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Inter- and intraspecific heterochromatin variation detected by restriction endonuclease digestion in two sibling species of the *Anopheles maculipennis* complex

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The sibling species *Anopheles atroparvus* and *Anopheles labranchiae* are cytogenetically almost indistinguishable. The chromosome complement ($2n = 6$) consists of two pairs of autosomes and two heteromorphic sex chromosomes with largely homologous heterochromatic long arms. Treatment of chromosome preparations with the restriction endonucleases, Alu I, Hae III, Mbo I, Hpa II, revealed species-specific differences of the sex chromosome banding pattern. These differences involved both amount and location of digested heterochromatin. Heterochromatin heterogeneity and a high level of intraspecific polymorphism, undetected with standard banding techniques, were observed in both species. Quantitative heterochromatin differences between the sex chromosomes did not inhibit their pairing and chiasmata formation. The endonuclease Msp I, which cleaves the same target sequence as Hpa II, did not digest heterochromatic as well as euchromatic regions in both species: inhibition of cleavage by methylation of the target sequence or limited access of the enzyme to the target could be involved in this response.

INTRODUCTION

The human malaria vectors *Anopheles labranchiae* Falleroni and *Anopheles atroparvus* Van Thiel (Diptera: Culicidae) are morphologically and genetically the most closely related species of the palaearctic *Anopheles maculipennis* group (Diptera: Culicidae). These sibling species are cytogenetically almost indistinguishable. The salivary gland polytene chromosomes are homosequential (Frizzi 1947; Kitzmiller *et al.*, 1967), although asynchronous replication of homologous bands has been observed in interspecific hybrids (Tiepolo *et al.*, 1974). The chromosome complement consists of two pairs of autosomes and two heteromorphic sex chromosomes. The karyotypes of the two species are comparable in terms of morphology, total chromosome length and DNA content (Jost and Mameli, 1972). The X and Y chromosomes (submetacentric and subtelocentric respectively) have largely homologous heterochromatic, late replicating, long arms and form chiasmata in meiosis (Fraccaro *et al.*, 1976; Jayakar *et al.*, 1982; Marchi, personal observation). C-positive heterochromatin is similarly distributed in *A. atroparvus* and *A. labranchiae* (Mezzanotte and Ferrucci, 1978), although qualitative differences of heterochromatin DNA between the two species have been inferred using fluorescent banding techniques (Mezzanotte and Ferrucci, 1978; Mezzanotte *et al.*, 1979).

Restriction endonucleases (RE) have been used on fixed chromosomes *in situ* to discriminate between regions with different distribution and frequency of RE cleavage sequences, *i.e.*, with different DNA composition (Mezzanotte *et al.*, 1983; Miller *et al.*, 1983). This technique has already provided new insight on the chromatin organization and DNA composition of various insect groups (Mezzanotte, 1986; Bianchi *et al.*, 1986; Bultmann and Mezzanotte, 1987; Gozalvez *et al.*, 1987; Mezzanotte and Marchi, 1987; Lopez-Fernandez *et al.*, 1988; Marchi and Mezzanotte, 1988). With the aim of revealing further chromatin/DNA differences between *A. atroparvus* and *A. labranchiae*, we tested five REs on the chromosomes of these closely related species. A large amount of heterochromatin resistant to RE digestion was present in both species. However, species-specific differences of RE banding patterns

were detected. A high level of intraspecific polymorphism, in many cases undetected with other banding methods and involving different types of constitutive heterochromatin, was also observed.

MATERIAL AND METHODS

Immature stages of *A. labranchiae* were collected from south-eastern Sardinia. *A. atroparvus* specimens were from a laboratory colony maintained at the Genetics Institute, University of Cagliari, Sardinia, for many years. Cephalic ganglia and gonads were dissected from IV instar larvae and pupae and fixed in methanol: acetic acid (3:1) for 30 s, transferred to 45–60 per cent acetic acid and squashed under a siliconized coverslip. Air dried slides were stored at 4°C for one day before processing. The following restriction enzymes were tested: Alu I, Mbo I, Hae III, Hpa II and Msp I (Boehringer, FDR, and New England Labs., U.K.). Twenty units of the enzyme were diluted in Tris-saline buffer (Maniatis *et al.,* 1982) to a final volume of 100 μl for each slide. A coverslip was placed on the preparation to distribute the enzyme evenly. Incubation with the enzyme was carried out overnight at 37°C in a moist chamber. After the treatment slides were washed in tap water and stained with 5 per cent Giemsa in distilled water. Control preparations were treated with the incubation buffer under the same experimental conditions.

RESULTS

The restriction endonucleases, Alu I, Hae III, Mbo I and Hpa II extensively digested all euchromatic regions and only partially altered the heterochromatic ones. Differences of banding pattern between *Anopheles atroparvus* and *A. labranchiae* were restricted to the sex chromosome heterochromatin (figs 1, 2, 3, 4). The digestion pattern of each enzyme did not present significant differences from the one produced by the other enzymes tested.

In *A. atroparvus*, the proximal and terminal regions of the X and Y long arms were resistant to RE digestion and stained darkly with Giemsa (figs 1; 2(a, c, e, g); 3(a); 4). An intercalary band, sometimes distinct in two subunits, was observed after digestion with Hae III, Mbo I and, less clearly, with Alu I and Hpa II (figs 2(a, g); 4(c, e). In some early metaphase stages the proximal band was resolved in two smaller adjacent bands (fig. 2(g)). The terminal band did not include the telomeric tip; however this differentiation was not appreciable in condensed metaphase chromosomes. The amount of heterochromatin resistant to RE treatment varied from 50 to 80 per cent of the X–Y long arm (mean = 63.1). This quantitative variation was due to a high level of heterochromatin polymorphism (figs 2(a, c, e, g); 4(a, c, e)). Two size variants of the proximal band and two of the terminal band were most frequently observed (fig. 1). These variants were shared by both sex chromosomes. However, the large variant of the proximal band was more frequent on the Y chromosome than on the X (figs 2(c, e, g); 4(a)). Of 11 males scored, eight had a larger proximal band on the Y chromosome. Variants of the distal band were equally distributed in the two sex chromosomes. The idiogram in fig. 1 shows the banding patterns most frequently observed on the X and Y chromosomes. Variants with extensive loss of digested heterochromatin (intercalary pale region) were also observed (fig. 4(e)).

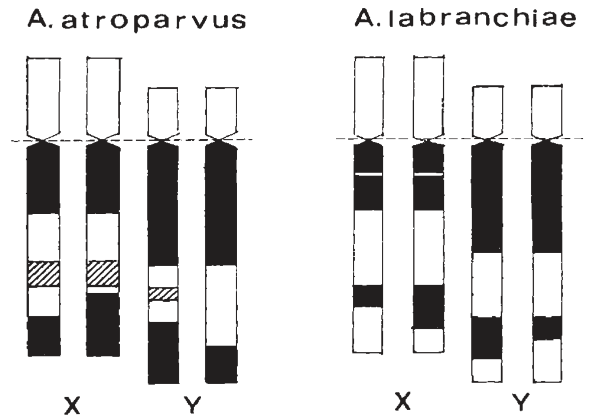

Figure 1 Main features and differences of the sex chromosome banding pattern in *Anopheles atroparvus* and *Anopheles labranchiae* after treatment with the restriction enzymes, Alu I, Hae III, Mbo I and Hpa II. Idiograms represent the patterns and band variants most frequently observed on the X and Y chromosomes. However, within each species, patterns found on the X are also shared by the Y and vice versa. Dark areas are the constant RE resistant bands, shaded areas are less conspicuous and variable bands. Presence of two proximal bands is only shown on the X chromosome of *A. labranchiae*, where the bands are very often distinct, while on the Y and on the sex chromosomes of *A. atroparvus* proximal bands are usually fused together.

In *A. labranchiae*, the X–Y banding pattern produced by Alu I, Mbo I, Hae III and Hpa II was different from the pattern of *A. atroparvus* in terms of band position and amount of heterochromatin digested by the enzymes (fig. 1). This pattern was

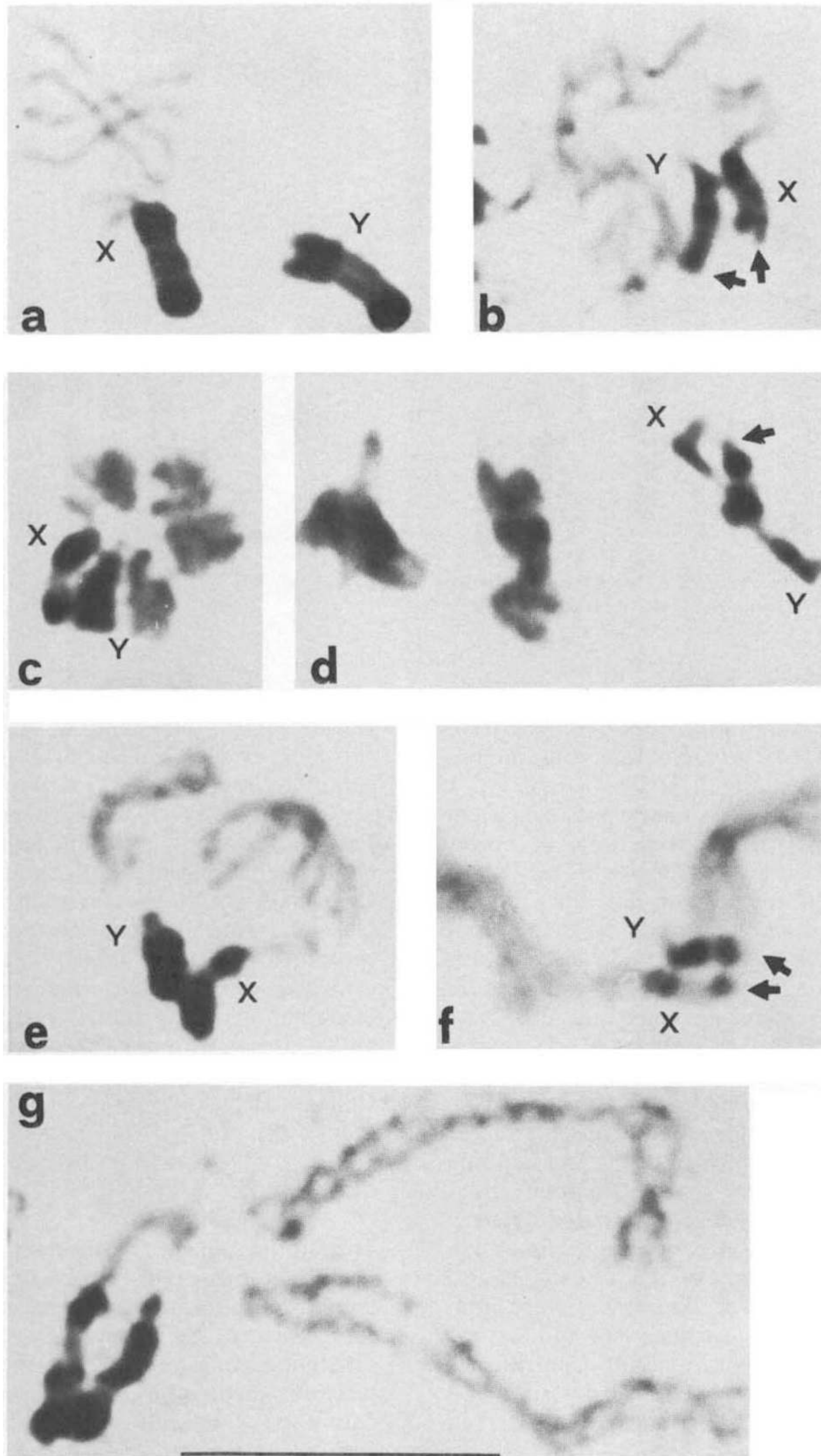

Figure 2 Banding pattern produced by Alu I (a, b), Hae III (c, d) and Mbo I (e, f, g) on the mitotic chromosomes of *A. atroparvus* (a, c, e, g) and *A. labranchiae* (b, d, f) male larvae: (e) and (g) are different mitotic stages from the same individual. Arrows point to the pale telomeric region of the sex chromosome long arm altered by the three enzymes in *A. labranchiae*. Bar represents 10 μm.

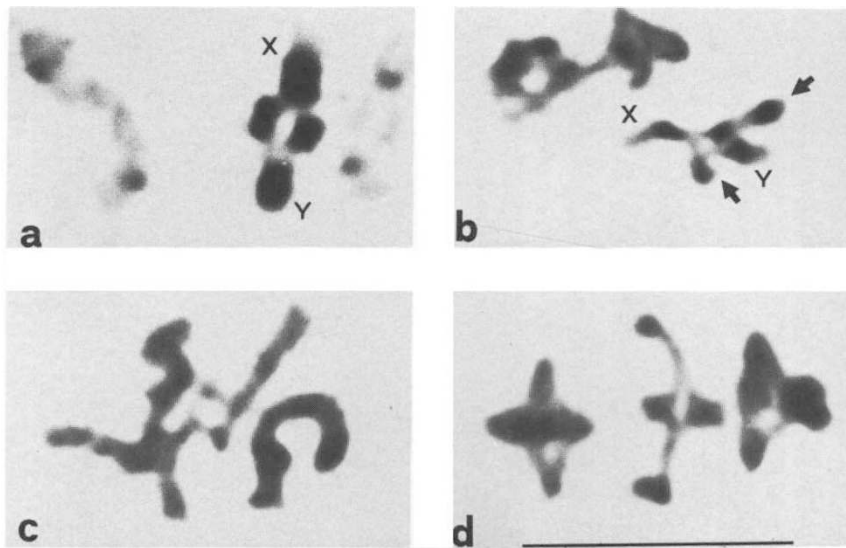

Figure 3 Banding pattern produced by the isoschizomers Hpa II (a, b) and Msp I (c, d) on meiotic chromosomes of *A. atroparvus* (right) and *A. labranchiae* (left). Bar represents 10 μm.

characterized by two proximal and one distal bands and by the digestion of the terminal 10–15 per cent of the heterochromatic long arm, including the telomere (figs 2(b, d, f); 3(b); 4(b, d, f)). A faint intercalary band, less sharp and consistent than in *A. atroparvus*, was sometimes observed. The amount of RE resistant heterochromatin was significantly lower (mean = 48 per cent) than in *A. atroparvus* and varied from 25 to 63 per cent of the long arm. Both proximal and distal bands were found polymorphic (fig. 1). Variants were common to both sex chromosomes (figs 2(b, d, f); 3(b); 4(b, d, f)). However, like in *A. atroparvus*, the Y chromosome carried the largest variant of the proximal band more frequently than the X (fig. 2(b, d, f)). Patterns most frequently observed in *A. labranchiae* are represented in fig. 1. The banding patterns of the two sibling species did not overlap, except in the infrequent case of terminal deletion in the X–Y long arm in *A. labranchiae* (fig. 4(b)).

The euchromatic arms of the autosomes and the short arm of the sex chromosomes, were completely digested by the four enzymes and stained faintly with Giemsa in both species. Centromeric regions were less sensitive to enzyme digestion and stained darker than the arms, particularly in meiosis.

Msp I produced almost no banding in either species (fig. 3(c, d)). In mitosis, the sex chromosomes sometimes showed a hint of differentiation similar to the one produced by the other four enzymes. Most frequently, and always in meiotic chromosomes, no banding was observed and chromosomes appeared darkly stained as in the control slides.

Quantitative heterochromatin differences between the X and Y chromosomes did not inhibit chromosome pairing and chiasmata formation. Chiasmata within the sex pair were observed in males carrying different heterochromatic variants (proximal and distal bands) on the X and Y long arms (fig. 5(a)). In these heterozygous males, recombination within the X–Y pair was visualized by the presence of sex chromosomes with heteromorphic chromatids in metaphase and anaphase II stages (fig. 5(b, c, d)).

DISCUSSION

The sibling species *Anopheles labranchiae* and *Anopheles atroparvus* possess typical *maculipennis* type sex chromosomes, with submetacentric X and subtelocentric Y. The two species have similar distribution of C-positive heterochromatin but are discriminated by the Q-banding pattern of the X-Y pair and by the differential response of the Y chromosome short arm after acid-alkaline treatment followed by Coriphosphine O staining (Mezzanotte and Ferrucci, 1978; Mezzanotte *et al.*, 1979). Structural chromosome differences between the two species were revealed by digestion with

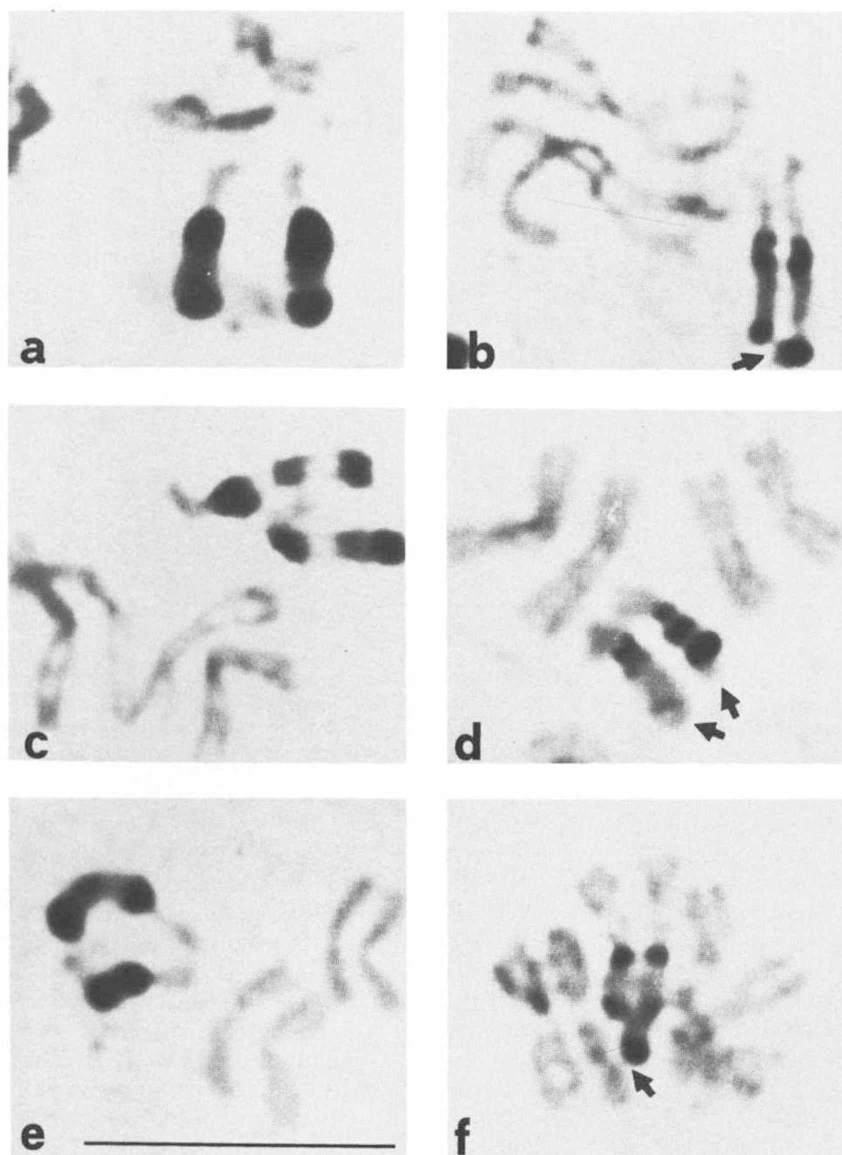

Figure 4 Intraspecific heterochromatic polymorphism in *A. atroparvus* (left) and *A. labranchiae* (right) revealed by digestion with Alu I (a, b), Hae III (c, d) and Mbo I (e, f). Mitoses from females heterozygous for different heterochromatic variants. Arrows point to the pale telomeric region of the X long arm altered by the three enzymes in *A. labranchiae*. Bar represents 10 μm.

four restriction endonucleases (Alu I, Hae III, Mbo I and Hpa II). The banding pattern produced by these enzymes on the sex chromosomes of the two species was distinctive in terms of localization of bands and amount of digested and undigested heterochromatin. A larger amount of heterochromatic material was altered in *A. labranchiae*, as compared with *A. atroparvus*, suggesting the presence, in the former species, of a higher number of DNA target sequences available for enzyme cleavage. Differences of banding pattern between the two species mainly concerned digestion of the terminal 10–15 per cent of the X-Y long arm in *A. labranchiae*, while the corresponding region in *A. atroparvus* was not altered by the four enzymes. These results confirmed that chromosome divergence between the two sibling species has involved changes, and probably rearrangements, in the heterochromatin DNA of the sex chromosomes, while the structural organization of the autosomes

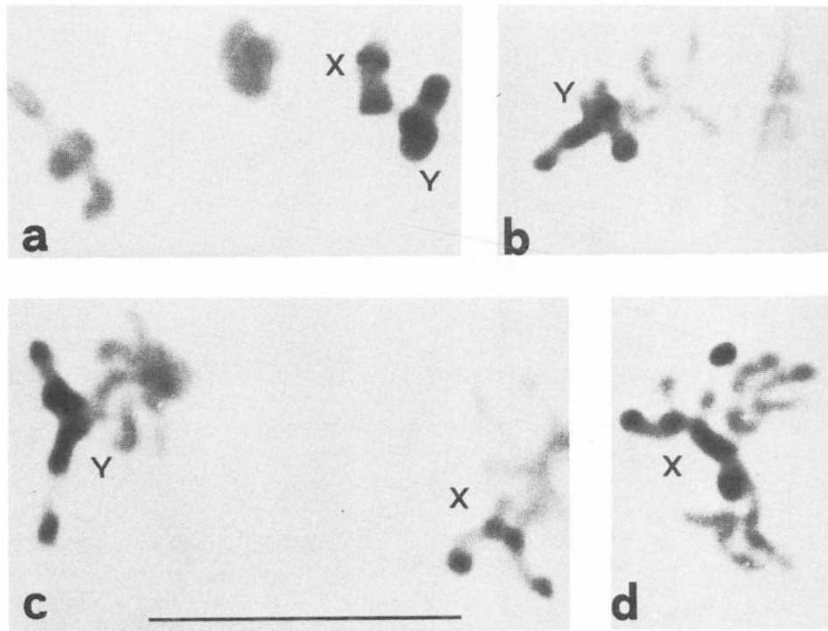

Figure 5 Meiotic chromosomes, digested with Hae III, from testes of *A. atroparvus* larva. (a): metaphase I with sex chromosomes carrying different heterochromatic variants; (b) and (c) anaphase II stages in which the sex chromosomes have heteromorphic chromatids with different banding patterns, as a consequence of a proximal crossing-over; (d) anaphase II showing the result of an interstitial crossing-over, the chromatids of each sex chromosome carry the two variants of the distal band. Bar represents 10 μm.

has not been dramatically modified, as suggested by the banding homologies of polytene chromosomes (Tiepolo *et al.*, 1974).

The sex chromosome heterochromatin of these sibling species was only partly digested by REs, resembling, particularly in *A. atroparvus*, the longitudinal differentiation generated by strong alkaline denaturation (NaOH), *i.e.*, proximal and distal C-positive bands on the long arm (Tiepolo *et al.*, 1975; Marchi, personal observation). Besides confirming the presence of these different heterochromatin types, digestion with REs revealed additional heterogeneity within the heterochromatic arm of the sex chromosomes (table 1). In *A. atroparvus*, the C-positive regions of the long arm are also bright fluorescent with quinacrine (Tiepolo *et al.*, 1975). These regions are resistant to RE digestion. However, the RE resistant heterochromatin includes also C and Q-negative regions. On the other hand, the C-positive heterochromatin of *A. labranchiae*, which fluoresces dull with quinacrine (Mezzanotte and Ferrucci, 1978), is not totally resistant to RE digestion. While correspondence between C-positive and RE resistant heterochromatin exists for the proximal bands, the terminal region, although C-positive, is altered by the three enzymes. These results confirm the usefulness of RE digestion in uncovering structural differences within heterochromatic regions.

Table 1 Heterochromatin heterogeneity revealed by standard C- and Q-banding techniques and by digestion with restriction enzymes. Re+ refers to the RE resistant heterochromatin

Species	*A. atroparvus*			*A. labranchiae*		
Banding	C	Q	RE	C	Q	RE
Heterochr. types	+	+	+	+	−	+
	+	+	−	+	−	−
	−	−	+	−	−	+
	−	−	−	−	−	−

Intraspecific heterochromatin polymorphism, undetected with standard banding techniques, was common in both *Anopheles* species after digestion with REs. Heterochromatin variation was quite high even in the highly inbred colony of *A. atroparvus*, although lower than in the field populations of *A. labranchiae*. This situation is quite different from that found in species of the *Anopheles gambiae* complex using fluorescent staining: the sex chromosomes of most laboratory populations were monomorphic and natural populations only possessed one or two heterochromatic variants

(Bonaccorsi *et al.*, 1980). It would be interesting to test whether or not additional polymorphism can be revealed also in these species using restriction enzymes. Variants in *A. labranchiae* and *A. atroparvus* were characterized by changes in the relative amount of digested and undigested heterochromatin, usually with little or no changes of total chromosome length; extensive loss of heterochromatin was observed in the two species only in a few cases. The X and Y chromosomes shared the same heterochromatic variants. However, difference in the frequency of the proximal band variants were observed between X and Y. The proximal band was usually larger in the Y than in the X chromosome, possibly due to low recombination frequency in the proximal region. This hypothesis is supported by data on chiasmata frequency in *A. atroparvus* males which indicate a low frequency of proximal chiasmata (0·03) as compared with interstitial chiasmata (0·28) (Jayakar *et al.*, 1982).

High heterogeneity and variability are characteristic features of heterochromatin. In the *Anopheles* genus the heteromorphic sex chromosomes are partly or totally heterochromatic (White, 1980) and intraspecific polymorphism of heterochromatic regions is quite common (Baimai and Traikpavasin, 1987; Bonaccorsi *et al.*, 1980; Vasantha *et al.*, 1982). Many, if not most, characteristics of constitutive heterochromatin are linked to the presence of high concentrations of satellite DNAs in this type of chromatin. In *Anopheles stephensi*, satellite DNA has been localized in the heterochromatic arms of the X and Y chromosomes by *in situ* hybridization (Redfern, 1981). Likewise, the heterochromatic, high polymorphic and RE-resistant heterochromatin of *A. labranchiae* and *A. atroparvus* may contain satellite DNAs which do not possess cleavage sequences of the REs tested. Intraspecific and interspecific variation in the sequence and abundance of highly repetitive DNA is also widespread in nature and has been reported in several mosquito species of the genus *Aedes* (McLain *et al.*, 1986; McLain *et al.*, 1987). The occurrence of such high polymorphism would imply that heterochromatin is not essential in basic cell functions and heterochromatic variations may be without any selective advantage. On the other hand, experimental observations support the adaptive significance of heterochromatin in recombination (John, 1981) and cell growth (Macgregor and Sessions, 1986). Although the evolutionary significance of these variations and, in general, of heterochromatin and associated DNA is still debated, they probably play an important role in evolution producing "most of the changes in chromosome size and structure during divergence of species" (Flavell, 1982), ultimately reinforcing reproductive isolation of populations. Heterochromatin variants in *A. labranchiae* and *A. atroparvus* probably arise quite frequently and spread in the populations by drift phenoman. Karyotypic divergence in the two sibling species could have derived from a common ancestor by fixation of different heterochromatic variants with progressive decrease of chromosome homology.

Differently from the other enzymes, Msp I did not produce any banding pattern on the chromosomes of *A. labranchiae* and *A. atroparvus*. Msp I and Hpa II are isoschizomers which cleave the same sequence, CCGG. Their activity is inhibited by methylation of the external (Msp I) or internal (Hpa II) cytosine (Maniatis *et al.*, 1982). The result obtained in the two sibling species would suggest that selective methylation of the external cytosine and consequent inhibition of the Msp I digestion occurs in euchromatic and heterochromatic regions. However, differential access of the enzymes to the target sequences could also play a role in the resulting pattern. Bianchi and coworkers (1986) obtained quite different pattern on a cell line of the mosquito, *Aedes albopictus*, indicating lack of methylation in most CCGG sequences. In another mosquito, *Culiseta longiareolata*, both enzymes produced the same banding pattern on larval brain and gonadal cells, with digestion of the euchromatic regions but not of the heterochromatic ones (Marchi and Mezzanotte, 1988). These results suggest that different levels of methylation, or different chromatin organization, exist in different species of the Culicidae family.

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