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Localization of NORs in spermatogonial metaphase chromosomes of six species of grasshoppers

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Abstract

The silver staining technique was employed to locate Nucleolar Organiser Regions (NORs) in six species of grasshoppers viz. *Aiolopus thalassinus* F. (Tryxalinae); *Oeodaleus abruptus* Thunb., *Gastrimargus transversus* Thunb., *Heteropternis respondens* Walk. (Oedipodinae); *Parahieroglyphus biliniatus* Bol. and *Spathosternum prasiniferum* Walk. (Catantopinae). Usually the NORs were located on the larger elements of the chromosomal complement. However, in *O. abruptus* NORs were found on autosomes S_8 and S_9. The salient observations were: (1) NORs were seen in only a few of the several spermatogonial metaphases examined; (2) Active NORs were mostly located either on one chromatid of the homologues or on the homologue depicting heteromorphism; (3) NORs showed either proximal, subproximal or interstitial locations. However, in *O. abruptus* and *P. bilineatus* NORs were located at two positions. Distribution of NORs in different species and their probable role in tracing the evolutionary pathways in Acridoidea are discussed.

Introduction

Nucleolus Organizer Regions (NORs) are defined as the areas of chromosomes that carry clusters of DNA coding for ribosomal RNA production. The use of silver staining for revealing cellular and chromosomal structures is quite old in practice (Ramon y Cajal, 1903). However, the recent development of a simple light-microscopical silver staining technique (Goodpasture & Bloom, 1975; Olert, 1979) stimulated the studies of NORs and nucleolus since the silver impregnation technique shows some nucleolar component positively stained and serves to mark the transcriptionally active NORs in metaphase chromosomes which were active during the previous interphase (Miller *et al.*, 1976). Biochemical studies have shown that the silver stained material consists mainly of acid proteins (Daskal *et al.*, 1980).

The concept of NOR that developed from studies on chromosomes of both plant and animal cells points that the nucleolus is a specific gene product formed by read-outs of rDNA and other genes localized in or near the NOR. The chromosomes that have these NORs are referred to as 'nucleolar chromosomes' (Busch, 1974). The interdependence between NOR and nucleolus is so complete that often a definite nucleolar morphology represents a metabolic state of the NOR (Gimenez-Martin *et al.*, 1977).

Employing silver staining, NORs have been successfully demonstrated in man (Hubbell & Hsu, 1977), other mammals (Dev *et al.*, 1977; Hofgartner *et al.*, 1979; Arnason, 1974, 1981) and amphibians (Schmid, 1978a and b); but although the cytogenetics of Orthoptera is far advanced in comparison with other groups of invertebrates (Hewitt, 1979), yet relatively few papers dealing with the NORs of grasshoppers available (Czaker, 1978; Garcia de la Vega *et al.*, 1982; Rufas & Gosalvez, 1982; Rufas *et*

al., 1985; Cabrero *et al.*, 1985). None of these reports pertains to the Indian fauna. During the present investigations NORs have been located in the spermatogonial metaphase chromosomes of six species of Indian grasshoppers.

Material and methods

Male individuals of *Aiolopus thalassinus* F. (Tryxalinae), *Oedaleus abruptus* Thunb., *Gastrimargus transversus* Thunb., *Heteropternis respondens* Walk. (Oedipodinae); *Parahieroglyphus biliniatus* Bol. and *Spathosternum prasiniferum* Walk. (Catantopinae) constituted the materials for the present investigations. All the hoppers were collected from the environs of the Kurukshetra University Campus during 1980–1983. Field collected hoppers were injected with 0.05% colchicine abdominally prior to dissecting out the testes. Slides were prepared by routine airdrying technique. Silver staining was done employing the slightly improved technique of Bloom & Goodpasture (1976). Usually 7–30 days old slides were treated with silver nitrate solution and incubated at 37 °C for 18–24 h. The slides were counterstained in 2% Giemsa (pH 6.9). After drying the slides were directly examined and selected spermatogonial metaphases as well as other stages were photomicrographed. The exact magnification is given with the figures.

Results

All the species have 2n = 23. The chromosomes are acrocentric. The male sex-determining mechanism is XO. Distinct size groups among autosomes (L, M & S), established elsewhere, have been followed in the description.

NORs were seen in only a few of the several spermatogonial metaphases examined. Specific details are as follows.

Aiolopus thalassinus

The largest pair of autosomes (L_1) carried the active NORs which were located proximally near the centromeric region (Fig. 1). One homologue of the L_1 pair had NORs on both chromatids while only one chromatid had NOR in the other homologue.

Oedaleus abruptus

Active NORs were located on autosomal pair M_8 and one of the homologues of autosome pair S_9 (Fig. 2). The S_9 pair was heteromorphic as one chromosome carried a large dark silver-positive grain towards the centromeric region which appeared to be the secondary constriction. In the M_8 pair active NORs were located interstitially in one homologue and subproximally in the other. This particular stage also showed one L-group chromosome bearing minute silver-positive grains towards the terminal end. However, this could not be confirmed from other spermatogonial metaphases. As such, this chromosome was not taken to be one of the 'nucleolar chromosomes' in the present report.

Gastrimargus transversus

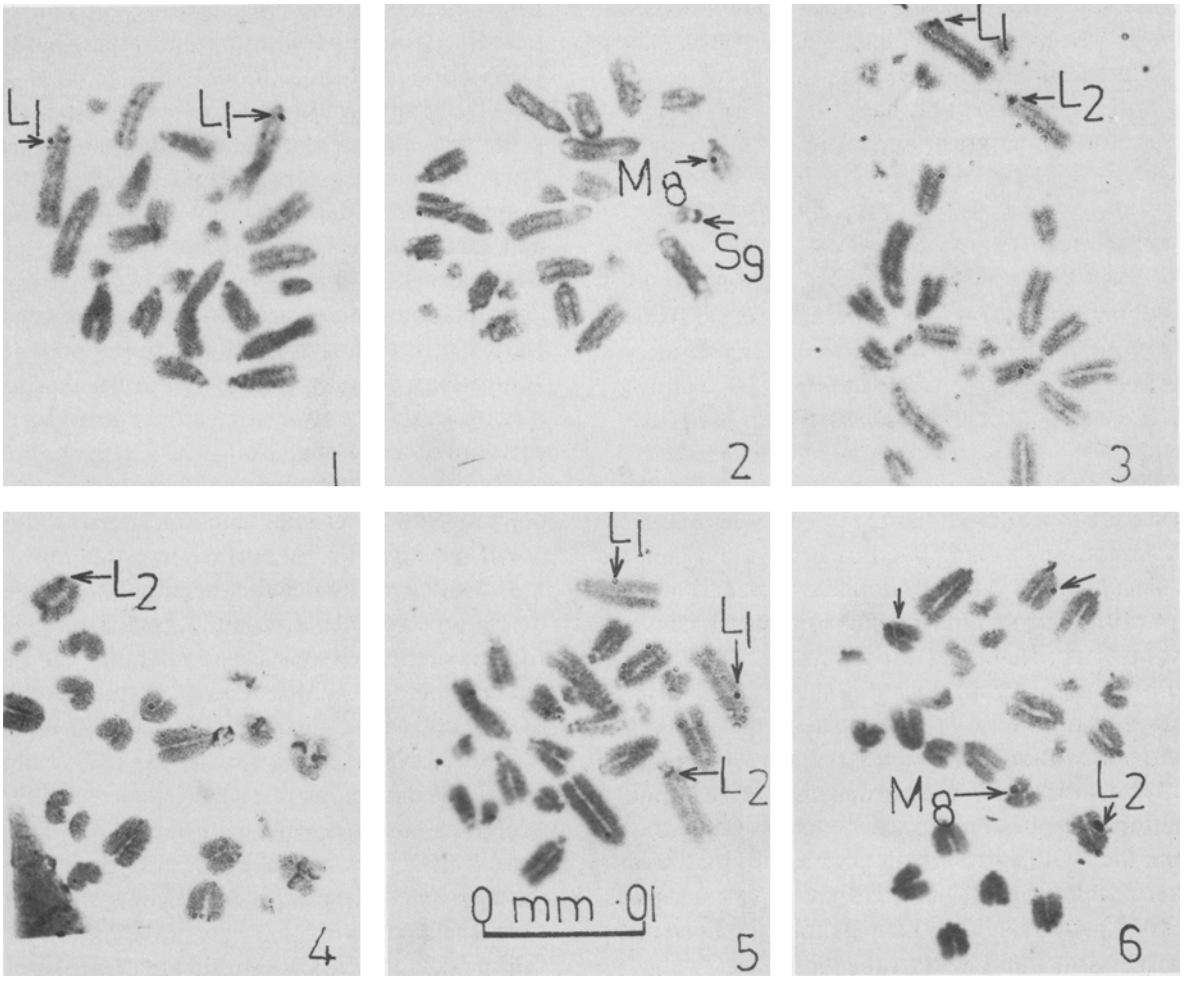
The L-group autosomes L_1 and L_2 pairs carried the active NORs (Fig. 3). In the former, one of the homologues showed three dark silver-positive grains on one chromatid near the centromeric region. In the L_2 pair, one chromosome showed a single silver-positive grain located proximally.

Heteropternis respondens

Autosome pair L_2 carried the active NORs which were located interstitially on both chromatids of both the homologues (Fig. 4).

Parahieroglyphus biliniatus

Active NORs were located proximally as well as interstitially on autosome pairs L_1 and L_2 (Fig. 5).



Figs. 1–6. Silver-stained spermatogonial metaphases: (1) *Aiolopus thalassinus*; (2) *Oedaleus abruptus*; (3) *Gastrimargus transversus*; (4) *Heteropternis respondens*; (5) *Parahieroglyphus biliniatus*; (6) *Spathosternum prasiniferum*.

Spathosternum prasiniferum

Autosome pairs L_2 and M_8 carried the active NORs which were located interstitially on only one chromatid of these chromosomes (Fig. 6). In the L_2 pair, the minute second arm also exhibited faint silver-positive reaction. This particular spermatogonial metaphase depicted two supernumerary or B-chromosomes. Silver-positive grains were not seen on the B-chromosomes.

Discussion

The following salient features emerged during the present investigations:

(1) NORs were seen in only a few of the several spermatogonial metaphases examined,

(2) Active NORs were mostly located either on one homologue or on one chromatid of the homologues showing heteromorphism, and

(3) NORs showed either proximal, subproximal or interstitial locations. However, in *Oedaleus abruptus* and *Parahieroglyphus biliniatus* NORs were located at two positions (Table 1).

Oud & Reutlinger (1981) observed that in mice Ag-NORs occur only incidentally in spermatogonial metaphases. This might explain the failure to mark a silver-positive chromosome in many spermatogonial metaphases during the present investi-

Table 1. Nucleolar chromosomes and position of NORs in six species of grasshoppers analysed during the present investigations.

Species	2n	Nucleolar chromosomes	Position of NORs
Aiolopus thalassinus	23	L_1 pair	proximal
Oedaleus abruptus	23	M_8 pair S_9 chromosome	subproximal interstitial
Gastrimargus transversus	23	L_1 and L_2 chromosomes	proximal
Heteropternis respondens	23	L_2 pair	interstitial
Parahieroglyphus biliniatus	23	L_1 and L_2 pairs	proximal interstitial
Spathosternum prasiniferum	23	L_2 and M_8 pairs	interstitial

gations. Moreover, silver only detects active NORs (Miller *et al.*, 1976). Minute NORs as well as NORs with low activity or both are, therefore, not easily detected when silver staining is employed. Moreover, the NOR number registered by this method is directly related to NOR activity, and this seems to depend particularly on the metabolic requirements of the cell (Flavell & Martini, 1982).

Variations in the amount of silver deposit between homologues Ag-stained NORs observed during the present investigations further support the opinion of Shi *et al.* (1982) that all the NORs, in a species with multiple NORs, may not be positively identifiable at a particular stage as some of these may be inactive in a given cell. Recently, Rufas *et al.* (1985), reporting on NORs in 21 species of acridoid grasshoppers, made similar observations – not all the NORs are active in a given cell. This differential expression implies that, even within the same cyst, the nucleolar remnants are not always associated to the same chromosome. This NOR-heteromorphism reflects the absence of rRNA genes activity in the preceding interphase since Miller *et al.* (1976) and Schmid *et al.* (1977) have postulated that the silver staining of NOR represents previous transcriptional activity of the ribosomal genes. However, because Ag-staining is based on a reaction with protein rather than with DNA (Goodpasture & Bloom, 1975), the absence of silver stain could indicate either that rRNA genes are not present or that they are inactive. Study of somatic cell hybrids (Dev *et al.*, 1977) has also provided evidence that not all NORs are active and that inactive NORs are not stained by silver-staining methods.

Intrachromosome variation in the expression of NOR activity was observed during the present investigations. It may have a molecular basis but nothing definite can be conjectured with the present state of knowledge. As such, why a chromatid exhibits a silver spot, while its sister chromatid does not show any silver impregnation, remains a moot point and a matter for further investigations.

The number of NORs per genome is characteristic (Busch, 1974). Bloom and Goodpasture (1976) supported the view that each individual has a modal number of NORs. Previous studies on orthopterans (Garcia de la Vega *et al.*, 1982; White *et al.*, 1982; Fernandez-Piqueras *et al.*, 1983; Cabrero *et al.*, 1985) demonstrated that there usually are variations among species with regard to the location of NORs and this character may be used as a good chromosome marker for distinguishing closely related species.

The position of NORs within the nucleolar chromosome is normally restricted to either proximal or distal regions (Rufas *et al.*, 1985). During the present investigations NORs were also found to be located interstitially. The general assumption is that this situation minimizes the harmful effects of interchange between rRNA genes on non-homologous chromosomes (Miller, 1981).

There is controversy about the possible correspondence of some C-bands and the NORs. Arnason (1974) reported the presence of C-heterochromatin adjacent to the secondary constrictions of the NOR pair in the general karyotype of Cetacea. This probably promotes the transposition of NOR sites between different chromosomes (Arnason, 1981). Schmid (1978a) claims that in amphibian species the NORs are always associated with heterochromatin. However, Mandahl (1979) in European hedgehogs and King (1980) in hylid

frogs, find considerable variation in the localization of NORs and the adjacent heterochromatic segment. Garcia de la Vega *et al.* (1982), describing the situation in *Chorthippus jucundus*, state that there exists a close correspondence between the localization of the nucleoli and the interstitial bands (C-bands) of the L_2 and L_3 pairs; it has not been possible to determine whether one or both bands on L_3 are related to the NOR. Still, they conclude that, at least in this species, it could be argued that heterochromatic zones hitherto found have no effect on the expression of NORs.

Yadav and Yadav (1983, 1984) have described C-bands in the spermatogonial metaphase chromosomes of 4 of the species under report. Details concerning localization of bands have been presented in Table 2. A comparison of Tables 1 and 2 shows a lack of correlationship between localization of NORs and C-bands.

The observations in *Spathosternum prasiniferum* indicate that active RNA genes were absent in B chromosomes.

The suggestion that some translocations require just one break (Bloom & Goodpasture, 1976), with the NORs having affinity for broken ends, can prove a useful tool in tracing the evolutionary pathways in Acridoidea where such mechanisms are well-known to occur in natural populations. In species having only one chromosome pair with NORs, as e.g. *Aiolopus thalassinus* and *Heteropternis respondens* (Table 1), biological difficulties may arise, if this chromosome pair suffers abrupt structural changes like translocations, deletions, etc. Therefore, karyotypic evolution should occur in such a way that the nucleolar chromosomes have a structure resistant to alterations.

Table 2. C-band distribution in 4 species of grasshoppers under report.

Species	Localization of bands in spermatogonial metaphase chromosomes	Reference
Gastrimargus transversus	Centromeric in all, additional telomeric bands in L_1, L_3, M_4 and X.	Yadav & Yadav 1984
Heteropternis respondens	Intercalary in L_1 and L_3, telomeric as well as centromeric in L_2 and M_7.	– ibid –
Parahieroglyphus biliniatus	Centromeric in all, additional intercalary bands in L_1 and M_7, telomeric in M_5.	Yadav & Yadav 1983
Spathosternum prasiniferum	Centromeric in all, additional telomeric in L_1, L_2, M_5, M_7 and X, in X, intercalary too.	– ibid –

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References

Arnason, U., 1974. Comparative chromosome studies in Cetacea. Hereditas 77: 1–36.

Arnason, U., Localization of NORs in cetacean karyotypes. Hereditas 95: 269–275.

Bloom, S. E. & Goodpasture, C., 1976. An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Hum. Genet. 34: 199–206.

Busch, H., 1974. The Cell Nucleus. Vol. I. Academic Press, Inc., London.

Cabrero, J., Camacho, J. P. M. & Pascual, F., 1985. Cytotaxonomic studies on pamphagids genus Eumigus. Detection of two chromosomal races in E. monticola (Rambur) (Insecta, Orthoptera). Caryologia 38(1): 1–12.

Czaker, R., 1978. Silver staining in transcriptionally active NORs of meiotic and mitotic cells in Acheta domesticus L. (Orthoptera). Chromosoma 68: 187–193.

Daskal, Y., Smetana, K. & Bush, H., 1980. Evidence from studies on segregated nucleoli that nucleolar silver staining proteins C23 and B23 are in the fibrillar component. Expl Cell Res. 127: 285–291.

Dev, V. G., Tantravahi, R., Miller, D. A. & Miller, O. J., 1977. Nucleolar organizer in Mus musculus subspecies and in the RAG mouse cell line. Genetics 86: 389–398.

Flavell, R. B. & Martini, G., 1982. The genetic control of nucleolus formation with special reference to common breadwheat. In: The Nucleolus, E. G. Jordan & C. A. Cullis (eds), pp. 153–177. Cambridge University Press, Cambridge.

Fernandez-Piqueras, J., Rodriguez Campos, A., Sentis Castano, C. & Rojo Garcia, E., 1983. Chromosomal location of the active NORs in the Steropleurus martorelli complex. Genetica 61: 9–12.

Garcia de la Vega, C., Lopez-Fernandez, C., Gosalvez, J. & Rufas, J. S., 1982. Cytogenetic studies on Chorthippus jucundus (Fisch.) (Othoptera). II. Heterochromatin variation and NOR localization. Cytobios 34: 7–14.

Gimenez-Martin, G., De la Torre, C., Lopez-Saez, J. F. & Esponda, P., 1977. Plant nucleolus: structure and physiology. Cytobiologie 14: 421–462.

Goodpasture, C. & Bloom, S. E., 1975. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma 53: 37–50.

Hewitt, G. M., 1979. Animal Cytogenetics. Vol. 3: Insecta 1. Orthopera. Borntraeger, Berlin/Stuttgart.

Hofgartner, F., Schmid, M., Krone, W., Zenzes, M. T. & Engel, W., 1979. Pattern of activity of nucleolar organizers during spermatogenesis in mammals as analyzed by silver staining. Chromosoma 71: 197–216.

Hubbell, H. R. & Hsu, P. C., 1977. Identification of nucleolar organizer regions (NORs) in normal and neoplastic human cells by the silver staining technique. Cytogenet. Cell Genet. 19: 185–196.

King, M., 1980. C-banding studies on Australian hylid frogs: Secondary constriction structure and the concept of euchromatin transformation. Chromosoma 80: 191–217.

Mandahl, N., 1979. Localization of nucleolar organizing regions in European hedgehogs (Insectivora: Mammalia). Hereditas 91: 149–161.

Miller, O. J., 1981. Nucleolar organizers in mammalian cells. In: Chromosomes Today, Vol. 7. M. D. Bennett, M. Bobrow & G. M. Hewitt (eds), George Allen & Unwin, London.

Miller, D. A., Dev, V. G., Tantravahi, R. & Miller, O. J., 1976. Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Expl Cell Res. 101: 235–243.

Olert, J., 1979. Interphase studies with a simplified method of silver staining of nucleoli. Experientia 35: 283–285.

Oud, J. L. & Reutlinger, A. H. H., 1981. The behavior of silver positive structures during meiotic prophase of male mice. Chromosoma 81: 569–578.

Ramon y Cajal, S., 1903. Un sencillo metodo de coloración selectiva del retículo protoplasmático. Trab. Lab. Invest. Biol. 2: 129–221.

Rufas, J. S. & Gosalvez, J., 1982. Development of silver stained structures during spermatogenesis of Schistocerca gregaria (Forsk.) (Orthoptera: Acrididae). Caryologia 35: 261–267.

Rufas, J. S., Esponda, P. & Gosalvez, J., 1985. NOR and nucleolus in the spermatogenesis of acricoid grasshoppers. Genetica 66: 139–144.

Schmid, M., 1978a. Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in Bufo and Hyla. Chromosoma 66: 361–388.

Schmid, M., 1978b. Chromosome banding in Amphibia. II. Constitutive heterochromatin and nucleolus organizer regions in Ranidae, Microhylidae and Rhacophoridae. Chromosoma 68: 131–147.

Schmid, M., Hofgartner, F. J., Zenzes, M. T. & Engel, W., 1977. Evidence for post meiotic expression of ribosomal RNA genes during male gametogenesis. Hum. Genet. 38: 279–284.

Shi, L., Pathak, S. & Hsu, T. C., 1982. Demonstration of kinetochores and centrioles in spermatocytes of two species of cockroaches by silver staining. Chromosoma 85: 421–426.

White, M. J. D., Dennise, S., Honeycutt, R. L., Contreras, N. & Peacock, W. J., 1982. Cytogenetics of the parthogenetic grasshopper Warramaba virgo and its bisexual relatives IX. The ribosomal RNA cistrons. Chromosoma 85: 181–199.

Yadav, J. S. & Yadav, A. S., 1983. Distribution of constitutive heterochromatin in catantopine grasshoppers. Adv. Ch. Genet. & Self Incompatibility: XV Int. Cong. Genet. Sat. Symp., Chandigarh, pp. 41–42.

Yadav, J. S. & Yadav, A. S., 1984. Karyological analysis of Oedipodine grasshoppers with banding techniques. III Oriental Entom. Symp., Kariavattom, Abstract No. 279.