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COMPARATIVE MORPHOMETRY AND MORPHOLOGY OF *Anopheles aconitus* FORM B AND C EGGS UNDER SCANNING ELECTRON MICROSCOPE

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SUMMARY

Comparative morphometric and morphological studies of eggs under scanning electron microscope (SEM) were undertaken in the three strains of two karyotypic forms of *Anopheles aconitus*, i.e., Form B (Chiang Mai and Phet Buri strains) and Form C (Chiang Mai and Mae Hong Son strains). Morphometric examination revealed the intraspecific variation with respect to the float width [36.77 ± 2.30 µm (Form C: Chiang Mai strain) = 38.49 ± 2.78 µm (Form B: Chiang Mai strain) = 39.06 ± 2.37 µm (Form B: Phet Buri strain) > 32.40 ± 3.52 µm (Form C: Mae Hong Son strain)] and number of posterior tubercles on deck [2.40 ± 0.52 (Form B: Phet Buri strain) = 2.70 ± 0.82 (Form B: Chiang Mai strain) < 3.10 ± 0.32 (Form C: Chiang Mai strain) = 3.20 ± 0.42 (Form C: Mae Hong Son strain)], whereas the surface topography of eggs among the three strains of two karyotypic forms were morphologically similar.

KEYWORDS: *Anopheles aconitus*; Karyotypic form; Egg; Fine structure; Scanning electron microscopy.

INTRODUCTION

So far, at least six *Anopheles* (*Cellia*) species, i.e., *An. aconitus* Donitz, *An. culicifacies* Giles, *An. jeyporiensis* James, *An. minimus* Theobald, *An. pampanai* Buttiker and Beales, and *An. varuna* Iyengar have been reported as the species member of the Myzomyia series in Thailand[8,9]. Among these species, *An. minimus* and *An. aconitus* are considered as respective primary and secondary vectors of malaria in Thailand[9,18]. Based on morphological differences[19], metaphase karyotype distinction[1] and isozyme divergences[7] the primary vectors, *An. minimus*, exhibits a species complex comprising two sibling species, A and C. The former is found throughout the country, while, the latter is limited to Kanchanaburi Province .

An. (*Cellia*) *aconitus* is one of the most abundant anophelines distributed throughout Thailand[9,18]. It is of medical importance because it has been implicated as a vector of malaria in the central plain of the country[6,18]. It was also incriminated as a vector of malaria in other countries, i.e., Indonesia[11,12], Bangladesh[14] and Malaysia[15] .

Studies of egg morphology and topography in several anopheline species under scanning electron microscope (SEM) have been documented[2,5,10,13,16,17,20], because they provide better descriptions of fine structures than those accomplished by a conventional light microscope. Recently, three karyotypic forms of *An. aconitus* [Form A (X_1, X_2, Y_1), B (X_1, X_2, Y_2), and C (X_1, X_2, Y_3)] have been reported sympatrically from Maetang District, Chiang Mai Province, northern Thailand, whereas Form D (X_3, X_4, Y_4) has been incriminated from only Java, Indonesia[1]. The Y_1-chromosome is small submetacentric, the Y_2-chromosome is medium submetacentric having an extra block of heterochromatin added into each arm of the Y_1-chromosome, and the Y_3-chromosome is clearly large submetacentric arised from the Y_2-chromosome by addition of an extra block of heterochromatin on the long arm. The X_1-chromosome is metacentric, in which the X_2-chromosome is large submetacentric arised from the X_1-chromosome by addition of a major block of heterochromatin. In view of the obviously cytological distinction among the three karyotypic forms of *An. aconitus* in the sympatric population of northern Thailand, one might expect some degree of variation and/or difference in the egg surface topography, and a reason for never having descriptions of these eggs by SEM reported before now. Here, we present comparative morphometry and detailed descriptions by SEM of the eggs of three strains of *An. aconitus* Form B (Chiang Mai Province, northern Thailand and Phet Buri Province, southwest Thailand) and C (Chiang Mai Province, northern Thailand and Mae Hong Son Province, northwest Thailand).

MATERIALS AND METHODS

The endemic areas of malaria in Thailand comprises three Provinces, i.e., Chiang Mai (Ban Pang Mai Daeng, Maetang District), Mae Hong Son (Ban Huai Pong Kan, Muang District) and Phet Buri (Ban Tha Salao, Nong Ya Plong District). These were the sites for mosquito collection using both human-baited and buffalo-baited traps (Fig. 1). The investigations of F_1-and/or F_2-progenies of 3, 4 and 89 iso-female lines of *An. aconitus* collected from Mae Hong Son, Phet Buri and Chiang

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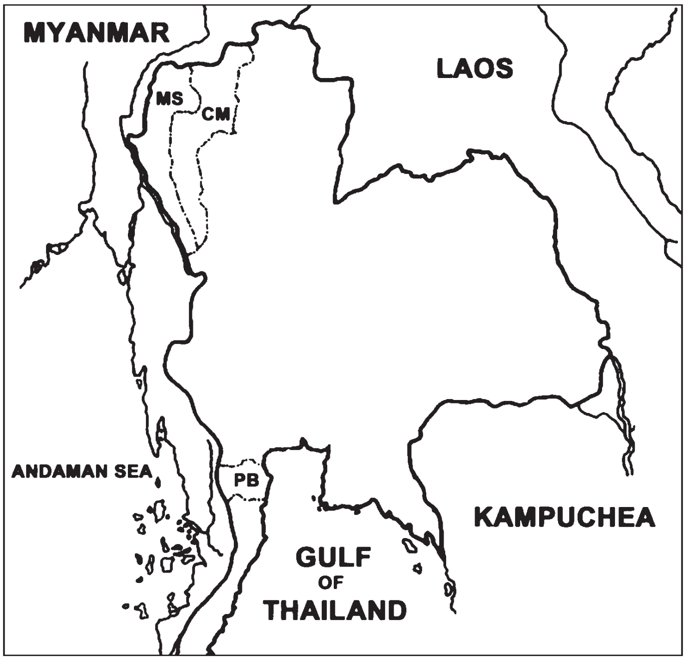


Fig. 1 - Map of Thailand showing Chiang Mai (CM), Mae Hong Son (MS) and Phet Buri (PB) Provinces, where mosquito collections were performed. Chiang Mai Province is situated on latitude 18 º 47' N and longitude 98 º 59' E in northern Thailand and is approximately 97 and 647 kilometers away from Mae Hong Son Province, northwest Thailand and Phet Buri Province, southwest Thailand, respectively.

Mai Province using the method to prepare metaphase chromosomes from newly-emerged adult females and males, as described by CHOOCHOTE *et al.*[3], revealed the two forms of metaphase karyotypes, i.e., Form B (X_1, X_2, Y_2), and C (X_1, X_2, Y_3) (Fig. 2). Form B was obtained in four and 48 iso-female lines from Phet Buri and Chiang Mai Province, respectively, and Form C was recovered in three and 41 iso-female lines from Mae Hong Son and Chiang Mai Province, respectively. Iso-female lines of the same karyotypic form and strain were pooled in order to establish the laboratory-colony strain. The four colonies (Form B: Chiang Mai and Phet Buri strains, Form C: Chiang Mai and Mae Hong Son strains) were successfully colonized for more than five consecutive generations in an insectarium (27 ± 2 ºC, 70-80% RH, 12 h illumination) using the method described by CHOOCHOTE *et al.*[4], and their eggs were used for the experiments. Embryonated eggs or 36-hour-old oviposited eggs of laboratory-raised *An. aconitus* Form B and C were placed in 2.5% glutaraldehyde in phosphate buffer (PB) pH 7.4 at 4º C, washed with PB (10 min, with two changes), and postfixed (1 h) in 1% osmium tetroxide at room temperature. The eggs 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 JOEL MED JSM 840-A SEM. The dimensions of the eggs and their surface features were given as a mean ± SD of 10 samples; one measurement from each egg.

RESULTS

Morphometric measurements and counts of float ribs and tubercles: Details of morphometric measurements and counts of float ribs and tubercles are shown in Table 1. Statistical analysis of egg dimensions at various sites, using the F-test for all tests and Kruskal-Wallis test for width including floats and number of posterior tubercles on deck, demonstrated that in most cases, i.e., entire length, width including floats, float length, number of float ribs and number of anterior tubercles, exhibited no significant differences ($p > 0.05$) among the three strains of *An. aconitus* Form B and C. Intraspecific variations with respect to the none correlation among the three strains of two karyotypic forms of *An. aconitus* were float width [36.77 ± 2.30 µm (Form C: Chiang Mai strain) = 38.49 ± 2.78 µm (Form B: Chiang Mai strain) = 39.06 ± 2.37 µm (Form B: Phet Buri strain) > 32.40 ± 3.52 µm (Form C: Mae Hong Son strain) (F = 11.73, $p < 0.05$)] and number of posterior tubercles on deck [2.40 ± 0.52 (Form B: Phet Buri strain) = 2.70 ± 0.82 (Form B: Chiang

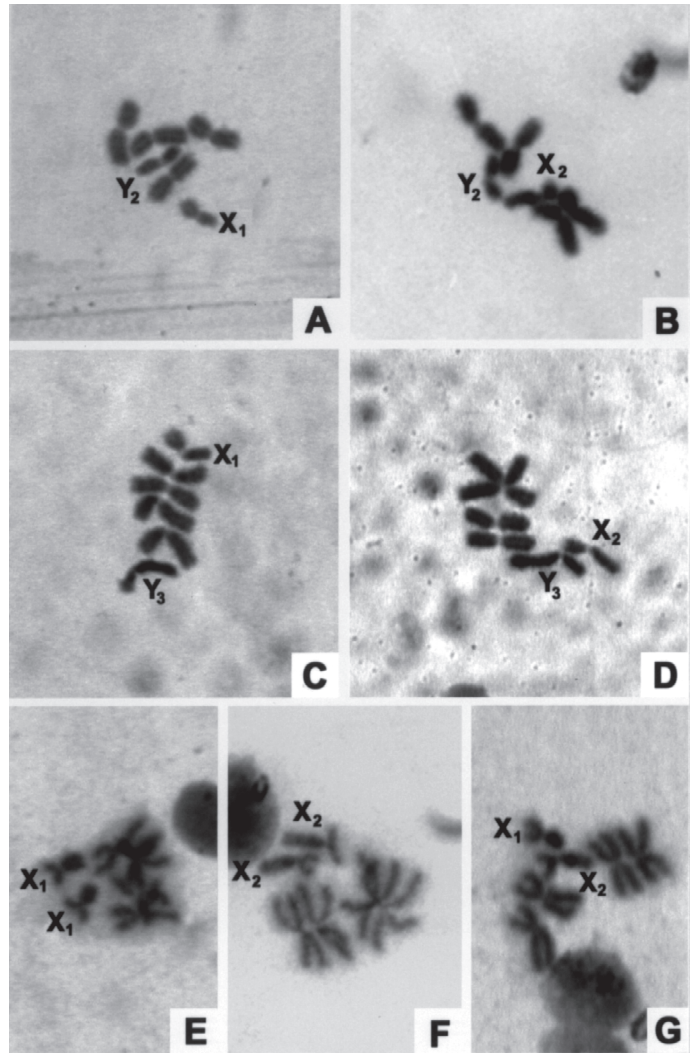


Fig. 2 - Metaphase karyotypes of *An. aconitus* Form B and C (Giemsa staining).Testis chromosomes; Form B: (A) Chiang Mai strain, showing X_1, Y_2-chromosomes; (B) Phet Buri strain, showing X_2, Y_2-chromosomes; Form C: (C) Chiang Mai strain, showing X_1, Y_3-chromosomes; (D) Mae Hong Son strain, showing X_2, Y_3-chromosomes. Ovary chromosomes: (E) showing homozygous X_1, X_1-chromosomes, (F) showing homozygous X_2, X_2-chromosomes, (G) showing heterozygous X_1, X_2-chromosomes. Note, all types of X-chromosomes were found in all forms and strains of *An. aconitus.*

Table 1
Morphometric measurements and counts of float ribs and tubercles of eggs of *An. aconitus* Form B (Chiang Mai and Phet Buri strains) and C (Chiang Mai and Mae Hong Son strains)

Experiments	Eggs of *An. aconitus* Form*			
	B		C	
	CM	PB	CM	MS
Measurements				
Entire length	361.99 ± 24.38 (334.80-404.20)	367.32 ± 19.22 (337.53-395.87)	359.61 ± 25.24 (329.19-400.03)	370.03 ± 21.66 (312.52-389.61)
Width including floats	141.10 ± 14.90 (126.09-179.18)	137.93 ± 16.58 (112.51-166.68)	142.51 ± 4.30 (137.51-150.01)	136.68 ± 5.57 (129.18-145.85)
Float length	309.05 ± 15.39 (280.45-331.28)	322.11 ± 9.63 (302.11-331.28)	316.48 ± 19.31 (279.19-337.53)	313.57 ± 18.77 (270.85-335.44)
Float width	38.49 ± 2.78 (34.78-43.75)	39.06 ± 2.37 (35.42-43.75)	36.77 ± 2.30 (33.34-39.59)	32.40 ± 3.52 (25.00-37.50)
Counts				
No. float ribs	16.90 ± 1.37 (15-19)	15.50 ± 1.51 (13-18)	16.40 ± 1.78 (13-19)	15.60 ± 1.26 (14-17)
No. anterior tubercles	2.40 ± 0.52 (2-3)	2.70 ± 0.48 (2-3)	3.00 ± 0.47 (2-4)	2.90 ± 0.32 (2-3)
No. posterior tubercles	2.70 ± 0.82 (2-4)	2.40 ± 0.52 (2-3)	3.10 ± 0.32 (3-4)	3.20 ± 0.42 (3-4)

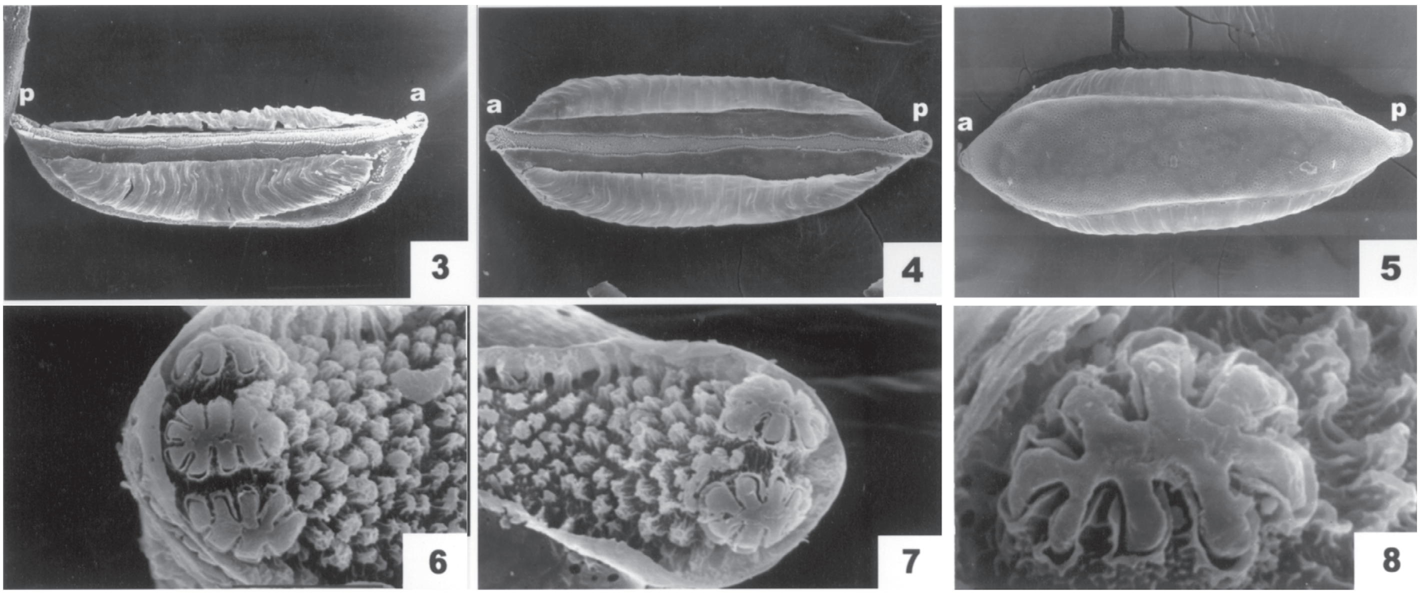
Mosquito strain; CM: Chiang Mai, MS: Mae Hong Son, PB: Phet buri; *Ten samples for each strain; Measurements in µm ± SD, range in parenthesis.

Mai strain) < 3.10 ± 0.32 (Form C: Chiang Mai strain) = 3.20 ± 0.42 (Form C: Mae Hong Son strain) (H = 11.43, $p < 0.05$)].

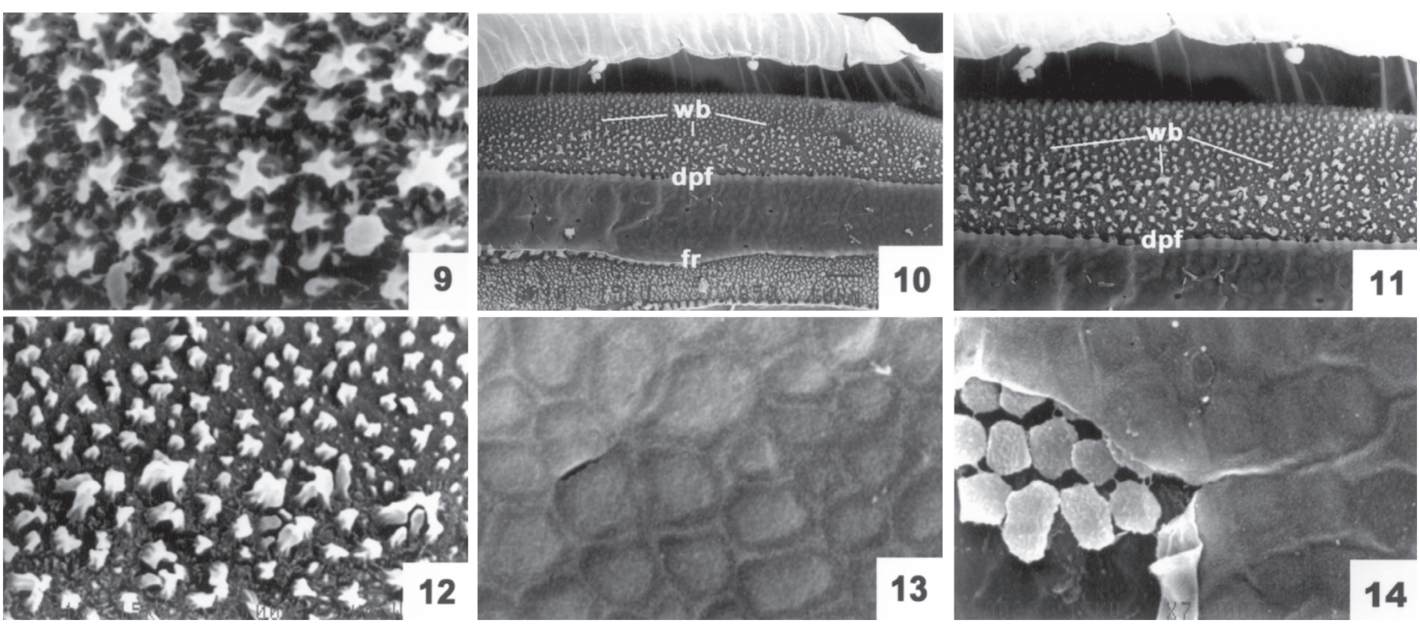
Eggs topography of three strains of *An. aconitus* Form B and C: The morphological feature and exochorionic sculpturing of the three egg strains of *An. aconitus* Form B (Chiang Mai and Phet Buri strains) and C (Chiang Mai and Mae Hong Son strains) were generally similar (Figs. 3-20), and no account of form specific characteristics that could be used to differentiate and/or characterize the forms under SEM. The eggs were boat-shaped, with a somewhat border anterior or head-end (Figs. 3-5). Viewed laterally, the contour of the entire egg was slightly concave on the morphologically dorsal surface and convex on the ventral surface. The middle region of each egg side was dominated by a float with approximately 16 (13-18) (Form B: Phet Buri strain), 17 (15-19) (Form B: Chiang Mai strain), 16 (13-19) (Form C: Chiang Mai strain) and 16 (14-17) ribs (Form C: Mae Hong Son strain). Viewed dorsally, there was a bare area, which was surrounded by the two longitudinal bands of a sclerotized ridge-like frill; this bare area is called the deck. The deck was continuous for the whole length of the egg and slightly constricted near the midline. Large-lobed tubercles that ranged from 2-4 in number were at each end of the egg on the ventral surface (Figs. 6, 7). Large-lobed tubercles on the anterior and posterior ends were rosette-shaped, giving rise to 7-9 lateral lobes, and surrounded by a sclerotized ridge and raised border (Fig. 8). The tubercles on either the deck (Fig. 9) or in areas covered by floats (observed from detached-float specimens) (Figs. 10-12) were irregularly jagged and surrounded by other much smaller, irregular tubercles. The cluster of tubercles adjacent to the detachment point of the float more or less formed a wavy border, and were apparently larger than other tubercles on the area covered by the float. The outer chorionic tubercles between the frill and detachment point of the float, were completely covered with a membrane-like sheet (Fig. 13). In a torn membrane-like sheet specimen, the tubercles were of an irregular base and a flattened-surface (Fig. 14). The inner surface of the frill was of a sclerotized, ridged-like texture and marked by picket like-ribs (Fig. 15); the outer surface was smooth with a parallel brick-like texture along its entire length (Fig. 16). At the anterior end, the micropylar orifice could be seen clearly. It was surrounded by a smooth collar that had an irregular outer margin and 4-6 spurs that extended radially toward the central orifice. One small central knob was seen clearly in unfertilized eggs (Fig. 17). Outer chorionic tubercles were present on the entire egg surface, except on the deck and the areas covered by floats. Tubercles, seen all over the eggs (lateral, dorsal, ventral surfaces), had an irregular base, with their surface partially covered with a membrane-like sheet at the anterior third and posterior third of the eggs (Fig. 18), and almost completely covered in the middle (Fig. 19). On observation of the torn membrane-like sheet specimens, the tubercles were seen clearly to have an irregular base and flattened-surface; these tubercles were arranged singularly (Fig. 20).

DISCUSSION

Biometry and 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 & BAIMAI[5] 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

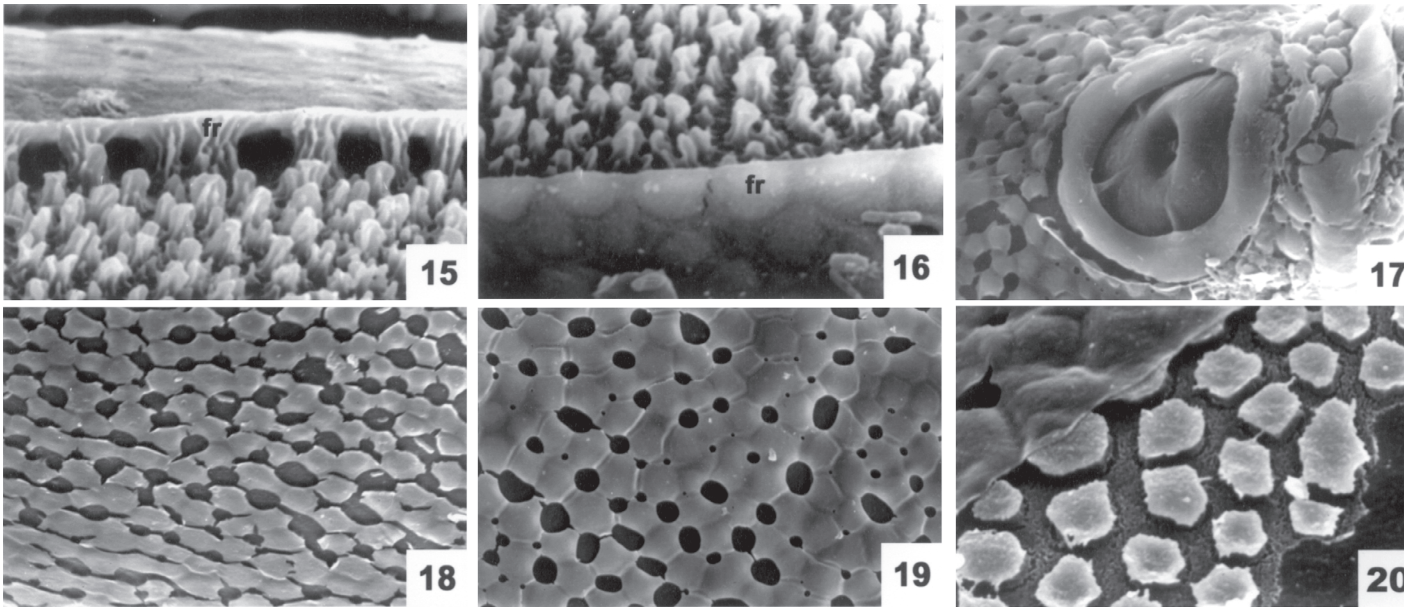


Figs. 3-8 - Whole eggs: (3) Lateral aspect, anterior end (a), posterior end (p)(x 270), (4) dorsal aspect, anterior end (a), posterior end (p)(x 270), (5) ventral aspect, anterior end (a), posterior end (p)(x 270). (6) Anterior end, showing irregularly jagged tubercles on the deck and three large, rosette-shaped tubercles (x 3,500). (7) Posterior end, showing irregularly jagged tubercles on the deck and two large, rosette-shape tubercles (x 3,500). (8) A higher magnification of the large, rosette-shaped tubercle, surrounded by a sclerotized ridge and raised border (x 10,000).



Figs. 9-14 - (9) A higher magnification of the irregularly jagged tubercles on the deck (x 12,000). (10) Irregularly jagged tubercles on the deck and area covered by the float, and outer chorionic tubercles covered with a membrane-like sheet between the frill (fr) and detachment point of the float (dpf). Note, that the cluster of tubercles adjacent to the detachment point of the float more or less form a wavy border (wb) (x 850). (11) A higher magnification of the irregularly jagged tubercles on the area covered by the float, and outer chorionic tubercles covered with a membrane-like sheet between the frill and detachment point of the float (x 1,500). (12) A higher magnification of the irregularly jagged tubercles on the area covered by the float (x 5,000). (13) A higher magnification of the outer chorionic tubercles covered with a membrane-like sheet between the frill and detachment point of the float (x 8,000). (14) Outer chorionic tubercles from the torn membrane-like sheet between the frill and detachment point of the float (x 540).

also distinct in different sibling species members. RODRIGUEZ *et al*.[16] 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.*[20] reported marked differences in shape and ornamentation of the eggs (deck, frill and micropylar) of two sibling species (A and C) in the *An.*



Figs. 15-20 - (15) The inner surface of the frill (fr), showing its sclerotized, ridge-like texture with picket-like ribs (x 8,000). (16) The outer surface of the frill (fr), showing its smooth surface and parallel brick-like texture along its entire length (x 8,000). (17) The anterior end, showing the micropylar orifice surrounded by a smooth collar with an irregular outer margin (x 3,700). (18) Outer chorionic tubercles at the anterior third of the egg, showing irregular bases partially covered with a membrane-like sheet (x 3,500). (19) Outer chorionic tubercles in the middle of the egg, showing irregular bases that were almost completely covered with a membrane-like sheet (x 3,500). (20) Outer chorionic tubercles from the torn membrane-like sheet in the middle of the egg, showing irregular bases and flattened-surface (x 7,000).

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 Phet Buri 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 Phet Buri 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[17] and *An. vagus* Form A and B[2]. 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[2,10,17,20], thus indicating the species-specific diagnostic characteristics.

RESUMO

Morfometria e morfologia comparadas de ovos de *Anopheles aconitus* formas B e C à microscopia eletrônica de varredura

Estudos comparativos morfométricos e morfológicos de ovos à microscopia eletrônica de varredura (SEM) foram efetuados nas três linhagens de duas formas cariotípicas de *Anopheles aconitus,* isto é, Forma B (linhagens Chiang Mai e Phet Buri) e Forma C (linhagens Chiang Mai e Mae Hong Son). Exame morfométrico revelou a variação intraespecífica com respeito à largura de superfície [36,77 ± 2,30 µm (Forma C: linhagem Chiang Mai) = 38,49 ± 2,78 µm (Forma B: linhagem Chiang Mai) = 39,06 ± 2,37 µm (Forma B: linhagem Phet Buri) > 32,40 ± 3,52 µm (Forma C: linhagem Mae Hong Son)] e número de tubérculos posteriores sobre a superfície livre [2,40 ± 0,52 (Forma B: linhagem Phet Buri) = 2,70 ± 0,82 (Forma B: linhagem Chiang Mai) < 3,10 ± 0,32 (Forma C: linhagem Chiang Mai) = 3,20 ± 0,42 (Forma C: linhagem Mae Hong Son)] embora a topografia de superfície dos ovos entre as três linhagens de duas formas cariotípicas tenham sido morfologicamente semelhantes.

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