CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Molecular study

- Adjustable automatic pipettes and tips
- Autoclave
- Beakers
- Bottles
- Classic Light Balances (Mettler-Toledo AG Laboratory and Weighing Technologies, Switzerland)
- Cylinder
- Dropper
- Electrophoresis (Mupid®-exu, Japan)
- Elite dry bath incubator (Major Science, U.S.A.)
- Erlenmeyer flask
- High speed Mini-centrifuge (Biosan, Latvia)
- Microwave oven
- Refrigerator and freezer (-20°C)
- Swift Minipro Thermal Cyclers (Esco Healthcare, Singapore)
- Vortex mixer (Biosan, Latvia)
- 1.5 ml micro-centrifuge tubes
- 0.2 ml PCR tubes

2.1.2 Mosquito rearing

- Black plastic sheet
- Bottle (3 cm in diameter and 6 cm in depth)
- Cages
  (10 x 10 x 10 cm, 20 x 20 x 20 cm, 30 x 30 x 30 and 40 x 40 x 40 cm)
- Cotton
- Dropper
- Filter paper
- Fine mesh nylon
- Fish food
- Garden grass (*Axonopus compressus*)
- Humid chamber
- Natural water
- Plastic cup (8.5 cm in diameter and 11 cm in depth)
- Plastic bag
- Plastic tray (25 x 36 x 6 cm)
- Towel
- 5% multivitamin solution
- 10% sucrose solution

2.1.3 Measurement

- Compound microscope (Olympus BX53, Japan)
- Cover glass
- Insect needle
- Petri dish
- Slides
- Stereoscopic microscope
- Surgical blade

2.2 Chemicals

2.2.1 Molecular study

- Agarose (Vivantis, Malaysia)
- BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Japan)
- Deoxyribonucleotide triphosphate (Invitrogen™, U.S.A.)
- De-ionized distilled water
- Distilled water
- DNeasy® Blood & Tissue kit (Qiagen, Japan)
- Ethidium bromide
- *Ex Taq* (Takara, Japan)
- Magnesium chloride ($MgCl_2$)
- Molecular weight marker
- Primers (Invitrogen™, U.S.A.)
- QIAquick® PCR Purification Kit (Qiagen, Japan)
- 6X loading dye (Qiagen, Japan)
- 10X PCR buffer (Wako, Japan)
- 70%, 100% ethanol

2.2.2 Measurement

- Hoyer's media
- 10% KOH

2.2.3 Scanning electron microscopy (SEM)

- phosphate buffer (pH 7.4)
- 35%, 70%, 80%, 95%, 100% ethanol

2.3 Methods

2.3.1 Field collection of the Hyrcanus Group

Eight species of the Hyrcanus Group were collected in six provinces of western and southern Thailand (Figure 2.1), where malaria and filariasis are endemic due to *Plasmodium falciparum* and *P. vivax,* and *W. bancrofti*, respectively (Manguin et al. 2010). The species and strains were as follows: *An. argyropus* (Nakhon Si Thammarat strain: 08° 29′ N, 100° 0′ E), *An. crawfordi* (Trang strain: 07° 33′ N, 99° 38′ E), *An. nigerrimus* (Songkhla strain: 07° 13′ N, 100° 37′ E), *An. nitidus* (Phang Nga strain: 08° 27′ N, 98° 31′ E), *An. paraliae* (Ratchaburi strain: 13° 30′ N, 99° 54′ E), *An. peditaeniatus* (Phang Nga strain: 08° 27′ N, 98° 31′ E), *An. pursati* (Ratchaburi strain: 13° 30′ N, 99° 54′ E) and *An. sinensis* (Chumphon strain: 10° 29′ N, 99° 11′ E). Wild-caught, fully engorged females of these species were collected from cow-baited traps.

2.3.2 Morphological species identification

Identification of wild caught-females followed standard illustrated keys (Reid 1968; Harrison and Scanlon 1975; Rattanarithikul et al. 2006). Subsequently, identification using intact morphology of eggs, larvae, pupal skins and adult females were performed intensively in F_1-progenies of iso-female lines.

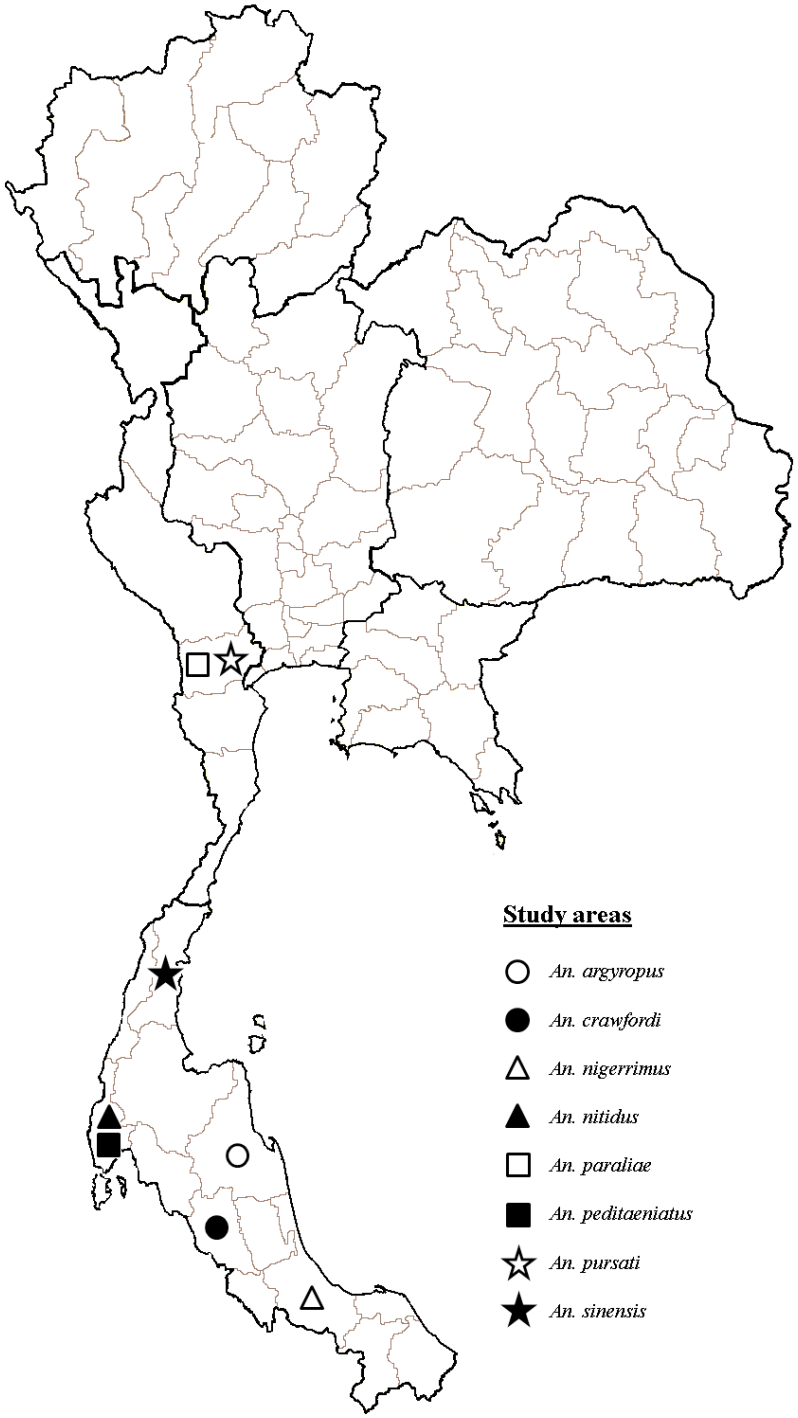

Figure 2.1 Map of Thailand showing six provinces where samples of eight species belonging to the Hyrcanus Group were collected

2.3.3 Molecular species identification

In order to guarantee the exact morphological identification of species, individual F_1-progeny adult females of each iso-female line were performed on DNA extraction and amplification. Genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Japan). The LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) barcoding primers of Folmer et al. (1994) were used to amplify the cytochrome *c* oxidase subunit I (COI) region of mitochondrial DNA (658 bp, excluding primers). Each PCR reaction was carried out in a 20 µl volume containing 0.5 U *Ex Taq* (Takara, Japan), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer, and 1 µl of the extracted DNA. The amplification profile comprised initial denaturation at 94°C for 1 minute, 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The amplified products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Lastly, the PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Japan) and their sequences directly determined using the BigDye® V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems of Life Technologies, Japan). The sequence data obtained were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database. The new COI sequences also were compared with those available in GenBank using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi and sequence data held in the Barcode of Life Data System (BOLD) at http://www.boldsystems.org/. Sequences were aligned with BioEdit version 7.0.5.3 (Hall 1999). Genetic distance was calculated using the Kimura two-parameter (K2P) model (Kimura 1980). Using the distances, construction of neighbor-joining trees (Saitou and Nei 1987) and the bootstrap test with 10,000 replications were performed with the MEGA version 4.0 program (Tamura et al. 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012) by using two replicates of 1 million generations with the nucleotide evolutionary model. The best-fit model was chosen using the Akaike Information Criterion (AIC) in MrModeltest version 2.3 (Nylander 2004). Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% of trees as burn-in.

2.3.4 Mosquito rearing procedures

Mosquito rearing procedures for the Hyrcanus Group (swamp-breeders) followed the detailed techniques described by Choochote and Saeung (2013). Wild-caught fully engorged adult females collected from animal-baited traps in the field were kept in a plastic cup (8.5 cm in diameter and 11 cm in depth, lined inside with filter paper), with a pad of cotton wool soaked with 10% sucrose solution placed on top of a covering screen. In order to keep humid conditions in the cup and delay the soaked cotton wool from drying rapidly, the covering screen was covered with a translucent plastic bag. The cup was stored in a humid chamber using a picnic foam-box (18 x 26 x 39 cm), in order to maintain humidity and temperature, before transporting it to the insectarium for colonization and biological studies. All of the experiments were performed in the insectarium at 27±2 ºC, 70-80% relative humidity, and with illumination from a combination of natural daylight from a glass window and fluorescent lighting provided for approximately 12 hours a day.

After the engorged adult female was maintained for 4-5 days and/or until gravid in the insectarium, it was placed in a screen-topped oviposition plastic-cup (6 cm in diameter and 7 cm in depth) containing 25 ml of natural water (brought from a basin that was used for tap water production). Wet filter paper lined the inside of the screen-topped was covered with a black plastic sheet. The eggs that were attached to the moist side of the filter paper and/or floating on the water's surface were rinsed and transferred to a white plastic tray (25 x 36 x 6 cm) containing 1,500 ml rearing water (equal parts of natural water and distilled water), with wet filter paper lining the inside. During the embryonation period, the eggs were exposed to a 40-watt light instead of sunlight, for warming the eggs until hatching.

After egg hatching, first instar larvae were transferred daily to a white plastic tray (25 x 36 x 6 cm) containing 2,000 ml rearing water and approximately 15 stems of garden grass (*Axonopus compressus*), and 80 first instar larvae were reared in each tray. The rearing tray was covered with a transparent plastic sheet for reducing the need to change and/or re-fill the tray with rearing water during the larval development process. Pulverized fish food was fed to the mosquito larvae. The larval food was sprinkled on the water in the larval trays from a bottle covered with a fine nylon screen.

First and 2nd instar larvae were fed twice daily and this schedule was increased to three times daily after most of the larvae had reached 3rd instar. Before each feeding, floating clumps of excess food were removed by dragging a paper towel across the water's surface. Any larvae trapped on the towel during the cleaning process were removed by rinsing the towel in a pan of clean water, before returning them to the appropriate rearing pan. Unconsumed food and dead larvae at the bottom of the tray were removed from the water with a pipette to avoid development of bacterial scum. The water in the trays was topped-up whenever evaporation decreased its level.

Pupae were removed from the rearing trays each morning and transferred to containers of water in emergence cages. Adults were obtained daily and the sexes were separated. Males were placed in paper cups covered with a nylon screen to enable ease in handling, and females were returned to the cages. Both sexes were provided continuously with 5% multivitamin solution. Five days after emergence, the females were fed on white rat blood, which was used as a source of blood meal for egg production. Subsequently, engorged females were mated with 4 to 5-day-old males using the insemination system described by Baker et al. (1962) and Ow Yang et al. (1963). After mating, the females were further maintained for 4-5 days before placed in an oviposited-plastic cup.

2.3.5 Establishment of a stock colony

After exact species identification, based on intact morphology of eggs, larvae, pupal skins and adult females, as well as molecular investigation of F_1-progenies, the stock colonies of eight species of the Hyrcanus Group were established by pooling five iso-female lines of each anopheline species that had been colonized consecutively for more than 10 generations. These stock colonies were used in the investigation of stenogamous behavior throughout the experiments.

2.3.6 Screening for stenogamous behavior and the establishment of self-mating colony

Mosquitoes of the 9th generation (F_9) were used to determine self-mating ability in a standard 30 x 30 x 30 cm size cage. The reason for using this mosquito generation was based on the fact that any mosquito colony, colonized for more than

eight consecutive generations, was of adaptive laboratory mosquito-strains, and easily maintained and mass produced for any experiments. Thus, 200 female and 300 male newly emerged mosquitoes (the 9^{th} generation), respectively, were introduced into the same cage to co-habit for one week by following the former procedures of Sucharit and Choochote (1983) and Choochote et al. (1983). This provided a density resting surface (or vertical resting surface per mosquito) of 7.2 (Gerberg et al. 1994). Subsequently, the fasted females were allowed to feed on white rat. Five days after feeding, 20 gravid females were allowed to lay eggs for two days in an oviposited-plastic cup, and later, the 100 spermathecae of females were investigated for the presence of sperms. All eggs obtained from copulation in the 30 x 30 x 30 cm size cage were processed for hatching, larval and adult rearing, and use for establishing the next stenogamous colony. This process was performed repeatedly from generation to generation until the stenogamous mating colony was stable.

2.3.7 Searching for possible mechanism(s) that control stenogamous mating

1) Mating ability of adult mosquitoes in various sized cubic cages and a density resting surface (DRS)

Comparing the mating ability of adult mosquitoes in a 10, 20 and 30 cubic cm cage at a DRS of 3.6 and that in a 10, 20, 30 and 40 cubic cm cage at a DRS of 7.2, was carried out using various numbers of female/male mosquitoes (F_9) (Figure 2.2). At a DRS of 3.6, the number of females/males (total) 44/67 (111), 178/267 (445) and 400/600 (1000) was introduced into a 10, 20 and 30 cubic cm cage, respectively. However, the 40 cubic cm cage was not used for the DRS of 3.6, since this experiment needs large total numbers of adult mosquitoes (1788). At a DRS of 7.2, the number of females/males (total) 22/34 (56), 89/133 (222), 200/300 (500) and 355/533 (888) was introduced into a 10, 20, 30 and 40 cubic cm cage, respectively, where they co-habited for one week. Then, both 10% sucrose and 5% multivitamin syrup solutions were provided as adult nutrients.

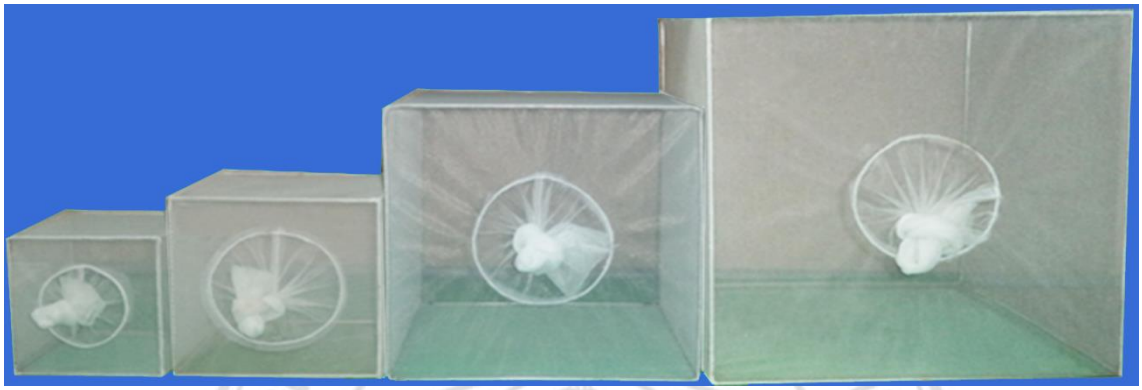

Figure 2.2 Four cages (left to right: 10, 20, 30 and 40 cubic cm cages) used for self-mating mosquitoes

The mean insemination rate was calculated from dissection of a total of 200 spermathecae (duplicate experiments, 100 spermathecae/experiment), and examined for evidence of insemination status. Movement of the long thread-like spermatozoa within the spermathecae was looked for under 100× magnification with a compound microscope (Olympus BX53, Japan). The spermatozoa appeared as fine concentric threads within the spermathecae and were often seen rotating as a cluster. After placing in a cover slip, the spermathecae were ruptured, and the surrounding field scanned for the spermatozoa. Then, they were graded as 0 (fairly transparent spermatheca or uninseminated), 1+ (25% of sperm in spermatheca), 2+ (50% of sperm in spermatheca), 3+ (75% of sperm in spermatheca) and 4+ (100% or spermatheca full of sperm) (Figure 2.3).

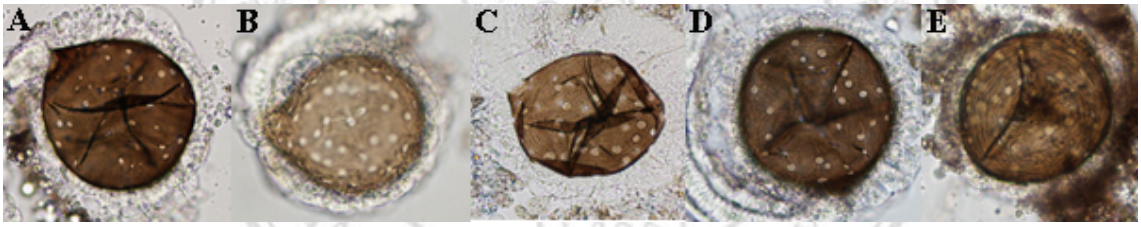

Figure 2.3 Grading of sperm within spermatheca of inseminated females of the eight species. A, 0; B, 1+; C, 2+; D, 3+ and E, 4+ (100X magnification)

2) Measurements and large sensilla coeloconica counts under light and scanning electron microscopy (SEM)

The measurements of male and female wings, female maxillary palpomeres and male genitalia, and large sensilla coeloconica (= pitted peg) counts of female antennae between stenogamous and eurygamous species were compared by using 36-hour post emerged females and males. Thirty samples from each species were immersed in a small bottle containing 10% potassium hydroxide (KOH) and left for 30–45 min in a hot oven. After clearing, they were washed with 80% ethanol and each structure was dissected using an insect needle. Each sample was slide-mounted with Hoyer's media. The measurements included: (1) length and width (ratio of length to width) of female and male wings (Figure 2.4); (2) maxillary palpomere index or palpomere ratio (calculated by dividing the combined length of palpomere 5 and 4 by length of palpomere 3) (Figure 2.5) (Mosha and Mutero 1982), and (3) male genitalia (length and width of the aedeagus, length between base of the aedeagus and origin of gonocoxite, width of gonocoxite at origin of last parabasal setae, length of gonocoxite and gonostylus) (Figures 2.6 and 2.7). In addition, the number of large sensilla coeloconica on the flagellum, which divided into 13 distinct flagellomeres (or segments) (Pitts and Zwiebel 2006) also was counted (Figure 2.8). All structures were measured and observed under a compound microscope (Olympus BX53, Japan).

Figure 2.4 The measurement of wing length and width of the eight female species. Scale bars: 0.2 mm

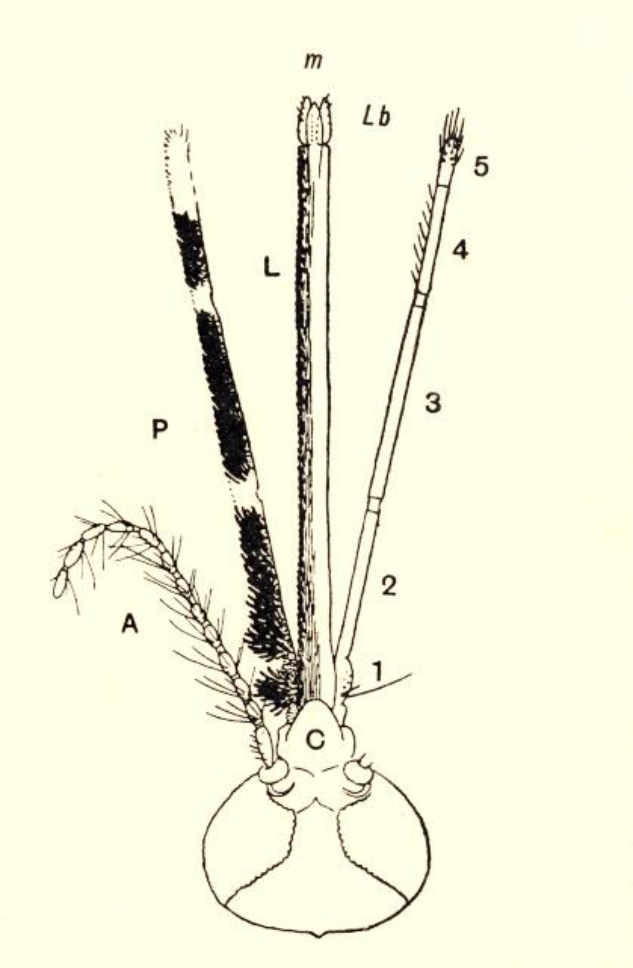

Figure 2.5 Schematic of the head (female anopheline). A, antenna; C, clypeus; L, labium (proboscis); Lb, labella; m, tip of mouthparts lying in labial sheath; P, palp (segments 1-5) (modified from Reid 1968)

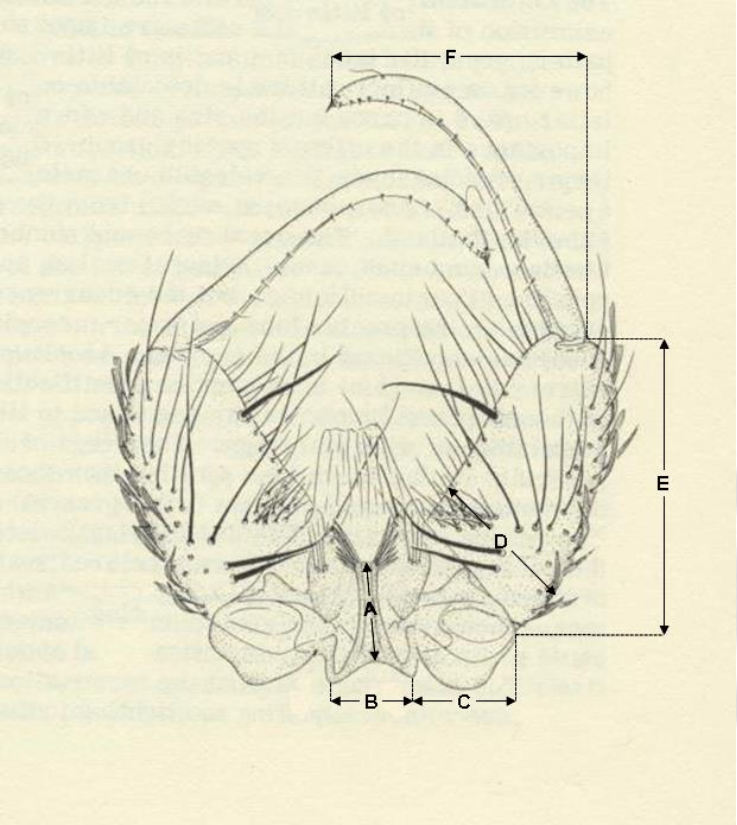

Figure 2.6 Schematic measurements of the male genitalia (ventral view) at various sites. A, length of the aedeagus; B, width of the aedeagus; C, length between the base of the aedeagus and origin of the gonocoxite; D, width of the gonocoxite at the origin of the parabasal seta; E, Length of the gonocoxite; F, length of the gonostylus (modified from Harrison and Scanlon 1975)

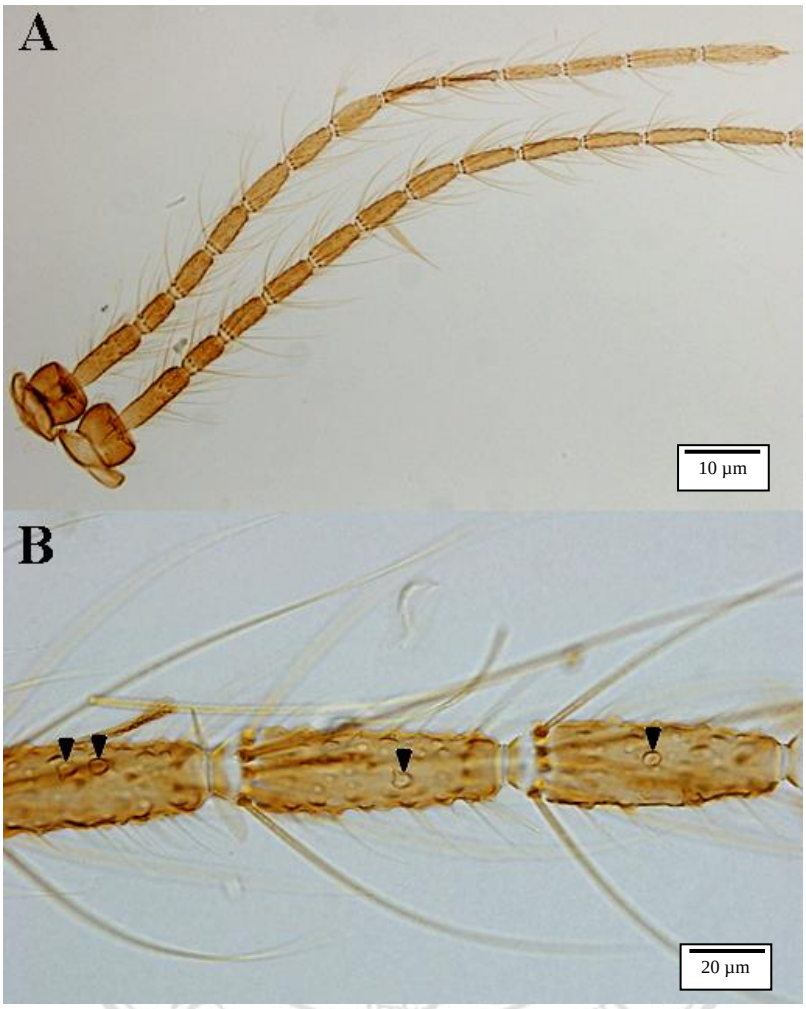

Figure 2.7 Representative female antennae of the eight species of the Hyrcanus Group. A, flagellomeres are typically numbered 1-13 from one pair of female antenna; B, large sensilla coeloconica (pitted peg) (arrow) on each flagellomere. Scale bars: 0.01 mm for A; 0.02 mm for B

Additionally, the ultrastructure of sensilla coeloconica of female antennae was investigated using scanning electron microscopy (SEM) with minor modifications to the method described by Saeung et al. (2013). Briefly, thirty heads of 4 to 5-day-old females of each species were excised under stereo microscope and immersed in phosphate buffer (pH 7.4) in order to remove surface debris. Then, the antennae were dissected from the head and dehydrated by passing through an ethanol series, i.e., 35%, 70%, 80% (10 minutes, with two changes), and 95% (15 minutes, with two changes), followed by absolute ethanol (10 minutes, with two changes). They were dried with a critical point dryer, mounted on stubs, sputter-coated with gold, and examined at 42 KV in a JEOL MED JSM 840-A SEM.

3) Frequency of clasper movement in male genitalia during induced copulation, and mating times

The frequency of clasper movement in male genitalia during induced copulation, and mating times between stenogamous and eurygamous species were compared by using 5-day-old females and males of each species. During induced copulation, the females were clasped initially with male claspers, and then remained coupled for a long period of time before a pumping motion started, with movement of their claspers (gonocoxite and gonostylus) until separation. The mating time and frequency of clasper movements were measured and counted using an electric watch under a stereoscopic microscope.

All results obtained in the topic numbers 1-3 were analyzed systematically and related to each other in order to elucidate on the possible mechanism(s) that control or affect stenogamous behavior. The summarized experimental design is shown in Figure 2.9.

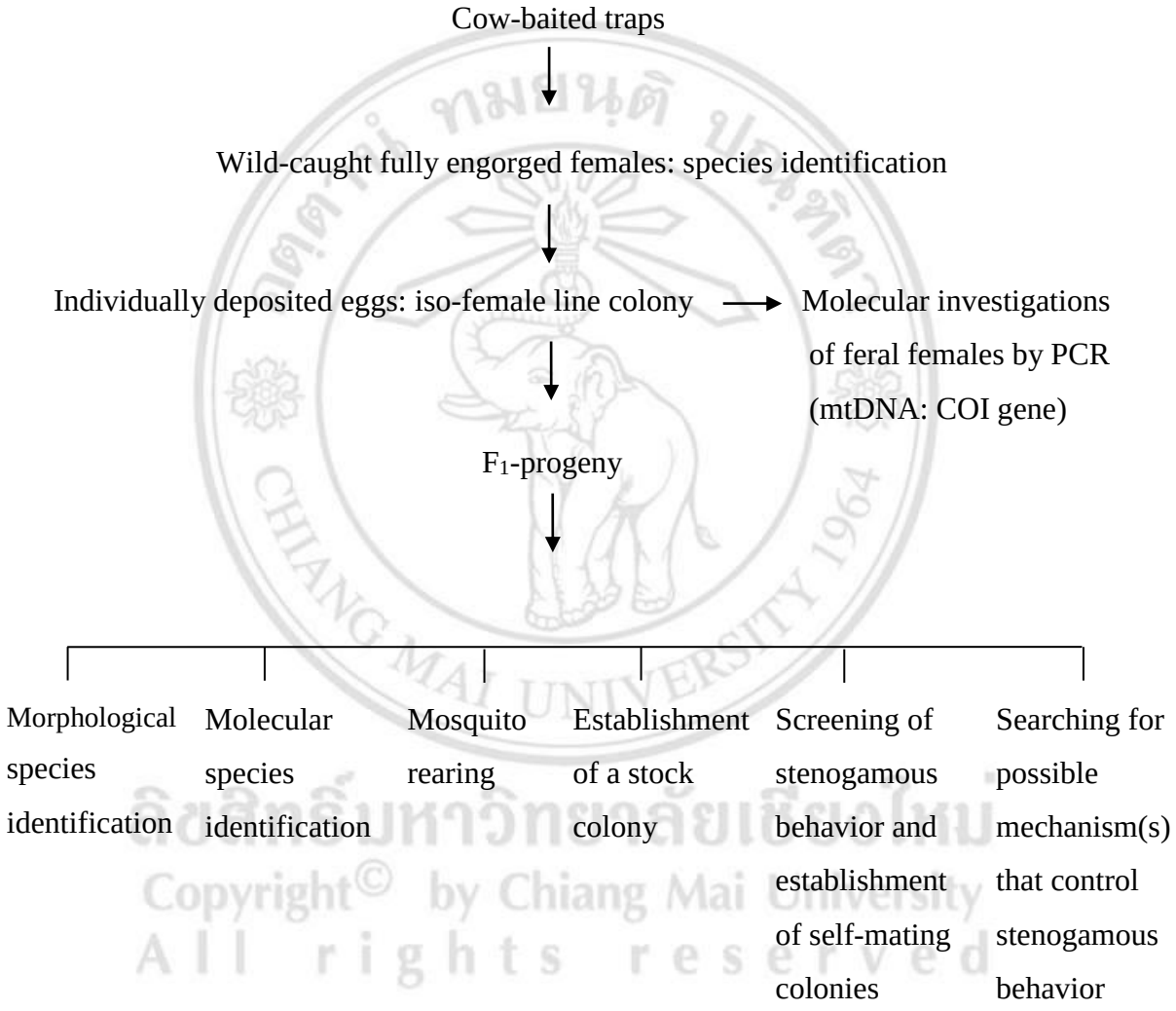

Figure 2.8 Summarized experimental design

2.4 Statistical analysis

The Chi-square test was used to determined insemination rates. Variation observed in anatomical features of adult females and males genitalia, number of large sensilla coeloconica on female antennae, and frequency of clasper movement in male genitalia during induced copulation and mating times was analyzed by one-way analysis of variance (ANOVA). Differences between stenogamous and eurygamous species were compared using Tukey's HSD (honest significant difference) test. All data were analyzed using SPSS v. 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.