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ORIGINAL PAPER

Genome balance in six successive generations of the allotetraploid *Festuca pratensis* × *Lolium perenne*

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Abstract In the allotetraploid, *Festuca pratensis* Huds. ($2n = 4x = 28$) × *Lolium perenne* L. ($2n = 4x = 28$) the balance of chromatin, as determined by GISH, changes over successive generations of open pollination in favour of *L. perenne*. There is extensive recombination between chromosomes of the two parental genomes, as well as substitution of whole *Festuca* chromosomes by whole *Lolium* chromosomes. The total number of *Lolium* chromosomes increased from a mean 14.36 in the F_2 to 16.26 in the F_6, and the total number of *Festuca* chromosomes decreased correspondingly from a mean of 13.57 to a value of 11.56. The number of recombinant chromosomes and recombination breakpoints per genotype also increased from generation to generation, although the respective values of both characters were higher for *Festuca* (0.86–8.41 and 1.14–15.22) than for *Lolium* (0.68–4.59 and 0.68–6.0). The proportion of total genome length contributed by the *L. perenne* chromatin increased from about 50% in F_2 to 59.5% in F_6. The results are based on the sample of 134 plants studied (26–28 plants per generation), and are discussed in terms of the dominance of *Lolium* chromosomes over those of *Festuca*, and possible mechanisms underlying this phenomenon of chromatin substitution.

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Introduction

The *Lolium–Festuca* complex contains a number of species of forage grasses which are well adapted to a wide range of ecological conditions and which have agriculturally desirable and complementary traits. *Lolium* species have high yield and excellent forage quality, while *Festuca* species express higher persistency and tolerance to abiotic and biotic stresses (Thomas and Humphreys 1991). Meadow fescue (*F. pratensis* Huds.) and the two cultivated ryegrass species, Italian ryegrass (*L. multiflorum* Lam.) and perennial ryegrass (*L. perenne* L.), are closely related, hybridise with relatively ease and their chromosomes pair (Jauhar 1975), and recombine freely in their hybrids and derivatives (King et al. 1998; Zwierzykowski et al. 1998a, b, 1999; Canter et al. 1999). The genomes of *Lolium* and *Festuca* are interchangeable, by chromosome substitution and recombination, and the gene pools of the two genera are therefore accessible for genetic manipulation (Zwierzykowski et al. 1998a, b, 1999). On the other hand, the relationship between the species of both genera is sufficiently distant that the dispersed repetitive DNA of *Festuca*

and *Lolium* chromosomes can be distinguished in intergeneric hybrids and their amphiploid and introgression derivatives, using genomic in situ hybridisation (GISH) (e.g. Thomas et al. 1994; Humphreys et al. 1995; Humphreys and Pašakinskienė 1996; King et al. 1998, 2002a; Zwierzykowski et al. 1998a, b, 1999; Canter et al. 1999; Armstead et al. 2001; Leśniewska et al. 2001; Kopecky et al. 2005a, b; Kosmala et al. 2006). The genome sizes of *L. perenne* (1C = 2,034 Mb) and *F. pratensis* (1C = 2,181 Mb) are close (RBG Kew Plant DNA C-values database: http://www.rbgkew.org.uk/cval/homepage.html). The possibility of combining the complementary traits of *Lolium* and *Festuca* species into a single genotype has for long time been the main incentive to undertake extensive investigations of synthetic amphiploids containing the complete sets of chromosomes of the species of both genera (Lewis 1980). However, the production of grass cultivars based on synthetic amphiploids has been limited because of homoeologous chromosome pairing leading to genetic instability in later generations (Thomas and Humphreys 1991). Intergeneric hybrids of diploid *L. multiflorum* and *L. perenne* with *F. pratensis* species are completely male sterile. Doubling the chromosome number of the diploid F_1 hybrids leads to the restoration of fertility. On the other hand, partly male and female fertile tetraploid F_1 hybrids can be obtained by crossing autotetraploid forms of *Lolium* sp. and *F. pratensis* (Zwierzykowski and Rybczyński 1981; Zwierzykowski et al. 1993). The first cultivars derived from intergeneric *Lolium–Festuca* hybrids (*Festulolium*), 'Elmet' (*L. multiflorum* × *F. pratensis*), and 'Prior' (*L. perenne* × *F. pratensis*) were produced in Wales, UK (Lewis et al. 1973). More recently, greater success in the amphiploid breeding of reciprocal hybrids of *L. multiflorum* ($2n = 4x = 28$) and *F. pratensis* ($2n = 4x = 28$) has been achieved and several cultivars developed: 'Paulita' in Germany (Netzband 1991), 'Perun', 'Achilles', and 'Perseus' in Czech (Fojtik 1994; Kopecky et al. 2005a; V. Černoch, personal communication), 'Felopa', 'Sulino', 'Rakopan', and 'Agula' in Poland (Zwierzykowski et al. 1998b; Zwierzykowski 2004), and 'Punia' in Lithuania (Pašakinskienė and Jones 2003). In the USA, the *Festulolium* tetraploid cv. 'Spring Green' has been developed from inter-crosses of *L. perenne* × *F. pratensis* and *L. multiflorum* × *F. pratensis* hybrids (Casler et al. 2001). *Festulolium* breeding has also been initiated in Japan (Yamada et al. 2005). Polish *Festulolium* cultivars are sufficiently fertile and have satisfactory levels of uniformity: they show high forage yield and quality (e.g. water soluble carbohydrates content and in vitro dry matter digestibility) compared with *L. multiflorum*, as well as higher levels of persistency, winter hardiness and drought resistance (Zwierzykowski et al. 1994; Zwierzykowski 2004). *Festulolium* cultivars can be used in both field monocultures and in pastures, as in mixtures with other grasses and clover or alfalfa (Fojtik 1994; Zwierzykowski 2004). Cytogenetic studies on the genome architecture of *Festulolium* cultivars performed by GISH have recently demonstrated that homoeologous chromosome pairing results in extensive recombination between chromosomes of the parental genomes (Thomas et al. 1994; Zwierzykowski et al. 1998b; Canter et al. 1999; Pašakinskienė and Jones 2003; Zwierzykowski et al. 2003; Kopecky et al. 2005a). Zwierzykowski et al. (1998b), for example, observed extensive recombination in four advanced (F_8) breeding populations registered as commercial *Festulolium* cultivars—'Felopa', 'Sulino', 'Agula' (*F. pratensis* × *L. multiflorum*), and 'Rakopan' (*L. multiflorum* × *F. pratensis*). These *Festulolium* cultivars represent the products of several generations of seed multiplication and have undergone considerable genome reconstruction. The outcome of this process is an almost perfect mixing of the genomes, with no deleterious effects in terms of hybrid fertility. Extensive chromosome substitutions and homoeologous recombination has also been shown for F_8 genotypes derived from the amphiploid *L. perenne* × *F. pratensis* cv. 'Prior' (Canter et al. 1999). High levels of intergeneric recombination have been found in the Lithuanian cv. 'Punia', derived from a *F. pratensis* × *L. multiflorum* hybrid combination (Pašakinskienė and Jones 2003), and in the Czech cv. 'Perun'—from a *L. multiflorum* × *F. pratensis* hybrid (Kopecky et al. 2005a). Recently, genomic in situ hybridisation (GISH) has been used to analyse genomic constitution in about 30 *Festulolium* cultivars registered and distributed worldwide (D. Kopecky et al., in preparation).

In this study, we present for the first time the genomic structure and results of homoeologous chromosome substitution and recombination revealed by GISH in successive generations (F_2–F_6) of breeding populations derived from allotetraploid *F. pratensis* ($2n = 4x = 28$) × *L. perenne* ($2n = 4x = 28$) hybrids.

Materials and methods

Plant material

Tetraploid hybrids of *F. pratensis* × *L. perenne* ($2n = 4x = 28$; *FpFpLpLp*) were generated in 1995 by intercrossing autotetraploid forms of both species. *F. pratensis* Huds. ($2n = 4x = 28$; *FpFpFpFp*), spontaneous tetraploids obtained from twin seedlings of

diploid cultivars (Sulinowski et al. 1982), was used as the female parent and *L. perenne* L. ($2n = 4x = 28$; *LpLpLpLp*) cv. 'Solen' as the pollinator. Five F_1 hybrid plants, partially male and female fertile, were intercrossed under controlled conditions and produced 240 F_2 progeny plants. Successive F_3–F_6 generations were produced at 2-year-intervals by inter-crossing of 10–20 genotypes selected for their agronomic traits (including vigour, ryegrass-type growth and inflorescence, winter hardiness, drought resistance and fertility) from field-grown populations consisting of 800–1,100 individuals. The breeding program is being developed at the Szelejewo Plant Breeding Station.

Genomic in situ hybridisation

Thirty randomly chosen plants, taken from each of F_2–F_6 generations of the breeding populations, were used for GISH analysis. To determine the number and genomic structure of chromosomes, root-tip chromosome spreads were prepared as described by Zwierzykowski et al. (1999).

The GISH procedure was carried out as described by Zwierzykowski et al. (1998b), with some modifications (Kosmala et al. 2006). To discriminate between *L. perenne (Lp)* and *F. pratensis (Fp)* chromosomes, total genomic DNA of *Lp* cv. 'Solen' was used as a probe and labelled with digoxigenin-11-dUTP by nick translation (according to the Roche protocol). Total genomic DNA of *Fp* cv. 'Westa' was used as a blocker and prepared by autoclaving for 10 min and applied at a ratio of 1:40 (probe:blocker). For signal detection the slides were incubated with anti-digoxigenin-fluorescein (Roche) at a final concentration of 2 μg/ml at 37°C for 40 min. Three to five cells per plant were analysed using a Nikon Optiphot-2 epifluorescence microscope, photographed on Fuji 800 film and the photographic images were then scanned into a computer.

The following observations were made for each plant studied: (1) the total number of chromosomes; (2) the number of parental chromosomes; (3) the number of recombinant chromosomes and recombinant segments, separately for *Lp* and *Fp*; (4) the number of recombination breakpoints calculated for terminal segments as a result of a single recombination event and interstitial segments resulting from two recombination events within the same chromosome arm; (5) the total lengths of *Lp* and *Fp* chromatin measured from 2 to 3 cells for each genotype studied. Measurements were taken directly from the computer screen.

Analysis of variance was carried out for verification of the general hypothesis of lack of significant differences among generations (F_2–F_6) for the number of parental chromosomes, the number of recombinant chromosomes, the number of recombination breakpoints, and the total lengths of *Lolium* and *Festuca* chromatin; least significant differences ($LSD_{0.001}$) for these characters were calculated.

Results

A total of 134 plants from the F_2–F_6 breeding generations were analysed by GISH (26–28 plants per generation), and of these the majority were tetraploid with $2n = 4x = 28$. A number of aneuploid plants with 26, 27, 29, and 30 chromosomes were also recorded, and their proportions ranged from 7.1% in F_2 to 29.6% in F_5 and F_6 (Table 1).

The tetraploid F_1, *F. pratensis* ($2n = 4x = 28$) × *L. perenne* ($2n = 4x = 28$) hybrids, used in this study expressed partly male and partly female fertility (an average of 45.0% of pollen stainability and 15.6% seed set). Selection for both agronomic characters (vigour, ryegrass inflorescence-type, winter hardiness, and drought tolerance) and for fertility made in each of F_2–F_6 generations has led to the development of the vigorous, uniform, and abiotic stress resistant F_6 plants. The level of male and female fertility increased slightly from generation to generation, and by then F_6 reached an average 65.0% for pollen stainability and 33.0% for seed set (Zwierzykowski et al., unpublished data). However, female fertility in F_6 derived from *F. pratensis* × *L. perenne* was significantly lower than that of F_6 plants derived from *F. pratensis* × *L. multiflorum*, where the mean seed set was 53.8% (Zwierzykowski et al. 1993).

In F_2, 17 (60.7%) individuals had an equal number of chromosomes: 14 *Lolium* and 14 *Festuca*. For the generation as a whole, the mean number of *Lolium* chromosomes (with *Lolium* centromeres) was 14.36 (range 14–16) and *Festuca* chromosomes (with *Festuca* centromeres) was 13.57 (12–14) (Table 2; Fig. 1a). The number of recombinant chromosomes was slightly higher for *Festuca* (on average 0.86 per genotype) than

Table 1 Chromosome numbers in plants of F_2–F_6 generations derived from *F. pratensis* (4*x*) × *L. perenne* (4*x*) hybrids

Generation	No. of plants studied	Chromosome number (2*n*)					Aneuploids (%)
		26	27	28	29	30	
F_2	28		2	26			7.1
F_3	26		3	19	4		26.9
F_4	26		4	20	2		23.1
F_5	27		1	19	7		29.6
F_6	27	2	4	19	1	1	29.6

for *Lolium* (on average 0.68). Seven plants (25%) showed no recombination events, 10 (35.7%) had either *Lolium* or *Festuca* recombinants and 11 (39.3%) showed recombination on both *Lolium* and *Festuca* chromosomes. The percentage of chromosomes with no recombination events was almost equal for *Lolium* (95.3) and *Festuca* (93.7). The same tendency was observed with respect to the number of recombination breakpoints. The mean number of breakpoints was 0.68 and 1.14 per genotype, and 1.0 and 1.33 per recombinant chromosome for *Lolium* and *Festuca*, respectively. In *Lolium* chromosomes all the segments of alien chromatin were terminally located in one of the chromosome arms, but for *Festuca* there were 72.7% terminal segments and 27.3% interstitial segments. The mean total chromatin length for each species over all genotypes was almost equal: 49.9% (46.1–55.7%) for *Lolium* and 50.1% (44.3–53.9%) for *Festuca* (Table 3).

The mean number of *Lolium* and *Festuca* chromosomes among the F_3 was 14.77 (13–17) and 13.27 (11–15), respectively (Table 2; Fig. 1b). The number of recombinant chromosomes was higher for *Festuca* (on average 2.85 per plant) compared with *Lolium* (on average 2.00). *Festuca* recombinant chromosomes were present in all the genotypes, whilst two genotypes had no *Lolium* recombinant chromosomes. The percentage of chromosomes with no recombination events was 86.5 for *Lolium* and 78.6 for *Festuca*. The mean number of breakpoints reached 2.23 (0–6) per *Lolium* and 3.77 (1–7) per *Festuca* genotype, and in both cases this value was significantly higher than in the F_2. The mean number of breakpoints per recombinant chromosome was 1.12 and 1.32 for *Lolium* and *Festuca*, respectively. The majority of the alien chromatin segments (92.6% for *Lolium* and 75.9% for *Festuca* chromosomes) were terminally located, with the remaining ones having an interstitial location. The mean length of total chromatin per genotype was slightly higher for *Lolium*, 51.4% (44.4–60.5%), than for *Festuca*, 48.6 (39.5–55.6%) (Table 3).

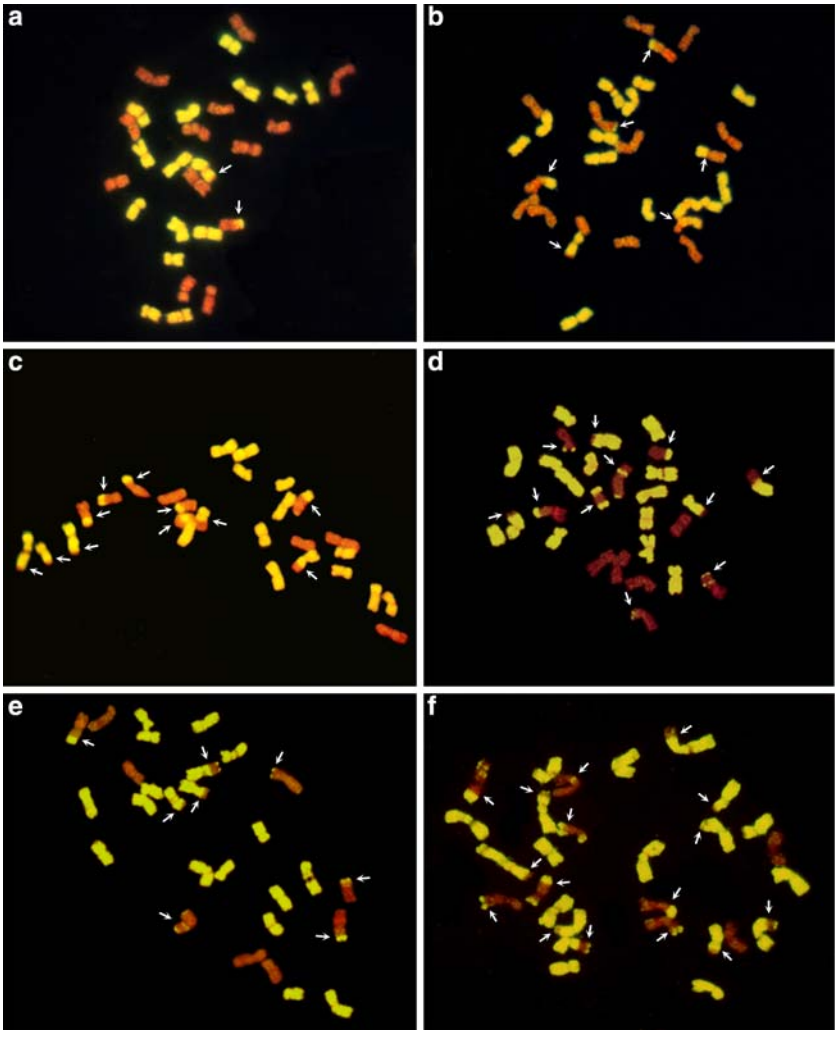

Fig. 1 GISH images of mitotic chromosome spreads from root-tip cells of F_2–F_6 plants derived from *F. pratensis* (*Fp*) × *L. perenne* (*Lp*) ($2n = 4x = 28$) hybrids. The images were created using total genomic DNA of *Lp* as a probe, labelled with digoxigenin and detected by anti-digoxigenin conjugated with fluorescein (*yellow*), blocking genomic DNA of *Fp* (*red*). Chromosomes were counterstained with propidium iodide. Recombinant (*R*) *Lp* and *Fp* chromosomes are indicated by *arrows*. **a** F_2 plant [15 *Lp* (1R) + 13 *Fp* (1R)]. **b** F_3 plant [15 *Lp* (2R) + 14 *Fp* (4R)]. **c** F_4 plant [17 *Lp* (5R) + 10 *Fp* (6R)]. **d** F_5 plant [17 *Lp* (4R) + 11 *Fp* (7R)]. **e** F_6 plant [19 *Lp* (3R) + 9 *Fp* (5R)]. **f** F_6 plant [19 *Lp* (9R) + 9 *Fp* (7R)]

Table 2 GISH analysis in the F_2–F_6 generations obtained from *F. pratensis* (4*x*) × *L. perenne* (4*x*) hybrids

Generation	No. of plants studied	*Lolium* chromosomes							*Festuca* chromosomes						
		Total no. of chromosomes		No. of recombinant chromosomes		No. of breakpoints			Total no. of chromosomes		No. of recombinant chromosomes		No. of breakpoints		
						per genotype		per RC[a]					per genotype		per RC[a]
		Mean	Range	Mean	Range	Mean	Range	Mean	Mean	Range	Mean	Range	Mean	Range	Mean
F_2	28	14.36	14–16	0.68	0–4	0.68	0–4	1.00	13.57	12–14	0.86	0–4	1.14	0–6	1.33
F_3	26	14.77	13–17	2.00	0–5	2.23	0–6	1.12	13.27	11–15	2.85	1–5	3.77	1–7	1.32
F_4	26	15.81	13–20	3.46	1–5	4.04	2–6	1.17	12.12	8–15	3.89	2–6	5.19	2–10	1.34
F_5	27	16.41	13–21	4.44	2–9	5.26	2–11	1.18	11.81	7–15	5.85	2–9	9.15	4–13	1.56
F_6	27	16.26	13–19	4.59	1–9	6.00	1–13	1.31	11.56	9–15	8.41	3–13	15.22	6–25	1.81
$LSD_{0.001}$		1.397		1.230		1.651			1.423		1.418		2.808		

[a] Recombinant chromosome

Table 3 The mean length of total chromatin contributed by *L. perenne* and *F. pratensis* genomes in plants of F_2–F_6 generations derived from *F. pratensis* (4*x*) × *L. perenne* (4*x*) hybrids

Generation	No. of plants studied	*Lolium* chromatin (%)		*Festuca* chromatin (%)	
		Mean	Range	Mean	Range
F_2	16	49.9	46.1–55.7	50.1	44.3–53.9
F_3	14	51.4	44.4–60.5	48.6	39.5–55.6
F_4	15	55.1	44.8–68.5	44.9	31.5–55.2
F_5	16	57.6	46.0–67.2	42.4	32.8–54.0
F_6	15	59.5	44.2–70.6	40.5	29.4–55.8
$LSD_{0.001}$		4.693		4.693	

In F_4, the mean number of *Lolium* chromosomes increased to 15.81 (13–20) while those of *Festuca* decreased to 12.12 (8–15) (Table 2; Fig. 1c). The mean number of recombinants was raised to 3.46 (1–5) for *Lolium* and 3.89 (2–6) for *Festuca*. In both the *Lolium* and the *Festuca* recombinant chromosomes the mean number of breakpoints per genotype also went up in comparison with F_3, and was significantly higher for *Festuca*, 5.19 (2–10), than for *Lolium*, 4.04 (2–6). The percentage of complete chromosomes reached 78.1 for *Lolium* and 67.9 for *Festuca*. The mean number of breakpoints per recombinant chromosome was 1.17 for *Lolium* and 1.34 for *Festuca*. Most of the alien recombined segments (94.9% for *Lolium* and 80.5% for *Festuca* chromosomes) had terminal locations. The mean length of total chromatin per genotype reached 55.1% (44.8–68.5) for *Lolium* and 44.9% (31.5–55.2%) for *Festuca* (Table 3).

In F_5, the mean number of *Lolium* and *Festuca* chromosomes was 16.41 (13–21) and 11.81 (7–15), respectively (Table 2; Fig. 1d). The number of recombinants increased again when compared with those of F_4, up to 4.44 (2–9) in *Lolium* and up to 5.85 (2–9) in *Festuca*. The percentage of complete chromosomes decreased to 72.3 for *Lolium* and 50.5 for *Festuca*. The mean number of recombination breakpoints reached 5.26 and 9.15 per genotype, and 1.18 and 1.56 per recombinant chromosome, for *Lolium* and *Festuca*, respectively. Terminal recombinant segments were more frequent than interstitial ones, and occurred in 89.9% of *Lolium* and in 78.2% of *Festuca* chromosomes. The mean length of total chromatin per genotype reached 57.6% (46.0–67.2%) for *Lolium* and 42.4% (32.8–54.0%) for *Festuca* (Table 3).

In F_6 generation, the total number of *Lolium* and *Festuca* chromosomes slightly decreased when compared with F_5 generation, and reached the mean values 16.26 (13–19) and 11.56 (9–15), respectively (Table 2; Fig. 1e–f). The mean number of recombinant chromosomes once again increased for *Lolium*, up to 4.59 (1–9), and for *Festuca*, 8.41 (3–13). Similarly, as in case of the F_2–F_5 generations, the terminal recombinant segments were more frequent than interstitial ones, and occurred in 80.0% of *Lolium* and in 77.3% of *Festuca* chromosomes. On average, 11.67 (8–16) *Lolium* and 3.15 (0–9) *Festuca* chromosomes showed no recombination events, i.e. 71.8% for *Lolium* and 27.2% for *Festuca*. The mean number of breakpoints per genotype increased in comparison with F_5, and was significantly higher for *Festuca*, 15.22 (6–25), than for *Lolium*, 6.0 (1–13). The mean number of breakpoints per recombinant chromosome was also higher than in F_5, and reached values 1.31 for *Lolium* and 1.81 for *Festuca*. The frequency of terminal and interstitial recombinant segments was almost the same: 80.0% for *Lolium* and 77.3% for *Festuca*. The mean total chromatin length per genotype was 59.5% (44.2–70.6%) for *Lolium* and 40.5% (29.4–55.8%) for *Festuca* (Table 3).

Discussion

Amphiploid breeding programs to combine valuable and complementary agronomic traits of ryegrasses and fescues into single *Festulolium* genotypes have been

developed in Europe (Netzband 1991; Thomas and Humphreys 1991; Ghesquière et al. 1993, 1996; Zwierzykowski et al. 1993, 1998b; Fojtik 1994; Pašakinskienė and Jones 2003; Zwierzykowski et al. 2003; Zwierzykowski 2004) and in the USA (Casler et al. 2001; Kopecky et al. 2005b). Until now, most of the allotetraploid *Festulolium* cultivars were produced from reciprocal *F. pratensis* × *L. multiflorum* and *L. multiflorum* × *F. pratensis* hybrids [× *Festulolium braunii* (K. Richter) A. Camus], and only a few cultivars from *L. perenne* × *F. pratensis* [× *Festulolium loliaceum* (Hudson) P.V. Fournier] (Zwierzykowski 2004; D. Kopecky et al., in preparation). In France (INRA, Lusignan), several *Festulolium* strains derived from tetraploid *L. multiflorum* × *F. arundinacea* var. *glaucescens* hybrids have been developed (M. Ghesquière, personal communication). In Poland, a *Festulolium* breeding program from *F. pratensis* (4*x*) × *L. perenne* (4*x*) has been carried out since 1995. Five successive (F_2–F_6) breeding populations were developed via selection for valuable agronomic characters of both species (establishment, growth and seedling vigour of perennial ryegrass, and stress resistance of meadow fescue) as well as fertility. Plant materials selected from these populations were analysed in the current work.

The analysis of the genomic structure of intergeneric hybrids was improved after the development of the GISH technique, to distinguish parental and recombined chromosomes within the *Lolium–Festuca* hybrids and their derivatives, more than a decade ago (Thomas et al. 1994, 2003; Pašakinskienė and Jones 2005, and references herein). In the studies of advanced generations (mostly F_8) of different *Festulolium* hybrids it was earlier revealed that extensive recombination had taken place between homoeologues of the *Festuca* and *Lolium* genomes, and that the balance of their chromatin was not equal (Zwierzykowski et al. 1998b; Canter et al. 1999; Pašakinskienė and Jones 2003; Jones and Pašakinskienė 2005; Kopecky et al. 2005a).

The first analysis of genome recombination in *Festulolium* cultivars was performed by Zwierzykowski et al. (1998b). The authors showed extensive intergeneric recombination within the genomes of 25 plants derived from four F_8 populations of reciprocal *F. pratensis* (4*x*) × *L. multiflorum* (4*x*) hybrids. Furthermore, it was shown that the proportion of the chromatin contributed by the parental species ranged from 49.2 to 66.7% for *Lolium* and from 33.3 to 50.8% for *Festuca*. Up to seven exchange points per recombinant chromosome and close to 40 breakpoints per genotype were observed in the individual plants. All four populations analysed have been registered in Poland as commercial cultivars of forage grasses ('Felopa', 'Sulino', 'Rakopan', and 'Agula'), having met all the requirements of fertility, uniformity, and productivity (Zwierzykowski et al. 1999; Zwierzykowski 2004). This suggested that the gene pools of *F. pratensis* and *L. multiflorum* were indeed interchangeable with the capacity to intermix freely in the hybrids, at least at the tetraploid level (Zwierzykowski et al. 1998b).

Detailed investigations of chromosome substitution and homoeologous recombination in the F_8 population of the amphiploid *L. perenne* × *F. pratensis* cv. 'Prior' ($2n = 4x = 28$) were later carried out by Canter et al. (1999). In the 12 genotypes analysed the authors observed that the substitution of *Festuca*-origin chromosomes by those of *Lolium*-origin resulted in a mean of 17.9 (15–21) *Lolium* and 9.7 (7–13) *Festuca* chromosomes per genotype. The mean length of total chromatin of the parental species per genotype comprised of 62.1% (53.7–71.2%) *Lolium* and 37.9% (28.2–46.3%) *Festuca*.

The genome constitution in the cv. 'Perun', derived from a tetraploid *L. multiflorum* × *F. pratensis*, has also been recently studied (Kopecky et al. 2005a). Among the 23 plants analysed, the mean number of recombinant chromosomes reached 14.70 per plant, but only 0.65 complete *Festuca* chromosomes were observed per plant, compared with 11.70 complete *Lolium* chromosomes. The mean number of recombination breakpoints was 21.30 per plant, with 1.45 breakpoints per recombinant chromosome. Pašakinskienė and Jones (2003) have also observed the *Lolium*-dominant behaviour in the Lithuanian cv. 'Punia', developed from a tetraploid hybrid of *F. pratensis* × *L. multiflorum*, but the proportion of complete and recombined chromosomes of *Lolium* and *Festuca* was not presented.

The results presented herein show for the first time the dynamics of changes in the genome balance over five successive generations (F_2–F_6) of open pollination in the allotetraploid hybrids *F. pratensis* ($2n = 4x = 28$) × *L. perenne* ($2n = 4x = 28$). The balance of chromatin, visible by GISH, becomes progressive to favour the dominant *Lolium* genome. The dominance of the *Lolium* chromatin results from extensive recombination between chromosomes of the parental genomes and from a substitution of *Festuca* chromosomes by *Lolium* chromosomes. From F_2 to F_6 generation, the total number of *Lolium* chromosomes increased (mean 14.36 in F_2 and 16.26 in F_6) and *Festuca* chromosomes decreased (mean 13.57 in F_2 and 11.56 in F_6). The number of recombinant chromosomes and recombination breakpoints per genotype also increased from genera-

tion to generation, although the values of both characters were higher for *Festuca* (0.86–8.41 and 1.14–15.22, respectively) than for *Lolium* (0.68–4.59 and 0.68–6.0, respectively). The proportion of total genome length occupied by the *L. perenne* chromatin increased from 49.9% in F_2 to 59.5% in F_6, and by the *F. pratensis* chromatin parallel decreased from 50.1 to 40.5%. Variation with respect to all the characters analysed within and among breeding generations was also found.

The observations made in the current study for the F_2–F_6 populations of the amphiploid *F. pratensis* (4*x*) × *L. perenne* (4*x*) (data not shown) confirmed earlier results obtained for the F_8 populations of amphiploid hybrids—*F. pratensis* (4*x*) × *L. multiflorum* (4*x*) (Zwierzykowski et al. 1998b) and *L. perenne* (4*x*) × *F. pratensis* (4*x*) (Canter et al. 1999)—that recombination breakpoints occur along the entire length of the chromosome arms, from the centromere to the telomere, and are more frequent in intercalary regions of the arms.

The mechanism leading to the substitution of *Festuca* chromosomes, and chromosome parts, by *Lolium* chromosomes, and the consequent and progressive change in the genome balance over successive cycles of sexual reproduction, in favour of the dominant *Lolium* genome, is unknown. How would the progression continue? Would it lead finally to a total *Lolium* GISH phenotype, or would recombination maintain a *Festuca* component? GISH is detecting the discriminating dispersed repeats, and we have no idea how chromosome recombination and substitution is influencing sequence-genotypes, or what is happening below the level of resolution of GISH. We lack sufficient knowledge of the genomics of *Festuloliums* to deal with these questions. What can be confident about, however, is that whatever is going on in these cultivars are making adjustments to their genomes of which the breeders will be unaware.

Various ideas have been proposed for the mechanism of genome adjustment including gametic competition, pollination effects, or selection for vigour in the early stages of seedling growth (Jones and Pašakinskienė 2005), but we still need to develop some sustainable hypotheses for this phenomenon. There are certain features of *Festuloliums* that can focus our thinking, and which probably make these hybrids unique from others. Reciprocal chromosome substitution, and homoeologous recombination, can take place between different *Lolium/Festuca* species in a seamless way, and without compromising the expression of the genome (King et al. 2002a, b). This suggests that while the dispersed repetitive DNAs are divergent enough for the application of GISH, the expressed sequence genomes must have similar identities, although there are doubtless allelic variations at many loci. Introgression forms with single segments of alien chromatin incorporated in the host genomic background are thought to be stable over generations, and may be an alternative for unstable allopolyploids for combining the complementary agronomic traits of both *Lolium* and *Festuca* species into a single genotype.

We too note that in these hybrid generations there is a low frequency of aneuploids, which suggests that some irregularity of meiosis may offer 'a window of opportunity' for parental chromosome sets to engage in competitive segregation. It seems unlikely that such a competitiveness would take place in newly formed zygotes, as happens in certain wide species crosses with the complete or partial elimination of sets of chromosomes, since this would compromise the relatively stable tetraploid status of the plants. We can, however, build a highly speculative hypothesis around the idea that univalents will sometimes be present at meiosis, based on the occurrence of aneuploidy, and that during the movement of half-bivalents at anaphase I, or chromatids at anaphase II, chromosomes or chromatids with *Lolium* centromeres will have the advantage of reaching the poles, especially if there is any asymmetry or spindle gradients at female meiosis, which we do not know about in this instance. This argument implies some difference between the centromeres of *Lolium* and *Festuca*, of which we lack knowledge, but we can draw on information of centromere organisation in general, and on some specific facts from other situations (see Jones and Pašakinskienė 2005 for a discussion and review on plant centromeres). The epigenetic mark that uniquely specifies the centromere locus in all eukaryotes is the particular form of H3 histone (CenH3) that is found in centromere chromatin, regardless of the underlying DNA sequences. The CenH3 histone is highly conserved, but nonetheless varies slightly for different species. In oat addition lines carrying a single added chromosomes of maize it is of interest that it is the oat form of CenH3 histone which is incorporated into the maize chromosome, indicating some allelic interaction, or could we say 'dominance' of oat over maize (Jin et al. 2004). This line of argument takes us far away from *Lolium* and *Festuca*, but leaves us close enough to speculate centromere 'drive' as possible component of the genome enabling chromatin substitution, and clearly this leaves us with a need to look at meiosis using GISH as a next step.

Our results, and those of others, as mentioned, raise the question of whether allotetraploids can be considered as true hybrids, or just a terminally driven and directional mix of parental genomes. However, our

thoughts on these issues finally land and we appreciate that reciprocal and extensive intergeneric recombination observed between the parental genomes of these hybrids are important for introgression breeding programs. The introgression forms with single segments of alien chromatin incorporated in the host genomic background are thought to be stable over generations and that they could provide an alternative for unstable allopolyploids for combining the complementary agronomic traits of both *Lolium* and *Festuca* species into single genotypes. That approach has been already applied by Grønnerød et al. (2004), who used an amphiploid *L. perenne* (4*x*) × *F. pratensis* (4*x*) cultivar 'Prior' as a starting point for the introgression of *F. pratensis* genes for freezing tolerance into *L. perenne* and by Kosmala et al. (2006), who used triploid F_1 hybrids *F. pratensis* (2*x*) × *L. multiflorum* (4*x*) for the introgression of freezing tolerance genes from *F. pratensis* into *L. multiflorum*.

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