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Comparison of genomes of eight species of sections *Linum* and *Adenolinum* from the genus *Linum* based on chromosome banding, molecular markers and RAPD analysis

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Abstract Karyotypes of species sects. Linum and Adenolinum have been studied using C/DAPI-banding, Ag-NOR staining, FISH with 5S and 26S rDNA and RAPD analysis. C/DAPI-banding patterns enabled identification of all homologous chromosome pairs in the studied karyotypes. The revealed high similarity between species *L. grandiflorum* ($2n = 16$) and *L. decumbens* by chromosome and molecular markers proved their close genome relationship and identified the chromosome number in *L. decumbens* as $2n = 16$. The similarity found for C/DAPI-banding patterns between species with the same chromosome numbers corresponds with the results obtained by RAPD-analysis, showing clusterization of 16-, 18- and 30-chromosome species into three separate groups. 5S rDNA and 26S rDNA were co-localized in NOR-chromosome 1 in the genomes of all species investigated. In 30-chromosome species, there were three separate 5S rDNA sites in chromosomes 3, 8 and 13. In 16-chromosome species, a separate 5S rDNA site was also located in chromosome 3, whereas in 18-chromosome species it was found in the long arm of NOR-chromosome 1. Thus, the difference in localization of rDNA sites in species with $2n = 16$, $2n = 30$ and $2n = 18$ confirms taxonomists opinion, who attributed these species to different sects. Linum and Adenolinum, respectively. The obtained results suggest that species with $2n = 16$, $2n = 18$ and $2n = 30$ originated from a 16-chromosome ancestor.

Keywords Ag-NOR-staining · C/DAPI-banding · FISH · 5S and 26S rDNA · Evolution · *Linum* · Chromosome identification · RAPD-analysis · Taxonomy

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Introduction

Domesticated about 6,000 years ago, the flax *Linum usitatissimum* L ($2n = 30$) is broadly used as an industrial crop (Vavilov 1926; Zohary and Hopf 1988; Lay and Dybing 1989). Its relative species *L. austriacum* ($2n = 18$) and *L. grandiflorum* ($2n = 16$) are employed as ornamental plants. Other flax relative species may be used as potential sources of genes necessary for creation of new flax

cultivars. This requires information about the relationships between the genomes of flax and its wild relative species. Such data are, however, rather negligible.

Based on morphological taxonomic characters, there are currently two versions of the taxonomy of *L. usitatissimum* L ($2n = 30$) and its relative species. Some taxonomists attribute to sect. *Linum*, together with species *L. usitatissimum* L ($2n = 30$), other closely related species with $2n = 30$ (*L. angustifolium* Huds., *L. bienne* Mill etc.) as well as relative species with $2n = 18$ (*L. leonii* F.W. Schultz, *L. perenne* L. ect.) and $2n = 16$ (*L. grandiflorum* Desf., *L. decumbens* Desf.) (Ockendon and Walters 1968). Other authors divided section *Linum* into two independent sections—*Linum* and *Adenolinum*. Sect. *Linum* includes species with $2n = 30$ and $2n = 16$. Sect. *Adenolinum* consists of the wild 18-chromosome species (Yuzepchuk 1949; Egorova 1996).

As for the origination of flax, most of authors have presumed that *L. angustifolium* as an ancestor of *L. usitatissimum* (Vavilov 1926; Zohary and Hopf 1988; Diederichsen and Hammer 1995). Up to now, there is no common opinion concerning the role of *L. bienne* Mill. in the origin of *L. usitatissimum* (Lay and Dybing 1989). Yuzepchuk (1949) classified *L. bienne,* along with *L. usitatissimum* and *L. angustifolium,* as separate species, and regarded *L. bienne* to be a primitive form of flax, being most closely related to the wild ancestor species *L. angustifolium.*

The taxonomic relationship between species within the genus *Linum* was also studied using different approaches. As a criterion for infrageneric classification of different *Linum* sections, the fatty acid composition and polypeptide analysis of seeds were assessed. It has been shown that species of sect. *Linum* differed from species of sect. *Adenolinum* (Kutuzova et al. 1999; Velasco and Goffman 2000). Some authors demonstrated the possibility to distinguish species of sect. *Linum* from sect. *Adenolinum* based on seed, pollen and leaf ultrastructure and seed coat anatomy (Moroz and Tsymbalyuk 2005; Optasyuk 2006; Svetlova and Yakovleva 2006).

Recently, methods used for the study of genome polymorphisms (RAPD and RFLP analysis) were successfully applied to the investigation of genetic mapping and variability in flax (Cullis et al. 1999; Lemesh et al. 1999; Mansby and Bothmer 2000; Oh et al. 2000). RAPD-analysis of genetic relationships in sect. *Linum* and *Adenolinum* showed that species with $2n = 30$, $2n = 18$ and $2n = 16$ were clustered in three separate groups (Lemesh et al. 2001; Fu et al. 2002). Due to the small size and similar morphology of *Linum* chromosomes (Ray 1944; Lewis 1964; Harris 1968; Chennaveeraiah and Joshi 1983), comparative chromosome analysis based on the use of different types of banding and molecular markers (physical chromosome mapping of ribosome genes by FISH) was not, until recently, applied. Meanwhile, such combined approach has been successfully applied to studies of different plant genomes. The phylogenetic relationship between cultivated species and their close relatives that are often used as donors of desirable genetic traits were established by this approach (Badaeva et al. 1998; Mukai et al. 2001; Hajdera et al. 2003).

To clarify the interspecific and evolutionary relationships of flax and its relatives in sections *Linum* and *Adenolinum,* we have undertaken in the present work an investigation of the genomes of species with $2n = 30$, $2n = 18$ and $2n = 16$ using several techniques, such as C/DAPI-banding, Ag-NOR-staining, FISH using probes for 5S and 26S rDNA and RAPD-analysis.

Materials and methods

Materials

The following specimens: *Linum usitatissimum* L. var. *usitatissimum* cultivar Orshanskii 2 ($2n = 30$) accession number 39; *L. angustifolium* Huds. ($2n = 30$) accession number 15; *L. bienne* Mill. ($2n = 30$) accession number 14; were obtained from the collection at the Institute of Genetics and Cytology of the Belarus National Academy of Sciences (Minsk, Belarus). In addition, flax specimens *L. grandiflorum* Desf. ($2n = 16$) accession number Lin 4/99, *L. decumbens* Desf. ($2n = 16$) accession number Lin 1913/98, *L. austriacum L.* subsp. *austriacum* ($2n = 18$) accession number Lin 6/76, *L. leonii* F.W. Schultz ($2n = 18$) accession number Lin 1672/92 and *L. perenne L.* ($2n = 18$) accession number Lin 1807/94 were kindly provided by the GenBank Gatersleben, Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany) (for details see Table 1).

Chromosome slide preparation

The seeds were germinated in Petri dishes on moist filter paper at room temperature. Root tips (of 0.5 cm) were excised and treated overnight (16–20 h) in an ice-cold water with 1 μg/ml 9-aminoacridine (Sigma) to harvest elongated chromosomes (Muravenko et al. 2003). After pretreatment, root tips were fixed in ethanol:acetic acid (3:1) for 3–24 h at room temperature. For C-banding and Ag-NOR-staining, the meristematic tissues were treated with 45% acetic acid for 2 min and squashed in 45% acetic acid onto glass slides. For FISH, the root tips were transferred from fixative solution into 1% carmine solution in 45% acetic acid for 40 min before squashing. The cover slips were removed after freezing in liquid nitrogen. The slides were dehydrated in 96% ethanol and then air-dried.

Table 1 Investigated species with chromosome numbers and sizes

Species	Our results			Literature data	
	Accession number, provenance	2*n*	Chromosome size, μm	2*n*	References
Sect. *Linum*					
L. bienne Mill.	14, Minsk	30	1–1.5	30	Ockendon and Walters (1968)
L. usitatissimum L.	39, Minsk	30	1–1.5	30	Ray (1944); Chennaveeraiah and Joshi (1983); Gill and Yermanos (1967)
L. angustifolium Huds.	15, Minsk	30	1–1.5	30	Ray (1944); Chennaveeraiah and Joshi (1983); Gill and Yermanos (1967)
L. grandiflorum Desf.	Lin 4/99, GenBank Gatersleben	16	1.9–4.3	16	Ray (1944); Seetharam (1972); Chennaveeraiah and Joshi (1983)
L. decumbens Desf.	Lin 1913/98, GenBank Gatersleben	16	1.9–4.3	30	Gill and Yermanos (1967)
Sect. *Adenolinum*					
L. austriacum L.	Lin 6/76, GenBank Gatersleben	18	1.7–3.9	18	Ray (1944); Chennaveeraiah and Joshi (1983); Gill and Yermanos (1967); Ockendon and Walters (1968);
				36	Seetharam (1972)
L. perenne L.	Lin 1807/94, GenBank Gatersleben	18	1.7–3.9	18	Ray (1944); Gill and Yermanos (1967);
				36	Ockendon and Walters (1968); Chennaveeraiah and Joshi (1983)
L. leonii F.W. Schultz	Lin 1672/92, GenBank Gatersleben	18	1.7–3.9	18	Ockendon and Walters (1968)

C-banding

Chromosome slides were hydrolyzed in 0.2 N HCl for 3–5 min at 60°C, washed in running water for 10 min, dehydrated in a series of 70, 85 and 96% ethanol and air-dried. The slides were incubated with a saturated solution of $Ba(OH)_2$ for 6 min at room temperature, rinsed in 1 N HCl for 5–10 s and washed in running water for 15 min. Slides were then incubated in 2 × SSC at 60°C for 1 h, washed in running water for 15 min and air-dried. The slides were stained with 1.5% Giemsa solution (Merck) in 0.125 M *Tris*–HCl buffer (pH = 6,8) for 3–10 min under control of the light microscope. The particular time of staining was selected for each species. Slides were washed with water, air-dried, soaked in xylene and mounted in enthellan (Merck). DAPI—banding was performed in parallel, the details of which are described below.

Ag-NOR staining

The silver staining procedure was carried out according to the modified method of Howell and Black (1980). Two solutions were prepared for Ag-staining: solution 1–50% of $AgNO_3$ (Sigma); solution 2–2% aqueous gelatin solution with 1% formic acid. Twenty microliters of solution 1 and 10 μl of solution 2 were mixed immediately before use, pipetted onto a slide and covered with a cover slip. After 10–20 min incubation at room temperature, the cover glass was rinsed off with tap water and the slide was washed with deionized water and air-dried. Slides were mounted in 0.125 μg/ml of DAPI (Serva) in Citifluor (UKC CHEM.-LAB) for simultaneous Ag-NOR staining and chromosome identification.

Probe preparation

Genomic DNA of *L. austriacum* L. was isolated from seedlings by the modified CTAB method and used as a template for PCR amplification of rDNA probes (Rogers and Bendich 1985). A set of PCR primers was designed on the basis of 26S and 5S rDNA sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/). 26S rDNA was amplified with primers 5′-GCA TAT CAA TAA GCG GAG GAA AAG AA-3′ and 3′-TAG TTG ATT CGG CAG GTG AGT TGT TA-5′. The primers for 5S rDNA amplification were 5′-GGTATG ATC GCA CCC GAA GAT TAA C-3′ and 3′-TCG TGT TGC ACC CCT TTT GTC-5′. The PCR program for rDNA amplification consisted of 2 min at 95°C for initial denaturation, followed by 30 cycles of 20 s at 95°C, 20 s at 62°C, and 2 min at 72°C, and completed by a 7-min final extension at 72°C. PCR products were separated in 0.8% agarose gels and rDNA bands were recovered and purified using a Gel Extraction Kit (Qiagen). 5S rDNA probe (272 bp) and 26S rDNA probe (820 bp) were then cloned into pTZ57R (Fermentas, St Leon-Rot, Germany) following the manufacturer's instructions. One microgram of each

rDNA probe was labeled using the Dig or Bio Nick mix translation kits (Roche). Biotin-16-dUTP was used for labeling 26S rDNA probes and digoxigenin (DIG)-11-dUTP for 5S rDNA labeling. Four microliters of 10 mg/ml sonicated salmon sperm DNA (Gibco BRL) were added to each labeled probe mix, which was precipitate with 100% ethanol and dissolved in 50 µl hybridization solution, consisting of 50% de-ionized formamide, 10% dextran sulfate, 1% Tween 20 and 2 × SSC.

FISH

Chromosome slides were pretreated with 1 mg/ml RNase A (Roche) in 2 × SSC at 37°C for 1 h and then washed three times for 10 min in 2 × SSC. The slides were dehydrated in a series of 70, 85 and 96% ethanol solutions and then air-dried. Fifteen microlitres of hybridization mixture containing 40 ng of each labeled probe were added to each slide. The slides were then coverslipped and sealed with rubber cement. Slides with DNA probes were co-denatured at 74°C for 5 min, placed in a moisture chamber and hybridized at 37°C overnight. After removing the coverslips, the slides were washed twice with 0.1 × SSC at 44°C for 10 min, followed by washing with 2 × SSC at 44°C for 2 × 5 min and 2 × SSC at room temperature for 5 min. Before detection, the slides were soaked in 4 × SSCT (0.1% Tween 20 in 4 × SSC) at room temperature for 3 min and then incubated in Detecting Buffer (5% fat-free dry milk in 4 × SSCT) at 37°C for 30 min. The slides were washed in 4 × SSCT at room temperature for 3 min. DIG-labeled probes (5S rDNA sites) were detected using anti-DIG-rhodamine (Roche), and biotin-labeled probes (26S rDNA sites) were detected using avidin-FITC (Vector). After incubation for 60 min at 37°C with the detection mixture, the slides were washed three times for 3 min with 4 × SSCT at room temperature, followed by a short rinse in PBS. Slides were dehydrated and air-dried in the dark.

DAPI-banding

After in situ hybridization, the slides were stained with 0.125 µg/ml of DAPI (Serva) in Citiflour (UKC CHEM.LAB).

RAPD-analysis

Total DNA for analysis of individual samples was isolated using a Plant Genomic DNA Miniprep Kit (V-gene biotechnology limited, China). RAPD-PCR with standard decamer primers OPA10 and OPY02 was performed according to Zelenina et al. (2006). PCR-products were fractionated in 6% polyacrylamide gels (PAAG). Gels were stained in ethidium bromide solution followed by scanning on variable mode imager Typhoon 8600 (Molecular dynamics, USA) under resolution of 100 microns per pixel. The RAPD profiles obtained were normalized against the molecular size scale. Calculation of RAPD profile similarity according to Pearson correlation coefficient and cluster analysis were carried out using Phoretix 1D Database software (Nonlinear Dynamics, UK). Application of the Pearson correlation coefficient instead of Dice one based on binary matrix calculation allowed us to take into account not only the presence or absence of bands, but also their intensity. An unrooted dendrogram of individual similarity of RAPD patterns was constructed in conformity with the UPGMA clustering procedure.

Chromosome analysis

Metaphase spreads were selected for investigation in accordance with principles defined earlier for small chromosomes. The main criterion for successful analysis of small chromosomes is the selection of convenient chromosome plates, chromosome length intervals 1.5–3 times greater than the minimal size of metaphase chromosomes (Popov et al. 2001). The analyses of chromosome plates were carried out using the Leitz Wetzlar (Germany) light and fluorescence microscope equipped with a QCoolSnep CCD camera (RoperScientific Inc., USA). The obtained images were processed with Adobe Photoshop 6.0 (Adobe Inc., USA) and VideoTest-Kario 1.5 (Ista-VideoTest, Russia) software. At least 10–15 metaphase plates were investigated for each species.

Results

The numbers and sizes of the chromosomes obtained by chromosome analysis of all studied species are given in Table 1 along with the literature data.

As shown in Table 1, the chromosome numbers in nearly all studied species correspond with published data, except those for *L. decumbens*, for which we determined the chromosome number ($2n = 16$), contrasting drastically with a previous publication.

C/DAPI-banding

Investigation of the chromosome C-banding patterns of all studied species demonstrated that the bigger heterochromatic bands are localized in pericentromeric regions of the majority of chromosomes, whereas medium-sized the middle size bands and small bands are detected presumably in telomeric and intercalary regions (Fig. 1a–c).

DAPI staining revealed banding patterns on chromosomes of all studied *Linum* species similar to those

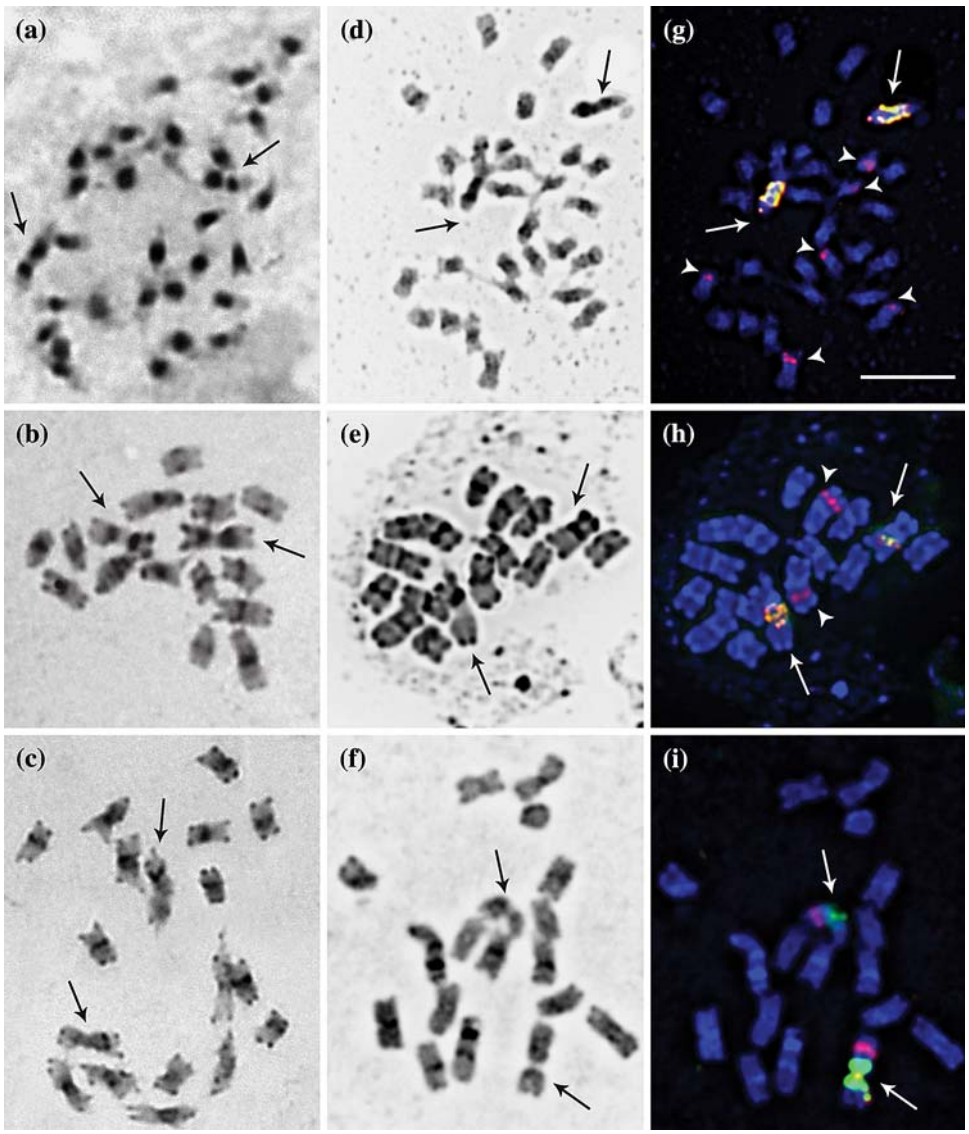

Fig. 1 Metaphase chromosome spreads of *L. angustifolium* ($2n = 30$), *L. grandiflorum* ($2n = 16$), *L. perenne* ($2n = 18$) after C-banding (**a, b, c**), DAPI-staining (*inverted*) (**d, e, f**) and FISH with 26S (*green signal*) and 5S rDNA (*red signal*) probes (**g, h, i**), respectively. The *arrows* show 26S and 5S hybridization signals in NOR-bearing chromosome 1. The *arrowheads* indicate 5S hybridization signals. *Bar* = 5 μm

obtained by C-staining (Fig. 1d–f), with the exception of a C-block located in the NOR region as well as some pale DAPI-staining intercalary C-blocks. Therefore, the C-blocks revealed by DAPI-staining were designated as C/DAPI bands. C/DAPI-banding patterns were chromosome-specific, which allowed for identification of all homologous chromosome pairs in all studied karyotypes. We created generalized idiograms for species with $2n = 18$, $2n = 16$ and $2n = 30$. Idiograms represent the schematic distribution of C/DAPI-banding patterns and rRNA gene localization in chromosomes (Fig. 2).

In sect. *Linum,* the largest amount of pericentromeric heterochromatin in the studied 30-chromosome species was found in the chromosomes of *L. angustifolium.* In the karyotypes of *L. usitatissimum* and *L. bienne,* the sizes of C/DAPI-bands were similar. *Linum angustifolium* chromosome 3 differed from that of two other species by a bigger centromeric index (see Fig. 2d, e).

In chromosomes of *L. grandiflorum* (2n = 16), telomeric C/DAPI-bands were slightly larger in size than those observed in *L. decumbens* ($2n = 16$) (Fig. 2b, c).

Among the 18-chromosome species of the sect. *Adenolinum* the *L. leonii* karyotype demonstrated the highest amount of heterochromatin, with the largest intercalary, pericentromeric and telomeric C/DAPI-bands. The size of these C/DAPI-bands decreased in the following order: *L. leonii, L. perenne, L austriacum*. The *L. perenne* chromosome 2 is characterized by a lower centromeric index and a larger C/DAPI-band in the median region of long arm as compared with other 18-chromosome species. In addition, a C/DAPI-band in 18-chromosome species is localized in the middle of the chromosome 1 arm, opposite to the satellite arm (Fig. 2a).

Comparison of karyotypes of 16- and 18-chromosome species revealed the similarity of species by C/DAPI-banding patterns in 1–8 chromosome pairs (Fig. 2a–c).

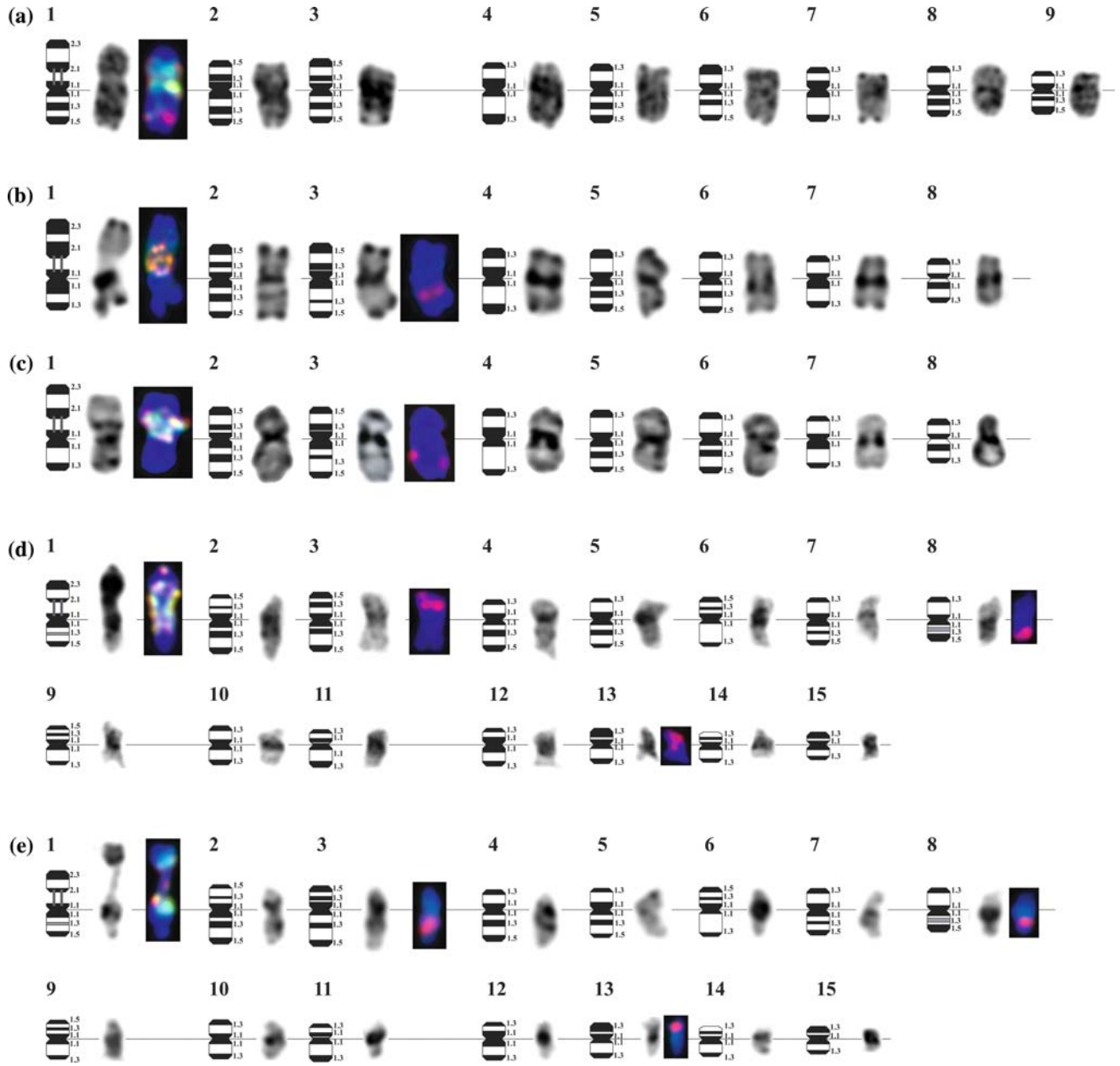

Fig. 2 Idiograms represent the schematic distribution of C/DAPI-banding patterns (*black*) and rRNA gene localization in chromosomes of *L. austriacum* ($2n = 18$) (**a**), *L. grandiflorum* ($2n = 16$) (**b**), *L. decumbens* ($2n = 16$) (**c**), *L. angustifolium* ($2n = 30$) (**d**) and *L. usitatissimum* ($2n = 30$) (**e**). 26S rDNA—*green signal*, 5S rDNA—*red signal*

However, some differences were found. Telomeric C/DAPI-bands in 16-chromosome species were larger than those in 18-chromosome species. In addition, a large C/DAPI-band was situated in the middle of the NOR chromosome 1 long arm in the 18-chromosome species. Chromosomes 8 and 9 of the 18-chromosome *Linum* species were similar in size and C/DAPI-banding patterns, but differed slightly by centromeric index and the size of C/DAPI-blocks. Regardless of their significantly smaller size, many chromosomes of the 30-chromosome species were also similar in C/DAPI-banding patterns to the 16- and 18-chromosome species, for example, the chromosome 1,2,3 (Fig. 2d, e). It is important to note that some chromosome pairs from the 30-chromosome species differ in size but are similar in banding patterns.

FISH

Physical mapping of chromosomes of the species under study was carried out. DAPI-banding not only allowed for the identification of all chromosomes possessing ribosomal genes, but also precisely localized the position of 26S and

5S rDNA. Metaphase plates of some representative 16-, 18- and 30-chromosome species are given in Fig. 1. A schematic distribution of ribosome genes on chromosomes is shown in generalized idiograms (Fig. 2).

The major sites of 26S and 5S rDNA in the *L. usitatissimum, L. angustifolium* and *L. bienne* karyotypes ($2n = 30$) sect. *Linum* were co-localized in NOR-chromosome 1 (Fig. 1g). The 26S rDNA sites varied in size and could occupy from half up to the whole chromosome arm. The 5S rDNA sites were located on three pairs of chromosomes. In the *L. angustifolium* karyotype, the major site of 5S rDNA was found in the distal C/DAPI-band of metacentric chromosome 3 in region 3S1.3 (Fig. 2d). In *L. usitatissimum* and *L. bienne* karyotypes, the major site of 5S rDNA was localized on the long arm of submetacentric chromosome 3 in region 3L1.3 (Fig. 2e). Additional loci of 5S rDNA genes were observed on chromosome 8 in proximal region 3L1.3 and in the subtelomeric part of chromosome 13 in region 13S 1.2. In some cases, FISH revealed a minor polymorphic site of 26S rDNA co-localized with the 5S rDNA site in karyotypes of all studied 30-chromosome species.

In the karyotypes of *L. grandiflorum* and *L. decumbens* ($2n = 16$), the major sites of 5S and 26S rDNA were co-localized in the nucleolus organizer region (NOR) of SAT chromosome 1 as described above for the 30-chromosome species (Fig. 1h). A rather large polymorphic signal for 5S rDNA was found in a median C/DAPI-band on the long arm of submetacentric chromosome 3 (3L1.3) (Fig. 2b, c).

In karyotypes of *L. austriacum*, *L. perenne* and *L. leonii* ($2n = 18$) of sect. *Adenolinum,* a large 26S rDNA site covered a part of the satellite, secondary constriction and proximal pericentromeric part of chromosome 1 (Fig. 1i). A small 5S rDNA site was co-localized with 26S rDNA, mainly in the satellite. On the other arm of chromosome 1 in the median C/DAPI-band, a large 5S rDNA site was observed simultaneously with a small 26S rDNA locus in region 1L1.3.

Comparative analysis of C/DAPI-banding patterns and ribosomal gene localization identified a pericentric inversion in chromosome 3, possessing the locus 5S rDNA (Fig. 2d, e). Based on this inversion, a distinction between species *L. angustifolium* and species *L. usitatissimum* and *L. bienne* could be determined. In addition, in the course of comparative analysis of C/DAPI-banding patterns and ribosomal 5S rDNA localization, a translocation between chromosome 1 and 3 was detected in 16- and 18-chromosome species (see Fig. 2a–c). This suggests that interchromosomal recombination between parts of chromosome arms with break points on the borders of region 1L 1.3 and 3L 1.3 occurred.

Ag-NOR staining

Ag-NOR staining revealed an intensive positive signal in the secondary constriction region of satellite chromosome 1 in the karyotypes of all species investigated. Ag-NOR stained metaphase spreads of 16-, 18- and 30-chromosome species are shown in Fig. 3.

RAPD-analysis

For estimation of genetic similarity, products of RAPD-PCR were fractionated by polyacrylamide gel electrophoresis. This technique gives better resolution than the use of agarose gels, which is generally accepted for this purpose (Weising et al. 2005). Using 6% PAAG allowed us to reveal numerous bands in each RAPD-spectrum and to obtain reliable results with just two appropriate primers, OPA10 and Y02. The total number of bands amounted to 137, 117 of which were polymorphic. In the UPGMA dendrogram, three clusters were identified. Each of these clusters pooled together *Linum* species with the same chromosome numbers: $2n = 16$, $2n = 18$ and $2n = 30$. RAPD spectra showed a small amount of variation within each cluster (Fig. 4).

Discussion

The pericentromeric type of the main C/DAPI-band pattern along chromosomes, as revealed in the studied *Linum* species, is also characteristic of other plants with low DNA

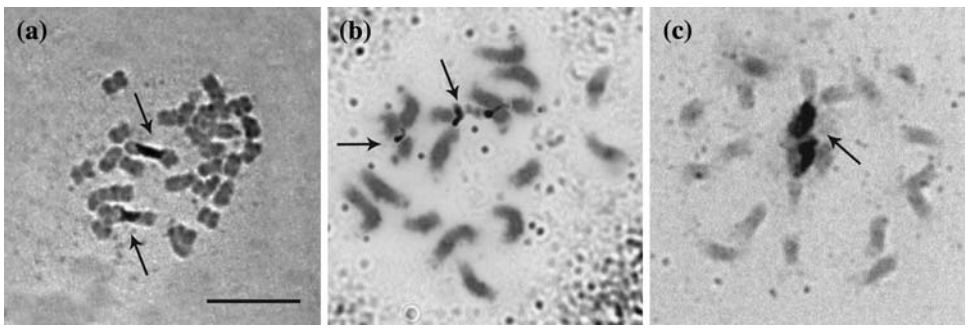

Fig. 3 Metaphase chromosome spreads of *L. bienne* ($2n = 30$) (**a**), *L. decumbens* ($2n = 16$) (**b**), *L. leonii* ($2n = 18$) (**c**) after Ag-NOR-staining. The *arrows* show active NORs. *Bar* = 5μm

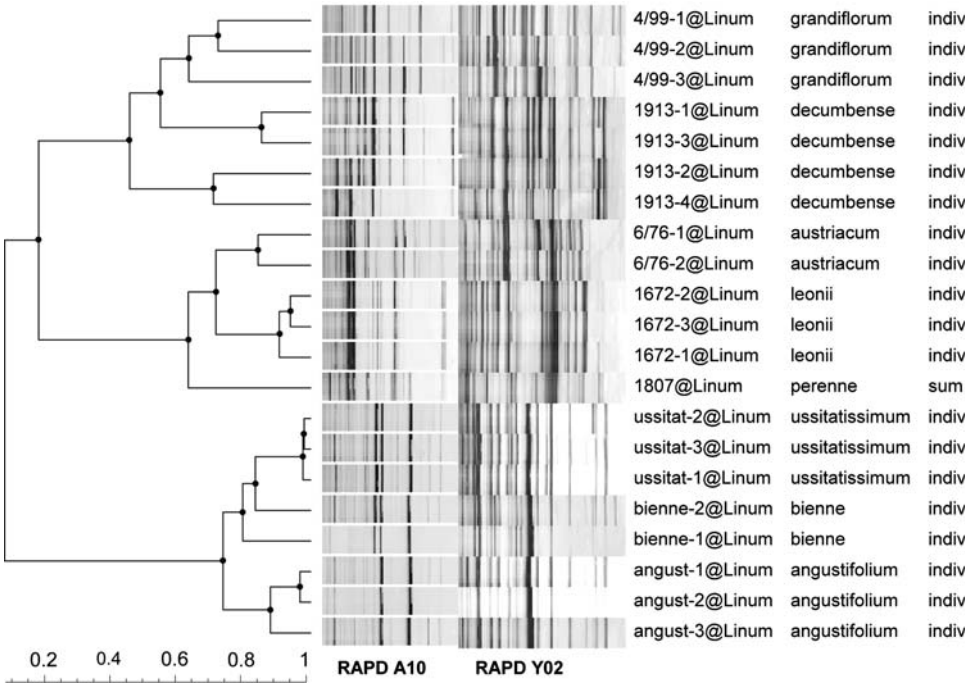

Fig. 4 The UPGMA-dendrogram of the similarity between *Linum* individual RAPD patterns. Pearson correlation coefficients are presented on the scale

content and small chromosomes (Olin-Fatih and Heneen 1992; Samatadze et al. 2001; Koo et al. 2002). The scarcity of chromosome banding patterns does not often allow for the precise identification of chromosomes in karyotypes of the small chromosome plant species (Pierozzi et al. 2001; Vanzela et al. 2002). We encountered a similar problem at the beginning of our investigation of the *Linum* chromosome. The use of DNA intercalator 9-aminoacridine increased the resolution of C/DAPI-banding and physical mapping of ribosomal genes. As a result, we performed a complete chromosome identification in the 16-, 30- and 18-chromosome species and compared C/DAPI-banding patterns and ribosomal gene localizations in different species.

The rDNA sites of the studied species were shown to localize in C-band regions (Fig. 2). A similar regularity has been previously found in other small chromosome plant species (Xu and Earle 1996; Dagne et al. 2000). Concordant changes in C-band size and the number of rRNA genes localized to this region allowed the authors to suggest that a part of the constitutive heterochromatin was associated with NORs, the ribosomal gene clusters dispersed within heterochromatic regions (Mukai et al. 1991; Zoldos et al. 1999). In most case, the bigger the C/DAPI-band sizes were, the more intense was the rDNA fluorescence signal. It should be noted that sometimes DAPI-bands (AT-rich) are rather pale (region 1S2.1 for example), which may be explained by an increased amount of GC-rich repeats.

All previous investigations of structure and localization of ribosomal genes were only carried out for cultivated *L. usitatissimum.* The 18-25S rDNA locus was mapped on one chromosome pair (Oh et al. 2000), which correlated with the presence of a single chromosome pair with the Ag-NOR-positive region in *L. usitatissimum* (Scweizer 1980). We confirmed these data concerning *L. usitatissimum,* and demonstrate similar results for other investigated species. In all studied species, the major loci of 26S rRNA genes were localized and found to be active in one chromosome pair (Figs. 2g and 3a). With the aid of radioactive ISH, 5S rDNA probes in interphase nuclei of *L. usitatissimum* were located dispersely (Schneeberger et al. 1989). More recent investigations by RFLP and RAPD analysis allowed for mapping of a pRS20.7 clone, which contained 5S rDNA from the *L. usitatissimum* genome, to the same chromosome as 45S rDNA (Oh et al. 2000). According to our data, the 5S and 26S rDNA co-localized in one pair of the NOR-chromosome 1 in the genomes of all species investigated. In addition, three separate 5S rDNA sites were presented in chromosomes 3, 8 and 13 in 30-chromosome species (Fig. 2d, e). A separate 5S rDNA site was also located in chromosome 3 in 16-chromosome species, whereas in 18-chromosome species—it is in the long arm of NOR-chromosome 1 (Fig. 2a–c).

The revealed similarity of C/DAPI-banding patterns in species with the same chromosome numbers is indicative of their close genetic relationship. This conclusion correlates with our data on RAPD-analysis, which showed clusterization of 16-, 18- and 30-chromosome species into three separate groups (see Fig. 4), as well as with the data of some other authors (Lemesh et al. 2001; Fu et al. 2002). It should be emphasized that we obtained these results by

means of only two appropriate primers and PAAG electrophoresis, whereas other authors used up to 20 primers and analyzed PCR products in agarose gels (Lemesh et al. 2001; Fu et al. 2002). Conformity of the obtained results demonstrates the advantages of the application of polyacrylamide gels for RAPD-analysis due to its high resolution.

The general similarity of chromosome C/DAPI-banding patterns in the karyotypes of the studied species of sects. *Linum* and *Adenolinum* with different chromosome numbers was revealed, being particularly evident in species with $2n = 16$ and $2n = 18$. This makes it likely that the genomes of all these species were derived from a common ancestor. This conclusion is proved by the similarity in localization of 26S rDNA genes and NOR-chromosome morphology in all species studied (Fig. 2).

According to the localization of 5S rRNA genes in chromosome 1, the studied species are divided into two groups. In 16- and 30-chromosome species, 5S rDNA is co-localized with 26S rDNA. In the 18-chromosome species, an additional site of 5S rDNA was revealed in the long arm of chromosome 1. Thus, the difference in the localization of 5S rDNA in chromosomes 1 and 3 suggests the existence of a translocation in the genome of species with $2n = 18$ (Fig. 2a–c).

Some taxonomists combined the 18-chromosome closely related species *L. perenne*, *L. leonii* and *L. austriacum* in one *L. perenne* group in sect. *Linum* (Ockendon and Walters 1968). Other taxonomists divided sect. *Linum* into two sections—*Linum* and *Adenolinum* (Yuzepchuk 1949; Egorova 1996). So, the difference in the localization of rDNA sites in chromosome 1 between species with $2n = 16$, $2n = 30$ and those with $2n = 18$ confirms the opinion of taxonomists who attributed these species to the different sections *Linum* and *Adenolinum*, respectively (Yuzepchuk 1949; Egorova 1996).

Comparison of C/DAPI-band size in the karyotypes of the studied 30-chromosome *Linum* species revealed a higher similarity between *L. usitatissimum* and *L. bienne*. In addition, the 5S rDNA site is localized in *L. usitatissimum* and *L. bienne* in the long arm of chromosome 3, whereas in *L. angustifolium*, it is localized in the short arm (Fig. 2d, e). This suggests a pericentric inversion in chromosome 3, including the 5S rDNA locus. This inversion could be indicative of a difference between species *L. angustifolium* and species *L. usitatissimum*, *L. bienne*. Thus, our data confirm the existence of a high degree of similarity between the *L. usitatissimum* and *L. bienne* genomes. At the same time, our results are contradictory to the assumption of authors who equate species *L. angustifolium* and *L. bienne* (Ockendon and Walters 1968) and correspond with the opinion of some taxonomists who classified *L. bienne* as a subspecies of *L. usitatissimum* (Chernomorskaya and Stankevich 1987) or regard these three species as separate species (Yuzepchuk 1949).

We found only one publication in which the chromosome number of *L. decumbens* was given as $2n = 30$ (Gill and Yermanos 1967). Our investigation does not coincide with these data; we found eight chromosome pairs in the karyotype of *L. decumbens*. We revealed a high similarity in chromosome C/DAPI-banding patterns, ribosomal gene localization (Fig. 2b, c) and RAPD-spectrum in polyacrylamide gels (Fig. 4) between species *L. grandiflorum* ($2n = 16$) and *L. decumbens,* proving the close relationship of their genomes. These results correspond with those of previous authors who studied these species by examining fatty acid patterns and RAPD-analysis in agarose gels (Fu et al. 2002; Velasco and Goffman 2000).

Evolution in sects. *Linum* and *Adenolinum*

Based on comparison of chromosome morphology (routine staining), the phylogenetic tree was built and species with $2n = 18$ were suggested to be the progenitor of the 18-, 16- and 30-chromosome species (Chennaveeraiah and Joshi 1983). Interspecific hybridization of *L. usitatissimum* × *L. grandiflorum* was more fruitful than *L. usitatissimum* × *L. austriacum* (Seetharam 1972). Other authors, based on the results of interspecific crossing, supposed that *L. usitatissimum* originated from progenitor species with $2n = 16$ via polyploidization, followed by reduction of chromosome number during the course of natural selection (Dubey and Kumar 1973). Our results for C/DAPI-banding patterns and ribosomal gene localization in 16-, 18- and 30-chromosome species of sects. *Linum* and *Adenolinum* suggest the origination of those species from a common 16-chromosome ancestor. Presumably, a translocation between chromosome 1 and 3 and duplication of chromosome 8 might have occurred in the genome of 18-chromosome species over the course of speciation. The presence of similar C/DAPI-banding patterns in some different chromosome pairs of 30-chromosome species acknowledges the role of polyploidy in their origination. Such an assumption was made previously as a result of the meiotic study of interspecific crossing of species from genus *Linum* (Gill and Yermanos 1967).

Linum angustifolium has been hypothesized to be a wild progenitor of *L. usitatissimum* (Zohary and Hopf 1988; Diederichsen and Hammer 1995; Fu et al. 2002). Until now, there is no consensus concerning the role of *L. bienne* Mill. in the origin of *L. usitatissimum* (Lay and Dybing 1989). *L. bienne* is considered as a primitive form of cultivated varieties and is most closely related to *L. angustifolium* (Yuzepchuk 1949). *Linum angustifolium* has dehiscing capsules, in contrast with *L. usitatissimum* and *L. bienne* (Yuzepchuk 1949), making it useless as a crop plant

but increasing its survival as a wild plant. Similar localization of 26S rDNA in species with $2n = 30$ suggest that all three species are closely related to each other and have a common progenitor. The inversion in chromosome 3 described above was probably involved in the divergence of these species.

The comparative chromosome analysis of two *Linum* sections points to a probable origin of species with $n = 15$ and $n = 9$ from an ancestor species with a basic chromosome number $n = 8$. Thus, our investigation made it possible to clarify the systematics and evolution of a number of *Linum* species and demonstrated the productivity of complex approaches to the comparative study of small chromosome species in general.

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