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Location and expression of ribosomal RNA genes in grasshoppers: Abundance of silent and cryptic loci

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Abstract

We investigate regularities and restrictions in chromosome location of ribosomal RNA genes, analysed by fluorescent *in situ* hybridization (FISH), and their phenotypic expression assessed by nucleolus formation at first meiotic prophase cells, analysed by silver impregnation, in 49 grasshopper species. High variation was found for rDNA location between species within most genera analysed. The mean haploid number of rDNA loci detected by FISH was 2.47, but some species had up to 10 loci. Chromosome distribution of rDNA loci differed between the Gomphocerinae and Oedipodinae subfamilies, most loci being proximal to the centromere in the former and distal to it in the latter. Chromosomes 2, 3 and X frequently carried rDNA in Gomphocerinae species with $2n♂ = 17$ chromosomes, whereas chromosomes 6 and 9 were the most frequent rDNA locations in the Oedipodinae. About 13% of the 126 rDNA loci detected by FISH were silent, although this figure might be even higher. The comparison of FISH and silver-impregnation results also suggested the existence of cryptic NORs, i.e. those forming small nucleoli with no apparent presence of rDNA revealed by FISH. This was especially clear after the same cells in two species were sequentially treated with both silver impregnation and FISH. The abundance of silent and cryptic loci might thus suggest that rDNA spreads through grasshopper genomes by the Dubcovsky and Dvorak mechanism—that is, the transposition of a few rRNA genes to new chromosome locations, their amplification giving rise to new NORs, and the elimination of the old NORs. The cryptic NORs might correspond to nascent NORs, i.e. a few rRNA gene copies moved to new locations, whereas the inactive rDNA loci might correspond to those being in the process of elimination.

Abbreviations and definitions

FISH	fluorescent *in situ* hybridization
NOR	nucleolus organizer region
rDNA	ribosomal DNA
rRNA	ribosomal RNA
Ectopic recombination	recombination between sites at different locations in the genome
Heteropycnosis	differential condensation of certain chromosomes, e.g. sex chromosomes
Orphon	dispersed, solitary genetic elements derived from tandem multigene families

Electronic supplementary material

The online version of this article (doi:10.1007/s10577-008-1214-x) contains supplementary material, which is available to authorized users.

Introduction

The three major classes of ribosomal genes in eukaryotes (28/26S, 18S, and 5.8S) are tandemly repeated in the genome, constituting a multigene family arranged into one or more loci located on one or several chromosomes. Each chromosome rDNA locus is called a nucleolus organizer region (NOR) since it is the place where nucleoli form (McClintock 1934). Notably, Childs *et al*. (1981) discovered orphons, a novel class of dispersed, solitary genetic elements derived from both protein-coding and non-protein-coding structural tandem multigene families, including those of histone and ribosomal genes. Ribosomal gene orphons were first reported in yeast and *Drosophila melanogaster* by Childs *et al*. (1981) and later in humans (Munro *et al*. 1986) and *Xenopus laevis* (Guimond & Moss 1999).

The chromosome location of NORs usually varies among closely related species and even within species. Several mechanisms have been proposed to explain this variation: (i) structural chromosome rearrangements (Maluszynska & Heslop-Harrison 1993, Castilho & Heslop-Harrison 1995, Thomas *et al*. 1997, Gu & Hua 2003, Shan *et al*. 2003), (ii) ectopic recombination between terminal chromosomal regions (Hanson *et al*. 1996, Pedrosa-Harand *et al*. 2006), and (iii) rDNA transposition, which was first suggested by Schubert (1984) on the basis of active NOR variability in size, number, and chromosomal position found after silver impregnation in *Allium cepa*, *A. fistulosum* and their interspecific hybrids. This researcher postulated that NORs in some onion species behave as mobile genetic elements, a suggestion that was strongly supported by *in situ* hybridization studies (Schubert & Wobus 1985). Subsequently, rDNA transposition was vindicated by a number of authors (Castro *et al*. 1994, 2001, Shishido *et al*. 2000, Cai *et al*. 2006, Datson & Murray 2006).

Dubcovsky & Dvorak (1995) proposed a mechanism to explain the mobility of NORs without chromosome structural rearrangements, deduced from comparative gene mapping in Triticeae genomes. They suggested that NOR loci may move within and among chromosomes via dispersion and magnification of minor loci consisting of a few rDNA copies. However, it is worth mentioning that this possibility had actually been anticipated by Schubert & Wobus (1985) when they stated that 'single orphon-like rDNA copies at different chromosomal sites (Childs *et al*. 1981)—undetectable by *in situ* hybridization or by $AgNO_3$ staining—could under certain conditions amplify very quickly to form functional NORs'. Dubcovsky and Dvorak's hypothesis has since gained support in several studies showing the existence of minor rDNA loci (in addition to major ones) in other Triticeae species such as *Thinopyrum* (Li & Zhang 2002) and *Hordeum* (Taketa *et al*. 2005), and has also been invoked to explain the origin of the variation in rDNA loci distribution found among closely related species in other plants such as the genera *Pinus* (Cai *et al*. 2006) and *Nemesia* (Datson & Murray 2006).

Remarkably, Raskina *et al*. (2004) demonstrated a direct involvement of *En*/*Spm* transposons in rDNA spreading through the genome of *Aegilops speltoides*, clearly supporting Dubkovsky and Dvorak's hypothesis. In addition, Rooney & Ward (2005) have recently hypothesized that 5S genes in filamentous fungi are capable of multiplying and integrating into other areas of the genome through a process the same as, or akin to, retroposition, a description resembling Dubkobsky and Dvorak's 'dispersion and magnification of minor loci'.

Silver impregnation has proved to be a simple and dependable technique for staining nucleoli and NORs (Goodpasture & Bloom 1975, Rufas *et al*. 1982). Some controversy has persisted about whether Ag-NORs, revealed on mitotic metaphase chromosomes, are true NORs, since silver can bind to chromosome regions not corresponding to NORs (see Dobigny *et al*. 2002). In grasshoppers, silver impregnation of mitotic chromosomes does not reveal reliable Ag-NORs, for which reason NOR location by this technique has most frequently been performed on meiotic bivalents at prophase, NOR activity being assessed by the presence of an attached nucleolus. Since the formation of a nucleolus is the direct result of ribosomal gene transcription (Oakes *et al*. 1993), there is no doubt about the reliability of NOR location through silver impregnation of nucleoli attached to chromosome bivalents in meiotic prophase I.

The combination of FISH for rRNA genes and silver impregnation of nucleoli in meiocytes constitutes an excellent option to both locating the chromosomes carrying these genes and ascertaining their phenotypic expression by the presence of an attached nucleolus. This has permitted the unambiguous

identification of inactive rRNA genes in some organisms, e.g. the grasshoppers *Eyprepocnemis plorans* (López-León *et al*. 1994) and *Stauroderus scalaris* (López-León *et al*. 1999) and plants of the genus *Muscari* (Cuñado *et al*. 2000). The combined use of FISH and silver impregnation also permits the detection of the contrary situation—that is, localization of chromosome regions forming nucleoli without apparent presence of rDNA—suggesting the possible existence of cryptic orphon-like rDNA loci.

The location of rRNA genes by FISH in grasshoppers has hitherto been a sporadic task, since only 25 grasshopper species had previously been analysed (see references in Supplementary Table S1). Silver impregnation, however, had been analysed in 59 species, most of them provided by four papers (Rufas *et al*. 1985, Cabrero & Camacho 1986, Santos & Fox 1988, Viseras *et al*. 1991). To compile a large sample of species with both types of information (physical location and activity of rDNA), we analyse here rDNA chromosome location by FISH in 30 grasshopper species, and NOR activity by silver impregnation in eight of these species where this information was unknown. This provides a database that includes 49 species with information concerning both subjects, on which we performed a comparative analysis to infer some regularities and restrictions in the evolution of the rRNA multigene family in grasshoppers. In addition, we performed sequential silver staining and FISH on the same cells in two species, which revealed that some nucleoli are formed in chromosome regions where no rDNA presence is revealed by FISH, suggesting the presence of cryptic rDNA loci.

Materials and methods

Adult males from 30 grasshopper species were collected in SE Spain, except *Ch. biggutulus* (NE Spain and Germany) and *Ch. eisentrauti* (Germany) (see localities in Table S1). Eleven species belonged to the subfamily Oedipodinae (*Acrotylus fischeri*, *A. patruelis*, *Aiolopus strepens*, *A. thalassinus*, *Locusta migratoria*, *Oedipoda charpentieri*, *O. coerulescens*, *Paracinema tricolor*, *Sphingonotus azurescens*, *S. coerulans* and *Tropydopola cylindrica*), one belonged to the subfamily Catantopinae (*Pezotetix giornae*), and 18 to the subfamily Gomphocerinae (*Dociostaurus jagoi*, *D. marocanus*, *Chorthippus apicalis*, *Ch. biguttulus*, *Ch. binotatus*, *Ch. dorsatus*, *Ch. eisentrauti*, *Ch. jacobsi*, *Ch. jucundus*, *Ch. nevadensis*, *Ch. parallelus erythropus*, *Ch. vagans*, *Omocestus bolivari*, *O. burri*, *O. panteli*, *O. raymondi*, *Stenobothrus bolivari* and *S. festivus*). Most Gomphocerinae species analysed here carry $2n♂ = 16 + X0$ chromosomes, the exception being the two *Dociostaurus* species with $2n♂ = 22 + X0$. The remaining species, in all subfamilies, carried $2n♂ = 22 + X0$ chromosomes.

The FISH technique with the 45S rDNA probe and silver impregnation were performed as previously described (Cabrero *et al*. 2003). Silver impregnation was carried out in the species *Aiolopus thalassinus*, *Oedipoda coerulescens*, *Tropydopola cylindrica*, *Pezotetix giornae*, *Dociostaurus jagoi*, *Dociostaurus marocanus*, *Chorthippus biguttulus* and *Chorthippus eisentrauti*. At least 10 meiocytes per male and three males per species were analysed.

In addition, we reviewed all available literature on active NOR location in grasshopper meiotic prophase I cells, as well as rDNA location. This included information on chromosome NOR location and activity, analysed by silver impregnation, in 59 species (see references in the Introduction section). Some species were analysed for NOR activity in two or more of these papers, so that we chose for our database the NOR data corresponding to the same population (or the nearest one) where the FISH analysis had been performed. In total, our database consisted of 49 species, with two species, i.e. *E. plorans* and *Ch. parallelus*, being considered twice because Eastern and Western populations of *E. plorans* differ in the number of chromosomes carrying rDNA (Cabrero *et al*. 2003), and the subspecies *Ch. parallelus parallelus* and *Ch. parallelus erythropus* differ for the presence of a rDNA locus in the X chromosome in the first one (Gosálvez *et al*. 1988).

Phylogenetic relationships among the 49 species analysed here are not known in detail. Two recent papers have provided molecular phylogenies in Gomphocerinae grasshoppers (Bugrov *et al*. 2006, Contreras & Chapco 2006), which constitute about the half of species included here (24), but only three and ten of our species, respectively, were included in these studies. This partial information suggests that both phylogenetic studies supported the taxonomic distribution of species within genera, since species within a same genus usually appeared in the same

cluster in both phylogenies, with the only exception of some *Chorthippus* species, which were discussed in terms of whether they belong to the subgenus *Glyptobothrus* (within *Chorthippus*) or whether they should constitute a separate genus (*Glyptobothrus*) (Bugrov *et al*. 2006, Contreras & Chapco 2006). In addition, in both phylogenetic studies, grasshopper species belonging to the Oedipodinae subfamily (the second best represented subfamily in our analysis) were used as outgroups and, in both cases, they were basal and well separated from the Gomphocerinae species. This indicates that grasshopper taxonomy

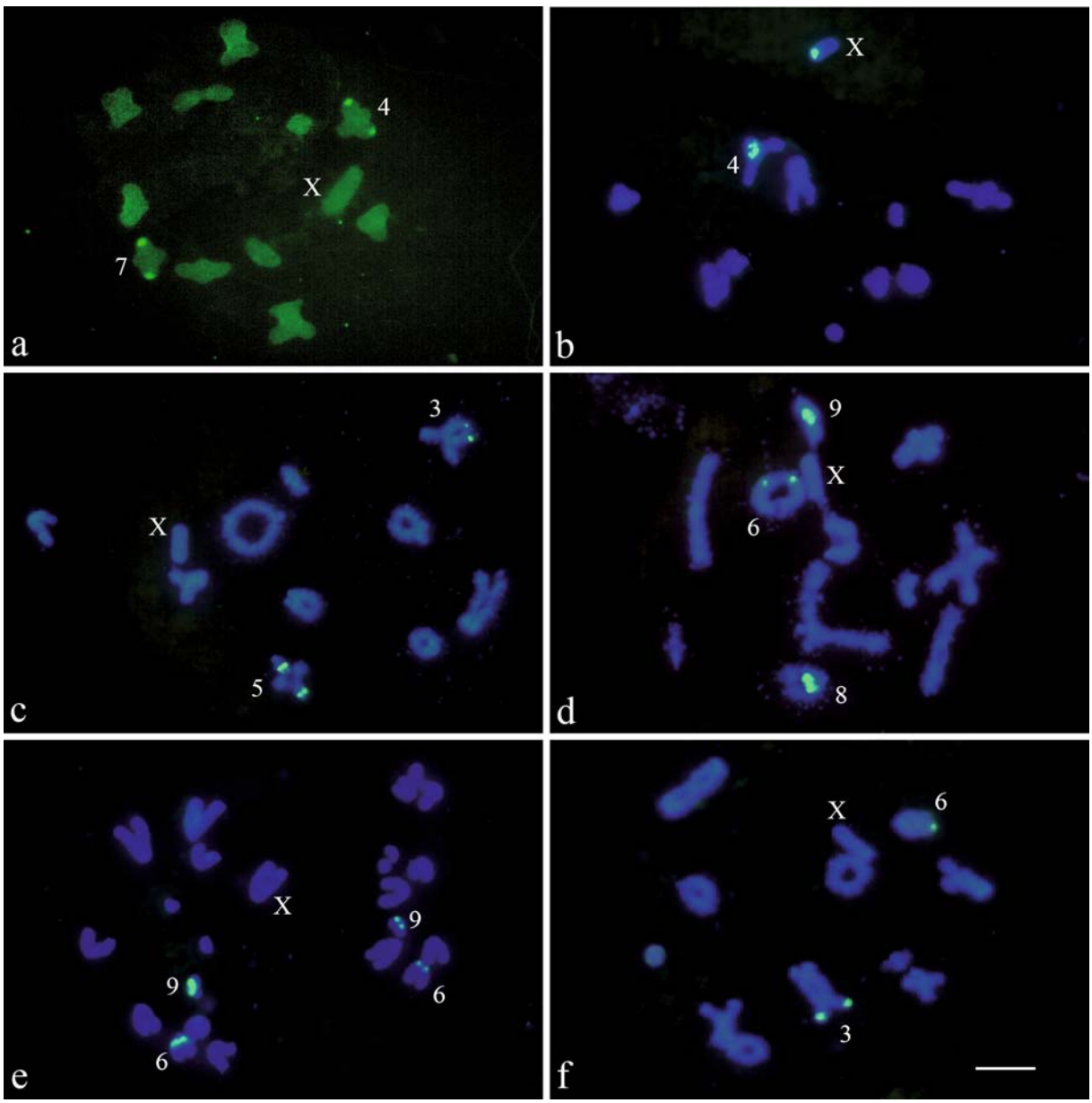


Figure 1. Fluorescent *in situ* hybridization (FISH), showing chromosome location of rDNA loci in six grasshopper species belonging to the subfamily Oedipodinae. **(a)** *Acrotylus fischeri*, **(b)** *Acrotylus patruelis*, **(c)** *Sphingonotus azurescens*, **(d)** *Sphingonotus coerulans*, **(e)** *Tropydopola cylindrica*, **(f)** *Paracinema tricolor*. Scale bar = 5 μm.

reflects evolutionary relationships, at least at the genus and subfamily levels. Therefore, these will be the levels used for interspecies comparisons in rDNA location.

Sequential silver staining and FISH were performed in *Oedipoda coerulescens* and *Dociostaurus maroccanus*. After making the silver impregnation, we took photographs from a number of cells and then removed silver deposits following the procedure described in Zurita *et al*. (1998). Afterwards, we applied the FISH technique. Photographs were taken in a cooled digital camera and optimized for brightness and contrast with the GIMP freeware. None of the variables used distributed normally (Shapiro-Wilks test: $p < 0.05$ in all cases) and we therefore used non-parametric Mann–Whitney tests

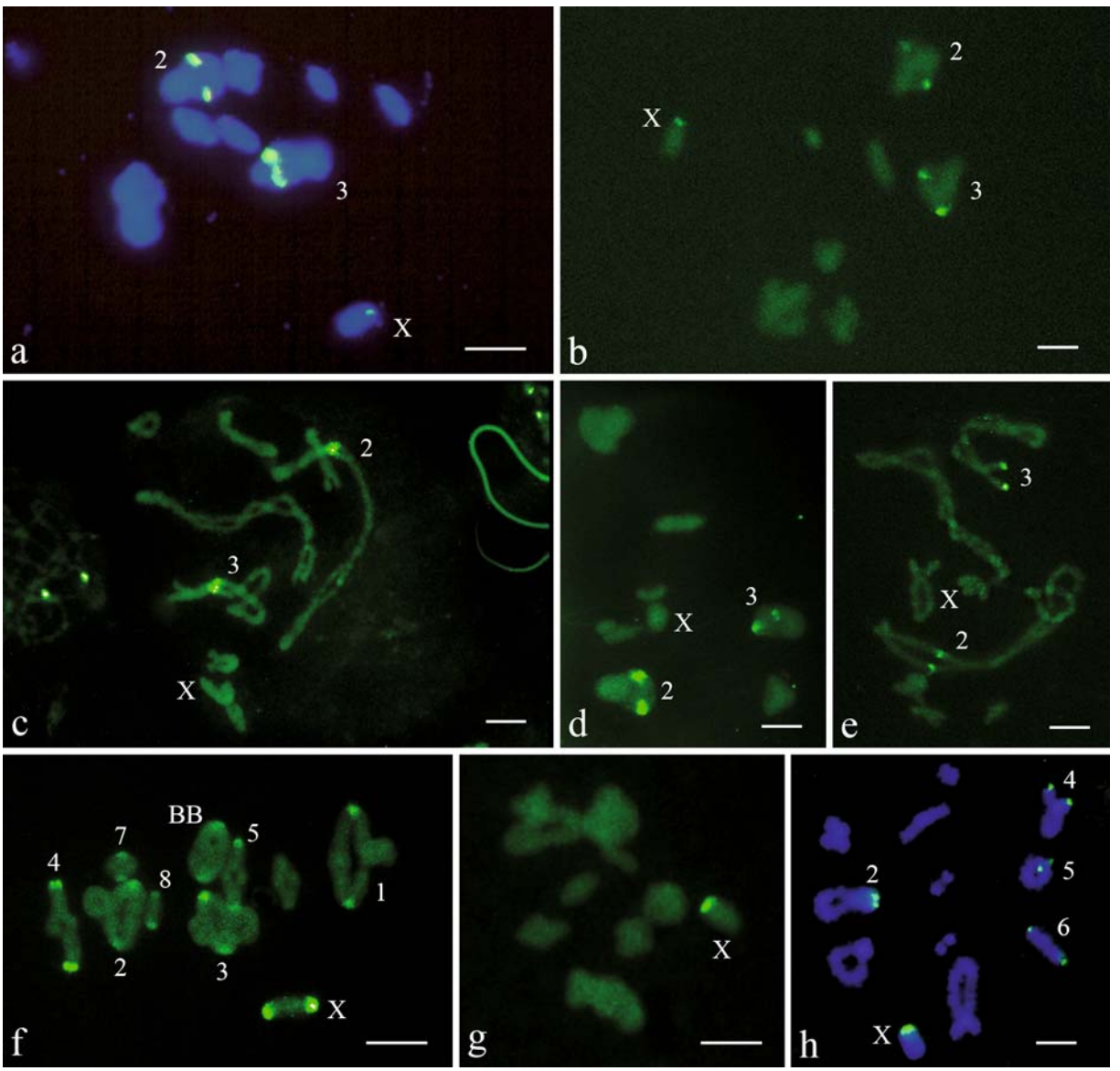
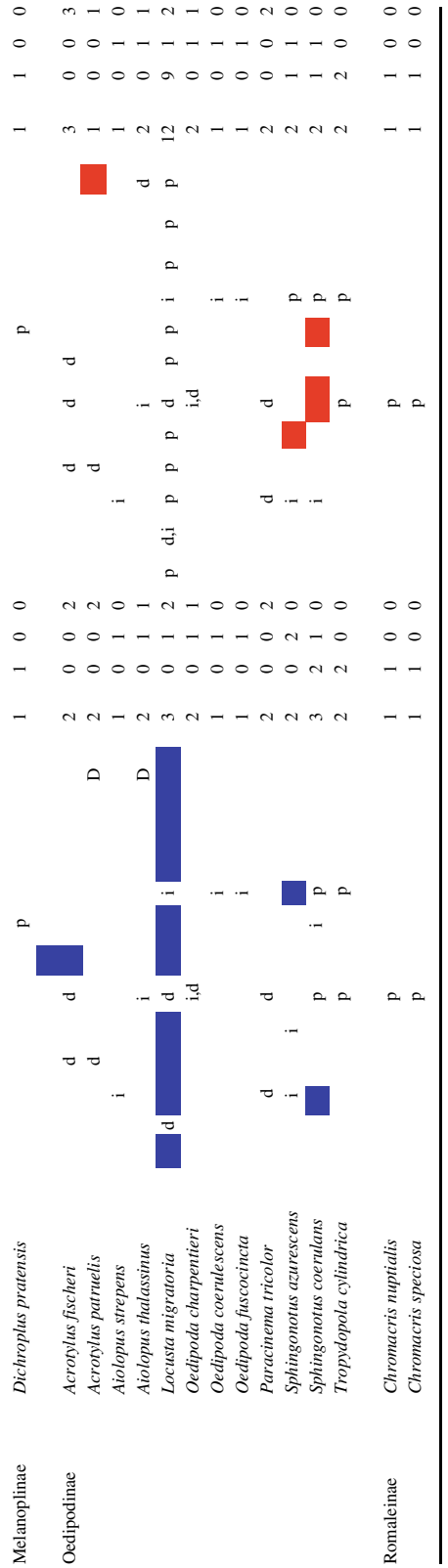


Figure 2. FISH showing chromosome location of rDNA in eight grasshopper species belonging to the subfamily Gomphocerinae, seven with $2n♂ = 17$ chromosomes **(a–g)** and one with $2n♂ = 23$ chromosomes **(h)**. **(a)** *Chorthippus eisentrauti*, **(b)** *Ch. bigguttulus*, **(c)** *Ch. jacobsi*, **(d)** *Ch. vagans*, **(e)** *Ch. parallelus*, **(f)** *Omocestus bolivari*, **(g)** *Stenobothrus festivus*, **(h)** *Dociostaurus maroccanus*. Scale bars = 5 μm.

Table 1. Chromosome location of rDNA by FISH and NOR activity by silver impregnation in 49 grasshopper species. The first 47 species belong to the Acrididae family and the two latter belong to the Romaleidae family. In *Eyprepocnemis plorans*, Eastern (E) and Western (W) populations were considered separately. Likewise, in *Chorthippus parallelus*, the *erythropus* (e) and *parallelus* (p) subspecies were considered separately. Red squares indicate inactive rDNA loci detected by FISH that were not active when analysed by silver impregnation; blue squares indicate active NORs in chromosome locations where FISH fails to reveal the presence of rDNA, and grey areas correspond to chromosome numbers that do not exist in 2n = 17 species. More details on populations and references in each species are provided in Table S1. p = proximal, i = interstitial, d = distal in respect to the centromere

Subfamily	Species	rDNA location																NOR activity															
		1	2	3	4	5	6	7	8	9	10	11	X	Total	p	i	d	1	2	3	4	5	6	7	8	9	10	11	X	Total	p	i	d
Catantopinae	*Pezotetix giornae*											p		1	1	0	0											p		1	1	0	0
Cyrtacanthacridinae	*Schistocerca flavofasciata*					i								1	0	1	0					i								1	0	1	0
	Schistocerca gregaria			i			i							2	0	2	0			i			i							2	0	2	0
	Schistocerca pallens					i	i							2	0	2	0					i	i							2	0	2	0
Eyprepocneminae	*Eyprepocnemis plorans* (E)									p		p		2	2	0	0									p		p		2	2	0	0
	Eyprepocnemis plorans (*W*)	p			p	p	p	p	p	i	p	p	P	10	9	1	0									i	p	p	p	4	3	1	0
	Eyprepocnemis unicolor									p	p			2	2	0	0									p	p			2	2	0	0
	Heterachris adspersa								p			p		2	2	0	0								p			p		2	2	0	0
	Shirakiacris shirakii									p	p	p		3	3	0	0									p	p	p		3	3	0	0
	Thisoicetrinus pterostichus									p				1	1	0	0									p				1	1	0	0
Gomphocerinae	*Amblytropidia* sp.									p				1	1	0	0									p				1	1	0	0
	Chorthippus apicalis				d		i							2	0	1	1				d	p	p		p					4	3	0	1
	Chorthippus biguttulus		i	i									P	3	1	2	0		i	i									p	3	1	2	0
	Chorthippus binotatus		i	i	p	p							P	5	3	2	0		i	i	p	p	p							5	3	2	0
	Chorthippus brunneus		i	i									D	3	0	2	1		i	i										2	0	2	0
	Chorthippus dorsatus		i	i										2	0	2	0		i	i										2	0	2	0
	Chorthippus eisentrauti		i	i									P	3	1	2	0		i	i									p	3	1	2	0
	Chorthippus jacobsi		i	i										2	0	2	0		i	i									p	3	1	2	0
	Chorthippus jucundus		i	i									P	3	1	2	0		i	i									p	3	1	2	0
	Chorthippus nevadensis		i											1	0	1	0		i		p								p	3	2	1	0
	Chorthippus parallelus (e)		i	d										2	0	1	1		i	d										2	0	1	1
	Chorthippus parallelus (p)		i	d									D	3	0	1	2		i	d									d	3	0	1	2
	Chorthippus vagans		i	i										2	0	2	0		i	i	d	p	p							5	2	2	1
	Dociostaurus jagoi	i								p				2	1	1	0	i								p				1	0	1	0
	Dociostaurus marocanus		p		p	p	p						P	5	5	0	0		p		p	p	p							4	4	0	0
	Omocestus bolivari	i	i	i	p	p		p	p				p,d	9	5	3	1		i		p	p	p		p				p,d	7	5	1	1
	Omocestus burri		i	i	p	p		p					p,i	7	4	3	0				p	p	p		p				i	5	4	1	0
	Omocestus panteli			i										1	0	1	0			i										1	0	1	0
	Omocestus raymondi												I	1	0	1	0												i	1	0	1	0
	Rhammatocerus brasiliensis				p		p			p				3	3	0	0				p		p			p				3	3	0	0
	Rhammatocerus brunneri									p				1	1	0	0									p				1	1	0	0
	Rhammatocerus palustris									p				1	1	0	0									p				1	1	0	0
	Rhammatocerus pictus									p				1	1	0	0									p				1	1	0	0
	Stauroderus scalaris	p	p	p	p	p	p	p	p				P	9	9	0	0			p										1	1	0	0
	Stenobothrus bolivari												P	1	1	0	0												p	1	1	0	0
	Stenobothrus festivus												P	1	1	0	0												p	1	1	0	0



for two-sample comparisons, and the Spearman rank test for correlations.

Results and discussion

Chromosome location of rDNA

Figures 1 and 2 present examples of rDNA location by FISH in Oedipodinae and Gomphocerinae species, respectively, and Table 1 shows a summary of all information known in grasshoppers regarding rDNA location (analysed by FISH) and activity (analysed by silver impregnation).

A quantitative analysis of data in Table 1 showed that the number of chromosomes (within the haploid genome) indicating the presence of rDNA ranged from 1 to 10 (mean = 2.47, SE = 0.29), although most species carried 1, 2 or 3 rDNA loci, and six species showed 5 to 10 loci (Figure 3). This might suggest the existence of some restrictions for high numbers of rDNA loci within species, as well as the spread of rDNA over the genome in some others, e.g. *S. scalaris* (López-León *et al*. 1999) and *E. plorans* (Cabrero *et al*. 2003). Such restrictions might simply be the lack, in most species, of the appropriate molecular mechanisms to move rDNA between non-homologous chromosomes.

Most of the 126 rDNA loci revealed by FISH were proximal to the centromere (52.4%), many were interstitial (34.9%), and a minority were distal (12.7%). The two most represented subfamilies were the Gomphocerinae, where the number of rDNA loci detected by FISH ranged from 1 to 9 (mean = 2.85, SE = 0.46, $n = 26$), and the Oedipodinae, where it ranged from 1 to 3 (mean = 1.92, SE = 0.19, $n = 12$). The difference between these two subfamilies did not reach significance (Mann–Whitney $Z = 0.74$, $p = 0.46$). They did not differ, either, for interstitial locations (about 39% in both; Mann–Whitney $Z = 1.01$, $p = 0.31$). However, proximal rDNA locations were significantly more frequent in Gomphocerinae (53%) than in Oedipodinae (17%) (Mann–Whitney $Z = 2.27$, $p = 0.023$), whereas distal rDNA locations were significantly more frequent in Oedipodinae (43%) than in Gomphocerinae (8%) (Mann–Whitney $Z = 2.17$, $p = 0.03$). Therefore, chromosome location of rDNA loci differs between acridid subfamilies, suggesting the existence of certain restrictions specific to different evolutionary lines.

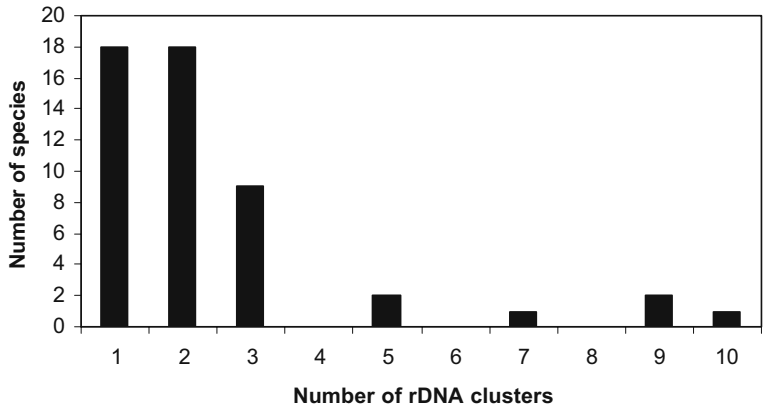


Figure 3. Distribution of rDNA loci number among 49 grasshopper species. Note that *E. plorans* and *Ch. parallelus* are considered twice because of their intraspecific variation (see text).

Correlation between rDNA and active NOR location

The 121 active NORs detected by silver impregnation of first meiocytes were located at one or more chromosomes (mean = 2.37, SE = 0.27), and showed chromosome locations very similar to rDNA (55.4% proximal, 31.4% interstitial and 13.2% distal) (contingency $\chi^2 = 0.35$, df = 2, $p = 0.84$). This indicates very high, although not complete, consistency between FISH and silver impregnation results. As expected, the number of rDNA loci per species was highly correlated with that of active NORs (Spearman $r_s = 0.776$, $r^2 = 0.602$, $p < 0.000001$), with only 60.2% of variation explained. This is due to two facts. First is the existence of inactive rDNA loci in some species, the most extreme case being *Stauroderus scalaris*, where the paracentromeric heterochromatin in all chromosomes contains much rDNA which is inactive in all chromosomes except L_3 (López-León *et al*. 1999). A similar phenomenon occurs in Spanish *Eyprepocnemis plorans*, where the rDNA in paracentromeric regions of most long and medium-sized chromosomes are usually inactive (López-León *et al*. 1995). In addition, Table 1 might suggest the existence of some apparently inactive rDNA loci, i.e. those failing to form a nucleolus in *Chorthippus binotatus*, *Ch. brunneus*, *Dociostaurus marocanus*, *Omocestus bolivari*, *O. burri*, *Acrotylus patruelis*, *Sphingonotus azurescens* and *S. coerulans*. Since Table 1 includes information compiled from different studies conducted at different times by different investigators, we cannot rule out that some of the 28 putatively inactive loci, found among the 126 loci detected by FISH, were actually artefacts caused by spatial or temporal differences in rDNA location and/or expression. In *S. scalaris*, *E. plorans*, *Ch. brunneus* and *D. maroccanus*, however, the same individuals were analysed by the same authors using both techniques, and they showed a total of 16 inactive rDNA loci. This indicates that 12.7–22% of all rDNA loci detected by FISH in the grasshopper sample analysed were inactive. In the 10 species carrying inactive rDNA, there was significant positive correlation between the total number of rDNA loci and the number of them that were inactive ($r_s = 0.81$, $p = 0.005$). It is also remarkable that B chromosomes in *Omocestus bolivari* carried rDNA at a pericentromeric location (see Figure 2f), but no NOR activity has been detected in them (Viseras & Camacho 1984).

A second explanation for the absence of complete correspondence between rDNA and NOR activity locations would be the existence of cryptic NORs giving rise to small nucleoli, in chromosome regions where no rDNA was revealed by FISH. For instance, the existence of NOR activity in all chromosomes of *Locusta migratoria* has been reported (Salcedo *et al*. 1988), but FISH reveals rDNA presence in chromosomes 2, 6 and 9 only, which usually yield the large, main nucleoli shown by silver impregnation (Salcedo *et al*. 1988). Likewise, in *Chorthippus apicalis*, *Ch. binotatus*, *Ch. jacobsi*, *Ch. nevadensis*, *Ch. vagans*, *Omocestus bolivari* and *O. burri*, Cabrero & Camacho (1986) reported the presence of active NORs in chromosome locations where our present FISH analysis has failed to detect rDNA (see

Table 1). Finally, in *Acrotylus fischeri*, *Sphingonotus azurescens* and *S. coerulans*, some active NOR locations previously revealed by silver impregnation (Viseras *et al*. 1991) are not corroborated by our present FISH analysis (see Table 1). A possible explanation for these contradictions would be the existence of orphon-like rDNA cryptic loci, at these chromosome locations, which are not detectable by FISH and are sometimes active.

To test this last possibility, we performed sequential silver staining and FISH in two species where we lacked previous evidence for cryptic NORs. As Figure 4a–c shows, the four autosomal rDNA clusters in *Dociostaurus maroccanus* are frequently active, forming conspicuous nucleolus material attached to the rRNA genes, whereas rDNA in the X chromosome was usually inactive. In addition, some small nucleoli can be observed emerging from chromosome regions where no evidence for rDNA presence is provided by FISH. In addition, a single rDNA cluster is always revealed by FISH in *Oedipoda coerulescens*, interstitially located in chromosome 9 (Figure 4d). However, nucleoli also frequently emerge from other chromosomes, in regions apparently lacking rDNA (Figure 4e,f). Therefore, the existence of orphon-like rDNA is conceivable at these locations.

Interspecies comparisons for chromosome rDNA location

Genetic maps are not available in grasshoppers, so that chromosome homeology relationships among the 49 species analysed here are not known. This represents a problem for interspecific comparative analyses since we cannot be sure that a given chromosome, e.g. the third in order of decreasing size, is homeologous in two or more species, because chromosomal rearrangements in the past might have changed size relationships among non-homologous chromosomes. However, it is expected that this problem is less serious when closely related species are compared.

Previous papers on active NOR location by silver impregnation (Rufas *et al*. 1985, Cabrero & Camacho 1986, Santos & Fox 1988, Viseras *et al*. 1991) performed interspecific comparisons under the assumption that homeology relationships coincide with those indicated by relative chromosome size. In order to compare our results with this previous information, and with the cautions remarked above, we will next perform interspecific comparisons to infer which chromosomes (arranged in decreasing size) are the ones carrying rDNA most frequently at genus and subfamily levels.

As shown in Figure 5, the chromosomes most frequently showing rDNA were 2, 3, 6, 9 and X. Chromosomes 2, 3 and X were especially frequent as rDNA carriers in Gomphocerine species with $2n♂ = 17$ chromosomes, whereas chromosomes 6 and 9 frequently carried rDNA in the Oedipodinae. In each subfamily, about half of species carried rDNA in the aforementioned chromosomes. It is thus presumable that these might represent the consensus rDNA locations in the ancestors of each subfamily, a contention consistent with previous information on active NOR location by silver impregnation (Rufas *et al*. 1985, Cabrero & Camacho 1986, Santos & Fox 1988, Viseras *et al*. 1991).

When we compared rDNA location among species within the same genus, three patterns emerged (see Table 1): (1) complete coincidence in rDNA location among species of the genera *Stenobothrus* and *Chromacris*; (2) no coincidence at all in *Dociostaurus*, *Aiolopus* and *Sphingonotus*; and (3) partial coincidence in the remaining genera analysed, with a different degree of consensus for different chromosome locations. Examples of consensus locations were chromosomes 2 and 3 in *Chorthippus*, chromosome 6 in *Chromacris*, chromosome 9 in *Rhamatocerus*, and the X chromosome in *Stenobothrus*.

Spread of rDNA through grasshopper genomes

The intra- and interspecific variation in chromosome location of 45S rDNA observed in grasshopper genomes could be explained by at least three different mechanisms: (i) structural chromosome rearrangements, such as translocations or inversions, (ii) ectopic recombination and (iii) transposition of a few rDNA repeats to new locations in the same or a different chromosome, amplification of these new minor loci, and deletion of original major loci (see Introduction).

The best example of chromosomal rearrangements in grasshoppers is the above-mentioned occurrence of three centric fusions giving rise to the Gomphocerinae species with $2n = 17$ (Hewitt 1979). Of course, other more cryptic rearrangements could have occurred, changing the rDNA location, such as a putative paracentric inversion which changed the interstitial rDNA locus in chromosome 3 to a distal position in *Ch. parallelus*.

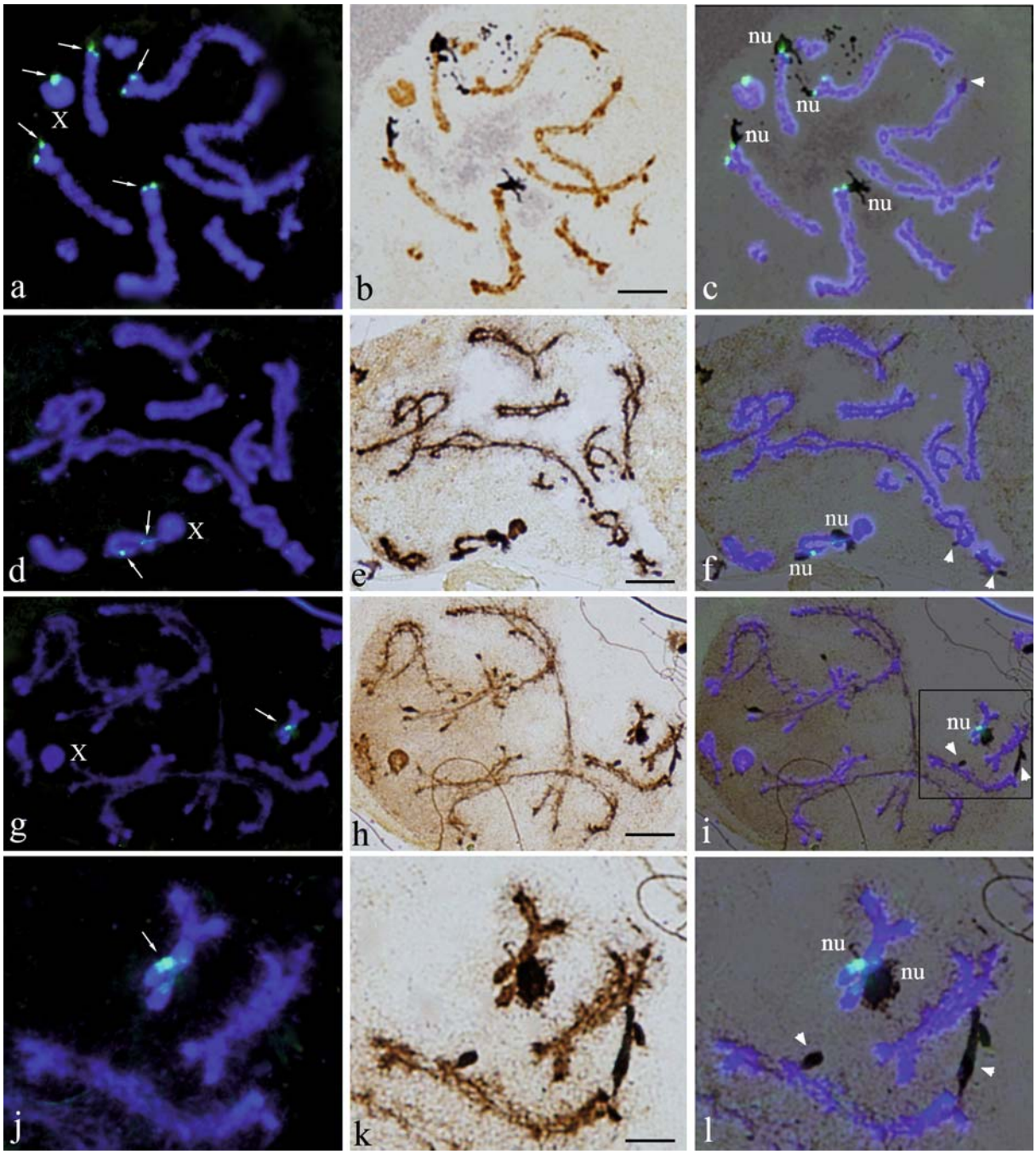


Figure 4. Sequential silver staining and FISH in *Dociostaurus maroccanus* **(a–c)** and *Oedipoda coerulescens* **(d–l)**. Chromosome location of rDNA (arrows) is shown by FISH in the left column, nucleolus formation is shown by silver impregnation in the central column, and both images are merged in the right column, which also shows nucleoli attached to rDNA (nu) and cryptic NORs (white arrowhead). Each row corresponds to an individual cell, except for the last one **(j–l)**, which shows part of the third-row cell at higher magnification. Scale bars represent 5 μm in **(b)**, **(e)** and **(h)**, and 2 μm in **(k)**.

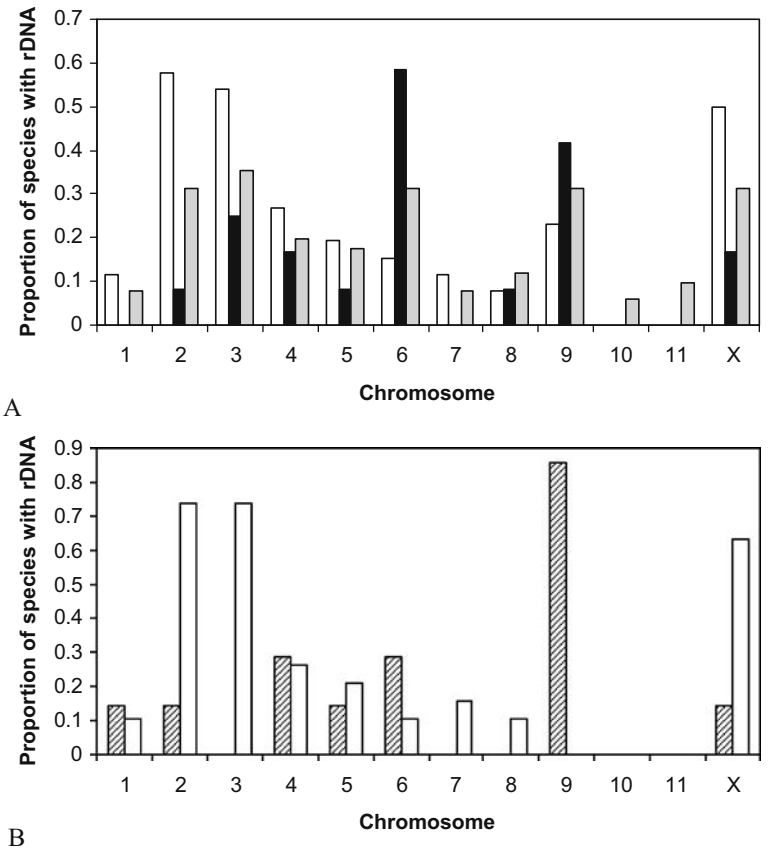


Figure 5. **(a)** Chromosome location of rDNA loci in 25 Gomphocerinae (white) and 12 Oedipodinae (black), and in all 49 grasshopper species analysed (grey). **(b)** Chromosome location of rDNA loci in Gomphocerinae grasshoppers with $2n♂ = 23$ (striped) and $2n♂ = 17$ (white).

The ectopic-recombination hypothesis was proposed to explain rDNA spread through *Gossypium* genomes (Hanson *et al*. 1996). This hypothesis predicts a higher number of rDNA loci in species with terminal rDNA loci, since it would permit rearrangements of rDNA with no deleterious effects at other loci. Our present data in 49 grasshopper species enable us to test this hypothesis since it would predict a positive correlation between the total number of rDNA loci per species and the number of those located in a distal position. However, correlation between these two parameters did not reach significance (Spearman $r_s = 0.27$, $p = 0.06$), whereas the total number of rDNA loci was significantly correlated with the number of proximal ($r_s = 0.40$, $p = 0.004$) and interstitial ($r_s = 0.40$, $p = 0.003$) locations. When the analysis was performed separately on the 26 Gomphocerinae and the 12 Oedipodinae species, the results did not change in the case of Gomphocerinae, but no correlation was found in the Oedipodinae. These results do not agree with predictions of the ectopic-recombination hypothesis.

It appears that Dubcovsky & Dvorak's mechanism (Dubcovsky & Dvorak 1995) has the highest explanatory power, since it is valid for every kind of rDNA mobility not changing the location of adjacent markers, as they found in Triticeae genomes. Unfortunately, no genetic maps are available in grasshoppers, so that no markers can be analysed other than rDNA chromosome location itself. However, our present results favour their hypothesis, since one of its predictions is the existence of minor rDNA loci spread over the genome, and these might correspond to the cryptic NORs revealed here.

Strong support for the Dubcovsky and Dvorak hypothesis has recently been provided in a cytogenetic analysis of the dynamics of *En*/*Spm* transposons in meiosis of the plant *Aegilops speltoides* (Raskina

et al. 2004), indicating that: (i) they are active during male gametogenesis; (ii) they form clusters in the hot spots of large chromosomal rearrangements, either separately or in conjunction with rDNA; and (iii) at least some of the mobile rDNA sites were connected with meiotic activity of *En*/*Spm* transposons. Recently, it has been suggested that 5S RNA genes in filamentous fungi are capable of multiplying and integrating into other areas of the genome through a process which is the same as, or akin to, retroposition (Rooney & Ward 2005). The intra- and interspecific variation in rDNA location observed in grasshoppers might be produced through the Dubcovsky and Dvorak mechanism, i.e. the transposition of a few rRNA genes to new chromosome locations, their amplification, and the elimination of the old NOR once the new one can supplant the old one in function. The observed inactive rDNA loci might correspond to those in the process of elimination, and the cryptic NORs might correspond to a few moved rDNA units before amplification.

The next step should be to demonstrate the presence of rDNA in these cryptic NORs and to investigate whether they are associated with transposable elements moving along with them through genomes. Species showing interpopulation variation in rDNA location, such as *Eyprepocnemis plorans* where a spread of rDNA throughout the genome seems to have taken place recently (Cabrero *et al*. 2003) constitute good material for a thorough analysis of possible spreading mechanisms.

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