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Nucleolus size variation during meiosis and NOR activity of a B chromosome in the grasshopper *Eyprepocnemis plorans*

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Abstract

The number of nucleoli and nucleolar area were measured in meiotic cells from males of the grasshopper *Eyprepocnemis plorans* collected in three natural populations. Number of nucleoli per cell showed no significant correlation among cells in different meiotic stages, but there was strong positive correlation for nucleolar area between leptotene and interkinesis cells in individuals from distant populations (Salobreña in Spain, and Smir in Morocco). No correlation was, however, observed for both parameters between the meiotic stages analysed in individuals from the population of Torrox (Spain). The number of nucleoli at leptotene was about double the number at interkinesis, as expected from the double ploidy level at leptotene and the corresponding double number of rDNA clusters. Leptotene nucleolar area, however, was about fourfold that in interkinesis, presumably due to higher requirements for ribosome biogenesis in meiosis I than meiosis II. In Torrox, diplotene cells showed a lower number of nucleoli but larger nucleolar area than in leptotene cells, suggesting an increase in nucleolus size during prophase I. Significant differences were found among populations for nucleolar area but not for number of nucleoli, the smallest nucleolar area being observed in Torrox, which is the population harbouring the most parasitic B chromosome variant. No clear effects on nucleolar area or number of nucleoli were associated with the B-chromosome number. However, B-chromosome effects on the nucleolar area were apparent in the Torrox population when data were analysed with respect to a B-chromosome odd–even pattern in leptotene and interkinesis cells. However, in diplotene cells no odd–even pattern was observed for both nucleolar parameters, suggesting that the increase in nucleolar size from leptotene to diplotene dilutes the leptotene odd–even pattern. The rDNA distally located in the B chromosome was associated with a nucleolus in 6.5% out of the 247 diplotene cells analysed. The implications of these findings are discussed in the context of B chromosomes as stress-causing genome parasites and the nucleolus as a sensor of stress.

Introduction

Nucleolus organizer regions (NOR) are the chromosome sites where the nucleolus is formed (McClintock 1934) through transcription of multiple copies of rDNA loci (28S, 18S and 5.8S). Ribosomes are assembled in the nucleolus from these three resulting rRNAs together with 5S rRNA and about 85 proteins coded by other loci (for review see Pikaard 2002). Nucleolus size increases and decreases in growing and resting cells, respectively, and it forms and disperses once every cell-division cycle (Pikaard 2002). It has been shown that the size of a nucleolus is proportional to the amount of rRNA synthesized (Caspersson 1950), that NOR size (the number of rRNA cistrons) is, in general, correlated with its

expression level (Shubert & Künzel 1990), that hypertrophy of the nucleolus is a state in which rRNA and ribosome synthesis has increased (Nakamoto *et al*. 2001), and that large nucleoli may correlate with cell-division activity and with cellular stages having high protein demand (Mosgoeller 2004). Therefore, the function of the nucleolus is tightly linked to cell growth and proliferation, and recent research suggests that the nucleolus also plays an important role in stress response (Rubbi & Milner 2003, Olson 2004).

A close relationship has been demonstrated between nucleolus area (visualized by silver staining) and the rate of cell proliferation: the shorter the cell-cycle time, the greater the amount of silver staining in the nucleoli, and this is especially useful to distinguish tumorous from non-tumorous cells (Derenzini *et al*. 1994, 2000, Trerè 2000). The size of silver-stained regions (AgNOR) is closely related to both nucleolar size and RNA polymerase I transcriptional activity (Derenzini *et al*. 1992, 1998, Derenzini 2000), it is a useful tool for obtaining information on the rate of ribosome biogenesis in *in-situ* cytohistological preparations (Derenzini 2000) and it is generally considered a sensitive marker of both nucleolar biosynthetic and cell-proliferation activities in individual cells (Busch *et al*. 1979, Okabe *et al*. 1991, Thiele & Fischer 1993, Busch 1997).

Nucleolus size is sensitive to factors such as starvation and feeding, since starvation for 24 h in rats produced a 50% decrease in the size of the nucleolus, but feeding of a high-protein diet led to a very rapid increase in nucleolar size (Lagerstedt 1949). It has been shown that the intracellular availability of certain amino acids can markedly influence the regulatory mechanisms of rRNA synthesis (Wannemacher 1972). Likewise, nucleolus size in hepatocytes from American bullfrogs (*Rana catesbeiana*) enlarges in response to oestrogen in parallel to an increase in the total amount of RNA in the nucleolus (Herbener & Bendayan 1998).

Nucleolus also changes in size in response to environmental contaminants. For instance, Hudson & Ciborowski (1996) submitted larvae of the mosquito *Chironomus salinarius* to several mixtures of contaminated and non-contaminated sediment and found that the proportion of larvae with reduced nucleolus size increased linearly with each doubling of concentration of contaminated sediment. This shows again that the nucleolus plays an important role in stress response (Olson 2004).

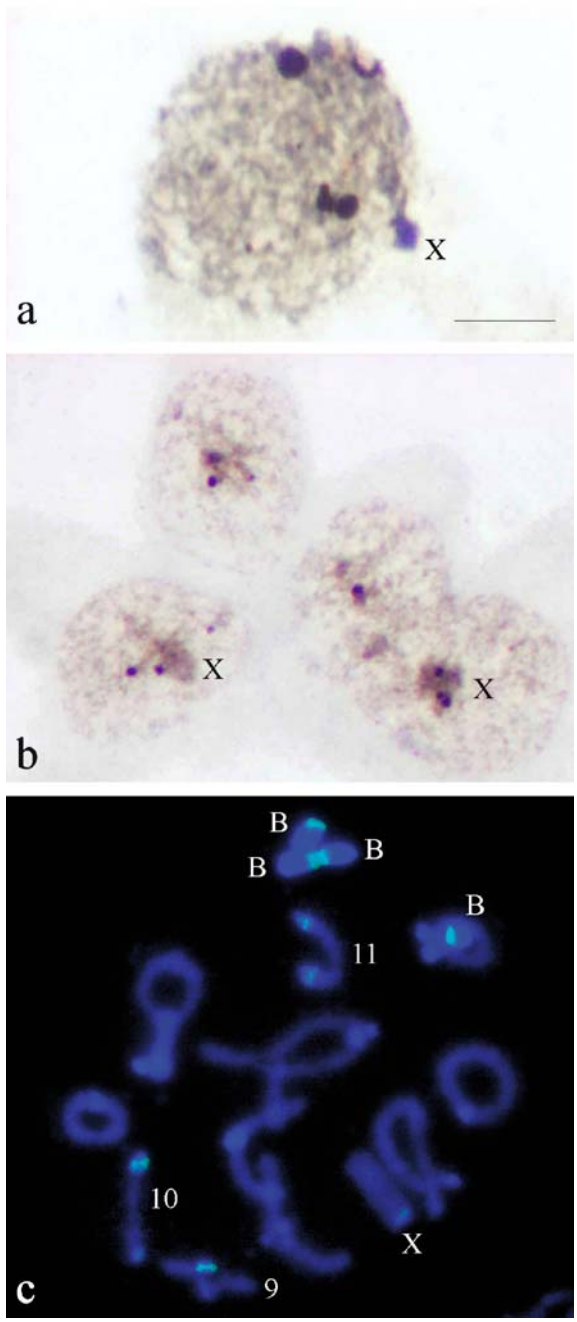

Figure 1. Silver-stained cells of the grasshopper *Eyprepocnemis plorans* at male meiotic leptotene (**a**) and interkinesis (**b**), and FISH of a diplotene cell with a rDNA probe (**c**). Scale bar = 5 μm.

During meiosis, in chordates, there is vigorous nucleolar activity during prophase I, with a maximum

at pachytene (Schmid *et al*. 1982, 1983, Wachtler & Stahl 1993). Ribosomal RNA synthesis ceases after pachytene in spermatogenesis but continues until diplotene in oogenesis. However, it is completely stopped from metaphase I until the end of the second meiotic division (Sumner 2003).

Meiosis is exploited by B chromosomes, i.e. additional chromosomes found in natural populations of many organisms, showing non-Mendelian accumulation mechanisms (drive) and thus behaving as genome parasites (for review see Camacho *et al*. 2000, Camacho 2005). The grasshopper *Eyprepocnemis plorans* shows a very widespread B-chromosome polymorphism with B chromosomes being highly mutable and composed mainly of two types of repetitive DNA, i.e. a 180 bp tandem repeat DNA and ribosomal DNA (Cabrero *et al*. 1999). The B-chromosome polymorphism of *E. plorans* shows B chromosomes passing through stages of parasitism (with drive) and neutralization (with no drive). These stages have been identified in natural populations from both Spain (Camacho *et al*. 1997, Zurita *et al*. 1998) and Morocco (Bakkali *et al*. 2002).

The grasshopper *E. plorans* carries rRNA genes in the paracentromeric region of almost all chromosomes, although NORs in chromosomes 9, 10, 11 and X are large while those in the remaining A chromosomes are small. The B chromosomes actually carry the largest rDNA cluster in a distal location (López-León *et al*. 1994, Cabrero *et al*. 1999).

NOR activity appears to be influenced by B chromosome presence in populations such as Salobreña (Granada, Spain) (Cabrero *et al*. 1987) and two Moroccan populations (Bakkali *et al*. 2001), where B-presence was associated with a higher number of active NORs per cell. Notably, Bakkali *et al*. (2001) observed that secondary NORs (those corresponding to small rRNA clusters) are frequently active in Moroccan *E. plorans* populations, in contrast to Spanish populations, where they have never been found to be active (Cabrero *et al*. 1987, López-León *et al*. 1995).

All these effects refer to the number of active NORs, but the nucleolus size was not controlled. Therefore, we do not know whether these changes in the number and types of active NOR imply differences in nucleolus size and, consequently, differences in the total amount of rRNA among individuals being associated to B chromosome presence or the geographical origin of grasshoppers. To investigate this question we measured nucleolus size and number in two different meiotic stages (leptotene and interkinesis) in males from three different populations. In addition, diplotene cells from Torrox males were scored because they showed NOR activity in the B chromosome.

Materials and methods

Fifteen adult males of the grasshopper *E. plorans* were collected at Salobreña (Granada, Spain) in 1997, Smir (Morocco) in 1997 and Torrox (Málaga, Spain) in 1999. Testes were fixed in freshly prepared 3:1 ethanol–acetic acid and stored at 4°C. Nucleoli were visualized by the silver impregnation technique developed by Rufas *et al*. (1982). To facilitate distinction between nucleolar material and chromatin in silver-stained squash preparations, slides were stained with 1% Giemsa for 1 min, yielding blue chromatin and brown nucleoli. Two meiotic stages

Table 1. Correlation analysis for the number of nucleoli (NN) and nucleolar area (NA) per cell between leptotene (L), interkinesis (I) and diplotene (D)

Population	Stages	N	Trait	*r*	*p*
Salobreña	L-I	15	NN	0.183	0.514
	L-I	15	NA	0.714	0.003
Smir	L-I	15	NN	0.390	0.151
	L-I	15	NA	0.563	0.029
Torrox	L-I	15	NN	0.296	0.284
	L-I	15	NA	0.217	0.436
	L-D	15	NN	0.088	0.756
	L-D	15	NA	0.162	0.564
	D-I	15	NN	−0.014	0.961
	D-I	15	NA	0.444	0.098

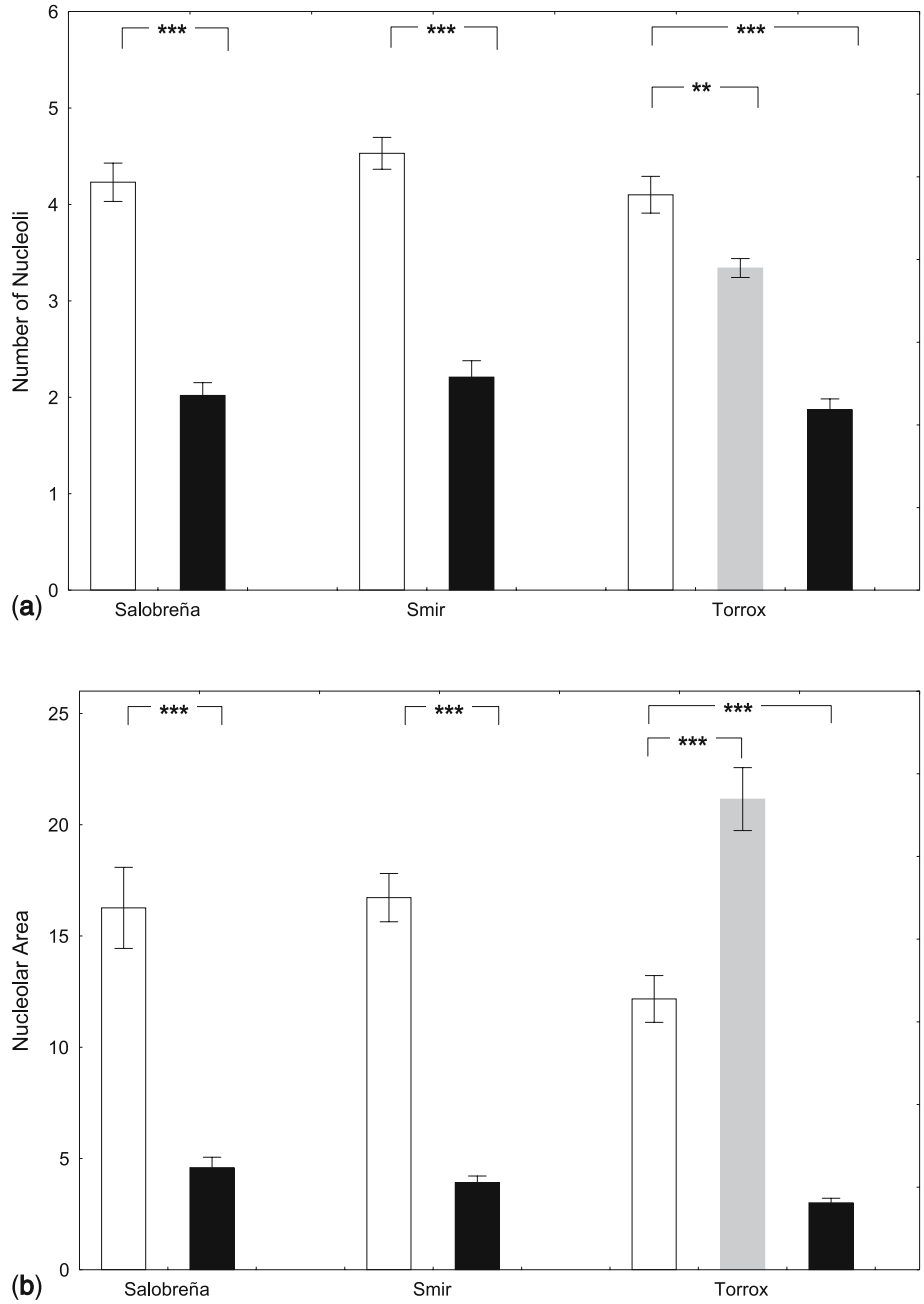

Figure 2. Comparison of the number of nucleoli (**a**) and nucleolar area (**b**) per cell between leptotene (white), interkinesis (black) and diplotene (grey). Deviation bars in the histograms indicate mean ± 1 standard error. Significance in matched-pairs *t*-tests is indicated by two ($p < 0.01$) or three ($p < 0.001$) asterisks.

were analysed in the three populations: leptotene and interkinesis. In Torrox, diplotene cells were also analysed. Ten leptotene and interkinesis cells and 20 diplotene cells per individual were photographed at 40× magnification with a digital camera (Olympus DP70, capture size of 2040×1536). Sizes were transformed to μm^2 after calibration with a scaled slide.

The number of nucleoli (NN) per cell was scored in all types of cells and populations. The nucleolus area was measured in arbitrary units (au), with the help of a

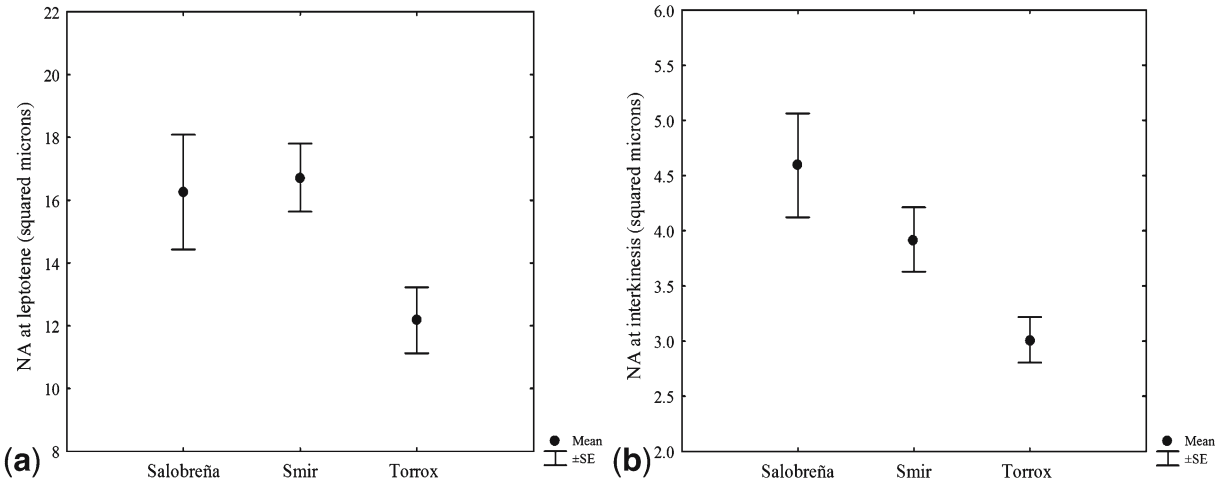

Figure 3. Population differences for nucleolar area (NA) at leptotene (**a**) and interkinesis (**b**) expressed in μm^2.

digital pen tablet and the ImageJ program (Rasband 1997), and then converted to μm^2 after calculating magnification scale. The sum of all nucleolar areas in a cell (NA) was used for statistical analyses. In diplotene cells it was possible to score and measure the nucleoli attached to each chromosome bivalent (autosomes) or univalent (X and B chromosome).

For statistical analyses we calculated the individual mean per male for both NN and NA prior to applying the Student *t*-test, one-way ANOVA and correlation analysis.

To ascertain the measurement precision of our experimental approach we first calculated the error for nucleolus size, which was determined by repeating the measurement in a subsample of 30 cells (122 nucleoli) randomly taken from several individuals. Following Yezerinac *et al*. (1992), a nested ANOVA was performed to calculate the percentage of measurement error (ME) as

$$\%ME = \frac{s^2_{\text{within}}}{s^2_{\text{within}} + s^2_{\text{among}}},$$

where s^2_{within} represents the within-individual variance (variance between measurements of the same nucleolus) and s^2_{among} the inter-individual variance (variance among different nucleoli).

To test for possible effects of squashing on area measurements, we measured nuclear area in all leptotene and interkinesis cells from Salobreña males, we inferred nuclear volume by assuming spherical shape for nuclei and compared it between these two stages. If squashing did not influence area

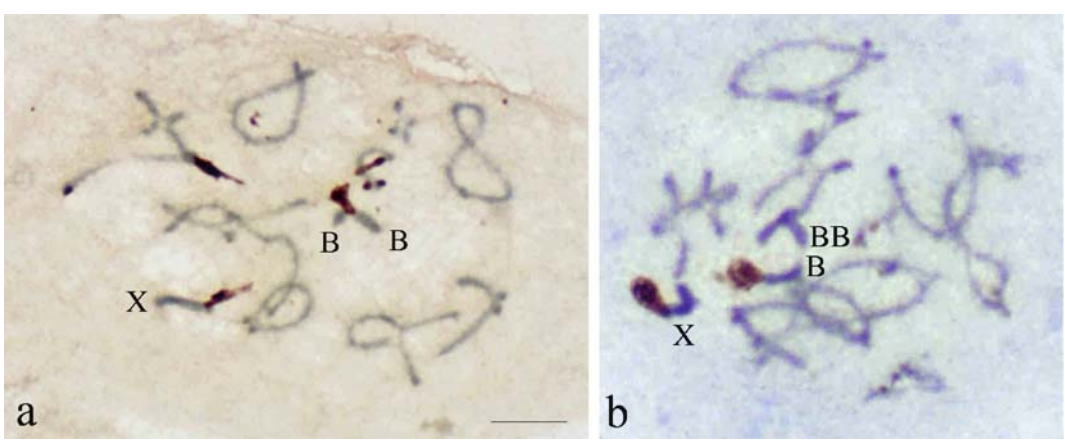

Figure 4. Examples of silver-stained diplotene cells from Torrox males carrying two (**a**) and three (**b**) B chromosomes, showing NOR activity in some B chromosomes. Scale bar = 5 μm.

Table 2. Frequency of activity of rRNA genes in the B chromosome in diplotene cells from the 13 B-carrying males collected at Torrox*

		B-NOR				
Id. no.	Bs	Inactive	Active	Total cells	Cells with B-NOR$^+$	B-NOR$^+$/B
1	1	20	0	20	0	0
2	1	20	0	20	0	0
3	1	20	0	20	0	0
4	1	20	0	20	0	0
5	1	18	0	18	0	0
6	1	20	0	20	0	0
7	2	20	0	20	0	0
8	2	9	1	10	0.100	0.050
9	2	20	0	20	0	0
10	2	20	0	20	0	0
11	3	14	6	20	0.300	0.100
12	4	15	4	19	0.211	0.053
13	6	15	5	20	0.250	0.042
Total or mean	2.08	231	16	247	0.065	0.031

*B-NOR$^+$/B in the last column indicates the proportion of B chromosomes showing NOR activity, and is calculated by dividing the proportion of cells with B-NOR$^+$ by the number of Bs, since NOR activity was rarely observed in more than one B in a same cell.

measurement, we should expect a volume at leptotene double that at interkinesis. A Student *t*-test showed no significant differences between leptotene and doubled interkinesis volumes inferred from area measurements ($t = 0.896$, $d.f. = 298$, $p = 0.371$). We therefore concluded that squashing is negligible in our area measurements.

Results

Changes in nucleolar area during meiosis

The silver-staining technique employed proved to be a convenient and reliable cytochemical technique with a measurement error for nucleolar area of 1.53%, which was acceptably low. Figure 1 shows some examples of silver-stained leptotene and interkinesis cells, as well as rDNA location by FISH. The number of nucleoli per cell (NN) showed no significant correlation between leptotene and interkinesis, but nucleolar area (NA) showed a positive correlation between these two meiotic stages in two (Salobreña and Smir) of the populations analysed (Table 1). This suggests that nucleolar area is more strictly subjected to cell control than the number of nucleoli, and that some difference exists among populations in this respect. In the Torrox population, however, both NN and NA were independent between the different meiotic stages analysed.

The two parameters measured, i.e. NN and NA, differed significantly between leptotene and interkinesis, with consistent results among populations (Figure 2). In leptotene, NN was about twice that in interkinesis, in accord with its double ploidy level. When we repeated the tests in Figure 2 but, in this case, doubling the interkinesis figures, no significant differences were found (p values 0.53, 0.73 and 0.16 for Salobreña, Smir and Torrox, respectively), suggesting that the number of nucleoli per cell is proportional to the number of rRNA gene clusters. NA, however, was approximately fourfold larger in leptotene than in interkinesis (see Figure 2).

In the Torrox population, NN was significantly higher at leptotene than at diplotene (Figure 2), indicating that some of the nucleoli produced by the two members of a pair of homologous chromosomes at leptotene are fused after synapsis and appear as a single nucleolus at diplotene. Most importantly, NA was significantly higher at diplotene than leptotene (Figure 2), suggesting that some changes in nucleolar size do occur between leptotene and diplotene which are not solely explained by nucleolar fusion.

Variation in number of nucleoli and nucleolar area per cell among populations

One-way ANOVA showed significant differences among the three populations for NA ($F_{2,42} = 3.35$, $p = 0.045$ at leptotene; $F_{2,42} = 5.42$, $p = 0.008$ at

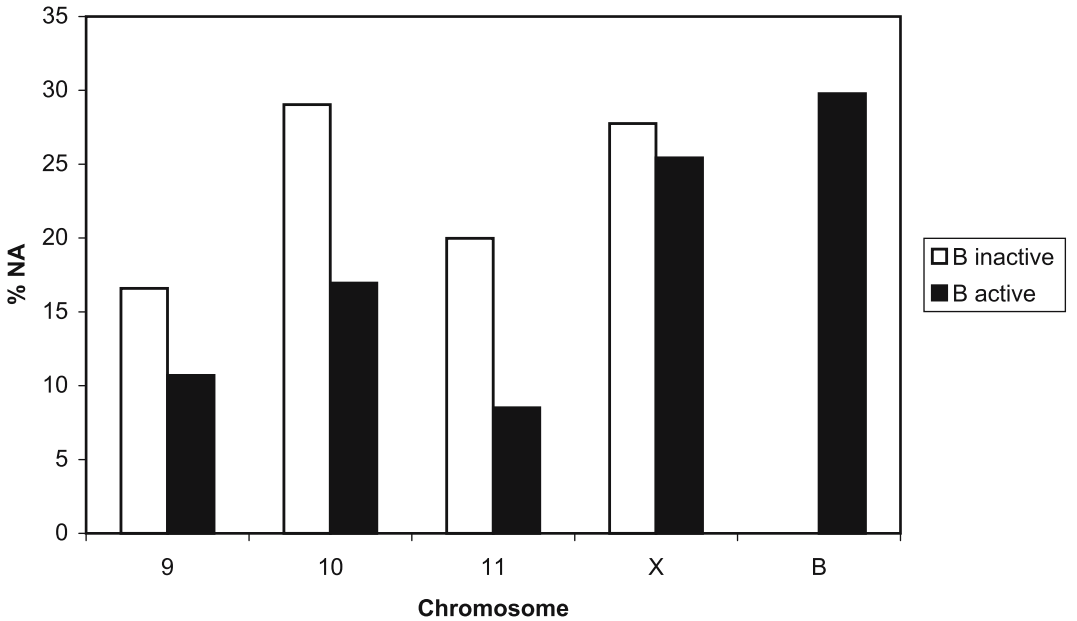

Figure 5. Relative nucleolar area (NA) associated with A chromosomes (9, 10, 11 and X) and B chromosomes in cells with the B-NOR inactive (white bars) and active (black bars), in 13 B-carrying males from Torrox. Note the decrease in nucleolar contribution by chromosomes 9, 10 and 11 when the B-NOR is active.

interkinesis) but not for NN ($F_{2,42} = 1.39$, $p = 0.261$ at leptotene; $F_{2,42} = 1.46$, $p = 0.243$ at interkinesis). Figure 3 shows that NA levels in both leptotene and interkinesis were lower in the Torrox population.

B-chromosome effects

Given the observed differences among populations, B-chromosome effects on NN and NA were separately analysed in each population. A series of one-way ANOVA tests showed no difference for either NN or NA in leptotene or interkinesis among individuals with different numbers of B chromosomes in Salobreña (p ranging from 0.47 to 0.97) and Torrox ($p = 0.18$–0.96). In Smir, however, NA at leptotene showed significant differences among males with different B number ($F = 4.19$, *d.f.* = 2,12, $p = 0.042$). Grouping B-carrying males according to whether they carried an odd or even B number, Student t tests showed the absence of effects in Salobreña ($p = 0.21$–0.92) and Smir ($p = 0.06$–0.33). In Torrox, however, males with odd B number showed significantly lower NA at leptotene ($t = 2.36$, *d.f.* = 11, $p = 0.038$) and higher NN at interkinesis ($t = 2.68$, *d.f.* = 11, $p = 0.021$).

Activity of rRNA genes in the B chromosome at Torrox

In diplotene cells from Torrox the rRNA genes located at the distal region of the B chromosome showed activity in four out of the 13 B-carrying males analysed (Figure 4). The B-NOR was active in 6.5% of diplotene cells, on average, but it was inactive in all six males with 1B and three of the four males with 2B, whereas NOR activity in at least one B was observed in all three males with three or more Bs (Table 2). On average, about 3.1% of the B chromosomes examined into diplotene cells displayed NOR activity.

One-way ANOVAs for NN and NA in diplotene cells (for total cell and chromosomes 9, 10, 11 and X, separately) comparing among males with 0B, 1B and 2B or more (grouped in a single class) showed no significant differences ($p = 0.26$–0.99), suggesting that B number does not influence these parameters. Likewise, no odd–even pattern was apparent for NN or NA, albeit expressed for total cell or for each NOR-carrying chromosome (9, 19, 11, X and B) separately ($p = 0.16$–0.82), suggesting that the observed odd–even pattern for NA at leptotene does not persist until diplotene.

In the 16 diplotene cells where the B chromosome displayed NOR activity, the nucleolus attached to the B measured 5.19 μm^2 on average, representing about 30% of total NA in the cell, the B thus being the chromosome with the highest contribution to NA in the cell (Figure 5). Total cell NA, however, did not differ between the cells where the B-NOR was inactive (mean = 21.87, SD = 16.69) and those where it was active (mean = 18.59, SD = 7.39) (t = 0.78, *d.f.* = 246, p = 0.44). This means that B-NOR activity is performed at the expense of that in A chromosomes, especially chromosome no. 11 (58% decrease), chromosome 10 (42% decrease) and chromosome 9 (36% decrease), the X chromosome (9% decrease) almost not being affected by the B-NOR activity (see Figure 5).

Discussion

Nucleolus size variation during meiosis

The strong positive correlation for nucleolar area between leptotene and interkinesis suggests that it is strictly subjected to cell control. In a species such as *E. plorans*, with rRNA genes in almost all chromosomes, the number of nucleoli formed appears to be irrelevant (since no correlation was found between leptotene and interkinesis), provided that the required amount of rRNA is assured. However, an increase in the number of nucleoli could increase the speed of interchange of molecules between the nucleolus and the surrounding nuclear environments leading to higher efficiency in the nucleolar function. The fact that nucleoli can fuse together or fragment, at different meiotic stages, could also explain the lack of correlation for NN among meiotic stages.

Little is known about how nucleolar size is controlled (Tuma 2005), but recent research suggests that nucleostemin, a nucleolar protein found preferentially in stem and cancer cells, might be involved in transmitting information on the surrounding cell environment to the nucleolus, thus linking growth signals with nucleolar size (Tsai & McKay 2005, Tuma 2005). We have shown that NN and NA vary significantly through meiosis, with nucleoli being present between leptotene and diplotene, during the first meiotic division, and at interkinesis and prophase II in the second division. Differences in NN between leptotene and interkinesis are explained simply by the double number of rDNA clusters at leptotene, in a way similar to that of the demonstrated increase in gene expression in proportion to gene dosage reported for maize ploidy series (Guo *et al.* 1996). However, differences in NA between these two stages are not explained by ploidy level or the number of rDNA clusters alone. The higher complexity and longer duration of meiosis I with respect to meiosis II, with the consequent greater needs for ribosome biogenesis, is a most likely explanation.

The finding that total cell nucleolar size at diplotene is significantly larger than that at leptotene indicates that some nucleolar material might be synthesized during meiotic prophase I, in close resemblance to chordates, where nucleolar activity reaches its maximum at pachytene (Schmid *et al.* 1982, 1983, Wachtler & Stahl 1993). However, we cannot rule out that the observed differences in NA between leptotene and diplotene might be an artefact yielded by higher squashing ease at diplotene because of the lack of a nuclear membrane which is, on the other hand, present at leptotene. This subject would need quantification of some nucleolar components in the two stages. The differences observed between leptotene and interkinesis, however, are not affected by this fact since the nuclear membrane is present at both stages.

Since diplotene is the last stage of the first meiotic division showing the presence of nucleoli, we can infer that the nucleoli present at interkinesis are the result of *de-novo* nucleolar activity at the onset of the second meiotic division, a fact that has not been reported in chordates (Sumner 2003).

Population differences in nucleolus size

Significant differences in NA were found among populations, due to lower values in the Torrox population. The absence of significant differences between specimens from Salobreña and Smir suggests that these differences might be due to reasons other than the geographical origin of the animals. A remarkable difference between Torrox and the two other populations is the type of B chromosome present and its frequency. Whereas Salobreña and Smir harbour neutralized B chromosomes (i.e. showing no drive; B_2 in Salobreña, see Camacho *et al.* 1997) or almost neutralized (B_1 in Smir, see Bakkali *et al.* 2002),

the Torrox population harbours the B_{24} chromosome, the most parasitic B variant we have so far found in *E. plorans* (i.e. showing drive and being detrimental to the host; Zurita *et al*. 1998). B frequency is also different among these populations, since the mean number of Bs per individual is about 0.8 in Salobreña (Camacho *et al*. 1997), 0.5 in Smir (Bakkali *et al*. 1999) and 1.5 in Torrox (Zurita *et al*. 1998). It is thus clear that the Torrox population is exposed to a higher B-chromosome load, which might lead to a higher degree of stress negatively affecting nucleolar size (see below).

B-chromosome effects

We found no effects of B-chromosome number (except for NA in Smir) but there were remarkable significant effects in Torrox when analysed in relation to the odd–even pattern. In the locust *Locusta migratoria*, Camacho *et al*. (2004) reported odd–even effects of B chromosomes on NN in diplotene cells, with NN being significantly lower in cells with B-odd numbers. We have observed the contrary pattern at interkinesis in *E. plorans* (i.e. higher NN in B-odd), which was also contrary to that observed for NA at leptotene (i.e. higher NA in B-odd), which might indicate some differences in how NN responds to B-chromosome effects between leptotene and interkinesis. In diplotene cells from *E. plorans* males from Torrox, however, we have not found any B effect associated to the odd–even pattern for either NN or NA. This indicates that the NA increase observed from leptotene to diplotene might be involved in the disappearance of the leptotene odd–even pattern.

It is conceivable that the odd–even patterns for B-chromosome effects are caused by some kind of disturbance associated with the presence of B univalents, which are logically more frequent in individuals with odd B numbers. It is noteworthy that Guo *et al*. (1996) found odd–even patterns for the expression of several genes (measured by their specific mRNA level per cell) in a ploidy series in maize, from monoploid to tetraploid, including the gene for the ribosomal protein S22. The pattern was, however, contrary to the one observed in *E. plorans*, since odd ploidy numbers showed higher expression levels of the several genes, whereas odd B numbers led to a smaller nucleolar area in *E. plorans*. Perhaps the way in which ploidy and B numbers influence gene expression is mechanistically different, but this issue requires more research.

Camacho *et al*. (2004) suggested that the odd–even effects of B chromosomes might be the result of the stressing effects of B chromosomes, as genome parasites. The observation of more intense effects in the Torrox population, which is suffering the higher B chromosome load (see above) is consistent with the former statement. Nucleolus size is indicative of many stress-causing factors, such as starvation (Lagerstedt 1949), hormonal changes (Herbener & Bendayan 1998) or environmental contaminants (Hudson & Ciborowski 1996), in all cases nucleoli being smaller in highly stressful conditions. This suggests that the nucleolus function is more complex than simply synthesizing rRNA and, among others functions, it is important in stress response (Olson 2004). The complexity of nucleolus function is demonstrated by the high number of endogenous proteins that can be isolated form it. Recently, Andersen *et al*. (2005) have characterized the flux of 489 endogenous nucleolar proteins in response to three different metabolic inhibitors affecting nucleolar morphology. The fact that these authors have demonstrated that the nucleolar proteome changes significantly over time, in response to changes in cellular growth conditions, suggests that it is a sensitive organelle for cell stress, such as that generated by B chromosomes.

NOR activity in B chromosomes

The finding of nucleolar activity in the B chromosomes in 6.5% of diplotene cells from Torrox males opens a series of noteworthy insights on B-chromosome evolution. The only previous evidence for activity of the B-chromosome rDNA was in a single male, collected at the Salobreña population, which carried its B_2 fused to the longest autosome (Cabrero *et al*. 1987). The situation in Torrox is different, since the expression of the B-chromosome NOR is a recurrent phenomenon which was observed in four out of the 13 B-carrying males analysed (see Table 2). We have previously shown that B chromosomes in *E. plorans* display a high mutation rate (i.e. about 0.001 per gamete per generation; see López-León *et al*. 1993, Bakkali & Camacho 2004). The Torrox population contains the B_{24} variant, which derived from B_2 by amplification of satDNA and deletion of some rDNA and replaced B_2 between

1984 and 1992 (Zurita *et al*. 1998). We have recently observed some new variants emerging in this population, suggesting that B-chromosome evolution is going on. It is thus conceivable that a new B variant has recently emerged in this population which is able to express its rDNA, and this might open the possibility for a new evolutionary pathway with the B providing rRNA material and thus becoming into a beneficial parasite.

Acknowledgements

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References

Andersen JS, Lam YW, Leung AK *et al*. (2005) Nucleolar proteome dynamics. *Nature* **433**: 77–83.

Bakkali M, Camacho JPM (2004) The B chromosome polymorphism of the grasshopper *Eyprepocnemis plorans* in North Africa. III. Mutation rate of B chromosomes. *Heredity* **92**: 428–433.

Bakkali M, Cabrero J, López-León MD, Perfectti F, Camacho JPM (1999) The B chromosome polymorphism of the grasshopper *Eyprepocnemis plorans* in North Africa. I. B variants and frequency. *Heredity* **83**: 428–434.

Bakkali M, Cabrero J, López-León MD, Perfectti F, Camacho JPM (2001) Population differences in the expression of nucleolus organizer regions in the grasshopper *Eyprepocnemis plorans*. *Protoplasma* **217**: 185–190.

Bakkali M, Perfectti F, Camacho JPM (2002) The B-chromosome polymorphism of the grasshopper *Eyprepocnemis plorans* in North Africa. II. Parasitic and neutralized B_1 chromosomes. *Heredity* **88**: 14–18.

Busch H (1997) Nucleolar and nucleolonemal proteins of cancer cells. *J Tumor Marker Oncol* **12**: 5–68.

Busch H, Daskal Y, Gyorkey F, Smetana K (1979) Silver staining of nucleolar granules in tumor cells. *Cancer Res* **39**: 857–863.

Cabrero J, Alché JD, Camacho JPM (1987) Effects of B chromosome on the activity of nucleolus organizer regions in the grasshopper *Eyprepocnemis plorans*: activation of a latent nucleolus organizer region on a B chromosome fused to an autosome. *Genome* **29**: 116–121.

Cabrero J, López-León MD, Bakkali M, Camacho JPM (1999) Common origin of B chromosome variants in the grasshopper *Eyprepocnemis plorans*. *Heredity* **83**: 435–439.

Camacho JPM (2005) B Chromosomes. In: Gregory TR, ed., *The Evolution of the Genome*. San Diego: Elsevier, pp. 223–286.

Camacho JPM, Sharbel TF, Beukeboom LW (2000) B chromosome evolution. *Phil Trans R Soc Lond B* **355**: 163–178.

Camacho JPM, Shaw MW, López–León MD, Pardo MC, Cabrero J (1997) Population dynamics of a selfish B chromosome neutralized by the standard genome in the grasshopper *Eyprepocnemis plorans*. *Am Nat* **149**: 1030–1050.

Camacho JPM, Perfectti F, Teruel M, López-León MD, Cabrero J (2004) The odd–even effect in mitotically unstable B chromosomes in grasshoppers. *Cytogenet Genome Res* **106**: 325–331.

Caspersson T (1950) *Cell Growth and Cell Function, a Cytochemical Study*. New York: WW Norton.

Derenzini M (2000) The AgNORs. *Micron* **31**: 117–120.

Derenzini M, Farabegoli F, Trerè D (1992) Relationship between interphase AgNOR distribution and nucleolar size in cancer cells. *J Mol Histol* **24**: 951–956.

Derenzini M, Sirri V, Trerè D (1994) Nucleolar organizer regions in tumour cells. *Cancer J* **7**: 71–77.

Derenzini M, Trerè D, Pession A, Montanaro L, Sirri V, Ochs RL (1998) Nucleolar function and size in cancer cells. *Am J Pathol* **152**: 1291–1297.

Derenzini M, Trerè D, Pession A, Govoni M, Sirri V, Chieco P (2000) Nucleolar size indicates the rapidity of cell proliferation in cancer tissues. *J Pathol* **191**: 181–186.

Guo M, Davis D, Birchler JA (1996) Dosage effects on gene expression in a maize ploidy series. *Genetics* **142**: 1349–1355.

Herbener GH, Bendayan M (1998) A correlated morphometric and cytochemical study on hepatocyte nucleolar size and RNA distribution during vitellogenesis. *Histochem J* **20**: 194–200.

Hudson LA, Ciborowski JJH (1996) Teratogenic and genotoxic responses of larval *Chironomus salinarius* group (Diptera: Chironomidae) to contaminated sediment. *Environ Toxicol Chem* **15**: 1375–1381.

Lagerstedt S (1949) Cytological studies on the protein metabolism of the liver in the rat. *Acta Anat Suppl* **9**: 1–140.

López-León MD, Cabrero J, Pardo MC, Viseras E, Camacho JPM, Santos JL (1993) Generating high variability of B chromosomes in *Eyprepocnemis plorans* (grasshopper). *Heredity* **71**: 352–362.

López-León MD, Neves N, Schwarzacher T, Heslop-Harrison JS, Hewitt GM, Camacho JPM (1994) Possible origin of a B chromosome deduced from its DNA composition using double FISH technique. *Chromosome Res* **2**: 87–92.

López-León MD, Cabrero J, Camacho JPM (1995) Changes in NOR activity pattern in the presence of supernumerary heterochromatin in the grasshopper *Eyprepocnemis plorans*. *Genome* **38**: 68–74.

McClintock B (1934) The relationship of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z Zellforch Mikrosk Anat* **21**: 294–328.

Mosgoeller W (2004) Nucleolar ultrastructure in vertebrates. In: Olson MOJ, ed., *The Nucleolus*. New York: Kluwer, pp. 10–20.

Nakamoto K, Ito A, Watabe K *et al*. (2001) Increased expression of a nucleolar Nop5/Sik family member in metastatic melanoma cells: evidence for its role in nucleolar sizing and function. *Am J Pathol* **159**: 1363–1374.

Okabe Y, Nakamura S, Okumura H *et al*. (1991) The relation of argyrophilic proteins of nucleolar organizer regions (AgNORs) to the proportions of Ki–67 or DNA polymerase á-reacting cells in non-Hodgkin's lymphomas. *Anticancer Res* **11**: 2031–2035.

Olson MOJ (2004) Sensing cellular stress: another new function for the nucleolus? *Sci STKE* 2004: e10.

Pikaard CS (2002) Transcription and tyranny in the nucleolus: the organization, activation, dominance and repression of ribosomal RNA genes. In: Somerville CR & Meyerowitz EM, eds., *The Arabidopsis Book*. Rockville: American Society of Plant Biologists, pp. 1–23.

Rasband WS (1997) *ImageJ*, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2006.

Rubbi CP, Milner J (2003) Disruption of the nucleolus mediates stabilisation of p53 in response to DNA damage and other stresses. *EMBO J* **22**: 6068–6077.

Rufas JS, Iturra P, de Souza W, Esponda P (1982) Simple silver staining procedure for the localization of nucleolus and nucleolar organizer under light and electron microscopy. *Arch Biol* **93**: 267–274.

Schmid M, Löser C, Schmidtke J, Engel W (1982) Evolutionary conservation of a common pattern of activity of nucleolus organizers during spermatogenesis in vertebrates. *Chromosoma* **86**: 149–179.

Schmid M, Müller H, Stasch S, Engel W (1983) Silver staining of nucleolus organizer regions during human spermatogenesis. *Hum Genet* **64**: 363–370.

Shubert I, Künzel G (1990) Position dependent NOR activity in barley. *Chromosoma* **99**: 352–359.

Sumner AT (2003) *Chromosomes: Organization and Function*. Oxford: Blackwell.

Thiele J, Fischer R (1993) Bone marrow tissue and proliferation markers: results and general problems. *Virchows Arch A Pathol Anat Histopathol* **423**: 409–416.

Trerè D (2000) AgNOR staining and quantification. *Micron* **31**: 127–131.

Tsai RYL, McKay RDG (2005) A multistep, GTP-driven mechanism controlling the dynamic cycling of nucleostemin. *J Cell Biol* **168**: 179–184.

Tuma RS (2005) A GTP signal to the nucleolus. *J Cell Biol* **168**: 172.

Wachtler F, Stahl A (1993) The nucleolus: a structural and functional interpretation. *Micron* **24**: 473–505.

Wannemacher RW (1972) Ribosomal ribonucleic acid synthesis and function as influenced by amino acid supply and stress. *Biochem J* **129**: 5P–6P.

Yezerinac SM, Lougheed SC, Handford P (1992) Measurement error and morphometric studies- Statistical power and observer experience. *Syst Biol* **41**: 471–482.

Zurita S, Cabrero J, López-León MD, Camacho JPM (1998) Polymorphism regeneration for a neutralized selfish B chromosome. *Evolution* **52**: 274–277.