CHAPTER I

INTRODUCTION

1. STATEMENT AND SIGNIFICANCE OF THE PROBLEM

Malaria is a major international public health concern. It is now endemic in more than 56 countries and threatens the health of about 49% of the world's population (350-500 million people/year), particularly, in tropical and subtropical regions (*e.g.*, parts of Africa, Asia, the Middle East, Eastern Europe, Central and South America, Hispaniola, and Oceania). The disease is caused by 6 *Plasmodium* spp., and anopheline mosquitoes are the important vectors (WHO, 1997; Singh *et al*, 2004; Guinovart *et al*, 2006; Vythilingam *et al*, 2006; Sutherland *et al*, 2010). In Thailand, five species of malaria parasites are found; the most common being *Plasmodium falciparum* (51.27%) and *P. vivax* (47.83%), while *P. malariae* (0.26%), *P. ovale* (one case reported from Chiang Mai province in 1996) and *P. knowlesi* (only 4 cases *i.e.,* first case from Prachuap Khiri Khan, 2 cases from Yala and 1 case from Chanthaburi) are rare, and 0.64% are mixed infections of *P. falciparum* and *P. vivax* (Jongwutiwes *et al*, 2004; Division of Malaria, 2010). Recently *P. ovale*, base on DNA samples from Ghana, Myanma, Nigeria, Sao Tome, Sierra Leone and Uganda at least two distinct new species, *i.e., P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type), have been described by Sutherland *et al*. (2010). However, the identity of these two new species in Thailand is still ambiguous and needs further

detailed investigation. The disease is generally limited to rural communities living in or near forested regions, mountains and foothills, particularly those people residing in newly opened land settlements of semi-forested areas, where they earn their living by growing agricultural crops. Regions near and along the borders with neighboring countries, *i.e*., Cambodia, Laos, Myanmar and Malaysia, are also affected.

Currently, there are at least 18 anopheline species playing an important role as primary, secondary and suspected vectors of malaria transmission in Thailand. The primary vectors are *Anopheles dirus* complex [*An. dirus* s.s. (species A)*, An. baimaii* (species D)], *An. minimus* complex [*An. minimus* s.l. (species A)] and *An. maculatus* complex [*An. maculatus* s.s. (species B)], while *An. aconitus* and *An. sundaicus* complex [species A = *epiroticus* (Linton *et al*, 2005)] are considered as secondary vectors (Gould *et al*, 1967; Scanlon *et al*, 1968; Harrison, 1980; Rosenberg *et al*, 1990; Rattanarithikul *et al*, 1996; Subbarao, 1998; Sallum *et al*, 2005a; 2005b). Subsequently, *An. pseudowillmori*, a member species of the *maculatus* complex, has been incriminated as a secondary vector (Green *et al,* 1991). Recently, *An. campestris* (identification was based only on the summation of seta 2-VI branches of pupal skins) was incriminated as a potentially natural vector of *P. vivax* in Pa Rai subdistrict of Aranyaprathet district, Sa Kaeo province (Apiwathnasorn *et al*, 2002). The remaining 11 species, *i.e.*, *An. annularis*, *An. karwari*, *An. kochi*, *An. nigerrimus*, *An. nivipes*, *An. peditaeniatus*, *An. philippinensis*, *An. sawadwongporni*, *An. sinensis*, *An. tessellatus* and *An. vagus*, are suspected vectors, since they were found positive by an ELISA method for oocysts in the midgut and/or circumsporozoite (CS) antigens (Baker *et al*, 1987; Harbach *et al*, 1987; Gingrich *et al*, 1990; Frances *et al*, 1996; Rattanarithikul *et al*, 1996).

2. LITERATURE REVIEWS

The discovery of genetic diversity and/or species complexes in *Anopheles* vectors adds a complication to vector control. More than 30 *Anopheles* taxa that are involved in the transmission of malaria in different parts of the world have been identified as species complexes. Members of a species complex commonly known as sibling species. The characteristics of sibling species members in the complex are genetically isolated at pre- and/or post-mating barriers with distinct gene pools but they have identical morphology (isomorphic species) or minimal morphological distinction that lead to cryptic differentiation among the members of the complex (Subbarao, 1998). Moreover, the significance of sibling species members are as follows: firstly, they may differ in distribution, *e.g.,* the distribution of *An. dirus* complex in Thailand: species A is widespread throughout Thailand except in the south. Species B is found only in the southern peninsular region of Thailand and extends into peninsular Malaysia. Species C is reported only from Kanchanaburi, Nakhon Si Thammarat and Phatthalung provinces. Species D is commonly found on the north-western side of Thailand, along the Thai-Myanmar border and found in sympatric association with species A (Baimai *et al*, 1988b). Baimai and Green (1988) reported the distribution of the sibling species of *An. minimus* complex in Thailand that species A is widespread in Thailand while, species C is presented in the Thai-Myanmar border areas. Secondly, they may differ in biological characteristics or behaviors, *e.g*., the nocturnal biting activity of the four sibling species members of the *dirus* complex in Thailand reveal different peak biting times throughout the night. The peak biting activities of species A is 2100-2300 hours. Species B exhibit slightly different feeding activity from species C with the peak period of outdoor biting time

around 1900-2100 hours, while species C is normally at a high level in early evening around 1800-2000 hours. The outdoor biting time activity for *An. dirus* D is even later than the other sibling species around 0100-0300 hours (Baimai *et al*, 1988b). In *An. barbirostris* complex: *An. campestris*-like prefer to bite on human, whereas *An. barbirostris* species A1, A2, A3 and A4 are zoophilic mosquitoes (Seaung *et al*, 2007; 2008; Suwannamit *et al*, 2009). Thirdly, they may be difference in malarial vector-competence, *e.g., An. culicifacies* species A and C were susceptible to *P. vivax*, while species B was refractory (Adak *et al*, 1999). In *An. oswaldoi* complex, *An. oswaldoi* was susceptible to *P. vivax*, while *An. konderi* was refractory (Marrelli *et al*, 1999). Fourthly, they may be different degree of sensitivity or resistance to insecticides, *e.g., An. culicifacies* complex: in areas where both species A and B are sympatric demonstrated that species A remains more susceptible to DDT than species B (Subbarao *et al*, 1988) and areas with species B and C sympatric association, species C developed resistance to malathion at a faster rate than did species B (Raghavendra *et al*, 1991). From the above information, differences in the biological characteristics of members of the complexes have an important bearing on malaria transmission dynamics. Thus, inability to identify individual members in the complexes of *Anopheles* vectors may result in failure to differentiate between a vector and non-vector species, and lead to unsuccessful vector control.

Complete systematic studies have been based on using multi-disciplinary approaches and/or the combination of old-fashioned techniques [genetic markers: behavioral trait, *e.g.,* anthropophilic or zoophilic, nocturnal biting activity, *etc*., microhabitat (breeding places), *e.g*., plane rice-paddy & forested foot-hills, *etc.*, morphological variants, cytogenetics (metaphase karyotypes); techniques use for

determining of pre-mating barriers in natural population: isoenzymes electrophoresis and polytene chromosome; the testing of post-mating barriers: crossing experiments] and new-fashioned techniques or molecular approach [ribosomal DNA (rDNA): internal transcribed spacer 1 (ITS1), internal transcribed spacer 2 (ITS2), intergenic spacer (IGS), the third domain (D3) of the 28S ribosomal gene; mitochondrial DNA (mtDNA): cytochrome c oxidase subunits I (COI) and II (COII), NADH dehydrogenase subunit 1 and 5 (*ND1* and *ND5*)]. Consequently, there are at least 107 sibling species comprising about 28 generalized species of *Anopheles* reported so far throughout the world (Subbarao, 1998; Sharpe *et al*, 1999; 2000; Somboon *et al*, 2001; Harbach, 2004; Linton *et al*, 2005; Alam *et al*, 2006; Walton *et al*, 1999; 2000; 2001; 2007; Saeung *et al*, 2007; 2008; Thongwat *et al*, 2008; Suwannamit *et al*, 2009).

In Thailand, significant progress has been made in the population genetic study of primary vectors; *i.e.*, *An. dirus* complex (Kanda *et al*, 1981; Baimai, 1988; Baimai *et al*, 1988a; 1988c; Sawadipanich *et al*, 1990; Kitthawee *et al*, 1995; Walton *et al*, 1999), *An. minimus* complex (Sucharit *et al*, 1988; 1995; Komalamisra, 1989; Green *et al*, 1990; Baimai *et al*, 1996a; Sharpe *et al*, 1999; Choochote *et al*, 2002b; Somboon *et al*, 2005), and *An. maculatus* complex (Sucharit *et al*, 1979; Takai *et al*, 1987; Chabpunnarat, 1988; Baimai *et al*, 1993; Rongnoparut *et al*, 1996; 1999; Thongwat *et al*, 2008); and secondary vectors, *i.e.*, *An. pseudowillmori* (Green *et al*, 1992; Thongwat *et al*, 2008), *An. sundaicus* complex (Baimai *et al*, 1996b; Sukowati and Baimai, 1996; Sukowati *et al*, 1999; Linton *et al*, 2005), and *An. aconitus* (Junkum *et al*, 2005b; Jariyapan *et al*, 2005).

The Myzorhynchus Series of *Anopheles* (*Anopheles*) in Thailand consists of at least 6 species, *i.e*., *An. barbirostris* Van der Wulp, *An. campestris* Reid, *An. donaldi* Reid, *An. hodgkini* Reid, *An. pollicaris* Reid and *An. barbumbrosus* Strickland and Chowdhury (Reid, 1962; 1968; Harrison and Scanlon, 1975; Harbach, 2004; Rattanarithikul *et al*, 2006). For *An*. *barbirostris*, two types were recognized in Malaysia as early as 1942 (Reid, 1942). The dark-winged type, with an abundance of pale sternal scales, was recognized by Reid (1962) as the distinct species, *An*. *campestris*, not only on morphological difference, but also behavioral traits and vector capabilities, which were quite distinct from those of *An*. *barbirostris*. Harrison and Scanlon (1975) suggested that the characters used by Reid to separate Malayan *An. campestris* and *An. barbirostris* were not valid in Thailand. They also reported that not only do many Thai *An*. *campestris* have light wings, but some specimens of *An*. *barbirostris* exhibit dark wings in areas where *An. campestris* does not exist. Moreover, *An*. *barbirostris* usually exhibits few pale sternal scales where found in areas with or near *An*. *campestris*, and often shows extensive pale sternal scaling in areas where *An*. *campestris* does not exist. They also recommended that the summation of seta 2-VI branches in the pupal skins of these two species could be used to separate them at about the 95-97% level.

Reproductive isolation between two strains of *An. barbirostris* from Chon Buri and Chumporn provinces were first demonstrated by Choochote *et al*. (1983). The results indicated that these two strains exhibit a possible presence of species complex. Subsequently, four karyotypic forms of *An. barbirostris* (Form A: X_2, X_3, Y_1; Form B: X_1, X_2, X_3, Y_2; Form C: X_2, X_3, Y_3; Form D: X_2, Y_4) have been reported sympartically and/or allopatrically populations in Thailand and only one karyotypic

form of *An. campestris* (X, Y) has been reported from Ayuttaya province. (Baimai *et al*, 1995; Suwannamit *et al*, 2009). Recently, two karyotypic forms of *An. campestris* Form B (X_2, Y_2) and E (X_2, Y_5) were obtained from Chiang Mai provinces. The morphology of these mosquito especially the pupal skins characters are in the range of *An. campestris* but the X_2 chromosome has the shape resembling to that of *An. barbirostris*. Thus, they were tentatively designated these mosquitoes as *An. campestris*-like Form B and E, respectively (Saeung *et al*, 2007). Interestingly, the hybridization between sympatric *An. campestris*-like Form B and E in Chiang Mai yielded genetical compatibility. The very low intraspecific variation of the sequence analysis of rDNA (ITS2) and mtDNA (COI, COII) between these two forms supported their conspecific relationship (Saeung *et al*, 2007). Additionally, the reproductive isolation and very large intraspecific divergence of the sequence analysis of rDNA (ITS2) and mtDNA (COI, COII) among three sympatric and/or allopatric strains of *An. barbirostris* Form A, strongly indicated the existence of at least 4 sibling species members in the taxon of *An. barbirostris* (species A1, A2, A3 and A4) (Saeung *et al*, 2008; Suwannamit *et al*, 2009). Due to the lack of complete, systematic information for *An. campestris*-like forms, these mosquito forms need to be investigated intensively, particularly by the use of multi-disciplinary approaches. It is anticipated that the multi-disciplinary results obtained from this proposed study will clarify the sibling species, subspecies and vector potential status of all *An. campestris*-like forms in Thailand.

3. OBJECTIVE

3.1 To investigate the existence of post-mating barriers by crossing among karyotypic forms.

3.2 To search for karyotypic form-specific polymerase chain reaction (PCR) products.

3.3 To search for the karyotypic form-specific biometric and morphology of eggs, larvae, pupae and adult stages under light and/or scanning electron microscopy.

3.4 To search for karyotypic form-specific polytene chromosomes and investigate the role of karyotype variation in generating pre-mating barriers.

3.5 To determine the vector potential of each karyotypic form by the malarial susceptibility test.

4. USEFULNESS OF THE STUDY

Due to an obvious genetic diversity in the *An. campestris*-like forms, the advantage of this study will be the elucidation of its karyotypic forms as sibling species or subspecies status. The exact karyotypic forms and ability to relate these to their behavioral traits, which are responsible for malaria transmission, will also be intensively clarified. Additionally, the molecular bearing of this study will enable a more detailed analysis to be performed on the molecular evolutionary aspects of all karyotypic *An. campestris*-like forms. Briefly, specific genomic, mitochondrial and genomic coding sequences will be analyzed in each form in order to create

evolutionary "gene trees" for these sequences. These will then be used in conjunction with biological data (behavior, survival, fecundity, level of malaria susceptibility, *etc*.) to provide insights into future changes in these forms and how this may affect malaria transmission.