CYTOGENETIC OBSERVATIONS ON *ANOPHELES DIRUS* OF THE *LEUCOSPHYRUS* COMPLEX[1]

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ABSTRACT. Cytogenetic observations were carried out on *Anopheles dirus*. Analysis of meiotic and mitotic chromosomes showed 2n = 6. Meiotic and mitotic polyploid chromosomes were found in one individual. A polytene chromosome map has been prepared from the salivary gland cells. No naturally occurring aberrations were observed in the polytene chromosomes in the laboratory strain of *dirus*. External morphological features were used for identification.

The *leucosphyrus* complex which includes *balabacensis, dirus, leucosphyrus, hackeri, pujutensis* and *riparis* (Reid 1968, Peyton and Harrison 1979) contains 3 important human malaria vectors. Since *balabacensis* is morphologically a highly variable species and similar to *dirus*, genetic relationships among the members of the complex become of importance from the standpoint of speciation and evolutionary genetics. Cytogenetic observations, cross mating experiments and biochemical analyses were attempted. Cytogenetic observations on the laboratory colony of *dirus* in the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia are reported in the present paper.

MATERIAL AND METHODS

The laboratory colony of *An. dirus* used for these observations has been maintained for 170 to 175 generations in the insectary of the Division of Medical Entomology, IMR. The colony was begun with ♀s attracted to human bait in the field in Perlis State, Malaysia. Fourth instar larvae were used for the preparation of salivary gland polytene chromosomes and also for the preparation of somatic karyotypes of mitotic cells from brain tissue. Meiotic karyotypes from the reproductive organs of pupae were also observed. The rearing methods described by Kanda (1979) and the induced mating described by Baker et al. (1962) were adopted.

DESCRIPTION OF THE CHROMOSOMES

MITOTIC AND MEIOTIC CHROMOSOMES. The karyotype of *dirus* consists of 1 pair of submetacentric heterosomes and 2 pairs of submetacentric autosomes. One longer pair of autosomes is considered to correspond with salivary chromosome 2 and the shorter pair of autosomes corresponds with salivary chromosome 3. The shortest pair of chromosome is considered to be the heterosomes; the X chromosome arms are more or less subequal length of arms, and are not subtelocentric as in many other anophelines. Chromosome lengths are quite variable in

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cells from different tissues. The ratio of the lengths of chromosome X, 2 and 3 is about 0.6 : 1 : 08. The X chromosome in the male is slightly longer than the Y (Fig. 1 & 2). The degree of heteromorphosis is variable and seems to be related, partially at least, to the precocious contraction of the Y chromosome. The latter shows a marked heteropycnosis, and the long arm can be supposed to be completely heterochromatic. The same arm shows, in the X chromosome, two subequal regions differentially condensed: one is supposedly heterochromatic near the centromere, the other, euchromatic in the distal part (Fig. 1, 2 & 3). The zone of contact between heterochromatin and euchromatin sometimes appears uncoiled and unstained in mitotic metaphase preparations, resembling a primary constriction.

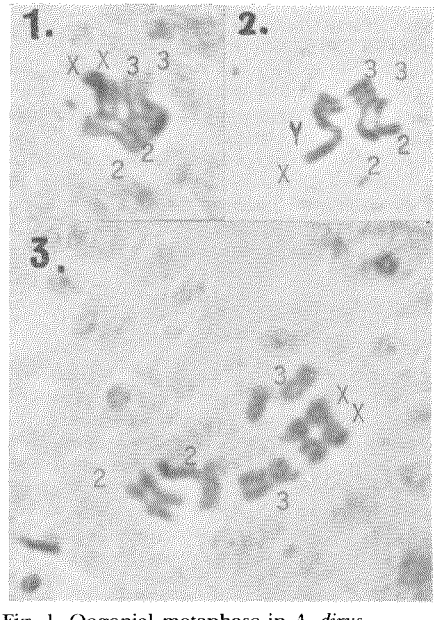


Fig. 1. Oogonial metaphase in *A. dirus*.
Fig. 2. Spermatogonial metaphase.
Fig. 3. Mitotic metaphase of brain cell.

Polyploidy was found in one individual as shown in Figs. 4 & 5. Nuclei from ovarian tissue show mitotic chromosome numbers of 2n = 36, and meiotic chromosomes show n = 18.

THE POLYTENE SALIVARY GLAND CHROMOSOMES. In salivary gland preparations the chromosomal complement consists of six arms weakly attached in the region of the centromere. The X chromosome is subtelocentric and consists of 1 long arm and 1 short arm in a ratio of 1 to 5. The length of the X(R + L) averages (x) 54.6 micra with a standard deviation (s) of 1.56 micra. The autosomal arms are designated 2R, 2L, 3R and 3L with the following average measurements: chromosome 2, right arm (2R) x = 236.2 micra, s = 11.95 micra; left arm (2L) x = 197.8 micra, s = 6.73 micra; chromosome 3, right arm (3R) x = 173.4 micra, s = 11.38 micra; left arm (3L) x = 153.8 micra, s = 11.19 micra. The ratios of respective arms against whole length are shown in Table 1. The average width of the synapsed chromosomal arms in the most developed nuclei, from which all the above measurements were taken, is 4.37 micra.

The X chromosome and the autosomal arms have been divided into zones and subdivisions by the same general numbering system as the one used for other species of the subgenus *Anopheles*. Thus, the long arm of the X chromosome (XR) contains zones 1 to 5 and the short arm (XL) only zone 6; chromosome 2 contains

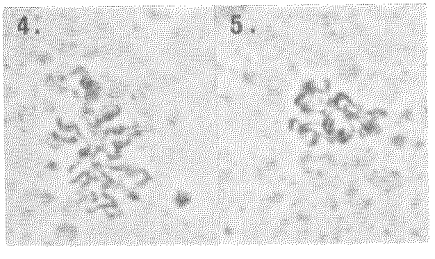


Fig. 4. Mitotic polyploid metaphase of brain tissue.
Fig. 5. Meiotic polyploid metaphse of oogonial cell.

Table 1. The length of the salivary gland polytene chromosomes of 25 individual cell sets.

Chromosome arm	Average length in micra	Standard deviation	Ratio against whole length %
X (R + L)	54.6	1.56	6.7
2R	236.2	11.95	29.0
2L	197.8	6.73	24.2
3R	173.4	11.38	21.3
3L	153.8	11.19	18.9

zones 7 to 19 in the right arm and 20 to 28 in the left arm; chromosome 3 contains 29 to 37 in the right arm and 38 to 46 in the left arm. The subdivisions of each zone into sections indicated by letters are arbitrary. A set of the salivary gland chromosomes is shown in Fig. 6 and a photo map and a drawn map are shown in Figs. 7 and 8.

X CHROMOSOME, RIGHT ARM (XR). The X chromosome may be immediately recognized by its length and by several distinctive recognition areas. Region 1A has a single dark band at the tip. This is followed by a light area with a group of 5 single light bands at 1B and 2 heavy bands at the beginning of 1C and 2

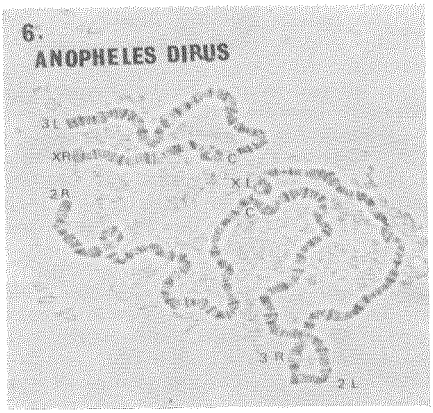
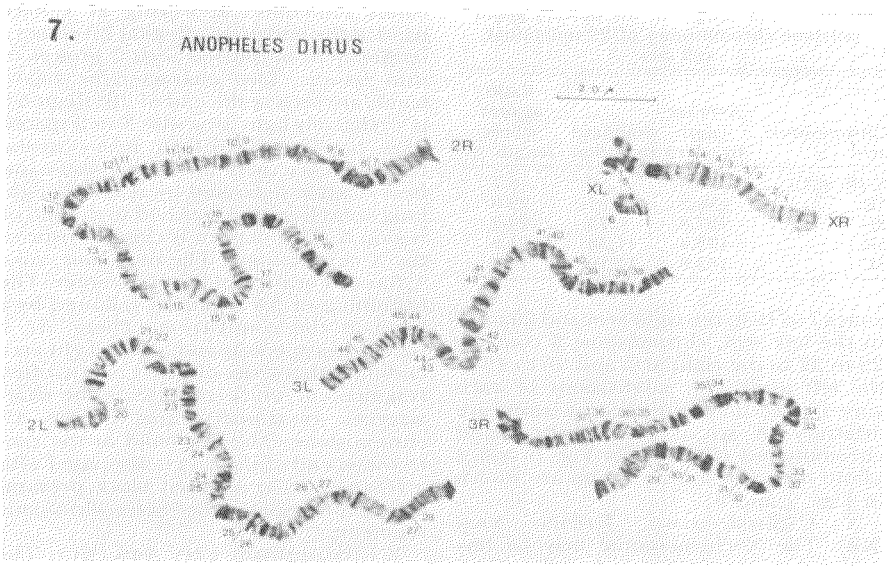


Fig. 6. Salivary gland polytene chromosome set.

bands sometimes appearing as 1 dark band at the end of 1C. Region 2A shows a tendency to be puffed with 2 groups of thin bands. Two characteristic heavy bands are seen at the end of 2B. Region 3 is a relatively light area with 3 well spaced dark bands and without heavy strong bands. Hence there are only 3 distinctive heavy bands separated by relatively light long areas from the free end to the end of region 3. This pattern is characteristic of the X chromosome. The next zone, zone 4, has two heavy bands in the center. The beginning of 5A is puffed followed by a band at the constriction. There are 2 paired heavy bands and a wide light area ending with a series of heavy bands, often fragmented, which represents the characteristic centromeric area of the XR chromosome. The last band at the centromeric end of the arm is associated with a fragmented condensed block presumably representing the heterochromatic segment.

X CHROMOSOME, LEFT ARM (XL). The shorter arm of the X chromosome consists mostly of lightly stained variable bands. The heavy dark band in 6A, two dark bands in the 1st half of 6B and the dark bands at the centromeric area in 6D are the most consistent features. This arm was often found in the shape of a loop, being attached at both ends to the centromeric region, as seen in *Anopheles gambiae* A and B. The distal end 6D showed a strong attraction for the centromeric end of chromosome 2L and in some preparations zone 6 may be absent.

CHROMOSOME 2, RIGHT ARM (2R). Easily recognizable as the longest arm in the complement, 2R also contains positive recognition areas at the free end, at several points in the middle of the arm and at the centromeric region. Region 7A contains 2 dark bands followed by 2 thin bands in 7B. A puff in 7C and D is bounded by strong dark bands at both ends with a lighter band in the center. Double heavy dark bands followed by a light area is usually consistent in the later half of 7D, bounded by double dark bands in the beginning end of 8A. A

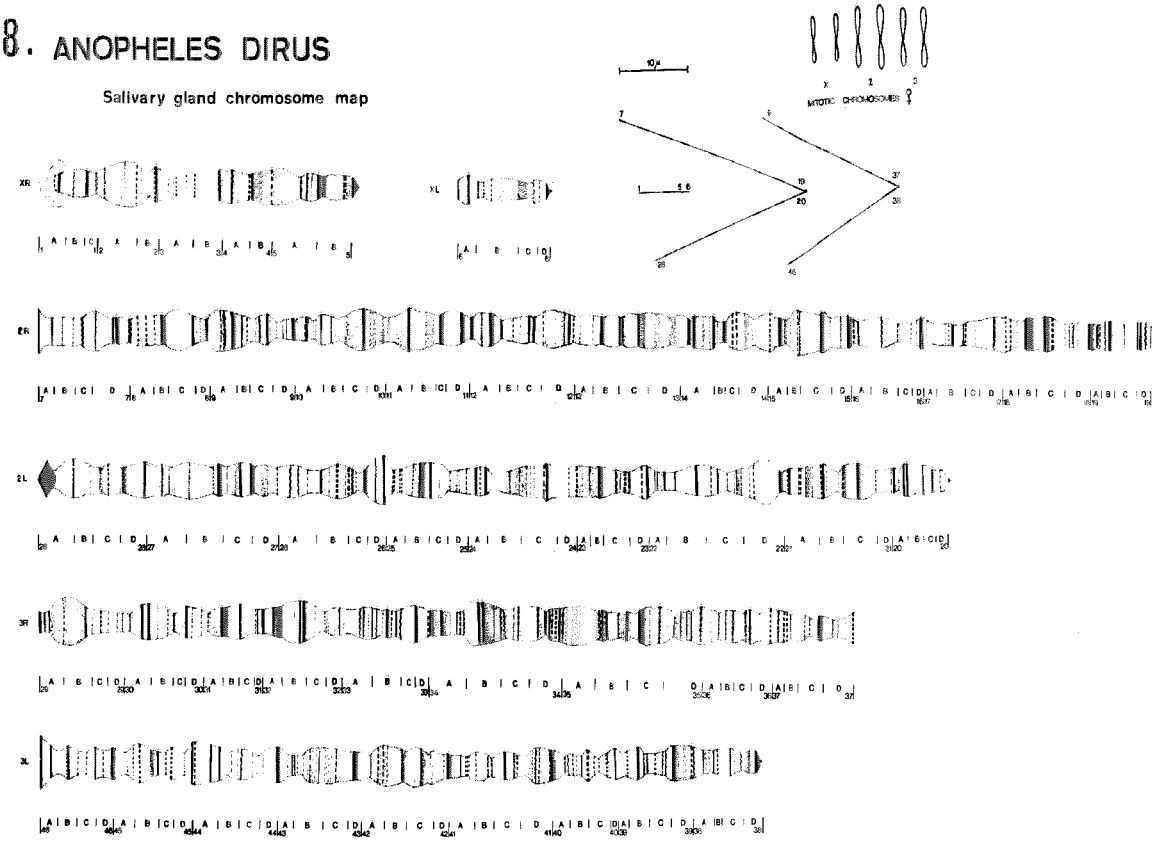


series of 3 dark bands in a darkly stained area preceded by a large light puff in 8C with a pair of heavy bands on either side is characteristic. The above areas are useful for identification of the distal end of 2R. This arm contains many light areas bounded by heavy dark bands. Region 9A is a puff and often appears as a weakly stained, stretched or twisted area. The band at the end of 9A is heavy. Region 9B has 2 diffused dark bands followed by a lighter region in 9C with a dark band at its end. The constricted necklike area of 9B is weakly stained. Region 10A commences with a double dark band, sometimes appearing as 1 thick dark band. Region 10A to 11C always has a series of dark bands. There are 5 dark bands separated by lightly stained areas of lighter bands. The light, bandless area in 11D is bounded at both ends by double heavy dark bands, then is followed by a small light area and another double heavy band. A dotted band and a double strong band in the puff of 12A, with a dark band which marks the beginning of 12B, is a characteristic area. A group of dark bands in the puff in 12D appears as a dark area bounded by a double dark band in 12C, and a heavy and dark band in 12D is consistent. The dark area in region 13 consists of several pairs of dark bands variably spaced, then followed by a puff, usually slightly extended, unmistakably marks the middle of the arm. The banding sequence in regions 14 through 17 can easily be followed by a series of double dark bands and light areas: 2 double heavy bands at the 1st half of 14A; double dark bands at the end of 14B; another series in the constricted area of 15A, 15C, 15D, 16D and 17B, while the light areas in 14D, 15C, 15D and 16A are easily recognized. A pair of dark triplets or quadruplets in 18B and 18C, two double bands in 19A, a single band in 19B, a long light area in 19C and D and double heavy dark bands followed by a double dark band in 19D mark the centromere end of the chromosome.

CHROMOSOME 2, LEFT ARM (2L): This arm can usually be identified by the dark

8. ANOPHELES DIRUS

Salivary gland chromosome map



stained free end. A very thick dark band is seen in 28A and the region that follows is almost always lightly stained and diffused in which three thin dark bands stand out. A dark band marks the end of region 28. Except for the double dark band at the end of 27B and the beginning 27D and a dark band at 27C, the whole region is almost always empty and diffuse. This pattern is easily recognized as marking the distal end of the arm. Region 26, 25 and 24 are also lightly stained. Two dark bands in 26A, a heavy band of the beginning of 26B followed by a single dark band, then a double lighter band in 26C are characteristic. However, the spindle shaped puff in 26D and 25A with a thin band then a dark band bounded by a dark band and a double light band are equally distinctive. There are also other recognizable bands: 2 dark bands followed by a dark band in 25B, a bandless area in which a thin band stands out in 25C; 3 dark bands in 24A sometimes appearing as 1 thick band; a lighter area, then a group of lightly stained bands in 24B and C; a light puff, usually stretched and bounded by a dotted band and a heavy band in 24C and a diffused band on the other side which marks the beginning of region 23A. Following this is a series of puffs, the light one in 22D sandwiched between a double light band and a double thick band. Another light puff in 21C is also bounded by a pair of long thick bands on either side serving as excellent landmark of the end of the chromosome. The distinctive areas and bands of regions 23 through 21 are as follows: a dark area in 23A and B; a double heavy band in 23B, two thick diffused bands in 23C; a thin dark band preceded by a thick band in 23D; the distinct dark band in 22B. The rest of the arm can easily be identified by 5 pairs of double dark bands in 21A; two in 21C and another in 20A. The 2 small light puffs with 2 dark bands in 20A followed by light area, and the 2 single dark bands in 20C mark the centromere end of arm 2L.

CHROMOSOME 3, RIGHT ARM (3R) The distinctive right arm of chromosome 3 contains many prominent areas. At the free end of the arm the tip contains a series of heavy dark bands, followed by a diffused puff in 29A and B with a lighter band in the center and a dark band at the constricted region. The puff with a pair of readily recognizable heavy bands in 30D, is separated by light areas that follow, one in 31B, another in 32A, from a dark band in the puff in 31B and C, and 2 diffused bands in 32D preceded by a heavy band in 32A. Also typical is a light area, sometimes puffed, with 2 heavy dark bands in 32B and C. Region 33 is always difficult to follow and the bands are variably spaced. However, the light bandless area followed by 2 dark, heavy bands in 33D mark the middle of the arm. They are followed by a group of light and heavy bands in 34A, these in turn are followed by a puffed light area and a double heavy band in the anterior half of region 34B. Region 34C and the 1st half of 34D consists of a group of undefined bands. A group of 6 heavy dark bands sometimes appear as a darkly stained area in the latter half of 34D; a stretched, lightly stained area in 35A and a group of 3 dark bands plus a single heavy band in 35B are consistent. The rest of the arm is lightly stained with indistinct series of bands except for a double heavy band in 35C and another pair of dark bands in 37C. These, together with an easily recognizable light puff in 35C and D, another in 37B and C provide recognition areas for the centromere end of the arm.

CHROMOSOME 3, LEFT ARM (3L). The distal three-fourths of 3L is lightly stained. At the free end, the 4 distinct dark bands in region 46 without any prominent puff, followed by a double heavy dark band at the end of 46D, are the best recognition areas. The puff in 45A and B with two broken bands of medium intensity provides positive recognition. There are 3 evenly spaced light bands in the latter half of region 45. This is followed by a puff in 44A and B. The rest of the arm except the proximal fourth is weakly and lightly stained. In the lightly stained areas, except for the

series of distinct double heavy bands, the rest of the bands are not easily distinguishable. Double dark bands in 43A, 43D, 42C, 42D and 41C stands out distinctly. The light area with indistinct bands in the whole of section 43, bounded by double heavy bands, is a characteristic feature.

The areas that follow are highly stained. The puff in 41D and 40A has 2 heavy dark bands, followed by a poorly stained area with a group of thin undefined bands; these in turn are followed by a series of single dark bands close together with a double dark band in 39D. A clear region with a dotted band in 39A and B bounded by a heavy dark band at both sides is consistent in this part of the arm. The light area in 38B and C sandwiched between double dark bands, followed by two bands in 38D are characteristic of the centromeric end of 3L.

DISCUSSION

This description of the polytene chromosomes was made of the species currently present in the insectary of the Division of Medical Entomology, IMR. Morphological studies were carried out to determine whether this strain is identical to *dirus, balabacensis* or any other. According to Peyton and Harrison (1979) the most significant morphological difference in the adult stage between *dirus* and *balabacensis* are the presector dark spot (Psd) on vein 1 and accessory sector pale spot (Asp) on the costa. Additional differences are found in setae 9-IV of the pupae. Identification of the specimens used for chromosomal observations were performed on 20 females, 20 males and 20 pupal skins. The results are shown in Table 2. Psd on vein 1 almost always extends basally beyond the corresponding dark spot on the costa. Asp spot on the costa was not found in 97.5% of females and in 62.5% of males; otherwise 2.5% of the females and 37.5% of the males had a few pale scales at that portion of Asp limited only to the inner half of the subcostan. This means that the Asp spots were incomplete ones. These characters of the adults are identical with *dirus*. The characters of sets 9-IV in pupae, however, were different, as shown in Table 2. In a comparison among *dirus, balabacensis* and the laboratory strain, the length of 9-IV in the present material gave data which overlap others (Table 2). The average length of the setae was 0.057 mm and standard deviation was 0.019 mm, ratios

Table 2. A comparison of the length of setae 9-IV and ratios of the 9th setae in the IIIrd, IVth and Vth abdominal segments of pupae among *dirus, balabacensis* and the laboratory strain.

	Length of setae 9 (in mm)		Ratios of 9-IV against that of III & V		
Specimens examined	III	IV		IV/III	IV/V
*dirus** 40		0.030–0.059 $\bar{x}$ = 0.043		1.50 –3.19 $\bar{x}$ = 2.14	0.28 –0.53 $\bar{x}$ = 0.41
*balabacensis** 28		0.056–0.089 $\bar{x}$ = 0.074		3.05 –5.54 $\bar{x}$ = 4.11	0.65 –1.05 $\bar{x}$ = 0.81
Present material 40	0.013–0.025 $\bar{x}$ = 0.018 s = 0.002	0.021–0.096 $\bar{x}$ = 0.057 s = 0.019	0.076–0.0110 $\bar{x}$ = 0.095 s = 0.007	1.30 –5.78 $\bar{x}$ = 3.179 s = 1.083	0.254–0.933 $\bar{x}$ = 0.599 s = 0.187
Values in t-distribution test					
dirus		3.21		4.18	4.41
balabacensis		3.90		3.74	4.92

degree of freedom (f) = 40 − 1, α = 0.05 $t_{0.05}$ (40 − 1) = 2.023.
* Peyton & Harrison, 1979, $\bar{x}$: average s: standard deviation.

of the length of the setae in IV and III (IV/III) were 1.30 – 5.78 ($\bar{x}$ = 3.179, s = 1.083) and the ones in IV/V were 0.254 – 0.933 ($\bar{x}$ = 0.599, s = 0.187). Those values are significant when compared to those of *dirus* and *balabacensis* in the t-distribution test, (degrees of freedom (f) = 40 – 1, α = 0.05 and $t_{0.05}$ (40 – 1) = 2.0). The pupal characters, therefore, suggest that the specimens observed should not be either *dirus* or *balabacensis*. Although genetic evaluations on these 3 entities should be carried out, the present paper tentatively treated the specimens as *dirus* due to the characters of the adult stage. The specific relationships of those 3 populations will be published in separate papers.

The preparation of the polytene chromosomes in the present paper were according to the methods described by Kanda (1979). The lengths of polytene chromosomes were quite variable as shown in Table 1. We recommend that average lengths and relative lengths of each arm be compared to total length from mathematically reliable chromosomes from individual karyotypes.

With respect to chromosome morphology, zone 6(XL) of the present strain generally appears similar to that of the *A. gambiae* complex described by Coluzzi and Sabatini (1967). The arm sometimes makes a loop being attached by both ends to the centromere region; sometimes 6A is attached to the centromeric end of chromosome 2L and sometimes may be mistaken as the basal part of that arm. Among the 350 slides of the preparations of salivary gland chromosomes almost all preparations were homologous in their whole chromosome complements without any chromosomal aberration or asynapsis. In this respect the materials can be considered to be cytologically homologous and a standardizable strain in spite of having a number of morphological variations within the strain.

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