ANOPHELES DIRUS SPECIES E: CHROMOSOMAL AND CROSSING EVIDENCE FOR ANOTHER MEMBER OF THE DIRUS COMPLEX[1]

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ABSTRACT. Cytogenetic and crossing data provide strong evidence for the existence of another species, *dirus* E in southwestern India, within the Dirus Complex of *Anopheles*. These findings are in accord with unpublished morphological observations. Our data suggest a significant genetic divergence between species E and its close relatives, *An. dirus* A, B and C in Thailand. These data also suggest that *dirus* E is an incipient sibling species of its geographically nearest relative, *dirus* D, and that it seemingly co-evolved through the process of allopatric speciation.

INTRODUCTION

The Dirus Complex of *Anopheles* subgenus *Cellia* consists of at least 7 morphologically similar species which occur from southwestern India across the Southeast Asian mainland to Con Son Island, Vietnam, Hainan Island, China and Taiwan (Peyton and Ramalingam 1988, Baimai 1988, Peyton 1990). These include 3 described species: *An. dirus* Peyton and Harrison, 1979 (species A), *An. nemophilous* Peyton and Ramalingam, 1988 (previously species F of Baimai et al. 1988a) and *An. takasagoensis* Morishita, 1946 (Peyton and Harrison 1980). Currently undescribed members of the complex are provisionally designated as species B, C, D (Baimai et al. 1987) and species E (Tewari et al. 1987). On August 24, 1981, a laboratory colony of the little-known *dirus* member from southwestern India was established by one of us (BAH) at the Department of Medical Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, from eggs provided upon request by Hari R. Bhat, National Institute of Virology, Pune, India. Preliminary cross-mating studies indicated that the southwest Indian *dirus* member was different from *An. balabacensis* Baisas, from Sabah, Malaysia (V. Baimai, unpublished data). For this reason, cytogenetic and crossing studies were performed to determine whether species E was really a member of the Dirus Complex. This paper presents the results of these investigations.

MATERIALS AND METHODS

Use of the designation "Dirus Complex" is taken from Peyton and Ramalingam (1988) and Peyton (1990). Although *An. dirus* is the correct name for what previously was designated species A, we have chosen to continue the use of "species A" for convenience in our crossing tables.

The original colony of *dirus* E was established from pooled egg-batches of 8 females collected in Shimoga District, Karnataka, southwestern India, during August 1981. This colony was maintained in the laboratory at 26°C by the artificial mating method (Ow Yang et al. 1963). All combinations of crosses (at least 10 pairs for each cross) between this colony and the laboratory stocks of 4 species of the Dirus Complex were performed by forced matings. The species strains used in this study were species A from Phet Buri (TL 33, 1983), species B from Phatthalung (PT 59, 1985), species C from Nakhon Si Thammarat (SC 28, 1984) and species D from Ranong (CP 25, 1983). These colonies were derived from isofemale lines and maintained in the insectary at Mahidol University.

Mitotic and salivary gland polytene chromosomes were prepared from fourth-instar larvae using the modified methods of Baimai et al. (1981). The male larva (XY), has a relatively thin polytene X chromosome, as compared with the normal thickness of a X chromosome of a female larva (XX). The fertility of F_1 hybrid progeny was determined by egg hatch success from the back- and self-crossing experiments, and from examination of the testes of F_1 hybrid males. Also, the degree of asynapsis in salivary gland polytene chromosomes of F_1 hybrids was taken to reflect the degree of genetic incompatibility.

RESULTS AND DISCUSSION

The mitotic karyotype of species E is very similar to that of *dirus* D (Baimai et al. 1987), particularly the sex chromosomes (cf. species A and B, Baimai et al. 1981). Giemsa staining revealed that the X chromosome of species E has a short telocentric shape with 2 separate dark bands of intercalary heterochromatin in the euchromatic section (Figs. 1–3). Only a small block of heterochromatin was observed at the centromeric region of the X chromosome of

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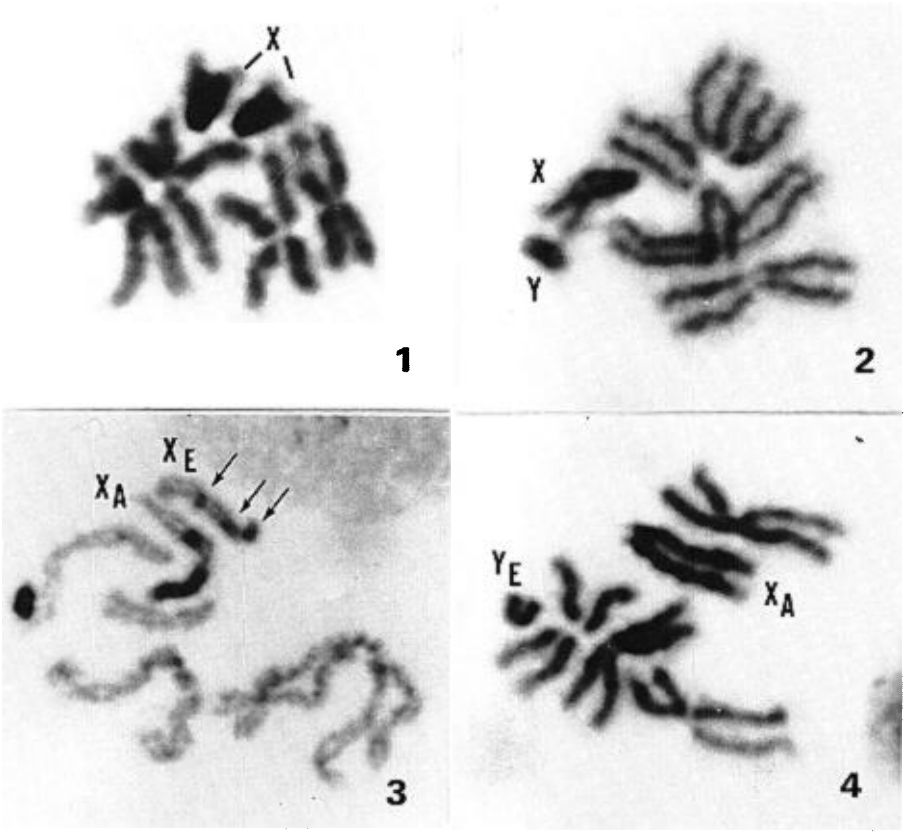


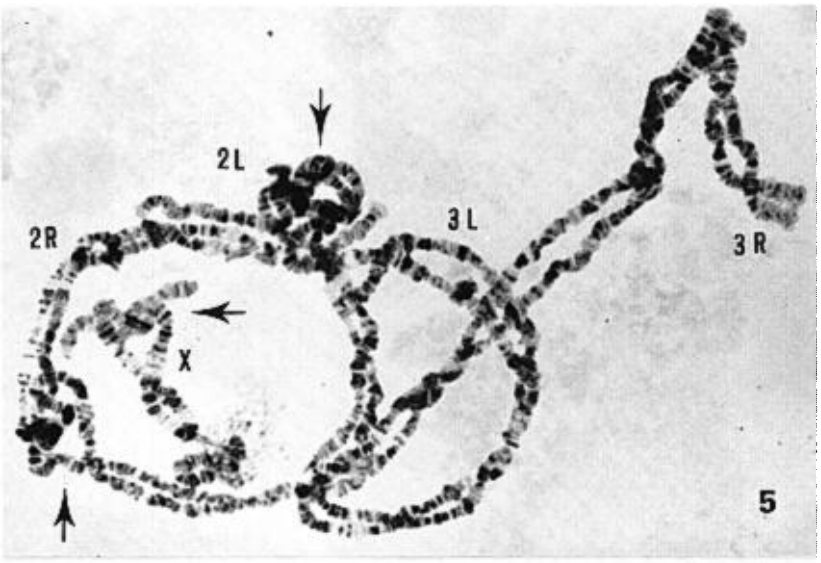
Fig. 1–4. Mitotic karyotype from larval neuroblast cells: 1, 2, female and male, respectively, of *Anopheles dirus* species E; 3, 4, F_1 hybrid female and male, respectively, from cross between *dirus* A female and *dirus* E male (× 670) (an arrow indicates a major block of heterochromatin).

species E, compared with that of species A (Fig. 3). The Y chromosome is small and rod-shaped and is mostly heterochromatin (Figs. 2, 4). This chromosome exhibits a large dotlike appearance in some preparations. Each of the autosomes have small blocks of pericentromeric heterochromatin similar to those of species A.

In the crossing studies, extensive asynapsis (over 90%) was observed in salivary gland polytene chromosomes of F_1 hybrid larvae derived from the crosses between species E and species A, B and C (Fig. 5) and heterozygous inversions were observed in the X chromosome and chromosome arms 2L and 2R in all cases (see Baimai et al. 1988b). However, F_1 hybrid larvae from the crosses between species E and D exhibited only small sections of asynapsis in the salivary gland polytene chromosomes, particularly at the very tips of chromosome arms 2L, 2R and 3R (Fig. 6), and nearly complete synapsis along the X chromosome except at the very tip. These observations suggest that a fixed simple inversion is likely to exist in the X chromosome of species E similar to that of species D. These data also suggest that species E possesses floating inversions in at least 2 autosome arms.

The fertility results of cross-mating experiments between species E and the 4 species of the complex from Thailand are summarized in Table 1. All combinations of crosses produced viable F_1 hybrids of both sexes. However, when F_1 hybrid progeny were backcrossed to the parental species only a very few progeny (larvae which subsequently died) were obtained in 3 crosses, and F_1 hybrids self-crossed failed to produce viable F_2 eggs (Table 2). The microscopic examination of testes of F_1 males from all of the crosses revealed that they were abnormal in size and shape (Figs. 7, 8) and contained either non-motile sperm or no sperm.

Crosses between species A and E produced a large number of eggs in both directions. The cross between females of species A and males of species E yielded relatively low percentages of hatched eggs (37.2%), but larval survival was comparable with that of the reciprocal cross



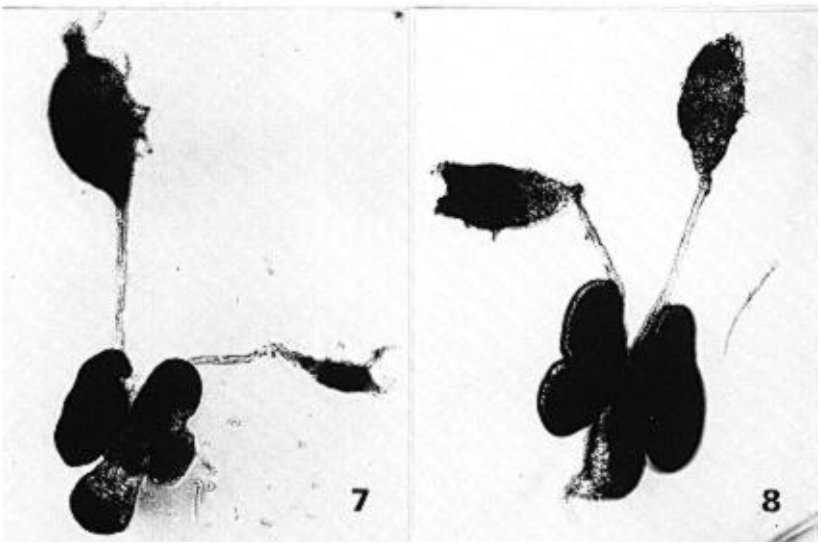


Fig. 5, 6. Larval polytene chromosomes from F_1 hybrid female larvae resulting from crosses between *Anopheles dirus* A female and *dirus* E male (× 260) and *dirus* D female and *dirus* E male (× 130), respectively (arrows indicate paracentric inversions). Figures 7, 8. Abnormal testes and accessory glands of F_1 hybrid males from the respective cross-matings in Figs. 5 and 6.

Table 1. Results of cross-mating of *Anopheles dirus* A, B, C and D from Thailand and species E from southwestern India.

Crosses female × male		No. of ovipositions hatched (total)	Mean no. of eggs per oviposition	% eggs hatched	% larval survival	% F_1 adults emerged	% F_1 males*
E	A	12 (15)	84.7	75.9 (772/1016)	73.8 (570)	89.1 (508)	48.6
A	E	10 (11)	91.2	37.2 (339/912)	65.8 (223)	53.3 (119)	58.8
E	B	4 (14)	71.3	2.5 (7/285)	71.4 (5)	60.0 (3)	33.3
B	E	10 (11)	81.9	71.3 (584/819)	22.1 (129)	70.1 (93)	52.7
E	C	13 (15)	96.6	56.8 (713/1256)	68.7 (490)	98.8 (484)	46.5
C	E	10 (11)	101.7	84.8 (862/1017)	83.8 (722)	93.9 (678)	53.7
E	D	4 (10)	81.0	51.5 (167/324)	49.1 (82)	81.7 (67)	56.7
D	E	5 (7)	103.6	76.1 (394/518)	43.2 (170)	94.7 (161)	57.8

* All F_1 male hybrids were sterile.

Table 2. Back- and self-crossing experiments with the F_1 hybrids from the crosses listed in Table 1.

Crosses female × male		No. of ovipositions (total pair matings)	% eggs hatched* (no.)
F_1 (EA)	E	0 (5)	—
F_1 (EA)	A	1 (5)	16.7 (10/60)
E	F_1 (EA)	0 (5)	—
A	F_1 (EA)	0 (5)	—
F_1 (EA)	F_1 (EA)	3 (5)	0 (0/228)
F_1 (AE)	E	0 (5)	—
F_1 (AE)	A	9 (11)	36.0 (180/500)
E	F_1 (AE)	0 (5)	—
A	F_1 (AE)	0 (5)	—
F_1 (AE)	F_1 (AE)	0 (10)	—
F_1 (BE)	E	0 (5)	—
F_1 (BE)	B	0 (5)	
E	F_1 (BE)	0 (5)	—
B	F_1 (BE)	0 (5)	—
F_1 (BE)	F_1 (BE)	0 (10)	—
F_1 (CE)	E	0 (5)	—
F_1 (CE)	C	0 (5)	—
E	F_1 (CE)	0 (5)	—
C	F_1 (CE)	0 (5)	—
F_1 (CE)	F_1 (CE)	0 (5)	—
F_1 (EC)	E	0 (5)	—
F_1 (EC)	C	3 (5)	7.5 (15/200)
E	F_1 (EC)	0 (5)	—
C	F_1 (EC)	0 (5)	—
F_1 (EC)	F_1 (EC)	3 (5)	0 (0/80)
F_1 (DE)	E	0 (5)	—
F_1 (DE)	D	0 (5)	—
E	F_1 (DE)	0 (5)	—
D	F_1 (DE)	0 (5)	—
F_1 (ED)	F_1 (DE)	0 (5)	—

* All larvae died before pupation.

(65.8 vs. 73.8%). The opposite result was obtained for the crosses between species C and E. Crosses between females of species E and males of species B were the least successful; only 2.5% of the eggs hatched and few larvae survived to become adults. The reciprocal cross gave a high percentage of hatched eggs (71.3%) but a low percentage of surviving larvae (22.1%). A greater degree of genetic compatibility was observed in the crosses between species D and E. Crosses in both directions produced fairly high percentages of hatched eggs (51.5 and 76.1%) and moderate survival (49.1 and 43.2%). These data suggest that there may be different degrees of genetic incompatibility at different stages of development of the F_1 hybrids derived from the crosses between species E and *dirus* A, B, C and D.

The above cytogenetic and crossing evidence clearly indicates that species E from southwestern India represents a distinct species, and is the seventh member recognized so far within the Dirus Complex. These findings are in complete accord with ongoing morphological studies (E. L. Peyton, unpublished data). Species E has been designated *dirus* E by Tewari et al. (1987), Peyton and Ramalingam (1988), Bhat (1988) and Peyton (1990). Our cytogenetic data suggest that *dirus* E is more closely related to *dirus* D than to the other members of the complex studied here. This is supported by data from recent studies of the geographic distribution of *dirus* D (Baimai et al. 1988c), apparently the predominant species throughout The Union of Myanmar [Burma] and Bangladesh. Populations of *dirus* E in southwestern India are the most western members of the Dirus Complex (see the comments of Bhat 1988, regarding a record of *balabacensis* from Kasauli in the western Himalayas), and are currently isolated from populations of *dirus* D. Thus, it seems probable that *dirus* E could have arisen from an ancestral stock of *dirus* D through the process of allopatric speciation. If this is the case, *dirus* E is probably the only representative of the Dirus Complex in southwestern India. The recognition of *dirus* E has proven to be of considerable importance in understanding the evolution of this medically important complex of Oriental *Anopheles*.

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