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THESIS

DISTRIBUTION OF *ANOPHELES* SPECIES COMPLEXES AND GROUPS OF PUBLIC HEALTH IMPORTANCE WITH EMPHASIS ON THE BIONOMICS, BEHAVIOR AND BACTERIAL BIODIVERSITY OF ANOPHELINES IN WESTERN THAILAND

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A Thesis Submitted in Partial Fulfillment of
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Krajana Tainchum 2015: Distribution of *Anopheles* Species Complexes and Groups of Public Health Importance with Emphasis on the Bionomics, Behavior and Bacterial Biodiversity of Anophelines in Western Thailand. Doctor of Philosophy (Entomology), Major Field: Entomology, Department of Entomology. Thesis Advisor: Professor Theeraphap Chareonviriyaphap, Ph.D. 150 pages.

The distribution of closely related *Anopheles* species (groups and complexes) of public health importance was investigated in Thailand. Based on use of molecular techniques for accurate species identification, countrywide malaria vector distribution maps using GIS coordinates were produced.

Species diversity, blood feeding behavior and host preference of *Anopheles* mosquitoes in two malaria endemic areas of Tak and Mae Hong Son Provinces were studied for a two-year period (2011-2012). Multiplex Allele Specific-PCR assays were used to differentiate species within respective *Anopheles* (*Cellia*) complexes or groups. Real-time PCR was performed for parasite detection (malaria/filaria) in mosquitoes. Eight species of mosquitoes were described, the most common being *Anopheles minimus*. Populations of *An. minimus* were comparatively high between February and April. In addition, the blood feeding behavior of important vectors was evaluated. One specimen of *An. minimus* (Mae Sot) was found infected with *Plasmodium vivax.*

Additionally, bacterial biodiversity of *Anopheles*abdomen was assessed by 16S rRNA gene PCR-Temporal Temperature Gradient Gel Electrophoresis (TTGE) profiling and sequence analysis.Nineteen bacteria genera were identified from eight field-caught *Anopheles* species. Seven genera are newly reported in *Anopheles* mosquitoes, suggesting that the abdominal bacterial diversity of *Anopheles* remains underestimated.

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Krajana Tainchum

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INTRODUCTION

In Thailand, malaria is a public health priority with a strong prevalence of this disease in forested regions, in particular along the international borders. Many previous studies on the distribution of malaria vector species have been based on morphological identification alone. Because the primary malaria vectors in Thailand belong to species complexes and are difficult to identify morphologically, several molecular-based species identification techniques have been developed and published elsewhere. The Ministry of Public Health in Thailand has classified malaria control areas in regard to the risk (A1, A2, B1 and B2, respectively) based on the presence or absence of malaria vector species. The accurate species identification of malaria vectors is essential to have a better understanding of each species' role in malaria transmission and for improving the effectiveness and proper targeting of disease control program activities.

In addition, along the Thailand-Myanmar border, where the highest malaria prevalence occurs, another parasitic disease Bancrofti lymphatic filariasis (LFB) for which only limited data are available. Malaria and LFB are mosquito-borne diseases due respectively to *Plasmodium* species, especially *P. falciparum* and *P. vivax*, and rural strains of *Wuchereria bancrofti* sharing the same *Anopheles* vector species. In Southeast Asia, *Anopheles* vectors belong to species complexes with different involvement in the transmission of pathogens. Several sibling species of the Dirus complex (8 species), the Minimus complex (3 species) and the Maculatus group (9 species) are involved in malaria and LFB, but specific role of each sibling species has

never been studied due to the lack of reliable methods for species identification, now available.

This study is based on these techniques for studying the distribution of each dominant *Anopheles* vector species in Thailand and estimating their specific role, as well as detecting and identifying the parasites present in mosquitoes. Another aspect of this study concerned the biodiversity of the microbitota in mosquito abdomen, which has been seldom studied in field collected *Anopheles*, although studies on laboratory strains data showed the role of specific bacterial on the inhibition of development of oocysts in mosquitoes. The bacterial biodiversity was evaluated by amplification of the 16S rRNA sequence and compared among specimens and species in relation to the collections site and most of all in relation to the positive or negative parasitic infestation in mosquitoes.

OBJECTIVES

The aim of this study is to define the vectorial capacities of *Anopheles* species along the Thai-Myanmar border and to study the biodiversity of bacteria in mosquito abdomen that may influence the infection of human malaria parasite (*Plasmodium spp*. and *Wuchereria bancrofti*).

1. To investigate the *Anopheles* species distribution in Thailand and more specifically those implicated as Public Health importancebased on morphological and molecular identification methods.

2. To describe the trophic behavior, biting activity, seasonal abundance, and parasite infection of individual specimens of sibling species by molecular technique, in Mae Sot, Tak Province and Sop Moei, Mae Hong Son Province, northwestern Thailand.

3. To estimate the abdomen bacterial biodiversity in *Anopheles* species, including malaria vectors from Thailand based on the amplification of the V3 region of the 16S rRNA gene, by separating the amplicon using TTGE then sequencing.

LITERATURE REVIEW

Malaria remains one of the world's most serious public health problems. Approximately 3.4 billion people (47% of the world's population) reside in areas at risk for malaria transmission. These endemic areas include regions where the most severe malaria problems have resulted from anthropogenic changes such as exploitation and development of natural habitats (e.g. jungle or forested areas), socio-political unrest, and lackof sufficient resources to control transmission in at risk populations. An estimated 500 million people reside in areas where endemic malaria remains unchanged and where malaria control programs have either partially failed or areabsent (World Health Organization [WHO], 2014). In 2012, approximately 207 million people contracted malaria with an estimated 627,000 deaths covering 100 endemic countries and territories (WHO, 2014).

In Southeast Asia, 1.6 billion people resided in high malaria risk areas (WHO, 2013). Malaria in this region has shown a variety of complex and diverse epidemiological characteristics such as forest/ forest fringe malaria. In addition, human migration across international borders plays a significant role for malaria transmission in the region. The number of cases was reduced approximately from 2.9 to 2 million between 2000 and 2012. Roughly 98% of malaria cases in the region were reported from India, Myanmar, and Indonesia. The other 7 countries, Bangladesh, Bhutan, Democratic People's Republic of Korea, Nepal, and Sri Lanka, have achieved >75% decrease in case incidence between 2000 and 2012. Thailand and Timor-Leste project to achieve this goal by 2015 (WHO, 2013).

1. Malaria in Thailand

Malaria remains one of the most important parasitic diseases in Thailand and has long been major cause of morbidity/mortality, especially along the international border areas (Bureau of Vector Borne Disease [BVBD], 2006). There are approximately 32 million people living along the Thailand borders,around 50% of the

total population, which are at risk for contracting malaria. The undeveloped border between Thailand- Myanmar remains the most heavily impact area (BVBD, 2013). Workers migrating across international borders represent the most vulnerable population. The frequent movement of these undocumented migrant workers has contributed to the spread of multi-drug resistant *Plasmodium falciparum* malaria (Wongsrichanalai *et al.*, 2001; McGready *et al.*, 2012; Phyo *et al.*, 2012; Das *et al.*, 2013; Ashley *et al.*, 2014). Three provinces along the Thai-Myanmar border, Kanchanaburi, Tak, and Mae Hong Son,are the epicenter where most malaria outbreaks occur (BVBD, 2013).

Thailand has experienced a 40% reduction in reported malaria cases between 2010 and 2013, with a decline of 49,911 cases to 27,104 cases respectively. Despite significant malaria control achievements in Thailand, between 27,000 and 50,000 confirmed cases occurred annually during (2007-2013) (BVBD, 2013).

2. Background

With the implementation of the national malaria control program in the 1950s, malaria incidence in Thailand has been reduced dramatically, with the only residual foci remaining in some remote areas and more extensive transmission along the international borders. Substantial reorganizations that occurred in 1996 and 2003 resulted in a 20-40% reduction in manpower throughout the country (Chareonviriyaphap *et al.*, 2000; BVBD, 2006).

In 2002, the Department of Disease Control (DDC) reformed the Vector-Borne Disease Control (VBDC) Program by combining the Malaria Control Unit (MCU) with other vector-borne disease control programs. The DDC was subsequently consolidated to contain other non-communicable diseases under its supervision. The restructuring helped ease constraints on governmental staffing, management, budget and equipment for all VBDC elements, and eliminated many of the redundancies and relatively high costs previously incurred by each of the former independent control programs. At the national level, the Bureau of Vector Borne

Disease (BVBD) is under the direction of the DDC, of the Ministry of Public Health (MOPH).

The program comprises 12 regions under the direction of a Medical Officer (Medical Doctor), Director of the Office of Disease Prevention and Control (DPC). Under the DPC, the Vector-Borne Disease Section (VBDS) was set up to respond to the major vector-borne diseases issues in each Regional DPC. In 2003, 39 Vector-Borne Disease Control (VBDC) Centers and 302 Vector-Borne Disease Control Units (VBDU) were set up at the provincial and village levels, respectively, but recently these local VBD sectors were reduced to 38 VBDCs and 165 VBDUs, respectively. In 2011, the 12 DPC regional offices underwent further restructuring, and the VBDSs were merged with and renamed either Technical Support Sections or Emergency Response for Public Health Disasters, depending on the individual DPCs. Further details of the history and current organization of the vector-borne disease control program have been published elsewhere (BVBD, 2006, 2007, 2013).

Thailand covers a total land area of 513,120 km² with a total population of nearly 65 million people in 2013 (Department of Provincial Administration: http://www.dopa.go.th/). Approximately 20% of the country is covered by forested mountains and hills, the inclines of which generally contain most forms of agriculture. Thailand shares borders with Myanmar on the north and west, Laos on the north and east, Cambodia on the east, and Malaysia in the south. Gem mining, hunting, logging, agriculture, road construction and other economic activities along Thailand's border areas attract migrant workers from neighboring countries. The regular migration of new workers and poor construction of their temporary residences facilitate cross border transmission of malaria and complicatecontrol efforts. The national malaria control program implementation in the 1950s, dramatically reduced malaria in Thailand, except along these borders. However, as in some other countries, in the past decade or more, Thailand has experienced a resurgence of malaria negating some of the past control progress and the significant reduction in indigenous and imported cases experienced decades ago (Chareonviriyaphap *et al.*, 2000). While deforestation has pushed malaria out of many regions in Thailand, this disease remains a

widespread threat in most areas along the international and undeveloped border regions of western Myanmar, southern Malaysia, and eastern Cambodia (BVBD, 2006, 2013). In general, there are two malaria transmission peak periods in Thailand, between June and August, and from October to November when the rainy season increases the availability of breeding habitats of important malaria vectors (BVBD, 2013).

In 2012, over 84% of malaria cases were documented from international border areas (Thai-Myanmar=49.1%, Thai-Malaysia=19.7%, Thai-Cambodia=11.9%, and Thai-Laos=3.8%) and are frequently associated with tribal, migratory populations in these areas (BVBD, 2013). The uncontrolled (undocumented) movement of these populations along border areas can exacerbate cross-border transmission and confound control efforts to combat malaria.

Based on the malaria surveillance activities in Thailand from 1999 to 2013, recorded malaria cases in Thailand were 208,323 in 1999 and declined thereafter to 57,592 and 54,920 cases in 2004 and 2005, respectively (Table 1). As a whole, reported malaria infections have greatly decreased during this decade compared to the years between 1985 and 1998 (BVBD, 2000, 2006, 2007,2013). This reduction in malaria infection has been partly attributed to an effective, well organized vector control program, other public health activities, and more recently the mass distribution of pyrethroid-impregnated bed nets (BVBD, 2013). However, in 2006, malaria cases actually increased to 66,651 cases, an increase of over 21% from the previous year. The cause for this increase in 2006 and 2007 remains controversial but some possible explanations include limitations imposed on health budgets for malaria control and manpower, an increase in uncontrolled population movement across the international borders, emergence of drug resistance (Ashley *et al.*, 2014), a weaken and unorganized state of vector control in some areas and socio-political unrest along the international borders, especially the southern part of the country. Since 2004, unrest among the southern most provinces appears to have contributed to an increase of nearly 4,000 cases per year in areas bordering Malaysia (BVBD, 2004).

At the same time, a rapid increase in rubber plantations in northeastern and eastern Thailand has presented a major concern for potential malaria reemergence in once controlled areas (Baimai, 1989; BVBD, 2013). Rubber plantations in the northeast have also opened job opportunities for lower paid migrant workers from neighboring countries to enter Thailand. Almost 2 million registered migrant workers from neighboring countries were involved in work in Thailand in 2011 (International Organization for Migration [IOM], 2011), while the number of undocumented workers is believed to remain substantial. Population movement across and near borders with Thailand has the potential to increase transmission of malaria in these and other areas of Thailand.

Since 2007, malaria morbidity declined dramatically from 51,510 reported cases in 2008 to 20,298 in 2013 (Table 1). This improvement has been attributed to the effective implementation of targeted and selective indoor residual spraying programs and an increased distribution of treated bed netting for vector control and bite prevention measures. Malaria reduction in Thailand is also attributed to expanded policies, prompt and accurate diagnosis, active case surveillance programs, and more effective treatments.

In addition, scientific knowledge gained from local and national universities on the complex interplay of the three primary components involved in malaria transmission; pathogenicity / virulence of the infectious agent, vector competence (infectivity), and host susceptibility have been used to assist the National Malaria Control Program. Successful control of human diseases requires an understanding of the interaction among these three components and the various other biological, environmental, and socio-economic factors that influence transmission. Such a task often requires the full participation of specialized university laboratories in the country, e.g., Mahidol, Kasetsart, Chiang Mai, and Chulalongkorn universities and other private universities, together with strong international collaborations with similar institutions. Additionally, active and passive case detection activities of local and provincial health staff supported by funding from various national and

international organizations and the strengthening existing health program infrastructure and vector control program have been part of the improvement.

Table 1 National population coverage, diagnostic exams and reported malaria cases in Thailand 1999-2013

Year	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**
Total population	56,706,163	57,356,571	57,823,000	58,681,371	59,884,424	60,452,157	60,846,656	62,006,741
Blood examined*	4,455,315	4,403,738	4,353,655	3,936,014	3,299,153	3,069,490	2,524,788	2,301,061
Positive slides	208,323	149,586	126,595	81,931	71,287	57,592	54,920	66,651
Mortality / 100,000 pop.	1.2	1.01	0.68	0.58	0.32	0.37	0.26	0.18
P. falciparum	63,902	36,881	29,061	20,644	18,864	13,008	11,565	14,123
P. vivax	60,910	40,709	34,154	28,436	18,293	13,397	12,587	15,991
Malaria clinics	525	526	515	526	NP	538	504	359

Table 1 (Continued)

Year	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
Total population	62,851,480	63,282,308	63,492,417	63,796,512	63,850,557	63,497,694	63,559,414
Blood examined*	2,093,101	1,971,564	1,982,795	1,763,231	1,392,489	1,324,740	935,432
Positive slides	63,354	51,510	49,423	49,911	34,002	28,244	20,298
Mortality / 100,000 pop.	0.15	0.16	0.11	0.13	0.07	0.06	0.07
Plasmodium falciparum	17,892	12,182	9,576	10,199	5,879	4,767	8,038
Plasmodium vivax	17,562	13,738	12,345	14,460	9,358	7,187	9,273
Malaria clinics	263	242		199	170	167	165

*Include both Thai and non-Thai cases

Source: BVBD (1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013).

All important malaria vectors in Thailand are known to be respective members of a complex of closely related, nearly identical, sibling species, which makes distinguishing individual species based on morphological criteria alone inaccurate. Other members in the complexes include non-vectors and potential vectors so that accurate discrimination of each species is important in order to determine control priorities (Saeung, 2012). Each member of species complexes usually exhibits important behavioral differences. Understanding the biological, behavioral, and ecological characteristics of each species is relevant to the pathogen transmission epidemiology and for selecting the appropriate disease control methods.

The primary malaria vectors are assembled within three groups, Leucosphyrus, Funestus and Maculatus. Within the Leucosphyrus Group, *An. dirus* and *An. baimaii* are considered to be the primary malaria vectors in Thailand. For the Funestus Group, only *An. minimus* has been incriminated as an important vector and is the most widespread across Thailand (Rattanarithikul *et al.*, 2006), while *An. aconitus*, a closely related species, is also involved in malaria transmission. In the Maculatus Group, *An. maculatus*, *An. sawadwongporni* and *An. pseudowillmori* have been implicated as important malaria vectors in southern, western and northwestern Thailand (Somboon *et al.*, 1998; Coleman *et al.*, 2002; Walton *et al.*, 2007). Several potentially important, but typically incidental (secondary) vectors are also present in the country, including *An. epiroticus* of the Sundaicus Complex (Ritthison *et al.*, 2014b), and within the *An. barbirostris* Subgroup with *An. campestris* incriminated as a potential vector of *P. vivax* in Thailand (Thongsahuan *et al.*, 2011).

Five species of malaria parasites can infect humans, including *Plasmodium falciparum* Welch, *Plasmodium vivax* Grassi and Feletti, *Plasmodium malariae* Grassi and Feletti, *Plasmodium ovale* Stephens and *Plasmodium knowlesi* Sinton and Mulligan, all present in Thailand (Putaporntip *et al.*, 2009). Historically, *Plasmodium falciparum* and *P. vivax* are the 2 most frequent species encountered (The Single Stream Funding Malaria [SSF-M]: http://www.biophics.org/malariar10/index.php).; whereas *P. malariae* and *P. ovale* are relatively rare, seldom reported and likely under diagnosed (BVBD, 2013). *Plasmodium knowlesi* is primarily a non-human primate

malaria parasite, reported to occasionally infect humans in Malaysia (Vythilingam *et al.*, 2008). *Plasmodium knowlesi* is also known to occur in humans in Thailand but the extent and importance of this zoonotic parasite in the country is unknow. However, in recent years, this species has been more commonly reported in Southeast Asia, including Thailand (Jongwutiwes *et al.*, 2004; Putaporntip *et al.*, 2009; Berry *et al.*, 2011; Jongwutiwes *et al.*, 2011; Sermwittayawong *et al.*, 2012; Ehrhardt *et al.*, 2013; Putaporntip *et al.*, 2013; Seilmaier *et al.*, 2014) . Since 1998, the overall proportion of *P. vivax* infections (from confirmed microscopy) has generally remained slightly greater than *P. falciparum* and from 2008 onwards, *P. vivax* has been consistently the more prevalent parasite species) compared to *P. falciparum*, such as respectively 56 % versus 44% (in 2013) (The Single Stream Funding Malaria [SSF-M]: http://www.biophics.org/malariar10/index.php).

3. Filariasis in Thailand

Bancroftian lymphatic filariasis (LFB) is endemic along the Thai-Myanmar border but of lowendemicity. The detection of this microfilaria parasite is commonly found in Burmese workers who are working in Thailand (Khamboonruang *et al.*, 1987; Jitpakdi *et al.*, 1998; Bhumiratana *et al.*, 1999; Bhumiratana *et al.*, 2002; Bhumiratana *et al.*, 2005; Bhumiratana *et al.*, 2013), with up to 54% of Thai-Karen blood samples found with antifilarial IgG4 antibodies (Nuchprayoon *et al.*, 2003).

The current, filariasis control program in Thailand has been integrated within the vector-borne diseases control program, mainly based on periodic mass treatment of the population with diethylcarbamazine (Bhumiratana *et al.*, 2010). The transmission is maintained within the at-risk local Thai population due to frequent cross-border migrants who re-introduce infection in this area where efficient mosquito vectors are widely distributed. Increase of temporary Myanmar-migrant workers in Thailand during peak agriculture periods is an important factor for maintaining disease circulation in the area. The prevalence of filariasis among the group of Myanmar migrant workers in the Tak Province (western Thailand) reaches 4.4 % (Triteeraprapab and Songtrus, 1999). In endemic areas where mosquito-borne

transmission is common, asymptomatic microfilaremia in high risk populations play an important role to enhance vector capacity for increase disease circulation and transmission.

The nocturnally subperiodic (NSP) form of *Wuchereria bancrofti*, is transmitted by several mosquito species including anopheline species (Pothikasikorn *et al.*, 2008). The microfilaria of *W. bancrofti* in experimentally induced infection could develop in two common malaria vectors, *An. minimus* and *Anopheles maculatus* (Pothikasikorn *et al.*, 2008). Very few studies on *W. bancrofti* detection in mosquitoes have been done. Malaria and LFB could share the same mosquito vectors (Manguin *et al.*, 2010).

4. *Anopheles* mosquitoes in Thailand

The first exhaustive list of *Anopheles* mosquitoes in Thailand was recorded by Scanlon et al, 1968, which at the time included 62 species-group taxa. Decades later, 74 species were reported (Rattanarithikul *et al.*, 2006; Somboon and Rattanarithikul, 2013).

5. *Anopheles* vectors of human malaria parasites

At least 74 species of *Anopheles* mosquitoes have been documented in Thailand, consisting of 73 formally named species and a closely related species of *Anopheles gigas* s.l. Giles (Somboon *et al.*, 2011; Somboon and Rattanarithikul, 2013). Moreover, 3 additional species in the *An. barbirostris* complex are being formally named (R.E. Harbach, per comm.). Several of the most important malaria vectors in Thailand are members of closely related sibling species that may differ considerably in their biology, behavior and other characteristics related to disease transmission, such as susceptibility to malaria parasites (Eamkum *et al.*, 2014) or insecticide resistances. The various sibling species may show distinct distribution patterns and population densities that can vary markedly according to the seasonal climatic changes. Better understanding of the biology and behavior of sibling species

is critically important to help identify their respective role in disease transmission. Such information helps to define vector capacity, relative risk for disease transmission, and assist in the design of appropriate vector prevention and control strategies.

The most important malaria vectors are in the subgenus *Cellia* and include members in the Leucosphyrus Group (Neomyzomyia Series), Maculatus Group (Neocellia Series), and the Minimus Subgroup (Myzomyia Series) (Gould *et al.*, 1967; Rosenberg *et al.*, 1990; Green *et al.*, 1991; Maheswary *et al.*, 1992; Rattanarithikul *et al.*, 1996a). Collectively, these include *Anopheles baimaii* Sallum & Peyton (Green *et al.*, 1991; Sallum *et al.*, 2005; Rattanarithikul *et al.*, 2006), *Anopheles dirus* Peyton & Harrison (Rosenberg *et al.*, 1990; Green *et al.*, 1991), *Anopheles minimus* Theobald (Coleman *et al.*, 2002; Rattanarithikul *et al.*, 2006), *Anopheles maculatus* Theobald (Green *et al.*, 1991; Coleman *et al.*, 2002), *Anopheles pseudowillmori* (Theobald) (Green *et al.*, 1991), *Anopheles sawadwongporni* Rattanarithikul & Green (Rattanarithikul *et al.*, 1996a; Somboon *et al.*, 1998; Coleman *et al.*, 2002), and *Anopheles aconitus* Doenitz (Gould *et al.*, 1967; Green *et al.*, 1991; Maheswary *et al.*, 1992). However, some species far exceed others in their overall capacity to transmit malaria and their relative importance and role may vary depending on locality, season and epidemiological circumstances. Additionally *Anopheles epiroticus* Linton & Harbach and *Anopheles campestris* Reid have been incriminated as secondary / incidental vectors in the country (Rattanarithikul *et al.*, 2006; Thongsahuan *et al.*, 2011). This review focuses on the distribution of these species and the methods used for their identification.

Out of 21 species (Harbach, 2013), the Leucosphyrus Group is represented by 10 species in Thailand, including *Anopheles baimaii*, *An. dirus*, *Anopheles cracens* Sallum & Peyton, *Anopheles hackeri* Edwards, *Anopheles introlatus* Colless, *Anopheles latens* Sallum & Peyton, *Anopheles macarthuri* Colless, *Anopheles nemophilous* Peyton & Ramalingam, *Anopheles pujutensis* Colless, and *Anopheles scanloni* Sallum & Peyton. *Anopheles baimaii* and *An. dirus* inhabit forest and forest-fringe environments and are considered highly anthropophilic (Baimai *et al.*, 1984;

Rattanarithikul *et al.*, 2006). The most favored larval habitats are shaded animal footprints, wheel-tracks, and temporary, shallow ground pools (Manguin *et al.*, 2008). Larvae have occasionally been found in water jars, cut tree stumps, and root holes. *Anopheles dirus* is the only species in the group that is found throughout Thailand and sometimes occurs in sympatry with *An. baimaii* in the Kanchanaburi Province (Rattanarithikul *et al.*, 1995; Tananchai *et al.*, 2012). The other 8 species in this group are regarded as non-vectors or poor vectors and occur primarily in southern (peninsular) Thailand (Baimai, 1989; Sallum *et al.*, 2005).

Within the Funestus Group, the Minimus Subgroup contains the *Anopheles* (*Cellia*) *minimus* complex, Theobald 1901, initially recognized as a complex of 5 sibling species, provisionally designated A, B, C, D and E. Currently, very limited information is available on species B (Sucharit *et al.*, 1988) and species D (Baimai, 1989). *Anopheles minimus* Theobald (formerly species A) (Harbach *et al.*, 2006; Harbach *et al.*, 2007) is the most common and widespread species of the complex throughout Thailand. *Anopheles harrisoni* Harbach & Manguin (formerly species C) is restricted to two districts of Kanchanaburi Province, western Thailand and occurs in sympatry with *An. minimus* (Garros *et al.*, 2006; Sungvornyothin *et al.*, 2006). *Anopheles harrisoni* has also been reported from Mae Sot in Tak Province and Mae Rim in Chiang Mai Province, northern Thailand but no definitive species confirmation had been made at the time (Rattanarithikul *et al.*, 2006). The fifth species, *An. minimus* species E, is restricted to Ishigaki Island in the Ryukyu Archipelago,a small group of islands in southern Japan (Somboon *et al.*, 2001) and was subsequently named *Anopheles yaeyamaensis* Somboon &Harbach (Somboon *et al.*, 2010). The three species within the Aconitus Subgroup are presented in Thailand including *An. varuna, An. pampanai* and *An. aconitus* (Sucharit and Choochote, 1982; Harrison *et al.*, 1990; Baimai *et al.*, 1996;Junkum *et al.*, 2005).

Four members of the Maculatus Group are important or local malaria vectors in the Oriental Region, including Thailand, Nepal, Indonesia, Malaysia and The Philippines (Reid, 1968). This group contains at least nine closely related species, and differentiated based on variabilities in morphology, behavior and genetic

characters (Green *et al.*, 1985; Rattanarithikul and Green, 1986; Takai *et al.*, 1987; Chiang *et al.*, 1991; Kittayapong *et al.*, 1993; Bangs *et al.*, 2002). In Thailand, seven species have been identified, including *An. maculatus, An. pseudowillmori, An. sawadwongporni, Anopheles dravidicus* Christophers, *Anopheles notanandai* Rattanarithikul and Green, and *Anopheles willmori* (Manguin *et al.*, 2008), and *Anopheles rampae* Harbach & Somboon (Upatham *et al.*, 1988; Green *et al.*, 1992; Rongnoparut *et al.*, 1999; Rongnoparut *et al.*, 2006; Walton *et al.*, 2007; Muenworn *et al.*, 2009)

Recent observations on biting cycles and host preferences have been made on various molecularly identified vector species, within the important species assemblages of the Dirus and Minimus Complexes and the Maculatus and Leucosphyrus Groups in Thailand (Sungvornyothin *et al.*, 2006; Tananchai *et al.*, 2012; Tisgratog *et al.*, 2012). All earlier ecological and behavior studies based exclusively on species identified using morphological characters are questionable as for the precise species observed (Figure 1). In the last decade, more detailed studies on anophelines have allowed recognition of additional *Anopheles* species and species complexes composed of closely related, morphologically indistinguishable sibling (cryptic) species (Rattanarithikul *et al.*, 2006; Walton *et al.*, 2007; Manguin *et al.*, 2008; Garros and Dujardin, 2013). These advance laboratory and molecular techniques have used cytogenetic / polytene chromosome compatibility and cross mating experiments, isozyme protein markers, species-specific DNA probes, polymerase chain reaction (PCR) technologies, DNA sequencing of ITS2, D3 genes, mtDNA COII sequences, Allele-Specific PCR (AS-PCR) and Random Amplified Polymorphic DNA(RAPD) markers to discern and accurately differentiate individual species from one another (WHO, 2007).

A better understanding of the biology and behavior of specific mosquito vectors is crucial to clarify each species' respective role in disease transmission in selected areas. For example, infrastructure development and deforestation along the Thai international borders with Myanmar and Cambodia in the last two to three decades has contributed to a significant reduction in malaria cases and yet some

malaria vectors appear to have adapted to the environmental changes (Chareonviriyaphap *et al.*, 2000). Using molecular methods for species identification, the trophic behavior of each species within the three primary malaria vector complexes in Thailand has been described from various localities. The biting activities of *An. minimus* and *An. harrisoni* have been described from two malaria endemic areas in Tak (Tisgratog *et al.*, 2012) and Kanchanaburi (Sungvornyothin *et al.*, 2006) Provinces, Thailand.

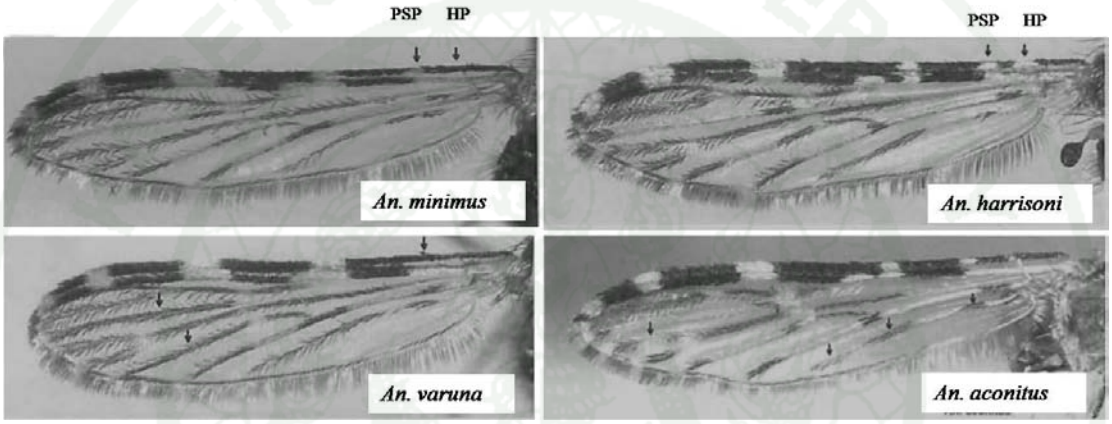

Figure 1 Species identification of Minimus Complex and related species by variable wing patterns

Source: Jaichapor *et al.*(2005).

Sungvornyothin *et al.* (2006) used the Garros *et al.* (2004a) a one-shot PCR for *An. minimus* and *An. harrisoni* species identification and for observing the trophic behavior of these two species. Kongmee *et al.* (2012) described the larval habitats of two sympatric Minimus Complex species, *An. minimus* and *An. harrisoni* in Kanchanaburi Province. Vector species diversity, biting activity patterns and trophic behavior of the Minimus Complex were observed in the Mae Sot District, Tak Province (Tisgratog *et al.*, 2012) that involved two sympatric, closely related malaria vector species, *An. minimus* and *An. aconitus*. With the availability of accurate molecular-based identification tools, more investigations on population biology and

behavior of malaria vector sibling species that live in sympatry should be carried out to determine the epidemiological role of each.

6. Geographical distribution and bionomics of malaria vectors

More than 540 species of *Anopheles* mosquitoes have so far been recognized throughout the world (Mosquito Taxonomic Inventory: www.mosquito-taxonomic-inventory.info) and approximately 80 species are regarded important vectors of malaria parasites, filarial nematodes and a few arthropod-borne viruses (Manguin *et al.*, 2011). Of 35 species assemblages within (complexes, subgroups and groups) in the genus *Anopheles,* including malaria vectors (Harbach, 2013), molecular identification assays have been developedfor > 90 sibling species (Garros and Dujardin, 2013), and 177 sibling species members have been recognized globally (Harbach, 2013). Out of 25 described *Anopheles* complexes worldwide, 11 occur in Asia, 10 of which are found in Thailand (Manguin and Boëte, 2011; Harbach, 2013). Incorrect species identification of individual members in species complexes may result in failure to distinguish between a vector and non-vector species, and thus lead to misdirect and ineffective vector control programs (Van Bortel *et al.*, 2001; Singh *et al.*, 2010). Understanding the bionomics and behavior of each mosquito species is crucial to determine its disease transmission role and devise the most efficient ways to mount a control strategy to combat it.

The combination of morphological and other methods have been essential in the differentiation of the species within the various species complexes. These methods have been detailed elsewhere (Choochote and Saeung, 2013; Garros and Dujardin, 2013) and include analysis techniques for studying morphological variations, use of cross-mating experiments, examining mitotic and meiotic karyotypes, use of cuticular hydrocarbon profiles, heterochromatin variations of polytene chromosomes, and electrophoretic protein variations. More recent molecular approaches i.e., DNA or RNA probes, use of Allele Specific Polymerase Chain Reaction (AS-PCR), Restriction Fragment Length Polymorphism PCR (RFLP-PCR), Random Amplified Polymorphic DNA PCR (RAPD-PCR), Single Strand

Conformational Polymorphisms (SSCP), and Hetero-Duplex Analysis (HDA) have added an array of tools that can differentiate individual sibling species easily and accurately.

7. Bacteria diversity in *Anopheles* abdomen

In malaria transmission cycle, a mosquito obtains infective stage of *Plasmodium spp.* from blood of an infected human. The parasite in the mosquito multiples and develops, and thenis transmitted to another human *via* mosquito feeding. The approximately volume of bloodingested by the female mosquito is 1-2 microliters. This may contain from 1 to 10,000 gametocytes. Of these, it is normal for just 12 to become successful macrogametes; 5-6 to develop as ookinetes, and 2 to develop into oocysts 2-7 days later. Of the 16,000 sporozoites produced from these two oocysts as few as 10-20 are inoculated by the malaria-infected female mosquito each time she probes when taking a subsequent bloodmeal. These significant population bottlenecks suggest that parasite differentiation is severely constrained by the environment in the mosquito, and therefore by the interactions between the parasite and the vector (Sinden, 1999).

Many insects contain large communities of diverse microorganisms that probably exceed the number of cells in the insect itself (Dillon and Dillon, 2004). More specifically, complex microbiota have been described in the mosquito abdomens including Gram-negative rods, like *Serratia marcescens, Klebsiella ozaenae, Pseudomonas aeruginosa, Escherichia coli*, and *Enterobacter* spp. (Azambuja *et al.*, 2005). Recently, three metagenomic studies provided a more comprehensive picture of the diversity of midgut microbiota in *Anopheles gambiae*, the main malaria vector in Africa (Wang *et al.*, 2011; Boissiere *et al.*, 2012; Osei-Poku *et al.*, 2012). In wild caught adults of *Anopheles* species, the microbiota showed common presence of *Pseudomonas* and *Aeromonas* species reported from at least 5 species among which malaria vectors. The following 5 genera, *Asaia, Bacillus, Chryseobacterium, Klebsiella*, and *Pantoea* were reported from 4 field collected *Anopheles* species, while *Serratia* and *Stenotrophomonas* were identified in 3 species.

At least 3 mosquito-specific bacterial species, isolated from the abdomen of main malaria vectors of the Gambiae Complex, have been described, such as *Thorsellia anophelis* (Kampfer *et al.*, 2006a), *Janibacter anophelis* (Kampfer *et al.*, 2006b) and *Elizabethkingia anophelis* (Kampfer *et al.*, 2011). The first of the three species represents a new genus and species found predominantly in *Anopheles arabiensis* (Briones *et al.*, 2008), the same *Anopheles* species from which *J. anophelis* was isolated. The third newly described species is closely related to *Elizabethkingia meningoseptica*, sharing 98.6% genetic similarity, and both species have been found in the midgut of *Anopheles gambiae* (Lindh *et al.*, 2008; Kampfer *et al.*, 2011). The latter species, *E. meningoseptica*, has also been isolated from diseased birds, frogs, turtles, cats, being most likely an agent of zoonotic infections, as well as a responsible agent for human meningitis especially in newborn infants (Bernardet *et al.*, 2005). Bacteria of the genus *Asaia* have also been associated with *Anopheles* species, in particular field-collected *An. gambiae*, *An. funestus*, *An. coustani* and *An. maculipennis*, as well as a colony of *An. stephensi* in which *Asaia* bacteria was dominant and persistently associated (Favia *et al.*, 2007). The presence of *Asaia* species in *Anopheles* could serve as a candidate for malaria control based on the production of antiparasite molecules for use in paratransgenic control of the disease (Straif *et al.*, 1998; Favia *et al.*, 2007;Damiani *et al.*, 2010). Other bacterial species have been defined as having properties conductive for antimalarial agents, especially those producing prodigiosin, a pigment produced by various bacteria, including *S. marcescens* (Azambuja *et al.*, 2005).

The number of bacteria not only varied between individuals but also changed markedly during the development, depending on both parameters, the stage of insect development and the blood-feeding status of the mosquitoes (Straif *et al.*, 1998). The normal abdomen microbiota of *Anopheles* mosquitoes need to be further identified (Chavshin *et al.*, 2012) as only few studies have reported the microbiota of wild caught malaria vectors (Jadin *et al.*, 1966; Jadin, 1967; Straif *et al.*, 1998; Gonzalez-Ceron *et al.*, 2003; Lindh *et al.*, 2005; Kampfer *et al.*, 2006a; Kampfer *et al.*, 2006b; Favia *et al.*, 2007; Briones *et al.*, 2008; Lindh *et al.*, 2008; Terenius *et al.*, 2008; Rani *et al.*, 2009; Damiani *et al.*, 2010; Dinparast Djadid *et al.*, 2011; Kampfer *et al.*, 2011;

Chavshin *et al.*, 2012; Osei-Poku *et al.*, 2012). Further investigations of gut microbiota, especially of wild-caught insect vectors, might contribute to understanding the seasonal and geographical variations recorded for vector transmitted diseases (Gonzalez-Ceron *et al.*, 2003) and yield novel vector-control strategies (Azambuja *et al.*, 2005).

Currently, the 16S gene is the most commonly used in molecular bacterial identification. It is a small subunit ribosomal RNA gene, originally introduced in phylogenetic analysis by Woese (1987). The gene contains conserved regions as well as variable and highly variable regions. The conserved regions can be used to infer relationship between distantly related species and the variable and highly variable regions between closely related species (Pettersson *et al.*, 1997). The conserved regions are also good sites for primer binding in PCR and sequencing reactions. The use of Temporal Temperature Gradient Electrophoresis (TTGE) has been reviewed by Muyzer and Smalla (1998) for studying microbial ecology. The technique is based on decreased electrophoretic mobility of partially melted double stranded DNA molecules (Muyzer, 1999). This allows for separation of DNA fragments according to sequence instead of size since different sequences will have different melting temperatures (Roudiere *et al.*, 2009). A GC-clamp attached to one of the specific primers used in the PCR preventsthe DNA fragment to melt completely and become single-stranded (Sheffield *et al.*, 1989). This is crucial since single-stranded DNA would migrate according to size and not according to sequence. The TTGE technique was utilized to group the bacterial isolates andall PCR amplicons migrating the same distance in the gel were assumed to be 16S gene fragments from the same bacterial species. This was later confirmed by sequencing several 16S rRNA genes (two-ten) from each group (Manguin *et al.*, 2013).

MATERIALAS AND METHOD

Part 1 Investigation the *Anopheles*species for presenting the current known distribution for all implicated malaria vector species in Thailand as identified by molecular methods

Illustrated keys exist to identify both female adult and larval stage of *Anopheles* mosquitoes in Thailand (Rattanarithikul *et al.*, 2006) as well as numerous publications describing molecular techniques that separate clearly morphologically identical sibling species and distribution maps (Sinka *et al.*, 2011). We have taken all available published information and new distribution data generated in this study to produce a series of tables and distribution maps illustrating the current known distribution of important malaria vectors in Thailand.

For this study samples reported herein, 10 provinces, evening adult mosquito collections were conducted as informed in Tables 2. The survey of *Anopheles* mosquito species was selected according to the malaria transmission area (BVBD 2013) (Figure 2A) and was examined from ten provinces of Thailand, along the Thai-Myanmar border (Figure 2B).

1.1 Collection sites

1.1.1 Chiang Mai is the second biggest province of Thailand and positioned at the northern part of Thailand. It is neighboring Shan State of Myanmar in north clockwise. The collection site at Mae Wang District is close to Doi Inthanon National Park which highest point of Thailand (2,560 meters above sea level). There are several kinds of forests in this province such as evergreen forests mixed deciduous forests and dry evergreen forests and pine forests. Malaria cases in Chiang Mai Province decreased significantly from 465 cases (*Pf*=182, *Pv*=275) in 2010 to only 3 cases (*Pf*=1, *Pv*=2) in 2011 (Source: Ministry of Public Health, 2010- 2011).

1.1.2 Tak Province is located in the northern part of Thailand and there is a long boundary with Kayin State of Myanmar. The transactional road between Thai - Myanmar is located in Mae Sot District. There are five Thailand‘s hill tribes; Yao, Akha, Lahu, Hmong, Lisu and the largest tribe is Karen, living in several part of this area. This province is a forested area covered by high trees, lots of vegetation as being an agricultural region and little streams along the agricultural fields. Also, it is a location with numerous Burma and Karen refugees that could be the reservoir of malaria. The malaria cases in Tak Province decreased from 18,495 cases (*Pf*=6,559, *Pv*=11,816) in 2010 to 1,605 cases (*Pf*=720, *Pv*=873) in 2011 (Source: Ministry of Public Health, 2010- 2011).

1.1.3 Mae Hong Son Province is located in northern part of Thailand. Most of Mae Hong Son Province is covered by rain forest with the complex mountain ranges of the Thai highlands. It is neighboring Shan Stateof Myanmar in north clockwise and the west borders with Kayin State andKayah State of Myanmar. This is the province with the lowest population density in Thailand. Most of the populations are Thai’s hill tribes; the Hmong, Yao, Lahu, Lisu, Akha, and Karen, which is also the biggest ethnic group is the Shan.Sop Moei District is the most southern district of Mae Hong Son Province and situated up north of Tak Province with the higher transmission period ranging from June to August, during the rainy season (Thimasarn *et al.*, 1995). In 2011, the number of malaria cases in this area reached 1,832 cases (*Pf*=840, *Pv*=934). The malaria cases werelittle reduced to 1,312 cases (*Pf*=588, *Pv*=689) in 2012 (Source: Ministry of Public Health, 2011-2012).

1.1.4 Kanchanaburi Province is the largest western province of Thailand. It is bordering Kayin State, Mon State and Tanintharyi Region of Myanmar. It is covered with timber and evergreen forests. Malaria cases in Kanchanaburi Province decreased from 1,306 cases (*Pf*=471, *Pv*=830) in 2010 to 1,095 cases (*Pf*=393, *Pv*=700) in 2011 (Source: Ministry of Public Health, 2010- 2011).

1.1.5 Prachuap Khiri Khan Province is located in the northern part of the Malay Peninsula, southern part of Thailand and the west borders of Tanintharyi

Division of Myanmar. The province contains the narrowest part of Thailand 13 km from the coast of the Gulf of Thailand to the border with Myanmar. The forest around Pa La-U Waterfall, Hua Hin District is a well known place for trekking. Malaria cases in Prachuap Khiri Khan Province were reduced from 416 cases (*Pf*=165, *Pv*=251) in 2011 to 130 cases (*Pf*=55, *Pv*=75) in 2012 (Source: Ministry of Public Health, 2011-2012).

1.1.6 Chumphon Province is one of the southern provinces of Thailand, along the shore of the Gulf of Thailand and also borders the Burmese province of Tanantharyi on the west. There are several hills of the Phuket mountain range and its northern continuation, the Tenasserim chain, while the east is the more flat land on the coast of theGulf of Thailand. In 2012, the number of malaria cases in this province wasof 506 cases with the prevalence of *Pf*=157 and *Pv*=341cases (Source: Ministry of Public Health, 2012).

1.1.7 Ranong Province is one of Thailand's southern provinces, located on the west coast along the Andaman Sea. On the west, it borders the Kawthaung and Tanintharyi Regions of Myanmar.The province is known for having the highest rainfall in Thailand, the rainy season last for about eight months. In 2010, 83 malaria cases found caused by *Pf*=44, *Pv*=37 cases (Source: Ministry of Public Health, 2011).

1.1.8 Surat Thani Province is the largest of the southern provinces of Thailand. Malaria cases increased from 261 cases (*Pf*= 138, *Pv*= 123) in 2011 to 603 cases (*Pf*=433, *Pv*=156) in 2012 (Source: Ministry of Public Health, 2012).

1.1.9 Phangnga Province is one of the southern provinces of Thailand, on the shore to the Amanda Sea. Malaria cases in this area reduced from 126 (*Pf*=60, *Pv*=65) cases in 2011 to 21 (*Pf*=5, *Pv*=16) cases in 2012 (Source: Ministry of Public Health, 2012).

1.1.10 Phuket Province is Thailand’s largest island and located in the Andaman Sea on the southern part of Thailand. This island is mostly mountainous

with a mountain range in the west of the island from the north to the south. It was hit by the Tsunami, caused by the 2004 Indian Ocean earthquake.Malaria cases in this island decreased each year from 2007 to 2011 (114 to 29 cases) (Source: Ministry of Public Health, 2011).

Table 2 *Anopheles* mosquito collection sites and time

Province	District	Observation period	Geo-coordinates
Chiang Mai	Mae Wang	11-12 March 2011	18°36′N 98°46′ E
	Mae Teang	20-21 May 2011	19°7′N 98°56′ E
Mae Hong Son	Sop Moei	22-23 February 2011	17 °52′ N 97°57′E
	Mae Sariang	19 July 2011	18°9′ N 97°56′ E
Tak	Mae Sot	28-29 October 2010	16°40′ N 98°40′ E
Kanchanaburi	Sai Yok	20-21 November 2010	14°17′N 99°11′E
Prachuap Khiri Khan	Hua Hin	11-12 September 2010	12°30′ N 99°31″E
Chumphon	Tha Sae	25-27 January 2012 23-25 April 2012 28-30 April 2012	10°39′ N 99°10′ E
	Phato	20-22 February 2012 18-20 July 2012	10° 42′N 99°19′ E
	Lamae	15-17 March 2012	9°46′ N 99°5′ E
Ranong	La-un	10-11 February 2012	10° 6′ N 98°45′ E
Surat Thani	Khiri Rat Nikhom	13-16 February 2012	9°1′ N 98° 57′ E
Phangnga	Thap Put	12-14 January 2012 7-8 March 2012 2-3 April 2012	8°30′ N 98°38′ E
	Mueang	7 March 2011 25-27 June 2011	8°26′ N 98°31′ E
Phuket	Thalang	17 May 2012	8°1′ N 98°20′ E

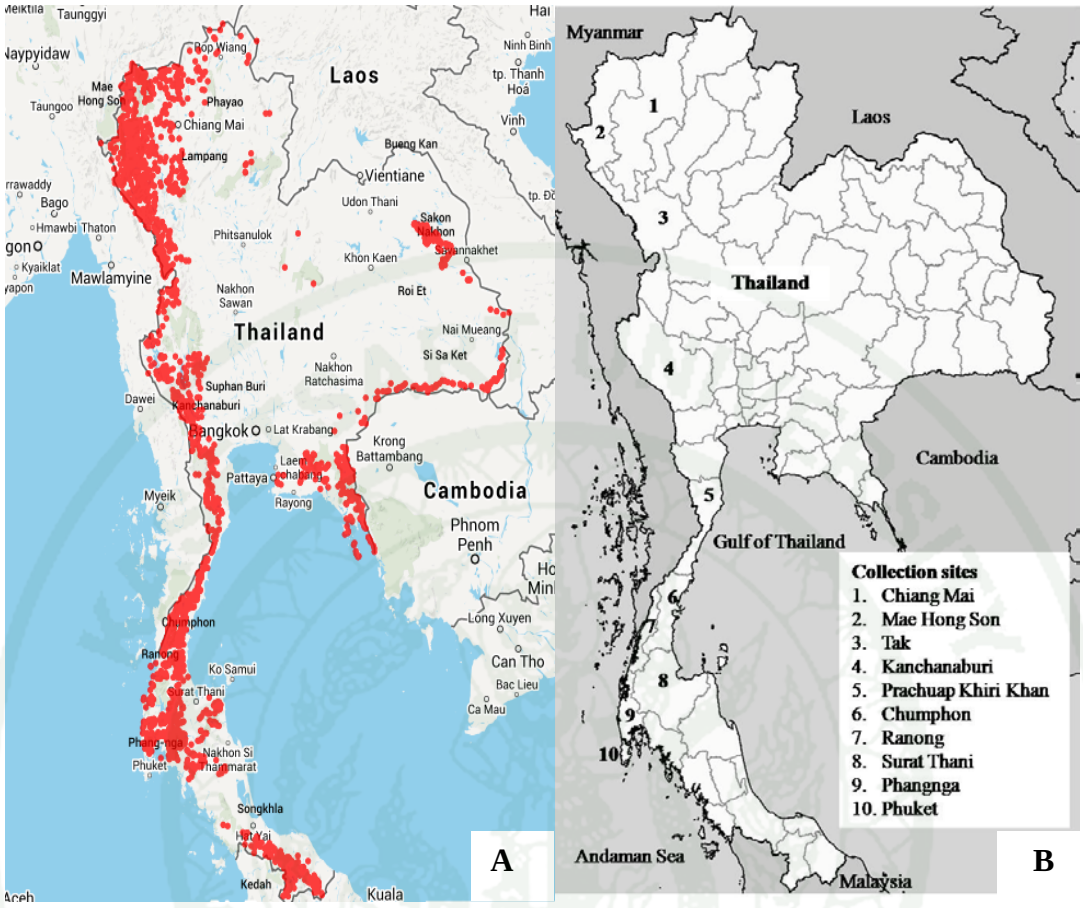

Figure 2 Malaria Transmission Area: A Zones 46/77 (59.7%) Provinces in 2013 along the international border (A) and ten study sites along Thailand-Myanmar border (B)

1.2 Species identification

Adult mosquito sample was identified using morphological keys (Rattanarithikul *et al.*, 2006) and individually stored in a clean, labelled 1.5 ml non-silicone tube. Mosquito samples were eventually brought back to the Department of Entomology, Faculty of Agriculture, Kasetsart University in Bangkok.

1.2.1 Mosquito DNA extraction

Individuals of *An. minimus* s.l., *An. dirus* s.l. and *An. maculatus* s.l. were placed in a DNA extraction tube and homogenized in 50 ml of extraction buffer (0.2 M sucrose, 0.1M Tris-HCl at pH 8.0, 50mM EDTA and 0.5% SDS). A volume of 11µl of 5mM KOAc (pH 9.0) was added and the tube placed on ice for 30 min. The sample was centrifuged at 12,000 rpm for 20 min and the supernatant was removed to a clean tube. Then, 100 µl of 100% ethanol was added and the samples placed at 4 ^{o}C for 30 min. Samples were spun at 12,000 rpm for 20 min at 4 ^{o}C. The supernatant was again cleaned using 150 µl of 70% ethanol and centrifuged at 12,000 rpm for 5 min at 4 ^{o}C, and again with 100% ethanol and centrifuged at 12,000 rpm for 5 min at 4 ^{o}C. The resultant pellet was dried at room temperature for 20 min before being re-suspended in 100 µl of TE buffer and store at -20 °C. This extraction procedure was based on Linton *et al.* (2001) and Manguin *et al.* (2002).

Species complex or group specific multiplex allele-specific polymerase chain reaction (AS-PCR) assays were used for molecular species identification within the 1) Minimus Complex and related species, including *Anopheles minimus, An. harrisoni, An. aconitus, An. varuna* and *An. pampanai* (Sungvornyothin *et al.*, 2006) using a protocol of Garros *et al.* (2004a), 2) Dirus Complex, including *Anopheles dirus, An. cracens, An. scanloni, An. baimaii* and *An. nemophilous* (Walton *et al.*, 1999), and 3) Maculatus Group, including *Anopheles maculatus, An. sawadwongporni, An. pseudowillmori, An. dravidicus, An. rampae* (former Form K) (Walton *et al.*, 2007; Somboon *et al.*, 2011).

1.2.2 Amplification by PCR

Minimus Complex: The ITS2 region was used to amplify the genomic DNA of Minimus Complex by the AS-PCR assay following the protocol of Garros *et al.* (2004a). In a final volume of 25 µl, PCR amplification conditions are as follows: 1X of 5x PCR reaction buffer (Bio-Rad Laboratories, Inc, Hercules, CA), 200 µM of each dNTP, 64 nmol of each primer (ITS2A; 5´- TGT GAA CTG CAG

GAC ACA T -3′, MIA; 5′- CCC GTG CGA CTT GAC GA-3′, MIC; 5′- GTT CAT TCA GCA ACA TCA GT-3′, ACO; 5′-ACA GCG TGT ACG TCC AGT-3′, VAR; 5′-TTG ACC ACT TTC GAC GCA-3′, PAM; 5′-TGT ACA TCG GCC GGG GTA-3′) (BioDesign Co., Ltd. Klong Luang Pathumthani, Thailand), 0.5 units of *Taq* DNA polymerase (i-Taq Plus ^{TM} DNA polymerase), and 1 µl of DNA template. The PCR cycles are as follows: one cycle at 94 °C for two min, follow by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 sec, and extension at 72 °C for 40 sec, and a final extension at 72°C for 5 min. Run electrophoresis of the PCR products on a 2.5% agarose gel.

Dirus Complex: The rDNA ITS2 was used to amplify the genomic DNA of Dirus complex by the AS-PCR assay following the protocol of Walton *et al.* (1999). In a final volume of 25 µl, PCR amplification conditions are as follows: 1x of 5x reaction buffer, 200 µM of each dNTP, 1.5 mM MgCl2, 0.2 mM of each primer (ITS2A; 5′- TGT GAA CTG CAG GAC ACA T-3′, D-U ;5′- GCG CGG GGC CGA GGT GG-3′, D-AC; 5′-CAC AGC GAC TCC ACA CG -3′, D-B; 5′-CGG GAT ATG GGT CGG CC 3′, D-D; 5′-GCG CGG GAC CGT CCG TT-3′, D-F;5′-AAC GGC GGT CCC CTT TG-3′) (BioDesign Co., Ltd. Klong Luang Pathumthani, Thailand), 0.5 units of *Taq* DNA polymerase, 10% dimethylsulphoxide (DMSO) and 2 µl of DNA template. The PCR cycles are as follows: one cycle at 94 °C for 5 min, follow by 32 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 15 sec, and extension at 72°C for 30 sec, and a final extension at 72 °C for 10 min. Run electrophoresis of PCR products on a 2.5% agarose gel.

Maculatus Group: The rDNA ITS2 was used to amplify the genomic DNA of Maculatus Group by the AS-PCR assay following the protocol ofWalton *et al.* (2007). In a final volume of 25 µl, PCR amplification conditions are as follows: 5 µl of 5x reaction buffer, 200 uM of each dNTP, 1.5 mM MgCl2, 0.2 mM of primers (5.8F; 5′-ATC ACT CGG CTC GTG GAT CG-3′, MAC; 5′- GAC GGT CAG TCT GGT AAA GT-3′, DRAV; 5′-GCC TAC TTT GAG CGA GAC CA-3′, K ; 5′-TTC ATC GCT CGC CCT TAC AA-3′) and 0.1 mM of primers SAW and PSEU (SAW; 5′-ACG GTC CCG CAT CAG GTG C-3′, PSEU; 5′-GCC CCC GGG TGT CAA

ACA G -3′) (BioDesign Co., Ltd. Klong Luang Pathumthani, Thailand), 0.5 units of *Taq* DNA polymerase, 2 µl of DNA template. The PCR cycles are as follows: one cycle at 94°C for 5 min, follow by 35 cycles of denaturation at 94 °C for 1 min, annealing at 61°C for 30 sec, and extension at 72 °C for 30 sec, and a final extension at 72 °C for 5 min. Run electrophoresis of the PCR products on a 2.5 % agarose gel.

Part 2 Trophic behavior, biting activity, seasonal abundance, and parasite infection of individual sibling species by molecular identification, inMae Sot (Tak Province) and Sop Moei (Mae Hong Son Province), northwestern Thailand.

2.1 Collection sites

Evening adult mosquito collections were conducted every two months during three consecutive nights, for a period of two years, February 2011-December 2012 in one village each in Mae Sot District (Tak Province) and Sop Moei District (Mae Hong Son Province) in northwestern Thailand (Figure 3).

2.1.1 The Mae Sot field site has a western boundary with Kayin State, Myanmar and located at approximately 471 meters above sea level. There are five isolated hill tribes living in the area, the most common ethnic group being Karen (> 85%). Rubber plantations, fruit orchards and other agricultural crops surround most the study site. A narrow, slow running stream, approximately 0.5 m in average depth and 2 m wide, runs across the village.

2.1.2 The Sop Moei study site is neighboring Myanmar, Shan State to the north and Kayin and Kayah States to the west. The field site is located approximately 50 m of distance from the Ngao River and one kilometer from Mae Ngao National Park. The study site is surrounded by secondary forest at approximately 126 m above sea level. There are several tribal groups in this area in which the most common is Thai.

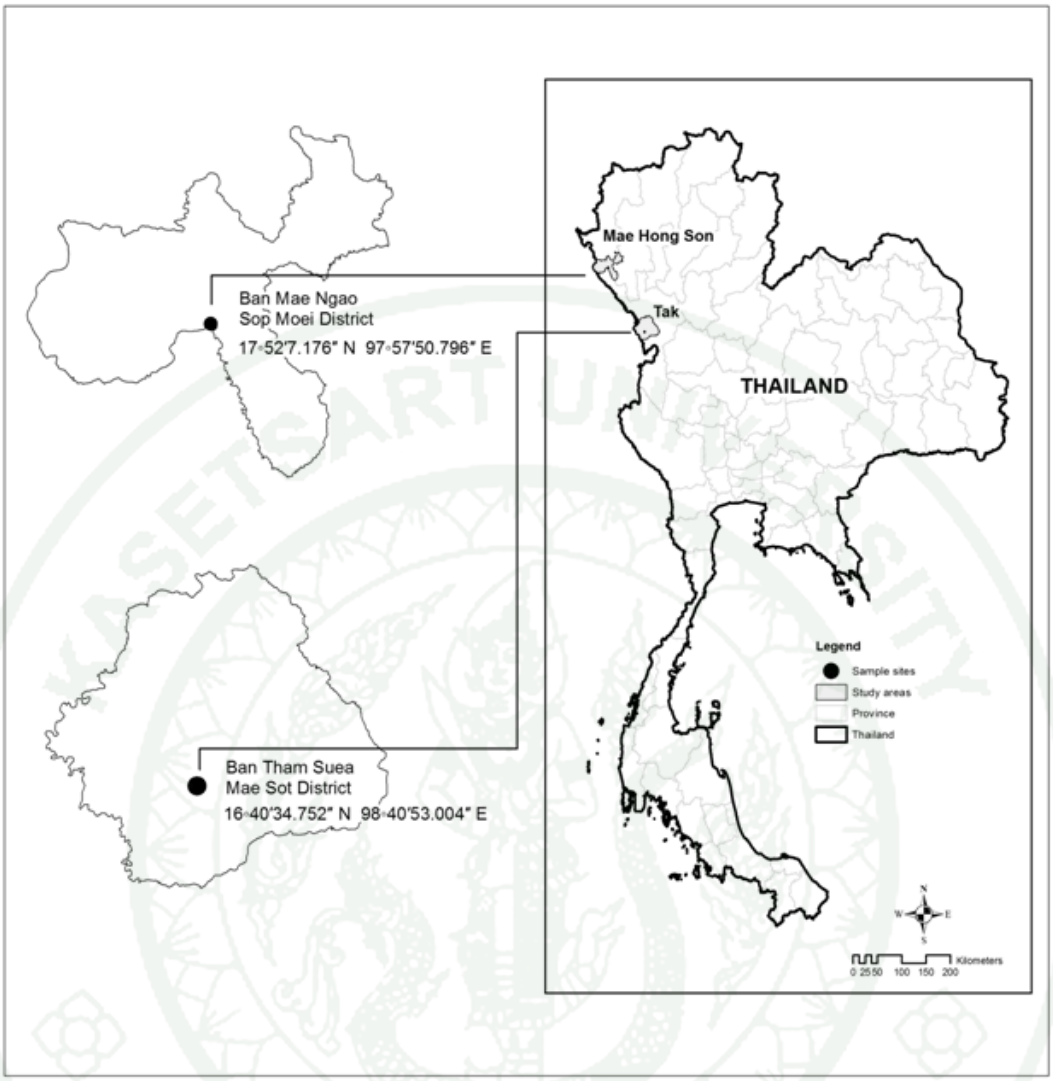

Figure 3 *Anopheles* collection sites in Tak and Mae Hong Son Provinces

2.2 Collection methods

Standard landing collection techniques were used such as indoor and outdoor human-baited landing captures and cattle-baited outdoor trap collections. Indoor and outdoor human landing collections (HLC) were conducted by two teams of four people each (Figure 4A-B). The first team worked from 18:00 to 24:00 hrs, followed by a second team from midnight to 06:00 hrs. Two people would sit inside the house, while the other two took up a position outside, approximately 100 m distance from the same hut. Human-landing collections occurred for 45 min each hour followed by a 15 min resting period. The collectors rotated hourly between

indoor and outdoor collections. Cattle bait collections were conducted by one separate collector for 15 min each hour, representing the equivalent of a 45 min resting collection (Figure 4C). Additional details on human landing collection methods were described previously (Tisgratog *et al.*, 2012). Collected mosquitoes were kept in a clean plastic cup covered with netting and provided 10% sugar solution soaked on cotton for sustenance. The following morning, cups were taken back to the field laboratory for morphological identification (Figure 4D). Hourly ambient outdoor air temperatures and relative humidity were recorded. Rainfall data was collected from the Mae Sot and Sop Moei District meteorological stations, located approximately 10 km apart from each village. The human and animal use protocols for this study were approved by the Ethical Research Committee, Chulalongkorn University, Bangkok, Thailand (No. 0961/56).

Figure 4 Three standard mosquito collections, such as: (A) indoor human landing, (B) outdoor human landing, and (C) cattle baited dwelling under the bed net, (D) the next morning all *Anopheles* were sorted to species complex and/or group level by morphological identification key

2.3 Mosquito species identification

Each female mosquito was intially sorted out using morphological keys (Figure 5A-C) (Rattanarithikul *et al.*, 2006) and individually stored in a clean, labelled 1.5 ml non-silicone tube for immediate freezing and storing in a liquid nitrogen tank. Mosquito samples were eventually brought back to the Department of Entomology, Faculty of Agriculture, Kasetsart University in Bangkok and samples were preserved in -80 ºC.

Figure 5 Photos inside a mosquito holding cup containing wild caught mosquitoes of the Minimus Complex (A), closed up of *An. dirus* (B) and *An. minimus* (C) Complexes for morphological sort out

DNA extraction was done based on Linton *et al.* (2001) and Manguin *et al.* (2002). Specific multiplex allele-specific polymerase chain reaction (AS-PCR)

assays were used for molecular species identification within the 1) Minimus Complex and related species, including *Anopheles minimus, An. harrisoni, An. aconitus, An. varuna* and *An. pampanai* (Garros *et al.*, 2004a; Sungvornyothin *et al.*, 2006), 2) Dirus Complex, including *Anopheles dirus, An. cracens, An. scanloni, An. baimaii* and *An. nemophilous* (Walton *et al.*, 1999), and 3) Maculatus Group, including *Anopheles maculatus, An. sawadwongporni, An. pseudowillmori, An. dravidicus, An. rampae* (former Form K) (Walton *et al.*, 2007; Somboon *et al.*, 2011).

2.4 Real-Time PCR detection for *Plasmodium* species and *Wuchereria bancrofti* in mosquitoes

Real-time PCR was performed on a Roche Light Cycler®480 (Software Version LCS480 1.5.0.39) using TaqMan reagents and hydrolysis probes for the detection of *Plasmodium falciparum, P. vivax,* and *P. knowlesi,* following a slightly modified methodology of Divis *et al.* (2010), and *Wuchereria bancrofti* immature stages using methods of Rao *et al.* (2006). Two reactions were separately performed including a first round of screening reaction for the detection of all *Plasmodium* species (Primers; Plasmo 1, Plasmo 2 and the *Plasmodium* screening probe) from Rougemont *et al.* (2004), *Plasmodium vivax* (Primers; VIV-F, VIV-R and probe VIV-PB) from Perandin *et al.* (2004) and *Wuchereria bancrofti* (Primers; LDR1-F, LDR2-R and probe WB-PB) from Rao *et al.* (2006) (Table 3). For the second round, a specific reaction was processed for the detection of *P. falciparum* (Primers; FAL-F, FAL-R and FAL probe) from Perandin *et al.* (2004) and *P. knowlesi* Primers; Plasmo 1 and 2 fromRougemont *et al.* (2004) and PK probe from Divis *et al.* (2010) (Table 4).

The Taqman probes were labelled with the reporter dyes FAM, TET, HEX or Cy5 at the 5′end, and the quencher dyes Black Hole Quencher 1 (BHQ1) and 2 (BHQ2) were added to the 3′ end. Primers and probes were commercially synthesized by Eurogentec (Angers, France). Extracted DNA used as positive controls for *P. falciparum* and *P. vivax* was provided by Daniel Parzy (Faculty pf

Phamacy, Marseille, France), and that used for *Wuchereria bancrofti* was provided by Adisak Bhumiratana (Mahidol University, Bangkok, Thailand).

The real-time PCR mix was prepared using the Platinum Taq polymerase Kit for each PCR run. Total volume (20 µl) of the mix containing (in final concentration) 1X PCR buffer, 4 mM MgCl2, 200 µM dNTP mixture, 0.225 µM of each primer, 0.1 µM of probe, 0.5 Unit Platinum Taq polymerase, and 2 µl of DNA template was used to fill a 384-well microplate (Roche Applied Science, Penzberg, Germany). Singleplex real-time PCR was preceded by positive and negative samples to detect the separated target and check the repeatability of the method. Multiplex assays were designed to simultaneously detect several parasite species, e.g. *Plasmodium* species, *P. vivax + W. bancrofti* and *P. falciparum + P. knowlesi*. Amplification consisted of one cycle of denaturation at 95°C for 15 min followed by 45 cycles at 95°C for 20 s, 60°C for 1 min and 40°C for 30 s. Analysis of the singleplex and multiplex data was performed with the LightCycler 480 software (Roche Applied Science, Penzberg, Germany). PCR grade water was used as no template control. DNA from *W. bancrofti, P. falciparum* and *P. vivax* served as positive controls, and DNA extracted from *Aedes aegypti* free of *Plasmodium* and *W. bancrofti* served as negative controls. All real-time PCR assays were carried out in duplicate, and samples that did not produce fluorescence before the threshold of 40 cycles (The International Center of Insect Physiology and Ecology) were considered negative. Questionable samples with a single positive well were sent for DNA sequencing (Ngo *et al.*, 2014).

2.5 Data analysis

Populations of collected mosquitoes from different collection methods were analyzed using non-parametric statistical methods. Wilcoxon test and Pearson's correlation analyses were used to investigate the interaction between the number of mosquitoes and environmental data. All data were analyzed with the accepted level of significance at 0.05% ($P< 0.05$), using the SPSS statistical package (version 17.0, SPSS, Chicago, IL).

Table 3 List of primers and probes for real-time PCR non-specific *Plasmodium spp.*, *Plasmodium vivax* and *Wuchereria bancrofti*

Species	Sequence	Citation
Plasmodium spp.	Forward primer : Plasmo 1: 5'-GTTAAGGGAG TGAAGACGA TCAGA-3' Reverse primer: Plasmo 2 :5'-AACCCAAAGACT TTGATTTC TCATAA-3' Probe: Plas prob (FAM) 5'-ACCGTCGTAATCTTAACCATAAACTATGC CGACTAG-TAMRA-3'	Rougemont *et al.* (2004)
P. vivax	Forward primer : VIV_F: 5'-ACGCTTCTAGCTTA ATCCACATAACT-3' Reverse primer: VIV_R: 5'-ATTTACTCAAAGTAA CAAGGACTTCCAAGC-3' Probe: VIV_PB (TET) 5'-TTCGTATCGACTTTGTGCGCATTTTGC-3'	Perandin *et al.* (2004)
W. bancrofti	Forward primer : LDR1_F: 5'-ATTTTGATCATCTG GGAACGTTAATA-3' Reverse primer: LDR2_R: 5'-CGACTGTCTAATCC ATTCAGAGTGA-3' Probe: WB_PB (Cy5) 5'ATCTGCCCATAGAAATAACTACGGTGGATCT CTG-3'	Rao *et al.* (2006)

Table 4 List of primers and probes for real-time PCR *Plasmodium knowlesi* and *Plasmodium falciparum* detections

Species	Sequence	Citation
P. knowlesi	Forward primer : Plasmo 1: 5'-GTTAAGGGAG TGAAGACGA TCAGA-3' Reverse primer: Plasmo 2: 5'-AACCCAAAGAC TTTGATTTC TCATAA-3' Probe: PK prob (FAM) 5'CTCTCCGGAGATTAGAACTCTTAGATTGCT-3'	Rougemont *et al.* (2004) Divis *et al.* (2010)
P. falciparum	Forward primer : FAL-F: 5'-CTTTTGAGAGGTT TTGTTACTTTGAGTAA-3' Reverse primer: FAL-R: 5'-TATTCCATGCTGTA GTATTCAAACACAA-3' Probe: FAL probe (HEX) 5'-TGTTCATAACAGACGGGTAGTCATGATTG AGTTCA-3'	Perandin *et al.* (2004)

Part 3 Bacterial biodiversity in abdomens of *Anopheles* mosquitoes in western Thailand

3.1 Mosquito collections and species identification

Populations of *Anopheles* mosquitoes were collected from two different sites located in a malaria endemic area along the Thai-Myanmar border; Mae Hong Son and Tak Provinces, using standard human-landing catch techniques (details in Materials and Methods in Parts 1-2). The following morning, each mosquito were sorted out using morphological criteria for initial identification at the complex or group level using published morphological keys (Rattanarithikul *et al.*, 2006). Subsequently, *Anopheles* mosquitoes were assayed using allele-specific PCR techniques for species identification within each complex. Each individual was split in two pieces, head-thorax for species identification and abdomen for bacteria analysis. Those abdomens were representative of species and location (8 *Anopheles* species and 2 locations). All specimens were kept at -80 ºC until laboratory processing.

3.2 DNA extraction from abdomen *Anopheles* mosquito

Each abdomen mosquito was rinsed twice in purified water (prepared from injectable solution) before abdomen was thoroughly disrupted using a tissue crusher device in 150 µL of TE buffer. DNA was extracted using the Master Pure Gram Positive DNA purification kit as recommended by the supplier (Epicentre Biotechnologics, Madison, USA).

3.3 PCR

The V2–V3 region of the 16S rRNA gene of bacteria in the samples was amplified using the primers HDA1 / HDA2 (Roudiere *et al.*, 2009); HDA1: 5'-ACTC CTA CGG GAG GCA GCA GT-3', HDA2: 5'-GTA TTA CCG CGG CTG CTG GCA-3'. A 40-bp clamp, named GC (5'-CGC CCG GGG CGC GCC CCG GGCGGG

GCG GGG GCA CGG GGG G-3') flanked the 5' extremity of HDA1 (Ogier *et al.*, 2002) in order to form HDA1-GC. PCR was performed using an Eppendorf thermal cycler® (Eppendorf, Le Pecq, France) and 0.5 ml tubes. The reaction mixture (50 µL) contained 2.5 units of Taq DNA Polymerase (FastStart High Fidelity PCR system, Roche, Meylan, France), 0.2 mM of each primer and 1 µl of DNA in the appropriate reaction buffer. Amplification was 95°C for 2 min, 35 cycles of 95°C for 1 min, 62°C for 30 s, 72°C for 1 min and 7 min at 72°C for final extension. To avoid contamination, solutions were prepared with sterile DNA-free water and preparation of the mastermix, addition of template DNA and gel electrophoresis of PCR products were carried out in separate rooms. PCR amplification was checked by DNA electrophoresis in 2% agarose gels containing ethidium bromide and visualized under ultraviolet light.

3.4 TTGE migration

TTGE was performed using the DCode universal mutation detection system (Bio-Rad Laboratories, Marne-la-Coquette, France) in gels that were 16 cm × 16 cm by 1 mm. The gels (60 mL) were composed of 8% (wt/vol) bisacrylamide (37.5:1), 7 M urea, 60 µL of N,N,N',N'-tetramethylethylenediamine (TEMED), and 0.1% (wt/vol) ammonium persulfate. Gels were run with 1X Tris–acetate–EDTA buffer at pH 8.4. The 5 µl of PCR product was loaded on gel with 5 µl of in house dye marker (saccharose 50%, Bromophenol Blue 0.1%) using capillary tips. Denaturing electrophoresis was performed at 46 V with a temperature ramp from 63°C to 70°C during 16 h. (increment 0.4°C/h) after a pre-migration of 15 min at 20 V and 63°C. Gels were stained with ethidium bromide solution (5 µg/mL) for 20 minutes, washed with de-ionized water, viewed using a UV transillumination system (Vilbert-Lourmat, France) and photographed.

3.5 TTGE band sequencing

TTGE bands were excised and the DNA was eluted with 50 µL of elution buffer EB of the Qiaquick PCR purification kit (Qiagen, Courtabeuf, France)

overnight at 37°C before PCR amplification with HDA1/HDA2 used without GC clamp. The reaction conditions were identical to those described above. PCR products were sequenced on an ABI 3730xl sequencer (Cogenics, Meylan, France). Each sequencing chromatograph was visually inspected and corrected. The sequences were analyzed by comparison with Genbank (http://www.ncbi.nlm.nih.gov/) and RDPII databases (http://rdp.cme.msu.edu/) using Basic Local Alignment Search Tool (BLAST) and Seqmatch programs, respectively. The reference sequence with the highest percentage was used for Operational Taxonomic Unit (OTU) affiliation. Operational taxonomic units (OTUs), which are clustered on the basis of DNA sequence identity alone, are the most commonly used microbial diversity unit. A sequence was affiliated to a species-level OTU when the percent of sequence similarity was > 99.0%, as proposed by Drancourt *et al.* (2000). This value is over the recognized cut-off value for the delineation of species (Stackebrandt and Goebel, 1994), but warrants high stringency for species-level OTU affiliation. Below 99.0%, the sequence is affiliated to the genus of the reference sequence with the highest percentage. When several species reference sequences match equally, affiliation was done to the genus level. For example, sequence with 99.5% in similarity to the species *Aeromonas caviae* and *Aeromonas hydrophila* was only assigned to the genus *Aeromonas*. Low cut-off is not defined for the genus delineation as affiliation to a higher taxonomic rank, such as family or order, was to be done considering the taxonomic frame of the clade using Greengenes database (McDonald *et al.*, 2012). On each TTGE gel, about 50% of the bands were sequenced, the others being affiliated to an OTU by comparison of their migration distance with that of sequenced bands.

RESULTS AND DISCUSSION

Part 1 Investigation the *Anopheles* species for presenting the current known distribution for all implicated malaria vector species in Thailand as identified by molecular methods

1. Distribution of *Anopheles*

A total of 3,127 *Anopheles* were identified by AS-PCR. Eleven species were found in this survey. There are two species in Minimus Complex; *An. minimus* and *Anopheles harrisoni*, and two related species; *An. aconitus* and *An. varuna*. The commonly found species, *An. minimus*, was present in all ten study sites (Table5).

1.1 Dirus Complex

The Dirus Complex belongs to the Leucosphyrus Group in the Neomyzomyia Series (Harbach, 2004). This group contains at least three important malaria vectors present in South and Southeast Asia, including *Anopheles dirus, Anopheles balabacensis* Baisis and *Anopheles leucosphyrus* Doenitz. However, the taxonomy of this diverse and widely distributed group remains difficult to discern. In 1989, 20 known species and two forms had been morphologically identified in the Leucosphyrus Group (Peyton, 1989). Peyton and Ramalingam (1988) provided the morphological and geographical descriptions of the Dirus Complex for the first time.

Evidence of sibling species within the Dirus Complex began with cytological observations of natural and laboratory populations, and crossing experiments between populations and morphological variations seen in natural populations. These populations comprised the chromosomally identified species A (*An. dirus*), B (*An. cracens*), C (*An. scanloni*) and D (*An. baimaii)* with an additional confirmation from electrophoretic variations of six enzyme systems associated with the chromosomal forms (Green *et al.*, 1992). Species E (*An. elegans*) was described

from a colony established from mosquitoes collected from southwestern India (Sallum *et al.*, 2005). Species F (*An. nemophilous*) was obtained from the mosquitoes collected from the Thai-Malaysia border (Peyton and Ramalingam, 1988). *Anopheles takasagoensis*, previously known as *An. balabacensis* Taiwan form, was subsequently elevated to species status by Peyton and Harrison (1980). As all sibling species have now been formally named (Sallum *et al.*, 2005), their official Latin names will be used from this point forward.

Table 5 Molecular identification of mosquito samples within the Minimus and Dirus Complexes and the Maculatus Group from the difference study sites

Locations	Sample size by species										
	An. minimus	*An. harrisoni*	*An. aconitus*	*An. varuna*	*An. maculatus*	*An. sawadwongporni*	*An. dravidicus*	*An. pseudowillmori*	*An. dirus*	*An. baimaii*	*An. nemophilous*
Mae Hong Son	265	-	8	11	67	203	15	6	2	4	-
Tak	1,164	-	7	51	236	59	30	4	15	3	-
Kanchanaburi	97	271	-	-	92	48	-	-	2	1	-
Prachuap Khiri Khan	86	-	15	-	2	4	-	-	-		-
Chumphon	211	-	-	-	16	-	-	-	4	5	-
Ranong	1	-	4	-	3	-	-	-	-	3	2
Phangnga	2	-	-	-	-	-	-	-	-	5	-
Phuket	11	-	-	-	-	-	-	1	-	-	-
Surat Thani	89	-	-	-	-	-	-	-	-	-	-
Chiang Mai	2	-	-	-	-	-	-	-	-	-	-
Total	1,928	271	34	62	416	314	45	11	23	21	2

Baimai (1989) used the mitotic karyotypes to demonstrate the variations in the structure and blocks of heterochromatin in chromosomes of all sibling species within the Dirus Complex. A standard map of the polytene

chromosomes of *An. dirus* has been used in comparative studies of the chromosomes of other closely related species (Baimai *et al.*, 1988c). Using DNA probes, Panyim *et al.* (1988) reported four species-specific DNA sequences, and Audtho *et al.* (1995) developed a simple system for the use of DNA probes, Horseradish peroxidase - labelled DNA probes and a chemoluminescent detection system. The sensitivity of the technique is high as it could detect 1-5 ng of target DNA, which was comparable to 32P labelled probes. This technique successfully identified *An. dirus, An. cracens, An. scanloni* and *An. baimaii* from field collections.

The ITS2 sequence analysis revealed two different sequences for *An. scanloni* from northern and southern Thailand (Walton *et al.*, 1999). The population genetic analysis using 11 microsatellite markers of northern and southern populations showed significant differences in the genetic structure between the two (Walton *et al.*, 1999; Walton *et al.*, 2001). From the sequence analysis of the ITS2 region, Walton *et al.* (1999) reported that *An. baimaii* is different from species D from China as reported by Xu *et al.* (1998), suggesting that the latter one from China may represent yet another species in this complex.

Several techniques and diagnostic features are available for the identification of sibling species. Twenty restriction enzymes were used to study restriction fragment length polymorphism (RFLP) (Yasothornsrikul *et al.*, 1988) in *An. dirus, An. cracens, An. scanloni* and *An. baimaii*. Seven enzymes (*Ava II, Alu I, Bgl II, Hae III, Hinf 1, Mbob* and *Sau 3A I*) produced unique patterns for each species while other enzymes produced unique patterns for one or two species only. An AS-PCR assay (Xu *et al.*, 1998) using three primers, one derived from highly conservative 5.8S coding sequences and two from the internal transcribed spacer (ITS2) region of ribosomal DNA (rDNA), could distinguish *An. dirus* from *A. baimaii* with 374 base pairs and 663 base pair length amplicons, respectively. Walton *et al.* (1999) used a multiplex PCR assay with primers designed from rDNA ITS2 sequences of the different sibling species that could identify *An. dirus, An. cracens, An. scanloni, An. baimaii* and *An. nemophilous* in Thailand using a single reaction.

Manguin *et al.* (2001) and Huong *et al.* (2001) also developed PCR assays which clearly distinguished *An. dirus, An. cracens, An. scanloni* and *An. baimaii*. The method of Huong *et al.* (2001) used a cocktail of four primer sets and requiring a small amount of DNA for identification. They also provide a simple and rapid method for the identification of field-collected specimens. Microsatellite markers have been used for population analysis (Walton *et al.*, 2000a) of *An. dirus* and to assess polymorphism in species *An. scanloni* and *An. baimaii*. Walton *et al.* (2000b) used these markers to study the gene flow in populations of *An. dirus, An. scanloni* and *An. baimaii*, where in all three species were found well differentiated using specific microsatellite loci. Based on the use of these techniques, specific distributions of *An. dirus* and *An. baimaii* are listed in Table 6 (from this study) and Tables 7-8 and illustrated in Figure 6A-B, respectively.

Anopheles dirus is found throughout the country (Figure 6A), whereas *An. baimaii* appears to have a more restricted distribution as only found in central, southern and mainly western parts of the country along the Thai-Myanmar border (Figure 6B). The other members of this complex, *An. cracens*, *An. scanloni*, and *An. nemophilous*, have been reported primarily in western and southern Thailand (Figure 6C) (Tables 9-11). From collections in this study along the Thailand-Myanmar border and based on AS-PCR assay species identification, higher numbers of *An. baimaii* were found compared to *An. dirus*, with only 2 specimens of *An. nemophilous* detected from Ranong Province.

Table 6 Molecular identification of mosquito samples within the Dirus complex in this study

Anopheles species	Province	District	Geographic coordinates (DMS)
An. dirus	Kanchanaburi	Sai Yok	14°17'N 99°11′E
	Chumphon	Phato	10°42′N 99°19′E
		Lamae	9°46′N 99°5′E
An. baimaii	Chumphon	Phato	10°42′N 99°19′E
	Ranong	La-un	10°6′N 98°45′E
	Phangnga	Thap Put	8°30′N 98°38′E
		Mueang	8°26′N 98°31′E
An. nemophilous	Ranong	La-un	10°6′N 98°45′E

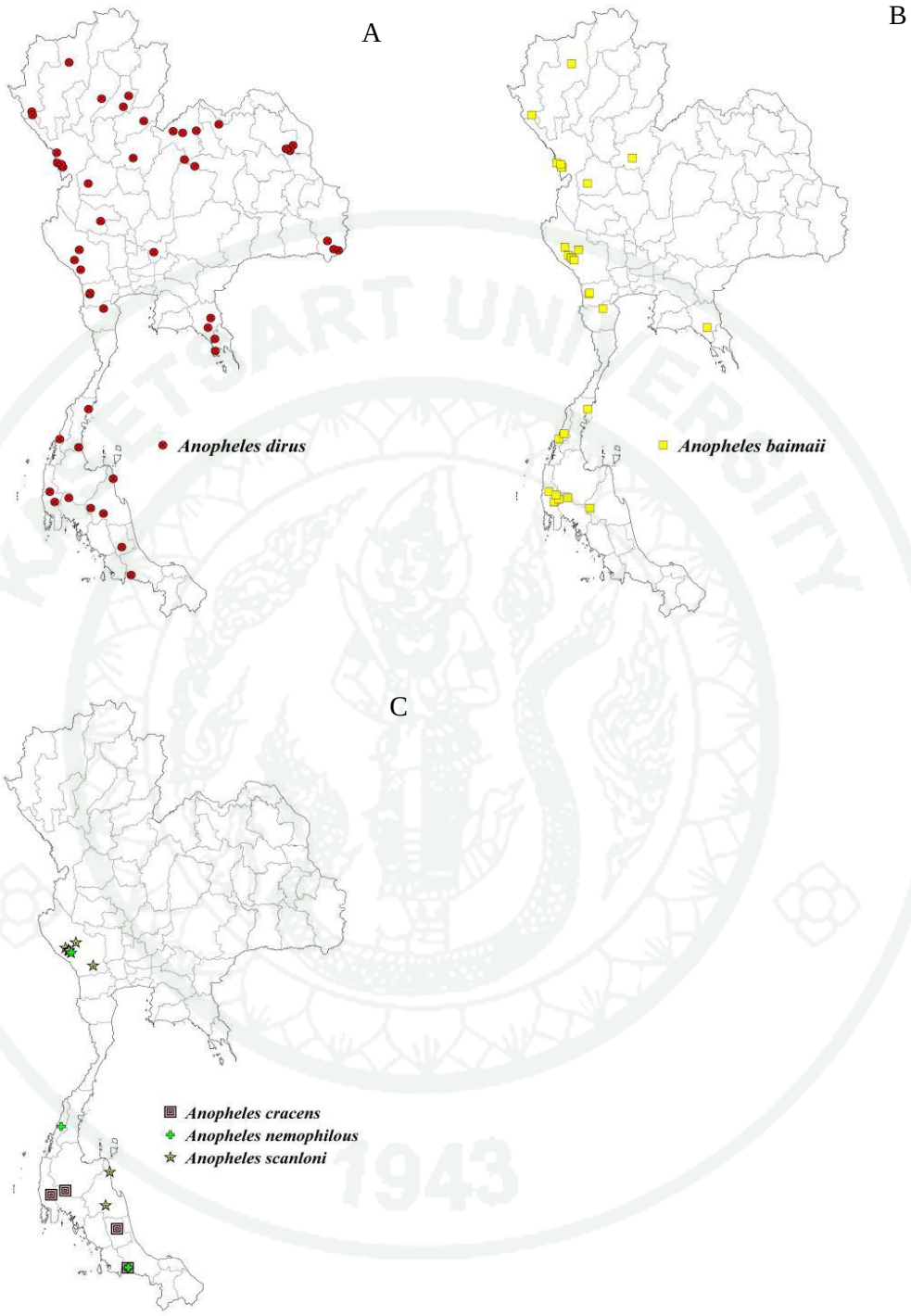

Figure 6 Known geographic distribution of *Anopheles dirus* (A), *Anopheles baimaii* (B) and 3 related species in the Dirus Complex (C) in Thailand based on molecular species identification

Table 7 *Anopheles dirus* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chanthaburi	NA	12°37′N102°9′E	AS-PCR	(Walton *et al.*, 1999)
Ubon Ratchathani		14°29′N105°21′E		
Sakon Nakhon		16°50′N104°16′E		
Loei		17°25′N101°38′E		
Phrae		18°4′N100°11′E		
Tak	Mae Ramat	16°57′N98°33′E		
Tak,	Mae sot	16°36′N98°42′E		
Ratchaburi	NA	13°30′N99°21′E		
Chiang Mai	Mae Taeng	19°9′N98°51′E	Polytene and/or mitotic chromosomes of their larval (4^{th} stage) progeny	(Baimai *et al.*, 1988b)
Mae Hong Son	Sop Moei	17°57′N97°56′E		
Lampang	Mae Mo	18°16′N99°39′E		
Phrae	Rong Kwang	18°20′N100°19′E		
Uttaradit	Nam Pat	17°43′N100°41′E		
Phetchabun	Nam Nao	16°46′N101°40′E		
Loei	Na Duang	17°28′N101°58′E		
Chaiyaphum	Khon San	16°36′N101°55′E		
Udon Thani	Ban Phue	17°41′N102°28′E		
Sakon Nakhon	Tao Ngoi	16°59′N104°10′E		
Ubon Ratchathani	Na Chaluai	14°31′N105°14′E		
Phitsanulok	Wang Thong	16°49′N100°25′E		
Tak	Mae Sot	16°42′N98°34′E		

Table 7 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kamphaeng Phet	Khlong Lan	16°12′N99°19′E	Polytene and/or mitotic chromosomes of their larval (4^{th} stage) progeny	(Baimai *et al.*, 1988b)
Kanchanaburi	Si Sawat	14°35′N99°6′E		
Phetchaburi	Nong Ya Plong	13°9′N99°41′E		
Uthai Thani	Huai Khot	15°17′N99°37′E		
Saraburi	Mueang	14°31′N100°54′E		
Chanthaburi	Pong Nam Ron	12°54′N102°15′E		
Ranong	Mueang	9°58′N98°38′E		
Phangnga	Kapong	8°41′N98°24′E		
Phangnga	Mueang	8°26′N98°31′E		
Krabi	Plai Phraya	8°32′N98°51′E		
Nakhon Si Thammarat	Sichon	9°0′N99°54′E		
	Thung Yai	8°17′N99°22′E		
	Thung Song	8°9′N99°40′E		
Phatthalung	Tamot	7°20′N100°6′E		
Songkhla	Sadao	6°39′N100°19′E		
	Sadao	6°39′N100°19′E	Cytogenetic and cross mating	(Baimai *et al.*, 1988a)
Chanthaburi	Makham	12°40′N102°11′E	Species-specific DNA probes	(Gingrich *et al.*, 1990)

Table 7 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi	Sai Yok	14°6′N99°8′E	AS-PCR	(Manguin *et al.*, 2002)
Sakon Nakhon	Tao Ngoi	16°59′N104°10′E		
Tak	Mae Sot	16°35′N98°40′E	AS-PCR	(O'Loughlin *et al.*, 2008)
Ratchaburi	NA	13°31′N99°21′E		
Loei		17°46′N101°4′E		
Sukon Nakhon		17°5′N104°18′E		
Ubon Ratchathani		14°43′N105°5′E		
Kanchanaburi	Sai Yok	14°17'N99°11'E	AS-PCR	(Tananchai *et al.*, 2012)
Trat	Ko Chang	12°6′N102°18′E	AS-PCR	(Ritthison *et al.*, 2014a)

Table 8 *Anopheles baimaii* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chiang Mai	Mae Taeng	19°09′N98°51′E	Polytene and/or mitotic chromosomes of their larval (4^{th} stage) progeny	(Baimai *et al.*, 1988b)
Mae Hong Son	Sop Moei	17°57′N97°56′E		
Phitsanulok	Wang Thong	16°49′N100°25′E		
Tak	Mae Sot	16°42′N98°34′E		
Kamphaeng Phet	Khlong Lan	16°12′N99°19′E		
Kanchanaburi	Si Sawat	14°35′N99°6′E		
Phetchaburi	Nong Ya Plong	13°9′N99°41′E		
Ranong	Mueang	9°58′N98°38′E		
Phangnga	Kapong	8° 41′N98°24′E		
	Mueang	8°26′N98°31′E		
Krabi	Plai Phraya	8°32′N98°51′E		
Nakhon Si Thammarat	Thung Yai	8°17′N99°22′E		
Chantaburi	Makham	12°40′N102°11′E	Species-specific DNA probes	(Gingrich *et al.*, 1990)
Tak	Mae Sot	16°36′N98°42′E	AS-PCR	(Walton *et al.*, 1999)
Phangnga	Thap Put	8°30′N98°38′E	Single multiplex PCR	(Manguin *et al.*, 2002)

Table 8 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi 1	NA	14°39′N98°46′E	AS-PCR	(Walton *et al.*, 1999)
Kanchanaburi 2		14°27′N98°51′E		
Kanchanaburi 3		14°24′N98°55′E		
Kanchanaburi 4		14°23′N98°56′E		
Kanchanaburi 5		14°20′N98°59′E		
Ratchaburi	NA	13°30′N99°21′E		
Tak	Mae Sot	14°43′N105°5′E	AS-PCR	(O'Loughlin *et al.*, 2008)
Ratchaburi	NA	13°31′N99°21′E		
Kanchanaburi		14°19′N98°59′E		
Ranong		10°04′N98°64′E		
Phangnga		8°36′N98°34′E		
Kanchanaburi	Sai Yok	14°17′N99°11′E	AS-PCR	(Tananchai *et al.*, 2012)

Table 9 *Anopheles cracens* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Nakhon Si Thammarat	Thung Song	8°11′N99°48′E	AS-PCR	(Walton *et al.*, 1999)
Phatthalung	NA	7°36′N100°4′E		
Phangnga	Mueang	8°26′N98°31′E	Polytene chromosome and/or mitotic chromosomes of their larval (4^{th} stage) progeny	(Baimai *et al.*, 1988b)
Krabi	Plai Phraya	8°32′N98°51′E		
Songkhla	Sadao	6°39′N100°19′E		
Nakhon Si Thammarat	Thung Song	8°11′N 99°48′E		
Phatthalung	Thung Yai Tamot	8°17′N99°22′E 7°20′N100°6′E		

Table 10 *Anopheles scanloni* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi	NA	14°1′N99°31′ E 14°27′N98°51′E 14°24′N98°55′E 14°23′N98°56′E 14°20′N98°59′E	AS-PCR	(Walton *et al.*, 1999)
Nakhon Si Thammarat	Thung Song	8°11′N99°48′E		
Kanchanaburi	Si Sawat	14°35′N99°6′E	Polytene and/or mitotic chromosomes of their larval (4^{th} stage) progeny	(Baimai *et al.*, 1988b)
Nakhon Si Thammarat	Sichon	9°0′N99°54′E		
	Thung Yai	8°17′N99°22′E		
Phatthalung	Tamot	7°20′N100°6′E		

Table 11 *Anopheles nemophilous* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identificationmethodology	Published sources
Songkhla	Sadao	6°39′N 100°19′E	Polytene chromosome and/or mitotic chromosomes of their larval (4^{th} stage) progeny	(Baimai *et al.*, 1988b)
Kanchanaburi	NA	14°20′N 98°59′E	AS-PCR	(Walton *et al.*, 1999)
Songkhla	Sadao	6°40′N 100°20′E		

1.2 Minimus Complex and closely related species

Initially, allozymes electrophpresis served as the gold standard to separate the two species A and C within the Minimus Complex (Green *et al.*, 1990). However, this technique is relatively impractical, as mosquito specimens need to be kept frozen to retain enzyme integrity. In addition, the entire specimen is generally required for this analysis, therefore precluding testing for *Plasmodium* infection (circumsporozoite antigen), blood meal identification, and metabolic mechanisms of insecticide resistance (Chareonviriyaphap *et al.*, 2004). In 1999, two polymerase chain reaction methods, an allele specific amplification (ASA-PCR) of the D3 variable region of the 28S rDNA gene and a single-strand conformation polymorphism (SSCP)PCR of the D3 amplified region, were developed to identify *An. minimus* (species A) and *An. harrisoni* (species C) and two other closely related species, *Anopheles aconitus* and *Anopheles varuna* (Sharpe *et al.*, 1999). The ASA-PCR was able to distinguish *An. minimus* from *An. harrisoni*, whereas the SSCP-PCR was used to separate *An. varuna* from *An. aconitus*. However, the electrophoresis run time for SSCP is comparatively very long (between 16 to 20 hrs), which is a limiting factor for processing large

number of specimens and for keeping relatively stable temperature during electrophoresis to avoid any conformational changes of ssDNA (Sharpe *et al.*, 1999). In 2000, a PCR-amplified rDNA ITS2 was designed by Van Bortel *et al.* (2000) and. in 2004, the two PCR assays, RFLP and AS, were developed, the latter being a one shot PCR method differentiating the members of the Minimus Complex and other related species (Garros *et al.*, 2004a; Garros *et al.*, 2004b). In this study, the use of AS-PCR for species identification is presented in Table 12. Based on the use of these techniques, specific distribution of *An. minimus* is listed in Tables 13-15 and illustrated in Figure 7.

The most common species of the complex found in Thailand is *Anopheles minimus* (Figure 7A, Tables12&13). This species is known as primary malaria vector commonly found in high densities throughout the country, especially in forestry endemic malaria areas (Rattanarithikul *et al.*, 2006). *Anopheles harrisoni* has been reported once in Chiang Mai and otherwise appears restricted within the Kanchanaburi Province, western Thailand (Figure 7B, Tables12&14). The other related species, *An. aconitus*, *An. varuna* and *An. pampanai*, have been found mainly in northern, western and southern locations of Thailand (Figure 7C, Tables12&15-17). *Anopheles pampanai* was found infected with *P. vivax*in Vietnam by Durnez *et al.* (2011) and Ngo *et al.* (2014) and may also play a role as a secondary malaria vector in Thailand.

Table 12 Molecular identification of mosquito samples within the Minimus Complex and closely related species in this study

Anopheles species	Province	District	Geographic coordinates (DMS)
An. minimus	Chiang Mai	Mae Wang	18°36′N98°46′E
		Mae Teang	19°7′N98°56′E
	Mae Hong Son	Sop Moei	17°52′N97°57′E
		Mae Sariang	18°9′N97°56′E
	Tak	Mae Sot	16°40′N98°40′E
	Kanchanaburi	Sai Yok	14°17′N99°11′E
	Prachuap Khiri Khan	Hua Hin	12°31′N99°32′E
	Chumphon	Tha Sae	10°39′N99°10′E
		Phato	10°42′N99°19′E
		Lamae	9°46′N99°5′E
	Ranong	La-un	10°6′N98°45′E
	Surat Thani	Khiri Rat Nikhom	9°1′N98°57′E
		Vibhavadi	9°14′N98°58′E
	Phangnga	Thap Put	8°30′N98°38′E
		Mueang	8°26′N98°31′E
	Phuket	Thalang	8°1′N98°20′E
An. harrisoni	Kanchanaburi	Sai Yok	14°17′N99°11′E
An. aconitus	Mae Hong Son	Sop Moei	17°52′N97°57′E
	Tak	Mae Sot	16°40′N98°40′E
	Prachuap Khiri Khan	Hua Hin	12°30′N99°31′E
	Chumphon	Lamae	9°46′N99°5′E
	Ranong	La-un	10°6′N98°45′E
	Phangnga	Thap Put	8°30′N98°38′E
An. varuna	Mae Hong Son	Sop Moei	17°52′N97°57′E
	Tak	Mae Sot	16°40′N98°40′E

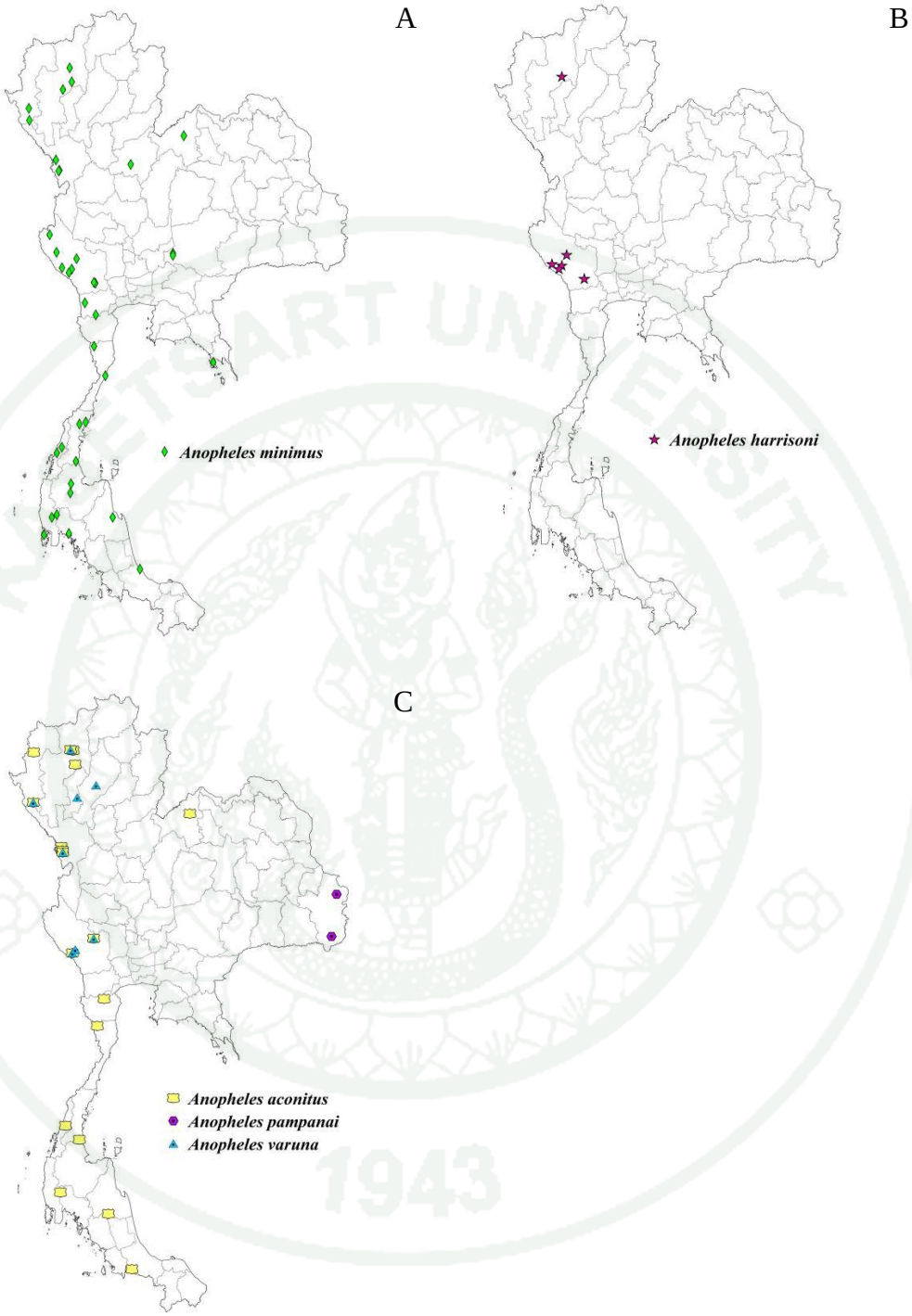

Figure 4 Known geographic distribution of *Anopheles minimus* (A) *Anopheles harrisoni* (B) and 3 related species (C) in Thailand based on molecular species identification

Table 13 *Anopheles minimus* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chiang Mai	NA	18°47′N98°59′E	SSCP / DNA sequencing; COII, ITS2, D3	(Sharpe *et al.*, 1999; Chen *et al.*, 2011)
Tak	NA	16°53′N90°07′E		
	Mae Sot	16°41′N98°41′E	AS-PCR	(Jaichapor *et al.*, 2005; Malaithong *et al.*, 2011; Tisgratog *et al.*, 2011; Tisgratog *et al.*, 2012)
	Mae Sot	16°41′N98°41′E	AS-PCR	(Jaichapor *et al.*, 2005)
Kanchana-buri	NA	14°1′N99°31′E	SSCP/DNA sequencing; COII, ITS2, D3	(Sharpe *et al.*, 1999; Chen *et al.*, 2011)
	NA	14°1′N99°31′E	RAPD marker	(Kengne *et al.*, 2001)
	Sai Yok	14°17′N99°11′E	AS-PCR	(Kengluecha *et al.*, 2005; Sungvornyothin *et al.*, 2006; Kongmee *et al.*, 2012)
	Sai Yok	14°15′N98°55′E	AS-PCR	(Kongmee *et al.*, 2012)

Table 13 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi	Mueang	14°0′N99°33′E	AS-PCR	(Kengluecha *et al.*, 2005; Rongnoparut *et al.*, 2005)
	Sangkhla Buri	15°9′N98°27′E	AS-PCR	(Kengluecha *et al.*, 2005; Rongnoparut *et al.*, 2005)
	Si Sawat	14°35′N99°6′E	AS-PCR	(Kengluecha *et al.*, 2005; Rongnoparut *et al.*, 2005)
	Thong Pha Phum	14°44′N98°37′E	AS-PCR	(Kengluecha *et al.*, 2005)
Nakhon Ratchasima	Pak Chong	14°42′N101°25′E		(Ratanatham *et al.*, 1988)
Loei	NA	17°29′N101°43′E	mtDNA COII sequences	(Chen *et al.*, 2011)
Prachuap Khiri Khan		11°48′N99°47′E		
Ranong		9°58′N98°38′E		
Krabi		8°3′N98°55′E		
Nakhon Si Thammarat		8°26′N99°57′E		
Songkhla		7°12′N100°35′E		
Ratchaburi	Pak Tho	13°8′N99°10′E	AS-PCR	(Eamkum *et al.*, 2014)
	Suan Phueng	13°32′N99°18′E		
Trat	Ko Chang	12°6′N102°18′E	AS-PCR	(Ritthison *et al.*, 2014a)

Table 13 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Phitsanulok Kanchanaburi Nakhon Ratchasima	Wang Thong Sai Yok PakChong	16°49′N100°25E 14°22'N98°45'E 14°39'N101°25'E	Metaphase karyotype preparations and chromosome analysis	(Baimai *et al.*, 1996)

Table 14 *Anopheles harrisoni* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi	Sai Yok	14°22'N98°45'E	Metaphase karyotype preparations and chromosome analysis	(Baimai *et al.*, 1996)
Chiang Mai	NA	18°47′N98°59′E	SSCP	(Sharpe *et al.*, 1999)
Kanchanaburi	NA	14°1′N99°31′E	SSCP	(Sharpe *et al.*, 1999)
	NA	14°1′N99°31′E	RAPD marker	(Kengne *et al.*, 2001)
	Sai Yok	14°17′N99°11′E	AS-PCR	(Sungvornyothin *et al.*, 2006; Malaithong *et al.*, 2011; Tisgratog *et al.*, 2011)
	Sai Yok	14°15′N98°55′E	AS-PCR	(Kongmee *et al.*, 2012)
	Si Sawat	14°35′N99°6′E	AS-PCR	(Kengluecha *et al.*, 2005)

Table 15 *Anopheles aconitus* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chiang Mai	Mae Taeng	19°8'N98°52'E	Metaphase karyotype preparations and chromosome analysis	(Baimai *et al.*, 1996)
Songkhla	Sadao	6°39′N100°19′E		
Chiang Mai	Mae Taeng	19°8'N98°52'E	Enzyme electromorph characteristics	(Green *et al.*, 1990)
Nakhon Si Thammarat	Thung Song	7°59'N99°45'E		
Kanchanaburi	Nong Prue	14°36'N99°26'E		
Chiang Mai	NA	18°47′N98°59′E	Single-strand conformation polymorphism (SSCP)	(Chen *et al.*, 2012)
Tak		16°53′N99°7′E		
Loei		17°29′N101°43′E		
Chiang Mai	Mae Taeng	19°7′N98°56′E	Sequence analysis; ITS1, ITS2 and D3 regions	(Jariyapan *et al.*, 2005)
Chiang Mai	Mae Taeng	19°7′N98°56′E	Metaphase karyotypes	(Junkum *et al.*, 2005)
Mae Hong Son	Mueang	18°47′N98°56′E		
Phetchaburi	Nong Ya Plong	13°9′N99°41′E		

Table 15 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Tak	Mae Sot	16° 4′N98°41′E	AS-PCR	(Tisgratog *et al.*, 2012)
Kanchanaburi	Sai Yok	14°15′N98°55′E	AS-PCR	(Kongmee *et al.*, 2012)

Table 16 *Anopheles varuna* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi	Sai Yok	14°15′N98°55′E	AS-PCR	(Kongmee *et al.*, 2012)
Lamphun	Thung Hua Chang	17°59'N99°2'E	Metaphase karyotype preparations and chromosome analysis	(Baimai *et al.*, 1996)
Chiang Mai	Mae Taeng	19°8'N98°52'E	Enzyme electromorph characteristics	(Green *et al.*, 1990)
Lampang	NA	18°17'N99°30'E		
Kanchanaburi	Nong Prue	14°36'N99°26'E		
Kanchanaburi	Sai Yok	14°17′N99°11′E	AS-PCR	(Sungvornyothin *et al.*, 2006)

Table 17 *Anopheles pampanai* in Thailand

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Ubon Ratchathani	Na Chaluai	14°34'N105°12'E	Enzyme electromorph characteristics	(Green *et al.*, 1990)
Ubon Ratchathani	Si Mueang Mai	15°34'N105°21'E	Metaphase karyotype preparations and chromosome analysis	(Baimai *et al.*, 1996)

1.3 Maculatus Group

Anopheles maculatus was reported as a species complex after studying the polytene and mitotic chromosomes from wild caught specimens. The members of the Maculatus Group can be identified easily from each other by examining polytene chromosomes for paracentric inversions on autosomes. 6-*Pgd* allelic variations can also be used to distinguish *An. sawadwongporni* (species A), *An. maculatus* (species B) and *An. pseudowillmori* (species I) (Green *et al.*, 1992). The two forms of species B, E and F can be distinguished by gas-liquid chromatographic analysis of cuticular lipids in association with a multivariate principal component analysis (Kittayapong *et al.*, 1990). Based on interspecific variation in the ITS2 region, a diagnostic PCR assay that distinguishes five members of the group found in China (*An. sawadwongporni*, *An. maculatus*, *An. willmori*, *An. dravidicus* and *An. pseudowillmori*) was developed (Ma *et al.*, 2006). Another PCR-based diagnostic assay was developed to facilitate field research in northern Thailand, which distinguishes *An. maculatus*, *An. dravidicus*, *An. pseudowillmori*,

An. sawadwongporni and *An. rampae* Harbach and Somboon (species K) (Walton *et al.*, 2007). About 23 microsatellite markers were identified from *An. maculatus* (species B) (Rongnoparut *et al.*, 1996), seven of which were used to study the genetic variations in eight widely dispersed localities in the western and peninsular Thailand (Rongnoparut *et al.*, 1999). In this study, the use of AS-PCR for species identification is presented in Table 18. Based on the use of these techniques, specific distribution in Thailand of seven species of the Maculatus Group is listed in Tables 18-25 and illustrated in Figure 8.

In the Maculatus Group, 3 speices have been described as important malaria vectors; including*An. maculatus*, *An. sawadwongporni* and *An. pseudowillmori* (Green *et al.*, 1991). The most common encountered species of the group in Thailand is *An. maculatus* (Figure 8A, Tables 18&19), followed by *An. sawadwongporni* (Figure 8B, Tables 18&20). *Anopheles pseudowillmori* does not present any evidence for its vectorial capacity status elsewhere in Thailand (Figure 8B, Tables18&21). A few other member species (*An. willmori, An. dravidicus, An. notanandai, An. rampae*) have been studied far less frequently and relatively little is known about their bionomics and vector status (Figure 8B-C, Tables22-25).

Table18 Molecular identification of mosquito samples within the Maculatus Group in this study

Anopheles species	Province	District	Geographic coordinates (DMS)
An. maculatus	Phangnga	Mueang	8°26′N98°31′E
	Phuket	Thalang	8°1′N98°20′E
	Kanchanaburi	Sai Yok	14°17′N99°11′E
	Prachuap Khiri Khan	Hua Hin	12°31′N99°29′E
	Chumphon	Tha Sae	10°39′N99°10′E
		Lamae	9°46′N99°5′E
An. sawadwongporni	Mae Hong Son	Sop Moei	17°52′N97°57′E
		Mae Sariang	18°9′N97°56′E
	Tak	Mae Sot	16°40′N98°40′E
	Prachuap Khiri Khan	Hua Hin	12°30′N99°31′E
An. pseudowillmori	Mae Hong Son	Sop Moei	17°52′N97°57′E
		Mae Sariang	18°9′N97°56′E
	Tak	Mae Sot	16°40′N98°40′E
An. dravidicus	Mae Hong Son	Sop Moei	17°52′N97°57′E
	Tak	Mae Sot	16°40′N98°40′E

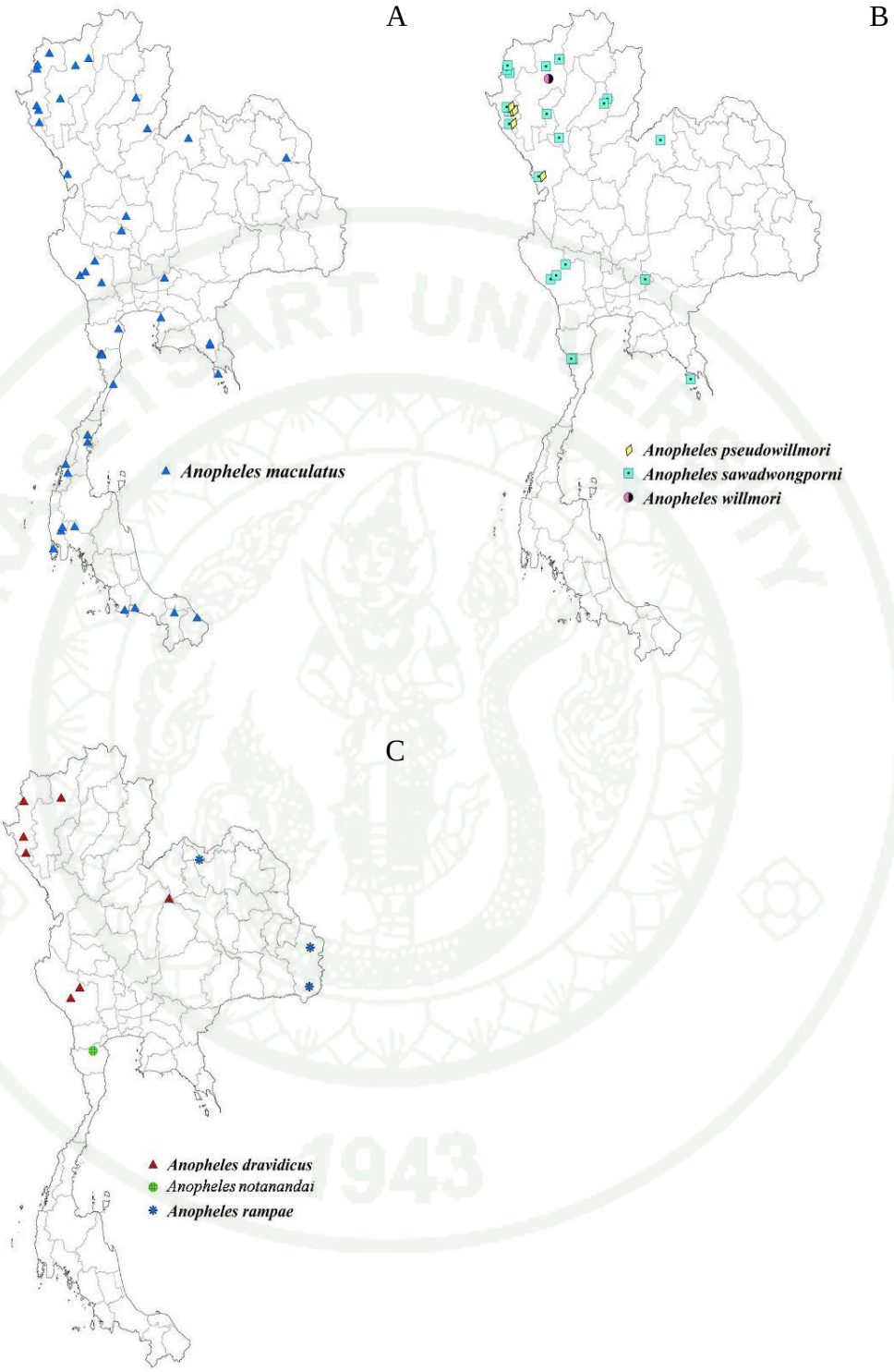

Figure 5 Known geographic distribution *Anopheles maculatus* (A) and six other members of Maculatus Group (B, C) in Thailand based on molecular species identification

Table 19 *Anopheles maculatus* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Nakhon Sawan	NA	15°42′N100°8′E	Microsatellite analysis	(Rongnoparut *et al.*, 1999)
Uthai Thani		15°22′N100°1′E		
Kanchanaburi		14°10′N99°31′E		
Phetchaburi		13°6′N99°56′E		
Prachuap Khiri Khan		11°49′N99°48′E		
Satun		6°36′N100°4′E		
Yala		6°32′N101°16′E		
Narathiwat		6°25′N101°49′E		
Uttaradit	Nam Pat	17°43′N100°41'E	Cytogenetic	(Baimai *et al.*, 1993)
Chanthaburi	Makham	12°40′N102°11′E		
Nakhon Nayok	Ban Na	14°16′N101°03′E		
Phangnga	Mueang	8°26′N98°31′E		
Krabi	Plai Phraya	8°32′N98°51′E		
Chiang Mai	Mae Taeng	19°11′N98°52′ E	Polytene chromosomes	(Green *et al.*, 1985)
Phrae	Rong Kwang	18°26′N100°24′ E		
Mae Hong Son	NA	19°12′N97°54′E		
Kanchanaburi		14°25′N99°7′E		
		14°40′N99°21′E		
Chumphon	Phato	9°46′N98°41′E		
Phangnga	NA	8°31′N98°33′E		

Table 19 (Continued)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chanthaburi	NA	12°45′N102°11′E	Polytene chromosomes	(Green *et al.*, 1985)
Chon Buri		13°21′N100°59′E		
Kanchanaburi	Sai Yok	14°17′N99°1′E	AS-PCR	(Tananchai *et al.*, 2012)
Mae Hong Son	Mae Sariang	18°15′N97°53′E	ITS2 sequences	(Walton *et al.*, 2007)
	Mueang	19°28′N98°12′E		
	Mueang	19°6′N97°53'E		
Chiang Mai	Chom Thong	18°35′N98°29′E		
	Phrao	19°21′N99°12′E		
Loei	Mueang	17°29′N101°43′E		
Sakon Nakhon	Tao Ngoi	17°29′N101°43′E		
Kanchanaburi	Sai Yok	16°59′N104°10′E		
Prachuap Khiri Khan	Hua Hin	12°34′N99°57′E		
Chumphon	Ban Noi Chok Kwa	10°29′N99°10′E		
Ranong	Ban Hing Chang	9°58′N98°37′E		
Songkhla	Sadao	6°39′N100°19′E		
Trat	Ko Chang	12°2'N102°23'E	AS-PCR	(Ritthison *et al.*, 2014a)

Table20 *Anopheles sawadwongporni* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Phrae	Rong Kwang	18°20′N100°19′E	Cytogenetic	(Baimai *et al.*, 1993)
Chiang Mai	Mae Taeng	19°11′N98°52′E	Polytene chromosomes	(Green *et al.*, 1985)
Mae Hong Son	NA	19°12′N97°54′E		
Phrae	Rong Kwang	18°26′N100°24′E		
Kanchanaburi	Wat Pratart	14°25′N99°7′E		
	Sam Larng	14°40′N99°21′E		
Nakhon Nayok	NA	14°19′N101°18′E		
Mae Hong Son	Mae Sariang	18°15′N97°53′E	ITS2 sequences	(Walton *et al.*, 2007)
	Mueang	19°18′N97°58′E		
	Mueang	19°6′N97°53'E		
Chiang Mai	Phrao	19°21′N99°12′E		
Lampang	Thoen	17°36′N99°1′E		
Lamphun	Thung Hua Chang	18°6′N98°53′E		
Prachuap Khiri Khan	Hua Hin	12°31′N99°32′E		
Loei	Mueang	17°29′N101°43′E	ITS2 sequences	(Walton *et al.*, 2007)
Kanchanaburi	Sai Yok	14°17 ′N99°11′E	AS-PCR	(Tananchai *et al.*, 2012)
Trat	Ko Chang	12°2'N102°23'E	AS-PCR	(Ritthison *et al.*, 2014a)

Table21 *Anopheles pseudowillmori* in Thailand

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Mae Hong Son	Mae Sariang	18°9′N97°56′E	Polytene chromosome and/or mitotic chromosomes of their larval (4th stage) progeny	(Baimai *et al.*, 1993)

Table 22 *Anopheles willmori* in Thailand

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chiang Mai	Mae Rim	18°54′N98°56′E	Polytene chromosome and/or mitotic chromosomes of their larval (4th stage) progeny	(Baimai *et al.*, 1993)

Table 23 *Anopheles dravidicus* in Thailand

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Kanchanaburi	Wat Pratart Sam Larng	14°25′N99°7′E 14°40′N99°21′E	Polytene chromosomes	(Green *et al.*, 1985)
Chiang Mai	Mae Taeng	19°11′N98°52′E		
Phetchabun	Nam Nao	16°46′N101°40′E	Polytene chromosome and/or mitotic chromosomes of their larval (4th stage) progeny	(Baimai *et al.*, 1993)
Mae Hong Son	Mueang	19°6′N97°53′E	ITS2 sequences	(Walton *et al.*, 2007)

Table 24 *Anopheles notanandai* in Thailand

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Phetchaburi	Nong Ya Plong	13°9′N99°41′ E	Polytene chromosome and/or mitotic chromosomes of their larval (4th stage) progeny	(Baimai *et al.*, 1993)

Table 25 *Anopheles rampae* in Thailand

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Ubon Ratchathani	Na Chaluai	14°31′N105°14′E	ITS2 sequences	(Walton *et al.*, 2007)
Udon Thani	Ban Phue	17°41′N102°28′E	Polytene chromosome	(Baimai *et al.*, 1993)
Ubon Ratchathani	Sri Muang Mai	15°29′N105°16′E	and/or mitotic chromosomes of their larval (4^{th} stage) progeny	

1.4 Sundaicus Complex (*Anopheles epiroticus*)

The SundaicusComplex is commonly found near brackish water habitats. The possible existence of a sibling species complex was first reported by Sukowati and Baimai (1996) based on chromosomal variations among populations in Thailand and Indonesia. The three cytological forms, A, B and C, differing in their polytene chromosome complement and heterochromatic variation in their Y-chromosomes, were described. In Trat and Phangnga Provinces, two areas in southern Thailand, only form A was found. In 1999, the isoenzyme *Mpi* locus variations were used to differentiate the three putative species (Sukowati *et al.*, 1999) in the Sundaicus Complex. Dusfour *et al.* (2004a) later analyzed 6 populations of*An. sundaicus* s.l. from two sites in Sarawak, Malaysia (Borneo), two in southern Thailand and two in southern Vietnam, comparing the mitochondrial DNA sequences of partial regions of cytochrome b (cyt-b) and cytochrome oxidase I (COI). Theirresults showed the presence of two sibling species including *An. sundaicus* s.s. from Sarawak, Malaysia and *An. sundaicus* species A from Vietnam and Thailand. *Anopheles sundaicus* species A was subsequently described and named *An. epiroticus* Linton & Harbach

(Linton *et al.*, 2005), based on ITS2, cyt-b and COI gene sequences; however there were no reliable morphological characters that could definitively distinguish adult, larval and pupal stages between *An.sundaicus* and *An. epiroticus*. Dusfour *et al.* (2004b) developed a multiplex PCR assay consisting of four species-specific SCAR (sequence characterized amplified region) primers derived from random amplified polymorphic regions (cyt-b and COII). Lately, Ritthison *et al*. (2014a; 2014b) reported that *An. epiroticus* was a common species in coastal areas of eastern and southern Thailand (Figure 9A, Table 26). Another recent work on the Sundaicus Complex also showed eight genetic and geographical groupings and evidence for recent gene flow among them (Zarowiecki *et al.*, 2014). Therefore, more work has to be done on this important species complex involved in malaria transmission along Asian coastal zones.

Table 26 *Anopheles epiroticus* in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Trat Pattani Phangnga	NA	12°14'N102°30'E 6°52'N101°15'E 8°26'N98°32'E	ITS2, cyt-b and COI gene sequences	(Linton *et al.*, 2005)
Rayong	Mueang	12°40′N101°16′E	COI, ITS2, D3 PCR amplification	(Sumruayphol *et al.*, 2010)
Trat	Ko Chang	12°2'N102°23'E	AS-PCR	(Ritthison *et al.*, 2014a)
Songkhla Surat Thani Phangnga	Hat Yai Chaiya Takua Pa	7°46′N100°28′E 9°24′N99°18′E 8°43′N98°14′E	AS-PCR	(Ritthison *et al.*, 2014b)

1.5 Barbirostris Subgroup *(Anopheles campestris*-like)

The Barbirostris Subgroup (Subgenus *Anopheles*, Myzorhynchus Series) in Thailand includes *Anopheles barbirostris*, *Anopheles campestris*, *Anopheles donaldi* Reid, *Anopheles hodgkini* Reid and *Anopheles pollicaris* Reid. Recently, at least five sibling species have been discovered within the *An. barbirostris*/ *campestris* group, i.e., *An. campestris*-like and *An. barbirostris* species A1, A2, A3 and A4. This grouping of isomorphic species was identified based on cytogenetic and molecular evidence and crossing experiments (Saeung *et al.*, 2007; Saeung *et al.*, 2008; Suwannamit *et al.*, 2009;Thongsahuan *et al.*, 2009). Previously, anthropophilic species within the *An. barbirostris/ campestris* complex have been incriminated as potential natural vectors of *P. vivax* in the Aranyaprathet District, Sa Kaeo Province, Thailand (Limrat *et al.*, 2001), Sangkhlaburi District, Kanchanaburi Province (Coleman *et al*., 2002) and Chantaburi Province (Sriwichai *et al.*, 2014), Thailand. The distribution map of *Anopheles campestris*–like species (Figure 9B, Table 27) indicates that this species can be found scattered across much of the country.

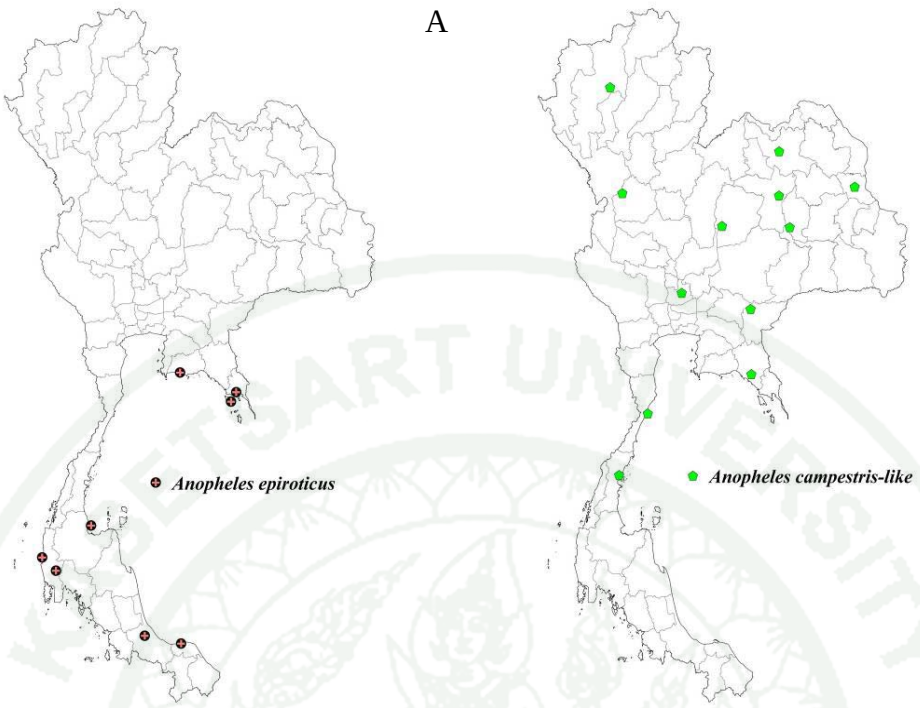

Figure 9 Known geographic distribution of *Anopheles epiroticus* (A) and *Anopheles campestris*-like species (B) in Thailand based on molecular species identification

Table 27 *Anopheles campestris*-like species in Thailand (NA= Not applicable)

Province	District	Geographic coordinates (DMS)	Species identification methodology	Published sources
Chiang Mai	NA	18º47′N98º59′E	DNA sequencing of the ITS2, COI and COII regions	(Thongsahuan *et al.*, 2009)
Kamphaeng Phet		16º50′N99º04′E		
Phra Nakhon Si Ayutthaya		14º01′N101º02′E		
Udon Thani		17º24′N102º47′E		
Khon Kaen		15º41′N101º45′E		
Maha Sarakham		15º45′N103º1′E		
Mukdahan		15º24′N103º16′E		
Chaiyaphum		15º48′N101º30′E		
Sa Kaeo		13º14′ N101º51′E		
Chanthaburi		12º37′N102º7’E		
Prachuap KhiriKhan		11º48′N99º49′E		
Chumphon		10º29′N99º11′E		

Part 2. Trophic behavior, biting activity, seasonal abundance, and parasite infection of individual sibling species by molecular identification, in Mae Sot (Tak Province) and Sop Moei (Mae Hong Son Province), northwestern Thailand.

2.1. Species composition and abundance

A total of 7,129 *Anopheles* females were collected during the two-year sampling period of February 2011 to January 2013. This sample included 22 species in the subgenus *Cellia* (6,156 specimens), and a single species and 3 groups in the subgenus *Anopheles* (n=973) (Table 28). Of 5,148 specimens within the two complexes and the Maculatus Group identified by molecular assays, 3,278 were collected in Mae Sot and 1,870 in Sop Moei field sites. The Minimus Complex and related species included *An. minimus*, *An. aconitus* and *An. varuna*; the Dirus Complex was composed of *An. dirus* and *An. baimaii*; and the Maculatus Group was represented by *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus* (Figure 10). Based on both human landing and cattle baited collections, the most abundant species, within the subgenus *Cellia* in Mae Sot and Sop Moei, was *An. minimus* accounting for 58.6% and 37.0% of all recorded anophelines, respectively and 49.6% of the total sample from both sites combined (Table 28). *Anopheles maculatus* represented 25.4% and 20.4% of the total anopheline sample by site in Mae Sot and Sop Moei, respectively, and 23.3% of total for both sites. In both sites, *An. dirus* and *An. baimaii* were rarely collected, 0.5% and 0.6%, respectively in Mae Sot and 0.4% for both species in Sop Moei. The other *Anopheles* taxa in the subgenus *Cellia* included *An. annularis* s.l., *An. culicifacies* s.l. (species B), *An. jamesii*, *An. jeyporiensis*, *An. karwari*, *An. kochi* s.l., *An. nivipes*, *An. philippinensis*, *An. splendidus*, *An. stephensi*, *An. subpictus*, *An. tessellatus* and *An. vagus*, all of which were identified by morphological criteria only. In the subgenus *Anopheles*, a species belonging to the *Anopheles hyrcanus* Group was the most prevalent with 46.7% and 45.0% collected in Mae Sot and Sop Moei, respectively and representing 45.6% of the total sample in the subgenus, followed by the *Anopheles barbirostris* Group (36.0% in Mae Sot, 21.9% in Sop Moei), for a total of 27% of collections for

both sites. In addition, specimens of the *Anopheles umbrosus* Group and *Anopheles peditaeniatus* were also found in both study sites.

Table 28 Numbers of *Anopheles* collected by species and collection methods divided by subgenus in Mae Sot and Sop Moei from February 2011 to December 2012

Species	Mae Sot (%)		Sop Moei (%)		Total
Anopheles (*Cellia)*	Human	Cattle	Human	Cattle	(%)
An. minimus	1,130 (31.5)	973 (27.1)	394 (15.3)	557 (21.7)	3,054 (49.6)
An. aconitus	-	16 (0.4)	3 (0.1)	16 (0.6)	35 (0.6)
An. varuna	-	60 (1.7)	1	11 (0.4)	72 (1.2)
An. dirus	17 (0.5)	1	5 (0.2)	6 (0.2)	29 (0.5)
An. baimaii	21 (0.6)	0	6 (0.2)	5 (0.2)	32 (0.5)
An. maculatus	454 (12.7)	457 (12.7)	89 (3.5)	436 (16.9)	1,436 (23.3)
An. sawadwongporni	72 (2.0)	44 (1.2)	36 (1.4)	238 (9.3)	390 (6.3)
An. pseudowillmori	0	4 (0.1)	39 (1.5)	12 (0.5)	55 (0.9)
An. dravidicus	10 (0.3)	19 (0.5)	1	15 (0.6)	45 (0.7)
An. annularis s.l.	0	0	0	8 (0.3)	8 (0.1)
An. culicifacies s.l.	0	2 (0.1)	0	83(3.2)	85 (1.4)
An. jamesii	3(0.1)	75 (2.1)	2 (0.1)	87 (3.4)	167 (2.7)
An. jeyporiensis	0	0	1	1	2 (0.0)
An. karwari	3 (0.1)	0	2 (0.1)	9 (0.4)	14 (0.2)
An. kochi s.l.	7 (0.2)	86 (2.4)	0	316 (12.3)	409 (6.6)
An. nivipes	1	8 (0.2)	0	6 (0.2)	15 (0.2)
An. philippinensis	3(0.1)	69 (1.9)	1	64 (2.5)	137 (2.2)
An. splendidus	0	2 (0.1)	1	1	4 (0.1)
An. stephensi	0	1	0	1	2 (0.0)
An. subpictus	0	1	0	9 (0.4)	10 (0.2)
An. tessellatus	0	0	7 (0.3)	31(1.2)	38 (0.6)
An. vagus	3 (0.1)	44(1.2)	0	70 (2.7)	117 (1.9)
Total (s.g. *Cellia*)	3,586 (58.3%)		2,570 (41.7%)		6,156

Table 28 (Continued)

Species	Mae Sot (%)		Sop Moei (%)		Total (%)
Anopheles (*Anopheles*)	Human	Cattle	Human	Cattle	
An. barbirostris Group	5 (1.4)	122 (34.6)	0	136 (21.9)	263 (27.0)
An. umbrosus Group	0	42 (11.9)	1 (0.2)	133 (21.5)	176 (18.1)
An. hyrcanus Group	29 (8.2)	136 (38.5)	14 (2.3)	265 (42.7)	444 (45.6)
An. peditaeniatus	1 (0.3)	78 (22.1)	0	71 (11.5)	90 (9.2)
Total (s.g. *Anopheles*)	353 (36.3%)		620 (63.7%)		973
Grand total	3,939 (55.3%)		3,190 (44.7%)		7,129

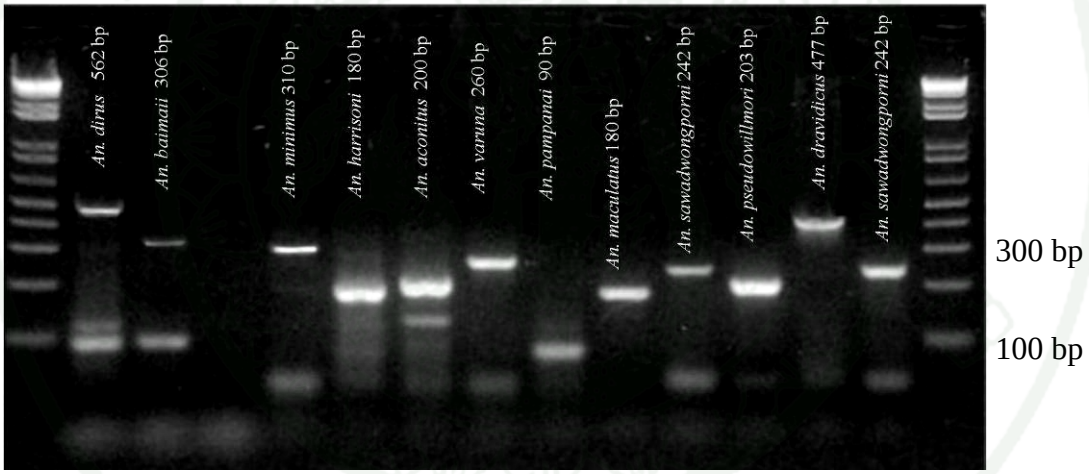

Figure 10 The amplification products from a single PCR reaction mix in 2.5% agarose gel under ultraviolet light illumination showing the species members of the Dirus,and Minimus Complexes and the Maculatus Group

2.2 Mosquito density and feeding behavior

2.2.1 Minimus Complex and related species

The monthly density of *An. minimus* and two closely related species, *An. aconitus* and *An. varuna,* collected from the two sites during the two years of collection is provided in Tables 29-30. *Anopheles minimus* was found throughout the year with the highest density during the 'summer' period, between February and April, in both sites and years. Comparatively, *An. aconitus*, and *An. varuna* were collected in much smaller densities in Mae Sot and Sop Moei, varying from 0.7% to 1.9% for *An. aconitus,* and from 2.7% to 1.2% for *An. varuna*, respectively. Both species showed a high zoophilic behavior as all were collected on cattle-baited traps in Mae Sot, and more than 80% were recorded on cattle in Sop Moei. Indoor and outdoor feeding behaviors of *An. minimus* in Mae Sot were quite similar with biting peak activity occurring between 21:00 and 23:00 hrs. In contrast, indoor and outdoor feeding behaviors of *An. minimus* from Sop Moei were markedly different with the outdoor peak between 02:00 and 04:00 hrs and theindoor peak after 05:00 hrs (Figures 11-12).

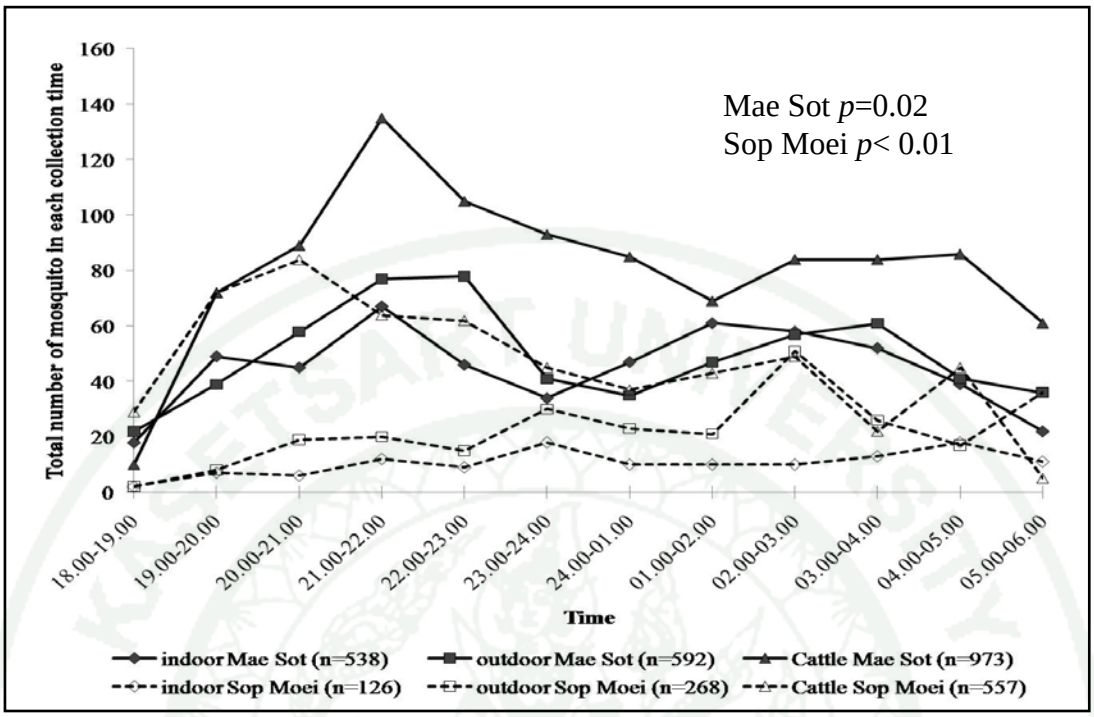

Figure 11 Total number of *An. minimus*specimens caught and statistical analysis between human landing and cattle baited collections

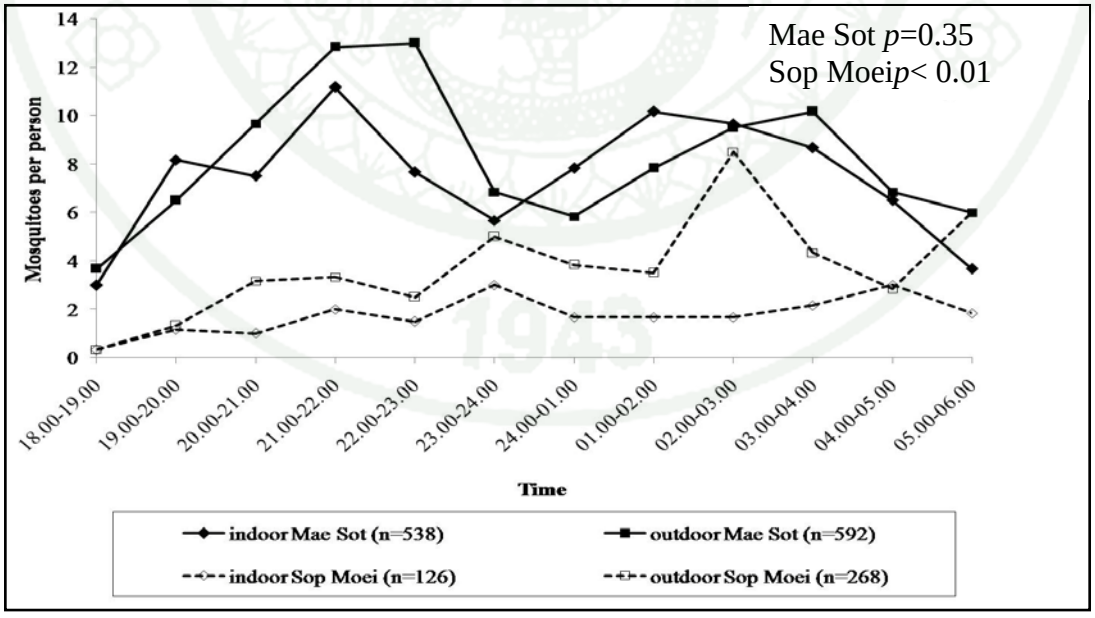

Figure 12 Mean hourly indoor and outdoor human-landing rates for *Anopheles minimus* in Mae Sot and Sop Moei

Table 29 Number of species members of the Minimus Complex and related species for 12 months collection from Mae Sot (I= indoor human landing, O=outdoor human landing, C= cattle baited)

Month*	Mae Sot								
	An. minimus			*An. aconitus*			*An. varuna*		
	I	O	C	I	O	C	I	O	C
Feb-11	134	128	207	-	-	3	-	-	24
Apr-11	121	111	220	-	-	3	-	-	33
Jun-11	65	106	30	-	-	-	-	-	-
Aug-11	-	5	2	-	-	-	-	-	-
Oct-11	23	26	33	-	-	-	-	-	2
Dec-11	30	36	61	-	-	-	-	-	-
Feb-12	41	31	111	-	-	-	-	-	-
Apr-12	59	79	183	-	-	2	-	-	-
Jun-12	38	37	78	-	-	1	-	-	1
Aug-12	-	3	10	-	-	-	-	-	-
Oct-12	16	6	21	-	-	1	-	-	-
Dec-12	11	24	17	-	-	6	-	-	-
Total	538	592	973	-	-	16	-	-	60

*****(Three seasons: Summer=February- May, Rainy=May-October, Dry=November-January)

Table 30 Number of species members of the Minimus Complex and related species for 12 months collection from Sop Moei (I= indoor human landing, O=outdoor human landing, C= cattle baited)

Month*	Sop Moei								
	An. minimus			*An. aconitus*			*An. varuna*		
	I	O	C	I	O	C	I	O	C
Feb-11	8	40	24	-	-	2	-	-	3
Apr-11	21	20	108	-	-	3	-	-	7
Jun-11	6	15	38	-	3	-	1	-	-
Aug-11	-	8	10	-	-	-	-	-	-
Oct-11	2	2	5	-	-	-	-	-	-
Dec-11	-	-	95	-	-	-	-	-	-
Feb-12	7	49	117	-	-	1	-	-	-
Apr-12	21	20	55	-	-	-	-	-	-
Jun-12	48	87	10	-	-	-	-	-	1
Aug-12	1	9	5	-	-	-	-	-	-
Oct-12	4	10	12	-	-	8	-	-	-
Dec-12	8	8	78	-	-	2	-	-	-
Total	126	268	557	-	3	16	1	-	11

*****(Three seasons: Summer=February- May, Rainy=May-October, Dry=November-January)

2.2.2 Dirus Complex

The monthly densities of *An. dirus* and *An. baimaii* collected from both study sites are provided in Table 31. Both species were found in quite similar proportions, respectively 18 and 21 specimens in Mae Sot and 11 specimens for each species in Sop Moei. Nearly twice as much specimens were captured in Mae Sot (n=39) compared to Sop Moei (n=22). For both years,the highest numbers of individuals were encountered in June. In Mae Sot, *An. dirus* and *An. baimaii* have a greater preference / attraction to humans with 97% (n=38) collected on human-bait compared to cattle. The indoor: outdoor (9:8) ratios were near identical (albeit overall numbers were small) for *An. dirus* (n=17), while *An. baimaii* was collected in greater number outdoors (n=16) than indoors (n=5) on humans. In Sop Moei, neither species showed any obvious anthropophilic behavior. Both species were collected as frequently on humans (n=11) as on cattle (n=11). In Mae Sot, *An. dirus* presented an early peak of biting activity indoors (19:00-20:00 hrs) and a later peak outdoors (23:00-midnight) (Figure 13). In Sop Moei, the numbers were too low to clearly define the precise biting pattern. For *An. baimaii*, outdoor and indoor collections showed nearly identical biting peaks from 20:00 to 22:00 hrs, with a second peak for outdoor collections (04:00-05:00). In Sop Moei, the biting (landing) peak activity was from 01:00 to 02:00 (Figure 14). Neither species was found in great abundance so that drawing any definitive conclusions regards these data should be done with caution.

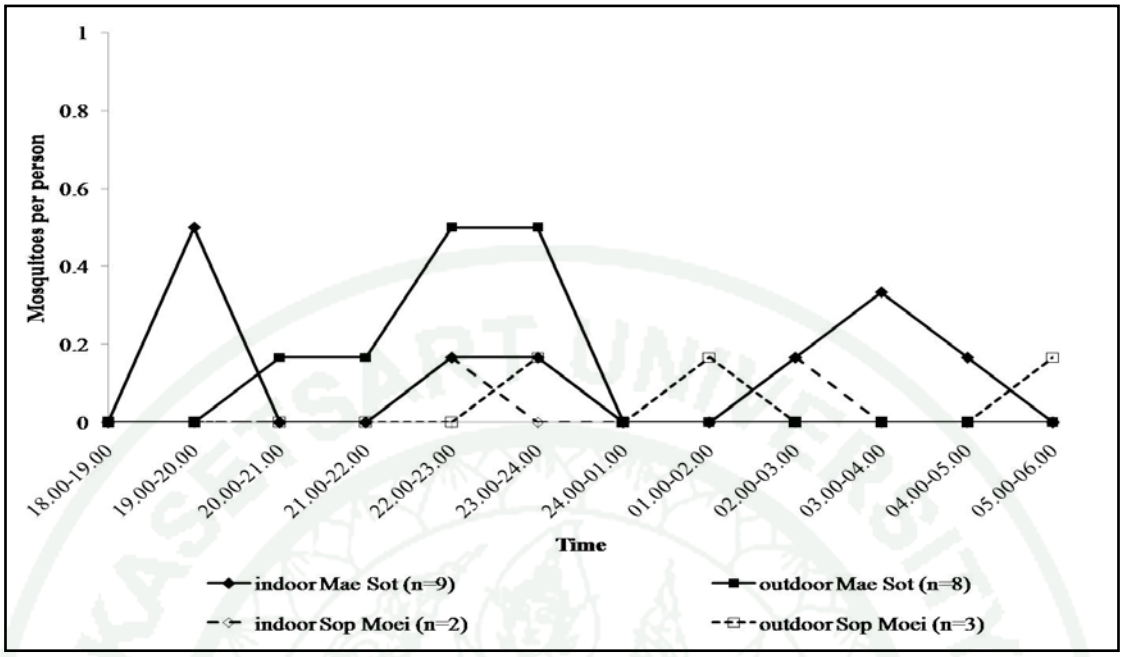

Figure 13 Mean hourly indoor and outdoor human-landing rates for *Anopheles dirus* in Mae Sot and Sop Moei

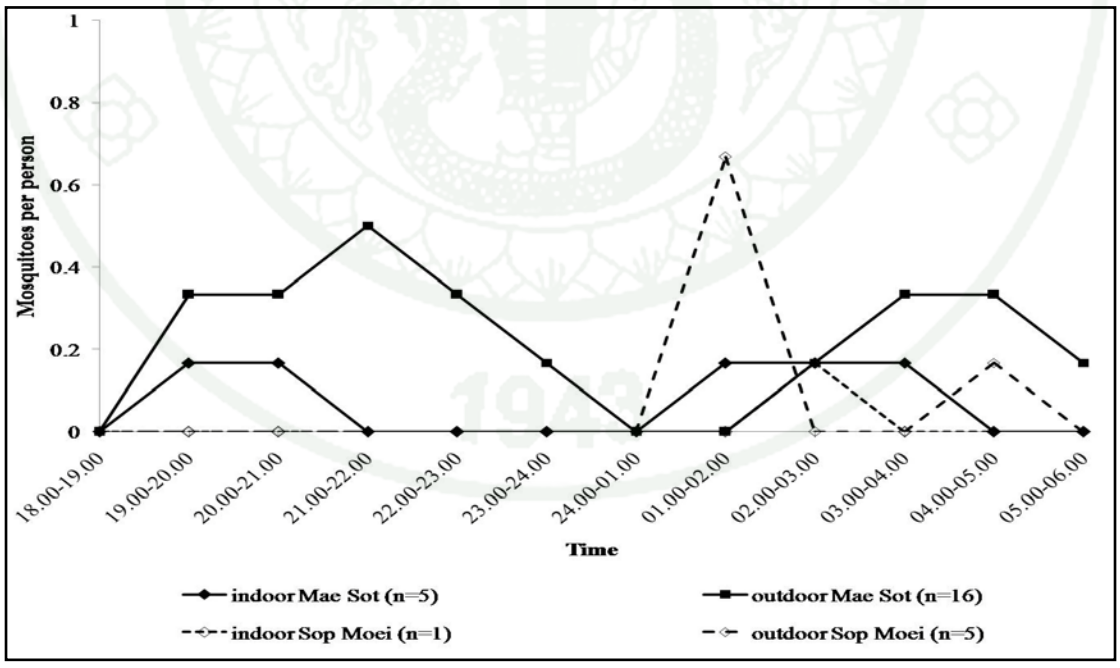

Figure 14 Mean hourly indoor and outdoor human-landing rates for *Anopheles baimaii* in Mae Sot and Sop Moei

Table 31 Number of species members of the DirusComplex for 12 months collection from Mae Sot and Sop Moei (I= indoor human landing, O=outdoor human landing, C= cattle baited)

Month*	Mae Sot						Sop Moei					
	An. dirus			*An. baimaii*			*An. dirus*			*An. baimaii*		
	I	O	C	I	O	C	I	O	C	I	O	C
Feb-11	-	-	-	-	-	-	-	-	-	-	-	-
Apr-11	1	1	-	-	1	-	-	1	-	-	-	-
Jun-11	6	7	1	2	2	-	1	-	1	1	1	2
Aug-11	-	-	-	-	2	-	-	-	-	-	-	-
Oct-11	-	-	-	-	-	-	-	1	1	-	-	-
Dec-11	-	-	-	-	1	-	-	-	-	-	-	-
Feb-12	-	-	-	-	-	-	-	-	-	-	-	-
Apr-12	-	-	-	-	-	-	-	-	-	-	-	-
Jun-12	1	-	-	-	5	-	1	1	2	-	4	2
Aug-12	-	-	-	3	1	-	-	-	-	-	-	-
Oct-12	1	-	-	-	4	-	-	-	2	-	-	-
Dec-12	-	-	-	-	-	-	-	-	-	-	-	1
Total	9	8	1	5	16	-	2	3	6	1	5	5

*****(Three seasons: Summer=February- May, Rainy=May-October, Dry=November-January)

2.2.3 Maculatus Group

Four species belonging to the Maculatus Group were identified in Mae Sot and Sop Moei: *An. maculatus, An. sawadwongporni, An. pseudowillmori* and *An. dravidicus* (Tables32-33). Collectively, the group was found throughout the year with the highest density during the rainy period between May and October in both sites. *Anopheles maculatus* was the most abundant and consistent species present in both sites (n=1,436) representing 74.6% of the species group, followed by *An. sawadwongporni* (n=390) with 20.2%. *Anopheles pseudowillmori* and *An. dravidicus* were captured in much smaller proportions, 2.9% and 2.3% of the total collections, respectively, and having periods (months) of little or no captures recorded. In general, all Maculatus Group members were collected in greater numbers from cow than human, except for *An. sawadwongporni* in Mae Sot and *An. pseudowillmori* in Sop Moei where higher numbers in outdoor HLC were seen (Tables 32-33). In Mae Sot, the biting activity on cattle peaked between 22:00 to 23:00 hrs, while in Sop Moei it was between 19.00 to 20.00 hrs (Figure 15). The activity of *An. maculatus* in Mae Sot showed a very clear early biting peak, between 18:00 and 21:00 hrs, for outdoor HLC (Figure 16).

The biting activity of *An. sawadwongporni* from outdoor HLC in both villages showed a small peak between 19:00 to 20:00 (Figure 17), the same for *An. pseudowillmori* that bited as early as 19.00 to 20.00 in Mae Sot (Figure 18). Like the Dirus Complex, neither species was found in great abundance so that drawing any definitive conclusions regards these data should be done with caution.

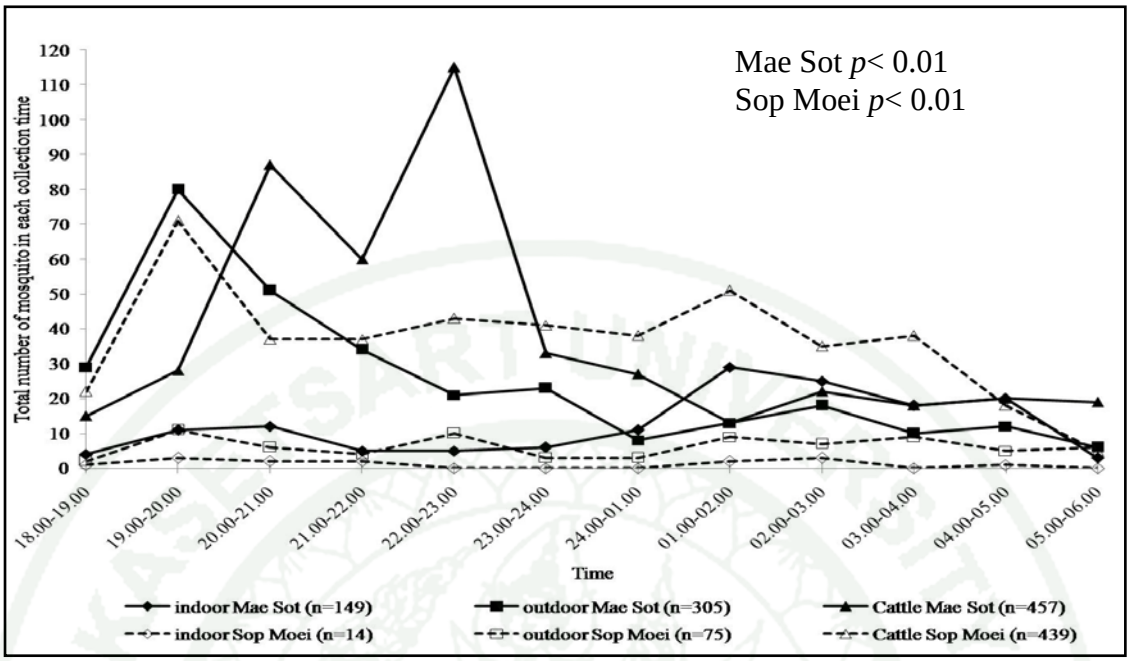

Figure 15 Total number of *An. maculatus* specimens caught and statistical analysis between human landing and cattle baited collections

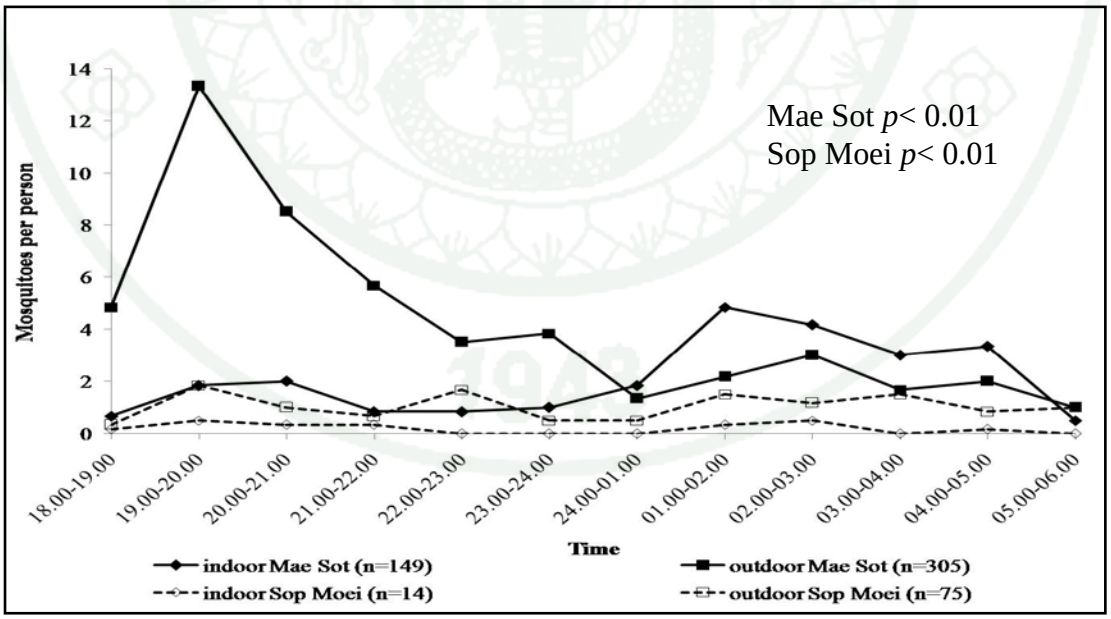

Figure 16 Mean hourly indoor and outdoor human-landing rates for *Anopheles maculatus* in Mae Sot and Sop Moei

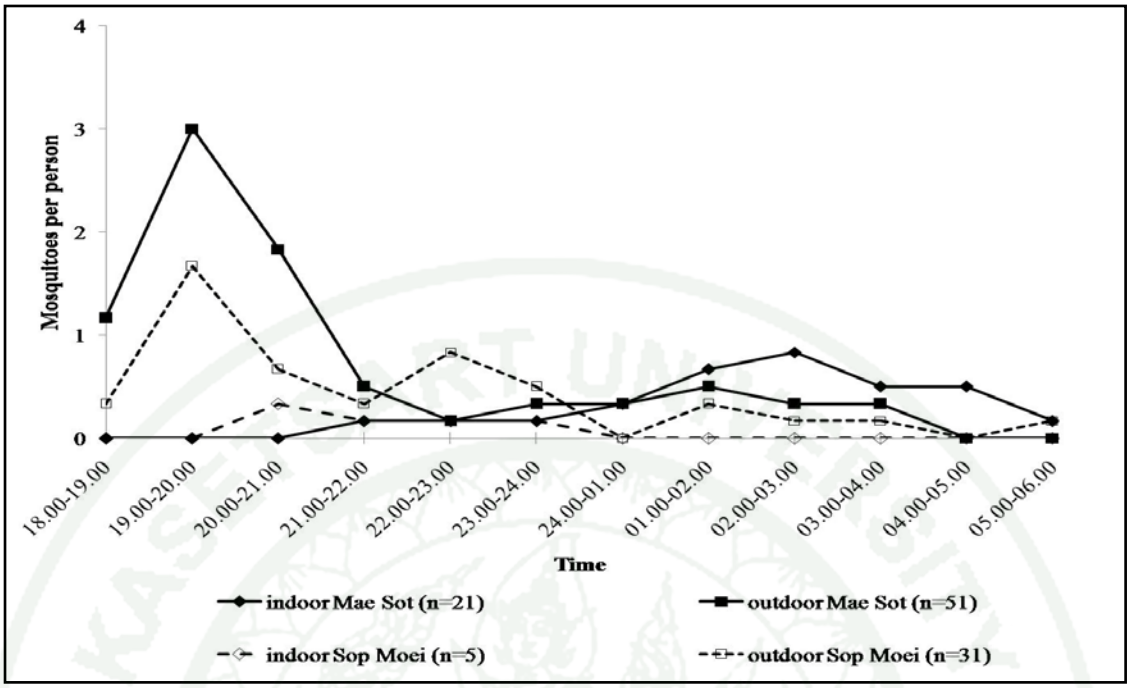

Figure 17 Mean hourly indoor and outdoor human-landing rates for *Anopheles sawadwongporni* in Mae Sot and Sop Moei

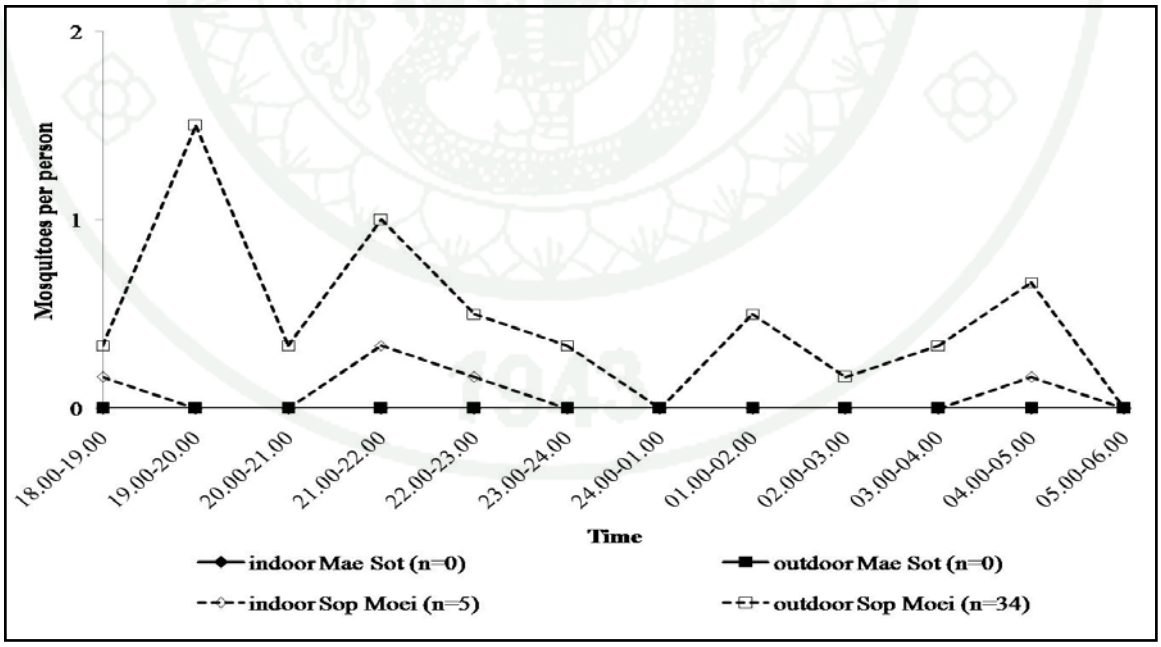

Figure 18 Mean hourly indoor and outdoor human-landing rates for *Anopheles pseudowillmori* in Mae Sot and Sop Moei

Table 32 Number of species members of the MaculatusGroup for 12 months collection from Mae Sot (I= indoor human landing, O=outdoor human landing, C= cattle baited)

Month*	Mae Sot											
	An. maculatus			*An. sawadwongporni*			*An. pseudowillmori*			*An. dravidicus*		
	I	O	C	I	O	C	I	O	C	I	O	C
Feb-11	1	6	10	2	6	14	-	-	-	-	3	-
Apr-11	3	3	21	-	2	15	-	-	4	-	3	17
Jun-11	9	53	39	1	3	12	-	-	-	-	2	2
Aug-11	3	11	-	-	2	-	-	-	-	-	-	-
Oct-11	26	32	193	7	7	-	-	-	-	1	-	-
Dec-11	20	20	38	-	-	-	-	-	-	-	-	-
Feb-12	8	8	-	-	1	-	-	-	-	1	-	-
Apr-12	4	4	-	1	4	-	-	-	-	-	-	-
Jun-12	17	20	114	-	2	2	-	-	-	-	-	-
Aug-12	5	25	42	-	3	1	-	-	-	-	-	-
Oct-12	23	45	-	1	6	-	-	-	-	-	-	-
Dec-12	30	78	-	9	15	-	-	-	-	-	-	-
Total	149	305	457	21	51	44	-	-	4	2	8	19

*****(Three seasons: Summer=February- May, Rainy=May-October, Dry=November-January)

Table 33 Number of species members of the MaculatusGroup for 12 months collection from Sop Moei (I= indoor human landing, O=outdoor human landing, C= cattle baited)

Month*	Sop Moei											
	An. maculatus			*An. sawadwongporni*			*An. pseudowillmori*			*An. dravidicus*		
	I	O	C	I	O	C	I	O	C	I	O	C
Feb-11	-	1	8	-	-	24	-	-	1	-	-	-
Apr-11	-	2	6	-	-	42	-	-	6	-	-	15
Jun-11	2	6	20	2	14	122	-	-	-	-	-	-
Aug-11	-	-	4	1	-	11	-	-	-	-	-	-
Oct-11	2	4	65	-	1	4	-	-	3	-	-	-
Dec-11	3	6	36	-	-	-	-	-	-	-	-	-
Feb-12	-	-	-	-	3	-	-	4	-	-	-	-
Apr-12	2	-	-	2	2	-	-	-	-	-	1	-
Jun-12	3	50	149	-	4	16	-	2	-	-	-	-
Aug-12	-	2	27	-	2	19	-	-	2	-	-	-
Oct-12	-	2	-	-	2	-	4	25	-	-	-	-
Dec-12	2	2	121	-	3	-	1	3	-	-	-	-
Total	14	75	436	5	31	238	5	34	12	-	1	15

*****(Three seasons: Summer=February- May, Rainy=May-October, Dry=November-January)

2.3 Trophic behavior

The non-parametric Wilcoxon analysis was used to compare the hourly landing rates of the two most abundant mosquito species, *An. minimus* and *An. maculatus*, using different collection methods and study locations. For *An. minimus*, no significant difference (Z=-0.94, p=0.348) was observed in Mae Sot between the indoor (3.76±0.3) and outdoor (4.11±0.41) human landing. However, *An. minimus* showed a significantly higher (Z=-2.43, p=0.02) anthropophilic (7.85±0.72) behavior compared to its attraction to cattle (6.76±0.72) (Table 31). In Sop Moei, *An. minimus* was significantly (p<0.01) more exophagic (Z=-5.72, p<0.01) and zoophilic (Z=-3.99, p<0.01) (Table 34). *An. maculatus* showed highly significant exophagic and zoophilic behavioral predilections in Mae Sot, while in Sop Moei its behavior washighly endophagic (Table 35).

2.4 Relationship of caught mosquito number and environmental condition

The Peason‘s correlation data between environmental variables (temperature and relative humidity) and number of *An. minimus* (Table 34) and *An. maculatus* (Table 35) were analyzed. Non-parametric analysis of densities of *Anopheles minimus* and *An. maculatus* in different collection sites, between the numbers of mosquitoes collected from indoor / outdoor human-baited collections and environmental data (temperature, relative humidity (RH) and rainfall), found no statistical association except for *An. maculatus* in Sop Moei regards adult densities and variations in mean ambient temperature.

Table 34 Non-parametric analysis for comparison of densities of *Anopheles minimus* in different collection sites and techniques deriving a correlation coefficient (*r*) between the numbers of mosquitoes collected from human-baited collections and environmental data (temperature, relative humidity (RH) and rainfall)

Location	Baited	Mean (SE)	Wilcoxon statistic	Mean Temp (ºC)	Mean RH (%)	Mean Rainfall
Mae Sot	Indoor	3.76(0.39)	*Z*=-0.94, *p*=0.348	21.61(0.59) *r*=0.74, *p*=0.37	79.85(2.14) *r*=0.37, *p*=0.66	3.87 (1.74) *r*=-0.12, *p*=0.35
	Outdoor	4.11(0.41)				
	Human	7.85(0.72)	*Z*=-2.43, *p*=0.02*			
	Cow	6.76(0.72)				
Sop Moei	Indoor	0.88(0.15)	*Z*=-5.72, *p*<0.01**	21.09(0.82) *r*=0.31, *p*=0.72	72.26(2.91) *r*=0.03, *p*=0.69	12.42(3.31) *r*=0.17, *p*=0.30
	Outdoor	3.57(0.40)				
	Human	2.74(0.33)	*Z*=-3.99, *p*<0.01**			
	Cow	5.80(0.57)				

Significant differences: * $p<0.05$; ** $p<0.01$

Table 35 Non-parametric analysis for comparison of the densities of *Anopheles maculatus* in different collection sites and techniques deriving a correlation coefficient (*r*) between the numbers of mosquitoes collected from human - baited collections and environmental data (temperature, relative humidity(RH) and rainfall)

Location	Baited	Mean (SE)	Wilcoxon statistic	Mean Temp (ºC)	Mean RH(%)	Mean Rainfall
Mae Sot	Indoor	1.03(0.13)	*Z*= -3.59, *p*<0.01**	21.61(0.59) *r*=0.13, *p*=0.13	79.85(2.14) *r*=0.08, *p*=0.34	3.87(1.74) *r*=-0.22, *p*=0.25
	Outdoor	2.12(0.28)				
	Human	3.15(0.32)	*Z*= -3.95, *p*<0.01**			
	Cow	5.19 (0.59)				
Sop Moei	Indoor	0.10(0.03)	*Z*= -3.62, *p*<0.01**	21.09(0.82) *r*=0.87, *p*<0.01**	72.26(2.91) *r*=0.15, *p*=0.08	3.87 (1.74) *r*= -0.43, *p*= 0.08
	Outdoor	0.05 (0.12)				
	Human	0.62(0.13)	*Z*= -6.40, *p*<0.01**			
	Cow	3.03(0.47)				

Significant differences: * *p*<0.05; ** *p*<0.01

2.5 Parasite detection

Among the *Anopheles* (*Cellia*) species from human landing collections in Mae Sot and Sop Moei, 1,447 specimens were assayed to determine the natural infectivity for three species of *Plasmodium* parasites: *Plasmodium falciparum, Plasmodium vivax, Plasmodium knowlesi* and one filarial parasite species (*Wuchereria bancrofti*). Eight *Anopheles* species from both collection sites in Mae Sot and Sop Moei were examined for parasite infection as follows: *An. minimus* (1,090, 132), *An. aconitus* (16, 10), *An. dirus* (18, 10), *An. baimaii* (14, 19), *An. maculatus* (55, 25), *An. sawadwongporni* (2, 39), *An. pseudowillmori* (2, 39)and *An. dravidicus* (5, 1), respectively. Only one specimen, *An. minimus* from Mae Sot from an outdoor HLC, collected between 00:00-01:00 h in April 2011, was found positive for *P. vivax* (Figure 19).

2.6 Entomological inoculation rates of *An.minimus*

Entomological inoculation rate (EIR) for *An. minimus* in the Mae Sot study site for the month of April 2011(only) was as follows:
April 2011 (HLC, indoor & outdoor) = 232 *An. minimus*
Sporozoite rate: 1 *P. vivax* / 232 tested = 0.00431, representing 0.43%

Human-landing (biting) density: 232 *An. minimus* / 4 collectors / 3 nights = 19.3 mosquitoes / person / night (1.6 mosquitoes / person/hr)
Adjusted to 60 min each hour = 24.125 mosquitoes / person / night

Therefore, the EIR for the month of April, 2011 is
24.125 x 0.00431 = 0.104 x 30 days = 3.12 infective bites / person / month.

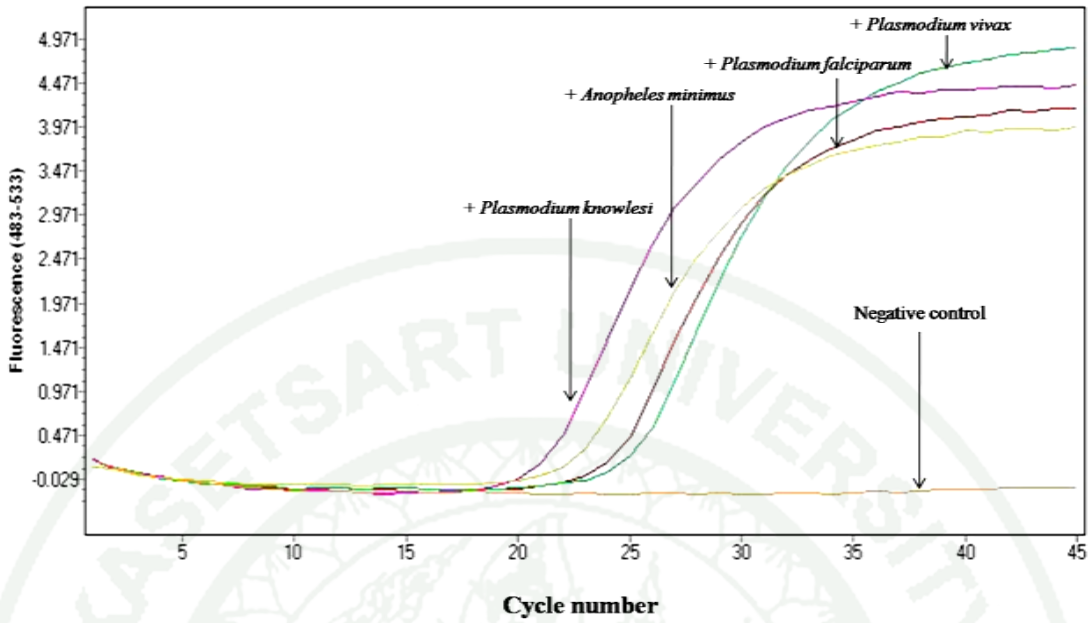

Figure 19 Detection of *Plasmodium* species by TaqMan Real-Time PCR assay. Plot of fluorescence readings against cycle number for *Plasmodium* spp. standard: *Plasmodium falciparum* DNA, *Plasmodium vivax* DNA (*Pv*) and *Plasmodium knowlesi* DNA, *Pv* positive mosquito DNA (*Anopheles minimus*) and negative control

This study represents the first detailed description on the diversity of *Anopheles* species based on a molecular identification in the two most malaria endemic areas of Thailand, Mae Sot District in Tak Province and Sop Moei District in Mae Hong Son Province, both located in the northwestern part of the country bordering the country of Myanmar. Results from this study provide accurate species identification as well as seasonal abundance and trophic behavior of *Anopheles minimus* and *Anopheles maculatus*, among the most important malaria vectors in northwestern Thailand.

In this study, six of the most important (primary and secondary) malaria vector species in Thailand were collected. During the 2 years collection, the most abundant species in Mae Sot and Sop Moei was *An. minimus* (42.8%), followed by *An. maculatus* (20.1%) and *An. sawadwongporni* (5.5%). Collectively, these species are responsible to varying degrees for maintaining malaria transmission in both areas which have been listed as 'A1' areas by the Thai Ministry of Public Health, areas where malaria occurs at least 6 months of the year,or longer, and regarded as epidemiologically 'high risk'(Chareonviriyaphap *et al.*, 2001; BVBD, 2013).

Anopheles minimus represented nearly 43% of the total *Anopheles* mosquitoes recorded from both areas during the two-year collection period, 53.4% in Mae Sot and 29.8% of all anophelines in Sop Moei. *Anopheles maculatus* and *An. sawadwongporni* were found in greater numbers compared to *An. pseudowillmori* and *An. dravidicus*, species within the Maculatus Group. These study findings are consistent with previous observations in Mae Sot District (Tum Sua Village) which found *An. minimus* by far the most abundant species (71%), followed by the *An. maculatus* Group (28%) and very few specimens of the *An. dirus* Complex (Tisgratog *et al.*, 2012). *Anopheles dirus* and *An. baimaii*, closely related and the most important forest-related malaria vectors in Thailand, were found in very low densities with only a total of 39 specimens collected in Mae Sot and 22 in Sop Moei during the two-year period. These two species were slightly more common during the June collection (wet season) both years with only a few specimens detected other the

months. The low numbers of the Dirus Complex collected complicate drawing any definitive conclusions on host seeking behavior in this study.

In Thailand, the biting activity and behavior of specific malaria vectors based on reliable molecular identification methods have been studied in details in relatively fewer instances compared to earlier studies that based all identification on morphological characters alone. For example, in 2006, two species within the Minimus Complex, *An. minimus* and *An. harrisoni* were studied from western Thailand using a molecular identification assay (Sungvornyothin *et al.*, 2006), and more recently, Kongmee *et al.* (2012) characterized the seasonal abundance, distribution and breeding sites of *An. minimus* and *An. harrisoni* from the same site in Sai Yok District, Kanchanaburi Province in western Thailand. Species diversity, biting activity and trophic behavior of the Minimus Complex were also observed previously in Mae Sot District (Tisgratog *et al.*, 2012) where the two closely related species, *An. minimus* and *An. aconitus* were collected. In this study, *An. varuna*, another genetically closely related species to *An. minimus*, was identified for the first time in both locations. Although *An. aconitus* and *An. varuna* are considered as low efficient vectors compared to *An. minimus*, the presence of these two species, especially *An. aconitus*, in Mae Sot and Sop Moei is of operational importance as these two species could possibly play a secondary, if not a primary role in malaria transmission (Scanlon *et al.*, 1968; Junkum *et al.*, 2007).

Tak Province is one of the most malaria-endemic area of Thailand, accounting for around 23% of the total malaria cases in the country in 2012 (5,199 cases) and 2013 (4,977 cases) (BVBD, 2013). Six important malaria vectors were identified in the study area using AS-PCR molecular assays. Our findings on the blood feeding habits of *An. minimus* in Mae Sot showed both zoophilic and anthropophilic behaviors with no strong preference for one host over the other which is similar to previous findings elsewhere in Mae Sot and Kanchanaburi (Sungvornyothin *et al.*, 2006; Tananchai *et al.*, 2012; Tisgratog *et al.*, 2012). In Sop Moei, *An. minimus* demonstrated a stronger zoophilic tendency (~2: 1) as a higher number of mosquitoes feed on cattle located near living structures. Generally, zoophilic behaviors are

considered less conductive for efficient malaria transmission as other animal hosts serve as alternative blood sources thereby reducing direct contact between vector and human and thus overall transmission risk. In Pu Teuy, Kanchanaburi Province, *An. minimus* and *An. harrisoni* are present, the latter species being predominant and with a distinct zoophilic behavior which is believed partly responsible for the typically low levels of malaria transmission in this area (Sungvornyothin *et al.*, 2006). *Anopheles minimus* in both study areas demonstrated variable endo / exophilic behaviors and host preference. Too few *An. dirus* and *An. baimaii* were collected to derive meaningful finding on host preference and feeding locations but what was observed appears in agreement with previous observations by Tananchai *et al.* (2012).

Only a few studies on species of the Maculatus Group in Thailand have been published (Green *et al.*, 1992; Walton *et al.*, 2007). Studies on this group have been hampered by previous lack of reliable tools to identify cryptic species, resulting in misidentification. From our study, four species within this group, *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus* were molecularly identified. The most common representative was *An. maculatus*, followed by *An. sawadwongporni*, both recognized malaria vectors in the southern Thailand (Rattanarithikul *et al.*, 1996b; Rattanarithikul *et al.*, 2006;Sinka *et al.*, 2011). This study showed that these two important putative vectors were present in both study locations in fair proportions, 20.1% (1,436/ 7,129) and 5.5%(390/ 7,129), compared to all other anophelines identified. The biology of these two malaria vectors, including trophic behavior, infection rate and parasite susceptibility (vector competence) deserves further study to determine their respective role, if any, in malaria transmission in northwestern Thailand.

Only one malaria parasite positive mosquito was detected during the dry season, accounting for a 0.092% (1/1,090) overall infection rate for *An. minimus*. This mosquito was collected from outdoor HLC from Mae Sot in the early part of the study (April 2011) during the wet season. The EIR for the month of April, 2011 is3.12 infective bites/ person/ month that is high risk of receiving potentially infected mosquito bites. Previous studies have reported the annual EIR of *An. dirus*, *An.*

minimus and *An. sundaicus*from Loas, Cambodia and Vietnam ranging from 0.6 to 1.00 (Trung *et al.*, 2004) and monthly EIR of *An. dirus*, *An. minius* and *An. sundaicus* from Loas ranging from 0.06 to 0.25 (Vythilingam *et al.*, 2003). Note that this is relatively high by most Southeast Asian standards but certainly not improbable. Our samples showed no evidence of malaria infection with *P. falciparum*, *P. malariae*, *P. knowlesi* or the filarial parasite, *W. bancrofti*. The vivax-positive *An. minimus* was captured during the normal malaria peak in Mae Sot in the late summer-wet months (April-May). The explanation for the low infection rate seen in mosquitoes tested is unclear, but the probability of finding an infection will have naturally decreased as the overall malaria incidence has been declining year after year in both provinces and likely where the study took place. For instance, there was a slight decline in reported malaria cases from 2012 to 2013 in Tak Province (0.98 to 0.93 per 1,000 population) and a 38% decline in Mae Hong Son Province (0.52 to 0.32/1000) (BVBD, 2013). This result is similar to the study by Trung *et al.* (2004) whom clearly explained that *An. minimus* is still efficient in transmitting malaria and that *An. minimus* should therefore be carefully followed-up, also in places where actual transmission is absent.

It is clear that environmental factors directly influence the distribution and behavior of the malaria vectors (Manguin *et al.*, 2008). For example, the *An. dirus* complex typically occurs in native forest type habitats but also has an ability to adapt to changing environmental conditions that allow it to successfully invade cultivated areas that simulate favored natural forest conditions. Each species within the Dirus Complex is dependent on forest cover heavy shade to some degree, and commonly occupies forest-fringe areas where it can interact with human populations to transmit malaria parasites. Habitats include natural dense forest (*An. dirus*), rubber plantations (*An. dirus* and *An. baimaii*) and secondary forest (*An. latens*) (Gingrich *et al.*, 1990; Rosenberg *et al.*, 1990; Suwonkerd *et al.*, 2002;Suwonkerd *et al.*, 2013). Vanwambeke *et al.* (2007a) found that land-use changes could selectively influence the diversity and likely density of mosquito species, thus having probably a direct impact on pathogen transmission.

Deforestation, the result of a wide variety of human activities, including land clearing for agricultural development, logging, population resettlement, road construction, mining and hydropower development, is one of the most potent factors either promoting or reducing infectious diseases like malaria and dengue in Southeast Asian, including Thailand (Guerra *et al.*, 2006; Vanwambeke *et al.*, 2007b; Yasuoka and Levins, 2007). Dramatic changes in environmental conditions are the direct result of modified land use, such as conversion of rice fields to rubber plantations or forest to urbanized zones. The extensive clearing of native and secondary forests has had enormous impacts on local ecosystems, in particular the critical microclimates of mosquitoes by reducing shade cover, changing the humidity regimen, and altering overall rainfall patterns (Reiter, 2001; Overgaard *et al.*, 2002). For example, anopheline species that prefer to use shaded water bodies for oviposition,will be effected by deforestation that can reduce dramatically acceptable breeding habitats, thus impacting their propagation and population densities (Overgaard *et al.*, 2002). In the future, the inevitable developmental changes that will occur to the landscape of northwestern Thailand needs to be monitored carefully regard its impact on potential malaria transmission and the adaptatability of vector species to overcome environmental changes.

Part 3 Bacterial biodiversity in abdomens of *Anopheles* mosquitoes in western Thailand.

3.1 *Anopheles* species

A total of 190*Anopheles* specimens for a total of 8 *Anopheles* species identified, obtained from Mae Sot, Tak Province and Sop Moei, Mae Hong Son Provinces, were used for evaluating the bacterial biodiversity in their abdomens. The molecular species identification was done for specimens belonging to the Minimus, Dirus Complexes or the Maculatus Group. Within the Minimus Complex, two species were identified, *An. minimus* and *An. aconitus*; two species in the Dirus Complex, *An. dirus* and *An. baimaii*; and four species in the Maculatus Group, *An. maculatus, An. sawadwongporni, An. pseudowillmori* and *An. dravidicus* (Table 36). The non-infected *Anopheles* abdomens included 103 specimens from Tak Province and 86 from Mae Hong Son Provinces. Only oneinfected mosquito, *An. minimus*, was detected with *P. vivax* and this specimen was obtained from Mae Sot, Tak Province.

3.2 PCR-TTGE profiles and diversity index in abdomen bacterial communities of *Anopheles*

The abdomen microbiota of 190 specimens of *Anopheles* mosquitoes was investigated by 16S rRNA gene PCR-TTGE anchored in the V3 hypervariable region. TTGE profiles were obtained for 97 samples, 93 samples (48.95%) giving no amplification in PCR or a faint PCR signals leading to non-detectable TTGE profiles. Negative results suggested a low bacterial inoculum rather than a total absence of bacteria in the corresponding samples. Most “negative” samples were *An. dirus* (n=16), *An. baimaii* (n=28) and *An. pseudowillmori* (n=26). Finally, V3 16S PCR-TTGE approach led to the description of a microbial community for about 51% of the specimens analyzed and therefore appears as an efficient tool to investigate bacterial diversity in large samplings of mosquitoes.

Table 36 Number and species of *Anopheles* tested for abdomen bacteria diversity in specimens found positive for bacteria showing number of Gram-negative bacteria genera

Collection sites	Species	No. specimens	Positive TTGE	Gram-negative
Mae Sot	*An. minimus*	22	12	9
	An. aconitus	1	-	-
	An. maculatus	23	6	4
	An. sawadwongporni	7	1	2
	An. pseudowillmori	1	-	-
	An. dravidicus	5	1	1
	An. dirus	19	7	5
	An. baimaii	25	3	4
		103	30	
Sop Moei	*An. minimus*	12	8	5
	An. aconitus	1	-	-
	An. maculatus	5	4	2
	An. sawadwongporni	12	3	-
	An. pseudowillmori	34	3	5
	An. dravidicus	1	-	-
	An. dirus	8	3	4
	An. baimaii	13	2	1
		86	23	

A raw diversity index that globally reflects the bacterial diversity in a sample is classically evaluated by counting the bands in TTGE profiles. At first glance, the number of bands on TTGE profiles (Figure20) ranged from 1 to 10 suggesting that the bacterial diversity per specimen ranged from 1 to 10 OTUs. However, sequencing showed that bands with different distance of migration could belong to the same OTU. This atypical phenomenon was observed for bacteria displaying sequence heterogeneity among their 16S rRNA gene copies. For instance,

members of the genus *Serratia*as well as most members of the genera affiliated to the family *Enterobacteriaceae* displayed a high level of 16S rRNA gene heterogeneity leadingto complex banding patterns in V3 16S PCR-TTGE. Considering that *Serratia* and *Enterobacteriaceae* were relatively common in our samples, the raw diversity index drastically overestimated the bacterial diversity. Therefore, a refined diversity index was calculated after affiliation of each band to an OTU by sequencing or by a comparative approach.

The refined diversity index showed a lower bacterial diversity with an average of 1.26 OTU per specimen. Three OTUs is the maximum biodiversity per specimen observed in this study. The number of OTUs per specimen differed slightly between populations in thetwo study sites, with an average of 1.33 and 1.17 OTU per specimen in Mae Sot and Sop Moei, respectively.

3.3 Bacterial diversity in the whole population of *Anopheles* mosquitoes

16S rRNA gene PCR-TTGE is focused on hypervariable region V3 produced sequences of about 200 bp, which are generally not informative enough for species-specific affiliation. Consequently, this study presents the bacterial diversity to the genus level. However, probable species affiliation was proposed for several genera when the phylogenetic signal of the V3 region was significant.

Contrasting with the low diversity per specimen, OTU diversity in the whole population was high with the detection of 19 different bacterial genera (Table 37) distributed in 4 phyla, *Proteobacteria, Bacteroidetes/ Chlorobi*, *Firmicutes* and *Actinobacteria*. *Proteobacteria* largely dominated the microbiota of *Anopheles* mosquitoes from this study. Their diversity encompassed the *Alpha- Beta-* and *Gamma* superclasses of *Proteobacteria*.

The gamma-proteobacterial genera, *Serratia, Enterobacter, Klebsiella, Pseudomonas, Acinetobacter* and *Raoultella* were commonly detected in the populations. The *Anopheles* abdomen microbiota included 3 main species, *Serratia*

marcescens, *Enterobacter cloacae*, *Klebsiella pneumonia*. *Enterobacteriales* was the second main order of gamma-proteobacteria represented in the microbiota of *Anopheles*. In *Enterobacteria*, the species affiliation could not be ascertained because genera are very similarin 16S rRNA gene phylogeny, particularly for *Enterobacter* and its relatives *Cronobacter* and *Pantoea*. Members of gamma-proteobacteria of the orders *Xanthomonadales* (*Stenotrophomonas*) and *Aeromonadales* (*Aeromonas*) were also detected showing the very wide diversity of gamma-proteobacteria in the abdomen microbiota of *Anopheles*. The diversity was lower in beta-proteobacteria with no finding for alpha- proteobacteria. However, the genus*Chromobacterium* was found in *An. minimus* and *Herbaspirillum* commonly found in *An. dirus*, two genera of *Beta-proteobacteria*. Besides*Proteobacteria*, the phyla *Bacteroidetes/ Flavobacteria*, and *Firmicutes* were represented by only a few genera: 3 (*Elizabethkingia*, *Pantoea* and *Chryseobacterium*) and 1 (*Staphylococcus*), respectively. Sequences affiliated to the genus *Elizabethkingia* could not be related with certainty to *Elizabethkingia anophelis* because the V3 region did not discriminate between this *Anopheles*-specific species and the human pathogen *Elizabethkingia meningoseptica*. The genus *Staphylococcus*was found in one mosquito abdomen. This genus were identical to those of a strain isolated in the abdomen of the ladybird *Harmonia axyridis* and were linked to the species *Staphylococcus sciuri* (Vilcinskas *et al.*, 2013).

Nineteen bacterial genera were detected from this study, among which 12 were previous reported (Manguin *et al.*, 2013) and 7 new bacterial genera were found, such as *Chromobacterium* in *An. minimus*, *Ferrimonas* in *An. minimus*, *Herbaspirillum* in *An. dirus*, *Pectobacterium* in *An. dirus*, *Raoultella* in *An. minimus* and *An. pseudowillmori*, *Shimwellia* in *An. minimus*, *An. dirus*, *An. baimaii* and *An. maculatus*, and *Stenotrophomonas* in *An. pseudowillmori*. The highest diversity with 9 bacterial genera was detected in *An. minimus* from Mae Sot, while 5 bacterial genera were detected in *An. minimus* and *An. pseudowillmori* from Sop Moei, and *An. dirus* from Mae Sot. In Mae Sot, the genus *Enterobacter* was the most frequently found in *An. minimus*, followed by the genus *Klebsiella* in *An. dirus* and *An. maculatus*. Three *Anopheles* species; *An. minimus*, *An. dirus* and *An. baimaii* shared

the same 2 bacterial genera *Serratia* and *Acinetobacter*. In Sop Moei, the genus *Serratia* was the most frequently found in *An. minimus*, followed by the genera *Pectobacterium* and *Klebsiella* in *An. dirus*, and the genus *Elizabethkingia* in *An. minimus* and *An. sawadwongporni*. The only one malaria-infected mosquito, *An. minimus*, (Figure 21) had 4 bacterial genera within Phylum *Firmicutes* including, *Aerococcus*, *Megaphaera*, *Streptococcus*, *Peptostreptococcus*, and one genus *Roseomonas* in the Phylum *Proteobacteria*.

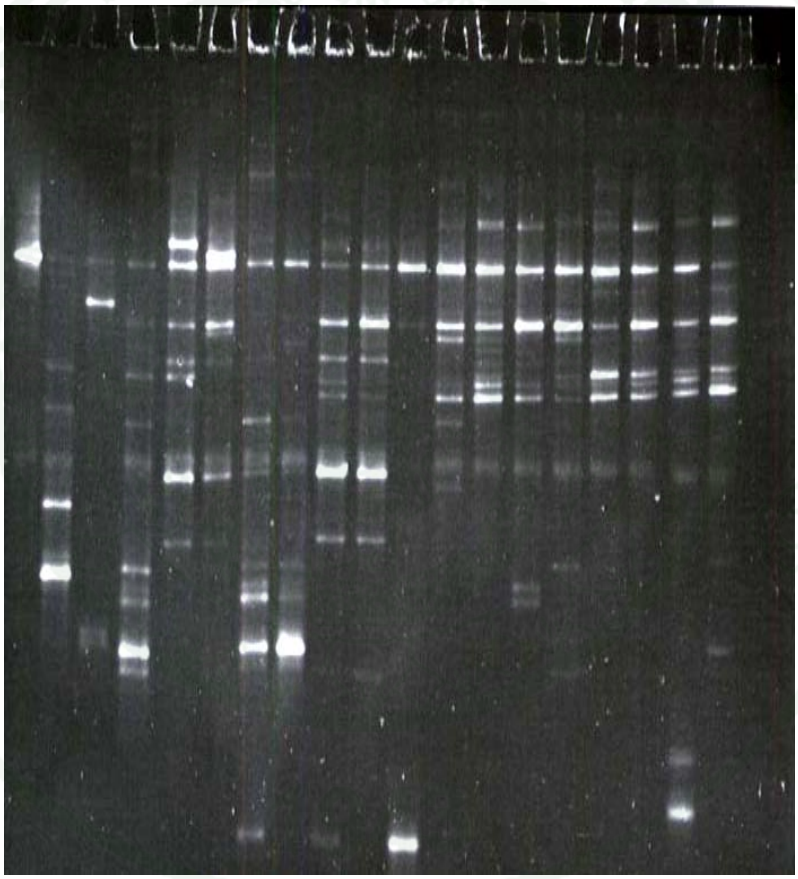

Figure 20 Representative TTGE analysis of V3 16S rRNA gene PCR products amplified from abdomen samples of *Anopheles* mosquitoes from Thailand. Each lane corresponded to a mosquito microbiota

Source: Manguin *et al.*(2013)

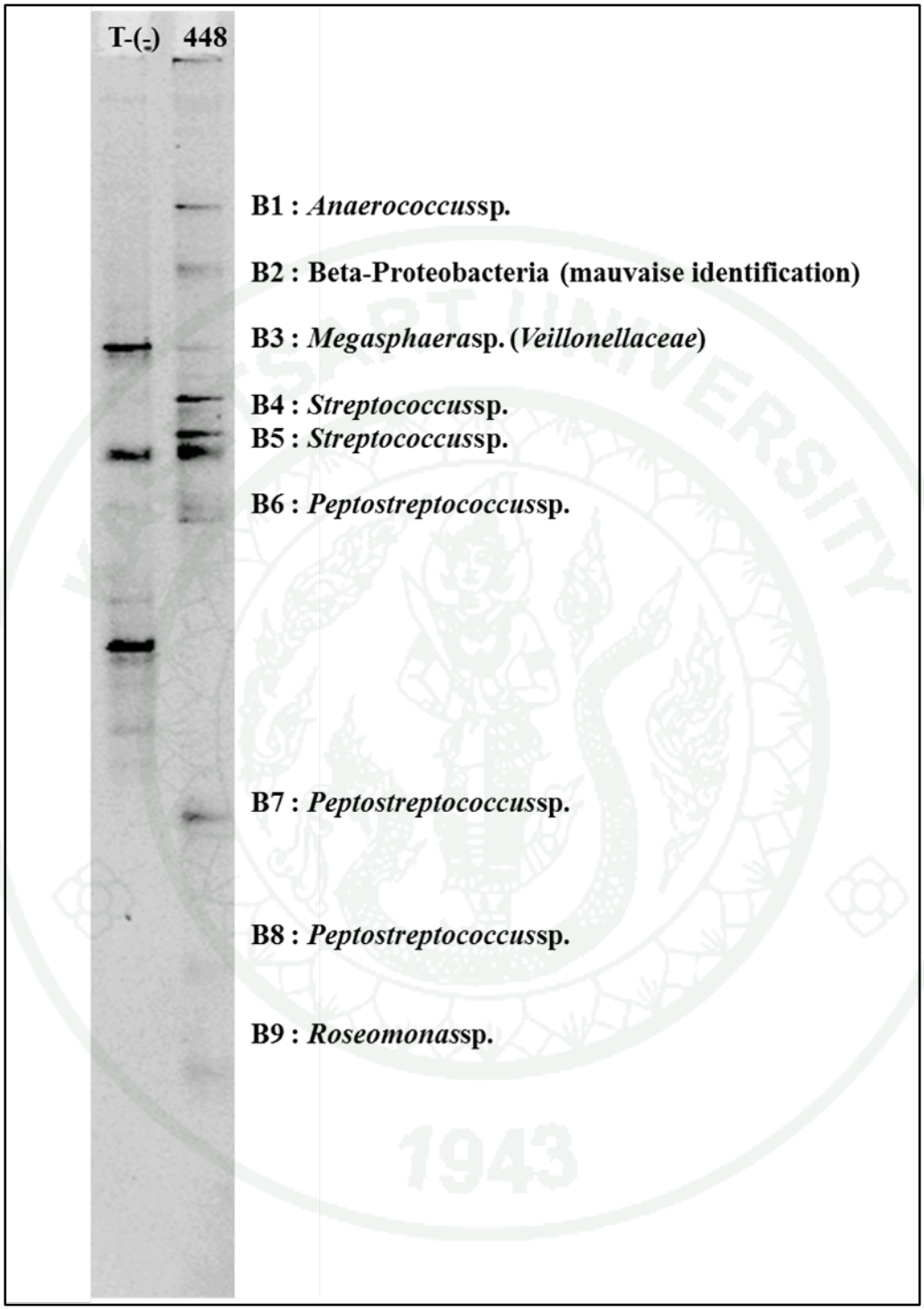

Figure 21 TTGE banding for the detection of bacteria genera in the abdomen of *P. vivax* infected *Anopheles minimus* (specimen no. 448) compared to control (T-)

Table 37 Bacterial genera detected in the abdomen of adult *Anopheles* species collected in Mae Sot and Sop Moei with the number of specimens carrying each genus.Genera described for the first time in *Anopheles* are marked with asterisk

Bacterial genera	*An. minimus*		*An. dirus*		*An. baimaii*		*An. maculatus*	
	M[a] (n=12)	S[b] (n=8)	M (n=7)	S (n=2)	M (n=3)	S (n=2)	M (n=6)	S (n=4)
Acinetobacter	1	-	-	1	1	-	-	-
Aeromonas	1	1	-	-	-	-	-	-
*Chromobacterium**	-	1	-	-	-	-	-	-
Chryseobacterium	-	-	-	-	-	-	-	-
Elizabethkingia	1	3	1	-	-	-	1	-
Enterobacter	11	-	-	-	-	-	-	1
Erwinia	1	-	-		-	-	-	-
*Ferrimonas**	-	1	-	-	-	-	-	-
*Herbaspirillum**	-	-	3	-	-	-	-	-
Klebsiella	-	-	6	3	-	-	6	1
Pantoea	-	-	-	-	2	-	1	-
*Pectobacterium**	-	-	-	3	-	-	-	-
Pseudomonas	2	-	-	-	-	-	-	-
*Raoultella**	1	-	-	-	-	-	-	-
Serratia	6	6	-	2	2	2	-	-
*Shimwellia**	4	-	1	-	1	-	2	-
Staphylococcus	-	-	-	-	-	-	-	-
*Stenotrophomonas**	-	-	-	-	-	-	-	-
Trabulsiella	1	-	1	-	-	-	-	-

Table 34 (Continued)

Bacterial genera	*An. sawadwongporni*		*An. dravidicus*		*An. pseudowillmori*	
	M[a] (n=1)	S[b] (n=3)	M (n=1)	S (n=0)	M (n=0)	S (n=3)
Acinetobacter	-	-	-	-	-	-
Aeromonas	-	-	-	-	-	-
*Chromobacterium**	-	-	-	-	-	-
Chryseobacterium	1	-	-	-	-	-
Elizabethkingia	1	4	1	-	-	-
Enterobacter	-	-	-	-	-	1
Erwinia	-	-	-	-	-	-
*Ferrimonas**	-	-	-	-	-	-
*Herbaspirillum**	-	-	-	-	-	-
Klebsiella	-	1	-	-	-	-
Pantoea	-	3	-	-	-	-
*Pectobacterium**	-	-	-	-	-	-
Pseudomonas	-	1	-	-	-	-
*Raoultella**	-	-	-	-	-	1
Serratia	-	-	-	-	-	2
*Shimwellia**	-	-	-	-	-	-
Staphylococcus	-	-	-	-	-	1
*Stenotrophomonas**	-	-	-	-	-	1
Trabulsiella	1	-	-	-	-	-

* Newly reported bacterial genera in *Anopheles* abdomens

[a]M= Mae Sot, Tak Province

[b]S= Sop Moei, Mae Hong Son Province

The effects of normal bacterial flora and invading microbes in mosquitoes and on the development of the parasite to employ them in the control of malaria transmission have been investigated. The review of some major findings of laboratory and field studies on the role of microbes in the biocontrol of malaria vectors and/ or parasites were recently summarized by Abdul-Ghani *et al.* (2012); Gendrin and Christophides (2013); Minard *et al.*, (2013); Hughes *et al.*, (2014); Sharma *et al.*, (2014); and Villegas and Pimenta, (2014).

The PCR-TTGE focused on the 16S rRNA gene, hypervariable region V3, proves its efficiency to study microbiota of *Anopheles* mosquitoes. This method, that presents a relative low to intermediate resolution, is efficient to follow bacterial communities with low to moderate diversities such as *Anopheles* samplesof this study. This limit is due to the number of bands that can be separated within the length of the gel. Optimization of TTGE conditions allows separation of bands by a minimum of 0.1 mm over the gel length. Therefore, the TTGE would be difficult to interpret if the diversity exceeds 25 to 30 OTUs (Roudiere *et al.*, 2009). Microbiotaof *Anopheles* displays TTGE profiles that do not exceed 10 bands but the profiles have been interpreted with difficulty due to heterogeneities in rRNA genes for most bacteria in the mosquito ecosystem. At the genomic level, rRNA genes are generally organized in multigene families (Acinas *et al.*, 2004) in which sequences show low variability within species, subspecies or genome level (Liao, 2000). However, TTGE remains useful to provide a snapshot of microbiota in large populations of hosts. Based onthe literature, the results obtained by pyrosequencing or Next Generation Sequencing (NGS) compared to fingerprint by TTGE demonstrated good correlation for the detection of majority OTUs in complex communities (Manguin *et al.*, 2013; Li *et al.*, 2014).

Thereby, the abdomen microbiota of 190 specimens of 8 *Anopheles* species with a sequencing effort of less than 150 reads was done compared to 5 million reads estimated for the same study by pyrosequencing. Twelve of the 19 genera have been previously detected in studies on field-collected *Anopheles*. However, the fact those7 bacterial genera were found herein for the first time

suggests that the bacterial diversity associated to *Anopheles* abdomens remains largely underestimated.

The gut microbiota of mosquitoes presented a large inter-specimen variability but was dominated by the genera, *Serratia, Pseudomonas, Enterobacter, Acinetobacter, Raoultella* and *Elizabethkingia*. This result shows that PCR-TTGE failed to detect minority and/ or low loaded populations. This low resolution is certainly a limit but we also see it as a benefit because the majority taxa detected by TTGE probably corresponds to true colonizers of the abdomen and not to transient or contaminant bacteria. Members of the genus *Elizabethkingia* detected in *Anopheles* from Thailand could not be identified as *E. anophelis*remaining to its close relatedness in 16S rRNA gene sequence with *E. meningosepticum*.

Antagonism against *Enterobacteria*is of particular interest because it has been suggested that mosquitoes harboring *Enterobacteriacae* are more likely to be infected by *P. falciparum* (Boissiere *et al.*, 2012). In addition, *Plasmodium vivax* infections in *An. albimanus*, co-infected with *Serratia marcescens*could inhibit the oocyst development (Gonzalez-Ceron *et al.*, 2003). In our collection, *An. minimus* specimen Mi488 from Mae Sot was infected with *P. vivax* and displayed a microbiota containing exclusively 5 genera, *Aerococcus, Megasphaera, Streptococcus, Peptostreptococcus* and *Rosomonas*, different from other non-infected samples. Those specimens displayed the highest enterobacterial diversity of the *Anopheles* collection, especially with the genus *Serratia*. Identification of the enterobacterial species in our samples will be the next step and the search for *Enterobacter* (Esp_Z), which was reported to inhibit *P.falciparum* development (Cirimotich *et al.*, 2011). As the microbiota might have an impact on pathogen development in *Anopheles* mosquitoes and disease transmission, more studies are needed for a better understanding of the role of specific bacteria in wild mosquito populations as a potential method of control.

CONCLUSION AND RECOMMENDATION

This studyhas compiled data regarding the known distribution of important malaria vectors in Thailand based on molecular identifications from 1994 to 2014. Using these various non-morphological tools that have been developed over the course of the last 3 decades, geo-referenced maps have been generated from published information or study site locations and more recent data in this study, illustrating the known countrywide distribution of primary and secondary malaria vector species in Thailand. Particularly with the advent of various molecular-based species identification methods, the distributions of the various sibling species have been more clearly defined in Thailand together with a far greater understanding of the epidemiological importance of each member. This effort has also helped to illustrate the current limitations of sampling efforts and how little we know about the distribution of species in large areas of Thailand that have not been sampled or have material not identified uto the species level. Other limitations from a number of published studies have included relatively low sample sizes, low number of collections attempted, temporal (seasonal) and spatial (geographical) boundaries of sampled locations and the collection techniques used that can bias observations on true diversity of mosquito populations in an area. Nevertheless, the tables and maps presented in this review provide the first update on the series of distribution maps on selected species published by Rattanarithikul *et al.*, (2006); however, the principal difference being all locations are based on methods other than morphological identification alone, including cytogenetic and molecular-based techniques. Recently, Saeung (2012) also provided a distribution map of species complexes in Thailand. In the current study, we produced four sets of distribution maps (11 total), 21 tables with GPS coordinates to more accurately show the *Anopheles* distribution in Thailand. Overall future research is requiredto conduct more work on mosquito fauna combining morphological and molecular methods for definitive (and confirmatory) species identification. These data will improve and update on a regular basis the anopheline map of Thailand for becoming more complete and useful to all operational and academic workers in this field.

Knowledge on mosquito behavior is important to understand the epidemiology of disease transmission and vector control. Detailed information on feeding and host seeking behavior helps to define vector competence of each species. It does also contribute to the relative risk for disease transmission in the human population. Precise species identification is needed and the PCR-based detection of parasites in mosquitoes allowed us to have a first estimation of the vectorial capacities of the *Anopheles* speciesin malaria and filariasis transmission areas. This is the first step to assist the design and implementation of appropriate vector prevention and control strategies. From this study results showed highly adaptable *Anopheles* species such as *An. minimus* with a large distribution. This species could bite both human and animal so the implementation and control should be adaptive to the place, host and time.

Based on the analysis of the abdomen microbiota of 8 field-caught *Anopheles* species from Thailand, 19 bacteria genera were detected, with 7 bacterial genera reported for the first time in *Anopheles* mosquitoes, suggesting that the bacterial diversity associated to abdomen of *Anopheles* remains underestimated. Low bacterial diversity ranging from 1 to 3 per specimen was found which contrasted with a high OTU diversity in the whole *Anopheles* population having3 phyla, *Proteobacteria*, *Bacteroidetes/ Chlorobi*, and *Firmicutes*. The comparison and analysis of the bacterial biodiversity in mosquitoes infected by parasites *versus* non-infected ones could not be done due to thelow number of infected mosquitoes (n=1). However, five bacteria genera were detected from the infected *An. minimus* specimen. The PCR-TTGE on the bacterial 16S rNRA provided a first basis estimation of the bacterial biodiversity present in *Anopheles* abdomens in Thailand. Microbiota might impact pathogen development in *Anopheles* and reduce disease transmission. More studies are needed to understand the biological role of bacteria in wild mosquito populations.

Vector control remains an important component of successful integrated malaria control programs. As demonstrated in this study, a better understanding of a specific vector species bionomics, its vector capacity and epidemiological importance, hinges on the accurate identification of sympatric sibling species in a given area. More investigations are needed in all remaining malaria endemic areas in Thailand so

as to assist in the development and refinement of more cost-effective and targeted vector control strategies based on evidence derived from well-design field studies.

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