Diversity of *Anopheles* species and trophic behavior of putative malaria vectors in two malaria endemic areas of northwestern Thailand

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ABSTRACT: We determined the species diversity, blood-feeding behavior, and host preference of *Anopheles* mosquitoes in two malaria endemic areas of Tak (Mae Sot District) and Mae Hong Son (Sop Moei District) Provinces, located along the Thai border with Myanmar, during a consecutive two-year period. Anopheline mosquitoes were collected using indoor and outdoor human-landing captures and outdoor cow-baited collections. Mosquitoes were initially identified using morphological characters, followed by the appropriate multiplex AS-PCR assay for the identification of sibling species within *Anopheles* (*Cellia*) complexes and groups present. Real-time PCR was performed for parasite-specific detection in mosquitoes (*Plasmodium* spp. and *Wuchereria bancrofti*). A total of 7,129 *Anopheles* females were captured, 3,939 from Mae Sot and 3,190 from Sop Moei, with 58.6% and 37% of all anophelines identified as *An. minimus*, respectively. All three malaria vector complexes were detected in both areas. One species within the Minimus Complex (*An. minimus*) was present along with two related species in the Funestus Group, (*An. aconitus*, *An. varuna*), two species within the Dirus Complex (*An. dirus*, *An. baimaii*), and four species within the Maculatus Group (*An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori*, and *An. dravidicus*). The trophic behavior of *An. minimus, An. dirus, An. baimaii, An. maculatus,* and *An. sawadwongporni* are described herein. The highest *An. minimus* densities were detected from February through April of both years. One specimen of *An. minimus* from Mae Sot was found positive for *Plasmodium vivax*. ***Journal of Vector Ecology* 39 (2): 424-436. 2014.**

Keyword Index: *Anopheles dirus*, *Anopheles minimus*, *Anopheles maculatus*, species complexes, malaria, Thailand.

INTRODUCTION

After years of intensive, well-organized malaria control activities in Thailand, malaria still remains prevalent in vulnerable areas, especially along the less developed international borders with Myanmar. Approximately 85% of all reported malaria cases in the country occur in this poorly monitored border region, primarily related to transient employment opportunities or occupational activities including agriculture, hunting, and gem mining. Several primary malaria vector species in Thailand are abundant in forested areas along the international border (Chareonviriyaphap et al. 2003, Corbel et al. 2013). Bancroftian filariasis is also endemic along the Thai-Myanmar border but of low prevalence. However, the detection of this parasite is not uncommon among Burmese working in Thailand (Khamboonruang et al. 1987, Jitpakdi et al. 1998, Bhumiratana et al. 2002). Nocturnally subperiodic (NSP) *Wuchereria bancrofti* is transmitted by a wide variety of mosquito species including anophelines also capable of malaria transmission (Pothikasikorn et al. 2008).

Of the 73 known *Anopheles* species in Thailand, various sibling species in the Minimus and Dirus Complexes and the Maculatus Group have been recognized as the main malaria vectors in the country (Rattanarithikul et al. 2006, Sinka et al. 2011). Molecular techniques based on polymerase chain reaction (PCR) have allowed for precise and reliable differentiation and identification of the sibling species of medical importance (Manguin et al. 2008, Sinka et al. 2011). Among these 73 species, five to six species, depending on the literature, are incriminated as primary malaria vectors in Thailand (Rattanarithikul et al. 2006), including *Anopheles baimaii* (previously *An. dirus* D) (Green et al. 1991) and *Anopheles dirus* (Rosenberg et al. 1990, Green et al. 1991) of the Dirus Complex, *Anopheles minimus* (previously *An. minimus* A) (Rattanarithikul et al. 1996a) of the Minimus Complex, and *Anopheles pseudowillmori* (Green et al. 1991), *An. maculatus* and *An. sawadwongporni* of the Maculatus Group (Saeung 2012). Additionally, *An. campestris* and *An. epiroticus* (Sundaicus Complex) have also been incriminated as potential malaria vectors in Thailand (Apiwathnasor et al. 2002). *Wuchereria bancrofti* develops experimentally in *An. minimus* and *An. maculatus* (Pothikasikorn et al. 2008). Natural infections of this parasite have also been found in *An. minimus, An. maculatus and An. vagus* in Thailand (Pothikasikorn et al. 2008).

While the number of cryptic species has increased, Thailand has been identifying sibling species within the complexes for several decades, yet information on the distribution, ecology and behavior of many of these species remains poor or lacking. This information is quite critical in defining the vector capacity of each species (Takken and Verhulst 2013). Knowledge on mosquito behavior is crucial to understanding the epidemiology of vector transmission throughout the range of each species and for assisting national and international efforts in vector-borne disease control. Numerous observations on trophic behavioral and seasonal abundance have been conducted on *Anopheles* complexes (*sensu lato*) or related groups (Ismail et al. 1974, Harbach et al. 1987, Ratanatham et al. 1988, Rattanarithikul et al. 1996a, Chareonviriyaphap et al. 2003). Unfortunately, most of these observations relied on species that were identified by morphological characters alone, as molecular assays capable of reliably identifying each sibling species were not readily available or still non-existent. During the past few decades, studies on malaria vectors in Thailand using molecular identification assays have increased and provided precise identification to the species level, as well as the recognition of additional *Anopheles* species and species complexes in Thailand (Baimai et al. 1984, 1988, Baimai 1989, Green et al. 1990, Green et al. 1992, Poopittayasataporn and Baimai 1995, Walton et al. 1999, Garros et al. 2004, Rattanarithikul et al. 2006, Dusfour et al. 2007, Walton et al. 2007, Poolprasert et al. 2008, Saeung et al. 2008, Thongsahuan et al. 2009, Eamkum et al. 2014).

Previous studies have described the biting activity of *An. minimus* and *An. harrisoni* in malaria-endemic Tak (Tisgratog et al. 2012) and Kanchanaburi Provinces (Sungvornyothin et al. 2006), and both biting activity and host preference of *An. dirus* and *An. baimaii* in Kanchanaburi Province (Tananchai et al. 2012). However, additional investigations on the bionomics and blood feeding activities of sympatric malaria vectors belonging to species complexes are needed. In the present study, molecular identification assays were combined with the systematic descriptions of the trophic behavior, biting activity, seasonal abundance, and parasite infections of individual sibling species in the two most malaria endemic areas of Mae Sot (Tak Province) and Sop Moei (Mae Hong Son Province), northwestern Thailand. Additionally, overall anopheline species diversity from these two areas along the Thai-Myanmar border is described.

MATERIALS AND METHODS

Collection sites

Adult mosquito collections were conducted every two months during three consecutive nights, for a period of two years in one village each in Mae Sot District (Tak Province) and Sop Moei District (Mae Hong Son Province) located in northwestern Thailand (Figure 1).

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

The Sop Moei study site neighbors Myanmar, Shan State to the north and Kayin and Kayah States to the west. The field site is located approximately 50 m 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.

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. The first team worked from 18:00 to 24:00, followed by a second team from midnight to 06:00. Two people sat inside the house, while the other two took up a position outside approximately 100 m 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. 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. The following morning, mosquitoes were taken back to the field laboratory for morphological identification. 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 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).

Mosquito species identification

Each female mosquito was intially sorted using morphological keys (Rattanarithikul et al. 2006), individually stored in a clean, labelled 1.5 ml non-silicone tube, and immediately frozen and stored in a liquid nitrogen tank. Mosquito samples were sent to the Department of Entomology, Kasetsart University, in Bangkok. Individual *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 7 µl of 8 mM 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° C for 30 min. Samples were spun at 12,000 rpm for 20 min at 4° C. The supernatant was again cleaned using 150 µl of 70% ethanol and centrifuged at 12,000 rpm for 5 min at 4° C, and again with 100% ethanol and centrifuged at 12,000 rpm for 5 min at 4° C. The resultant pellet was dried at room temperature for 20 min before being re-suspended in 100 µl of TE buffer and stored at -20°C, 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

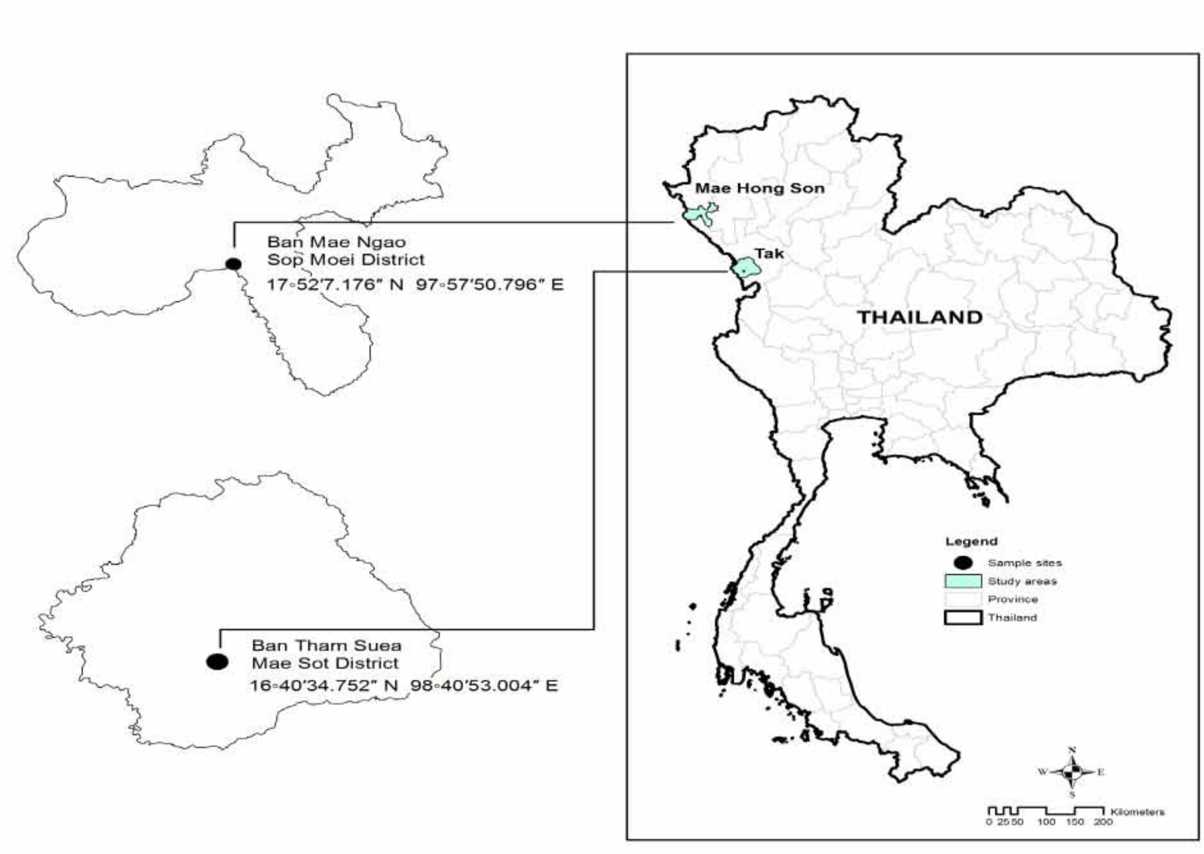

Figure 1. Study site locations in Mae Sot District, Tak Province and Sop Moei District, Mae Hong Son Province.

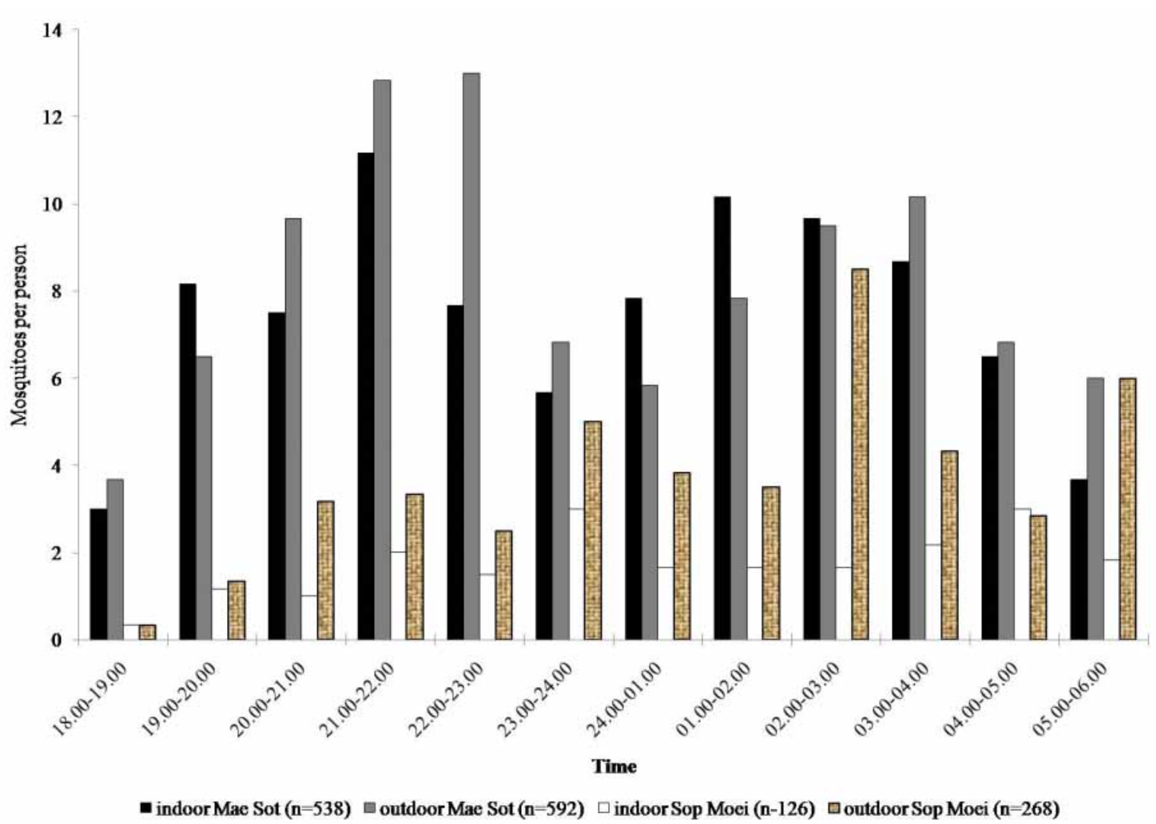

Figure 2. Mean indoor and outdoor human-landing rates by hour for *Anopheles minimus* in Mae Sot and Sop Moei.

Complex and related species, including *Anopheles minimus*, *An. harrisoni*, *An. aconitus, An. varuna* and *An. pampanai* (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).

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

Real-time PCR was performed with a Roche LightCycler®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). 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 from Rougemont et al. (2004) and PK probe from Divis et al. (2010).

Data analysis

Numbers of primary malaria vectors collected by different collection methods were analyzed using non-parametric tests. A Wilcoxon test and Pearson's correlation analyses were used to investigate the interaction between the number of mosquitoes and environmental parameters. All data were analyzed with a level of significance set at 0.05% ($p < 0.05$), using a SPSS statistical package (ver. 17.0, SPSS, Chicago, IL).

RESULTS

Species composition and abundance

A total of 7,129 *Anopheles* females was 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 three groups in the subgenus *Anopheles* (n=973) (Table 1). Of 5,148 specimens within the two complexes and the Maculatus Group identified by molecular assays, 3,278 were derived from Mae Sot and 1,870 from 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*. Within the subgenus *Cellia*, the most abundant species 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 1). *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., *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 collection for both sites. In addition, specimens of the *Anopheles umbrosus* group and *Anopheles peditaeniatus* were also found in both study sites.

Mosquito density and feeding behavior

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 shown in Table 2. *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 (data not shown). Both species were highly zoophilic as all were collected on cattle-baited traps in Mae Sot, and more than 80% were attracted to cattle in Sop Moei. Indoor and outdoor feeding behaviors of *An. minimus* in Mae Sot were quite similar, with peak biting activity occurring between 21:00 and 23:00. 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 and indoor peak after 05:00 (Figure 2).

The monthly densities of *An. dirus* and *An. baimaii* collected from both study sites are shown in Table 2. Both species were found in similar proportions, with 18 and 21 specimens, respectively, in Mae Sot and 11 specimens for each species in Sop Moei. Nearly twice as many specimens were captured in Mae Sot (n=39) compared to Sop Moei (n=22). In both years, the greatest numbers of individuals were encountered in June. In Mae Sot, *An. dirus* and *An. baimaii* had a preference/attraction to humans with 97% (n=38) collected on human-bait compared to cattle. The indoor:outdoor (9:8) ratios were nearly identical, although the overall numbers were small, while *An. baimaii* was collected in greater number outdoors (n=16) than indoors (n=5) on humans. In Sop Moei, neither species showed any significant 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) and a later peak outdoors (23:00-midnight)

Table 1. Numbers of *Anopheles* collected by species and percentage divided by subgenus in Mae Sot and Sop Moei from February, 2011 to January, 2013.

Species	Mae Sot (%)	Sop Moei (%)	Total (%)
Anopheles (Cellia)			
An. minimus	2,103 (58.6)	951 (37.0)	3,054 (49.6)
An. aconitus	16 (0.5)	19 (0.7)	35 (0.6)
An. varuna	60 (1.7)	12 (0.5)	72 (1.2)
An. dirus	18 (0.5)	11 (0.4)	29 (0.5)
An. baimaii	21 (0.6)	11 (0.4)	32 (0.5)
An. maculatus	911 (25.4)	525 (20.44)	1,436 (23.3)
An. sawadwongporni	116 (3.2)	274 (10.7)	390 (6.3)
An. pseudowillmori	4 (0.1)	51 (2.0)	55 (0.9)
An. dravidicus	29 (0.8)	16 (0.6)	45 (0.7)
An. annularis s.l.	-	8 (0.3)	8 (0.1)
An. culicifacies s.l.	2 (0.1)	83 (3.2)	85 (1.4)
An. jamesii	78 (2.2)	89 (3.5)	167 (2.7)
An. jeyporiensis	-	2 (0.1)	2 (0.0)
An. karwari	3 (0.1)	11 (0.4)	14 (0.2)
An. kochi s.l.	93 (2.6)	316 (12.3)	409 (6.6)
An. nivipes	9 (0.3)	6 (0.2)	15 (0.2)
An. philippinensis	72 (2.0)	65 (2.5)	137 (2.2)
An. splendidus	2 (0.1)	2 (0.1)	4 (0.1)
An. stephensi	1 (0.0)	1 (0.0)	2(0.0)
An. subpictus	1 (0.0)	9 (0.4)	10 (0.2)
An. tessellatus	-	38 (1.5)	38 (0.6)
An. vagus	47 (1.3)	70 (2.7)	117 (1.9)
Total (s.g. *Cellia*)	3,586 (58.3%)	2,570 (41.7%)	6,156
Anopheles (Anopheles)			
An. barbirostris Group	127 (36.0)	136 (21.9)	263 (27.0)
An. umbrosus Group	42 (11.8)	134 (21.6)	176 (18.1)
An. hyrcanus Group	165 (46.7)	279 (45.0)	444 (45.6)
An. peditaeniatus	19 (5.4)	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

Table 2. Number, distribution and frequency of four primary *Anopheles* malaria vector species collected in Mae Sot and Sop Moei (I= indoor human landing, O=outdoor human landing, C= cattle baited).

	Mae Sot												Sop Moei											
Month	*An. minimus*			*An.dirus*			*An. baimaii*			*An.maculatus*			*An. minimus*			*An.dirus*			*An. baimaii*			*An.maculatus*		
	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C	I	O	C
Year 1																								
Feb-11	134	128	207	-	-	-	-	-	-	1	6	10	8	40	24	-	-	-	-	-	-	-	1	8
Apr-11	121	111	220	1	1	-	-	1	-	3	3	21	21	20	108	-	1	-	-	-	-	-	2	6
Jun-11	65	106	30	6	7	1	2	2	-	9	53	39	6	15	38	1	-	1	1	1	2	2	6	20
Aug-11	-	5	2	-	-	-	-	2	-	3	11	-	-	8	10	-	-	-	-	-	-	-	-	4
Oct-11	23	26	33	-	-	-	-	-	-	26	32	193	2	2	5	-	1	1	-	-	-	2	4	65
Dec-11	30	36	61	-	-	-	-	1	-	20	20	38	-	-	95	-	-	-	-	-	-	3	6	36
Year 2																								
Feb-12	41	31	111	-	-	-	-	-	-	8	8	-	7	49	117	-	-	-	-	-	-	-	-	-
Apr-12	59	79	183	-	-	-	-	-	-	4	4	-	21	20	55	-	-	-	-	-	-	2	-	-
Jun-12	38	37	78	1	-	-	-	5	-	17	20	114	48	87	10	1	1	2	-	4	2	3	50	149
Aug-12	-	3	10	-	-	-	3	1	-	5	25	42	1	9	5	-	-	-	-	-	-	-	2	27
Oct-12	16	6	21	1	-	-	-	4	-	23	45	-	4	10	12	-	-	2	-	-	-	-	2	-
Dec-12	11	24	17	-	-	-	-	-	-	30	78	-	8	8	78	-	-	-	-	-	1	2	2	121
Total	538	592	973	9	8	1	5	16	-	149	305	457	126	268	557	2	3	6	1	5	5	14	75	436

*Summer= February, March, April, May; Wet=May, June, July, August, September, October; Dry= November, December, January.

(Figure 3). 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 4). Neither species was found in great abundance, so that drawing any definitive conclusions about these data should be done with caution.

Four species belonging to the Maculatus Group were identified in Mae Sot and Sop Moei: *An. maculatus*, *An. sawadwongporni*, *An. pseudowillmori* and *An. dravidicus* (Tables 1, 2). 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) representing 20.2%. *Anopheles pseudowillmori* and *An. dravidicus*, were captured in much smaller proportions, 2.9% and 2.3% of 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 (data not shown). The activity of *An. maculatus* in Mae Sot showed a very clear early biting peak between 18:00 and 21:00 for outdoor HLC (Figure 5). The biting activity of *An. sawadwongporni* from outdoor HLC in both villages showed a small peak between 19:00 to 20:00 (data not shown). Like the Dirus Complex, neither species was found in great abundance, so drawing any definitive conclusions regarding these data should be done with caution.

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.

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 differences (Z=-0.94, p=0.348) were observed in Mae Sot between the indoor (3.76$\pm$0.3) and outdoor (4.11$\pm$0.41) human landing. However, *An. minimus* showed significantly greater (Z=-2.43, p=0.02) anthropophilic (7.85$\pm$0.72) behavior compared to its attraction to cattle (6.76$\pm$0.72) (Table 5). In Sop Moei, *An. minimus* was significantly more exophagic (Z=-5.72, p<0.01) and zoophilic (Z=-3.99, p<0.01) (Table 5). *An. maculatus* showed similar and highly significant exophagic and zoophilic behavior in both study sites.

Parasite detection

Eight *Anopheles* species (n=1,447) from both collection sites in Mae Sot and Sop Moei were examined for parasite infection rates 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 in an outdoor HLC, 00:00-01:00 in April, 2011, was found positive for *P. vivax.*

DISCUSSION

This study is the first detailed description of 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 that borders 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. 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 and longer (Chareonviriyaphap et al. 2001, BVBD 2013).

Anopheles minimus represented 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*, all species within the Maculatus Group. These findings are consistent with previous observations in Mae Sot District (Tum Sua Village) that found *An. minimus* to be the most abundant species (71%), followed by *An. maculatus* Group (28%) and 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, were found in very low densities with only a total of 39 specimens collected in Mae Sot and 22 in Sop Moei during a two-year period. These two species were slightly more common during the June collection both years with only a few specimens detected other months. Low collection numbers of the Dirus Complex 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, has been studied in detail in relatively fewer instances compared to earlier studies that based all identification on morphological characters alone. For example, two species

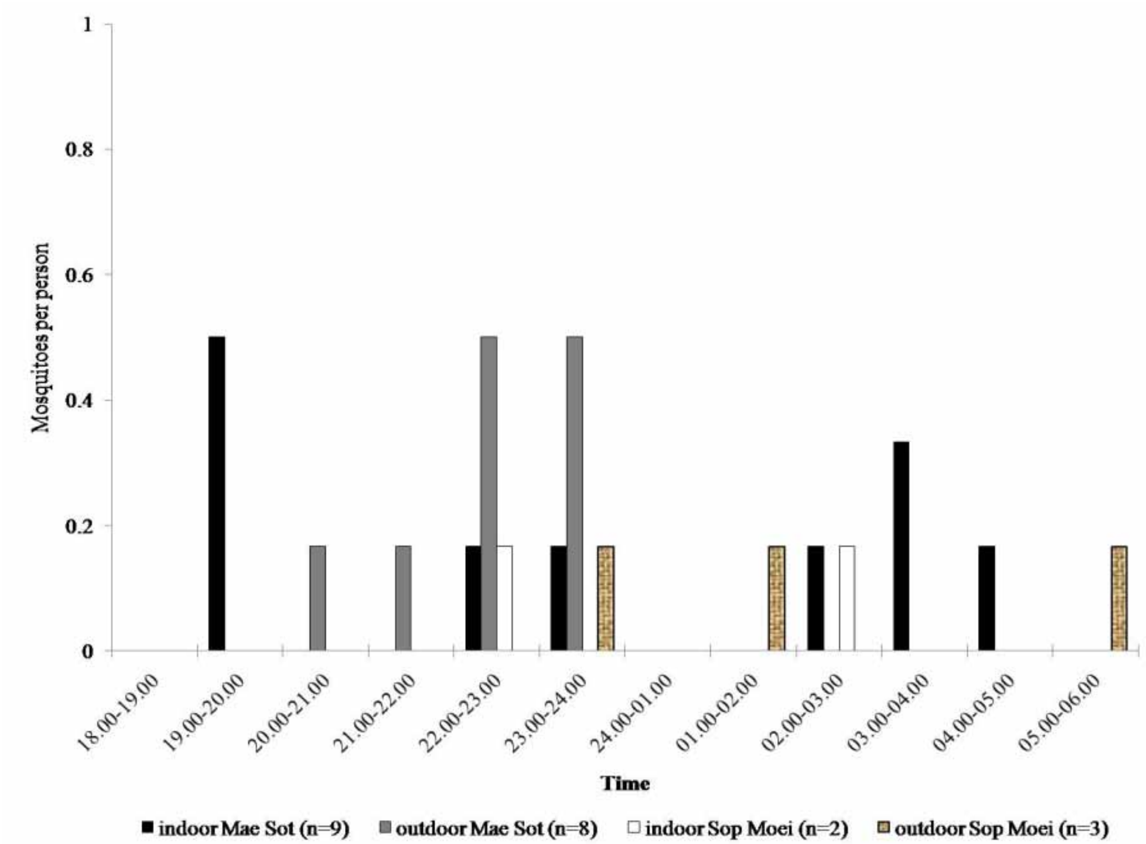

Figure 3. Mean indoor and outdoor human-landing rates by hour for *Anopheles dirus* in Mae Sot and Sop Moei.

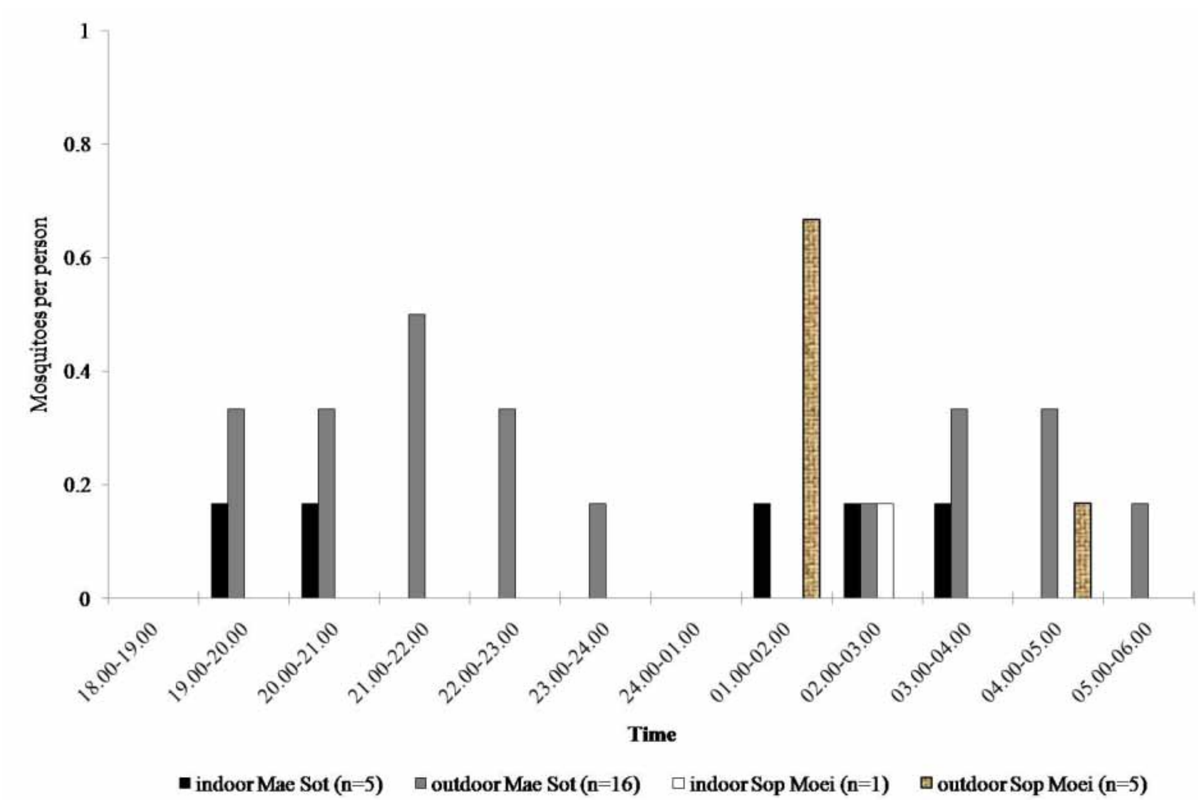

Figure 4. Mean indoor and outdoor human-landing rates by hour for *Anopheles baimaii* by hour in Mae Sot and Sop Moei.

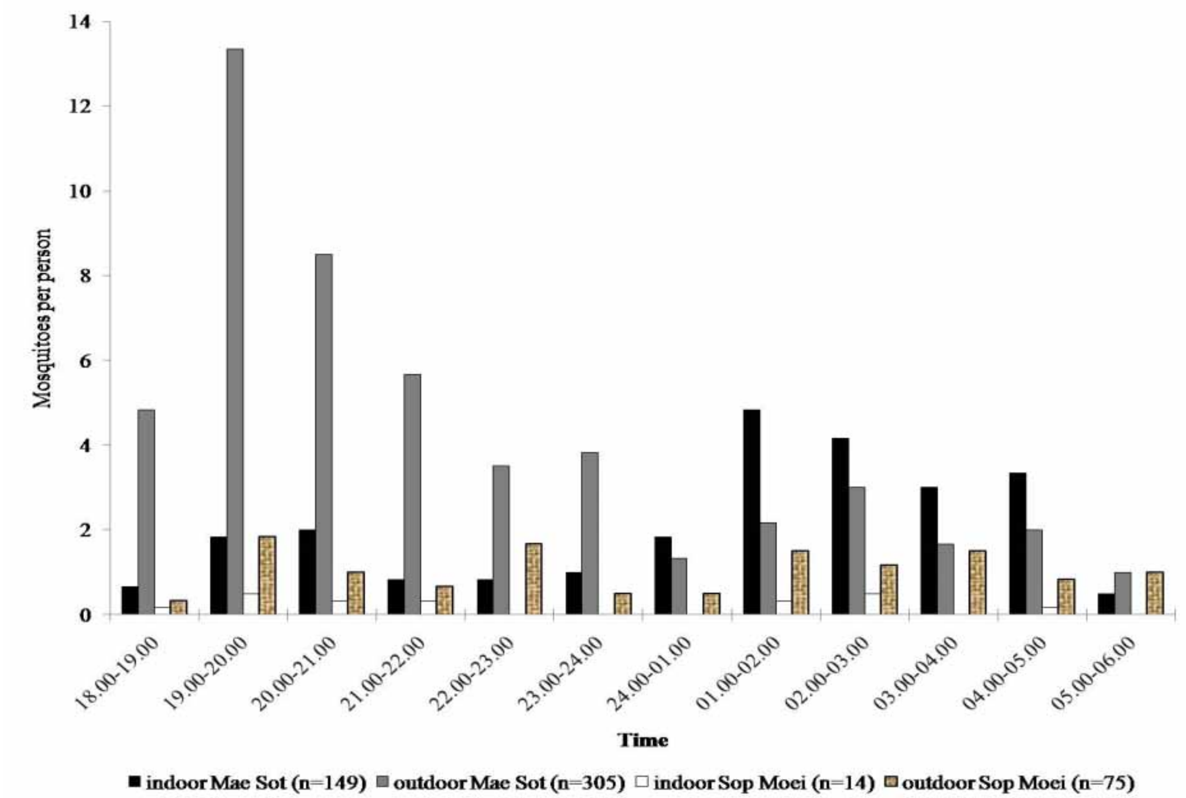

Figure 5. Mean indoor and outdoor human-landing rates by hour for *Anopheles maculatus* in Mae Sot and Sop Moei.

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 related species to *An. minimus,* was identified for the first time in both locations. Although *An. aconitus* and *An. varuna* are not as efficient vectors compared to *An. minimus*, the presence of these two species, especially *An. aconitus*, in Mae Sot and Sop Moei is of importance as these two species could possibly play a secondary role in malaria transmission (Gould et al. 1967, Scanlon et al. 1968, Junkum et al. 2007).

Tak Province is one of the most malaria-endemic areas of Thailand, accounting for around 23% of 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 as more mosquitoes feed on cattle located outside houses. 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 vector capacity. In Pu Teuy, Kanchanaburi Province, *An. minimus* and *An. harrisoni* are present, the latter species being predominant and with generally distinct zoophilic behavior that is believed partly responsible for the typically low levels of malaria transmission in this area (Sungvornyothin et al. 2006). *Anopheles minimus*, *An. dirus* and *An. baimaii,* found in both study areas demonstrated exophagic and zoophilic behaviors which are in agreement with previous observations by Tananchai et al. (2012).

Few studies on species of the Maculatus Group in Thailand have been published (Takai et al. 1987, Upatham et al. 1988, Green et al. 1992, Rongnoparut et al. 1996, 1999, Walton et al. 2007, Muenworn et al. 2009). Studies on this group have been hampered by the 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 identified molecularly. The most common representative was *An. maculatus*, followed by *An. sawadwongporni*, both malaria vectors in the southern Thailand (Rattanarithikul et al. 1996b, Sinka et al. 2011). Our findings showed that these two important putative vectors were present in study locations in fair proportions, 20.1% and 5.5%, respectively, 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 mosquito positive for the malaria parasite was detected during the dry season, accounting for a 0.092% (1/1,090) infection rate for *An. minimus*. This mosquito was collected from outdoor HLC from Mae Sot in the early part of the study (April 2011). There was no evidence of malaria infection with *P. falciparum*, *P. malariae, P. knowlesi* or the filarial parasite, *W. bancrofti*. The one positive *An. minimus* was captured during the normal malaria peak in Mae Sot in the late summer months (April-May). The explanation for the low infection rate 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 to year in both provinces and 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).

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 (shading) 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). Vanwambeke et al. (2007a,b) found that land-use changes could selectively influence the diversity and likely density of mosquito species, thus having 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 programs, road construction, mining and hydropower development, is one of the most potent factors either promoting or reducing infectious diseases like malaria and dengue in the southeast Asian region, including Thailand (Guerra et al. 2006, Vanwambeke et al. 2007a,b, 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 rainfall patterns (Reiter 2001, Overgaard et al. 2002). For example, anopheline species that prefer to use shaded water bodies for oviposition, deforestation can reduce dramatically breeding habitats, thus affecting their propagation (Overgaard et al. 2002). In the future, the inevitable developmental changes that will occur to the landscape of northwestern Thailand will need to be monitored carefully to assess their impacts on potential malaria transmission.

Vector control remains an important component of successful integrated malaria control programs. As demonstrated in this study, a better understanding of the bionomics of a specific vector species, 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 develop more cost-effective and targeted vector control strategies based on evidence derived from well-design field studies.

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