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THESIS

POPULATION STUDIES OF THE *ANOPHELES MINIMUS* COMPLEX, VECTOR OF MALARIA IN THAILAND: GENETICS AND BEHAVIORAL ASPECTS

PISIT POOLPRASERT

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Entomology)
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Pisit Poolprasert 2007: Population Studies of the *Anopheles minimus* Complex, Vector of Malaria in Thailand: Genetics and Behavioral Aspects.
Master of Science (Entomology), Major Field: Entomology, Department of Entomology. Thesis Advisor: Associate Professor Pongthep Akratanakul, Ph.D. 65 pages.

Six-field populations of *Anopneles minimus s.l.* from Kanchanaburi Province were compared using isozyme starch gel electrophoresis to study the gene flow rate between and among populations. From eight enzyme systems, 9 loci with 6 polymorphism were detected. Small levels of genetic differentiation were observed when all populations were compared (F_{ST} = 0.053). The highest percent polymorphic loci were recorded in Bong Ti Noi, whilst the lowest was in Tha Kradan. Gene flow among the six local populations varied from 4.30 to 62.25 reproductive migrants per generation. However, overall gene flow migration was only 4.47 when all populations were studied. Isolation by distance among all populations showed no correlation between genetic and geographical distance (P>0.05).

Additionally, an investigation of the house entering behavior of *An. minimus s.l.* into the experimental huts was performed using human landing collection method. High proportion of *An. minimus s.l.* entered the huts was found shortly after sunset and continued throughout the night with an obvious peak during 1900-2100 hr. Additional trials were conducted after huts were sprayed either with DDT and deltamethrin. Comparative results showed DDT had even more powerful repellency effect over deltamethrin.

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Pisit Poolprasert

May, 2007

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POPULATION STUDIES OF THE *ANOPHELES MINIMUS* COMPLEX, VECTOR OF MALARIA IN THAILAND: GENETICS AND BEHAVIORAL ASPECTS

INTRODUCTION

The *Anopheles minimus* complex, Theobald 1901, is known as an important malaria vector throughout the Asian and Southeast Asian countries (Reid, 1968). In Thailand, this complex consists of at least three related species including species A (presently = *An. minimus s.s.*), *An. minimus* C (species C) and *An. minimus* D (species D) (Sucharit *et al*. 1988, Baimai 1989, Green *et al*. 1990). *An. minimus s.s.* is the predominant species and is found throughout the country. This species is generally claimed as the most important vector of malaria in Thailand (Ministry of Public Health (MOPH), 2006). *Anopheles minimus* C is commonly present along the western Thai-Myanmar border, particularly in Kanchanaburi Province (Sucharit *et al*., 1988, Baimai 1989, Green *et al*., 1990, Sungvornyothin *et al*., 2006a and 2006b). Although *An. minimus* C is not considered to be a serous vector in Thailand, several reports demonstrated that this species remains an important vector of malaria in many areas in Asian countries (Chen *et al*., 2000). *Anopheles minimus* D has been reported in Thailand, but insufficient information on this vector has been supported the proposed sibling species status (Baimai, 1989, Somboon *et al*., 2005). Furthermore, species D has been claimed a chromosomal variant of *An. minimus s.s*. (Sungvornyothin *et al*., 2006).

Knowledge on biology, ecology and behavior of vector is paramount importance to identify the epidemiological role in disease transmission. Such information may help in searching cryptic species, defining the vector capacity and developing vector prevention and control operations. Additionally, population movement and population size of efficient malaria vectors are known as the critical factors to support the success of vector control. Currently, none has been reported on the patterns of movement of *An. minimus s.s*., and *An. minimus* C, from Kanchanaburi Province.

Anopheles minimus s.s. and *An. minmius* C have been found in Kanchanaburi Province, specifically in Pu Teuy area, Sai Yok District, Kanchanaburi Province. Rwegoshora *et al*. (2002) reported the species ratio between *An. minimus s.s.* and *An. minimus* C from Pu Teuy area was 1: 3. Recent observation from molecular basis indicated that 96% of *An. minimus s.l.* from Pu Teuy area belongs to *An. minimus* C (Sungvornyothin *et al*., 2006a and 2006b). The reason for this discrepancy remains unclear and questionable but it could partly result from the changes in local environmental and climatic factors that support a competitive advantage to *An. minimus* C in the area (Kengluecha *et al*. 2005). Demographic changes from increased deforestation and urbanization are invariably claimed as primary contributors to affect in species expansion and distribution. Therefore, genetic relationship between *An. minimus* C from Pu Teay and *An. minimus s.s.* from other areas within Kanchanaburi need to be clarify. In this study, we analyze genetic relationships among *An. minimus* populations in two levels, one being among six populations of *An. minimus s.l.*, and the other being between *An. minimus* C and 6 local populations of *An. minimus s.s.* from Kanchanaburi Province in an attempt to define the genetic variations and genetic differentiation among test populations.

In addition, behavioral responses of *An. minimus s.s.* to DDT and deltamethrin are a significant component in vector control operation. Several studies reported that DDT and deltamethrin exert behavioral responses in many insect vectors (Ismail *et al*., 1974, 1975, Roberts *et al*., 2000, Chareonviriyaphap *et al*., 2004). During the past ten years, behavioral responses of several malaria vectors by DDT and deltamethrin have been reported from Thailand (Chareonviriyaphap *et al*. 1997, 2001 and 2004, Sungvornyothin *et al*., 2001, Muenworn *et al*., 2006). However, little has been reported on the entering behavior of *An. minimus s.l.* using experimental huts treated with DDT and deltamethrin. In this study, the responses of DDT and deltamethrin to field *An. minimus s.l.* were conducted using the experimental hut.

OBJECTIVES

1. To determine the genetic relationship among populations of *Anopheles minimus s.l.* and among *Anopheles minimus s.s.* and *An. minimus* C from Kanchanaburi Province using enzyme assay technique.

2. To observe the behavioral responses of DDT and deltamethrin by *An. minimus s.l.* from Pu Teuy area using the experimental hut.

LITERATURE REVIEW

1. Malaria situation

Malaria is known as the most serious vector borne disease in the tropic and subtropics of the world with the estimate of over 105 countries (Figure 1). In recent years, approximately 60% of malaria cases were found in the African countries whereas 30% remain in Asia. In Thailand, malaria remains one of the important infectious diseases, despite years of successful disease control programs and significant reductions in both mortality and morbidity (Ministry of Public Health (MOPH), 2006). The disease is prevalent along the dispersal rural communities along the borders of eastern Myanmar, western Cambodia and northern Malaysia (Figure 2) (Chareonviriyaphap *et al*., 2000). Since 1999, the number of malaria cases has declined from 130,000 to 45,000 in 2002 (Roll Back Malaria (RBM), 2005). As a consequence, the reduction in mortality and morbidity rate has been observed (Figure 3). Nevertheless, malaria remains a major infectious disease with approximately 30,000 to 60,000 cases annually. Recent surveillance data has demonstrated that malaria case report has been fluctuated with the slight increase in 2005 (MOPH, 2006). The reason for the small increase is unclear. It could partly be the consequence of the human migration and trade activities as well as a political crisis along the international boundaries.

In addition, increase in rubber plantation and fruit orchards in the country are also considered to be sensitive and highly conductive to the transmission and therefore human population in such areas are a special group at risk. In 2006, a total of 22,599 malaria cases were reported with 42 deaths (Table 1). Although significant reduction in cases, malaria is still a serious parasitic disease of the country and better understanding on epidemiological complex need to be elucidated.

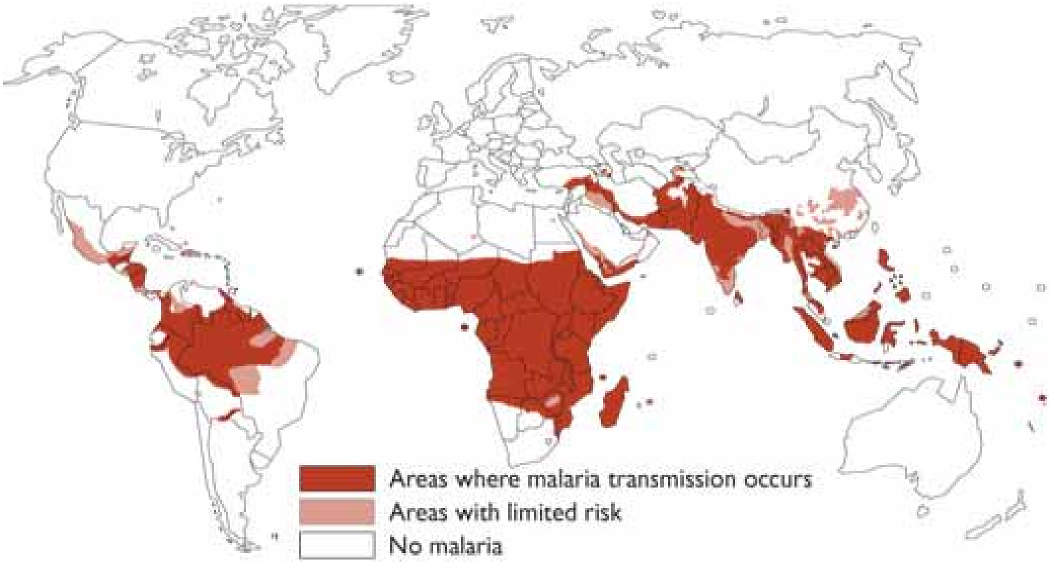


Figure 1 World map depicting endemic areas of malaria in 2006.
Source: World Health Organization (2006)

Figure 2 The ranges with risk of Malaria in these regions.

Source: Centers for Disease Control and Prevention (2006)

Figure 3 Morbidity and mortality rates (per 1000,000 populations) of malaria (1972-2005)

Source: Center of Epidemiological Information, Bureau of Epidemiology, Ministry of Public Health (2006).

Table 1 Case fatality rates of malaria situation in Thailand from January 2006 to December 2006.

Month	**Case**	**Deaths**
January	1,603	3
February	1,152	0
March	1,453	5
April	2,500	7
May	4,622	16
June	3,374	2
July	2,992	1
August	2,110	5
September	1,583	2
October	1,054	1
November	156	0
December	Non applicable	Non applicable
Total	**22,599**	**42**

Source: Center of Epidemiological Information, Bureau of Epidemiology, Ministry of Public Health (2006).

2. Malaria vectors

From a total of 73 *Anopheles* species found in Thailand, only three species are considered to be important malaria vectors i.e. *Anopheles dirus*, *An. minimus, An. maculatus*. All three belong to the species complex of which can not be morphological identified (Rattanarithikul and Panthusiri, 1994). *Anopheles dirus* is composed of ten species, i.e. *An. baimaii*, *An. cracens, An. dirus, An. hackeri, An. introlatus, An. latens, An. macarthuri, An. nemorphilous, An. pujutensis,* and *An. scanloni*. (Rattanarithikul *et al*., 2006). Only four species in the Dirus complex are served as malaria vectors. Those are *An. latens, An. cracens, An. scanloni*, and *An. baimaii*. *Anopheles minimus* group contains at least three genetically related species, *An. minimus s.s.*, *An. minimus* C and *An. minimus* D and three closely, related species i.e. *Anopheles varuna*, *Anopheles aconitus*, *Anopheles pampani* (Rattanarithikul *et al*., 1995). Recent report indicated that *Anopheles fluviatilis*, an important vector of malaria from India is genitically similar to *An. minimus* C (Singh *et al*., 2006). *Anopheles maculatus* group includes *An. dravidicus An. maculatus*, *An. notanandai, An. pseudowillmori*, *An. sawadwongporni,* and *An. willmori* (Rattanarithikul and Green, 1986 and Bangs *et al*., 2002)

3. *Anopheles minimus* complex

Anopheles minimus belongs to Myzomyia Series of subgenus *Cellia* in the Family Culicidae of Order Diptera. Detail background of the zoological classification of this species is given below (Service, 1993).

Phylum Arthropoda
Order Diptera
Suborder Nematocera
Family Culicidae
Genus *Anopheles*
Species *minimus*

Anopheles minimus Theobald 1901 taxon is regardes as a primary vector of malaria in forest fringe and hill areas of East Asia, including Thailand (Ismail *et al*., 1978). Previous studies on distribution patterns, morphology, genetics, biting behavior and host preferences have demonstrated the occurrence of complex within *An. minimus* (Baimai, 1989; 1996, Green *et al*., 1990; Rattanarithikul and Punthusiri 1994; Van Bortel *et al*., 1999). Ismail *et al*. (1978) observed the differences in outdoor biting activity from different populations of *An. minimus* in Thailand. This phenomenon was subsequently confirmed by Ratanatham *et al*. (1988), Green *et al*., 1996, Rattanarithikul *et al*. (1996), and Chareonviriyaphap *et al.* (2003). Difference in wing patterns on the costa vein has also been reported from different geographical areas and resulted into two phenotypic groups. The marked differences in such various characteristics confirmed the presence of species complex within *An. minimus* species (Rattanarithikul and Punthusiri, 1994; Van Bortel *et al*., 1999; Sungvornyothin *et al*., 2006a and 2006b).

Currently, the *Anopheles minimus* complex contains at least nine recognized species in Oriental Region and one species in Africa, *Anopheles funestus* (Green, 1982; Sungvornyothin *et al*., 2006). The *An. minimus* complex in Thailand consists of three closely related species, *An. minimus s.s., An. minimus* C, and *An. minimus* D which remain difficult to accurately identified from one another morphologically (Baimai, 1989; Rattanarithikul and Panthusiri, 1994; Van Bortel *et al*., 1999; Sungvornyothin *et al*., 2006a and 2006b). *Anopheles minimus s.s.* is an important malarial vector that is found throughout most of Thailand; whereas species C seems to be along the western Thai-Myanmar border, especially in Kanchanaburi Province (Kengluecha *et al*., 2005 and Sungvornyothin *et al*., 2006). *Anopheles minimus* D has been reported in Thailand but information is limited on the distribution patterns and medical significance of this sibling species in the transmission of malaria (Baimai 1989; Somboon *et al*., 2005 and Sungvornyothin *et al*., 2006). Previous reports on behavioral differences between *An. minimus s.s*. and C in Vietnam has demonstrated species C exhibiting predominently exophagic and zoophilic behavioral characteristics compared to *An. minimus s.s*. (van Bortel *et al*., 1999).

Rwegoshora *et al*. (2002) and Sungvornyothin *et al*. (2006) have observed the greater outdoor feeding activity of *An. minimus* C in Thailand.

4. Vector control

One of the reliable methods that used to combat the malaria is a vector control. This method remains the most possible method in the flight against malaria. Thailand had many years of success in its national control program based primary on vector control and the application of indoor residual DDT to control malaria transmission (Prasittisuk, 1985; Chareonviriyaphap *et al*., 2000). DDT supported by various international donor was first used in Chiang Mai Province from 1949-1951. From the successful field trials, DDT appeared to be the insecticide of choice for malaria control in the country. Due to negative response to spraying and the adverse long term impact on environment, the use of DDT in malaria control was terminated in 2000. Although banned, DDT had been reported as the chemical of choice in malaria control worldwide (Brown, 1976). Several published reports the potential function of DTT in disease control (Robert *et al*., 1997; Bang, 2000). Such reports presented the wonderful action of DDT in repelling mosquitoes and later referred too an excito-repellency (Kennedy, 1946; Roberts, 1993). Roberts *et al* (2000) examined DDT in malaria control and provides compelling the dominant action on action on mosquitoes in reducing human-vector contract inside sprayed house.

Synthetic pyrethroids are currently an insecticide of choice for malaria control in Thailand. Pyrethroids have known great promise for pest control due to their low mammalian toxicity and remarkable potency at low level that quickly immobilizes kills and repels insects (Prasittisuk, 1994 and Chareonviriyaphap *et al*., 1997). WHO described pyrethroids and accepted it as terrific compound in vector control worldwide (WHO, 1995). Patipong (2000) reported that pyrethroids gained general acceptance for use in impregnating bed nets (permethrin) and for indoor residual spraying (deltamethrin).

Deltamethrin, odorless compound, is a synthetic insecticide based structurally on pyrethins, which rapidly paralyze the insect nervous system giving a quick knockdown effect (EXTOXNET, 1995). Deltamethrin has been used worldwide, ranging from agricultural used to home pest control. Several trade names under deltamethrin product including Butolin, Butoss, Cislin, Crrackdow, Decis, Decis-Prime, K-Okytek (EXTOXNET, 1995). Deltamethrin has been sold in many countries for agricultural public health and livestock application (Thomson, 1995). In 1995, deltamethrin impregnated bed nets provides a high degree of personal protection against all the local species of human biting mosquitoes (Jana-Kara *et al*., 1995).

MATERIALS AND METHODS

1. Genetic relationship between *Anopheles minimus s.s.* populations

1.1 Collection sites

Based on entomological records, mosquito prevalent, and malaria cases, *Anopheles minimus s.l.* was collected from six different locations in Kanchanaburi Province, western Thailand (Figure 4). Detail background of locations was given below.

1. Bong Ti Noi population (BTN), a village in Sai Yok Kanchanaburi Province (14^{o}17’N, 98^o 56’E.). This area is located in mountainous area, surrounded by dry forest on one site and vegetable field on the other. There is a stream, running across the village during the dry season. The stream becomes the river during the wet season.

2. Huai Khayeng population (HK), a village in Thong Pha Phum District, Kanchanaburi Province (14^{o}68’N, 98^o 59’E). This area is located in the south of Khao Laem or Wachiralongkon dam, surrounded by the evergreen trees and dry forest. There are natural streams, running from foot of the hill to dam.

3. Na Suan population (NS), a village in Si Sawat District, Kanchanaburi Province (14^{o}70’N, 99^o 09’E). This area is located the east of Si Nakharin dam, surrounded by seasonal evergreen forest, cassava, and vegetable field. The stream runs from the deep forest to the dam.

4. Tha Kradan population (TK), a village in Si Sawat District, Kanchanaburi Province (14^{o}40’N, 99^o 08’E). The site is located in the west of Si Nakharin dam and is covered by hill evergreen forest and corn field.

5. Pra Jedee population (PJ), a village in Sangkhla Buri District, Kanchanaburi Province (14^{o}66’N, 98^o 59’E). This area is located in the far west of

the province, at the boundary to Myanmar near the Wachiralongkon dam. The area is surrounded by deep forest and rubber plantation. Natural running stream runs through the site and remains the water source for villagers.

6. Pu Teuy population (PT), a village in Sai Yok District, Kanchanaburi Province ($14^{o}17'$N, 99^{o} 11'E.). The site is located in mountainous terrain area, surrounded by deep forest. There is a slow running stream, coving with native vegetation along its margin near the collection site. This stream is found to be the main habitat of *An. minimus s.l.* (Baimai, 1989; Chareonviriyaphap *et al*. 2003)

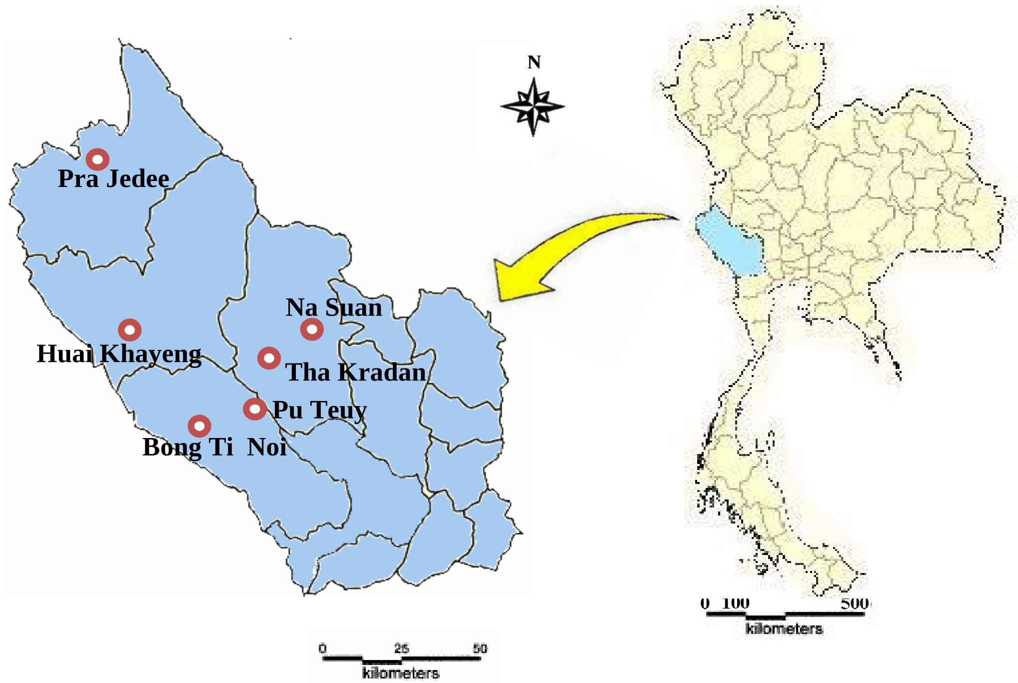


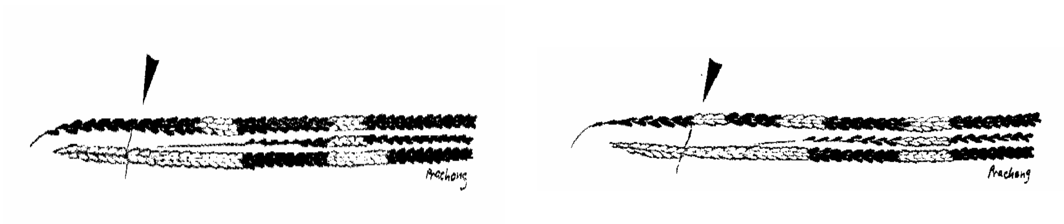
Figure 4. Collection sites locate within Kanchanaburi Province

1.2 Mosquito population

All *An. minimus s.l.* were collected as larvae or pupae and reared to adults in a protected insectary at the Department of Entomology, Kasetsart University. As it is difficult to obtain the larvae and pupae of *An. minimus s.l.*, an additional collection from human bait was also performed to increase the sample size. Strict segregation of field specimens was maintained in the insectary to prevent potential contamination from the others. Adult mosquitoes were either tested immediately or shortly frozen (- 20^{o} C) before processing.

1.3 Mosquito identification

Anopheles minimus s.l. mosquitoes were identified by morphological keys (Harrison, 1980; Peyton and Scanlon, 1966; Rattanarithikul *et al*., 2005). Species were separated by the presence or absence of the humeral pale spot on the costa wing vein. *Anopheles minimus s.s.* was determined by the presence of a wing costa without the humeral pale on both wings whereas *An. minimus* C has the humeral pale spot at least on one wing (Figure 5).



Anopheles minimus s.s. *Anopheles minimus* C

Figure 5 Wing pattern between *Anopheles minimus s.s.* and *An. minimus* C.

1.4 Starch gel electrophoresis

An average of 20-30 adults from each population was processed and tested by electrophoresis. Starch gel electrophoresis was performed on the nine potential enzyme systems using the Morpholine (Morph) buffer system (Pasteur *et al.*, 1988). Horizontal starch gel electrophoresis was conducted as described in Harris and Hopkinson (1976), Manguin *et al.* (1995) and Chareonviriyaphap and Lerdthusnee (2002).

Electrophoresis was carried out on starch gel using 55 g of Sigma's potato starch (Sigma chemical Co., St. Louis, MO), 25 g of sucrose and 550 ml of the appropriate gel buffer. Each mosquito was ground in 25 μl of grinding buffer (25 μl/3 wicks) and the homogenate was absorbed onto 0.4 x 1.4 cm cellulose polyacetate wicks (Gelman Sciences Inc., Ann Arbor, MI). The Morph was run for approximately 5 hours at a constant power of 16 volts/cm (Manguin *et al.*, 1995). Each gel was stained and incubated at 37^{o}C for 15-60 minutes.

1.5 Data analysis

Analysis of allele frequencies, heterozygosity per locus, conformity to Hardy-Weinberg expectations and genetic distances were calculated using BIOSYS-1 (Swofford and Selander, 1989). Differentiation among populations was determined using F-statistics (F_{ST}). The number of effective migration rate ($N_e m$) among populations were estimated from the F_{ST} values with equation $N_e m = (1-F_{ST})/4F_{ST}$ (Wright, 1978). The exchange of genes was estimated from the number of effective migration rate. GENEPOP-3.1 was used to analyze the isolation by distance between populations (Rousset, 1997). This was measured by the relationship between pairwise estimates of F_{ST} and logarithms of geographical distance to determine whether geographical distance among populations serves as a barrier to gene flow. Cavalli-Sforza & Edwards (1967) chord distance and modified Rogers distance (Wright, 1978) were used for the cluster analysis by unweighted pair group method arithmetic averaging (UPGMA) to produce the phenogram.

2. Entering behavior of *Anopheles minimus s.l.* into the experimental hut treated with DDT and deltamethrin

2.1 Study site

The study was conducted at Ban Pu Teuy, Sai Yok District, Kanchanaburi Province, western Thailand (NE 14 99 CA), 14°17'N, 99° 11'E. The study site is mountainous and surrounded by deep forest, approximately 500 m from the nearest house at Ban Pu Tuey. The main occupation of people is logging, plant and animal hunting, and forest reservation labors. This area is a potential habitat for *An. minimus s.l.* and other important Anopheline vectors. There is a long, slow moving stream (approximately 2 m-width and 1 m-dept) with natural vegetables along the edge of the low hill zones runs across the villages. Deltamethrin has been used as intradomicillary spray for malaria prevention since 1994 (Chareonviriyaphap *et al*., 1999).

2.2 Experimental hut

Two experimental huts with identical patterns were used for the study of entering behavior of *An. minimus s.l.* (Chareonviriyaphap *et al*., 2006). Huts were built using locally acquired materials. Hut frames were made of iron pipe and custom-welded galvanized pipes (Figure 6). Pieces of non-treated wood planks, measuring 1 m x 2.5 m in length served as the side walls. Floors were adjusted and aligned with cement blocks and an A frame style zinc roof was put in place. The top of the angled roof measured 3.5 m from the ground level. There are four eaves between the top of the wall and roof on the front (2 eaves) and rear (2 eaves) walls. The eaves are sealed by 1/12-in aluminum wire mesh fastened across the eave opening. All three windows, one on each of three side-walls, and one door were affixed with traps (Figure 7) which remained open during the period of collections. Two huts were positioned 100 m apart in an isolated area, surrounded primarily by mountainous terrain and agricultural fields (Figure 8).

Figure 6 Hut frames from iron pipe and custom-welded galvanized pipes

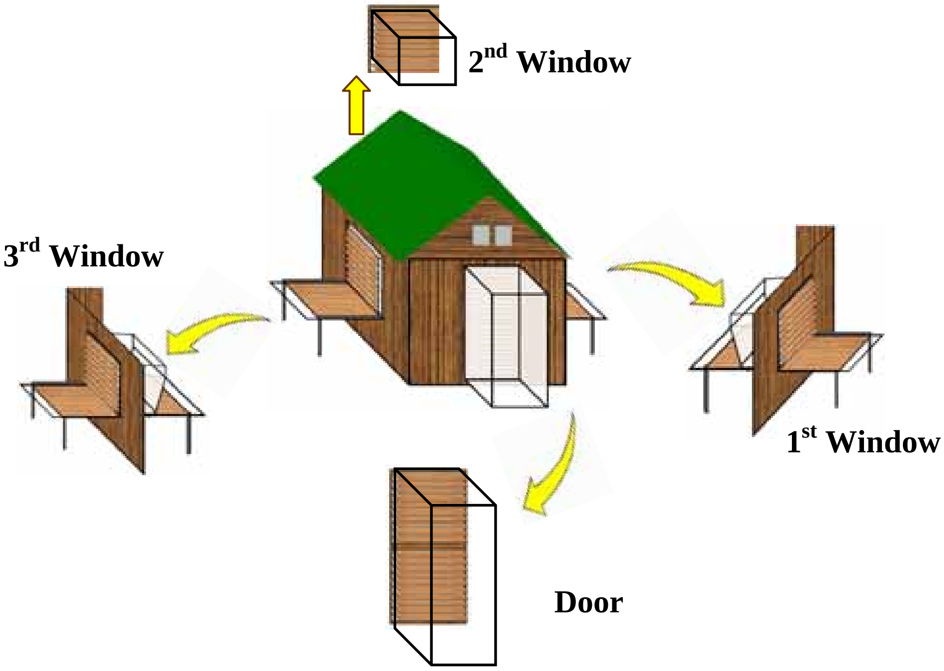


Figure 7 All three windows, one on each of three side-walls, and one door affixed with traps

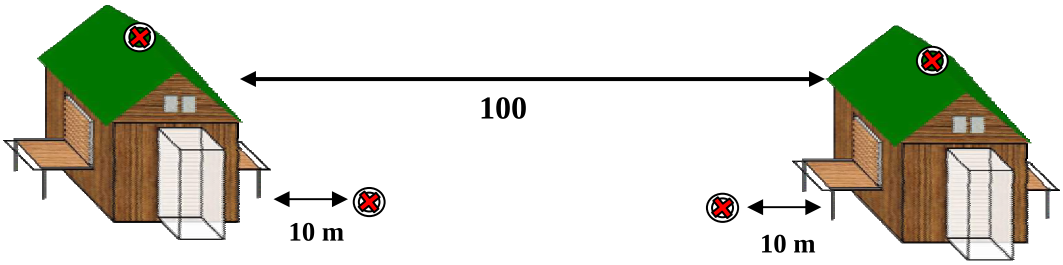


Figure 8 Two huts positioning 100 m apart in an isolated area, surrounded by mountainous terrain and agricultural fields

2.3 Pre spray collection

Two untreated experimental huts were used during the pre-spray period (June – July 2006). Simultaneous indoor collections were performed on the two untreated huts to obtain the baseline data on the normal pattern of entering behavior of *An. minimus s.l.* into the experimental huts. Human-landing collectors were divided into two teams of four persons each. The first team worked from 1800-2400 h for each hut with two collectors inside the hut, followed by the second team beginning from 0000 h to 0600 h. Human-landing collection occurred for 45 min with 15-min break each hour. On the following night, collectors who worked in the same period (either early or late collection periods) were rotated in order to avoid the bias. Each collector exposed their lower legs and collected all landing mosquitoes by mouth aspirator. Collected mosquitoes were retained in plastic cups labels by hour and hut of collection and covered with netting and cotton soaked with a 10% sugar solution placed on the top of the netting. Specimens were transferred to the field laboratory for morphological identification the following morning. Additional details on human-landing collection methods are given in previous work (Sungvornyouthin *et al*., 2006). Hour ambient outdoor temperature and humidity records were made during the period of mosquito collection.

2.4 Post spray collection

After the pre-spray collections were completed, we began the second phase of post spray period from August to October 2006. During the post-spray collection, the first hut served as a control and the second hut was prepared for a treatment.

In order to test chemicals in the treated hut without applying test compound directly to the wall surfaces, a series of panels were developed for holding treated netting which could be positioned around the interior surface of the hut (Figure 9). The aluminum frame that houses the netting contained holes in each corner and were placed onto bolts that are attached to hut walls. There is a 9 cm gap between the aluminum panel and the wood planks to prevent the netting from touching the interior walls. Wing nuts were also used to prevent aluminum panels from touching the wood and to assure the netting did not lean on the bolts due to wind or unexpected accidents.

Treatment hut was lined with treated netting materials either with $2 g/m^2$ DDT or $0.02 g/m^2$deltamethrin on the interior wall whereas the control hut was sealed with untreated netting. Windows and one door were open during the period of collection to allow female mosquitoes to enter.

Figure 9 A series of panels were developed for holding treated netting which could be positioned around the interior surface of the hut

2.5 Data analysis

Pre - spray Collections: Human landing collection (HLC) of *An. minimus s.l.* was obtained and demonstrated as a graphic presentation. Collection periods were further grouped into four categories, evening period (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr) and dawn (0400-0600 hr). The mean number of HLC from hut 1 and hut 2 was compared using Independent-Sample T Test, One-Way Analysis of Variance (ANOVA), and test of normality of HLC of *An. minimus s.l.* in each hut was tested using Normal Probability Plot and Komogorov–Smirnov Test (K-S Test) or Shapiro–Wilk Test using SPSS program. (SPSS version 15.0. Inc., Chicago, IL).The accepted level for all significance's was determined at 0.05% (*P*-value < 0.05).

Post – spray Collections: HLC of *An. minimus s.l.* was obtained and demonstrated as a graphic presentation. The mean number of HCL from unsprayed hut and treated hut with DDT or deltametrhin was compared using Paired–Sample T Test and One - Way Analysis of Variance (ANOVA) using SPSS program. (SPSS version 15.0. Inc., Chicago, IL).

RESULTS

1. Genetic relationship between *Anopheles minimus s.l.* populations

Of eight enzyme systems, 9 putative loci were detected (Table 2). The number of allelic polymorphic loci was 6 in Na Suan, 5 in Bong Ti Noi and Pu Teuy, 4 in Pra Jedee, 3 in Huai Khayeng, and 2 in Tha Kradan (Table 3). Seven loci, *Got-1, Got-2, Had-1, Idh-1, Mdh-2, Pgm-1,* and *Pgd-1*, were polymorphic in all test populations, whereas the other two loci, *Hk-1* and *Tpi-1* showed allelic monomorphism in all populations. The allelic frequencies of all polymorphic loci are given in Table 3.

Chi-square test performed to observe if there are any significant differences between observed and expected allelic frequencies. Out of 54 comparisons, there are 4 significant deviations from the Hardy-Weinberg equilibrium ($P<0.05$), representing a value of approximately 7% of expected deviations by chance only. Two loci were found from Bong Ti Noi and 1 was detected from Pra Jedee and Pu Teuy (Table 2).

The isozyme comparison of 9 loci (Table 3) among six test populations revealed that the expected heterozygosity ranged from 0.036 to 0.136 with an average ($\pm$SEM) of 0.085 ($\pm$) 0.036 across all 6 populations (Table 4). The greatest expected heterozygosity was observed in population Bong Ti Noi with the value of 0.136$\pm$0.050 whereas the smallest was found in Tha Kradan with the value of 0.036$\pm$0.024 (Table 4). Frequency of polymorphic loci varied from 22.2 in Tha Kradan to 55.6 in Bong Ti Noi (Table 4). The observed heterozygosities from all populations were not significantly different from all Hardy-Weinberg expected heterzygosity ($t_{0.025}$ =, df = -1.744, P> 0.05)

Mean value of F_{ST} calculated from all polymorphic loci was 0.053. The greatest F_{ST} value (0.083) was found at *Pgd-1* whereas the lowest was observed at *Got-1* (0.029). Three loci, *Got-2, Pgm-1, and Pgd-1*, demonstrated small F_{ST} values

(0.15≥ F_{ST} ≥0.05) whereas the other four, *Got-1, Had-1*, *Idh-1, and Mdh-2* showed negligible genetic differentiation (Table 4). The F_{IS}, the fixation indices of individuals relative to the total subpopulations, is 0.087.

Table 2 Enzyme systems used in electrophoretic studies on the adult *An. minimus s.l.*

Enzyme abbreviation	Enzyme name	E.C. number	Number of loci
Got	Glutamate oxaloacetate transamirase	2.6.1.1	2
Had	β-Hydroxyacid dyhydrogenase	1.1.1.30	1
Hk	Hexokinase	3.7.1.1	1
Idh	Isocritate dehydrogenase	1.1.1.42	1
Mdh	Malate dehydrogenase	1.1.1.37	1
Pgm	Phosphoglucomutase	2.7.5.1	1
6-Pgd	6-Phosphogluconate dehydrogenase	1.1.1.44	1
Tpi	Triose phosphate isomerase	5.3.1.1	1

Table 3 Allele frequencies and sample sizes (*n*) at 6 populations of *An. minimus s.l.*

Locus / allele	Kanchanaburi population					
	BTN[1]	HK[2]	NS[3]	TK[4]	PJ[5]	PT[6]
Got-1						
n	30	33	11	12	18	39
127	0.050	0.152	0.091	0.083	0.167	0.026
100	0.917	0.833	0.864	0.917	0.806	0.974
73	0.033	0.015	0.045	0.000	0.028	0.000
Got-2						
n	30	18	11	12	18	39
-120	0.117	0.000	0.091	0.000	0.000	0.000
-100	0.833	0.861	0.909	1.000	0.889	1.000
-40	0.050	0.139	0.000	0.000	0.111	0.000
Had-1						
n	30	18	11	12	18	39
166	0.000	0.000	0.045	0.000	0.000	0.000
100	1.000	1.000	0.955	1.000	1.000	1.000
Hk-1						
n	30	18	11	12	18	39
100	1.000	1.000	1.000	1.000	1.000	1.000
Idh-1						
n	30	18	11	12	18	33
127	0.000	0.000	0.045	0.000	0.000	0.121
100	1.000	1.000	0.955	1.000	1.000	0.879

Table 3 (Continued)

Locus / allele	Kanchanaburi population					
	BTN[1]	HK[2]	NS[3]	TK[4]	PJ[5]	PT[6]
Mdh-2						
n	30	18	11	12	18	39
100	0.067	0.000	0.091	0.000	0.000	0.077
-67	0.933	1.00	0.909	1.000	1.000	0.923
Pgm-1						
n	30	12	11	12	18	39
115	0.050	0.083	0.045	0.042	0.056	0.051
100	0.750	0.917	0.955	0.917	0.944	0.963
77	0.200	0.000	0.000	0.042	0.000	0.000
Pgd-1						
n	30	18	11	12	18	17
138	0.067	0.000	0.000	0.000	0.056	0.176
100	0.867	1.000	1.000	1.000	0.944	0.824
63	0.067	0.000	0.000	0.000	0.000	0.000
Tpi-1						
n	30	15	11	7	15	23
100	1.000	1.000	1.000	1.000	1.00	1.000
H_{exp}**	0.136	0.077	0.097	0.036	0.083	0.080
	(0.050)	(0.040)	(0.030)	(0.024)	(0.039)	(0.034)

[1] Bong Ti Noi population [2] Huai Khayeng population [3] Na Suan population

[4] Tha Kradan population [5] Pra Jedee population [6] Pu Teuy population

* Deviation from Hardy-Weinberg equilibrium ($P < 0.05$)

** Average expected genetic heterozygosity and the standard error in parenthesis

Table 4 Genetic variability at 9 loci of pooled populations of *An. minimus s.l.*

Population	Average alleles per locus	% polymorphic loci [1]	Mean heterozygosity	
			H_{obs}	H_{exp} [2]
Bong Ti Noi	2.0±0.3	55.6	0.104 ±0.039	0.136±0.050
Huai Khayeng	1.4±0.2	33.3	0.073 ±0.038	0.077±0.040
Na Suan	1.8±0.2	33.3	0.101 ±0.032	0.097±0.030
Tha Kradan	1.3±0.2	22.2	0.037 ±0.024	0.036±0.024
Pra Jedee	1.6±0.2	44.4	0.068 ±0.037	0.083±0.039
Pu Teuy	1.6±0.2	44.4	0.070 ±0.030	0.080±0.034
			$t_{0.025} = -1.744^{ns}$	

[1] A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.

[2] Unbiased estimate and standard error (Nei, 1978).

Gene flow between populations was calculated by estimating the number of effective migration rate (N_em) where Ne is the effective population size and m is the migration rate between populations. As m is the proportion of migrants (number of migrants / Ne), N_em is actually an estimate of the number of migrants regardless of population size that would be allowed and still permit the observed degree of genetic differentiation between the tested populations. N_em between any of six provincial collections ranges from 4.30 to 62.25. The greatest N_em was seen from pair of Huai Khayeng and Pra Jedee (62.52) whereas the lowest was between Huai Khayeng and Pu Teuy (4.30) (Table 6).

Analyses of isolation by distance on pairwise F_{ST} / (1-F_{ST}) among populations were tested against the geographic distance to determine the correlation between gene flow and geographic distance. Result indicated that there were no correlations ($P > 0.05$) between genetic and geographic distance among *An. minimus s.l.* populations (Table 6)

Average genetic distances between all population (Table 7) supported the lack of genetic differentiation on *An. minimus s.l.* in Kanchanaburi. The Nei (1978) unbiased genetic distance and unbiased genetic identity (Nei, 1978) of any two among all 6 populations of *An. minimus s.l.* varied from 0.000 to 0.007 (Table 7). Phenograms produced by several methods showed nearly identical branching patterns (Figure 1).

The matrix of genetic distances based on Nei (1978) unbiased genetic distance between 6 *An. minimus s.l.* population in Kanchaburi showed that the Pu Teuy vs. Huai Kayeng populations produced the largest genetic differences (0.007), whereas the least were seen in Pra Jedee vs. Huai Kayeng as well as in Pra Jedee vs. Na Suan populations, Tha Kradan vs. Na Suan, and Na Suan vs. Bong Ti Noi (0.000).

Table 5 *F*-statistic analysis of polymorphic loci in 6 populations of *An. minimus*

Locus	F_{IS} [1]	F_{ST} [2]
Got-1	0.092	0.029
Got-2	-0.139	0.062
Had-1	-0.048	0.038
Idh-1	-0.063	0.041
Mdh-2	-0.086	0.042
Pgm-1	0.257	0.060
Pgd-1	0.315	0.083
Mean	0.087	0.053
		$N_em = 4.47$

[1] F_{IS} = In breeding coefficient

[2] $F_{ST} > 0.25$ Great genetic differentiation among the subpopulations

$0.25 > F_{ST} > 0.15$ Moderately great differentiation

$0.15 > F_{ST} > 0.05$ Small differentiation

$F_{ST} \leq 0.05$ Negligible differentiation

Table 6 Pairwise F-statistics at all loci between any of 6 populations of *An. minimus*

Populations compared	F_{ST}[1]	Effective migration rate ($N_e m$)	Distance (Km)
Bong Ti Noi : Huai Khayeng	0.037	6.51	59.42
Bong Ti Noi : Na Suan	0.031	7.81	52.69
Bong Ti Noi : Tha Kradan	0.043	5.56	22.78
Bong Ti Noi : Pra Jedee	0.036	6.69	125.80
Bong Ti Noi : Pu Teuy	0.039	6.16	10.44
Huai Khayeng : Na Suan	0.020	12.25	54.27
Huai Khayeng : Tha Kradan	0.027	9.01	61.22
Huai Khayeng : Pra Jedee	0.004	62.25	71.81
Huai Khayeng : Pu Teuy	0.055	4.30	57.59
Na Suan : Tha Kradan	0.021	11.65	33.75
Na Suan : Pra Jedee	0.019	12.91	91.44
Na Suan : Pu Teuy	0.032	7.56	42.84
Tha Kradan : Pra Jedee	0.025	9.75	118.00
Tha Kradan : Pu Teuy	0.044	5.43	12.55
Pra Jedee : Pu Teuy	0.041	5.85	121.50
Coefficient of determination of isolation by distance between populations, $r^2 = 0.052^{ns}$			

[1]$F_{ST} > 0.25$ Great differentiation

$0.25 > F_{ST} > 0.15$ Moderately great differentiation

$0.15 > F_{ST} > 0.05$ Small differentiation

$F_{ST} \leq 0.05$ Negligible differentiation.

Table 7 Matrix of genetic distance between 6 *An. minimus s.l* populations

Populations	Populations					
	Bong Ti Noi	Huai Khayeng	Na Suan	Tha Kradan	Pra Jedee	Pu Teuy
1. Bong Ti Noi	*****	0.994	0.996	0.995	0.994	0.993
2. Huai Khayeng	0.006	*****	1.000	0.999	1.000	0.992
3. Na Suan	0.004	0.000	*****	1.000	1.000	0.997
4. Tha Kradan	0.005	0.001	0.000	*****	0.999	0.996
5. Pra Jedee	0.006	0.000	0.000	0.001	*****	0.995
6. Pu Teuy	0.007	0.008	0.003	0.004	0.005	*****

Values above the diagonal correspond (Nei, 1978) unbiased genetic identity, and values below the diagonal correspond Nei (1978) unbiased genetic distance.

The Wright (1978) cord distances were clustered by the UPGMA to produce the phenogram as shown in Figure 11. The Haui Kayeng was closely related to the Pra Jedee population, and Na Suan population was genetically similar to the Tha Kradan population. The Bon Ti Noi and Pu Teuy population were markedly different from all other populations (Figure 10).

Anopheles minimus s.l. mosquitoes were also identified using morphological character, resulting in two sibling species, *An. minimus s.s.* and *An. minimus* C. Among six locations, seven populations were obtained, including six local populations of *An. minimus s.s.* and one local population of *An. minimus* C. The modified Roger distances were clustered by UPGMA to the produce the phenogram (Figure 11). The phenogram showed similar branching patterns population as seen in Figure 10, with minor difference from Bong Ti Noi is far apart from the others. Haui Kayeng was closely related to Pra Jedee, Na Suan was closely related to The Kradan, whereas *An. minimus* C was distantly related to *An. minimus s.s.* from Pu Teuy. *An. minimus* C is more closely related to those from Pu Teuy, Haui Kayeng, Pr Jedee, Na Suan and the Tha Kradan (Figure 11).

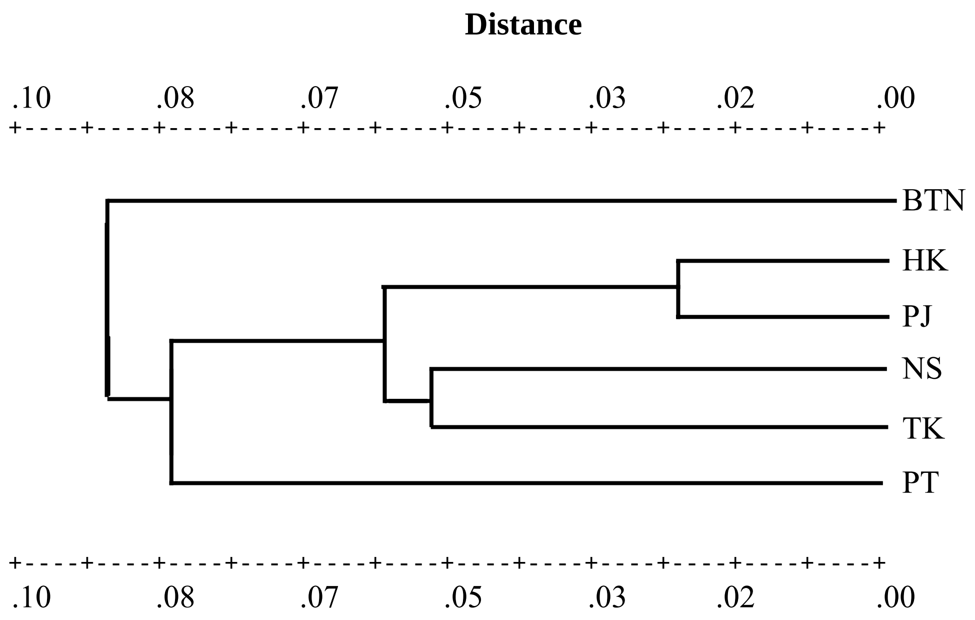


Figure 10 Unweighted pair group method averaging phenogram from Wright (1978) unbiased genetic distance matrix among the collections of *An. minmimusi s.l.* (cophenetic correlation = 0.969)

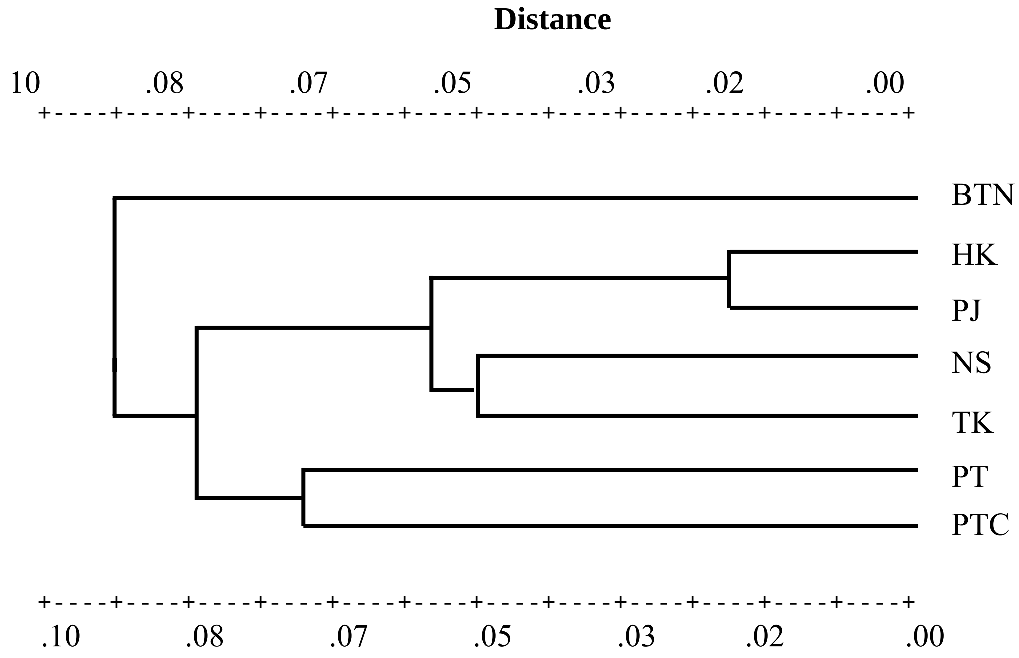


Figure 11 Unweighted pair group method averaging phenogram from Wright (1978) unbiased genetic distance matrix among the collections of *An. minmimus s.s* and *An. minimus* C (cophenetic correlation = 0.901)

2. Entering behavior of *Anopheles minimus s.l.* into the experimental hut treated with DDT and deltamethrin

1.1 Pre-pray collections

Entering behavior of *An. minimus s.l.* into the experimental huts was observed during the rainy season (August-September 2006) (Figure 12). From a total of twenty night collections, 260 and 248 *An. minimus s.l.* females were captured from huts 1 and 2, respectively. One prominent peak was obtained during 1900-2100 hr whereas a very week peak was observed at 0400-0500 hr. When collection times were tabulated into four periods, evening (1800-2100 hr), late night (2100-2400 hr), before dawn (0000-0300 hr) and dawn (0400-0600 hr), the lowest proportion of *An. minimus s.l.* females, entering the two huts was found during before dawn period (33 for hut 1 and 39 for hut 2) (Table 8). Majority of *An. minimus s.l.* females entered the hut was observed during the early evening (119 hut 1 and 104 hut 2). Ratio of females entering the two huts was 1:0.95. The Levene's test for equality or homogeneity of variances was showed that the two experimental huts had equal variances without any significant differences in entering behaviors of *An. minimus s.l.* (Student's t-test, t = -0.263, 38df, $p>0.05$).

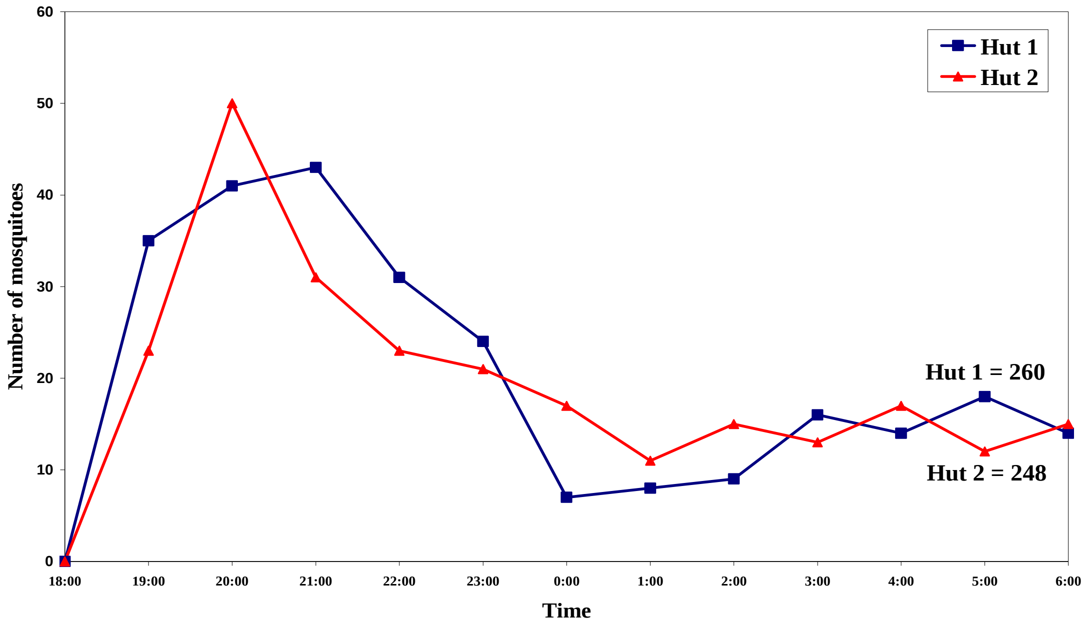


Figure 12 Number of *An. minimus s.l.* collected from human landing collections during 20 collection nights in huts 1 and 2

Table 8 Number of *An. minimus s.l.* in four periods collect from human landing collections during 20 collection night in hut 1 and 2

Hut / Time	Number of *An. minimus s.l.* (N)					
	1800-2100	**2200-2400**	**0000-0300**	**0400-0600**	**Total (N)**	**Ratio**
Hut 1	119	62	33	46	**260**	**1**
Hut 2	104	61	39	44	**248**	**0.95**

In addition, tests of normality by Sig. of Kolmogorov-Smirnov and Shapiro-Wilk were performed, resulting in the value of 0.2 00 and 0.405 for hut 1 and, 0.125 and 0.053 for hut 2, respectively (Figure 13). This indicated that *An. minimus* females had equal chances to entrance either huts 1 or 2.

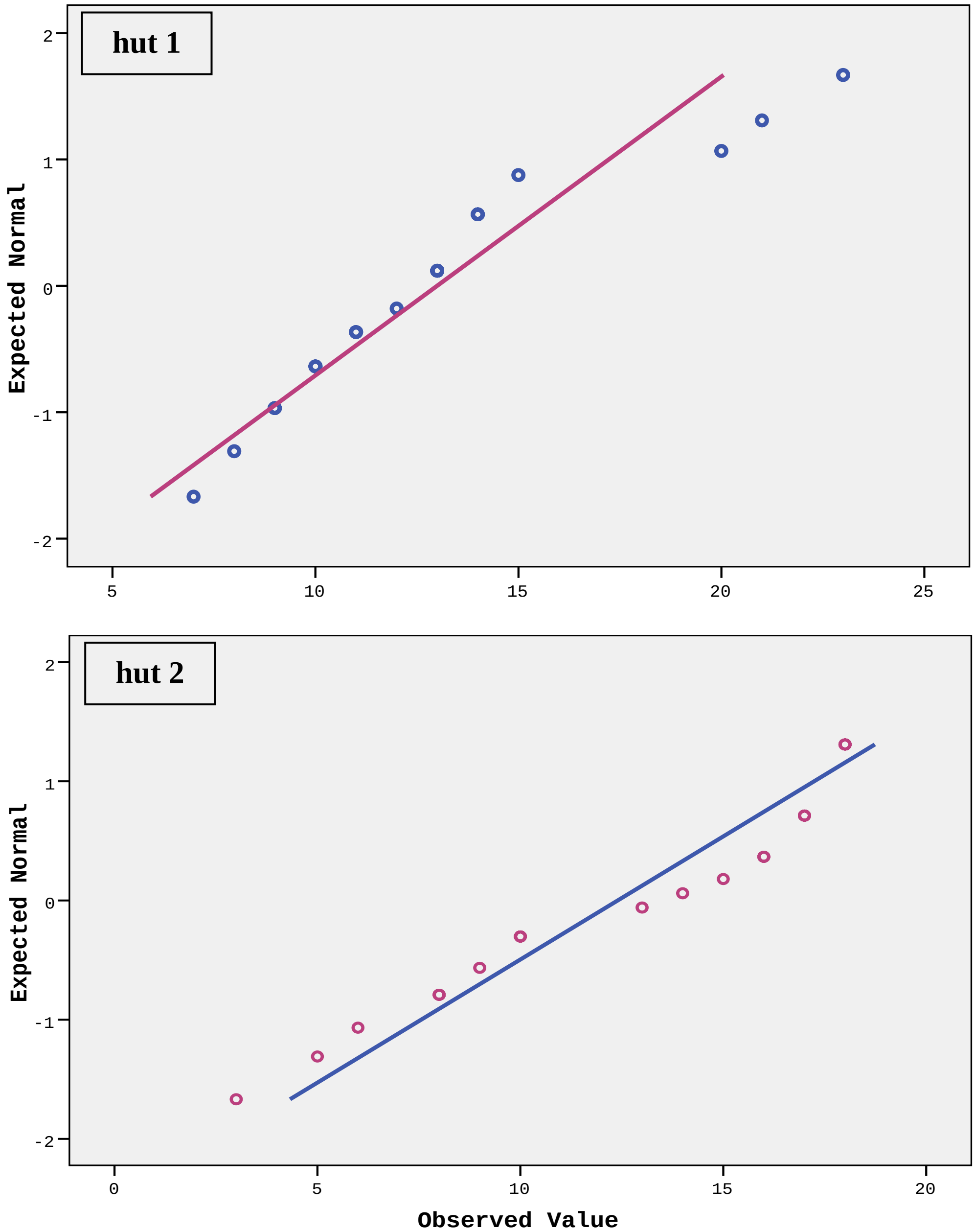


Figure 13 Normal Q-Q Plot in pre-spray of entering behavior of *Anopheles minimus s.l.* using experimental huts (hut 1 and hut 2)

1.2 Post-Spray Collections

After the initial phase of the pre spray, one hut was lined with DDT treated nettings and the other, the control hut, was covered with untreated nettings. Ten night human landing collections were performed to assess the entering behavior of *An. minimus s.l.* 601 *An. minimus s.l.* females were caught from the untreated hut (control) whereas 179 were captured from the DDT treated huts (Table 9). Significant reduction of mosquito population entering the treated hut was observed throughout the night with a major reduction in number of mosquitoes during the early evening period (1800-2100 hour).

After DDT netting materials were removed from the interior wall, the hut frames were chemically cleaned. Subsequently, the study of entering behavior of *An. minimus s.l.* deltamethrin was conducted. Two huts, deltamethrin treated hut and control hut, were set for human landing collections during the period of ten nights. 509 females were collected from the deltamethrin treated hut whereas 291 were caught from the untreated hut (Table 9).

Table 9 Number of *Anpheles minimus s.l.* in four time periods collected from human during 10 collection night in hut treated with DDT, hut treated with deltamethrin, and untreated hut

Treated hut / Time	Number of *An. minimus s.l.* (N)				
	1800-2100	**2200-2400**	**0000-0300**	**0400-0600**	**Total (N)**
DDT	110	46	18	5	179
Unsprayed	292	186	91	58	601
Deltamethrin	86	77	82	46	291
Unsprayed	172	115	130	92	509

This comparison of the average number of *An. minimus s.l.* collected under post-spray conditions indicated that the mean number of *An. minimus s.l.* collected at two huts, hut treated with DDT (13.77) and hut treated with deltamethrin (22.38), were significantly different (t value = -2.179; $p<0.05$); hut treated with DDT (13.77) and unsprayed hut (46.19), were significantly different (t value = -5.006: $p<0.05$). Moreover, comparison of mean number of *An. minimus s.l.* between hut tread with deltamethrin (22.38) and unsprayed hut (39.15) were significantly (t value = -5.313: $p<0.05$).

The sprayed huts exhibited a reduction in population level from unsprayed trails. Number of *An. minimus s.l.* collected in unsprayed hut compared to the number sprayed hut documented a 70.22% reduction in the DDT hut and a 42.83% reduction in deltamethrin hut. In the number of *An. minimus s.l.* that entered the

DDT post-spray was very low and small peaks of entrance activity occurred during 2000 – 0500 (Figures 14 and 15).

DDT shows the repellent action by reducing a significant number of *An. minimus* from entering the house whereas deltamethrin tend to reduced entering the house of *An. minimus s.l.* number.

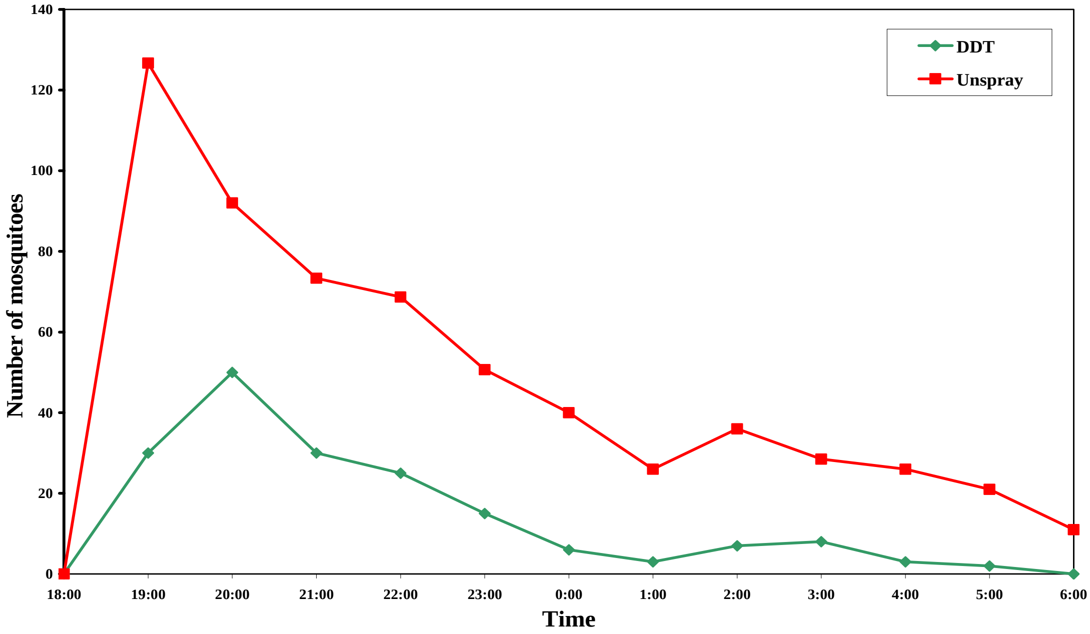


Figure 14 Comparison of the average number of *An. minimus s.l.* collected from human landing collection (HLC) during 20 collection nights inside DDT treated huts compared with unsprayed hut

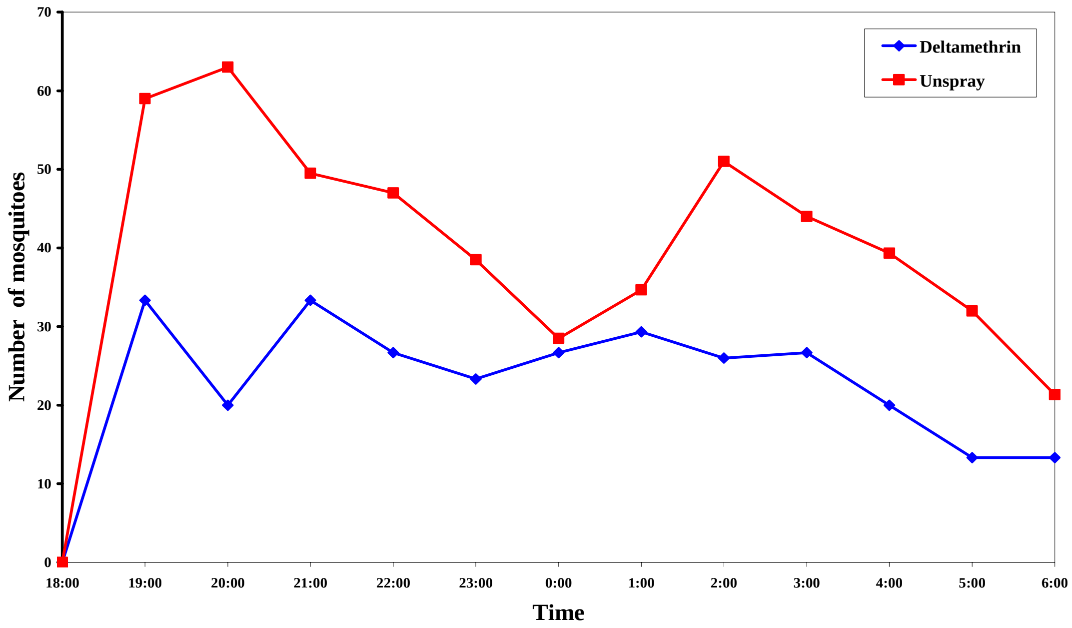


Figure 15 Comparison of the average number of *An. minimus s.l.* collected from Human landing collections (HLC) during 10 collection nights inside deltamethrin treated huts compared with unsprayed hut

DISCUSSION

1. Genetic relationship between *Anopheles minimus s.l* populations

This study was conducted at Kanchanaburi Province, west Thailand, the area where at least three sibling species of the Minimus group were previously reported (Baimai *et al.*, 1984; Sucharit *et al.*, 1988; Baimai, 1989; Green *et al.*, 1990). Information of the species complex of malaria vector is extremely important, especially for each group within the complex. Such information could facilitate the vector control program to precisely combat the mosquito target. In this study, genetic variation and gene flow of the six local populations of *An. minimus s.l.* collected from various localities within Kanchanaburi Province were discussed using information from comparable allele variation frequencies. In addition, the genetic relationship between *An. minimus s.s.* and *An. minimus* C, identified by morphological and geographical criteria, was analyzed.

Anopheles minimus is recognized as an important vector of malaria in Thailand (Harrison, 1980; Baimai, 1989; Green *et al*., 1990, Ministry of Public Health, 2005). At least three sibling species within this group occur in Thailand, *Anopheles minimus* s.s. (previously species A), species C and species D (Baimai, 1989; Green *et al*., 1990). Although three sibling species were previously documented along the Thai-Mynmar borders, only two confirmed species have been recognized (Green *et al*., 1990; Kengluecha *et al*., 2005). *Anopheles minimus s.s.* is found throughout Thailand; whereas, species C is appears confined to Kanchanaburi Province in western Thailand. Within Kanchanaburi Province, *An. minimus s.s.* and species C has been reported from Pu Teuy Village, Sai Yok District near the Thai-Myanmar border (Green *et al*., 1990; Sungvornyothin *et al*., 2006). Interestingly, recent report documented the first presence of *An. minimus* C in Sri Sawat District, Kanchanaburi Province where it has never been reported (Kengluecha *et al.*, 2005). In the same study, 36 water bodies of breeding habitats of the Minimus group within Kanchanaburi Province were investigated and indicated that *An. minimus s.s.* has recently been reported at Pu Teuy area where previously it was not collected

(Kengluecha *et al*., 2005). Sungvornyothin *et al*., (2006) found that approximately 4% of *An. minimus s.s.* were obereved in Pu Teuy area during the two years period of collection. The reasons for these unstable and dynamic changes in favor of species C remain unclear but could be due to differences in species misidentification or to dramatically changes in surrounding environmental or climatic factors over time. Shifts in human population and movement in and out of the area have gradually resulted in greater deforestation and urbanization. The relatively intact natural environment of the collection site may also have had a significant role in species composition. The inevitable evolutionary process occurring and influenced by many biotic and abiotic factors may account for varying rates of species adaptation or extinction in an area resulting in the apparent changes in species frequency over time (Dombeck and Jaenike, 2004).

The level of genetic differentiations among six *An. minimus s.l.* obtained from Kanchanaburi Province was examined on eight protein loci. In general, small genetic differentiation was observed among six local populations (*Fst* =0.053). The *Fis* (0.087) indicates the occurrence of random mating among local populations. The phenograme produced by UPGMA based on the data of genetic distance is given in Figure 2. Mosquitoes collected from the same district or adjacent areas appear genetically similar. Specifically, the Huai Khayeng (Thong Pha Phum District) is closely related to the Pra Jedee (Sung Khla Buri District) whereas Na Suan (Sri Sawat District) is closely similar to the Tha Kradan (Sri Sawat District). This may be due to the geographical proximity of adjacent populations. Two *An. minimus s.l.* populations from Sai Yok District (Pu Teuy and Bong Ti Noi Villages) are markedly different from the other four populations. We know that the breeding sites of *An. minimus s.l.* in Pu Teuy Village, Sai Yok District are somewhat different from the common breeding habitats for other *An. minmius s.l.* populations. These differences in larval ecology may translate into intraspecific differences.

The highest gene flow (62.25 reproductive migrations per generation) was observed between the two adjacent populations from Thong Pha Phum (Huai Khayeng) and Sung Khla Buri (Pra Jedee), the two districts closed to Thai-Mynmar border. Gene flow is quite limited when Pu Teuy population was compared to the others. Pu Teuy, an isolated, elevated hill area, is surrounded by mountain ranges. Geographical isolation by mountain ranges may contribute to genetic divergence in *An. minimus* complex. In addition, breeding site at Pu Teuy is quite unique when compared to the others in terms of water body. At Pu Teuy, breeding site is originated right after the lime stone (spring water). The stream body is in a zigzag form and native vegetations are really dense on both sites, running through the village. Conversely, breeding sites of all other locations where specimens were collected are part of the stream body where water is stem from deep forest area. Both sites of the stream body cover with shrub tree with less native vegetations.

Genetic analysis was further performed based on morphological and geographical criteria, resulting in seven local populations, one population of *An. minimus* C and six populations of *An. minimus s.s*. Two sympatric populations from Pu Teuy, *An. minimus* C and *An. minimus s.s.*, remain the most genetically similar. We surmise that *An. minimus* C from Pu Teuy may evolve from *An. minimus s.s.*.

2. Entering behavior of *Anopheles minimus s.l.* into the experimental hut treated with DDT and deltamethrin.

Millions of people in the tropical and subtropical world are at risk of infection of malaria. Each year, 300 to 500 million cases are reported as being infected with malaria worldwide (WHO, 2005). Malaria parasites are transmitted by Anopheles mosquitoes (Bruce Chwatt, 1970). In Thailand malaria remains an important vector borne disease, despite decades of successful control programs and significant reduction in malaria mortality and morbidity. Most cases have been found along the national borders of eastern Myanmar, western Cambodia and northern Malaysia. Prevention of this disease remains almost entirely dependent on using vector control, the most possible means of reducing malaria transmission in all endemic areas (Roberts *et al*., 2000).

In Thailand, vector control is the mainstay in the flight against malaria and claimed as the most successful means for malaria abatement, having relied mostly on the indoor residual spray with insecticide. DDT had been the first used insecticide in Thailand national malaria control and continued to use until 2000. Because of changing human response to the DDT spraying and its implied adverse long term impact on surrounding environment, the use of DDT was eventually replaced by synthetic pyrethroids, especially deltamethrin.

Most pyrethroids are known to elicit behavioral responses in insects (Threlkeld, 1985). Mosquito control by using deltamethrin has been initiated in Thailand since 1994 (Chareonviriyaphap *et al*., 1999). Although years of DDT and deltamethrin applications as the intradomicillary, the true impact of both compounds on mosquito vectors, in terms of behavioral responses and disease transmission, remains poorly understood.

Behavioral responses of DDT and deltamethrin by several malaria vectors were reported from Thailand (Pothikasikorn *et al*., 2005; Sungvornyothin *et al*., 2001; Chareonviriyaphap *et al*., 2001, 2004; Prasittisuk *et al*., 1996; Ismail *et al*., 1975; Suwonkerd *et al*., 1997). Most works were relied on the excito-repellency test system (Pothikasikorn *et al*., 2005; Sungvornyothin *et al*., 2001; Chareonviriyaphap *et al*., 2001, 2004) and a few field trials on responses of Anopheles mosquitoes to insecticides were performed using experimental hut (Prasittisuk *et al*., 1996; Ismail *et al*., 1975; Suwonkerd *et al*., 1997). In this study, we observed the entering behavior of *An. minimus* into the experimental huts treated either DDT or deltamethrin. DDT strongly reduced *Anopheles minimus* females from entering the hut. Almost 70.22% of *An. minimus* females entering the hut treated with DDT were reduced as compared to the control, indicating strong repellency action of DDT. Hut studies with Anopheline vectors from Belize provided the same conclusion that DDT produced the irritant and repellent actions (Grieco *et al*., 2006). Similarly, Roberts *et al*. (1987) observed that *Anopheles darlingi* females from Brazil completely disappeared after experimental hut was sprayed by DDT.

In addition to DDT, deltamethrin also prevents *An. minimus* from entering the experimetnal hut. However, the efficacy of deltamethrin to repel *An. minimus* females from entering the hut was not as effective as was for DDT. There are 1.92 times greater of *An. minimus* females to enter the deltamethrin treated hut than those from DDT treated hut, indicating lower powerful repellency of deltamethrin compared with DDT. Similar result was seen from works on house entering behavior of *An. vestitipennis* after huts were sprayed with deltamethrin and DDT (Grieco *et al*., 2000). They found that higher proportion of female mosquitoes entered the hut treated with deltamethrin than hut treated with DDT, indicating the powerful repellency of DDT over deltamethrin. In other word, there was a 97% reduction of mosquitoes entering to hut treated with DDT whereas a reduction was only 66% of *An. vestitipennis* to enter the hut treated with deltamethrin.

In conclusion, without a better understanding the relationship between insecticide residual and mosquito behavior, vector control activities has never been success. Study on avoidance behavior of *An. minimus* using insecticide treated huts provides significant baseline data and critical information on how gravid female mosquitoes react in natural surrounding. Such information will facilitate the vector control program. Additional works on behavioral responses to insecticides using the experimental huts need to be performed and huts should be equipped with both entrance and exit traps.

CONCLUSION

1. Genetic relationship between *Anopheles minimus* s.s. populations

Small genetic differentiation was obtained when all populations were compared (F_{ST} = 0.053). Bong Ti Noi showed the greatest percent polymorphic loci (55.6%), whereas Tha Kradan demonstrated the lowest percent polymorphic loci (22.2%).

Results revealed that level of gene flow rate between/among *An. minimus s.l.* population varied, depending upon the locations, epidemiological background and other related factors. Gene flow was 4.47 reproductive migrations per generation when all populations were compared. Among the studied populations, the greatest gene flow was observed between Huai Khayeng and Pra Jedee (N_em = 62.25). Traffics between the sites of study may help to increase the movement of *An. minimus s.l.* mosquitoes between these two populations.

2. Entering behavior of *Anopheles minimus s.l.* into the experimental hut treated with DDT and deltamethrin

Anopheles minimus in Kanchanaburi, western Thailand exhibited a partial endophagic behavior. This species fed indoor and outdoor, and was a persistent biter throughout the night. The deltamethrin showed an irritancy effect, although the insecticide did not alter the pattern of house entering activity. DDT, on the other hand proved to have a very powerful repellency effect. Deltamethrin only exhibited a 42.83% reduction, while DDT showed a reduction of 70.22%. The results strongly suggests that *Anopheles minimus* in this area shows clear behavioral avoidance to DDT at the concentration of 2 g/m^2.

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APPENDIX

Appendix Table 1 Morbidity, mortality and case fatality rates of malaria situation in Thailand, 2006.

Reporting areas	Cases	Deaths	Morbidity per 100,000	Mortality per 100,000	*CFR (%)
Total	**22599**	**42**	**36.21**	**0.07**	**0.19**
North Region	**6765**	**16**	**53.61**	**0.13**	**0.24**
Zone:1	**3081**	**7**	**56.93**	**0.12**	**0.23**
Chiang Mai	281	6	17.03	0.36	**2.14**
Chiang Rai	74	0	6.04	0.00	**0.00**
Lampang	40	0	5.15	0.00	**0.00**
Lamphun	61	0	15.07	0.00	**0.00**
Mae Hong Son	2594	0	1022.83	0.00	**0.00**
Nan	11	1	2.30	0.21	**9.09**
Phayao	5	0	1.03	0.00	**0.00**
Phrae	15	0	3.18	0.00	**0.00**
Zone:2	**3451**	**6**	**100.16**	**0.17**	**0.17**
Phetchabun	44	2	4.39	0.20	**4.55**
Phitsanulok	25	2	2.97	0.24	**8.00**
Sukhothai	43	0	7.05	0.00	**0.00**
Tak	3320	2	635.78	0.38	**0.06**
Uttaradit	19	0	4.05	0.00	**0.00**
Zone:3	**233**	**3**	**8.66**	**0.11**	**1.29**
Kamphaeng Phet	129	2	17.71	0.09	**1.55**
Nakhon Sawan	67	1	6.22	0.00	**1.49**
Phichit	1	0	0.18	0.27	**0.00**
Uthai Thani	36	0	11.02	0.00	**0.00**
Central Region	**5770**	**17**	**27.89**	**0.08**	**0.29**
Bangkok	106	0	1.87	0.00	**0.00**
Zone:4	**49**	**1**	**1.74**	**0.04**	**2.04**
Ang Thong	8	1	2.82	0.00	**0.00**
Nonthaburi	20	0	2.06	0.35	**12.50**

Appendix Table 1 (Continued)

Reporting areas	Cases	Deaths	Morbidity per 100,000	Mortality per 100,000	*CFR (%)
P.Nakhon S.Ayutthaya	1	0	0.13	0.00	**0.00**
Pathum Thani	20	0	2.45	0.00	**0.00**
Zone:5	**25**	**0**	**1.31**	**0.00**	**0.00**
Chai Nat	17	0	0.29	0.00	**0.00**
Lop Buri	1	0	0.80	0.00	**0.00**
Saraburi	1	0	2.82	0.00	**0.00**
Sing Buri	6	0	0.46	0.00	**0.00**
Zone:6	**2622**	4	**79.42**	**0.12**	**0.15**
Kanchanaburi	2215	3	268.10	0.36	**0.14**
Nakhon Pathom	60	0	7.42	0.00	**0.00**
Ratchaburi	287	1	34.85	0.12	**0.35**
Suphan Buri	60	0	7.12	0.00	**0.00**
Zone:7	**1324**	**4**	**83.38**	**0.25**	**0.30**
Phetchaburi	371	1	81.7	0.22	**0.27**
Prachuap Khiri Khan	880	2	180.77	0.41	**0.23**
Samut Songkhram	56	0	12.39	0.00	**0.00**
Samut Sakhon	17	1	8.71	0.51	**5.88**
Zone:8	**430**	**1**	**14.51**	**0.03**	**0.23**
Chachoengsao	75	0	11.58	0.00	**0.00**
Nakhon Nayok	34	0	13.56	0.00	**0.00**
Prachin Buri	30	0	6.67	0.00	**0.00**
Sa Kaeo	58	0	43.39	0.00	**0.00**
Samut Prakan	233	1	5.58	0.09	**1.72**
Zone:9	**1214**	**7**	**49.57**	**0.29**	**0.15**
Chanthaburi	659	1	132.29	0.20	**0.58**
Chon Buri	133	0	11.34	0.00	**0.00**
Rayong	116	1	20.75	0.18	**0.86**
Trat	306	5	139.64	2.28	**1.63**

Appendix Table 1 (Continued)

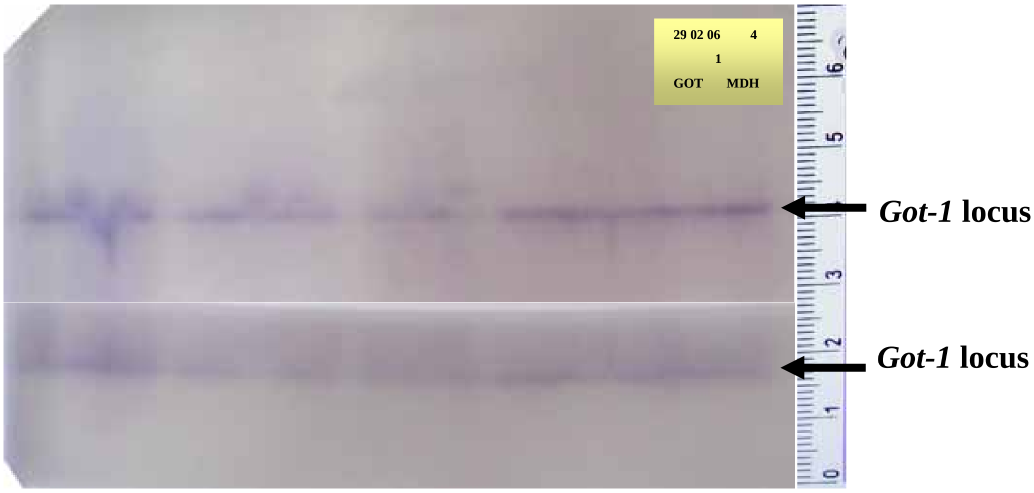
Reporting areas	Cases	Deaths	Morbidity per 100,000	Mortality per 100,000	*CFR (%)
North-Eastern Region	**678**	**1**	**3.18**	**0.00**	**0.15**
Zone:10	**20**	**0**	**0.57**	**0.00**	**0.00**
Loei	5	0	0.82	0.00	**0.00**
Nong Bua Lam Phu	2	0	0.40	0.00	**0.00**
Nong Khai	3	0	0.33	0.00	**0.00**
Udon Thani	10	0	0.66	0.00	**0.00**
Zone:11	**59**	**0**	**1.90**	**0.00**	**0.00**
Kalasin	13	0	1.34	0.00	**0.00**
Mukdahan	8	0	2.39	0.00	**0.00**
Nakhon Phanom	5	0	0.72	0.00	**0.00**
Sakon Nakhon	33	0	2.99	0.00	**0.00**
Zone:12	**11**	**0**	**0.28**	**0.00**	**0.00**
Khon Kaen	8	0	0.46	0.00	**0.00**
Maha Sarakham	0	0	0.00	0.00	**0.00**
Roi Et	3	0	0.23	0.00	**0.00**
Zone:13	**144**	**0**	**2.19**	**0.00**	**0.00**
Buri Ram	19	0	1.24	0.00	**0.00**
Chaiyaphum	6	0	0.54	0.00	**0.00**
Nakhon Ratchasima	32	0	1.26	0.00	**0.00**
Surin	87	0	6.33	0.00	**0.00**
Zone:14	**444**	**0**	**10.75**	**0.02**	**0.23**
Amnat Charoen	4	0	1.08	0.00	**0.00**
Si Sa Ket	228	0	15.79	0.07	**0.44**
Ubon Ratchathani	201	0	11.33	0.00	**0.00**
Yasothon	11	0	2.03	0.00	**0.00**
South Region	**3894**	**8**	**110.20**	**0.09**	**0.09**
Zone:15	**9386**	**6**	**243.19**	**0.37**	**0.15**
Chumphon	1002	0	210.61	0.00	**0.00**

Appendix Table 1 (Continued)

Reporting areas	Cases	Deaths	Morbidity per 100,000	Mortality per 100,000	*CFR (%)
Ranong	2616	5	1468.66	2.81	**0.19**
Surat Thani	276	1	29.13	0.11	**0.36**
Zone:16	**141**	**0**	**5.41**	**0.00**	**0.00**
Nakhon Si Thammarat	113	0	7.51	0.00	**0.00**
Phatthalung	5	0	1.00	0.00	**0.00**
Trang	23	0	3.82	0.00	**0.00**
Zone:17	**378**	**2**	**40.67**	**0.22**	**0.53**
Krabi	77	0	19.46	0.00	**0.00**
Phangnga	236	2	97.75	0.83	**0.85**
Phuket	65	0	22.24	0.00	**0.00**
Zone:18	**3935**	**0**	**218.73**	**0.00**	**0.00**
Narathiwat	1400	0	199.85	0.00	**0.00**
Pattani	131	0	20.65	0.00	**0.00**
Yala	2404	0	517.97	0.00	**0.00**
Zone:19	**1038**	**0**	**65.68**	**0.00**	**0.00**
Satun	3	0	1.08	0.00	**0.00**
Songkhla	1035	0	79.47	0.00	**0.00**

*CFR = Case Fatality Rate of Malaria

Source: Center of Epidemiological Information, Bureau of Epidemiology, Ministry of Public Health (2006).



Appendix Figure 1 Example of eletrophoretic pattern of Glutamate oxaloacetate transamirase (Got) from *Got-1* and *Got-2* loci

Appendix Figure 2 Example of eletrophoretic pattern of Phosphoglucomutase (Pgm) from *Pgm-1* locus