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N-bands and nucleolus expression in *Schistocerca gregaria* and *Locusta migratoria*

D. P. Fox* and J. L. Santos†

*** Department of Genetics, University of Aberdeen, 2 Tillydrone Avenue, Aberdeen, AB9 2TN and † Department of Genetics, Universidad Complutense, Madrid**

A new technique is described for simultaneous N-band and nucleolus identification using acridine orange staining following incubation of chromosome squashes in 50 per cent formamide. This technique, together with silver deposition, has been used to clarify the chromosomal locations of N-bands and nucleolus organisers in the two locust species *Schistocerca gregaria* and *Locusta migratoria*. *S. gregaria* consistently shows interstitial nucleolus organisers in chromosomes L3 and M6 while *L. migratoria* has telomeric nucleolus organisers in L2 and M6 and an interstital nucleolus organiser in S9 close to the centromere. All nucleolus organisers also N-band and C-band but in addition both species have a large interstitial N-band on chromosome M8 which does not act as a nucleolus organiser. These data are discussed in relation to the mechanism of N-banding, the conflicting reports in the literature of different nucleolus organiser locations in these species and the significance of chromosome regions which N-band but do not act as nucleolus organisers.

INTRODUCTION

The nucleoli of eukaryotic cells characteristically form at specific chromosomal sites known as nucleolus organisers (NORs) (McClintock, 1934). Nucleoli may be detected in interphase or prophase either by phase-contrast microscopy or by specific staining techniques such as acetic carmine (McClintock, 1934; John and Henderson, 1962) or by silver deposition techniques (Rufas and Gosalvez, 1982). The NOR described by McClintock in maize is a compact, deeply staining structure attached to the nucleolus and a similar structure has been described in *Schistocerca gregaria* by John and Henderson (1962). In contrast, the site of the NOR at metaphase is often marked by a constriction. The correspondence between nucleolus number and position at interphase and secondary constriction number and position at metaphase and anaphase was first demonstrated by Heitz (1931).

In mammalian cells, bands (N-bands) may be produced by differential staining which correspond in position to NORs (Matsui and Sasaki, 1973). Further, NORs which have been active in the previous interphase may be demonstrated by various silver deposition techniques (Miller *et al.*, 1976; Goodpasture and Bloom, 1975; Bloom and Goodpasture, 1976; Hofgartner, *et al.*, 1979).

Several techniques have been used in an attempt to localise the NORs in the locust *Schistocerca gregaria*. John and Henderson (1962), using aceto-carmine staining of male dipolotene cells, claimed the positions of the NORs to be on chromosome L3 about 70 per cent of the length of the chromosome away from the terminal centromere and on chromosome M6 in a median position. In contrast Hagele (1978), using the N-banding technique of Matsui and Sasaki (1973) reported the presence of single N-bands at the telomeres of L2 and M6, one or two interstitial bands on M8 and a further band near the centromere on S9. Finally, Rufas and Gosalvez (1982), using a silver deposition technique in male meiotic cells, reported nucleoli attached to the M6 and S9 bivalents at diplotene.

We have attempted to resolve the conflict between the results reported above and to clarify the relationship between N-bands, secondary constrictions and NORs in *Schistocerca gregaria* and *Locusta migratoria* using silver staining of nucleoli and a newly-developed N-band technique which allows simultaneous visualisation of both N-bands and any nucleoli attached to them.

MATERIALS AND METHODS

Schistocerca gregaria and *Locusta migratoria* adults and egg pods were obtained from three sources, the Welsh Mountain Zoo, Bioserv Ltd. and the Zoology Department, Aberdeen University. Other grasshoppers mentioned were either collected in the wild or were gifts from Dr G. M. Hewitt and Dr G. H. Jones.

Silver deposition was carried out using the technique of Rufas and Gosalvez (1982) but with minor variations in the *p*H of the silver nitrate solution.

The N-banding technique was discovered by accident while attempting to produce controlled *in situ* denaturation of chromosomal DNA. The protocol is given below.

1. Fix 1 hour in 3 parts alcohol: 1 part acetic acid.
2. Store in 70 per cent alcohol at −20°C. Material can be stored for several months in this way.
3. Make squash preparations in 45 per cent acetic acid, freeze on dry ice, remove coverslips and plunge into 100 per cent alcohol. Air dry slides from alcohol.
4. Incubate slides at 65°C in a solution containing 50 per cent formamide and 1 ×SSC for 1 hour. The parameters employed in this stage may need to be varied for different species.
5. Wash slides in water.
6. Staining may be achieved in a number of different ways, *e.g.* Feulgen reaction, gallocyanin-chrome alum (Sandritter *et al.*, 1966) 2 per cent Giemsa in phosphate buffer (*p*H 6·8) or 0·05 per cent acridine orange in McIlvaine's buffer, *p*H 7·0 for 5 minutes.

RESULTS

N-bands

The N-band pattern of *S. gregaria* is shown in fig. 1 (gallocyanin-chrome alum staining) and fig. 3 (acridine orange staining). The band patterns are identical in both cases, with bands on L3, M6 and M8. Similarly, the *L. migratoria* N-band pattern is illustrated in fig. 2 (gallocyanin-chrome alum) and fig. 4 (acridine orange) and shows bands on L2, M6, M8 and S9. N-band patterns are also shown for three other grasshopper species, *Stethophyma grossum* (fig. 6), *Myrmeleotettix maculatus* (fig. 8) and *Chrothippus brunneus* (fig. 9). There is a clear differentiation of bands in all cases except *L. migratoria* where bands can still be seen but are less clearly delimited by this technique. In addition to N-bands, in some cases dots can be seen at the position of the centromere (see figs. 1 and 9). In *S. gregaria* and *L. migratoria* the N-bands all correspond to marked secondary constrictions at mitotic metaphase (see fig. 5, this paper and figs. 4–6 Fox *et al.* (1975) respectively). In the case of *Ch. brunneus* where there is a double band in the short arm of L3 there is also a double constriction at metaphase (see figs. 9 and 10 respectively).

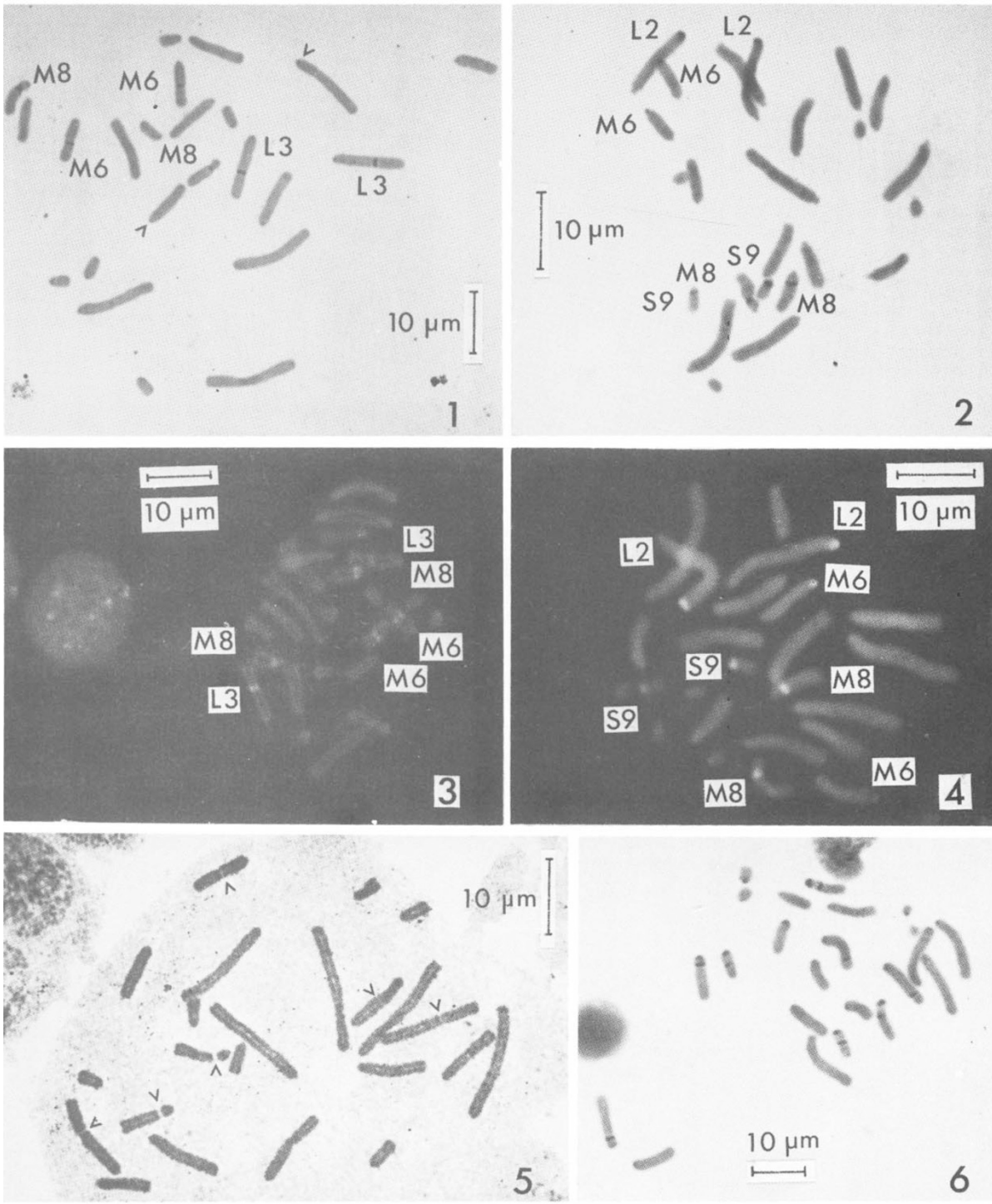
The locations of the bands for the species used have been found consistently to be in the same positions. This is particularly true for *S. gregaria* and *L. migratoria* which have been obtained from three different commercial sources. The locations are summarised in table 1.

Acridine orange-stained metaphases, when viewed in the fluorescence microscope, show bright green fluorescence in the N-bands and less bright yellow-green, orange or red-brown fluorescence in the non-N-banded parts of the chromosomes. In interphase (figs. 11–14) the N-bands remain compact and usually are distinctly separated from each other. They have the same staining properties as at metaphase. Attached to some bands is a small, red, nucleolus-like body (figs. 11 and 13). The number of these bodies is more or less constant from cell to cell within one species. In *S. gregaria* usually only 4 of the 6 N-bands have bodies attached to them (fig. 11) while in *L. migratoria* usually only 6 of the 8 N-bands have bodies (fig. 13). In the case of *S. gregaria* it is possible to identify which bands do not carry bodies because of a frequent N-band polymorphism on chromosome M8. The cell shown in fig. 1 is heterozygous, with one M8 homologue having a large band and one a small band. In figs. 11 and 12 the interphase cell is also from a heterozygous animal and the two green dots which lack nucleolus-like bodies are heteromorphic.

The N-band technique reported here is also applicable to all meiotic cells and can be used to locate nucleoli as well as the N-bands to which they are attached. Fig. 15 shows an early dipolotene from *Omocestus panteli* with a single N-band in the short arm of chromosome L3. Acridine orange-stained bodies following the N-band procedure and silver-stained nucleoli correspond precisely in number and position.

Silver-stained nucleoli

Figs. 16–20 show that the nucleoli of male dipolotene cells stain intensely with the silver deposition technique. The positions of attachment of nucleoli to the diplotene bivalents, which we have found consistently in all our preparations for



Figures 1–4 N-bands in *Schistocerca gregaria* embryo cells (figs. 1 and 3) and *Locusta migratoria* embryo cells (figs. 2 and 4). Cells in figs. 1 and 2 are stained with gallocyanin-chrome alum while cells in figs. 3 and 4 are stained with acridine orange. The N-banded chromosomes (L2, L3, M6, M8, S9) are indicated. Arrows point to centromere dots visible in some chromosomes.

Figure 5 Orcein-stained neuroblast metaphase of *Schistocerca gregaria* showing secondary constrictions (arrows) in L3, M6 and M8 chromosomes.

Figure 6 N-bands in *Stethophyma grossum* embryo cell. Magnifications figs. 1, 3 × 1000, figs. 2, 4, 5 × 1200, fig. 6 × 800.

Table 1 Secondary constrictions, Nucleolus organisers, N-bands, C-bands and R-DNA at specific sites in the mitotic and meiotic chromosomes of *Schistocerca gregaria* and *Locusta migratoria*

Property	Reference†	Chromosome and band position*									Species
		L1 (100)	L2 (100)	L3 (70)	M6 (50)	M6 (100)	M8 (100)	M8 (20)	M8 (30)	S9 (20)	
L. migratoria Mitotic constriction	1		+			+		+		+	*L. migratoria*
Mitotic NOR	2					(6 of 8 N-bands are NORs)					
Pachytene NOR	3, 10	+					+			+	
Diplotene NOR	2		+			+				+	
N-bands	2		+			+		+		+	
C-bands	11		+			+				+	
R-DNA	4		+			+				+	
S. gregaria Mototic constriction	5, 2			+	+			+			*S. gregaria*
Mitotic NOR	2			+	+						
Pachytene NOR	3			+	+						
Diplotene NOR	2, 7			+	+						
	6					+				+	
N-bands	2			+	+			+			
	8		+			+		+	+	+	
C-bands	9			+	+						

* Band position (in brackets) expressed as per cent distance from centromere.
† References: 1, Fox, Mello-Sampayo and Carter (1975), 2. This paper, 3. Jones, Croft and Wallace (1983), 4. W. Kunz—personal communication, 5. Fox (1966), 6. Rufas and Gosalvez (1982), 7. John and Henderson (1962), 8. Hagele (1978), 9. Fox, Carter and Hewitt (1973) and unpublished work, 10. Moens (1973), 11. Fox unpublished work.

Schistocerca gregaria and *Locusta migratoria*, are shown in figs. 17 and 18. These positions are summarised in table 1.

At early pachytene (fig. 16) the nucleoli from a pair of homologues are invariably fused but at early-mid dipolotene (figs. 17 and 18) each may separate into two bodies, one attached to each homologue and at this stage the site of attachment can be defined precisely. This separation into two nucleoli may be equal or unequal (fig. 20) and non-homologous nucleoli are rarely fused (fig. 19). It is important to choose cells which are not later than mid-diplotene in order to get accurate observations of attachment sites because the nucleoli become detached from the bivalents at late diplotene (fig. 20) and remain compact but free in the cytoplasm for a short while. Almost invariably nucleoli can no longer be seen at diakinesis, attached or free, presumably due to dispersion.

DISCUSSION

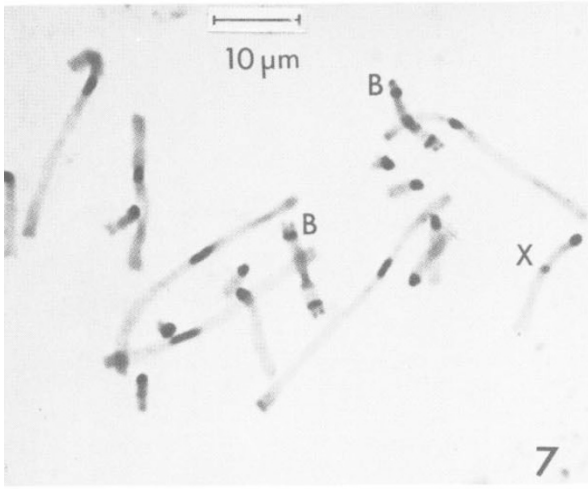
The mechanism of N-band staining

The dyes listed in the protocol above for demonstrating N-band staining all react with DNA. Further, although quantitative measurements have not

Figures 7 and 8 *Myrmeleotettix maculatus* embryo cells. Fig. 7 2B, 1X (male) is C-banded and fig. 8 2B, 2X (female) is N-banded. The interstitial region of the X chromosome which is C-banded in fig. 7 is N-banded in fig. 8 but the interstitial regions of the B-chromosome which are C-banded in fig. 7 are not N-banded in fig. 8. Chromosome L3 (arrows) carries the other N-band in the karyotype.

Figures 9 and 10 *Chorthippus brunneus* embryo cells. Fig. 9 is N-banded and centromere dots are also present. Note the double bands on the short arm of chromosome L3 and the single bands on the short arm of chromosome L1. Fig. 10 is orcein stained. The arrows indicate double constrictions in L3 and single constrictions in L1.

Figures 11–14 Black and white prints of colour positive photographs of interphase cells stained with acridine orange following the N-banding technique. Figs. 11 and 12—same cell of *Schistocerca gregaria*, figs. 13 and 14—same cell of *Locusta migratoria*. Figs. 12 and 14 printed using a red filter and showing the nucleoli clearly, figs. 11 and 13 printed using a green filter and showing the condensed N-bands clearly. Note that in *Schistocerca gregaria* figs. 11 and 12 one heteromorphic pair of N-bands (arrows) lacks nucleoli while the other four bands have nucleoli. In *Locusta migratoria* (figs. 13 and 14) six of the eight N-bands have nucleoli (two N-bands share a fused nucleolus) and two lack them (arrows). Magnifications figs. 7–9, 10 × 850, figs. 9, 11–14 × 700.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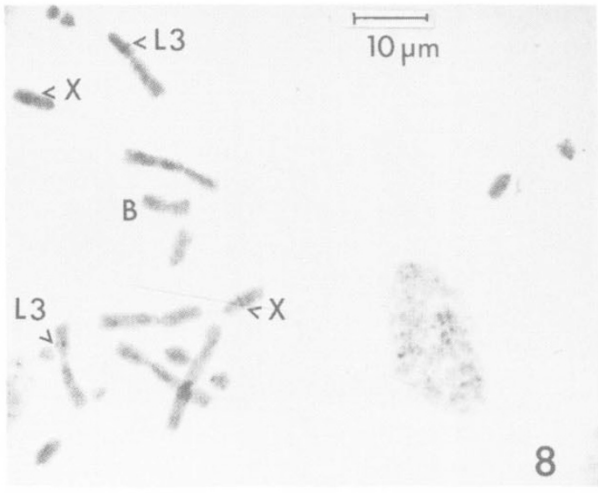
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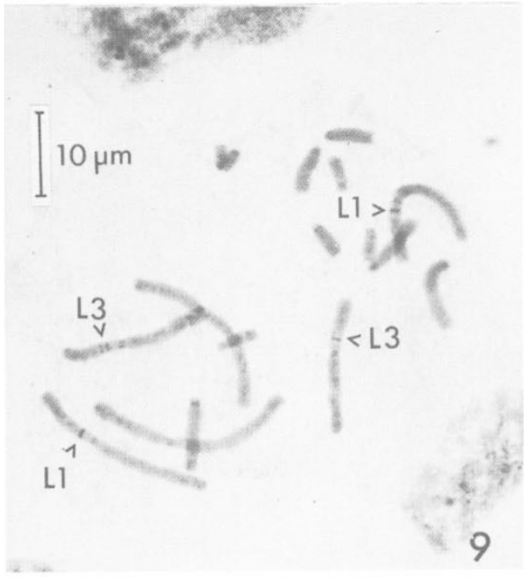
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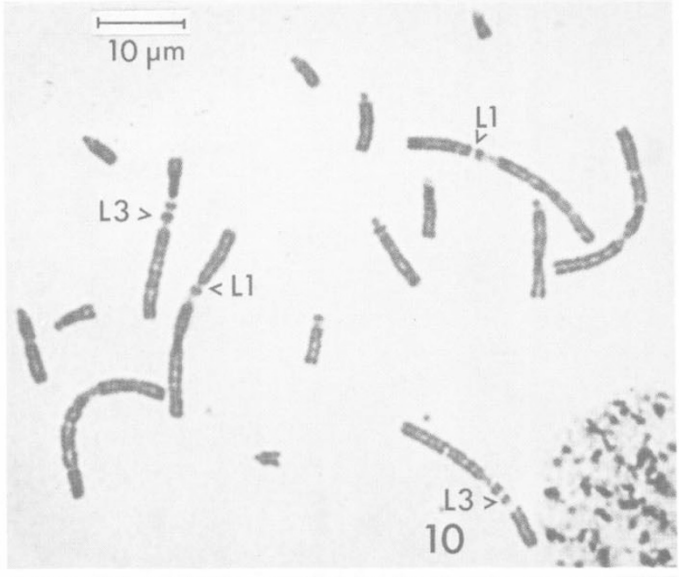
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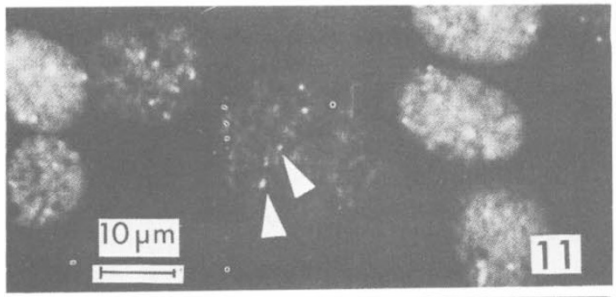
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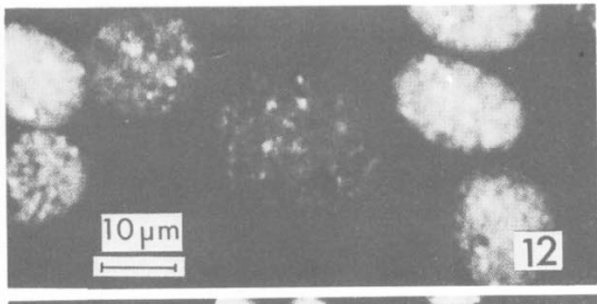
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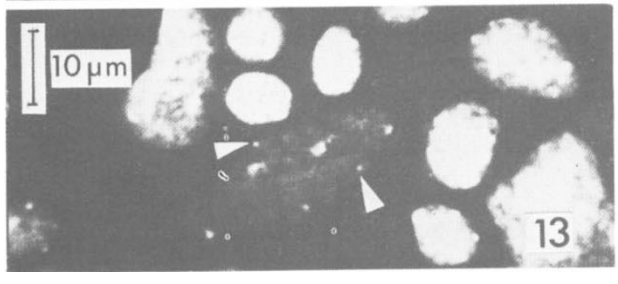
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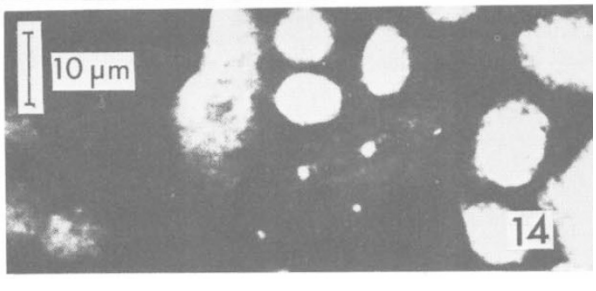
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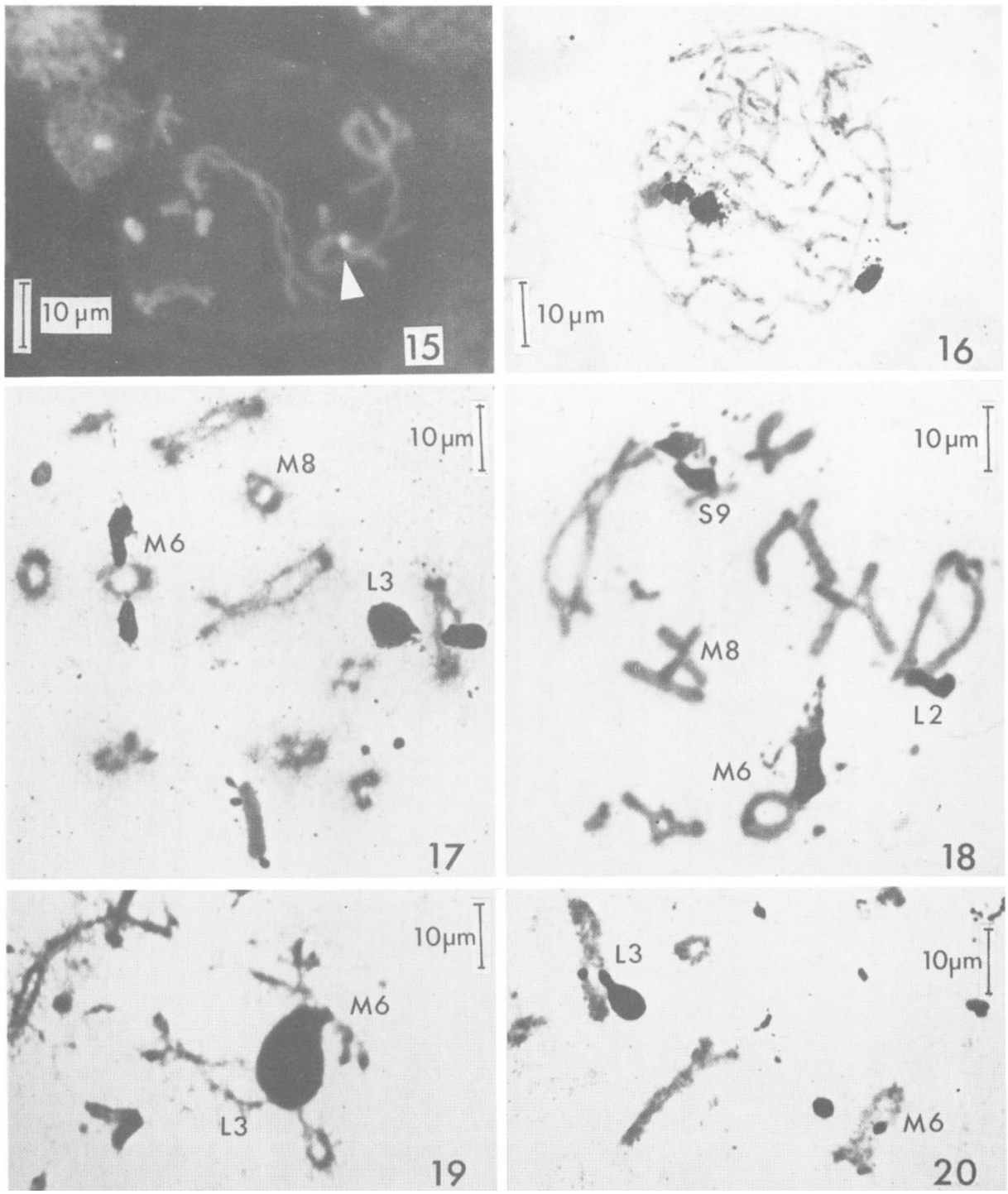

Figure 15 Early diplotene of *Omocestus panteli* showing a single N-band in bivalent L3 stained with acridine orange. In the fluorescence microscope a small red nucleolus can be seen attached to the green N-band.

Figures 16–20 Silver staining of nucleoli in male meiotic cells of *Locusta migratoria* (figs. 16 and 18) and *Schistocerca gregaria* (figs. 17, 19, 20). Fig. 16 early pachytene—note three fused nucleoli. Fig. 17 note pairs of nucleoli on L3 and M6 bivalents. Fig. 18 note telomeric nucleoli in bivalents L3 and M6 and interstitial nucleolus in S9. In figs. 17 and 18 no nucleoli are present in bivalents M8. Fig. 19 note fusion of non-homologous nucleoli in L3 and M6 bivalents of *Schistocerca gregaria*. Fig. 20. In this cell the nucleoli are in the process of detaching from the bivalents. Magnifications figs. 15–20 × 1000.

been made, the intensity of staining drops markedly at incubation temperatures above 65°C. It seems likely that the mechanism of differential staining involves the differential removal of DNA from the fixed chromosomes during the incubation in formamide. This explanation is similar to that proposed by Comings (1978) to account for the production of R-banding in mammalian chromosomes and it is interesting to note that human lymphocyte chromosome preparations, processed according to our N-band technique and stained in acridine orange, give R-banding patterns identical to those generated by the technique of Verma and Lubs (1976). The protection of DNA from removal at the sites of N-bands could be due to a high *GC* content of the DNA which is resistant to removal by formamide because of its higher melting temperature.

The N-bands are not simply a subset of C-bands. While NORs appear to react with both the C- and N-banding techniques, some N-bands do not C-band, in particular the N-bands on chromosomes M8 in both *S. gregaria* and *L. migratoria.* It is also interesting to note that the C-bands in both arms of the B isochromosome of *Myrmeleotettix maculatus* (Fig. 7) which were postulated to be derived from the C-band at the NOR on the X-chromosome of that species (Hewitt, 1973; Gallagher *et al.*, 1973) do not in fact give the N-band reaction, while the NORs on the short arm of L3 and the X-chromosome do. This observation does not support the hypothesis that the B-chromosome of *M. maculatus* is derived from a deleted X-chromosome of that species.

What are the N-bands?

The term "N-band" is here used to denote a band produced by the technique which we have described as an N-band technique. While it certainly reveals NORs it also reveals centromere dots (Eiberg, 1974) and some bands which appear not to organise nucleoli. Reference to table 1 shows that both for the data reported in the literature and in this paper there is a close correlation between nucleolus organisation at mitosis or meiosis, the presence of an N-band after N-band staining and the presence of secondary constrictions. There are some discrepancies in table 1 between the conclusions of different authors (see also Introduction). Jones, Croft and Wallace (1983) and Moens (1973) report that pachytene bivalents L1 and M8 in *Locusta migratoria* have telomeric NORs while S9 has an NOR near to the centromere. In contrast we find terminal NORs in L2 and M6 and an NOR proximal to the centromere in S9 for diplotene bivalents. In all three cases the identification of the long and medium chromosomes is made solely on the basis of length and in mitotic and meiotic chromosomes L1 and L2 are similar in length, as are M6–M8. Since the NORs reported by all three sets of authors in the L and M group chromosomes are telomeric it seems likely that slight differences in relative degree of contraction between pachytene (when the synaptonemal complex is present) and diplotene (when it is not) are responsible for the disagreement. Indeed in the paper by Moens, synaptonemal complex length at late pachytene places the NOR chromosomes in the same order as reported in this paper for mitotic NOR chromosomes *i.e.*, early pachytene L1 becomes late pachytene or mitotic L2 and early pachytene M8 becomes late pachytene or mitotic M6. In *Schistocerca gregaria*, Rufas and Gosalvez (1982) report the presence of one NOR in a distal position on M6 and a second NOR on S9. Our results and those of most other authors (see table 1) are in disagreement. Two possibilities may account for this difference. Firstly, there may be a polymorphism for NOR locations within this species. Arguing against this explanation is the fact that we have examined animals from three separate sources and found no such variation. Secondly, we have shown (fig. 20) that the nucleoli detach, sometimes asynchronously, from the diplotene bivalents and remain as discrete bodies in the nucleoplasm for a short while before becoming dispersed. Such detachment might account for the apparent location of a distal NOR on bivalent M6 and a second NOR on bivalent S9 in Fig. 1(d) of Rufas and and Gosalvez's (1982) paper. The results of Hagele (1978) on N-bands in *Schistocerca gregaria* are difficult to reconcile with the other data reported in the second part of table 1. There is no correlation with bivalent or position within bivalent for the L and M chromosomes between his NOR locations and those reported here though there is partial agreement with Rufus and Gosalvez (1982). While chromosomal polymorphism must again be considered as a possible expalantion it is also the case that there is a close similarity between the sites of N-bands reported by us for *Locusta migratoria* and Hagele's results for *Schistocerca gregaria.*

In both *S. gregaria* and *L. migratoria* mitotic chromosomes the most prominent secondary constriction in the complement is that located close to the centromere on M8. In both species this site is marked by an N-band. We have shown directly in *S. gregaria* that this N-band is not an NOR in

embryonic cells or male meiotic cells. In *L. migratoria* this can be shown indirectly. Only six of the eight N-bands in the diploid complement of embryonic cells form nucleoli (figs. 13 and 14). Kunz (personal communication) has shown by *in situ* hybridisation that 18s and 26s *r*-DNA sequences exist at only three of the four N-band positions, M8 lacking them. Thus the M8 N-band in *L. migratoria* also cannot be a nucleolus organiser.

The bands produced by our N-banding technique (ignoring centomere dots) are clearly of two types, sites of NORs and sites which are not NORs. We have suggested that all these sites have one property in common, a relatively high $G+C$ content in the DNA. It is not surprising that NORs should stain using this technique since they seem generally to contain DNA of high $G+C$ composition. Using chromomycin *A*3 staining, which indicates regions of relatively high $G+C$ content (Schweitzer, 1981), NORs of several animal and plant species stain brightly (Schmid, 1982; Appels, 1982; Deumling and Greilhuber, 1982; Schweitzer, 1976, 1980; Schweitzer *et al.*, 1983; Schwartzacher and Schweitzer, 1982). Not only are the 18*s* and 26*s* *r*-RNA genes of relatively high $G+C$ content but they often are associated in insect species with a $G+C$ rich satellite DNA (Gall *et al.*, 1969; Lima-de-Faria, 1969).

What is the nature of the M8 N-bands in *S. gregaria* and *L. migratoria* which do not organise nucleoli? There are several possibilities.

(i) Fortuitous concentration of high $G+C$ DNA at these sites which has no homology with 18*s* or 26*s* *r*-DNA or adjacent sequences.

(ii) Degenerate NORs which have either inactive *r*-DNA sequences or have lost *r*-DNA sequences but retained a high $G+C$ linked DNA.

(iii) 5*s* *r*-RNA genes.

The M8 N-band in *Locusta migratoria* lacks detectable 18*s* and 26*s* *r*-DNA sequences (Kunz, personal communication) though it could still retain homology with the functional NORs through the adjacent $G+C$ rich DNA. This seems unlikely since the M8 N-band in both *L. migratoria* and *S. gregaria* is the site of the most prominent secondary constriction in embryonic cells and this would suggest a functional rather than an inactive locus. However, in several genera of 17-chromosome grasshoppers (Santos and Fox unpublished work) N-bands which do not organise nucleoli exist at the primitive locus of nucleolar organisation on chromosome L3, indicating that in some species degenerate NORs exist which still N-band.

In the genus *Warramaba* (White *et al.*, 1982) clusters of 18*s*+26*s* *r*-RNA cistrons and also clusters of 5*s* *r*-RNA cistrons stain with an N-banding technique. This suggests, at least for the M8 chromosome in *S. gregaria* and *L. migratoria*, that the non-nucleolus organising N-bands may be the sites of the 5*s* *r*-RNA genes. This hypothesis remains to be tested.

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