CYTOGENETIC EVIDENCE FOR TWO SPECIES WITHIN THE CURRENT CONCEPT OF THE MALARIA VECTOR *ANOPHELES LEUCOSPHYRUS* IN SOUTHEAST ASIA[1]

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ABSTRACT. Karyotypes and crossing relationships were investigated for three allopatric populations of *Anopheles leucosphyrus* in Southeast Asia: South Kalimantan, Sumatra and Thailand. The mitotic karyotypes of these populations were similar to those previously observed in other species of the *An. leucosphyrus* group. Populations from Thailand and South Kalimantan exhibited telocentric and subtelocentric sex chromosomes, respectively, with a distinctive band of intercalary heterochromatin in the X chromosome. Strikingly different submetacentric X and Y chromosomes were observed in the population from Sumatra, and it seems likely that the evolution of these chromosomes occurred through the acquisition of constitutive heterochromatin. Sterile F_1 males were observed in crosses between the Sumatra population and the populations from South Kalimantan and Thailand. No genetic incompatibility was observed in crosses between the latter two populations. We believe that the present concept of *An. leucosphyrus* includes two allopatric species, one inhabiting Borneo, West Malaysia and southern Thailand and one confined to Sumatra.

INTRODUCTION

Anopheles leucosphyrus Dönitz is the nominotypical member of the widely distributed *An. leucosphyrus* species group. This group contains several important vectors of human malaria in the forested areas of Southeast Asia. *Anopheles leucosphyrus* occurs in Sumatra, Kalimantan, Sarawak, Sabah, West Malaysia and southern Thailand. There is no published record of the true *An. leucosphyrus* (current concept) in Thailand, but the presence of this species in the southern part of the country has been known for some time from collections made by the senior author and previous investigators (E. L. Peyton and Bruce A. Harrison) of the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. At AFRIMS, collections containing *An. leucosphyrus* date back to 1965.

Anopheles leucosphyrus is a vector of human malaria in Sumatra and Sarawak (Colless 1956), and probably also Kalimantan (Harbach et al. 1987). It is a vector of non-human primate malaria in West Malaysia (Wharton et al. 1962). This species is known to attack man in both West Malaysia and southern Thailand, but has not been incriminated as a vector of human malaria in these areas.

The taxonomic history of the *An. leucosphyrus* group reveals a proliferation of species and subspecies concepts within what was originally regarded as a single species. Ten species and five subspecies are currently recognized within the group (Table 1) (Colless 1957; Reid 1968; Peyton and Harrison 1979, 1980). Among these species is *Anopheles dirus* Peyton and Harrison which recent cytogenetic study has shown to be a complex of at least four distinct species on the Southeast Asian mainland (Baimai et al. 1987). This paper, the result of ongoing cytogenetic studies being conducted in conjunction with the development and testing of DNA probes for distinguishing members of the *An. leucosphyrus* group in Thailand, presents evidence for the existence of at least two species within the prevailing concept of *An. leucosphyrus*.

MATERIALS AND METHODS

Specimens of *An. leucosphyrus* were collected on human bait from three geographically isolated populations: (1) Bukit Baru (near Muarabungo), Bungo Tebo Regency, Jambi

Table 1. Species and subspecies formally recognized within the *Anopheles leucosphyrus* group.

Taxon	Year described
An. leucosphyrus Dönitz	1901
An. elegans (James)	1903
An. hackeri Edwards	1921
An. balabacensis balabacensis Baisas	1936
An. balabacensis baisasi Colless	1957
An. balabacensis introlatus Colless	1957
An. cristatus King and Baisas	1936
An. riparis riparis King and Baisas	1936
An. riparis macarthuri Colless	1956
An. takasagoensis Morishita	1946
An. pujutensis Colless	1948
An. sulawesi Koesoemawinangoen	1954
An. dirus Peyton and Harrison	1979

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

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Province, Sumatra; (2) Salaman (near Kintap), Tanah Laut Regency, South Kalimantan; and (3) Padang Besar, Songkla Province, Thailand (Fig. 1, Table 2). Female mosquitoes were allowed to engorge at the time of capture, maintained in a cool, humid environment and carried to Bangkok for egg laying and colonization (Harbach et al. 1987). For some unknown reason the survival rate of captured females is much lower for this species than for either *Anopheles balabacensis* Baisas or members of the *An. dirus* complex. Only a few isofemale lines were successfully obtained as a result of higher than expected mortality among the wild-caught females (Table 2).

Chromosome preparations were obtained from fourth-instar larvae derived from eggs laid by wild-caught females. Mitotic brain chromo-

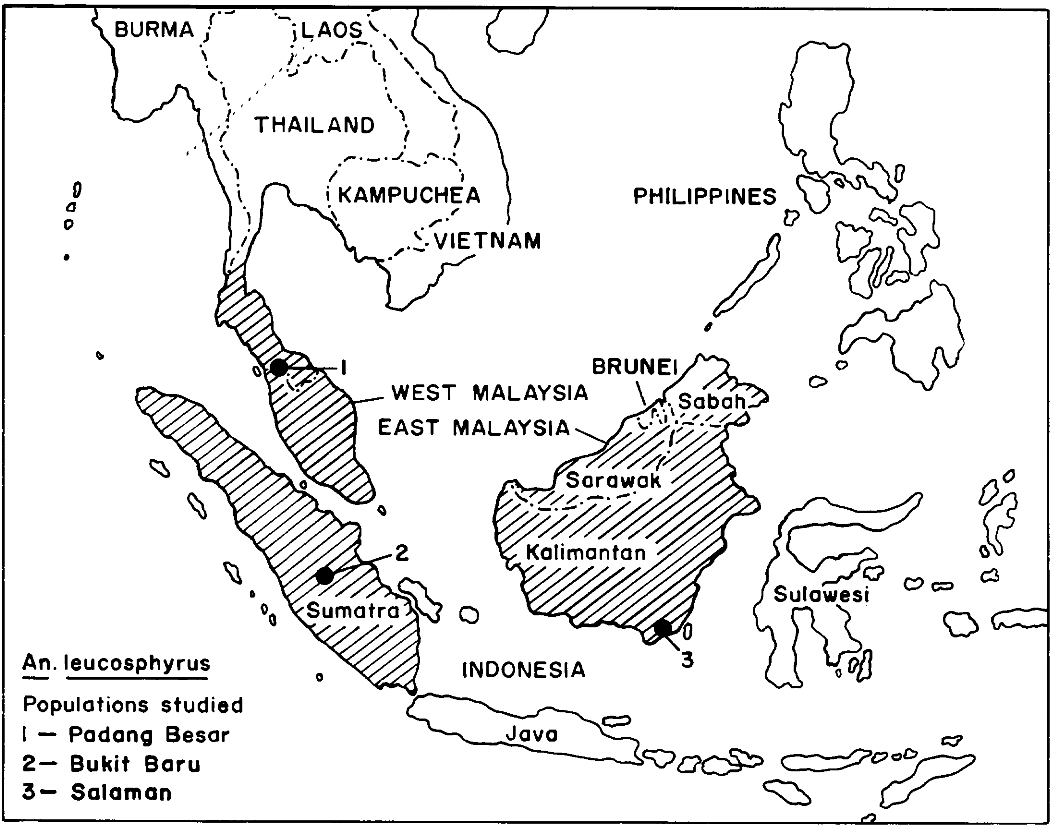


Fig. 1. Map of Southeast Asia showing the distribution of *An. leucosphyrus* and locations of the three allopatric populations investigated. The distribution is based on published reports (see Harbach et al. 1987) and collection records from Thailand maintained at AFRIMS laboratory in Bangkok.

Table 2. The number of female (isolines) of *Anopheles leucosphyrus* collected and examined cytologically from three allopatric populations in Southeast Asia.

Locality	Stock no.	Date of collection	No. of successful isolines (females collected)	No. of isolines examined
Bukit Baru, Jambi Province, Sumatra	IKS 18	April 1986	29 (86)	25
Salaman, South Kalimantan*	IDK 43	September 1986	10 (112)	6
Padang Besar, Songkla Province, Thailand**	PB 96	December 1986	45 (72)	45

* Collected in sympatry with *An. balabacensis.*

** Collected in sympatry with *An. dirus* species B.

somes were prepared by using a Giemsa staining method (Baimai 1977). Salivary gland polytene chromosomes were prepared in lacto-aceto-orcein in the manner described previously by Baimai et al. (1980).

Laboratory colonies were established from isofemale lines of the three allopatric populations: IDS-18 from Sumatra, IDK-43 from South Kalimantan and PB-96 from southern Thailand. Unlike *An. balabacensis* and members of the *An. dirus* complex, laboratory colonies of *An. leucosphyrus* are very difficult to maintain. Therefore, crossing experiments among the three isolines were performed as soon as sufficient material became available. Crosses were made later between the *An. leucosphyrus* stocks from Sumatra and Thailand and species A, B and D (Baimai et al. 1987) of the *An. dirus* complex. All crosses were made by induced copulation (Ow Yang et al. 1963). Eight to ten females were mated in each direction for all possible reciprocal crosses. Salivary gland polytene chromosomes of F_1 hybrid larvae were examined for the degree of synapsis. The fertility of F_1 hybrids was determined by mating male and female progeny from each cross. Sterile F_1 hybrid males were confirmed later by the presence of abnormal testes bearing no spermatozoa. In cases where fully fertile hybrids were obtained, matings between progeny were continued for many generations (10 as of July 1987).

RESULTS

Mitotic chromosomes. The mitotic karyotype (2n = 6) of members of the *Neomyzomyia* series of the subgenus *Cellia* of *Anopheles* consists of two pairs of autosomes and one pair of sex chromosomes. Generally, the sex chromosomes are virtually telocentric as exemplified by the *An. dirus* complex (Baimai et al. 1981, 1984b). The Y chromosome is almost totally heterochromatic. The X chromosome consists of a large block of centromeric heterochromatin and two distinct intercalary heterochromatic bands located approximately at the middle of the chromosome and an euchromatic segment located distally. All *An. leucosphyrus* examined from Thailand and South Kalimantan generally share this mitotic karyotype (Figs. 2–5). The distal block of intercalary heterochromatin of the X chromosome is very conspicuous compared with that found in *An. balabacensis* and members of the *An. dirus* complex. This character appears to be diagnostic for *An. leucosphyrus*.

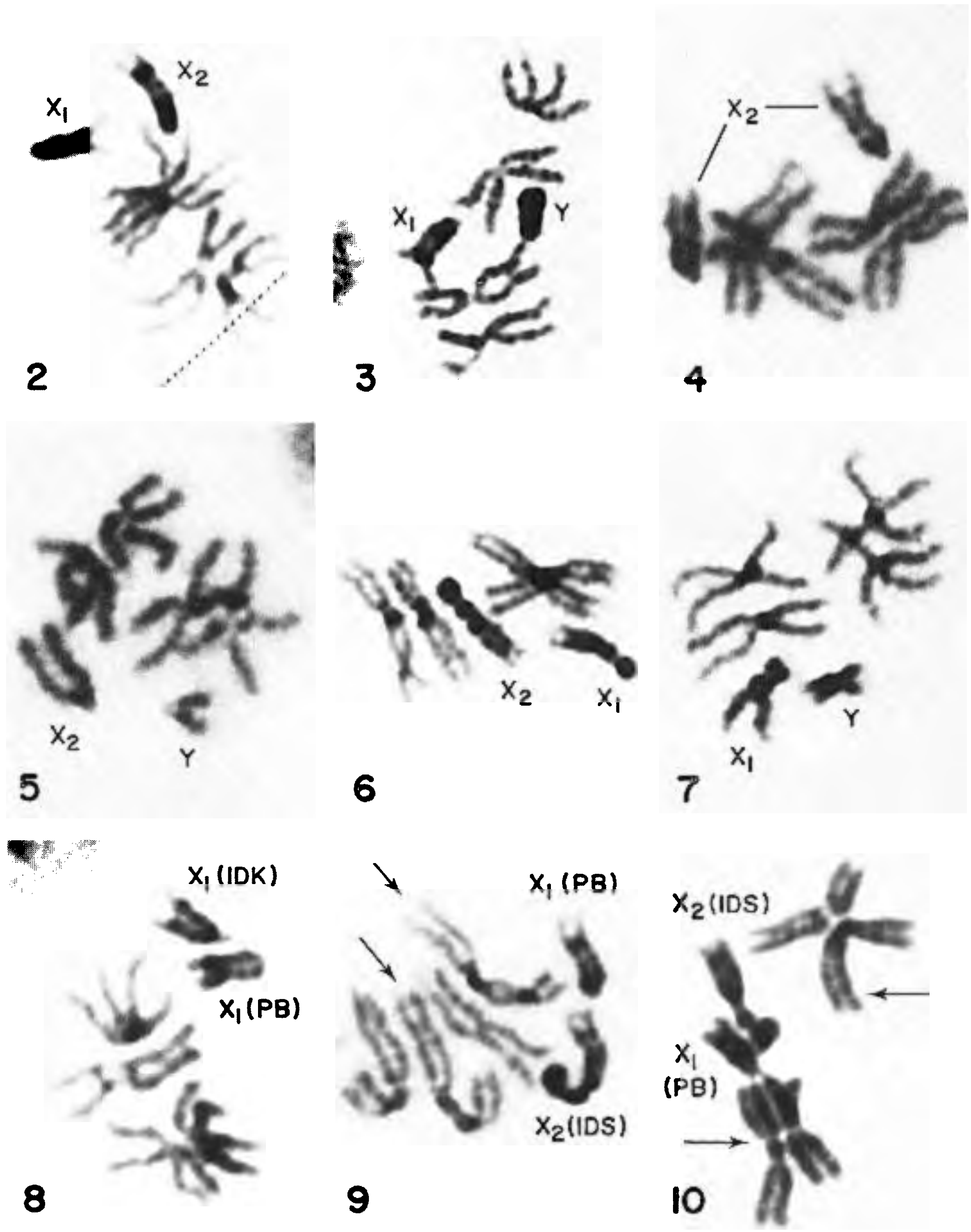
The X and Y chromosomes of specimens from South Kalimantan possess a very small segment of extra heterochromatin at the centromeric region. This characteristic gives the chromosomes a subtelocentric configuration which is not apparent in specimens from Thailand (Figs. 4, 5). This minor difference between the two populations is noticeable in the sex chromosomes of F_1 hybrid larvae (Fig. 8).

A striking difference is evident in the mitotic karyotype, especially in the sex chromosomes, of specimens from Sumatra. Here the X chromosome is submetacentric. The short arm is totally heterochromatic with a distinct secondary constriction at the middle (Figs. 6, 7). The long arm is similar to that observed in specimens from Thailand and Kalimantan. The Y chromosome is also uniquely submetacentric in shape and composed almost entirely of heterochromatin (Fig. 7). Each of the autosomes bears a prominent segment of pericentric heterochromatin which is lacking in specimens from the other populations examined (Figs. 9, 10). These observations suggest that *An. leucosphyrus* from Sumatra has undergone extensive chromosomal differentiation with respect to the constitutive heterochromatin of the karyotype. The populations from Thailand and South Kalimantan closely resemble the karyotype of other members of the *An. leucosphyrus* group, i.e., *An. balabacensis* and members of the *An. dirus* complex. Quantitative differences in the heterochromatin of the X chromosomes of specimens from Thailand and Sumatra are illustrated in Figs. 2 and 6, respectively. The characteristics and relationships of the mitotic chromosomes of the three populations are illustrated and compared diagrammatically in Fig. 11.

Hybridization experiments. All combinations of crosses between the three isoline colonies, except IDK and PB, showed some degree of reproductive isolation. Crosses between IDS female X PB male and IDS female X IDK male produced sterile F_1 males and the F_1 females exhibited low fertility when backcrossed to the parental stocks. The salivary gland polytene chromosomes of F_1 larvae showed some degree of asynapsis, particularly on chromosome arms 2R and 3R. Zones 1–5 of the X chromosome showed good synapsis, but zone 6 was completely asynapsed (Fig. 12a).

There is no evidence for reproductive isolation between the Thailand and South Kalimantan populations. Crosses in both directions between IDK and PB yielded fully fertile F_1 offspring. Successful crossmatings of hybrid progeny had continued through 10 generations as of July, 1987.

Some crosses were attempted between *An. leucosphyrus* and species A, B and D of the *An. dirus* complex. Crosses between IDS female X *An. dirus* B male gave small numbers of hybrid



Figs. 2–10. Photomicrographs of mitotic chromosomes from larval neuroblasts of *An. leucosphyrus*. 2,3. Species A X_1X_2 female and X_1Y male, respectively, from Thailand (PB). 4,5. Species A X_2X_2 female and X_2Y male, respectively, from South Kalimantan (IDK). 6,7. Species B X_1X_2 female and X_1Y male, respectively, from Sumatra (IDS). 8. F_1 X_1X_1 female hybrid of IDK female × PB male. 9. F_1 X_1X_2 female hybrid of IDS female × PB male. 10. F_1 X_1X_2 female hybrid of IDS female × IDK male. Autosomes of species B are indicated by arrows in Figs. 9 and 10.

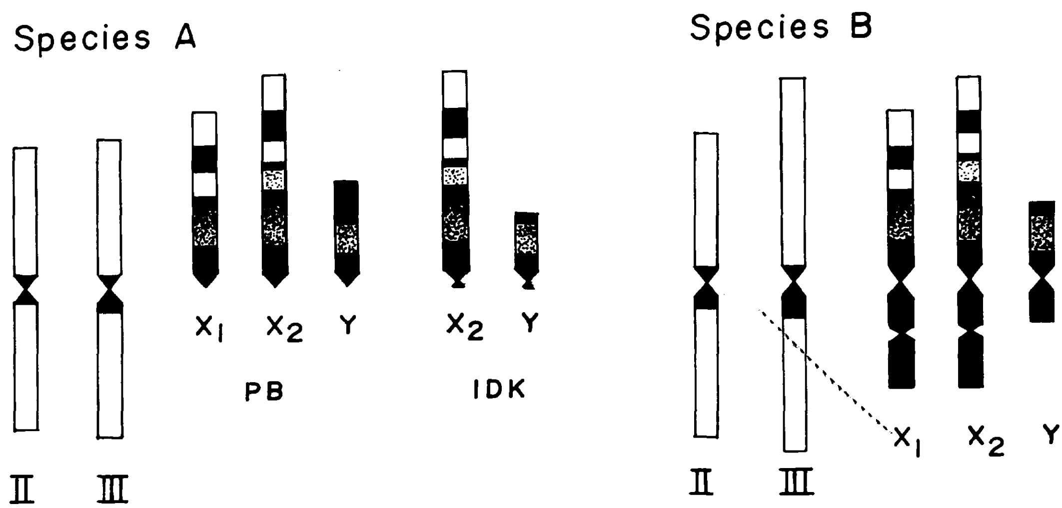


Fig. 11. Diagrammatic representation of mitotic chromosomes of the two species currently confused within the concept of *An. leucosphyrus* in Southeast Asia—species A from Padang Besar (PB) and South Kalimantan (ISK); species B from Sumatra. Black and shaded areas represent heterochromatin.

progeny, but the F_1 males were completely sterile. The salivary gland polytene chromosomes of the F_1 larvae resulting from this cross exhibited almost complete asynapsis (Fig. 12b). Similar results were obtained when the Thailand stock of *An. leucosphyrus* was crossed with species A and D of the *An. dirus* complex. The F_1 males were sterile and the larval polytene chromosomes showed a greater degree of synapsis. This may indicate that the Thailand population (and presumably the population from South Kalimantan) has a closer genetic affinity to members of the *An. dirus* complex than does the population of *An. leucosphyrus* from Sumatra.

DISCUSSION

The results of this study clearly indicate that the present concept of *An. leucosphyrus,* an important malaria vector in Southeast Asia, includes at least two distinct species. The Thailand and South Kalimantan populations are genetically similar and appear to represent a single species, provisionally designated here as *An. leucosphyrus* species A. The Sumatra population, although currently morphologically indistinguishable from the other populations examined, is a distinct species provisionally designated as *An. leucosphyrus* species B. Species A appears to be more widespread, with widely disjunct populations occurring in West Malaysia and Thailand and the island of Borneo. Species B occurs on the island of Sumatra.

It is suggested that genetic isolation between *An. leucosphyrus* A and B may have arisen as a consequence of genetic drift or as a by-product of genetic divergence within isolated populations on the island of Sumatra. It appears that the mitotic karyotype of *An. leucosphyrus* B has undergone extensive differentiation via the acquisition of constitutive heterochromatin in the sex chromosomes and the centromeric regions of the autosomes. This supports the general idea that gain of heterochromatin in the genome is a common phenomenon in karyotype evolution (John and Miklos 1979), a fact which seems true of the species of oriental *Anopheles* studied thus far (Vasantha et al. 1982, Baimai et al. 1984b). Variation observed in the X chromosomes of these species is also likely to be due to the acquisition of heterochromatin, similar to that observed in the four species currently confused under the name of *An. dirus* in Thailand (Baimai et al. 1984a; Baimai and Traipakvasin 1987).

Only a slight gain of heterochromatin is evident in the X chromosomes of *An. leucosphyrus* A when compared with *An. balabacensis* and members of the *An. dirus* complex. If a greater degree of synapsis in the polytene chromosomes of F_1 hybrids implies a closer relationship between species, then the present data strongly suggest that *An. leucosphyrus* A is more closely related to *An. balabacensis* and the *An. dirus* complex than is *An. leucosphyrus* B. Additional cytogenetic study is needed to more fully elucidate the karyotypic, genetic and

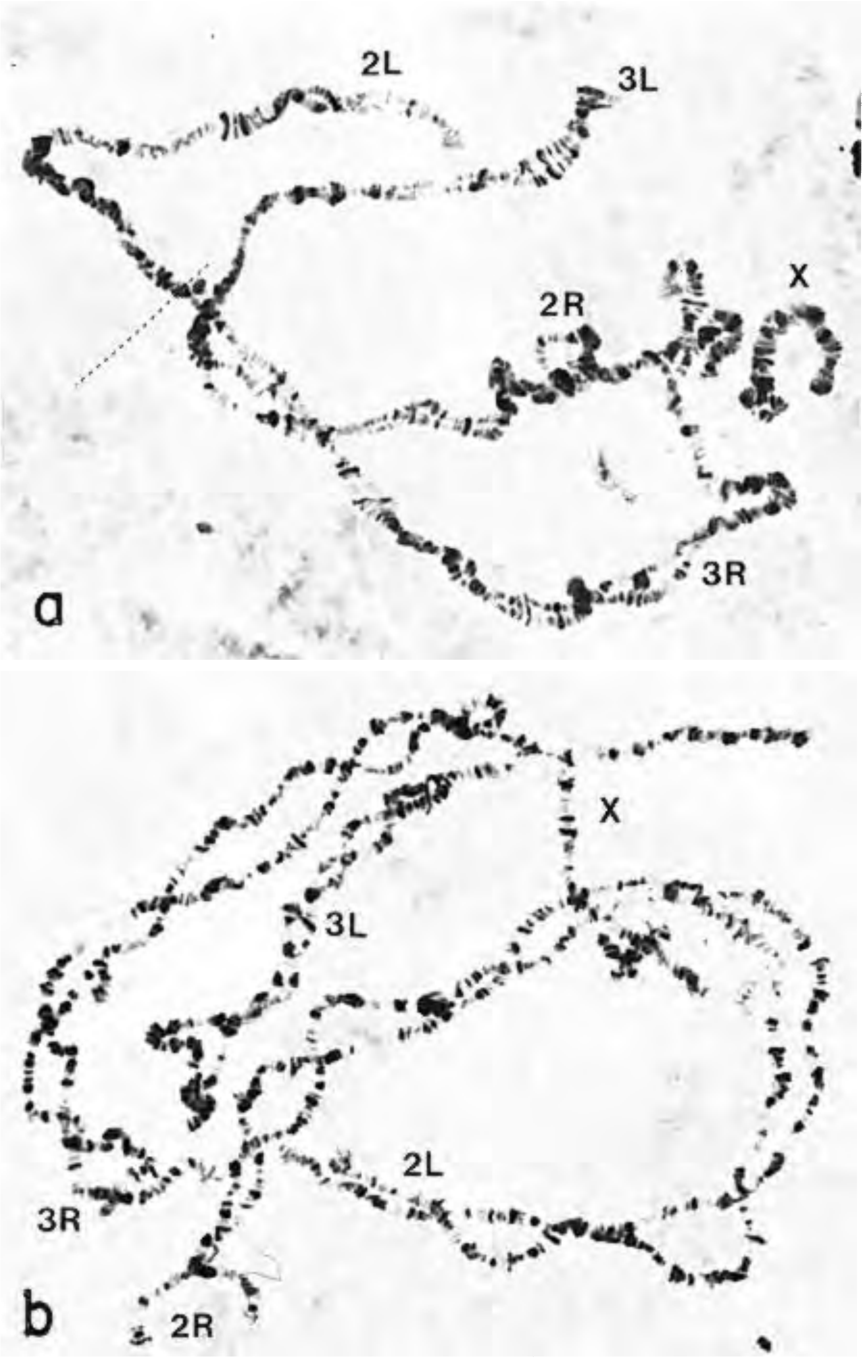


Fig. 12. Salivary gland polytene chromosomes of F_1 female hybrids showing degrees of asynapsis: (a) *An. leucosphyrus* Sumatra female × *An. leucosphyrus* Thailand male, (b) *An. leucosphyrus* Sumatra female × *An. dirus* B male.

evolutionary relationships of members of the *An. leucosphyrus* group. Of special importance in this regard is an investigation of genetic variation in natural populations relative to vectorial capacity for malarial parasites. This information may be important in the design of future malaria control programs.

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