CHAPTER IV

DISCUSSION

Comparative morphological and morphometrical studies of eggs, larvae, pupae and adults are still primarily effective tools for the detection of anopheline mosquito variants in the intra-taxon, when suspected from distinct evidence of biology and/or ecology, cytogenetics and molecular genetics (White and Muniss, 1972; Rodriguez *et al.*, 1992; Nguyen *et al.*, 1993, 2000; Lounibos *et al.*, 1997). The investigations of major diagnostic points on 4th instar larvae, pupal skins and adult females of *An. aconitus* Form B (X_1, X_2, Y_2) and C (X_1, X_2, Y_3), which were obviously cytological differences of the Y-chromosomes, were generally similar to the standard values as described by Harrison (1980). That is to say, the number of branches on seta 2-C, 3-C, 4-C, 3-T, O-IV, O-V, O-VI of 4th instar larvae; number of branches on seta O-III-VII, 4-IV, 7-III-IV of pupal skins; dimension of wings, morphology of palpus, proboscis, wings and legs of females demonstrated all major diagnostic characters of *An. aconitus* Form B and C. In addition, the separation of *An. aconitus* Form B and C by using coeloconic sensillae and palpal ratio of adult females could be separated these two karyotypic forms at a 20% level. These combinative characters were reported as an efficient tool to separate sibling species members of *An. gambiae* complex, *i.e.*, *An. gambiae* s.s and *An. merus*, at about a 94% level (White, 1979). Nonetheless, it has been proven as subsequently not valid to separate *An. gambiae* s.l. from *An. merus* by yielding only 40.9% of separation (Mosha and

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Mutero, 1982). The low degree (20%) of separation between *An. aconitus* Form B and C may not result from the unreliable tool of using combinative characters of coeloconic sensillae and palpal ratio, but it appears to be dependent upon the minimal morphological difference by themselves.

Scanning electron microscopic studies of mosquito eggs not only provide descriptions of far greater accuracy and fidelity than achieved by traditional light microscope, but they can also be used to aid in the differentiation of species, sibling species or varieties of *Anopheles* mosquitoes. Damrongphol and Baimai (1989) conducted comparative scanning electron microscopic studies of four isomorphic egg species of *An. dirus* complex, *i.e.*, species A, B, C and D. The results indicated that the eggs of species A and C were similar in size and shape. Their size was intermediate, in between egg species B, which was the largest, and species D, the smallest. The patterns of outer chorionic cells between the frills and floats and the arrangement of deck tubercles were also distinct in different sibling species members. Rodriguez *et al.* (1992) continued light and scanning electron microscopic studies of the eggs of five strains of *An. albimanus*, which had morphological differences in pupae and behavioural distinction in adults. The authors reported four different types of eggs in respect to the size and shape of the floats, whereas the ornamentation under SEM was similar. Sucharit *et al.* (1995) reported marked differences in shape and ornamentation of the eggs (deck, frill and micropylar) of two sibling species (A and C) in the *An. minimus* complex.

Given the marked differences between the metaphase karyotypes of *An. aconitus* Form B (X_1, X_2, Y_2) and C (X_1, X_2, Y_3) in sympatric (Chiang Mai province, northern Thailand) and allopatric (Mae Hong Son province, northwest Thailand and

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Phetchaburi province, southwest Thailand) populations, comparative egg morphometry and surface topography studies by SEM were carried out in order to elucidate the intraspecific differences and/or variations between the two karyotypic forms. The result of this study indicated that three strains of *An. aconitus* Form B (Chiang Mai and Phetchaburi strains) and C (Chiang Mai and Mae Hong Son strains) had intraspecific variations in float width and number of posterior tubercles on deck, whereas the entire egg surface topography was morphologically identical. Similar results were found in two cytologically polymorphic races of *An. sinensis* Form A and B (Rongsriyam *et al.*, 1996) and *An. vagus* Form A and B (Choochote *et al.*, 2002a). Additionally, the egg surface topography under SEM of *An. aconitus* Form B and C in this and/or the first study was morphologically distinct from the other *Anopheles* species (subgenus *Anopheles* and *Cellia*) formerly reported in Thailand, *i.e.*, *An. barbirostris, An. donaldi, An. minimus* A and C, *An. sinensis* Form A and B, and *An. vagus* Form A and B (Sucharit *et al.*, 1995; Rongsriyam *et al.*, 1996; Jitpakdi *et al.*, 1998; Choochote *et al.*, 2002a), thus indicating the species-specific diagnostic characteristics.

Several intra-taxa of the Asian anopheline species, which were primarily detected with morphological, biological, and cytological differences and/or variations, led to the doubtful status of sibling species and/or subspecies. Subsequently, they were confirmed by polytene chromosome and isoenzyme investigations, and hybridization experiments, *e.g.*, *An. culicifacies* complex (Green and Miles, 1980; Subbarao *et al.*, 1988; Adak *et al.*, 1994), *An. dirus* complex (Kanda *et al.*, 1981; Baimai *et al.*, 1987; Sawadipanich *et al.*, 1990; Green *et al.*, 1992a), *An. maculatus* complex (Takai *et al.*, 1987; Chabpunnarat, 1988; Green *et al.*, 1992b), *An. minimus*

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complex (Komalamisra, 1989; Green *et al*., 1990; Choochote *et al*., 2002b) and *An. sundaicus* complex (Sukowati and Baimai, 1996; Sukowati *et al*., 1999). Thus, ovarian nurse cell polytene chromosome and isoenzyme investigations, and hybridization experiments were performed to determine the degree of genetic proximity between *An. aconitus* Form B and C strains from Phetchaburi, Mae Hong Son and Chiang Mai provinces. Additionally, their comparative DNA sequences of ITS2, COI and COII were included in this study.

The examination of polytene chromosomes of wild-caught adult females and/or progenies of isolines provides unequivocal evidence for the existence of different specific mate recognition systems (SMRS) (Paterson, 1980). The total absence or significantly deficient number of heterozygotes for an inversion in a population indicates entirely the presence of reproductive isolation within a taxon (Green *et al*., 1992b; Subbarao, 1998). The results of no major chromosomal rearrangements from the comparative investigations of ovarian nurse cell polytene chromosomes of standard mapping, *An. aconitus* Form B (Chiang Mai strain), to sympatric *An. aconitus* Form C (Chiang Mai strain), and allopatric *An. aconitus* Form B (Phetchaburi strain) and Form C (Mae Hong Son strain), indicate no pre-mating isolation between *An. aconitus* Form B and C.

Enzyme electrophoresis is being used extensively in studies of species complexes. Electrophoretic variations at enzyme loci are not only useful for the identification of isomorphic species, but they can also be used for the correct identification of morphologically cryptic *Anopheles* species. Variations at a locus thus enable the detection of the reproductive isolation within populations, resulting from positive assortative matings within a population (Green *et al*., 1990; Subbarao, 1998).

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The similar allelic frequencies observed at 10 isoenzymes 16 loci in 4th instar larvae, and 11 isoenzymes 13 loci in adult females of sympatric *An. aconitus* Form B and C (Chiang Mai strain), and allopatric *An. aconitus* Form B (Phetchaburi strain) and C (Mae Hong Son strain), suggest negative assortative mating between *An. aconitus* Form B and C.

Hybridization experiments and/or the testing of reproductive isolation at the post-mating barriers are still efficient and reliable diagnostic tools for the differentiation of intra-taxon of anopheline species to a sibling species. Hybrid inviability, sterility, or breakdown are the criteria for genetic incompatibility, including lack of insemination, embryonation, hatchability, larva survival, pupation, emergence, adult sex distortions, abnormal morphology and reproductive system (Kanda *et al*., 1981). Nonetheless, a point to be remembered is that colonies established from species-specific diagnostic characteristics of progeny from isolines have to be used. A laboratory colony established from a mixed, natural population may be a mixture of two or three species (Subbarao, 1998). The results of genetic compatibility, providing viable progenies and complete synaptic salivary gland polytene chromosomes from the crossing studies among four isolines of *An. aconitus* Form B (Chiang Mai and Phetchaburi strains) and C (Chiang Mai and Mae Hong Son strains), revealed no post-mating barriers between *An. aconitus* Form B and C both in sympatric and allopatric populations.

Molecular investigation of some specific genomic markers, *e.g*., ribosomal DNA (ITS1, ITS2, D3) and mitochondrial DNA (COI, COII), has been used extensively as a tool to characterize and/or diagnose the sibling species and/or cryptic members in the intra-taxa of anopheline mosquitoes (Mitchell *et al*., 1992; Paskewitz

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et al., 1993; Sharpe *et al.*, 2000; Van Bortel *et al.*, 2000; Manonmani *et al.*, 2001; Min *et al.*, 2002; Park *et al.*, 2003; Phuc *et al.*, 2003; Sawabe *et al.*, 2003; Chen *et al.*, 2006). From the molecular evidences of genetic distances and nucleotide divergences between isoline sequences of *An. aconitus* Form B and C, a conspecific relationship of these two *An. aconitus* forms was well supported.

Based on the above evidences, it confidently concludes that *An. aconitus* Form B and C are conspecific cytological races in the Thai population. Similar results have been found previously in *An. maculatus* Form B and E (Chabpunnarat, 1988), *An. vagus* Form A and B (Choochote *et al.*, 2002a), *An. sinensis* Form A and B (Choochote *et al.*, 1998, Min *et al.*, 2002), and *An. pullus* Form A and B (Park *et al.*, 2003).

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). 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. Nevertheless, 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. According to the vectorial status of *An. aconitus* to *Plasmodium falciparum* and *P. vivax* as determined by the susceptibility tests using a laboratory-bred, clean mosquito colony has never been done and/or reported before this time. The high oocyst and sporozoite rates of *An. aconitus* Form B strains from Chiang Mai and Phetchaburi provinces to infection with *P. falciparum*, and Form B strains from Chiang Mai and Phetchaburi provinces and Form C strains from Chiang Mai and Mae

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Hong Son provinces to infection with *P. vivax* in the present study, confirming the secondary vector status of *An. aconitus* as reported by Gould *et al.* (1967). Nonetheless, further investigations on the oocyst and sporozoite rates of wild-caught female *An. aconitus* in an endemic area of malaria in Chiang Mai province and/or other suspected areas should be done intensively to determine its role as a naturally transmissive vector.

Many Thailand *Anopheles* species have been reported positive ELISA for circumsporozoite (CS) antigens of *P. falciparum* and *P. vivax* by using the whole body and/or head and thorax of mosquitoes (Baker *et al.*, 1987; Harbach *et al.*, 1987; Gingrich *et al.*, 1990; Frances *et al.*, 1996; Rattanarithikul *et al.*, 1996). This diagnostic tool did not definitely incriminate the mosquito as the natural vector, since it could be detected CS protein from the developing oocysts (Beier *et al.*, 1987), soluble CS protein shed from oocysts and sporozoites (Verhave *et al.*, 1988) and CS protein in various body parts (Verhave *et al.*, 1988)). In addition, false positive *P. falciparum* and *P. vivax* detections by ELISA were reported (Somboon *et al.*, 1993). However, the mosquito species which were highly susceptible to malarial infections could not be incriminated as the potential vectors, since sporozoites did not invade salivary glands (Rosenberg, 1985). Judged from the above evidence, therefore, the combining of positive ELISA for CS antigens with sporozoite rate of a laboratory-bred, clean *Anopheles* colony should be the important evidences prior to the incrimination of potentially natural vector. Additionally, the sporozoite-like crystal found in the median lobe of salivary glands of *An. aconitus* Form B and C might be one of the important, missed leading factor in the identification of true sporozoites in salivary glands of the laboratory susceptibility experiments and/or wild-caught

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Anopheles females. Similar results have been reported in *An. sinensis* Form A and B (Rongsriyam *et al.*, 1998).

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