CHAPTER IV

DISCUSSION

Throughout the larval rearing period, the number of larvae, rearing conditions in the tray, and food were the most important factors, not only for routine rearing, but also special rearing in order to obtain a high yield of metaphase and polytene chromosomes, which were necessary for population genetic study. Stressful rearing-conditions, *e.g.*, the overcrowding of larvae in a rearing tray (in this study, 80 larvae per 25 x 36 x 6 cm tray was an appropriate number for *An. campestris*-like Form E), and the use of inappropriate water medium and food would lead to a rapid drop in and/or loss of a colony. Also, this would result in low larval and pupal survival, adult F_1-progenies refusing to take bloodmeal, difficulty in artificial mating of adult males and/or failure to inseminate sperm into mated-female spermathecae, short life span of adult females and males, mated gravid adult females laying fewer numbers of eggs and/or failure to lay eggs, and low egg-hatchability. Thus, any rearing system, which is an important first step that leads to obtaining healthy larvae, would be a promising method for successfully establishing a colony, particularly an iso-female line colony, which is more difficult and complicated to establish than a mixed colony. As mentioned previously, food was one of the most important factors for obtaining healthy larvae, thus, several kinds of larval food were tried for use and comparison, *e.g.*, mouse pellets, cat and dog biscuits and various formulas of fish food. The results

indicated that the extra and/or standard formula of fish food, consisting of protein 47.5%, oil 6.5%, fibre 2.0%, ash 10.5%, moisture 6.0% and additives of vitamin A 29,770 IU/kg, vitamin D3 1,860 IU/kg, vitamin E 200 mg/kg, L-ascorbyl-2-polyphosphate 137 mg/kg, lecithin, l-lysine monochlorhydrate, and citric acid proved to be excellent larval food for *An. campestris*-like Form E. This food was also ideal for other species with rearing difficulties, particularly in the iso-female line colony of *An. sinensis* Form A and B (Choochote *et al*, 1998; Min *et al*, 2002), *An. vagus* Form A and B (Choochote *et al*, 2002a), *An. pullus* Form A and B (Park *et al*, 2003), and *An. aconitus* Form B and C (Junkum *et al*, 2005b). The use of filtered natural water (brought from a basin used for tap-water production) as the larval rearing medium also proved to be promising. Trials using boiled tap-water, filtered tap-water, polarized water and deionized water yielded unsatisfactory outcomes by providing low larval survival, particularly through subsequent progenies. The addition of garden grass to the larval rearing tray, as stated by Choochote *et al*. (1983), resulted in high larval survival for *An. campestris*-like Form E. Using few stems of garden grass, or withdrawing it, would lead to low larval survival and/or weak larvae for rearing subsequent generations. Using slightly more or less than 15 stems of garden grass, depending upon the size of the stems, and size and number of leaves, proved to improve conditions to a suitable level for larval rearing, since the grass provided a resting place for larvae, rendered shade as in natural breeding sites (rice paddy, ponds and swamps associated with water plants) (Reid, 1968; Harrison and Scanlon, 1975), and aerated the medium. Its roots were also very important for maintaining clear and clean rearing medium by using larval waste products and unconsumed food as fertilizer, which determined the obvious active growth of grass in the rearing tray.

Esah and Scanlon (1936) emphasised that the survival rate of early stage *An. balabacensis* larvae was affected by the lack of aeration, and consequently, they tried to use sand substrate in the rearing medium, which seemed helpful but not essential for larval development.

One of the difficulties in the colonization of anopheline-mosquitoes in the laboratory might be due to adults not being capable of copulation in a small and/or standard cage (30 x 30 x 30 cm). The determination of adaptive stenogamy of *An. campestris*-like Form E by gathering females and males in a standard cage for one week indicated that *An. campestris*-like Form was strong eurygamy indicated by yielding a 0% insemination rate. Additionally, in current studies of various strains, *An. campestris*-like Form B (Kamphaeng Phet: F_{11}) and E (Sa Kaeo: F_{22}, Chanthaburi: F_{11}, Ayutthaya: F_{21}, Maha Sarakham: F_{16}**,** Udon Thani: F_{11}, Khon Kean: F_{11}, Mukdahan: F_{10}, Prachuap Khiri Khan: F_{10} Chumphon: F_{10}) failed to copulate freely in a standard cage, also suggesting strongly eurygamous behavior. Thus, the artificial mating methods as described by Baker *et al*. (1962) and Ow Yang *et al*. (1963) were used. The best age for artificial mating in male *An. campestris*-like Form E was 5-days-old. Nonetheless, males aged 4 to 8 days old could be used satisfactorily.

The engorged females that derived from 2 feeding methods, *i.e.*, direct feeding ability on white rat in a 30 x 30 x 30 cm cage, and artificial feeding ability on bovine heparinized-blood in a paper cup (8.5 cm in diameter and 11 cm in depth) were used satisfactorily for the maintenance of an iso-female line laboratory-raised colony of *An. campestris*-like Form E. One difficulty and/or failure in rearing mosquitoes in the laboratory was the subsequent generation's refusal to feed on blood, particularly from small laboratory animals such as guinea pig, white rat, golden hamster, *etc*. This leads

to direct feeding from human volunteers, especially at the beginning of the 1st to 5th generations of the colony. However, to solve this problem, forced artificial feeding on bovine heparinized-blood by *An. campestris*-like Form E was successful in this study. Nevertheless, a point to be kept in mind is that only the healthy progenies of laboratory-raised colonies could be used successfully. Additionally, the use of direct blood feeding of subsequent mosquito progenies from human volunteers is a potentially dangerous method and should be given up entirely, since many reports have declared that some mosquito genera such as *Aedes* and *Culex* could be capable of transmitting many viruses vertically (Khin and Than, 1983; Ronsen *et al*, 1983; 1989; Dhanda *et al*, 1989; Mitchel and Miller, 1990; Bosio *et al*, 1992; Baqar *et al*, 1993; Ahmad *et al*, 1997; Thenmozhi *et al*, 2000). As for the genus *Anopheles*, at least 3 species, *i.e.*, *An. peditaeniatus* (Mourya *et al*, 1989), *An. subpictus* (George *et al*, 1987; Thenmozhi *et al*, 2006) and *An. barbirostris* (Chakravarty *et al*, 1975) have been incriminated as secondary vectors of Japanese encephalitis virus, which is possibly transmitted vertically.

Many anopheline-colonies have been reported to adapt easily to oviposit eggs in the cage on various types of simple ovipots, *e.g.*, petridish, crystallizing dish, terra-cotta bowl, white plastic cup, black cup, *etc*. (Chomcharn, 1979; Gerberg *et al*, 1994; Bangs *et al*, 2002; Kim *et al*, 2003; Da Silva *et al*, 2006). In the case of allowing gravid adult females of *An. campestris*-like Form E to oviposite eggs in a standard cage, fewer numbers of eggs were obtained from a plastic cup ovipot, whereas the forced laying of eggs by placing gravid adult females in a plastic cup, the same size and conditions as used in the cage, a massive number of eggs were recovered (Figure

3A). Thus, in rearing *An. campestris*-like Form E and other strains and/or karyotypic forms in our laboratory, this method has been used routinely up until now.

Observation of the life cycle and developmental period of *An. campestris*-like Form E from the laboratory-raised iso-female line colony (F_1, F_5, F_{10}, F_{20}, F_{30}), demonstrated that the average eggs per deposited female, duration of egg-hatching, embryonation and hatchability rates, larval and pupal duration, pupation and emergence rates were generally in accordance with other anopheline-mosquito development (Chomcharn, 1979; Gerberg *et al*, 1994).

Comparative analysis of metaphase chromosomes of *An. barbirostris* species complex in Thailand revealed that there were four karyotypic forms in *An. barbirostris*, *i.e.*, Form A [X_2, X_3, Y_1 (subtelocentric or acrocentric)], Form B [X_1, X_2, X_3, Y_2 (submetacentric)], Form C [X_2, X_3, Y_3 (large submetacentric or metacentric)] and Form D [X_2, Y_4 (medium metacentric)]; and one karyotypic form in *An. campestris* [X, Y (telocentric)] was found indigenously in Thai populations (Baimai *et al*, 1995; Suwannamit *et al*, 2009). Recently, Saeung *et al*. (2007) reported two karyotypic forms, *i.e.*, Form B [X_2, Y_2 (submetacentric)] and E [X_2, Y_5 (small metacentric)] in sympatric strains of *An. campestris*-like from Chiang Mai province, northern Thailand. The morphological character of the summation of branches of seta 2-VI pupal skins (22.4-24.5 branches) were in the range of *An. campestris* (17-58 branches), whereas the X_2 and X_3 chromosome were similar in characters to all forms of *An. barbirostris*. Thus, they were tentatively designated as *An. campestris*-like Form B and E, respectively. In addition to the above results concerning *An. campestris*-like, the X_2, X_3, Y_6 (large subtelocentric or acrocentric) sex chromosome in *An. campestris*-like (summation of branches of seta 2-VI pupal skins: 21.3-30.0

branches), discovered in this study, was tentatively designated as Form F. The Y_6 chromosome of this karyotypic form was obviously different from Y_1, Y_2, Y_3 and Y_4 chromosomes of *An. barbirostris* Form A, B, C and D, respectively, and the Y chromosome of *An. campestris*, and Y_5 chromosome of *An. campestris*-like Form E.

Crossing experiment and/or testing of post-mating reproductive isolation is still an efficient and reliable diagnostic tool for determining the intra-taxon of anopheline species to a sibling species and/or other. Hybrid non-viability, sterility, or breakdown are the criteria for genetic incompatibility, including lack of insemination, embryonation, hatchability, larval survival, pupation, emergence, adult sex distortion, abnormal morphology, and reproductive system (Kanda *et al*, 1981; Baimai *et al*, 1987; Subbarao, 1998). Nonetheless, a point worth noting is that an isoline colony established from the combinative characters of morphological, cytological (polytene and mitotic chromosomes) and/or molecular markers has to be used. A laboratory colony established from a naturally mixed population should be omitted, since it may be a mixture of two or three sibling species (Subbarao, 1998). Despite the obvious difference in characteristics of the metaphase karyotypes of *An. campestris*-like Form B, E and F, the results of genetic compatibility, by providing viable progenies from the crossing studies among three karyotypic forms derived from twelve isoline strains in Thailand, revealed no post-mating reproductive isolations in either sympatric or allopatric populations.

Molecular investigation of some specific genomic markers, *e.g.*, ribosomal DNA (ITS1, ITS2, D3) and mitochondrial DNA (COI and COII), has been used extensively as a supportive tool to determine and/or characterize the sibling species and/or cryptic species member in the intra-taxon of anopheline speices

(Mitchell *et al*, 1992; Sharpe *et al*, 2000; Min *et al*, 2002; Park *et al*, 2003; Junkum *et al*, 2005b; Saeung *et al*, 2007; 2008; Suwannamit *et al*, 2009; Choochote, 2011). Apart from the molecular evidence of very low intraspecific variation (genetic distance < 0.005) of the nucleotide sequences of the ITS2 of rDNA, and COI and COII of mitochondrial DNA among twenty-eight isoline strains of *An. campestris*-like Form B, E and F, a conspecific relationship of these three karyotypic forms was well supported.

Based on the above evidence, it was concluded confidently that *An. campestris*-like Form B, E and F were conspecific cytological races in the Thai mosquitos' populations. Similar results have been reported in *An. sinensis* Form A and B (Choochote *et al*, 1998; Min *et al*, 2002), *An. vagus* Form A and B (Choochote *et al*, 2002a), *An. pullus* Form A and B (Park *et al*, 2003), *An. aconitus* Form B and C (Junkum *et al*, 2005b), *An. campestris*-like Form B and E (Saeung *et al*, 2007) and *An. peditaeniatus* Form B, C, D and E (Choochote, 2011).

Comparative morphological studies of egg, larva, pupa and adult are still primarily reliable tools for the recognition of morphological variants within the taxon anophelines, particularly when the distinct evidence of biology, microhabitat, cytogenetics, biochemical and molecular genetics were pointed out (Peyton and Harrison, 1979; Sucharit and Choochote, 1983). The investigation of major diagnostic characters of egg, larva, pupa and adult among 71 strains of *An. campestris*-like Form B, E and F in the present study confirmed that karyotypic variation has not an effect on the generating of morphological variant.

The morphological features and fine structure of mosquito eggs examined under scanning electron microscopy (SEM) are species specific (Iwaki and Choochote,

1991; Iwaki *et al*, 1992; 1994; Jitpakdi *et al*, 1998), and may be helpful in detection the difference some sibling species members in the taxon anopheline species complex (Damrongphol and Baimai, 1989; Rodriguez *et al*, 1992; Sucharit *et al*, 1995). Given the marked karyotypic difference among *An. campestris*-like Form B, E and F, thus, their comparative egg morphometry by light microscope and surface topography study by SEM were performed. The result of study revealed that their eggs were intraspecific morphometric variation, whereas the entire egg surface topography was morphological identical. Similar results were obtained in two cytologically polymorphic races of *An. sinensis* Form A and B (Rongsriyam *et al*, 1996), *An. vagus* Form A and B (Choochote *et al*, 2002a) and *An. aconitus* Form B and C (Junkum *et al*, 2004).

Polytene chromosome investigation by searching for fixed paracentric inversion on chromosomal arms has been used as a robust tool for the recognition of sibling species members or isomorphic species within the taxon anopheline species complex (Green and Baimai, 1984; Green *et al*, 1985; 1992). The present study demonstrated that marked difference in kaytotypic Fome B, E and F of *An. campestris*-like was not generated the rearrangement of polytene chromosomes in the speciation process. Similar results were also found in *An. aconitus* Form B and C that have homosequential banding of polytene chromosomes (Junkum *et al*, 2005b).

In order to incriminate a mosquito vector in an endemic area of mosquito-borne human diseases, it is necessary to confirm the susceptibility rate in a laboratory-bred, clean mosquito colony that has been fed on a carrier blood containing pathogens (Sasa, 1976; Choochote *et al*, 2001; Junkum *et al*, 2005a). Thus, by using this criterion, the susceptibility test in an experimental laboratory is still a useful tool

when suspecting the potential vector of a certain mosquito species. Nonetheless, the susceptibility alone does not imply an important role in the transmission of disease in nature, whereas a refractory one can entirely rule out its significance. The susceptibility test of *An. campestris*-like to *P. falciparum* malaria was determined by using a laboratory-bred, clean mosquito colony. There was absolutely no development of oocysts, and 0% sporozoite rates obtained from *An. campestris*-like Form E (Chiang Mai strain) and F (Udon Thani strain) indicated that they were an entire refractory vector for *P. falciparum*. The results were in agreement with the previous reports by Somboon *et al*. (1994) that *An. barbirostris* (Mae Hong Son, northern Thailand strain) was non-susceptible to local strain of *P. falciparum*.

According to the vector-potential status of *An. campestris*-like to *P. vivax* has been reported only from *An. campestris* (identification was based only branches summation of seta 2-VI pupal skin) (Apiwathnasorn *et al*, 2002). The high oocyst and sporozoite rates of *An. campestris*-like Form B and E strains from Chiang Mai provinces, confirming the potentially natural vector status of *An. campestris*-like as proposed by Limrat *et al*. (2001), Apiwathnasorn *et al*. (2002) and Sattabongkot *et al*. (2004). Nonetheless, further investigations on the oocyst and sporozoite rates of wild-caught female *An. campestris*-like in an endemic area of malaria in Chiang Mai province should be done intensively to determine its role as a naturally transmissive vector. Interestingly, the other strains of *An. campestris*-like forms were entirely refractory to *P. vivax* although their genetic proximity are identical and/or nearly identical. Similar results were also obtained from different strains of *An. atroparvus* to *P. berghei* (Sluiters *et al*, 1986), *An. stephensi* to *P. gallinaceum* (Frizzi *et al*, 1975), *An. gambiae* to *P. gallinaceum* (Vernick *et al*, 1995) and *Aedes aegyti* to *P.*

gallinaceum (Shahabuddin *et al*, 1995; Morlais *et al*, 2003). These strains appear to be involved through a genetically controlled mechanism, *i.e.*, malaria susceptibility (*Pif-B*^{s}*/Pif-B*^{r}) and refractoriness (*Pif-C*^{s}*/Pif-C*^{r}) genes (Vernick *et al*, 1989), in which mostly refractoriness is manifested by malaria-mosquito recognition during midgut invasion and the mechanism by which the mosquito defends against the malaria induced (Abraham and Jacobs-Lorena, 2004).

The 0% sporozoite rates recovered from *An. campestris*-like Form E (Sa Kaeo strain) in this study were contrary to the 23.80% sporozoite rate obtained from *An. campestris* (Sa Kaeo strain) reported by Apiwathnasorn *et al*. (2002). Whereas, different strains of *P. vivax* gametocyte were used in the susceptibility tests, *i.e.*, a northern strain (Chiang Mai province) was used in this study, and an eastern (Sa Kaeo province) one by Apiwathanasorn *et al*. (2002). Even though the immunophenotypic strains of *P. vivax* (Vk-210 and Vk-247 variants) (Rosenberg *et al*, 1989) were not determined in this study; two reports from western and eastern Thailand demonstrated that Vk-210 was detected in 2/478 *An. campestris* strains from a western region (Tak province) (Coleman *et al*, 2002) and Vk-247 in 2/42 *An. barbirostris* strains from an eastern area (Chanthaburi province) (Frances *et al*, 1996). Thus, a crucial question is still whether the immunophenotypic strains of malaria gametocytes are specifically susceptible to different strains of the *An. barbirostris/campestris* group.