INTRASPECIFIC HYBRIDIZATION OF TWO KARYOTYPIC FORMS OF *ANOPHELES VAGUS* (DIPTERA: CULICIDAE) AND THE RELATED EGG SURFACE TOPOGRAPHY

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Abstract. Hybridization tests of the two karyotypic forms (Form A and B) of laboratory-raised, isolines of *Anopheles vagus*, were conducted by induced copulation. The results of reciprocal- and back-crosses indicated that they were genetically compatible, providing viable progeny. Comparative egg morphometry and morphology, aided by scanning electron microscopy (SEM), revealed that the eggs of the two karyotypic forms were morphometrically and morphologically similar.

INTRODUCTION

Anopheles (*Cellia*) *vagus* Donitz is a member of the Subpictus group belonging to the Pyretophorus series. It has been identified as an experimental and natural malaria vector in other countries: in Vietnam (Tran-Thi-Minh-Phuong *et al*, 1972) and Bangladesh (Maheswary *et al*, 1994). In addition, the Japanese encephalitis virus (JEV) has been isolated from this mosquito in Indonesia (Olson *et al*, 1985). In Thailand, *An. vagus* seems to be of little medical importance (Scanlon *et al*, 1968). However, an experiment of highly susceptible wild-caught *An. vagus* from Mae Sariang District, Mae Hong Son Province, northern Thailand to indigenous strains of *Plasmodium falciparum* and *P. vivax* was reported by Somboon *et al* (1994).

Little is known of the evolutionary genetics of *An. vagus.* Two karyotypic forms have been reported from three provinces in Thailand: Form A (X_1, X_2, Y_1: 3 wild-caught females from Nakhon Nayok Province, central Thailand) and Form B (X_1, X_2, Y_2: 2 wild-caught females from Chiang Mai Province, northern Thailand, 2 wild-caught females from Songkhla Province, southern Thailand) (Baimai *et al*, 1996). Given this karyotypic diversity, and the evidence of a possible cryptic species of *An. vagus*, the intraspecific hybridization of *An. vagus* Form A and B and the related egg morphometry and surface topography were taken as the subjects of thus study.

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MATERIALS AND METHODS

Isolines of *An. vagus* Form A and B

Two karyotypic isolined colonies of *An. vagus* were established based on metaphase karyotypes. The investigation of F_1-progenies of 6 and 8 isolines of *An. vagus* collected from San Sai and San Kamphaeng Districts, Chiang Mai Province, northern Thailand, using the techniques described by Choochote *et al* (2001), revealed the two forms of metaphase karyotypes: Form A (X_1, X_2, Y_1), of which the Y_1-chromosome is a normal submetacentric shape (Fig 1A) and Form B (X_1, X_2, Y_2), of which the Y_2-chromosome is a larger submetacentric chromo-some due to the presence of an extra block of heterochromatin in the short arm (Fig 1B) (Baimai *et al*, 1996). These forms have been found sympatrically, *ie* 1/6 and 2/8 of Form A/Form B were obtained from San Sai and San Kamphaeng districts respectively. In order to use the slightly allopatric *An. vagus*, therefore, the isoline of *An. vagus* Form A was established by using a single wild-caught female from San Sai district (plane area), whereas the Form B colony was established from a single wild-caught female from San Kamphaeng district (foothills), which lies some 60 km from San Sai.

Hybridization study

Intraspecific crossing experiments between isolines of *An. vagus* Form A and B were conducted by following the methods reported by Choochote *et al* (1998). Briefly, the reciprocal- and back-crosses were carried out using virgin females and males; the viability of these crosses (hatching rates, survival rates, pupation rates, emergence rates, adult sex-ratios) was compared with that of the parental crosses. F_2-progeny failure to survive was the criterion for reproductive isolation.

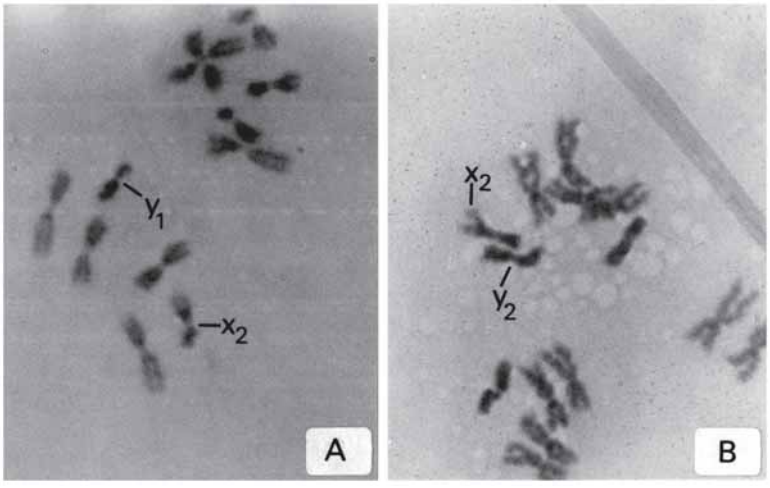

Fig 1- Metaphase karyotypes from male testes of *An. vagus* (Giemsa stained): [A] Form A, showing X_2 and Y_1-chromosomes; [B] Form B, showing X_2 and Y_2- chromosomes.

SEM study

Eggs were allowed to embryonate on distilled water for 48 hours, and then processed for scanning electron microscopy (SEM) as described by Iwaki and Choochote (1991). The dimensions of the eggs and their surface features were expressed as means ± SD of 10 samples (one measurement from each egg).

RESULTS

Hybridization study

Details of hatchability, pupation, and the emergence of parental, reciprocal-and back-crosses between *An.vagus* Form A and B are shown in Table 1. The hatchability, pupation, emergence, and adult sex-ratio of parental, reciprocal- and back-crosses revealed that all the crosses yielded viable progeny; no evidence of genetic incompatibility was found between *An. vagus* Form A and B. The hatchability, pupation, emergence rates, and ratio of adult female/male of the 1) parental, 2) reciprocal, and 3) back-crosses were 1) 91.74-93.83%, 93.12-100%, 100% and 0.98-1.17, 2) 88.18-97.01%, 95.00-100%, 96.36-98.97% and 0.72-0.79, and 3) 88.04-100%, 93.48-97.21%, 98.18-100% and 0.87-1.42.

SEM study

Comparative morphometry of 10 eggs of each of *An. vagus* Form A and B under SEM revealed similar egg dimensions [overall length 468.99 ± 35.17 μm (Form A), 466.22 ± 33.57 μm (Form B) (t=0.18, p>0.05); maximum width (including floats) 168.75 ± 11.12 μm (Form A), 173.24 ± 6.66 μm (Form B)(t=1.09, p>0.05)]; a similar number of float ribs: 27.30 ± 2.26 (Form A), 25.30 ± 2.16 (Form B)(t=2.02, p>0.05); a similar number of anterior tubercles: 6.30 ± 1.25 (Form A), 6.50 ± 1.18 (Form B)(t=0.37, p>0.05); and a similar number of posterior tubercles: 5.50 ± 0.71 (Form A), 6.10 ± 1.20 (Form B)(t=1.36, p>0.05).

The morphological features and external chorionic sculpture of the eggs of *An. vagus* Form A and B were generally similar (Figs 2A-L), and no distinct specific characteristics, which could be used to differentiate and/or characterize the forms under SEM. The eggs were boat-shaped, with a somewhat broader anterior or head-end (Figs 2A,B,C). Viewed laterally, the contour of the entire egg was slightly convex on the morphologically dorsal surface and concave on the ventral surface. The middle region of each side of the eggs was dominated by a float with either approximately 27 (24-32) ribs (Form A) or 25 (22-29) ribs (Form B). There was a bare area on the dorsal surface, 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 length of whole egg, albeit slightly constricted near the midline and, in most specimens, tapered slightly toward the extremities of the egg. At each end of the egg on the dorsal surface were large-lobed tubercles that ranged from 4-8 in number (Fig 2D). The tubercles that were either on the deck or in areas covered by floats (observed from detached-float specimens) were irregularly jagged and were surrounded by much smaller, irregular tubercles (Figs 2E,F). The large-lobed tubercles at the extremities were rosette-shaped, gave rise to 4-7 lateral lobes, and were surrounded by

Table 1
Cross-mating between isolines of *An. vagus* Form A and B.

Cross[a] Female x male	Total eggs (range)	Embryonation rate (No.)	No. hatched (%)	No. pupated (%)	No. emerged (%)	No. females and males from total emerged (%)		Sex ratio Female/male
						Female	Male	
Parental crosses								
A x A	206 (82-124)	100 (30/30)	189 (91.74)	176 (93.12)	176 (100)	87 (49.43)	89 (50.57)	0.98
B x B	162 (70-92)	96.67 (29/30)	152 (93.83)	152 (100)	152 (100)	82 (53.95)	70 (46.05)	1.17
Reciprocal crosses								
A x B	268 (132-136)	100 (30/30)	260 (97.01)	247 (95.00)	238 (96.36)	100 (42.02)	138 (57.98)	0.72
B x A	220 (104-116)	93.33 (28/30)	194 (88.18)	194 (100)	192 (98.97)	85 (44.27)	107 (55.73)	0.79
Back crosses								
A x (A x B)F_1	146 (68-78)	100 (30/30)	146 (100)	141 (96.58)	141 (100)	72 (51.06)	69 (48.94)	1.04
B x (B x A)F_1	234 (76-158)	100 (30/30)	227 (97.01)	220 (96.92)	216 (98.18)	102 (47.22)	114 (52.78)	0.89
(A x B)F_1 x B	209 (86-123)	96.67 (29/30)	184 (88.04)	172 (93.48)	172 (100)	101 (58.72)	71 (41.28)	1.42
(B x A)F_1 x A	231 (71-160)	96.67 (29/30)	215 (93.07)	209 (97.21)	209 (100)	97 (46.41)	112 (53.59)	0.87

[a] Two selective egg-batches of inseminated females for each cross.
A: *vagus* Form A; B: *vagus* Form B

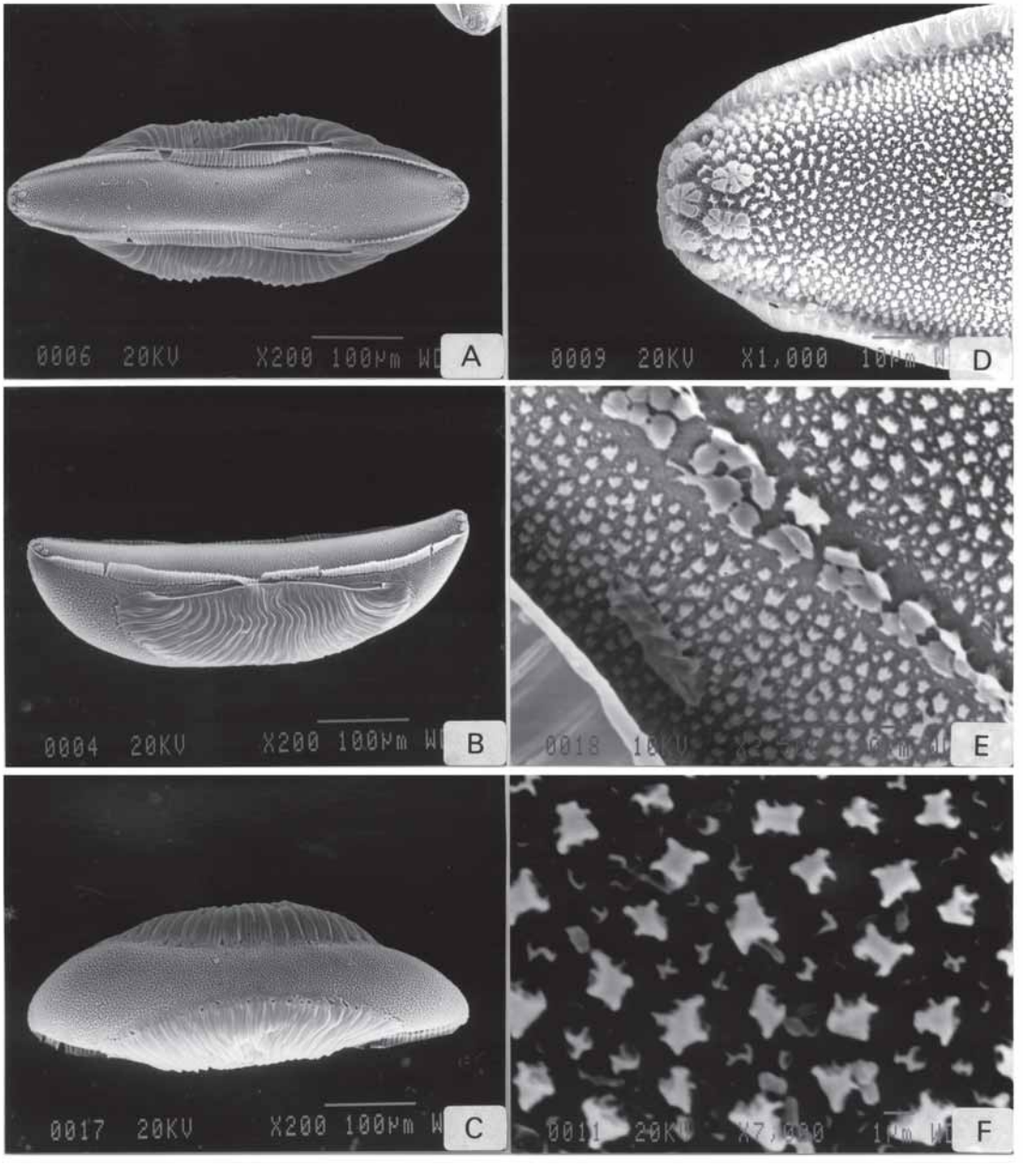

Fig 2- Egg surface topography of *An. vagus* Form A and B. Note that the egg surface topographic characteristics of the two forms were morphologically similar. Whole eggs: [A] dorsal aspect (x200); [B] lateral aspect (x200); [C] ventral aspect (x200); [D] Posterior end, showing irregularly jagged tubercles on the deck and large, rosette-shaped tubercles (x1,000). [E] Irregularly jagged tubercles on the deck and the areas covered by floats (x2,500); [F] A higher magnification of the irregularly jagged deck tubercles(x7,000).

a sclerotized ridge and a raised border (Fig 2G). The inner surface of the frill was of a sclerotized, ridged texture and was marked by picket like-ribs and a bumpy upper surface (Fig 2H); the outer surface was smooth, with a parallel groove-like texture along its entire length (Fig 2I). 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 7 spurs that extended radially inwards towards the central orifice. One small central knob was seen clearly in unfertilized eggs (Fig 2J). Outer chorionic tubercles were present on the entire egg surface, except on the

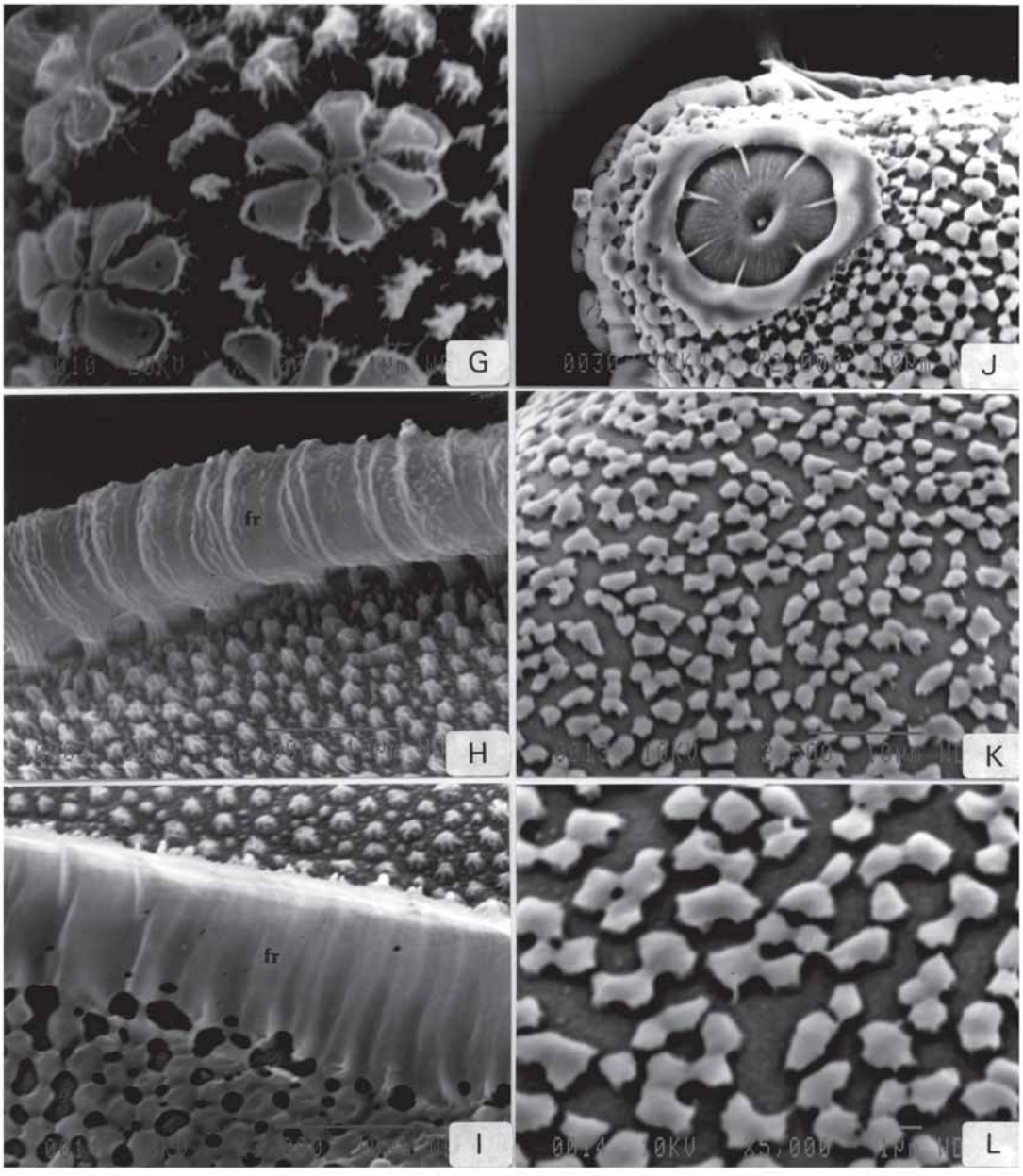

Fig 2 - [G] A higher magnification of the large, rosette-shaped tubercles, surrounded by sclerotized ridge and a raised border (x5,000); [H] The inner surface of the frill (fr), showing its sclerotized, ridge-like texture with picket-like ribs (x3,000); [I] The outer surface of the frill (fr), showing its smooth surface and parallel groove-like texture along its entire length (x3,000); [J] The anterior end, showing the micropylar orifice surrounded by a smooth collar with a irregular outer margin and 7 spurs extending radially inwards towards the orifice (x2,000); [K] Outer chorionic tubercles with their irregular bases and smooth surfaces; these came as either single tubercles or as clusters of two or more (x2,500); [L] A higher magnification of the outer chorionic tubercles (x5,000).

deck and the areas covered by floats. Tubercles, seen all over the eggs, were of an irregular base and a smooth surface; these tubercles were arranged singly or in clusters of two or more (Figs 2K,L).

DISCUSSION

Hybridization experiments and/the testing of reproductive isolation at the postmating barrier are still

efficient and reliable diagnostic tools for the differentiation of intra-taxons of anopheline species to a sibling species. Hybrid inviability, sterility, or breakdown are the criteria for genetic incompatibility; these criteria include insemination, embryonation, hatchability, larva survival, pupation, emergence, adult sex ratio, abnormal morphology, and reproductive system (Kanda *et al*, 1981). Nonetheless, the genetic compatible one does not entirely rule out its sibling species status, since the investigation of assortative mating or premating barrier (Paterson, 1980) by using pericentric and paracentric inversions of polytene chromosomes, biochemical and molecular genentics should be done intensively prior to the definite conclusion (Subbarao, 1998), whereas a genetic incompatible one can absolutely differentiate its sibling species status. The hybridization of *An. vagus* Forms A and B was conducted in order to determine whether the two karyotypic forms of *An. vagus* were in reproductive isolation. The results of reciprocal- and back-crosses between two laboratory-raised, isolined colonies of *An. vagus* Form A and B revealed that they were genetically compatible, indicating two cytologically polymorphic races of *An. vagus*. Similar results were found for two forms of the *An. maculatus* complex (Form B and E) (Chabpunnarat, 1988) and *An. sinensis* (Form A and B) (Choochote *et al*, 1998), in which there were karyotypic differences.

Biometry and surface topography studies of anopheline eggs can be used to assess specific morphological and ultrastructural differences; these techniques have been used efficiently to differentiate varieties or cryptic species of some anopheline species, *eg* the *An. dirus* complex (Damrongphol and Baimai, 1989), the *An. subpictus* complex (Reuben and Suguna, 1983; Suguna *et al*, 1994), and *An. albimanus* (Rodriguez *et al*, 1992). Given the marked differences between the metaphase karyotypes of *An. vagus* (Form A: X_1, X_2, Y_1; Form B: X_1, X_2, Y_2) in sympatric populations in Chiang Mai Province, northern Thailand, comparative egg morphometry and surface topography studies by SEM were carried out in order to establish the intraspecific differences and/variations between the two karyotypic forms. The results of this study indicated that the eggs of *An. vagus* Form A and B were morphometrically and morphologically identical. Similar results were found in sibling species members of the *An. oswaldoi* complex (Lounibos *et al*, 1997) and *An. sinensis* Form A and B (Rongsriyam *et al*, 1996).

Our findings of genetic compatibility and morphometrical and morphological egg similarity were not sufficients to determine whether *An. vagus* Form A and B enjoy the status of sibling species. Nevertheless, the present results call for further investigation of the two *An. vagus* forms and warrant a determintation of the premating barrier in the wild population.

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