CHAPTER II

MATERIALS AND METHODS

1. MATERIALS

1.1 For metaphase chromosome preparation

One percent normal saline-extracted *Gloriosa superba* rhizomes solution; 1% sodium citrate solution; concentrated carnoy's fixative solution; 60% acetic acid; Giemsa stain; and Permount® medium.

1.2 For light and scanning electron microscopic studies

Hoyer's media, 2.5% glutaraldehyde in phosphate buffer (PB) pH 7.4; 1% osmium tetroxide; 35%, 50%, 70%, 80%, 95% and absolute ethyl alcohol.

1.3 For polytene chromosome preparation

One percent sodium citrate solution; 15% and 45 % acetic acid; aceto-lactic orcein; and clear nail vanish.

1.4 For molecular study

DNeasy Tissue kit (Qiagen); *Ex Taq* (Takara); QIAquick® Gel Extraction Kit (Qiagen); pCR2.1⁻ TOPO (Invitrogen); QIAquick® PCR Purification Kit (Qiagen); and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

2. METHODS

2.1 Field collection and establishment of isoline colonies

Wild-caught, fully engorged female mosquitoes of *An. campestris*-like (Saeung *et al*, 2007) were collected from human-baited and buffalo-baited traps at twelve localities in Thailand, *i.e*., Ban Nong Chom, San Sai district, Chiang Mai, northern Thailand; Ban Salok Bat, Khanu Woralaksaburi district, Kamphaeng Phet; Ban Prem, Bang Pa In district, Ayuttaya, central Thailand; Ban Chiang Phin, Mueang district, Udon Thani; Ban Nong Nam Kliang, Mueang district, Khon Kaen; Ban Pang Daeng, Dong Luang district, Mukdahan; Ban Mad, Mueang district, Maha Sarakham; Ban Huai Chan, Mueang district, Chaiyaphum, northeastern Thailand; Ban Nong Ya Plong, Aranyaprathet district, Sa Kaeo; Ban Phang Ngon, Pong Nam Ron district, Chanthaburi, eastern Thailand; Ban Nong Kok, Mueang district, Prachuap Khiri Khan; and Ban Don Khiam, Tha Sae district, Chumphon province, southern Thailand (Figure 1). A total of seventy-one isoline colonies, derived from both human-baited and animal-baited traps, were established the colonies and used for metaphase karyotype identification, crossing study, molecular investigation, morphological and salivary gland polytene chromosome examinations and malaria susceptibility test. The flow charts of operational works are summarized in Figure 2.

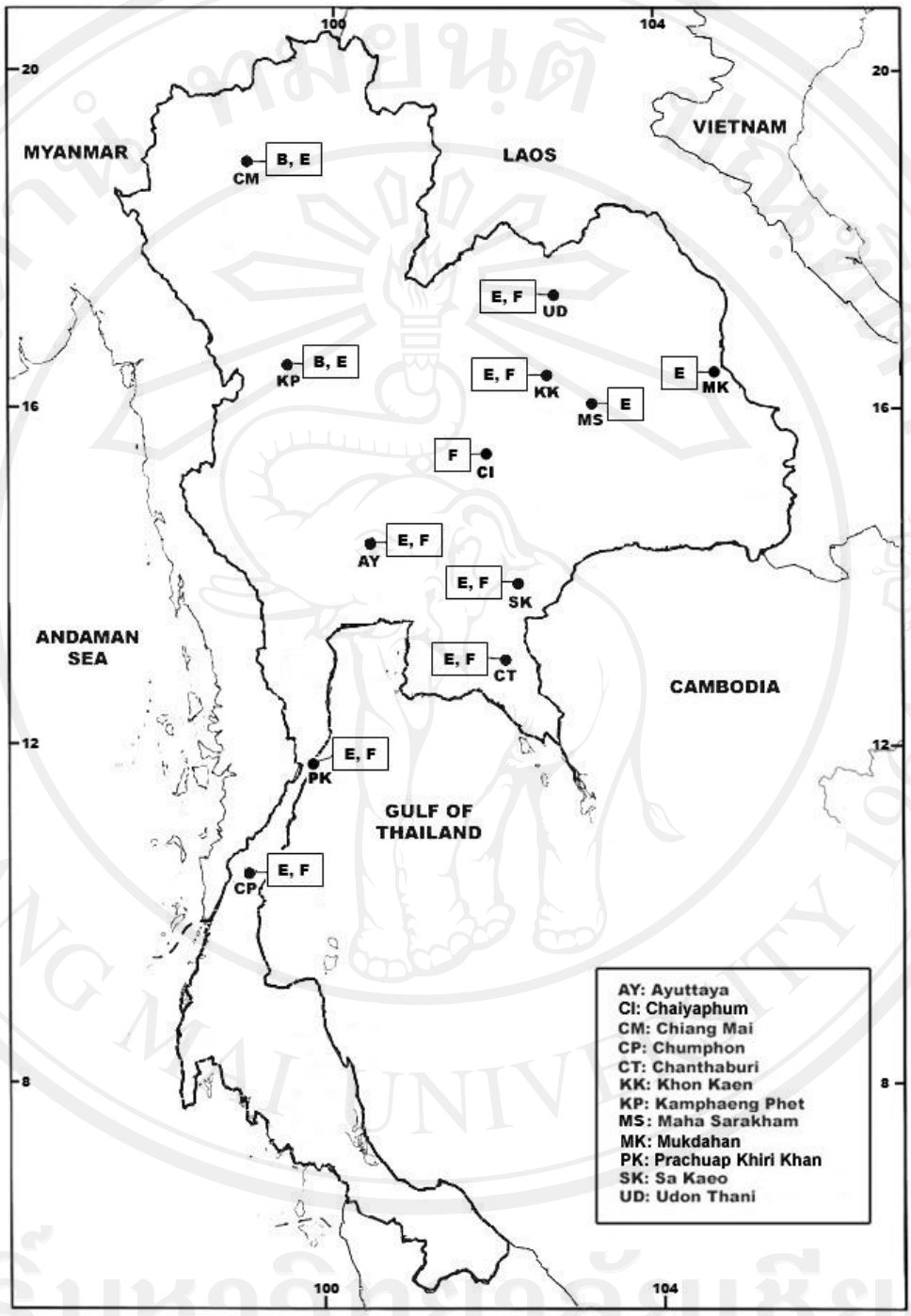

Figure 1 Map of Thailand showing distribution of *An. campestris*-like Form B, E and F.

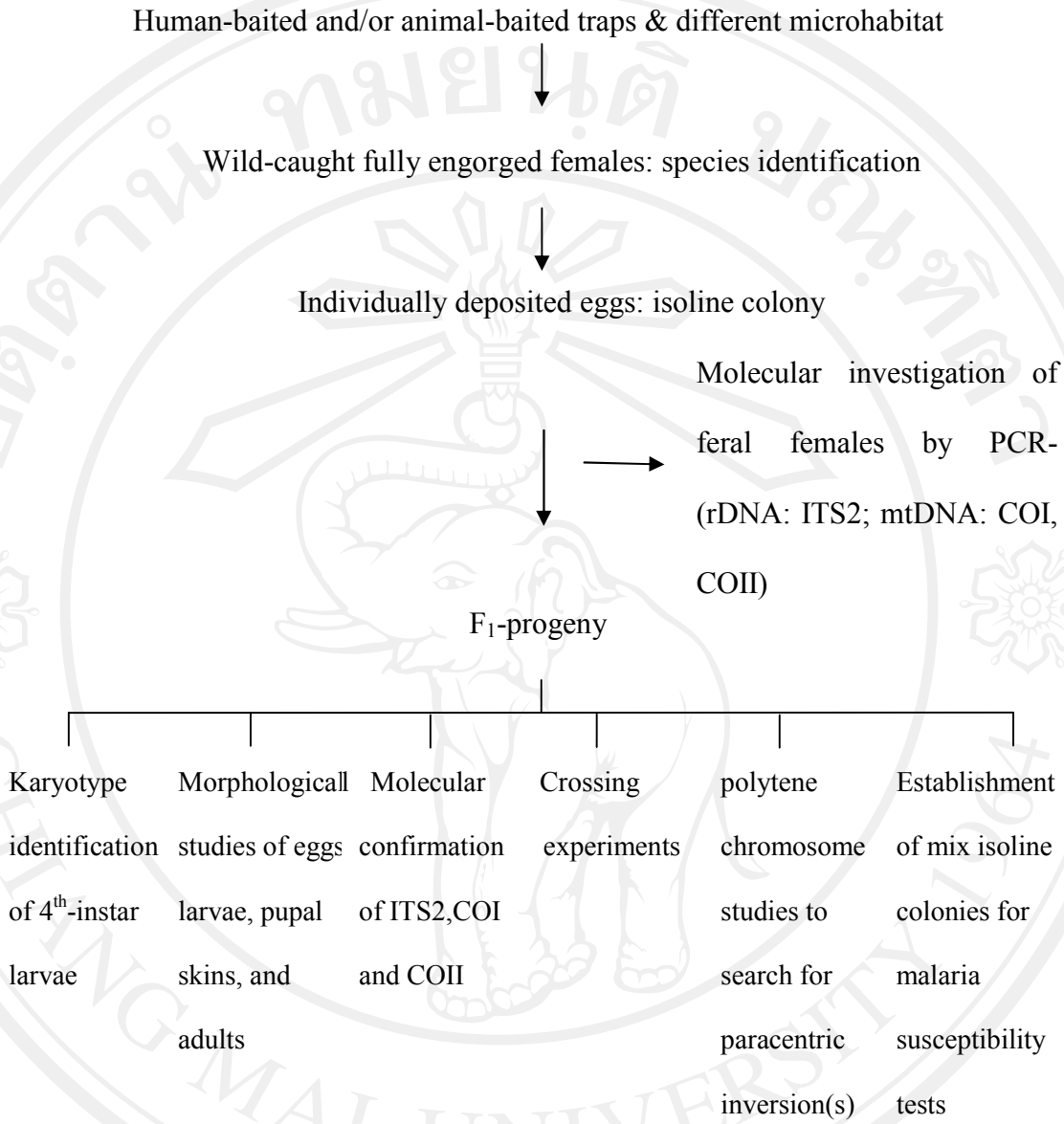

Figure 2 Flow chart for rapid systematic procedure.

2.2 Mosquito rearing

2.2.1 Wild-caught *An. campestris*-like Form E

An iso-female line colony of *An. campestris*-like Form E, Thai strain (Saeung *et al*, 2007) was used as model for the established of laboratory colony by using 1 wild-caught fully engorged adult female, collected from a human-baited trap at Ban Nong Chom, San Sai district, Chiang Mai province, northern Thailand. The engorged adult female was kept in a paper cup (8.5 cm in diameter and 11 cm in depth), with a pad of cotton wool soaked with 10% sucrose solution placed on top of the covering screen. It was stored in a humid chamber using a picnic cooler to maintain humidity and temperature. Then it was transported to the insectarium of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai province, northern Thailand, for colonization and biological studies. Identification of the specimen to *An. campestris*-like Form E was based on the combinative characteristics of morphology (Harrison and Scanlon, 1975); metaphase karyotypes (Baimai *et al*, 1995); and nucleotide sequences of internal transcribed spacer 2 (ITS2), and mitochondrial cytochrome c oxidase subunits I and II (COI and COII) (Saeung *et al*, 2007; 2008).

2.2.2 Insectarium condition

All of the experiments were performed in the insectarium at 27 ± 2 °C, 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.

2.2.3 Egg laying

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 (6.4 cm in diameter and 7.2 cm in depth) containing 25 ml of filtered 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 (Figure 3A). The eggs attached to the moist side of the filter paper and/or floating on the water surface were rinsed and transferred to white plastic tray (25 x 36 x 6 cm) containing 1,500 ml filtered natural 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 (Figure 3B).

2.2.4 Rearing of larva, pupa and adult

After egg hatching, first instar larvae were transferred daily from an ovipot to a white plastic tray (25 x 36 x 6 cm) containing 2,000 ml of filtered natural 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 water during the larval development process (Figure 3C). An extra and/or a standard formula of fish food consisting of protein 47.5%, oil 6.5%, fibre 2.0%, ash 10.5%, moister 6.0% and additives of vitamins A (29,770 IU/kg), D3(1,860 IU/kg), E (200 mg/kg), L-ascorbyl-2-polyphosphate (138 mg/kg), lecithin, l-lysine monochlorhydrate, and citric acid was used as larval nutrient. Fine fish food was placed in a vial covered with a nylon screen (34 x 43 threads per cm^2) and sprinkled on the water until the food

particles stopped spreading across the water's surface. First and second instar larvae were fed twice daily, and this schedule was increased to 3-5 times daily after most of the larvae reached third and fourth instar, respectively. Before each feeding, floating clumps of excess food were removed by dragging a sheet of typing-paper across the water's surface. Any larvae trapped on the paper during the cleaning process were dislodged by rinsing the paper in a tray of filtered natural water and returning it to the rearing tray. After pupation, approximately 100 pupae placed in a plastic cup (14.5 cm in diameter and 6 cm in depth) containing 150 ml of filtered natural water were kept in a 30 x 30 x 30 cm cage, and the emerged adults were provided with both 10% sucrose and 5% multivitamin syrup solutions saturated in cotton wool coiled around a small piece of wood and placed in a small bottle. Increased humidity to promote adult survival was provided by covering the cage with a wet towel overlaid with a transparent plastic sheet (Figure 3D). One-day-old males were removed daily from the cage and kept in a screen-topped paper cup where they were provided with a 5% multivitamin syrup solution through a pad of soaked cotton wool, which was placed on top of the screen and changed daily. In order to keep humid conditions in the cup and delay rapid drying of the cotton wool soaked in 5% multivitamin syrup solution, the screen-top was covered with a translucent plastic bag (Figure 3D).

2.2.5 Searching for a suitable blood-feeding condition

In order to determine the optimal age of adult females and a suitable method for feeding, the feeding ability of females at different ages, ranging from 1 to 10 days old, was investigated both in a 30 x 30 x 30 cm cage and a paper cup (8.5 cm in diameter and 11 cm in depth). The feeding duration was between 1800-2100 hours,

which was compatible to the nocturnal biting habit of the *An. campestris*-like Form E collected from the field. For feeding in the cage, each of 100 adult females at various ages, having fasted for 12-16 hours, were kept in the cage and allowed to feed on white rat restrained in elastic plastic sleeves, with an expanded opening to allow access for easy feeding. For feeding in the paper cup, each of 50 adult females at various ages (2 replicates), having fasted for 12-16 hours, were kept in the paper cup covered at the top with a nylon screen and allowed to feed artificially on bovine heparinized-blood (10 IU of heparin/ml of blood) obtained from a slaughter house. These methods followed those described by Chomcharn *et al*. (1980), except for the use of thin-spread paraffin as a feeding membrane instead of bovine intestine pad-skin (Long & Long Co., Belleville, N.J.) (Figure 3E). The number of adult females fed in both experiments was recorded and a total of 1,000 adult females were used in each experiment.

2.2.6 Ability of free mating in a 30 cm cubed cage

An experiment was designed to determine the degree of adaptive stenogamy in a 30 x 30 x 30 cm cage. One hundred and fifty and 300 newly emerged adult females and males, respectively, were transferred into a cage where they co-habitated for one week. Both 10% sucrose and 5% multivitamin syrup solutions were provided as adult nutrients. Subsequently, the spermathecae of 100 adult females were examined for evidence of insemination.

2.2.7 Male ability to mate artificially

The mating ability of males, at different ages ranging from 1 to 10 days old, with 5-day-old adult females, and/or anopheline mosquitoes of laboratory blood feeding-age, was investigated by using the methods of Baker *et al*. (1962) and Ow Yang *et al*. (1963). Thirty adult males of each age were used, with a total of 300 adult males used in the experiment. Completely mated adult females were reared for 24 hours before being dissected and examined for the presence of sperm in their spermathecae.

2.2.8 Searching for a suitable oviposition-condition

In order to determine the suitable condition for oviposition of gravid adult females, comparative oviposition in a 30 x 30 x 30 cm cage and a plastic cup (9.7 cm in diameter and 11.5 cm in depth) were performed. Firstly, for oviposition in the cage, 20 gravid adult and/or 5 day post-bloodmeal females were introduced into the cage, and a plastic cup containing 80 ml of filtered natural water and filter paper lining the inside was used as an ovipot. Secondly, for oviposition in a plastic cup, 20 gravid adult females were placed in a plastic cup screen-topped with a black plastic sheet and containing 80 ml of filtered natural water and filter paper lining the inside where the mosquitoes were allowed to oviposit (Figure 3A). The oviposition duration was 12 hours starting from 1800-0600 hours, and after that the number of eggs obtained from both methods were counted and compared.

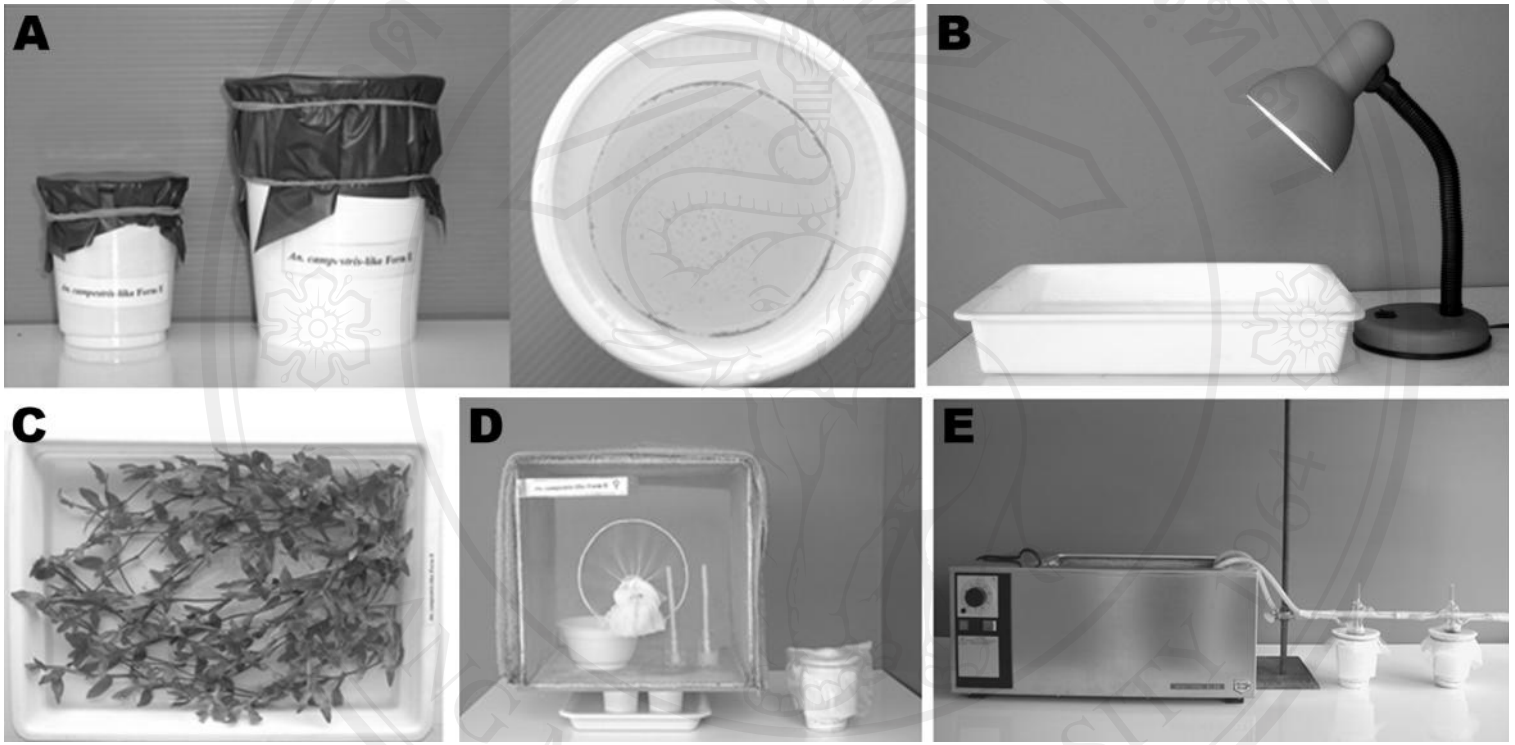

Figure 3 Mosquito equipment used in this study. (A) Ovipot derived from a screen-topped plastic cup with the top covered by a black plastic-sheet (left) for single gravid wild-caught adult females to lay eggs, (middle) for 20 gravid adult females to lay eggs, and (right) top view of the plastic cup showing massive egg-batches after 12-hours-oviposition of the 20 gravid adult females; (B) eggs placed in a white plastic tray and exposed to a 40 watt light.

Figure 3 (continued) Mosquito equipment used in this study. (C) White plastic rearing tray containing 2,000 ml of filtered natural water, 15 stems of garden grass and 80 larvae, with the top of the tray covered with a transparent plastic-sheet; (D) Left: adult rearing cage partially covered with a wet towel and a transparent plastic-sheet with plastic container for holding pupae, and two bottles with cotton wicks, one containing 10% sucrose solution and the other 5% multivitamin syrup solution. Right: adult males being kept in a screen-topped paper cup with 5% multivitamin syrup solution, and the top covered with a translucent plastic bag to maintain humidity; (E) Artificial feeding system with thin paraffin-membrane at the bottom tip of the feeding apparatus containing bovine heparinized-blood that is in close contact with 50 fasted adult female *An. campestris*-like Form E inside a screen-topped paper cup.

2.2.9 Establishment of the laboratory-colony strain

Suitable conditions from all of the above experiments were selected and used for rearing an iso-female line colony of *An. campestris*-like Form E for at least 40 consecutive generations. The biological aspects of a laboratory iso-female line colony strain of *An. campestris*-like Form E, *i.e*., average eggs deposited per gravid adult female; embryonation rate; hatchability rate; egg-, larva- and pupa-durations; adult female and male longevity; and sex ratio, were recorded on the 1st, 5th, 10th, 20th and 30th generations. For adult longevity, 30 of each newly emerged adult females and males were kept separately in a screen-topped paper cup (8.5 cm in diameter and 11 cm in depth). Cotton wool pads soaked with 5 % multivitamin syrup solution were provided regularly and changed every day until all of the adult females and males died. Both cups were checked daily, and the dead mosquitoes were counted and removed until all of the mosquitoes had died.

2.2.10 Rearing condition for chromosome preparation

The methods for rearing conditions were generally routine as mentioned above, except 10 first instar larvae per tray were used to obtain a high yield of metaphase chromosomes from larval brains and polytene chromosomes of larval salivary glands for comparison with routine rearing conditions, by using 80 first instar larvae per tray. The methods used for metaphase and polytene chromosome preparations followed those of Saeung *et al*. (2007) and Kanda (1979), respectively.

2.3 Metaphase karyotype preparation

Metaphase chromosomes were prepared from the early fourth-instar larval brains of F_1- and/or F_2-progenies of each isoline using previous techniques, as described by Saeung *et al*. (2007, 2008). Identification of karyotypic forms followed the cytotaxonomic key of Baimai *et al*. (1995).

2.4 Crossing study

Crossing studies were conducted among three karyotypic forms of twelve laboratory-raised isolines, *i.e*., *An. campestris*-like Form B (AKpB1: Kamphaeng Phet), E (HCE6: Chiang Mai, AKkE4: Khon Kaen, AMkE1: Mukdahan, AMsE3: Maha Sarakham, HSkE3: Sa Kaeo, ACpE6: Chumphon) and F (AUdF5: Udon Thani, ACiF1: Chaiyaphum, AAyF2: Ayuttaya, HCtF4: Chanthaburi, APkF1: Prachuap Khiri Khan) in order to determine post-mating reproductive isolation by employing the techniques reported previously by Saeung *et al*. (2007, 2008).

2.5 DNA extraction, amplification and sequencing

Genomic DNA was extracted from a whole adult mosquito of each isoline using a DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The amplification was done with primers and conditions, as described previously (Saeung *et al*, 2007; 2008) for ITS2, COI and COII regions. PCR was carried out using 20 µl volumes containing 0.5 units of *Ex Taq* (Takara), 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 amplified products were electrophorized through 1% agarose gel. PCR products of ITS2 were gel purified with the QIAquick® Gel Extraction Kit (Qiagen) and cloned into pCR2.1˙TOPO (Invitrogen). Sequences of several clones

from each isoline were determined. PCR products of COI and COII were purified with the QIAquick® PCR Purification Kit (Qiagen) and directly sequenced. Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit and run on a 3130 Genetic Analyzer (Applied Biosystems). The sequence data have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database.

2.6 DNA sequence and phylogenetic analysis

Sequences of ITS2 were aligned using the CLUSTALW multiple alignment program (Thompson *et al*, 1994). Gap sites were excluded from the following analysis. Genetic distances were estimated using 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 program (Tamura *et al*, 2007). The bootstrap values in percent are indicated above the branches of the trees. For the phylogenetic trees of COI and COII, *An. gambiae* and *An. pullus* were used as outgroups (NC_002084, AY444349, AY444350). The phylogenetic tree of ITS2 was constructed as an unrooted tree because an outgroup with easily aligned ITS2 was not available. The published data of *An. campestris*-like and *An. barbirostris* described by Saeung *et al*. (2007, 2008) were also used for phylogenetic analysis.

2.7 Morphological studies of eggs, larvae, pupae and adults under light and/or scanning electron microscopy

Eggs, larvae, pupae and adults of *An. campestris*-like obtained from different karyotypic forms of laboratory-raised F_1 and/or F_2-progenies were studied under light and/or scanning electron microscopy (SEM) to search for morphological differences among karyotypic forms.

For the light microscope study, the eggs, 4th instar larvae and pupae were mounted in Hoyer's media on slides, and examined under a compound microscope, while the adults aged 24-48 hours were pinned and investigated under a dissecting microscope.

For the SEM study, the eggs were placed in 2.5% glutaraldehyde in phosphate buffer (PB) pH 7.4 at 4 ^{o}C, washed with PB (10 min, with two changes), and post fixed (1 hour) in 1% osmium tetroxide at room temperature. The specimens were dehydrated by passage through an ethanol series, *i.e.*, 35%, 50%, 70%, 80% (10 min), and 95% (15 min, with two changes), followed by absolute ethanol (10 min, 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. A comparative study to search for karyotypic form-specific morphometry and morphology were performed.

2.8 Salivary gland polytene chromosomes preparation

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 coverslip for 1 min. After that, one drop of 2% aceto-lactic orcein stain was added to the coverslip and left for 15 min. Then a clean microscopic slide was placed on the coverslip and gently squashed. The coverslip edges were sealed with transparent nail varnish. The graceful polytene chromosomes were compared with the standard chromosome, as described by Chowdaiah *et al.* (1970) and White *et al.* (1975).

2.9 Malaria susceptibility test

Four to five-day-old adult females of all karyotypic forms of *An. campestris*-like obtained from laboratory-raised isoline colonies (No. 2.1) and the control mosquito, *An. cracens* [formerly *An. dirus* B, which was proven as an efficient laboratory vector to both *P. falciparum* and *P. vivax* (Sallum *et al*, 2005a; Junkum *et al*, 2005a)], were fasted for 12 hours and then allowed to feed on heparinized blood containing gametocytes of *P. falciparum* and *P. vivax* using the artificial membrane feeding techniques as described by Chomcharn *et al*. (1980). The fully engorged females were separated into small paper cups (6.5 cm in diameter and 8 cm in depth), with 6-8 mosquitoes per cup, and maintained in an insectarium at 27$\pm$ 2 ^{o}C and 70-80% relative humidity (RH). Seven to nine days after feeding, approximately one to third of the live mosquitoes was dissected for oocyst rates and the remainders were dissected for oocyst and sporozoite rates on days 10 to 15.

For the oocyst rates in midgut of mosquitoes in the experiment, normal and abnormal development, such as retaining stage, melanin forming inside a cyst *etc*., were investigated and compared with the development of the control mosquito (*An.*

cracens). As for sporozoite rates, the smeared salivary glands (6 lobes) obtained from both experiment and control groups, were examined. The invaded sporozoite found inside the salivary gland (Rosenberg, 1985) was the criteria for susceptible karyotypic forms.

2.10 Statistical analysis

2.10.1 The Fisher exact test and/or student t test were used for malarial susceptibility.

2.10.2 The Kimura two-parameter method was used for calculating the genetic distances.

2.10.3 The MEGA version 4.0 program was used for construction of neighbor-joining trees and bootstrap test.