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Numerical changes of nucleolar organiser regions and nucleolar activity in *Lathyrus*

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Summary. The transcriptional activity of the nucleolar organiser regions was compared in 3 *Lathyrus* species having different numbers of secondary constrictions in their complements. The 3 species differed significantly. It is also shown that the distribution of ribosomal RNA gene copies varied between pairs of satellited chromosomes within a complement.
Key words. NOR; nucleolar activity; ribosomal DNA; chromosomal distribution; *Lathyrus*.

The nucleoli which appear in the interphase and prophase nuclei of eukaryotic cells are rich in RNA and are formed by one or more specific regions in the haploid complement called nucleolar organisers. The nucleolar organiser regions (NORs) are well differentiated regions of chromatin DNA which transcribes 5.8S, 18S and 28S ribosomal RNA sequences. At metaphase the nucleoli disappear and the NORs appear as constrictions (secondary constrictions) at the distal end of metaphase chromosomes. The NOR is highly variable in number and location among species but within a complement it is relatively constant[1]. The genes for 5.8S, 18S and 28S rRNA occur in the form of tandemly repeated units separated by transcribed spacer regions[2]. While the rRNA genes are highly conserved among higher organisms the spacer sequences may be heterogeneous within a species and highly diverged among related species.

A survey among *Lathyrus* species has shown large variation in the number of secondary constrictions in the cromosome complements. The number ranged from one pair to six pairs among the satellited complements even though all species investigated were diploids with the same chromosome number, 2n = 14. The aim of this investigation was to find out a) whether the numerical changes of secondary constrictions has any significant effect upon the nucleolar activity at interphase and prophase and b) whether the distribution of ribosomal RNA gene copies varies among satellited chromosomes within a complement.

Materials and methods. *L. articulatus* L. and *L. tingitanus* L., were from the Aberystwyth collection. *L. pisiformis* L. was supplied by Hortus Botanicus, Sweden.

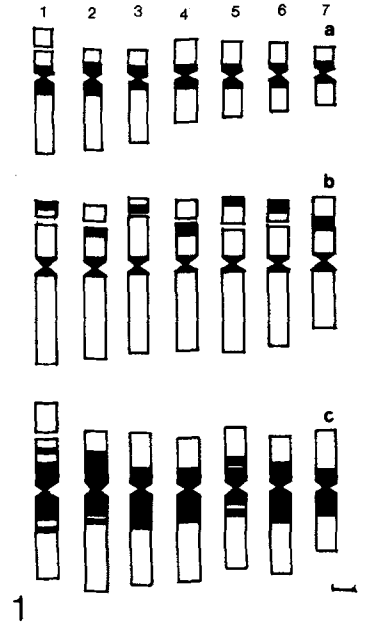
1) Silver staining of interphase nuclei. The procedure for staining interphase nuclei was essentially the same as described by Bloom and Goodpasture[3]. Cytological preparations were made from the meristematic tissue dissected out from root tips which were fixed in 1:3 acetic alcohol. Squash preparations were stored in absolute ethanol at −20 °C until use. Slides were air dried and 2–3 drops of freshly prepared silver nitrate solution (75%, w/v) was applied to each slide. The coverslips were sealed with rubber solution and incubated in a moist black box at 50 °C for 24 h. The slides were rinsed in distilled water and treated with 3% (v/v) formaldehyde for 20–40 min. The slides were rinsed briefly first in distilled water and then in 0.04 M phosphate buffer (equimolar mixture of Na_2HPO_4 and NaH_2PO_4, pH 6.8) and stained in 1% Giemsa solution (Gurr's improved, R66). After differentiating in 0.04 M phosphate buffer the slides were made permanent by mounting in euparal.

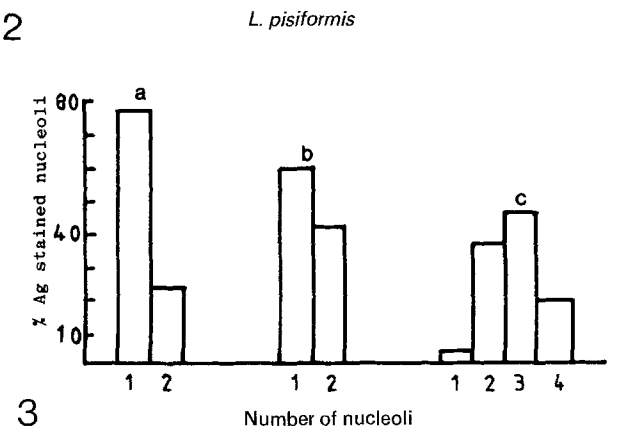
2) Silver staining of metaphase chromosomes. The method of Howel and Black[4] was used for staining metaphase chromosomes. Two stock solutions of reagents were made and stored in dark bottles in a refrigerator. They were: a) a solution containing 1% (w/v) gelatin and 1% (v/v) formic acid in distilled water, b) 50% (w/v) silver nitrate made in 0.04% (v/v) formaldehyde. Metaphase chromosome preparations were made from meristematic tissue of root tips as described above. Two drops of solution (a) and 4 drops of (b) were placed on each slide. The slides were warmed for 3–5 sec on a hot plate maintained at 50 °C. The slides were rinsed in deionised distilled water and transferred to 'photo fix' (1:3 dilution of Kodak fixative) for 1 min. The slides after washing in distilled water were air dried and mounted in euparal. The chromosomes were observed under oil immersion using a phase contrast microscope.

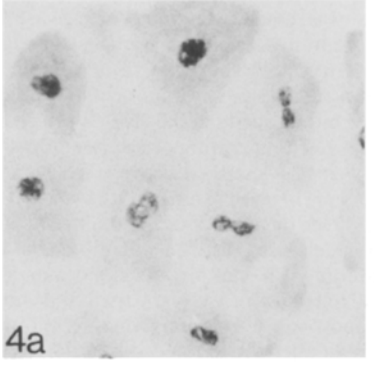
3) Measurement of 2C nuclear DNA amount and chromosome volume. The methods have already been reported[5]. 2C nuclei in root meristems quantitatively stained with Feulgen were measured in a Vickers M85 microdensitometer. The 2C DNA values are the means of at least 3 replications. *Allium cepa* (2C = 33.5 pg) was used as a standard to convert DNA estimates to absolute amounts (pg). At least 4 Feulgen-stained full metaphase plates at c-mitosis were used for measuring chromosome volume and the mean values are given in the text.

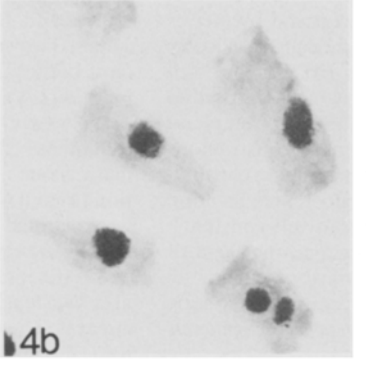
4) C-banding of metaphase chromosomes. The method suggested by Vosa[6] was used in this investigation.

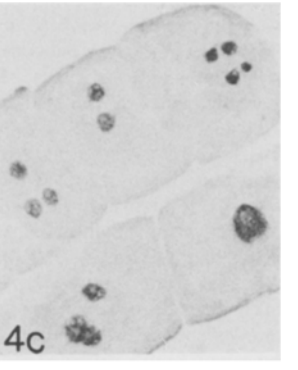
5) In situ hybridisation. The cloned *Pisum sativum* ribosomal DNA probe used in this experiment was a gift from Dr Jan-Peter Nap, Department of Molecular Biology, Agricultural University, Wageningen, The Netherlands. The procedure for the transcription of complementary RNA (cRNA) and in situ hybridisation has already been reported[7]. H^3-labeled ATP (32 Ci/mMol) and UTP (46 Ci/mMol) were purchased from Amersham Radiochemicals, UK. *E. coli* RNA polymerase was from Sigma Ltd. The unlabeled nucleotides were from Boehringer Mannheim Ltd. The H^3-labeled cRNA had a specific activity of 10^8 cpm/µg. It was hybridised in situ to the chromosomal DNA using the method of Dennis et al.[8]. 8 µl of cRNA (100,000 cpm) in 3 × SSC and 50% formamide was placed under a coverslip on each slide. It was sealed with rubber solution. The chromosomal DNA was denatured by dipping the slides for 3 min in a water bath maintained at 75 °C. The slides were then incubated for 24 h at 37 °C. After removing the coverslips the excess cRNA was removed by extensive washing in several changes of SSC (0.15 M NaCl+0.015 M Na citrate pH 7). Non-specifically bound cRNA

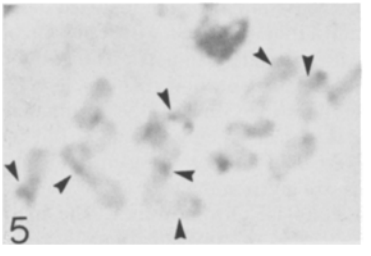












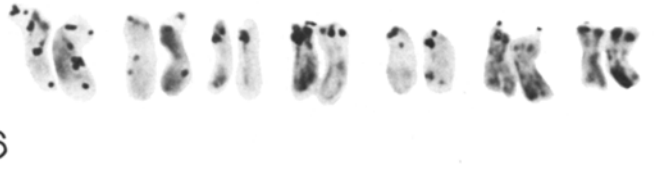


Figure 1. The C-banded karyotypes of a) *L. articulatus*, b) *L. pisiformis*, c) *L. tingitanus*. Bar = 1 µm.

Figure 2. The metaphase chromosomes of *L. pisiformis*.

Figure 3. The distribution of nucleoli in the interphase nuclei a) *L. articulatus*, b) *L. tingitanus*, c) *L. pisiformis*.

Figure 4. Silverstained nucleioli at interphase in a) *L. articulatus*, b) *L. tingitanus*, c) *L. pisiformis*.

Figure 5. Silverstained metaphase chromosomes of *L. pisiformis*. Arrows point to bands seen under phase contrast.

Figure 6. Metaphase chromosomes of *L. pisiformis* hybridised with H^3-labeled rRNA of *Pisum sativum*.

was removed by digesting with RNAse. The slides were dehydrated stepwise in 75% and 95% ethanol and air dried. The slides were dipped in Ilford photographic emulsion (K_2 emulsion diluted 1:1 with distilled water) and exposed in dark boxes for 14–21 days. The slides were developed in Kodak D-19 developer and stained with Giemsa. The chromosome preparations were made permanent by mounting in euparal.

Results and discussion. 1) Chromosome differentiation between *L. articulatus*, *L. pisiformis* and *L. tingitanus*. The C-banded karyotypes of the 3 species investigated are in figure 1. The total chromosome volume, 2C nuclear DNA amount, number of satellited pairs of chromosomes and the average number of nucleoli found in the interphase nuclei of each species is given in the table.

In figure 1 the chromosomes are arranged in the order of decreasing size and numbered 1–7. The three complements are made up only of median and submedian chromosomes. The secondary constrictions are on the short arms of the largest

chromosomes (chromosome 1) of *L. tingitanus* and *L. articulatus*. *L. pisiformis* is exceptional among diploid species of higher plants. It contains six chromosomes out of the seven with secondary constrictions on the short arms. The smallest chromosome in the complement is without a secondary constriction. In 3 pairs of satellited chromosomes the NORs are elongated and the terminal satellites appear as knobs (fig. 2).

All three species have constitutive heterochromatin at or very near the centromeres of all chromosomes. In *L. tingitanus* interstitial bands are also found in the short and long arms of chromosomes 1, 2 and 5. In *L. pisiformis* interstitial or terminal c-bands are found in the short arms of all chromosomes (fig. 1). There are significant differences between the three species in chromosome size, and 2C nuclear DNA amounts. *L. tingitanus* has approximately twice as much DNA in its genome than *L. articulatus*. *L. pisiformis* has an intermediate value. It would be seen from the table that the variation in the number of satellited chromosomes is not correlated with changes either in the total chromosome volume or in 2C nuclear DNA content. If we make the assumption that the rRNA cistrons are distributed approximately evenly in all satellited chromosomes, then *L. pisiformis* would be expected to have six times as many ribosomal gene copies in its genome as in *L. articulatus* or *L. tingitanus*. This hypothesis may be tested by comparing the nucleolar activity in the interphase nuclei of the three species.

2) Nucleolar activity in the interphase nuclei of *L. articulatus*, *L. tingitanus* and *L. pisiformis*. At the cytological level the function and composition of the NORs are investigated by silver staining. Several reports have confirmed that silver staining is localised to the nucleolar organiser regions of chromosomes during mitosis[9,10] and to nucleoli during interphase[11–13]. The silver stained interphase nuclei are compared in figure 4. *L. articulatus* which has a pair of satellited chromosomes has either one or two silver stained nucleoli at interphase. 77% of the interphase cells had one nucleolus and the remainder had 2 nucleoli (fig. 3). The single nucleolus was bigger in size and some of them presumably have originated by the fusion of 2 smaller nucleoli. In fact, the dumb-bell shaped nucleoli seen in several cells (fig. 4a) confirm the nucleolar fusion in *L. articulatus*. Nucleolar fusions are reported to occur in barley as a regular process during cell growth and differentiation[14]. 59% of the interphase nuclei investigated in *L. tingitanus* contained one nucleolus and the remainder (41%) had 2 nucleoli. No dumb-bell shaped nucleolus was observed in *L. tingitanus*. *L. pisiformis* had by far the greater number of nucleoli in the interphase nuclei. 44% of the total cells analysed had 3 nucleoli, 35% 2 nucleoli, 18% 4 nucleoli, and 3% had a single nucleolus (fig. 3). The average number of nucleoli in the interphase cells although significantly greater in *L. pisiformis* has not increased in proportion to the total number of secondary constrictions in the metaphase complements (table).

3) Transcriptional activity of the NOR regions of metaphase chromosomes. The transcriptional activity of the NOR regions in metaphase chromosomes was compared by silver staining[4]. In *L. articulatus* and *L. tingitanus* only the satellited pair of chromosomes were silver stained even though the intensity of staining varied between metaphase plates. In *L. pisiformis* silver staining was not visible on all nucleolar organiser regions. Moreover there was substantial variation in the intensity of staining between chromosomes. The variation may be due to heritable differences in the stainability of the NORs or due to differences in the activity of nucleolar organisers. Variation in the copy number of rRNA cistrons can also cause differences in the activity of nucleolar organisers (fig. 5).

Partial suppression of NOR activity as evidenced by the production of nucleoli of reduced size is reported in reconstructed plant genomes[15]. Wallace and Longbridge[16] compared the number of nucleoli formed at interphase in plant hybrids with the number of secondary constrictions and reported complete suppression of activity of some nucleolar organiser regions. Methylation of the ribosomal DNA is known to be a mechanism which regulates the ribosomal gene activity. The onset of rRNA gene activity in the early development of *Xenopus laevis* is accompanied by a loss of methylation of the rDNA[17]. Similarly, it has been shown that genetically active NORs are not methylated whereas inactive NORs are hyper methylated[18]. The regulatory mechanisms responsible may involve differences in the number of active DNA polymerases[19] or in the elongation rate of the growing ribosomal RNA chains[20]. To find out whether the copy number of rRNA cistrons varied betwen satellited chromsomes the rDNA of *Pisum sativum* was used as a molecular probe. The cRNA transcribed from the rDNA was hybridised in situ to the chromosomal DNA of the 3 *Lathyrus* species.

4) The distribution of ribosomal DNA in the chromosome complements. The ribosomal DNA probe, clone ppsλR1 is a 4.0 *kb* ECORI subclone in pACYC184. It was isolated from a partial genomic library of *Pisum sativum* variety Rondo.

H^3-labeled cRNA transcribed from the cloned rDNA of *Pisum sativum* hybridised to the chromosomal DNA of all 3 species. In *L. tingitanus* and *L. articulatus* the silver grains were few and its distribution was restricted to the satellited chromosomes. In *L. pisiformis* the cRNA hybridisation was more extensive. The silver grains were heavily deposited at the NOR regions of a pair of satellited chromosomes. The deposition of silver grains was less on other satellited chromosomes. The conclusion is that the rDNA copy number vary substantially between satellited chromosomes in *L. pisiformis*. Grain count in the interphase nuclei of the 3 species confirmed that on average *L. pisiformis* DNA had greater hybridisation with the cRNA probe. This may be explained as due to the variation in ribosomal RNA gene copy number between related species[22]. The sequence which code for rRNA are homogeneous within and between the species whereas the spacer sequences, particularly the nontranscribed spacers, may be highly variable between species[21]. There is also sequence length heterogeneity reported among cereal plants which is attributable to the variation in the larger spacer sequences[23]. Repeat length heterogeneity of the rDNA sequences can also cause the observed variation in the amount of cRNA hybridised to the chromosomal DNA of different species.

Detailed analysis of several metaphase plates have shown that silver grains are located, though sparsely, at chromosome sites other than the NORs. This suggests that rDNA sequences may be present at regions other than the NORs.

2C nuclear DNA amount, total chromosome volume, number of pairs of satellited chromosomes and average number of nucleoli at interphase for 3 *Lathyrus* species

	L. articulatus	*L. pisiformis*	*L. tingitanus*
2C nuclear DNA amount (pg)	12.15	19.69	22.08
Total chromosome volume (μm^3)	24.85	50.26	43.05
Number of pairs of satellited chromosomes	1	6	1
Average number of nucleoli	1.23	2.77	1.41

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Isozyme studies in *Nicotiana suaveolens, N.glutinosa* and their interspecific hybrid: Genetic control of phosphoglucomutase and glutamic-oxaloacetic-transaminase

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Summary. Two isozyme systems, phosphoglucomutase and glutamic-oxaloacetic-transaminase, have been studied in leaves of *Nicotiana suaveolens, N.glutinosa* and their interspecific hybrid. By analyzing the different isozyme patterns in the hybrid a model for the genetic control of these systems has been proposed. Phosphoglucomutase appears to be controlled by a single locus and glutamic-oxaloacetic-transaminase behaves as a dimeric isozyme system, being controlled by at least two loci.
Key words. Nicotiana suaveolens; N.glutinosa; interspecific hybrid; glutamic-oxaloacetic-transaminase; phosphoglucomutase.

Except in rare cases[2,3] it is very difficult to study the genetic control of certain isozyme systems in species of the genus *Nicotiana* through the methods of formal genetics, which use the crossing among plants of different isozyme patterns, and statistical analysis of the hybrid plant segregations. The major reason for this difficulty is the absence of variability in these species[4]. This problem has been avoided in other species[5] through aneuploid genetics, which analyzes the effects produced by dosage differences in chromosomes or chromosome segments to obtain genetic information[5]. The reasoning followed in our study of the structure and the genetic control of some isozymes in two *Nicotiana* species, was similar to that used by Hart and other authors. In our case, different genomes have been joined in one individual, the interspecific hybrid, which is used to analyze the relative intensities of isozyme patterns and the presence or absence of bands with intermediate migration.

According to the results obtained and having regard to the fact that the hybrid pattern always correspond to the sum of the parental ones, a model of genetic control for these species is proposed.

Material and methods. Two species of *Nicotiana* were used, *N.glutinosa (glu)*, which is a diploid species (2n = 24), and *N.suaveolens (sua)*, which is an allopolyploid species (2n = 32), the parental species of which are unknown. Seeds of *glu* were obtained from: The Indian Agricultural Research Institute, The University of London Botanical Supply Unit, The Botanic Gardens of the University of Birmingham and The Hortus Botanicus Bergianus of Stockholm. In all cases, *glu* plants showed the same isozyme patterns. Seeds of *sua* were also obtained from different sources, but only in one case, namely seeds received from Instituto Tecnológico del Tabaco de Sevilla, did *sua* plants have 32 chromosomes; in the other cases they have 64 chromosomes. These latter ones were not used. The interspecific hybrid *(sua × glu)* was obtained from crossing *sua* from Instituto Tecnológico del Tabaco and *glu* from Hortus Botanicus Bergianus. Isozyme analysis was carried out with a crude extract of leaves. Phosphoglucomutase (PGM) and glutamic-oxaloacetic-transaminase (GOT) isozymes were studied using 0.015 M Tris-citric acid, pH 7.75 as the gel buffer (12% starch) and 0.3 NaOH-boric acid, pH 7.0 as the electrode buffer. All the crude leaf extracts were analyzed simultaneously for all the above mentioned systems, by using 1 cm thick gels, spliced into 2-mm slabs before staining.

The slabs were stained after Bewer and Sing[6] (PGM), Selander et al.[7] and Schwartz et al.[8] (GOT).

Each gel activity zone has been called by the name of the isozyme system studied and numbered in order from the faster to the slower mobility. The implicated loci have been given the same name as the activity zones, and their alleles have sub-indices added to the locus name, the number 1 corresponding to the allele with the faster mobility, 2 to the next one and so on. Each sub-index is followed by the letter s or g to indicate whether the allele corresponds to *sua* or *glu* loci.

Results and discussion. Phosphoglucomutase (PGM): One activity zone has been observed in both species. *Sua* showed two bands numbered 1 and 2 and *glu* only band 1 (fig.). The electrophoretic pattern of the hybrid shows both isozymes. In rye[9] and barley[10] this isozyme system has been described as monomeric with a monogenic control.

It seems likely that the system is controlled by only one locus (PGM) with at least two alleles (PGM_{1s} and PGM_{2s}) for *sua* and one (PGM_{1g}) for *glu*. In the case of *glu*, PGM_{1g} appeared to be fixed, while *sua* had two alleles, being a fixed heterozygote. This last species has been previously described as an amphiploid; therefore it can maintain the heterozygosity in this locus, in spite of its autogamous character; as Adams and Allard[11] demonstrated by electrophoretic techniques, allopolyploids can maintain heterozygosity in homozygous autogamous plants because they can have different alleles fixed in each genome.

Glutamic-oxaloacetic-transaminase (GOT): In both parental species it is possible to distinguish the isozyme pattern into two different activity zones, namely GOT-1 and GOT-2 (fig.).

GOT-1 presented three bands, numbered 1, 2 and 3. *Sua* showed all the three isozymes, band 2 always being more intense than 1