

Chromosome pairing in allotetraploid hybrids of *Festuca pratensis* × *Lolium perenne* revealed by genomic *in situ* hybridization (GISH)

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Abstract

Genomic *in situ* hybridization (GISH) was used to make a detailed study of chromosome pairing at metaphase I (MI) of meiosis in six F₁ hybrid plants of the allotetraploid *Festuca pratensis* × *Lolium perenne* (2n = 4x = 28; genomic constitution *FpFpLpLp*). The mean chromosome configurations for all hybrids analysed were 1.13 univalents + 11.51 bivalents + 0.32 trivalents + 0.72 quadrivalents, and the mean chiasma frequency was 21.96 per cell. GISH showed that pairing was predominantly intragenomic, with mean numbers of *L. perenne* (*Lp/Lp*) and *F. pratensis* (*Fp/Fp*) bivalents being virtually equal at 5.41 and 5.48 per cell, respectively. Intergenomic pairing between *Lolium* and *Festuca* chromosomes was observed in 33.3% of *Lp/Fp* bivalents (0.62 per cell), in 79.7% of trivalents – *Lp/Lp/Fp* and *Lp/Fp/Fp* (0.25 per cell), and in 98.4% of quadrivalents – *Lp/Lp/Fp/Fp* and *Lp/Lp/Lp/Fp* (0.71 per cell). About 4.0% of the total chromosome complement analysed remained as univalents, an average 0.68 *Lp* and 0.45 *Fp* univalents per cell. It is evident that in these hybrids there is opportunity for recombination to take place between the two component genomes, albeit at a low level, and this is discussed in the context of compromising the stability of *Festulolium* hybrid cultivars and accounting for the drift in the balance of the genomes over generations. We speculate that genotypic differences between hybrids could permit selection for pairing control, and that preferences for homologous versus homoeologous centromeres in their spindle attachments and movement to the poles at anaphase I could form the basis of a mechanism underlying genome drift.

Introduction

Species within the *Lolium-Festuca* complex, namely Italian ryegrass (*Lolium multiflorum* Lam., 2n = 2x = 14), perennial ryegrass (*Lolium perenne* L., 2n = 2x = 14) and meadow fescue (*Festuca pratensis* Huds., 2n = 2x = 14), offer many desirable and complementary traits, including rapid establishment, good forage quality and high yield of ryegrasses, and

persistency, winter hardiness and freezing tolerance of meadow fescue (Thomas & Humphreys 1991). These closely related species of both genera hybridize naturally, and their chromosomes pair and recombine at high frequency in hybrids (e.g. Jauhar 1975, 1993, King *et al.* 1998, 1999, Zwierzykowski *et al.* 1998, 1999, Canter *et al.* 1999, Naganowska *et al.* 2001, Kosmala *et al.* 2006b). Chromosome pairing in intergeneric diploid (2n = 2x = 14) hybrids

of *L. perenne* $\times$ *F. pratensis* and *L. multiflorum* $\times$ *F. pratensis* is regular, with seven bivalents being mostly formed at metaphase I, and a chiasma frequency only slightly lower than in the parents (Jauhar 1975). In spite of regular pairing, the hybrids are male sterile (with indehiscent anthers), suggesting a genetic basis of male sterility (Jauhar 1993). However, in some cases the diploid F_1 hybrids can express a small level of female fertility which enabled their backcrossing to the parental species (Werner 1983, Connolly & Wright-Turner 1984).

The sterility of diploid hybrids has led to a widespread use of tetraploid ($2n = 4x = 28$) F_1 *Lolium* $\times$ *Festuca* hybrids in amphiploid and introgression breeding programmes, but there is only fragmentary information on their chromosome pairing relationships based on conventional cytological staining procedures, including *L. perenne* $\times$ *F. pratensis* (Thomas & Thomas 1973), *L. multiflorum* $\times$ *F. pratensis* (Jauhar 1975), *L. multiflorum* $\times$ *F. apennina* (Thomas *et al.* 1983), and *L. multiflorum* $\times$ *F. arundinacea* var. *glaucescens* (Thomas *et al.* 1983, Ghesquière *et al.* 1993).

Stabilizing the genome and phenome composition of *Lolium* $\times$ *Festuca* hybrids in order to combine the complementary traits found in species from the two genera has become an increasingly important research objective, especially given the increasing priority for developing more sustainable grassland systems requiring both the maintenance of forage quality and improved crop persistency and resource-use efficiency (Thomas & Humphreys 1991, Humphreys *et al.* 2006). Chromosome doubling to achieve hybrid fertility, and at least a modicum of genome stability, has had some success, and amphidiploid *Festulolium* cultivars have been increasingly developed and marketed (Lewis *et al.* 1973, Zwierzykowski *et al.* 1998, Casler *et al.* 2001, Kopecký *et al.* 2006). For continued stability and maintenance of hybrid genome integrity over generations, preferential pairing between homologous chromosome sets, and regular segregation of *Lolium* and *Festuca* chromosomes leading to disomic inheritance, is required, but this has proved difficult to achieve (Zwierzykowski *et al.* 2006). Despite the presence in the amphidiploid hybrid of two sets of homologous chromosomes, homoeologous chromosomes of *Lolium* and *Festuca* pair and recombine at significant frequencies (e.g. Zwierzykowski *et al.* 1998, 1999, Canter *et al.* 1999), and this has

compromised both the maintenance of hybrid integrity and fertility (Thomas *et al.* 2003). In addition to this promiscuity of recombination there is also a drift in the balance between the two genomes over generations, so that the *Lolium* chromatin gradually comes to replace that of *Festuca*, whether the chromosomes are recombined or not (Zwierzykowski *et al.* 2006).

Genomic *in situ* hybridization (GISH) has been used successfully to differentiate genetically close genomes such as *L. multiflorum* and *F. pratensis* (Thomas *et al.* 1994, Zwierzykowski *et al.* 1998, 1999, Kosmala *et al.* 2006a), *L. perenne* and *F. pratensis* (King *et al.* 1998, Zwierzykowski *et al.* 2006), and *L. multiflorum* and *F. arundinacea* (Humphreys & Pašakinskienė 1996). This technique was also used to discriminate among the ancestral genomes in the allohexaploid *F. arundinacea* (Humphreys *et al.* 1995), and to identify alien chromosomes and chromosome segments in allopolyploid and introgression forms of *Lolium-Festuca* (e.g. Thomas *et al.* 1994, Humphreys & Pašakinskienė 1996, King *et al.* 1998, 1999, Zwierzykowski *et al.* 1998, 1999, 2006, Canter *et al.* 1999, Cao & Slepser 2001, Morgan *et al.* 2001, Humphreys *et al.* 2005, Kopecký *et al.* 2006, Kosmala *et al.* 2006a, 2007).

Until now only a small number of papers have been published on *Lolium* $\times$ *Festuca* hybrids where GISH was used to study meiosis, i.e. triploid ($2n = 3x = 21$) hybrids – *L. perenne* $\times$ *F. pratensis* (King *et al.* 1999), *L. multiflorum* $\times$ *F. pratensis* (Kosmala *et al.* 2006b), and *L. perenne* $\times$ *F. mairei* (Cao *et al.* 2000), and tetraploid ($2n = 4x = 28$) hybrids – *L. multiflorum* $\times$ *F. arundinacea* var. *glaucescens* (Humphreys *et al.* 2005), and these studies have been targeted almost exclusively at introgression mapping.

In the present investigation we used GISH to study chromosome pairing in six allotetraploid ($2n = 4x = 28$) F_1 hybrids *F. pratensis* ($4x$) $\times$ *L. perenne* ($4x$). This is the first detailed report of the specificity of intragenomic (intraspecific) pairing (within *Lolium* chromosomes and *Festuca* chromosomes) and intergenomic (intergeneric) pairing (between *Lolium* and *Festuca* chromosomes) in tetraploid *Festulolium* hybrids. The aim was to provide detailed information on the presence and position of *Lp* and *Fp* chromosomes in a sample of metaphase I configurations, as a rational basis for understanding the breakdown of genome integrity in allotetraploid hybrid populations.

Materials and methods

Plant materials

Tetraploid hybrids of *F. pratensis* × *L. perenne* ($2n = 4x = 28$; *FpFpLpLp*) were produced by intercrossing autotetraploid forms of both species. Spontaneous tetraploids of *F. pratensis* Huds. ($2n = 4x = 28$; *FpFpFpFp*), obtained from twin seedlings of diploid cultivars (Sulinowski *et al.* 1982), or plants of cv. Westa, were used as the female parent, and *L. perenne* L. ($2n = 4x = 28$; *LpLpLpLp*) cv. Solen as the pollinator. Immature embryos (10–14 days after pollination) were cultured on Gamborg's B₅ medium, as described by Ślusarkiewicz-Jarzina *et al.* (1994). A total of 16 F₁ hybrids were obtained and six of them, nos. 59/1/1, 59/2/1, 59/2/3, 59/3/3, 83/1/4 and 83/1/5, were used for meiotic analyses. The hybrids were produced in 1995 and 1996, and when the meiosis work was started in 2006 only 8 plants still existed. We used a sample of 6 from the four cross combinations. To determine the chromosome number and genomic constitution of F₁ plants, root-tip chromosome spreads were prepared as described by Zwierzykowski *et al.* (1999). Male fertility (pollen stainability) was analysed in a mixture of acetocarmine and glycerine (1:1). Female fertility was determined on the basis of seed set under open pollination of F₁ plants.

Preparation of slides for meiosis

For meiotic analyses, anthers at metaphase I (MI) were collected and fixed in 3:1 ethanol–acetic acid, and then placed in 1% acetocarmine for 2 hours before use. Preparations were made from pollen mother cells (PMCs) by squashing pieces of anther in 45% acetic acid. Slide preparations were then examined under phase-contrast microscopy. Slides with MI cells were placed on dry ice to flip the coverglass. Chromosome pairing and chiasma frequency were determined for 30 PMCs per genotype, after the application of GISH.

Genomic in situ hybridization (GISH)

Total genomic DNA was extracted from actively growing young leaves of *Lp* cv. Solen and *Fp* cv. Skra, as described by Doohan *et al.* (1998). *Lp* DNA

was used as a probe and labelled with digoxigenin-11-dUTP by nick translation (according to the Roche protocol). *Fp* DNA to be used as a blocker was prepared by boiling 400 µg DNA (1 µg/µl) in 0.4 mol/L NaOH for 45 min, and then combined in the hybridization mixture at a ratio of 1:60 (probe: blocker). The GISH protocol was as given in Masoudi-Nejad *et al.* (2002) with some minor modifications: the hybridization mixture was applied to the chromosome preparations (10 µl/slide) and the slides were placed on a hotplate at 76°C for 2 min to denature the chromosomes and the probe, and then kept in a moist box overnight at 37°C. The probe signal was detected by incubation with anti-digoxigenin-fluorescein (Roche) at a final concentration of 2 µg/ml at 37°C for 40 min. Chromosomes were counterstained with 2.0 µg/ml propidium iodide (PI)/Vectashield antifade solution (Vector Laboratories). Chromosome preparations were analysed using a Nikon Optiphot-2 epifluorescence microscope and photographed on Fuji 800 film.

Statistical analyses

The data concerning the number of bivalents, trivalents and quadrivalents for all PMCs of six F₁ hybrids studied were considered as binomial responses, with the appropriate totals, obtained in a one-way classification. They were analysed by the generalized linear model with logit link function to estimate mean values for plants and to test the significance of differences between plants. The numbers of chiasmata were analysed correspondingly but using a linear model with assumption of normality. All computations were done in Genstat Ed. 9 (VSN International Ltd).

Results

A total of 1082 florets of *F. pratensis* (4x) were emasculated and pollinated with *L. perenne* (4x) in five cross-combinations with using embryo rescue (Table 1). The overall crossability level was very low, and ranged from 0.47% to 4.27% (average 1.48%), for the 16 F₁ hybrids obtained. All F₁ plants had $2n = 28$ chromosomes, and the GISH analysis confirmed the presence of a complete set of 14 *Fp* and 14 *Lp* chromosomes. Twelve of the F₁ were

Table 1. Hybridization results between tetraploid ($2n = 4x = 28$) forms of *F. pratensis* and *L. perenne*

Cross no.	Cross combination		No. of florets emasculated	No. of embryos cultured ^a	No. of plants obtained	Crossability (%)
	Female parent	Male parent				
	<i>F. pratensis</i> (4x)	<i>L. perenne</i> (4x)				
59/1	ST-6/1 ^b	cv. Solen-12	214	11	1	0.47
59/2	cv. Westa-10	cv. Solen-8	259	46	3	1.16
59/3	cv. Westa-1	cv. Solen-6	240	32	3	1.25
59/4	cv. Westa-11	cv. Solen-2	205	22	2	0.98
83/1	ST-4/7 ^b	cv. Solen-11	164	24	7	4.27
	Total/mean		1082	135	16	1.48

^aImmature embryos (10–14 days after pollination) cultured on Gamborg's B₅ medium.

^bStrains derived from spontaneous tetraploids.

partially male and female fertile (pollen stainability ranged from 49.3% to 83.7%, and seed set from 4.0% to 20.2%), and four plants were completely sterile.

Analysis of chromosome pairing

Chromosome pairing and chiasma frequency at MI in 180 PMCs from six of tetraploid F₁ hybrids used were examined (Table 2; Figure 1a to l). Using GISH, it was possible to distinguish the *Lp* and *Fp* chromosomes and to record the presence and relationship of the *Lp* and *Fp* chromosomes in each configuration at MI. All the F₁ hybrids showed

extensive pairing that was characterized by the formation of bivalents (II), trivalents (III) and quadrivalents (IV). Some unpaired univalents (I) were also observed. In a total of 180 PMCs studied, 96.0% of the chromosome complement paired. The pairing was mostly as bivalents, with mean frequency ranging from 10.80 to 12.23 per cell. The maximum number of 14 bivalents occurred in 39 (21.7%) cells, with only 23 (12.8%) cells having fewer than 10 bivalents per cell (the minimum number was 3). The mean number of ring bivalents was significantly higher than that of rod bivalents, and reached the values 7.12 (6.20–7.90) and 4.39 (3.36–5.03),

Table 2. Chromosome pairing in six tetraploid F₁ hybrids *F. pratensis* (4x) × *L. perenne* (4x)

Plant no.	No. of PMCs studied	Mean and range of chromosome configurations at metaphase I						Mean and range of chiasma frequency/cell
		I	II			III	IV	
			Total	Ring	Rod			
59/1/1	30	0.87 (0–4)	10.80 (6–14)	7.43 (2–10)	3.36 (1–6)	0.60 (0–3)	0.93 (0–4)	22.97 (20–25)
59/2/1	30	1.13 (0–6)	11.67 (8–14)	7.90 (2–11)	3.77 (1–8)	0.33 (0–2)	0.63 (0–3)	22.83 (17–26)
59/2/3	30	0.57 (0–2)	12.23 (4–14)	7.60 (1–12)	4.63 (2–8)	0.23 (0–1)	0.57 (0–5)	22.33 (19–26)
59/4/1	30	0.80 (0–4)	11.06 (3–14)	6.20 (2–11)	4.86 (1–8)	0.27 (0–1)	1.06 (0–4)	21.56 (17–26)
83/1/4	30	2.07 (0–5)	11.80 (6–14)	7.10 (3–10)	4.70 (1–9)	0.20 (0–2)	0.43 (0–3)	20.83 (17–24)
83/1/5	30	1.33 (0–6)	11.50 (8–14)	6.47 (3–11)	5.03 (2–10)	0.33 (0–2)	0.67 (0–2)	21.20 (18–25)
Mean (standard error)		1.13 (0.08)	11.51 (0.10)	7.12 (0.12)	4.39 (0.12)	0.32 (0.04)	0.72 (0.06)	21.96 (0.14)
<i>p</i> -Value for lack of differences between plants		<0.001	0.006	<0.001	<0.001	0.086	0.012	<0.001

respectively. A low frequency of trivalents, average 0.32 (0.20–0.60), and quadrivalents, average 0.72 (0.43–1.06) per cell, was observed. Univalents were observed in 84 (46.7%) cells, average 1.13 (0.57–2.07) per cell. The mean chromosome configurations for six hybrids analysed were 1.13 I + 11.51 II (7.12 ring + 4.39 rod) + 0.32 III + 0.72 IV. The mean chiasma frequency per cell was 21.96 (20.83–22.97).

Differences among F_1 plants with respect to the number of univalents, bivalents and quadrivalents were statistically significant, as were the differences with respect to the mean number of chiasma events per cell. Differences among plants with respect to the number of trivalents were not significant. The variation between plants derived from one cross (59/1/1, 59/2/1, 59/2/3, 59/4/1) was no different from that for plants from the other cross (83/1/4, 83/1/5) (Table 2).

GISH analysis of specificity of chromosome pairing

The results of the pairing specificities in six F_1 hybrid plants are summarized in Tables 2 and 3. The meiotic analysis revealed predominantly intragenomic pairing, with a low frequency of intergenomic chromosome associations.

Bivalents

The mean number of *Lp/Lp* and *Fp/Fp* bivalents was almost equal, 5.41 (5.17–5.76) and 5.48 (5.13–5.87) per cell, respectively (Table 3). In both the *Lp/Lp* and the *Fp/Fp* bivalents the mean number of ring associations was higher, 3.20 (2.60–3.53) and 3.72 (3.13–4.24) per cell, respectively, than the number of rod associations, 2.21 (1.17–2.60) and 1.76 (1.36–2.17) per cell, respectively (Fig. 1a–c). Intergenomic *Lp/Fp* bivalents were found in 60 (33.3%) cells. Out of 39 (21.7%) cells with 14 bivalents, 27 cells had only intragenomic bivalents – 7 *Lp/Lp* and 7 *Fp/Fp* (Fig. 1a), and 12 remaining cells had 5–6 *Lp/Lp*, 5–6 *Fp/Fp* and 2 or 4 *Lp/Fp* bivalents. The mean number of *Lp/Fp* bivalents was 0.62 (0.27–1.00) per cell. Among the intergenomic bivalents, in contrast to intragenomic bivalents, rods occurred more frequently – on average 0.42 (0.20–0.70) per cell, than rings – on average 0.20 (0.07–0.37) per cell (Fig. 1d–f). The mean chiasma frequency per bivalent was 1.62 (1.56–1.69), but it varied for different types of bivalents: *Lp/Lp* – 1.59, *Fp/Fp* – 1.68, and *Lp/Fp* – 1.45.

Trivalents

In 46 (25.6%) cells analysed, a low frequency of trivalent formation was observed (Table 4). Fifty-nine trivalents were found, including 47 (79.7%) intergenomic (26 *Lp/Fp/Fp* and 21 *Lp/Lp/Fp*) and 12 (20.3%) intragenomic (7 *Fp/Fp/Fp* and 5 *Lp/Lp/Lp*) associations. The mean number of trivalents for both groups was 0.25 and 0.07 per cell, respectively. The maximum of three trivalents was observed in two PMCs studied, which had the following pairing configurations: 1 IV (*Lp/Lp/Fp/Fp*) + 3 III (1 *Lp/Fp/Fp* + 1 *Lp/Lp/Fp* + 1 *Fp/Fp/Fp*) + 7 II (4 *Lp/Lp* + 3 *Fp/Fp*) + 1 I (*Lp*), and 3 III (2 *Lp/Fp/Fp* + 1 *Lp/Lp/Lp*) + 9 II (4 *Lp/Lp* + 5 *Fp/Fp*) + 1 I (*Lp*). Different types of trivalents, frying pan-, chain-, V- and T-shaped were observed, but in both intergenomic and intragenomic trivalents frying pan associations occurred more frequently than other types, and in these a single *Lp* or *Fp* chromosome was mostly located in the handle. In most chain-, V- and T-shaped trivalents, a single *Lp* or *Fp* chromosome was located at the end of the configuration (Fig. 1e, f), and sporadically at the middle position between two chromosomes of *Fp* or *Lp*, respectively (Fig. 1l). The mean chiasma frequency per trivalent was 2.45 (2.14–2.71).

Quadrivalents

In 88 (48.9%) PMCs analysed quadrivalents were found (Table 4). The total number of quadrivalents was 129, including mostly intergenomic associations – *Lp/Lp/Fp/Fp* (124), *Lp/Fp/Fp/Fp* (2) and *Lp/Lp/Lp/Fp* (1), and only two intragenomic *Fp/Fp/Fp/Fp* associations. The mean number of quadrivalents per cell was 0.72 (0.43–1.06) (Table 2), and in *Lp/Lp/Fp/Fp* quadrivalents the proportion of rings and chains was similar, 0.32 and 0.37, respectively (Table 4). In both ring and chain quadrivalents, chromosomes paired mostly as ***Lp-Lp-Fp-Fp*** configurations (Fig. 1g, h, j, l), and only rarely as ***Lp-Fp-Lp-Fp*** (Fig. 1k). A maximum of five quadrivalents was observed in one PMC with the total pairing configuration of 5 IV (5 *Lp/Lp/Fp/Fp*) + 4 II (1 *Lp/Lp* + 1 *Fp/Fp* + 2 *Lp/Fp*). The mean chiasma frequency per quadrivalent was 3.48 (3.20–3.75).

Univalents

In 84 (46.7%) cells analysed there were unpaired chromosomes. In total 203 chromosomes, comprising of 4.0% of the total chromosome complement studied, remained as univalents. 122 *Lp* and 81 *Fp*

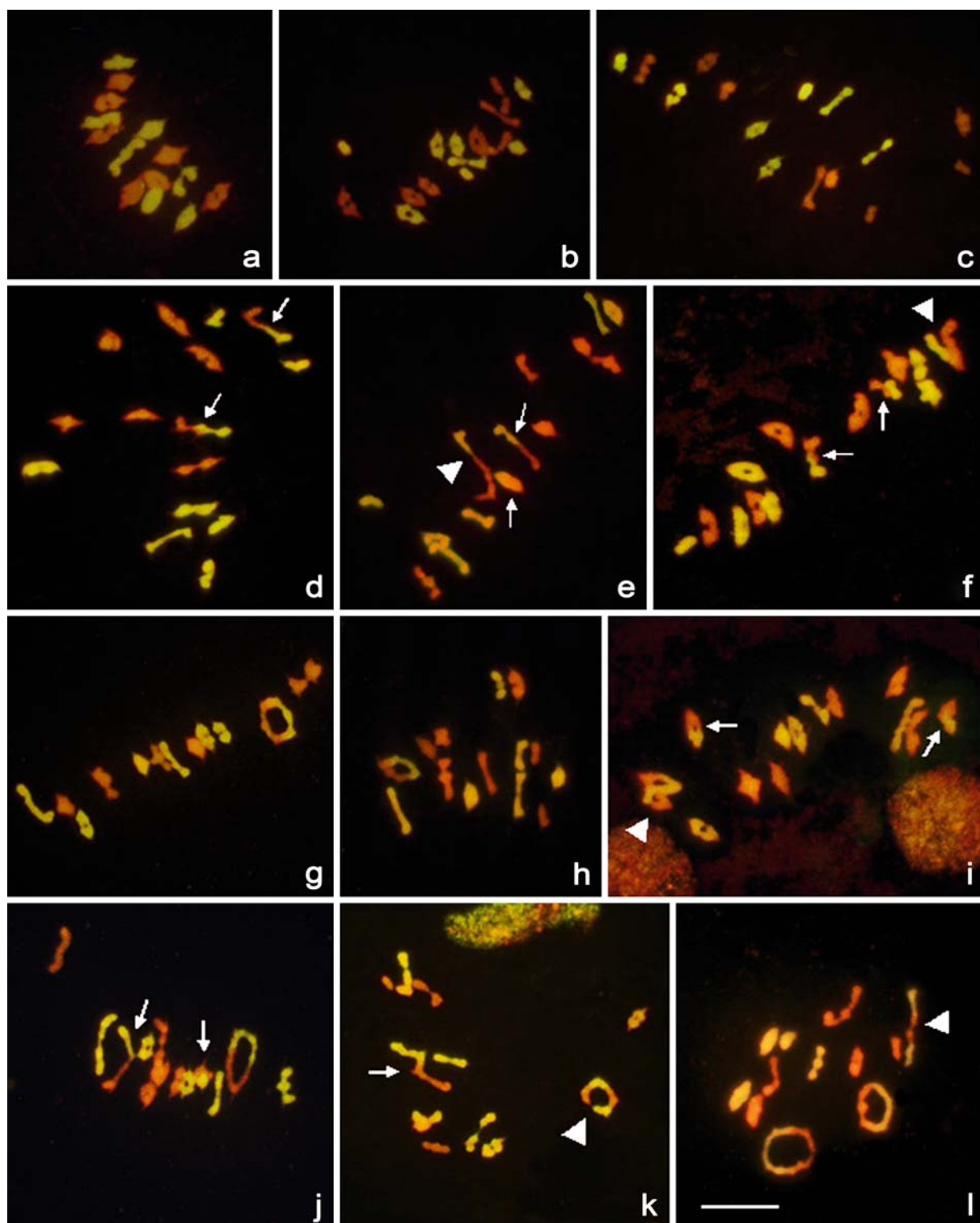


Figure 1. Chromosome associations at metaphase I of meiosis in allotetraploid (2n = 4x = 28) F₁ hybrids *F. pratensis* (Fp) × *L. perenne* (Lp). GISH images created using *Lp* genomic DNA as a probe labelled with digoxigenin and detected by anti-digoxigenin conjugated with fluorescein (yellow), with blocking genomic DNA of *Fp* (red). Chromosomes were counterstained with propidium iodide. (a) 7 Lp/Lp II + 7 Fp/Fp II. (b) 6 Lp/Lp II + 7 Fp/Fp II + 2 Lp I. (c) 7 Lp/Lp II + 6 Fp/Fp II + 2 Fp I. (d) 5 Lp/Lp II + 6 Fp/Fp II + 2 Lp/Fp II (thin arrows) + 2 Lp I. (e) 1 Lp/Fp/Fp III (arrowhead) + 5 Lp/Lp II + 5 Fp/Fp II + 2 Lp/Fp II (thin arrows) + 1 Lp I. (f) 1 Lp/Fp/Fp III (arrowhead) + 5 Lp/Lp II + 5 Fp/Fp II + 2 Lp/Fp II (thin arrows) + 1 Lp I. (g) 1 Lp/Lp/Fp/Fp IV + 6 Lp/Lp II + 6 Fp/Fp II. (h) 1 Lp/Lp/Fp/Fp IV + 6 Lp/Lp II, 5 Fp/Fp II + 2 Fp I. (i) 1 Lp/Lp/Fp/Fp IV (arrowhead) + 5 Lp/Lp II + 5 Fp/Fp II + 2 Lp/Fp II (thin arrows). (j) 1 Lp/Lp/Fp/Fp IV + 5 Lp/Lp II + 5 Fp/Fp II + 2 Lp/Fp II (thin arrows). (k) 2 IV – Lp/Lp/Fp/Fp chain (thin arrow) and Lp/Fp/Lp/Fp ring (arrowhead) + 5 Lp/Lp II + 5 Fp/Fp II. (l) 2 Lp/Lp/Fp/Fp IV + 1 Lp/Fp/Lp III (arrowhead) + 4 Lp/Lp II + 4 Fp/Fp II + 1 Fp I. Bar = 10 µm.

univalents were recorded (Fig. 1b–f and l), average 0.68 (0.37–1.17) and 0.45 (0.20–0.90) per cell, respectively.

Discussion

Diploid (2n = 2x = 14) hybrids *L. multiflorum* (2x) × *F. pratensis* (2x), and *L. perenne* (2x) × *F. pratensis* (2x) are male sterile, although they can sporadically show some female fertility (Werner 1983, Connolly & Wright-Turner 1984). It is possible to restore the fertility through chromosome doubling (by colchicine treatment) of sterile F₁

hybrids, or by crossing autotetraploid *Lolium* spp. with autotetraploid *F. pratensis* (Zwierzykowski & Rybczyński 1981, Zwierzykowski & Zwierzykowska 1994). This latter procedure was used to synthesize the allotetraploid (2n = 4x = 28) hybrids in the present work. The autotetraploid forms of *F. pratensis* and *L. perenne* can be hybridized in controlled conditions, but only with much difficulty. To obtain a good supply of F₁ hybrids it is necessary to use *in vitro* culture of very young embryos, 10–14 days after pollination (Ślusarkiewicz-Jarzina *et al.* 1994, Zwierzykowski 1996). In this study the level of crossability was still very low, and its value ranged from 0.47% to 4.27%; a low level of crossability comparable to that recorded in our earlier studies for the same cross combination (Zwierzykowski & Zwierzykowska 1994).

Diploid (2n = 2x = 14) hybrids of *L. perenne* × *F. pratensis* and *F. pratensis* × *L. perenne* are male-sterile, but in some cases they may express a low level of female fertility in backcrossing to their parental species (Jauhar 1975, 1993, and references therein; Connolly & Wright-Turner 1984, Zwierzykowski 1996, and references therein). The tetraploid (2n = 4x = 28) hybrids, in contrast, derived from reciprocal crosses of autotetraploid forms of *L. perenne* and *F. pratensis*, show both male and female partial fertility (Zwierzykowski & Zwierzykowska 1994, Zwierzykowski *et al.* 2006). In the current study, out of 16 F₁ hybrids obtained, 12 were male- and female-fertile (pollen stainability ranged from 49.3% to 83.7%, and seed set ranged from 4.0% to 20.2%), and four hybrids were male-sterile. A comparable level of male and female fertility (an average of

Table 3. GISH analysis of specificity of chromosome pairing (in bivalents) in six tetraploid F₁ hybrids *F. pratensis* (4x) × *L. perenne* (4x)

Hybrid no.	No. of PMCs studied	Mean no. of bivalents per cell ^a									Mean chiasma frequency/bivalent	
		Total	<i>Lp/Lp</i>			<i>Fp/Fp</i>			<i>Lp/Fp</i>			
			Total	Ring	Rod	Total	Ring	Rod	Total	Ring		Rod
59/1/1	30	10.80	5.17	3.53	1.64	5.13	3.77	1.36	0.50	0.13	0.37	1.69
59/2/1	30	11.67	5.43	3.50	1.93	5.64	4.24	1.40	0.60	0.17	0.43	1.68
59/2/3	30	12.23	5.76	3.23	2.53	5.80	4.17	1.63	0.67	0.20	0.47	1.61
59/4/1	30	11.06	5.20	2.60	2.60	5.16	3.23	1.93	0.70	0.37	0.33	1.57
83/1/4	30	11.80	5.66	3.26	2.40	5.87	3.77	2.10	0.27	0.07	0.20	1.60
83/1/5	30	11.50	5.20	3.03	1.17	5.30	3.13	2.17	1.00	0.30	0.70	1.56
Mean		11.51	5.41	3.20	2.21	5.48	3.72	1.76	0.62	0.20	0.42	1.62

^a*Lp* = *L. perenne* chromosomes; *Fp* = *F. pratensis* chromosomes.

Table 4. GISH analysis of specificity of chromosome pairing (in trivalents and quadrivalents) in six tetraploid *F. pratensis* (4x) × *L. perenne* (4x)

Hybrid no.	No. of PMCs studied	Mean no. of trivalents per cell				Mean chiasma frequency/ trivalent	Mean no. of quadrivalents per cell				Mean chiasma frequency/ quadrivalent		
		Total					Total						
		Lp/Lp/Fp and Lp/Fp/Fp ^a		Chain	V- and T-shaped		Lp/Lp/Fp/Fp		Ring	Chain		Fp/Fp/Fp/Fp	
		Frying-pan											
59/1/1	30	0.60	0.23	0.03	0.20	0.13	2.42	0.93	0.36	0.50	0.07	3.32	
59/2/1	30	0.33	0.20	0.00	0.07	0.06	2.71	0.63	0.39	0.24	0.00	3.75	
59/2/3	30	0.23	0.03	0.13	0.07	0.00	2.14	0.57	0.30	0.23	0.03	3.55	
59/4/1	30	0.27	0.10	0.03	0.14	0.00	2.37	1.06	0.40	0.63	0.03	3.40	
83/1/4	30	0.20	0.10	0.03	0.00	0.07	2.67	0.43	0.10	0.33	0.00	3.20	
83/1/5	30	0.33	0.10	0.06	0.03	0.13	2.43	0.67	0.37	0.27	0.03	3.60	
Mean		0.32	0.12	0.05	0.08	0.07	2.45	0.72	0.32	0.37	0.03	3.48	

^aLp = *L. perenne* chromosomes; Fp = *F. pratensis* chromosomes.

61.5% pollen stainability and 15.4% seed set) was observed in tetraploid hybrids of *F. pratensis* (4x) × *L. multiflorum* (4x) (Zwierzykowski et al. 1993).

Chromosome pairing using conventional cytogenetic analysis (acetocarmine staining) has been described in a number of interspecific and intergeneric hybrids of the *Lolium-Festuca* complex (e.g. Jauhar 1975, Lewis 1980, Zwierzykowski 1980, Springer & Buckner 1982, Kleijer 1984, Morgan et al. 1988, Humphreys 1989, Kopyto et al. 1989, Thomas & Morgan 1990, Naganowska et al. 2001). However, chromosome pairing in allotetraploids between tetraploid *Lolium* (*L. multiflorum* and *L. perenne*) and *Festuca* (*F. pratensis* and *F. arundinacea* var. *glaucescens*) species has been described only in a few cases, including *L. perenne* (4x) × *F. pratensis* (4x) (Thomas & Thomas 1973, Jauhar 1975, Osborne et al. 1976), *L. multiflorum* (4x) × *F. pratensis* (4x) (Osborne et al. 1976), *L. multiflorum* (4x) × *F. glaucescens* (4x) (Thomas et al. 1983, Ghesquière et al. 1993), and *L. multiflorum* (4x) × *F. apennina* (4x) (Thomas et al. 1983). All intergeneric *Lolium* × *Festuca* hybrids are characterized by a high degree of chromosome pairing, including the tetraploids. A high frequency of bivalent formation and regular meiosis was reported by Thomas & Thomas (1973) in *L. perenne* (4x) × *F. pratensis* (4x) hybrids, where the mean chromosome configurations per cell were 1.33 I + 11.95 II + 0.21 III + 0.55 IV, and by Ghesquière et al. (1993) in *L. multiflorum* (4x) × *F. arundinacea* var. *glaucescens* (4x) hybrids – 1.40 I + 11.02 II + 0.62 III + 0.67 IV.

These high levels of pairing in all the *Lolium-Festuca* hybrids reflect the homology between the *Lolium* chromosomes and their corresponding homoeologues in *Festuca* species, as well as the structural similarity of the chromosomes of the component genomes of the allopolyploid species (Thomas et al. 1983). These relationships hold despite the discrimination that can be achieved with GISH, and they are confirmed by the relative ease with which whole chromosomes, or parts of chromosomes, can be introgressed from one species into another, for example from *F. pratensis* into *L. perenne* (King et al. 1998, 2002a, b) or *L. multiflorum* (Zwierzykowski et al. 1999, Kosmala et al. 2006a). It seems that within this species complex the chaos, in terms of genome restructuring, which often follows when related genomes are brought together in a new situation of hybridity, does not always apply

here, although we are still lacking the detailed information on any possible DNA sequence changes which may be occurring. The most detailed studies that we do have are those relating to polyploid hybrids between *L. multiflorum* and *F. arundinacea*, where newly made octoploids restructure their genomes and revert to being diploid with somatic recombination taking place to restructure the genome, and with *F. pratensis* forming the basis of the novel diploids so produced during the process of elimination of other chromosomes from the components of *F. arundinacea* (Jones & Pašakinskienė 2005, Pašakinskienė & Jones 2005).

Humphreys *et al.* (2005) reported results of meiosis using GISH in one F_1 hybrid of *L. multiflorum* (4x) × *F. glaucescens* (4x). The meiotic analysis revealed predominantly *Lm/Lm* and *Fg/Fg* intraspecific chromosome pairing. The mean number of configurations was $2.40 \text{ I} + 9.64 \text{ II} + 0.40 \text{ III} + 1.28 \text{ IV}$. The mean chiasma frequency was 23.0 per cell, including 20.75 for intraspecific *Lm/Lm* and *Fg/Fg* associations and 2.25 for intergeneric *Lm/Fg* associations. Incidents of pairing between *Lm* and *Fg* chromosomes were found among quadrivalents (*Lm/Lm/Fg/Fg* or *Lm/Lm/Lm/Fg*), trivalents (*Lm/Lm/Fg* or *Fg/Fg/Lm*) and bivalents (*Lm/Fg*), the latter at a low frequency (0.57 per cell).

In the current study, concerning six F_1 *F. pratensis* (4x) × *L. perenne* (4x) hybrids, GISH analysis revealed predominantly intragenomic *Lp/Lp* and *Fp/Fp* chromosome pairing in bivalents. The mean number of bivalents per cell reached the value 11.51, and was almost equal for *Lp/Lp* and *Fp/Fp* configurations, 5.41 and 5.48, respectively. Intergenic pairing between *Lp* and *Fp* chromosomes were observed in 33.3% of *Lp/Fp* bivalents (0.62 per cell), in 79.7% of trivalents – *Lp/Lp/Fp* and *Lp/Fp/Fp* (0.25 per cell), and in 98.4% of quadrivalents – *Lp/Lp/Fp/Fp* and *Lp/Lp/Lp/Fp* (0.71 per cell). These data are instructive, and provide us with precise information on the level of intergenomic pairing, and the potential for recombination which can occur between the two genomes concerned.

As six F_1 hybrid plants were studied in our experiment, we were able to observe the nonhomogeneity of the hybrids with respect to the number of chromosome configurations and the number of chiasmata. Except for the number of trivalents, the hybrids were statistically not homogeneous. We cannot explain these observed differences in terms

of any characteristics of the plants, and at the moment we only ascribe these differences to genotypic variation between plants used to synthesize the hybrids. However, the results suggest that a similar experimental design could be followed in future studies on chromosome pairing within the *Lolium-Festuca* complex.

The observations fit well with earlier studies on somatic root tip cells of F_2 – F_6 plants of *F. pratensis* (4x) × *L. perenne* (4x), where the recombinant chromosomes are visible by GISH (Zwierzynski *et al.* 2006), and show how genome integrity can break down over successive cycles of open pollination. Despite the accumulation of evidence we now have in these hybrids on mechanisms leading to the erosion of genomes, we are still lacking an explanation for the basis of changes in genome balance in favour of *Lolium* over *Festuca*, and further studies on GISH might reveal how segregation of chromosomes takes place at the anaphase stages and whether there are any preferences for homologous versus homoeologous centromeres in their spindle attachments and movement to the poles. It will also be instructive to have sequence data and high-resolution maps to identify possible regions of synteny or hotspots of homology, which may offer the opportunity to facilitate intergenomic pairing and recombination. Some progress is already being made in this direction by comparative mapping between *F. pratensis*, *L. perenne* and the Triticeae (Alm *et al.* 2003). As far as the stability of cultivars (derived from *Lolium-Festuca* hybrids) is concerned, we already know that there is genetic variation for pairing preferences in certain hybrids (Evans & Davies 1983), and that selection for pairing control is one promising avenue to follow.

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