CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Metaphase chromosome preparation

- Glass slide
- Insect needle
- Pasteur pipette
- Phosphate buffer pH 7.2
- Siliconized slide
- Test tube
- Warming plate

2.1.2 Polytene chromosome preparation

- Cover slip
- Insect needle
- Pencil
- Filter paper
- Siliconized slide

2.1.3 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)
- 0.2 ml PCR tubes
- 1.5 ml micro-centrifuge tubes

2.1.4 Mosquito rearing

- Black plastic sheet
- Cages (30 x 30 x 30 cm)
- Cotton
- Filter paper
- 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)
- 5% multivitamin solution
- 10% sucrose solution

2.2 Chemicals

2.2.1 Metaphase chromosome preparation

- Carnoy's fixative solution
- Giemsa
- 0.1% sodium citrate solution
- 0.5% percent normal saline (0.85% sodium chloride)-extracted *Gloriosa superba* seed solution
- 60% acetic acid

2.2.2 Polytene chromosome preparation

- Aceto-lactic orcein
- Clear nail vanish
- 1% sodium citrate solution
- 15% and 45 % acetic acid
- 50% propionic acid

2.2.3 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)
- Isopropanol
- Magnesium chloride ($MgCl_2$)
- Molecular weight marker

- Primers (Invitrogen™, U.S.A.)
- QIAquick® Gel Extraction Kit (Qiagen, Japan)
- QIAquick® PCR Purification Kit (Qiagen, Japan)
- 6X loading dye (Qiagen, Japan)
- 10X PCR buffer (Wako, Japan)
- 70% ethanol
- 99.5% ethanol

2.3 Methods

2.3.1 Field collection of *Anopheles nigerrimus* and *Anopheles nitidus*

Samples of wild-caught, fully engorged females of *An. nigerrimus* were collected from cow-baited traps in 4 provinces of Thailand, i.e., Lampang province, northern region; Ubon Ratchathani province, northeastern region; Songkhla and Nakhon Si Thammarat provinces, southern region and 1 location in Ratanakiri province of Cambodia.

Anopheles nitidus were collected from cow-baited traps at 2 allopatric locations in Thailand, i.e., Ubon Ratchathani province and Phang Nga provinces in northeastern and southern region, respectively.

2.3.2 Mosquito identification and processing

The wild-caught, fully engorged females were kept in paper cups, with a screen and pad of water-soaked cotton wool placed on top. The mosquitoes were stored in a humid chamber placed inside a picnic cooler to maintain humidity and temperature. Then, they were transported for colonization to the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Identification of mosquito species was based on the keys of Rattanarithikul et al. (2006). The identified wild-caught females were processed following the summarized experimental design shown in Figure 2.1

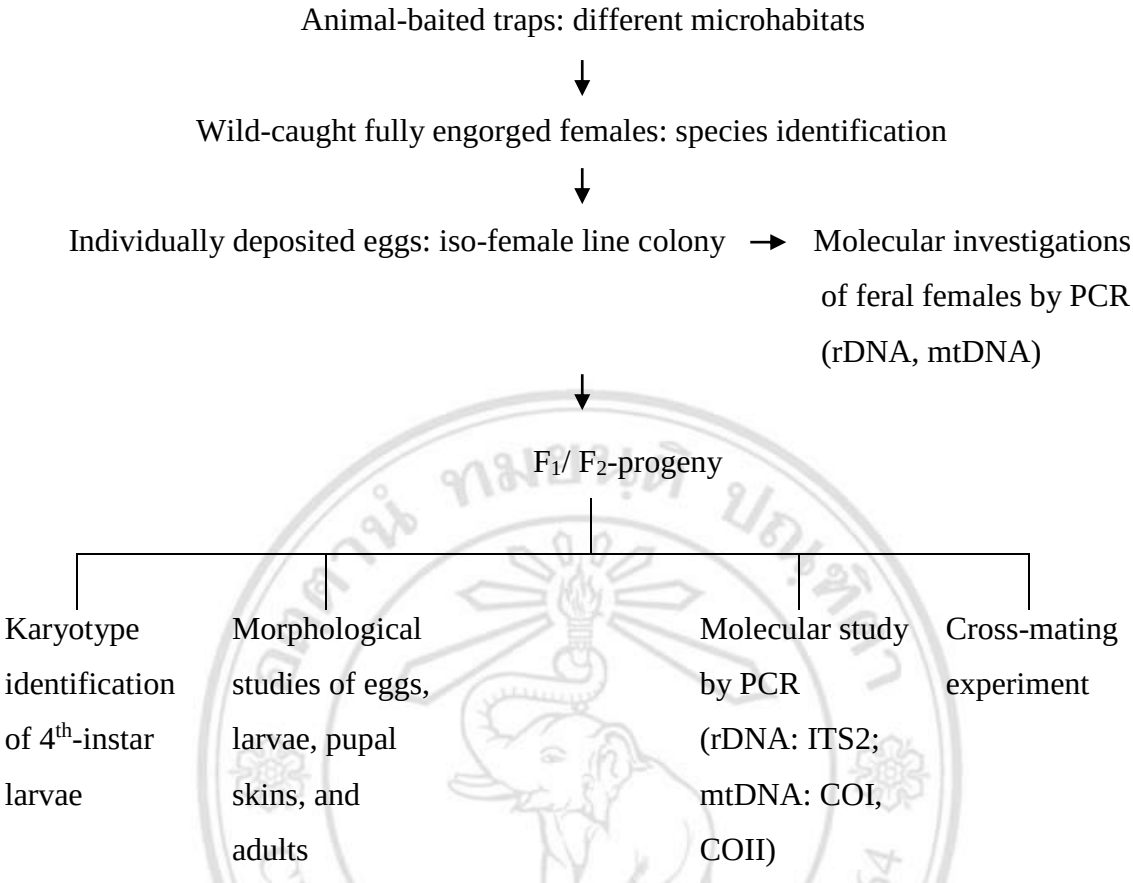

Figure 2.1 Summarized experimental design

2.3.3 Mosquito rearing and establishment of isoline colonies

The methods for rearing mosquitoes and establishment of isoline colonies were performed as described by Choochote and Saeung (2013). Briefly, 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 the covering screen. It was covered with a translucent plastic bag in order to keep humid conditions in the cup and delay rapid drying of the soaked cotton wool. It was stored in a humid chamber using a picnic foam-box (18 x 26 x 39 cm) to maintain humidity and temperature. Then it was transported 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 illumination from a combination of natural daylight from a glass window and fluorescent lighting was 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 3 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 10% sugar and 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.

2.3.4 Metaphase and polytene chromosome preparation

Metaphase chromosomes were prepared from 10 samples of the early fourth-instar larval brains of F_1- and/or F_2-progenies of each isoline, using techniques previously described by Saeung et al. (2007). The excised heads of 4th instar larvae were incubated with a 1 ml filtrate of 1% solution of dried *Gloriosa superba* seed powder in a 1.5 ml microcentrifuge tube (Eppendorf®) for two hours at room temperature. The incubated heads were left in 1% hypotonic sodium citrate solution on a siliconized slide for 10 minutes, and then the brains were removed and transferred to a small drop of Carnoy's fixative (1 part glacial acetic acid and 3 parts absolute ethanol) on a siliconized slide for at least 2 minutes. Then, a drop of 60% acetic acid was added, and the organs were torn and mixed well with dissecting needles. A drop of cell suspension was placed on a clean microscopic slide on a warming plate at about 45-50 °C. Droplets of cells were released slowly from a Pasteur pipette to form a circular trail of monolayer cells. The dried slides were stained with 10% Giemsa in phosphate buffer pH 7.2 for 60 minutes, rinsed with deionized water, air-dried at room temperature and mounted in Permount® (Fisher, Fairlawn, NJ, USA). Identification of karyotypic forms were followed the cytotaxonomic key of Baimai et al. (1993b).

For polytene chromosome preparation, the early 4th instar larvae were removed from the rearing tray and rinsed in clean distilled water. The excess water was removed by filter paper. The larvae were placed on the cavity slide filled with 1% hypotonic sodium citrate and dissected under the dissecting microscope. Bilobated salivary glands were removed from the thorax using fine needles. Only the whitish anterior lobe of each salivary gland was transferred into small drops of 15% and 45% acetic acid on a siliconized slide for 1 minute. After that, one drop of 2% aceto-lactic orcein stain was added to the slide and left for 15 minutes. Then, a clean coverslip was placed on the microscopic slide and gently squashed. The coverslip edges were sealed with

transparent nail varnish. The graceful polytene chromosomes were compared with the standard chromosome of White et al. (1975).

2.3.5 Molecular study

1) Amplification and sequencing of the rDNA (ITS2) and mtDNA (COI, COII)

Molecular analysis of 3 specific genomic loci [second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II (COII) of mitochondrial DNA (mtDNA)] were performed to determine intra-specific sequence variation in all karyotypic forms of *An. nigerrimus* and *An. nitidus*. An individual F_1 adult female from each isoline of *An. nigerrimus* (Lp1A, Ur1A, Ur7A, Ur20D, Ur26A, Ns1B, Ns2B, Ns3A, Sk2A, Sk3A,Rt2C, Rt3C, Rt4A) and *An. nitidus* (Ur2D, Ur5E, Ur8E, Ur11D, Ur12D, Ur15D, Ur16E, Ur19D, Ur22E, Ur23E, Ur24D, Ur25D, Ur27D, Ur28E, Ur30E, Ur31D, Ur33E, Ur34D, Pg2A, Pg4C, Pg5B) were used for DNA extraction and amplification. Genomic DNA was extracted from adult mosquito using DNeasy® Blood and Tissue Kit. The primers used for PCR amplification and sequencing are shown in Table 2.1. Amplifications were performed in a total of 20 µl volumes containing 0.5 U *Ex Taq*, 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. For ITS2, the conditions for amplification consisted of initial denaturation at 94ºC for 1 minute, 30 cycles at 94ºC for 30 second, 55ºC for 30 second, and 72ºC for 1 minute, and a final extension at 72ºC for 5 minutes. The amplification profile of COI and COII comprised initial denaturation at 94ºC for 1 minute, 30 cycles at 94ºC for 30 second, 50ºC for 30 second, and 72ºC for 1 minute, and a final extension at 72ºC for 5 minutes. The amplified products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Finally, the PCR products were purified using the QIAquick® PCR Purification Kit and their sequences directly determined using the BigDye® Terminator Cycle Sequencing Kit and 3130 genetic analyzer. The sequence data of this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database. The ITS2, COI and COII sequenced obtained from this study were also compared with sequences available in

GenBank using the Basic Local Alignment Search Tool (BLAST) available at (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2.1 PCR primers (f = 'forward'; r = 'reverse')

Primers	**Sequences (5′–3′)**	**References**
ITS2 primers		
ITS2A (f)	TGTGAACTGCAGGACACAT	Beebe and Saul (1995)
ITS2B (r)	TATGCTTAAATTCAGGGGGT	
COI primers		
LCO1490 (f)	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO2198 (r)	TAAACTTCAGGGTGACCAAAAAATCA	
COII primers		
LEU (f)	TCTAATATGGCAGATTAGTGCA	Sharpe et al. (2000)
LYS (r)	ACTTGCTTTCAGTCATCTAATG	

2) Sequence alignment and phylogenetic analysis

Sequences of ITS2, COI and COII were aligned using the CLUSTAL W multiple alignment program (Thompson et al. 1994) and edited manually in BioEdit version 7.0.5.3 (Hall 1999). Gap sites were excluded from the following analysis. Genetic distances were estimated with the Kimura two-parameter method (Kimura 1980). Construction of neighbor-joining trees (Saitou and Nei 1987) and the bootstrap test with 1,000 replications were conducted with the MEGA version 4.0 programme (Tamura et al. 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012) by using two replicates of one million generations with the nucleotide evolutionary model, GTR+I, which was selected by MrModeltest version 2.3 (Nylander 2004) as the best-fit model for ITS2, COI and COII. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burn-in.

2.3.6 Cross-mating experiments

The 8 laboratory-raised isolines of *An. nigerrimus* were selected arbitrarily from the 13 isoline colonies, which were representative of 4 karyotypic forms, i.e., Form A (Lp1A, Ur1A, Ns3A, Sk2A, Rt4A), B (Ns1B), C (Rt2C) and D (Ur20D). Moreover, the 5 laboratory-raised isolines of *An. nitidus* were selected arbitrarily from the 21 isoline colonies as representatives of the 5 karyotypic forms, i.e., Form A (Pg2A), B (Pg5B), C (Pg4C), D (Ur2D), and E (Ur5E). These isolines were used for cross mating-experiments in order to determine post-mating barriers by employing the techniques previously reported by Saeung et al. (2007).

Adult females and males emerged from pupae that placed individually into test tubes were used for the experiments. The crosses were performed by induced mating soon after the mosquitoes have been fed on blood. The gravid females were allowed to oviposit in a single colony, and the eggs were counted and placed in hatching pans. The spermathecae of mated females were examined for evidence of insemination. The details of hatching, larval survival, pupation, adult emergence and sex ratios were recorded. The remaining F_1-hybrids were conducted to reciprocal or back crosses with their parental forms in order to observe genetic relationships. The salivary gland polytene chromosomes of 4^{th} instar larvae from the crosses were investigated using the techniques described by Kanda (1979) and White (1975). The complete and complete synapses of polytene chromosomes from all crosses were recorded.