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Molecular and cytogenetic characterization of repetitive DNA sequences from *Lolium* and *Festuca*: applications in the analysis of *Festulolium* hybrids

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Summary. A set of species-specific repetitive DNA sequences was isolated from *Lolium multiflorum* and *Festuca arundinacea*. The degree of their species specificity as well as possible homologies among them were determined by dot-blot hybridization analysis. In order to understand the genomic organization of representative *Lolium*- and *Festuca*-specific repetitive DNA sequences, we performed Southern blot hybridization and in situ hybridization to metaphase chromosomes.

Southern blot hybridization analysis of eight different repetitive DNA sequences of *L. multiflorum* and one of *F. arundinacea* indicated either tandem and clustered arrangements of partially dispersed localization in their respective genomes. Some of these sequences, e.g. LMB3, showed a similar genomic organization in *F. arundinacea* and *F. pratensis*, but a slightly different organization and degree of redundancy in *L. multiflorum*. Clones sequences varied in size between 100 bp and 1.2 kb. Estimated copy number in the corresponding haploid genomes varied between 300 and 2×10^4. Sequence analysis of the highly species-specific sequences from plasmids pLMH2 and pLMB4 (*L. multiflorum* specific) and from pFAH1 (*F. arundinacea* specific) revealed some internal repeats without higher order. No homologies between the sequences or to other repetitive sequences were observed. In situ hybridization with these latter sequences to metaphase chromosomes from *L. multiflorum*, *F. arundinacea* and from symmetric sexual *Festulolium* hybrid revealed their relatively even distribution in the corresponding genomes. The in situ hybridization thus also allowed a clearcut simple identification of parental chromosomes in the *Festulolium* hybrid.

The potential use of these species-specific clones as hybridization probes in quantitative dot-blot analysis of the genomic make-up of *Festulolium* (sexual and somatic) hybrids is also demonstrated.

Key words: *Lolium multiflorum* – *Festuca arundinacea* – Repetitive DNA sequences – In situ hybridization – *Festulolium* hybrids

Abbreviations: bp, Base pair (s); CMA, chromomycin A_3; DAPI; 4,6-diamidino-2-phenylindole; IPTG, isopropyl β-D-thio-galactopyranoside; kb, kilobase pair (s); NBT, nitroblue tetrazolium chloride; X-gal, 5-bromo-4-chloro-3-inonyl β-D-galactopyranoside

Introduction

Repeated DNA sequences contribute considerably to the genome size of higher eukaryotes. In higher plants, repetitive DNA is mainly responsible for the wide variations in genome size (Bennett et al. 1982; Flavell 1980). Both highly and middle repetitive DNA sequences have been described in higher plants (Flavell 1980), and molecular studies on their function, localization and distribution are available (Kato et al. 1985; Sakowicz et al. 1986; Leclerc and Siegel 1987; Junghans and Metzlaff 1988; Ganal et al. 1988; Iwabuchi et al. 1991).

Detailed studies on repetitive DNA sequences from Gramineae have been reported for important cereal crops, e.g. wheat (Dennis et al. 1980; Hutchinson and Lonsdale 1982; Metzlaff et al. 1986), rye (Bedbrook et al. 1980; Appels et al. 1981), barley (Dennis et al. 1980; Ananiev et al. 1986; Junghans and Metzlaff 1988), maize (Peacock et al. 1981; Viotti et al. 1985) or rice (Wu and Wu 1987; Zhao et al. 1989). In contrast to the well-characterized cereal repeated sequences (size, abundance,

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chromosomal distribution, and nucleotide sequence) there is a general lack of information on the repetitive sequences of forage grasses.

Repetitive DNA sequences of *Lolium* and *Festuca* have not yet been studied. Although the function and origin of these repetitive DNA sequences remain to be elucidated, they have proven useful in studying genome evolution and species divergence at the molecular level. In addition, they represent valuable tools for analysing hybrid genomes, as has been demonstrated for rye-wheat sexual hybrids (Appels 1982; Rayburn and Gill 1986) or *Brassica* somatic hybrids (Itoh et al. 1991).

In this article we report the isolation and molecular and cytological characterization of repetitive DNA sequences in *Lolium multiflorum* (Italian ryegrass) and *Festuca arundinacea* (tall fescue), two important forage grass species in temperate pastures. In addition, their potential use for the genome analysis of intergeneric sexual and somatic *Festulolium* hybrids is discussed.

Materials and methods

Plant material

Hexaploid (2n=42) tall fescue (*Festuca arundinacea* Schreb.) populations 'Alpes' and 'Bière' and diploid (2n=14) Italian ryegrass (*Lolium multiflorum* Lam.) cv 'Turilo' were used. F_1 hybrids from a *L. multiflorum* × *F. arundinacea* cross were made. Part of the set of these *Festulolium* sexual hybrids used in this study was produced at the Swiss Federal Research Stations of Changins and Zürich-Reckenholz and was made available to us by Drs. G. Kleijer and B. Nüesch.

DNA isolation, restriction and gel electrophoresis

Total genomic cellular DNA was isolated from freeze-dried leaf material from shoot cultures or greenhouse-grown plants. The isolation and digestion of genomic DNA was performed according to Lichtenstein and Draper (1985). Restriction enzyme analysis and agarose gel electrophoresis were carried out following standard protocols (Sambrook et al. 1989).

Isolation of species-specific repetitive DNA sequences

The construction of DNA probes and the isolation of *L. multiflorum*- and *F. arundinacea*-specific repetitive clones was basically performed according to Saul and Potrykus (1984). One µg of total DNA isolated from either *L. multiflorum* or *F. arundinacea* leaves was cut with *Hin*dIII or *Bam*HI, mixed with 0.2 µg of *Hin*dIII- or *Bam*HI-cut pBluescript SK$^-$ (Stratagene Cloning Systems, La Jolla, USA) and ligated overnight at 14 °C. Aliquots of this ligation were transformed into *E. coli* strain XL1Blue and plated on LB agar medium containing 40 µg/ml X-gal, 48 µg/ml IPTG with 50 µg/ml ampicillin. White colonies, which contained insertions of plant DNA, were picked at random and used for colony hybridization screenings. Two replicates of the total bank of clones were produced on Biodyne (Pall, Glen Cove, USA) filters and hybridized with *L. multiflorum* and *F. arundinacea* probes created by random priming of total DNA partially digested with *Hin*dIII. Clones preliminarily identified as Italian ryegrass specific or tall fescue specific were further tested. Plasmid DNA was isolated from the bacterial clones found to be species specific in the colony hybridization screening – pLMH1, pLMH2, etc (for plasmid *Hin*dIII inserts from *L. multiflorum*); pLMB3, pLMB8 etc. (for plasmid *Bam*HI inserts from *L. multiflorum*); and pFAH1 (for plasmid *Hin*dIII insert from *F. arundinacea*) – and used for further analysis.

Southern blot and dot-blot hybridizations

Southern blot hybridization experiments using digested (*Eco*RI, *Bam*HI or *Hin*dIII) total genomic DNA from *L. multiflorum* and *F. arundinacea* were performed following standard protocols as described in Sambrook et al. (1989). Dot-blot hybridizations using inserts from cloned species-specific DNA sequences or total genomic DNA were performed following the instructions of the manufactor (Schleicher & Schüll GmbH, Dassel, FRG).

Hybridization probes were labeled with [^{32}P]dATP by random priming (Feinberg and Vogelstein 1983). Fragments corresponding to cloned species-specific sequences were isolated from the respective plasmids following Dretzen (1981) and were used as hybridization probes.

DNA sequences and computer sequence analysis

Inserts from species-specific repetitive DNA clones were sequenced by the dideoxynucleotide chain termination procedure of Sanger et al. (1980) using the T7 sequencing kit (Pharmacia, Sweden). DNA sequences were analysed using the corresponding software from the University of Wisconsin Genetics Computer Group (University Research Park, Madison, USA).

Chromosome preparation and in situ hybridization

Chromosome preparations were made from shoot meristems isolated and pre-treated following de Lautour and Cooper (1971) with modifications. After propionic acid-ethanol fixation and enzymatic treatment, meristems were squashed, fluorochrome-stained with CMA/DAPI solution and embedded in glycerol with mercapto-ethanol (Franklin and Filion 1985).

In situ hybridization with digoxigenin-labeled probes and detection was done with modifications (L. Petris et al. in preparation) according to the manufactor's instructions (Boehringer Mannheim, FRG).

Results

Isolation and molecular characterization of L. multiflorum- and F. arundinacea-specific repetitive DNA sequences

In order to isolate repetitive species specific DNA sequences, about 250 recombinant plasmid clones, containing random sequences from either *L. multiflorum* or *F. arundinacea*, respectively, were screened using labeled total DNA from both species as hybridization probes. About 9% of the *L. multiflorum* clones and 5% of the *F. arundinacea* clones showed strong hybridization signals when probed with DNA from the same species, as was expected for multicopy sequences. For these clones, little or no hybridization signal was detectable when colony hybridization filters were probed with total DNA from the other species.

Representative recombinant plasmids containing inserts from either *F. arundinacea* or *L. multiflorum* were

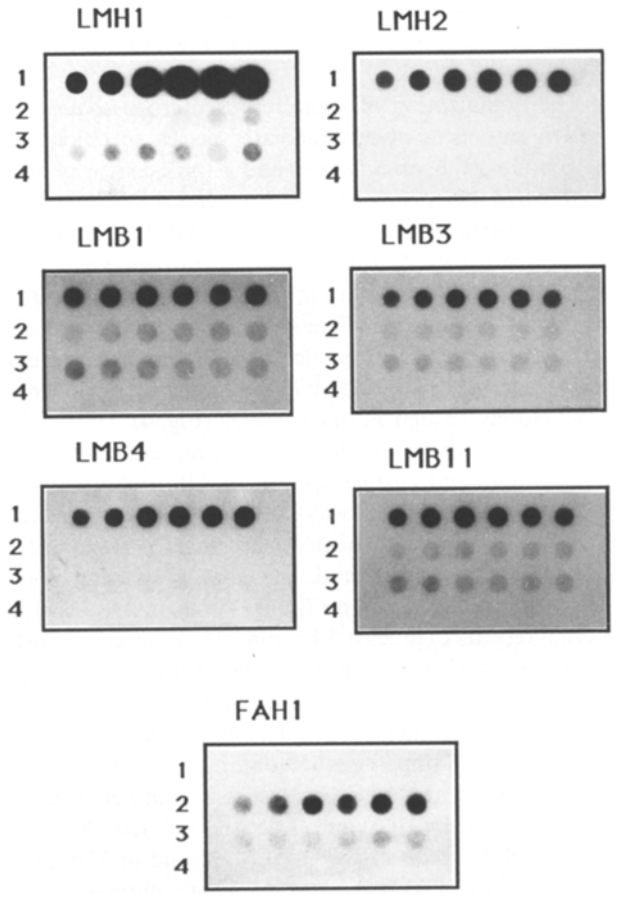

Fig. 1. Test for species-specificity of repetitive DNA sequences isolated from *L. multiflorum* and *F. arundinacea*. Labeled probes were obtained from DNA fragments isolated from *L. multiflorum* (LMH1, LMH2, LMB1, LMB3, LMB4 and LMB11) or *F. arundinacea* (FAH1) and hybridized to dot blots containing (from left to right) 0.5, 1, 2, 3, 4 and 5 μg of *L. multiflorum* (*1*), *F. arundinacea* (*2*), *F. pratensis* (*3*) and calf thymus (*4*) DNA

selected for further characterization. DNA inserts from 1 *F. arundinacea* clone and from 8 *L. multiflorum* clones showing differential hybridization were isolated and their species specificity was tested by dot-blot hybridization analysis to total DNA from *L. multiflorum*, *F. arundinacea* and *F. pratensis* using the isolated inserts as hybridization probes (Fig. 1). Sequences designated LMH2 and LMB4 were only present in *L. multiflorum*; sequence FAH1 cloned from *F. arundinacea* cross-hybridized to *F. pratensis* DNA, but was absent in the *L. multiflorum* genome. Based on densitometric measurements performed with non-saturated autoradiograms, the degree of cross-hybridization to *F. arundinacea* or to *F. pratensis* DNA observed for the *Lolium*-specific sequences LMH1, LMB1, LMB3 and LMB11 was never higher than 10% of the hybridization spot signal obtained with *L. multiflorum* DNA. Insert size and copy number of the cloned

Table 1. Characteristics and nucleotide sequence of repetitive DNA clones from *L. multiflorum* and *F. arundinacea*. **A** Size of the cloned repetitive DNA sequence was estimated by comparing the electrophoretic mobility of the corresponding inserts to size standards. The estimated number of copies per haploid genome was determined by comparison of the extent of hybridization of the indicated sequence to defined amounts of genomic DNA and of the corresponding DNA sequence. **B** Nucleotide sequence of *L. multiflorum*-specific repetitive clone LMH2 and *F. arundinacea*-specific repetitive clone FAH1. Direct repeats (at least 5 bp long) showing more than 75% homology to the repeated sequence are *underlined*.

A

Sequence	Approximate size (bp)	Estimated copy number per haploid genome	Characteristics	
			Lolium[a]	*Festuca*[b]
LMH2	170	$1.3 \cdot 10^4$	Dispersed	–
LMB4	300	$2.0 \cdot 10^3$	Dispersed	–
FAH1	100	$2.0 \cdot 10^4$	–	Clustered
LMB1	1000	900[a]	N.D.	Tandemly arranged
LMB3	750	$5.0 \cdot 10^3$[a]	N.D.	Tandemly arranged
LMB8	1200	300[a]	N.D.	Tandemly arranged

N.D., Not determined

[a] Data from *L. multiflorum*

[b] Data from *F. arundinacea* and *F. pratensis*

B

LMH2

AGACTTTGTG CAATGTCAGA AGTGTTAAGA ATGATTATGT CACCTCTGAA

TGTATGAATT TTITATTATG CACTAACCCT CTAATGAGTT TGCTTGAAGT

TTGGTGTGGA GGAAGTTTTC AAGGGTCAAG AGAAGAGGAT GATACAATAT

GATCAAGAAG AGTGAAAGGT CTA

FAH1

AGCTTGGCTA GAGCTGCTTG CCTCCTGACC TTTTCCGGTT CCGGCCTTGG

GAGCAGAGGG GGAGGCACTC ATCCGGTCGA TCTCGGCTTC AAGCCCGTCG

GA

sequences were estimated to range from 100 bp to 1.2 kb and from 300 to 20 000 copies per haploid genome, respectively (Table 1).

Cross-hybridization experiments were performed for assessing possible homologies among the *L. multiflorum* cloned sequences. The highly *Lolium*-specific sequences LMH1, LMH2 and LMB4 did not cross-hybridize to any other tested sequence (results not shown). However, sequences with an intermediate degree of species specificity, such as LMB1, LMB3, LMB8 and LMB11, shared

some degree of homology. Nevertheless, homology was not evenly shared, since sequence LMB1 and LMB8 preferentially hybridized to clones LMB3 and LMB11, respectively, while sequence LMB3 hybridized to all of them with varying intensity (Fig. 2).

Southern blot hybridization studies were performed to gain information on the organization of the sequences in the *Festuca* and *Lolium* genomes. When Southern blots of *L. multiflorum* DNA digested with three different restriction enzymes were probed with *Lolium*-specific sequences LMH2, LMB4 and LMH1, the strongest hybridization was found to occur to a few bands in the low size range. However, hybridizing bands in the range of 2–9 kb could be detected on longer exposures of autoradiograms, which might suggest a partially dispersed arrangement of these sequences in the genome (Fig. 3 A). The *Festuca*-specific sequence FAH1 showed a banding pattern similar to the former ones with the small-sized band in the *Hin*dIII digest probably corresponding to the cloned insert. The *L. multiflorum* sequences LMB1, LMB3, LMB8 and LMB11, which share some homology among each other (Fig. 2), showed complex multiple band patterns in Southern blot hybridization analysis. Despite evident similarities in the banding patterns of these sequences, some differences concerning the stoichiometry of specific bands in the small size range could be observed (Fig. 3 B). These differences enable us to discriminate LMB3 from LMB1 and LMB8 or LMB11. The sequence LMB7, which did not cross-hybridize to the ones just mentioned, also showed a complex banding pattern, but one that was clearly distinguishable from the others. This is evidenced by the lack of hybridization signals in the range of 2–4 kb for the *Hin*dIII digest (Fig. 3 B).

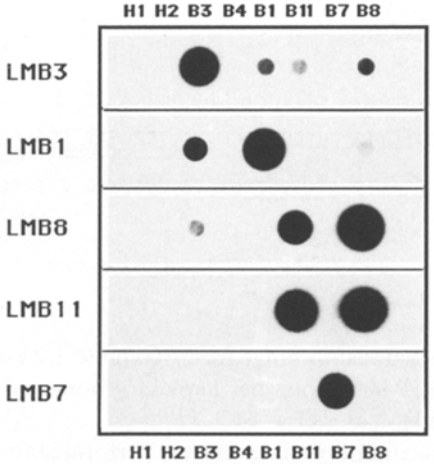

Fig. 2. Cross-hybridization analysis of repetitive DNA sequences isolated from *L. multiflorum*. Dot blots loaded with 50 ng of each *Lolium* repetitive sequence were hybridized to labeled probes from the same sequences. *H1* to *B8* at the *top* and *bottom* of the figure represent the sequences applied to the filter (LMH1 to LMB8). *LMB3* to *LMB7* on the *left* represent the sequences used as probes

The organization of some representative *Lolium* repetitive sequences of intermediate species specificity in the genome of *F. arundinacea* and *F. pratensis* was also investigated by means of Southern hybridization. The *Lolium* repetitive sequences LMB1, LMB7, LMB8 and LMB11, when used as hybridization probes, showed a ladder-like banding pattern in both *F. arundinacea* and *F. pratensis* DNA digests. The same held true for the *L. multiflorum* repetitive sequence LMB3, thus indicating a tandem arrangement of these *Lolium* repetitive sequences in the closely related *Festuca* species (Fig. 4).

The molecular characterization of representative isolated *Lolium*- and *Festuca*-specific middle and highly repetitive DNA sequences was extended to their sequence analysis. Complete sequences of the *Lolium*-specific clone LMH2 (173 bp) and the *Festuca*-specific clone FAH1 (102 bp) are shown (Table 1). When screened for internal repeats of at least 5 bp and 75% homology, both LMH2 and FAH1 sequences displayed a number of direct repeats up to 10 bp long that were not organized in higher level structures (Table 1). Similar features were found in the 286-bp sequence obtained for the LMB4 clone (not shown). The estimated copy number of their inserts was 1.3×10^4 and 2.0×10^3 in the haploid genomes of *L. multiflorum* for pLMH2 and pLMB4, respectively. For pFAH1 the estimated copy number in the haploid genome of *F. arundinacea* was 2×10^4 (Table 1).

Computer screening of the plant DNA sequences stored in the EMBL data bank failed to detect any significant homology to the LMH2, FAH1 and LMB4 sequences. An 86% homology in a 22-bp overlap was detected between LMH2 (position 54–75) and the *del1* retrotransposon (position 4450–4471), a repetitive dispersed sequence from *Lilium henryi* (Smyth et al. 1989; Smyth 1991). This correlation is probably coincidental.

In situ hybridization and quantitative dot-blot analysis of Festuca and Lolium repetitive DNA sequences

To gain further understanding of the genomic organization and distribution of the species specific repetitive DNA sequences we carried out an in situ hybridization analysis with metaphase chromosomes of *L. multiflorum*, *F. arundinacea* and symmetric *Festulolium* F_1 hybrids. *Lolium* (LMB4, LMH2)-and *Festuca* (FAH1)-specific sequences were chosen for this type of analysis. This analysis also allowed us to evaluate particular highly specific *Lolium* and *Festuca* sequences for the characterization of the nuclear composition of sexual and somatic *Festulolium* hybrids (Takamizo et al. 1991).

In situ hybridization analysis using symmetric *Lolium-Festuca* sexual hybrids (such as BB5 and KrII) carrying

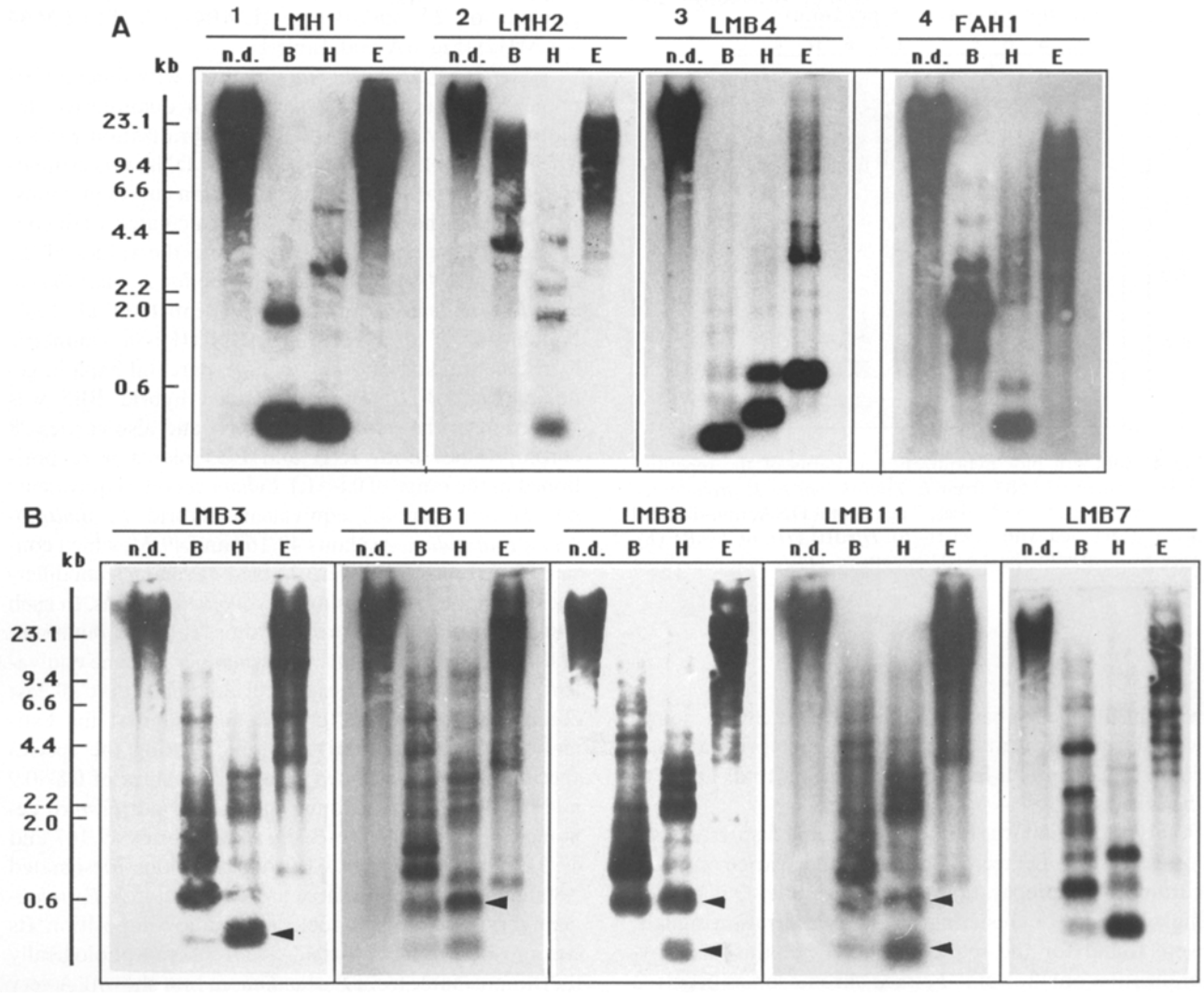

Fig. 3 A, B. Southern blot hybridization patterns of repetitive DNA sequences from *L. multiflorum* and *F. arundinacea*. **A** Southern blots of total DNA from *L. multiflorum* (*1, 2, 3*) or *F. arundinacea* (*4*) were probed with the repetitive DNA sequences of high species specificity (LMH1 to FAH1). **B** Southern blots of total DNA from *L. multiflorum* were probed with repetitive DNA sequences of intermediate species specificity (LMB3 to LMB7). In all cases, 7 µg of total DNA, non-digested (n.d.) or digested with *Bam*HI (*B*), *Hin*dIII (*H*) or *Eco*RI (*E*), were loaded per lane

one complete genome of each parent was performed in order to determine the degree of dispersion of the selected sequences, their chromosomal localization as well as their ability to discriminate chromosomes from each species. These *Festulolium* materials are symmetric F_1 hybrids from a *L. multiflorum* × *F. arundinacea* sexual cross and contain 28 chromosomes (Fig. 5). The *Lolium* chromosomes were clearly identified by a detailed cytogenetic analysis after staining with DAPI and CMA dyes (Fig. 5A, B), and by in situ hybridization using total DNA from *L. multiflorum* as probe (Fig. 5C). Three larger chromosomes of *L. multiflorum* showed distinctive heterochromatic bands when stained with DAPI and CMA (Fig. 5A). An identification of all of the *Lolium* chromosomes in *Festulolium* hybrids is feasible if based on image analysis systems creating division images of double-stained preparations (Fig. 5 A, B). Hybridization of digoxigenin-labeled total DNA from *Lolium* lead to an unequivocal identification of all 7 chromosomes from *L. multiflorum* in this hybrid: 4 large and 3 small chromosomes appeared densely stained (Fig. 5C). However, the resolution achieved with this method was not optimal since the chromosomes from *F. arundinacea* also showed weak staining (Fig. 5C).

Both the LMH2 (Fig. 5D) and LMB4 (Fig. 5E) sequences hybridized to all *Lolium* chromosomes in metaphase spreads from the *Festulolium* F_1 hybrids. A less intense staining of the *Lolium* chromosomes together

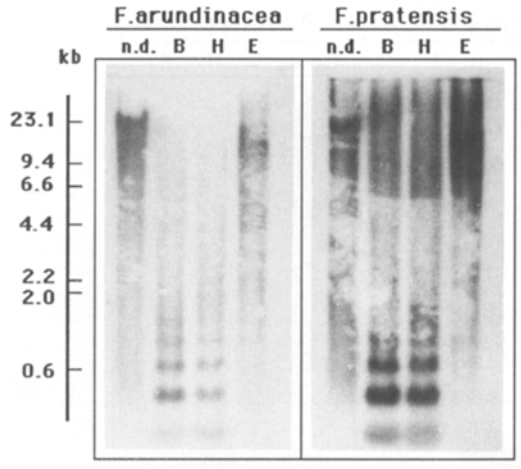

Fig. 4. Southern blot hybridization patterns of the repetitive DNA sequence LMB3 from *L. multiflorum* to *F. arundinacea* and *F. pratensis*. In both cases, 7 µg of total DNA, non-digested (*n.d.*) or digested with *Bam*HI (*B*), *Hin*dIII (*H*), or *Eco*RI (*E*), were probed with the LMB3 repetitive DNA sequence from *Lolium*

with the absence of background in *Festuca* chromosomes were the main advantages of this type of hybridization as compared to in situ hybridizations using total DNA as the label.

Hybridization was found to occur in a dispersed fashion with some degree of clustering that varied with the chromosome preparation. Similar results, but with a higher degree of clustering of the hybridization signals, were found for the sequence FAH1. In this case, hybridization only to some *Festuca* chromosomes occurred (Fig. 5F).

In addition to the analysis using the *Festulolium* hybrid, species specific in situ hybridization of the cloned sequences LMH2, LMB4 and FAH1 was confirmed on separate preparations of metaphase chromosomes of *L. multiflorum* and *F. arundinacea*, respectively (data not shown).

Once the even representation of both LMB4 and LMH2 sequences in the *L. multiflorum* genome was confirmed, their use for quantitative determination of the amounts of each parental DNA in a *Festulolium* hybrid could be tested by quantitative dot blots. Calibration mixes representing a range of concentrations of *L. multiflorum* DNA in a strong background of *F. arundinacea* DNA were blotted onto filters and probed with radioactively labeled LMB4, LMH2 or a mix of both. Calibration lines obtained by plotting the bound radioactivity against the amount of DNA applied showed acceptable linearity within the range of concentrations used (Fig. 6A). Increasing slope values were obtained depending on the degree of representation of each probe in the genome of *L. multiflorum* (LMB4 < LMH2 < LMB4 + LMH2) (Fig. 6A and Table 1).

A set of sexual *Festulolium* hybrids with different genome compositions were analyzed in quantitative dot blots using LMH2 and LMB4 as hybridization probes. The relative content of *L. multiflorum* DNA was estimated by extrapolation from the calibration curves previously obtained. The amounts of DNA calculated were converted into genome equivalents using the values of 2.1 and 6.7 pg DNA per haploid genome for *L. multiflorum* and *F. arundinacea*, respectively (Bennett et al. 1982; Hutchinson et al. 1979) (Fig. 6B). KrII is a symmetric hybrid containing one copy of each parental haploid genome (Fig. 5B-D). The *Festulolium* hybrid BB5 was derived from the same cross as KrII and also carries 28 chromosomes. Both KrII and BB5 plants were positioned in the range of 0.8–1.1 *Lolium* genome equivalents per *Festuca* genome equivalent. Hybrid *L. multiflorum* × *F. arundinacea* plants 49/167 and 49/31, which contain 52 chromosomes, were derived by selfing from different symmetric amphidiploid *Festulolium* hybrids. In each case 4 chromosomes were lost after selfing. A minimum of 0.7 genome equivalents of *Lolium* per genome equivalent of *Festuca* is thus expected, assuming that all lost chromosomes belonged to the *Lolium* parental line. Estimations from quantitative dot blots using the species specific probes mentioned resulted in values of 0.8–0.9 and 0.7–0.8 *Lolium* genome equivalents per *Festuca* genome equivalent for the *Festulolium* hybrids 49/167 and 49/31, respectively (Fig. 6B). A plant clone (designated A8001b) was made available to us as a putative *Festulolium* hybrid of unknown nuclear genomic composition. Its chromosome number was 42, and it was morphologically indistinguishable from *F. arundinacea* (not shown). A very low or zero content or *Lolium* DNA was estimated for this material after performing quantitative dot-blot hybridization analysis using the previously characterized species specific repetitive dispersed *L. multiflorum* sequences LMH2 and LMB4. The *Festulolium* hybrid FeLiLi (28 chromosomes) was derived from a cross between hybrid line FeLi (28 chromosomes) and a tetraploid *L. multiflorum* (28 chromosomes). The hybrid FeLi was obtained by selfing a symmetric amphidiploid (56 chromosomes) *Festulolium* hybrid. A total of 28 chromosomes was lost during the selfing process that lead to the *Festulolium* line FeLi. Our estimations on the nuclear make-up for this line resulted in values of *Lolium*/*Festuca* genome equivalents in the range of 3–10 depending on the hybridization probe used (Fig. 6B).

These results thus demonstrate the potential use of some of the cloned *Lolium* and *Festuca* species specific repetitive DNA sequences in the characterization of the nuclear composition of sexual *Festulolium* hybrids. Their suitability for similar studies involving symmetric or asymmetric somatic hybrids derived from different pro-

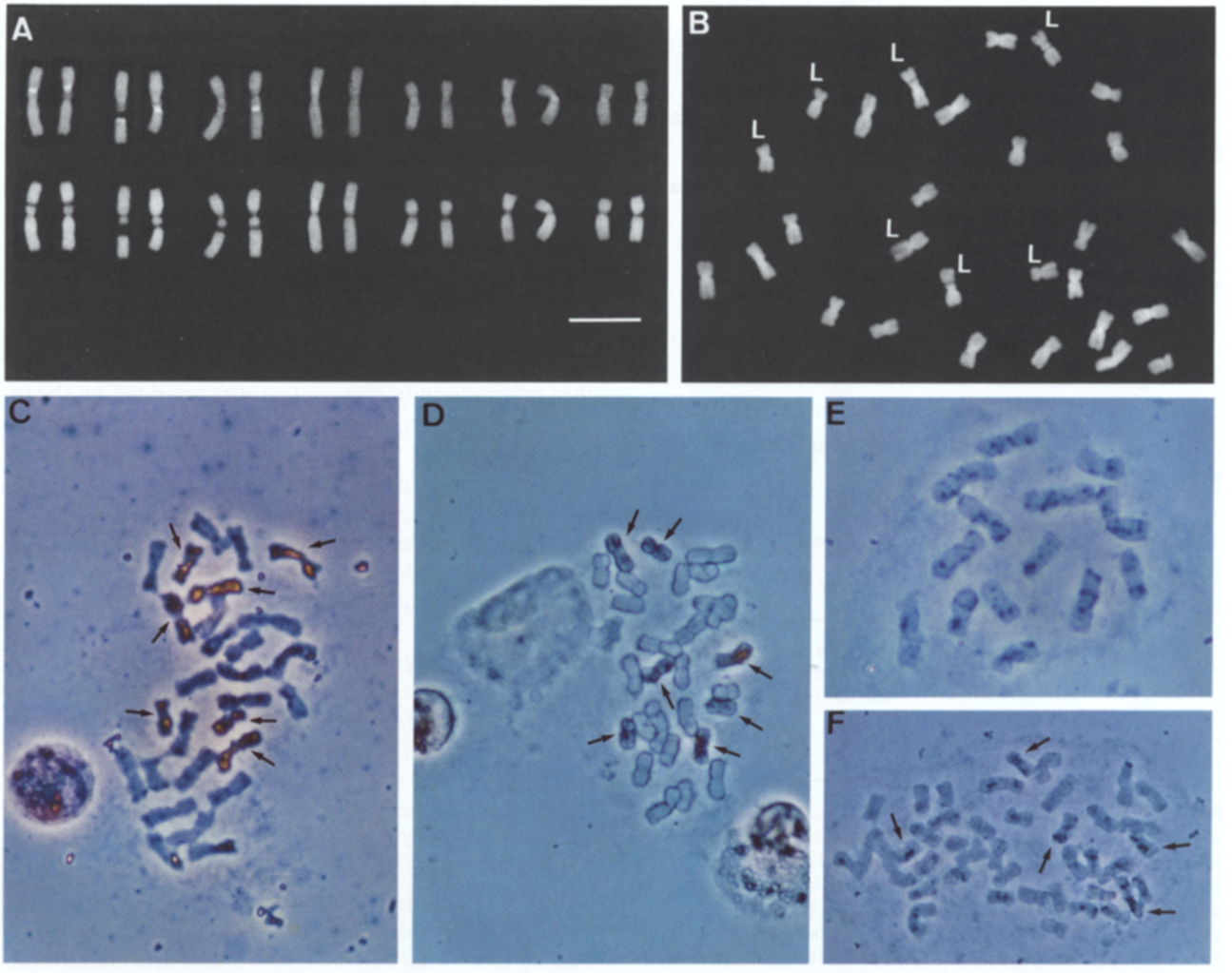

Fig. 5 A–F. Cytogenetic analysis of *Lolium*- and *Festuca*-specific DNA sequences. **A** *L. multiflorum* karyotype: homologous chromosomes were paired according to their banding pattern after CMA (*upper row*) or DAPI (*lower row*) staining. *Bar*: 10 μm. **B** Metaphase chromosomes from *Festulolium* sexual F_1 hybrid stained with DAPI. Preliminarily identified *L. multiflorum* chromosomes are marked (*L*). **C** In situ hybridization of total *L. multiflorum* DNA to metaphase chromosomes from *Festulolium* F_1 hybrid. Digoxigenin-labeled total DNA from *L. multiflorum* and non-labeled total DNA from *F. arundinacea* in a 1:100 ratio was used as probe. Strongly stained *L. multiflorum* chromosomes are indicated with *arrows*. **D** In situ hybridization on metaphase chromosomes from symmetric *Festulolium* F_1 hybrid using digoxigenin-labeled sequence LMH2 as probe. *L. multiflorum* chromosomes are marked with *arrows*. **E** In situ hybridization on metaphase chromosomes from *L. multiflorum* using digoxigenin-labeled sequence LMB4 as probe. **F** In situ hybridization on metaphase chromosomes from *F. arundinacea* using digoxigenin-labeled sequence FAH1 as probe. Stained chromosomes are marked with *arrows*. The extent of hybridization of the digoxigenin-labeled probes was revealed by NBT reduction catalyzed by alkaline phosphatase coupled to anti-digoxigenin antibodies. Pictures were taken using phase-contrast optics

toplast fusion combinations with other *Festuca* and *Lolium* species as well as with other forage grasses remains to be tested. A preliminary cross-hybridization analysis showed the absence of these or homologous sequences (e.g. LMH2, LMB4 and FAH1) in *F. rubra, Agropyron repens, Bromus inermis* and *Poa annua* as well as the presence of related sequences (e.g. LMH2 and LMB4) in *L. perenne*, but in a copy number corresponding to less than 1% of the respective value observed for *L. multiflorum*.

Discussion

Different types of repetitive sequences from *L. multiflorum* and *F. arundinacea* were isolated and studied for their copy number, species specificity, sequence analysis and distribution in the corresponding genomes. These results are summarized in Table 1. In addition, their potential use as molecular tools for characterizing the nuclear composition of sexual hybrids from the closely re-

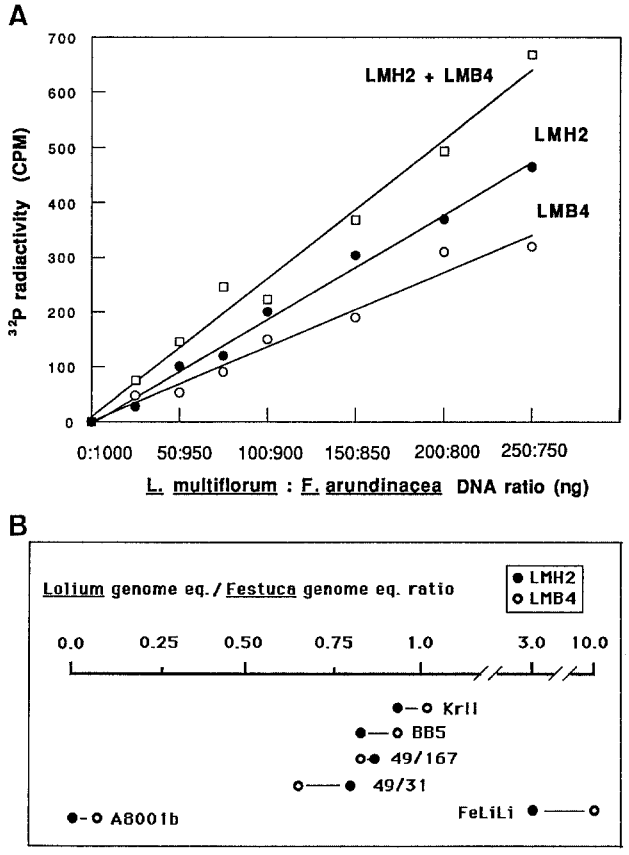

Fig. 6 A, B. Dot-blot analysis of *L. multiflorum* × *F. arundinacea* sexual hybrids. One µg DNA of each one of the following *Festulolium* genotypes was blotted in duplicate dots: KrII, BB5, 49/167, 49/31, FeLiLi and A8001b. Calibration mixes containing *L. multiflorum* and *F. arundinacea* DNA at the ratios indicated in the figure were blotted (1 µg DNA per dot) on the same filters. Filters were probed with labeled LMH2, LMB4 or a 1:1 mix of both probes. Dots were cut out, radioactivity per dot was counted by liquid scintillation, and the CPM values (Minus background) were plotted against the amount of *Lolium* DNA blotted to obtain the calibration lines showed in **A**. *Lolium* DNA contents of each of the above-mentioned genotypes were extrapolated from the LMB4 or LMH2 calibration lines and expressed as *Lolium* genome equivalents per *Festuca* genome equivalent in **B**. Presence of *Festuca* DNA (its amount was estimated by the difference to the corresponding *Lolium* values) was confirmed by previous hybridization of the same blots with FAH1

lated species *L. multiflorum* and *F. arundinacea* has been tested.

The use of species-specific dispersed repetitive DNA sequence for the identification of intergeneric fusion products (Saul and Potrykus 1984) and for a molecular characterization of the genomic make-up of asymmetric somatic hybrids in intra- and intergeneric combinations has previously been demonstrated for non-graminaceous plant species (Imamura et al. 1987; Piastuch and Bates 1990; Itoh et al. 1991). Similarly, species specific dispersed repetitive sequences can be used to follow wide crosses in conventional breeding programmes in order to assess donor genome elimination, chromosome translocations, etc.

The *Lolium* sequences of high species specificity, LMH1, LMH2 and LMB4, did not show homology to each other, while the sequences LMB1, LMB3, LMB8 and LMB11 of intermediate species specificity seem to belong to related families, as indicated by the dot-blot and Southern hybridization analyses.

The relatively simple banding patterns observed in Southern blots for *L. multiflorum* sequences LMH2 and LMB4 did not reflect their truly dispersed nature. Further information in this context was provided by the in situ hybridization studies. High stringency conditions were used in the Southern hybridizations, which may explain, at least in part, the lack of smear-type signals expected for a hybridization to dispersed sequences. However, dispersed repetitive sequences from *Lupinus luteus* have been shown to hybridize almost exclusively to a single band corresponding to the cloned insert (Sakowicz et al. 1986).

It is interesting to note that the situation found for the *Lolium* sequences of intermediate species specificity, LMB1, LMB3, LMB7, LMB8 and LMB11, was different in the closely related genera *Festuca* and *Lolium*. Southern analysis data strongly suggested a tandem arrangement for all five sequences in both *F. arundinacea* and *F. pratensis*, while in *L. multiflorum* a complex multi-band pattern was obtained with no indication of a ladder of bands representing exact multiples. The present organization of these sequences in *Lolium* may be explained in part by their being derived from tandem arrays by loss of particular restriction sites, although larger genomic rearrangements and sequence relocations, as has been suggested for the generation and evolution of repetitive plant DNA sequences (Flavell 1980), might also have been involved. Since all three species are derived from a common ancestor (Bulinska-Radomska and Lester 1988), a more intense modification of this particular set of repetitive sequences might have occurred in *L. multiflorum* than in *F. arundinacea* or *F. pratensis*, although the genus *Festuca* is considered to be more ancient than *Lolium* (Malik and Thomas 1966).

The nucleotide sequences obtained for *L. multiflorum*-specific clones (LMH2 and LMB4) and the *F. arundinacea*-specific clone (FAH1) did not display any clear internal sequence periodicity; however, a number of short (5–10 bp) imperfect direct repeats appeared scattered along the sequence in all three cases. Short scattered repeats of 4–12 bp seem to be common features of repetitive DNA sequences and have been reported for dispersed repetitive elements in *Lupinus luteus* (Sakowicz et al. 1986) and for non-tandemly arranged repetitive sequences in *Hordeum vulgare* (Junghans and Metzlaff

1988). Although the length of the sequence being examined may play an important role in the search for subrepeats, repetitive DNA clones from *Vicia faba* as long as 1.7 kb have been shown to lack simple internal patterns and to contain many short (4–8 bp) repeats distributed throughout the entire sequence (Kato et al. 1985).

The repetitive sequences LMB4, LMH2 and FAH1, which were selected by their species specificity in the *L. multiflorum* versus *F. arundinacea* comparison, are also highly species specific and absent if tested with other members of the subfamily Pooideae (Macfarlane 1986), with the exception of *F. pratensis* (for FAH1) and *L. perenne* (for LMB4 and LMH2). The copy number of sequences homologous to LMB4 and LMH2 in *L. perenne* was found to be low. In contrast, FAH1-homologous sequences seem to be present in higher copy number in *F. pratensis* than in *F. arundinacea*. This result is not unexpected since the hexaploid *F. arundinacea* line used is likely to contain a complete genome of *F. pratensis* (Borill 1976; Sleper and Nelson 1990). The conservation of repetitive DNA sequences among related species of genera might be disturbed by the high divergence observed for these elements (Zamir and Tanksley 1988). Homologous copies of a given element are frequently limited to plant species within the same genus or closely related genera (Ganal et al. 1988; Leclerc and Siegel 1987). In this context, the lack of any significant homology of LMH2, LMB4 and FAH1 to other plant sequences in the EMBL data bank is also not unexpected.

The in situ hybridization studies using the cloned repetitive DNA probes (LMH2, LMB4 and FAH1) showed the species specificity of the probes at the chromosome level, thus confirming the results obtained from the dot-blot and Southern hybridization analyses. In spite of slight variations from sample to sample, the evaluation of different metaphases in both parental forms and in a cytogenetically well-characterized symmetric *Festulolium* F_1 hybrid after in situ hybridization with either the *Lolium*-specific probes (LMH2 and LMB4) or the *Festuca*-specific probe (FAH1) supported the view that these sequences are represented in all or some chromosomes, respectively, in the corresponding parental genome. Experiments using the *Lolium*-specific repetitive probes resulted in a better resolution and no or significantly lower cross hybridization to *Festuca* chromosomes than did analogous experiments using labeled total genomic *Lolium* DNA with an excess of unlabeled *Festuca* DNA as hybridization probe. In addition, the in situ hybridization experiments performed allow the identification of candidates for both *Lolium*- and *Festuca*-specific repetitive sequences with an even distribution or a good representation in their corresponding parental genomes.

Similar species specific repetitive sequences have recently been used individually or in mixes as hybridization probes for the chromosomal analysis of asymmetric somatic hybrids in *Nicotiana* (Piastuch and Bates 1990) and *Brassica* (Itoh et al. 1991), respectively. Since some of the repetitive sequences fulfill the requirements of high species specificity and random localization in the corresponding genomes, their suitability for a fast measurement of the relative amounts of parental DNAs found in asymmetric somatic hybrids by quantitative dot blotting can be envisaged. Such cloned repetitive DNAs from dicots (Saul and Potrykus 1984) were first used for this purpose by Imamura et al. (1987). In this report we have demonstrated that repetitive *L. multiflorum*- and *F. arundinacea*-specific DNA sequences can be used in dot blots to measure the amount of *Lolium* DNA in a set of *Festulolium* sexual hybrids with a reasonable degree of accuracy. With the production of the first flowering intergeneric somatic hybrids in Gramineae, after protoplast fusion between *F. arundinacea* and *L. multiflorum* (Takamizo et al., 1991), similar studies on the quantification of donor genome elimination upon asymmetric somatic hybridization in these monocot species becomes feasible. Work on this line is in progress.

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