CHAPTER II

MATERIALS AND METHODS

1. MATERIALS

1.1 For metaphase chromosome preparation

One percent ethanol-extracted *Gloriosa superba* solution; 0.1% 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%, 70%, 80%, 95% and absolute ethyl alcohol.

1.3 For polytene chromosome preparation

One percent sodium citrate solution; 15% and 45 % acetic acid; 50% propionic acid; aceto-lactic orcein; and clear nail vanish.

1.4 For isoenzyme study

Nineteen enzymes [aldehyde oxidase (Aldox), acid phosphatase (Acp), alkaline phosphatase (Alp), esterase (Est), fumerase (Fum), hexokinase (Hk), isocitrate dehydrogenase (Idh), leucine aminopeptidase (Lap), lactate dehydrogenase (Ldh), malic dehydrogenase (Mdh), malic enzyme (Me), glucose phosphomutase (Gpm), glucose phosphate isomerase (Gpi), xanthine dehydrogenase (Xdh), α-glycerophosphate

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dehydrogenase (α-Gpdh), peptidase (Pep), hydroxybutyrate dehydrogenase (Hbdh), octanol dehydrogenase (Odh), and 6-phosphogluconate dehydrogenase (6-Pgd)]; substrates; polyacrylamide gel; 10% sucrose solution; 0.005 M Tris-0.0384 M Glycine pH 8.3; and 0.067 M Tris-0.022 M Citric acid pH 7.1.

1.5 For molecular study

DNeasy Tissue kit (Qiagen, Co.); GeneClean kit (Q·BIOgene, Co.); pGEM®-T Easy Vector Systems (Promega, Co.); QIAprep miniprep kit (Qiagen, Co.); and Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Co.).

2. METHODS

2.1 Collection of mosquitoes

The endemic areas of malaria in Thailand comprise three provinces, *i.e.*, Chiang Mai (Ban Pang Mai Daeng, Maetang district), Mae Hong Son (Ban Huai Pong Kan, Muang district) and Phetchaburi (Ban Tha Salao, Nong Ya Plong district). These were the sites for mosquito collection using both human-baited and/or buffalo-baited traps (Figure 1). The wild-caught, fully engorged females were kept in paper cups with a screen and pad of water-soaked cotton wool placed on the tops. The mosquitoes were stored in a humid chamber placed inside a picnic cooler to maintain humidity and temperature. Then they were transported to the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, for colonization. Identification of mosquito species was based on the keys of Reid (1968) and Harrison (1980). The identified, wild-caught females were processed following the summarized experimental design shown in Figure 2.

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Figure 1. Map of Thailand showing Chiang Mai (CM), Mae Hong Son (MS) and Phetchaburi (PB) provinces, where mosquito collections were performed. Chiang Mai province is situated on latitude 18° 47′ N and longitude 98° 59′ E in northern Thailand and is approximately 97 and 647 kilometers away from Mae Hong Son province, northwest Thailand and Phetchaburi province, southwest Thailand, respectively.

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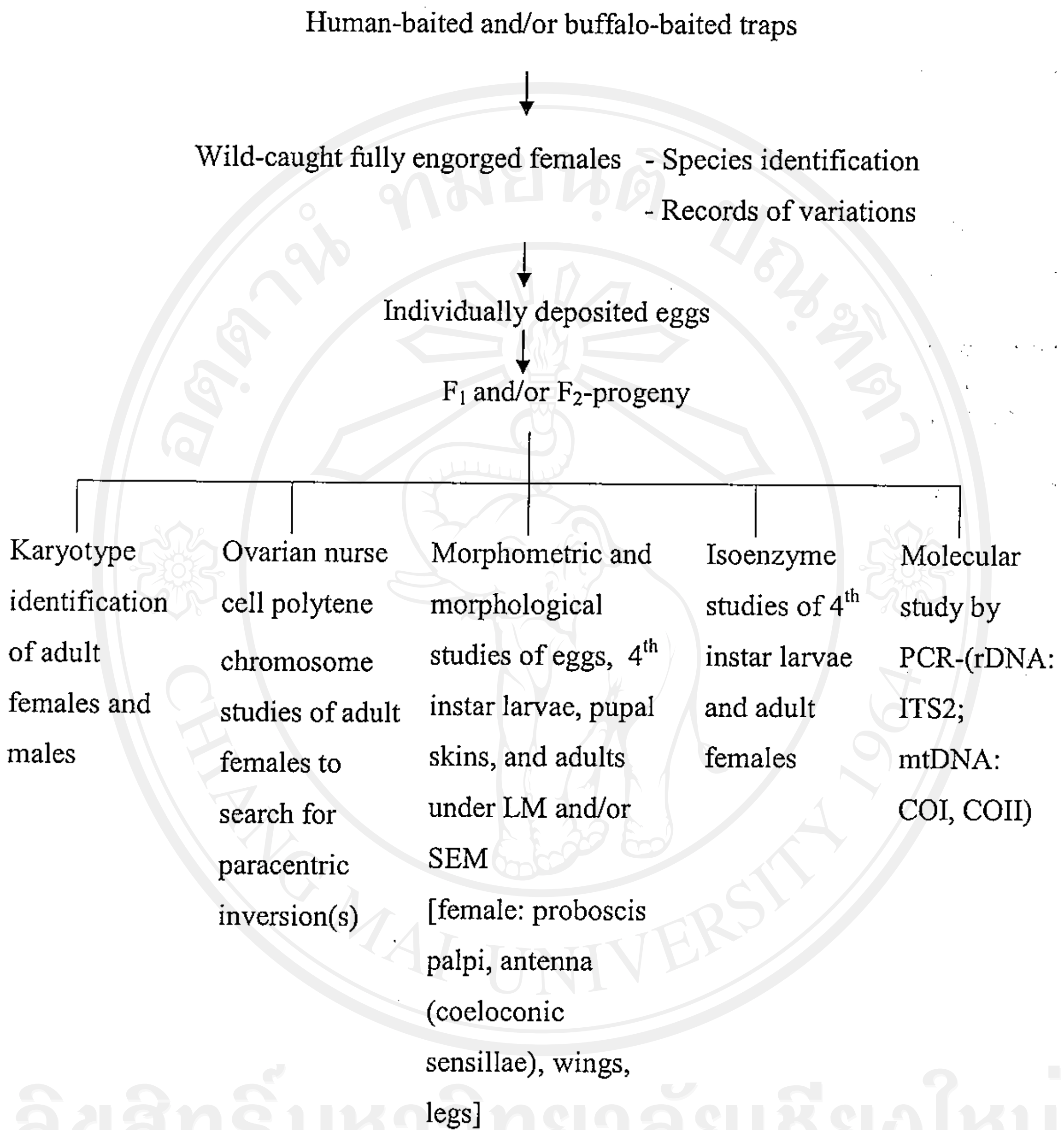

Figure 2. Summarized experimental design

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2.2 Rearing mosquitoes

The wild-caught females were refed on an anaesthetized golden hamster using a forced blood-feeding technique (Jitpakdi *et al.*, 2005). The fully engorged females were reared with 10% sucrose solution for 4-5 days in the insectory (27 ± 2°C, 70-80% relative humidity, and illuminated with a combination of natural daylight from a glass-window and fluorescent lighting for approximately 12 hr a day) until gravid. In order to establish the iso-female line (isoline), each gravid female was individually oviposited in a plastic cup (6 cm in diameter, 13 cm in depth) containing 30 ml of filtrated natural water (brought from a basin that has been used for tap-water production) with wet filter paper lining the inside. After hatching, larvae were processed for rearing following the methods described by Choochote *et al.* (1985) and Kim *et al.* (2003).

In order to obtain a high yield of metaphase chromosomes, salivary gland and ovarian nurse cell polytene chromosomes from healthy larvae and adults and successful establishment a laboratory colony of *An. aconitus*. Therefore, the procedures for larval rearing were as follows. Eighty first instar larvae were placed in a white plastic tray (25 x 35 x 6 cm) containing 1,500 ml of filtrated natural water. Ten stems of garden grass, (*Axonopus compressus*) were added in the rearing tray as larval resting places. The rearing tray was covered with a transparent plastic sheet to eliminate the need for changing and/or re-filling the water during the process of larval development. Standard formula fish food consisting of protein 49%, oil 5%, fiber 2%, ash 11% and moisture 8%, and an addition of vitamins A (30,200 IU), D3 (1,850 IU), E (200mg), and L-ascorbyl-2-polyphosphate (138 mg per Kg) was used for larval rearing. Pulverized fish food was placed in a vial covered with a fine mesh nylon screen (34 x 43 threads per cm^2) and sprinkled on the water until the food particles cease to spread across the water's surface.

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First and second instar larvae were fed twice daily, and this schedule was increased to 3-5 times daily after most of the larvae reach third and fourth instar. By using this non-stressful environmental for rearing the larvae, the colony of *Anopheles* could be easily colonized under laboratory conditions for many generations (Choochote *et al*., 1983, 1985; Kim *et al*., 2003).

2.3 Metaphase chromosome preparation

The technique for chromosome preparation in the adult mosquitoes was as described by Choochote *et al.* (2001). The newly emerged adults of laboratory-raised *An. aconitus* isolines, aged about 3-6 hr, were intra-thoracically inoculated with 0.30 μl and 0.50 μl of 1% ethanol-extracted *Gloriosa superba* solution for adult males and females, respectively. The inoculated mosquitoes were held in an insectary at 27 ± 2°C, 70-80% relative humidity for 3 hr and then dissected in a small drop of 1% hypotonic sodium citrate solution on a siliconized slide. Reproductive organs were excised from the last abdominal segment. They were left in 1% hypotonic sodium citrate solution for 10 min, and then transferred to a small drop of Carnoy's fixative (equal parts of 45% acetic acid and 95% ethanol) on a siliconized slide for 2 min before a drop of 60% acetic acid was added. 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. The droplet of cells was released slowly from the Pasteur pipette to form a circular trail of monolayer cells. The dried slides were stained with 10% Giemsa in phosphate buffer pH 6.8 for 30 min, rinsed with deionized water. air-dried at room temperature, mounted in Permount® (Fisher, Fairlawn, NJ), and examined under a compound microscope.

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Identification of karyotypic forms followed the cytotaxonomic key of Baimai *et al.* (1996a).

2.4 Morphometric and morphological studies of eggs, larvae, pupae and adults under light and/or scanning electron microscopy

Eggs, larvae, pupae and adults of *An. aconitus* 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 the morphometric and morphological differences among three karyotypic forms.

For the light microscope study, the eggs, 4th instar larvae, pupae and adults were mounted in Hoyer's media on slides, and examined under a compound microscope, while the adults aged 24-48 hr 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 °C, washed with PB (10 min, with two changes), and post fixed (1 hr) in 1% osmium tetroxide at room temperature. The specimens were dehydrated by passage through an ethanol series, *i.e.*, 35%, 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.

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2.5 Ovarian nurse cell polytene chromosome preparation

The technique for chromosome preparation was as described by Green (1972). Females of the F_1 and/or F_2-progenies from each isoline of *An. aconitus* were allowed to feed from golden hamsters. Engorged females at the semi-gravid stage with Christopher's stage-III ovaries were harvested in a drop of 5% propionic acid on a cavity slide. The harvested ovaries were transferred to a drop of 50% propionic acid and onto a grease-free slide for 2 min before a drop of 2% aceto-lactic orcein was added and mixed well with dissecting needles. After 5 min of staining, a grease-free 22 mm^2 coverslip was placed on the stained tissues. The preparation was wrapped firmly in filter paper and gently pressed with a thumb to squash and spread the chromosomes. Then, the coverslip was sealed with clear nail vanish. The prepared chromosomes were scrutinized under a compound microscope using a green filter (Olympus: VANOX-AH2-PC) to search for the inverted heterozygote and homozygote of paracentric inversions.

2.6 Isoenzyme study

The method of electrophoresis followed that of Komalamisra (1989). Nineteen isoenzymes of 4th instar larvae and adult females from each isoline of *An. aconitus* were investigated. The enzymes were aldehyde oxidase (Aldox), acid phosphatase (Acp), alkaline phosphatase (Alp), esterase (Est), fumerase (Fum), hexokinase (Hk), isocitrate dehydrogenase (Idh), leucine aminopeptidase (Lap), lactate dehydrogenase (Ldh), malic dehydrogenase (Mdh), malic enzyme (Me), glucose phosphomutase (Gpm), glucose phosphate isomerase (Gpi), xanthine dehydrogenase (Xdh), α-glycerophosphate

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dehydrogenase (α-Gpdh), peptidase (Pep), hydroxybutyrate dehydrogenase (Hbdh), octanol dehydrogenase (Odh), and 6-phosphogluconate dehydrogenase (6-Pgd).

Sample preparation and polyacrylamide vertical slab gel electrophoresis was as described by Takai (1986), with some modifications (Table 1). The gel was consisting of two layers, lower and upper, laminated vertically in the slit of gel molds. Sample constituents were concentrated in the upper or stacking gel, and then separated in the lower or separating gel. Table 1 summarizes the buffer systems and the enzymes were examined with reference to other electrophoretic conditions. The two buffer systems, I and II, were used. In each system, acrylamide gel concentration in the separation gel was adjusted to result in a better resolution of allelic bands of isoenzymes. Individual mosquitoes were homogenized in 10% sucrose solution. The homogenates were centrifuged and the supernatants used for loading into the polyacrylamide gel for electrophoresis. Electrophoresis was carried out at a constant voltage of 300 V at 6°C. After electrophoresis, the gel was treated in staining solution, which consists of staining buffer, substrate specific to the investigated-enzyme and dye. The gel-developed, color-banding patterns were photographed or allowed to dry and kept as a thin film for further investigations. Isoenzymes were numbered with respect to increasing anodal migration. Allozymes were named numerically according to their mobility relative to the most common allele (=100).

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Table 1. Electrophoretic procedures

Enzyme (Abbrev.)	Enzyme commission number	Buffer system*	Stage of sample
Aldehyde oxidase (Aldox)	EC 1.2.3.1	I	Adult
Acid phosphatase (Acp)	EC 3.1.3.2	I	Larva, adult
Alkaline phosphatase (Alp)	EC 3.1.3.1	I	Larva
Esterase (Est)	EC 3.1.1.1	I	Larva, adult
Fumerase (Fum)	EC 4.2.1.2	I	Adult
Hexokinase (Hk)	EC 2.7.1.1	II	Larva, adult
Isocitrate dehydrogenase (Idh)	EC 1.1.1.42	II	Larva, adult
Leucine aminopeptidase (Lap)	EC 3.4.1.1	I	Larva
Lactate dehydrogenase (Ldh)	EC 1.1.1.27	I, II	Larva, adult
Malic dehydrogenase (Mdh)	EC 1.1.1.37	I	Adult
Malic enzyme (Me)	EC 1.1.1.40	I	Larva, adult
Glucose phosphomutase (Gpm)	EC 2.7.5.1	I	Adult
Glucose phosphate isomerase (Gpi)	EC 5.3.1.9	I	Larva, adult
Xanthine dehydrogenase (Xdh)	EC 1.2.1.37	I	Adult
α-Glycerophosphate dehydrogenase (α-Gpdh)	EC 1.1.1.8	II	Larva
Peptidase (Pep)	EC 3.4.1.1	I	Larva
Hydroxybutyrate dehydrogenase (Hbdh)	EC 1.1.1.30	I	Adult
Octanol dehydrogenase (Odh)	EC 1.1.1.73	I	Adult
6-Phosphogluconate dehydrogenase (6-Pgd)	EC 1.1.1.44	I, II	Adult

*Buffer system: I = 0.005 M Tris-0.0384 M Glycine pH 8.3; II = 0.067 M Tris-0.022 M Citric acid pH 7.1.

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2.7 Molecular study

2.7.1 DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA was extracted from individual adult mosquitoes using a DNeasy Tissue kit (Qiagen, Co.) according to manufacturer's instructions. The rDNA ITS2, and mitochondrial COI and COII were amplified using the primers described in Park *et al.* (2003) and Folmer *et al.* (1994): 18S+1600 (5'-GCG TTG ATT ACG TCC CTG CCC TTT G-3') and 28S-60 (5'-GTT GGT TTC TTT TCC TCC-3') for ITS2; 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') for COI; AnoCO2+1 (5'-GAT TAG TGC AAT GAA TTT AAG C-3') and AnoCO2END (5'-GAG ATC ATT ACT TGC TTT CAG TC-3') for COII (Table 2). PCR conditions were as follows: one cycle of 2 min at 94°C; 35 cycles of 15 s at 95 °C, 30 s at 56 °C and 2 min at 72 °C and final cycle of 5 min at 72 °C. The PCR fragments were gel purified using a GeneClean kit (Q·BIOgene, Co.). The PCR-amplified DNA fragments were cloned using the pGEM®-T Easy Vector Systems (Promega, Co.). Positive clones were selected by clonal PCR using identical primers used in original PCR amplifications. Plasmid DNAs from selected clones were extracted using a QIAprep miniprep kit (Qiagen, Co.). The purified samples were subjected to sequencing in an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Co.) using a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Co.). The newly reported sequences were deposited in the GenBank nucleotide sequence database library under accession numbers DQ000241-DQ000276.

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Table 2. Primer used for PCR amplification.

Region	Oligonucleotide name and sequence		Reference
ITS2	18S+1600 28S-60	5'-GCG TTG ATT ACG TCC CTG CCC TTT G-3' 5'-GTT GGT TTC TTT TCC TCC-3'	Park *et al.* (2003)
COI	LCO1490 HCO2198	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	Folmer *et al.* (1994)
COII	AnoCO2+1 AnoCO2END	5'-GAT TAG TGC AAT GAA TTT AAG C-3' 5'-GAG ATC ATT ACT TGC TTT CAG TC-3'	Park *et al.* (2003)

2.7.2 Sequence data analysis

Both strands were sequenced and aligned using the CLUSTALX multiple alignment program (Thompson *et al.*, 1997). Geographical and cytological types of specimens, symbols used in figures, and their sequence accession numbers within Genbank are denoted in Table 11. Estimates of Kimura two-parameter distances (Kimura, 1980) were determined with the MEGA version 3.0. To explore the conflict between data sets (Farris *et al.*, 1994), the partition homogeneity test was applied to the combined data matrix (100 randomizations) with PAUP ver. 4.0 b10 (Swofford, 1999). The DnaSP version 3.99 software (Rozas *et al.*, 2003) was used for the analysis of polymorphism and genetic differentiation.

The level of polymorphism was estimated as the number of polymorphism (S), the average number of pairwise nucleotide differences (k), nucleotide diversity (p) (Nei, 1987), and the average number of nucleotide substitutions per site between groups or populations (d_{xy}). The statistical significance of genetic differentiation between groups, as estimated by K_s^* was established by the permutation test (Hudson *et al.*, 1992).

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2.8 Hybridization experiments

Crossing experiments were conducted among the two representative karyotypic forms of four laboratory-raised isolines of three strains (Chiang Mai, Mae Hong Son and Phetchaburi) of *An. aconitus*. Hybridization followed the method reported by Choochote *et al.* (2002a). Adult females and males of the two karyotypic forms that emerge from pupae were placed individually into test tubes and 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 rates for hatching, larval survival, pupation, and adult emergence, and sex ratios and abnormal morphology were recorded. The remaining F_1-hybrids were conducted to reciprocal and back crosses with their parental forms in order to observe genetic relationships. The F_2- progeny that fails to survive were the criterion for reproductive isolation. The salivary gland polytene chromosomes of 4th instar larvae from the crosses were investigated using the techniques described by Kanda (1979). The incomplete and complete synapsis of polytene chromosomes from all crosses was recorded.

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

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added to the coverslip and left for 5 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 Sharma *et al.* (1980) and White *et al.* (1975).

2.9 Establishment of mixed colonies of the three karyotypic forms

After karyotypic identification of individual isolines, laboratory-raised colonies of *An. aconitus* were established based on metaphase karyotypes and localities. Each colony was established by pooling 20 identified isolines of the same karyotypic form and locality.

2.10 Malarial susceptibility test

2.10.1 Laboratory-raised *An. aconitus* Form B and C

Two colonies of Form B, Phetchaburi and Chiang Mai strains, were established by pooling 4 and 20 isolines, respectively. Two colonies of Form C, Mae Hong Son and Chiang Mai strains, were established by pooling 3 and 20 isolines, respectively. These colonies were successfully reared by using the method of Choochote *et al.* (1983) in an insectary room at 27 ± 2°C, 70-80% relative humidity, illuminated with a combination of natural daylight from glass-window and fluorescent lighting (approximately 12 hr a day) for more than five consecutive generations, and were used for malarial susceptibility test throughout the experiments.

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2.10.2 Outgroup and ingroup control vectors

The outgroup, *An. dirus* B, a species member of *An. dirus* complex belongs to the Leucosphyrus Group, and the ingroup, *An. minimus* A and C, the same taxon as *An. aconitus* Form B and C in the Myzomyia Series, were used as the control vectors in the malarial susceptibility experiments. *An. dirus* B was obtained originally from Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, and the free-mating colony was established in the insectarium of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand for more than two decades. The free-mating colony of *An. minimus* A (CM strain) was from northern Thailand (Somboon and Suwonkerd, 1997). The CM strain was confirmed as species A by metaphase karyotypes and DNA sequence analysis of the D3 region of rDNA (Somboon *et al.*, 2001). For artificial mating colony of *An. minimus* C, it was established by pooling 10 isolines of *An. minimus* C strain from Kanchanaburi province, central Thailand. All 10 isolines were identified to species C by using the combination characteristics of adults (Sucharit *et al.*, 1988b) and metaphase karyotypes (Baimai *et al.*, 1996a). Subsequently, additional evidence of species C was confirmed by DNA sequences of D3 region (Sharpe *et al.*, 1999).

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2.10.3 *P. falciparum* and *P. vivax* gametocytes

The gametocytes of *P. falciparum* and *P. vivax* were obtained from malaria patients, whom got infection from Maetang and/or other districts in Chiang Mai province. Ten ml of blood containing gametocytes of the above malaria species were collected by venepuncture into a heparinized syringe, kept in ambient temperature (Somboon and Morakote, 1990), and used for infection of mosquitoes within 12 hr.

2.10.4 Infection of mosquitoes with *P. falciparum* and *P. vivax* gametocytes

After emergence, all adult female mosquitoes were provided with 5% sucrose solution until age of 4-6 days, subsequently, they were fasted for 12 hr prior to the infections. The 12-hr fasted females of *An. aconitus* Form B and C, outgroup control mosquito-vector (*An. dirus* B), and ingroup control mosquito-vectors (*An. minimus* A and C) were put in a paper cup size 8.5 cm in diameter and 11 cm in depth (50 fasted females per cup for each species), and allowed to feed on heparinized blood containing gametocytes (gametocyte density of *P. falciparum* = 21 per 1 µl; *P. vivax* = 28, 17 and 34 per 1 µl in experiment 1, 2 and 3, respectively) using artificial membrane feeding techniques as described by Chomcharn *et al.* (1980). The fully engorged females were separated to small paper cups (diameter 6.5 cm, depth 8 cm) with 10 mosquitoes per cup and maintained in an incubator at 27 ± 2°C, 70-80% relative humidity. Eight and twelve days after feeding, the infected mosquitoes were dissected and examined for oocysts in midguts and sporozoites in salivary glands, respectively.

2.11 Statistical analyses

The F-test and H-test were used for morphometry of egg, larva, pupa and adult stages.

The t-test was used for ascertaining the number of oocyst in the midgut.

The Fisher exact test and Chi-square test were used for malarial susceptibility.

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