CHAPTER 4

Discussion

In Thailand, investigations on the metaphase karyotypes of the Hyrcanus Group have been reported on only 6 species, which were *An. sinensis* Forms A (X, Y_1) and B (X, Y_2), *An. nigerrimus* Forms A (X_1, Y_1) and B (X_2, Y_2), *An. crawfordi* Forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), *An. argyropus* Forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), *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), and 2 types of X (X_1, X_2) and 1 type of Y chromosomes in *An. nitidus* (Baimai et al. 1993b). These species exhibited genetic diversity at the chromosomal level. However, the metaphase karyotype of *An. paraliae* has never been reported. Therefore, this study extensively investigated 16 *An. paraliae* isolines from 4 provinces in 3 regions (western, eastern, southern) of Thailand. The results demonstrated that typical metaphase karyotypes (2n = 6) comprise two pairs of autosomes (submetacentric and metacentric) and one pair of heteromorphic sex chromosomes. These metaphase karyotypes can be distinguished on the basis of size, shape, amount and distribution of constitutive heterochromatin, similar to that of the 6 species mentioned above. Five distinct karyotypic forms, i.e., 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), of *An. paraliae* are due to additional heterochromatin block(s) on sex chromosomes (X, Y), which means that this study is in keeping with Baimai's hypothesis. Baimai (1998) stated that this phenomenon is an important mechanism in the speciation process of Oriental anophelines. It also could be used effectively as a primary genetic marker for further recognitions of sibling species and subspecies members within the taxon of *Anopheles*.

Cross-mating experiments using iso-female lines of closely related Oriental *Anopheles* species have proven to be a robust systematic procedure for clarifying

species status on, for example, *An. minimus* and *An. aconitus* (Harrison 1980; Sucharit and Choochote 1982), *Anopheles annularis* and *Anopheles philippinensis* (Choochote et al. 1984), *Anopheles nivipes* and *An. philippinensis* (Klein et al. 1984) and *An. minimus* and *Anopheles flavirostris* (Somboon et al. 2000). These methods are useful for solving taxonomic problems of some sibling species complexes, e.g., *An. dirus* (Baimai et al. 1987), *Anopheles maculatus* (Thongwat et al. 2008), *An. minimus* (Somboon et al. 2001, 2005; Choochote et al. 2002b) and *An. barbirostris* (Saeung et al. 2007, 2008; Suwannamit et al. 2009). Likewise, the status of subspecies or cytological races of *Anopheles* can be elucidated by the same approach of cytogenetic study, as exemplified in *An. pullus* (= *Anopheles yatsushiroensis*) (Park et al. 2003), *An. vagus* (Choochote et al. 2002a), *An. aconitus* (Junkum et al. 2005), *An. sinensis* (Choochote et al. 1998; Min et al. 2002; Park et al. 2008b), *An. barbirostris* species A1 (Saeung et al. 2007; Suwannamit et al. 2009), *Anopheles campestris*-like taxon (Thongsahuan et al. 2009) and *An. peditaeniatus* (Choochote 2011).

Due to the markedly distinct characteristics of X (X_1, X_2, X_3) and Y (Y_1, Y_2, Y_3, Y_4, and Y_5) chromosomes among the 5 karyotypic forms of *An. paraliae*, as presented in this study, the role of these karyotypic variants in generating post-mating barriers was determined by using cross-mating experiments, relating to their comparative DNA sequences of ITS2, COI, and COII. The results of no post-mating reproductive isolation, by yielding viable progenies through F_2-generations and synaptic salivary gland polytene chromosomes, indicated a conspecific nature, comprising 5 cytological races within this taxon. The very low intra-specific sequence divergence (average genetic distance = 0.000- 0.002) of the nucleotide sequences of the ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII) of the 5 karyotypic forms was strong supportive evidence. The results obtained from this study were similar to those in several previous reports on *An. vagus* Forms A and B (Choochote et al. 2002a), *An. pullus* Forms A and B (=*An. yatsushiroensis*) (Park et al. 2003), *An. sinensis* Forms A and B (Choochote et al. 1998; Min et al. 2002; Park et al. 2008b), *An. aconitus* Forms B and C (Junkum et al. 2005), *An. barbirostris* species A1 (Forms A, B and C) and species A2 (Forms A and B) (Saeung et al. 2007; Suwannamit et al. 2009), *An. campestris*-like Forms B, E, and F (Thongsahuan et al. 2009), and *An. peditaeniatus* Forms A, B, C, D, E and F (Choochote 2011; Saeung et al. 2012).

The intraspecific variation of morphological characteristics within the *Anopheles* species is common and results may risk misidentifications in vector control programs. The wing markings provide important characteristics for anopheline identification, but they are highly variable in some groups. Consequently, identifiers should anticipate intraspecific variation when attempting to identify specimens to species (Ngo et al. 2013).

Several investigators have reported the effect of environmental conditions on expression of the morphological characteristics observed in *Anopheles* (Harrison 1980; Van Bortel et al. 1999; Hwang et al. 2004; Singh et al. 2010). For example, Park et al. (2003) reported that variation in the pattern of dark scaling on the wings; a characteristics used previously to differentiate *An. pullus* and *An. yatsushiroensis* (= *An. pullus*) in Korea, was attributed to seasonal variation. More recently, Ngo et al. (2013) showed that *An. dangi* is merely a morphological variant of *An. crawfordi* and is deemed to be a synonym of that nominal species, based on the low variation of COI, COII and Cyt-*b* genes of mtDNA, and the D3 gene of rDNA sequences. They found that the humeral pale spot, which is sometimes represented by only a few pale scales, is present occasionally in *An. crawfordi*. Their results are in agreement with those of Reid (1953) and Harrison and Scanlon (1975), who noted that specimens of *An. crawfordi* occasionally exhibit two or three pale scales or a distinct humeral pale spot on the costa; usually on one wing and particularly in males. Likewise, the species identity of *An. paraliae* and *An. lesteri* is 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}), which has led so far to taxonomic problems. Thus, the genetic relationships between *An. lesteri* and *An. paraliae* were determined intensively in order to clarify their specific species status. The findings in this study showed no post-mating reproductive isolation between *An. lesteri* from South Korea and *An. paraliae* from Thailand. These results were supported clearly by cytological evidence (polytene chromosome) and DNA analysis. Therefore, complete synapsis of salivary gland polytene chromosomes without inversion loops along the entire length of all chromosome arms, was observed in the F_1-hybrid larvae of *An. lesteri* and *An. paraliae*, which strongly indicated genetic compatibility between them.

ITS2 sequence analyses of *An. lesteri* from South Korea (ilG1, ilG2, ilG3) revealed identical sequences to *An. lesteri* from China (= *An. anthropophagus*) and Japan (genetic distance = 0.000), although they showed some difference from those of the Philippines (genetic distance = 0.007) (Wilkerson et al. 2003; Ma and Yang 2005; Park et al. 2008a; Sawabe et al. unpublished data). The results of this study were in agreement with those previously reported by Ma and Xu (2005). In addition, the low level of pairwise distance (0.040) detected between *An. lesteri* from South Korea and *An. paraliae* from Thailand, based on ITS2 sequences, was in accordance with previous reports from different groups of *Anopheles*, e.g., the *Anopheles gambiae* complex (0.4-1.6%) (Paskewitz et al. 1993), *Anopheles dunhami* and *Anopheles nuneztovari* (mean genetic distance = 0.025) (Ruiz et al. 2010), *Anopheles fluviatilis* S and *An. minimus* C (pairwise distance = 0.036) (Singh et al. 2006), *An. kunmingensis* and *An. liangshanensis* (pairwise distance = 0.0381) and *An. pullus* (= *An. yatsushiroensis*) and *Anopheles junlianensis* (pairwise distance = 0.03081) (Hwang 2007). Currently, Calado et al. (2008) show that *An. nuneztovari* A is not conspecific with *An. nuneztovari* B/C, based on COI sequences (genetic distance = 0.00818-0.02071), and *An. dunhami* has been reported as newly recorded in the Brazilian Amazon by comparing sequences with those of *An. nuneztovari* A (genetic distance = 0.01436-0.03343). Similarly, comparative sequences for COI and COII between *An. lesteri* and *An. paraliae* revealed low average genetic distance between them (0.008-0.011). The phylogenetic trees and low values of genetic distances suggest that these 2 species have closer genetic relationships than the other species members of the Hyrcanus Group.

Remarkably, controversy over taxonomic problems, with respect to fully-fledged species, sibling species and subspecies within the taxon of *Anopheles*, has occurred when only data of comparative DNA sequence analyses of certain specific genomic regions were used as first hand criteria for their separation. For example, *An. fluviatilis* S and *An. minimus* C were considered a synonymous, based on comparison of the D3 domains of 28S (28S-D3) (Harbach 2004; Garros et al. 2005; Chen et al. 2006). However, Singh et al. (2006) carried out molecular analysis on ITS2 and D2-D3 domains of the 28S rDNA regions of *An. fluviatilis* S and *An. minimus* C. The authors suggest that these *Anopheles* species do not deserve synonymous status. Hence, cross-

mating experiments between *An. fluviatilis* S and *An. minimus* C, using iso-female lines, are essential prior to a definite conclusion as to their conspecificity.

This study has clearly indicated that *An. lesteri* from South Korea and *An. paraliae* from Thailand are conspecific within the taxon *An. lesteri* by using crossing experiments incorporated with data on species distributions, morphological variants, cytology (polytene chromosome) and comparative DNA sequence analyses.