CHAPTER 4

Discussion

4.1 Molecular species identification

Prior to the study on biology, ecology and behavior of mosquito-borne disease vectors, accurate species identification is the main key for success in obtaining robust information. Members of the eight species of the Hyrcanus Group collected in Thailand exhibit overlapping morphological characteristics in the adult stages, which leads to the misidentification of females, particularly when using the traumatic scales of wild-caught specimens from epidemiology and vector control approaches (Hempolchom et al. 2013). Consequently, ribosomal (ITS2) and mitochondrial (COI and COII) DNA have been used intensively and widely in the Oriental region for recognition of the species of Hyrcanus Group (Paredes-Esquivel et al. 2011; Min et al.2002; Park et al. 2003; Wilkerson et al. 2003; Ma and Xu 2005; Rueda 2005; Hwang 2007; Park et al. 2008a, b; Joshi et al. 2010; Choochote 2011; Saeung et al. 2012). However, COI barcode sequences for identification of the eight species of the Hyrcanus Group in Thailand are not available. Thus, we initiated this approach to develop DNA barcodes for these species. Our phenotypic analyses here separated the eight species into eight distinct clusters. In accordance with Harbach (2015), *An. crawfordi*, *An. paraliae* and *An. peditaeniatus* are classified as members of the Lesteri Subgroup of the Hyrcanus Group, *An. nigerrimus*, *An. nitidus* and *An. pursati* are classified in the Nigerrimus Subgroup, and *An*. *argyropus* and *An*. *sinensis* are unplaced species of Group. However, interestingly, based on this study, *An*. *sinensis* shares a closer relationship to species of the Lesteri Subgroup and *An*. *argyropus* appears to be more closely related to species of the Nigerrimus Subgroup. Furthermore, the intra-specific genetic distances less than 1%, and the inter-specific genetic distances greater than 2% that were obtained during the study are in agreement with the threshold value for distinguishing species based on COI barcode sequences (Hebert et al. 2003; Kumar et al. 2007; Laboudi et al. 2011; Ruiz-Lopez et al. 2012). In contrast to the previously reported for Neotropical species of *Anopheles* (*Nyssorhynchus*) by Foster et al. (2013), this study showed non-overlapping of intra- and inter-specific genetic distances based on COI sequences. In addition, the COI sequences of *An. paraliae*, *An. peditaeniatus* and *An. sinensis* showed little difference (mean genetic distances=0.002-0.005) from sequences in GenBank, i.e., *An. paraliae* (accession number AB733031) (Taai et al. 2013), *An. peditaeniatus* (accession numbers AB539069 and AB715091) (Saeung et al. 2012) and *An. sinensis* (accession number AY444351) (Saeung et al. 2012). It is interesting to note that *An. lesteri* from Korea (accession number AB733028) exhibits a low level (0.011) of inter-specific genetic distance with our *An. paraliae*, which is congruent with the current results of Taai et al. (2013), who suggested that these two morphological species were conspecific based on results of no post-mating reproductive isolation from hybridization experiments, together with the molecular data of low pairwise genetic distances of COI (0.007-0.017). Thus, the results of this study show that COI-based DNA barcoding is a very effective tool for identifying field-collected Hyrcanus Group mosquitoes, not only in Thailand but also in other countries.

4.2 Screening of stenogamous behavior and establishment of self-mating colony

Establishment of colonies is a fundamental for mosquito-borne-disease research, and the inability to maintain a healthy colony of difficult-to-rear species is a principal cause of many failed research efforts. It has long been known that the anopheline mosquitoes have difficulty copulating naturally under laboratory conditions, especially in small spaces, such as a 30 x 30 x 30 cm size cage. However, some species can successfully copulate in small cages, e.g., *An. quadrimaculatus* (Heal and Pergrin 1945; Casanges et al. 1949), the Gambiae Complex (Davidson 1969), *An. earlei* (Kreutzer and Kitzmiller 1969), *An. sinensis* (Oguma and Kanda 1976; Li et al. 1999; Kim et al. 2003), *An. farauti* No. 1 (Bryan 1977), *An. albimanus* (Bailey et al. 1980), *An. subpictus* (Panicker and Geetha Bai 1980), *An. cracens* (Sucharit and Choochote 1983; Sallum et al. 2005), *An. annularis* (Choochote et al. 1983), *An. dirus* (Lianzhu et al. 1986), *An. barberi* (Copeland 1987), *An. sergentii* (Beier et al. 1987), *An. freeborni* (Fritz et al.

1989), *An. barbirostris* (Soelarto et al. 1995), *An. minimus* (Somboon and Suwonkerd 1997), *An. albitarsis* (Horosko et al. 1997), *An. maculatus* (Bangs et al. 2002), *An. aquasalis* (Da Silva et al. 2006), *An. stephensi* (Grech et al. 2007) and *An. pseudopunctipennis* (Lardeux et al. 2007). Therefore, artificial mating techniques have been developed by previous investigators in order to solve the mating problems for maintaining laboratory colonies (Baker et al. 1962; Ow Yang et al. 1963).

In laboratory conditions, the limited space in a 30 x 30 x 30 cm size cage appears to prevent swarming, and therefore copulation. However, there are many species of *Aedes*, *Culex* and *Mansonia* that can copulate without males forming a swarm, and they mate easily in small spaces (Wharton 1953; Sasa et al. 1967; Clements 1999). In this study, three species of the Hyrcanus Group (*An. argyropus*, *An. crawfordi* and *An. nitidus*) failed to copulate naturally in a 30 x 30 x 30 cm cage (triplicate experiments). After selection a colony of self-mating stenogamous *An. peditaeniatus* thrived for more than 20 generations, with insemination rates ranging from 61-86%. However, *An. sinensis*, *An. pursati*, *An. nigerrimus* and *An. paraliae* failed to copulate naturally in 30 x 30 x 30 cm cages, with a 0% insemination rate at F_{11}, F_{12}, F_{15} and F_{16} after selection. The success in selecting a a colony of stenogamous *An. peditaeniatus* strongly suggest that an artificially mated colony of this anopheline species can easily modify its behavior to reproduce naturally in a 30 x 30 x 30 cm size cage. The first self-mating colony of *An. peditaeniatus* in Thailand was established during this study.

4.3 Searching for possible mechanism(s) that control stenogamous behavior

In view of the success in establishing a stenogamous colony of *An. peditaeniatus*, the possible mechanism that controls its stenogamous behavior was investigated intensively and compared with the behavior of the seven eurygamous species (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae, An. pursati* and *An. sinensis*) included in the study.

The study of stenogamous behavior in adult mosquitoes when mating naturally in 10, 20, 30 and 40 cubic cm cages, with a Density Resting Surface (DRS) of 3.6 and 7.2, was carried out using a procedure similar to that detailed by Choochote et al. (1983), who used a stenogamous colony and DRS of 7.2 (vertical resting surface) for

An. annularis. Three hundred males were deemed appropriate for copulation with 200 females, since male anophelines are monogamous in their mating behavior. In the present study, among the eight species, the highest insemination rates (70-97) were obtained from *An. peditaeniatus* in all cage sizes at both DRS 3.6 and 7.2, whereas *An. crawfordi* had the lowest rate (0-4). Remarkably, more than 50% of inseminated females of *An. peditaeniatus* had high sperm density (3+ and 4+) in their spermathecae in all experiments, and no statistically significant difference using various cage sizes and DRS. Thus, it appears that neither cage size nor DRS influenced the mating success of this stenogamous species.

Basically, males within dancing swarms give a mating response when stimulated by flight tones (wing-beat sound) of a conspecific female flying nearby (Belton and Costello 1979; Tamarina et al. 1980; Brogdon 1994; Wekesa et al. 1998). The studies on size assortative mating by Yuval et al. (1993) demonstrated that a bigger size of male *An. freeborni* can mate more often with females than the smaller male. Similarly, Maïga et al. (2012) found that mated male *Anopheles gambiae* was significantly bigger than non-mated ones. Comparative measurements of wings in adult females and males between the stenogamous and eurygamous species showed that the wings of males were statistically significant difference. *Anopheles crawfordi* has the largest size of males, whereas, *An. peditaeniatus* is an intermediate-sized male. However, the male size of *An. peditaeniatus*, which may involve its mating behavior, corresponds with studies by Ng´habi et al. (2008), who reported that intermediate-sized males mate more successfully, either due to being more agile in flight or because they can make and maintain contact with females faster and longer within swarms.

The mean palpomere ratios in females were different with statistical significance between stenogamous and eurygamous species. In contrast to this study, Junkum (2006) reported no significant difference in the palpomere ratios of adult females between *Anopheles aconitus* Forms B and C. The palpal ratios have been used as taxonomic tools for distinguishing members of the Gambiae Complex, *Anopheles melas*, in field studies (Gillies and Wilkes 1969). Subsequently, Mosha and Mutero (1982) reported that the combined values for sensilla coeloconica numbers and palpal ratio could separate only 40.9% of specimens of *Anopheles merus* from freshwater *An. gambiae* s.l.

It is interesting to note that *An. peditaeniatus* was longer in palpomere 4 size than other species. However, certain relationships between the length of palpomere 4 and mating behavior for this stenogamous species are still unclear.

The olfactory receptor neurons are located in cuticular sensilla on the antennae and maxillary palpi of mosquitoes. Antennae are the major site of the chemoreceptors that detect and discriminate between air-borne stimuli and guide the females to suitable hosts or to an oviposition site. Hence, it has been assumed that antennal sensilla that most olfaction-driven behaviours, such as host-seeking, oviposition, sources for nectar-feeding, are mediated by these sensilla. Subsequently, other sensory structures on other parts of the body, labellum, tarsi, genitalia, etc., also play an important role in mosquito behaviour (McIver 1982). The sensory mechanism plays a significant role in host-seeking and oviposition behavior of mosquitoes, which enable them to transmit various diseases to human (Seenivasagan et al. 2009).

This study is the first to reveal under light microscopy the variation in the number of sensilla coeloconica on the antennal flagellum of females of the eight species of the Hyrcanus Group. However, it was not possible to count individual sensilla contained within sacculi on the flagellomeres of *An. argyropus* and *An. peditaeniatus*, as clearly revealed by using the SEM. The number of sensilla coeloconica on female antennae varied from 45/antenna for *An. crawfordi* to 89 for *An. paraliae*, which is greater than those of *An. maculipennis* (28) and *An. stephensi* (32) (Ismail 1962, 1964). Also, the mean number of sensilla coeloconica per antennae of each of the eight species is greater than the number found on the antennae of *An. gambiae* s.s. (21.6) and *An. quadriannulatus* (29) (Pitts and Zwiebel 2006). Nevertheless, the comparisons of the mean numbers of these particular sensilla per antennae revealed no statistically significant difference between them.

Sensilla coeloconica are small, thick-walled sensilla that occur in small and elarge forms in the anophelines (McIver 1982). Small sensilla coeloconica are also have a peg set into the bottom of a pit but the peg did not protrude from the opening (McIver 1982). These sensilla are volcano-like structure with an opening at the peak and have a much smaller cuticular opening than the large coeloconica. Large sensilla coeloconica are commonly called pitted pegs and are absent in the culicines. These sensilla appeared as round openings in the cuticle, with single peg-shaped setae projecting from within, and parallel to the walls of the pit. The pegs of large sensilla coeloconica were grooved lengthwise but had more grooves than sensilla basiconica (Pitts and Zwiebel 2006). This type of sensilla usually locates in a sunken depression of the integument called a saccalus (Sukontason et al. 2004). The sunken group of sensilla basiconica both in individual sockets and group pit of the antennae of female *An. barbirostris* was also reported by Kaur (2005). In this study, SEM observations revealed the cluster of 7-15 large sensilla coeloconica located in sacculi on flagellomeres 3-7 of *An. peditaeniatus*. *Anopheles argyropus* also bore a cluster of large sensilla coeloconica on flagellomeres 3-7, whereas the other six species have simple large sensilla coeloconica (pit with a single sensillum) on their flagellomeres instead of clusters of sensilla. In addition to sensilla coeloconica, three other types of sensilla i.e., sensilla chaetica (spine-like organs), sensilla trichodea (typical hair-like setae) and sensilla basiconica (grooved pegs), occur on the antennae of the eight species included in the present study. The types of sensilla found in these species also occur in other mosquito species (Ismail 1962, 1964; Seenivasagan et al. 2009; Kaur 2005; Pitts and Zwiebel 2006), flies (Sukaontason et al. 2004, 2007) and biting midges (Urbanek et al. 2014).

Ismail (1962) and McIver (1982) suggested that large sensilla coeloconica are probably olfactory sensilla. In this study, the sunken group of large sensilla coeloconica that are borne on the antennal flagellomeres of females might contribute to the successful mating of the stenogamous *An. peditaeniatus* in small cages. However, detailed SEM and electrophysiological studies of the different types of sensilla and their distributions on the antennae of females of the eight species of the Hyrcanus Group must be must be conducted before definitely drawing conclusion about their function.

The behavioral polymorphism, stenogamy/eurygamy, of anophelines has been shown to be inherited and controlled by one or more genes located on the Y-chromosome (Fraccaro et al. 1977). Additionally, differences in male genital morphology and frequency of clasper movements have been reported as possibly being involved in the stenogamous behavior of mosquitoes, e.g., stenogamous *An. cracens* and eurygamous *An. dirus* of the Dirus Complex of subgenus *Cellia* (Sallum et al. 2005) (Sucharit and Choochote 1983). The genitalia of *An. cracens* are larger than those of *An.*

dirus. This study found differences in the size of the male genitalia of the stenogamous and eurygamous species. The gonostyli of *An*. *peditaeniatus* were significantly shorter than the gonostyli of the eurygamous species, in concert with the findings of Sucharit and Choochote (1983). However, no significance difference was found in size of the gonocoxites. Hence, it might be supposed that a shorter gonostylus could contribute to decrease mating duration (clasping during copulation) for *An*. *peditaeniatus*.

The frequency of clasper movement during induced copulation and mating duration was observed for stenogamous and eurygamous species. The frequency of clasper movement of the stenogamous *An. peditaeniatus* was lower than that in the eurygamous species. Also, the duration of copulation of this species was shorter than that in the other species, except for *An. argyropus*. These findings are consistent with those of Sucharit and Choochote (1983) who found that the stenogamous *An. cracens* has a lower frequency of clasper movement and and shorter period of copulation than *An. dirus*. The shorter duration of pumping motion associated with clasper movement in *An. punctipennis* compared to that of *An. perplexens* was also observed by Kreutzer and Kitzmiller (1971). Kanda and Oguma (1976) reported that the frequency of clasper movement can be used to distinguish various strains of *An. sinensis* (Japan Strain), which are morphologically highly variable. In addition, Sucharit and Choochote (1983) suggested that the morphology of male genitalia and the frequency of clasper movements during induced copulation might be used as a tool for distinguishing *An. cracens* and *An. dirus*.