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Molecular cytogenetic analysis of genome structure in *Lupinus angustifolius* and *Lupinus cosentinii*

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Abstract Molecular cytogenetic analysis of *Lupinus angustifolius* and *Lupinus cosentinii* was performed using flow cytometry, fluorescence in situ hybridisation (FISH) and differential chromosome staining. Genome size was determined as 2.07 pg for *L. angustifolius* and 1.54 pg for *L. cosentinii*. Analysis of nuclear DNA amount in cells during plant development has shown endopolyploidisation in different organs. The highest level of endopolyploidy was in cotyledons and reached 32C in *L. angustifolius* and 64C in *L. cosentinii*. Both of the investigated *Lupinus* species belong to the polysomatic type of plants. Double FISH with rDNA probes provided chromosomal landmarks for 10 out of 40 chromosomes for *L. angustifolius* and 8 out of 32 chromosomes for *L. cosentinii*. In *L. angustifolius*, the number and localisation of 25S rDNA hybridisation signals precisely corresponded to the chromomycin A3 (CMA^+) bands, while in *L. cosentinii* both 25S and 5S rDNA loci overlapped with CMA^+ bands. Silver staining revealed that only 45S rRNA genes located in secondary constriction regions were transcriptionally active. FISH with *Arabidopsis*-type telomeric arrays revealed the presence of signals at termini of all chromosomes. Despite the application of different DNA probes for FISH and different chromosome staining, a relatively small proportion of chromosomes in the *Lupinus* karyotypes can be distinguished. Identification of all chromosomes requires the use of more chromosome-specific markers.

Keywords FISH · Flow cytometry · *Lupinus* · rDNA · Telomere

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Introduction

The genus *Lupinus* (Fabaceae) contains around 300 species of annual and perennial herbaceous plants and small trees. The taxonomy of this genus is not fully understood. During the past several decades, considerable effort has been made including molecular analysis, to improve the taxonomy and systematics of lupines (Aïnouche and Bayer 1999). Among the Old World species two distinct groups have been recognised on the basis of the seed coat texture: the smooth-seeded and the rough-seeded species, *Lupinus angustifolius* and *Lupinus cosentinii*, respectively. Some *Lupinus* species, including *L. angustifolius*, are important crops as a source of protein for humans and farm animals. Furthermore, some lupine crops are cultivated on poor and contaminated soils, and are widely used in sustainable agriculture as crops for soil enrichment in nitrogen.

The genome size of *Lupinus* is relatively small. The DNA amount ranges from 0.6 pg/1C in *L. albus* to 1.0 pg/1C in *L. luteus*, which is slightly larger than *Arabidopsis thaliana* (http://www.rbgkew.org.uk/cvalues/CvalServlet?querytype=2). The chromosomes are small, numerous and rather morphologically uniform. To date, cytogenetic analysis has mainly been focused on a determination of chromosome number and size (Naganowska and Ladon 2000), and DNA content in some *Lupinus* species (Obermayer et al. 1999). Recently, Naganowska and Zielinska (2002) roughly determined the number of 5S and 18S-25S rDNA loci in some of the *Lupinus* species. It was also established that endopolyploidy occurred in different organs during plant development and could reach the level of 32C in *L. luteus* (Olszewska and Legocki 1989; Sakowicz and Olszewska 1997) and 64C in *L. albus* (Le Gal et al. 1986). rDNA methylation and nucleolar activity have also been studied in different organs of *L. luteus* (Sakowicz and Olszewska 1997).

The continuous progress observed in molecular cytogenetics created new opportunities to study both the chromosomes and interphase nuclei, including their structure and function. Such techniques, as multicolour

fluorescence in situ hybridisation (mFISH) have allowed simultaneous application of several DNA probes and their localisation. rRNA genes have been widely used as chromosome markers for karyotyping (e.g. in *A. thaliana*—Murata et al. 1997; Fransz et al. 1998, *Hordeum vulgare*—Leitch and Heslop-Harrison 1993; *Trifolium repens*—Ansari et al. 1999), and for studying the evolutionary relationship within many genera (e.g. *Phaseolus*—Moscone et al. 1999; *Lathyrus*—Ali et al. 2000; *Brassica*—Hasterok et al. 2001; *Glycine*—Singh et al. 2001). The work on *Beta vulgaris* represents a good example of the efficient use of both tandem and dispersed repeats to saturate cytogenetic maps in plant species with small and morphologically uniform chromosomes (Schmidt and Heslop-Harrison 1998). However, the modern molecular cytogenetic methods have not been extensively applied to study *Lupinus* chromosomes.

The aim of the present study was to localise the chromosomal position of 5S rRNA and 25S rRNA genes by FISH, along with the fluorescent CMA/DAPI banding pattern and silver staining. The study also utilised FISH with telomeric $(TTTAGGG)_n$ arrays to check if and where they are distributed in the chromosomes of *Lupinus* species. In addition, the nuclear DNA content and pattern of ploidy level in different organs of young plants of *L. angustifoius* and *L. cosentinii* were determined.

Materials and methods

Plant material and chromosome preparation

Seeds of *L. angustifolius* L. cv Emir (2n = 40) and *L. cosentinii* Guss. (2n = 32) were hydrated with running tap water at room temperature (RT—approximately 22 °C) for 24 h. After removing seed coats the seeds were germinated on moist filter paper in Petri dishes. To induce development of secondary roots, the tip of the primary root was removed. Secondary roots 1.5–2 cm long were treated with 2 mM 8-hydroxyquinoline for 2 h at RT and then 1 h at 4 °C, and finally fixed for 4 h in freshly prepared 3:1 methanol:glacial acetic acid. Fixed roots were washed in 0.01 M citric acid—sodium citric buffer (pH 4.8) and digested in a mixture of 40% (v/v) pectinase (Sigma), 2% (w/v) cellulase (Calbiochem) and 2% (w/v) cellulase "Onozuka R-10" (Serva) for 4 h (*L. angustifolius*) or 3 h (*L. cosentinii*) at 37 °C. Single root-tips were transferred into a drop of 45% acetic acid on a microscope slide and squashed. The coverslips were removed after freezing and the preparations were briefly post-fixed in 3:1 pre-chilled ethanol:glacial acetic acid and dehydrated for 30 min in absolute ethanol. The air-dried slides were stored at 4 °C before use.

Staining methods

Chromosome analysis was carried out using double fluorescent staining with chromomycin A_3 (CMA) and 4′, 6-diamidino-2-phenylindole (DAPI) (Schweizer 1976). Slides were stained with 0.5 mg/ml of CMA solution (Serva) in the dark for 1.5 h, briefly rinsed in distilled water, air-dried and mounted in Vectashield (Vector Laboratories—H-1000) containing 2.5 μg/ml of DAPI (Serva). After staining, for better band differentiation, the slides were 'matured' at 37 °C for 3–4 days prior to examination. When the images of CMA fluorescent staining were taken, the coverslips were gently removed and the preparations were de-stained in 3:1 ethanol:glacial acetic acid, and then used for FISH.

Transcriptional activity of 45S (18-5.8–25S) rRNA genes was determined using silver staining (Hizume et al. 1980). Slides were immersed in a borate buffer (pH 9.2), dried, and several drops of freshly prepared 50% (w/v) silver nitrate (Merck) in re-distilled water were applied. Then the slides were immediately covered with a nylon mesh, incubated in a moisture chamber for about 15 min at 42 °C, washed in distilled water, air-dried and mounted in DPX.

Probe labelling

The following probes were used in this study:

(1). Telomeric probe (HT100.3): arrays of *Arabidopsis*-type telomeric repeats $(TTTAGGG)_n$ were assembled by annealing oligonucleotides TELAC2 (5′-CTA AAC CCT AAA CCC TAA ACC CTA A-3′) and TELGT2 (5′-GGT TTA GGG TTT AGG GTT TAG GGT TTA G-3′). Following gap repair with DNA polymerase (Klenow fragment) and T4 DNA ligase, arrays were tailed with *Taq* DNA polymerase and cloned into pGEM-T Easy (Promega) following manufacturer's instructions. A clone containing approximately 30 copies of the repeat was used as the template for subsequent PCR labelling reaction with rhodamine-4-dUTP (Amersham—RNP 2122). Oligonucleotides and conditions used for PCR labelling were as follows: universal M13 forward (5′-CAG GGT TTT CCC AGT CAC GA-3′) and reverse (5′-CGG ATA ACA ATT TCA CAC AGG A-3′) sequencing primers, 93 °C × 5 min, 35 cycles of 94 °C × 30 s, 55 °C × 30 s, 72 °C × 90 s, and 1 cycle of 72 °C × 5 min.

(2). 5S rDNA (pTa794): a 410-bp clone was isolated from *Triticum aestivum* (Gerlach and Dyer 1980). Label, oligonucleotides and conditions used for PCR labelling were the same as for the telomeric probe.

(3). For 45S rDNA a 2.3-kb *Cla*I fragment of the 25S rRNA gene isolated from *A. thaliana* (Unfried and Gruendler 1990) was labelled with digoxigenin-11-dUTP (Roche—1093 088) using a nick translation kit (Roche—976 776) according to the manufacturer's protocol.

Fluorescence in situ hybridisation

FISH was carried out according to the standard method (Hasterok et al. 2001). The slides were pre-treated with RNase (100 μg/ml) in 2 × SCC at 37 °C for 1 h, washed in 2 × SCC and dehydrated in ethanol. The hybridisation mixture (50% de-ionised formamide, 10% dextran sulphate, 2 × SSC, 0.5% SDS, salmon sperm blocking DNA in 75–100 × the excess of labelled probe and 2.5–3 ng/μl of probe DNA) was pre-denatured at 75 °C for 10 min and applied to the chromosome preparations. Slides and the DNA probe were then denatured together at 75 °C for 5 min in an in situ Thermal Cycler (Hybaid) and then allowed to hybridise overnight in a humid chamber at 37 °C. After stringent washes (20% formamide in 0.1 × SSC at 42 °C) the immunodetection of a digoxygenated DNA probe was carried out with FITC-conjugated anti-digoxygenin primary antibodies (Roche—1 207 741) and FITC-conjugated anti-sheep secondary antibodies (DAKO—F135). Dehydrated preparations were counterstained and mounted in 2.5 μg/ml of DAPI in Vectashield antifade buffer.

Image capturing and processing

All pictures were taken using a Hamamatsu (C5810) CCD camera attached to an Olympus (Provis AX) microscope, then uniformly processed and superimposed using Micrografx Picture Publisher software.

Flow cytometry

For flow cytometry analyses, suspensions of nuclei of both *L. angustifolius* and *L. cosentinii* were prepared from 4-day old seedlings grown on moist filter paper in Petri dishes or young plants grown in soil. Young leaves were used for measuring DNA content in absolute units. The seedlings were used to determine the ploidy pattern in different organs. The samples were individually chopped with a sharp razor blade in nuclei extraction buffer (Dolezel et al. 1998), filtered through a 30 μm nylon sieve (Partec CellTrics) and stained. Nuclei were stained with DAPI for ploidy level determination and with PI (propidium iodide) for DNA content measurement. In both cases a Partec high-resolution DNA kit was used. For each sample at least 5,000 nuclei were analysed. Samples were measured with a DAKO Galaxy flow cytometer equipped with an HBO-100 mercury lamp and an air-cooled argon ion laser. A mercury lamp with the set of filters and mirrors (KG1, UG1, BG38, TK420, GG435) was used for DAPI-stained samples. The laser with an RG 590 filter and a TK 560 mirror was used for PI-stained nuclei.

Ploidy pattern was determined for ten individual plants from each species. Seedlings were divided into several parts corresponding to different organs: shoot tip with immature leaves, hypocotyl, cotyledons, upper part of root and root tip. Each part from each seedling was measured individually. The DNA content for each of the two species was calculated from three independent measurements of five plants. The nuclear DNA content of *L. cosentinii* was calculated using nuclei isolated from young leaves of *Glycine max* cv Polanka—2C DNA, 2.50 pg (Dolezel et al. 1994), and *Lycopersicon esculentum* cv Stupicke—2C DNA, 1.96 pg (Dolezel et al. 1992), as internal standards. *G. max* cv Polanka and *L. cosentinii* (2C 1.54 pg, estimated in this work) were used as the standards for measuring the DNA amount in *L. angustifolius* nuclei. The FloMax software processed data were plotted on a semi-logarithmic (for ploidy pattern) and linear (for DNA content) scale. The value of the DNA content was estimated as an average of 30 measurements for each species.

Results and discussion

Genome size

Results presented for *L. cosentinii* and *L. angustifolius* enrich the list of *Lupinus* species with an estimated nuclear DNA content. Flow cytometric analysis of nuclear DNA content was done using two standards for each *Lupinus* species. The genome size of *L. cosentinii* calculated using *L. esculentum* cv Stupicke (2C = 1.96 pg) as an internal standard was 1.51 ± 0.04 pg. When *G. max* cv Polanka (2C = 2.50 pg) was used as a standard this value was 1.57 ± 0.02 pg, so we assume that the 2C DNA content in the *L. cosentinii* genome is 1.54 ± 0.04 pg. We also used *L. cosentinii* and the value of its nuclear DNA content as a standard for the *L. angustifolius* genome-size calculation. The 2C DNA of *L. angustifolius* calculated on that basis was 2.04 ± 0.02 pg. The second standard was as previously *G. max* and obtained a value of 2.11 ± 0.02 pg. We assume that the genome size (2C) of *L. angustifolius* is therefore 2.07 ± 0.04 pg.

L. cosentinii has a smaller genome than *L. angustifolius* which is directly correlated with the number of chromosomes, because an average DNA content of a single chromosome of *L. cosentinii* and *L. angustifolius* is similar and amounts to 0.048 pg and 0.051 pg respectively. The nuclear DNA content of the investigated species is in a range of known data for other *Lupinus* species. Genome size determinated by propidium iodide flow cytometry ranged between 1.12 pg (2C) in *Lupinus anatolicus* and 2.36 pg (2C) in *L. luteus* (Obermayer et al. 1999).

Data presented in the Angiosperm C-values Database Query Results (http://www.rbgkew.org.uk/cvalues/CvalServlet?querytype=2) includes the genome size of *L. angustifolius* (2C = 1.85 pg), which is slightly different from results obtained in this work. This difference could be due to the different cultivars of *L. angustifolius* used for investigation, the different method of genome size estimation or, more likely, to the different standards selected for genome size calculation. There is no data for *L. cosentinii* in the above database.

Polyploidy pattern

Nuclear DNA amount is known to change from 2C to 4C during each cell cycle as a result of DNA replication, and then back to 2C again after mitosis. Somatic cells have an ability to modify the classical cell cycle and undergo DNA synthesis independently from mitosis. This partial cell cycle, called endoreduplication, leads to an increase of ploidy level resulting in mixoploid tissue. This phenomenon is common in most angiosperm plants and is under developmental regulation (Joubes and Chevalier 2000).

The pattern of endopolyploidization of two *Lupinus* species was determined by flow cytometric analysis of nuclei released from seedling tissues stained with DAPI. Different ploidy levels were observed in five selected parts of a seedling: root tip, differentiated part of root, hypocotyl, cotyledon and shoot tip (Fig. 1). In root and shoot tips mainly two peaks were distinguished, 2C and 4C corresponding to G1 and G2 cells in these meristematic tissues, while in other parts, especially in hypocotyls and cotyledons, more peaks were observed. It should be noted that in general the pattern of endoreduplication was very similar for both species although the participation of the particular peaks in the whole population of nuclei was slightly different. The highest level of ploidy was observed in cotyledons and reached 64C in *L. cosentinii* and 32C in *L. angustifolius*. This means that some cells of *L. cosentinii* have undergone more endocycles than in *L. angustifolius*. A very high level of polyploidy in cotyledons seems to be typical for seed storage organs (Larkins et al. 2001), which could be connected with high metabolic activity of the tissue.

The histograms (Fig. 1) indicated that the level of polyploidy was increasing with age of the tissue. In the root tip only 2C and 4C cells were seen. In the upper part of the root, where cell differentiation has already started, the peak corresponding to 8C nuclei appeared. In hypocotyls, an 8C peak was higher than in the root and additionally 16C nuclei were present. Of interest is the comparison of 2C and 4C nuclei proportion in both

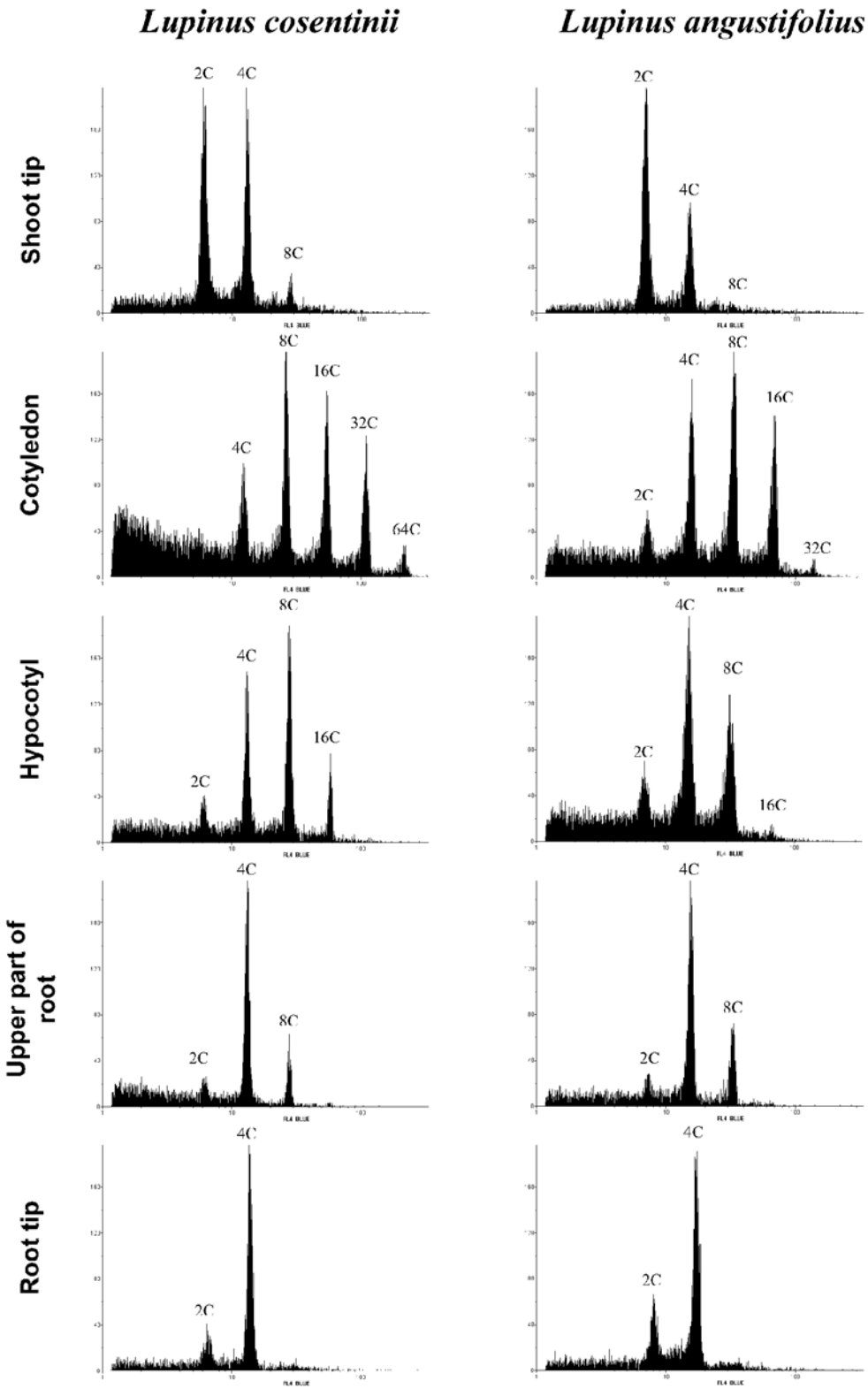

Fig. 1 Flow cytometric analysis of endopolyploidy pattern in *L. cosentinii* and *L. angustifolius* seedlings. X-axis, intensity of fluorescence; Y-axis, number of nuclei

meristematic tissues. In roots the highest peak corresponded to cells in G2 (4C) while in the shoots the highest was peak G1 (2C) and a small peak at the 8C level appeared. This small 8C peak probably came from young leaves that started differentiation. The characteristic diploid level of the shoot meristem can be related to the prevention of genetic unstable germ line cells, originating from the shoot apical meristem tissue. This is in agreement with the results of investigations on *A. thaliana*

(Galbraith et al. 1991) where multiploidy is restricted to non-reproductive tissues.

The results of flow cytometric analysis of the ploidy level in the two *Lupinus* species showed that these species, like most angiosperms, are polysomatic types of plants. Endopolyploidization has been earlier reported for *L. luteus* (Sakowicz and Olszewska 1997) and *L. albus* (Le Gal et al. 1986), and the maximum of ploidy was similar, 64C and 32C, respectively. Analysis of endoreduplication during pod wall development in *L. albus* has also shown a maximum level of polyploidy as 32C (Lagunes-Espinoza et al. 2000). Similar patterns of endopolyploidy were also observed in *Lycopersicon esculentum* seedlings (Smulders et al. 1994), and *Brassica rapa* and *Brassica oleracea* plants (Kudo and Kimura 2001).

Karyotype

Somatic metaphase chromosomes of *L. angustifolius* (2n = 40) and *L. cosentinii* (2n = 32) are shown in Fig. 2. The chromosomes of both species are relatively small and morphologically uniform. Their length ranges from 0.6 to 1.3 μm. Despite rather well defined centromeric regions, which are often visible as a negative DAPI band in the chromosomes of both species, the chromosome identification based on chromosome or arm lengths is very difficult. As in the genera *Brassica* (Hasterok and Maluszynska 2000a), *Solanum* (Srebrniak et al. 2002) and in other species with small chromosomes, morphometric analyses of *Lupinus* chromosomes allow only their rough classification into groups according to their centromere positions. An exact determination of homologous chromosomes was restricted to one pair of NOR chromosomes in each complement. These chromosomes can be easily recognised on the basis of the secondary constriction present in the short arm. All other chromosomes were unclassified and require additional landmarks.

rRNA gene distribution and activity

The distribution of ribosomal RNA gene families, 5S and 25S, differed between the two investigated *Lupinus* species. Double FISH with rDNA probes to metaphase chromosomes showed that two types of rRNA genes were located on different chromosomes. The FISH of *L. angustifolius* chromosomes tagged 10 out of 40 chromosomes (Fig. 2A, G). One pair of chromosomes carried the 25S rRNA gene cluster, which occupied the large distal part of the short arm and fully distended the secondary constrictions of NOR chromosomes. All 5S rDNA sites of varying size were localised on the short arms of four pairs of chromosomes, and were distributed in either pericentric or distal parts of the relevant chromosome arm. One pair of 5S rDNA loci was significantly bigger than the others. The results obtained in this study contradict these reported by Naganowska and Zielinska (2002), who observed only the biggest pair of 5S rDNA sites. The difference in a number of rDNA loci can be explained by intraspecific variation. Such variation has already been observed in some other species, for example in the genus *Brassica* (Kulak et al. 2002). Another explanation can be technical differences in the FISH procedure and image capturing, which can increase resolution and therefore allow visualisation of smaller hybridisation signals.

Contrary to *L. angustifolius*, FISH analysis of somatic chromosomes of *L. cosentinii* (Fig. 2D, H) revealed the presence of only two 5S rDNA sites and six 25S rDNA sites, which tagged 8 out of 32 chromosomes. The former are significant in size, indicating a high copy number in the locus and seem to occupy the terminal part of the long arm, whilst the latter display a polymorphism in size (Fig. 2H). The most distinct 25S rDNA hybridisation signals were these in the secondary constriction region of NOR chromosomes. Two other pairs of chromosomes bear significantly smaller 25S rDNA loci in their pericentric regions.

Differential staining of *L. angustifolius* chromosomes with chromomycin A_3 (CMA, Fig. 2B), the fluorochrome which binds preferentially to G-C rich regions (Schweizer 1976), revealed the presence of two bright CMA^+ bands in secondary constriction regions, which closely correspond to the FISH signals utilising a 25S rDNA probe. This indicated that, as in many other plant genomes, e.g. *Cucumis sativus* (Hoshi et al. 1999), *Solanum* (Srebniak et al. 2002) and *Brassica* (Hasterok and Maluszynska 2000a), the regions occupied by genes for 18S-5.8S–25S rRNA are the only large G-C rich blocks of chromatin in the *L. angustifolius* genome. Results of CMA staining and FISH signals with rDNA in the same metaphase plate of *L. cosentinii* (Fig. 2D, E) revealed the presence of eight bright CMA^+ bands. Six of them corresponded to the localisation of 25S rDNA hybridisation sites, while two further ones obviously overlapped with the signals of 5S rDNA, suggesting a G-C rich composition of these regions. This is a particularly interesting observation, which has so far probably not been reported for other plant species.

As was expected, the only pair of 25S rDNA loci in the *L. angustifolius* genome was transcriptionally active and gave positive results with silver staining (Fig. 2C). In the *L. cosentinii* genome the Ag^+ bands were also limited to secondary constrictions only (Fig. 2F), which indicated that the two remaining pericentromeric pairs of 25S rDNA loci were either transcriptionally inactive or their expression was below detection. A similar situation was observed in the species of *Brassica*, where the only active 18S-5.8–25S rRNA genes were these at the secondary constrictions, whilst all others did not express possibly due to DNA methylation or other expression-regulating mechanisms (Hasterok and Maluszynska 2000b).

Ribosomal genes are housekeeping genes that occur in many copies per genome, and are present and express in each cell that contains a nucleolus, regardless of tissue-type and developmental stage (Maluszynska et al. 1998). The chromosomal distribution of rDNA has been exten-

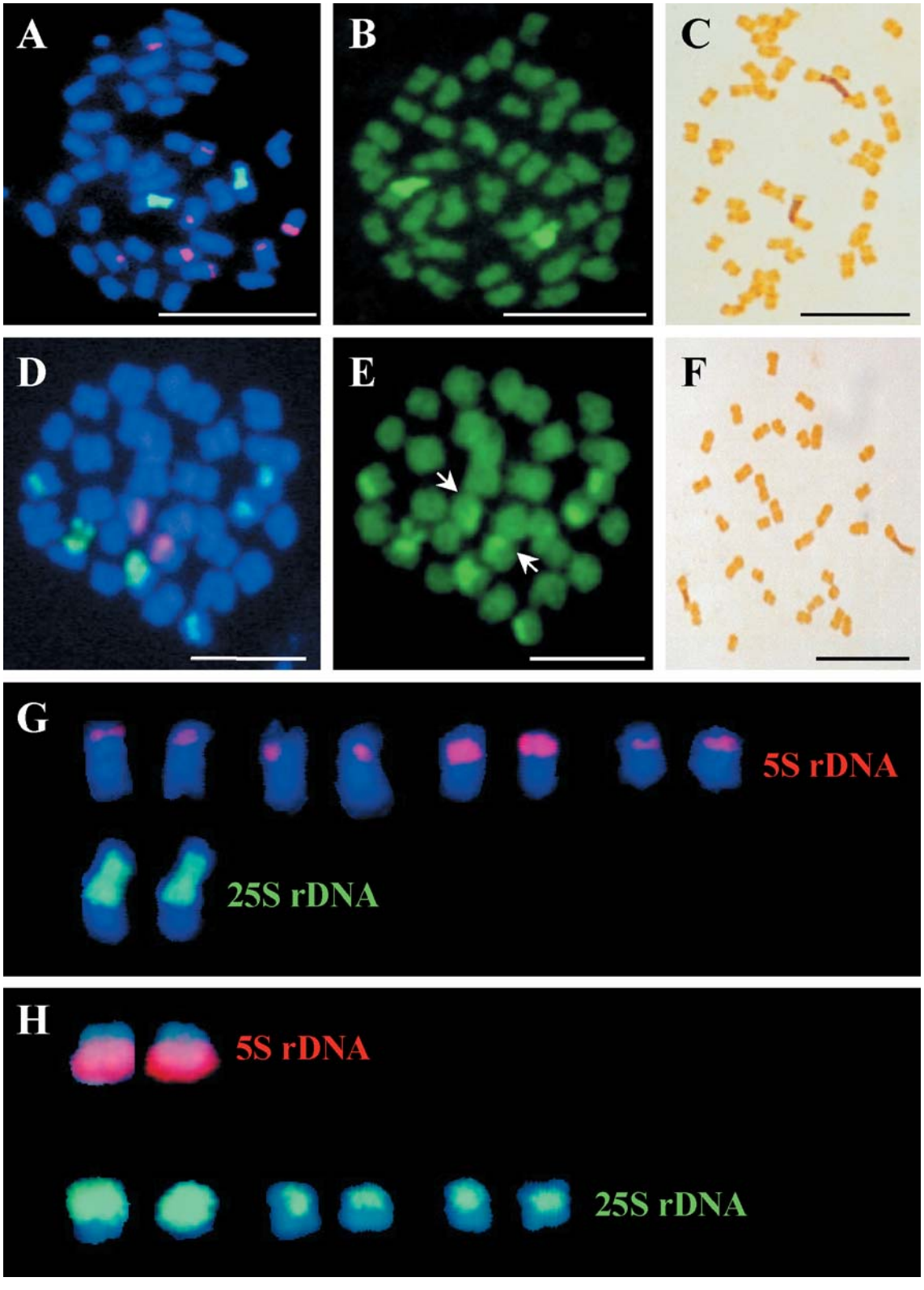

Fig. 2 Somatic metaphase chromosomes of *L. angustifolius* (**A, B, C, G**) and *L. cosentinii* (**D, E, F, H**); **A and D**, localisation of rRNA genes by double-target fluorescence in situ hybridisation (FISH) of 5S rDNA (*red*) and 25S rDNA (*green*) probes; **B and E**, differential fluorescent staining with chromomycin A3, CMA^+ bands indicate large G-C rich regions of chromatin, *arrows* in (**E**) show CMA^+ bands overlapping with 5S rDNA hybridisation sites; **C and F**, detection of active 18S-5.8S–25S rRNA genes by silver staining; **D–E**, FISH and CMA staining of the same chromosome complement; **G–H**, partial karyotype showing 25S rDNA and 5S rDNA carrying chromosomes. Bar = 5 μm

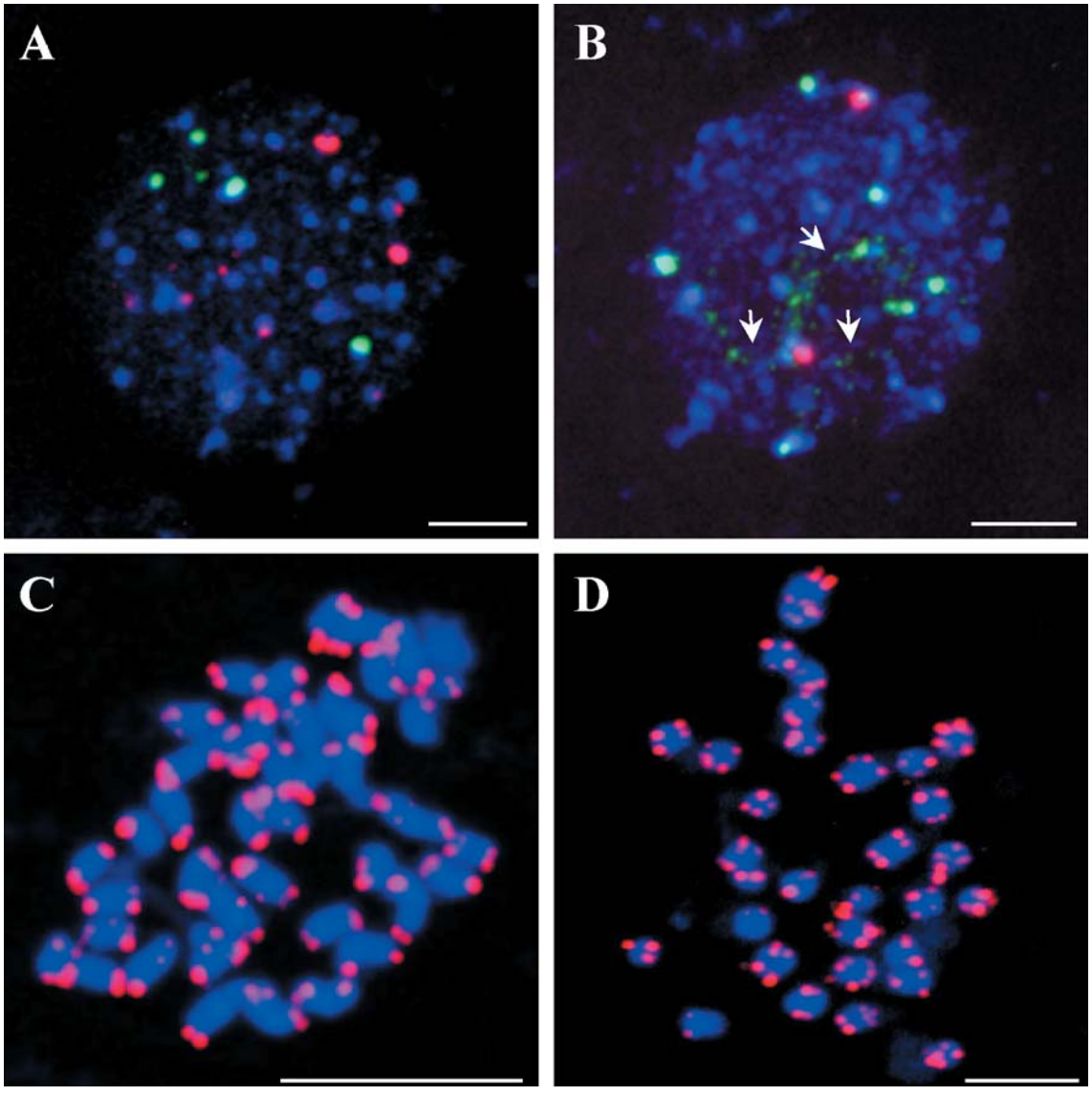

Fig. 3 Fluorescence in situ hybridisation of 5S rDNA (*red*) and 25S rDNA (*green*) probes to interphase nuclei of *L. angustifolius* (**A**) and *L. cosentinii* (**B**). *Arrows* in (**B**) indicate decondensed 'ribbon-like' nucleolar organising region. The plant telomeric $(TTTAGGG)_n$ probe hybridising to chromosomes of *L. angustifolius* (**C**) and *L. cosentinii* (**D**). Bar = 5 μm

sively studied in vast number of plant species providing the information about the phylogeny of closely related taxa (e.g. Kitamura et al. 2001; Li et al. 2001; Raina et al. 2001; Singh 2001). Often rDNA localisation has been used in karyotyping as landmarks for chromosome identification (e.g. Moscone et al. 1999; Kulak et al. 2002). However, the efficiency of rDNA as chromosome markers highly depends on the number of rDNA loci in comparison with the total number of chromosomes in the complement, on their variation in size and their chromosomal distribution. In some plant species, such as *H. vulgare* (Leitch and Heslop-Harrison 1993), *Pinus elliottii* (Doudrick et al. 1995), *A. thaliana* (Murata et al. 1997) and some of the species of *Brassica* (Hasterok et al. 2001), simultaneous FISH with 5S and 25S rDNA probes has enabled the identification of all or many chromosomes in the complement. However, the results of this study (Fig. 2) show clearly that this was not the case for both studied species of *Lupinus*, where relatively low polymorphic loci of both types of rDNA allowed identification of only 25% of chromosomes in the genome, which is not efficient for karyotyping. Additional markers are required to identify chromosomes for karyotype construction or phylogenetic analysis.

The number and distribution of hybridisation sites after FISH with rDNA are useful not only for chromosome identification, but also in analysis of interphase nucleus structure. The position of chromosomes carrying rRNA genes can be determined in each stage of the cell cycle by FISH. The number of hybridisation sites in the nucleus can help to determine the ploidy level of the cell or even detect chromosome rearrangements. Figure 3A and B

shows the organisation of rRNA genes in interphase nuclei of root-tip cells of *L. angustifolius* and *L. cosentinii*. In both species the nucleolus is hardly visible as an unstained area within DAPI-stained chromatin. In the nuclei of *L. angustifolius* the number and size of 5S rDNA signals corresponded to those observed on the metaphase chromosomes (Fig. 3A). The number of 25S rDNA signals was consistently four, which was twice the number of sites observed on the chromosomes. This apparent discrepancy is most likely caused by the characteristic pattern of de-condensation of the nucleolar organising region in the interphase nucleus. The secondary constriction is highly de-condensed and therefore is almost or completely invisible, whilst the distal part of the short arm and the satellite were highly compact and appeared as two seemingly separate signals. In *L. cosentinii* interphase nuclei (Fig. 3B) the number of 5S rDNA and 25S rDNA signals usually reflects the number of respective loci in the metaphase chromosomes. In most nuclei four sites were compact and two others highly de-condensed and could be seen as a dotted ribbon-like structure twisted in the nucleolus. The de-condensed status of rDNA can reflect its high activity, which has been reported for other species (Maluszynska and Heslop-Harrison 1991).

Distribution of telomeric arrays

The *Arabidopsis*-type telomeric repeat sequence (TRS) comprising the $(TTTAGGG)_n$ motif is known to hybridise with chromosomes of numerous plant species, e.g. *Secale cereale* (Schwarzacher and Heslop-Harrison 1991), *Crepis capillaris* (Maluszynska and Schweizer 1993) and *Vigna unguiculata* (Galasso et al. 1995). However, there are some genera like *Allium, Aloe, Nothoscordum* and *Tulbaghia* (Fay and Chase 1996; Adams et al. 2000), in which chromosomes do not hybridise with $(TTTAGGG)_n$ repeats and most-likely contain alternative telomeric sequences (Pich et al. 1996; Pich and Schubert 1998). Recently Weiss and Schertan (2002) reported the presence of vertebrate-like telomeric sequences at the ends of *Aloe* chromosomes.

In both species of *Lupinus*, FISH with TRS revealed distinct signals at termini of all chromosomes (Fig. 3C, D), but the size of the signals varied. This can be the result of a different number of repeats at the telomere of each arm, but the difference observed is too faint and inconsistent to be used as a landmark for chromosome identification. Similar variation was observed in rye chromosomes (Schwarzacher and Heslop-Harrison 1991). Chromosomal distribution of TRS was restricted exclusively to telomere regions, suggesting lack of chromosome fusions and translocations of terminal fragments in *Lupinus* genomes, while the non-telomeric distribution of these sequences was observed in the chromosomes of *A. thaliana* and *Vicia faba* (Richards et al. 1992; Schubert 1992).

Further identification of chromosomes and the development of cytogenetic analysis of *Lupinus* species will require chromosome-specific markers. A similar problem was solved with bacterial artificial chromosomes (BAC) in potato. The BACs were used as probes for FISH and a set of 12 clones specific for each chromosome was isolated (Dong et al. 2000). Also recently, Howell et al. (2002) successfully used BAC-FISH to identify individual chromosomes of *B. oleracea*.

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References

Adams SP, Leitch IJ, Bennet MD, Leitch AR (2000) *Aloe* L., a second plant family without $(TTTAGGG)_n$ telomeres. Chromosoma 109:201–205

Ali HBM, Meister A, Schubert I (2000) DNA content, rDNA loci and DAPI bands reflect the phylogenetic distance between *Lathyrus* species. Genome 43:1027–1032

Aïnouche A-K, Bayer RJ (1999) Phylogenetic relationships in *Lupinus* (Fabaceae: Papilionoideae) based on internal transcribed spacer sequences (ITS) of nuclear ribisomal DNA. Am J Bot 84:590–607

Ansari HA, Ellison NW, Reader SM, Badaeva ED, Friebe B, Miller TE, Williams WM (1999) Molecular cytogenetic organization of 5S and 18S-16S rDNA loci in white clover (*Trifolium repens* L.) and related species. Ann Bot 83:199–206

Dolezel J, Sgorbati S, Lucretti S (1992) Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol Plant 85:625–631

Dolezel J, Dolezelova M, Novak FJ (1994) Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*. Biol Plant 36:351–357

Dolezel J, Greilhuber J, Lucretti S, Meister A, Lysak MA, Nardi L, Obermayer R (1998) Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot 82:17–26

Dong F, Song J, Naess SK, Helgeson JP, Gebhardt C, Jiang J (2000) Development and application of a set of chromosome-specific cytogenetic DNA markers in potato. Theor Appl Genet 101:1001–1007

Doudrick RL, Heslop-Harrison JS, Nelson CD, Schmidt T, Nance WL, Schwarzacher T (1995) Karyotype of slash pine (*Pinus elliottii* var. *elliottii*) using patterns of fluorescence in situ hybridisation and fluorochrome banding. J Hered 86:286–296

Fay MF, Chase MW (1996) Resurrection of Themidaceae for the Brodiaea alliance and re-circumscription of Alliaceae, Amaryllidaceae and Agapanthoideae. Taxon 45:441–451

Fransz P, Armstrong S, Alonso-Blanco C, Fisher TC, Torres-Ruiz R, Jones G (1998) Cytogenetics for the model system *Arabidopsis thaliana*. Plant J 13:867–876

Galasso I, Schmidt T, Pignone D, Heslop-Harrison JS (1995) The molecular cytogenetics of *Vigna unguiculata* (L.) Walp: the physical organization and characterization of 18S-5.8S–25S rRNA genes, 5S rRNA genes, telomere-like sequences, and a family of centromeric repetitive DNA sequences. Theor Appl Genet 91:928–935

Galbraith DW, Harkins KR, Knapp S (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol 96:985–989

Gerlach WL, Dyer TA (1980) Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Res 11:4851–4865

Hasterok R, Maluszynska J (2000a) Cytogenetic analysis of diploid *Brassica* species. Acta Biol Cracov Ser Bot 42:145–153

Hasterok R, Maluszynska J (2000b) Nucleolar dominance does not occur in root tip cells of allotetraploid *Brassica* species. Genome 43:574–579

Hasterok R, Jenkins G, Langdon T, Jones RN, Maluszynska J (2001) Ribosomal DNA is an effective marker of Brassica chromosomes. Theor Appl Genet 103:486–490

Hizume M, Sato S, Tanaka A (1980) A highly reproducible method of nucleolus organizer regions staining in plants. Stain Technol 55:87–90

Hoshi Y, Plader W, Malepszy S (1999) Physical mapping of 45S rRNA gene loci in the cucumber (*Cucumis sativus* L.) using fuorescence in situ hybridization. Caryologia 52:49–57

Howell EC, Barker GC, Jones GH, Kearsey MJ, King GJ, Kop EP, Ryder CD, Teakle GR, Vicente JG, Armstrong SJ (2002) Integration of the cytogenetic and genetic linkage maps of *Brassica oleracea*. Genetics 161:1225–1234

Joubes J, Chevalier C (2000) Endoreduplication in higher plants. Plant Mol Biol 43:735–745

Kitamura S, Inoue M, Shikazono N, Tanaka A (2001) Relationships among *Nicotiana* species revealed by the 5S rDNA spacer sequence and fluorescence in situ hybidization. Theor Appl Genet 103:678–686

Kudo N, Kimura Y (2001) Flow cytometric evidence for endopolyploidy in seedlings of some *Brassica* species. Theor Appl Genet 102:104–110

Kulak S, Hasterok R, Maluszynska J (2002) Karyotyping of *Brassica* amphidiploids using 5S and 25S rDNA as chromosome markers. Hereditas 136:144–150

Lagunes-Esponoza LC, Huyghe C, Bousseau D, Barre P, Papineau J (2000) Endoreduplication occurs during pad wall development in temperate grain legumes. Ann Bot 86:185–190

Larkins BA, Dilkes BP, Dante RA, Coelho CM, Woo Y, Liu Y (2001) Investigating the hows and whys of DNA endoreduplication. J Exp Bot 52:183–192

Le Gal MF, Lecocoq FM, Hallett JN (1986) The reserve proteins of mature cotyledons of *Lupinus albus* var. Lucky. II. Relationship with the nuclear DNA content. Protoplasma 130:128–137

Leitch IJ, Heslop-Harrison JS (1993) Physical mapping for four sites of 5S rDNA sequences and one side of the alpha-amylase gene in barley (*Hordeum vulgare*). Genome 36:517–523

Li R, Taylor S, Jenkins G (2001) Unravelling the phylogeny of tetraploid *Vicia amoena* (Fabaceae) and its diploid relatives using chromosomal landmarks. Hereditas 134:219–224

Maluszynska J, Heslop-Harrison JS (1991) Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J 1:159–166

Maluszynska J, Schweizer D (1993) B chromosomes of *Crepis capillaris*: structure and behaviour. First B-Chromosome Conference, Universidad Autonoma, Madrid, p 52

Maluszynska J, Hasterok R, Weiss H (1998) rRNA genes—their distribution and activity in plants. In: Maluszynska J (ed) Plant cytogenetics. Wyd. US, Katowice pp 75–95

Moscone EA, Lein F, Lambrou M, Fuchs J, Schweizer D (1999) Quantitative karyotyping and dual-color FISH mapping of 5S and 18S–25S rDNA probes in the cultivated *Phaseolus* species (Leguminosae). Genome 42:1224–1233

Murata M, Heslop-Harrison JS, Motoyoshi F (1997) Physical mapping of the 5S ribosomal RNA genes in *Arabidopsis thaliana* by multi-color fluorescence in situ hybridization with cosmid clones. Plant J 12:31–37

Naganowska B, Ladon D (2000) Chromosomes of *Lupinus hispanicus* subsp. *hispanicus* Boiss. et Reut., *L. luteus* L. and their hybrids. J Appl Genet 14:167–170

Naganowska B, Zielinska A (2002) Physical mapping of 18S–25S rDNA and 5S rDNA in *Lupinus* via fluorescent in situ hybridisation. Cell Mol Biol Lett 7:665–670

Obermayer R, Swiecicki WK, Greilhuber J (1999) Flow cytometric determination of genome size in some old world *Lupinus* species (Fabaceae). Plant Biol 1:403–407

Olszewska MJ, Legocki A (1989) Changes in DNA content during rhizobial nodule development in *Lupinus luteus* L. Cytophotometry and autoradiography. Biol Zentralbl 108:221–230

Pich U, Schubert I (1998) Terminal heterochromatin and alternative telomeric sequences in *Allium cepa*. Chrom Res 6:315–321

Pich U, Fuchs J, Schubert I (1996) How do Alliaceae stabilize their chromosome ends in the absence of TTTAGGG sequences? Chrom Res 4:207–213

Raina SN, Mukai Y, Kawaguchi K, Goel S, Jain A (2001) Physical mapping of 18-5.8–26S and 5S ribosomal RNA gene families in three important vetches (*Vicia* species) and their allied taxa constituting three species complexes. Theor Appl Genet 103:839–845

Richards EJ, Chao S, Vongs A, Yang J (1992) Characterization of *Arabidopsis thaliana* telomeres isolated in yeast. Nucleic Acids Res 20:4039–4046

Sakowicz T, Olszewska MJ (1997) DNA content, interphase AgNOR-area, number of ^{3}HrDNA hybridization signals and the methylation level in coding rDNA sequence in different organs of *Lupinus luteus* L. Genetica 99:67–72

Schmidt T, Heslop-Harrison JS (1998) Genome, genes and junk: the large-scale organization of plant chromosomes. Trends Plant Sci 3:195–199

Schubert I (1992) Telomeric polymorphism in *Vicia faba*. Biol Zentralbl 111:164–168

Schwarzacher T, Heslop-Harrison JS (1991) In situ hybridisation to plant telomeres using synthetic oligomers. Genome 34:317–323

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58:307–324

Singh RJ, Kim HH, Hymowitz T (2001) Distribution of rDNA loci in the genus *Glycine* Willd. Theor Appl Genet 103:212–218

Smulders MJM, Rus-Kortekaas W, Gilissen LJW (1994) Development of polysomaty during differentiation in diploid and tetraploid tomato (*Lycopersicon esculentum*) plants. Plant Sci 97:53–60

Srebrniak M, Rasmussen O, Maluszynska J (2002) Cytogenetic analysis of an asymmetric potato hybrid. J Appl Genet 43:19–31

Unfried I, Gruendler P (1990) Nucleotide sequence of the 5.8S and 25S rRNA genes and the internal transcribed spacers from *Arabidopsis thaliana*. Nucleic Acids Res 18:4011

Weiss H, Scherthan H (2002) *Aloe* spp.—plants with vertebrate-like telomeric sequences. Chromosome Res 10:155–164