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Original article

Variability of ribosomal DNA sites in *Festuca pratensis, Lolium perenne*, and their intergeneric hybrids, revealed by FISH and GISH

T. Książczyk, M. Taciak, Z. Zwierzykowski

Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland

Abstract. This study focuses on the variability of chromosomal location and number of ribosomal DNA (rDNA) sites in some diploid and autotetraploid *Festuca pratensis* and *Lolium perenne* cultivars, as well as on identification of rDNA-bearing chromosomes in their triploid and tetraploid *F. pratensis* × *L. perenne* hybrids. The rDNA loci were mapped using fluorescence *in situ* hybridization (FISH) with 5S and 25S rDNA probes, and the origin of parental genomes was verified by genomic *in situ* hybridization (GISH) with *L. perenne* genomic DNA as a probe, and *F. pratensis* genomic DNA as a block. FISH detected variation in the number and chromosomal location of both 5S and 45S rDNA sites. In *F. pratensis* mostly additional signals of 5S rDNA loci occurred, as compared with standard *F. pratensis* karyotypes. Losses of 45S rDNA loci were more frequent in *L. perenne* cultivars and intergeneric hybrids. Comparison of the *F. pratensis* and *L. perenne* genomes approved a higher number of rDNA sites as well as variation in chromosomal rDNA location in *L. perenne*. A greater instability of *F. pratensis*-genome-like and *L. perenne*-genome-like chromosomes in tetraploid hybrids was revealed, indicating gains and losses of rDNA loci, respectively. Our data indicate that the rDNA loci physically mapped on chromosomes 2 and 3 in *F. pratensis* and on chromosome 3 in *L. perenne* are useful markers for these chromosomes in intergeneric *Festuca* × *Lolium* hybrids.

Keywords: *Festuca* × *Lolium* hybrids, 5S rDNA, 45S rDNA, GISH, rDNA-FISH.

Introduction

Species of the *Lolium-Festuca* complex, including 2 important forage grasses – meadow fescue (*F. pratensis* Huds.) and perennial ryegrass (*L. perenne* L.) – have agriculturally desirable and complementary traits. *Festuca* species express better persistency and tolerance to abiotic and biotic stresses, while *Lolium* species have high yields and excellent forage quality (Thomas and Humphreys 1991). *F. pratensis* and *L. perenne* are closely related and can hybridize at various ploidy levels, producing diploid, triploid and tetraploid intergeneric hybrids (Jauhar 1975; King et al. 1998; Zwierzykowski et al. 2006), in which homoeologous chromosomes pair (Jauhar 1975; King et al. 1999; Zwierzykowski et al. 2008) and recombine freely (King et al. 1998; Canter et al. 1999; Kopecký et al. 2006; Zwierzykowski et al. 2006, 2008).

Genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization with rDNA probes (rDNA-FISH) are very effective techniques to analyse genome homoeology and organization as well as chromosome karyotyping in a large number of plant species. FISH also provides a tool for constructing physical maps and analysing chromosome structure and aberrations. The most frequently mapped genes for these purposes are repetitive and tandemly organized ribosomal RNA (rRNA) genes, 5S rDNA and 45S rDNA, which are located at one or more sites per genome. Their

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Correspondence: T. Książczyk, Institute of Plant Genetics, Polish Academy of Sciences, Laboratory of Cytogenetics and Molecular Biology, Strzeszyńska 34, 60–479 Poznań, Poland; e-mail: tksi@igr.poznan.pl

map positions provide chromosome landmarks that can reveal similarities and differences between chromosomes of related species. rDNA-FISH has been used in several cereal and grass species, such as *Hordeum vulgare* (Leitch and Heslop-Harrison 1992), *Triticum* spp. (Mukai et al. 1993; Jiang and Gill 1994), *Aegilops* spp. (Badaeva et al. 1996), *Oryza* spp. (Chung et al. 2008), as well as in forage grasses, including *Festuca* spp. (Thomas et al. 1997; Harper et al. 2004), *Lolium* spp. (Thomas et al. 1996), and *Agrostis* spp. (He et al. 2009). Those studies have provided useful information about the physical location of rDNA sites and evolutionary and phylogenetic relationships between species.

The 5S and 45S rRNA gene loci have been mapped by FISH in *F. pratensis* (Thomas et al. 1997; Harper et al. 2004) and *L. perenne* (Thomas et al. 1996) as well as in various amphiploid and introgression forms of *Festulolium* (Kopecký et al. 2006; Kosmala et al. 2006). Such studies have revealed that the diploid *F. pratensis* genome ($2n = 2x = 14$; 1C = 2.23 pg according to http://www.kew.org/cvalues/homepage.html) has 7 chromosome pairs in the standard karyotype: one with a secondary constriction and a 45S rDNA locus (known as chromosome 2 according to Thomas 1981, or chromosome 3, consistent with its orthologous Triticeae chromosome counterpart, according to Jones et al. 2002) and 6 pairs numbered 1, 3, 4, 5, 6, and 7 (Thomas 1981). The *F. pratensis* chromosome 3 (or no. 2 in the Triticeae numbering system) carries a 5S rDNA locus on its short non-satellited arm (Thomas et al. 1997). Among *Festuca* chromosomes, nos. 1, 3, and 4 are metacentric, 2, 5, and 6 are submetacentric, and 7 is subtelocentric (Malik and Thomas 1966; Thomas et al. 1997).

The standard karyotype of the diploid *L. perenne* genome ($2n = 2x = 14$; 1C = 2.08 pg according to http://www.kew.org/cvalues/homepage.html) comprises 3 chromosomes (nos. 1, 2, and 3 according to Thomas 1981 or nos. 6/7, 3, and 2 in the Triticeae numbering system according to Jones et al. 2002) with secondary constrictions and 45S rDNA loci, and 4 other chromosomes, numbered as 4, 5, 6 and 7 (Thomas 1981). The *L. perenne* chromosome 3 (no. 2 in the Triticeae numbering system) has a 5S rDNA locus on its short non-satellited arm (Thomas et al. 1996), while chromosome 7 (no. 5/7 in the Triticeae numbering system) carries a 45S rDNA locus on its short arm (Thomas et al. 1996; Huang et al. 2008). Among *Lolium* chromosomes, nos. 1, 2, and 4 are metacentric, 3, 5, and 6 are submetacentric, and 7 is subtelocentric (Malik and Thomas 1966; Thomas et al. 1996). In the later parts of the article, the chromosome nomenclature proposed by Thomas (1981) is applied.

Variation in the number and location of sites of rDNA loci has been documented in *L. rigidum*, suggesting extensive chromosome changes resulting from paracentric and pericentric inversions and reciprocal translocations (Thomas et al. 2001). Recently, 5S and 45S rDNA sites have been counted in successive generations of *Festulolium* cultivars (Lideikytė et al. 2008), and some differences in the number of rDNA sites have been found. A loss of rDNA loci has apparently occurred in some amphiploid *Brassica* species, compared with their diploid progenitors (Hasterok et al. 2006). This suggests their genome imbalance following hybridization, but also different origin of the material studied and differences in approach sensitivity, which cannot be excluded when drawing conclusions.

In the present study, our main objectives were: (i) to verify the number and location of chromosomal sites of 5S and 45S rDNA in several diploid and autotetraploid cultivars of *F. pratensis* and *L. perenne*; (ii) to analyse rDNA-bearing chromosomes in 2 intergeneric hybrids: triploid *F. pratensis* ($2n = 2x = 14$) × *L. perenne* ($2n = 4x = 28$) and tetraploid *F. pratensis* ($2n = 4x = 28$) × *L. perenne* ($2n = 4x = 28$); and (iii) to use GISH for determination of the parental genomes and an assignment of chromosomal markers to corresponding genomes in these hybrids.

Materials and methods

Plant materials

Plant materials used for the study consist of: (i) *F. pratensis* (*Fp*) cultivars – diploid 'Skra' and 'Fure' ($2n = 2x = 14$), and autotetraploid 'Westa' and '3S' ($2n = 4x = 28$); (ii) *L. perenne* (*Lp*) cultivars – diploid 'Arka' ($2n = 2x = 14$), and autotetraploid 'Solen' ($2n = 4x = 28$); and (iii) intergeneric F_1 hybrids: triploid ($2n = 3x = 21$) *F. pratensis* ($2x$) × *L. perenne* ($4x$) (*FpLpLp*) and tetraploid ($2n = 4x = 28$) *F. pratensis* ($4x$) × *L. perenne* ($4x$) (*FpFpLpLp*) (Zwierzykowski et al. 2006). As the information about the parental plants used for the initial crossings to obtain triploid and tetraploid F_1 hybrids analysed herein was unavailable, the other plants derived from the available *Festuca* and *Lolium* cultivars as men-

tioned above were selected as control plants in our analyses.

Chromosome preparations

Root tips of cultivars and hybrids were collected in ice water, refrigerated for 24 h, fixed in ethanol with glacial acetic acid (3:1, v/v), and then stored at –20°C until use. Further treatment was carried out as described by Hasterok et al. (2001). Briefly, excised roots were washed in 0.01 M citric acid – sodium citrate buffer (pH 4.8) and digested in an enzyme mixture of 40% (v/v) pectinase (Sigma), 2% (w/v) cellulase (Calbiochem), and 2% (w/v) cellulase 'Onozuka R-10' (Serva) for 3 h at 37°C. Meristems were dissected out from root tips, squashed in drops of 45% acetic acid, and the preparation was frozen. Cover slips were removed, followed by air-drying. Chromosome preparations were made from 3 individual roots per plant of the cultivars and hybrids. Chromosome analysis was carried out on 3–5 well-spread metaphases. Each chromosomal preparation derived from a different single root tip.

DNA probes

Three kinds of probes were used: (i) total genomic DNA from *F. pratensis* and *L. perenne* was extracted from young leaves according to Lombard and Delourme (2001); genomic DNA from *L. perenne* was mechanically sheared to fragments of 5–10 kb by boiling for 30-45 min, and labelled by nick translation with digoxigenin-11-dUTP (Roche). Blocking DNA from *F. pratensis* was sheared by the same technique and used at a ratio of 1: 90 (probe:block); (ii) the 5S rDNA probe was generated from the wheat clone pTa794 (Gerlach and Dyer 1980) by PCR amplification and labelled also by PCR with tetramethyl-rhodamine-5-dUTP (Roche) by using 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. The thermal cycling program was as follows: 94°C for 1 min, 39 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 90 s, and finally 72°C for 5 min; (iii) the 25S rDNA probe was made by nick translation of a 2.3-kb *Cla*I sub-clone of the 25S rDNA coding region of *Arabidopsis thaliana* (Unfried and Gruendler 1990) with digoxigenin-11-dUTP (Roche). The latter probe was used for detection of 45S rDNA loci. The conditions for this reaction were as follows: 15°C for 95 min and 65°C for 10 min.

Fluorescence *in situ* hybridization (FISH)

The FISH procedure was adapted from Hasterok et al. (2001) with minor modifications. For the cloned probes, the hybridization mixture for FISH consisted of 50% formamide, 10% dextran sulphate, 2×SSC, 0.5% SDS, and 10 μg of salmon sperm DNA. The ribosomal DNA probes were mixed to a final concentration of 100-120 ng/slide, predenatured (75°C for 10 min) and applied to the chromosomal preparations. The slides were then denatured along with the hybridization mixture (70°C for 4 min; MJ Research). Stringent washes (an equivalent of 79% stringency) were followed by immunodetection of the digoxigenated probe by FITC-conjugated anti-digoxigenin antibodies (Roche). The preparations were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5 g mL^{-1} of DAPI (Sigma).

Genomic *in situ* hybridization (GISH)

The selected slides of F_1 hybrids were analysed by using the total genomic DNA of *L. perenne* and *F. pratensis* as a probe and/or block, respectively, to distinguish the 2 subgenomes of intergeneric hybrids. Before GISH, incubation of slides previously subjected to FISH experiments was carried out as follows: 37°C for 30 min, 4×SSC with Tween 20 (Sigma) for 5 min, twice in 2×SSC for 5 min, and then the slides were dehydrated in an ethanol series at room temperature. The GISH procedure was adapted from Kosmala et al. (2006), with minor modifications. In short, post-hybridization washes (an equivalent of 73% stringency) were followed by immunodetection of the digoxigenated probe by FITC-conjugated anti-digoxigenin antibodies (Roche). The preparations were mounted and counterstained in Vectashield (Vector Laboratories) containing 1.5 g mL^{-1} of propidium iodide (Sigma).

Image capturing and processing

All images were acquired using either an Olympus XM10 CCD camera attached to an Olympus BX 61 automatic epifluorescence microscope or an F-View II CCD camera attached to an Olympus BX 60 epifluorescence microscope. Image processing and superimpositions were done using Olympus Cell-F imaging software and Micrographx Picture Publisher software.

Results

Number and position of rDNA sites in *F. pratensis* and *L. perenne* cultivars

Diploid *Fp*. Two large interstitial 5S rDNA sites were found, on the pair of chromosomes 3 in all plants studied. In 5 plants additional distal 5S rDNA sites were observed on one (Table 1, Figure 1a) or 2 chromosomes (Table 1, Figure 1b). In all diploid *Fp* plants, 2 large 45S rDNA sites were observed on the pair of chromosomes 2 at the secondary constrictions (Table 1).

Tetraploid *Fp*. Four large 5S rDNA sites were found on 4 homologues of chromosome 3 in all plants studied, and were located interstitially. Eleven plants had an additional distal 5S rDNA site on 1-2 (Table 1, Figure 1c) or 3 (Table 1, Figure 1d) or 4 metacentric/submetacentric chromosomes (Table 1). In all tetraploid *Fp* plants, 4 large 45S rDNA sites were observed and located on 4 homologues of chromosome 2 at the secondary constrictions.

Diploid *Lp*. In all plants studied, 2 large interstitial 5S rDNA sites were found (Table 1) in the short arms of a pair of chromosomes 3 (chromosomes with both 5S and 45S rDNA loci). Different numbers of 45S rDNA sites were observed in most diploid *Lp* plants studied (Table 1). In one plant, large 45S rDNA sites were found on 4 long chromosomes at secondary constrictions and on one short chromosome with a similar size of a signal at a proximal position, close to the centromere (Table 1, Figure 1e). In this plant, the lack of 45S

Table 1. Number and chromosomal position of rDNA sites in diploid and tetraploid cultivars of *Festuca pratensis* and *Lolium perenne*

Plant no.	2*n*	No. of rDNA sites (position*)		No. of chromosomes with both rDNA sites
		5S	45S	
1	2	3	4	5
F. pratensis ‘Skra’				
06/23	14	2 (is)	2 (sc)	0
06/33	14	2 (is)	2 (sc)	0
06/152	14	2 (is)	2 (sc)	0
07/2	14	2 (is)	2 (sc)	0
08/3	14	2 (is)	2 (sc)	0
08/17	14	2 (is)	2 (sc)	0
08/43	14	2 (is)	2 (sc)	0
8/48	14	2 (is)	2 (sc)	0
08/51	14	2 (is) + 1 (d)	2 (sc)	0
08/54	14	2 (is)	2 (sc)	0
F. pratensis ‘Fure’				
05/11	14	2 (is) + 1 (d)	2 (sc)	0
05/32	14	2 (is) + 1 (d)	2 (sc)	0
07/6	14	2 (is)	2 (sc)	0
07/8	14	2 (is)	2 (sc)	0
7/10	14	2 (is) + 1 (d)	2 (sc)	0
07/11	14	2 (is)	2 (sc)	0
07/14	14	2 (is)	2 (sc)	0
07/15	14	2 (is)	2 (sc)	0
07/23	14	2 (is)	2 (sc)	0
07/25	14	2 (is) + 2 (d)	2 (sc)	0
F. pratensis ‘3S’				
05/7	28	4 (is)	4 (sc)	0
05/11	28	4 (is) + 4 (d)	4 (sc)	0
05/15	28	4 (is) + 3 (d)	4 (sc)	0
05/21	28	4 (is) + 3 (d)	4 (sc)	0
05/22	27	4 (is)	4 (sc)	0
05/25	28	4 (is) + 2 (d)	4 (sc)	0
05/35	28	4 (is) + 2 (d)	4 (sc)	0
06/3	28	4 (is)	4 (sc)	0
06/10	28	4 (is) + 3 (d)	4 (sc)	0
09/41	28	4 (is) + 2 (d)	4 (sc)	0
F. pratensis ‘Westa’				
06/44	28	4 (is)	4 (sc)	0
06/48	28	4 (is) + 3 (d)	4 (sc)	0
07/19	28	4 (is)	4 (sc)	0
07/32	27	4 (is) + 1(d)	4 (sc)	0
08/38	28	4 (is)	4 (sc)	0
08/40	28	4 (is)	4 (sc)	0
08/41	28	4 (is)	4 (sc)	0
08/43	27	4 (is) + 1 (d)	4 (sc)	0
08/45	28	4 (is) + 2 (d)	4 (sc)	0
09/44	28	4 (is)	4 (sc)	0
L. perenne ‘Arka’				
06/5	14	2 (is)	6 (sc/p)	1
06/24	14	2 (is)	7 (sc/p)	1
06/25	14	2 (is)	7 (sc/p)	1
07/106	14	2 (is)	5 (sc/p)	0
07/115	14	2 (is)	7 (sc/p)	2
07/117	14	2 (is)	6 (sc/p)	2
07/119	14	2 (is)	6 (sc/p)	2
08/8	14	2 (is)	6 (sc/p)	1
08/31	14	2 (is)	6 (sc/p)	2
08/48	14	2 (is)	6 (sc/p)	2
L. perenne ‘Solen’				
04/60	28	4 (is)	12 (sc/p)	3
07/7	28	4 (is)	12 (sc/p)	4
07/16	29	4 (is)	14 (sc/p)	4
07/17	29	4 (is)	14 (sc/p)	4
07/18	28	4 (is)	15 (sc/p)	4
07/20	28	4 (is)	13 (sc/p)	3
07/29	27	4 (is)	14 (sc/p)	4
07/30	28	4 (is)	15 (sc/p)	4
08/38	29	5 (is)	14 (sc/p)	4
08/40	29	4 (is)	14 (sc/p)	4

*Position of rDNA sequences is shown in brackets: is = interstitial; d = distal; sc = secondary constriction; p = proximal

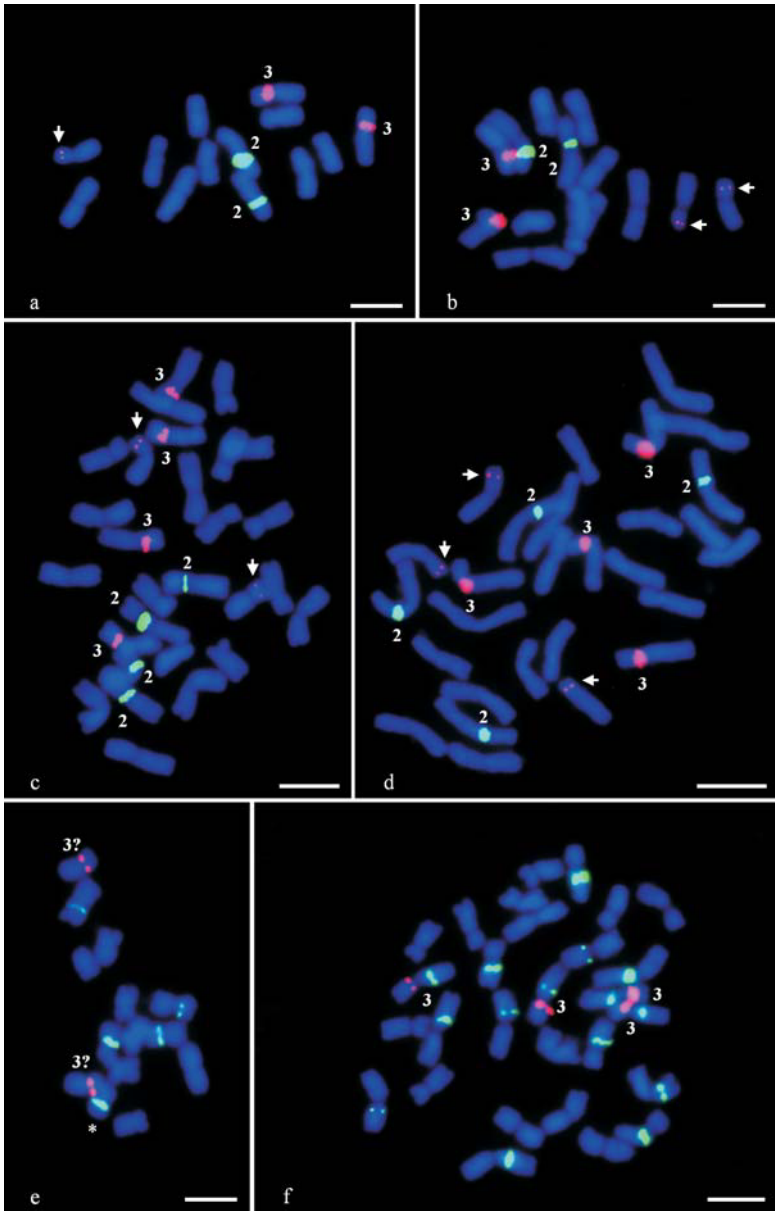

Figure 1. FISH analyses of somatic metaphase chromosomes of *F. pratensis* 'Skra' plant 08/51 (**a**); 'Fure' plant 07/25 (**b**); '3S' plant 05/35 (**c**); 'Westa' plant 06/48 (**d**); *L. perenne* 'Arka' plant 07/106, with possible chromosome 7 marked by an asterisk (**e**); and 'Solen' plant 07/30 (**f**). FISH images (**a**-**f**) were created using as a probe (i) 5S rDNA labelled with rhodamine (red) and (ii) 25S rDNA labelled with digoxigenin and detected by anti-digoxigenin conjugated with FITC (green). The chromosomes were counterstained with DAPI (blue). The chromosome nomenclature of rDNA-bearing chromosomes (Arabic numerals) follows Thomas (1981). White arrows = unknown variant chromosomes; ? = known variant chromosomes. Scale bar = 5 μm.

rDNA sites on both chromosomes 3 is worth mentioning. Nine remaining plants had six and seven 45S rDNA sites. Half of the diploid *Lp* plants studied had various numbers of chromosomes 3 carrying both kinds of rDNA or lacked rDNAs in these chromosomes (Table 1).

Tetraploid *Lp*. In all plants studied, 4 large interstitial 5S rDNA sites were found in the short arms of 4 homologues of chromosome 3 (chromosomes with both 5S and 45S rDNA loci). One exception was found in a plant that had 5 homologues of chromosome 3 with interstitial 5S rDNA sites. Half of the tetraploid *Lp* plants studied had fourteen 45S rDNA sites. The remaining plants had various numbers of chromosomes carrying 45S rRNA gene loci (Table 1) – 12, 13 and 15 (Table 1, Figure 1f) 45S rDNA sites differing in size. The presence or absence of 45S rDNA sites involves long chromosomes 1 and/or 2 and/or 3, in which the 45S rRNA gene loci were located at the secondary constrictions, and short chromosome 7 with 45S rDNA site located at proximal position, close to the centromere. In most plants studied, both rDNA loci were observed in 4 homologues of

Table 2. Number and chromosomal position of rDNA sites in triploid and tetraploid hybrids of *Festuca pratensis* × *Lolium perenne.*

Plant no.	2*n*	No. of rDNA sites (position*)		No. of chromosomes with both rDNA sites
		5S	45S	
F. pratensis (2*x*) × *L. perenne* (4*x*)				
105/1/3	21	3 (is)	9 (sc/p)	2
105/1/4	21	3 (is)	8 (sc/p)	2
105/1/11	21	3 (is)	7 (sc/p)	2
105/1/13	21	3 (is)	7 (sc/p)	2
105/1/16	21	3 (is)	8 (sc/p)	2
105/1/18	21	3 (is)	9 (sc/p)	2
105/1/20	21	3 (is)	8 (sc/p)	2
105/1/23	21	3 (is)	7 (sc/p)	2
105/1/24	21	3 (is)	9 (sc/p)	2
105/1/28	21	3 (is)	7 (sc/p)	2
F. pratensis (4*x*) × *L. perenne* (4*x*)				
59/1/1	28	4 (is)	9 (sc/p)	2
59/2/1	28	4 (is) + 1 (d)	8 (sc/p)	2
59/2/3	28	4 (is) + 1 (d)	8 (sc/p)	2
59/3/2	28	4 (is)	9 (sc/p)	1
59/3/3	28	4 (is) + 1 (d)	8 (sc/p)	2
83/1/3	28	4 (is)	7 (sc/p)	1
83/1/4	27	4 (is)	9 (sc/p)	2
83/1/5	28	4 (is) + 1 (d)	9 (sc/p)	2

*Position of rDNA sequences is shown in brackets: is = interstitial; d = distal; sc = secondary constriction; p = proximal

chromosome 3, although in the 2 plants studied only 3 homologues of chromosome 3 and a chromosome with 5S rDNA at the interstitial position were identified.

Number and position of rDNA sites in *F. pratensis* × *L. perenne* hybrids

The origin of chromosomes in triploid and tetraploid hybrids was verified by GISH. In triploid hybrids, the Fp genome contributes 7 complete chromosomes, and the Lp genome contributes 14 (Table 2, Figure 2a and c). In tetraploid hybrids, both genomes contribute 14 complete chromosomes (Table 2, Figure 2e and g).

Triploid *FpLpLp* hybrids. In all plants studied, 3 large interstitial 5S rDNA sites were found (Table 2) in one *Fp* chromosome 3, and in the short arms of both *Lp* homologues of chromosome 3 (Fig. 2b and d). In contrast, different numbers of 45S rDNA sites (7–9) were observed in 10 triploid plants (Table 2). The variability of 45S rDNA sites involves *Lp* chromosomes 1 or 2, in which large 45S rRNA gene loci were located at the secondary constrictions, and also the *Lp* chromosome 7 with a similar size of 45S rDNA site, which was located proximally, close to the centromere. Three plants had the expected, eight 45S rDNA sites, consisting of one *Fp* chromosome 2 with a large 45S rDNA locus, and 7 undifferentiated *Lp* chromosomes (besides both homologues of chromosome 3) with 45S rDNA (Figure 2b). Four plants had one *Fp* chromosome 2 as well as 6 undifferentiated Lp chromosomes (besides both homologues of chromosome 3) with large 45S rDNA sites. In the 3 remaining plants, one *Fp* chromosome 2 as well as 8 undifferentiated *Lp* chromosomes (besides both homologues of chromosome 3) with large 45S rDNA sites were observed (Figure 2d). All *FpLpLp* plants had the 2 expected *Lp* homologues of chromosome 3 bearing both 5S and 45S rRNA gene loci (Table 2).

Tetraploid *FpFpLpLp* hybrids. Four large interstitial 5S rDNA were observed in both *Fp* homologues of chromosome 3 and in the short arms of both *Lp* homologues of chromosome 3. In some plants small additional 5S rDNA sites were found in a distal region of one *Fp* metacentric/submetacentric chromosome (Table 2, Figure 2h). Like in the triploids, various numbers of 45S rDNA sites (7–9) were found in tetraploid plants studied (Table 2). The presence or absence of ribosomal 45S DNA sites involves Lp chromosomes 1 or 2, as well as chromosome 3, in which large 45S rDNA sites were located at the secondary constrictions, and also *Lp* chromosome 7 with 45S rDNA site, located proximally close to the centromere. Some plants showed, as would be expected, nine 45S rDNA sites, consisting of both *Fp* homologues of chromosome 2, with large 45S rDNA sites and 7 undifferentiated *Lp* chromosomes (besides both homologues of chromosome 3) with 45S rDNA sites. In one plant of this group, one out of the pair of homologues of chromosome 3 was observed (Figure 2f). It is worth mentioning that one plant had both *Fp* homologues of chromosome 2 with 45S rDNA loci and 5 undifferentiated *Lp* chromosomes (besides one chromosome 3) with 45S rDNA sites. Another plant had both *Fp* homologues of chromosome 2 with 45S rDNA, one *Fp* metacentric/submetacentric chromosome with an additional, distal 5S rDNA site, and 7 undifferentiated *Lp* chromosomes (besides both homologues of chromosome 3) with 45S rDNA sites (Table 2, Figure 2h). In 6 plants, the expected number of 2 homologues of chromosome 3 bearing both 5S and 45S rRNA gene loci was observed.

Discussion

FISH mapping using the 2 ribosomal DNA probes showed considerable interspecific and intraspe-

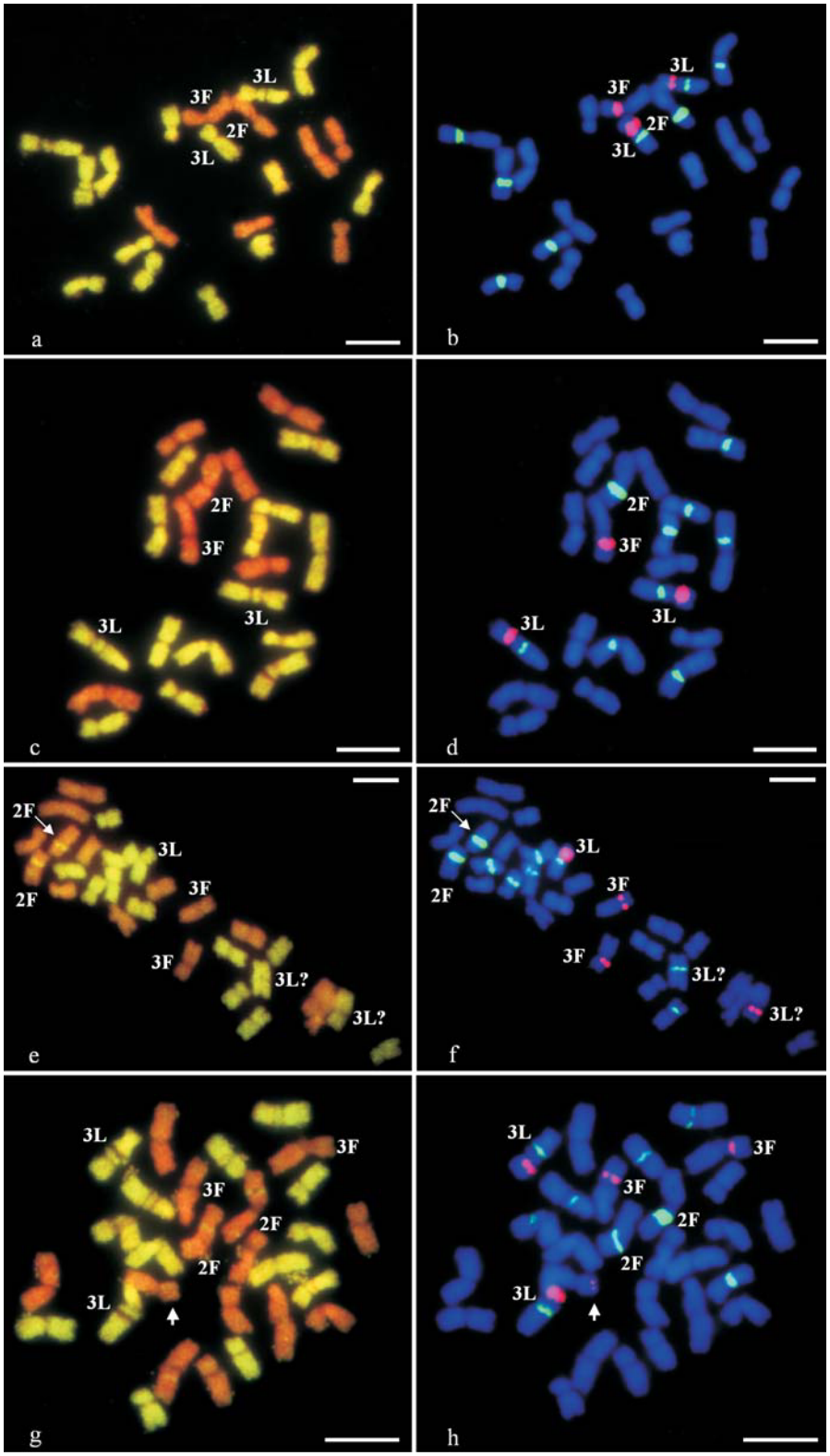

Figure 2. GISH (**a**, **c**, **e**, **g**) and FISH (**b**, **d**, **f**, **h**) analyses of the same somatic metaphase chromosome spreads of F_1 hybrids: triploid *FpLpLp* plant 105/1/20 (**a**-**b**) and plant 105/1/18 (**c**-**d**); as well as tetraploid *FpFpLpLp* plant 59/3/2 (**e**-**f**) and plant 83/1/5 (**g**-**h**). GISH images (**a**, **c**, **e**, **g**) were created after FISH hybridization using total genomic DNA from *Lp* as a probe labelled with digoxigenin and detected by anti-digoxigenin conjugated with fluorescein (green/yellow), with blocking genomic DNA of *Fp* (orange/red); chromosomes were counterstained with propidium iodide. FISH images (**b**, **d**, **f**, **h**) were created using as a probe (i) 5S rDNA labelled with rhodamine (red) and (ii) 25S rDNA labelled with digoxigenin and detected by anti-digoxigenin conjugated with FITC (green). Chromosomes for FISH experiments were counterstained with DAPI (blue). The nomenclature of rDNA-bearing chromosomes (Arabic numerals) follows Thomas (1981). White arrows = unknown variant chromosomes; ? = known variant chromosomes. Uppercase letters denote the genomic origin of tagged chromosomes. Scale bar = 5 μm.

cific variation in numbers and chromosomal location of ribosomal DNA sites in *Fp* and *Lp* cultivars. The polymorphism of rRNA gene loci may indicate that inter- and intrachromosomal variation could have occurred during speciation within *Festuca* and *Lolium* species. In general, 53 changes in 5S and 45S rDNA loci patterns (all changes to the standard number and/or location of rDNA loci) were found among the studied cultivars and hybrids.

Number and position of rDNA sites in *F. pratensis* and *L. perenne* cultivars

All the cultivars that contained only the *Fp* genome in their chromosome complement displayed variation in number and location of 5S rDNA sites. The chromosomes of diploid and tetraploid *Fp* genotypes carried 1–4 additional 5S rDNA sites, which were always located distally. In part, distally located 5S rDNA sites were also observed by Harper et al. (2004) in diploid *F. drymeja*. Those authors described the chromosome complement including a pair of chromosomes with an interstitial 45S rDNA site flanked by two 5S rDNA sites. The same authors observed a different number of rDNA sites in other diploid *Festuca* species, namely *F. scariosa*, *F. altissima*, *F. donax*, and *F. lasto*, in which rDNA sites in more than 2 pairs of chromosomes have been localized. Our subsequent research revealed the presence of 2 pairs of rDNA-bearing chromosomes in most *Fp* plants, which is concurrent with data obtained by Thomas et al. (1997). The numbers of 5S rDNA sites observed in the colchicine-induced autotetraploid *Fp* plants were in most cases not exactly twice the numbers found in the diploid plants. This observation could indicate that chromosome changes involving rDNA-containing regions and/or the creation of new rDNA sites had happened. In contrast, the number of 5S rDNA sites in the hexaploid *F. arundinacea* is equal to the sum of those found in its progenitors (Thomas et al. 1997). Numerical changes of rDNA sites were reported for some other species, e.g. in the colchicine-induced autotetraploid of *A. thaliana* (Weiss and Maluszynska 2000), indicating the 45S rDNA-bearing chromosome rearrangement, as an object of intragenomic translocation, and in tetraploid *Centaurea jacea* (Dydak et al. 2009), in which the deletion of one pair of 45S rDNA loci was detected. Variation in 5S rDNA locus number among individuals derived from the same cultivar was also observed in *Secale* species (Cuadrado and Jouve 1997, 2002) and in *Crocus vernus* (Frello and Heslop-Harrison 2000). Pontes et al. (2004) reported missing 5S rDNA loci in a study of di- and allopolyploid *Arabidopsis* species. Their cytogenetic studies revealed that the loss, gain, and/or rearrangements of 5S and 45S rDNA located at the secondary constriction site may have occurred in the early generations after formation of the allotetraploid *A. suecica* genome. However, in contrast to 45S rDNA sites, the variability of 5S rDNA sites is rather difficult to explain and its regulation seems to be dependent on another mechanism yet to be well uncovered. One of possible explanations is that 5S rDNA loci are often embedded in pericentromeric heterochromatin that is typically rich in transposable elements, so a transposon-mediated rearrangement might contribute to a loss or transposition of rDNA sequences. It is worth mentioning that among diploid *Fp* plants, cultivar 'Skra' seems to be more stable than cultivar 'Fure', and tetraploids seem to be less stable than diploids.

Polymorphism of rDNA sites was also observed in diploid and tetraploid *Lp* cultivars. Unlike in *Fp* plants, changes in the number of 45S rDNA sites seem to have occurred more frequently than for 5S rRNA in *Lp* cultivars. Since diploid *Lp* genotypes were shown to have seven 45S rRNA gene loci (Thomas et al. 1996; Huang et al. 2008), tetraploid genotypes were expected to have 14, but not all plants had exactly the same number, as observed in different *Lp* genotypes studied by Lideikytė et al. (2008). The intraspecific variation in the number of 45S rDNA does not seem to be rare in plants (Hayasaki et al. 2001). This observation has led to the hypothesis that rDNA tandem repeats are mobile, with different mechanisms believed to be involved in this mobility (Datson and Murray 2006; Pedrosa-Harand et al. 2006), suggesting the transposition of the rDNA loci (Frello and Heslop-Harrison 2000) activated by *En/Spm*-like transposons (Raskina et al. 2004a, b; Datson and Murray 2006).

Variation in chromosome patterns of rDNA loci is common and has also been observed in other plant genera, providing valuable information about the phylogenetic relationship between related taxa, e.g. in *Oryza* species (Chung et al. 2008). Those authors revealed a polymorphism in the number of rDNA loci and in chromosomal positions among the *Oryza* species, suggesting that chromosomal inversion, amplification, and retrotransposition might occur during *Oryza* species evolution. These data also showed that some rDNA loci are species-specific and "represent" a certain subgenome in *Oryza* allotetraploids. It can be concluded that homologous pairing and rDNAs transposition as well as gene amplification could

have happened before genomic stability in polyploids was achieved. The rRNA gene copy number evolves rapidly and quickly, resulting in repositioning or loss of some sites as well as the reduction in copy number below the detection threshold or mapping resolution (Jiang and Gill 1994; Raskina et al. 2004a). This seems to be the case also for *F. pratensis* and *L. perenne*. To account for these differences some mechanisms have been already proposed, such as various chromosome rearrangements, unequal crossing-over, gene transposition (gene mobility), and conversion (Schubert and Wobus 1985; Dubcovsky and Dvorak 1995; Thomas et al. 2001). There is some evidence that rDNA sites may change chromosomal location without the involvement of translocations (Dubcovsky and Dvorak 1995) or with the activity of transposable elements (Schubert and Wobus 1985; White et al. 1994; Hayasaki et al. 2001; Raskina et al. 2004b). The latter process may indicate chromosomal repatterning of rDNA, which seems to be activated by *En/Spm*-like transposons being responsible for travelling of (r)DNA to a new site (Raskina et al. 2004a). Unfortunately, there is no clear evidence what mechanism can be involved in rDNA loci variation in the plants studied, so further efforts are required for critical FISH analyses on variation patterns of rDNA loci.

Number and position of rDNA sites in *F. pratensis* × *L. perenne* hybrids

The number of 45S rDNA loci in the genomes of the triploid and tetraploid hybrids was in some individuals lower than the sum of the rDNA sites observed in their putative diploids. Uniparental elimination of rDNA may be a part of a broader process of DNA deletion induced by allopolyploidy. A decrease in the expected number of 45S ribosomal DNA sites during polyploid formation was also observed in *Brassica napus* and *B. juncea* (Maluszynska and Heslop-Harrison 1993), *Nicotiana tabacum* (Volkov et al. 1999), *Aegilops sharonensis* × *Ae. umbellulata* (Shaked et al. 2001), and *Arabidopsis suecica* (Pontes et al. 2004). The variation of tandem repetitive sequences during the evolution of polyploid genomes is thought to be a part of diploidization process (Mishima et al. 2002), i.e. a result of genome imbalance following hybridization and the reduction of dispensable ancestral-genome-like sequences as well as duplicated rRNA gene loci. The polymorphism of rDNA loci can be connected with chromosome number reduction, as proposed by Lysak et al. (2006) in the genus *Arabidopsis*. It highlights the role of specific pericentric inversions and translocations as well as elimination of chromosomal fragments after chromosome fusions. Such a scenario could have accounted for deletion of some terminal NOR regions, but also broad rearrangements leading to varying location of rDNA loci in different chromosomes and their spreading within the genome.

The tendency to reduce the number of 45S rDNA loci in amphiploid species can be accompanied by the amplification of 5S rRNA gene loci in *Fp*-genome-like chromosomes in the F_1 plants studied. Additional 5S rDNA site were observed in *FpFpLpLp* plants. However, the origin and extent of 5S rDNA variation remained unclear. Detailed analysis of *Lp* 45S rDNA distribution in the F_1 hybrids revealed that the chromosomes in which 45S rDNA sites were located in the secondary constrictions of metacentric chromosomes 1, 2 and submetacentric chromosome 3, and at the proximal pericentromeric region of subtelocentric chromosome 7, were found to be polymorphic. In this group of chromosomes, the *Lp* chromosome 3 is worth mentioning, because it includes both 5S and 45S rDNA. In a few *FpFpLpLp* plants, the *Lp* chromosome 3 occurred as a single chromosome instead of the expected pair of homologues of this chromosome. These plants had different numbers of 45S rDNA sites, suggesting a loss of 45S rDNA in *Lp* chromosome 3 and in one of the chromosomes belonging to the *Lp* group of metacentric or subtelocentric chromosomes with 45S rDNA, or translocation of a particular *Lp* 45S rDNA locus from chromosome 3 to another chromosome in this complement. It should be pointed out that this modification can lead to the new variant of chromosome 3 without either 45S rDNA or 5S rDNA loci or without both sites. Similar rearrangements are postulated for chromosomal type II (carrying 5S and 45S rDNA in the pericentromeric region) present in the A-genome of *Brassica rapa*, indicating an occasional loss of both 5S and 45S rDNA or only 5S rDNA (Hasterok et al. 2006).

The number and location of 45S rDNA sites was found to be stable in the chromosome complement of diploid and tetraploid *Fp* plants, while the number and position of 5S rDNA sites varied. The *Lp* plants were found to have a variable number of 45S rDNA sites both in diploids and tetraploids, but the number of 5S rDNA sites appears to be stable. The *Lp* chromosome 3 with both markers was much more stable in tetraploid *Lp* plants. The

triploid *FpLpLp* and tetraploid *FpFpLpLp* hybrids had various numbers of 45S rDNA sites, but the number of 5S rDNA sites and the number of *Lp* homologues of chromosome 3 was stable in triploid hybrids. The gaining of 5S rRNA genes appears to have occurred more frequently (*Fp* genome) than for 45S rRNA genes, but losses of 45S rDNA gene loci has occurred only in *Lp* chromosomes in cultivars and *Lp*-genome-like chromosomes in hybrids. It was estimated that among 53 changes to the standard number and/or location of rDNA sites, 21 involved signal amplification in the *Fp* and *Fp*-genome-like chromosomes, and 32 characterized the *Lp* and *Lp*-genome-like chromosomes (6 signal amplifications, 23 losses, and 3 others, including translocations/transpositions). Losses in the rDNA locus (mainly *Lp*-genome) were slightly more common than locus gains (mainly *Fp*-genome). The *Lp*-genome-like 45S rDNA loci could be sensitive to allopolyploidy-associated rearrangements (12 cases), compared with the *Fp*-genome loci (no case). In the tetraploid F_1 hybrids, the *Lp* 45S rDNA locus losses were often compensated by *Fp* 5S rDNA locus gains. This "reliance" is difficult to explain; rather different rDNA loci patterns can be due to intraspecific rDNA loci variation, which has been observed in the cultivars studied.

A uniform labelling of the *Lp* chromosomes in *FpLpLp* and *FpFpLpLp* hybrids indicated that their genomic integrity has been retained in the allotri- and allotetraploid background. Such genomic integrity, i.e. a lack of intergenomic chromosome translocations, was also observed in the naturally occurring grass species *Milium montianum* (Bennett et al. 1992), confirming the allopolyploid origin of this species.

The absence of intergenomic translocations in intergeneric *Festuca* × *Lolium* hybrids seems to contrast with the findings obtained by Zwierzykowski et al. (2006) and Kopecký et al. (2006), reporting on intergenomic chromosome translocations in the successive generations of *Festulolium* hybrids. One of possible explanations is that homoeologous chromosome pairing can lead to genetic instability in advanced generations (Thomas and Humphreys 1991), indicating extensive recombination between chromosomes of the parental genomes in various types of *Festulolium* hybrids (Canter et al. 1999; Zwierzykowski et al. 2006; Kopecký et al. 2006; Lideikytė and Pašakinskienė 2007). The results presented herein may also suggest that we are not able to discriminate parental dispersed repeats because of the low level of resolution of GISH in the F_1 generation of *Festuca* × *Lolium* hybrids. However, the presence of intergenomic chromosome translocations in the hybrid genome, indicating that the 2 parental genomes may have undergone some rearrangements following hybridization, was revealed in polyploid *Avena* spp. (Hayasaki et al. 2000), *Poa jemtlandica* (Brysting et al. 2000), or in the first generation of newly synthesized *Brassica* allopolyploids (T. Książczyk, unpubl. data).

Chromosome evolution is very often thought to occur by structural rearrangements, and there are many examples of species differentiated by deletion, duplication or translocations. It would seem also reasonable to assume that rDNA rearrangements mediated by gene travelling through DNA transposition differentiating homoeology of subgenomes in intergeneric *Festuca* × *Lolium* hybrids. In *F. pratensis* and *L. perenne,* we could demonstrate some variation of the rDNA-bearing chromosomes by rDNA-FISH, especially when combined with GISH in F_1 hybrids, but we were unable to characterize all rDNA-bearing chromosome pairs because of undifferentiated rDNA loci patterns in some *Lp* chromosomes. At the moment, chromosomes 2 and 3 in *F. pratensis*, and chromosome 3 in *L. perenne* can be easily identified by rDNA-FISH/GISH. Therefore, the use of a set of BAC clones and genome- or chromosome-specific DNA sequences might allow broader identification of more chromosomes of these genomes and more precise assignation of rDNA loci to particular chromosomes. BAC-FISH experiments are now being carried out and the combined use of several kinds of probes should further facilitate parental chromosome identification in intergeneric *Festuca* × *Lolium* hybrids as well as genome reconstruction in their successive generations.

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