CHAPTER I

INTRODUCTION

1. GENERAL INTRODUCTION

Malaria remains a major health problem of the world, particularly in the tropics (WHO, 1999). In Thailand, four species of malaria parasites are found; the most common being *Plasmodium vivax* (50.74%) and *P. falciparum* (48.61%), while *P. malariae* (0.20%) and *P. ovale* (one case reported from Chiang Mai province in 1996) are rare, and 0.45% are mixed infections (Division of Malaria, 2005). 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.*, Kampuchea, Laos, Myanmar and Malaysia, are also affected.

So far, at least 18 anopheline species have been incriminated as primary, secondary and suspected vectors of malaria 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, b). Subsequently, *An. pseudowillmori*,

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a member species of the *maculatus* complex, has been incriminated as a secondary vector (Green *et al.,* 1991). Recently, the *An. barbirostris/campestris* group (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 (Limrat *et al.*, 2001; Apiwathnasorn *et al.*, 2002). The remaining 10 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 for oocysts in the midgut and/or circumsporozoite (CS) antigens by an ELISA method (Baker *et al.*, 1987; Harbach *et al.*, 1987; Gingrich *et al.*, 1990; Frances *et al.*, 1996; Rattanarithikul *et al.*, 1996).

Although vector control programs have been established in Thailand for a long time, the diseases continue to be endemic year by year. The partial failure to control vectors has many components, *i.e.*, no and/or incomplete spraying of insecticide in the household; the vector's change in biting habits; its tolerance or resistance to insecticides; and its exhibition of species complexes. The last factor appears to be important and presumably affects all the others, since populations within a species sometimes exhibit distinct differences with reference to resting habitats, preference to the host they feed from, the rate they develop resistance to insecticides, and their susceptibility to acquiring infection (Ismail *et al.*, 1978; WHO, 1992; Subbarao, 1998). Due to the partial failure of vector control, various other aspects of malaria research have been the subject of investigation for more than 2 decades. These include the development of an immunological or biochemical method

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for stopping the parasite in the human host or stopping the insect from transmitting the parasite.

2. LITERATURE REVIEWS

The discovery of genetic diversity and/or species complexes in *Anopheles* vectors adds a complication to vector control. The sibling species members of the complex are genetically isolated at pre- and/or post-mating barriers, and hence, differ in biological characteristics, *e.g.*, resting habitat, biting behavior, sensitivity or resistance to insecticide, being susceptible or refractory to malaria parasites, *etc.*, which determine their ability to transmit malaria (Rao, 1984; Joshi *et al.*, 1988; Baimai *et al.*, 1988c; Subbarao *et al.*, 1988; Subbarao, 1998). 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 [morphological variants, cytogenetics (metaphase karyotypes and polytene chromosomes), biology (or ecology) and behavior, isoenzymes, and crossing experiments] and new-fashioned [rDNA (ITS1, ITS2, IGS, D3), mtDNA (COI, COII, ND5)] techniques. Consequently, there are at least 102 sibling species comprising 28 original and/or holotypic 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, 2006).

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, c; Sawadipanich *et al.*, 1990; Kitthawee *et al.*, 1995; Walton *et*

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al., 1999), *An. minimus* complex (Sucharit *et al*., 1988b, 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); and secondary vectors, *i.e.*, *An. pseudowillmori* (Green *et al*., 1992b), and *An. sundaicus* complex (Baimai *et al*., 1996b; Sukowati and Baimai, 1996; Sukowati *et al*., 1999; Linton *et al*., 2005).

2.1 *An. dirus* complex

The Leucosphyrus Group was classified in the Neomyzomyia Series of subgenus *Cellia* of *Anopheles* (Harbach, 2004). It consists of three Subgroups, *i.e.*; the Leucosphyrus Subgroup, which includes Dirus Complex: *An. baimaii* (*dirus* D), *An. cracens* (*dirus* B), *An. dirus* s.s. (*dirus* A), *An. elegans* (*dirus* E), *An. nemophilous* (*dirus* F), *An. scanloni* (*dirus* C) and *An. takasagoensis*, and Leucosphyrus Complex: *An. baisasi*, *An. balabacensis*, *An. introlatus*, *An. latens* (*leucosphyrus* A) and *An. leucosphyrus* s.s. (species B); the Hackeri Subgroup (formerly named Elegans Subgroup), which includes *An. hackeri*, *An. pujutensis*, *An. sulawesi*, *An. recens* (previously known as *An. leucosphyrus* Sumatra Form) and *An. mirans* (previously misidentified as *An. elegans*); and the Riparis Subgroup, which includes *An. cristatus*, *An. macarthuri* and *An. riparis* (Peyton, 1990; Subbarao, 1998; Harbach, 2004; Sallum *et al*., 2005a, b). The geographical distribution of the Leucosphyrus Group ranges from the southern islands of the Philippines through most of mainland Southeast Asia to the Chinese islands of Hainan and Taiwan in the east, and westward to southern India and Sri Lanka (Peyton, 1990).

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Regarding Dirus complex in Thailand, only five species, *i.e.*, *An. baimaii*, *An. cracens*, *An. dirus* s.s., *An. nemophilous* and *An. scanloni* were found indigenously (Rattanarithikul *et al.*, 2006). Peyton and Ramalingam (1988) provided morphological and geographical descriptions of the *An. dirus* complex for the first time. They described the morphological characters of this complex, which distinguish it from other members of the Leucosphyrus Group. Morphological variations observed in natural populations, biological and behavioral difference of laboratory-bred colonies, cytological (metaphase karyotypes and polytene chromosomes) analyses of laboratory colonized and natural populations, and crossing experiments between populations have led to the recognition of members of this complex.

The reproductive isolation by providing sterile F_1-hybrid males from crosses of female *An. balabacensis* (Perlis Form) (*dirus* B) and male *An. dirus* (Bangkok Colony Strain) (*dirus* A), and the difference of metaphase karyotypes and salivary gland polytene chromosomes were the first evidence that *An. dirus* exhibited a species complex (Peyton and Harrison, 1979; Baimai *et al.*, 1981; Hii, 1985). These two sibling species were morphometrical and morphological different in only the pupal seta 9-IV, *i.e.*, *dirus* A: stout and simple with a length of 0.030-0.059 (0.043) mm, and *dirus* B: long, slender and usually with side branches of 0.056-0.089 (0.074) mm (Peyton and Harrison, 1979; Choochote *et al.*, 1987). Additionally, biological distinction is the fact that *An. dirus* A is strongly eurygamous, whereas *An. dirus* B is highly stenogamous with an insemination rate of 67.2% (Sucharit and Choochote, 1983).

An additional two sibling species, *i.e.*, *An. dirus* C (Kanchanaburi strain) and *An. dirus* D (Ranong and Phangnga strains), were based on the genetic incompatibility

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from the crosses of *An. dirus* C with *An. dirus* A and B, and *An. dirus* D with *An. dirus* A, B and C, and their distinction of metaphase karyotypes and banding of salivary gland polytene chromosomes (Wibowo *et al.*, 1984; Baimai *et al.*, 1987). Similar results of cytogenetic and crossing data provided strong evidence for the existence of other sibling species, *i.e.*, *An. dirus* E in southwestern India (Sawadipanich *et al.*, 1990) and *An. dirus* F strain from the Thai-Malaysia border (Baimai *et al.*, 1988a).

Observation on the biting activity of *An. dirus* A, B, C, and D revealed that these four isomorphic species feed at different times during the night (Baimai *et al.*, 1988b). The case of early biting in species C at Nakhon Si Thammarat province is strikingly different from that of the other species, although all of them are anthropophilic. Outdoor biting activity of *An. dirus* C is normally at a high level in early evening, at around 1800-2000 hr, then it declines sharply and is maintained at a very low level throughout the second half of the night. Species B at Phatthalung province exhibited a slightly different feeding activity from species C, with a peak period of outdoor biting time at around 1900-2100 hr, and a low level maintained throughout the second half of the night. In contrast, the outdoor biting activity of species A at Phitsanulok province started somewhat later in the first half of the night, with a peak period at around 2100-2300 hr. The outdoor biting activity of species D at Krabi province was even later than the others, beginning at a low level and gradually increasing to a peak period in the second half of the night at around 0100-0300 hr. This difference in feeding behavior probably has a significant epidemiological implication that still has to be investigated.

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Evidence of positive assortative mating for six enzyme-electromorph loci of *An. dirus* A, B, C and D mixtures in natural populations supporting *An. dirus* A, B, C and D, represented four distinct biological species within the *An. dirus* complex (Green *et al.*, 1992a). Furthermore, comparative scanning electron microscopic studies of four isomorphic eggs of *An. dirus* A, B, C and D revealed that the eggs of species A and C were similar in size and shape. Their size was intermediate, in between egg species B, which was the largest and species D, the smallest. The patterns of outer chorionic cells between the frills and floats and the arrangement of deck tubercles were also distinct in different sibling species members (Damrongphol and Baimai, 1989). Recently, ribosomal DNA (rDNA), mitochondrial DNA (mtDNA) and microsatellite genotyping have been used extensively as efficient tools for both separating the sibling species member of *An. dirus* A, B, C and D, and determining their population structure and history (Walton *et al.*, 1999, 2000, 2001).

2.2 *An. maculatus* complex

An. maculatus belongs to the Maculatus Group (formerly named Theobaldi Group) of the Neocellia Series. It consists of two Subgroups and one Unassociated Species, *i.e.*; the Maculatus Subgroup, which includes *An. dravidicus* (species C), and *An. maculatus* s.s. (species B plus genetic Forms E, K); the Sawadwongporni Subgroup, which includes *An. notanandi* (species G), and *An. sawadwongporni* (species A); and the Unassociated Species, which includes *An. dispar* (species J), *An. greeni* (species D), *An. pseudowillmori* (species I) and *An. willmori* (species H) (Subbarao, 1998; Harbach, 2004; Rattanarithikul *et al.*, 2006). Only 6 species, *i.e.*, *An. dravidicus*, *An. maculatus* s.s., *An. notanandi*, *An. sawadwongporni*, *An.*

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pseudowillmori and *An. willmori* were found indigenously in Thailand, whereas *An. dispar* and *An. greeni* were confined to the Philippines.

As early as 1931, *An. maculatus* was regarded as a single species based on studies of morphological variation in adults, and recognized in two vectorial forms, *i.e.*, one with reduced abdominal scaling, var. *maculatus*, and the other with heavy abdominal scaling, var. *willmori.* Thus, *An. pseudowillmori* (the non-scaly form), *An. dravidicus* and *An. hanabusai* were considered as synonymous to var. *maculatus*, and *An. dudgeonii*, *An. indica* and *An. maculosa* were considered as synonymous to var. *willmori* (Christophers, 1931). Nevertheless, subsequent studies by Christophers (1933), Crawford (1938), Reid *et al.* (1966), Reid (1968), and Rattanarithikul and Green (1986) stated that the morphological concept and formal taxonomy of this group remained unchanged. Comparative morphological and cytological studies together have now unequivocally identified eight biological species in this complex (Green, 1982; Green and Baimai, 1984; Green *et al.*, 1985; Rattanarithikul and Green, 1986; Rattanarithikul and Harbach, 1990; Green *et al.*, 1992b; Rattanarithikul *et al.*, 1994; Rattanarithikul *et al.*, 2006). Consequently, morphological keys of eggs and adults have been constructed to identify eight sibling species members of the complex, of which 3 species-named were followed by the morphological variation-recorded by Christophers (1931), *i.e.*, *An. dravidicus* of var. *maculatus*; *An. willmori* and *An. pseudowillmori* of var. *willmori.*

There is evidence that *An. maculatus* exhibited a species complex, which has come from the studies of metaphase chromosomes and ovarian nurse cell polytene chromosomes (Green and Baimai, 1984; Green *et al.*, 198; Baimai *et al.*, 1993). For polytene chromosomes, no variation has been seen in the chromosome arms, 3(2L),

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4(3R) and 5(3L), whereas both fixed and floating inversions were seen on X and arm 2(2R). The remarkable point is that all rearrangements in *An. maculatus* complex are referred to *An. stephensi*, which serves as an arbitrary standard chromosome. The total absence and/or significantly deficient number of heterozygotes for paracentric inversions in populations indicated the entire presence of reproductive isolation within a taxon, *i.e.*, species A, B and C, were evidently the first to recognize a species complex of *An. maculatus* (Green *et al.*, 1985). Similar events of a total heterozygote absence from fixed paracentric inversions on chromosomes X and 2 provided strong evidence of additional biological species, *i.e.*, species G (Green and Baimai, 1984), species D and J (Green *et al.*, 1985; Rattanarithikul and Harbach, 1990; Green *et al*, 1992b), and species H and I (Rattanarithikul and Green, 1986; Green *et al.*, 1992b). Interestingly, the floating inversions of two allopatric populations that are close to species B, but differ from it in the frequency of inversions identified as E and F Forms (Green and Baimai, 1984; Green *et al.*, 1985), are considered as conspecific cytological races of species B (Rattanarithikul and Green, 1986; Baimai *et al.*, 1993). Additionally, the crosses between *An. maculatus* Form B (species B) and E provide healthy and fertile hybrids through a number of generations (Chabpunnarat, 1988). The status of Form K, which differs morphologically from Form B and E (Rattanarithikul, personal communication), was questioned until the current study by Walton *et al.* (2006) clarified it by using rDNA (ITS2). The results of this study indicated that a doubtful Form K from eastern Thailand has a unique ITS2 sequence, *i.e.*, 3.7% divergence from the next most closely related taxon, *An. sawadwongporni*, suggesting that the Form K is most probably a distinct species. This PCR-based identification method was successfully developed for the differentiation of five sibling

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species, *i.e.*, *An. maculatus* s.s., *An. dravidicus*, *An. pseudowillmori*, *An. sawadwongporni* and Form K. As for metaphase chromosomes, heterochromatic variation in X-chromosomes independently supported the finding that *An. maculatus* is a species complex. In addition, a Y-chromosome polymorphism was observed within each species, but a particular polymorphism to a specific species was not noted (Baimai *et al.*, 1993).

Additional evidence of enzyme polymorphism (6-phosphogluconate dehydrogenase: 6-*pgd*) was supported by the biological species status of *An. sawadwongporni*, *An. maculatus* s.s. and *An. pseudowillmori* (Tan *et al.*, 1986). Three allelomorphs were found associated with three species, *i.e.*, *An. pseudowillmori* ($6\text{-}pgd^{70}$), *An. maculatus* ($6\text{-}pgd^{100}$) and *An. sawadwongporni* ($6\text{-}pgd^{130}$), with a total absence of heterozygotes. Furthermore, the results of hybridization between two morphological forms of *An. maculatus*, *i.e.*, sparsely scaled type (*maculatus*) and densely scaled type (*willmori*), revealed that Thai *An. maculatus* involved at least 2 forms that differentiated morphologically, as well as by incomplete reproductive isolation (Takai *et al.*, 1987), even through the parental broods of these two forms were not identified biochemically (Tan *et al.*, 1986) and/or cytologically (Green and Baimai, 1984; Green *et al.*, 1985, 1992b; Baimai *et al.*, 1993).

Bionomics and vector potential of the species members of *An. maculatus* complex were first studied by Upatham *et al.* (1988) in Pakchong district, Nakhon Ratchasima province, central Thailand and Sadao district, Songkhla province, southern Thailand. In Pakchong district, *An. maculatus* A was the most dominant species, followed by species B (Form F) and species C, which were rare. The densities of species A and species B (Form F) were high between July and November,

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with their peaks in October. Biting activities of both species occurred throughout the night, with a major peak during the first quarter of the night in all seasons. In Sadao district, only *An. maculatus* species B (Form E) was obtained with peak densities between February and June. Biting activities of this species varied according to the season. All species identified in the study were found to be predominantly zoophagic and preferred to bite humans outdoors, rather than indoors. No sporozoite positive glands (4,472 wild-caught females) were found in any species, but a very low oocyst rate (0.23%) was recovered. In both areas of the same study, *An. dirus* complex was found positive for *P. falciparum* sporozoites, indicating that *An. maculatus* A, B (E and F Forms) and C play no role in transmission.

Currently, modernized diagnostic techniques for the characterization and/or identification of sibling species members of the *An. maculatus* complex have been proposed by many investigators. Hitherto, it has been known that the cuticular hydrocarbon analysis for sibling species identification involves determining species-specific differences in the hydrocarbons contained in the wax layer of the insect cuticle, which applied mainly to the *Simulium* species complex, an important vector of river blindness due to *Onchocerca volvulus* (Carlson and Walsh, 1981; Phillips *et al.*, 1985; Millest, 1992; Mafuyai *et al.*, 1994). Kittayapong *et al.* (1990) were pioneers, who applied this technique for the differentiation of some chromosomal forms in *An. maculatus* complex, *i.e.*, Form E and F of species B. The results of investigation revealed a principal component analysis which substantiated that the vector Form E has very similar cuticular lipid profiles to Form B, and is well separated from the non-vector Form F. In addition, the application of PCR-based assays by using rDNA sequence markers [ITS2, D3 (28S gene)] (Torres *et al.*, 2000; Walton *et al.*, 2006),

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and microsatellite analysis (Rongnoparut *et al.*, 1996, 1999) were proven to be effective tools for distinguishing sibling species members and/or determining their genetic proximities within a taxon.

2.3 *An. minimus* complex

An. minimus belongs to the Funestus Group of the Myzomyia Series. It consists of one unplaced Subgroup and four Subgroups, *i.e.*; *An. jeyporiensis*; the Aconitus Subgroup, which includes *An. aconitus*, *An. filipinae*, *An. mangyanus*, *An. pampanai* and *An. varuna*; the Culicifacies Subgroup, which includes *An. culicifacies* complex; the Funestus Subgroup, which includes *An. aruni*, *An. confusus*, *An. funestus*, *An. parensis* and *An. vaneedeni*; and the Minimus Subgroup, which includes *An. flavirostris* and *An. leesoni*, the Fluviatilis Complex: *An. fluviatilis* complex, and the Minimus Complex: *An. minimus* A, C and E (Harbach, 2004; Somboon *et al.*, 2001; Rattanarithikul *et al.*, 2006). Regarding the Funestus Group, at least six species are indigenous to Thailand, *i.e.*, *An. aconitus*, *An. culicifacies* complex, *An. jeyporiensis* complex, *An. minimus* complex, *An. pampani* and *An. varuna* (Reid, 1968; Harrison, 1980; Rattanarithikul *et al.*, 2006).

Harrison (1980) reported 12 morphological variations in *An. minimus* populations from Thailand. Subsequently, Yuan (1987) reported two morphological forms, Form A and B, from the hilly regions of China. Based on three morphological variant forms in Thailand, *i.e.*, typical *minimus* [M form: wing with presector pale (PSP) on costa], *varuna* form [V form: wing without prehumeral pale (PHP), humeral pale (HP) and PSP on costa], and *pampanai* form [P form: wing with HP and PSP on costa], isoenzyme divergences (Esterase: $Est\text{-}2^{98}$ for P form, $Est\text{-}2^{100}$ for M form), and

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reproductive isolation from the crosses between M form and P form, Sucharit *et al* (1988a, b) declared M and V forms as *An. minimus* A, and P form as *An. minimus* C.

The evidence of a sympatric occurrence of homozygotes for two electromorphs controlled by a locus for octanol dehydrogenase (*Odh*$^{100, 134}$), and the absence of heterozygotes, indicates two isomorphic species within the taxon of *An. minimus* in Thailand (Green *et al.*, 1990). Following the nomenclature given by Sucharit *et al* (1988b) to sibling species in this taxon, Green *et al.* (1990) equated *Odh*^{100} to species A, and *Odh*^{134} to species C. Interestingly, the authors reported that the morphological forms M and P, described by Sucharit *et al.* (1988b), occurred both in species A and C in the samples from Ban Phu Rat, Kanchanaburi province. They also mentioned that if the morphological characteristic, which distinguished these two forms, is used for the identification there would be a 37% error. The metaphase karyotype analysis of the wild specimens genetically identified as *An. minimus* A and C (Green *et al.*, 1990) revealed that these sibling species were remarkably different in mitotic sex chromosomes as well as in the amount of pericentric heterochromatin in both pairs of autosomes (Baimai *et al.*, 1996a). Additionally, as supportive evidence, the reproductive isolation from reciprocal crosses among species C females (P form) and species A males (V and M forms), and partial reproductive isolation from reciprocal and back crosses among species A females (V and M forms) and species C males (P form), strongly indicated the sibling species status of *An. minimus* species C (Choochote *et al.*, 2002b). The parental iso-female lines of *An. minimus* A and C were established with respect to the three morphological variants (Sucharit *et al.*, 1988b) and two characteristics of metaphase karyotypes, *i.e.*, species A: V form (X_1, Y_1), M form (X_2, Y_1); and species C: P form (X_3, Y_2) (Baimai *et al.*, 1996a).

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The extensive investigation of intra- and inter-specific molecular variations at rDNA (ITS2, D3) and mtDNA (COII) regions in four members of the Minimus Group, *i.e.*, *An. aconitus*, *An. minimus* A and C, and *An. varuna* confirmed the presence of two cryptic species of *An. minimus* A and C within a taxon, and provided evidence for the possible existence of a third species dependent upon only the single wild specimen collected from Kanchanaburi province, central Thailand, tentatively designated species D (Baimai, 1989; Sharpe *et al.*, 1999, 2000). Recently, *An. minimus* E was declared from Ishigaki Island, the Ryukyu Archipelago, Japan according to the distinction of morphology, reproductive isolation, and difference of sequence for the rDNA (D3) region from *An. minimus* A (Somboon *et al.*, 2001, 2005). Interestingly, the characteristics of metaphase karyotypes of these two sibling species were identical, although some intra-specific variations were observed on the X-chromosomes of *An. minimus* E.

An. minimus A is the predominant species of the complex in Thailand (Green *et al.*, 1990). This species is probably widely distribution in the Oriental region and it has been suggested as Theobald's species (Harrison *et al.*, 1990). Ever since it was reported in India, Vietnam, China and Taiwan (Subbarao, 1998; Van Bortel *et al.*, 1999; Chen *et al.*, 2002; Harbach *et al.*, 2006). *An. minimus* C was commonly recorded in Kanchanaburi province and found in sympatry with *An. minimus* A, but absent or rare in other provinces (Sucharit *et al.*, 1988b; Green *et al.*, 1990; Sharpe *et al.*, 1999). Based on recent enzyme electrophoresis, *An. minimus* C has been reported in Vietnam (Van Bortel *et al.*, 1999), where it occurs in sympatry with *An. minimus* A in varying proportions depending upon locality, host preferences and season. Little is known about the distribution of these 2 sibling species in other countries. As for *An.*

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minimus E, it has so far been reported from only islands of the Ryukyu Archipelago, Japan (Somboon *et al.*, 2001).

Observation by Sucharit *et al.* (1988b) on the biting activities of *An. minimus* A and C at Ban Phu Toei, Sai Yok district, Kanchanaburi province revealed that *An. minimus* A bit more on humans than animals, while *An. minimus* C bit mainly on animals. These results were contrary to the study by Rwegoshora *et al.* (2002), although the same area of interest was investigated in these two studies. The latter found that both *An. minimus* A and C tended to feed from cows rather than humans, and they did not find any preference for indoor, outdoor or forest-biting in either species. Both species had a peak biting density in October/November, at the end of the rainy season. Nonetheless, the vector status of *An. minimus* C in transmitting malaria has not been determined up to this time.

2.4 *An. sundaicus* complex

An. sundaicus belongs to the Sundaicus Complex of the Pyretophorus Series (Harbach, 2004; Rattanarithikul *et al.*, 2006). Within this complex, at least four sibling species members, *i.e.*, *An. epiroticus* (*sundaicus* A), *An. sundaicus* s.s. (species B and C) and *An. sundaicus* D were identified in Oriental regions (Sukowati *et al.*, 1999; Sukowati and Baimai, 1996; Nanda *et al.*, 2004; Alam *et al.*, 2006).

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Three karyotypic forms of *An. sundaicus* (designated Form A, B and C) identified from Thailand and Indonesia were the first evidence of *An. sundaicus* that exhibited as a possible species complex. Form A is widely distributed in Thailand and Indonesia, and Form B has been found in north Sumatra and central Java. Form C, however, has been found only in Asahan, north Sumatra, Indonesia. The analysis of

ovarian nurse cell polytene chromosomes of wild-caught females collected from different localities in Thailand and Indonesia was compared with the standard mapping, *An. sundaicus* Form A (Phangnga province, Thailand strain), which indicated that there were no inversion polymorphisms. All the wild samples examined demonstrated chromosomal arrangements similar to the standard mapping, except for the banding patterns at the tip of chromosome X (1) and at the proximal region of chromosome arm 2R (2) (Sukowati and Baimai, 1996). The distinct banding patterns at the tip of chromosome X (Xb) compared with the standard sequence (Xa), and the distinct loosely diffuse bands in zone 19 of chromosome arm 2R (2Rb) compared with the standard banding patterns (2Ra), were found only from Indonesia. In addition, the existence of the 2Rb pattern correlates perfectly with the presence of an extra block of centromeric heterochromatin in autosome 2, as revealed by metaphase karyotype analysis (Sukowati and Baimai, 1996). These cytological differences have confirmed the recognition of 3 distinct forms, *i.e.*, A, B and C within the taxon *An. sundaicus*. These were subsequently designated as biological species of *An. sundaicus* A, B and C according to the results of positive preferential mating for 12 enzyme-electromorph loci and created phylogenetic dendrogram mixtures of *An. sundaicus* A, B and C in natural populations. Based on the 2.1% mean sequence divergence in both COI and cytochrome b (Cyt b) genes of mtDNA between *An. sundaicus* A and *An. sundaicus* s.s., Linton *et al.* (2005) proposed the formerly named *An. sundaicus* A as *An. epiroticus*. Recently, a new sibling species in this taxon, *An. sundaicus* D, has been reported from Car Nicobar island, Nicobar district, India. The evidence to support *An. sundaicus* D is the discovery of a new cytogenetic variant (cytotype D), which was raised from the combinative characteristics of the ovarian nurse cell

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polytene chromosome of *An. sundaicus* A and C, *i.e.*, Xa and 2Rb chromosomal-typed (Nanda *et al.*, 2004). Molecular identification by using ITS2 and D3 regions, which could separate *An. sundaicus* D from *An. sundaicus* A and *An. sundaicus* s.s., formed strongly supportive evidence (Alam *et al.*, 2006).

An. sundaicus is widely distributed in the Oriental region. The distribution extends from India, east to China through Bangladesh, Myanmar, Indochina, Thailand, Malaysia, Singapore and Indonesia (Rao, 1984). It is generally a brackish water breeder, except *An. sundaicus* A from South Tapanuli, North Sumatra, Indonesia (Sukowati and Baimai, 1996; Sukowati *et al.*, 1999), and *An. sundaicus* D from Teressa, Nancowry, Car Nicobar and Katchal islands, India (Alam *et al.*, 2006), are freshwater breeders. In Thailand, only *An. sundaicus* A has been recorded and it is widespread along the coastal areas of Trat and Phangnga provinces (Sukowati and Baimai, 1996; Sukowati *et al.*, 1999).

2.5 *An. aconitus*

Little is known about *An. aconitus* from the population genetic point of view. Three karyotypic forms were reported from Maetang district, Chiang Mai province [Form A (X_1, X_2, Y_1: 2 wild-caught females), Form B (X_1, X_2, Y_2: 5 wild-caught females), and Form C (X_1, X_2, Y_3: 2 wild-caught females) (Baimai *et al.*, 1996a)]. Studies of malate dehydrogenase-1^{157} (Mdh-1^{157} locus) (126 wild-caught females, Chiang Mai province; 34 wild-caught females, Nakhorn Si Thammarat province; 8 wild-caught females, Kanchanaburi province) showed that *An. aconitus* was similar to *An. varuna*, but different from *An. minimus* (fixed Mdh-1^{100} locus) (Green *et al.*, 1990). Two haplotypes (haplotype 1: 1 wild-caught female, Tak province; 1 wild-

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caught female, Chiang Mai province; 2 wild-caught females Kanchanaburi province; haplotype 2: 1 wild-caught female Kanchanaburi province) were described, but without relating to the three karyotypic forms (Sharpe *et al.*, 1999, 2000). Due to the lack of complete, systematic information in the above text, this mosquito species needs to be investigated intensively, particularly by the use of multi-disciplinary approaches in order to clarify its cytological and/or molecular status.

3. PURPOSES OF THE STUDY

3.1 To search for karyotypic form-specific morphometry and morphology of eggs, 4th instar larvae, pupae and adults under light and/or scanning electron microscopy.

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

3.3 To search for karyotypic form-specific isoenzyme(s).

3.4 To search for karyotypic form-specific polymerase chain reaction (PCR) products and devise a specific PCR identification tool.

3.5 To investigate the existence of post-mating barriers by hybridization among three karyotypic forms.

3.6 To determine the vector potential of the three karyotypic forms by the malarial susceptibility test.

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4. SIGNIFICANT OF THE RESEARCH

Prior to the application of mosquito control methods in an endemic area of malaria, it is necessary to understand the exact mosquito ecology and behavior, particularly the behavior of different members of genetic species or forms responsible for malaria transmission. The advantage of this study will be the elucidation of exact karyotypic forms of *An. aconitus* and the ability to relate these to their behavioral traits, which are responsible for malaria transmission. This is necessary for the formation of low cost and highly effective strategies for successful control of the *An. aconitus* vector.

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