

# INTRASPECIFIC HYBRIDIZATION OF *ANOPHELES MINIMUS* (DIPTERA: CULICIDAE) SPECIES A AND C IN THAILAND

Wej Choochote<sup>1</sup>, Yupha Rongsriyam<sup>2</sup>, Somjai Leemingsawat<sup>2</sup>, Atchariya Jitpakdi<sup>1</sup>, Narumon Komalamisra<sup>2</sup>, Kamhaeng Surathin<sup>2</sup>, Pradya Somboon<sup>1</sup>, Bin Chen<sup>3</sup>, Sirijit Wongkamchai<sup>4</sup>, Narissara Jariyapan<sup>1</sup>, Pongsri Tippawangkosol<sup>1</sup>, Benjawan Pitasawat<sup>1</sup> and Doungrat Riyong<sup>1</sup>

<sup>1</sup>Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai;

<sup>2</sup>Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok; <sup>3</sup>School of Biology, University of Leeds, United Kingdom; <sup>4</sup>Department of Parasitology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

**Abstract.** Hybridization tests of laboratory-raised, isolines of *Anopheles minimus*, species A and C were conducted by induced copulation. The three isolines were established based on three morphological variants of wild-caught, fully engorged females and two distinct types of metaphase chromosomes. They were *An. minimus* species A: V form ( $X_1, Y_1$ ), M form ( $X_2, Y_1$ ); species C: P form ( $X_3, Y_2$ ). The results of reciprocal and back crosses indicated that the two morphologically variant forms of species A were genetically compatible, providing viable progeny and completely synaptic salivary gland polytene chromosomes, whereas they were genetically incompatible with species C and/or the P form. Hybrid progeny was only obtained from both forms of species A females x species C males, but asynaptic salivary gland polytene chromosomes on 3L and partial development of ovarian follicles in females were seen. Back crosses of  $F_1$  hybrid males with parental species A females provided viable progeny, while back crosses of  $F_1$  hybrid females with parental species C males provided progeny of low viability and adult males with abnormal spermatozoa, suggesting the partial reproductive isolation of *An. minimus* species A and C.

## INTRODUCTION

The *Anopheles minimus* species group belongs to the subgenus *Cellia* and the *Myzomyia* series, at least six species of which are indigenous to Thailand, *ie aconitus* Donitz, *culicifacies* Giles, *jeyporiensis* James, *minimus* Theobald, *pampanai* Buttiker and Beales, and *varuna* Iyengar (Reid, 1968; Harrison, 1980). Among these species, *An. minimus* and *An. aconitus* are considered to be the primary and secondary vectors respectively of malaria in Thailand (Harrison, 1980; Scanlon *et al*, 1986). The primary vector has a species complex that comprises two sibling species, A and C. The former is found throughout the country, whereas the latter is confined to the province of Kanchanaburi (Sucharit *et al*, 1988a; Green *et al*, 1990; Baimai *et al*, 1996).

Sucharit *et al* (1988a) were the pioneers who discovered the species complex of *An. minimus* (species A and C); the species' differentiation is based on three major variants of wing morphology, *ie* typical *minimus* [M: wing with presector pale (PSP) on costa], *varuna* form [V: wing without prehumeral pale (PHP),

humeral pale (HP) and PSP on costa], *pampanai* form [P: wing with HP and PSP on costa], and isoenzyme studies. Additional work by Sucharit *et al* (1988b) revealed the reproductive isolation from the crossing studies between *An. minimus* species A (M form) and species C (P form). We report on the hybridization among three laboratory-raised, isolines of *An. minimus* species A (M and V forms) and species C (P form).

## MATERIALS AND METHODS

### Isolines of *An. minimus*

Three isolines of *An. minimus* were established based on the three morphological variants of wild-caught, fully engorged females, and two distinct types of metaphase karyotypes. For *An. minimus* species A, V and M forms, they were established by using a single wild-caught female collected from baited water buffalos in Ban Tha Lam Yai, Kanchanaburi Province, and Ban Pang Mai Daeng, in Chiang Mai Province, respectively; *An. minimus* species C (P form) was established by using a single wild-caught female collected from a baited water buffalo in Ban Phu Toei, Kanchanaburi Province. The identified gravid females of the two sibling species and/or three morphological variants were allowed to separately oviposit eggs. Subsequent  $F_1$  4<sup>th</sup> larvae, pupal skins, and adult females of each isolate were confirmed for species by using the standard key (Harrison, 1980). Metaphase

Correspondence: Wej Choochote, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.  
E-mail: wchoocho@mail.med.cmu.ac.th

Table 1  
Cross-mating among isolines of *An. minimus* species A and C.

Cross Female x Male	No. egg <sup>a</sup> batches (range)	Total eggs	Embryonation rate (No.)	No. hatched (%)	No. pupation (%)	No. emergence (%)	No. females and males from total emergence(%)	
							Female	Male
Parental crosses								
AV x AV	2 (61-97)	158	86.67 (26/30)	129 (81.65)	118 (91.47)	109 (92.37)	58 (53.21)	51 (46.79)
AM x AM	2 (49-116)	165	83.33 (25/30)	133 (80.61)	109 (81.95)	104 (95.41)	48 (46.15)	56 (53.85)
CP x CP	2 (110-124)	234	90 (27/30)	201 (85.89)	179 (89.05)	173 (96.65)	83 (47.98)	90 (52.02)
Reciprocal crosses								
AV x AM	2 (74-105)	179	90 (27/30)	158 (88.27)	145 (91.77)	142 (97.93)	79 (55.63)	63 (44.37)
AM x AV	2 (63-82)	145	76.67 (23/30)	108 (74.48)	99 (91.67)	97 (97.98)	56 (57.73)	41 (42.27)
AV x CP	2 (97-113)	210	90 (27/30)	189 (90)	168 (88.89)	159 (94.64)	78 (49.06)	81 (50.94)
CP x AV	2 (61-122)	183	57.14 (92/161)	-	-	-	-	-
AM x CP	2 (66-91)	157	93.33 (28/30)	131 (83.44)	123 (93.89)	119 (96.75)	58 (48.74)	61 (51.26)
CP x AM	2 (85-118)	203	21.17 (29/137)	-	-	-	-	-
Back crosses								
AV x (AV x AM) F1	2 (82-95)	177	80 (24/30)	136 (76.84)	121 (88.97)	118 (97.52)	56 (47.46)	62 (52.54)
AM x (AM x AV) F1	2 (91-94)	185	86.67 (26/30)	127 (68.65)	120 (94.49)	116 (96.67)	47 (40.52)	69 (59.48)
(AM x AV) F1 x AV	2 (56-79)	135	90 (27/30)	124 (91.85)	105 (84.68)	92 (87.62)	43 (46.74)	49 (53.26)
(AV x AM) F1 x AM	2 (78-86)	164	96.67 (29/30)	160 (97.56)	143 (89.38)	139 (97.20)	61 (43.88)	78 (56.12)
AV x (AV x CP) F1	2 (98-121)	219	63.37 (64/101)	89 (40.64)	76 (85.39)	71 (93.42)	38 (53.52)	33 (46.48)
AM x (AM x CP) F1	2 (69-85)	154	43.94 (58/132)	31 (20.13)	29 (93.55)	28 (96.55)	16 (57.14)	12 (42.86)
(AV x CP) F1 x CP	9 (16-44)	302	38.53 (42/109)	9 (2.98)	5 (55.56)	4 (80)	3 (75)	1 (25)
(AM x CP) F1 x CP	5 (11-53)	129	29.66 (35/118)	3 (2.33)	2 (66.67)	2 (100)	1 (50)	1 (50)

AV: *minimus* species A (V form; X<sub>1</sub>,Y<sub>1</sub>), AM: *minimus* species A (M form; X<sub>2</sub>,Y<sub>1</sub>), CP: *minimus* species C (P form; X<sub>3</sub>,Y<sub>2</sub>).

<sup>a</sup>Selective egg-batches from inseminated females.

chromosomes of each isolated family were prepared from newly emerged adult females and males using the method of Choochote *et al* (2001). Finally, the three isolines were set up with respect to the morphological variants and characteristics of metaphase karyotypes (Baimai *et al*, 1996). They were *An. minimus* species A; V form [male: X<sub>1</sub>,Y<sub>1</sub> (Fig 1A); female: homozygous X<sub>1</sub>,X<sub>1</sub> (Fig 1B)]; M form [male: X<sub>2</sub>,Y<sub>1</sub> (Fig 1C); female: homozygous X<sub>2</sub>,X<sub>2</sub> (Fig 1D)] and *An. minimus* species C; P form [male: X<sub>3</sub>,Y<sub>2</sub> (Fig 1E)]. The X<sub>1</sub> chromosome is submetacentric, consisting of short and long arms. The X<sub>2</sub> chromosome is also submetacentric, similar to the X<sub>1</sub> chromosome, but the long arm is relatively much longer due to the acquisition of a major block of heterochromatin in the distal region. The X<sub>3</sub> chromosome is large and has a submetacentric shape. The Y<sub>1</sub> chromosome is a normal submetacentric figure, whereas the Y<sub>2</sub> chromosome is a very large submetacentric shape.

#### Hybridization study

Intraspecific crossing experiments among three isolines of *An. minimus* complex were conducted using

the method of Choochote *et al* (1998). The reciprocal and back crosses were carried out by using virgin females and males, whose viability was compared with that of the parental crosses. The salivary gland polytene chromosome of the 4<sup>th</sup> larvae from the crosses were also investigated using the technique as described by Kanda (1979).

#### RESULTS

Details of embryonation, hatchability, pupation, and the emergence of parental, reciprocal and back crosses among three laboratory-raised, isolines of *An. minimus* species A and C are shown in Table 1.

The results of reciprocal and back crosses indicated that the two isolines of *An. minimus* species A, V form (X<sub>1</sub>,Y<sub>1</sub>) and M form (X<sub>2</sub>,Y<sub>1</sub>), were genetically compatible, producing viable heterozygous progeny (Fig 1F) and completely synaptic salivary gland polytene chromosomes (Fig 2A), whereas they were genetically incompatible with species C, P form (X<sub>3</sub>,Y<sub>2</sub>). From the crosses of P form (X<sub>3</sub>,Y<sub>2</sub>) females

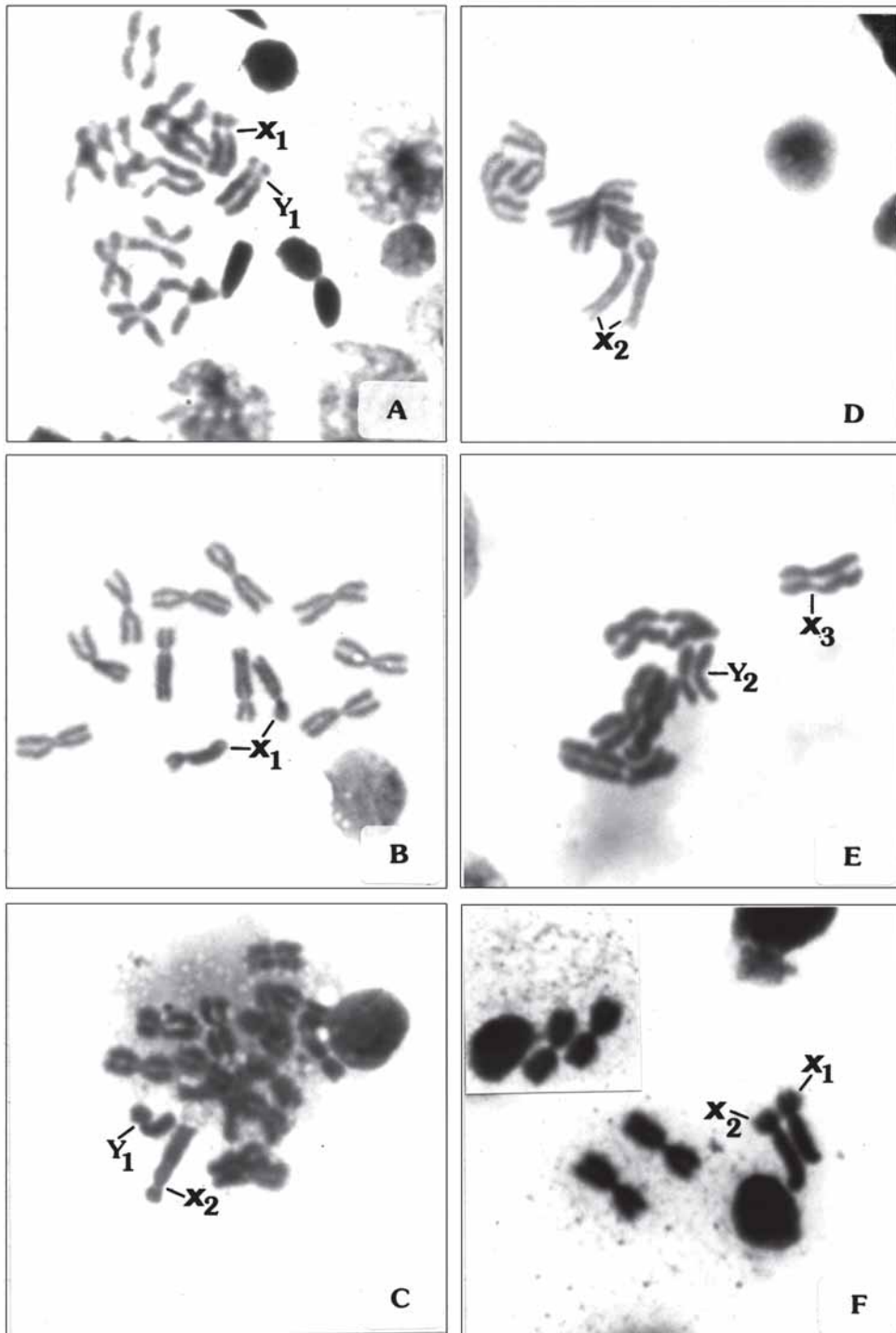


Fig1- Metaphase karyotype of *An. minimus* species complex (Giemsa staining). *An. minimus* species A; V form: [A] testis chromosomes, showing  $X_1, Y_1$ -chromosomes, [B] ovary chromosomes, showing homozygous  $X_1, X_1$ -chromosomes; M form: [C] testis chromosomes, showing  $X_2, Y_1$ -chromosomes, [D] ovary chromosomes, showing homozygous  $X_2, X_2$ -chromosomes. *An. minimus* species C; P form: [E] testis chromosomes, showing  $X_3, Y_2$ -chromosomes. [F] ovary chromosomes from  $F_1$  hybrid of V form female x M form male, showing heterozygous  $X_1, X_2$ -chromosomes.

with M form ( $X_2, Y_1$ ) and V form ( $X_1, Y_1$ ) males, embryonation rates were 21.17 and 57.14%, respectively; these rates were lower than those obtained in the control crosses (83.33-90%), and all the embryonated eggs failed to hatch. Hybrid progenies were only obtained from the crosses of P form ( $X_3, Y_2$ ) males with V form ( $X_1, Y_1$ ) and M form ( $X_2, Y_2$ ) females, but incomplete asynapsis of salivary gland polytene chromosomes on 3L (Fig 2B) and partial development of ovarian follicles in females (Fig 3A) were seen. Back crosses of  $F_1$  hybrid males with parental species A females, V form ( $X_1, Y_1$ ) and M form ( $X_2, Y_1$ ), yielded viable progenies, indicating the fertile

$F_1$  hybrid males. Back crosses of  $F_1$  hybrid females with parental species C males, P form ( $X_3, Y_2$ ), yielded low embryonation (29.66-38.53%) and hatchability (2.33-2.98%) rates, and adult males with abnormal sperms (Fig 3B), suggesting the infertility of  $F_1$  hybrid females.

## DISCUSSION

Hybridization experiments have been used widely as a tool to diagnose sibling species of some anopheline vectors. Several intra-taxa of anopheline species which were primarily detected by morphological, cytological, and biochemical differences and/or variations, led to the doubtful status of sibling species. Subsequently, it was confirmed by hybridization experiments, *eg An. barbirostris* complex (Choochote *et al*, 1983), *An. maculatus* complex (Takai *et al*, 1987) and *An. dirus* complex (Baimai *et al*, 1987; Sawadipanich *et al*, 1990). 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 mixed, natural population may be a mixture of two or three species (Subbarao, 1998).

The hybridization among three isolines of *An. minimus* species A and C that was established with respect to the three morphological variants (Sucharit *et al*, 1988a) and two characteristics of metaphase karyotypes (Baimai *et al*, 1996), *ie* species A: V form ( $X_1, Y_1$ ), M form ( $X_2, Y_1$ ); species C: P form ( $X_3, Y_2$ ), was done to determine whether these three isolines had reproductive isolation. The evidence of reproductive isolation from reciprocal crosses among species C females [P form ( $X_3, Y_2$ )] and species A males [V form ( $X_1, Y_1$ ) and M form ( $X_2, Y_1$ )] and partial reproductive isolation from reciprocal and back crosses among species A females [V form ( $X_1, Y_1$ ), M form ( $X_2, Y_1$ )] and species C males [P form ( $X_3, Y_2$ )] strongly indicated the sibling species status of *An. minimus* species C. The sequence for the D3 region of the 28S gene of ribosomal DNA (Sharpe *et al*, 1999) of the individuals V form ( $X_1, Y_1$ ) and P form ( $X_3, Y_2$ ) had sequences identical with those of *An. minimus* species A and C, respectively (unpublished data), confirming the morphological (Sucharit *et al*, 1988a) and cytological (Baimai *et al*, 1996) differentiations of the two sibling species. In addition, each of *An. minimus* from Ban Pang Mai Daeng, Chiang Mai Province, the same strain as M form ( $X_2, Y_1$ ) in our studies had a sequence identical to that of *An. minimus* species A (Somboon *et al*, 2001). However, Green *et al* (1990) reported that the morphological forms M and P, described by Sucharit *et al* (1988a), occurred both in species A and

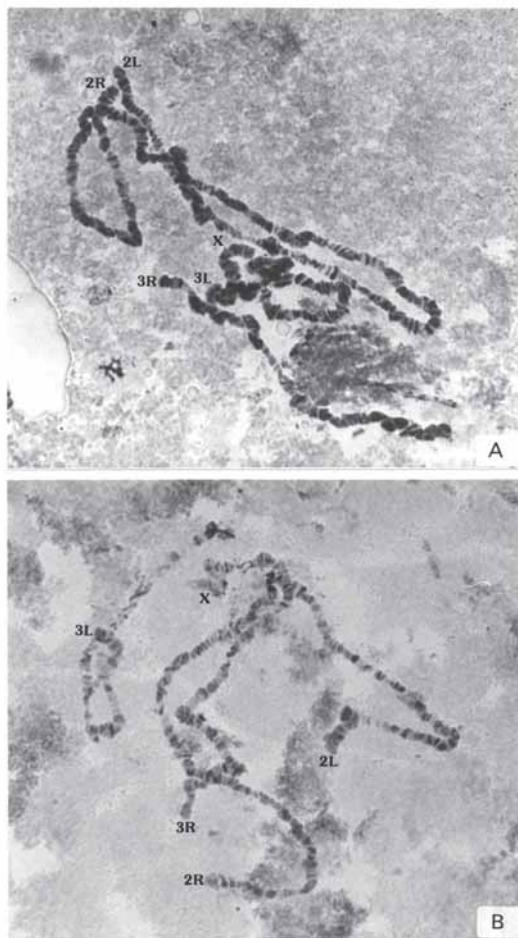


Fig 2- [A] Salivary gland polytene chromosome of  $F_1$  hybrid 4<sup>th</sup> larva of *An. minimus* species A: V form female ( $X_1, Y_1$ ) x M form male ( $X_2, Y_1$ ), showing complete synapsis in all arms. [B] Salivary gland polytene chromosome of  $F_1$  hybrid 4<sup>th</sup> larva of *An. minimus* species A: V form female ( $X_1, Y_1$ ) x *An. minimus* species C: P form male ( $X_3, Y_2$ ), showing incomplete asynaptic in chromosome arm 3L.



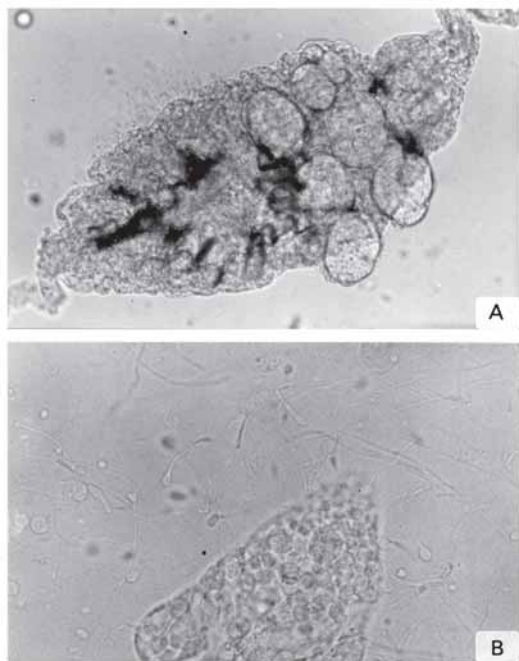


Fig 3- [A] An ovary of  $F_1$  hybrid of *An. minimus* species A: M form female ( $X_2, X_2$ ) x *An. minimus* species C: P form male ( $X_3, Y_2$ ), showing partial development of ovarian follicles. [B] A ruptured testis of back cross of  $F_1$  hybrid female [*An. minimus* species A: M form female ( $X_2, X_2$ ) x *An. minimus* species C: P form male ( $X_3, Y_2$ )] x *An. minimus* species C: P form male ( $X_3, Y_2$ ), showing abnormal spermatozoa with enlarged-head.

C in the samples from Ban Phu Rat, Kanchanaburi, according to studies of electrophoretic variations of six enzyme systems. They also mentioned that if the morphological characteristic, which distinguishes these two forms, is used for the identification there would be a 37% error.

Our study was concerned with only one wild-caught mosquito sample for each morphological variants related to the two types of metaphase karyotypes; this is the first report of the hybridization of *An. minimus* species A and C using two combinative characteristics.

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