

## G banding in two species of grasshopper and its relationship to C, N, and fluorescence banding techniques

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A G banding technique combining trypsin and hot saline treatments was used to analyze the chromosomes of two grasshopper species, *Eyprepocnemis plorans* and *Locusta migratoria*, both of which contain both standard and supernumerary heterochromatin. Although this technique does not produce G bands like those in mammalian chromosomes, it serves to characterize heterochromatic regions whose nature has been inferred from other banding techniques (C, N, CMA, and DAPI banding). The light regions revealed by G banding contain GC-rich DNA sequences, the more prominent of which coincide with nucleolus organizer regions (NORs). Furthermore, the proximal heterochromatin in *E. plorans* was heterogeneous, and the standard and supernumerary heterochromatin showed conspicuous differences in organization. Supernumerary heterochromatin is an exception to the regular patterns shown by the standard heterochromatin. The findings are related to the mechanism of action of these banding techniques.

*Key words*: banding techniques, grasshoppers, *Eyprepocnemis plorans*, *Locusta migratoria*.

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Une technique de révélation des bandes G, combinée à des traitements à la trypsine et des traitements salins à chaud, a été utilisée pour analyser les chromosomes de deux espèces de sauterelles, l'*Eyprepocnemis plorans* et le *Locusta migratoria*, espèces qui toutes deux contiennent soit de l'hétérochromatine standard et de l'hétérochromatine excédentaire. La technique utilisée, bien qu'elle ne révèle pas les bandes G telles qu'observées chez les chromosomes des mammifères, permet de caractériser des régions hétérochromatiques dont la nature a été inférée par d'autres techniques de révélation des bandes, savoir les bandes C, N, CMA et DAPI. Les régions claires révélées par les bandes G contiennent des séquences d'ADN riches en G-C, dont la plus proéminente coïncide avec les régions organisatrices de nucléoles. De plus, l'hétérochromatine proximale chez l'*E. plorans* s'est avérée hétérogène et la chromatine standard tout comme l'excédentaire ont présenté d'importantes différences organisationnelles. L'hétérochromatine excédentaire, par rapport à l'hétérochromatine standard, constitue une exception. Ces observations sont reliées aux mécanismes d'action de ces techniques de révélation des bandes.

*Mots clés* : techniques de révélation des bandes, sauterelles, *Eyprepocnemis plorans*, *Locusta migratoria*.

[Traduit par la rédaction]

### Introduction

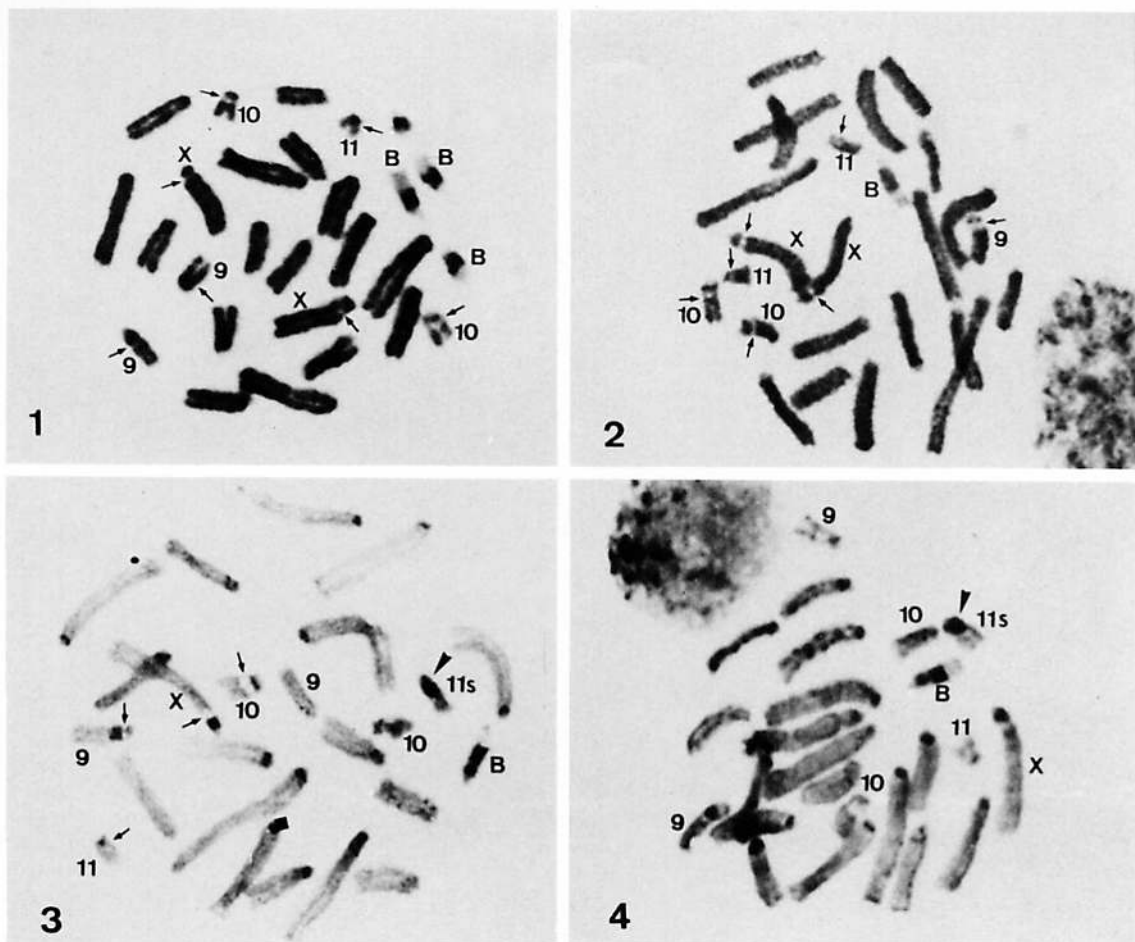
The most widely accepted explanation for the nature of G bands is based on the enhancement of the chromomere pattern pre-existing in the metaphase chromosome by inducing a rearrangement of the chromatin fibers from interbands to G bands, possibly with some extraction of DNA from interbands, and covering the remaining DNA in the interbands with denatured nonhistone proteins, followed by enhancement of this pattern with Giemsa staining (Comings 1978). The comparison of the G banding pattern with Q and R banding patterns, obtained with AT- and GC-specific fluorochromes, indicates that G bands contain AT-rich DNA and interbands have GC-rich DNA (Comings 1972, 1978). Merrick et al. (1973) demonstrated that trypsin produced G or C bands in human chromosomes, depending on the intensity of the treatment. A technique combining trypsin and hot saline treatments to yield G bands of very high quality in mammalian chromosomes was recently developed by Burgos et al. (1986). We now describe the results of this technique in grasshopper chromosomes and compare these findings with those obtained with C, N, CMA, and DAPI banding techniques. We investigated two species,

*Eyprepocnemis plorans* and *Locusta migratoria*, both of which carry supernumerary heterochromatin in the form of B chromosomes and supernumerary segments.

### Materials and methods

Adult males and females of *Eyprepocnemis plorans* and *Locusta migratoria* were caught at several localities in the province of Granada (Spain) and reared in laboratory cages to obtain egg pods, which were incubated at 27°C for 6 (*L. migratoria*) or 10 (*E. plorans*) days. The embryos were dissected out of the eggs and immersed in 1 mL 0.05% colchicine in insect saline solution for 90 (*L. migratoria*) or 120 (*E. plorans*) min. Afterward, 1 mL distilled water was added for hypotonic shock for 15 min, and the embryos were fixed in ethanol - acetic acid (3:1).

Cytological preparations of the embryos were made using a method based on Meredith's technique (1969) for preparing meiotic chromosomes from mammalian testes. Embryos were immersed for 3 min in 25 µL 70-80% acetic acid in a glass tube with a conical base, and then dispersed and suspended with the aid of a micropipette. After 3 min the cell suspension was taken up into the micropipette and one drop was transferred to a clean grease-free slide kept at 60°C on a flat surface. The drop was immediately



FIGS. 1-4. Embryonic mitotic metaphase cells of *E. plorans* submitted to the G banding technique at various intensities: weak (Fig. 1), mild (Fig. 2), and strong (Figs. 3 and 4). Note the differential staining of B chromosomes in all cases. Arrows in Figs. 1-3 point to interstitial light G bands ( $G^-$ ) on chromosomes X, 9, 10, and 11 coinciding with the location of standard NORs in this species. Arrowheads in Figs. 3 and 4 point to the supernumerary segment proximally located on chromosome 11.

withdrawn into the micropipette and expelled onto a different region of the same slide, where it was picked up again. This procedure was repeated until four or six drops had been placed on several slides, a single embryo preparation yielded enough material for three to four slides.

C banding was performed as described in Camacho et al. (1984). G banding was performed according to the technique of Burgos et al. (1986) with slight modifications: slides were immersed for 15-25 s in 0.0125% trypsin in  $Ca^{2+}$ - and  $Mg^{2+}$ -free phosphate-buffered saline solution, washed under running tap water, and immersed in  $2\times$  SSC ( $1\times$  SSC: 0.15 M NaCl plus 0.015 M sodium citrate) at  $65^\circ C$  for 5-10 min. Slides were then washed in distilled water, stained for 30-60 s with 20% Giemsa, washed in distilled water, dried under hot air, and mounted in DPX.

N banding was done using a simplified version of Fox and Santos's technique (1985): slides were immersed in a 50% mixture of formamide and  $2\times$  SSC at  $60^\circ C$  for 1 min, washed under running tap water, stained with 10% Giemsa, and mounted in DPX.

CMA and DAPI banding were performed by triple fluorescent staining with chromomycin  $A_3$  (CMA), distamycin A (DA), and 4'-6-diamidino-2-phenylindole (DAPI), based on the method of Schweizer (1980, 1981). This technique reveals the chromomycin  $A_3$  R bands in addition to DA-DAPI bands on the same chromosome. A few drops of CMA were placed on the slides, which were then covered with cover slips and incubated in the dark at  $37^\circ C$  for 60 min. After this, they were washed first in tap water and then

in distilled water, and dried under a warm air stream. Counterstaining in the dark by DA at the same temperature took 15 min. After a second washing the slides were stained with DAPI during 40 min at room temperature, followed by washing and drying, and mounting in McIlvaine's buffer pH 7 - glycerol (1:1). All slides were stored at  $37^\circ C$  in the dark for a minimum of 48 h before observation with a Nikon fluorescence microscope.

## Results

### G banding

Although this technique does not yield G bands like those in mammalian chromosomes, it gave us considerable information about the nature of many chromosomal regions in both species of grasshopper, making it possible to distinguish certain chromosome regions even with very brief treatments. Different times of treatment gave different patterns of G banding in *E. plorans* chromosomes. The first zones to appear with weak G banding were light regions similar to secondary constrictions in the X, 9, 10, and 11 standard chromosomes at sites where NOR activity has been demonstrated (Cabrero et al. 1987), in addition to a grey part in the B chromosome (Fig. 1). This G banding pattern makes the B chromosomes easily identifiable for routine studies.

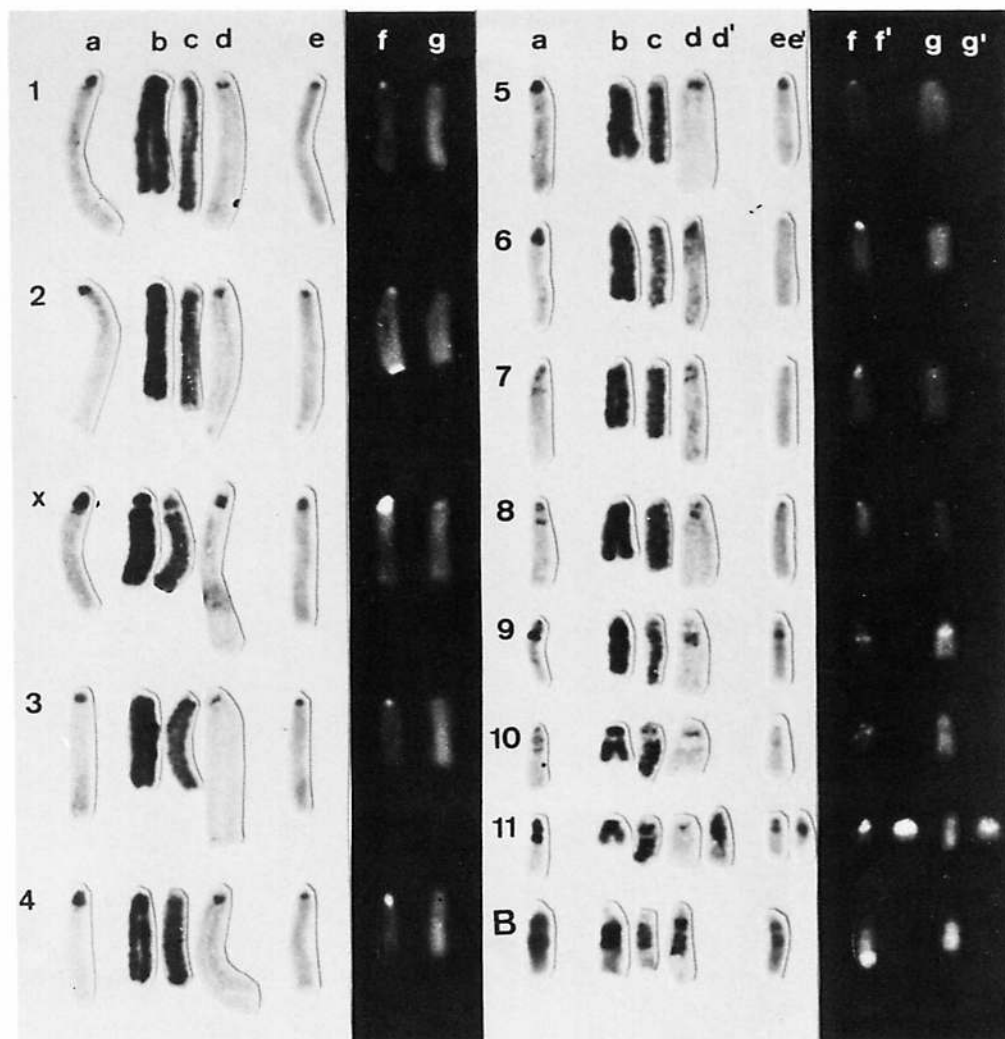


FIG. 5. Haploid karyogram of *E. plorans* chromosomes submitted to C banding (a), weak G banding (b), mild G banding (c), strong G banding (d), N banding (e), chromomycin A<sub>3</sub> (CMA) banding (f), and DAPI banding (g). d', e', f', and g' show G, N, CMA, and DAPI banding, respectively, of the supernumerary segment on the chromosome 11.

Mild treatment yielded a slightly more pronounced pattern, so that in addition to the clear regions, many narrow grey interbands appeared in the standard chromosomes. The appearance of the B chromosome was similar to that seen with weak G banding (Fig. 2).

Longer treatment produced a banding pattern that resembled C banding; the chromosomes showed a very limited number of dark and light bands and interbands (Figs. 3 and 4). However, light zones in the X, 9, 10, and 11 standard chromosomes were still present, whereas with C banding in these regions was not visible (see below). After intense treatment, standard chromosomes usually showed three types of chromatin: dark presumably corresponding to some type of heterochromatin; clear, corresponding to NORs; and grey, in most places. The B chromosome also showed three different regions: the same dark interstitial zone as was revealed by weaker treatments; a grey centromeric region, which included the short arm and part of the long arm; and a clear distal region. Thus, increasing the intensity of G banding only affected this latter distal zone. Chromosome pair 11 is heterozygous in Figs. 3 and 4 for a proximally located supernumerary heterochromatic segment, which was darkly stained by intense G banding.

#### Comparative study of G banding with C, N, CMA, and DAPI banding techniques

##### (i) *Eyprepocnemis plorans* (Fig. 5)

###### Proximal bands

C banding reveals conspicuous dark bands in proximal regions of most standard chromosomes. However, these C bands may be subdivided into two different regions in chromosomes 1, 3–8, 10, and 11, on the basis of their differential response to the other banding techniques. While the pericentromeric region, including the centromere, is C<sup>+</sup>, G<sup>-</sup>, N<sup>+</sup>, CMA<sup>+</sup>, and DAPI<sup>-</sup>, the paracentromeric part of the proximal C band is C<sup>+</sup>, G<sup>+</sup>, N<sup>0</sup>, CMA<sup>0</sup>, and DAPI<sup>0</sup> (see Fig. 5, a, d, e, f, and g). This demonstrates that proximal C heterochromatin is heterogeneous in nature (Fig. 7). The X chromosome shows a similar pattern for the proximal C band, with the only peculiarity being that both proximal subregions are CMA<sup>+</sup>.

###### Interstitial bands

Scarce interstitial bands are present in the standard chromosomes of *E. plorans*. They are only present in chromosomes X and 7–11, and most (excepting only those in chromosomes 7 and 8) coincide with the localization of NORs.

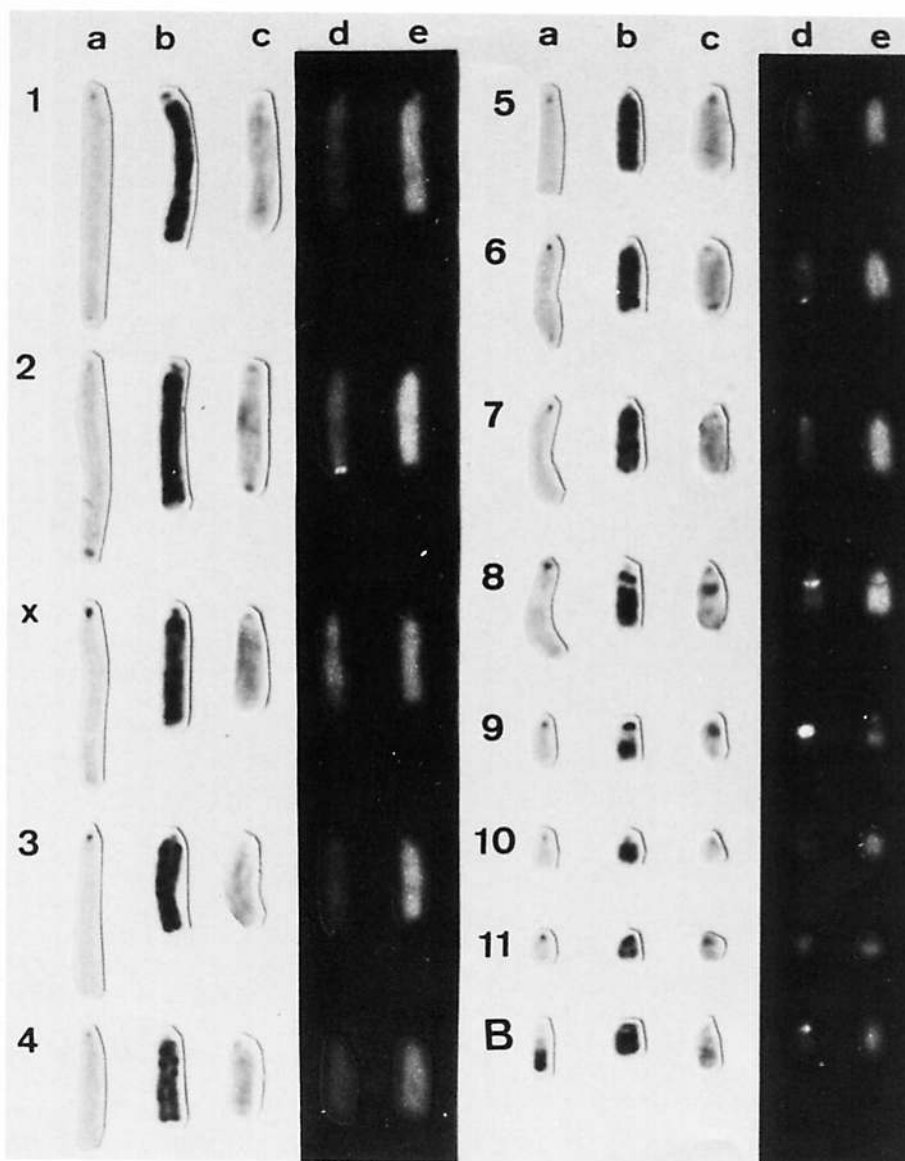


FIG. 6. Haploid karyogram of *L. migratoria* chromosomes submitted to C (a), G (b), N (c), CMA (d), and DAPI (e) banding techniques. Note the presence of distal G<sup>-</sup>, N<sup>+</sup>, CMA<sup>+</sup>, and DAPI<sup>-</sup> bands on chromosomes 2 and 6, in addition to an interstitial G<sup>-</sup>, N<sup>+</sup>, CMA<sup>+</sup>, and DAPI<sup>-</sup> band on chromosome 9. These three bands coincide with the location of primary NORs in this species. The interstitial band on chromosome 8, however, does not contain any active NOR despite the fact that it shows the same banding responses as NORs on chromosomes 2, 6, and 9.

Two types of interstitial bands may be distinguished, those containing a NOR (in chromosomes X and 9–11) and those without (in chromosomes 7 and 8), and the banding responses of them were conspicuously different: whereas the bands containing a NOR in chromosomes X, 9, 10, and 11 showed C<sup>+</sup>, G<sup>-</sup>, N<sup>+</sup>, CMA<sup>+</sup>, and DAPI<sup>-</sup> responses, those in the chromosomes 7 and 8 (without an active NOR) showed C<sup>+</sup>, G<sup>+</sup>, N<sup>0</sup>, CMA<sup>0</sup>, and DAPI<sup>0</sup> responses.

#### Distal bands

The only distal band observed was in chromosome 2, and it was very small and associated with a subdistal secondary constriction. This small satellite shows a greyish response to C, G, and N banding and an intensely positive response to CMA banding. The secondary constriction is particularly evident with the strong G banding technique (Fig. 5, d).

Chromosome 11 was frequently polymorphic for a prox-

imally located supernumerary chromosome segment, which was C<sup>+</sup>, G<sup>+</sup> (Fig. 5, d'), N<sup>+</sup> (Fig. 5, e'), CMA<sup>+</sup> (Fig. 5, f'), and DAPI<sup>+</sup> (Fig. 5, g').

The B chromosome showed two large interstitially located C bands, but the remaining chromatin appeared darker than the euchromatin of standard chromosomes, being almost totally heterochromatic, with the possible exception of the short arm and the zone between the two dark C bands (Fig. 5, a). G banding gave a very similar banding pattern, but the difference between dark and light zones was much more apparent even with weak treatments, the dark C bands being more resistant to G banding treatment than the distal greyish C banded regions (see Fig. 5, b–d). G banding also yielded a clearer definition of the bands than C banding (compare Fig. 5, a with d). The N banding pattern also coincided with C and G patterns, although the more distal interstitial N band seemed to be shorter than the correspond-

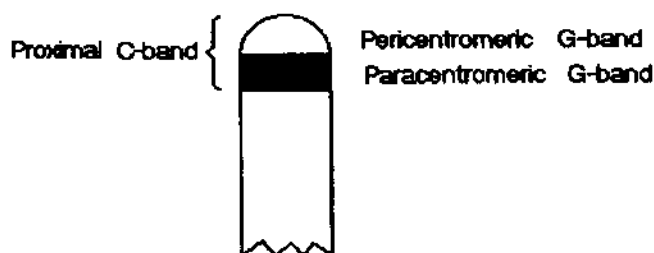


FIG. 7. A schematic representation of the proximal region of most chromosomes in *E. plorans*. Two regions are distinguishable in proximal C bands, a pericentromeric light G band and a paracentromeric dark G band.

ing C and G bands. The B chromosome may be divided into two different regions on the basis of its response to fluorochromes, the distal third being CMA<sup>+</sup>, DAPI<sup>-</sup> and the two proximal thirds, including the two dark C bands, being CMA<sup>-</sup>, DAPI<sup>+</sup> (see Fig. 5, f and g).

### (iii) *Locusta migratoria* (Fig. 6)

This species shows scarce heterochromatin, so that the bands from the different banding techniques are usually small.

#### Proximal bands

C banding yielded very small bands in all chromosomes (Fig. 6, a), which correspond to faint N bands (Fig. 6, c) but not to CMA bands, with the single exception of the B chromosome (Fig. 6, d).

#### Interstitial bands

There were no interstitial C bands other than the negative one in chromosome 8 (Fig. 6, a) which sometimes appears darkly stained (Viseras et al. 1991). G banding revealed a negative interstitial band in both chromosomes 8 and 9 (Fig. 6, b). The band in chromosome 9 corresponded to an active NOR, but that in chromosome 8 did not (Salcedo et al. 1988). Both negative G bands were N<sup>+</sup> (Fig. 6, c), CMA<sup>+</sup> (Fig. 6, d) and DAPI<sup>-</sup> (Fig. 6, e).

#### Distal bands

Only chromosomes 2, 6, and B showed distal bands. The distal bands in chromosome 2 and 6 were C<sup>+</sup> (Fig. 6, a), G<sup>-</sup> (Fig. 6, b), N<sup>+</sup> (Fig. 6, c), CMA<sup>+</sup> (Fig. 6, d), and DAPI<sup>-</sup> (Fig. 6, e) and coincided with the location of active NORs (Salcedo et al. 1988). The B chromosome showed a large C band occupying its entire distal half (Fig. 6, a). This C band was N<sup>+</sup> (Fig. 6, c) but did not respond to CMA (Fig. 6, d) or DAPI (Fig. 6, e). G banding in the B chromosome gave a pattern of three dark G bands (proximal, interstitial, and distal), alternating with two light interbands (Fig. 6, b).

## Discussion

The G banding technique used to analyze the chromosomes of two species of grasshopper, *L. migratoria* and *E. plorans*, did not produce true G bands in the sense used for mammalian chromosomes (Burgos et al. 1986), since a sequence of dark bands and light interbands was not observed. It nevertheless provides considerable information about heterochromatic regions in both standard and B chromosomes. The findings are comparable with those obtained in the grasshopper *Chortoicetes terminifera* by Webb (1976) and Webb and Nehaus (1979): A and B chromosomes

showed different patterns, the former being characterized by only a few gaps (light interbands) and the latter showing dark bands and light interbands. Some authors do not consider these types of banding patterns G bands, on the basis that they appear only in heterochromatic regions (Greilhuber 1977) and give homogeneous staining rather than crossbanding in most chromosomes (Zhan et al. 1984). These latter authors were able to produce true G bands in a species of grasshopper by using the high resolution G banding technique. We agree with Webb and Nehaus (1979) in considering that G bands are produced by G banding techniques, despite their conspicuous differences from those produced in mammalian chromosomes.

The nature of the G bands we obtained in *L. migratoria* and *E. plorans* was investigated by comparing them with the banding patterns produced by C, N, CMA, and DAPI banding techniques. This approach also made it possible to identify a series of chromosome regions with particular banding patterns. These are exclusively heterochromatic regions, most of which C band darkly. The only regions that did not C band (interstitially located in chromosomes 8 and 9 of *L. migratoria*) reacted positively with N and CMA banding, which demonstrated their heterochromatic nature.

Some general concluding remarks can be made from the present results as follows.

(i) The close correspondence between different banding patterns in standard chromosomes indicates that these banding techniques may serve to demonstrate particular types of heterochromatin in grasshoppers. The light G interbands correspond to dark N bands and bright CMA bands. Since it is widely accepted that CMA binds to GC-rich DNA (Schweizer 1981), light G bands and dark N bands probably also correspond to GC-rich DNA.

(ii) Strong G banding in *E. plorans* demonstrates the heterogeneity of the heterochromatin proximal to the centromere in standard chromosomes. This segment comprises a pericentromeric G<sup>-</sup> zone containing GC-rich DNA and a paracentromeric G<sup>+</sup> region presumably containing AT-rich DNA, although this was not demonstrated by DAPI staining. The correspondence observed between C bands and strong G banding (Merrick et al. 1973; Comings et al. 1973; Wang and Shoffner 1974; Comings 1978) could therefore be due to the prevalence of AT-rich DNA sequences in the C-heterochromatin, which G bands darkly. This condition is fulfilled in some human chromosomes with larger paracentromeric heterochromatic blocks, e.g., 1, 9, 16, and Y, which are DAPI<sup>+</sup> (Verma and Babu 1989). The DAPI<sup>0</sup> nature of the remaining C-heterochromatin may be a consequence of the limited sensitivity of this fluorochrome to detect minute amounts of AT-rich DNA sequences.

(iii) Supernumerary heterochromatin reacts differently from standard heterochromatin to some banding techniques. Thus the extra segment located proximally on chromosome 11 of *E. plorans* contains mostly GC-rich DNA sequences, since it is CMA<sup>+</sup>, and some AT-rich DNA, since it is partly DAPI<sup>+</sup>. Being a standard chromosome it should be G<sup>-</sup>, not G<sup>+</sup>. The B chromosomes of *E. plorans* and *L. migratoria* also fail to show the same correspondence between the different banding techniques as in standard heterochromatin. In *E. plorans* the B chromosome shows the same G and N banding patterns, which contradicts observations in standard heterochromatin, where G<sup>-</sup> regions corresponded to N<sup>+</sup> regions. G<sup>-</sup> regions in the B chromo-

some are CMA<sup>+</sup> and DAPI<sup>-</sup>, like GC-rich DNA sequences in standard heterochromatin. In the B chromosome of *L. migratoria* similar C and N banding patterns were observed, although these patterns were not consistent with G, CMA, and DAPI ones. These observations indicate that supernumerary heterochromatin must have a different molecular organization from standard heterochromatin. Although it presumably evolved from chromatin in the chromosomes of the standard complement, the extra heterochromatin in the form of supernumerary segments or B chromosomes must have undergone remarkable random mutational changes which were innocuous in this dispensable chromatin, thus leading to conspicuously different chromatin organizations in each particular case of supernumerary heterochromatin. This would explain the inconsistency between the banding patterns of standard and supernumerary heterochromatin, as well as between different cases of the latter.

(iv) Dark G bands correspond to chromatin containing AT-rich DNA sequences, while light interbands reflect GC-rich DNA sequences (Comings 1972, 1978). Moreover, it seems clear that nonhistone proteins and their interactions with DNA are involved in the production of G bands (Burkholder and Ducek 1980). The G banding technique used in the present report involves trypsin treatment, which causes protein denaturation, and 2 × SSC treatment, which causes DNA extraction. The dark bands observed after strong G banding in *E. plorans* must result from no DNA extraction, whereas light interbands must result from considerable DNA extraction at these zones after trypsin + 2 × SSC treatment. This could indicate that the nonhistone proteins bound to GC-rich DNA sequences (in the light interbands) are less resistant to denaturation than those bound to AT-rich DNA sequences (in the dark bands). If so, it is peculiar that AT-rich DNA, which is less resistant to denaturation than GC-rich DNA, is complexed with nonhistone proteins which are more resistant to denaturation than those of GC-rich DNA chromatin. This produces a fine balance in the composition of the chromatin along the chromosomes.

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