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

Anuluck JUNKUM(1), Atchariya JITPAKDI(1), Narumon KOMALAMISRA(2), Narissara JARIYAPAN(1), Pradya SOMBOON(1), Paul A. BATES(3) & Wej CHOOCHOTE(1)

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)] 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. varana Iyengar have been reported as the species member of the Myzomyia series in Thailand. Among these species, An. minimus and An. aconitus are considered as respective primary and secondary vectors of malaria in Thailand. Based on morphological differences, metaphase karyotype distinction and isozyme divergences 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. It is of medical importance because it has been implicated as a vector of malaria in the central plain of the country. It was also incriminated as a vector of malaria in other countries, i.e., Indonesia, Bangladesh and Malaysia.

Studies of egg morphology and topography in several anopheline species under scanning electron microscope (SEM) have been documented, 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 (X1, X2, Y1), B (X1, X2, Y2), and C (X1, X2, Y3)] have been reported sympatrically from Maetang District, Chiang Mai Province, northern Thailand, whereas Form D (X2, X2, Y3) has been incriminated from only Java, Indonesia. The Y1-chromosome is small submetacentric, the Y2-chromosome is medium submetacentric having an extra block of heterochromatin added into each arm of the Y1-chromosome, and the Y3-chromosome is clearly large submetacentric arised from the X2-chromosome by addition of an extra block of heterochromatin on the long arm. The X1-chromosome is metacentric, in which the X2-chromosome is large submetacentric arised from the X2-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 Form 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 Hua Pong Kan, Muang District) and Phet Buri (Ban Tha Sala, Nong Ya Plong District). These were the sites for mosquito collection using both human-baited and buffalo-baited traps (Fig. 1). The investigations of F1- and/or F2-progenies of 3, 4 and 89 iso-female lines of An. aconitus collected from Mae Hong Son, Phet Buri and Chiang

(1) Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
(2) Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.
(3) Molecular and Biochemical Parasitology Group, Liverpool School of Tropical Medicine, University of Liverpool, Liverpool, United Kingdom.
Correspondence to: Wej Choochote, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. Fax: + 66-53-217144. E-mail: wchoocho@mail.med.cmu.ac.th
Mai Province using the method to prepare metaphase chromosomes from newly-emerged adult females and males, as described by CHOOCHOTE et al., revealed the two forms of metaphase karyotypes, i.e., Form B (X₁, X₂, Y₂), and C (X₁, X₂, Y₃) (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 °C, 70-80% RH, 12 h illumination) using the method described by CHOOCHOTE et al., 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 4°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*. Intraspécific 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 Mai strain)].
Counts

Measurements

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

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

Experiments

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)

<table>
<thead>
<tr>
<th></th>
<th>Experiments</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CM</td>
<td>PB</td>
</tr>
<tr>
<td>Measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire length</td>
<td></td>
<td>361.99 ± 24.38</td>
<td>367.32 ± 19.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(334.80-404.20)</td>
<td>(337.53-395.87)</td>
</tr>
<tr>
<td>Width including floats</td>
<td></td>
<td>141.10 ± 14.90</td>
<td>137.93 ± 16.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(126.09-179.18)</td>
<td>(112.51-166.68)</td>
</tr>
<tr>
<td>Float length</td>
<td></td>
<td>309.05 ± 15.39</td>
<td>322.11 ± 9.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(280.45-331.28)</td>
<td>(302.11-331.28)</td>
</tr>
<tr>
<td>Float width</td>
<td></td>
<td>38.49 ± 2.78</td>
<td>39.06 ± 2.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(34.78-43.75)</td>
<td>(35.42-43.75)</td>
</tr>
<tr>
<td>Counts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. float ribs</td>
<td></td>
<td>16.90 ± 1.37</td>
<td>15.50 ± 1.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15-19)</td>
<td>(13-18)</td>
</tr>
<tr>
<td>No. anterior tubercles</td>
<td></td>
<td>2.40 ± 0.52</td>
<td>2.70 ± 0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2-3)</td>
<td>(2-3)</td>
</tr>
<tr>
<td>No. posterior tubercles</td>
<td></td>
<td>2.70 ± 0.82</td>
<td>2.40 ± 0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2-4)</td>
<td>(2-3)</td>
</tr>
</tbody>
</table>

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\textsuperscript{c} 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*. 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*. reported marked differences in shape and ornamentation of the eggs (deck, frill and micropylar) of two sibling species (A and C) in the *An.*

Given the marked differences between the metaphase karyotypes of An. aconitus Form B (X₁, X₂, Y₂) and C (X₁, X₂, Y₃) 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 B17 and An. vagus Form A and B2. Additionally, the egg surface topography under SEM 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 B17 and An. vagus Form A and B2. 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 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 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 Mae Hong Son)].

ACKNOWLEDGEMENTS

The authors sincerely thank the Thailand Research Fund (TRF: BRG/14/2545) and the Thailand Research Fund through the Royal Golden Jubilee Ph.D Program (Grant No. PHD/0044/2546) for financially supporting this research project, Supot Wudhikarn, Dean of the Faculty of Medicine, Chiang Mai University, for his interest in this research, and the Faculty of Medicine Endowment Fund for Research Publication for its financial support in defraying publication costs.

REFERENCES


Received: 8 June 2004
Accepted: 13 September 2004