CHAPTER 4

Discussion

Metaphase chromosome investigations of 4 *An. nigerrimus* isolines from 2 allopatric locations (2 isolines/each location) in Thailand (Nachaluai District, Ubon Ratchathani and Bangpa-in District, Ayutthaya provinces) were performed first by Baimai et al. (1993b). The results revealed that this anopheline species exhibited karyotypic variation via a gradual increase in the extra heterochromatin on X (X_1, X_2) and Y (Y_1, Y_2) chromosomes. In this study, a total of 13 *An. nigerrimus* isolines obtained from 4 and 1 locations in Thailand and Cambodia, respectively, demonstrated 3 types of X (X_1, X_2, X_3) and 4 types of Y (Y_1, Y_2, Y_3, Y_4) chromosomes, thus forming 4 karyotypic forms, which were designated as Form A (X_1, X_2, X_3, Y_1), B (X_2, X_3, Y_2), C (X_1, Y_3) and D (X_3, Y_4). The newly discovered Form C and D from Ratanakiri, Cambodia and Ubon Ratchathani, northeastern Thailand, were based on the unique characteristics of small telocentric Y_3 and small subtelocentric Y_4 chromosomes, respectively, which were clearly different from the former 2 types of Y chromosomes (large subtelocentric Y_1, submetacentric Y_2) previously reported by Baimai et al. (1993b). Apparently, the 4 distinct karyotypic forms of *An. nigerrimus* were due to the gradual addition of extra heterochromatin on sex chromosomes. Thus, the accumulation of heterochromatin in the genome elucidates the possible cytological mechanism for karyotypic evolution of Oriental anophelines as proposed by Baimai (1998). Regarding the distribution of *An. nigerrimus* cytological forms, it is worth noting that a new karyotypic Form C was detected in only 2 isoline colonies from Ratanakiri, Cambodia, whereas Form A was common in both Thailand and Cambodia. Interestingly, Form B and D were recovered rather specific in Nakhon Si Thammarat province, southern region and Ubon Ratchathani province, northeastern region of Thailand, respectively. However, additional surveys are expected in order to obtain greater numbers of isolines

from both countries, and this would bring about understanding of the exact distributed-pattern of *An. nigerrimus* cytological forms.

Hybridization experiments using isoline colonies of *Anopheles* mosquitoes, which relate to data of cytogenetic and molecular investigations to elucidate post-mating barriers, have been proven so far as robust traditional techniques for recognizing sibling species and/or subspecies members within the taxon *Anopheles* (Kanda et al. 1981; Baimai et al. 1987; Subbarao 1998; Junkum et al. 2005; Somboon et al. 2005; Saeung et al. 2007, 2008; Thongwat et al. 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009; Choochote 2011; Saeung et al. 2012). The genetic diversity at the chromosomal level of *An. nigerrimus* found in this study, warrants intensive hybridization experiments among the 4 karyotypic forms. The results showed no post-mating reproductive isolation. All crosses yielded viable progenies through F_2-generations and synaptic salivary gland polytene chromosomes, suggesting conspecific nature comprised 4 cytological forms within this taxon. The low intraspecific sequence variations (average genetic distance = 0.002-0.007) of the nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII) of the 4 karyotypic forms in both Thai and Cambodian *An. nigerrimus* populations were good supportive evidence.

It has long been known that misidentification of species leads to failure in controlling target vectors, especially the sibling species and/or subspecies members of *Anopheles* species complexes in sympatric areas. Several studies reported misidentification of malaria vectors, due to overlapping and/or variations based on morphological characters (Van Bortel et al. 2001; Singh et al. 2010). Paredes-Esquivel et al. (2011) reported that field workers had misidentified mosquitoes of the Hyrcanus Group as belonging to species of the Barbirostris Complex. In order to overcome unresolved taxonomic questions on members of the Hyrcanus Group, the evidence from molecular markers was combined with morphological and hybridization experiments that identified the exact species status of 4 karyotypic forms of *An. nigerrimus* for the first time in two different geographical localities. Furthermore, three ITS2 published sequences of specimens from south Kalimantan, Indonesia (K13: GenBank accession number HM488261, K22: GenBank accession number HM488263, K26: GenBank

accession number HM488267) were identified as the Hyrcanus Group by Paredes-Esquivel et al. (2011) and retrieved and compared with sequences of this study. It was interesting to note that these sequences were placed within the same clade of the Thai and Cambodian *An. nigerrimus* in the phylogenetic tree and the low level of intra-specific divergence (0.001-0.006) was found among them. This study confirms the presence of *An. nigerrimus* in Kalimantan, Indonesia, which corresponds to previous findings by O'Connor (1980).

Regarding *An. nitidus*, a cytogenetic investigation of *An. nitidus* in Thailand was carried out first by Baimai et al. (1993b), who demonstrated two types of X (X_1, X_2) and one type of Y chromosomes which obtained from 2 isoline colonies caught from Muang district, Phang Nga province and Sadao district,Songkhla province, southern Thailand. The results indicated that this anopheline species exhibited genetic diversity at the chromosomal level via a gradual increase in the extra block(s) of constitutive heterochromatin in the X chromosome (X_1, X_2), whereas this event was not detected in the Y chromosomes, possibly due to the limited number of isolines used. Herein, the 21 *An. nitidus* isolines from 2 allopatric locations [Phang Nga province, southern region; Ubon Ratchathani province, northeastern region] in Thailand revealed 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, which were designated as Form A (X_1, Y_1), Form B (X_1,Y_2), Form C (X_2, Y_3), Form D (X_1, X_3, Y_4) and Form E (X_1, X_2, X_3, Y_5), depending upon the uniquely distinct characteristics of Y chromosomes. The five different karyotypic forms of *An. nitidus* found in this study were due clearly to the addition of extra block(s) of constitutive heterochromatin on sex chromosomes (X, Y), which is in keeping with Baimai's hypothesis (Baimai 1998). Baimai suggested that the quantitative differences in heterochromatin of mitotic chromosomes could be used as a genetic marker for further identification of cryptic (isomorphic) or closely related species, as exemplified in the population cytogenetic studies of the Dirus Complex and the Maculatus Group (Baimai et al. 1984a, 1984b; Baimai et al. 1988; Baimai et al. 1993a). Interestingly, investigation of the 18 isolines from Ubon Ratchathani province, northeastern region, revealed only 2 karyotypic forms (Form D: 10 isolines; Form E: 8 isolines), whereas that of the three isolines from Phang Nga province, southern region, yielded 3 distinct karyotypic forms (Forms A, B and C) in each isoline, even though these 2 allopatric locations were placed approximately 800

km apart. The climate of these 2 provinces is quite different, i.e., Ubon Ratchathani province has a tropical wet and dry climate whereas Phang Nga province is located on the shore to the Andaman Sea, and has heavy rain. Our results are in accordance with Saeung et al. (2014). These authors showed that *An. crawfordi* Form A was detected only in Phang Nga province, whereas Forms A, B, C and D were found from 8 isolines in Trang province, which placed approximately 190 km apart. This phenomenon appeared to elucidate the difference in ecological diversity, which favored specific microhabitats for the karyotypic forms of *An. nitidus*. However, additional surveys are expected in order to obtain greater numbers of isolines from both provinces and/or other locations across 6 regions (northern, western, central, northeastern, eastern and southern) of Thailand. This would bring about understanding of the population-genetic structure of this anopheline species.

Cross-mating experiments among the five karyotypic forms of *An. nitidus* showed no post-mating reproductive isolation. They yielded viable progenies through F_2 generations and synaptic salivary gland polytene chromosomes, along the entire length of autosomes and the X chromosome. Thus, our results indicated that the five karyotypic forms were conspecific. Quantitative changes in constitutive heterochromatin in mitotic chromosomes of *An. nitidus* observed in this study were likely intraspecific chromosomal variation which may lead to interspecific difference in the process of speciation. Our results are agreed with previous cross-mating experiments among sympatric and/or allopatric karyotypic forms of other anopheline species, i.e., *An. vagus* (Choochote et al. 2002a), *An. pullus* (Park et al. 2003), *An. sinensis* (Choochote et al. 1998; Min et al. 2002; Park et al. 2008b), *An. aconitus* (Junkum et al. 2005), *An. dissidens*, *An. saeungae* (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Taai and Harbach 2015), *An. wejchoochotei* (Thongsahuan et al. 2009; Taai and Harbach 2015), *An. peditaeniatus* (Choochote 2011; Saeung et al. 2012), *An. paraliae* (Taai et al. 2013b), *An. argyropus* and *An. pursati* (Thongsahuan et al. 2014).

Furthermore, this study incorporated a nuclear and mitochondrial DNA sequence to increase the exact identification of this species from other species members of the Hyrcanus Group (Min et al. 2002; Park et al. 2003; Park et al. 2008a; Choochote

2011; Taai et al. 2013a). The monophyletic trees and very low intra-specific sequence variations (average genetic distances = 0.002-0.008) of the ITS2, COI and COII of the five karyotypic forms are good supportive evidence, which confirms that these forms represent a single species of *An*. *nitidus*. It is interesting to note that the three specimens (TR2, TR3 and TR6) collected from Trat province, eastern Thailand, and identified as the Hyrcanus Group by Paredes-Esquivel et al. (2011), based on ITS2 sequences, were clustered together with five karyotypic forms of *An. nitidus*, and are presumed to belong to that species.