# CHAPTER 1

# Introduction

## 1.1 Statement and Significance of the Problem

The evolution of species complexes in anopheline vectors leads to difficulty in precisely identifying sibling species (isomorphic species) and/or subspecies (cytological races) members that possess identical morphology or minimal morphological distinction. Inability to identify individual members within the taxon *Anopheles* species complex may result in failure to distinguish between a vector and non-vector, and result in complication of a vector control approach.

Regarding morphological differentiation, *Anopheles nigerrimus* Giles, 1900 (a suspected vector of *Plasmodium vivax* and secondary vector of filarial *Wuchereria bancrofti*) and *Anopheles nitidus* Harrison, Scanlon, and Reid, 1973 (a vicious biter of humans and an economic pest to livestock) exhibit overlapping morphological characteristics in the adult stages that lead to the misidentification of adult females, particularly in the traumatic scales of wild-caught specimens in the study of epidemiology and vector control measurement. Some of the identical characteristics listed are, for example, base of costa with pale scales, humeral crossvein with a patch of dark scales, remigium with dark scales, midtarsi with narrow apical pale bands, hind tarsomere with wide apical and basal pale-bands and CuA with apical fringe. However, at least 2 distinct characteristics were used routinely to separate these 2 morphological species, i.e., CuA long basal dark mark and Rs poorly dark mark at their origin for *An. nigerrimus*, and CuA short basal dark mark and Rs dark mark at their origin for *An. nitidus*. Nonetheless, the question of limitation in the use of these characteristics has been raised. Only a non-traumatic scale of wild-caught specimens and/or perfect specimens obtained from laboratory-raised progenies were required intensively to perform exact morphological differentiation.

As for cytogenetic investigation, *An. nigerrimus* and *An. nitidus* exhibited genetic diversity at the chromosomal level via a gradual increase in the extra block of heterochromatin on X and Y chromosomes, resulting in markedly chromosomal variations. Thus, 2 karyotypic forms of *An. nigerrimus* [Form A ($X_1$, $Y_1$) and B ($X_2$, $Y_2$)], and 2 types of X ($X_1$, $X_2$) and 1 type of Y chromosomes of *An. nitidus* were recovered. The markedly genetic variation at the chromosomal level of each intra-species member potentially results in the existence of species complexes, which brings about the difficulty for a vector control approach, as mentioned above.

Even though the genetic diversity at the chromosomal level of *An. nigerrimus* and *An. nitidus* has been reported extensively since 1993, there is still a complete lack of directed systematic knowledge of genetic proximity among chromosomal and/or karyotypic variants within these taxa. It is anticipated that additional cytogenetic investigations on a number of isolines from various provinces across 6 regions of Thailand will bring about the recovery of new karyotypic variants that are realistic enough for performing multidisciplinary studies (hybridization experiments using cytological markers together with the data of comparative DNA sequence analyses). All outcomes yielded from this proposed study will enable development of robust identification tools for *An. nigerrimus* and *An. nitidus* species, including sibling species and/or subspecies members within each taxon.

## 1.2 Literature Review

### 1.2.1 Distributions and medical important

Throughout the world, there are 472 formally named species and many unnamed members of *Anopheles* species complexes are discovered, and approximately 80 of them play an important role as vectors of malaria, filarial nematode and viral diseases. Among these, at least 25 species exhibit species complexes, which comprise about 150 sibling species members (Harbach 2015).

The Hyrcanus Group has a wide distribution range extending from Iberia in Europe to East and Southeast Asia, including some of the off-lying islands of the Indian and Pacific Oceans. Until today, 26 species have been reported within this group, and it

is well known that some members of it are involved in the transmission of human diseases, i.e., malaria due to *Plasmodium vivax*, filariasis caused by *Brugia malayi*, and Japanese encephalitis virus, particularly in the Oriental Region and contiguous parts of the eastern Palaearctic Region (Harbach 2015). In Thailand, at least 8 species of the Hyrcanus Group have been reported so far, i.e., *Anopheles argyropus* (Swellengrebel, 1914), *Anopheles crawfordi* Reid, 1953, *An. nigerrimus*, *An. nitidus*, *Anopheles paraliae* Sandosham, 1959, *Anopheles peditaeniatus* (Leicester, 1908), *Anopheles pursati* Laveran, 1902, and *Anopheles sinensis* Wiedemann, 1828 (Reid 1968; Harrison and Scanlon 1975; Rattanarithikul et al. 2006).

Among these, *An. peditaeniatus* and *An. sinensis* are considered as a suspected vector of *P. vivax* in Thailand (Baker et al. 1987; Harbach et al. 1987; Gingrich et al. 1990; Frances et al. 1996; Rattanarithikul et al. 1996), and 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 cryptic question, which needs to be investigated more intensively (Scanlon et al. 1968; Harrison and Scanlon 1975). Moreover, the Hyrcanus Group has also been incriminated as a primary vector of malaria in northern Afghanistan (Faulde et al. 2007).

*Anopheles nigerrimus* is distributed widely in Thailand and other countries, i.e., India (Assam, Bihar and Punjab), Sri Lanka, Bangladesh, China (Hainan Island), Myanmar, Laos, Cambodia, Vietnam, Malaysia (Malaysian Peninsular, Sabah and Sarawak), Indonesia (Java and Sumatra) and Brunei (Scanlon et al. 1968; Reid 1968; Harrison and Scanlon 1975; Knight and Stone 1977; Harbach 2015). It was incriminated as suspected vector of *P. vivax* in Thailand (Rattanarithikul et al. 1996), and *Plasmodium falciparum* and *P. vivax* in Bangladesh (Alam et al. 2010, 2012). In addition, it was incriminated a potentially natural vector of *W. bancrofti* in Phang Nga province, southern Thailand (Division of Filariasis 1998) and recently considered as a secondary or incidental vector of *W. bancrofti* in Asia (Manguin et al. 2010). Additional experiments by Saeung et al. (2013) indicated that this anopheline species could serve

as a potential vector of the filarial nematode, nocturnally subperiodic *Brugia malayi*, in Thailand as determined by 50-65% susceptibility rates and 4.20-9.77 average number of $L_3$ larvae per infected mosquito. *Anopheles nitidus* is a foothill anopheline species, and has a wide distribution range extending from India (Assam) to Vietnam, Cambodia, Thailand (a cosmopolitan species), Malaysia (Malaysian Peninsular and Sarawak) and Indonesia (Sumatra) (Reid 1968; Harrison and Scanlon 1975; Rattanarithikul et al. 2006; Harbach 2015). Although *An. nitidus* acts as a vicious biter of humans in some localities of Thailand, it has never been incriminated as a natural and/or suspected vector of any human-diseases, unlike other species of the Hyrcanus Group as mentioned above.

### 1.2.2 Cytological study

Regarding cytogenetic studies, 8 species of the Hyrcanus Group (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis*) have been reported in Thailand. The results revealed that they exhibited 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. argyropus* Forms A ($X_1$, $X_2$, $Y_1$) and B ($X_1$, $X_2$, $Y_2$), *An. crawfordi* Forms A ($X_1$, $X_2$, $X_3$, $Y_1$), B ($X_1$, $X_2$, $X_3$, $Y_2$), C ($X_2$, $Y_3$) and D ($X_2$, $Y_4$), *An. nigerrimus* Forms A ($X_1$, $Y_1$) and B ($X_2$, $Y_2$), *An. nitidus* [2 types of X ($X_1$, $X_2$) and 1 type of Y chromosome], *An. paraliae* Forms A ($X_3$, $Y_1$), B ($X_1$, $X_2$, $X_3$,$Y_2$), C ($X_3$, $Y_3$), D ($X_1$, $X_2$, $X_3$, $Y_4$) and E ($X_3$, $Y_5$), *An. peditaeniatus* Forms A ($X_3$, $Y_1$), B ($X_1$, $X_2$, $X_3$, $Y_2$), C ($X_3$, $Y_3$), D ($X_1$, $X_2$, $X_3$, $Y_4$), E ($X_1$, $X_2$, $X_3$,$Y_5$) and F ($X_2$, $X_3$, $Y_6$), *An. pursati* Forms A ($X_1$, $X_2$, $Y_1$), B ($X_1$, $X_2$, $Y_2$) and C ($X_2$, $Y_3$) and *An. sinensis* Forms A (X, $Y_1$) and B (X, $Y_2$) (Baimai et al. 1993b; Choochote 2011; Saeung et al. 2012; Taai et al. 2013b; Saeung et al. 2014; Thongsahuan et al. 2014) (Figure 1.1)

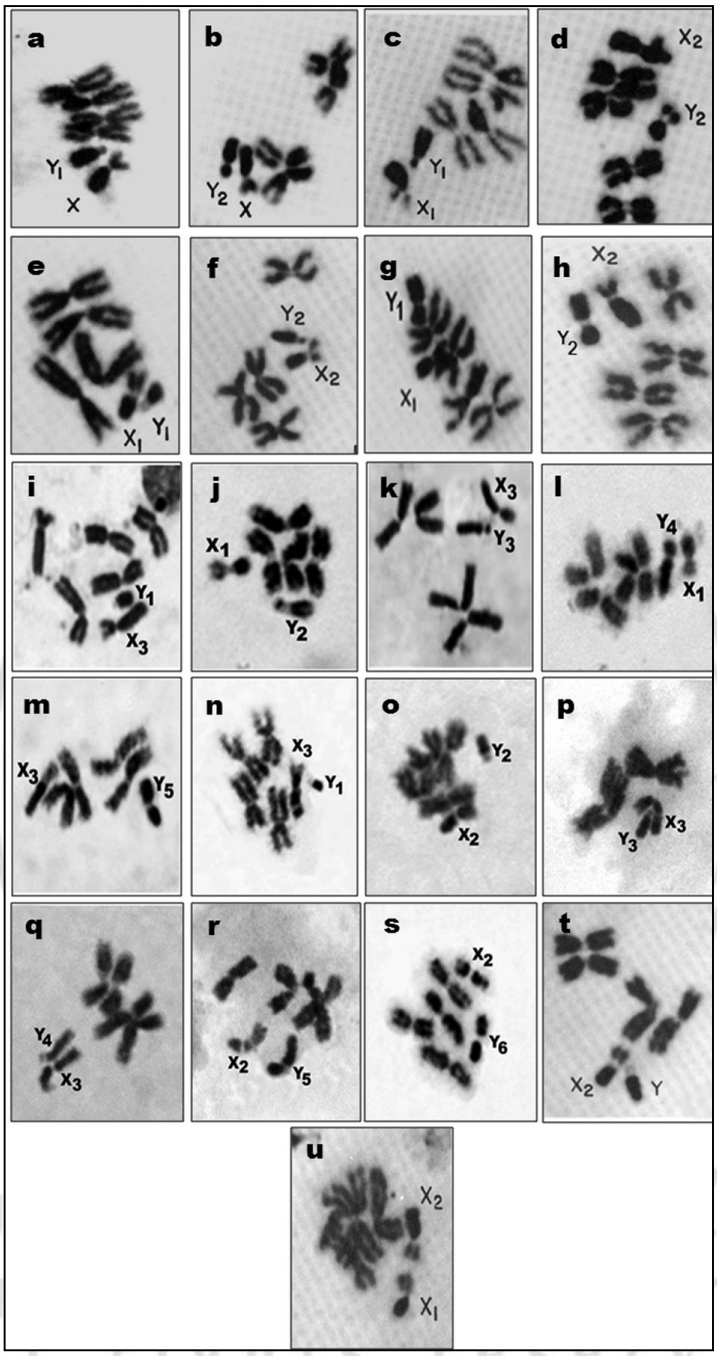




**Figure 1.1** Metaphase karyotypes of the Hyrcanus Group. *An. sinensis* Form A (a) and B (b). *An. nigerrimus* Form A (c) and B (d). *An. crawfordi* Form A (e) and B (f). *An. argyropus* Form A (g) and B (h). *An. paraliae* Form A (i), B (j), C (k), D (l) and E (m), *An. peditaeniatus* Form A (n), B (o), C (p), D (q), E (r) and F (s). *An. nitidus*: Y and X chromosomes (t) and (u)

### 1.2.3 Molecular study

Until now, sequence comparisons of the second internal transcribed spacer (ITS2) of ribosomal DNA, and cytochrome *c* oxidase subunit I and II [COI (DNA barcoding) and COII)] of the mitochondrial DNA have been used widely and effectively to distinguish a total of 6 species, i.e., *An. argyropus*, *An. crawfordi*, *An. paraliae* (= *An. lesteri*), *An. peditaeniatus*, *An. pursati* and *An. sinensis*, which belong to the Hyrcanus Group in Thailand (Choochote et al. 1998; Min et al. 2002; Park et al. 2008a; Taai et al. 2013b; Choochote 2011; Saeung et al. 2012, 2014; Thongsahuan et al. 2014). Hempolchom et al. (2013) developed the multiplex-PCR assay successfully, based on ITS2 sequences for species identification of the 8 species belonging to Hyrcanus Group in Thailand. In addition, Wijit et al. (2013) also used DNA barcoding successfully for identifying these species.

### 1.2.4 Systematic techniques for the recognition of *Anopheles* species complexes

The existence of species complexes in *Anopheles* vectors leads to difficulty in the correct identification of sibling species (isomorphic species) and/or subspecies (cytological races) members that have identical morphology or minimal morphological distinction. In addition, those members may differ in biological characteristics (e.g., microhabitats, geographic distribution, trophic behavior, sensitivity or resistance to insecticides, susceptible or refractory to pathogens and/or parasites, etc.), which can be used to determine their potential for transmitting diseases. Incorrect identification of individual members within the taxon *Anopheles* species complex may lead to the failure to recognize a vector and non-vector, and result in difficulty and/or unsuccessful vector control (Harbach 2015; WHO 2007).

In Thailand, significant progress has been made in the recognition of sibling species and/or subspecies members within 5 taxa of *Anopheles* mosquitoes, i.e., the Dirus Complex (Kanda et al. 1981; Baimai 1988; Sawadipanich et al. 1990; Kitthawee and Edman 1995; Walton et al. 1999), the Leucosphyrus Complex (Baimai 1998), the 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) and the Sundaicus Complex (Baimai et al. 1996b; Sukowati and Baimai 1996, 1999; Linton et al. 2005) during the past 25 years.

So far, at least 1 and 2 traditional techniques have been used widely for the recognition of sibling species and/or subspecies members at post- and pre-mating barriers. For post-mating barriers; the hybridization or crossing experiment, using the artificial mating technique to determine hybrid non-viability, sterility or breakdown, is still a useful tool for recognizing *Anopheles* species complexes. Detailed genetic incompatibility, including lack of insemination, embryonation, hatchability, larval survival, pupation, emergence, adult sex distortion, abnormal reproductive system and complete or incomplete (some cases only at the inversion heterozygote regions) asynaptic salivary gland polytene chromosomes are useful criteria for elucidating sibling species and subspecies status. However, a point worth noting is that an isoline colony established from the combinative characters of morphological and/or cytological markers has to be considered seriously. A laboratory raised colony established from a naturally mixed population should be omitted, since it may be a mixture of cryptic species (Kanda et al. 1981; Baimai et al. 1987, 1988; Sawadipanich et al. 1990; Subbarao 1998). As for pre-mating barriers; examination of the polytene chromosomes in wild-caught adult females, and/or progenies of iso-female lines, provides clear evidence that different specific mate recognition systems (SMRS) exist. The total absence or significantly deficient number of heterozygotes for an inversion in a sympatric population entirely indicates the presence of reproductive isolation within a taxon (Subbarao 1998; Peterson 1980; Green et al. 1992). Nonetheless, at least 4 problems have been raised regarding this matter, i.e., (1) a skilled person is needed to prepare a perfect chromosome and make an identification, (2) homosequential banding species cannot be employed, e.g., the Maculipennis Group (Harbach 2015) and the Barbirostris Complex (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Saeung 2012; Taai and Harbach 2015), (3) a relatively large amount of sample materials are required to perform the Hardy-Weinberg equilibrium, which cannot be applied to small numbers of rare species specimens that are caught during specific seasons, and (4) it cannot be performed in allopatric anopheline populations. Electrophoretic variations at enzyme

loci are not only useful for identification of sibling species, but also for the correct identification of morphologically cryptic *Anopheles* species. Variations at a locus thus enable detection of reproductive isolation within populations, resulting from positive assortative (preferential) mating (Subbarao 1998; Peterson 1980; Green et al. 1990). Nevertheless, at least 2 problems have been raised regarding this technique, i.e., (1) specimens must be fresh or frozen until analysis, and (2) its use must be similar to that of the polytene chromosome, as it requires a relatively large amount of sample materials to perform the Hardy-Weinberg equilibrium and cannot be performed in allopatric anopheline populations, as previously described (Subbarao 1998; Saeung 2012; Choochote and Saeung 2013).

Regarding the modernized technique; molecular investigation of some specific genomic markers, e.g., ribosomal DNA (ITS2, D2, D3, IGS) and mitochondrial DNA (COI, COII, Cyt b, ND5), has been used extensively as a tool to characterize and/or diagnose cryptic members in the intra-taxa of *Anopheles* mosquitoes (WHO 2007), and the advantage of this PCR-based technique is that few nanograms of DNA are required from preserved specimens (Collins and Paskewitz 1996).

Nonetheless, controversy arose when only comparative DNA sequence analyses of some specific genomic regions were used as first hand criteria to differentiate between the status of specific species, sibling species and subspecies within the taxon *Anopheles*. For example, based on a comparison of the D3 domains of 28S (28S-D3), *Anopheles fluviatilis* S has been considered as synonymous to *Anopheles harrisoni* (Harbach 2004; Garros et al. 2005; Chen et al. 2006). However, subsequent investigation of the conspecificity of these two species, based on ITS2 and D2-D3 domains of 28S rDNA regions, suggests that *An. fluviatilis* S and *An. harrisoni*, do not deserve to be synonymous (Singh et al. 2006). Similar results were also obtained in the determining on specific species status between *An. lesteri* and *An. paraliae* (Taai et al. 2013b). Remarkably, prior to reach a definite conclusion of specific species, sibling species and subspecies status within the taxon *Anopheles*, crossing experiments need to be carried out intensively using iso-female lines established from sympatric and/or allopatric populations, which relate to morphological variants, cytogenetic forms and/or

comparative DNA sequence analyses of some specific regions (Taai et al. 2013a; Choochote and Saeung 2013).

Formation of robust systematic procedures: in light of the advantages and disadvantages of the techniques mentioned above, 3 techniques, i.e., the hybridization or cross-mating experiment, molecular investigation and cytogenetic markers (characteristics of metaphase karyotypes) were selected, and they formed the rapid systematic procedures for the recognition of *Anopheles* species complexes (Choochote and Saeung 2013) (Figure 1.2).

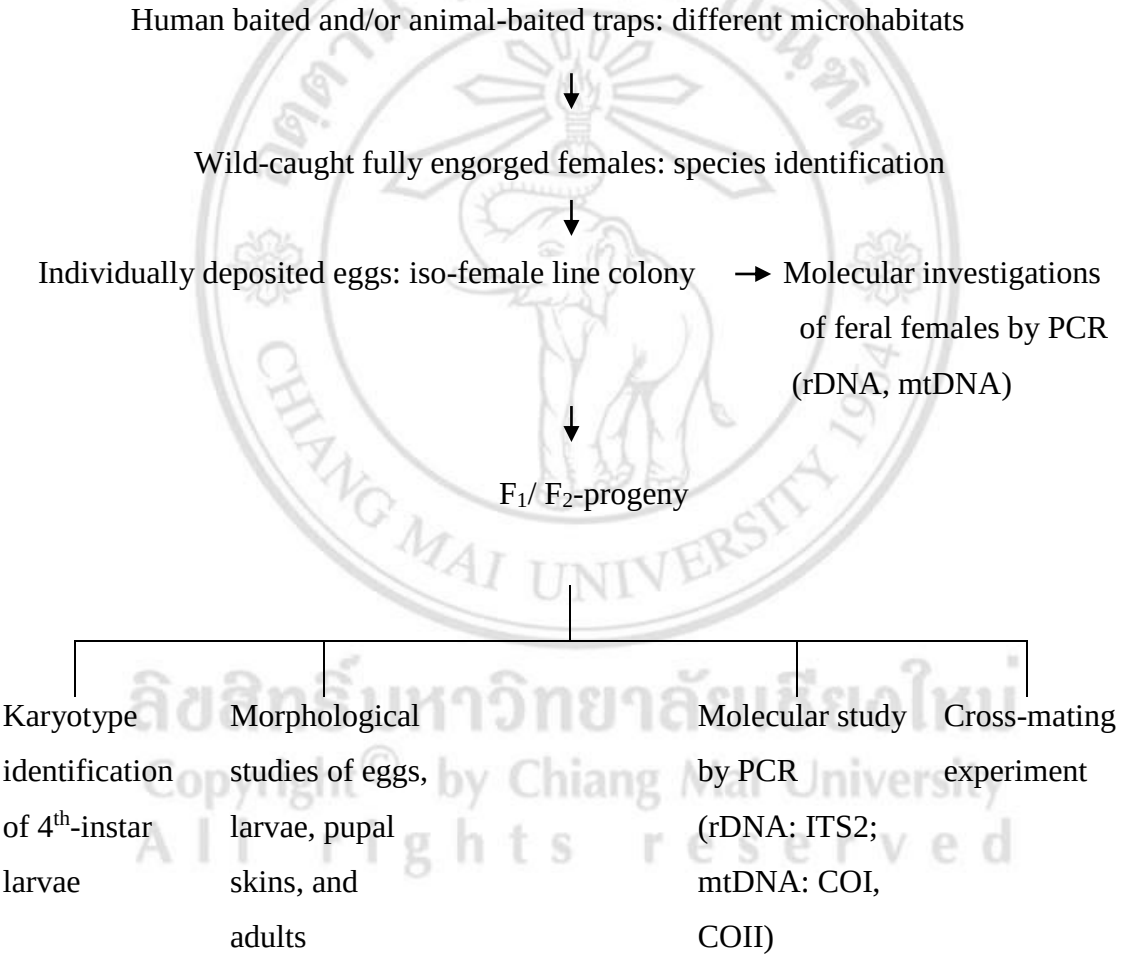


**Figure 1.2** Summarized flow chart for rapid systematic procedure

By following the flow chart: (1) try to collect anopheline mosquitoes that are distinct in their behavior (e.g., biting humans or animals with relation to different microhabitats and/or locations), (2) try to record morphological variation (s) as far as

possible during the species identification process of wild-caught females, (3) establish an isoline colony by allowing gravid females to lay eggs individually, (4) conduct molecular investigation of laid-egg feral females to obtained a robust DNA marker, with this step usually taking about 1 week. Since development of the $F_1$-progeny usually takes about 2 weeks from first instar larvae to adults, (5) the metaphase karyotype investigation of fourth instar larvae is performed in order to obtain a cytogenetic marker (karyotypic form), (6) if molecular investigation fails in the step of laid-egg feral female it will be performed in $F_1$-progeny, (7) carry out morphometric and morphological investigations of eggs, larvae, pupal skins and adults to confirm precise species identification, and (8) perform the important step of cross-mating experiments among isoline colonies by using a karyotypic marker (or form) related to a DNA marker (large sequence divergence or very low intraspecific sequence variation) of each isoline colony.

The application of this robust systematic procedure, 3 sibling species members have been recognized recently in the taxon *An. barbirostris* species complex within 2 years (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Taai and Harbach 2015). In addition, 11 species comprising a total of 35 subspecies (cytological races) have been recognized during the past decade, i.e., *Anopheles vagus* Forms A and B (Choochote et al. 2002a), *Anopheles pullus* Forms A and B (=*Anopheles yatsushiroensis*) (Park et al. 2003), *An. sinensis* Forms A and B (Choochote et al. 1998; Min et al. 2002; Park et al. 2003), *Anopheles aconitus* Forms B and C (Junkum et al. 2005), *Anopheles dissidens* Forms A, B, C and D and *Anopheles saeungae* Forms A and B (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Taai and Harbach 2015), *Anopheles wejchoochotei* Forms B, E, and F (Thongsahuan et al. 2009; Taai and Harbach 2015), *An. peditaeniatus* Forms A, B, C, D, E and F (Choochote 2011; Saeung et al. 2012), *An. paraliae* (= *An. lesteri*) Forms A, B, C, D and E (Taai et al. 2013a), *An. crawfordi* Forms A, B, C and D (Saeung et al. 2014), *An. argyropus* Forms A and B and *An. pursati* Forms A, B and C (Thongsahuan et al. 2014).

## 1.3 Purpose of This Study

1.3.1 To search for new karyotypic forms within the taxon *An. nigerrimus* and *An. nitidus*.

1.3.2 To evaluate the role of karyotypic variants in generating post-mating barriers by means of hybridization experiments.

1.3.3 To compare intraspecific sequence variation of ribosomal DNA (ITS2) and mitochondrial DNA (COI, COII) among karyotypic variants.

## 1.4 Usefulness of the Study

Exact identification of species, sibling species and subspecies members within the taxon *Anopheles* species complex is a main key of importance necessary for success in controlling anopheline vectors. Inability to identify exact species, sibling species and/or subspecies members within the taxon is the principle cause of failure in control efforts. Regarding to *An. nigerrimus* and *An. nitidus*, they not only exhibit mostly identical adult morphology, but also marked genetic diversity at chromosomal levels. The last factor potentially results in existing species complexes bringing about difficulty in recognition of sibling species and/or subspecies members. Thus, it is largely believed that the outcomes obtained from multidisciplinary approaches will enable construction of robust identification tools for *An. nigerrimus* and *An. nitidus* species, including sibling species and/or subspecies members within each taxon. This is an important key necessary for the formation of a reliable and effective strategy for vector control measurement.