THESIS

ECOLOGICAL ASPECTS AND CHARACTERIZATION OF MALARIA MOSQUITO, *ANOPHELES MINIMUS* COMPLEX IN THAILAND

SUNGSIT SUNGVORNNYOTHIN

GRADUATE SCHOOL, KASETSART UNIVERSITY

2007

THESIS

ECOLOGICAL ASPECTS AND CHARACTERIZATION OF MALARIA MOSQUITO, *ANOPHELES MINIMUS* COMPLEX IN THAILAND

SUNGSIT SUNGVORNYOTHIN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Entomology)
Graduate School, Kasetsart University
2007

Sungsit Sungvornyothin 2007: Ecological Aspects and Characterization of Malaria Mosquito, *Anopheles minimus* Complex in Thailand. Doctor of Philosophy (Entomology), Major Field: Entomology, Department of Entomology. Thesis Advisor Associate Professor Theeraphap Chareonviriyaphap, Ph.D. 80 pages.

The aim of this study was to perform the analyses on species identification of the Minimus complex using data from previous report along with a large sample of wild specimens from Thailand. The Minimus complex is composed of at least three sibling species, *An. minimus*, species C and species E. *Anopheles minimus* and species C are widespread throughout the Southeast Asian region whereas species E is found in Ryukyu islands, Japan. *Anopheles minimus* and species C are known as malaria vectors and appear sympatric with some specific behaviors. The first presents a presector pale spot (PSP) on the wing costa whereas the latter have both presector and humeral pale spots (HP). No acceptable diagnostic power on species identification has been established over large temporal and geographic investigation. This present study explored nine populations throughout Southeast Asia, including published data and wild caught populations from two sites in Thailand, indicating unreliable of the two morphological characters in identification of the two species, *An. minimus* and species C. Therefore, molecular identification remains the most significant tool for the species identification of the Minimus complex.

In addition, the trophic behavior of *An. minimus* and species C was described by using a molecular identification assay over a two year period at Pu Teuy Village, Sai Yok District, Kanchanaburi Province. Feeding activity of species C was unique compared to *An. minimus* from other localities in Thailand. Outdoor feeding of species C occurred throughout the night with one prominent peak (1800 hour) whereas indoor feeding showed two small peaks at 2000 and 2400 hours. The small number of *An. minimus* collected during the study precluded a determination of peak activity patterns. A better understanding of biology and behavior of a single species is critically important to help identify the respective role in disease transmission.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and deep appreciation to Assoc. Prof. Dr. Theeraphap Chareonviriyaphap, my thesis advisor for invaluable teaching guidance and suggestion. I would sincerely like to thank Assoc. Prof. Dr. Somsak Apisitwanich, Assoc. Prof. Dr. Chitapa Ketavan, and Col. Pote Aimpun my thesis committee for their helpful suggestions and valuable advice. Sincere appreciation is also due to Prof. Dr. Sylvie Manguin for her kindness and technical support during my field works and molecular training. Special thanks are also due to Dr. Claire Garros and Dr. Isabelle Dusfour for their excellent enthusiastic and excellent cooperation which enable me to complete this thesis.

I would like to express my appreciation to the Thai Research Funds; the Development and Promotion of Science and Technology Talent Project; Department of Entomology, Kasetsart University; Institute of Research for Development; and Thai-French Cooperation Program for Higher Education and Research for their financial support to conduct part of the research at the Centre de Biologie et Gestion des Populations (CBGP), Montpellier, France.

Finally, I would like to thank my friends in Department of Entomology for strongly assistance, the villagers at Ban Pu Teuy and Ban Tha Sao for their warming cooperations and the officers of the Bureau of Vector Borne Disease for helping in field work and malaria and entomological data.

Sungsit Sungvornyothin

September 2007

TABLE OF CONTENTS

Page

TABLE OF CONTENTS i
LIST OF TABLES iii
LIST OF FIGURES iv
INTRODUCTION 1
OBJECTIVES 5
LITERATURE REVIEWS 6
1. Overview 6
2. Malaria in Thailand 7
3. Malaria vectors 12
4. Malaria parasites 15
5. Species complexes and identification 17
5.1. Background 17
5.2. Cytogenetics 18
5.3. Allozyme analysis 19
5.4. DNA based techniques 20
5.4.1. Mitochondrial DNA 21
5.4.2. Ribosomal DNA 21
5.4.3. Highly polymorphic markers 22
5.4.3.1. Microsatellite DNAs 22
5.4.3.2. RAPDs and SCARs 23
6. The Minimus Complex 23

TABLE OF CONTENTS (CONTINUED)

Page

MATERIALS AND METHODS 27

Part 1. Diagnostic character for the identification of the two sibling species of the Minimus Complex 27

1. Study Area 27

2. Mosquito Collections 31

3. Morphological Identification 31

4. Molecular Identification 32

5. Reliability test of the presence/absence of the HP spot for the identification. 33

Part 2. Host seeking behavior and biting activity between the two siblings of the Minimus Complex based on molecular identification 34

1. Study Area 34

2. Mosquito Collection 35

3. Morphological Identification. 36

4. Molecular Identification 36

5. Data Analysis 36

RESULTS 38

Part 1. Possible diagnostic character for the identification of the two sibling species of the Minimus Complex 38

Part 2. Host seeking behavior and biting activity between the two sibling species of the Minimus Complex based on molecular identification 43

DISCUSSION 50

Part 1. Possible diagnostic character for the identification of the two sibling species of the Minimus Complex 50

Part 2. Host seeking behavior and biting activity between the two sibling species of the Minimus Complex based on molecular identification 52

RECOMMEND FOR FURTHER STUDIES 56

LITERATURE CITED 57

APPENDIX 76

LIST OF TABLES

Table		Page
1	Malaria surveillance statistics in Thailand 1999-2006	11
2	Recognized Anopheline vectors and potential vectors of malaria in Thailand and neighboring countries	13
3	Characteristics of test populations	40
4	Identification results and indexes	41
5	Significative test for the positive predictive (upper table) and the negative predictive values (lower table)	42
6	Monthly frequency of *Anopheles* mosquitoes at Ban Pu Teuy, Sai Yok District, Kanchanaburi Province, for 2 years (February 2004-January 2006)	45
7	Total monthly collections from three collection methods of *Anopheles minimus* and species C	46

LIST OF FIGURES

Figure		Page
1	Map of Thailand depicting the general distribution of the ten most malarious provinces in Thailand, 2006	9
2	Total cases of malaria from Thai and non-Thai groups from 1999-2006	10
3	Proportion of malaria parasite species in relation to the National drug policy, Thailand	17
4	Taxonomic hierarchy of *Anopheles minimus* complex	26
5	Map of collection site	30
6	HP phenotype and PSP phenotype	32
7	Map of collection sites at Ban Pu Teuy and Ban Tha Soa, Sai Yok District, Kanchanaburi Provinc	35
8	Rate of human indoor collections by hour of *Anopheles minimus* and species C	47
9	Rate of human outdoor collections by hour of *Anopheles minimus* and species C	48
10	Rate of Cow bait collections by hour of *Anopheles minimus* and species C	49

ECOLOGICAL ASPECTS AND CHARACTERIZATION OF MALARIA MOSQUITO, *Anopheles minimus* COMPLEX IN THAILAND

INTRODUCTION

Malaria remains one of the most significant vector-borne parasitic diseases in the tropics and subtropics with the estimate of 107 endemic countries and territories. At the end of 2004, the estimated world's clinical malaria cases were 402 million (range 350-500 million). Approximately 57% occur in Africa, 30% in Asia and around 5% in the Americas (Thiel, 2005). About 311 million cases (range 270-400 million) were estimated from *Plasmodium falciparum*, the most aggressive and fatal malaria species. This species is distributed globally but is especially common in Africa (72%) and in Asia (19%) of the world's cases (Korenromp, 2005). Between 1 and 2 million deaths are reported annually and most malaria deaths result from *P. falciparum* (Korenromp, 2005; Thiel, 2005).

Before the end of the 20th century, World Health Organization (WHO) developed the Global Strategy for Malaria Control emphasized on the early diagnosis and prompt treatment of malaria cases while the vector control was selective operations which choice of control based on epidemiological, economic and political considerations of each country (WHO, 1993a, b). Using this strategy, the epidemiological, socio-economic and entomological research data have to be integrated for implementation of cost-effective control measures. The entomological studies on biological features and on response to insecticidal application of malaria vector should be an important reason of alternative decision (Meek, 1995). However, the strategy developed by WHO remains unclear and has never been accepted by any single malaria endemic countries as a choice of malaria control (Roberts *et al*., 2000). Vector control using the indoor residual spray (IRS) and insecticide impregnated net (IIN) seem to be the most appropriated method for malaria control as described in several published

reports (Roberts and Andre, 1994; Klun *et al*., 2000; Roberts *et al*., 2000; Chareonviriyaphap *et al*., 2004).

In Southeast Asia, level of antimalarial resistance of *P. falciparum* is the highest in the world and the cost of multi-drug treatment is extremely high (Chareonviriyaphap *et al*., 2000, Bureau of Vector Borne Disease (BVBD, 2006)). Resistance to antimalarial drug was first discovered in Thailand in 1963 (BVBD, 2000). Since then, malaria parasites resistant to drugs have been spread all over the endemic zones, especially in Southeast Asian countries. In Thailand, malaria remains a major infectious disease with more or less 30,000-70,000 reported cases each year. Surveillance data indicate malaria has been increased over 60,000 since 2006 (BVBD, 2004, 2005, 2006). The explanation for the increase would appear to be a consequence of human and economic activities as well as a political crisis along the southern frontier international boundaries. The disease is transmitted by anopheline mosquitoes. *Anopheles minimus* complex is currently considered to be one of the most important malaria vectors throughout Thailand (BVBD, 2005). This taxon represents a complex of closely related species that are impossible to distinguish morphologically (Garros *et al*., 2004a). The accuracy of mosquito species identification is the essential basic evidence for the entomological studies, especially for studies of the *An. minimus* complex.

The *Anopheles minimus* complex in Thailand is composed of two closely related species, *An. minimus* (former=species A) and species C which are very difficult to accurately distinguish from one another morphologically (Sucharit *et al*., 1988; Green *et al.*, 1990; Rattanarithikul and Panthusiri 1994; Van Bortel *et al*., 1999; Somboon *et al*., 2001; Chareonviriyaphap *et al*., 2002; Garros *et al*., 2004a, b). *Anopheles minimus* is the predominant species found throughout most of Thailand whereas, species C appears confined along the western Thai-Myanmar border, most notably in Kanchanaburi Province (Sucharit *et al*., 1988; Baimai, 1989; Green *et al*., 1990). Previous studies on behavioral differences between *An. minimus* and species C in Vietnam (Van Bortel *et al*., 1999) have shown that species C is exhibiting more important exophagic and zoophilic behavioral propensities than *An. minimus.*

Rwegoshora *et al*., (2002) has also demonstrated greater outdoor feeding activity of species C in Thailand. Another sibling species within the Minimus Complex, species E, was recently recognized from the Ishigaki Island, Japan by using morphological, cytogenetic, molecular and hybridization evidence (Somboon *et al*., 2001, 2005).

Better understanding of the biology and behavior of a vector 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 correct identification of the species under study is also critical, especially within a vector complex like *An. minimus* subgroup. Sucharit *et al*., (1988) provided morphological characteristics that could apparently separate *An. minimus* and species C. The presence of the wing presector pale spot (PSP) and humeral pale spot (HP) on the costa vein were defined as diagnostic characters for species C. In contrast, Rattanarithikul *et al*., (1995) considered only the PSP sufficient to identify species C. Use of two different phenotype wing patterns to identify either species have been reported (Green *et al*., 1990; Van Bortel *et al*., 1999; Rwegoshora *et al*., 2002); however, the use of morphological characters alone to distinguish between *An. minimus* and species C in Thailand has also resulted in 37% misidentification of the time (Green *et al*., 1990). Until recently, use of morphological features in combination with isozyme electrophoresis has served as the gold standard to separate these two sympatric members of the complex (Green *et al*., 1990). Isozyme electrophoresis; however, mandates specimens be either assayed fresh or frozen and further requires complete destruction of the specimen in the process making it impossible to conduct other assays.

In addition to isozyme analysis, molecular identification using polymerase-chain reaction (PCR)-based methods for identifying members in the Minimus Complex have been developed (Sharpe *et al*., 1999; Kengne *et al*., 2001; Chen *et al*., 2002; Garros *et al*., 2004 a, b). A restriction fragment length polymorphism (RFLP)-PCR technique was developed to separate the sibling species within the Minimus Complex and related species (Van Bortel *et al*., 2000, Garros *et al*., 2004a), and more recently, a one-shot and rapid multiplex allele-specific PCR technique (AS-PCR) was used to identify

species in the taxon (Garros *et al*., 2004b). The AS-PCR method can easily distinguish members of the Minimus Complex, *An. minimus* and species C, and three other related species, *An. aconitus*, *An. varuna* and *An. pampanai*, as well as closely related species that occur in tropical Africa. A PCR-Allele-Specific Amplification (ASA) has also proven more simple and accurate to classify species *An. minimus* and species C than isozyme analysis (Zheng *et al*., 2005). However, the most efficient and rapid assay used nowadays for this complex is the multiplex PCR developed by Garros *et al*. (2004b).

Anopheles minimus and species C can occur in sympatry but few investigations have been conducted on each sibling species regarding feeding activity and resting behaviors, host preference (degree of anthropophily), and other bionomical factors that may influence their vector capacities, respectively. Rwegoshora *et al*., (2002) reported biting activity of *An. minimus* and species C from Thailand with species identification based exclusively on morphological criteria. Additionally, their study did not observe behavior throughout the entire night (dusk to dawn), but rather in relation to seasonal climatic variations during the year. As mentioned, upwards of 37% of *An. minimus* and species C may be misidentified based on morphological criteria alone (Green *et al*., 1990). Recently, night biting activity of *An. minimus* complex was reported from Kanchanaburi Province, but these observations did not distinguish between *Anopheles minimus* and species C (Chareonviriyaphap *et al*., 2003). Therefore, by using molecular taxonomic identification techniques, the feeding activity and behaviors that might distinguish *An. minimus* and species C were accurately described.

OBJECTIVES

1. Comparison of the two techniques, morphological method and DNA-based assays, to identify *Anopheles minimus* and species C.

2. Comparison of biting activity and host prevalence between *Anopheles minimus* and species C.

LITERATURE REVIEW

1. Overview

Despite years of control success and a competent network of country-wide health infrastructure, malaria is still one of the most important health treat in Thailand, especially along the undeveloped borders of eastern Myanmar, northern Malaysia, and western Cambodia (BVBD, 2007). In the central plain areas, malaria has been eliminated for over two decades (Chareonviriyaphap *et al*., 2000). Of four known human malaria parasites, *Plasmodium falciparum* and *P. vivax* are prominent with the approximate ratio of 1:1 (BVBD, 2007). The existence of multi-drug resistance *P. falciparum* is the most serious development to occur for decades. Recently, known malaria vectors were updated, including *Anopheles baimaii (=dirus D), Anopheles minimus (previously = An. minimus* species A*), Anopheles pseudowillmori* and *Anopheles aconitus* (Rattanarithikul *et al*., 2006). In addition, several secondary and suspected vectors of malaria are progressively reported, representing almost 15 known vector species throughout the country (Rattanarithikul *et al*., 2006) (Table 2).

The most effective proven methods for malaria prevention has been by vector control, mainly by use of routine residual insecticide spray inside houses and distribution of pyrethroid-impregnated bed nets. For years, DDT was used for malaria control as an indoor residual spray (IRS) in Thailand. DDT was completely stopped for public health use in 2001 although phase out period was from 1995 to 1999 (Chareonviriyaphap *et al*., 2000). The reasons for the removal of DDT from malaria control in Thailand were due mainly to a perceived adverse impact on environment and a public negative attitude towards DDT. However, the true impact of DDT on mosquito vectors in terms of behavioral responses and disease transmission remains poorly understood and needs further clarification. In 2000, the mathematical framework for understanding the repellent, irritant and toxic properties of insecticides on mosquito populations and how they function in control of malaria has been proposed (Roberts *et al*., 2000). In addition, a new classification system for the actions of IRS chemicals

used for malaria control has been suggested. In this study, DDT primarily functioned as the most repellent compound that keep mosquitoes outside of huts.

DDT was replaced by two potential synthetic pyrethroids, deltamethrin and permethrin. The first has been primary used as the IRS whereas the latter been applied as impregnated treated netting (ITN). The increased use of pyrethroids to IRS and ITN are generally more accepted by human populations. The organized malaria control activities have reduced malaria morbidity from 286/1000 population in 1947 to 1.5/1000 population by 1996. Recently, the increase of malaria morbidity has been recorded with malaria morbidity of 0.49/1000 in 2006 (BVBD, 2007).

2. Malaria in Thailand

Malaria remains one of the most important infectious diseases throughout the tropical and subtropical regions (Campbell, 1997). Many countries, including Thailand have experienced a dramatic resurgence of malaria negating routine malaria control progress and significant reduction in indigenous and imported cases (Baird 2000, Chareonviriyaphap *et al*., 2000). While deforestation has pushed malaria out of many regions in Thailand, malaria is widespread in most areas along the international and undeveloped borders of western Myanmar, southern Malaysia, and eastern Cambodia (BVBD, 2006). The current status of malaria in Thailand is shown in Figure 1 and Table 1. Over 70% of malaria cases are documented from Thai-Myanmar border and frequently associated with tribal populations that are mostly migratory due to temporally occupation and illegal activities. The un-controlled movement as the nomadic nature of these tribal groups confounds the critical problems associated with cross border transmission and control. Based on the malaria surveillance activities in Thailand from 1999-2006 (Table 1), recorded malaria cases in Thailand were approximately 200,000 in 1998, peaking to 208,323 cases in 1999, and declined thereafter to 57,592 and 54,920 cases in 2004 and 2005, respectively. As a whole, malaria cases have greatly decreased during this decade. This significant improvement in malaria situation has been partly as a consequence of effective, well organized vector control program, other public health activities and more recently pyrethroid-

impregnated bed nets (BVBD, 2006). However, beginning from 2006, malaria cases have actually increased (66,651) (Table 1). The reason and cause of this significant increase in 2006 and 2007 remain controversial. Limited health budgets for malaria control, increased population movement across the malaria endemic borders, drug resistance, weaken and unorganized vector control and political unrest along the international borders, especially along the southern frontier international boundary, have been provided the opportunity for malaria to increase. However, unknown malaria cases from three southern provinces along the Thai-Malaysia border (the ongoing insurgency in the far South) remain another portion that may contribute to the increase of malaria in 2006 and 2007. In addition, reorganizations in 1996 and 2003 have resulted in a 20-40% reduction in manpower throughout the country (Chareonviriyaphap *et al*., 2000; Roll Back Malaria (RBM), 2006; BVBD, 2006).

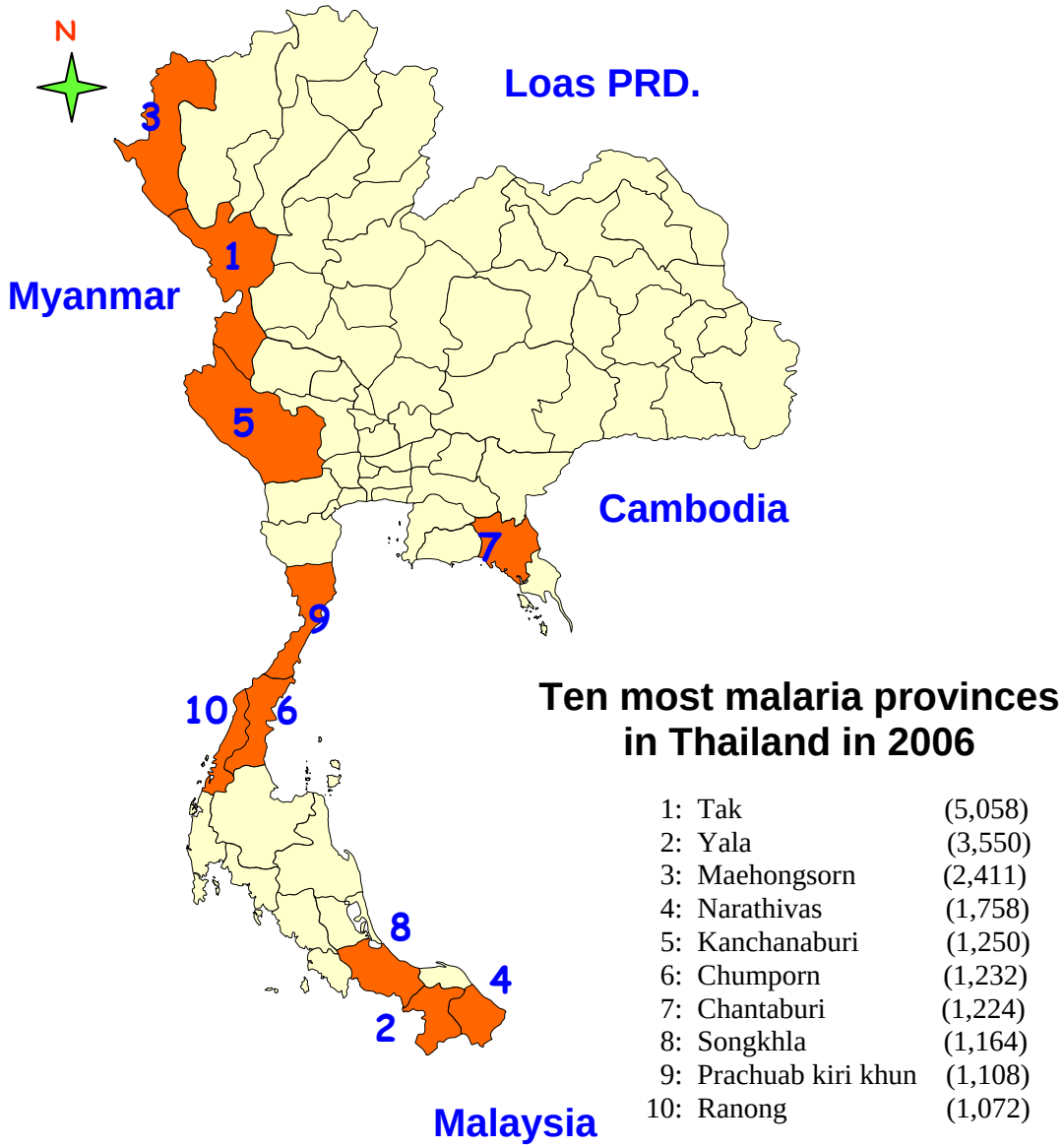


Figure 1. Map of Thailand, depicting the general distribution of the ten most malarious provinces in Thailand, 2006 (Source: BVBD, 2007)

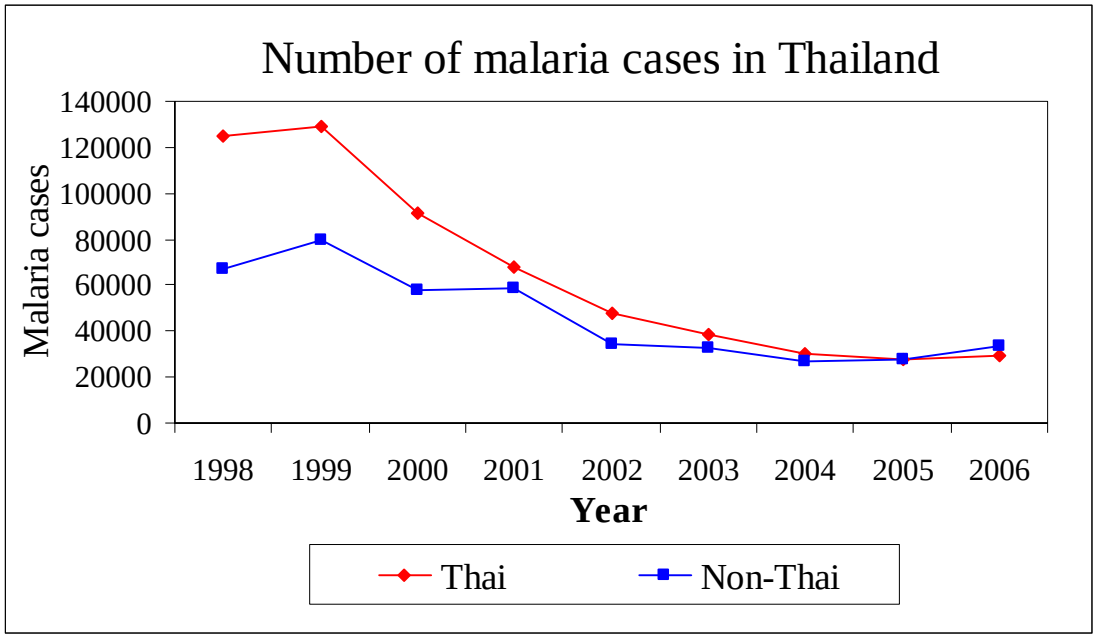


Figure 2. Total cases of malaria from Thai and non-Thai groups from 1998-2006 (Source: BVBD, 2007)

In summary, recent observation on malaria situation strongly indicates that malaria may be re-emerging in Thailand after years of successful control program (Fig 2). The emerging is mainly a consequence of uncontrolled and political crisis along the international borders as well as limited health budget and manpower (RBM, 2006). Although most malaria cases have been recorded from western Thai-Myanmar, hundreds of malaria cases remain undetectable due to a severely political situation from the southern border of Thailand and Malaysia (BVBD, Pers. Com.).

Table 1. Malaria surveillance statistics in Thailand 1999-2006

Categories	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 slide	208,323	149,586	126,595	81,931	71,287	57,592	54,920	66,651
Death rate / 100,000	1.2	1.01	0.68	0.58	0.32	0.37	0.26	0.18
ABER	7.43	7.53	7.44	6.78	5.44	4.76	4.04	3.68
API	1.55	1.36	1.10	0.77	0.62	0.44	0.49	0.49
Pf	63,902	36,881	29,061	20,644	18,864	13,008	11,565	14,123
Pv	60,910	40,709	34,154	284,36	18,293	13,397	12,587	15,991
IRS	643,493	544,800	648,106	543,255	709,706	400,823	300389	263225
Bed net	543,728	636,573	863,643	579,908	863,643	337,645	111,927	107,553
Malaria clinics	525	526	515	526	530	538	504	359

*Include both Thai and non-Thai cases

ABER = Annual Blood Examination Rate

API = Annual Parasite Incidence

Pf = *Plasmodium falciparum*

Pv = *Plasmodium vivax*

IRS = Insecticide Residual Spraying

Source: BVBD, 2000-2007

3. Malaria vectors

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. Recently, 73 known species of Anopheline in Thailand were documented, consisting of 71 named species (Rattanarithikul *et al*., 2006).

Of approximately 73 *Anopheles* species recognized in Thailand, two groups, Leucosphyrus group (Neomyzomyia series) and Maculatus group (Neocellia series), and one subgroup, Minimus (Myzomyia series), consist of important malaria vectors. Specifically, 5 species within 2 groups and one subgroup are incriminated as malaria vectors in Thailand, including *An. baimaii* (previously= *dirus* D*)* (Green *et al*., 1991), *An. dirus* (Rosenberg *et al*., 1990; Green *et al*., 1991), *An. minimus* (previously=species A) (Rattanarithikul *et al*., 1996a), *An. pseudowillmori* (Green *et al*., 1991) and one individual species, *An. aconitus* (Gould *et al*., 1967, Green *et al*., 1991; Maheswary *et al*., 1992) are discriminated as a malaria vectors in Thailand (Table 2).

Table 2. Recognized Anopheline vectors and potential vectors of malaria in Thailand and neighboring countries (Rattanarithikul *et al.*, 2006)

Mosquito Vectors	Thailand	Elsewhere	References
An. aconitus	X	X	Gould *et al*., 1967; Green *et al*., 1991; Maheswary *et al*., 1992
An. anularis	(X)	X	Ghosh *et al*., 1985; Baker *et al*., 1987
An. campestris	(X)	-	Coleman *et al*., 2002
An. culicifacies s.l.	-	X	Ramachandra, 1984; Subbarao, 1988
An. dirus	X	X	Scanlon and Sandhinand, 1965 as *balabacensis*; Rosenberg *et al*., 1990; Green *et al*., 1991
An. baimaii (dirus D)	X	-	Green *et al*., 1991
An. hodgkini	(X)	-	Coleman *et al*., 2002
An. karwari	[X]	-	Rosenberg *et al*., 1990
An. kochi	(X)	X	Wattal, 1961; Baker *et al*., 1987
An.maculatus	[X]	X	Scanlon *et al*., 1968; Delorme *et al*., 1989; Green *et al*., 1991
An. minimus	X	X	Harrison, 1980; Green *et al*., 1991; Rattanarithikul *et al*., 1996a
An. minimus C	X	X	Van Bortel *et al*., 1999; Kengne *et al*., 2001
An. nivipes	(X)	-	Harbach *et al*., 1987; Rattanarithikul *et al*., 1996a
An. philippinensis	[X]	X	Elias *et al*., 1987; Rosenberg *et al*., 1990
An. pseudowillmori	X	-	Green *et al*., 1991
An. stephensi	-	X	Ramachandra, 1984
An. subpictus	-	X	Kirnowardoyo, 1985
An. epiroticus (*sundaicus* A)	[X]	X	Scanlon *et al*., 1968; Reid, 1968
An. tessellatus	[X]	X	Harinasuta *et al*., 1976; Ramachandra, 1984
An. sawadwongporni	(X)	-	Rattanarithikul *et al*., 1996a; Somboon *et al*., 1998; Coleman *et al*., 2002
An. vagus	(X)	X	Ramachandra, 1984; Baker *et al*., 1987
An. willmori	-	X	Pradhan *et al*., 1970
Barbirostris Group	[X]	X	Harrison and Scanlon, 1975; Rattanarithikul *et al*., 1996a
Hyrcanus Group	(X)	X	Harrison and Scanlon, 1975; Rattanarithikul *et al*., 1996a
Umbrosus Group	-	X	Harrison and Scanlon, 1975; Khoon, 1985

X = sporozoites in the salivary glands (X) = ELISA

[X] = oocysts - = no evidence

Leucosphyrus Subgroup: This subgroup consists of 12 species, including *An. baisasi* Colless and 2 complexes: Dirus complex and Leucosphyrus Conmplex. The Dirus Complex consists of 7 species including *An. dirus* (species A), *An. cracens* (species B), *An. scanloni* (species C), *An. baimaii* (species D), *An. elegans* (species E present in India only), *An. nemophilous* and *An. takasogoensis* (restricted to Taiwan). The Leucosphyrus Complex consists of 4 species including *An. leucosphyrus* (species B present in Indonesia), *An. latens* (species A), *An. balabacensis* (occurrence in Malaysia) and *An. introlatus* (Sallum *et al.,* 2005). *Anopheles baimaii* and *An. dirus,* are considered to be primary malaria vectors in Thailand (Rattanarithikul *et al*., 2006). Both are forest and forest-fringe inhabiting mosquitoes that are considered highly anthropophilic (Baimai *et al*., 1984; Rattanarithikul *et al*., 2006). The most favored breeding habitats are animal footprints, wheel-tracks, temporary ground pools. In addition, larval habitats have occasionally been found in water jar, cut tree stumps, and root holes. *Anopheles dirus* is the only species that is found throughout Thailand and sometime occurs in sympatry with *An. baimaii* (Rattanarithikul *et al*., 1995).

Minimus Subgroup: The *Anopheles minimus* Complex is composed of at least three sibling species, *An. minimus* (former species A), *An. minimus* species C and species E. A neotype for *An. minimus* (species A) has recently been designated (Harbach *et al*., 2006), therefore this species is now recognized as *An. minimus*. *Anopheles minimus* is the most common that is widespread throughout Thailand (Baimai, 1989). Species C is restricted in two districts of Kanchanaburi Province, western Thailand and occurs in sympatry with *An. minimus* (Kengluecha *et al*., 2005). Species C was previously collected from Mae Sot in Tak Province and Mae Rim in Chiangmai Province, north Thailand but no clear confirmation was made at the time (Rattarithikul *et al*., 2006). The third species, *An. minimus* species E, is restricted to the Ishigaki Island in the Ryukyu Archipelago, Japan (Somboon *et al*., 2001, 2005).

Maculatus Group: Members of the *Anopheles* (*Cellia*) *maculatus* group are important vectors of malaria throughout the Oriental Region, including Thailand, Indonesia, Malaysia and the Philippines (Reid 1968). This group contains 4 species and 2 subgroups, Maculatus and Sawadwongporni, with 2 species each for a total of eight

closely related species (Harbach, 2004). These species can be differentiated based on variability in morphological, behavioral and genetic characters (Green *et al*., 1985; Rattanarithikul and Green 1986; Kittiyapong *et al.*, 1993; Chiang *et al*., 1991; Bangs *et al*., 2002). In Thailand, six species have been reported, including *An*. *maculatus* Theobald and *An. dravidicus* Christophers from the Maculatus Subgroup; *An. sawadwongporni* Rattanarithikul and Green and *An. notanandai* Rattanarithikul and Green from the Sawadwongporni Subgroup; along with *An. willmori* (James) and *An. pseudowillmori* (Theobald) (Green *et al*., 1985, Rattanarithikul and Green 1986, Rattanarithikul and Harbach 1990, Kittiyapong *et al.*, 1990, Green *et al*., 1992). One species, *Anopheles pseudowillmori* (Theobald) has been incriminated as important malaria vector in Thailand (Green *et al*., 1991, Rattanarithikul *et al*., 2006). *Anopheles sawadwongporni* is a common species often found in high density throughout Thailand, especially along the border provinces with Myanmar and Malaysia (BVBD, 2005) and this species could be a potential vector of malaria in Thailand (Rattanarithikul *et al*., 2006).

Several secondary potential malaria vectors are present in Thailand. These vectors have a close association with human, including *An. epiroticus* (=*sundaicus* A), *An. karwari, An. maculatus, An. philippinnensis* and *An. tessellatus*. In addition, the Barbirostris Group is considered to include one of the most potential malaria vectors in Thailand (Rattanarithikul *et al*., 2006).

4. Malaria parasites

There are 4 generally recognized species of human malaria parasite in Thailand, including *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* (Chareonviriyaphap *et al*., 2000). *Plasmodium falciparum* and *P. vivax* are relatively common compared to *P. malariae* and *P. ovale* (BVBD, 2006). Despite control efforts and encouraging trends in dramatically reducing malaria, proportion of *P. falciparum* (from confirm microscopy) remains significantly greater than *P. vivax* (BVBD, 2003). In 2004, the distribution of malaria parasites originated from the confirmed microscopy for *P. falciparum* and *P. vivax* in Thai populations were 18,864 and 18,293, respectively. Recent observation in 2006 has documented approximately equal ratio of

those two main malaria parasites in the population with 14,123 for *P. falciparum* and 15,991 for *P. vivax* (Table 1).

Parasites resistant to drug remains one of the most serious problems in malaria controls. The highest rate of drug resistance has been recognized in Southeast Asia, including Thailand. For years, multi-drug resistance has contributed to the occurrence of malaria emergence in Thailand and has been attributed primarily to strong selection pressures on *P. falciparum* due to poor and incomplete compliance, indiscriminate or inappropriate therapy, and personal treatment (Chareonviriyaphap *et al*., 2000, BVBD, 2006). *Falciparum* malaria was first detected resistant to chloroquine in 1961 (Harinasuta *et al*., 1965; Kain *et al*., 1994; ter Kuile *et al*., 1992). Drug combination of sulfadoxine-pyrimethamine (S-P) and 4-aminoquinoline has been used in place of chloroquine in 1994 (Thaithong *et al*., 1988) (Figure 3). These drugs became less effective in malaria treatment and showed high level of resistance to *falciparum* malaria throughout the country, especially along the border areas near Cambodia (Prasittisuk, 1985; Tan-ariya *et al*., 1995). Consequently, a seven-day course of quinine-tetracycline was claimed as drug of choice in many malaria endemic areas where S-P resistance was detected (Pinichpongse *et al*., 1982). Melfloquine and S-P were strongly recommended for the treatment of *falciparum* malaria. Due to the prolong course of treatment, administrative problem, poor compliance, a single dose of mefloquine was launched. A few years later, partial resistance to mefloquine was found in *falciparum* malaria before spreading out in 1998, particularly along the Thai-Myanmar and Thai-Cambodia borders (Suebsaeng *et al*., 1986; Fevre *et al*., 1999). Since 1999, several drugs have been tested either alone or in combination to flight against *P. falciparum* strains.

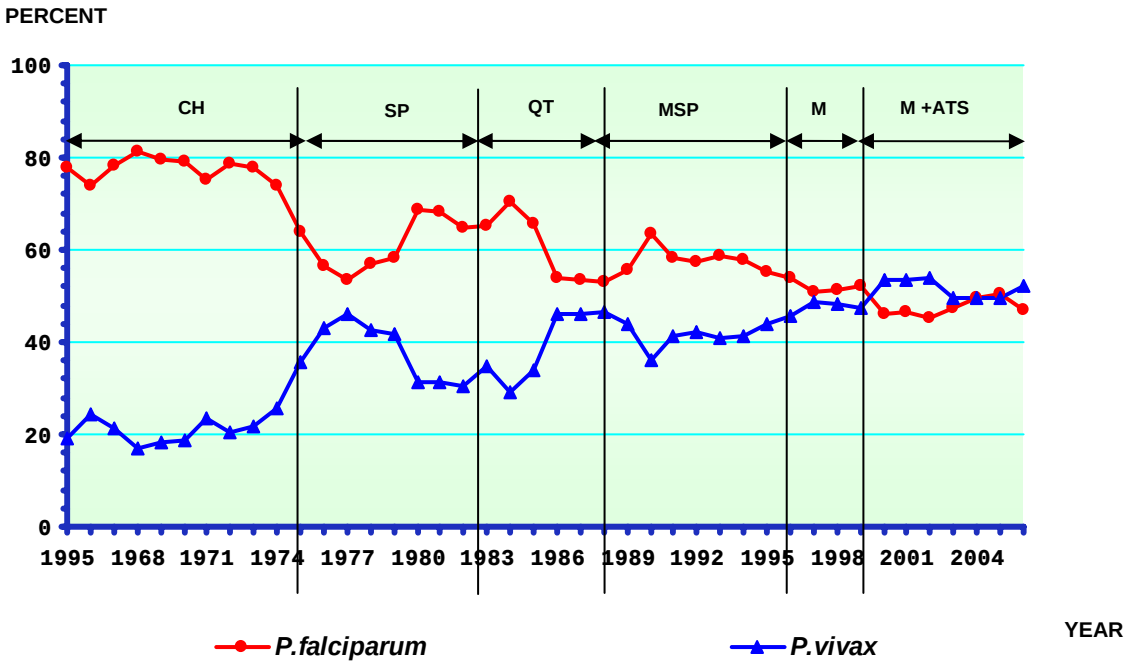


Figure 3. Proportion of malaria parasite species in relation to the National drug policy, Thailand (BVBD, 2007)

5. Species complexes and identification

5.1. Background

Species complexes include closely related species that are impossible to distinguish by morphological traits. The existence of cryptic species of *Anopheles* was first realized in the 1930s after it was ignored for years (Hackett, 1937). When some parts of Europe was malaria epidemiological paradox by absent of malaria whereas present of large quantity of mosquito vector, *Anopheles maculipennis*. This phenomenon is known as 'Anophelism without malaria' (White, 1978). In the laboratory investigating *An. maculipennis* Complex was discovered that included at least several vector and non-vector species. Both differed in their capacity for malaria transmission (Norris, 2002).

Difficulties in the study of species complex of mosquitoes include not exclusively strong morphological similarity among species, but also pronounced

morphological variation within species. Accurate species identification usually requires both adult and immature for morphological comparison. Harrison (1980) made extensive study of intra- and inter-specific variation in the morphology of *An. minimus* and its close relatives. On the basis confirmed identification by diagnostic morphological characters of the immature stages, 5-10% of the adult females would be misidentified (Harrison, 1980).

To facilitate this conclusion, there are a number of advance methods developed for identifying the species of individual specimens from these complexes as alternative tools to morphological diagnosis (Walton, 1999; Norris, 2002; Krzywinski and Besansky, 2003).

Ideal method should be fast, cost-effective, easy to implement, and applicable to both sexes and to all developmental stages. The method should be consistent and meaningful and produces the same result species-wide. This requires that the marker(s) on which the method is based is fixed within, and exclusive to, the species of interest. The difficulty in finding such markers increases with the recently of lineage splitting, larger descendant population sizes, and evolutionary conservation of the marker (Krzywinski and Besansky, 2003).

5.2. Cytogenetics

Cytogenetics involving comparison of mitotic and meiotic karyotypes and polytene chromosomes is known as the earliest tool for the study anopheline genetics (Norris, 2002). It is often useful in determining the phylogenetic affinities of closely related Dipteran species, and may suggest the direction and mechanisms of chromosomal evolution (White, 1978). Analysis of the banding pattern of polytene chromosome often reveals fixed differences between sibling species due to chromosomal inversions.

Chromosomal inversions provide the advantages to address phylogenetic issues regarding the origin, maintenance and introgression of inversions between

sympatric populations and taxa among the anopheline groups (Coetzee *et al*., 1999). Green *et al*., (1985) conducted a cladistic analysis of polytene chromosome rearrangements of six oriental anopheline mosquitoes including *An. minimus* Complex and provided a meaningful result for further population analysis.

Among the disadvantages of this technique in *Anopheles* mosquitoes, polytene chromosome preparations must be made only from ovarian tissue of the half gravid stage of adult females or stage of the fourth instar larvae (Norris, 2002). Interpretation of the banding pattern is time consuming and needs long term practices. The markers are not abundant or particularly informative in some species (Lounibos and Conn, 2000). Additionally, not all anophelines have discernible banding patterns and not all anopheline sibling species differ in banding patterns (Hunt *et al.*, 1998; Somboon *et al*., 2001). Despite many limitations, this method remains powerful for much of the contemporary work.

5.3. Allozyme analysis

Protein electrophoresis has been used extensively to discover and diagnose cryptic species through fixed allozyme differences (Krzywinski and Besansky 2003). Initial work utilizing these biochemical markers in *Anopheles albimanus* were able to document significant amounts of polymorphism among laboratory colonies that were used in linkage mapping studies (Narang *et al*., 1981). In Thailand, this technique was used to characterize the two sibling species of *An. minimus* complex and found that Octanol dehydrogenase (*Odh)* is the diagnostic marker in separating the two species (Green *et al*., 1990). In addition, *An. minimus* and species C were separated based on enzyme electrophoresis in Vietnam (Van Bortel *et al*., 1999). As a consequence, *Odh* enzyme is considered to be a standard marker in separating the two species of the Minimus complex.

The need to preserve enzyme activity dictates that specimens must be fresh or stored frozen and this requirement is sometimes difficult to meet under field condition. Moreover, this technique could not apply for those species that have not

been reproductively isolated for long period of time, given that it only detects the small fraction of variation causing amino acid replacements that affect net charge (Krzywinski and Besansky 2003).

5.4. DNA based techniques

Several approaches have been used in the study the species complex, including DNA technology. There has been a shift toward DNA-based methods of species identification because DNA is easily preserved by desiccation or immersion in alcohol and offers virtually unlimited polymorphic markers in coding and non-coding regions. This technique relies on probe hybridization or the polymerase chain reaction (PCR). The hybridization assays employ species-specific probes complementary to the families of highly repetitive DNA that differ in copy number or nucleotide sequence among related species (Crampton and Hill, 1997). The PCR assay strategies for species identification are to amplify anonymous regions of the genome or target specific sequences.

A significant advantage of PCR over other scientific approaches is the minute amount of template DNA usually required. Only a few scales or a leg segment is sufficient (Paskewitz and Collins, 1997), specimens can be kept alive for other experiments, submitted to other analyses, or preserved as morphological confirmation. Species-specific differences among PCR products similar in size may be detected by restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), or heteroduplex analysis (HDA).

Allele specific amplification (ASA) is based on Taq DNA polymerase. It will not extend primers that are mismatched to their template DNA (Ugozzoli and Wallace, 1992). PCR primers can therefore be designed in order to match one allele and, providing that the allele is specific to a certain species, a PCR product will exclusively be generated with that primer from the genomic DNA of the corresponding species. Because of the need to design such species-specific primers, sequence information is required for this method. The great advantage of ASA over the previous

two methods is that identification can be a one step procedure needing only a single PCR reaction to distinguish a number of different species.

5.4.1. Mitochondrial DNA

The use of mitochondrial DNA (mtDNA) sequence data has become standard for many phylogenetic studies (Caterino *et al*., 2000). The sequence and genome organization of the *An. gambiae* mitochondrial genome was published for decades ago (Beard *et al*., 1993). Among the many mitochondrial genes that have been studied, cytochrome oxidase subunit II (COII) region has been extensively used for phylogenetic inference by itself or in combination with other sequences, and has proven phylogenetically informative in *Anopheles* mosquitoes (Sharpe *et al*., 1999, 2000; Chen *et al*., 2002,2003; Garros *et al*., 2006). Sharpe *et al*., (2000) confirmed the presence of species complex of *An. minimus* in Thailand and inferred the phylogenetic relationships among six taxa of the minimus group based on the COII locus. The 685 bp consistent length of COII sequences are also reported. However, the mitochondrial DNA has failed to differentiate cryptic taxa within the *An. maculipennis* complex (Collins *et al*., 1990) and *An. gambiae* s.l. (Caccone *et al*., 1996; Besansky *et al*., 1997; Lehmann *et al*., 1997; Thelwell *et al*., 2000).

5.4.2. Ribosomal DNA

The most widely applied PCR assays target nuclear ribosomal DNA (rDNA), present at a single X-linked locus in anophelines (Rai *et al*., 1999). It is organized as a tandem-repeated array of conserved genes (18S, 5.8S, and 28S) punctuated by rapidly evolving noncoding spacers: internal transcribed spacers 1 and 2 (ITS1, ITS2) and the intergenic spacer (IGS). Researchers have discovered that the nucleotide sequence of these spacer regions are often much more polymorphic among species than within species. This makes this region of the genome useful for delineating molecular differences among cryptic species by length or sequence polymorphisms (Fritz *et al*., 1994; Marrelli *et al*., 1999; Hackett *et al*., 2000). Most recently developed species identification assays on the Minimus Complex are based on differences within

the ITS2 (Sharpe *et al*., 1999, 2000; Van Bortel *et al*., 1999, 2000; Phuc *et al*., 2003; Garros *et al*., 2004a, 2004b, 2005a). The D3 variable region of the 28S gene also have been exploited successfully (Sharpe *et al*., 1999, 2000; Chen *et al*., 2002, 2003; Somboon *et al*., 2001; Garros et al., 2005a, 2005b).

5.4.3. Highly polymorphic markers

5.4.3.1. Microsatellite DNAs

Microsatellites are abundant, tandem repetitive and highly polymorphic DNA within anopheline genomes become a useful tool for population genetic study. This tool facilitates researchers to easily select microsatellite markers that represent the entire mosquito genome or to target specific regions of the genome, including qualitative and quantitative traits (Zheng *et al*., 1997; Lanzaro *et al*., 1998; Wang *et al*., 2001). Many investigators have taken advantage of the high polymorphism of these markers for study of the population structure within this complex of mosquitoes, both at the micro- and macro-geographic scales (Lanzaro *et al*., 1995). The vast majority of this work continues to focus on populations of *An. gambiae* Complex (Lehmann *et al*., 1996, 1997; Lanzaro *et al*., 1998; Kamau *et al*., 1998). Lehmann *et al*. (2003) found more differences between *An. gambiae* from each side of the Rift Valley and in paradox little differences between populations from northwestern and southeastern Africa.

Concerns have been raised to the potentiality of mutations in the flanking regions causing inaccuracies in determination of the fragment sizes (Walton *et al*., 1998). Although such mutations are likely to occur, the frequency is expected to stay low and the solution for population analyses where sequencing each allele would be unreasonable, is to analyze a larger set of microsatellite loci.

5.4.3.2. RAPDs and SCARs markers

Random amplified polymorphic DNA (RAPD-PCR) relies on the analysis of banding patterns generated with arbitrary decamer primers, it requires no prior knowledge of the genome and tends to target repetitive and rapidly evolving genome regions. Random amplified polymorphic DNA (RAPD) markers have been utilized to distinguish between *An. gambiae* and *An. arabiensis* (Wilkerson *et al*., 1993). The notoriously inconsistent of RAPD-PCR technique for the identification the *An. minimus* Complex were reported (Sucharit and Komalamisra, 1997; Kengne *et al*., 2001). Nevertheless, RAPD fragments specific for *An. minimus* A and C, also called SCAR (sequence characterized amplified region) were cloned and sequenced to design and develop more robust allele-specific primers for species identification.

6. The Minimus Complex

Anopheles minimus Theobald was described in 1901, and currently the Minimus Complex is composed of the 3 sibling: *Anopheles minimus*, species C, and species E (Harbach, 2004; Somboon *et al*., 2001). The Minimus Complex is now classified in the Minimus Subgroup, within the Funestus Group of Myzomyia Series (Harbach 2004; Garros *et al*., 2005a, b) (Figure 4).

Anopheles minimus is considered one of the principal malaria vectors in tropical Asia. *Anopheles minimus* has wide distribution in Asia. Its range extends from India and Srilangka across mainland southeastem Asia to Japan and from China (under 30°N latitude) to Thai-Malaysian border. *An. minimus* does not penetrate far into the equatorial rain forest of Malaysia and Bomeo Island, probably because these countries are too uniformly wet throughout the year and lack any marked dry season (Reid 1968). *Anopheles minimus* Complex occurs sympatrically on the Asian continent with 8 other closely related species: *An. aconitus*, *An. filipinae*, *An. flavirostris*, *An. fluviatilis*, *An. jeyporensis*, *An. mangyanus*, *An. pampanai*, and *An. varuna* which have highly morphological variation. It is only 90-95% level of identifable by adul morphology (Harrison, 1980). Associated immature skins can be useful for differentiating the

species, except those of the species complex. *An. minimus* was suspected to be a species complex based on differences in egg morphology (Baba 1950), and ecological and behavioral heterogeneities (Nutsathapana *et al*., 1986). Two forms, designated A and B, were described in China based on morphological features in larvae, pupae and adults (Yu and Li, 1984; Yu, 1987) and form B was subsequently regarded as a species. However, molecular identification showed no taxonomic significance between these forms and were concluded to be only morphological variants of the same biological species. (Chen *et al.*, 2002)

The genetic evidence for the Minimus Complex was first provided for populations in Thailand (Green *et al*., 1990), then confirmed in Vietnam (Van Bortel *et al*., 1999), where sympatric homozygotes at the *Odh* (Octanol deshydrogenase) locus occurred in the absence of heterozygotes. The species were informally named species A and C (Green *et al*., 1990), the latter designated to distinguish it from form B previously described in China (Yu and Li, 1984). The Minimus Complex was confirmed using molecular markers. The single-strand conformational polymorphism-polymerase chain reaction (SSCP-PCR) assay (Sharpe *et al*., 1999). Recently, Chen *et al*., (2002) showed that forms A and B in China are morphological variants of *An. minimus*. In Japan, Somboon *et al*., (2001, 2005) provided morphological, cytogenetic, molecular and hybridization evidence for the recognition of another sibling species, designated species E, on the Ishigaki Island of the Ryukyu Archipelago. Crossing experiments between species E and either species A or C showed that F1 crosses were sterile (Somboon *et al*., 2001, 2005). Both studies showed hybrid male sterility, which is generally accepted as very clear evidence of specific status. There is now no reason to cast doubt on the specific status of species E (Van Bortel and Coosemans, 2003; Walton and Somboon, 2004). However, the complex may include two other species, species D (Baimai, 1989) and specimen no. 157 (Sharpe *et al*., 1999) in Thailand. The specific status of these two entities is uncertain and needs further study. It seems that species D is a chromosomal variant of *An. minimus*. It is difficult to assess the status of specimen no. 157 because only a single individual of this molecular variant (variation of two nucleotides of 313 on D3 domain of the 28S subunit, ribosomal DNA) is known.

The habitat of *An. minimus* is foothill and hilly regions with small, cool, clear-water streams. The larvae and immature adults can usually collected from stream margins, rock pools, sand pools next to streams, seepage pools in spring and fallow rice fields in hilly region (Harrison, 1980, Rattanarithikul et al. 1996).

In Thailand, *An. mmimus* was recorded in 1923 and has been recognized as a primary vector of malaria in the country since 1935 (Harrison, 1980). *An. minimns* has been known as primarily anthropophilic, endophagic and sometimes endophilic habit. It is no longer found in the plains but only in forested foothill areas Recent studies, however, indicate behavioral changes of *An. minimus* towards zoophilic and exophagic in areas where DDT has been applied. Densities of *An. minimus* vary from month to month and are associates mostly with the availability of its breeding places. Ismail *et al*., (1974) reported from forest fringe areas near perennial streams on northem-central Thailand that the species appeared in high densities at the beginning (November) and remained at high density for the major part of the dry season (December-February). The density dropped during the later part of the dry season (March-April). A second peak ofhigh density appeared in the earlier part of the rainy season (May-June), then declined to reach the lowest density at the second half of the rainy season (August-September). These authors explained the first drop in population density of *An. minimus* by the partial drying up of and second drop by the washing out of breeding places.

Genus *Anopheles*

Subgenus *Cellia*

Myzomyia series

Funestus Group (Garros *et al.*, 2004a,b2005a,b)

jeyporiensis James

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

aconitus Donitz

filipinae Manalang

mangyanus (Banks)

pampanai Btittiker & Beales

varuna Iyengar

Culicifacies Subgroup (Garros *et al.*, 2004a, b, 2005a,b)

culicifacies Giles (species A, B, C, D and E) (Kar, 1999)

Funestus Subgroup (Garros *et al.*, 2004a, b, 2005a,b)

aruni Sobti

confusus Evans & Leeson

funestus Giles

parensis Gillies

vaneedeni Gillies & Coetzee

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

flavirostris (Ludlow)

leesoni Evans

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

fluviatilis James (species T and U)

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

minimus Theobald

minimus C (Green *et al.*, 1990)

minimus E (Somboon *et al.*, 2001)

Rivulorum Subgroup (Garros *et al.*, 2004a, b)

brucei Service

fuscivenosus Leeson

rivulorum Leeson

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

Figure 4. Taxonomic hierarchy of *Anopheles minimus* Complex (Harbach, 2004)

MATERIALS AND METHODS

Part 1. Diagnostic character for the identification of the two sibling species of the Minimus Complex

1. Study Area

The study was conducted from twelve locations in Thailand (Figure 5), including 2 villages from Chiang Mai and Tak in the northern part, 5 villages from Kanchanaburi in the western part, 2 villages from Chantaburi the eastern part, and 3 villages from Surat Thani in the southern part. Detail of locations was given below.

Location #1. Ban Pu Teuy (14º17′N, 99º11′E) is a village in Sai Yok District, Kanchanaburi Province. The site is located in mountainous terrain surrounded by deep and intact forest. There is a narrow (2 m wide), slow running stream, covered with native vegetation along its margin near the collection site (Chareonviriyaphap *et al*., 2003). This stream is known as a potential habitat for *An. minimus* larvae (Baimai, 1989). IRS using by DDT was applied in this area for 60 years (1940-2000). No IRS was carried out in the area during the course of our observation period.

Location #2. Ban Tha Sao (14º6′N, 99º24′E), a village in Saiyok District, Kanchanaburi Province. This area is comparatively dry but has a temporal stream in wet season. The site is 1.6 km away from the Khew River and there are many ethnic groups living in this area. Fruit orchards and bamboo vegetation are prevalence in the area. No IRS was applied in this area.

Location #3. Ban Mae Kanin Nua (18º47′N, 98º43′E) is located in Hangdong District, Chieng Mai Province. The area is surrounded by rice field. There are many streams around the area. IRS using DDT was applied in this area and completely stopped during the period of collection.

Location #4. Ban Nam Dip (16º 41′N, 98º 41′E) is a village in Mae Sot District, Tak Province. It is surrounded by rice field on the east and forest on the west. There is a 3 m-width running stream bordered by a variety of plants all along its margins. IRS using DDT was applied in this area.

Location #5. Ban Tai Maung (14º6′N, 99º0′E) is a village in Bong Tee canton, Saiyok District, Kanchanaburi Province. The site is located in mountainous terrain, surrounded by rice field and forest. There are many streams around the area. IRS using deltamethrin was applied in this area.

Location #6. Ban Mae Num Noi (14º25′N, 98º50′E) is a village in Thongpaphum District, Kanchanaburi Province. The area is surrounded by rice field and forest. There are many streams around the area. IRS using deltamethrin was applied in this area.

Location #7. Ban Bong Tee Noi (14º7′N, 99º8′E) is a village in Vang Kra Jae canton, Saiyok District, Kanchanaburi Province. There is a seasonal running stream bordered by grasses along its margins in dry season. The stream becomes a river in wet season. The site is surrounded by fruit orchards and forest.

Location #8. Soidao District, Chantaburi Province, Eastern Thailand. The collection site is surrounded by fruit orchard. There is an irrigating stream near the site.

Location #9. Kang Hang Meo District, Chantaburi Province (12º58′N, 101º54′E). The collection site is surrounded by rubber tree orchard. There is slow running stream near the site.

Location #10. Nasan District, Surat Thani Province, Southern Thailand (8º48′N, 99º22′E). The collection site is located in mountainous terrain surrounded by fruit orchard. Close to the site, there is slow running stream covered with native vegetation.

Location #11. Panom District, Surat Thani Province (8º49′N, 98º49′E). There are two collection sites in this area. One is located near the main road, surrounded by grass field. Near the site, there is a slow running stream covered with shed trees. The other is surrounded by rubber tree orchard. There is slow running stream near the site.

Location #12. Donsak District, Surat Thani Province (9º18′N, 99º40′E). The collection site is surrounded by fruit, palm oil tree, rubber tree and fruit orchards. There is an irrigating stream near the site. IRS using bifenthrin was applied in this area. No IRS has been conducted since 1999.

Figure 5. Map of mosquito collection sites

2. Mosquito Collections

For Ban Pu Teuy (location #1) and Ban Tha Sao (location #2), female mosquitoes were collected during three consecutive nights per month. Each night, three collection methods were done, including indoor and outdoor human landing and cattle collections from 1800 and 0600 hr. The collectors were divided into two teams, one working from 1800 to 2400 hr and the second team from 2400 to 0600 hr. Indoor collectors were placed at the corridor of the house and the outdoor collectors were 10 meters from the house. Hourly period of collecting on human was 45 min with 15 min break. The collector on cattle aspirated the mosquitoes resting on the cow net, 4 x 4 x 3 m size, for 15 min every hour. All mosquito cages were covered with cotton soaked with sugar and kept in plastic bag for morphological identification until the following morning.

For the other ten locations, female mosquitoes were collected by either outdoor human biting or cow traps for two to three consecutive nights, depending on the budget and time. Each site, one collection was made in the wet season. The purpose of these collections was to do a preliminary survey on the distribution of the Minimus Complex on the other plain areas of Thailand.

3. Morphological identification

Female mosquitoes were identified in the field in the next morning using the morphological keys of Peyton and Scanlon (1966) and Rattanarithikul and Punthusiri (1994). Specimens belonging to *An. minimus* Complex were identified as *Anopheles minimus* if the PSP phenotype was present, while specis C was identified with the presence of the HP phenotype (Figure 6)

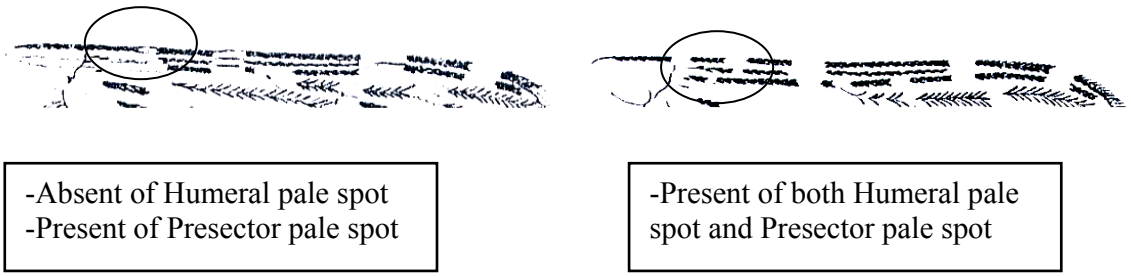


Figure 6. HP phenotype and PSP phenotype (Rattanarithikul and Punthusiri, 1994)

4. Molecular identification

Following morphological identification, specimens of *Anopheles minimus* Complex were shipped to the Center of Biology and Management of Population, Institute of Research for Development, Montpellier, France. Molecular analysis was performed using an allele-specific multiplex assay (Garros *et al*., 2004b). Specimens were individually ground and genomic DNA was extracted from the whole body of mosquito according to the procedure of Collins *et al*., (1987). After extraction, 3 μL of each DNA sample was diluted 20 times as DNA template. The ITS2 regions of DNA were amplified using a multiplex PCR method described by Garros *et al*., (2004b) and species-specific primers were used in the one-step reaction to differentiate *An. minimus* and species C. In a final volume of 25 μL, PCR amplification conditions were as follows: 2.5 μL of 10x reaction buffer (Qiagen), 200 μM of each dNTP, 0.16 nmol of each primer, 0.5 units of *Taq* polymerase (Qiagen), and 2 μL of DNA template. The PCR cycles were as follows: one cycle at 94°C for two minutes, followed by 40 cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 40 seconds each. An additional autoextension cycle at 72°C for five minutes was included at the end. The PCR products were subjected to electrophoresis on a 3% agarose gel at 100 V for 30 minutes and stained with ethidium bromide.

5. Reliability test of the presence/absence of the HP spot for the identification

Reliability test of the presence and absence of the HP spot for identification was conducted on test populations. Five test populations were obtained from Ban Pu Teuy, Sai Yok District, Kanchanaburi Province (location# 1) and one test population was collected from Ban Nam Dip, Mae Sot District, Tak Province (TT) (location #4). Test populations collected from location#1 include TKM, TKi04, Tko04, Tkc04, and Tko03. Three test populations from Thailand (Green *et al*., 1991), Vietnam (Van Bortel *et al*., 1999) and China (Chen *et al.*, 2002) were included and served as control point. Detail of each test population is given below.

TKM population was the adult males of *An. minimus* Complex emerged from larvae and pupae collected along the margins of the slow-running stream in September 2003.

TKi04 population was the adult females of *An. minimus* Complex obtained from indoor human landing collections during January to August 2004.

TKo04 population was the adult females of *An. minimus* Complex obtained from outdoor human landing collections during January to August 2004.

TKc04 population was the adult females of *An. minimus* Complex obtained from cow bait collections during January to August 2004.

TKo03 population was the adult females of *An. minimus* Complex obtained from outdoor human landing collections in August 2003.

TT population was the adult females of *An. minimus* Complex obtained from outdoor human landing collections in June and August 2003 from the Tak Province.

To test the reliability of the presence/absence of the HP spot to clearly identify the species of the Minimus Complex, biomedical tests was used (Altman 1991)

to evaluate diagnostic power of the morphological character. Several values provide insights on the reliability of the test. The sensitivity and specificity were calculated, by comparing the observed test outcome with the produce of the golden standard, i.e. the molecular identification. Another way to characterize a diagnostic test was to calculate the proportion of correctly classified individuals as an index of validity (Iv). The index of validity is the probability of agreement between the molecular and the morphological identifications. The positive predictive value (PPV) provides the probability of having the species C specimen if the HP phenotype is present. There is a corresponding negative predictive value (NPV) predicting the probability of rightly identifying *An. minimus* if the PSP phenotype is present.

Part 2. Host seeking behavior and biting activity between the two sibling species of the Minimus Complex based on molecular identification

1. Study Area

The study was conducted from Ban Pu Teuy (location# 1) and Ban Tha Sao (location# 2) from Saiyok District, Kanchanaburi Province (Figure 7).

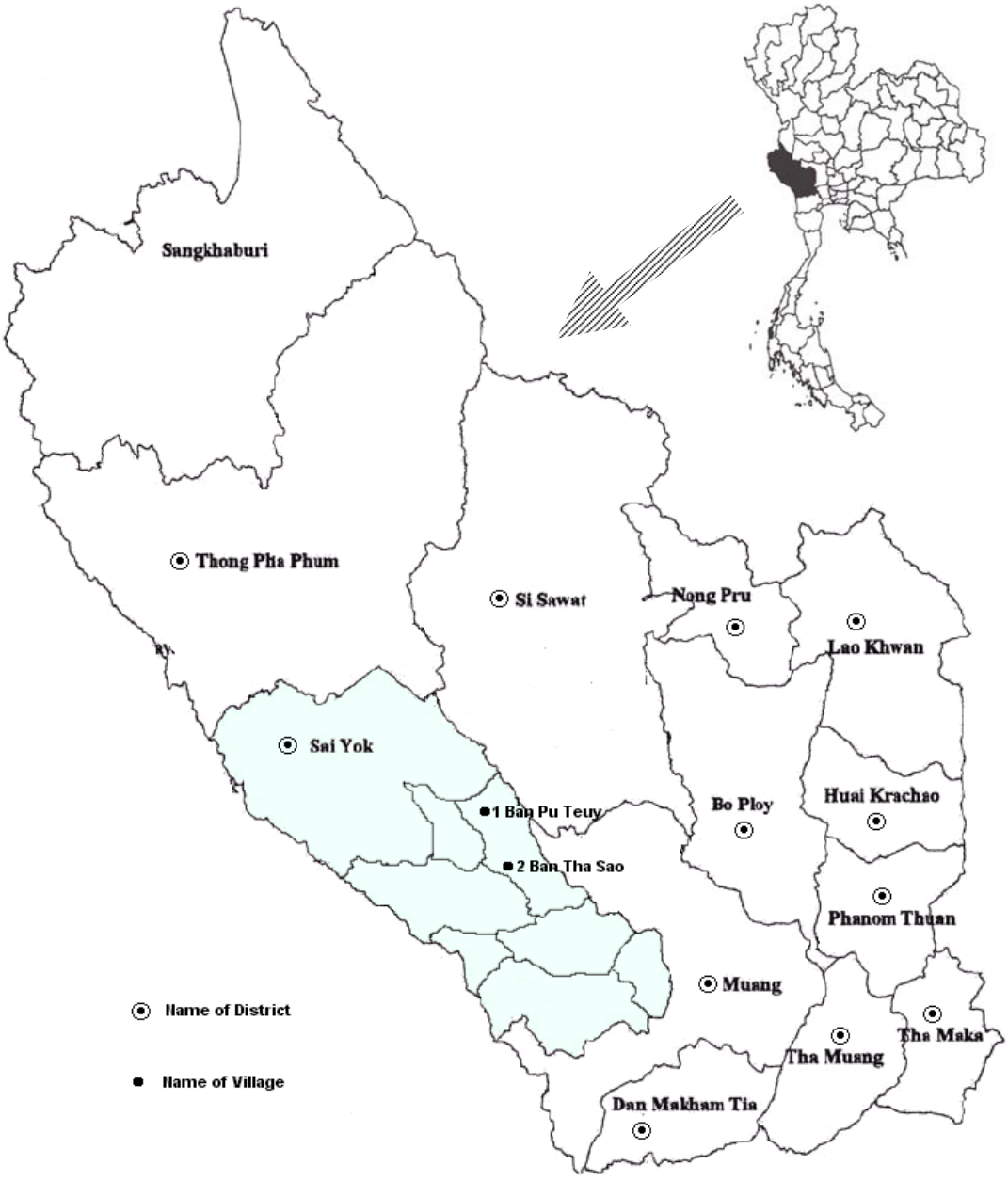


Figure 7. Map of collection sites at Ban Pu Teuy and Ban Tha Soa, Sai Yok District, Kanchanaburi Province (modified from Kengluecha *et al*., 2005)

2. Mosquito Collection

Adult female mosquitoes were collected during three consecutive nights per month during February 2004 to January 2006. Three collection methods, indoor human

landing, outdoor human landing, and cow bait collections, were employed. The collectors were divided to two teams of four persons each. The first team worked from 1800 to 2400 hours and second team worked from 0000 to 0600 hours. Method of human landing collection has already been described in Part 1. Cow bait collection was done by two collectors at 10 min each hour. Mosquitoes collected were kept in a plastic cup covered with cotton soaked sugar and placed in plastic bag for morphological identification in the following morning. Hourly ambient outdoor temperature and relative humidity were recorded. Rainfall data was obtained from the local Metrological Station located in the Sai Yok District, Kanchanaburi Province.

3. Morphological Identificatio**n**

Mosquitoes were identified using the morphological keys of Peyton and Scanlon (1966) and Rattanarithikul and Panthusiri (1994). Specimens belonging to *An. minimus* complex were identified as *An. minimus* when the humeral pale spot (HP) is absent on costal vein of both wing whereas as species C when the HP is present at least on one wing.

4. Molecular Identification

Specimens of *An. minimus* complex were individually subject to DNA extraction following the technique of Collins *et al*., (1987). Molecular analysis was performed by the Allele-Specific assay as previously described in Part 1.

5. Data analysis

Seasons, time periods and biting habits were selected as the main factors in human/cow landing collections. Seasons were composed of 3 periods: dry (December to February), hot (March to May) and wet (June to November). Time periods were divided in 4 including evening (1800-2100 hours), late night (2100-2400 hours), before dawn (2400-0300 hours) and dawn (0300-0600 hours). Biting habit of *An. minimus* was classified as human indoor, human outdoor and cow bait. Nocturnal blood-feeding

cycles of *An. minimus* were tabulated by number per human per night for indoor and outdoor collections and by number per cow per night for cattle collection. The differences in number for mosquitoes landing were analyzed by three–way analysis of variance, with year as block factor and differences among groups determined by Duncan multiple range tests. All data were analyzed using SAS program package (SAS Release 6.10, SAS Institute, Cary, NC).

RESULTS

Part 1. Possible diagnostic character for the identification of the two sibling species of the Minimus Complex

Of the twelve locations, two locations (locations# 1 in Kanchanaburi Province and 4 in Tak Province) were found positive for both *An. minimus* and species C. Location# 1 includes five test populations as follow; TKM, TKi04, TKo04, TKc04, and TKo03 and the location #4 is TT.

A total of 400 mosquitoes were DNA extracted and identified with both morphological and molecular methods (Table 3). Only one specimen was morphologically identified as *An. minimus* but was molecularly identified as *An. varuna* (location #1), therefore it was deleted from the analysis. Three test populations from previously described studies were used as references (Chen *et al*., 2002; Green *et al*., 1990; Van Bortel *et al*., 1999), representing an additional sample of 1,430 *An. minimus s.l*., for a total of 1,830 specimens. All the indexes were calculated and are presented in Table 3.

All the specimens of the male population (TKM) from Kanchanaburi Province were misidentified, leading to an index of validity (Iv) of 0. For the other populations, the index of validity ranged from 0.543 (CC population) to 0.961 (TKo04 population). The positive predictive value was maximum (PPV=1) for the 3 populations TKi04, TKo04 and TKc04. This value ranged from 0.690 (CC population) to 0.976 (TKo03 population) for the 5 remaining populations. The negative predictive value (NPV) indicated the correct identifications of *An. minimus* when the PSP phenotype is present. This value fluctuated between 0.283 (TG population) and 0.667 (VVB population), with a value of 0 for the TT population. This extreme value was most likely due to the low number of identified samples. The mean probability of having a correct identification of *An. minimus* based on the PSP phenotype (mean NPV) is significantly lower than the mean probability of carrying a good identification of species C if the HP phenotype (mean PPV) is present ($p<0.01$).

The PPV was not significantly different between the four populations collected in the Kanchanaburi Province in 2003 and 2004 (TKi04, TKo04, TKc04, and TKo03). No statistical difference was noted between PPVs of the three collection methods used in Kanchanaburi Province. Therefore, the HP phenotype is not linked to the trophic behavior. The TG population, the collected in 1984 and 1987 (Green *et al*., 1990) was not different from the five populations collected in Thailand (the four latter ones and TT). This may indicate that there are little temporal variations in the HP phenotype in this region. However, the PPV of the TT population was significantly different from the one of TKo04 and TKo03 populations, both collected by outdoor human landing. Moreover, the PPV of the VVB and CC populations, respectively from Vietnam and China, were significantly different from all the other populations, except VVB with TT. No difference between the PPV values has been noted for the VVB and CC populations. These differences may represent spatial variations of the phenotypes.

The negative predictive value (NPV) was not significantly different between the three methods of collections in Kanchanaburi Province (TKi04, TKo04, and TKc04) (Table 4). The PSP phenotype does not appear to be linked to the trophic behavior, nor is the HP phenotype. Temporal variations of the PSP phenotype are revealed between the TG and TKi04; TKo04 and TKc04 populations, TKi04 and TKo03 populations but not between the TG and TKo03;. Large spatial variations of the PSP phenotype were noted between the populations from Vietnam (VVB) and China (CC), and between the CC and VVB populations and the Thai populations.

Table 3. Characteristics of *An. minimus* Complex test populations

Population Code	Country, Locality	Collection Date and Methods	Sex	Number	Molecular Identification	Reference
TKM	Thailand, Kanchanaburi Province	September 2003 Immature collections	M	37	AS-PCR	Present work
TKi04	Thailand, Kanchanaburi Province	January to August 2004 Monthly indoor human landing collections	F	50		
TKo04	Thailand, Kanchanaburi Province	January to August 2004 Monthly outdoor human landing collections	F	102		
TKc04	Thailand, Kanchanaburi Province	January to August 2004 Monthly cattle collections	F	121		
TKo03	Thailand, Kanchanaburi Province	August 2003 Outdoor human landing collections	F	63		
TT	Thailand, Tak Province	June and August 2003 Outdoor human landing collections	F	27		
			Total	400		
TG	Thailand, Kanchanaburi Province	1984 and 1987 Human landing, cattle, and immature collections	F	263	Isozyme	Green *et al*., 1990
VVB	Vietnam, Hoa Binh Province	June-November 1995 Indoor and outdoor human landing, cattle and indoor resting	F	911		Van Bortel *et al*., 1999
CC	China, several localities in southern China	July-september 2000 Human landing, cattle and immature collections	M	256	SSCP-PCR	Chen *et al*., 2002
			Grand Total	1,830		

Table 4. Identification results and indexes

Population Code[1]	Morphological identification	Molecular identification		Specificity	Sensitivity	Iv	PPV	NPV
		Species C	*An. minimus*					
TKM	Species C	0	0	xx	0	0	xx	xx
	An. minimus	37	0					
TKi04	Species C	43	0	1	0.915	0.920	1	0.429
	An. minimus	4	3					
TKo04	Species C	92	0	1	0.958	0.961	1	0.600
	An. minimus	4	6					
TKc04	Species C	89	0	1	0.840	0.860	1	0.469
	An. minimus	17	15					
Tko03	Species C	40	1	0.875	0.727	0.746	0.976	0.318
	An. minimus	15	7					
TT	Species C	23	1	0	0.885	0.852	0.958	0
	An. minimus	3	0					
TG	Species C	131	5	0.878	0.590	0.635	0.963	0.283
	An. minimus	91	36					
VVB	Species C	26	6	0.990	0.082	0.672	0.813	0.667
	An. minimus	293	586					
CC	Species C	20	9	0.930	0.156	0.543	0.690	0.524
	An. minimus	108	119					
MEAN						0.774 ± 0.148	0.880±0. .125	0.470±0.140

[1] Details of the test populations were described in Table 3

[2] xx = cannot be calculated

Part 2. Host seeking behavior and biting activity between the two siblings of the Minimus Complex based on molecular identification

Adult survey on Anopheline diversity was obtained from February 2004 to November 2006 at Ban Pu Teuy, Saiyok District, Kanchanaburi Province, west Thailand and results are presented in Table 6. *Anopheles* identification was made following the morphological keys to adult of Thailand (Harrison 1980, Rattanarithikul and Panthusiri 1994). Member of three Anopheline vectors was collected in the study area with the majority of *An. minimus* Complex throughout the year. Within *An. minimus* complex, molecular method was used to finalize species identification, resulting in the two species of *An. minimus* and species C. *Anopheles minimus* and species C were found sympatrically, with high proportion of species C (94%) as compared to *An. minimus* (6%). In addition to *An. minimus* s.l., *An. maculates* s.l. and *An. dirus* s.l. mosquitoes, important malaria vectors in Thailand, were also found but in a small proportion as compared to *An. minimus*. These two species were found more abundant during the wet season, especially from June to September of the year (Table 6). *Anopheles maculatus* Complex and *An. dirus* Complex consisted of 20.69 and 10.69% of the total mosquito collected, respectively. Among *An. maculatus* Complex, three related species were observed, including *Anopheles maculatus*, *Anopheles sawadwongporni*, and *Anopheles notanandai* .

Anopheles minimus s.l. was found to be the most common taxon encountered, comprising 68.57% of the total anopheline mosquitoes. Since comparatively low numbers of *An. maculatus* s.s. and *An. dirus* s.l. were collected, investigation targeted mainly *An. minimus.* Results described the biting behavior and host prevalence between *An. minimus* and species C, important malaria vectors in Thailand.

Specimens of *An. minimus* Complex were identified by morphological method and later confirmed by molecular methods, a multiplex PCR assay, to obtain the precise identification. Among the *An. minimus* Complex, the species C was collected in a high proportion (94%) whereas relatively low number of *An. minimus* was observed, comprising 6% of the total population throughout the year (Table 6).

Landing rates of *An. minimus* and species C were observed from human indoor, outdoor and cow bait collections during one-year observation period (Figures 8-10). Generally, species C exceeded *An. minimus* from all types of landing collection methods. For species C, two peaks indoor biting activities were observed, with a major peak activity from 1800-2100 hours and a minor peak from 2300 to 0200 hours (Figure 8). An outdoor peak activity of species C was observed right before dusk, reaching a peak at 1900 hours, before dramatically declining onward (Figure 9). Cow bait catches showed a prominent peak of species C at 1900 hours prior to decreasing throughout the period of the night (Figure 10). With *An. minimus*, no distinct indoor and outdoor peaks was observed throughout the night (Figure 8 and 9). Both *An. minimus* and species C were found more attractive to cow than to human with a peak at 1800-2000 hours.

Total numbers landing per hour were used in a three way analysis of variance, with seasons, collection methods (indoors, outdoors, and cow), and time periods as key factors. With species C, significant differences were found among seasons ($F=15.23$; $df=2, 11$ $P<0.0001$), among collection methods ($F=8.95$; $df=1, 11$, $p=0.0007$) and among time periods ($F=12.98$; $df=1,11$, $P=0.0007$). Strong interactions between seasons and collection methods were observed ($F=5.87$; $df=2, 23$, $P=0.0007$). There was no interaction between seasons and time periods ($F=1.89$; $df=6$, $P=0.123$), between time periods and collection methods ($F=1.56$; $df=1,11$, $P=0.24$), and among seasons, time periods and collection methods ($F=1.09$; $df=6, 23$, $P=0.123$). Due to small number of specimen of *An. minimus*, interaction between factors involved can not be assessed.

Table 6. Monthly frequency of *Anopheles* mosquitoes at Ban Pu Teuy, Sai Yok District, Kanchanuburi Province, for 2 years (February 2004-January 2006).

Month	*An. minimus*		Species C		*An. dirus* Complex.		*An. maculatus*		Total
	No	%	No	%	No	%	No	%	
Year 1									
Feb	46	11.9	335	86.6	1	0.3	5	1.3	387
Mar	15	6.9	192	88.6	0	0.0	9	4.2	216
Apr	7	12.3	46	80.7	3	5.3	1	1.8	57
May	21	6.0	215	66.4	47	13.4	50	14.2	333
Jun	28	4.3	256	39.0	139	21.2	233	35.5	656
Jul	21	4.5	150	32.0	42	9.0	256	54.6	469
Aug	8	6.4	63	50.4	38	30.4	16	12.8	125
Sep	4	3.9	39	38.2	47	46.1	12	11.8	102
Oct	3	3.8	61	77.2	8	10.1	7	8.9	79
Nov	7	4.1	132	78.1	1	0.6	29	17.2	169
Dec	15	6.8	195	88.2	0	0.0	11	5.0	221
Jan	9	4.1	206	94.9	0	0.0	2	0.9	217
Year 2									
Feb	16	6.9	214	93.1	0	0.0	0	0.0	230
Mar	20	3.7	513	95.6	0	0.0	4	0.7	537
Apr	20	3.7	444	78.2	0	0.0	2	0.1	466
May	13	5.7	178	83.5	5	2.3	18	8.5	214
Jun	19	3.2	380	65.2	63	10.7	123	20.9	585
Jul	3	2.3	45	34.4	47	35.8	36	27.5	131
Aug	3	0.7	97	25.4	79	19.5	221	54.4	400
Sep	6	4.8	44	35.5	74	59.7	0	0.0	124
Oct	13	5.3	156	64.8	73	29.9	0	0.0	242
Nov	22	7.1	263	92.6	1	0.3	0	0.0	286
Dec	9	4.5	218	95.5	0	0.0	0	0.0	227
Jan	13	5.7	216	94.3	0	0.0	0	0.0	229
Total	**341**	**5.6**	**4658**	**76.7**	**668**	**11.0**	**1035**	**6.7**	**6702**
Frequency per complex									
	Total 4999		**74.6%**		**10.0%**		**15.4%**		

Table 7. Total monthly collections from three collection methods of *Anopheles minimus* and species C during February 2004 – January 2006

Month	Indoor			Outdoor			Cattle bait		
	An. minimus	**Species C**	**% Species C**	***An. minimus***	**Species C**	**% Species C**	***An. minimus***	**Species C**	**% Species C**
Year1									
Feb	1	3	75.0	6	76	92.7	39	256	86.8
Mar	0	12	100.0	3	28	90.3	12	152	92.7
Apr	2	8	80.0	0	5	100.0	5	33	86.8
May	2	21	91.3	6	26	81.3	13	168	92.8
Jun	0	9	100.0	3	29	90.6	25	218	89.7
Jul	0	10	100.0	2	15	88.2	19	125	86.8
Aug	2	4	66.7	0	10	100.0	6	49	89.1
Sep	0	8	100.0	0	4	100.0	4	27	87.1
Oct	0	0	0.0	3	14	82.4	0	47	100.0
Nov	0	2	100.0	1	19	95.0	6	111	94.9
Dec	0	4	100.0	4	67	94.4	11	124	91.8
Jan	0	1	100.0	3	31	91.2	6	174	96.7
Year2									
Feb	0	0	0.0	0	20	100.0	16	194	92.4
Mar	0	2	100.0	4	76	95.0	16	435	92.4
Apr	0	1	100.0	1	17	94.4	19	426	95.7
May	1	2	66.7	0	15	100.0	12	161	93.1
Jun	1	27	96.4	4	67	94.4	14	186	95.3
Jul	0	2	100.0	0	4	100.0	3	39	92.9
Aug	0	9	100.0	0	38	100.0	3	50	94.3
Sep	1	0	0.0	0	1	100.0	5	43	89.6
Oct	0	4	100.0	3	24	88.9	10	128	92.8
Nov	0	1	100.0	8	51	86.4	14	211	93.8
Dec	0	3	100.0	1	45	97.8	8	170	95.5
Jan	0	1	100.0	2	34	94.4	11	181	94.3
Total	**10**	**134**	**93.1**	**54**	**716**	**92.9**	**277**	**3808**	**93.2**

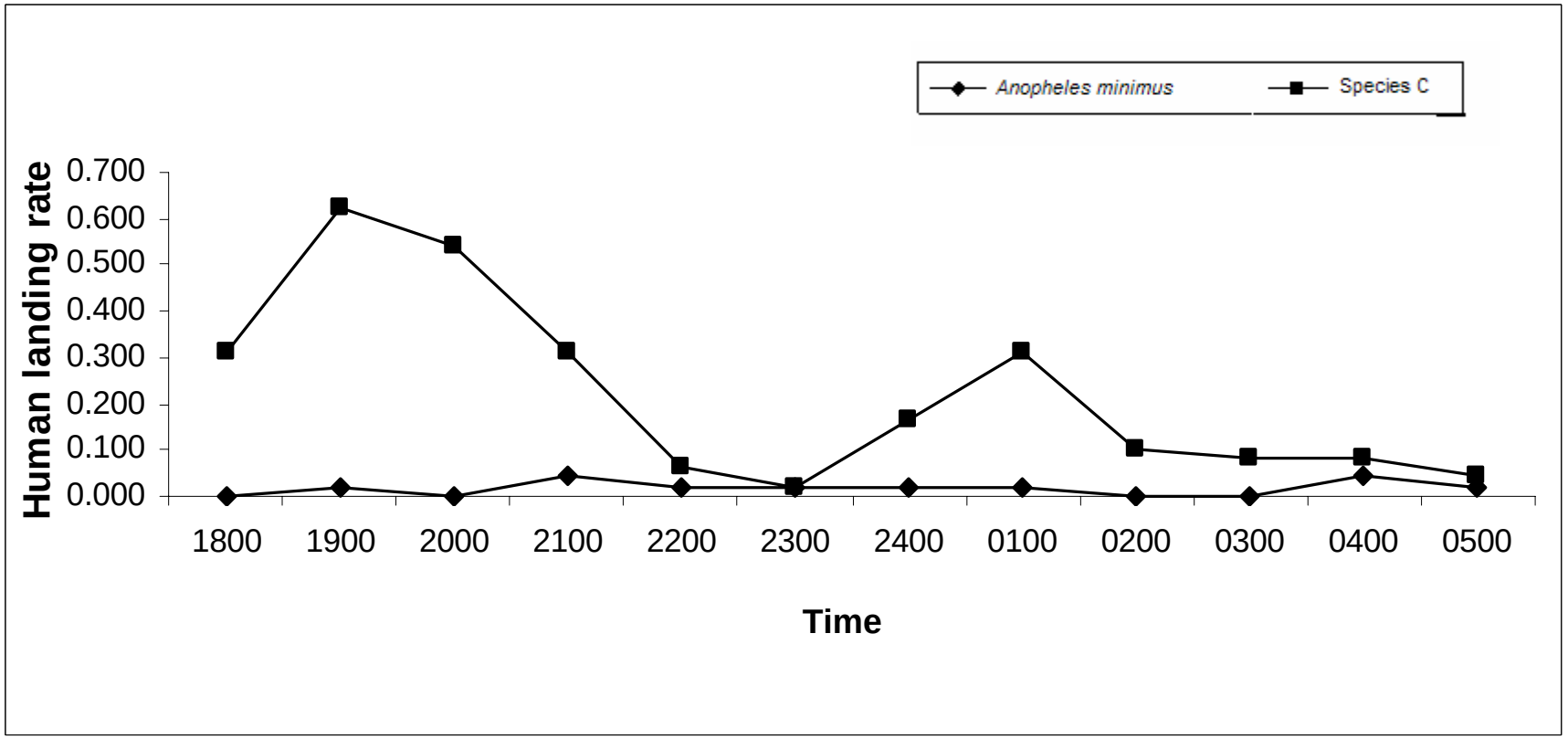


Figure 8. Rate of human indoor collections by hour of *Anopheles minimus* and species C.

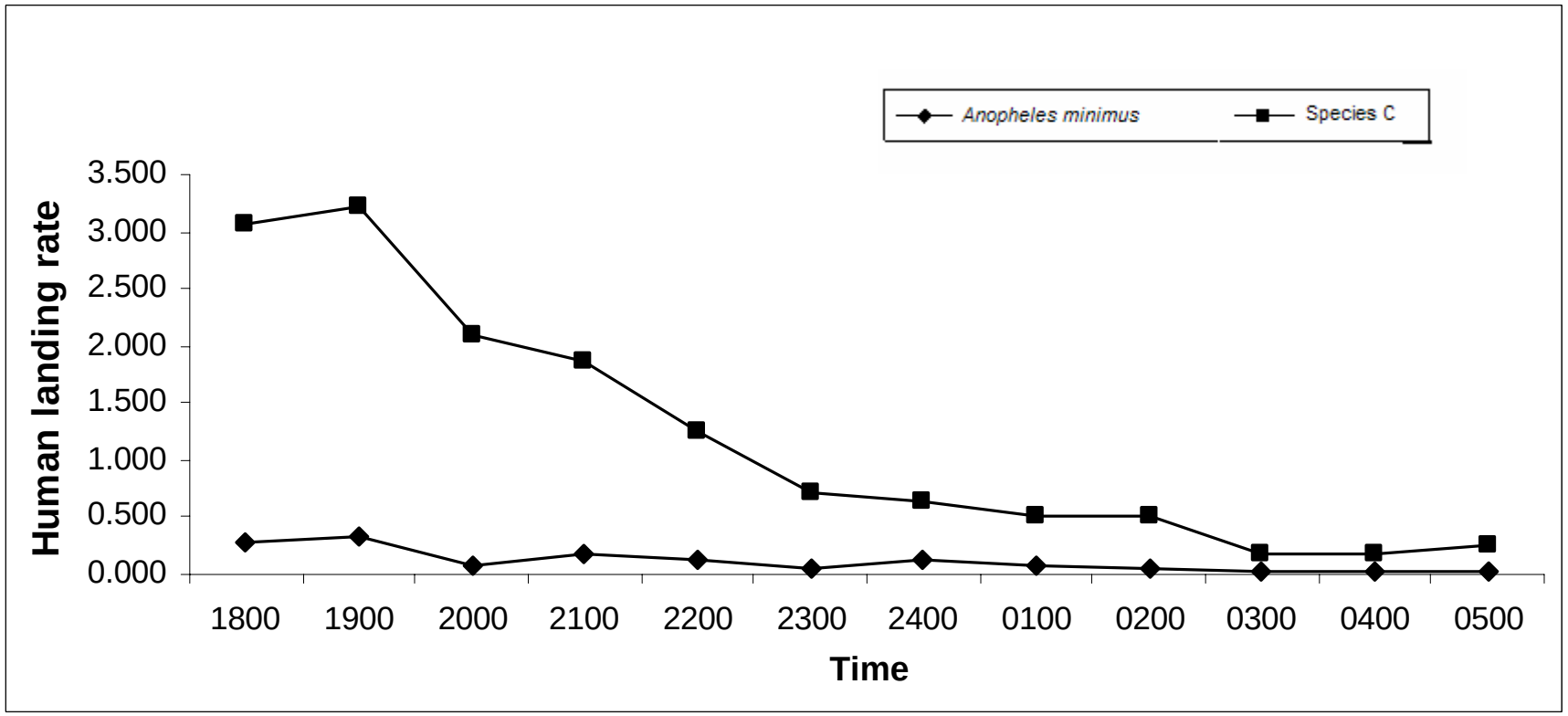


Figure 9. Rate of human outdoor collections by hour of *Anopheles minimus* and species C

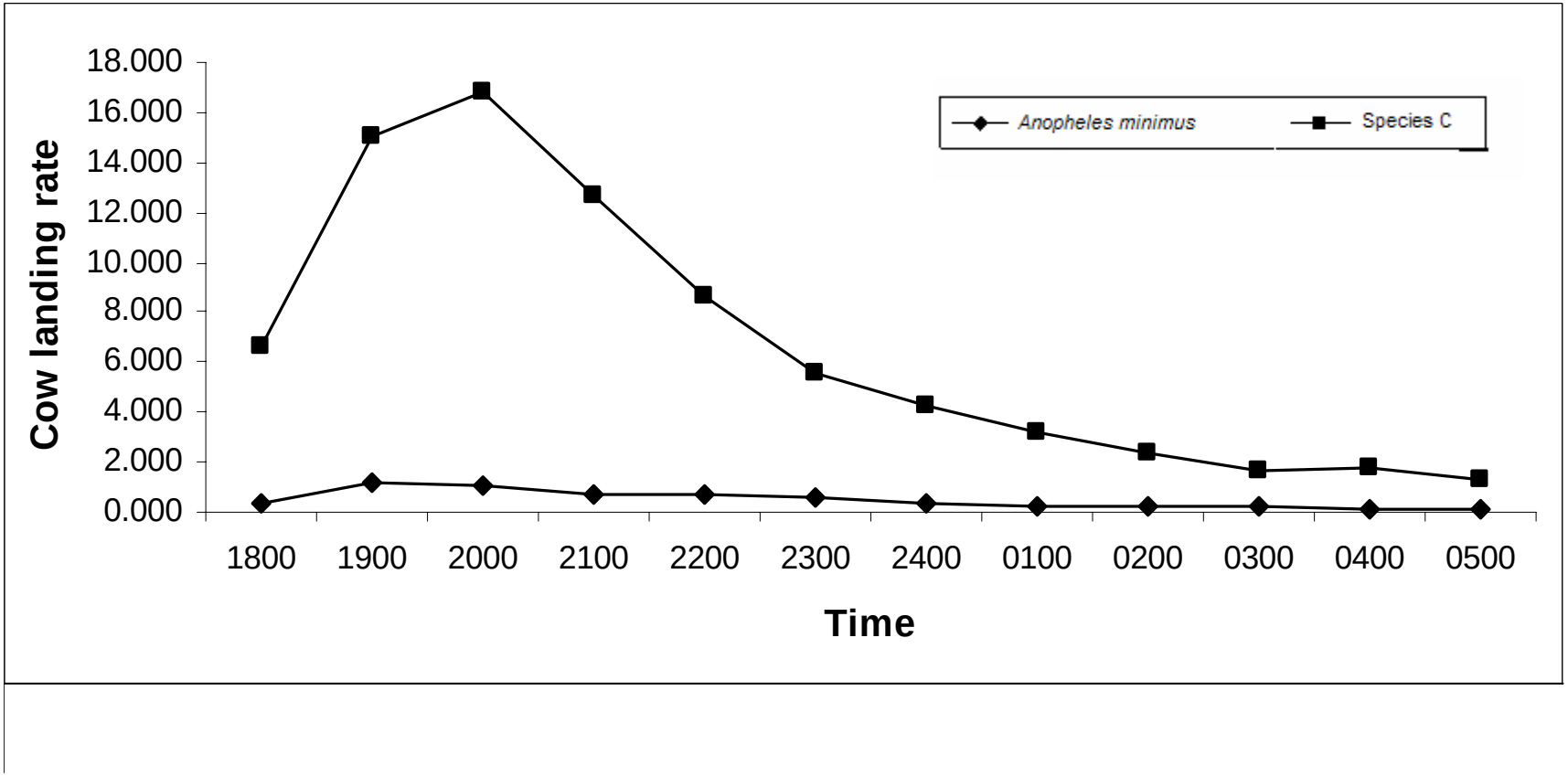


Figure 10. Rate of Cow bait collections by hour of *Anopheles minimus* and species C

DISCUSSION

Part 1. Possible diagnostic character for the identification of the two sibling species of the Minimus Complex

Precise identification of Anopheline mosquitoes is essential for a better understanding of their potential role in malaria transmission, as well as for improving the effectiveness of vector control strategies. Molecular identification assays are really useful tools because they allow rapid and easy identification of numerous mosquitoes in one shot PCR reaction. However, molecular laboratories may not be available and chemicals and consumables represent an important budget, especially when a large number of specimens need to be identified. Therefore, the presence of a diagnostic morphological character is very important and useful for rapid identification in the field.

Excluding the TKM population, a total 86.8% of the *An. minimus s.l.* were correctly identified, with a high probability of 0.880 of identifying species C but with a low probability of 0.470 to have a correct identification of *An. minimus*, based on the two PSP and HP phenotypes. Green *et al*., (1990) found 63% of correct identifications, and Van Bortel *et al*., (1999) a higher percentage of 67%. Therefore, even if the HP phenotype appears to be present in species C with a high reliability, this phenotype is also present in *An. minimus*, with high spatial variations. Based on these results, and in agreement with Chen *et al.*, (2002), The PSP phenotype was suggested not using to identify the species of the Minimus Complex. Moreover, the phenotype HP is present in *An. aconitus*, *An. jeyporiensis* and *An. pampanai* (Harrison 1980), species of the Funestus Group (Harbach 2004; Garros *et al.*, 2005a, b). Since these five *Anopheles* species are closely related, especially at the adult stage, morphological identification based on this polymorphic character will lead to misidentifications.

Natural populations live under different climates which raises the problem of the influence of the temperature on phenotypes. This link has already been revealed in other natural populations of Dipterans (Dombeck and Jaenike 2004, Katz and Foley

1993) and altitudinal or latitudinal clines were demonstrated (Gibert *et al*., 2004, Karan *et al*., 1998, 2000). Our results exhibited spatial variations within the Thai populations, and among the populations of the three countries (China, Thailand, Vietnam), which may reflect different ecological and climatic conditions. The divergence is less marked when closed populations are compared, especially for the HP phenotype. The relative high homogeneity of the population in Kanchanaburi over the time could be explained by the enclosed environment in which is localized the site. The function and role of the wing spots are unknown. They might play a role in (i) the camouflage, (ii) in the communication and recognition between and within species, or (iii) the protection against solar radiations. Anyway, the morphological variations suggest response to local conditions.

Moreover, several authors have suggested the influence of seasonality on the colour patterns observed on *Anopheles* adults. Davis (1928) working on the *Nyssorhynchus* species in South America concluded that melanism was correlated to seasons, the darker patterns being dominant during colder months. In the Afrotropical region, Leeson (1930), Gillies (1963) and Service (1964) also concluded that the dark/pale scaling on wing vein may be governed by the seasons. Cold climatic variants of *Anopheles fluviatilis s.l.* were also found (Rahman *et al*., 1960). Recently, Van Bortel *et al*., (1999) noted a change in the relative importance of the different morphotypes in each of the *An. minimus* species during the study period (from wet season to cool dry season). In Thailand, three periods are recognized: from late November to early February with cool and dry weather, from February to May with hot and dry weather and from June to November with the rainy season. Unfortunately, it was not possible to test the hypothesis neither with our samples nor on the samples of Green *et al*., (1990) (collected in January 1984 and May-June 1987) because the data were pooled.

Green *et al*., (1990) and Van Bortel *et al*., (1999) checked the morphological identifications with an isozyme assay (Green *et al*., 1990), whereas Chen *et al*., (2002) used the SSCP-PCR test (Sharpe *et al*., 1999). These techniques presented potential reading errors, which could also explain significant differences among the populations.

In agreement with Chen *et al*., (2002), the wing spot patterns is unreliable to clearly identify both *Anopheles minimus* and species C of the Minimus Complex. Between such cryptic species, where no reliable morphological character is diagnostic, molecular identification remains indispensable and the more appropriate and robust way to obtain an unambiguous differentiation.

Part 2. Host seeking behavior and biting activity between the two sibling species of the Minimus Complex based on molecular identification

Two sibling species of the Minimus Complex occur in Thailand, *An. minimus* and species C, which are known for their sympatry in Kanchanaburi Province. These two sibling species are impossible to accurately distinguish based on immature or adult morphological characters, which has complicated interpretation of previous findings based only on morphological identification (Kengluecha *et al.*, 2005). Mosquitoes reported in this study were subjected to a multiplex AS-PCR, thus providing accurate species identification and describing with reliability the trophic behavior, seasonal abundance, and biting activity of *An. minimus* and species C in the PuTuey village in Kanchanaburi Province.

Species C represented 93.2% of the *An. minimus* complex. collected during the two-year period, which is consistent with previous observations in the same locality based on morphological identifications only. Species C was found to comprise 73-95% of the *An. minimus* complex captured in Pu Teuy (Green *et al.,* 1990, Sucharit *et al.,* 1988), and Rwegoshora *et al.,* (2002) reported a species ratio of approximately 3:1 in favor of species C. Why this particular environment favors a significantly higher frequency of species C in the area is unknown but is likely related to local environmental or climatic factors that lend a competitive advantage to species C. Demographic changes resulting in increased deforestation and urbanization are often cited as contributors to changes in species distribution. However, the study site has remained in a natural environment, thus maintaining the same species composition over time. In the past, *An. minimus*.complex populations have been reduced significantly in

peninsular and southern Thailand and are also considered rare in the central plains of the country (Nutsathapana *et al.,* 1986). Regular indoor residual spraying (IRS) for malaria control has been cited as a way to greatly reduce populations (Nutsathapana *et al.,* 1986). This was also observed in the Terai and Himalayan foothills of Nepal where *An. minimus* Complex was once considered the primary vector of hyperendemic malaria until DDT residual spraying reportedly eliminated the species completely from the area (Haworth 1988). Garros *et al.,* (2005) also reported drastic and rapid changes in *An. minimus* complex species composition in central Vietnam following the introduction of permethrin-treated bednets, producing a significant reduction of *An. minimus* along with the sudden increase of species C. In Thailand, *An.minimus* complex remains abundant in many foothill and forest fringe areas of the country, possibly the result of incomplete IRS coverage or inherent biological/behavioral differences (lower indoor resting and feeding behavior) in adult mosquitoes compared to other areas (Chareonviriyaphap *et al.*, 2000, 2003, Potikasikorn *et al.*, 2005). In general, there have been fewer environmental changes in foothill and forested areas that serve as stable habitats for *An. minimus* populations regardless of degree of IRS coverage.

Unfortunately, the paucity of information on larval ecology of different members in the Minimus complex confound analysis and does not provide plausible explanations for species spatial distribution (Rattanarithikul *et al.*, 1995, Kengluecha *et al.*, 2005). Despite an intensive effort of larval habitat survey in Kanchanaburi Province, including Sai Yok District. Kengluecha *et al.*, (2005) were unable to identify key environmental factors associated with *An. minimus*. or species C. Their results implied that species distribution may be more associated with location of habitat rather than habitat type.

Pu Teuy village is considered nearly malaria-free, and only a few cases are documented each year. The results indicated that feeding habits of both species present a clear zoophilic behavior as they mainly feed on cattle located outside of living structures. In general, such feeding behavior, zoophily and exophagy, is considered less conducive to efficient and stable malaria transmission. Because *An. minimus* complex, especially species C, was the predominant anopheline in Pu Teuy village during the

two-year study, the low levels of malaria transmission in this area are likely the result of poor vectorial capacity, in particular because of the strong zoophilic tendency of both species. Actually, *An. minimus* is considered a relatively more efficient malaria vector than species C based on observed differences in host feeding behaviors (Green *et al.,* 1990; Van Bortel *et al.*, 1999;, Trung *et al.*, 2004). However, this study confirms that *An. minimus* and species C exhibited behavioral heterogeneities and are opportunist mosquitoes. In any case, the vectorial status of species C remains uncertain and the bionomics of this species requires further investigation. A low anthropophilic index and a strong tendency towards exophagy is in agreement with most studies on feeding behavior of *An. minimus* complex. in Thailand (Ismail *et al.*, 1978; Harrison, 1980, Nutsathapana *et al.*, 1986, Rwegoshora *et al.*, 2002, Chareonviriyaphap *et al.*, 2003).

In Thailand, biting activity of *An. minimus* complex has been studied but never at the specific status. Harbach *et al.*, (1987) observed a single biting peak between 2100-2200 h, whereas Ratanatham *et al.*, (1988) reported two peaks, one in the early evening (1900-2200 h) and another before dawn (0500-0600 h). Rattanarithikul *et al.*, (1996) found two prolonged feeding periods, the first wave occurring from 1800 to 2300 h, followed by a second wave from midnight until the pre-dawn hours. The results of indoor human collections also showed two peaks for species C, similar to previous studies. In a sympatric area of northern Vietnam, the relative risk of being bitten before 2200 h was higher for species C compared to *An. minimus*, whose peak feeding activity occurred after 2200 h (Trung *et al.*, 2005). The limited number of *An. minimus* collected there did not allow an estimation of the feeding activity pattern. Our study took advantage of PCR technology to identify the species of the Minimus Complex and thus describe individual biting cycles and blood-feeding activities. This information on the behavior of vector populations is crucial to explain the different levels of malaria risk based on the species in an area, which is essential for defining the most appropriate vector control strategies. A distinct biting pattern for species C was observed demonstrating a pronounced outdoor activity peak beginning around 1800 h until 1900 h, followed by a steady decline in landing numbers thereafter. Indoor activity was nearly 6-fold less than outdoor human landing counts, showing two modest peaks compared to outdoor populations, the largest at 1900-2000 h and a second,

smaller peak around midnight- 0100 h. Timing of indoor counts can be explained by an early evening delay in mosquito entry into dwellings followed by varying periods of pre-feed resting behavior before attacking a host (Roberts *et al.*, 2000). Although similar behavioral patterns with *An. minimus*, the low numbers of specimens captured in Pu Teuy village precluded any definitive statistical descriptions about this member of the complex.

RECOMMEND FOR FURTHER STUDIES

Unreliable wing spot patterns to clearly distinguish between such cryptic species, *Anopheles minimus* and species C need the alternative techniques for accurately identification. This is particularly important in study areas where the species present sympatrically with other in the Minimus Complex or with other closely related species: *An. aconitus*, *An. pampanai*, and *An. varuna.* For long term of morphological identification of adult female of malaria vector, reports of vector survey and insecticide susceptibility tests might overlapping identified that might cause ambiguous results and interpretations. Allele-Specific PCR assays are really useful tools because they allow rapid and easy identification of numerous mosquitoes in one shot PCR reaction, which reduce consumption of chemicals and budget, especially when a large number of specimens need to be identified.

Revision of biology and ecology of *Anopheles minimus* complex and the other related species are also needed. By more confidential methods, investigation of behavioral heterogeneities should be study to reveal data of human-mosquito relationship and vectorial status in particular area, to design the suitable vector control measure.

LITERATURE CITED

Altman, D.G. 1991. **Some common problems in medical research. Pratical statistics for medical research.** Chapman & Hall, London.

Baba, K. 1950. A study of anopheline mosquitoes in relation to the epidemiology of malaria in Canton Delta, South China. **Trans. Kansai Entomol. Soc.** 15: 1-11.

Baimai, V. 1989. Speciation and species complexes of the *Anopheles* malaria vectors in Thailand. pp. 146-162. **Proceeding of the 3rd conference on malaria research,** Thailand.

_____, C.A. Green, R.G. Andre, B.A. Harrison, and E.L. Peyton. 1984. Cytogenetic studies of species complexes of *Anopheles* in Thailand and Southeast Asia. **Southeast Asian J. Trop. Med. Public. Health** 15: 536 - 546.

Baker, E.Z., J.C. Beier, S.R. Meek and R.A. Wirtz. 1987. Detection and quantification of *Plasmodium falciparum* and *P. vivax* infections in Thai-Kampuchean *Anopheles* by enzyme-linked immunosorbent assay. **J. Med. Entomol.** 24: 536-541

Baird, J.K. 2000. Resurgent malaria at the millennium: control strategies in crisis. **Drugs** 59: 719-743.

Bangs M.J., S. Rusmiarto, Y.R. Gionar, A.S. Chan, K. Dave, J.R. Ryan. 2002. Evaluation of a dipstick malaria sporozoite panel assay for detection of naturally infected mosquitoes. **J. Med. Entomol.** 39: 324-330.

Beard, C.B., D. Mills Hamm and F.H. Collins. 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. **Insect Mol. Biol.** 2: 103–124.

Besansky, N.J., T. Lehmann, G.T. Fahey, D. Fontenille, L.E.O. Braack, W.A. Hawley and F.H. Collins. 1997. Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae* and *A. arabiensis*, suggest extensive gene flow. **Genetics** 147: 1817–1828.

Bureau of Vector Borne Disease (BVBD), Ministry of Public Health. 2000 - 2007.**Annual Report** (Thai)

Caccone, A., B.A. Garcia and J.R. Powell. 1996. Evolution of the mitochondrial DNA control region in the *Anopheles gambiae* complex. **Insect Mol. Biol.** 5: 51–59.

Campbell, C.C. 1997. Malaria: An emerging and re-emerging global plague. **Immunol. Med. Microbiol.** 18: 325-331.

Caterino, M.S., S. Cho and F.A. Sperling. 2000. The current state of insect molecular systematics: a thriving Tower of Babel. **Annu. Rev. Entomol.** 45:1–54

Chareonviriyaphap, T., A. Prabaripai and M.J. Bangs. 2004. Exito-repellency of deltamethrin on the malaria vectors, *Anopheles minimus*, *Anopheles dirus*, *Anopheles swadiwongporni*, and *Anopheles maculates*, in Thailand. **J. Am. Mosq. Control Assoc.** 20: 45-54.

_____, _____ and S. Sungvornyothin. 2002. An improved excito-repellency test chamber for mosquito behavioral tests**. J. Vector Ecol.** 27: 250-252.

Chareonviriyaphap, T., A. Prabaripa, M.J. Bangs and B. Aum-Aung. 2003. Seasonal abundance and blood feeding activity of *Anopheles minimus* Theobald in Thailand. **J. Med. Entomol.** 40: 876-881.

_____, S. Ratanatham and M. J. Bang. 2000. Status of malaria in Thailand. **Southeast Asian J. Trop. Med. Public Health** 31: 225-237.

Chen, B., R.K. Butlin, and R.E. Harbach. 2003. Molecular phylogenetics of the Oriental members of the Myzomyia Series of *Anopheles* subgenus *Cellia* inferred from nuclear and mitochondrial DNA sequences. **Syst. Entomol.** 28: 57-69.

_____, B., R.E. Harbach, and R.K. Butlin. 2002. Molecular and morphological studies on the *Anopheles minimus* group of mosquitoes in southern China: taxonomic review, distribution and malaria vector status. **Med.Vet. Entomol.** 16: 253-265.

Chiang, G.L., K.P. Loong, S.T. Chan, K.L. Eng, H.H. Yap.1991. Capture-recapture studies with *Anopheles maculatus* Theobald, the vector of malaria in peninsular Malaysia. **Southeast Asian J. Trop. Med. Public Health.** 22: 643-647.

Coetzee, M., J.G. Estrada-Franco, C.A. Wunderlich and R.H. Hunt. 1999. Cytogenetic evidence for a species complex within *Anopheles pseudopunctipennis* Theobald. **Am. J. Trop. Med. Hyg.** 60: 649–653.

Cohuet, A., F. Simard, J.C. Toto, P. Kengne, M. Coetzee and D. Fontenille. 2003. Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species. **Am. J. Trop. Med. Hyg.** 69: 200-205.

Coleman, R.E., R. Sithiprasasna, P. Kankaew, C. Kiaattiut, S. Ratanawong, B. Khuntirat, J. Sattabongkot. 2002. Naturally occurring mixed infection of *Plasmodium vivax* VK210 and *P. vivax* VK247 in *Anopheles* mosquitoes in western Thailand. **J. Med. Entomol.** 39: 556-559.

Collins, F.H., C.H. Porter and S.E. Cope. 1990. Comparison of rDNA and mtDNA in the sibling species *Anopheles freeborni* and *A. hermsi*. **Am. J. Trop. Med. Hyg.** 42: 417–423.

_____, M.A. Mendez, M. O. Rasmussen, P. C. Mehaffey, N. J. Besansky, and V. Finnerty. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. **Am. J. Trop. Med. Hyg**. 37: 37-41.

Crampton, J.M. and S.M. Hill. 1997. Generation and use of species-specific DNA probes for insect vector identification, pp. 384–98. In J.M. Crampton, C.B. Beard and C. Louis, eds. **The Molecular Biology of Insect Disease Vectors: A Methods Manual.** Chapman & Hall. London.

Davis, N.C. 1928. A consideration of variability in the *Nyssorhynchus* group of the genus *Anopheles*. **Am. J. Hyg.** 8: 539-563.

Delorme, D.E., R.A. Wirtz, K.P. Loong, and G.E. Jr. Lewis. 1989. Identification of sporozoites in *Anopheles maculatus* from Malaysia by enzyme-linked immunosorbent assays. **Trop. Biomed.** 6: 21-26.

Dombeck, I. and J. Jaenike. 2004. Ecological genetics of abdominal pigmentation in *Drosophila falleni*: a pleiotropic link to nematode parasitism. **Evolution Int. J. Org. Evolution.** 58: 587-596.

Delorme, D.E., R.A. Writz, K.P., Loong and G.E. Lewis. 1989. Identification of sporozoites in *Anopheles macualtus* from Malaysia by enzyme–linked immunosorbent assays. **Trop. Biomed.** 6: 21-26.

Dusfour, I., J.R. Michaux, R.E. Harbach, S. Manguin. 2007. Speciation and phylogeography of the Southeast Asian *Anopheles sundaicus* complex. **Infect. Genet. Evol.** 7: 484–493.

Elias, M., A.J. Rahman, Ali M. Mobarak, J. Begum and A.R. Chowdhury. 1987. The ecology of malaria carrying mosquito *Anopheles philippinensis* Ludlow and its relation to malaria in Bangladesh. **Bangladesh Med. Res. Counc. Bull.** 13: 15-28.

Fevre, E.M., G. Barnish, P. Yamokgul and W. Rooney. 1999. Sensitivity in vitro of *Plasmodium falciparum* to three currently used antimalarial drugs on the western border of Thailand. **Trans. R. Soc. Trop. Med. Hyg.** 93:180-184.

Fritz,G.N., J. Conn, A. Cockburn and J. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari*. **Mol. Biol. Evol.** 11: 406–416.

Garros, C., R.E. Harbach and S. Manguin. 2005a. Systematics and biogeographical implications of the phylogenetic relationships between members of the *funestus* and *minimus* groups of *Anopheles (Cellia)*. **J. Med Entomol.** 42: 7–18.

_____, R.E. Harbach and S. Manguin. 2005b. Morphological assessment and molecular phylogenetics of the *funestus* and *minimus* Groups of *Anopheles (Cellia)*. **J. Med. Entomol.** 42: 522–536.

Garros, C., L.L. Koekemoer, L. Kamau, T.S. Awolola, W. Van Bortel, M. Coetzee, M. Coosemans and S. Manguin. 2004a. Restriction fragment length polymorphism method for the identification of major African and Asian malaria vectors within the *Anopheles funestus* and *An. minimus* groups. **Am. J. Trop. Med. Hyg.** 70: 260-265.

_____, _____, M. Coetzee, M. Coosemans and S. Manguin. 2004b. A single multiplex assay to identify major malaria vectors within the African *Anopheles funestus* and the Oriental *An. minimus* groups. **Am. J. Trop. Med. Hyg.** 70: 583-590.

_____, W. Van Bortel, H.D. Trung, M. Coosemans and S. Manguin. 2006. Review of the Minimus Complex of *Anopheles*, main malaria vector in Southeast Asia: From taxonomic issues to vector control strategies. **Trop. Med. Int. Health.** 11: 102-114.

Ghosh, K.K., S. Chakraborty, S. Bhattachara, A. Palit, N. Tandon and A.K. Hati. 1985. *Anopheles annularis* as a vector of malaria in rural West Bengal. **Indian J. Malariol.** 22: 65-69.

Gibert, P., B. Moreteau and J.R. David. 2004. Phenotypic plasticity of body pigmentation in *Drosophila melanogaster*: genetic repeatability of quantitative parameters in two successive generations. **Heredity** 92: 499-507.

Gillies, M.T., 1963. A note on the identification of *Anopheles rivulorum* Leeson. **Proc. R. Entomol. Soc. London (B)** 32: 86-88.

Gould, D.J., S. Esah and U. Pranith. 1967. Relation of *Anopheles aconitus* to malaria transmission in the central pain of Thailand. **Trans. R. Soc. Trop. Med. Hyg.** 61: 441-442

Green, C. A., R.F. Gass, L.E. Munstermann and V. Baimai. 1990. Population-genetic evidence for two species in *Anopheles minimus* in Thailand. **Med. Vet. Entomol.** 4: 25-34.

_____, B. A. Harrison, T. Klein and V. Baimai. 1985. Cladistic analysis of polytene chromosome rearrangements in Anopheline mosquitoes, subgenus *Cellia*, series Neocellia. **Can. J. Genet. Cytol.** 27: 123-133.

_____, R. Rattanarithikul, A. and Charoensub. 1992. Population genetic confirmation of species status of the malaria vectors *Anopheles willmori* and *An. pseudowillmori* in Thailand and chromosome phylogeny of the Maculatus group of mosquitoes. **Med. Vet. Entomol.** 6: 335-341.

_____, _____, S. Pongparit, P. Sawadwongporn and V. Baimai. 1991. A newly-recognized vector of human malarial parasites in the Oriental region, *Anopheles (Cellia) pseudowillmori* (Theobald, 1910). **Trans. R. Soc. Trop. Med. Hyg.** 85: 35-36.

Hackett, B.J., J. Gimnig, W. Guelbeogo,C. Costantini, L.L. Koekemoer, M. Coetzee, F.H. Collins and N.J. Besansky. 2000. Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *A. rivulorum*, and uncover a cryptic taxon. **Insect Mol. Biol.** 9: 369–374.

Hackett, L.W. 1937. *Malaria in Europe*. Oxford. Oxford University Press.

Harbach, R.E., J.B. Gingrich and L.W. Pang. 1987. Some entomological observations on malaria transmission in the remote village in northwestern Thailand. **J. Am. Mosq. Control Assoc.** 3: 296-301.

_____ 2004. The classification of genus *Anopheles*: A working hypothesis of phylogenetic relationships. **Bull. Entomol. Res.** 94: 537-553.

Harbach, R.E., E. Parkin, B. Chen and R.K. Butlin. 2006. *Anopheles (Cellia) minimus* Theobald neotype designation, characterization, and systematics. **Proc. Entomol. Soc. Wash.** 108: 198-209.

Harinasuta, T., H.M. Gilles and A.A. Sandosham. 1976. Malaria in Southeast Asia. **Southeast Asian J. Trop. Med. Public Health** 7: 645-678.

_____, P. Suntharasamai and C. ViraVan. 1965. Chloroquine-resistant *falciparum* malaria in Thailand. **Lancet** 2: 657-660.

Harrison, BA. 1980. The Myzomyia Series of *Anopheles* (*Cellia*) in Thailand, with emphasis on intra-interspecific variations. Medical entomology studies-XIII. **Contrib. Am. Entomol. Inst. (Ann Arbor)** 17: 1-195.

_____ and J.E. Scanlon . 1975. Medical entomology studies: II. The subgenus *Anopheles* in Thailand. **Contrib. Am. Entomol. Inst. (Ann Arbor)** 12: 1-307.

Hunt, R.H., M. Coetzee and M. Fettene. 1998. The *Anopheles gambiae* complex: A new species from Ethiopia. **Trans. R. Soc. Trop. Med. Hyg.** 92: 231-235

Ismail, I.A.H., S. Phinichpongse and P. Boonrasri. 1978. Responses of *Anopheles minimus* to DDT residual spraying in a cleared forested foothill area in central Thailand. **Acta Tropica** 35: 69-82.

_____, V. Notananda and J. Schepens. 1974. Studies on malaria and responses of *Anopheles balabacensis balabacensis* and *Anopheles minimus* to DDT residual spraying in Thailand. Part I. Pre-spraying observations. **Acta Tropica** 31: 129-63.

Kain, K.C., R. Gopinath, Y. Yau, T. Temahivong, and C. Wongsrichanalai. 1994. In vivo response of *falciparum* malaria to chloroquine in southern Thailand. **J. Infect Dis.** 170: 258-259.

Kamau, L., W.A. Hawley, T. Lehmann, A.S.S. Orago, A. Cornel, Z. Ke and F.H. Collins. 1998. Use of short tandem repeats for the analysis of genetic variability in sympatric populations of *Anopheles gambiae* and *Anopheles arabiensis*. **Heredity** 80: 675–82.

Kar, I., S.K. Subbarao, A. Eapen, J. Ravendaran, T.S. Satyanarayana, K. Raghavendra, N. Nanda and V.P. Sharma. 1999. Evidence for a new malaria vector species, species E, within the *Anopheles culicifacies* complex. **J. Med. Entomol.** 36: 595-600.

Karan, D., A. K. Munjal, P. Gibert, B. Moreteau, R. Parkash, and J. R. David. 1998. Latitudinal clines for morphometrical traits in *Drosophila kikkawai*: A study of natural populations from the Indian subcontinent. **Genet. Res.** 71: 31-38.

_____, S. Dubey, B. Moreteau, R. Parkash, and J. R. David. 2000. Geographical clines for quantitative traits in natural populations of a tropical drosophilid: *Zaprionus indianus*. **Genetica** 108: 91-100.

Katz, A.J., and T.A. Foley. 1993. Effect of temperature on frequencies of spots in *Drosophila* wing-spot assay. **Environ. Mol. Mutagen** 22: 54-58.

Kengluecha, A., P. Rongnoparut, S. Boonsuepsakul, R. Sithiprasasna, P. Rodpradit and V. Baimai. 2005. Geographical distribution of *Anopheles minimus* species A and C in western Thailand. **J. Vector Ecol.** 30: 225-230.

Kengne, P., H.D. Trung, V. Baimai, M. Coosemans, and S. Manguin. 2001. A multiplex PCR-based method derived from random amplified polymorphic DNA (RAPD) markers for the identification of species of the *Anopheles minimus* group in Southeast Asia. **Insect Mol. Biol.** 10: 427-435.

Khoon, CC. 1985. Status of malaria vectors in Malaysia. **Southeast Asian J. Trop. Med. Public Health.** 16: 133-138.

Kirnowardoyo, S. 1985. Status of Anopheles malaria vectors in Indonesia. **Southeast Asian J. Trop. Med. Public Health.** 16: 129-132.

Kittayapong, P., J.M. Clark, J.D. Edman, T.L. Potter, B.K. Lavine, J.R. Marion and M. Brooks. 1990. Cuticular lipid differences between the malaria vector and non-vector forms of the *Anopheles maculatus* complex. **Med. Vet. Entomol.** 4: 405-413.

Kittayapong, P., J.M. Clark, J.D. Edman, B.K. Lavine, J.R. Marion and M. Brooks. 1993. Survey of the *Anopheles maculates* complex in Peninsular Malaysia by analysis of cuticular lipids. **J. Med. Entomol.** 30: 969-974.

Klun, J.A., D. Ma, R. Gupta. 2000. Optically active arthropod repellents for use against disease vectors. **J. Med. Entomol.** 37:182-186.

Korenromp, E.L. 2005. Malaria incidence estimates at country level for the year 2004 – proposed estimates and draft report. Roll Back Malaria, World Health Organization. Available Source: http://mosquito.who.int/docs/incidence _estimations2. pdf, March 23, 2006.

Krzywinski, J. and N.J. Besansky. 2003. Molecular systematics of *Anopheles*: from subgenera to subpopulations. **Annu. Rev. Entomol.** 48:111-139.

Lanzaro, G.C., Y.T. Toure, J. Carnahan, L. Zheng, G. Dolo, S. Traore, V. Petrarca, K.D. Vernick and C.E. Taylor. 1998. Complexities in the genetic structure of *Anopheles gambiae* populations in west Africa as revealed by microsatellite DNA analysis. **Proc. Natl. Acad. Sci. U. S. A.** 95: 14260-14265.

Leeson, H. S. 1930. Variations in the wing ornamentation of *Anopheles funestus* Giles. **Bull. Entomol. Res.** 21: 421-425.

Lehmann, T., N.J. Besansky, W.A. Hawley, T.G. Fahey, L. Kamau and F.H. Collins. 1997. Microgeographic structure of *Anopheles gambiae* in western Kenya based on mtDNA and microsatellite loci. **Mol. Ecol.** 6: 243–253.

_____, W.A. Hawley, L. Kamau, D. Fonenille, F. Simard and F.H. Collins. 1996. Genetic differentiation of *Anopheles gambiae* populations from East and West Africa: comparison of microsatellites and allozyme loci. **Heredity** 77: 192–200.

_____, M. Licht, N. Elissa, B.T. Maega, J.M. Chimumbwa, F.T. Watsenga, C.S. Wondji, F. Simard, and W.A. Hawley. 2003. Population structure of *Anopheles gambiae* in Africa. **J. Hered.** 94: 133-147.

Lounibos, L.P. and J.E. Conn. 2000. Malaria vector heterogeneity in South America. **Am. Entomol.** 46:238–249.

Maheswary, N.P., M.A. Habib and M. Elias. 1992. Incrimination of *Anopheles aconitus* Donitz as a vector of epidemic malaria in Bangladesh. **Southeast Asian J. Trop. Med. Public Health**. 13: 798-801.

Marrelli, M.T., R.S. Malafronte, C. Flores-Mendoza, R. Lourenco-de-Oliveiro, J.K. Kloetzel and O. Marinotti. 1999. Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi*. **J. Med. Entomol.** 36: 679–684.

Meek, S.R. 1995. Vector control in some countries of Southeast Asia: Comparing the vectors and the strategies. **Ann. Trop. Med. Parasitol.** 85:135-147.

Narang, S., J.A. Seawright and J.B. Kitzmiller. 1981. Linkage relationships and assignment of esterase-4 and esterase-8 loci to chromosome 3 in *Anopheles albimanus*. **J. Hered.** 72: 157-160.

Norris, D.E., 2002. Genetic markers for study of the Anopheline vectors of human malaria. **Int. J. Parasitol.** 32: 1607-1615.

Nutsathapana S., P. Sawasdiwongphorn, V. Chiprarop and J.R. Cullen. 1986. The behavior of *Anopheles minimus* Theobald subjected to differing levels of DDT selection pressure in northern Thailand. **Bull. Entomol. Res.** 76: 303-312.

Paskewitz, S.M. and Collins, F.H. 1997. PCR amplification of insect ribosomal DNA, pp. 374–83. In J.M. Crampton, C.B. Beard and C. Louis, eds. **The Molecular Biology of Insect Disease Vectors: A Methods Manual.** Chapman & Hall. London.

Peyton, E. L., and J. E. Scanlon. 1966. **Illustrated key to the female *Anopheles* mosquitoes of Thailand.** U. S. Army Med. Component South East Asia Treaty Organization, Bangkok, Thailand.

Phuc, H.K., A.J. Ball, L. Son, N.V. Hanh, N.D. Tu, N.G. Lien, A. Verardi, and H. Townson. 2003. Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. **Med Vet Entomol.** 17: 423-428.

Pinichpongse,S., E.B. Doberstyn, J.R. Cullen, L. Yisunsri, Y. Thongsombun and K. Thimasarn. 1982. An evaluation of five regimens for the outpatient therapy of *falciparum* malaria in Thailand 1980-81. **Bull. World Health Organ.** 60: 907-912.

Potikasikorn, J., T. Chareonviriyaphap, M.J. Bangs, and A. Prabaripai. 2005. Behavioral responses to DDT and pyrethroids between *Anopheles minimus* species A and C, malaria vector in Thailand. **Am. J. Trop. Med. Hyg.** 73: 343-349

Pradhan, J.N., S.L. Shrestha and R.G. Vaidya. 1970. Malaria transmission in high mountain valleys of west Nepal including first record of *Anopheles willmori* (James) as a third vector of malaria. **J. Nepal Med. Assoc.** 8: 89.97.

Prasittisuk, C. 1985. Present status of malaria in Thailand. **Southeast Asian J. Trop. Med. Public Health** 16: 141-145.

Rai K.S. and W.C. IV Black. 1999. Mosquito genomes: Structure, organization, and evolution. **Adv. Genet.** 41: 1–33.

Ramachandra, T., 1984. **The Anophelines of India.** (Revised Edition). Malaria Research Center, Indian Council of Medical Research, Delhi. 518 pp.

Rahman, J., M.V. Singh and N.N. Singh. 1960. A note on the study of the morphology, prevalence, and host preference of an ecocline of *An. fluviatilis* in Nainital Tarai (UP). **Bull. Nat. Soc. Indian Malariol.** 8: 137-142.

Ratanatham, S., S.E. Upathem, C. Prasittisuk, W. Rojanasunan, N. Theerasilp, A. Tremongkol and V. Viyanant. 1988. Bionomics of *Anopheles minimus* and its role in malaria transmission in Thailand. **Southeast Asian J. Trop. Med. Public Health** 19: 283—289.

Rattanarithikul, R. and CA. Green. 1986. Formal recognition of the species of the *Anopheles maculatus* group occurring in Thailand including the description of two new species and preliminary key to females. **Mosq. Syst.** 18:246-278.

_____, _____, S. Panyim, C. Noigamol, S. Chanaimongkol, and P. Mahapibul. 1995. Larval habitats of malaria vectors and other *Anopheles* mosquitoes around a transmission focus in northwestern Thailand**. J. Am. Mosq. Control Assoc.** 11: 428-433.

_____ and R.E. Harbach. 1990. *Anopheles macualtus* from the type locality of Hong Kong and two new species of the Maculatus Complex from the Philippines. **Mosq. Syst.** 22: 160-83.

_____, B.A. Harrison, R.E. Harbach, P. Panthusiri, R.E. Coleman and P. Panthusiri. 2006. Illustrated keys to the mosquitoes of Thailand. IV. *Anopheles*. **Southeast Asian J. Trop. Med. Public Health** 37 (Suppl 2): 1-128.

_____, E. Konishi and K.J. Linthicum. 1996. Detection of *Plasmodium vivax* and *Plasmodium falciparum* circumsporozoite antigen in anopheline mosquitoes collected in southern Thailand. **Am. J. Trop. Med. Hyg.** 54: 114-121.

_____ and P. Punthusiri. 1994. Illustrated keys to the medically important mosquitoes of Thailand. **Southeast Asian J. Trop. Med. Public Health** 25: 1-66.

Reid, J.A., 1968. *Anopheles* mosquitoes of Malaya and Borreo. **Stud. Inst. Med. Res. Malaya** 31: 310-325.

Roberts D.R. and R.G. Andre.1994. Insecticide resistance issues in vector-borne disease control. **Am. J. Trop. Med. Hyg.** 50 (Suppl 6): 21-34.

_____, S. Manguin, and J. Mouchet. 2000. DDT house spraying and re-emerging malaria. **Lancet** 356: 330-332.

Roll Back Malaria (RBM). (2006) World Malaria Report. Available Source: http://www.rollbackmalaria.org/, February 12, 2007.

Rosenberg, R., R.G. Andre and L. Somchit. 1990. Highly efficient dry season transmission of malaria in Thailand. **Trans. R. Soc. Trop. Med. Hyg.** 84: 22-28.

Rwegoshora, R.T., R.G. Sharpe, K.J. Baisley, and P. Kittayapong. 2002. Biting behavior and seasonal variation in the abundance of *Anopheles minimus* s.s. and C in Thailand. **Southeast Asian J. Trop. Med. Public Health** 33: 694-701.

Sallum, M.A., E.L. Peyton and R.C. Wilkerson. 2005. Six new species of the *Anopheles leucosphyrus* group, reinterpretation of *An. elegans* and vector implications. **Med. Vet. Entomol.** 19: 158-199.

Sarala, K.S., N. Nutan, K. Vasantha, V.K. Dua, M.S. Malhotra, R.S. Yadav and V.P. Sharma. 1994 Cytogenetic evidence for three sibling species in *Anopheles fluviatilis*. **Ann. Entomol. Soc. Am.** 87: 116-121.

Scanlon, J.E., E.L. Peyton, and D.J. Gould. 1968. An annotated checklist of the *Anopheles* of Thailand. **Thai Natl. Sci. Pap. Fauna Ser.** 2: 1-35.

Scanlon, J.E. and U. Sandhinand. 1965. The distribution and biology of *Anopheles balabacensis* in Thailand. **J. Med. Entomol.** 47: 61-69.

Service, M.W. 1964. An of individual and seasonal analysis variations occurring in the wing ornamentation of *Anopheles (Cellia) funestus* Giles. **Proc. R. Soc. Lond., B, Biol. Sci.** 33:139-143.

Sharpe, R.G., R.E. Harbach. and R.K. Butlin. 2000. Molecular variation and phylogeny of the Minimus Group of *Anopheles* subgenus *Cellia.* **Syst. Entomol.** 25: 265-272.

_____, R.G., M.M. Hims, R.E. Harbach and R.K. Butlin. 1999. PCR-based methods for identification of species of the *Anopheles minimus* group: allele-specific amplification and single-strand conformation polymorphism. **Med. Vet. Entomol.** 13: 265-273.

Somboon, P., A. Aramrattana, J. Lines, R. Webber. 1998. Entomological and epidemiological investigations of malaria transmission in relation to population movements in forest areas of north-west Thailand. **Southeast Asian J. Trop. Med. Public Health** 29: 3-9.

_____, D. Thongwat, P. Somwang, H.J. Teng, Y. Tsuda and M. Takagi. 2005. The specific status of *Anopheles minimus* s.l. collected from Taiwan. **Southeast Asian J. Trop. Med. Public Health** 36: 605-608.

_____, C. Walton, R. G. Sharpe, Y. Higa, N. Tuno, Y. Tsuda, and M. Takagi. 2001. Evidence for a new sibling species of *Anopheles minimus* from the Ryukyu Archipelago, Japan. **J. Am. Mosq. Control Assoc.** 17: 98-113.

Subbarao, S.K. 1988. The *Anopheles culicifacies* complex and control of malaria. **Parasitol. Today** 4: 72-75.

Sucharit S and Komalamisra N. 1997. Differentiation of *Anopheles minimus* species complex by RAPD-PCR technique. **J. Med. Assoc. Thai.** 80: 598–602

_____, _____, S. Leemingsawat, C. Apiwathnasorn and S. Thongrungkiat. 1988. Population genetic studies on the *Anopheles minimus* complex in Thailand. **Southeast Asian J. Trop. Med. Public Health** 19: 717-723.

Suebsaeng, L., W.H. Wernsdorfer and W. Rooney. 1986. Sensitivity to quinine and mefloquine of *Plasmodium falciparum* in Thailand. **Bull. World Health Organ.** 64: 759-765.

Tan-ariya, P., K. Na-Bangchang, T. Tin, L. Limpaibul, C.R. Brockelman, J. Karbwang. 1995. Clinical response and susceptibility in vitro of *Plasmodium vivax* to the standard regimen of chloroquine in Thailand. **Trans. R. Soc. Trop. Med. Hyg.** 89: 426-429.

ter Kuile, F.O., F. Nosten, M. Thieren, C. Luxemburger, M.D. Edstein, T. Chongsuphajaisiddhi, L. Phaipun, H.K. Webster and N.J. White. 1992. High-dose mefloquine in the treatment of multidrug-resistant *falciparum* malaria**. J. Infect. Dis.** 166: 1393-1400.

Thaithong, S., L.Suebsaeng, W. Rooney and G.H. Beale. 1988. Evidence of increased chloroquine sensitivity in Thai isolates of *Plasmodium falciparum*. **Trans. R. Soc. Trop. Med. Hyg.** 82: 37-38.

Thiel, L., 2005. Gathering of data on the global epidemiology of the malaria threat, applied interventions and socio-economic impact. African Centre for Gene Technologies. Available Source: http://www.acgt.co.za/pdfs/malaria_report_sec01.pdf, January 1, 2006

Ugozzoli, L. and R.B. Wallace. 1992. Application of an allele-specific polymerase chain reaction to the direct determination of ABO blood group genotypes. **Genomics** 12: 670-674.

Van Bortel, W. and M. Coosemans. 2003. Suggesting new species? Comments on "Evidence for a new species of *Anopheles minimus* from the Ryukyu Archipelago, Japan". **J. Am. Mosq. Control Assoc.** 19: 261-264.

_____, H.D. Trung, N.D. Manh, P. Roelants, P. Verle and M. Coosemans. 1999. Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioural divergences. **Trop. Med. Int. Health** 4: 257-265.

_____, _____, P. Roeiants, R.E. Harbach, T. Backeljau and M. Coosemans. 2000. Molecular identification of *Anopheles minimus* s.l. beyond distinguishing the members of the species complex. **Insect Mol. Biol.** 9: 335-340.

Walton, C., N.J. Thelwell, A. Priestman and R.K. Butlin. 1998. The use of microsatellites to study gene flow in natural populations of *Anopheles* malaria vectors in Africa: potential and pitfalls. **J. Am. Mosq. Control Assoc.** 14, 266–272.

Walton, C., R.G. Sharpe, S.J. Pritchard, N.J. Thelwell and R.K. Butlin. 1999. Molecular identification of mosquito species. **Biol. J. Linn. Soc.** 68: 241–256.

Wang, R., L. Zheng, Y.T. Toure´, T. Dandekar and F.C. Kafatos. 2001. When genetic distance matters: Measuring genetic differentiation at microsatellite loci in whole-genome scans of recent and incipient mosquito species. **Proc. Natl. Acad. Sci. USA** 98: 10769–10774.

Wattal, B.L. 1961. Dissection records of wild caught anopheline mosquito other than the reported vector of malaria "Vectors of malaria in India". **Nat. Soc. Ind. Mal. Mosq. Dis.** 1: 187-237.

White, G. B., 1978. Systematic reappraisal of the *Anopheles maculipennis* complex. **Mosq. Syst.** 10: 13-44.

Wilkerson, R.C., T.J. Parsons, D.G. Albright, T.A. Klein and M.J. Braun. 1993. Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species. **Insect Mol. Biol.** 1: 205–211.

World Health Organization (WHO) 1993a. **A global strategy for malaria control.** World Health Organization, Geneva.

_____b. **Implementation of the global malaria control strategy. Technical Report Series No. 839.** World Health Organization, Geneva.

Yu, Y. 1987. Studies on the two forms of *Anopheles (Cellia) minimus* Theobald, 1901 in China. **Mosq. Syst**. 19: 143-145.

_____ and M.X. Li. 1984 Notes on the two forms of *Anopheles (Cellia) minimus* Theobald, 1901 in Hainan Island. **J. Parasitol. Parasit. Dis.** 2: 95-98.

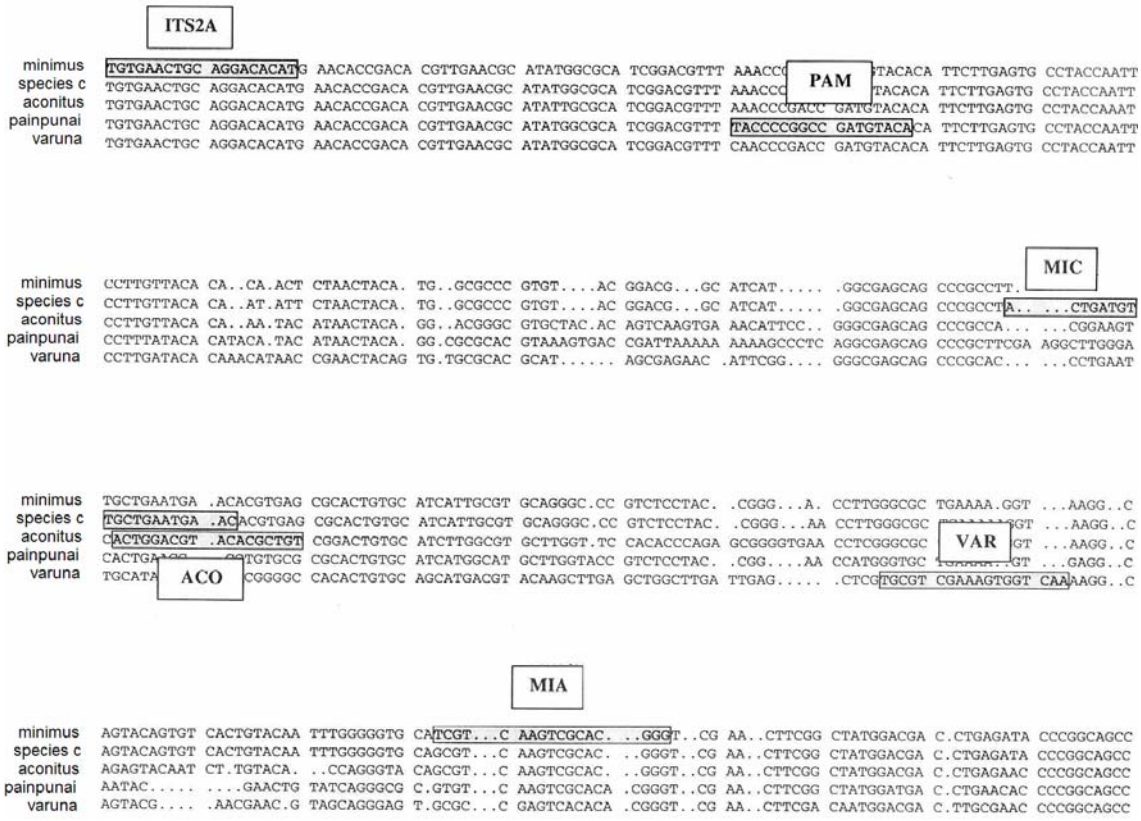
Zheng, B., L.H. Tang, Y.J. Ma, W.Q. Shi, S.S. Zhou and X.Z. Wang. 2005. Comparative study on the resting habit of *Anopheles minimus* A and *Anopheles minimus* C in Yunnan Province. **Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi** 23: 146-149. (Chinese)

Zheng, L., A.J. Cornel, R. Wang, H. Erfel, H. Voss, W. Ansorge, F.C. Kafatos and F.H. Collins. 1997. Quantitative trait loci for the refractoriness of *Anopheles gambiae* to *Plasmodium cynomolgi* B. **Science** 276: 425–428.

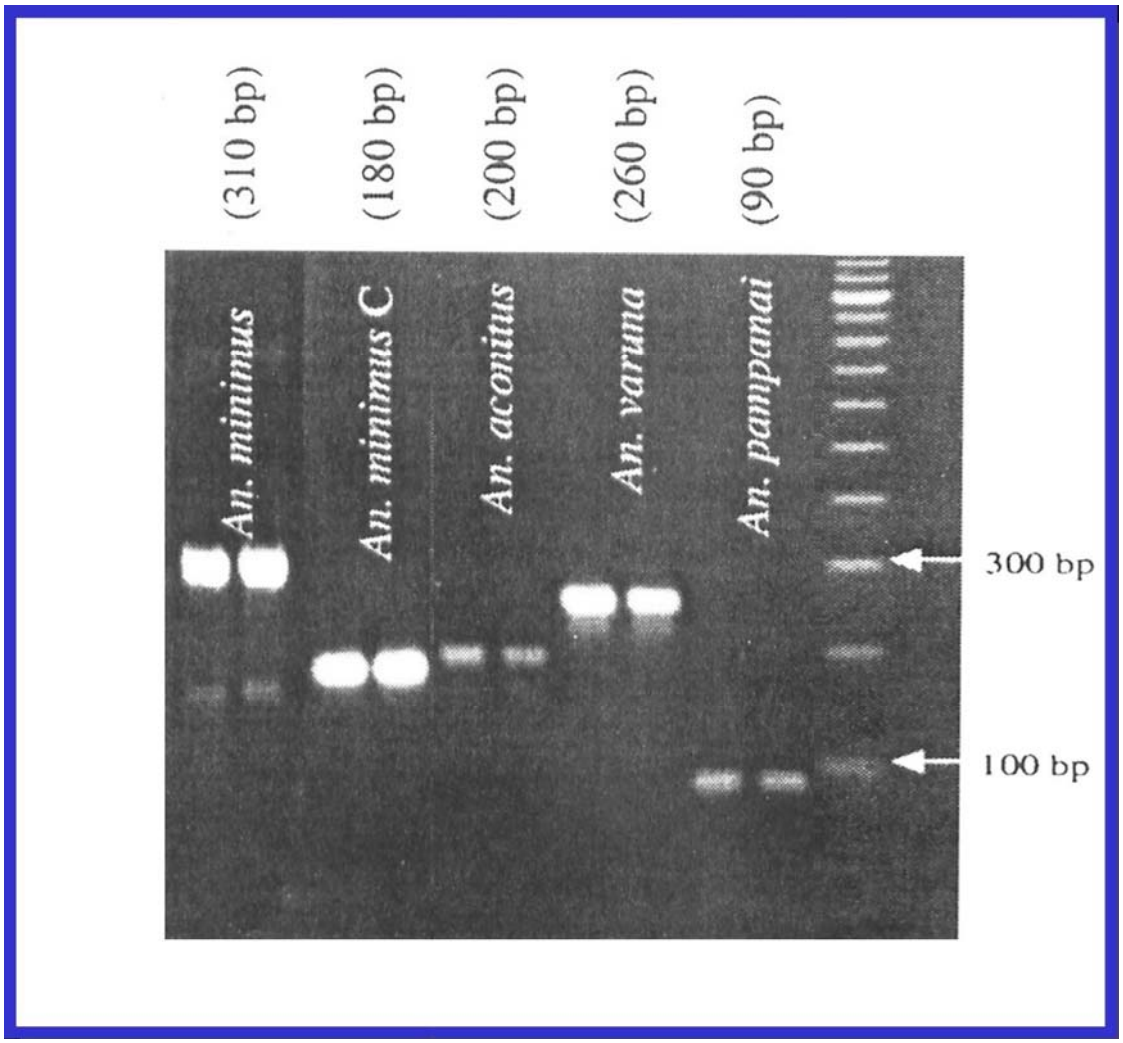
APPENDIX

Appendix Table 1. Primers designed for *Anopheles species* diagnostic assay (Garros *et al.*, 2004b)

Species	Genbank accession number	Primer name	Primer sequence 5′ to 3′	Size of product (bp)	Tm (°c)
Universal forward primer		ITS2A	TGT GAA CTG CAG GAC ACA T	-	54.5
An. minimus	AY255108	MIA	CCC GTG CGA CTT GAC GA	310	57
Species C	AY255109	MIC	GTT CAT TCA GCA ACA TCA GT	180	57.6
An. aconitus	AY255106	ACO	ACA GCG TGT ACG TCC AGT	200	53.2
An. varuna	AY255111	VAR	TTG ACC ACT TTC GAC GCA	260	53.7
An. painpunai	AY255110	PAM	TGT ACA TCG GCC GGG GTA	90	58.2



Appendix Figure 1. Alignment of the nucleotide sequence of the internal transcribed spacer 2 (ITS2) from 5 species of the Oriental *Anopheles* species, shaded boxes indicate primer selection sites.



Appendix Figure 2. Electrophorogram of ITS2 of 5 species of the Oriental *Anopheles* species in the Myzomyia series.

Appendix Table 2. Morphological keys to Oriental *Anopheles* species in the Myzomyia series female (Harrison, 1980).

1.	Center of scutum covered with fairly broad white scales back onto scutellum; hindtarsomeres with broad pale bands, or some fore-tarsomeres with pale bands nearly 2.0 the width of tarsomere diameter (females and males) …….	2
	Center of scutum appearing nearly bare except for setae, or with slender seta-like pale scales back to scntellum; legs entirely dark or some tarsomeres with apical pale bands or dorsal patches not wider than tarsomere diameter …….……	3
2(1).	Hindtarsomeres with broad pale bands, tarsomere 5 entirely pale; scutum with supraalar row of pale scales just above wing root …….	*majidi*
	Hindtarsomeres with narrow pale bands, tarsomere 5 black; scutum with only setae in supraalar row over wing root ……..	*jeyporiensis*
3(1).	Base of vein R next to remigium with patch of gray or black scales ….....	4
	Base of R with only white or yellow-white scales…….……..……..	5
4(3).	Female preapical dark palpal band much longer than apical pale band (females and males hereafter): remigium usually entirely dark- scaled; foretarsomeres dark scaled; vein R4+5 usually dark except at base ……..……..	*culicifacies*
	Female preapical dark palpal band approximately equal or shorter than length of apical pale band (females and males hereafter): remigium with dark scales only at apex; foretarsomeres 1-3 (often 4) with narrow apical pale bands or dorsal patches; vein R4+5 with dark spots near base and apex, middle pale …….	*pampanai*
5(3).	Preapical dark palpal band longer than apical pale band, and 3.0-5 0 longer than small preapical pale band……	*.fluviatilis*
	Preapical dark palpal band variable, from slightly longer than nearly equal apical and preapical pale bands to much smaller than pale bandsor even absent with apical 0.33-0.40 of palpus pale ……..……	6
6(5).	Hind margin of wing with pale fringe spot at vein 1A…….	7
	Hind margin of wing without pale fringe spot at vein 1A……	8

Appendix Table 2. (Continued).

7(6).	Proboscis with distal 0.33-0.60 pale-scaled on dorsum and venter ……..…….	*aconitus*
	Proboscis entirely dark-scaled (confined to Philippines)… …….	*filipinae*
8(6).	Costa with humeral and presector pale spots (confined to Philippines) ……..…….	*mangyanus*
	Costa usually with presector pale spot or without pale sector basal to sector pale spot ……...……	9
9(8).	Foretarsomeres 1-4 with very small dorsoapical pale patches or pale bands (mainland Southeast Asia and Indian subregions)……..	*minimus*
	Foretarsomeres entirely dark-scaled…….....	10
10(9)	Costa without pale spot or scales basal to sector pale spot; vein Cu1 often with one long dark spot distal to m-cu crossvein (widespread in India subregion and the western mainland part of the Southeast Asian)..…..…….	*varuna*
	Costa with or without pale scales basal to sector pale spot; vein Cu1 usually with 2 dark spots distal to m-cu crossvein (confined to Philippines and Indonesia)……..	*.flavirostris*

CURRICURUM VITAE

NAME : Mr. Sungsit Sungvornyothin

BIRTH DATE : January 10, 1975

BIRTH PLACE : Bangkok, Thailand

EDUCATION :

YEAR	INSTITUTION	DEGREE
1993-1997	Mahidol Univ.	B.Sc. (Biology)
1997-2002	Mahidol Univ.	M.Sc.(Environmental Biology)

SCHOLARSHIP : The Development and Promotion of Science and Technology Talent Project 2002-2006