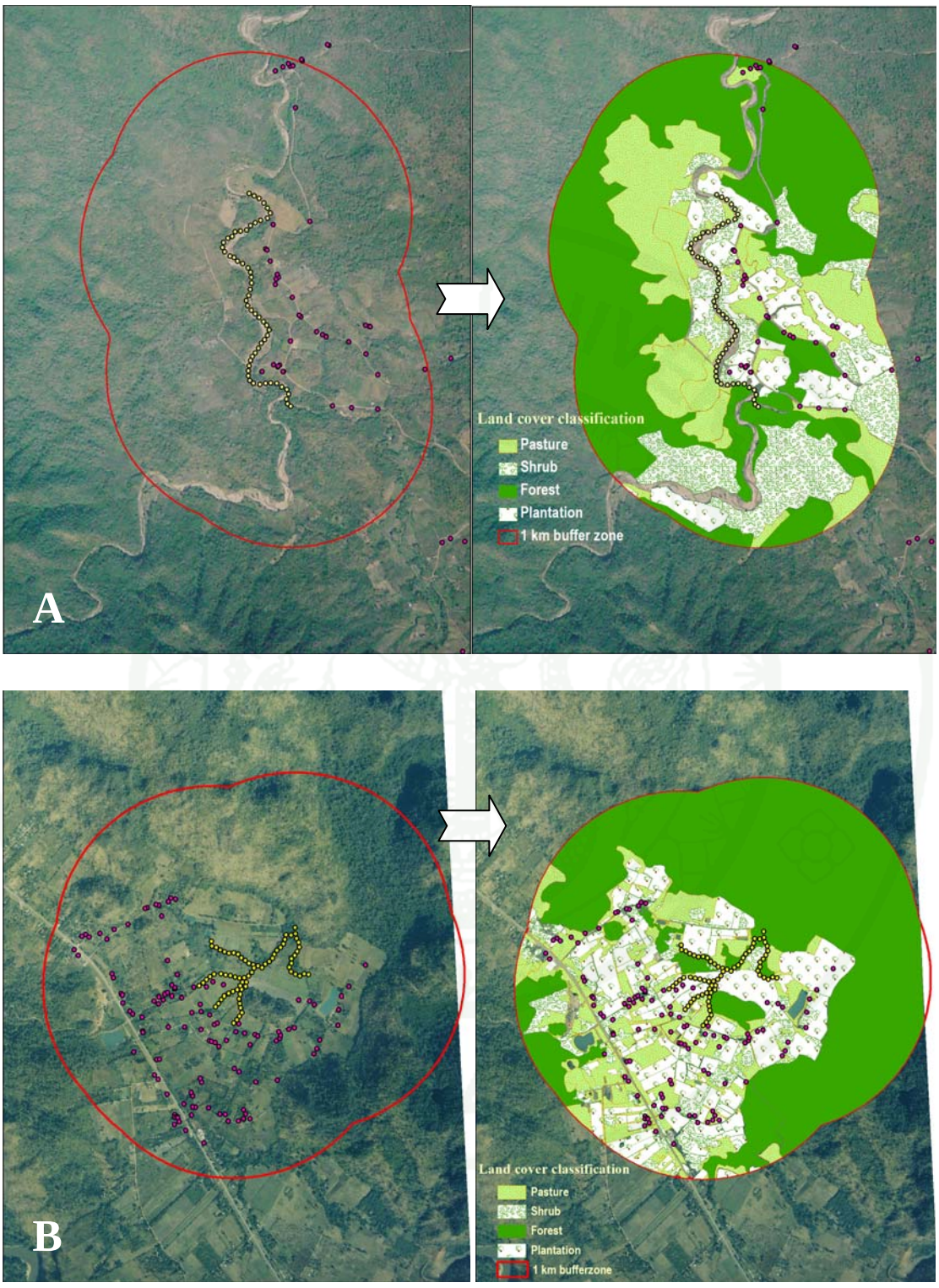

Figure 12 The manual digitization of land use classification on aerial photograph of (A) Bong Ti Noy and (B) Pu Teuy sites

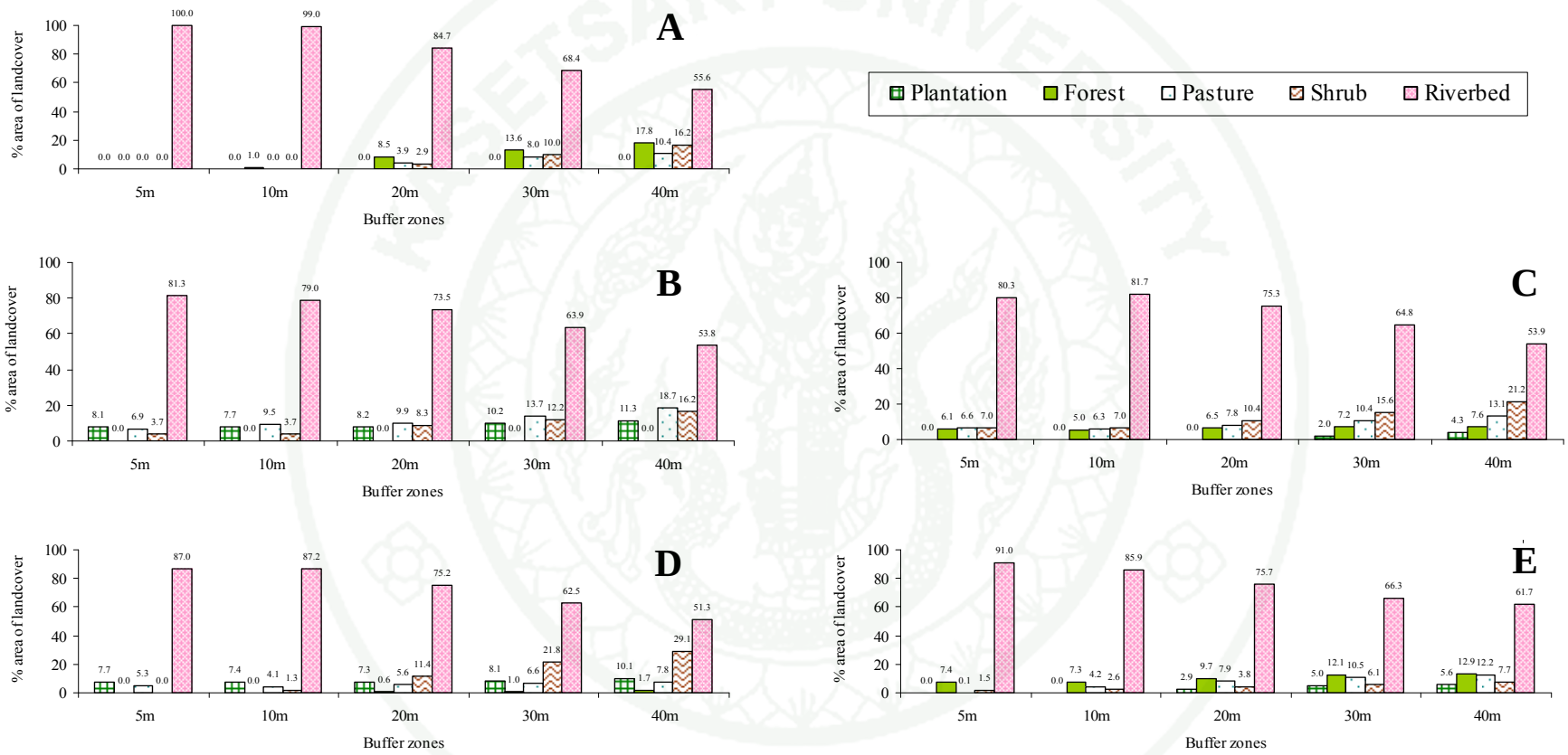

Figure 13 Percentage of land cover class (% pixel) within habitats of *Anopheles harrisoni* (A), *Anopheles minimus* (B = high density habitat and C = low density habitat), and *Anopheles maculatus* (D = high density habitat and E = low density habitat) in Bong Ti Noy

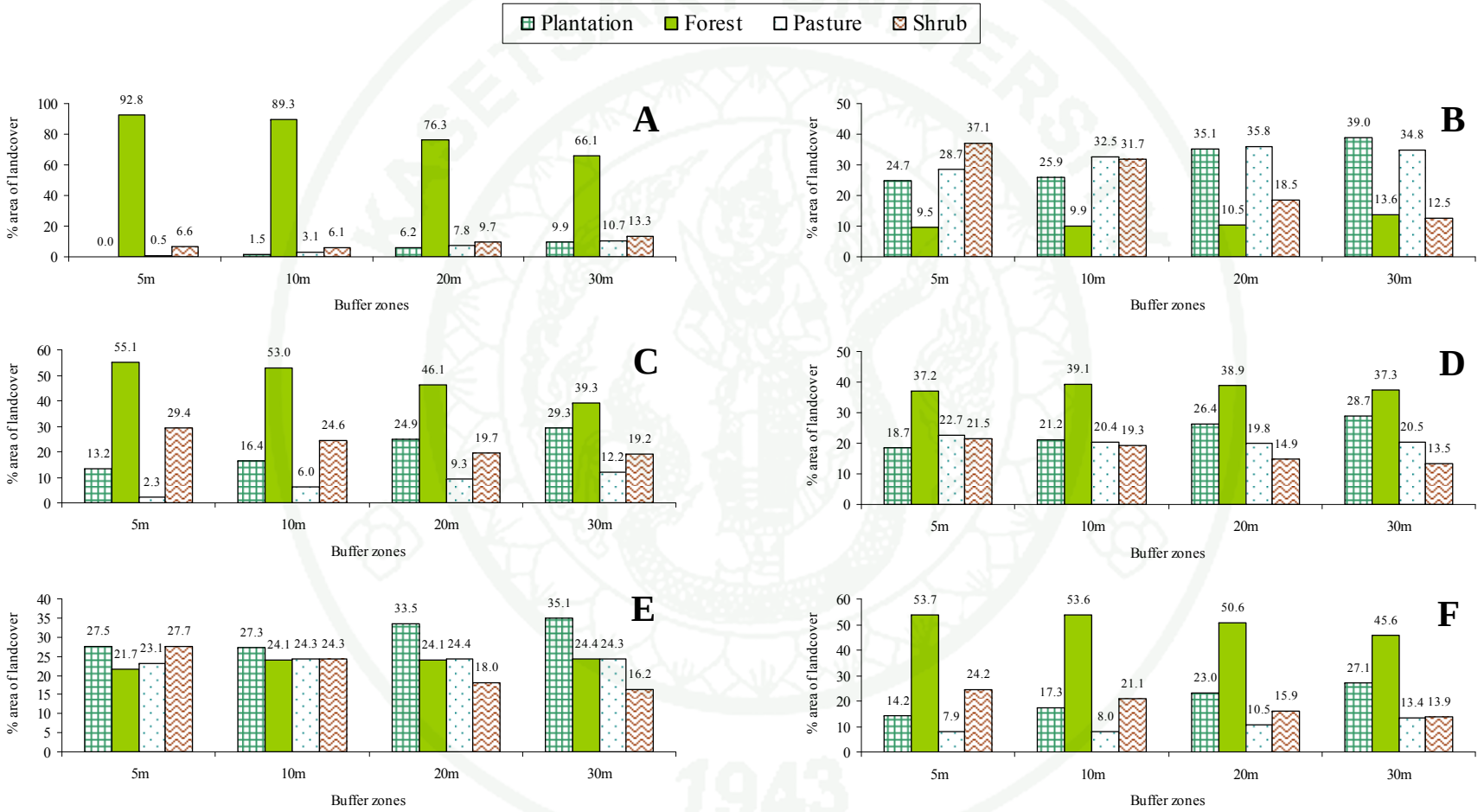

Figure 14 Percentage of land cover class (% pixels) within high and low habitats of *Anopheles harrisoni* (A, B), *Anopheles minimus* (C, D), and *Anopheles maculatus* (E, F) in Pu Teuy

Part 2 Seasonal abundance of larvae of *Anopheles harrisoni* and *Anopheles minimus* in two different potential breeding sites

2.1 Bong Ti Noy

A total of 3,513 anopheline larvae from all 12 sampling periods in BTN Village during the two-year study period from January 2007 to November 2008 were identified to species (Table 4). During this same period of time, average 39 malaria-infected village residents were reported each year, representing approximately 8% of the total population (Table 2). Field collections consisted of *Anopheles* 9 species. Approximately three out of four morphologically-identified mosquitoes (75.75%) belonged to the Minimus Complex and related species in the Funestus Group. A total of 2,657 specimens of the Minimus Complex and Aconitus Subgroup were morphologically identified and confirmed by an established molecular method (Table 5). Proportionally, they were *An. minimus* (69.93%), *An. harrisoni* (0.06%) and two genetically-related species belonging in the Aconitus Subgroup, *An. aconitus* (0.63%) and *An. varuna* (5.13%). The Maculatus Group (*An. maculatus*) accounted for 20.47 percent of the total study collection, Barbirostris Group (*An. barbirostris*) 0.48 percent and the remaining of 3.31% consisted of *An. culicifacies* (3.05%), *An. philippinensis* (0.17%), and *An. vagus* (0.09%) (Figure 15). *Anopheles pampani* (Aconitus Subgroup) was not found in this study site.

In general, both *An. minimus* and *An. maculatus* were collected in relatively high numbers (2,453 and 719, respectively). The monthly proportion of the presence of collected anopheline larvae varied (Figure 16) and were not found homogenous among the different species (χ^2 = 1,009.83; df = 88, $P < 0.0001$). *Anopheles minimus*, *An. varuna*, *An. maculatus* and *An. culicifacies* were regularly collected all year-round, exceptions only occurring during a few periods of the wet season.

Regression analysis of the relationship between the density of all these species and the monthly rainfall (Table 6) indicated rainfall had little or no affect on

the monthly larval population density of *An. minimus* ($P = 0.059$), *An. maculatus* ($P = 0.255$), *An. culicifacies* ($P = 0.517$), *An. philippinensis* ($P = 0.287$), *An. vagus* ($P = 0.459$), and *An. harrisoni* ($P = 0.572$). On the other hand, rainfall influenced the presence of *An. aconitus* ($P = 0.013$), *An. varuna* ($P = 0.018$) and *An. barbirostris* populations ($P = 0.005$). There was an inverse correlation between rainfall and population density of *An. minimus*. This relationship was re-analyzed by calculating data by year of each survey. Findings revealed that there was a significant association between rainfall and larval population density in the first year ($r^2 = 0.703$, $P = 0.037$), but not in the second half of the study ($r^2 = 0.532$, $P = 0.100$). The two-way ANOVA was used to investigate the variations of the primary study species (*An. aconitus*, *An. harrisoni*, *An. minimus*, and *An. varuna*) collected by year and season and by season between each year (Year * Season) (Table 7). Meaningful analysis was not possible for *An. harrisoni* and *An. aconitus* as both species were collected in low number in all seasons in both years. However, the higher numbers of *An. varuna* (n = 180) were significantly correlated with year and season of sampling ($P = 0.006$). The number of *An. minimus* (n = 2,453) was not significantly associated with season ($P = 0.195$), while high significance was observed by year ($P = 0.000$) and by season between each year ($P = 0.001$). In addition, the ratio of *An. minimus* density between seasons showed a higher ratio in the dry season of both years and similar to *An. varuna* population densities (Table 8 and Figure 17).

All physical and chemical measurements of water including mean values and difference between seasons are shown in Table 8. As expected, during the wet season, the water flow velocity, turbidity and water depth were higher than seen in both the hot and dry seasons, while the pH and conductivity in the wet season were lower than other seasons. The water characteristics at the same sampling points (n = 58) in each season were compared between years by paired *t*-tests. All measured parameters for each season showed significant difference between years ($P < 0.05$), except for water temperature in hot season ($P = 0.850$).

Table 4 Collection of larval *Anopheles* species from a stream environment at Bong Ti Noy Village, SaiYok District, Kanchanaburi Province (January 2007-December 2008)

Month	Minimus Complex & Aconitus Subgr.	Maculatus Group	Barbirostris Group	*An. culicifacies*	*An. philip-pinensis*	*An. vagus*	Subtotal
Year 1							
Jan	349	62	4	5	0	0	420
Mar	193	124	2	44	6	2	371
May	19	85	0	22	0	0	126
Jul	0	1	0	0	0	0	1
Sep	0	0	0	1	0	0	1
Nov	18	7	0	1	0	0	26
Year 2							
Jan	475	62	5	4	0	0	546
Mar	350	210	3	16	0	0	579
May	216	47	0	11	0	1	275
Jul	557	8	1	0	0	0	566
Sep	2	0	0	0	0	0	2
Nov	478	113	5	4	0	0	600
Subtotal	2,657	719	20	108	6	3	
	(75.74%)	(20.47%)	(0.48%)	(3.05%)	(0.17%)	(0.09%)	
Total	3,513						

Table 5 Total larvae of the Minimus Complex and related species identified by allele-specific PCR (Bong Ti Noy)

Month	Minimus Subgroup				Aconitus Subgroup			
	An. minimus		*An. harrisoni*		*An. aconitus*		*An. varuna*	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
Jan	280	452	0	0	8	4	61	19
Mar	178	339	0	0	1	1	14	10
May	5	202	0	0	2	0	12	14
July	0	540	0	1	0	0	0	16
Sep	0	2	0	0	0	0	0	0
Nov	18	437	0	1	0	6	0	34
Subtotal	481	1,972	0	2	11	11	87	93
Total	2,453		2		22		180	
	(92.32%)		(0.08%)		(0.83%)		(6.77%)	
Grand total	2,657							

Table 6 Relationship between monthly rainfall and abundance of mosquito species using regression statistics (Bong Ti Noy)

Species	*r*	r^2	F	*P*-value
An. minimus	-0.559	0.312	4.535	0.059
An. harrisoni	-0.182	0.033	0.342	0.572
An. aconitus	-0.692	0.479	9.210	0.013*
An. varuna	-0.667	0.445	8.003	0.018*
An. maculatus group	-0.357	0.127	1.458	0.255
An. barbirostris group	-0.793	0.629	16.985	0.002*
An. culicifacies	-0.216	0.047	0.489	0.500
An. philippinensis	-0.335	0.112	1.267	0.287
An. vagus	-0.237	0.056	0.594	0.459

Table 7 Two-way ANOVA of total number of each species collected within the Minimus Complex and Aconitus Subgroup by, season (dry, hot, and wet), and year (year 1 and 2) as discriminating factors (Bong Ti Noy)

Source		*An. minimus*		*An. harrisoni*		*An. aconitus*		*An. varuna*	
	df	F	*P*	F	*P*	F	*P*	F	*P*
Intercept	1	156.133	0.000	4.000	0.069	10.083	0.008	40.399	0.000
Year	1	57.684	0.000*	4.000	0.069	0.000	1.000	0.045	0.836
Season	2	1.879	0.195	4.000	0.047	1.083	0.369	1.122	0.357
Year * Season	2	13.243	0.001*	4.000	0.047	1.750	0.215	7.960	0.006*

By Year (of surveyed), By Season (of 2-year-surveyed), Year * Season (season between each year)

Table 8 Mean number of each species collected form Bong Ti Noy linked with mean stream characteristics during different seasons

Season	Dry (Dec-Feb)		Hot (Mar-May)		Wet (Jun-Nov)		Total		Ratio(Dry:Hot:Wet)	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year1	Year2	Year 1	Year 2
An. minimus	280	452	92	271	6	326	378	1,049	46.7:15.3:1	1.7:1:1.2
An. harrisoni	0	0	0	0	0	1	0	1	-	0:0:1
An. aconitus	8	4	2	1	0	2	10	7	4:1:0	4:1:2
An. varuna	61	19	13	12	0	17	74	48	4.7:1:0	1.6:1:1.4
Total	349	475	107	284	6	346	462	1,105	58.2:17.8:1	1.7:1:1.2
Grand total	824 (52.58%)		391 (24.95%)		352 (22.46%)		1,567		2.3:1.1:1	
Velocity (m/s)	0.32±0.06	0.36±0.09	0.36±0.09	0.42±0.14	0.48±0.12	0.46±0.09				
Turbidity (m)[1]	clear	clear	clear	clear	0.27±0.13	0.28±0.16				
Depth (m)	0.15±0.05	0.19±0.05	0.19±0.04	0.22±0.07	0.40±0.08	0.36±0.07				
Water temp. (°C)	25.84±2.45	24.66±2.35	31.50±2.11	31.48±1.69	27.49±1.99	26.43±2.80				
pH[2]	-	7.91±0.05	-	7.94±0.06	-	7.74±0.08				
Conductivity (μS/cm)[2]	-	296.2±9.9	-	263.8±21.7	-	246.9±15.1				

[1] Higher turbidity occurred in July and September 2007 and in September 2008.

[2] pH and conductivity were measured in January (dry), May (hot), July (wet), and September (wet) 2008.

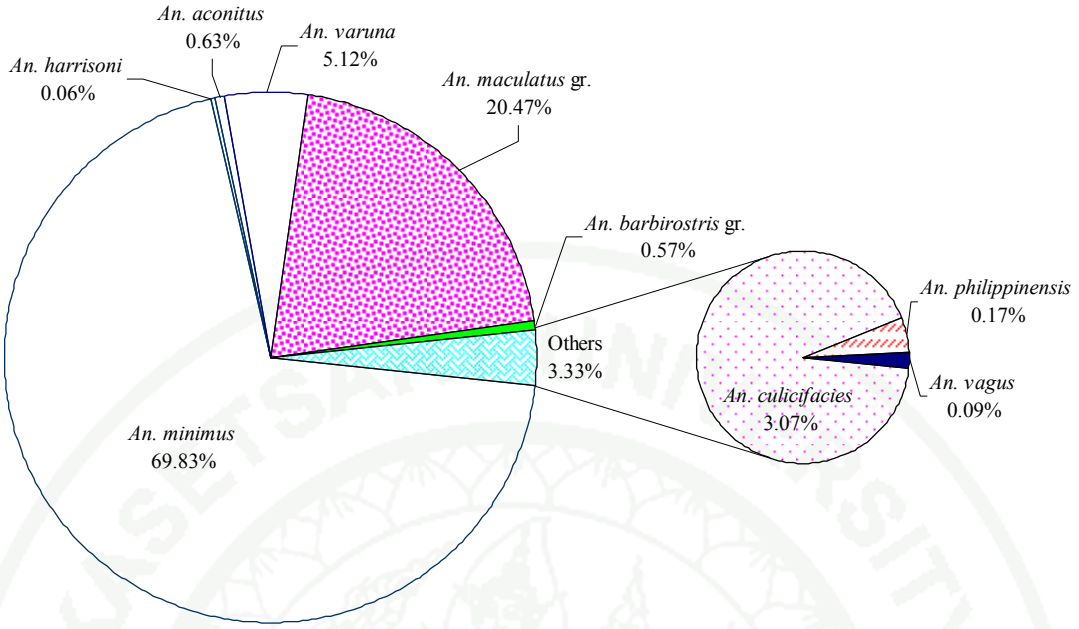

Figure 15 Overall relative proportion of anopheline larvae by species collected from a stream in Bong Ti Noy, Sai Yok District, Kanchanaburi Province during a two year period

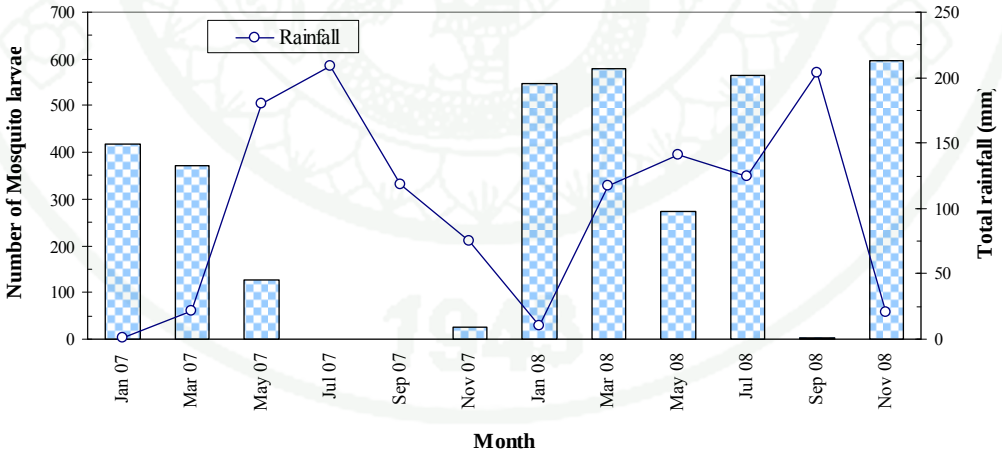

Figure 16 Total number (by collection month) of *Anopheles* larvae collected from Bong Ti Noy stream compared to rainfall patterns in the area

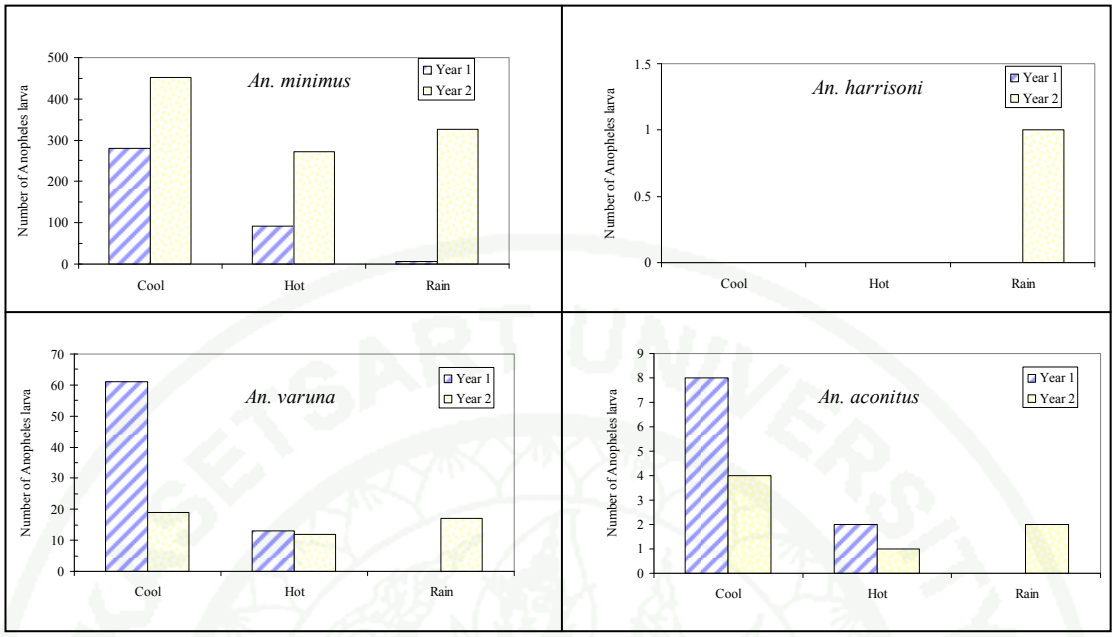

Figure 17 Mean number of Minimus Subgroup and Aconitus Subgroup (*Anopheles minimus*, *Anopheles harrisoni*, *Anopheles aconitus*, and *Anopheles varuna*) collected from Bong Ti Noy stream compared by seasons

2.2 Py Teuy

A total of 7,012 anopheline larvae from all 24 sampling periods in PT Village during the two-year study period from December 2006 to November 2008 were identified to species (Table 9). Field collections consisted of *Anopheles* 8 species. Mostly morphologically-identified mosquitoes (97.10%) belonged to the Minimus Complex and related species in the Funestus Group. A total of 6,809 specimens of the Minimus Complex and Aconitus Subgroup were morphologically identified and confirmed by an established molecular method (Table 10). Proportionally, they were *An. harrisoni* (96.71%), *An. minimus* (2.92%) and 2 genetically-related species belonging in the Aconitus Subgroup, *An. aconitus* (0.12%) and *An. varuna* (0.25%). The Maculatus Group (*Anopheles maculatus*) accounted for 1.74 percent of the total study collection, Barbirostris Group (*Anopheles barbirostris*) 0.46 percent and the remaining of 0.70% consisted of *An. aikenii* (0.64%), and *An. jamesii* (0.06%) (Figure 18). *Anopheles pampani* (Aconitus Subgroup) was not found in this study site.

Anopheles harrisoni was the most common species collected in relatively highest numbers (6,585). The monthly proportion of the presence of collected anopheline larvae varied (Figure 19) and were not found homogenous among the different species (χ^2 = 742.75; df = 161, $P < 0.0001$). *Anopheles harrisoni*, *An. minimus*, and *An. maculatus* were regularly collected all year-round, nevertheless in lower numbers of *An. minimus* and *An. maculatus* (199 and 122, respectively).

Regression analysis of the relationship between the density of all these species and the monthly rainfall (Table 11) indicated rainfall had little or no affect on the monthly larval population density of *An. minimus* ($P = 0.050$), *An. harrisoni* ($P = 0.112$), *An. aconitus* ($P = 0.384$), *An. varuna* ($P = 0.184$), *An. maculatus* ($P = 0.747$), *An. barbirostris* ($P = 0.053$), and *An. jamesii* ($P = 0.069$). On the other hand, rainfall influenced the presence of *An. aitkenii* population ($P = 0.517$) which was a positive correlation between rainfall and population density. The two-way ANOVA was used to investigate the variations of the primary study species (*An. aconitus*, *An. harrisoni*,

An. minimus, and *An. varuna*) collected by year and season and by season between each year (Year * Season) (Table 12). Meaningful analysis was not possible for *An. aconitus* and *An. varuna* as both species were collected in low number in all seasons in both years. However, the higher numbers of *An. minimus* (n = 199) was not significantly correlated with any sampling ($P > 0.05$). The number of *An. harrisoni* (n = 6,585) was not significantly associated with year ($P = 0.942$) and by season between each year ($P = 0.294$), while high significance was observed by season ($P = 0.015$). In addition, the ratio of *An. minimus* density between seasons showed a higher ratio in the dry and hot seasons of both years whereas *An. harrisoni* population showed a highest ratio in the hot season of both years (Table 13 and Figure 20).

All physical and chemical measurements of water including mean values and difference between seasons are shown in Table 13. The stream was clear throughout the study period. The water characteristics at the same sampling points (n = 73) in each season were compared between years by paired *t*-tests. There was significant difference of the velocity between seasons of first year ($P < 0.01$) with the highest value occurred in dry season, followed by wet season. Contrary, in the second year there was no significant difference between seasons ($P > 0.05$). In addition, the velocity of dry ($P < 0.001$) and hot ($P = 0.034$) seasons of the first and second year was significant difference while no difference of wet seasons between years ($P = 0.473$). Depth of water body was not difference between seasons of first year ($P > 0.05$). Whereas significant difference between seasons of second year and also between years with a higher depth of the first year than the second year ($P < 0.05$). The water temperature each season showed significant difference between years and seasons ($P < 0.05$). The pH and conductivity showed significant difference between seasons with lowest pH and highest conductivity in dry season.

Table 9 Collection of larval *Anopheles* species from the stream at Pu Teuy Village, Sai Yok District, Kanchanaburi Province (December 2006-November 2008)

Month	Minimus Complex and the related species	Maculatus Group	Barbirostris Group	*An. aitkenii*	*An. jamesii*	Subtotal
Year 1						
Dec	252	0	0	0	0	252
Jan	140	0	0	0	0	140
Feb	419	1	0	0	2	422
Mar	488	0	0	0	0	488
Apr	435	4	0	0	0	439
May	217	11	0	0	0	228
Jun	365	12	0	3	0	380
July	249	4	4	2	0	259
Aug	130	1	0	1	0	132
Sep	224	1	1	2	0	228
Oct	270	2	0	1	0	273
Nov	329	5	0	1	0	335
Year 2						
Dec	357	5	2	0	1	365
Jan	193	0	0	0	0	193
Feb	470	13	0	0	1	484
Mar	322	8	0	0	0	330
Apr	503	9	0	0	0	512
May	395	13	5	7	0	420
Jun	278	3	4	4	0	289
July	82	0	1	0	0	83
Aug	166	1	1	9	0	177

Table 9 (Continued)

Month	Minimus Complex and the related species	Maculatus Group	Barbirostris Group	*An. aitkenii*	*An. jamesii*	Subtotal
Sep	73	0	3	4	0	80
Oct	144	1	7	6	0	158
Nov	308	28	4	5	0	345
Subtotal	6,809	122	32	45	4	
	(97.10%)	(1.74%)	(0.46%)	(0.64%)	(0.06 %)	
Total	7,012					

Table 10 Total larvae of the Minimus Complex and related species identified by allele-specific PCR (Pu Teuy)

Month	Minimus Subgroup				Aconitus Subgroup			
	An. minimus		*An. harrisoni*		*An. aconitus*		*An. varuna*	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
Dec	12	22	240	335	0	0	0	0
Jan	4	4	136	187	0	0	0	2
Feb	8	16	408	454	1	0	2	0
Mar	6	9	482	312	0	0	0	1
Apr	6	20	428	482	1	1	0	0
May	11	14	206	381	0	0	0	0
Jun	6	11	359	265	0	2	0	0
July	4	1	244	81	1	0	0	0
Aug	1	0	129	164	0	2	0	0
Sep	2	0	222	73	0	0	0	0
Oct	0	2	270	141	0	0	0	1
Nov	5	35	324	262	0	0	0	11
Subtotal	65	134	3,448	3,137	3	5	2	15
Total	199 (2.92%)		6,585 (96.71%)		8 (0.12%)		17 (0.25%)	
Grand total	6,809							

Table 11 Relationship between monthly rainfall and abundance of mosquito species using regression statistics (Pu Teuy)

Species	r	r^2	F	*P*-value
An. minimus	-0.404	0.163	4.823	0.050
An. harisoni	-0.333	0.111	2.748	0.112
An. aconitus	0.186	0.035	0.788	0.384
An. varuna	-0.281	0.079	1.880	0.184
An. maculatus group	-0.069	0.005	0.107	0.747
An. barbirostris group	0.400	0.160	4.181	0.053
An. aitkenii group	0.489	0.239	6.909	0.015*
An. jamesii	-0.378	0.143	3.662	0.069

Table 12 Two-way ANOVA of total number of each species collected within the Minimus Complex and Aconitus Subgroup by, season (dry, hot, and wet), and year (year 1 and 2) as discriminating factors (Pu Teuy)

Source		*An. minimus*		*An. harrisoni*		*An. aconitus*		*An. varuna*	
	df	F	*P*	F	*P*	F	*P*	F	*P*
Intercept	1	26.166	0.000	170.360	0.000	4.445	0.049	1.396	0.253
Year	1	2.734	0.116	0.005	0.942	0.037	0.850	0.565	0.462
Season	2	1.261	0.307	5.394	0.015*	0.276	0.762	0.242	0.788
Year * Season	2	0.017	0.983	1.311	0.294	0.827	0.454	0.444	0.649

Note: By Year (of surveyed), By Season (of 2-year-surveyed), Year * Season (season between each year)

Table 13 Mean number of each species collected form Pu Teuy linked with mean stream characteristics during different seasons

Season	Dry (Dec-Feb)		Hot (Mar-May)		Rain (Jun-Nov)		Total		Ratio (Dry:Hot:Rain)	
	Year1	Year2	Year1	Year2	Year1	Year2	Year1	Year2	Year1	Year2
An. minimus	8	14	8	14	3	8	19	37	2.7:2.7:1	1.8:1.8:1
An. harrisoni	261	325	372	392	258	164	891	881	1:1.4:1	2:2.4:1
An. aconitus	0.3	0	0.3	0.3	0.2	0.7	0.8	1	1.5:1.5:1	-
An. varuna	0.7	0.7	0	0.3	0	2	0.7	3	-	2.3:1:6.7
Total	270	340	380	407	261	175	912	922	1:1.5:1	1.9:2.3:1
Grand total	610 (33.28%)		787 (43.00%)		436 (23.82%)		1,833		1.4:1.8:1	
Velocity (m/s)	0.33±0.13	0.28±0.10	0.27±0.09	0.29±0.10	0.29±0.11	0.29±0.07				
Turbidity (m)	clear	clear	clear	clear	clear	clear				
Depth (m)	0.28±0.14	0.25±0.13	0.28±0.14	0.24±0.12	0.28±0.15	0.23±0.10				
Water temp (°C)	24.79±0.54	24.90±0.49	25.46±0.79	25.63±0.53	24.87±0.30	25.47±0.35				
pH[1]	-	7.05±0.24	-	7.18±0.19	-	7.12±0.23				
Conductivity (μS/cm) [1]	-	761.7±13.8	-	758.6±22.7	-	749.5±18.9				

[1] The pH and conductivity was measured in December 2007 and January 2008 (dry), April and May 2008 (hot), July - September 2008 (rain)

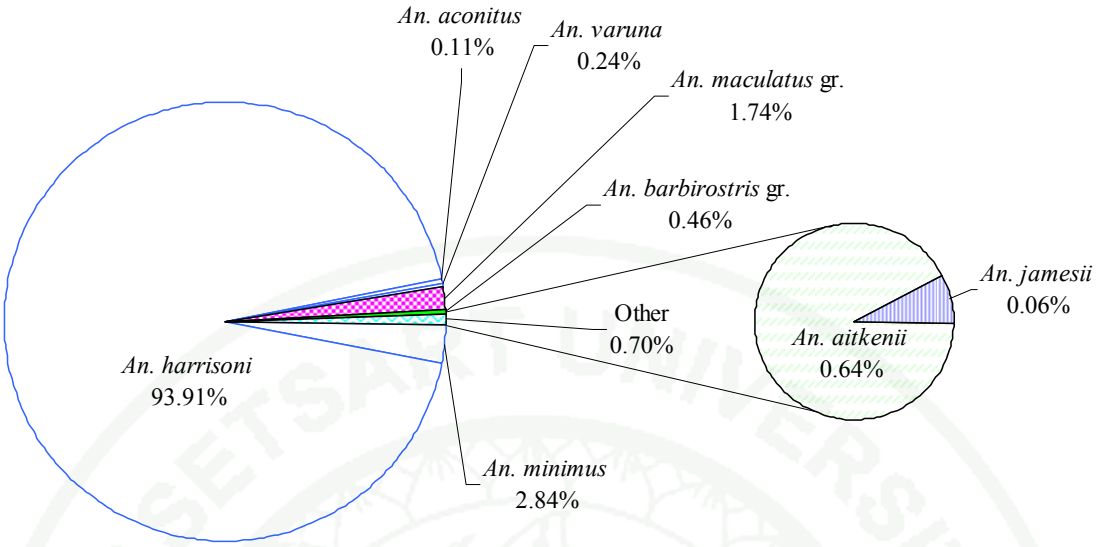

Figure 18 Overall relative proportion of anopheline larvae by species collected from a stream in Pu Teuy, Sai Yok District, Kanchanaburi Province during a two year period

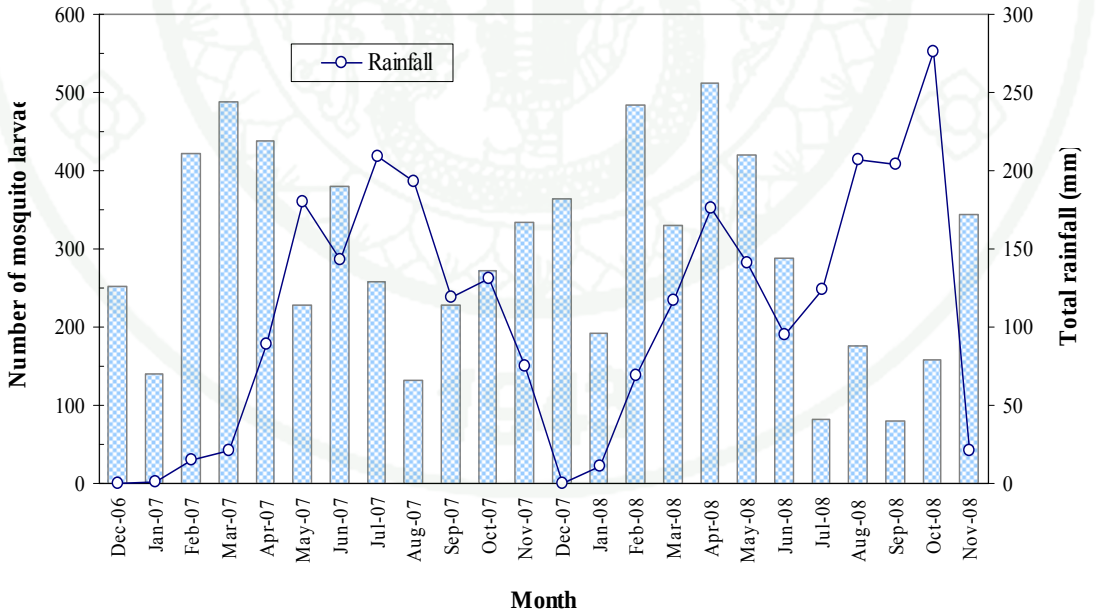

Figure 19 Total number (by collection month) of *Anopheles* larvae collected from Pu Teuy stream compared to rainfall patterns in the area

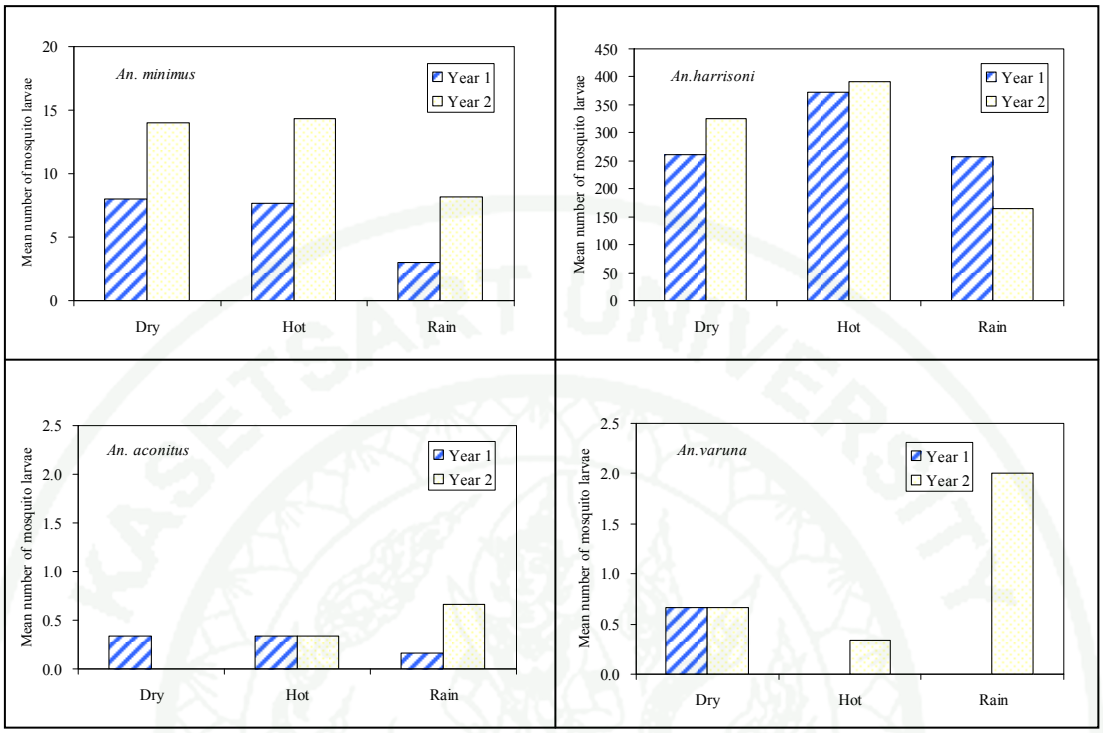

Figure 20 Mean number of Minimus Subgroup and Aconitus Subgroup (*Anopheles minimus*, *Anopheles harrisoni*, *Anopheles aconitus*, and *Anopheles varuna*) collected from Pu Teuy stream by seasons

2.3. Mortality of larval mosquitoes

Transfer of field collected larval mosquitoes from field to insectary at Kasetsart University can cause loss some larvae and also during rearing in insectary (Table 14). The collected larvae from BTN had the lower mortality that may be adaptability of the larvae to laboratory condition. Because of their habitat was uncertain environment when compare with the collected larvae from Pu Teuy which live in the rather stable environment. In addition, mostly of mortality was first two early stage of larval mosquito (>50% of total larval number).

Table 14 Mortality of the larvae during transfer from Kanchanaburi to Bangkok and rearing at Kasetsart University insectary

Study sites	Number of immature stages						Number of adults	% Mortality
	L1 (%)	L2 (%)	L3 (%)	L4 (%)	P (%)	Total		
Pu Teuy	13,035 (41.8)	9,086 (29.2)	5,185 (16.6)	3,660 (11.8)	196 (0.6)	31,162	7,012	77.50
Bong Ti Noy	3,104 (32.3)	2,457 (25.5)	1,871 (19.4)	1,872 (19.5)	318 (3.3)	9,622	3,513	66.49

Part 3 Identification of the two species of the Minimus Complex, *Anopheles harrisoni* and *Anopheles minimus* collected from two different ecological breeding habitats by morphological and molecular identification

In total, 2,657 mosquitoes from BTN were identified with both morphological and molecular methods. Comparison of identification methods revealed agreement (accuracy) of the morphological method (Table 15) was 100%, 97%, and 96%, for *An. aconitus*, *An. varuna*, and *An. minimus*, while only 4% with *An. harrisoni* identification. Of the 51 misidentified specimens of *An. harrisoni*, 36 were actually *An. minimus* (68%), and 15 (28%) *An. varuna*.

A total of 6,809 mosquitoes from PT were identified with both moethods revealed that 100%, 99%, 50% and 34% was agreement (accuracy) of the morphological method for *An. aconitus*, *An. harrisoni*, *An. varuna*, and *An. minimus*. Of the 307 misidentified specimens of *An. minimus*, 296 were actually *An. harrisoni* (64%), and 11 (2%) *An. varuna* (Table 16).

The reliability test of the presence/absence of the specific characteristics to identify the species in a group of closely related species, all the indexes (both female and male) were calculated and are presented in Tables 17 and 18. Identification for separate both female and male *An. aconitus* form other closely related species in both locations, the index of validity was more than 0.99, showing a highly probability of agreement between the morphological and molecular identification. The PPV was 1, indicating that all the *An. aconitus* had pale scales on distal half (dorsum and venter) of the proboscis. The NPV was more than 0.99, indicating highly probability of the correct identification of other closely related species when proboscis entirely dark or ventral pale patch was present.

For the identification to separate both female and male *An. varuna* from Minimus Complex, the index of validity ranged from 0.9428 to 0.9976, showing a high probability of agreement between the morphological and molecular identification. The PPV, probability of having a correct identification of *An. varuna*

base on the absence of the PSP phenotype of BTN population (0.9762 for female and 0.9667 for male) is higher than PT population (0.6667 for female and 0.3333 for male). The NPV was more than 0.9, indicating high probability of rightly identifying Minimus Complex species when PSP was present.

The index of validity of the identification both female and male between *An. harrisoni* and *An. minimus* more than 0.9 (lowest value was 0.9116, male mosquitoes in PT). The PPV of BTN population (0.0741 for female and 0 for all misidentified male) was extremely lower than PT population (PPV > 0.99), showing lowest probability of having a correct identification of *An. harrisoni* base on the presence of the HP phenotype of BTN population. The NPV was maximum (NPV = 1) for BTN population, indicating highly probability of rightly identifying *An. minimus* of this population if the PSP was present. For PT population, NPV was 0.1915 for male *An. minimus*, indicating the probability of the correct identification of *An. minimus* when the PSP was present lower than female *An. minimus* of this population (NPV = 0.7541) and both sexes of *An. minimus* BTN population.

Table 15 Number and comparison of mosquitoes identified by morphological and molecular method (Bong Ti Noy)

Morphological identification		Molecular identification			
		An. minimus	*An. harrisoni*	*An. aconitus*	*An. varuna*
An. minimus	2,516	2,415 (95.99%)*	0	6 (0.24%)	95 (3.78%)
An. harrisoni	53	36 (67.92%)	2 (3.77%)	0	15 (28.30%)
An. aconitus	16	0	0	16 (100%)	0
An. varuna	72	2 (2.78%)	0	0	70 (97.22%)
Total	2,657	2,453	2	22	180

*Percent of morphologically identified sample corrected by molecular analysis.

Table 16 Number and comparison of mosquitoes identified by morphological and molecular method (Pu Teuy)

Morphological identification		Molecular identification			
		An. minimus	*An. harrisoni*	*An. aconitus*	*An. varuna*
An. minimus	462	155 (33.55%)	296 (64.07%)	0	11 (2.38%)
An. harrisoni	6,330	40 (0.63%)	6,287 (99.32%)	3 (0.05%)	0
An. aconitus	5	0	0	5 (100%)	0
An. varuna	12	4 (33.33%)	2 (16.67%)	0	6 (50.00%)
Total	6,809	199	6,585	8	17

Table 17 Identification results and the reliability test indexes of female mosquitoes

Sites[1]	Morphological identification	Molecular identification		Sensitivity	Specificity	Iv	PPV	NPV
		An. aconitus	Minimus Complex & *An. varuna*					
BTN	*An. aconitus*	12	0	0.7500	1	0.9971	1	0.9971
	Minimus Complex & *An. varuna*	4	1,380					
PT	*An. aconitus*	4	0	0.6667	1	0.9994	1	0.9994
	Minimus Complex & *An. varuna*	2	3,500					
		An. varuna	Minimus Complex					
BTN	*An. varuna*	41	1	0.3445	0.9992	0.9428	0.9762	0.9417
	Minimus Complex	78	1,260					
PT	*An. varuna*	4	2	0.3636	0.9994	0.9974	0.6667	0.9980
	Minimus Complex	7	3,487					
		An. harrisoni	*An. minimus*					
BTN	*An. harrisoni*	2	25	1	0.9801	0.9802	0.0741	1
	An. minimus	0	1,233					
PT	*An. harrisoni*	3,350	15	0.9911	0.8598	0.9871	0.9955	0.7541
	An. minimus	30	92					

[1] PT = Pu Teuy, BTN = Bong Ti Noy

Table 18 Identification results and the reliability test indexes of male mosquitoes

Sites[1]	Morphological identification	Molecular identification		Sensitivity	Specificity	Iv	PPV	NPV
		An. aconitus	Minimus Complex & *An. varuna*					
BTN	*An. aconitus*	4	0	0.6667	1	0.9984	1	0.9984
	Minimus Complex & *An. varuna*	2	1,255					
PT	*An. aconitus*	1	0	0.5000	1	0.9997	1	0.9997
	Minimus Complex & *An. varuna*	1	3,301					
		An. varuna	Minimus Complex					
BTN	*An. varuna*	29	1	0.4754	0.9992	0.9737	0.9667	0.9739
	Minimus Complex	32	1,193					
PT	*An. varuna*	2	4	0.3333	0.9988	0.9976	0.3333	0.9988
	Minimus Complex	4	3,291					
		An. harrisoni	*An. minimus*					
BTN	*An. harrisoni*	0	12	xx[2]	0.9899	0.9899	0	1
	An. minimus	0	1,181					
PT	*An. harrisoni*	2,237	25	0.9170	0.7159	0.9116	0.9916	0.1915
	An. minimus	266	63					

[1] PT = Pu Teuy, BTN = Bong Ti Noy

[2] xx = can not be calculated

Part 4 Behavioral responses of *Anopheles harrisoni* and *Anopheles minimus* to bifenthrin and deltamethrin using an excito-repellency system and a live host

4.1 Percentage of escape response

The percentage of escape responses (movement from the chamber with impregnated papers to the receiving chamber) of both *An. harrisoni* and *An. minimus* to bifenthrin or deltamethrin in the presence and absence of a live host were measured using contact and noncontact exposure test conditions.

The results indicate that escape patterns of both mosquito species under each host condition (with and without host) in contact trials to bifenthrin or deltamethrin were considerably higher than those from matched controls and all test conditions in noncontact trials (Table 19). Overall, there was a lower percentage escape response from *An. harrisoni* than from *An. minimus* after contact with bifenthrin (51-58% and 42-75%, respectively) or deltamethrin (49-54% and 95-99%, respectively) in both the presence and absence of hosts. The highest percentage escape response of *An. minimus* was observed when exposed to deltamethrin in the absence of host (99%), followed by in the presence of host (95%) with the same compound. In addition *An. minimus* exhibited greatest escape response to bifenthrin in the absence of host (75%) as compared to when a host was present (42%) (Table 19).

4.2 Mortality rates of tested mosquitoes

The 24-h mortality rates of *An. harrisoni* and *An. minimus* exposed to either bifenthrin or deltamethrin for each experimental treatment is shown in Table 18. Overall, mortality rates of escaped and nonescaped test populations were higher in *An. harrisoni* (escaped, 16-20%; nonescaped, 10-18%) than in *An. minimus* (escaped, 2-3%; nonescaped, 2-4%) after exposure to bifenthrin. On the contrary, the mortality rates of escaped and nonescaped mosquitoes after exposure to deltamethrin were higher in *An. minimus* (escaped, 34-38%; nonescaped, 80-100%) as compared to *An.*

harrisoni (escaped, 8-18%; nonescaped, 40%). For *An. minimus* mosquitoes that remained in the chamber after 30-min exposure to deltamethrin, 24-h mortality rates higher than those exhibited in test populations that had successfully escaped and higher in the absence of a host than when a guinea pig was present in the test system (100 vs. 80%, respectively) (Table 20). Mortality rates of *An. harrisoni* following exposure to deltamethrin indicated similar results with greatest mortality occurring in nonescaped test populations than in those that moved to the receiving chamber but the opposite was true when exposed to bifenthrin. Overall, lowest mortality was seen in noncontact trials and all matched controls in contact trials, with no more than 1 death from each cohort of escaped and nonescaped populations.

4.3 Escape time

Escape time (ET), measured at 1-min intervals, was designated based on the percentage of the mosquitoes escaping from chambers treated with bifentrhin or deltamethrin in contact trials with and without host, into the receiving chamber at 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of the total test population with the system during the 30-min exposure period (Table 21). A delay in escape response to both insecticides in contact trials was clearly indicated using *An. minimus* mosquitoes in the presence of a host, unlike in *An. harrisoni* where a host presence had no minimal delay effects. Bifenthrin produced a greater duration in time until escape using *An. minimus* under both host treatment conditions, especially for ET_{50} and ET_{75} with a host present which could not be calculated because of insufficient numbers of mosquitoes exiting the treatment chamber during the testing period. For *An. harrisoni*, there was longer time duration to escape from treated chambers containing either bifentrhin or deltamethrin under both host conditions (presence or absence) when compared with escape times of *An. minimus*.

Table 19 Summary of the percentage of escape response of female *Anopheles harrisoni* and *Anopheles minimus* in contact and noncontact test trials with bifenthrin (0.025 g/m^2) or deltamethrin (0.02 g/m^2)

Trial	Insecticides	Conditions	*An. harrisoni*		*An. minimus*	
			Tested	% escaped	Tested	% escaped
Contact	Control	with host	99	3.03	99	2.02
	Bifenthrin	with host	99	50.51	98	41.84
	Control	w/o host	99	5.05	100	6.00
	Bifenthrin	w/o host	98	58.16	99	74.75
	Control	with host	98	4.08	99	2.02
	Deltamethrin	with host	98	54.08	99	94.95
	Control	w/o host	100	4.00	100	8.00
	Deltamethrin	w/o host	99	49.49	98	98.98
Noncontact	Control	with host	99	0	99	1.01
	Bifenthrin	with host	100	2.00	100	5.00
	Control	w/o host	100	2.00	100	5.00
	Bifenthrin	w/o host	100	4.00	99	9.09
	Control	with host	98	1.02	98	3.06
	Deltamethrin	with host	99	2.02	99	8.08
	Control	w/o host	100	3.00	102	5.88
	Deltamethrin	w/o host	99	4.04	101	11.88

Table 20 Summary of the 24-h mortality rates of female *Anopheles harrisoni* and *Anopheles minimus* in contact and noncontact trials with bifenthrin and deltamethrin

Trial	Insecticides	Conditions	*An. harrisoni*		*An. minimus*	
			Escaped[1]	Notescaped[2]	Escaped[1]	Notescaped[2]
Contact	Control	with host	0	0	0	0
			(0/3)	(0/96)	(0/2)	(0/99)
	Bifenthrin	with host	20.00	18.37	2.44	1.75
			(10/50)	(9/49)	(1/41)	(1/57)
	Control	w/o host	0	1.06	0	0
			(0/5)	(1/94)	(0/6)	(0/94)
	Bifenthrin	w/o host	15.79	9.76	2.70	4.00
			(9/57)	(4/41)	(2/74)	(1/25)
	Control	with host	0	0	0	1.03
			(0/4)	(0/94)	(0/2)	(1/97)
	Deltamethrin	with host	7.55	40.00	34.04	80.00
			(4/53)	(18/45)	(32/94)	(4/5)
	Control	w/o host	0	1.04	0	0
			(0/4)	(1/96)	(0/8)	(0/92)
	Deltamethrin	w/o host	18.37	40.00	38.14	100.00
			(9/49)	(20/50)	(37/97)	(1/1)

Table 20 (Continued)

Trial	Insecticides	Conditions	*An. harrisoni*		*An. minimus*	
			Escaped[1]	Not-escaped[2]	Escaped[1]	Not-escaped[2]
Noncontact	Control	with host	0 (0/0)	0 (99/0)	0 (1/0)	0 (98/0)
	Bifenthrin	with host	0 (2/0)	0 (98/0)	0 (5/0)	0 (95/0)
	Control	w/o host	0 (2/0)	0 (98/0)	0 (5/0)	1.05 (95/1)
	Bifenthrin	w/o host	0 (4/0)	0 (96/0)	0 (9/0)	1.11 (90/1)
	Control	with host	0 (1/0)	1.37 (97/1)	0 (3/0)	0 (95/0)
	Deltamethrin	with host	0 (2/0)	0 (97/0)	12.50 (8/1)	0 (91/0)
	Control	w/o host	0 (3/0)	0 (97/0)	0 (6/0)	0 (96/0)
	Deltamethrin	w/o host	0 (4/0)	0 (95/0)	0 (12/0)	0 (89/0)

[1] Mortality rate (%) of escaped mosquitoes (number of dead mosquitoes /number of escaped mosquitoes)

[2] Mortality rate (%) of not-escaped mosquitoes (number of dead mosquitoes /number of escaped mosquitoes)

Table 21 Escape time (in minutes) for 25%, 50% and 75% of *Anopheles harrisoni* and *Anopheles minimus* test populations to escape from excito-repellency chambers containing either bifenthrin (0.025 g/m^2) or deltamethrin (0.02 g/m^2) within a 30-min exposure period in contact trials

Species	Conditions	Bifenthrin			Deltamethrin		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
An. harrisoni	with host	11	30	-	7	22	-
	w/o host	14	25	-	9	-	-
An. minimus	with host	17	-	-	4	7	11
	w/o host	11	18	30	3	4	9

-, Indicates insufficient number escaped from exposure chambers to estimate ET_{25}, ET_{50} and ET_{75} during the 30-min exposure period.

4.4 Escape patterns

Figures 21 and 22 showed escape patterns of *An. harrisoni* and *An. minimus* in response to bifenthrin or deltamethrin in contact and noncontact trials with and without host treatments. There were highly significant differences in observed escape response of both mosquito species for all treatment pairs comparing contact to noncontact trials ($P < 0.0001$). Highly significant differences in escape responses were also observed when the control was compared to contact trials ($P < 0.0001$), but not significant when the matched control was compared to noncontact treatment trials ($P > 0.05$) (Table 22).

The escape patterns following exposure to bifenthrin or deltamethrin using *An. harrisoni* in contact trials were not significantly different when comparing between trials with hosts vs. contact trials without hosts ($P > 0.05$), whereas *An. minimus* exhibited highly significant differences in escape responses for the same comparison ($P < 0.01$). No significant differences in escape responses to bifenthrin or deltamethrin were observed in noncontact trials with host vs. noncontact trials without host for either mosquito species ($P > 0.05$) (Table 23).

When comparing escape probabilities between *An. harrisoni* and *An. minimus* in contact trials against deltamethrin in the presence of host, *An. minimus* exhibited significantly higher escape probability than *An. harrisoni* ($P < 0.0001$) (Table 24). The same was true in contact trials against both chemicals in the absence of a host where response in *An. minimus* was significantly higher than that exhibited by *An. harrisoni* (bifenthrin, $P = 0.0076$; deltamethrin, $P < 0.0001$). In addition, the escape response of *An. minimus* against deltamenthrin under both host conditions was significantly higher than when the mosquitoes were exposed to bifenthrin ($P < 0.0001$) (Table 25). In contrast, there was no significant difference in the escape response of *An. harrisoni* to bifenthrin vs. deltamethrin under host presence or absence treatment conditions (with host, $P = 0.2230$; without host, $P = 0.8204$).

Table 22 Comparison of escape patterns of *Anopheles harrisoni* and *Anopheles minimus* against bifenthrin or deltamethrin in the presence or absence of a host between paired control and treatment test systems in contact, noncontact, and paired contact and noncontact trials

Species	Insecticides	With host			Without host		
		Contact control vs. contact treatment	Noncontact control vs. noncontact treatment	Contact treatment vs. noncontact treatment	Contact control vs. contact treatment	Noncontact control vs. noncontact treatment	Contact treatment vs. noncontact treatment
An. harrisoni	Bifenthrin	<0.0001*	0.1583	<0.0001*	<0.0001*	0.4107	<0.0001*
	Deltamethrin	<0.0001*	0.5677	<0.0001*	<0.0001*	0.6952	<0.0001*
An. minimus	Bifenthrin	<0.0001*	0.0987	<0.0001*	<0.0001*	0.2637	<0.0001*
	Deltamethrin	<0.0001*	0.1318	<0.0001*	<0.0001*	0.1377	<0.0001*

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior

Table 23 Comparison of escape patterns of *Anopheles harrisoni* and *Anopheles minimus* from control and treatment chambers against bifenthrin or deltamethrin between paired host conditions in contact and noncontact trials

Species	Insecticides	Control		Treatment	
		Contact trial w/ host vs. contract trial w/o host	Noncontact trial w/ host vs. noncontract trial w/o host	Contact trial w/ host vs. contract trial w/o host	Noncontact trial w/ host vs. noncontract trial w/o host
An. harrisoni	Bifenthrin	0.4789	0.1583	0.4151	0.4005
	Deltamethrin	0.9768	0.3254	0.5840	0.4130
An. minimus	Bifenthrin	0.1462	0.0985	<0.0001*	0.2652
	Deltamethrin	0.0553	0.3448	0.0055*	0.3838

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior

Table 24 Comparison of escape patterns between paired *Anopheles harrisoni* and *Anopheles minimus* escape patterns, in contact and noncontact trials, with host and without host, against bifenthrin or deltamethrin

Trials	Host conditions	*An. harrisoni* vs. *An. minimus*	
		Bifenthrin	Deltamethrin
Contact	with host	0.1576	<0.0001*
	without host	0.0076	<0.0001*
Noncontact	with host	0.2449	0.0550
	without host	0.1526	0.0510

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior

Table 25 Comparison of escape patterns between paired bifenthrin and deltamethrin insecticides in contact and noncontact trials, with host and without host, of *Anopheles harrisoni* and *Anopheles minimus*

Trials	Host conditions	Bifenthrin vs. Deltamethrin	
		An. harrisoni	*An. minimus*
Contact	with host	0.2230	<0.0001*
	without host	0.8204	<0.0001*
Noncontact	with host	0.9858	0.3873
	without host	0.9914	0.5256

* Identifies results of log-rank tests with statistically significant ($P < 0.05$) differences in patterns of escape behavior

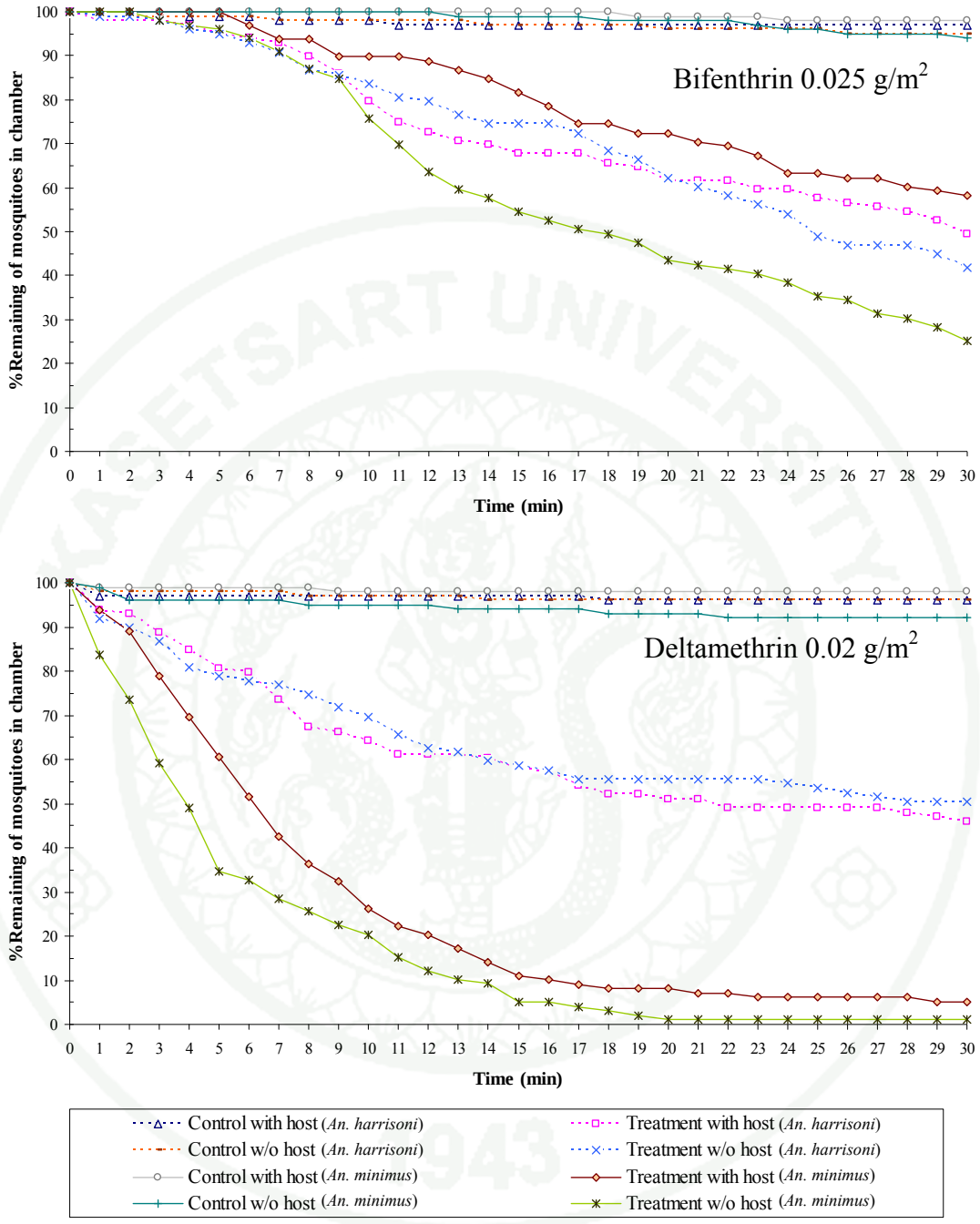

Figure 21 Escape patterns of *Anopheles harrisoni* and *Anopheles minimus* to bifenthrin 0.025 g/m^2 and deltamethrin 0.02 g/m^2 in contact trial

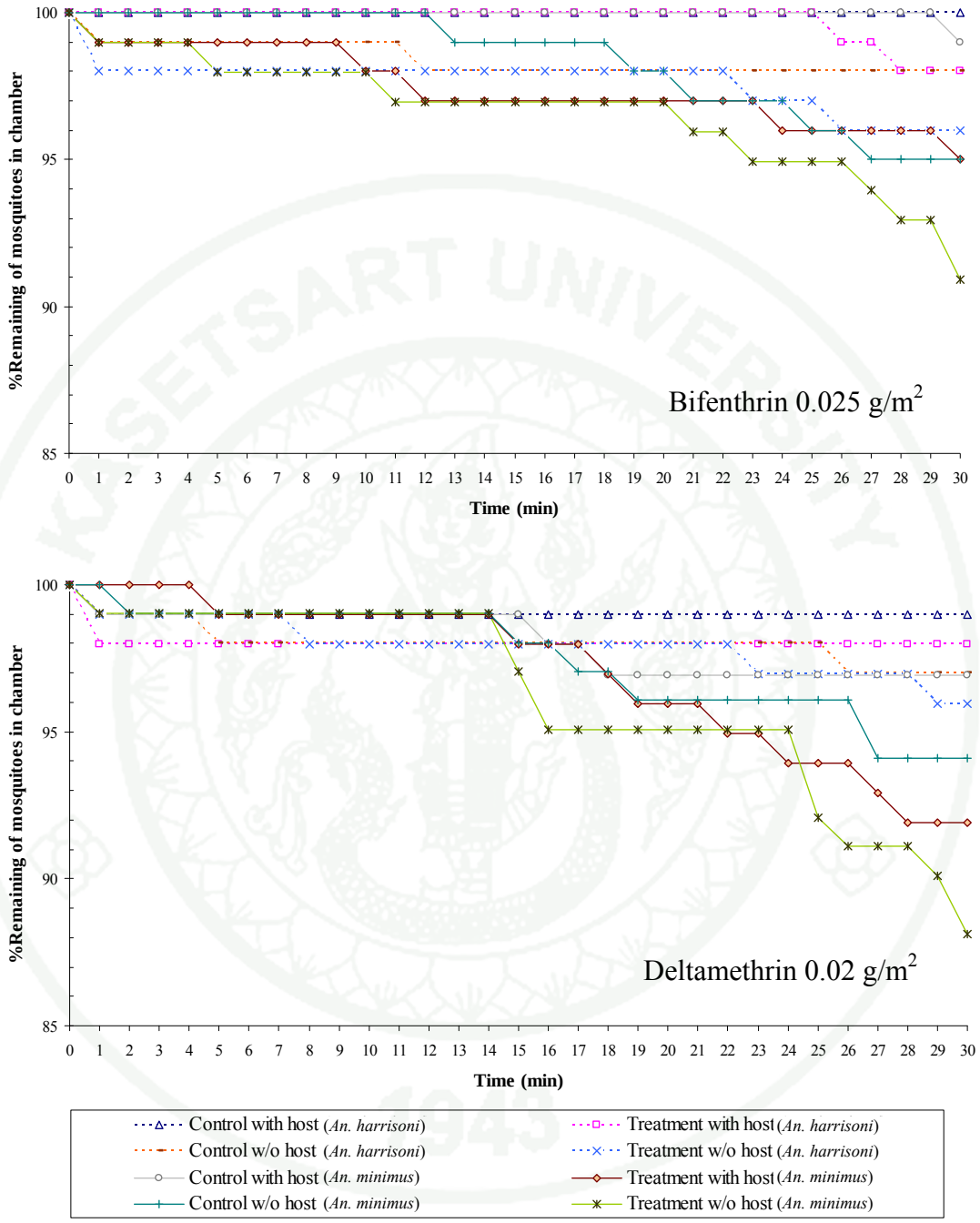

Figure 22 Escape patterns of *Anopheles harrisoni* and *Anopheles minimus* to bifenthrin 0.025 g/m^2 and deltamethrin 0.02 g/m^2 in noncontact trial

DISCUSSION

Part 1 Survey of two species in the Minimius Complex, *Anopheles harrisoni* and *Anopheles minimus* from two different potential breeding sites

Overall, there were more *An. harrisoni* higher density habitats in PT than in BTN although PT had less *An. minimus* than BTN. In BTN, *An. minimus* was associated with pasture land cover with the highest density habitats being present at short distances from a human habitation (< 180 m). While in PT, *An. harrisoni* was associated with forest land cover with high density habitats being present at further distances from inhabited houses (> 320 m) than low density habitats. In addition, *An. maculatus* in BTN was associated with shrub and with plantation in PT with the higher density habitats being present at short distances from a human habitation as well (< 208 m).

Data from this study are supported by the biologies of the target vectors as *An. harrisoni* is more zoophagic than *An. minimus* (Van Bortel *et al*. 1999; Trung *et al*. 2005; Sungvornyothin *et al*., 2006b) therefore more productive habitats of this species may be found further from human host-sources and closer to forest land cover. The closer distance between *An. minimus* high density habitats and houses reflects this species anthropophagic nature.

Species density differences existed between the two study sites indicating that surrounding land cover may be associated with species-specific productive larval habitats. Studies should be repeated at other locations within the geographic range of *An. harrisoni* and *An. minimus* to generate a broader dataset and control for potential land cover artifact.

Part 2 Seasonal abundance of larvae of *Anopheles harrisoni* and *Anopheles minimus* in two different potential breeding sites

2.1 Bong Ti Noy

The Minimus Subgroup is represented in Thailand by two sibling species of *An. minimus* and *An. harrisoni*. While *An. minimus* was the predominant species detected, both species were found sympatric in BTN Village in Kanchanaburi Province. Only a few studies have examined the biology and habitats of *An. minimus s.l.* larval mosquitoes in Thailand (Rattanarithikul *et al.,* 1995; Overgaard *et al.,* 2002).

Fluctuations of mosquito population densities can be highly dependent on environmental factors, such as climate and habitat availability (Dutta and Dutt, 1978; Laird, 1988; Zhou *et al.,* 2007; Teng *et al.,* 1998). The results indicated that the larval population densities of different species were affected by rainfall patterns, which led to the changes of the water movement (velocity) and other physical/chemical parameters that might have influenced increasing or decreasing larval numbers. In particular, strong water current can naturally impact amount of floating materials (turbidity) and aquatic vegetation along the stream banks, therefore either causing anopheline larvae to be physically washed away, eliminating vegetation and debris that may serves as protection against natural predators, or sites becoming less preferential for gravid females to oviposit. This appears to be the explanation for dramatic reductions in larval densities during periods of much higher rainfall in the first year. However, in the second year of collections, larval population densities were not greatly affected by increases in overall rainfall. This may have been due, at least partly, to the construction of a series of small water control check-dams in the upstream areas for agricultural purposes during the study period. The dams reduced the water velocity and diminished the magnitude of fluctuations in depth of water. With a more 'stable' environment, sheltered from extremes in precipitation, the riparian/littoral vegetation and floating debris could maintained for sufficiently longer periods of time, thus protecting immature mosquitoes from both adverse water

current and potential predators. However, only two larvae were collected in September of the second year, a month which recorded more than 200 mm of rainfall. In the dry season of both years, *An. minimus* and *An. varuna* larvae were relatively more common than other times of the year. Similarly, *An. minimus* was found more abundant during the dry season in Pang Mai Daeng Village, Chiang Mai Province, northern Thailand (Overgaard *et al.,* 2002). The mean levels of turbidity, water velocity and temperature, were found to be lower in the dry season than other periods of the year which appear associated with higher larval densities and more ideal habitat for development. During the wet period of the year, higher flow rate and turbidity were evident.

As with almost any field study, certain limitations apply to the design, analysis and interpretation of the data collected. The fact that approximately two-thirds of the collected larvae did not survive to eclosion (adulthood) and thus were not identified either morphologically or using PCR potentially biases the findings as different species and stage of instar will determine probability of rearing success. Species identification by examining DNA found both *An. harrisoni* and *An. aconitus* too few in numbers to justify any meaningful analysis on these two species. Further study in BTN could determine if the relatively scarcity of both species is a normal year-to-year distribution or simply just what was observed during the two years of sampling would be worthwhile. It would have possibly been more useful to collect daily rainfall data at the study site to allow more accurate examination of the frequency and amount of rainfall over time. Lastly, more detailed monitoring of the larval habitat and various stream parameters using a continuous electronic measuring device would have increased understanding of the changing dynamics between survey points.

2.2 Pu Teuy

Pu Teuy Village in Kanchanaburi Province is the sympatric area of the two sibling species, *An. minimus* and *An. harrisoni* (Sucharit *et al*., 1988; Chareonviriyaphap *et al*., 2003; Sungvornyothin *et al*., 2006a, b). In the study, *An. harrisoni* was the most commonly collected mosquito species along the stream margins and represented more than 97% of the total larvae collected in two year period. *Anopheles minimus* larvae were collected lower proportion than *An. harrisoni* in this study area. Corresponding to a study in the past 20 years by Sucharit *et al*. (1988) found that 0.05% of all collected *An. minimus s.l.* was *An. minimus* M form (*An. minimus*) and 94.72% *An. minimus* P form (*An. harrisoni*) in the same area.

Low numbers of larvae in January of both years were attributed to the removal of vegetation along the stream to improve drainage. This activity commonly was conducted at the end of wet season and temporarily modified larval habitats. Similarly previous study in the same area by Chareonviriyaphap *et al.* (2003) reported that the activity was conducted in the different months, November and December. Other than, some months in wet season of the second year (July to October 2008), the vegetation were removed for agricultural purposes in several sampling points which close to agriculture areas, particularly the sampling point that had high numbers of larvae before. Fluctuations of mosquito population densities can be highly dependent on environmental factors, such as climate and habitat availability (Dutta and Dutt, 1978; Laird, 1988; Zhou *et al.,* 2007; Teng *et al.,* 1998). The results speculated that the larval population densities may affected by changing environmental habitat associated with human activity. Environmental modification, e.g. changing the banks of water by vegetation removal has been shown to reduce the mosquito population (Grieco *et al*., 2005; Lawler *et al*., 2007), such a small stream in PT.

Moreover, *Anopheles maculatus* group is also an important malaria vector (Chareonviriyaphap *et al*., 2000; Sinka *et al.*, 2011), was the second most common species (20.5 % collected, BTN; and 1.74% collected, PT) encountered during two years. In fact, actual adult densities could have been much higher than

represented in the larval sampling as *An. maculatus* can utilize other aquatic habitats such as ground pools, ditching, and flooded rice fields (Rattanarithikul *et al.,* 1995; Ndoen *et al.,* 2010; Rohani *et al.,* 2010) which were not sampled in the study.

The findings of this study indicated the one environmental variable that had the greatest influence on larval population density in similar stream in BTN was "rainfall". This proxy variable could be used to forecast larval population densities of species that typically inhabit riparian environments in western Thailand. Information derived from this type of study can be used to predict mosquito species distributions by season and prevailing climatic activity and may prove useful in forecasting malaria transmission in a given area and assist in the timing of recommended control measures. In addition, the data further in PT was supporting evidence that environmental modification of larval habitats is a potentially important strategy for anopheline larval control.

Part 3 Identification of the two species of the Minimus Complex, *Anopheles harrisoni* and *Anopheles minimus* collected from two different ecological breeding habitats by morphological and molecular identification

Correct and precise identification of mosquito vectors is important in many respects including development of vector control strategies. Especially the vector species complex, consist of a number of morphologically almost identical species that are very closely related. Vectorial and behavioural variations found among these species groups or complexes constitute the major reason that needs accurate and precise identification (Garros *et al*., 2004).

Morphological misidentifications of closely related sympatric species are common (Van Bortel *et al.,* 2001). Within the Minimus Complex and other closely related species in the Funestus Group, accurate identification cannot be made by morphological characters alone. Morphological identification of *An. harrisoni* BTN population was found to have a very high percentage (96%) of misidentification compared to AS-PCR confirmation. The majority of *An. harrisoni* specimens were molecularly identified as *An. minimus* (68%), followed by *An. varuna* (28%), a member of the Aconitus Subgroup. In all, this resulted in only two specimens of *An. harrisoni* being detected only during the second year of collections. Whereas PT, morphological identification of *An. minimus* was found to have a high percentage (66%) of misidentification, the majority of *An. minimus* specimens were molecularly identified as *An. harrisoni* (64%), followed by *An. varuna* (2%).

Correct identification of both sexes of *An. aconitus* in both locations were more than 99% with a high probability of identifying *An. aconitus* and other three closely related species correctly base on proboscis characteristics (PPV = 1, NPV > 0.99). The results revealed that pale scales on the distal half of the proboscis can reliably separate *An. aconitus* and other closely related species. Harrison (1980) found that 1.3% and 6.1% of *An. minimus* specimens from Hong Kong and Thailand, respectively, had pale scales on the proboscis (uncommon characteristic in *An. minimus*). Similarly in recent study in Vietnam by Cuong *et al*. (2008) found that

99.9% of specimens that had pale scaling on the distal half of the proboscis were *An. aconitus*; this characteristic was found in only 0.1% of An. *minimus* C (*An. harrisoni*) specimens and 6.6% of *An. minimus* A (*An. minimus*) specimens. In this study, no specimens of other closely related species had this characteristic. However, the specimens had no this characteristic but identified as *An. aconitus* by molecular method, 0.22% in BTN and 0.04% in PT. There was indistinct to separate *An. varuna* from *An. minimus* complex of PT population by using only the absence of PSP on costa vein. Especially male mosquitoes showed low PPV (0.333 and 0.6667 for female). Similarly a study in Vietnam by Van Bortel *et al*. (2001) found that the most specimens of *An. varuna* species (92%) misidentified as *An. minimus* by morphology. Differ from BTN, the using this characteristic was higher reliable identification of *An. varuna* species. PT population, 98% in female and 91% in male of the *An. minimus* complex were correctly identified, with a high probability (0.99 in both sexes) of identifying *An. harrisoni* correctly but a low probability (0.7541 in female and 0.1915 in male) of identifying *An. minimus* correctly, based on the 2 PSP and HP phenotypes. Contrary in BTN, the *An. harrisoni* specimens that present HP phenotypes was unusual morphotype of *An. minimus* as showed with a lowest PPV (0.0741 in female and 0 in male) and maximum NPV. Green *et al*. (1990) obtained 63% correct identifications, Van Bortel *et al*. (1999) 67%, and Sungvornyothin *et al*. (2006) a higher percentage (86.8%). Therefore, even if the HP phenotype seems to be present in *An. harrisoni* with high reliability, this phenotype also may be present in *An. minimus*, with high spatial variations.

As pointed out by Harrison (1980), no morphological characters are completely reliable for distinguishing the adults of these species. The characteristics of specific species were varied in different ecological habitat. In addition, the identification would seem be more accurate if the species is present prevalence in an area.

Part 4 Behavioral responses of *Anopheles harrisoni* and *Anopheles minimus* to bifenthrin and deltamethrin using an excito-repellency system and a live host

Bifenthrin and deltamethrin are pyrethroid insecticides recommended by the World Health Organization for indoor residual spraying against malaria vectors. Deltamethrin has a relatively high irritant and knockdown effect on mosquitoes when compared to bifenthrin, whereas bifenthrin, which is considered to elicit low irritant effects, consistently provides a high kill due to the ability of mosquitoes to rest on treated surfaces for longer periods of time (WHO, 2001). However, the kill effect of insecticides also depends on the level of resistance/tolerance of the target vector population to the chemical being applied. Some studies have reported that physiological and biochemical resistance can influence behavioral avoidance as well (Sparks *et al.*, 1989).

The excito-repellency test system reported in the current study with subsequent design modifications has been previously used to evaluate the behavioral responses of mosquitoes to insecticides and provide information on both contact irritancy and noncontact repellency behavioral responses (Chareonviriyaphap *et al.*, 1997; Sungvornyothin *et al.*, 2001; Kongmee *et al.*, 2004; Potikasikorn *et al.*, 2005; Sathantriphop *et al.*, 2006; Pothikasikorn *et al.*, 2007). By adding another variable-a live host-to this test system were able to measure the effect of host cues on escape response to insecticides under contact or noncontact test conditions with chemicals.

The results demonstrated that contact irritancy was the primary action of both deltamethrin and bifenthrin to *An. harrisoni* and *An. minimus* test populations with no noncontact repellency effect from either insecticide. Greatest irritancy effects for both chemicals were observed in *An. minimus*, with deltamethrin producing stronger responses than bifenthrin. Overall, 24-h mortality rates of both *An. minimus* and *An. harrisoni* were higher when exposed to deltamethrin than with exposure to bifenthrin. In addition, the mortality rates from exposure to bifenthrin of *An. minimus* were lower than exhibited in *An. harrisoni* populations. As both mosquito populations are

susceptible to the chemicals evaluated, these differences may be due to innate behavioral characteristics of each species to avoid or contact insecticide-treated surfaces, resulting in different mortality rates. Previous observations have indicated different responses to DDT, deltamethrin, lambda-cyhalothrin, bifenthrin, and DEET between *An. minimus* and *An. harrisoni* (Potikasikorn *et al.* 2005, Tisgratog *et al.* 2011). Such behavioral avoidance to bifenthrin may result in mosquitoes moving to a chemical-free surface or area before acquiring a lethal dose (Kennedy 1947; Muirhead-Thomson 1960; Roberts *et al.* 2000).

For *An. harrisoni*, the presence or absence of host had no significant effect on escape responses to both insecticides. The physiological state (mating condition) of test populations could be an influential factor in escape movements because *An. harrisoni* is normally colonized in the laboratory using forced-mating techniques. The tested *An. harrisoni* mosquitoes were mostly unmated mosquitoes. However, in similar evaluations conducted by Pothikasikorn *et al.* (2007) using the same test system and field-collected *An. harrisoni* exposed to deltamethrin but without any host involved, escape responses were similar to those reported in this study, (51% escape in field populations, 49% in colony populations) but with lower mortality (2%) as compared to current results (18-40% mortality).

For *An. minimus*, the presence or absence of a host had significant effects on behavioral responses to both insecticides with the presence of a host delaying mosquito escape from the treated chamber. This phenomenon may occur when test populations originate from different colonies due to variations in physiological condition of mosquitoes (i.e., mated or unmated) and attractive substances produced by the host (guinea pig) that preferentially attract mated-female mosquitoes. However, all mosquito test cohorts in the study were populations from the same free-mating colony, thereby representing mixed physiological status.

In conclusion, the results clearly demonstrate that deltamethrin elicited stronger irritant effects in both *An. harrisoni* and *An. minimus* than bifenthrin, even in the presence of a host. Such information is beneficial for designing future behavioral studies, either under controlled laboratory or field conditions, to examine responses of other anopheline mosquitoes following exposure to insecticidal compounds used in vector control.

CONCLUSION

Two different ecological breeding habitats of vectors from the Minimus Complex in Bong Ti Noy (BTN) and Pu Teuy (PT) Villages, Sai Yok District, Kanchanaburi Province were characterized using a Geographical Information System (GIS) in combination with remote sensing technology and mosquito field data. The study site, BTN is located at an altitude of ~100 m above sea level and has reported an annual average of 38 cases of malaria during 2006 to 2008. The village of PT, on the other hand is located at an altitude of ~300 m above sea level and few malaria cases are reported. In this study, nine mosquito species were obtained from BTN and eight species identified from PT. *Anopheles harrisoni* existed at higher density habitats in PT than in BTN, although PT had less *An. minimus* than BTN. In BTN, *An. minimus* was associated with pasture land cover with the highest density habitats being present at short distances from a human habitation (< 180 m). While in PT, *An. harrisoni* was associated with forest land cover with high density habitats being present at further distances from inhabited houses (> 320 m). Species density differences existing between the two study sites indicate that surrounding land cover may be associated with species-specific productive larval habitats.

Fluctuations of larval population densities at both sites indicate that the larval population densities of the various species collected in BTN were affected by rainfall patterns, while in PT, the larval population densities seemed more affected by changing environmental conditions associated with human activity. Given this information, rainfall could be used to forecast larval population densities of species that typically inhabit riparian environments in western Thailand. In addition, the data also supports evidence that environmental modification of larval habitats is a potentially important strategy for anopheline larval control.

The morphological characteristics of specific species varied from BTN and PT collection sites and no morphological characters were completely reliable for distinguishing *An. harrisoni* and *An. minimus* of the Minimus Complex species. In addition, the identification seemed more accurate when the species was present at

high prevalence in a given area. Therefore, molecular identification remained the most efficient method to obtain an unambiguous differentiation of these two species. Correct species identification is essential and mandatory for any relevant study on the Minimus Complex and for the application of successful control strategies.

Behavioral responses of *An. harrisoni* and *An. minimus* were evaluated following exposure to two pyrethroid insecticides, bifenthrin and deltamethrin, using an excito-repellency test system in the presence and absence of live host cues. The results demonstrated that contact irritancy is the primary action of bifenthrin or deltamethrin in both mosquito species. There was no non-contact repellency effect elicited by either insecticide. *Anopheles minimus* showed rapid escape response with high mortality rates following direct contact with deltamethrin in the absence of a host and delayed escape responses when a host was present. Similarly, exposure of *An. minimus* to bifenthrin also elicited a delayed escape response in the presence of a host but with lower mortality rates. For experiments using *An. harrisoni*, the presence or absence of a host had no significant effect on behavioral responses to either insecticide ($P > 0.05$). The result revealed that deltamethrin elicited stronger irritant chemical effects than bifenthrin but that behavioral responses in vector populations are dampened in the presence of an available host. This information is useful for estimating probability of pathogen transmission when using irritant chemicals in proximity to a blood-meal source.

RECOMMEDATION

The following recommendations have been developed following completion of the currenty study. Overall, further research is required to provide a better understanding of site-specific characteristics of mosquito vectors of medical importance. This includes ensuring surveys of larval habitats and larval population densitites are conducted monthly within specified seasons of the year to ensure that information is not missing for some months. In addition, any remotely-sensed data (i.e. aerial photgraphs; satellite images) of land use around larval habitats utilized in a GIS analysis should reflect no more than a two year difference in time from the survey being conducted and multiple images of the same area should be included to increase data reliability. Transfer of larvae from the field to the laboratory should be minimized to avoid excess mortality during transport and thereby data loss - especially those within the first and second instar stage. Where possible, rearing to later stage larvae should be conducted prior to transport or most optimal is to rear adults on site. Additional behavioral studies of these two species, *An. harrisoni* and *An. minimus*, with the same insectcides should be conducted with corresponding field populations to be clear, in mechanism of behavioral responses (irritancy and/or repellency) to insecticides of both laboratory and field populations that are in the same or different. The information would be useful to serve as a baseline for evaluating the response to insecticides in the future field studies.

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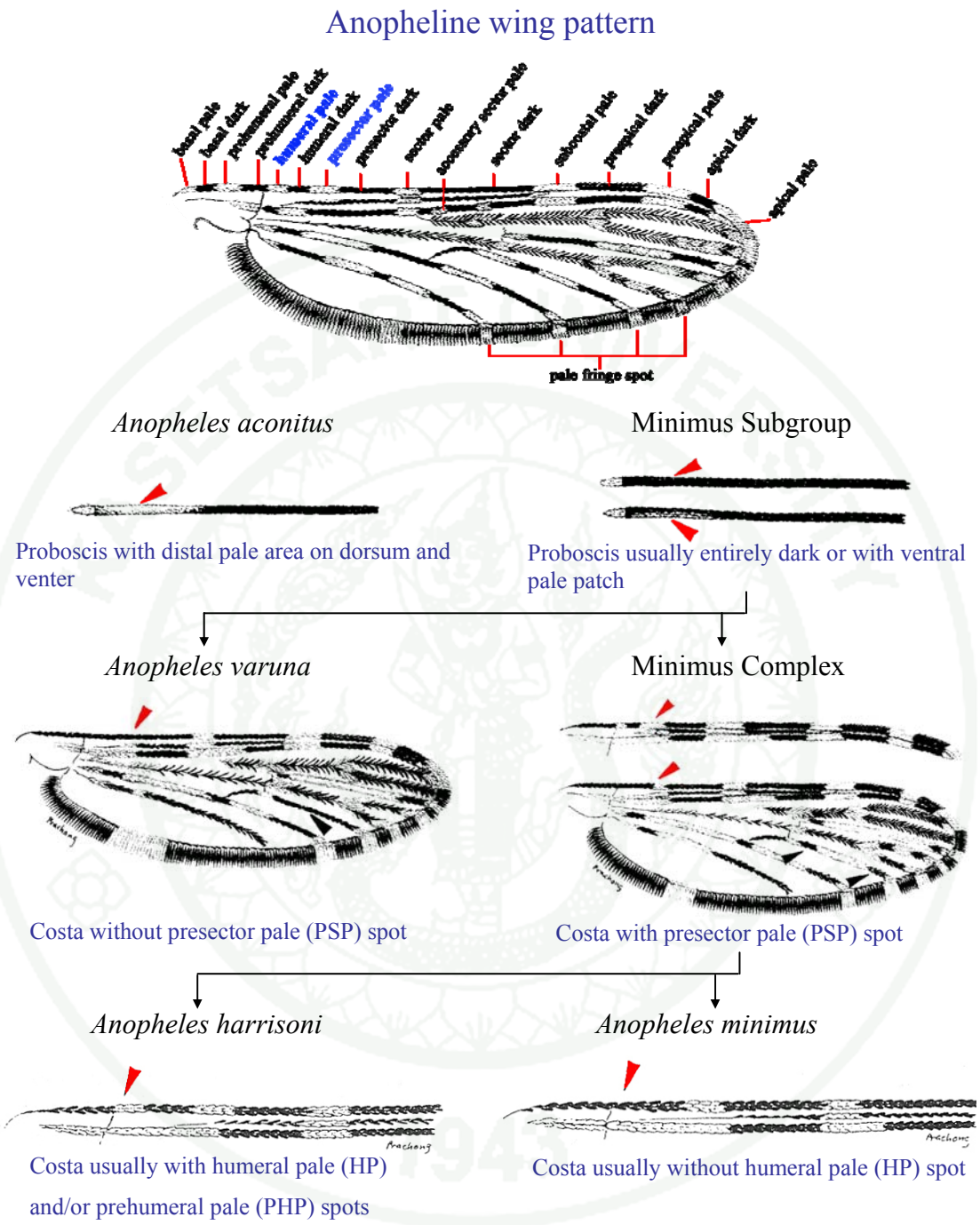
APPENDIX



Appendix Figure 1 Anopheline wing patterns and morphological characters of mosquito wing use to identify adult mosquitoes, *Anopheles aconitus*, *Anopheles varuna*, *Anopheles harrisoni*, and *Anopheles minimus*

Source: Modified from Rattanarithikul *et al.* (2006)

		Condition (as determined by "Gold standard")		
		Condition Positive	Condition Negative	
Test Outcome	Test Outcome Positive	**(a)** True Positive	**(b)** False Positive	Positive predictive value = a / (a + b)
	Test Outcome Negative	**(c)** False Negative	**(d)** True Negative	Negative predictive value = d / (c + d)
		Sensitivity = a / (a + c)	Specificity = d / (b + d)	Index of validity = (a + d) / (a + b + c + d)

Appendix Figure 2 Diagram illustrates of related calculation of the reliability test indexes, the positive predictive value (PPV), negative predictive value (NPV), sensitivity, specificity, and index of validity

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