Theor Appl Genet (2006) 113:731–742
DOI 10.1007/s00122-006-0341-z

ORIGINAL PAPER

Genome constitution and evolution in *Lolium* × *Festuca* hybrid cultivars (Festulolium)

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Received: 21 February 2006 / Accepted: 3 June 2006 / Published online: 11 July 2006

Abstract Festulolium hybrids are being increasingly used worldwide as forage grasses. This is due to their superior agronomic characteristics, which combine yield performance of ryegrasses (*Lolium multiflorum* and *L. perenne*) and tolerance against abiotic stress of fescues (*Festuca pratensis*, *F. arundinacea* and *F. arundinacea* var. *glaucescens*). Despite the widespread use, only fragmentary information exists on their genomic constitution. We used genomic in situ hybridization (GISH) to analyze genomic constitution of over 600 plants from almost all commercially available cultivars of Festulolium. Our results revealed a surprisingly large range of variation in the proportions of parental genomes and in the extent of intergenomic recombination. Using fluorescence in situ hybridization (FISH) with probes for ribosomal DNA, we assessed the frequency of recombination and elimination of particular chromosomes and chromosome groups in three contrasting Festulolium cultivars. This study provides novel information that will aid in understanding the relationship between a genetic make-up and the phenotype of Festulolium hybrids. Our results indicate that GISH might be a useful tool to aid in Festulolium breeding and provide data for a more detailed description of registered cultivars.

Communicated by B. Friebe

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Introduction

Festulolium hybrids derived from crossings of grass species belonging to two genera, fescue (*Festuca* L.) and ryegrass (*Lolium* L.) are being increasingly grown worldwide. The uses range from forage to turf. Considering the area on which *Festuca* and *Lolium* are grown, there is a great potential for the expansion of Festulolium, which is in many aspects superior to the existing cultivars of fescue and ryegrass.

The superiority of Festulolium hybrids is due to successful combination of desirable traits of both parental species. Ryegrasses, namely Italian ryegrass (*Lolium multiflorum* Lam., $2n = 2x = 14$) and perennial ryegrass (*L. perenne* L., $2n = 2x = 14$) are two of the most dominant species used for forage production and turf development. They are characterized by high yield, good palatability and digestibility, rapid establishment from seed, deep green color, relatively fine texture, good density and uniformity. However, they are sensitive to abiotic and biotic stress. On the other hand, *Festuca arundinacea* Schreb. ($2n = 6x = 42$), *F. pratensis* Huds. ($2n = 2x = 14$) and *F. arundinacea* var. *glaucescens* Boiss. ($2n = 4x = 28$; referred here to as *F. glaucescens*)

are known for good persistence and tolerance to stress conditions. *F. arundinacea* is known for its deep root system and excellent drought tolerance while *F. pratensis* carries genes for freezing tolerance and winter hardiness (Jauhar 1993).

Lolium and *Festuca* hybridize in nature, but their hybrids are sterile (Lewis 1975). A possibility to obtain fertile hybrids under controlled conditions of crossing opened a way to breed improved grass cultivars, and more than 30 years ago the first Festulolium cultivars 'Prior' and 'Elmet' were released (Lewis et al. 1973) in Europe, followed by cv. 'Kenhy' in the USA (Buckner et al. 1977). 'Kenhy' became very popular for high yield and has been used for a long time with or instead of tall fescue. Since then, breeding programs throughout the world produced a number of commercially successful Festulolium hybrids.

Commercial cultivars of Festulolium are being produced from diverse intergeneric hybrids. Most originate from two types of tetraploid hybrids: *L. multiflorum* (4*x*) × *F. pratensis* (4*x*) and *F. pratensis* (4*x*) × *L. multiflorum* (4*x*). All cultivars developed from these hybrids are perennial and combine good yield quality of Italian ryegrass with adequate persistency (3–4 years) and winter hardiness of meadow fescue. They are used for conservation, temporary meadows and pastures. Their high productivity was verified in pure cultures as well as in mixtures with other grass species and clovers (Fojtík 1994; Zwierzykowski 2004).

Another group of Festulolium includes cultivars developed from hybrids of *L. multiflorum* (2*x*) × *F. arundinacea* (6*x*). Tetraploid F_1 hybrids thus obtained are backcrossed either to *F. arundinacea* (6*x*) or *L. multiflorum* (4*x*). Cultivars developed from backcrosses to *F. arundinacea* are hexaploid ($2n = 6x = 42$) and morphologically similar to *F. arundinacea*, however they tend to have superior seed yield. 'Korina' and 'Lesana' are turf cultivars, whereas other cultivars, such as 'Kenhy', 'Johnstone', 'Hykor' and 'Felina' are used for forage and have a high yield potential. They provide a higher feeding value relative to tall fescue (measured by the water-soluble carbohydrates and energy content). These cultivars are suitable for multi-cut usage and for waterlogged soils (Buckner et al. 1977, 1983; Fojtík 1994, 1998; V. Černoch, pers. comm.).

The second group consists of two cultivars, 'Bečva' and 'Lofa', developed from backcrosses of *L. multiflorum* (2*x*) × *F. arundinacea* (6*x*) hybrids to *L. multiflorum* (4*x*) (Fojtík 1994). These cultivars are mainly used for silage, but can also be used in temporary meadows and pastures because of persistence and re-growth capacity after cutting. High yields were observed in pure cultures as well as in mixtures with other grass species and/or grass-clover mixtures. They show high digestibility, palatability and seed yield, which are higher than in both parents. Persistence is prolonged to 2 years in case of 'Bečva' and 3–4 years in case of 'Lofa' (Fojtík 1994, 1998).

Other parental combination used in Festulolium breeding programs involves *L. perenne* (4*x*) × *F. pratensis* (4*x*). Cultivars produced from these hybrids, e.g. 'Prior', generally show agronomic characteristics similar to those originating from *L. multiflorum* × *F. pratensis* hybrids. They combine desirable traits of both parents, such as tolerance against drought and cold inherited from meadow fescue and overall forage quality, establishment rate, high tillering density and resistance to treading from *L. perenne* (Casler et al. 2001; Thomas and Humphreys 1991).

Currently, there are no commercially available cultivars derived from hybrids *L. multiflorum* × *F. glaucescens*. However, successful hybridization of the two species has been reported (Ghesquière et al. 1993, 1996; Morgan et al. 2001). Strain '99–01' was developed at INRA Lusignan from hybrids between tetraploid biannual varieties of *L. multiflorum* and various natural populations of *F. glaucescens* collected in the French Alps (Ghesquière et al. 1996). Strain '99–01' has been included in official tests in France and combines high disease tolerance to rust, helminthosporium, and bacterial wilt, high seed yield and high tolerance under severe water deficit.

Despite a widespread use and increasing economic importance of Festulolium cultivars, their genomic constitution remains largely unknown. This is surprising as it may be determined relatively easily by genomic in situ hybridization (GISH) (Thomas et al. 1994). The method is based on hybridization of DNA of chromosomes fixed on a microscopic slide and labeled genomic DNA of parental species (Schwarzacher et al. 1989). After Thomas et al. (1994), the method was applied to identify introgressed *Festuca* chromosome segments carrying genes for tolerance against abiotic stress (Canter 2000; Humphreys and Pašakinskienė 1996; Humphreys et al. 1997; Kosmala et al. 2006), to determine genomic constitution of androgenic progeny of hybrids (Humphreys et al. 1998; Kopecky et al. 2005a; Zwierzykowski et al. 1998a), and in phylogenetic analyses (Humphreys et al. 1995). Only scarce data on chromosome constitutions in some registered cultivars have already been published (Canter et al. 1999; Kopecký et al. 2005b; Zwierzykowski et al. 1998b).

Here we summarise results of a comprehensive study on the genomic constitution of almost all

commercially available cultivars of *Lolium* × *Festuca* hybrids. We used GISH to determine the numbers of parental chromosomes in individual plants and we quantified the number of intergeneric recombination events fixed by selection on per plant and per population basis. We further combined GISH and FISH (fluorescence in situ hybridization) with locus-specific probes to get the first insights on the frequency of recombination and elimination of individual chromosomes and chromosome groups.

Materials and methods

Plant materials

Seed samples of Festulolium cultivars were obtained from the Institute of Plant Genetics PAS, Poznań, Poland (cv. 'Agula', 'Sulino', 'Rakopan', 'Felopa', 'Elmet' and 'Prior'), INRA, Lusignan, France (*L. multiflorum* × *F. glaucescens* strain '99-01', '01-1' and '99-04'), and Plant Breeding Station Hladké Životice, Czech Republic (cv. 'Perseus', 'Achilles', 'Punia', 'Paulita', 'Kemal', 'Spring Green', 'Duo', 'Matrix', 'Bečva', and a strain 'HŽ14DK', which has been included in official tests for registration).

Chromosome preparations

Seeds were germinated in Petri dishes on wet filter paper and seedlings were planted in 30-mm pots in the greenhouse. After 3–4 weeks, plantlets were transferred to a hydroponic culture with aerated solution of Hydroponex at 0.9 g/l (Hu-Ben, Čerčany, Czech Republic). Mitotic metaphase spreads were prepared from root tips according to Masoudi-Nejad et al. (2002).

Genomic in situ hybridization

GISH was done according to Masoudi-Nejad et al. (2002). In most of the experiments, total genomic DNA of *L. multiflorum* was labelled with digoxigenin using DIG-Nick Translation Kit according to manufacturer's recommendation (Roche) and used as a probe. Genomic DNA of *F. arundinacea*, *F. pratensis* and *F. glaucescens*, which were used as blocking DNA, were sheared to 200–500-bp fragments by boiling for 45 min. In some experiments, DNA of one species was labeled by biotin (Biotin-Nick Translation Kit, Roche) and used as a second labelled probe. In other experiments, DNA of *Lolium* and *Festuca* were used reciprocally, where DNA of *Lolium* was used as a block and DNA of *Festuca* was used as a probe. The probe to block ratio was 1:150 with minor variation. Sites of probe hybridization were detected either by the Anti-DIG-FITC conjugate (Roche) or by streptavidin-Cy3 conjugate (Amersham). Chromosomes were counterstained either with 1.5 μg/ml propidium iodide (PI) or 1.5 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI) made in Vectashield antifade solution (Vector Laboratories). Slides were evaluated with Olympus AX70 microscope equipped with epi-fluorescence and a SensiCam B/W camera. ScionImage and Adobe Photoshop software were used for processing of color pictures.

Fluorescence in situ hybridization with probes for 5S and 45S rDNA

After the microscopic evaluation of GISH, coverslips were carefully removed from selected slides, the slides were soaked for 5 min in 4× SSC, twice in 4× SSC for 45 min and then for 5 min in 2× SSC and air dried. DNA clone pTa71 (Gerlach and Bedbrook 1979) containing a 9 kb *Eco*RI fragment of wheat ribosomal DNA, which carries the 18S-5.8S-26S cluster of ribosomal RNA genes (here referred to as 45S rDNA), was labelled by biotin. Digoxigenin-labelled probe for 5S rDNA was prepared using PCR with a pair of specific primers (RICRGAC1, RICRGAC2), which amplify 303 bp in rice (Fukui et al. 1994), using rice genomic DNA as a template. In situ hybridization and detection was done as described for GISH.

Results

L. multiflorum × *F. pratensis* and *F. pratensis* × *L. multiflorum* cultivars

Cultivars originating from both crosses are tetraploid (2n = 4x = 28) with a high frequency of aneuploids. Somatic chromosome numbers of individual plants ranged from 26 to 32 (Table 1). While the cultivars originating from *F. pratensis* (4x) × *L. multiflorum* (4x) hybrids appeared to have chromosome numbers close to the expected 28, the average chromosome number was lower among cultivars originating from reciprocal crosses *L. multiflorum* (4x) × *F. pratensis* (4x). The average frequency of aneuploidy was 38 and 33% in *L. multiflorum* × *F. pratensis* and *F. pratensis* × *L. multiflorum* cultivars, respectively.

The cultivars were not homogenous in terms of chromosome number and genomic structure. They could be classified into three groups based on the presence of parental chromosomes and chromosomes with

Table 1 GISH analysis of Festulolium cultivars

Cultivar	Hybrid	Country of origin	No. of analysed plants	No. of chromosomes		No. of Lolium chromosomes		No. of Festuca chromosomes		No. of translocated chromosomes		No. of breakpoints	
				Range	Mean	Range	Mean	Range	Mean	Range	Mean	Mean	Per TC
Achilles	LmFp	CZ	28	26–30	27.61	4–18	11.18	0–2	0.32	8–24	16.11	30.39	1.89
Barfest[b]	LmFp	USA											
Elmet	LmFp	GB	26	26–30	27.69	5–17	7.69	1–7	3.88	6–20	16.12	23.73	1.47
Emrys[b]	LmFp												
HŽ14DK	LmFp	CZ	10	26–29	27.60	9–15	12.00	0–4	0.40	11–19	15.20	27.50	1.81
Kemal	LmFp	USA	29	26–28	27.59	26–28	27.59	0	0.00	0	0.00	0.00	0.00
Lifema[b]	LmFp	GER											
Perseus	LmFp	CZ	28	26–29	27.64	8–26	16.39	0–1	0.07	2–20	11.21	18.61	1.66
Perun[a]	LmFp	CZ	23	26–28	27.04	8–19	11.70	0–2	0.65	7–21	14.70	21.30	1.45
Rakopan	LmFp	POL	28	27–28	27.71	4–15	7.50	0–4	2.14	12–22	18.11	31.64	1.75
Tandem[b]	LmFp	USA											
Spring Green	LmFp[c]	USA	25	25–28	27.24	16–26	22.44	0–2	0.24	0–10	4.56	7.56	1.67
Agula	FpLm	POL	27	26–29	27.64	2–8	4.93	0–4	1.96	17–25	20.74	36.96	1.73
Felopa	FpLm	POL	26	26–30	27.88	2–10	6.81	0–4	2.27	13–23	18.81	31.04	1.65
Paulita	FpLm	GER	26	27–29	27.88	0–16	7.19	0–2	0.50	12–29	20.19	37.62	1.86
Punia	FpLm	LIT	27	26–29	27.74	6–15	8.93	0–4	1.52	11–21	17.30	28.30	1.64
Sulino	FpLm	POL	23	26–32	28.00	2–11	7.09	0–7	2.70	14–25	18.22	31.87	1.75
Duo	LpFp	USA	25	26–30	27.96	26–30	27.96	0	0.00	0	0.00	0.00	0.00
Matrix	LpFp	NZ	22	14	14.00	14	14.00	0	0.00	0	0.00	0.00	0.00
Prior	LpFp	GB	29	27–30	28.10	8–19	12.00	1–6	2.90	7–18	13.28	19.97	1.50
Felina[a]	LmFaFa	CZ	29	38–42	41.24	3–10	6.69	18–32	25.79	5–15	8.76	17.17	1.96
Hykor[a]	LmFaFa	CZ	19	40–42	41.05	0–10	4.95	21–32	25.89	5–15	10.21	22.11	2.16
Johnstone[b]	LmFaFa	USA											
Kenhy[b]	LmFaFa	USA											
Korina[a]	LmFaFa	CZ	22	40–42	41.45	3–11	6.86	22–32	27.73	3–10	6.86	15.45	2.25
Lesana[a]	LmFaFa	CZ	24	39–42	41.25	4–12	7.50	24–32	27.42	4–9	6.33	13.13	2.07
Bečva[a]	LmLmFa	CZ	27	26–28	27.67	26–28	27.63	0	0.00	0–1	0.04	0.04	–
Bečva selected[c]	LmLmFa	CZ	17	26–30	27.65	24–29	26.94	0	0.00	0–2	0.71	0.88	1.25
Lofa[a]	LmLmFa	CZ	29	27–28	27.52	23–28	27.21	0	0.00	0–4	0.31	0.41	1.33
99–01	LmFg	FRA	28	26–28	27.46	7–16	12.68	5–12	9.93	2–9	4.86	6.46	1.33
99–04	LmLmFg	FRA	10	27–28	27.60	8–15	11.50	0	0.00	12–20	16.10	27.90	1.73
01–1	LpLmFg	FRA	10	26–29	27.40	10–19	15.30	0–1	0.50	8–15	11.60	16.80	1.45

Lm Lolium multiflorum, *Lp* L. Perenne, *Fp* Festuca pratensis, *Fg* F. glaucescens, *TC* translocated chromosome

[a] Data published in Kopecký et al. (2005b)

[b] No screened cultivars

[c] Origin described in results

detectable translocations. The first group involved three Czech cultivars 'Perun', 'Achilles', 'Perseus' (Fig. 1a) and the strain 'HŽ14DK', with a high proportion of intact *Lolium* chromosomes, ranging from 11.2 in 'Achilles' to 16.4 in 'Perseus'. Individual plants possessed less than one complete *Festuca* chromosome (ranging from 0.1 chromosome in cv. 'Perseus' to 0.7 chromosome in cv. 'Perun').

The second group of cultivars originated from *F. pratensis* (4x) × *L. multiflorum* (4x) hybrids ('Paulita', 'Punia', 'Felopa', 'Sulino' and 'Agula') and *L. multiflorum* (4x) × *F. pratensis* (4x) hybrids ('Elmet' and 'Rakopan'). Plants from these cultivars averaged approximately seven complete *Lolium* chromosomes and more than one complete *Festuca* chromosome. The only exception being 'Paulita', where on average only a 0.5 complete *Festuca* chromosome was detected. The remaining chromosomes were recombined (see Fig. 1b).

The third type of karyotype was represented by cv. 'Spring Green' and 'Kemal'. In 'Spring Green', which was developed from three *L. multiflorum* × *F. pratensis* cultivars ('Kemal', 'Tandem' and 'Elmet') and one *L. perenne* × *F. pratensis* cultivar ('Prior'), GISH revealed prevalence of *L. multiflorum* chromatin. On average, there were more than 22 complete chromosomes of *Lolium* and only 0.2 complete *Festuca* chromosomes. Also, the number of recombined chromosomes was low compared to other cultivars originating from similar crosses (on average, 4.6 recombined chromosomes per plant; see Fig. 1c). In cv. 'Kemal' we were unable to detect chromatin of *F. pratensis* in any of the analyzed

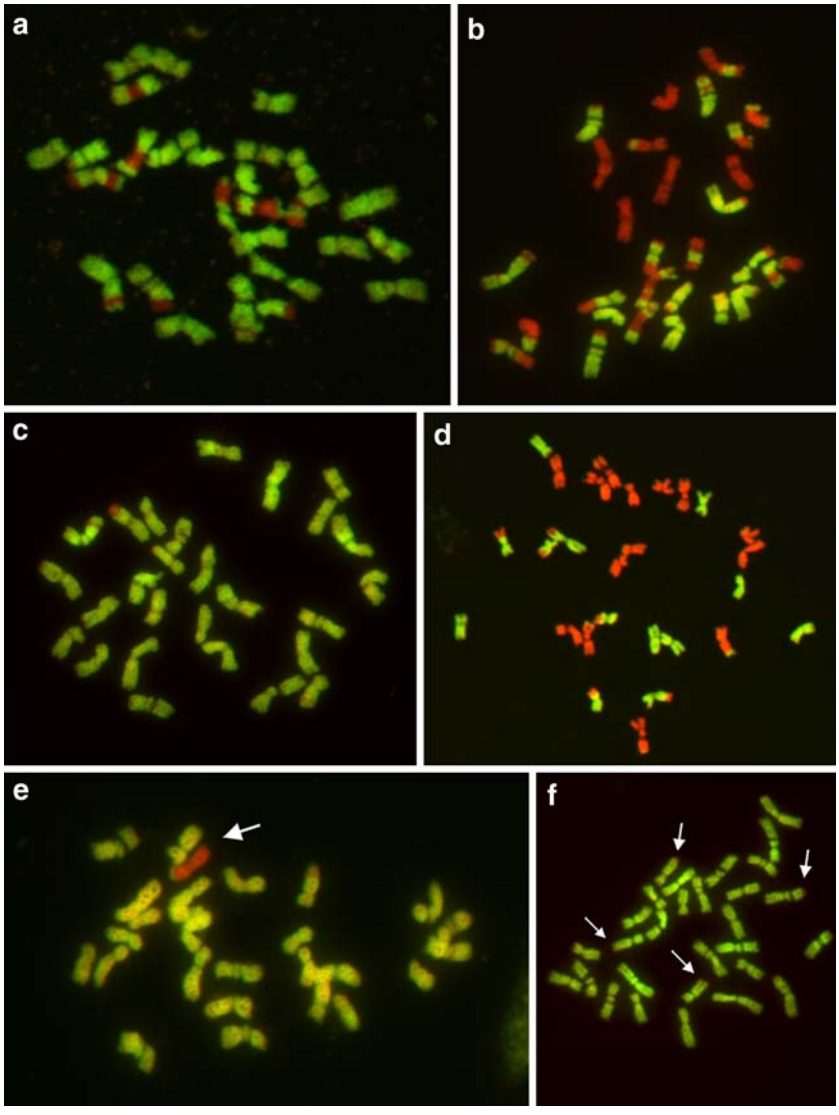


Fig. 1 Molecular cytogenetic analysis of Festulolium cultivars. GISH in *L. multiflorum* × *F. pratensis* cultivars 'Perseus' (**a**), 'Rakopan' (**b**), and 'Spring Green' (**c**). Total genomic DNA of *L. multiflorum* was labelled with FITC and used as a probe (*yellow-green color*); genomic DNA of *F. pratensis* was used to block hybridization of common sequences. **d** GISH in a hybrid strain '99–01' of *L. multiflorum* × *F. glaucescens*. Total genomic DNA of *F. glaucescens* was labeled with FITC and used as a probe *(yellow-green color*); genomic DNA of *L. multiflorum* was used to block hybridization of common sequences. **e** GISH on mitotic metaphase plate of *L. multiflorum* × *F. arundinacea* cv. 'Bečva'. DNA of *L. multiflorum* was used as probe (*green color*) and DNA of *F. pratensis* was used as block. One chromosome is of *Festuca* origin with terminal translocation of *L. multiflorum* (*arrow*). Chromosomes were counterstained using propidium iodide (*red color*). **f** GISH on metaphase spread of cv. 'Duo'. DNA of *L. multiflorum* was used as labeled probe (*green color*) and DNA of *F. pratensis* was used as blocking DNA. Chromosomes were counterstained by DAPI (shown in *red pseudocolor*). Four chromosomes show no hybridization signal of terminal regions (*arrows*)

29 plants. Each plant seemed to possess only the genome of *L. multiflorum*.

While the cultivars could be divided into three groups based on the proportions of parental chromatin, no distinct grouping was evident based on the number of breakpoints per recombined chromosome. The lowest number of breakpoints per recombined chromosome was observed in cultivars 'Perun' and 'Elmet' (1.45 and 1.47, respectively), whereas 'Achilles' averaged 1.89 breakpoints per recombined chromosome (Table 1).

L. multiflorum × *F. arundinacea* cultivars

There are two groups of cultivars that were developed from *L. multiflorum* (2x) × *F. arundinacea* (6x) hybrids. The first originated from a backcross of F_1 progeny to *F. arundinacea*. All cultivars were found hexaploid (2n = 6x = 42) with chromosome number of individual plants ranging from 38 to 42 and no presence of hyperploids. GISH indicated a prevalence of the *F. arundinacea* genome in all cultivars (Table 1) and a

relatively high number of breakpoints per recombined chromosome (ranging from 2.0 to 2.2).

The second group includes cultivars derived from backcrosses of *L. multiflorum* (2x) × *F. arundinacea* (6x) hybrids to *L. multiflorum* (4x). Cultivars 'Lofa' and 'Bečva' showed prevalence of *L. multiflorum* chromatin (Table 1). We selected 17 plants of 'Bečva' whose morphology differed from the rest of the population and analyzed them using GISH (see 'Bečva selected' in Table 1). In eight plants the *Festuca* genome was absent; one or more recombined chromosomes were detected in nine plants (Fig. 1e).

L. multiflorum × *F. glaucescens* cultivars

As there is no registered cultivar derived from this cross, we analyzed plants of the strain '99–01'. We also screened two additional strains originating from the same type of cross. The strains '99–04' and '01–1' were obtained from backcrosses between the same F_1 hybrids as those, which led to the strain '99–01', and tetraploid varieties of *L. multiflorum* and *L. perenne*, respectively.

Strain '99–01' (2n = 4x = 28) showed a prevalence of the *Lolium* genome, similar to *L. multiflorum* × *F. pratensis* cultivars, but in a significantly lower degree. The number of complete *Lolium* chromosomes ranged from 7 to 16, averaging 12.7. The number of complete *Festuca* chromosomes ranged from 5 to 12 and averaged 9.9 (Table 1). The number of translocated chromosomes was significantly lower than in the *L. multiflorum* × *F. pratensis* cultivars (4.86 recombined chromosomes in '99–01' compared with over 11 recombined chromosomes in *L. multiflorum* × *F. pratensis* cultivars) (Fig. 1d).

The two strains '99–04' and '01–1' (2n = 4x = 28) produced from '99–01' by backcrossing to *L. multiflorum* and *L. perenne*, respectively, showed a significant drift towards the *Lolium* genome (Table 1). Following a backcross to *L. multiflorum* ('99–04'), the number of translocated chromosomes and the number of breakpoints per translocated chromosome increased. The majority of chromosomes present were recombined and no complete *Festuca* chromosomes were present. Surprisingly, a similar drift towards the *Lolium* genome occurred after a backcross to *L. perenne* ('01–1'). Regrettably, it was not possible to discriminate between chromosomes *L. multiflorum* and *L. perenne* using GISH. Thus, we were not able to determine a genomic origin of the *Lolium* chromosomes.

L. perenne × *F. pratensis* cultivars

The genome constitution of tetraploid cultivar 'Prior' showed prevalence of the ryegrass genome over that of fescue but to a lesser extent when compared to *L. multiflorum* × *F. pratensis* cultivars. On average, 12.0 complete *Lolium* chromosomes and 2.9 complete *Festuca* chromosomes were present with the remaining 15.1 chromosomes being recombined (Table 1). The number of breakpoints per recombined chromosome was also lower than in *L. multiflorum* × *F. pratensis* cultivars (with the exception of 'Perun' and 'Elmet').

In a tetraploid cultivar 'Duo' and in a diploid cultivar 'Matrix', GISH did not reveal any *Festuca* chromosomes and/or chromosome segments (Table 1; Fig. 2c, d). Although aneuploidy occurs very frequently among tetraploid and hexaploid Festulolium cultivars, all 23 analyzed plants of diploid 'Matrix' were found euploid with 14 chromosomes (Table 1).

The application of GISH led to an interesting observation in cultivar 'Duo'. When DNA of *L. multiflorum* instead of *L. perenne* was used as a probe for GISH, the fluorescent signal was detected all over the chromosomes except in the distal regions of some chromosomes (see Fig. 1f). When we used *F. pratensis* DNA as a probe and *L. multiflorum* as blocking DNA, distal segments were not labeled, indicating that the distal parts were not introgressed from *F. pratensis*. One explanation could be a variability of species-specific repetitive DNA in subtelomeric regions (evolving more rapidly). Unfortunately, it is not possible to discriminate chromatin of *L. multiflorum* and *L. perenne* from each other by genomic in situ hybridization (Pašakinskienė et al. 2000).

Chromosome elimination in hybrid cultivars

Four *L. multiflorum* × *F. pratensis* cultivars ('Elmet', 'Sulino', 'Perun' and 'Spring Green'), which differ in the proportions of parental genomes, were used to analyze elimination of specific chromosomes and chromosome groups (see Fig. 2e–h). Based on the presence of rDNA loci, three groups of *L. multiflorum* chromosomes and three groups of *F. pratensis* chromosomes could be distinguished (Table 2). In *L. multiflorum*, the G1L group consisted of one pair of chromosomes carrying both 45S rDNA and 5S rDNA (chromosome 2 in the Triticeae numbering system), the G2L group comprised two chromosome pairs with a 45S rDNA locus (chromosomes 6 or 7, and 3 in the Triticeae numbering system), and the G3L group comprised four pairs of chromosomes with no rDNA. In *F. pratensis*, the G1F group contained one pair of chromosomes carrying 45S rDNA (chromosome 3 in the Triticeae numbering system); the G2F group comprised one chromosome pair with 5S rDNA (chromosome 2 in Triticeae numbering system), and the G3F group consisted of five pairs of chromosomes with no rDNA (Table 2).

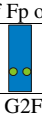
The analysis of a set of 47 plants from 4 cultivars revealed uneven elimination of specific groups of chromosomes. Thus, in cultivars with more balanced parental contribution, such as 'Perun', 'Sulino' and 'Elmet', the chromosomes belonging to group G3L were 1.1 to 1.4 times more frequent than other *Lolium* chromosomes. G1L chromosomes were present 1.1× more frequently than the G2L chromosomes. However, no statistically significant preference in elimination of *Festuca* chromosomes was observed (Table 3).

Chromosome recombination in hybrid cultivars

The same set of four cultivars that was used to evaluate chromosome elimination was analysed for chromosome recombinantion. The results showed that the recombination frequency of individual chromosomes did not appear to be random (Table 4, Fig. 3). Chromosomes with *Festuca* centromere recombined at a higher frequency than chromosomes with *Lolium* centromere: 74 (of chromosomes with a *Festuca* centromere were recombined and 51 (of chromosomes with a *Lolium* centromere were recombined ($\chi^2_{1\ df} = 67.26$; $P < 0.001$). *Festuca* chromosomes seemed to display more breakpoints per recombined chromosome. Among recombined chromosomes with *Festuca* centromere, the average number of breakpoints was 1.88 ± 0.32 as compared to 1.71 ± 0.23 breakpoints in recombined chromosomes with *Lolium* centromeres.

The presence of exchanges between parental genomes was confirmed in subsequent analysis of 28 euploid plants (2n = 28) that were selected from 47 plants of the four hybrid cultivars ('Elmet', 'Sulino', 'Perun' and 'Spring Green'). The ability to identify one pair of homoeologous chromosomes (G1L and G2F) allowed us to verify the presence and assess the extent of non-homoeologous exchanges. In 6 out of the 28 euploid hybrids, we found exchanges of segments between non-homoeologous parental chromosomes (Fig. 2g, h). This corresponds on average to 3.7 (of all recombination events between parental genomes.

Table 2 The presence of particular chromosomes and chromosome groups in four cultivars of *L. multiflorum* × *F. pratensis*

Cultivar	Chromosomes of Lm origin			Chromosomes of Fp origin		
						
	G1L	G2L	G3L	G1F	G2F	G3F
Expected	2	4	8	2	2	10
Spring Green	3.92	7.69	14.77	0.23	0.08	0.92
Perun	2.98	5.68	12.60	1.20	0.60	4.30
Sulino	2.29	4.08	10.83	1.67	2.08	6.46
Elmet	2.08	3.58	10.00	2.00	1.83	8.08

Lm – *Lolium multiflorum*

Fp – *Festuca pratensis*

- 45S rDNA

- 5S rDNA

Table 3 Summary of analysis of chromosome elimination in four hybrid cultivars ('Elmet', 'Sulino', 'Perun' and 'Spring Green'). The homogeneity test among the four cultivars was not significant ($\chi^2_{6\ df} = 5.15$; $P < 0.53$)

Chromosome group	Expected number of chromosomes[a]	Actual number of chromosomes[b]	Significance
G1L	2.88	2.79	$\chi^2_{2\ df} = 2.69$; $P < 0.26$
G2L	5.76	5.34	
G3L	11.53	12.04	
Sum	20.17		
G1F	1.05	1.26	$\chi^2_{2\ df} = 3.68$; $P < 0.16$
G2F	1.05	1.17	
G3F	5.22	4.89	
Sum	7.32		

[a] Average somatic chromosome number was established from the four hybrid cultivars, and the expected number of chromosomes in each chromosome group was determined based on average ratio of parental chromosomes in the hybrid cultivars and frequency of chromosomes of particular chromosome groups in parental species

[b] Actual number of chromosomes in each chromosome group was determined after GISH and FISH analysis of the same metaphase plates

Table 4 The frequency of recombination in particular chromosomes and chromosome groups in three cultivars of *L. multiflorum* × *F. pratensis*

Chromosome group	Frequency of recombined chromosomes			No. of breakpoints per recombined chromosome		
	Elmet	Sulino	Perun	Elmet	Sulino	Perun
G1L	0.32	0.29	0.74	1.38	1.33	1.38
G2L	0.33	0.51	0.55	1.47	1.96	1.90
G3L	0.49	0.57	0.48	1.47	1.82	1.86
G1F	0.83	0.95	0.92	1.65	2.40	2.18
G2F	0.82	0.72	1.00	1.72	1.45	1.40
G3F	0.68	0.75	0.81	1.59	2.20	2.03

Discussion

Currently there are about thirty Festulolium cultivars registered and distributed worldwide. Most of them originate from hybrids *L. multiflorum* × *F. pratensis* (× *Festulolium braunii* (K. Richter) A. Camus). There are only three cultivars originating from hybrids *L. perenne* × *F. pratensis* (× *Festulolium loliaceum* (Hudson) P.V. Fournier), whereas nine cultivars were derived from hybrids *L. multiflorum* × *F. arundinacea* (×*Festulolium holmbergii* (Dörfl.) P. Fournier).

GISH has been used for more than 10 years for identification of parental chromatin in *Lolium-Festuca* hybrids (Thomas et al. 1994). The results from different laboratories seem to be comparable, thus indicating a reliability of the method. For example, our results obtained in cv. 'Prior' are similar to those of Canter et al. (1999). The authors analyzed 12 plants by GISH and detected 9.3 complete *Lolium* chromosomes and 3.5 complete *Festuca* chromosomes. After analyzing 29 plants, we determined 12.0 complete *Lolium* chromosomes and 2.9 complete *Festuca* chromosomes, on average. The number of recombination events and number of breakpoints per recombined chromosome were also comparable in both studies. Higher numbers of complete *Lolium* chromosomes observed in our work could be due to a more advanced stage of seed multiplication (higher number of generations). A change in genome balance in successive generations of *Lolium* × *Festuca* hybrids, in favor of the dominant *Lolium* genome was also described by Zwierzykowski et al. (2003; in preparation).

Until recently, a relatively laborious, time-consuming and costly GISH protocol was used. Masoudi-Nejad et al. (2002) developed a simplified procedure that is especially suitable for large-scale screening. In our previous report (Kopecký et al. 2005b), we used the method to screen over 170 plants from 7 Czech Festulolium cultivars and in this work report the analysis of over 600 plants. In addition to describing genomic constitutions in almost all commercially available hybrid cultivars, our work indicates that it is realistic to incorporate GISH into particular phases of breeding programs. A combined use of GISH and FISH to identify particular parental chromosomes in hybrids as demonstrated in this work, could facilitate early selection of hybrids with chromosome carrying desired agronomic characters.

Our study revealed a striking intravarietal variation in the genomic constitution. Differences in the proportion of parental genomes between plants within cultivars were often larger than those between cultivars that originated from the same type of cross (e.g. *L. multiflorum* × *F. pratensis*). The variation could be due to a specific breeding strategy, particular genomic constitution and/or a random drift. It may also be a consequence of an early stage of the hybrid genome development, which is known to be accompanied by genomic instability, ranging from a complete elimination of one of the genomes as is the case of a cross *Hordeum vulgare* × *H. bulbosum* (Bennett et al. 1976) to a more subtle change at DNA level as observed in *Triticum* (Feldman and Levy 2005). It is intriguing to note that despite the variation in genomic constitution, the cultivars appear morphologically uniform. Whatever the reason for the variability, it needs to be considered when characterizing genomic constitution of hybrid cultivars.

Intra- and inter-varietal variation in genomic constitution could be affected by the number of generations that passed since the initial hybridization. Unfortunately, there is only a limited data available in this regard. All Polish and Czech cultivars and the French strains '99–01' and '99–04', which were analyzed in this study, were in the F9–F10 generation, while the French strain '01–1' was in the F5 generation. Information about the generation level and in some cases about the parents used for initial crossings was unavailable for the remaining cultivars studied.

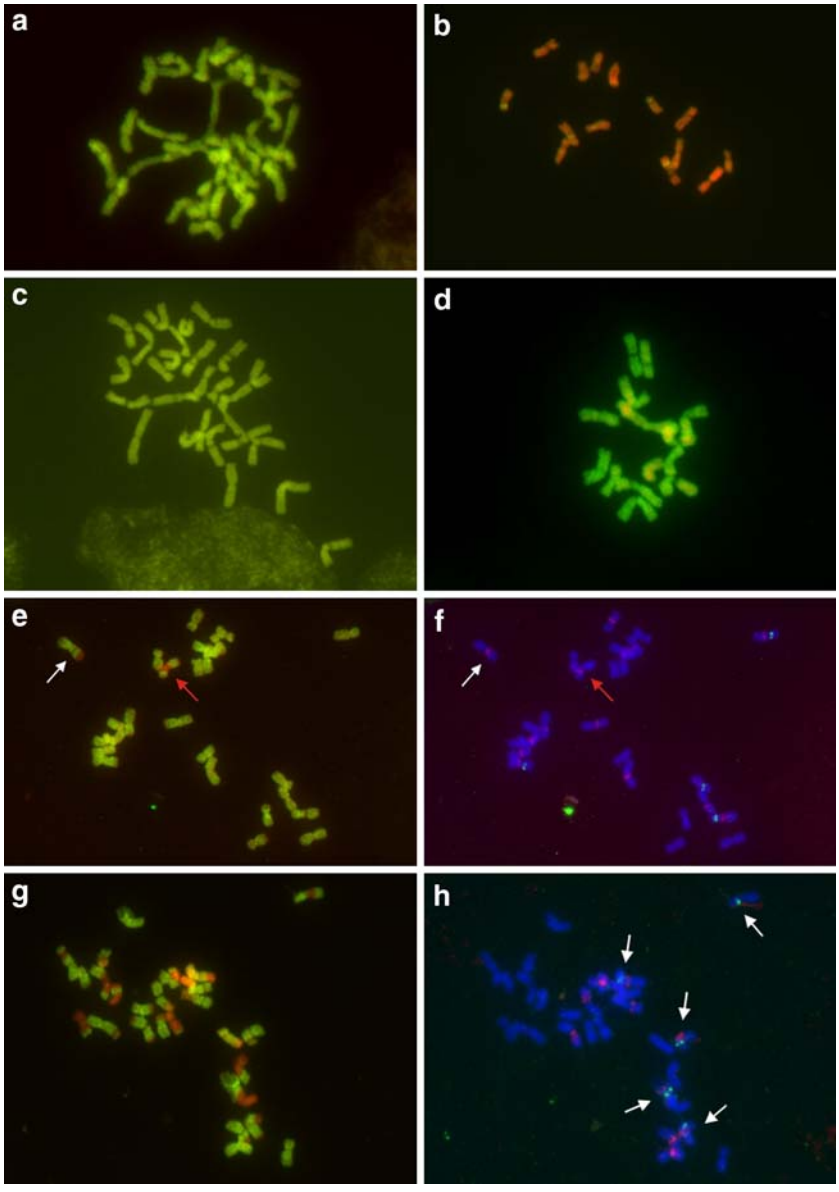


Fig. 2 Molecular cytogenetic analysis of Festulolium cultivars and parental species. GISH on mitotic metaphase plates of *L. perenne* (**a**), *F. pratensis* (**b**) and two *L. perenne* × *F. pratensis* cultivars 'Duo' (**c**) and 'Matrix' (**d**). In situ hybridization was performed on one slide in order to assure identical reaction conditions for all four genotypes. Total genomic DNA of *L. multiflorum* was labelled with FITC and used as a probe (*yellow-green color*); genomic DNA of *F. pratensis* was used as blocking DNA. Chromosomes were counterstained using propidium iodide (*red color*). The results indicate the absence of *Festuca* chromatin in cvs 'Duo' (**c**) and 'Matrix' (**d**). *Yellow-green* bands on some chromosomes of *F. pratensis* are NORs (**b**). Successive GISH and FISH analysis of mitotic metaphase plates from cultivars, 'Spring Green' (**e**, **f**) and 'Elmet' (**g**, **h**). **e**, **g** GISH was done with FITC-labelled genomic DNA of *L. multiflorum* (*green color*) and using genomic DNA of *F. pratensis* as blocking DNA. Chromosomes were counterstained using propidium iodide (*red color*). **f**, **h** FISH on the same metaphase plates with probe for 45S rDNA (*red color*) and probe for 5S rDNA (*green color*). Chromosomes were counterstained with DAPI (*blue color*). Note the presence of two recombined chromosomes in 'Spring Green' (**e**, **f**). On one of the chromosomes (*white arrow*), the segment carrying 45S rDNA is of the *Lolium* origin. On the second chromosome (*red arrow*), the segment carrying 45S rDNA is of *Festuca* origin. The other metaphase spread of one selected plant of cv. 'Elmet'. Five hybridization signals of 5S rDNA were detected (*arrows*)

Despite the enormous value of GISH for characterization of Festulolium hybrids, the method may have some limitations. Previously, we encountered difficulties in detecting chromatin of one of the parental genomes in cv. 'Lofa' and 'Bečva', which originated from a cross of *L. multiflorum* × *F. arundinacea*, followed by backcrossing to *L. multiflorum* (Kopecký et al. 2005b). Out of 29 plants of cv. 'Lofa', only 6 plants were found carrying *Festuca* segments in the *Lolium* background. This could be a consequence of

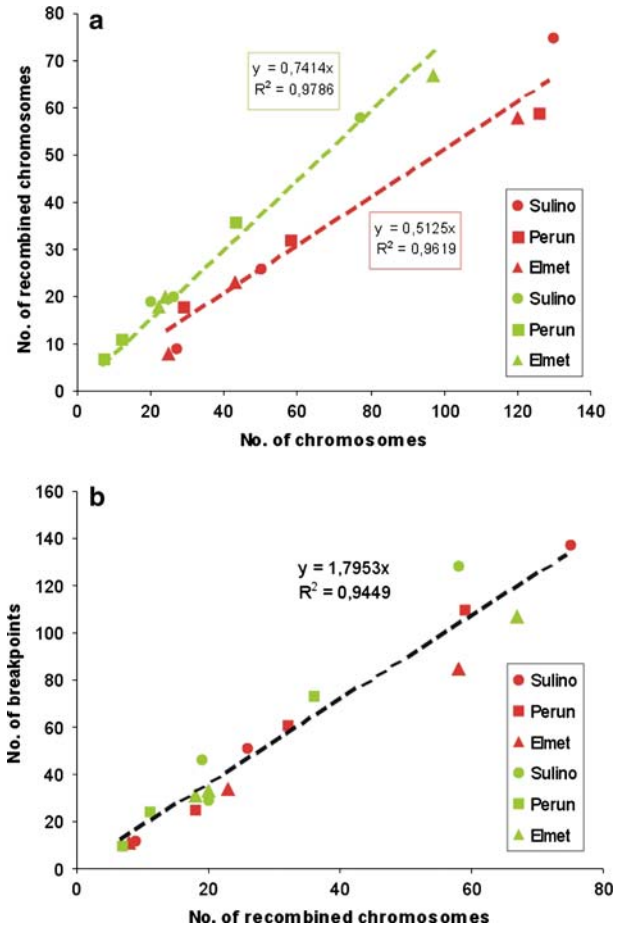


Fig. 3 Recombination between chromosomes of parental species in *L. multiflorum* × *F. pratensis* cultivars 'Elmet', 'Sulino' and 'Perun'. **a** Frequency of recombined chromosomes is higher in chromosomes with *Festuca* centromere (*green color*) than in chromosomes with *Lolium* centromere (*red color*). *X*-axis: sum of all chromosomes in individual chromosome groups; *Y*-axis: sum of recombined chromosomes in individual chromosome groups. **b** The number of breakpoints per recombined chromosome is similar in chromosomes of both species. The color-coding is the same as in (**a**). *X*-axis: sum of recombined chromosomes in individual chromosome groups; *Y*-axis: sum of breakpoints in individual chromosome groups. Each data point represents one chromosome group in both graphs. Chromosome groups within parents are not distinguished

backcrossing and selection towards the *Lolium* type. This explanation seems to be supported by GISH analysis of cv. 'Perseus', where we detected 16.4 complete *Lolium* chromosomes (compare with other *L. multiflorum* × *F. pratensis* cultivars in Table 1). The selection strategy of this cultivar was for the *Lolium* plant type (I. Houdek, pers. comm.). Despite this, cultivars 'Lofa' and 'Bečva' represent a valuable combination of desired characters of *Festuca* and *Lolium*.

We were unable to detect fescue chromatin in cultivars 'Duo' and 'Matrix', which were obtained after crossing *L. perenne* with *F. pratensis* and among 29 plants of cv. 'Kemal', selected from *L. multiflorum* × *F. pratensis* hybrids. Hybrid cultivar 'Spring Green', which was developed from 'Kemal' and three other cultivars ('Elmet', 'Prior' and 'Tandem'), carried few chromosome segments of *Festuca* origin. Thus, if the genomes of 'Elmet' and 'Prior' consisted of almost equal proportions of parental genomes, 'Kemal' and/or 'Tandem' had to carry a very small part of the *Festuca* genome, if any. One way to explain the apparent lack of chromatin in one of the parental species by GISH may be the sensitivity of the method. In wheat-rye hybrids, megabase stretches of DNA appeared to be below the resolution limit of GISH (Lukaszewski et al. 2005). Another possible explanation may be a real absence of one of the parental genomes, which in the case of cv. 'Kemal' is supported by the analysis of genomic constitution of cv. 'Spring Green'.

Genetic mapping revealed that chromosomes 3, 4 and 5 of fescue carried important genes for winter hardiness and drought tolerance (Humphreys et al. 1997; King et al. 2002). Nevertheless, there is no study estimating the frequency of recombination and elimination of particular chromosomes or their segments in hybrid cultivars. The main obstacle was the inability to identify cytologically individual chromosomes of *Festuca* and *Lolium*. One possibility is to identify individual parental chromosomes using chromosome-specific molecular markers. For the analysis of large numbers of hybrids, PCR markers would be preferred. While genetic maps of *Lolium* species with PCR markers have been developed (Inoue and Cai 2004; Jensen et al. 2005) no such map exists for *F. pratensis*. Moreover, high frequency of recombination and elimination of parental chromosomes implies that many markers would be needed for a detailed characterization of chromosome complements in hybrids.

In this work, we were able to discriminate two chromosome pairs in *F. pratensis* and one chromosome pair in *L. multiflorum* and *L. perenne* based on presence of rDNA (Thomas et al. 1996, 1997). Our results indicate that the frequency of recombination of particular chromosomes does not appear to be random. Chromosomes with a *Festuca* centromere recombined at higher frequency than those with a *Lolium* centromere, which could be due to a higher frequency of elimination of *Festuca* chromosomes. The absence of *Festuca* homologues could stimulate pairing between chromosomes of *Festuca* and *Lolium* in a homoeologous and non-homoeologous manner. Because our study focused on analysis of genomic constitution in somatic cells, we were not able to analyze pairing and recombination of parental chromosomes during meiosis. A high frequency of bivalent formation was observed in early

generations of allotetraploid *L. multiflorum* × *F. pratensis* and *L. perenne* × *F. pratensis* (Osborne et al 1977) and *L. multiflorum* × *F. glaucescens* (Ghesquière et al. 1993). However, the authors were not able to discriminate parental chromosomes and hence assess the extent of homologous and homoeologous pairing. Our future work will address these questions.

Acknowledgments We are grateful to Dr. Vladimír Černoch for providing plants of Festulolium hybrids and to Dr. Marie Kubaláková for providing the probe for 5S rDNA. This work was supported by the Academy of Sciences of the Czech Republic (grant award no. S5038104).

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