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 subtelo-centric 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 F1 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 Dönnitz is the nomenotypic 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.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Year described</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. leucosphyrus Dönnitz</td>
<td>1901</td>
</tr>
<tr>
<td>An. elegans (James)</td>
<td>1903</td>
</tr>
<tr>
<td>An. hackeri Edwards</td>
<td>1921</td>
</tr>
<tr>
<td>An. balabacensis balabacensis Baisas</td>
<td>1936</td>
</tr>
<tr>
<td>An. balabacensis baisasi Colless</td>
<td>1957</td>
</tr>
<tr>
<td>An. balabacensis introlatus Colless</td>
<td>1957</td>
</tr>
<tr>
<td>An. cristatus King and Baisas</td>
<td>1936</td>
</tr>
<tr>
<td>An. riparis riparis King and Baisas</td>
<td>1936</td>
</tr>
<tr>
<td>An. riparis macarthuri Colless</td>
<td>1956</td>
</tr>
<tr>
<td>An. takasagoensis Morishita</td>
<td>1946</td>
</tr>
<tr>
<td>An. pujutensis Colless</td>
<td>1949</td>
</tr>
<tr>
<td>An. sulawesi Koesoemawinangoen</td>
<td>1954</td>
</tr>
<tr>
<td>An. dirus Peyton and Harrison</td>
<td>1979</td>
</tr>
</tbody>
</table>
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.

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

* Collected in sympathy with An. balabacensis.
** Collected in sympathy 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 F₁ hybrid larvae were examined for the degree of synapsis. The fertility of F₁ hybrids was determined by mating male and female progeny from each cross. Sterile F₁ 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 F₁ 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 isolate 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 F₁ males and the F₁ females exhibited low fertility when backcrossed to the parental stocks. The salivary gland polytene chromosomes of F₁ 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 F₁ 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 X₁X₂ female and X₁Y male, respectively, from Thailand (PB). 4,5. Species A X₁X₂ female and X₂Y male, respectively, from South Kalimantan (IDK). 6,7. Species B X₁X₂ female and X₁Y male, respectively, from Sumatra (IDS). 8. F₁ X₁X₁ female hybrid of IDK female × PB male. 9. F₁ X₁X₂ female hybrid of IDS female × PB male. 10. F₁ X₁X₂ female hybrid of IDS female × IDK male. Autosomes of species B are indicated by arrows in Figs. 9 and 10.
progeny, but the F1 males were completely sterile. The salivary gland polytene chromosomes of the F1 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 F1 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 F1 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 F₁ 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.

ACKNOWLEDGMENTS

We wish to thank U. Kijchalao, K. Vejsanit and K. Jayana for technical assistance; C. A. Green, R. G. Andre and E. L. Peyton for commenting on the manuscript; and P. Panthusiri for preparing Fig. 1. This work was supported in part by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases and the Walter Reed Army Institute for Research (In-House Laboratory Research Project No. 3M161101A91C).

REFERENCES CITED


