CYTOGENETIC EVIDENCE FOR A FIFTH SPECIES WITHIN THE TAXON *ANOPHELES DIRUS* IN THAILAND[1]

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ABSTRACT. Crossbreeding and chromosomal evidence are presented for the existence of a fifth sibling species within the taxon of *Anopheles dirus* in Thailand. The new species is morphologically identifiable as *Anopheles balabacensis* "Fraser's Hill form." Structural differences in mitotic chromosomes and extensive asynapsis in hybrid polytene chromosomes indicate that significant genetic divergence exists between this species and its closest relatives, *An. dirus* species A, B, C and D and *An. balabacensis*.

INTRODUCTION

Anopheles dirus Peyton and Harrison is the predominant mainland taxon of the complicated *An. leucosphyrus* species group in Southeast Asia. This group includes several species that are consistent and dangerous vectors of human malarial parasites. *Anopheles leucosphyrus* Dönitz and *An. balabacensis* Baisas are important vector species in parts of Indonesia and Malaysia (Colless 1956, Harbach et al. 1987), while the primary mainland vector previously regarded as *An. balabacensis* (Eyles et al. 1964, Ismail et al. 1975, Wilkinson et al. 1978) is known to be *An. dirus* (Peyton and Harrison 1979, Rosenberg and Maheswary 1982).

Present knowledge of *An. dirus* shows that it consists of four genetically distinct species which have been informally designated as species A, B, C and D (Baimai et al. 1981, 1984, 1987; Wibowo et al. 1984, Hii 1985). The exact involvement of these species in malaria transmission is unknown and cannot be resolved until all members of the group have been identified. In this paper we present cytogenetic evidence for the existence of a fifth species within the group in Thailand. This species was collected in an area adjacent to the Thai-Malaysia border and is morphologically congruous with the Fraser's Hill form of *An. balabacensis* recognized by Colless (1956, 1957) for specimens from central West Malaysia.

MATERIALS AND METHODS

Specimens belonging to the *An. leucosphyrus* group were collected on human bait in a densely forested and hilly area adjoining the Thai-Malaysia border near Padang Besar in Songkla Province, Thailand. Mosquitoes were collected on three consecutive nights at two sites located about 5 km from one another. At the first site, two men collected on a platform built in a tree about 12 m above the ground while two men collected at ground level beneath the tree. At the second site, two men collected outside houses in a small village. Specimens were given bloodmeals, tentatively identified to species by morphology and transferred to Bangkok for egg laying. Larvae from each wild-caught female were examined cytologically for confirmation of the species (Baimai et al. 1984, 1988b). Salivary gland polytene chromosomes and mitotic karyotypes were prepared from fourth-instar larvae (Baimai et al. 1981). Isofemale lines of a cytotype previously unrecognized within the *An. leucosphyrus* group were maintained in the laboratory for further study. Isoline number PB136 was used to represent the new cytotype in reciprocal crossmatings with *An. dirus* species A, B, C and D from Thailand and *An. balabacensis* from East Malaysia. The other species were represented by the following isolines: *An. dirus* species A (KS14 from Chaiyaphum Province), species B (PT59 from Patthalung Province), species C (KA70 from Kanchanaburi Province), species D (PG30 from Phangnga Province) and *An. balabacensis* (SAB10 from Sabah). Crossmatings were performed using the force-mating method of Ow Yang et al. (1963). Three to five pair-matings were made in each direction of each cross. The fertility of F_1 hybrid progeny was determined by self-mating among themselves. Genetic incompatibility was inferred from the degree of asynapsis observed in the salivary gland polytene chromosomes of F_1 hybrid larvae. Testes of F_1 hybrid males were also examined.

RESULTS

Ninety-seven females belonging to the *An. leucosphyrus* group were collected at Padang Besar (Table 1). Seventy of these were identified as *An. leucosphyrus* species A (Baimai et al. 1988a), 17 isofemale lines conformed to the karyotype of *An. dirus* species B (Baimai et al.

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[1] The views of the authors do not purport to reflect the positions of the supporting agencies.

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1984) and 10 families possessed mitotic chromosomes that were different from any known species of the *An. leucosphyrus* group. Larvae and adults of the new cytotype were examined in detail and tentatively identified by one of us (REH) as *An. balabacensis* "Fraser's Hill form" of Colless (1956, 1957). This identification was later confirmed by E. L. Peyton, Walter Reed Biosystematics Unit, Smithsonian Institution, who is currently completing a comprehensive taxonomic revision of the *An. leucosphyrus* group in Southeast Asia. We agree with E. L. Peyton that the Fraser's Hill form is a member of the *An. dirus* species complex. The cytogenetic evidence described below shows that the new cytotype represents a distinct species within the group. The new cytotype is informally recognized here as *An. dirus* species F (Investigators in India plan to use the letter E for another species within the taxon).

Hybridization tests. The results of the crossmating experiments are summarized in Table 2. All combinations of crosses between *An. dirus* F and the other species produced F_1 hybrid progeny of both sexes. No eggs were obtained when the F_1 males and females resulting from each cross were mated among themselves. The testes of F_1 hybrid males were subsequently examined and found to be unusually small or absent and without sperm in all cases. This strongly indicates that the F_1 males were sterile and that genetic incompatibility exists between *An. dirus* F and the other species. These findings are further supported by the cytological investigations of F_1 larval salivary gland polytene chromosomes reported below.

Cytological observations. The mitotic karyotype of *An. dirus* F most closely resembles that of *An. dirus* species B. The prominent shared feature of these species is the acrocentric development of the sex chromosomes (Figs. 1, 2, 10). Two distinctive characters serve to distinguish the mitotic karyotype of *An. dirus* F from that of species B. First, the heterochromatic short arm of the X and Y chromosomes is relatively smaller in *An. dirus* F (Figs. 6, 7) and second, autosome III of *An. dirus* F is metacentric rather than submetacentric as in species B and the other members of the group. The difference in the shape of autosome III is due to the presence of a large block of centromeric heterochromatin which is unique to *An. dirus* F. The character of autosome III is clearly diagnostic for this species (Figs. 1–10). These cytological differences are readily recognized in the mitotic karyotypes of F_1 hybrid larvae (Figs. 3–9). The mitotic karyotypes of *An. dirus* F, *An. dirus* species A, B, C and D and *An. balabacensis* are compared diagrammatically in Fig. 10.

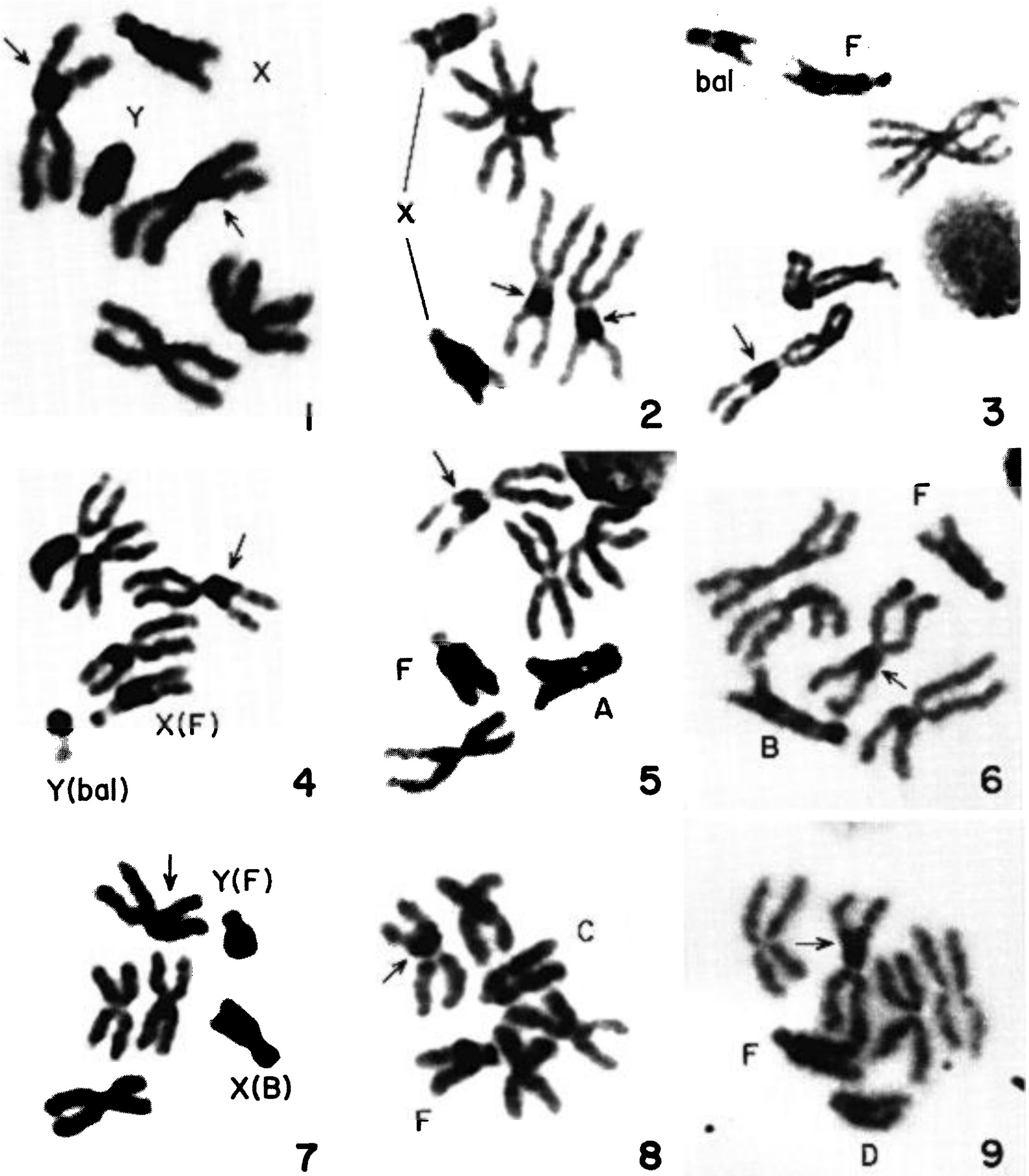
Considerable asynapsis was observed on all arms of the salivary gland polytene chromosomes of F_1 hybrid female larvae derived from all of the crosses (Table 2). The large degree of asynapsis is exemplified in the crosses between

Table 1. Numbers of females of the *Anopheles leucosphyrus* group captured on human bait at Village No. 5, Padang Besar, in December 1986.

	Forest		Village	
Species	Platform	Ground	Outside houses	Totals
An. leucosphyrus A	37	1	32	70
An. dirus B	7	1	9	17
An. dirus F	8	1	1	10
Totals	52	3	42	97

Table 2. Results of reciprocal crossmatings between *Anopheles dirus* F and five closely related species of the *An. leucosphyrus* group.

Cross		F_1 progeny	
Male	Female	Female polytene chromosomes	Male testes
balabacensis *dirus* F	*dirus* F *balabacensis*	almost complete asynapsis	atrophied, no sperm
dirus A *dirus* F	*dirus* F *dirus* A	~90% asynapsis	atrophied, no sperm
dirus B *dirus* F	*dirus* F *dirus* B	~90% asynapsis	atrophied, no sperm
dirus C *dirus* F	*dirus* F *dirus* C	almost complete asynapsis	atrophied, no sperm
dirus D *dirus* F	*dirus* F *dirus* D	>90% asynapsis	atrophied, no sperm



Figs. 1–9. Mitotic karyotypes from larval neuroblast cells: 1,2, male and female, respectively, of *An. dirus* F; 3, 4, F_1 female and male, respectively, from *dirus* F female × *balabacensis* male; 5, F_1 female from *dirus* F female × *dirus* A male; 6, 7, F_1 female and male, respectively, from *dirus* F male × *dirus* B female; 8, F_1 female from *dirus* F female × *dirus* C male; 9, F_1 female from *dirus* F female × *dirus* D male. Arrows indicate large blocks of constitutive heterochromatin in autosome III. The letters A, B, C, D, F and bal denote chromosomes contributed by *An. dirus* species A, B, C, D, and F and *An. balabacensis*, respectively.

An. dirus F × *An. dirus* B (Fig. 11) and *An. dirus* F × *An. dirus* C (Fig. 12). These results show that extensive genetic incompatibility exists between *An. dirus* F and its closely related species within the *An. leucosphyrus* group.

DISCUSSION

The crossing evidence and chromosomal observations presented here strongly support separate species recognition for *An. dirus* F. Based

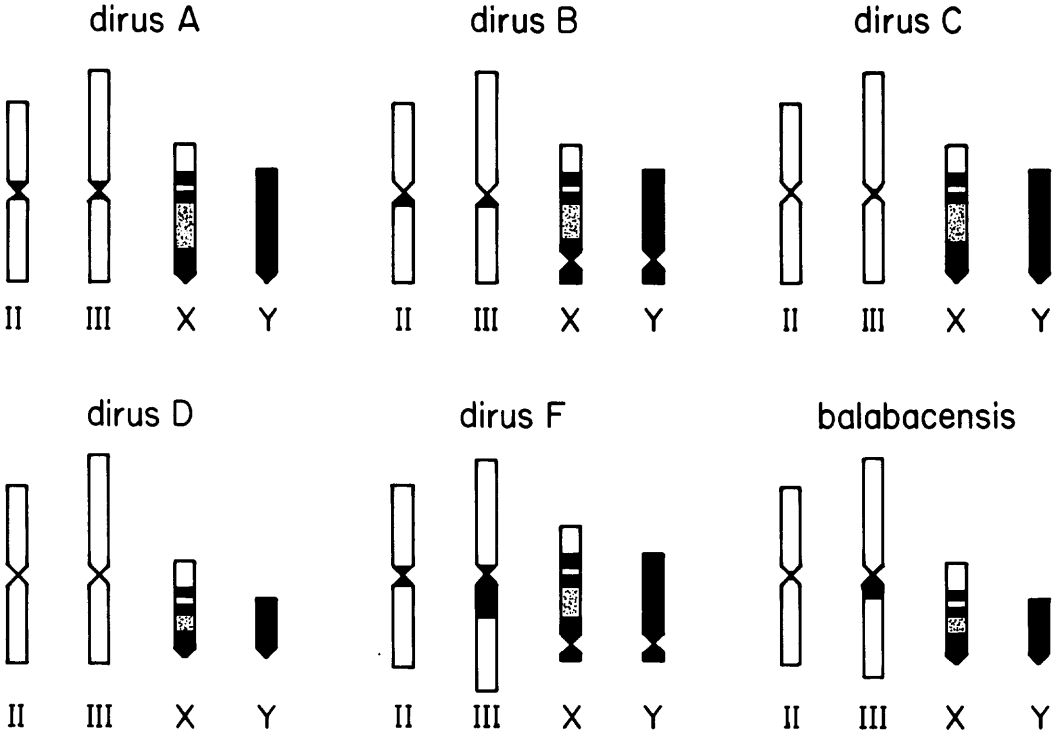


Fig. 10. Diagrammatic representation and comparison of mitotic karyotypes of *An. dirus* species A, B, C, D, and F and *An. balabacensis*. *Anopheles dirus* F is characterized by the short arm of the acrocentric sex chromosomes and the large block of centromeric heterochromatin (black) in autosome III which gives the chromosome a metacentric configuration. Autosome III has a submetacentric configuration in the other species.

on the form of the mitotic chromosomes, this species appears to have undergone an extensive acquisition of constitutive heterochromatin in autosome III and to a lesser extent in the sex chromosomes compared with the closely related *An. dirus* species B and the other species under consideration. *Anopheles dirus* F and species B could have arisen from a common ancestral stock which had relatively little centromeric heterochromatin in autosome III and the sex chromosomes. If this is the case, then speciation in the *An. leucosphyrus* group would appear to be marked by the acquisition of novel constitutive heterochromatin. The findings in this study provide additional support for the notion that the sex chromosomes of oriental species of *Anopheles* are prone to gain extra heterochromatin during the evolutionary process. This seems to be a general phenomenon in the karyotypic evolution of eukaryote organisms (John and Miklos 1979).

The recognition of *An. dirus* F as a separate species within the *An. leucosphyrus* group will be useful in determining the individual roles of the included species in malaria transmission. These species may have different ecological and behavioral characteristics that influence their epidemiological importance, vectorial capacity and susceptibility to control measures. Judging by the apparent rarity of *An. dirus* F (Colless 1956, 1957) and the limited information presented in Table 1, it appears that this mosquito feeds primarily above ground level, perhaps on monkeys or other small mammals in the forest canopy; however, its potential for involvement in human malaria transmission should not be discounted.

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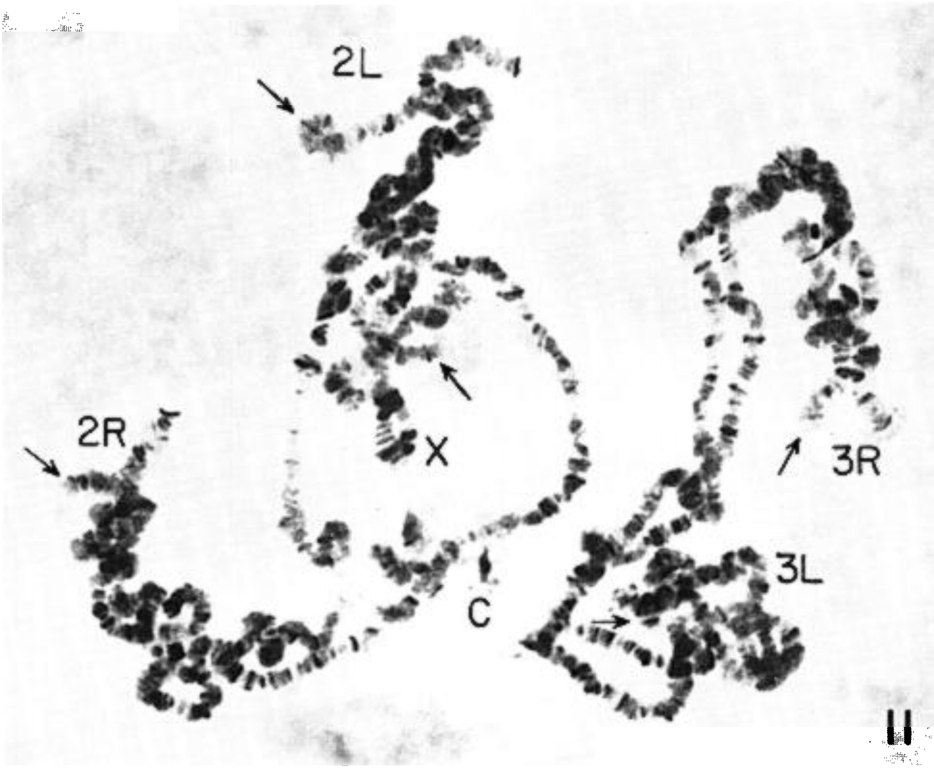

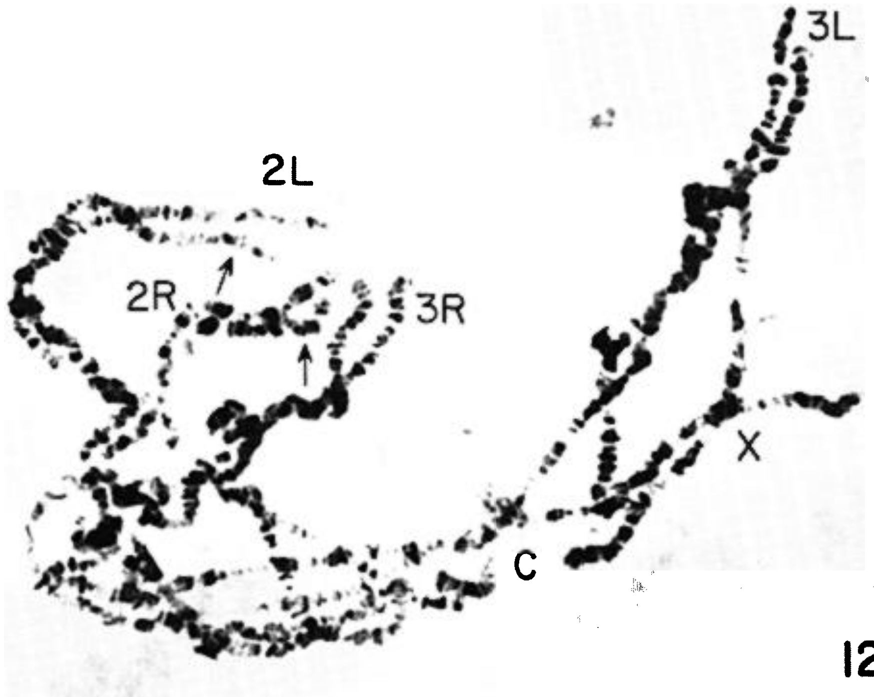

Figs. 11, 12. Salivary gland polytene chromosomes of F_1 hybrid female larvae from crossmatings: 11, *dirus* F male × *dirus* B female; 12, *dirus* F female × *dirus* C male. A large degree of asynapsis is evident. The chromosome arms of *An. dirus* F are indicated by arrows. The letter C indicates chromocenters.

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