CHAPTER 1

Introduction

1.1 Statement and Significant of the Problem

Exact identification is the backbone of the vector control system, whereas inaccurate identification of species, sibling species (isomorphic species) and/or subspecies (morphologically/cytologically polymorphic races) members of the *Anopheles* species group/complexes is the principle cause of failure in control efforts. At least 2 problems have been raised regarding identification of the *Anopheles hyrcanus* group in Thailand, as follows: (1) the overlapping morphological characters of adult females among 8 inter-species members (*Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nigerrimus*, *Anopheles nitidus*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pursati* and *Anopheles sinensis*) have created confusion regarding their exact identity, and (2) at least 6 inter-species members demonstrated genetic diversity at the chromosomal level, resulting in markedly karyotypic variations via a gradual increase in the extra block of heterochromatin on X and Y chromosomes, i.e., *An. sinensis* Form A (X, Y_1) and B (X, Y_2), *An. nigerrimus* Form A (X_1, Y_1) and B (X_2, Y_2), *An. crawfordi* Form A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), *An. argyropus* Form A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), 2 types of X (X_1, X_2) and 1 type of Y chromosomes of *An. nitidus*, and 3 types of X (X_1, X_2, X_3) and 5 types of Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes of *An. peditaeniatus*. The markedly genetic variation at the chromosomal level of each intra-species member potentially results in the existence of species complex and causes difficulty in exactly identifying sibling species and/or subspecies members that result from identical morphology or minimal morphological distinction. Due to the lack of a proper identifying tool, vector species cannot be separated from non-vectors. Also, many other studies of vectors on aspects like resting habitats, biting behavior, sensitivity or resistance to insecticide, susceptibility or refractory to pathogens, etc.

, which are all necessary for the formation of a reliably effective control strategy, are poorly understood.

Among 8 inter-species members of the Thai *An. hyrcanus* group, significant progress has been made in multidisciplinary study (combined on related-aspects of morphology, cytology, molecular investigation and cross-mating experiment) of 2 species members (*An. peditaeniatus* and *An. sinensis*) in order to elucidate the role of karyotypic variants in generating species complex within each taxon. Obviously, there is still a gap in the knowledge of genetic proximity among karyotypic variants at the intra-species level of the 6 remaining species, particularly the complete lack of cytogenetic evidence of *An. paraliae*. Regarding *An. paraliae*, its taxonomic ambiguity was raised as early as 1959. Unlike *Anopheles lesteri* that is found in the Philippines and Palaearctic region (China, Korea and Japan), the form of this species, which occurs in coastal areas of Malaysia (Malaysian peninsular, Sabah and Sarawak), Brunei, Vietnam and Thailand, has a narrow apical fringe spot on the wing, whereas the immature stages could not be distinguished from each another. Consequently, *An. paraliae* was considered to be the subspecies, *An. lesteri paraliae*. However, based on only its distinct characteristics of the adult wing and use of immature habitats in lowly elevated coast (brackish and/or peaty), this subspecies was raised to the species status of *An. paraliae* in 1991. As pointed out by the above information, there is a shortage of knowledge on the genetic proximity among karyotypic variants of *An. paraliae* in a systematic direction. Thus, it is largely believed that the outcomes obtained from current multidisciplinary study of this anopheline mosquito will enable elucidation of sibling species and/or subspecies members within taxon. Additionally, the crucial specific species status of *An. paraliae* in Thailand and/or Malaysian peninsular also was clarified clearly in this study.

1.2 Literature Review

1.2.1 Distributions and medical important

The Hyrcanus Group of the Myzorhynchus Series of the subgenus *Anopheles* comprises a large number of species that occur widely in Asia. Up until now, at least 26

species have been reported within this group (Harbach 2014). It is well known that some species of the Hyrcanus Group are involved in transmission of human diseases, particularly in the Oriental and contiguous parts of the eastern Palaearctic regions. At least 8 species of this series, i.e., *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis* are recorded in Thailand (Reid 1968; Harrison and Scanlon 1975; Rattanarithikul et al. 2006). *Anopheles lesteri* has been found in the Philippines (type locality) and the Palaearctic region (China, Korea and Japan) whereas *An. paraliae* has been detected in the coastal areas of Peninsular Malaysia, Sabah and Sarawak states, Brunei, Vietnam and Thailand.

The human malaria *Plasmodium vivax* was detected in *An. sinensis*, *An. lesteri*, *Anopheles kleini*, *Anopheles pullus* and *Anopheles belenrae* (Harrison 1973; Ree et al. 2001; Whang et al. 2002; Ma and Xu 2005; Lee et al. 2007; Joshi et al. 2009, 2011; Rueda et al. 2010). Moreover, *An. nigerrimus*, *An. peditaeniatus* and *An. sinensis* are considered as a suspected vector of *Plasmodium vivax* in Thailand (Baker et al. 1987; Harbach et al. 1987; Gingrich et al. 1990; Frances et al. 1996; Rattanarithikul et al. 1996), while *An. sinensis* and *An. peditaeniatus* have been incriminated as natural vectors of *P. vivax* in China and Korea (Mourya et al. 1989; Liu 1990; Chai 1999; Ree et al. 2001; Whang et al. 2002; Lee et al. 2007; Joshi et al. 2009) and Japanese encephalitis virus in China and India (Zhang 1990; Kanojia et al. 2003), respectively. Although *An. peditaeniatus* has been found abundantly and widely distributed throughout Thailand, its status as a vector of the Japanese encephalitis virus is still a crucial question, which needs to be clarified more thoroughly (Scanlon et al. 1968; Harrison and Scanlon 1975). Furthermore, the *An. hyrcanus* group has also been incriminated as a primary vector of malaria in northern Afghanistan (Faulde et al. 2007). In addition, *An. nigerrimus* was incriminated as a potentially natural vector of *Wuchereria bancrofti* in Phang Nga province, southern Thailand (Division of Filariasis 1998), whereas, *An. sinensis* and *An. lesteri* are considered as a vectors of *Brugia malayi* (Sasa 1976). Likewise, the *An. hyrcanus* group was also considered as an economic pest of cattle because of its vicious biting-behavior and ability to transmit cervid filariae of the genus *Setaria* (Reid 1962; Reid 1968; Harrison and Scanlon 1975).

1.2.2 Morphological study

Morphologically, some members among the 8 species members of the *An. hyrcanus* group exhibit overlapping morphological characteristics in the adult stages that lead to the misidentification of adult females, particularly the traumatic scales of wild-caught specimens in the study of epidemiology and vector-control. Some of the listed characteristics are, e.g., highly variable hindtarsal banding (among *An. sinensis*, *An. crawfordi* and *An. nigerrimus*, and between *An. argyropus* and *An. peditaeniatus*); similar humeral crossvein with a patch of dark scales, remigium with dark scales, and midtarsi with narrow apical pale bands (between *An. argyropus* and *An. nigerrimus*); similar wing patterns (between *An. crawfordi* and *An. nitidus*); and similar narrow apical fringe spot on the wing and narrow tarsal bands (between *An. paraliae* and *An. pursati*) (Reid 1953, 1968; Harrison and Scanlon 1975). Likewise, the species identity of *An. paraliae* and *An. lesteri*, based on only morphological variation of the apical fringe spot (AP) on the wing (*An. paraliae*: narrow AP: R_{1-3}, *An. lesteri*: wide AP: R_{1-4+5}) and distinct immature habitats (*An. paraliae*: brackish water breeding-habitat, *An. lesteri*: fresh water breeding-habitat), has led so far to taxonomic problem. The taxonomic ambiguity of *An. paraliae* was raised as early as 1959. Consequently, *An. paraliae* was considered to be a subspecies, *An. lesteri paraliae*, by earlier authors (Sandosham 1959; Reid 1963, 1968; Harrison and Scanlon 1975). Nevertheless, this subspecies was elevated subsequently to species status, i.e., *An. paraliae*, based on distinct characteristics of the adult wings and immature habitats (brackish and/or peaty water) (Harrison et al. 1991).

In addition, most sibling species and subspecies members within the taxon *Anopheles* species complex have identical morphology (isomorphic species) or minimal morphological distinction, which lead frequently to the cryptic differentiation among the sibling species and subspecies members in the complex (Baimai et al. 1988; Green et al. 1990; Subbarao 1998). Thus, it would be reasonable to assume that if any inter-species members of the *An. hyrcanus* group exhibit species complex, there is a high chance of misidentifying the sibling species and/or subspecies members within the complexes, when morphological criteria are set to differentiate between them.

1.2.3 Cytological study

Cytologically, 2 karyotypic forms of *An. sinensis* [Form A (X, Y_1) and B (X, Y_2)] were obtained from Mae Hong Son province; 2 karyotypic forms of *An. nigerrimus* [Form A (X_1, Y_1) and B (X_2, Y_2)] were recovered from Ubon Ratchathani and Ayutthaya provinces; 2 karyotypic forms of *An. crawfordi* [Form A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2)] were found in Chanthaburi and Phang Nga provinces; 2 karyotypic forms of *An. argyropus*, i.e., Form A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2) were detected in Chiang Mai and Phrae provinces, and 1 karyotypic form [Form B (X_1, X_2, Y_2)] was obtained from Chiang Mai and Chanthaburi provinces; 1 karyotypic form of *An. nitidus* (X_1, X_2, Y) was recovered from Phang Nga and Songkhla provinces; and 3 types of X (X_1, X_2, X_3) and 5 types of Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes of *An. peditaeniatus* were found in Chanthaburi, Chiang Mai and Phrae provinces (Figures 1.1, 1.2). Although the metaphase karyotype investigation was limited to only 6 species (the remaining 2 species: *An. paraliae* and *An. pursati*) and 8 localities and/or provinces in Thailand, the results obviously indicated that the genetic diversity at chromosomal levels may play a role in generating pre- and/or post-mating barriers in the speciation process of the *An. hyrcanus* group.

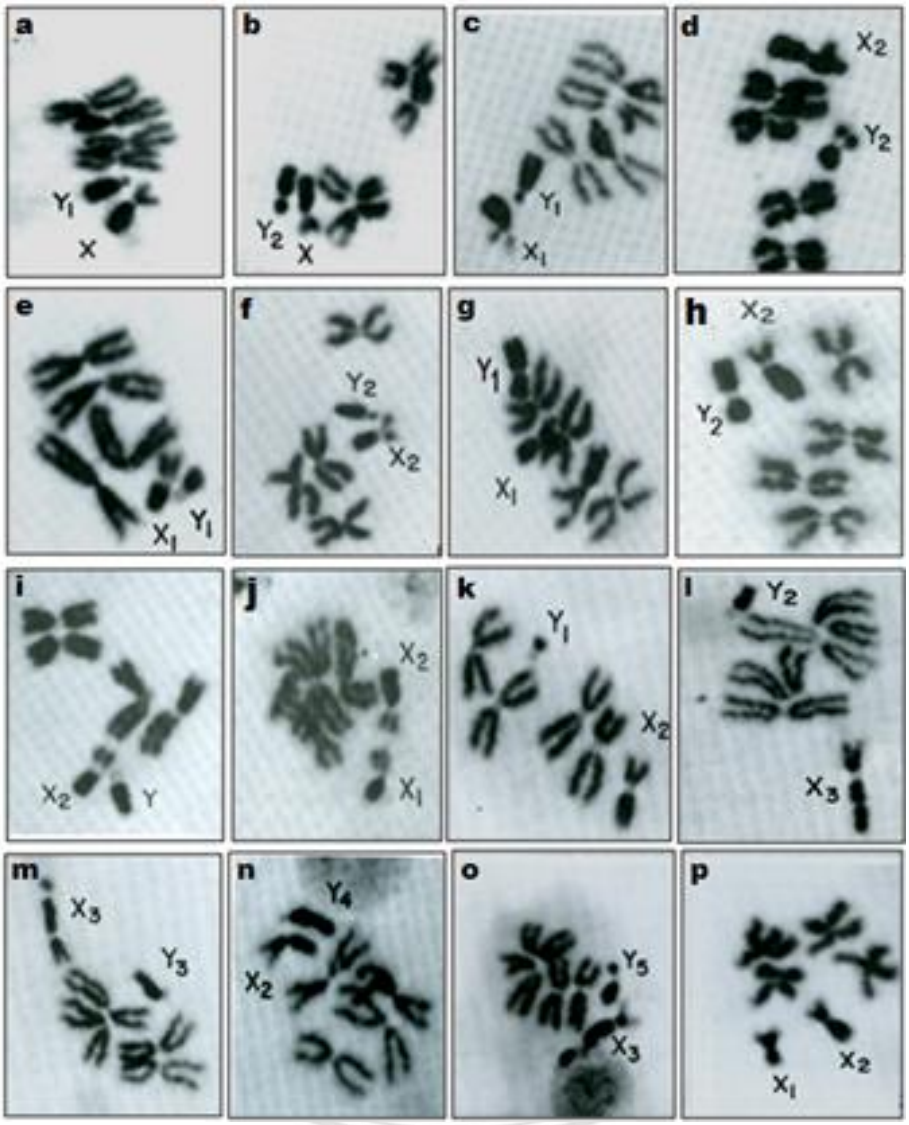

Figure 1.1 Metaphase karyotypes of the *An. hyrcanus* group. *An. sinensis* Form A (a) and B (b). *An. nigerrimus* Form A (c) and B (d). *An. crawfordi* Form A (e) and B (f). *An. argyropus* Form A (g) and B (h). *An. nitidus*: X and Y chromosomes (i) and (j). *An. peditaeniatus*: X and Y chromosomes (k), (l), (m), (n), (o) and (p) (Baimai et al. 1993b)

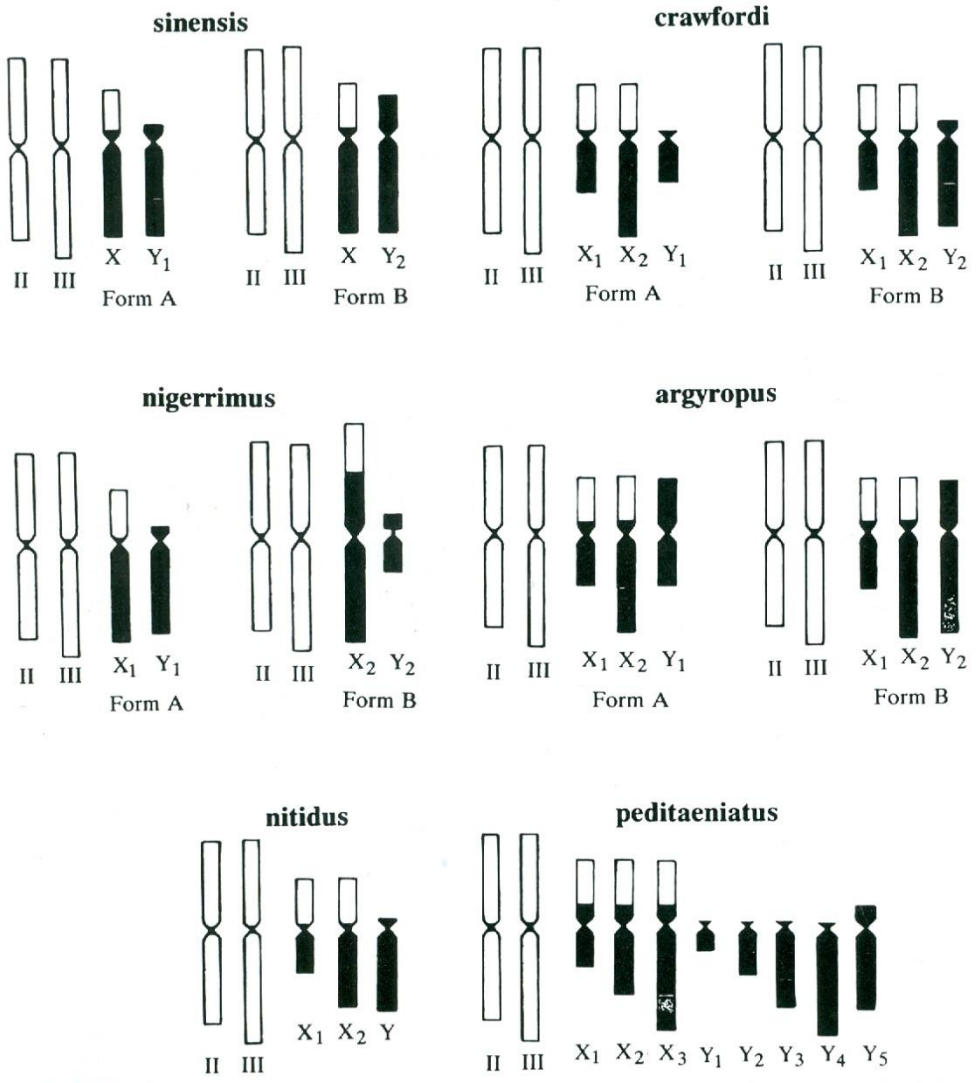

Figure 1.2 Schematic representations of metaphase karyotypes of *An. sinensis*, *An. nigerrimus*, *An. crawfordi*, *An. argyropus*, *An. nitidus* and *An. peditaeniatus* (Baimai et al. 1993b)

1.2.4 Molecular study

To date, at least 3 DNA regions (ITS2, COI, COII) have been used widely to investigate a total of 13 species of the *An. hyrcanus* group in the Oriental and Palaearctic regions, i.e., *An. belenrae* [ITS2 (Rueda 2005; Hwang 2007; Park et al. 2008a; Joshi et al. 2010)], *An. crawfordi* [ITS2 (Ma and Xu 2005)], *Anopheles. junlianensis* [ITS2 (Ma and Xu 2005)], *An. kleini* [ITS2 (Li et al. 2005; Rueda 2005; Joshi et al. 2010)], *Anopheles kunmingensis* [ITS2 (Ma and Xu 2005)], *Anopheles kweiyangensis* [ITS2 (Ma and Xu 2005)], *Anopheles liangshanensis* [ITS2 (Ma and Xu 2005)], *An. lesteri* [ITS2 (Sawabe et al. 2003; Wilkerson et al. 2003; Gao et al. 2004; Ma and Xu 2005; Hwang et al. 2006; Park et al. 2008a)], *An. nigerrimus* [ITS2 (Gao et al. 2004)], *An. peditaeniatus* [ITS2 (Gao et al. 2004; Ma and Xu 2005); ITS2, COI (Parades-Esquivel et al. 2009); ITS2, COI, COII (Choochote 2011; Saeung et al. 2012)], *An. pullus* [ITS2 (Wilkerson et al. 2003; Hwang et al. 2004; Ma and Xu 2005; Park et al. 2008a); ITS2, COI, COII (Park et al. 2003)], *An. sinensis* [ITS2 (Gao et al. 2004; Ma and Xu 2005; Hwang et al. 2006); ITS2, COII (Min et al. 2002; Park et al. 2008b; Parades-Esquivel et al. 2009); ITS2, COI, COII (Park et al. 2003)], *Anopheles sineroides* [ITS2 (Sawabe et al. 2003; Ree et al. 2005; Park et al. 2008a)]. However, the study relating DNA information on morphology, cytology and hybridization experiments in a systematic direction, as a multidisciplinary approach within intra-species, was limited in only 3 species, i.e., *An. peditaeniatus* Form B, C, D and E (Choochote 2011; Saeung et al. 2012), *An. pullus* Form A and B (Park et al. 2003) and *An. sinensis* Form A and B (Min et al. 2002; Park et al. 2008b). More recently, Hempolchom et al. (2013) have successfully developed the multiplex-PCR assay based on ITS2 sequences for species identification of 8 species members of the Hyrcanus Group in Thailand.

Several intra-taxa of Thai anopheline species, which were primarily detected with metaphase karyotype differences or variations, led to the doubtful status of sibling species or subspecies. Subsequently, they were confirmed by salivary gland and/or ovarian nurse cell polytene chromosome investigations, isoenzyme electrophoresis, DNA sequence analysis of some specific genomic regions and hybridization experiments, i.e., *Anopheles dirus* complex (Kanda et al. 1981; Baimai 1988;

Sawadipanich et al. 1990; Kitthawee and Edman 1995; Walton et al. 1999), *Anopheles minimus* complex (Sucharit et al. 1988, 1995; Komalamisra 1989; Green et al. 1990; Baimai et al. 1996a; Sharpe et al. 1999; Somboon et al. 2001; Choochote et al. 2002b; Somboon et al. 2005), the Maculatus Group (Sucharit et al. 1979; Takai et al. 1987; Chabpunnarat 1988; Baimai et al. 1993a; Rongnoparut et al. 1999; Thongwat et al. 2008; Somboon et al. 2008), *Anopheles sundaicus* complex (Baimai et al. 1996b; Sukowati and Baimai 1996, 1999; Linton et al. 2005), *An. sinensis* Form A and B (Rongsriyam et al. 1996; Choochote et al. 1998; Min et al. 2002; Park et al. 2008b), *Anopheles vagus* Form A and B (Choochote et al. 2002a), *Anopheles aconitus* Form B and C (Junkum et al. 2005), *Anopheles barbirostris* complex (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009) and *An. peditaeniatus* Form B, C, D and E (Choochote 2011). According to directed-systematic genetic study, in Thai *An. hyrcanus* group was limited in only 2 inter-species members (*An. peditaeniatus* and *An. sinensis*). Therefore, it becomes important to conduct further studies because these species are in numerous ways considered important, as either disease vectors or vicious biters of humans and/or livestock.

1.3 Purpose of This Study

1.3.1 To search for new karyotypic forms within the taxon *An. paraliae*.

1.3.2 To search for karyotypic form-specific sequence variation of ribosomal DNA (ITS2) and mitochondrial DNA (COI, COII).

1.3.3 To investigate the role of karyotypic forms in generating post-mating barriers by means of cross-mating experiments.

1.3.4 To clarify the specific species status of *An. paraliae* by comparing DNA sequence analysis (ITS2, COI and COII) and cross-mating with *An. lesteri.*

1.4 Usefulness of the Study

There is an obvious shortage of multidisciplinary knowledge from directed-systematic genetic study of *An. paraliae*, and taxonomic ambiguity between *An. paraliae* and *An. lesteri*. Thus, the advantage yielded from this study provided first hand information to assist in vector control and management projects by furnishing an exact picture of the distribution, composition and relative abundance of sibling species and

subspecies members within the taxon *An. paraliae*. Also, the specific species status of *An. paraliae* in Thailand and/or other parts of the continent where this anopheline species is found.