CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Collection of mosquitoes

1.1.1 *Anopheles minimus* species C

Wild caught blood-fed *An. minimus* females were collected from Ban Phu Toei, Amphur Sai Yoke, Kanchanaburi Province, in February 2001, by using bovine and human baits. They were kept in paper cups with a towel soaked with water placed on the top of screen and stored in a picnic cooler to maintain humidity and temperature, and then transported to the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai Province, Thailand, for colonization. The insectarium was maintained at 25-27^{0}C and 70-80% relative humidity (RH) with a photoperiod of 14:10 h. L:D. The mosquitoes were identified morphologically following Sucharit *et al.* (1988b). Only those having the presector pale spot (PSP) and humeral pale spot (HP) on the costa of the wing (a presumptive diagnosis of species C) were selected for further studies. The selected mosquitoes were kept in paper cups provided with cotton wools soaked with 5% sugar mixed with 5% multivitamin syrup solution. They were maintained in a humid chamber for 4-5 days and allowed to lay eggs individually in plastic cups lined with a strip of filter paper (Whatman No.1). Well water from Ban Don Gaw, Amphur Mai Rim, Chiang

Mai Province, was used for oviposition and rearing of immature stages. The plastic cups were covered with a black plastic sheet to facilitate oviposition. After laying eggs, the mosquitoes were killed by chloroform and put into a small vial. A number of vials were kept in a plastic bag with siliga gel and sent to Dr. Catherine Walton, School of Biology, University of Leeds, England, who confirmed the species status by DNA sequencing of the D3 rDNA using the method described by Sharpe *et al.* (1999). The DNA-confirmed *An. minimus* species C were pooled to establish the colony which is designated as KAN strain.

1.1.2 *Anopheles minimus* species E (ISG strain) colony

A sub-colony of the ISG strain identified previously as species E by Somboon *et al.* (2001) was taken from Japan and maintained in the insectary in Chiang Mai.

1.2 Chemicals and reagents

1.2.1 For polytene chromosome study

1.2.1.1 sodium citrate solution 1% :

- 1 gm of *tri*-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in 100 ml of distilled water

1.2.1.2 acetic acid 15% and 45%

1.2.1.3 aceto-lactic orcein stain:

- Stock stain (2% by weight of synthetic orcein powder in 1 part of 85% lactic acid and 1 part of glacial acetic acid)
- Working stain [1 part of concentrated orcein stain mixed with 1-3 parts of aceto-lactic acid (1:1)]

1.2.1.4 Siliconizing fluid

- Chlorinated organopolysiloxane in heptane

2. Methods

2.1 Rearing method of mosquitoes

2.1.1 Rearing method of *Anopheles minimus* species C

The method for rearing of mosquitoes followed the procedure described by Kanda (1979) and Somboon *et al.* (2001). The first stage larvae were reared in a tray (25x35x6 cm) containing about 1.5 litres of well water. Approximately 5 stems of a garden grass were put into the tray to serve as a resting-place for the larvae and aerated the medium. Progeny from each female mosquito were placed in each rearing tray.

A special food was required to insure healthy and normal development of the larval stages. The following food formulation was modified from Kanda (1979):

1. Dry yeast	1,000 mg
2. Oat meal	1,000 mg
3. Wheat germ	1,000 mg
4. Nestum	750 mg
5. Corn starch	250 mg
6. Liver	3 mg

Each ingredient was ground as fine as possible to encourage the spread of the food over the water surface. The young larval stages (first and second instars) were fed twice daily, and increased to 3 times daily when most of the larvae had reached the third instar. Unconsummated food and dead larvae at the bottom of the trays were removed with a pipette because it favored the development of bacterial scum.

The pupae were transferred to plastic cups covered with the net. The emerging adult mosquitoes were transferred to another cup, provided with cotton wool soaked with sugar and multivitamin syrup solution. To maintain humidity, the cups were placed in a plastic tray with towel soaked with water at the bottom and covered with a transparent plastic sheet. After 4-5 days, the female mosquitoes were fed on a blood-meal by exposing them to an aneastizied hamster for 1-3 hour, using Nembutal® (0.1 ml /100g mouse-weight) injected through the abdominal cavity. After that, the artificial mating between the blood-fed female and male mosquitoes was done following the method described by Ow-Yang *et al.* (1963). The mated female mosquitoes were maintained with sugar and multivitamin syrup solution until they were gravid (about 5 days).

For oviposition, the gravid females were placed in a small rearing tray (20x 30x5 cm) containing about 1 liter of well water. The inner site of tray was lined with a strip of filter paper and the top covered with a plastic sheet. The filter paper was served as egg laying surface and maintained humidity inside the rearing tray. After laying eggs, the female mosquitoes were taken off from the tray. Eggs were reared to adults to produce the laboratory colony.

The adult mosquitoes were maintained in a cage measuring 30x30x30 cm which have a 20 cm diameter hole, with 30 cm sleeve that was securely tied when not in use. The cotton wool pads soaked with sugar and multivitamin syrup solution were provided and changed 3 times a week. The humidity was provided by covering the cage with a wet towel which was changed twice daily. A plastic sheet was placed on top of the towel to minimize evaporation of water. When the mosquitoes were 4-days old, the females were fed a blood meal on a white mouse confined in a small cage and

placed into the mosquito cage for 1-3 hours. After that, the artificial mating between blood-fed female and male mosquitoes was done. The mated female mosquitoes were provided with sugar and multivitamin syrup solution for 5 days, after that they were allowed to lay eggs in the small laying tray as mentioned above.

2.1.2 Rearing method of *Anopheles minimus* species E

The method for rearing *An. minimus* species E mosquitoes was similar to that of species C with a few exceptions. Because this species has stenogamous behavior (mating ability in cage), no artificial mating is required. The gravid females laid eggs readily on the water in a cup which was placed into the mosquito cage.

Because *An. minimus* species E mosquitoes was the imported mosquitoes, special cares were taken to prevent them releasing to the environment, such as the rearing trays were always treated with hot water before washing, the experiments concerning this species were done only in the insectarium, and all stages of this mosquito were not allowed to be taken from the insectarium.

2.2 Biological study of *Anopheles minimus* species E and C

2.2.1 Observation on life duration

Embryonation period: The duration of eggs developing to hatching first instar larvae was observed. One hundred eggs were randomly selected from the oviposition tray of each mosquito colony in the morning. They were placed into a plastic cup containing about 300 ml. of well water. The inner site of the cup was lined with a strip of paper. Eggs were checked and counted for the emerging first instar larvae daily.

Larval period: The duration of larvae developing to pupae was observed. One hundred of newly emerged first instar larvae were randomly selected from the oviposition tray of each mosquito colony. They were reared in a tray (25x35x6 cm) containing about 1.5 litres of well water, and were fed twice daily on 5 mg of larval food until pupated. Pupae were collected and counted daily.

Pupation period: The duration of pupae developing to emerging adults was observed. One hundred of newly developing pupae were randomly selected. They were placed into a plastic cup covered with netting. The emerging adults were counted daily.

Adult longevity: One hundred of each newly emerged females and males were kept separately in cages covered with wet towels. Cotton wool pads soaked with sugar and multivitamin syrup solution were provided and changed every two days. Dead mosquitoes were checked daily, counted and removed until all of the mosquitoes died.

2.2.2 Ability of free mating in a 30x30x30 cm cage

To observe if there is preferential mating behavior in the laboratory, a crossing experiment was carried out in which virgin females or males of *An. minimus* species E were confined with the opposite sex of species C in a 30x30x30 cm cage (30-cm cage). In each cage, 100 pairs of 1-4 days old adults were released. Sugar and multivitamin syrup solution were provided for adult nutrients and were changed every two days. Ten days after their release, the insemination rate was determined by checking spermathecae for the presence of spermatozoa. As the control, 100 pairs of

An. minimus species E and species C mosquitoes were released in separate cages, and their insemination rates were compared.

2.3 Hybridization study

Anopheles minimus species E was crossed with species C to determine genetic compatibility. Virgin females separated at the pupal stage were placed in paper cups provided with sugar and multivitamin syrup solution for 5 days. Subsequently, they were fasted for 12-18 hours and permitted to feed on hamster blood. After the females took 1 bloodmeal, reciprocal-crossed were carried out by the artificial mating technique. After mating, the females were placed in the paper cup provided with sugar and multivitamin syrup solution. The cup was placed in a humid chamber for 5 days and then each female was isolated in an oviposition vial. Eggs were counted and left until they hatched. Following oviposition, the females were dissected to check for spermatozoa in their spermatheca, and eggs from uninseminated females were excluded. Newly hatched larvae from each egg batch were counted and placed in rearing trays until they pupated. Egg batches with no or little hatching were allowed to stand for another 3 days, after which they were examined for embryonation. Pupae were removed daily, sexed, and placed separately in cups until the adults emerged. The F_1 hybrid adults that emerged were counted. Their fertility and viability were observed by further crosses among the hybrids and backcrosses with the parental colonies. The testes and ovaries of the hybrids were also dissected to check fertility. The crosses were made in the same rooms housing the colonies, and the test specimens were kept in these rooms under identical laboratory conditions to those of the colonies. Every data of experiment were compared with parental colonies.

2.4 Polytene chromosomes study

Siliconization of coverslips and slides

Clean, dry coverslips and slides were dipped in the siliconizing fluid (SIGMACOTE®) for at least 5 seconds, after that they were dried at room temperature for 10 minutes and cleaned with clean cloth.

Polytene chromosomes preparation

The polytene chromosomes were prepared by the techniques described by French *et al.* (1962), and Kanda (1979). The best salivary gland preparations were made from large and active larvae of the early fourth instars.

The larvae were removed from the rearing tray and rinsed in distilled water. Excess water was removed by filter paper. The larvae were then placed on a cavity slide filled with 1% hypotonic sodium citrate and dissected under dissecting microscope. Salivary glands were removed from the thorax by fine dissection needles. To open the thorax, a fine dissection needle was inserted into the dorsal aspect of the thorax directly under the cuticle. A second fine dissection needle, placed on the outer surface of the cuticle, is then rubbed against the first needle so as to split the cuticle in an anterior-posterior direction along the dorsal surface of the thorax. Only whitish anterior lobe of each salivary glands were transferred into a small drop of 15% and 45% acetic acid on a siliconized slide for 1 minute each, respectively. After that, one drop of aceto-lactic orcein stain was added and left for 10-15 minutes. Then a siliconized cover slip was placed on the slide. The prepared slide was firmly wrapped in filter paper to absorb the exceed stain. To squash the salivary gland tissue

and spread the polytene chromosomes. The coverslip was tapped gently several times with a hard material, such as the blunt tip of pencil.

In order to preserve the prepared slide and prevent it from drying out, a semi-permanent preparation was made by sealing the coverslip edges with transparent nail varnish. The slide could be preserved for one year or more when kept at -20° C.

A large number of specimens were needed for chromosomal preparations and were selected for clearly chromosomal banding pattern. Nicely spread chromosome arms were examined under light microscope and photographed.

2.5 Statistical analysis

For quatitative data, the comparison of normal distribution data between two groups was analyzed using Student *t* Test, more than two groups of sample were analyzed using Analysis of Variance (ANOVA). The comparison of high variable data between two groups was analyzed using Mann-Whitney U Test, more than two groups of sample were analyzed using Kruskal-Wallis ANOVA. Quanlitative data was compared using Chi-Square test. All significant tests accepted at α and β errors = 0.05.