METAPHASE KARYOTYPES OF *ANOPHELES* OF THAILAND AND SOUTHEAST ASIA: I. THE HYRCANUS GROUP

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ABSTRACT. Metaphase karyotypes of 6 species of the Hyrcanus Species Group of the subgenus *Anopheles* show constitutive heterochromatin variation in X and Y chromosomes. *Anopheles peditaeniatus* exhibits the most extensive variation in the size and shape of heterochromatic sex chromosomes, with 3 types of X and 5 types of Y chromosomes. *Anopheles nitidus* shows the least variation, with only 2 types of X chromosomes. *Anopheles sinensis* and *An. crawfordi* each have 2 forms of metaphase karyotype in the heterochromatin of the Y chromosome. It is not known whether the 2 forms of metaphase karyotype in these 2 species represent inter- or intraspecific differences. The 2 forms of heterochromatic sex chromosomes observed in *An. argyropus* and *An. nigerrimus* may suggest the existence of sibling species complexes within each of these species.

INTRODUCTION

The genus *Anopheles* is one of the most important groups of mosquitoes in terms of disease transmission in the world. This genus consists of more than 400 described species (Colless 1956, Reid 1968, Harrison and Scanlon 1975, Harrison 1980, Harrison et al. 1991). The subgenera *Anopheles* and *Cellia* are the 2 largest of the 6 subgenera belonging to the genus. Harrison et al. (1991) recently updated the checklist of *Anopheles* found in Thailand, and included 35 and 37 species in the subgenera *Anopheles* and *Cellia*, respectively. Although considerable knowledge on the morphology, systematics and geographic distribution of the known species of *Anopheles* in the Southeast Asian Region has been documented since the publication of Harrison and Scanlon (1975), relatively few species are known cytologically. Of those species that are known chromosomally, mitotic chromosome information is notably sparse (Kitzmiller 1967, Kanda 1968, Vasantha et al. 1982, Avirachan et al. 1969).

During our studies on the population cytogenetics of human malaria vectors in Thailand and Southeast Asia, we had an opportunity to study metaphase karyotypes in 40 anopheline species collected from natural populations. Our aim in this series of publications is to provide data on metaphase karyotypes of the *Anopheles* of Thailand and neighboring countries for a better understanding of chromosomal evolution in this area of anopheline diversity. Even more importantly, karyotypic differentiation due to the different amount and distribution of constitutive heterochromatin can be a useful cytotaxonomic tool in separating closely related species, particularly homosequential species as exemplified in the Leucosphyrus Group (Baimai et al. 1981, 1988a, 1988b; Baimai 1988).

We report here on the metaphase karyotype of 6 species, including various forms, of the Hyrcanus Group of the subgenus *Anopheles*, which occur in Thailand. Morphological identification of species belonging to the Hyrcanus Group has been a major problem for mosquito taxonomists of this region (Reid 1968, Harrison 1972, Harrison and Scanlon 1975, Kanda et al. 1981).

MATERIALS AND METHODS

The 6 species of the Hyrcanus Group studied are *An. sinensis* Wiedemann, *An. nigerrimus* Giles, *An. crawfordi* Reid, *An. nitidus* Harrison, Scanlon and Reid, *An. argyropus* (Swellengrebel) and *An. peditaeniatus* (Leicester) (Table 1). Adult females of these species were collected from animal and/or human bait at different localities during our research program, which was initiated in 1980. Fully blood-fed females were identified morphologically to species as far as possible. The healthy specimens were brought back and reared individually in our laboratories at Mahidol University and at the Armed Forces Research Institute of Medical Sciences to obtain single families (isofemale lines). The brain ganglia of fourth-instar larvae were used for metaphase chromosome preparations employing a modified method as described by Baimai (1977). The air-dried slides were stained with Giemsa and mounted in Permount (Fisher) for permanent preparations. The best prophase and/or metaphase spreads of mitotic karyotypes were photographed with Kodak Technical Film using an Olympus Photomicroscope with a green filter and an oil immersion (×670) lens.

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Table 1. The number of females (isolines) of 6 species (including karyotypic forms) within the Hyrcanus Group of the subgenus *Anopheles* collected and examined cytologically from different wild populations in Thailand, Taiwan and Indonesia.

Species/Form	Locality	No. of isolines examined	Date of collection
sinensis			
Form A	Mae Sariang, Mae Hong Son Province	3	September 1985
	Chiayi County, Taiwan	2	August 1986
Form B	Mae Sariang, Mae Hong Son Province	4	September 1985
	Chiayi County, Taiwan	3	August 1986
nigerrimus			
Form A	Jambi Province, Sumatra, Indonesia	1	April 1986
	Nachaluai, Ubon Ratchathani Province	1	January 1987
Form B	Bangpa-in, Ayutthaya Province	1	February 1983
crawfordi			
Form A	Makarm, Chanthaburi Province	2	July 1982
Form B	Tung Ka Ngok, Phangnga Province	2	June 1982
argyropus			
Form A	Maetang, Chiangmai Province	3	November 1983
	Rongkwang, Phrae Province	3	February 1984
Form B	Maetang, Chiangmai Province	8	November 1983
	Makarm, Chanthaburi Province	3	November 1983
nitidus			
	Tung Ka Ngok, Phangnga Province	1	June 1982
	Sadao, Songkhla Province	1	January 1986
peditaeniatus			
	Makarm, Chanthaburi Province	2	November 1983
	Maetang, Chiangmai Province	4	November 1983
	Makarm, Chanthaburi Province	4	November 1983
	Maetang, Chiangmai Province	9	November 1983
	Makarm, Chanthaburi Province	1	November 1983
	Rongkwang, Phrae Province	3	February 1984
	Makarm, Chanthaburi Province	2	November 1983
	Maetang, Chiangmai Province	2	November 1983

RESULTS

The chromosomal analysis of the Hyrcanus Group has revealed that the metaphase karyotype ($2n = 6$) usually consists of one pair of metacentric and one pair of submetacentric autosomes and one pair of heteromorphic sex chromosomes (XX in females and XY in males). The X chromosome is generally either metacentric or submetacentric. One arm clearly consists of euchromatin while the opposite arm is totally heterochromatic. Secondary constriction(s) may sometimes be observed in the heterochromatic arm. Variation of the X chromosome due to different amounts of major block(s) of constitutive heterochromatin has also been observed in 5 species. The Y chromosome, on the other hand, is almost entirely heterochromatic. The Y may be telocentric, subtelocentric (acrocentric), submetacentric, or metacentric of different sizes. Specific differences of the metaphase chromosomes of the 6 species of the Hyrcanus Group are briefly described below.

Anopheles sinensis: Twelve families of this species from Mae Sariang, Mae Hong Son Province in northern Thailand and Chiayi County in Taiwan were examined cytologically. The X chromosome is submetacentric, comprising the euchromatic short arm with a considerable block of pericentric heterochromatin and the totally heterochromatic long arm with a conspicuous secondary constriction present in some preparations (Figs. 2, 4, 5). The 2 types of Y chromosomes have been observed in the same populations at both localities (Table 1). The Y_1 chromosome is subtelocentric or acrocentric with only a small portion of the short arm present (Fig. 1). The long arm is approximately the same length as that of the X chromosome. It occasionally shows a secondary constriction similar to the X. Chromosome Y_2 is clearly a submetacentric with the short arm approximately one-half the length of the long arm (Figs. 3, 5). The Y_2 appears to have arisen from the presumed ancestral Y_1 simply by means of the addition of an extra block of heterochromatin onto the short arm, transforming it to a submetacentric configuration. Based on the different types of Y chromosomes, two forms of metaphase karyotypes have been recognized in *An. sinensis*, viz., form A (X, Y_1) and form B (X, Y_2). These 2 forms of metaphase karyotype have

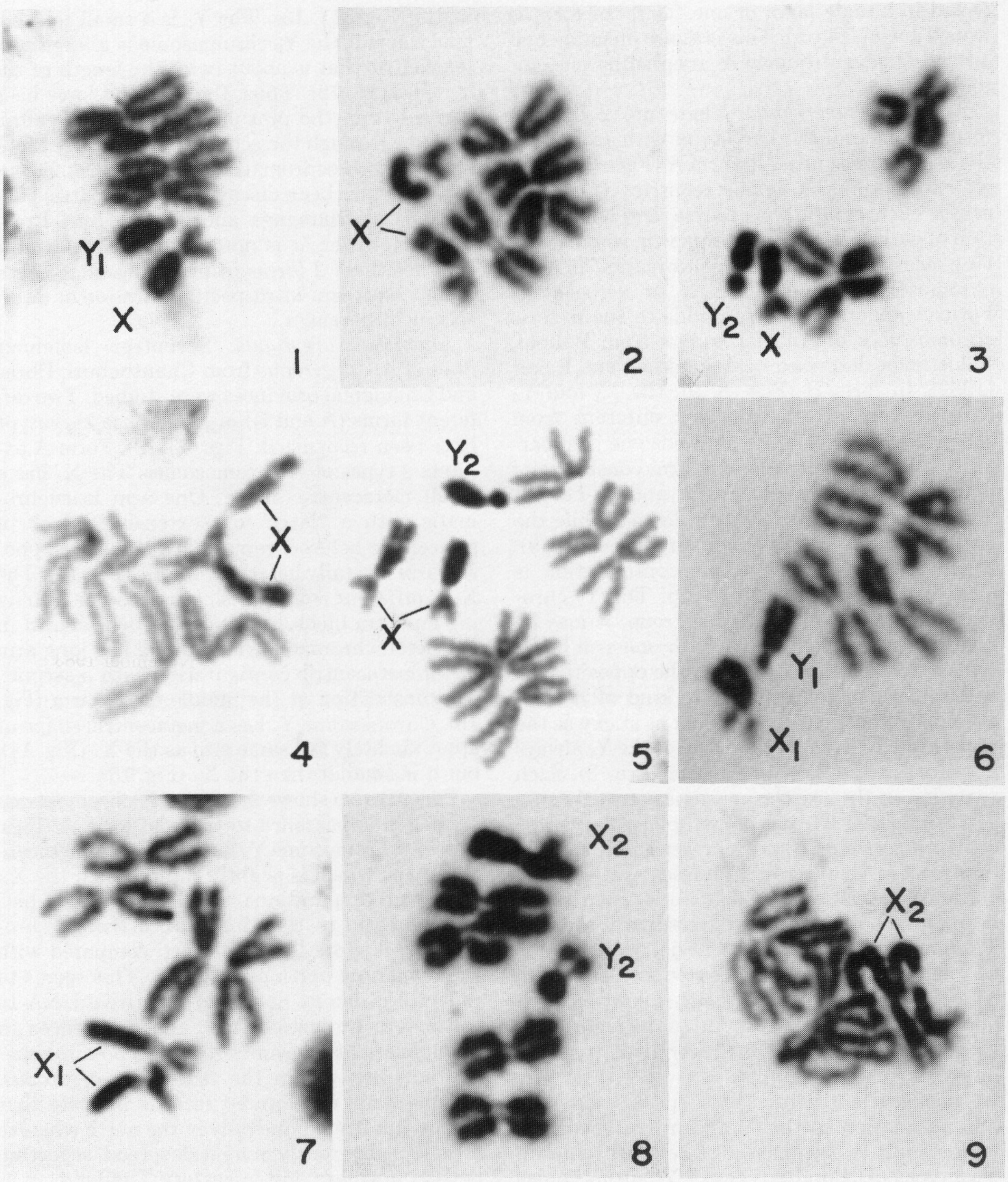

Fig. 1–9. Metaphase karyotypes from larval neuroblast cells. 1–5, *Anopheles sinensis*: 1, 2, male and female, respectively, of form A; 3, 4, male and female, respectively, of form B; 5, triploidy of a male larva of form B. 6–9, *Anopheles nigerrimus*: 6, 7, male and female, respectively, of form A; 8, 9, male and female, respectively, of form B.

been found in the same populations at both localities in Thailand and Taiwan. Thus, these 2 forms of metaphase karyotype may reflect interspecific differences within the *An. sinensis* complex. Further cytogenetic study is required before a definite conclusion can be made. Form B corresponds with the mitotic karyotype of *An. sinensis s. l.* from the Simao District of Yunnan Province, China recently reported by Xu Shubi and Qu Fengyi (1991). However, the photomicrographs of the karyotype of "*An. sinensis*" reported by Kanda (1968) were too small to be used for comparison with our present data. In addition, a triploid condition ($3n$) has been detected in a male larva of one family of form B from Taiwan (Fig. 5). This is a rare phenomenon occurring adventitiously in anopheline mosquitoes.

Anopheles nigerrimus: There are 2 distinct forms of metaphase karyotype with respect to the sex chromosomes. In form A (Figs. 6, 7), the X_1 chromosome is submetacentric. The short arm is euchromatic with only a very small portion of pericentric heterochromatin, whereas the long arm is totally heterochromatic. The Y_1 chromosome is subtelocentric or acrocentric. Form A apparently corresponds to the mitotic chromosomes of *An. nigerrimus* from Balloki, Pakistan as described by Aslamkhan and Baker (1969). In form B (Figs. 8, 9), the X_2 and Y_2 chromosomes are distinctively different from those of form A. The X_2 chromosome is apparently a metacentric with one arm consisting of approximately ⅓ euchromatic and ⅔ heterochromatic portions of the arm length while the opposite arm is totally heterochromatic (Fig. 9). This type of X chromosome configuration is quite peculiar within this group. The Y_2 chromosome is also unique to this group. It may be regarded as submetacentric with one arm being typical heterochromatin while the opposite arm is a somewhat lighter-staining kind of heterochromatin that is apparently not as sticky as the other arm. Thus the second arm of the Y_2 always appears as a satellite chromosome (Fig. 8). Such variation in the sex chromosomes strongly suggests that these 2 forms of metaphase karyotype of *An. nigerrimus* could represent interspecific differences. Among the 3 families available for chromosome analysis in this study, form A has been found both in Ubon Ratchathani Province, Thailand and Sumatra, Indonesia, while form B was detected in Ayutthaya Province, Thailand. The 2 forms of *An. nigerrimus* found in Thai populations do not correspond to the metaphase karyotype of *An. hyrcanus nigerrimus* reported by Avirachan et al. (1969).

Anopheles crawfordi: This species also exhibits variation in the X and Y chromosomes with respect to the amount of heterochromatin. Two types of X and 2 types of Y chromosomes have been observed, representing 2 forms of metaphase karyotype in *An. crawfordi*, viz., form A (X_1, X_2 and Y_1) and form B (X_1, X_2 and Y_2). The X_1 is a small metacentric. One arm of the X_1 is euchromatic with a very small portion of pericentric heterochromatin present, whereas the opposite arm is entirely heterochromatic of approximately equal length (Fig. 10). The X_2 is submetacentric with a euchromatic short arm similar to that of the X_1. However, the heterochromatic long arm is approximately twice the length of that of the X_1, apparently due to the acquisition of an extra portion of heterochromatin (Figs. 11, 13). The Y_1 is a small telocentric, whereas the Y_2 chromosome is a large subtelocentric that is about twice the length of the Y_1 (Figs. 10, 12). Thus, the Y_2 could have been derived from the presumed ancestral Y_1 chromosome through the acquisition of a large block of distal heterochromatin in the long arm. Forms A and B have been discovered in allopatric populations at Phangnga and Chanthaburi Provinces. However, it is not possible to determine whether these 2 forms of metaphase karyotype merely represent intraspecific variation or interspecific differences.

Anopheles argyropus: Seventeen isofemale lines of *An. argyropus* from Chanthaburi, Phrae and Chiangmai provinces were studied. Two different forms (A and B) of metaphase karyotype have been recognized (Figs. 14-19). Form A exhibits 2 types of X chromosomes. The X_1 has a small metacentric shape. One arm is euchromatic with a "large" or "extensive" block of pericentric heterochromatin, whereas the opposite arm is totally heterochromatic (Fig. 14). The X_2 is different from the X_1 chromosome in having an extra block of distal heterochromatin in the heterochromatic arm, making it a long arm of submetacentric configuration with a secondary constriction at the middle of the arm (Fig. 16). Chromosome Y_1 has a metacentric shape of approximately the same size as the X_1 (Fig. 14), but it is smaller than the X_2 (Fig. 15).

Form B also shows 2 types of X chromosomes similar in appearance to those of form A (Figs. 17-19). Chromosome Y_2 has a large submetacentric shape which is slightly shorter than the X_2. In certain preparations, the distal block of heterochromatin in the long arm of the Y_2 chromosome is more lightly stained compared with the remaining portions (Fig. 19). This seems to reflect a different nature of repetitive DNA in the heterochromatic arm. Such differences in metaphase chromosomes suggest a possible species complex within the taxon *An. argyropus*. Furthermore, our limited records indicate that form A has been found only in the north whereas form B is apparently more widespread, occurring both in northern and in eastern Thailand.

Anopheles nitidus: This species occurs in southern Thailand. Specimens from Songkhla and Phangnga Provinces show 2 types of X and one type of Y chromosome. The X_1 is metacentric while X_2 appears as a large submetacentric (Fig. 21), similar to the X_1 and X_2 chromosomes of *An. crawfordi* mentioned above. The Y chromosome is apparently subtelocentric (Fig. 20). Thus, the Y chromosome of *An. nitidus* is also similar in some ways to the Y_2 of *An. crawfordi*. Hence, it is rather difficult to distinguish *An. nitidus* from *An. crawfordi* form B on the basis of metaphase chromosomes even though they

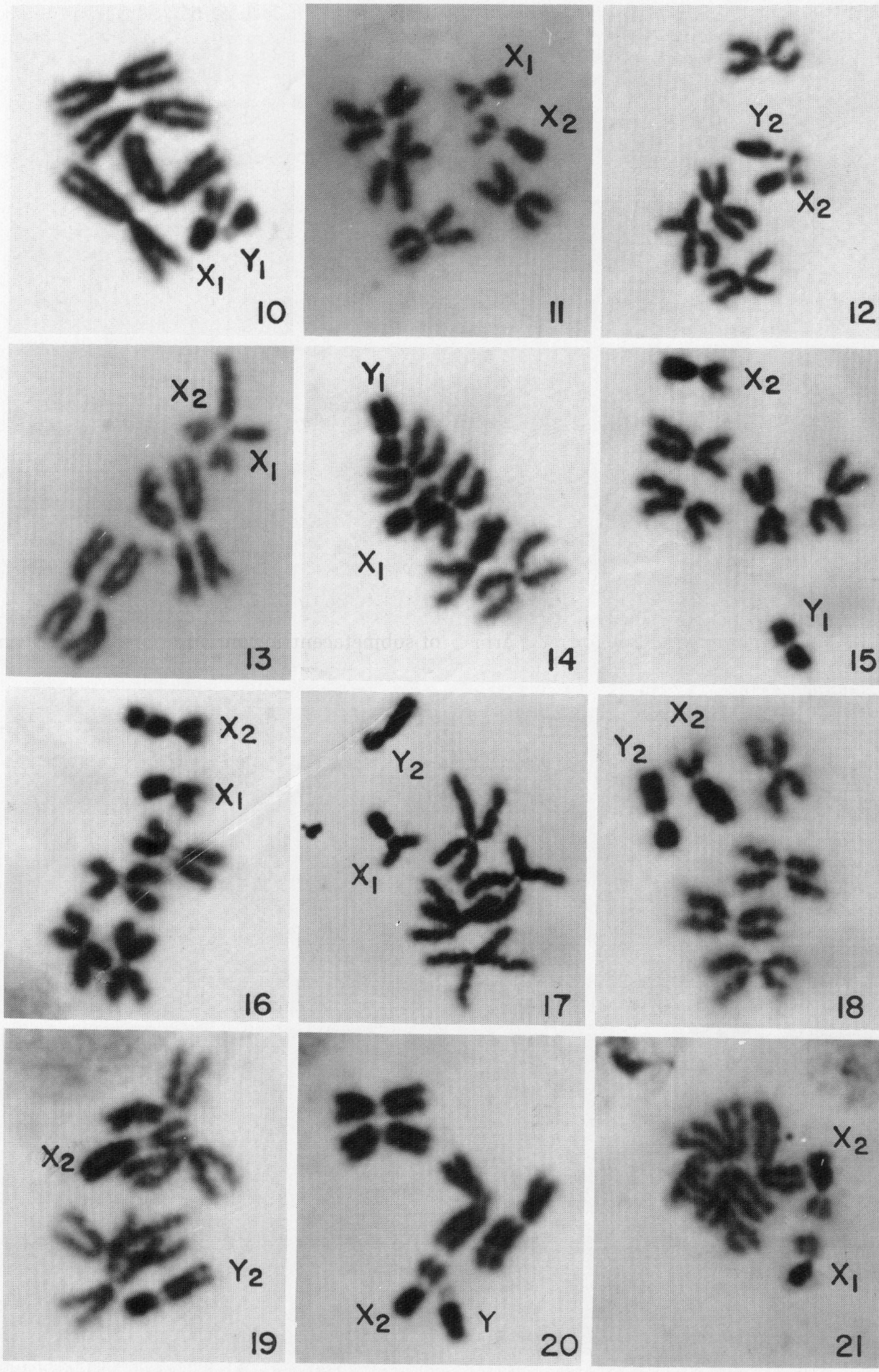

Fig. 10–21. Metaphase karyotypes. 10–13, *Anopheles crawfordi*: 10, 11, male and female, respectively, of form A; 12, 13, male and female, respectively, of form B. 14–19, *Anopheles argyropus*: 14 and 15, 16, males and female, respectively, of form A; 17–19, males of form B. *Anopheles nitidus*: 20, 21, male and female, respectively.

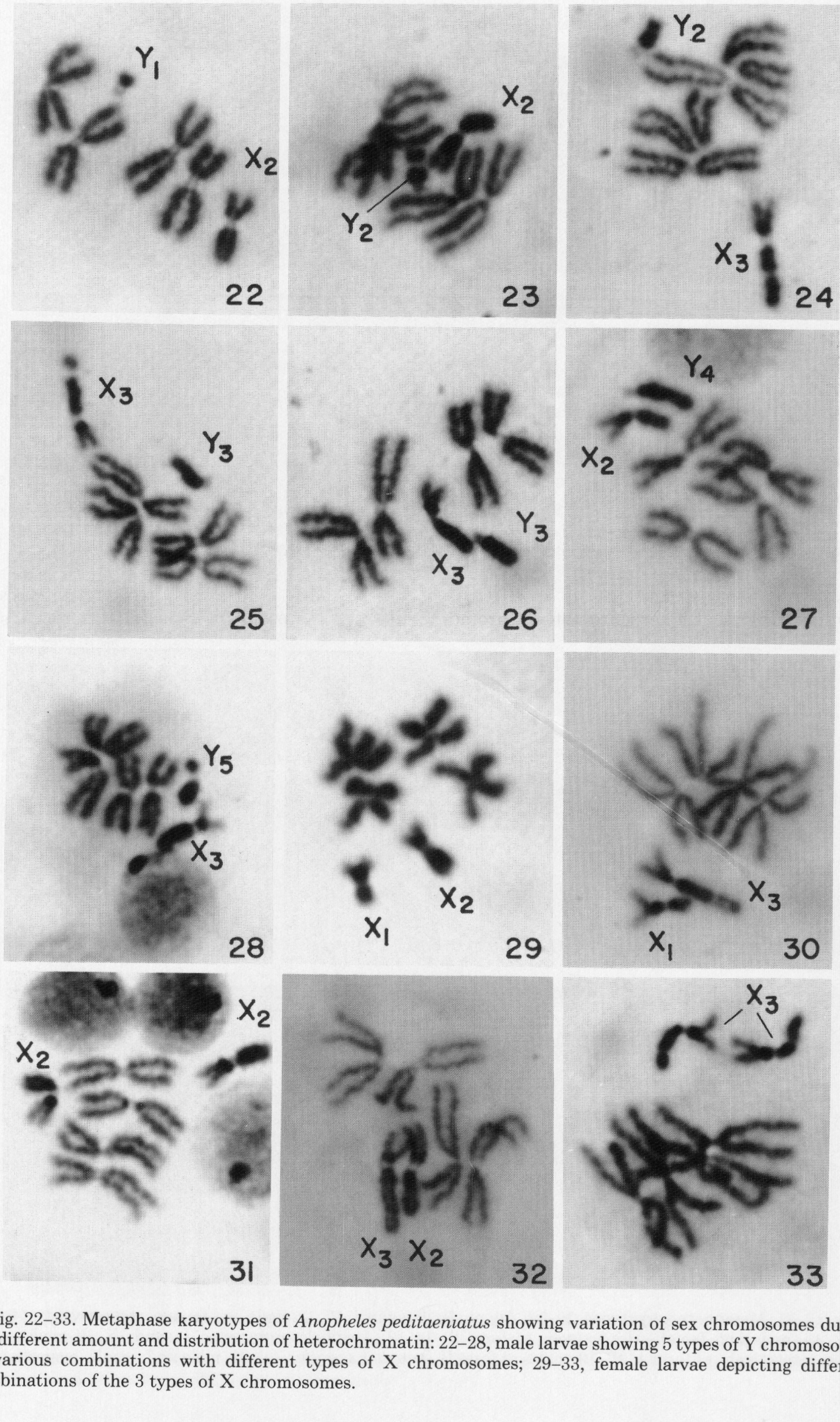

Fig. 22–33. Metaphase karyotypes of *Anopheles peditaeniatus* showing variation of sex chromosomes due to the different amount and distribution of heterochromatin: 22–28, male larvae showing 5 types of Y chromosomes in various combinations with different types of X chromosomes; 29–33, female larvae depicting different combinations of the 3 types of X chromosomes.

can be readily separated by morphological criteria.

Anopheles peditaeniatus: Twenty-seven wild-caught females morphologically identified as *An. peditaeniatus* collected at Chiangmai, Phrae and Chanthaburi provinces show remarkable variation in the sex chromosomes. Three types of X and 5 types of Y chromosomes have been detected in these families. Chromosomes X_1, X_2 and X_3 differ from each other in the number and amount of major block(s) of heterochromatin present in the heterochromatic arm, making them appear as metacentric or small or large submetacentric (Figs. 29-33). The euchromatic arm of each X chromosome type exhibits a large block of pericentric heterochromatin. The variation in the X chromosomes in this case is likely due to the acquisition of extra heterochromatin. Likewise, the evolution of Y chromosome types in *An. peditaeniatus* could have arisen via the process of gain, rather than loss, of major block(s) of heterochromatin. Thus, the very small telocentric Y_1 chromosome could be taken as an ancestor (Fig. 22). Chromosome Y_2 (Figs. 23, 24) could have been derived from the presumed ancestral Y_1 simply through the addition of an extra block of heterochromatin. Chromosomes Y_3, Y_4 and Y_5 (Figs. 25-28) could also have arisen in a similar fashion. Since there are various combinations of X and Y chromosomes occurring within a population, e.g., X_2 or X_3 with Y_1 or Y_2 or Y_3, X_2 or X_3 with Y_4, and X_3 with Y_5, these types of X and Y chromosomes may merely represent intraspecific variation with respect to the amount and distribution of heterochromatin.

A diagrammatic representation of mitotic karyotypes of the 6 species and forms within the Hyrcanus Group is shown in Fig. 34.

DISCUSSION

Previous studies of the metaphase karyotype of *Anopheles* have shown that in most cases, if not all, the typical karyotype ($2n = 6$) consists of 2 pairs of autosomes and one pair of sex chromosomes. The autosomes are generally submetacentrics and/or metacentrics. The sex chromosomes are obviously heteromorphic. Thus, the X is usually subtelocentric or submetacentric with short and long arms. The Y chromosome is usually subtelocentric. The X and Y chromosomes can be distinguished on the basis of their size and shape. Nonetheless, little attention has been paid to the constitutive heterochromatin present in the sex chromosomes and, to a lesser extent, in the centromeric region of the autosomes. The heterochromatin differentiation as revealed in metaphase karyotype is sometimes useful as a diagnostic character of closely related species of *Anopheles* (Baimai et al. 1988a, 1988b, Baimai 1988, Green et al. 1985).

The present chromosomal evidence indicates that the 6 morphological species of the Hyrcanus Group examined generally exhibit a similar autosomal configuration. On the other hand, remarkable differences have been encountered in the X and Y chromosomes, primarily due to the amount and distribution of constitutive heterochromatin. It is generally believed that an acquisition of extra heterochromatin is a common phenomenon in the chromosomal evolution of higher organisms (White 1973, John and Miklos 1979). Our findings support this.

The position of the centromere in the X and Y chromosomes varies from telocentric to subtelocentric, or acrocentric to submetacentric and metacentric, making the chromosomes variable in total length as extensively demonstrated in *An. peditaeniatus* and, to a lesser extent, in *An. nitidus*. It is not known whether heterochromatin variation in the sex chromosomes of *An. crawfordi* is an indication of inter- or intraspecific difference. Heterochromatin variation observed in the sex chromosomes of *An. sinensis*, *An. argyropus* and *An. nigerrimus* is, however, likely to represent interspecific difference, suggesting the presence of sibling species complexes in each of these taxa. This karyotypic approach may prove an excellent tool to explore the chromosomal interrelationships of various closely related species groups within the subgenera *Anopheles* and *Cellia*, or among the subgenera of the genus *Anopheles* as a whole. Further chromosomal data on the metaphase karyotype of other species groups of the *Anopheles* in Thailand will be published in separate papers.

Gain of heterochromatin has played an important role in chromosome evolution in the oriental *Anopheles* (Vasantha et al. 1982, Baimai 1988). In all cases observed in our studies, the direction of chromosomal change has been from telocentric or acrocentric to submetacentric or metacentric depending on the location of the acquired block(s) of heterochromatin. A good example is the sex chromosome variation seen in *An. peditaeniatus* described here, and in the *An. dirus* complex (Baimai et al. 1984, Baimai and Traipakvasin 1987). The biological significance of having one or more major blocks of heterochromatin in the genome, particularly in sex chromosomes, is an unsolved problem (Baimai 1988). Why is it so common in the *Anopheles* of Southeast Asia? Could it be very useful in species differentiation in *Anopheles* and other dipteran insects as a whole? The phenomenon of acquisition of heterochromatin is of interest in its own right and deserves further investigation.

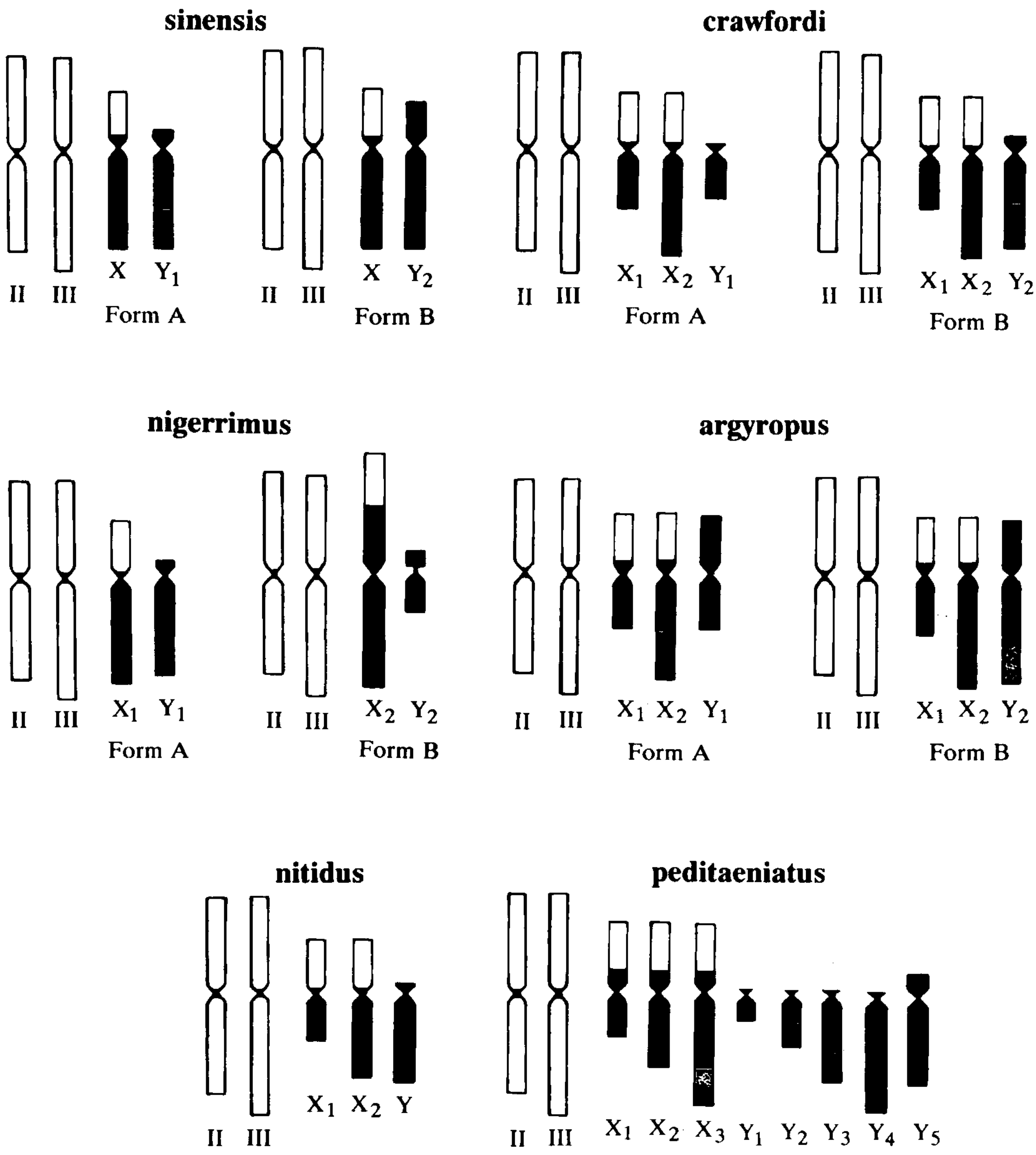

Fig. 34. Diagrammatic representation and comparison of metaphase karyotypes of the 6 species (including forms) of the Hyrcanus Group of *Anopheles* (*Anopheles*). Only one set of autosomes II and III is present. Black and shaded areas represent variable heterochromatic portions. The centromeres are indicated by constrictions of each chromosome. Chromosome lengths, arm ratios and heterochromatic portions are shown in proportion.

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