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Meiotic behaviour of individual chromosomes of *Festuca pratensis* in tetraploid *Lolium multiflorum*

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Abstract

Intergeneric hybrids of fescues (*Festuca* spp.) and ryegrasses (*Lolium* spp.) are unique for the ability of their chromosomes to pair essentially freely in meiotic metaphase I (MI). At the same time, their chromosomes can be readily recognized by genomic in-situ hybridization (GISH). Past genome-wide observations suggested that this homoeologous pairing was not completely random. In this study we extend the analysis to all seven individual chromosomes of *F. pratensis* introgressed into autotetraploid *L. multiflorum* and show that for any *F. pratensis* chromosome the choice of an MI pairing partner depends on the identity of the remaining chromosomes present in the quadruplet. In monosomic introgressions, the choice of a homologous or homoeologous partner was completely random; in disomics there was a slight preference for homologous pairing. Pairing preference was similar for each chromosome, suggesting that pairing affinity of all chromosomes is essentially the same and no structural rearrangements differentiate the two genera. Homoeologous crossover rates for individual chromosomes were similar and they were consistently lower than expected on the basis of the MI pairing. High homoeologous MI pairing in these hybrids may be due to a very permissive system of chromosome pairing control that overlooks differences between the parental chromosomes. Given the ease of genome discrimination by GISH in the *Lolium–Festuca hybrids*, the differences in repetitive DNA sequences must be substantial. On the other hand, it appears just as likely that while the DNA repeats diverged markedly during evolution, the sequences involved in chromosome pairing have been conserved enough to facilitate regular pairing partner recognition and crossing-over.

Abbreviations

AI	anaphase I
BAC	bacterial artificial chromosome
DAPI	4′,6-diamidino-2-phenylindole
FISH	fluorescent in-situ hybridization
Fp	*Festuca pratensis* Huds.
GISH	genomic in-situ hybridization
Lm	*Lolium multiflorum* Lam.
MI	metaphase I
PCR	polymerase chain reaction
PI	propidium iodide
PMC	pollen mother cell
rDNA	ribosomal DNA
SC	synaptonemal complex

Introduction

Since the early days of genome analyses in polyploids, pairing of chromosomes during meiotic

metaphase I (MI) has been used to assess genome affinity with the aim of establishing the origin and relationships of genomes in related species and identifying donors of diploid genomes in allopolyploids (Kimber 1983). Given that the success of the MI pairing is a function of DNA sequence similarity and the length of homology available for crossover establishment (Shen & Huang 1986, Sant'Angelo *et al*. 1992, Datta *et al*. 1997), more closely related chromosomes are more likely to pair in a hybrid. Although this approach is subject to several assumptions, such as the absence of genetic systems that may control pairing stringency, and the absence of structural differences that make proper synapsis impossible, it was successfully used to identify diploid donor species in a wide range of allopolyploids, from *Triticum* and *Aegilops* to *Brassica* and *Gossypium* (for recent review, see Jenczewski & Alix 2004). This general approach has been extended to different ploidy levels and genome configurations, and numerical models for testing genome affinities have been developed (Alonso & Kimber 1981, Kimber & Alonso 1981). The critical factor in these analyses is the chromosome competition for pairing partners. Hence, a diploid wide hybrid may not be informative in the context of genome affinity, as in absence of competition for pairing partners homoeologous bivalents may form with frequencies much higher than their true affinity would suggest or permit (Jauhar & Joppa 1996).

Wide hybrids in the *Lolium–Festuca* complex are a notable exception to the general rule that homoeologues from more closely related species pair more readily than those from more distantly related species (but as the MI pairing is a measure of phylogenetic distance, the argument is somewhat circular). In intergeneric hybrids *Lolium* × *Festuca*, chromosome MI pairing is usually high, approaching the levels of homologues (for review, see Jauhar 1993). For this very reason these hybrids have been of considerable interest to cytogeneticists for many years. Unfortunately, there are only a few reports analysing phylogenetic relationships between *Festuca* and *Lolium* using DNA polymorphism (Charmet *et al*. 1997, Catalan *et al*. 2004), and no comparison at the whole-genome level has been done, so the actual phylogenetic distance of the genera is somewhat ambiguous. It can be argued that classification of the taxa into separate genera is correct: chromosomes of ryegrasses and fescues must be so different at the DNA level that the most rudimentary method of in-situ hybridization with the total genomic DNA readily discriminates the genomes, chromosomes and even very small chromosome segments (Humphreys *et al*. 1995, Canter *et al*. 1999, Zwierzykowski *et al*. 1999). Because this method (called genomic in-situ hybridization, GISH) relies on gross differences in dispersed repetitive DNAs, there can be little doubt of chromosome divergence. On the other hand, almost complete or complete MI pairing, with regular crossing-over, suggests that either the system of chromosome pairing control is very lax, permitting crossing-over between highly divergent DNA sequences, or that sequences involved in crossing over in the *Lolium–Festuca* complex are highly conserved.

Still, some effort has been invested over the years to quantify the affinity of the entire genomes of *Lolium* and *Festuca* (Jauhar 1975, Evans & Davies 1985). Here we attempt to extend this effort to individual chromosomes tested for their meiotic pairing affinity in different configurations and proportions.

Material and methods

Plant material

Single chromosome substitutions of *F. pratensis* Huds. into tetraploid *L. multiflorum* Lam. cv. 'Mitos' were developed at University of California, Riverside, USA, by backcrosses from triploid F_1 hybrids with genomic constitution LmLmFp, (where Lm and Fp are used to denote the genomes of *L. multiflorum* and *F. pratensis*, respectively) to tetraploid *L. multiflorum* cv. 'Mitos'. The F_1 hybrids and BC_1 progenies were produced and kindly provided by Dr Z. Zwierzykowski (Institute of Plant Genetics, Poznan, Poland). These BC_1 plants were individually screened by GISH according to Masoudi-Nejad *et al*. (2002); selected plants were grown and backcrossed to cv. 'Mitos' again. This process was repeated until all seven Fp chromosomes were identified as individual single chromosome introgressions in tetraploid *L. multiflorum*. The selected $BC_{4–5}$ plants containing the same single complete Fp chromosome were intercrossed in isolation from all other hybrids, their progenies were screened by GISH, and disomic and other types of introgressions for this study were selected.

Tetraploid F_1 hybrids *F. pratensis* × *L. multiflorum* were also made available for this study by Dr Z. Zwierzykowski and the specimens from his field-grown plants were collected in May 2006. Dr Zwierzykowski also provided samples of a commercial Festulolium cultivar 'Sulino' and tetraploid cultivar 'Mitos' of *L. multiflorum*, while Dr V. Cernoch from the Plant Breeding Station Hladké Zivotice, Czech Republic, kindly provided samples of Festulolium cv. 'Spring Green' and tetraploid cultivar 'Patra' of *F. pratensis* (Table 1). Plant material obtained as seed was established at the Institute of Experimental Botany, Olomouc, Czech Republic, and sampled for this study as needed.

Chromosome constitutions of analysed plants

A total of 48 plants of hybrid origin were analysed at meiotic metaphase I (MI). Among these there were 40 plants of tetraploid *L. multiflorum* with introgressions of Fp chromosomes, in various combinations, two tetraploid F_1 plants with chromosome constitution FpFpLmLm, and six plants were selected from commercial cultivars of Festulolium (Table 2). In addition, meiotic pairing was scored in two plants of autotetraploid *F. pratensis* cv. 'Patra' and three plants of autotetraploid *L. multiflorum* cv. 'Mitos'.

The 40 introgression plants represented various constitutions of all seven different chromosomes of *F. pratensis*. As only two chromosomes (2 and 3) out of seven could be assigned unequivocally to homoeologous groups (M. W. Humphreys *et al*., personal communication) we used letter designations of chromosomes throughout the study. Out of the 40 introgression plants, 17 were monosomic introgressions of chromosomes labelled A, B, C, D, F, G and N, and 15 plants were disomic introgressions of chromosomes A, C and N. The other three plants had one pair of a complete Fp chromosome plus another copy of the same chromosome recombined in one arm (this means that there were two pairs of homologues for one arm and three homologues and

Table 1. Pairing of individual introgressed *F. pratensis* chromosomes in different *L. multiflorum* × *F. pratensis* hybrids

Genotype	No. of plants/PMCs	Homologous pairing[a]	Homoeologous pairing[a]	Expected ratio
F_1	2/50	3.02	0.21	1:2**
A′	5/474	1.80	1.70	1:1
A″	6/253	1.57	1.92	1:2**
A+T_A	2/104	1.38	1.63	5:7
A″+T_A	1/48	1.10	2.23	5:7*
B′	2/100	1.35	1.36	1:1
B+T_B	2/150	1.62	2.07	5:7
C′	3/177	1.85	1.64	1:1
C″	5/234	1.29	1.91	1:2**
D′	1/25	1.60	1.76	1:1
F′	2/82	1.54	1.50	1:1
F+T_F	1/50	1.22	1.34	5:7
G′	1/35	1.86	1.34	1:1*
N′	3/300	1.78	1.73	1:1
N″	4/167	1.48	2.16	1:2**
N″+T_N	2/99	1.57	1.88	5:7
Chromosome B				
Sulino 197	1/50	2.04	1.82	1:1
Spring Green 483	1/50	2.74	0.94	3:1
Chromosome N				
Sulino 387	1/50	1.54	2.06	1:1
Sulino 401	1/50	2.88	0.82	3:1
Spring Green 516	1/38	1.76	1.42	1:1

*Significant deviation from the expected proportion of homologous : homoeologous pairing at $p \leq 0.05$.

**Significant deviation from the expected proportion of homologous : homoeologous pairing at $p \leq 0.01$.

[a]Homologous, respectively homoeologous, pairing is represented by the number of paired arms within an individual quadruplet with introgressed Fp chromosome(s) in homologous, respectively homoeologous, manner. The total of 4 paired arms can be detected within one quadruplet in case of complete pairing.

Table 2. Frequency of homoeologous recombination *Lolium-Festuca* among progeny from monosomic introgressions backcrossed as female to cv. 'Mitos' or intercrossed to generate disomic introgressions

Chromosome present	Type of progeny	Number of plants	Number of single exchanges	Number of double exchanges	Crossover rate per gamete
A	Backcross	48	21	1	0.48
A	Intercross	84	75	9	0.55
B	Intercross	108	118	3	0.57
C	Intercross	40	37	3	0.54
F	Intercross	57	74	4	0.72
N	Backcross	36	20	1	0.61
N	Intercross	44	40	1	0.47

one homoeologue of the other arm of the same chromosome) for chromosomes A and N. Moreover, five plants had a single complete chromosome of Fp and a translocation of the same chromosome (this means two pairs of homologues in one arm and three homologues and one homoeologue for the other arm of the same chromosome) for chromosomes A, B and F.

Six plants of Festulolium selected for this study represented two contrasting genotypes: cv. 'Spring Green' has a low level of *Festuca* chromatin introgression, whereas cv. 'Sulino' has about equal proportions of the parental genomes (Kopecký *et al*. 2006). All analysed plants of cv. 'Spring Green' were euploid with 28 chromosomes. Of the three plants of cv. 'Sulino' analysed, two had 28 and the other 30 chromosomes.

Chromosome preparations

For meiotic analyses, plants with various introgressions of Fp chromosomes were sampled in Riverside, CA, USA; the tetraploid F_1 hybrids *FpFpLmLm* were sampled in Poznan, Poland; and those of Festulolium cultivars in Olomouc, Czech Republic. Individual anthers, confirmed to be in MI, were fixed in the Carnoy's solution I (3:1 absolute ethanol–glacial acetic acid) at 37°C for 7 days. Meiotic metaphase spreads were prepared from anthers according to Masoudi-Nejad *et al*. (2002).

Fluorescence in-situ hybridization (FISH)

GISH and FISH were done according to Masoudi-Nejad *et al*. (2002). For GISH, the total genomic DNA of *F. pratensis* was labelled with digoxigenin using DIG-Nick Translation Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's recommendation and used as a probe. Total genomic DNA of *L. multiflorum* cv. 'Mitos' was sheared to 200—500 bp fragments and used as a block. In all experiments, the probe to block ratio was about 1:150. The sites of probe hybridization were detected by the Anti-DIG-FITC conjugate (Roche Applied Science) and by the streptavidin-Cy3 conjugate (Amersham, Piscataway, NJ, USA).

For FISH, probes were made of 5S and 45S rDNA, and of a BAC 1G18 clone of *F. pratensis*. The 5S rDNA probe was prepared by PCR with a pair of specific primers (RICRGAC1, RICRGAC2) which amplify a 303 bp fragment in rice (Fukui *et al*. 1994), in the presence of biotin labelled nucleotides and the rice genomic DNA as a template. For the 45S rDNA probe, clone pTa71 (Gerlach & Bedbrook 1979) containing a 9 kb *Eco*RI fragment of wheat ribosomal DNA was labelled with biotin by nick translation. This 9 kb fragment carries the 18S-5.8S-26S cluster of ribosomal RNA genes (here referred to as 45S rDNA). BAC 1G18 was selected from a partial *F. pratensis* BAC library, made available for this study by Dr H. Šimková (Institute of Experimental Botany, Olomouc, Czech Republic). Among 74 clones tested, clone 1G18 was the only one producing specific hybridization patterns on each *F. pratensis* chromosome and showing virtually no cross-hybridization to *L. multiflorum* chromosomes. DNA of clone 1G18 was isolated and labelled by digoxigenin using the DIG-Nick Translation Kit (Roche Applied Science) according to the manufacturer's recommendation.

Following FISH or GISH, chromosomes were counterstained either with 1.5 μg/ml propidium iodide (PI) or 1.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in the Vectashield antifade solution (Vector Laboratories, Oberkochen, Germany). Observations were made under a Zeiss Axioscope 20 equipped with epifluorescence, recorded with a SPOT

RT Color digital camera (Diagnostic Instruments Inc., Oberkochen, Germany), and processed using the SPOT Advanced and Adobe Photoshop v. 6 software.

Identification of individual F. pratensis chromosomes

Individual Fp chromosomes introgressed into *L. multiflorum* were identified based on their overall morphology (total length, arm ratio, presence of secondary and tertiary constrictions), the presence/absence of the 5S rDNA and 45S rDNA loci, and the hybridization pattern with BAC 1G18. The 5S and 45S rDNA loci in *F. pratensis* are located on chromosomes B and N, or chromosomes 2 and 3 according to the Triticeae numbering system (Thomas *et al.* 1997). Additionally, chromosomes A and C failed to pair with each other in MI of a hybrid and were always involved in different quadrivalents.

Scoring of the MI chromosome pairing configuration

Chromosome pairing was scored only in MI. Whenever possible, 50–100 pollen mother cells (PMCs) were scored on each preparation. The standard measure of pairing was 'arms paired per chromosome'(abbreviated as appc); a chromosome in a ring bivalent has both arms paired (2.0); one arm pairs in a rod bivalent (1.0), and a univalent has zero arms paired. This scoring method does not discriminate between pairing in bivalents or quadrivalents. Therefore, to detect any possible effects of the Fp introgressions on general pairing in the analysed plants, the overall levels of MI pairing were scored in subsamples of PMCs, and the frequencies of quadrivalents were noted, including the pairing status of quadruplets involving the introgressed chromosome(s). For a particular quadruplet, pairing is always expressed in terms of the labelled Fp chromosome. Depending on the chromosome constitution of each plant analysed, the introgressed chromosome may only be involved in homoeologous pairing (as in a monosomic introgression), or be involved in homologous or homoeologous pairing (as in a disomic introgression).

The measure of 'arms paired per chromosome' ignores possible multiple crossovers per arm. The estimate of the frequency of such crossovers was based on the frequency of chromosome arms with more than one Fp-Lm translocation breakpoint recovered among progeny. The progenies were produced either by backcrosses to *L. multiflorum* using the *F. pratensis* chromosome-bearing plants as female, or by intermating plants with the same introgression. Given that a double crossover need not involve the same two chromatids, the frequencies of such multiple translocation arms were converted into MI chiasma frequencies by multiplication by a factor of 4.

Results

Identification of individual F. pratensis chromosomes

To ensure that the MI pairing data were indeed for different chromosomes, each of the seven Fp chromosomes had to be reliably identified. However, until this work the molecular karyotype of *F. pratensis* was not available. In our study, FISH with a set of three DNA probes enabled identification of individual chromosomes and establishment of the karyotype (Figure 1). Chromosome A is the longest

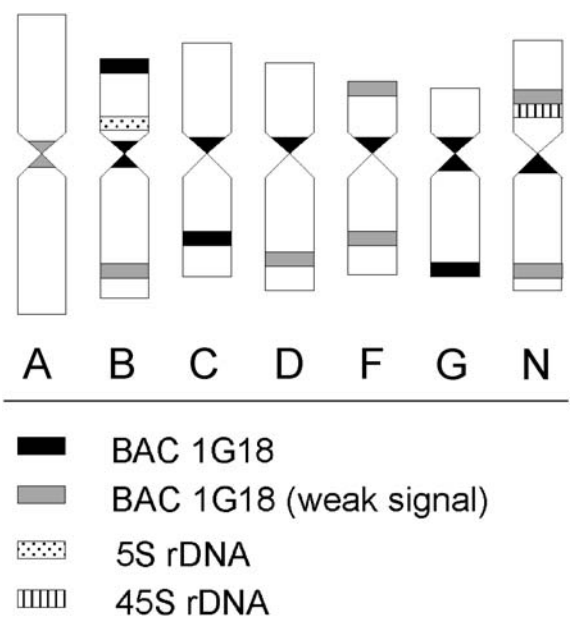


Figure 1. Identification of individual chromosomes of *F. pratensis* in the *L. multiflorum* background based on chromosome length, arm ratio and the pattern of hybridization with probes for 5S rDNA, 45S rDNA and BAC 1G18. Chromosomes are drawn to scale.

in the genome and the most metacentric, with a minor centromeric band of BAC 1G18. Chromosome B is of medium length, metacentric, and carries the 5S rDNA locus. Chromosome C is long, almost metacentric with a strong signal of BAC 1G18 distally located on the long arm. Chromosome D is a medium length submetacentric with a strong centromeric signal of BAC 1G18 and a minor one on the long arm. Chromosome F is short with a high arm ratio (ca. 1.6) and with BAC 1G18 signals on the long arm, in the centromere and at the distal part of the short arm. Chromosome G is the shortest in the karyotype with the highest arm ratio (ca. 2.0); it has a centromeric band of BAC 1G18 and another one at the end of the long arm. Chromosome N is long, almost metacentric, with a clear secondary constriction that has the 45S rDNA locus.

Overall chromosome pairing

The average MI pairing in tetraploid *L. multiflorum* was 1.84±0.11 appc (mean±S.D.) and the frequency of quadrivalents was 4.89±1.24 per PMC. The remaining chromosomes were paired mostly in bivalents; trivalents and univalents were rare (data not shown). In autotetraploid *F. pratensis*, the average MI pairing was lower (1.66±0.20 appc) with a corresponding lower frequency of quadrivalents (3.87±1.55). In the introgression lines (all disomic and monosomic plants for chromosomes A, C and N), the overall level of pairing did not significantly deviate from the *L. multiflorum* control, ranging from 1.78±0.13 to 1.85±0.11 appc, with the average number of quadrivalents per PMC ranging from 4.50±1.32 to 5.09±1.29. No significant plant-to-plant differences were observed. The overall pairing in the F_1 hybrids FpFpLmLm (1.61±0.13 appc and 0.68±0.93 quadrivalents per PMC) was significantly lower than in the Lm introgression lines. As these figures indicate, pairing was mostly in bivalents. The overall pairing levels among individual plants of the two cultivars sampled were more variable, ranging from 1.56±0.16 to 1.92±0.07 appc, with a parallel variation in the frequency of quadrivalents per PMC, ranging from 3.08±1.43 to 5.78±0.99.

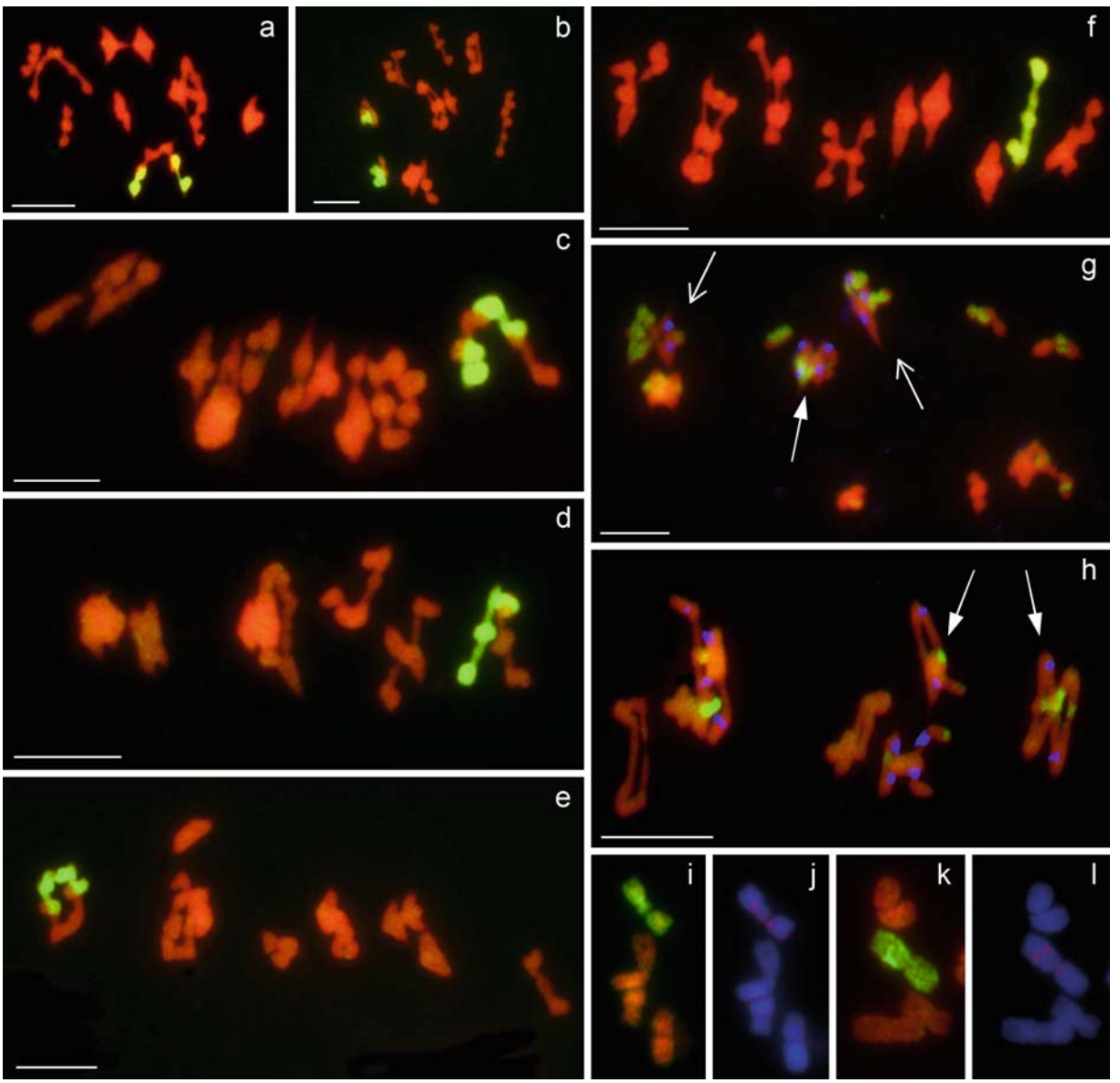
MI pairing of individual F. pratensis chromosomes

In a tetraploid, meiotic behaviour of one or more copies of an introgressed Fp chromosome can be evaluated within the context of four chromosomes (quadruplet) of which some are homologues and some are homoeologues. In a monosomic introgression, the quadruplet of chromosomes containing the introgressed chromosome consists of three homologues and one homoeologue. In a disomic introgression, there are two pairs of homologues present. In a plant with one complete and one translocated Fp chromosome, there are two pairs of homologous arms, and three homologous copies plus one homoeologous copy of the other arm. Four chromosomes of the quadruplet containing the introgression can pair only homologously, or in any combination of homologously and homoeologously. The expected ratios of homologous to homoeologous pairing depend on the chromosome constitution of the

Figure 2. The analysis of chromosome pairing during meiotic metaphase I in *L. multiflorum* × *F. pratensis* substitution lines **(a–f)** and hybrid cultivars (*L. multiflorum* × *F. pratensis*) 'Perun' **(g)** and 'Spring Green' **(h)** using GISH and FISH. Genomic DNA of *F. pratensis* was labelled with digoxigenin and detected with anti DIG-FITC (green colour), and genomic DNA of *L. multiflorum* was used as blocking DNA (no label). The chromosomes were counterstained with DAPI (red pseudocolour). In (g) and (h), biotin-labelled probe for 45S rDNA was also used and detected by streptavidin-Cy3 (blue pseudocolour). **(a–f)** Disomic substitution line of chromosome A with different configurations in metaphase I. Two homoeologous chromosomes are involved in ring **(e)** and rod quadrivalents **(a, c, d)** and in ring and rod bivalents **(b, f)**. Only homoeologous pairing **(b, c)**, only homologous pairing **(f)**, and both homologous and homoeologous pairing **(a, d, e)** was observed within quadruplet of this chromosome. **(g)** Chromosome pairing during metaphase I in a hybrid cultivar 'Sulino' (Sulino 387). The presence of the 45S rDNA locus enabled to study the pairing of chromosome N (chromosome 3 in the Triticeae numbering system), in a quadruplet consisting of three chromosomes of *Lolium* origin and one chromosome of *Festuca* origin (arrow). There are two more chromosome quadruplets, which are identified by presence of rDNA loci in only *Lolium* homoeologues (open arrows). **(h)** Chromosome pairing during metaphase I in a hybrid cultivar 'Spring Green' (Spring Green 550). Pairing of chromosomes resulted in two quadrivalents (arrows), in which only two chromosomes carry a 45S rDNA locus. Note that quadrivalents of homo(eo)logous chromosomes are expected to carry four 45S rDNA loci (one per each chromosome). This was probably caused by large reconstruction of the hybrid genome. **(i–l)** Identification of individual chromosomes using BAC FISH and GISH. FISH with a BAC clone 1G18 was used to identify chromosome F **(i, j)** and chromosome C **(k, l)** of *F. pratensis* origin. Sites of probe hybridization were detected using Cy3 (red signals). The chromosomes were counterstained with DAPI (blue colour). After BAC FISH, slides were washed and reprobed with genomic DNA of *F. pratensis* (green colour) and counterstained with DAPI (green pseudocolour). Sheared unlabelled DNA of *L. multiflorum* was used as blocking DNA. Scale bar in each image represents 10 μm.

quadruplet. Assuming random pairing, the expected ratio of homologous to homoeologous pairing in a monosomic introgression is 1:1 (of the three Lm chromosomes, two will pair homologously, leaving the third one free to pair homoeologously with the chromosome of Fp). In a disomic introgression, this ratio is 1:2, and in a plant with one complete and one translocated chromosome it is 5:7 (arms). The combination with two complete and one recombined chromosome is an inverse of one complete and one translocated chromosome.

Among the monosomic introgressions of all seven different Fp chromosomes, only the chromosome G deviated significantly from the 1:1 ratio of homologous to homoeologous pairing as expected for random selection of a pairing partner. When the frequencies for all seven individual chromosomes were combined, the deviation from random pairing was not significant. On the other hand, in each of the disomic introgressions (chromosomes A, C and N), significant deviations from the expected 1:2 ratio were present in favour of homologous pairing.



Introgressions of one or two complete and one recombined *F. pratensis* chromosome were of intermediate character, in that in some combinations there was a statistically significant deviation from random in favour of homologous pairing while in other combinations pairing was random (Table 1).

The largest deviation from the assumed random pairing of homologues and homoeologues was observed in FpFpLmLm F_1 hybrids. The ratio of homologous vs. homoeologous pairing was 1:14.5 for the entire genome. For chromosome N, which could be scored individually due to the presence of the 45S rDNA locus, the ratio was 1:6. Both for the entire genome, and for chromosome N, this deviation from random in favour of homologous pairing was highly significant ($p \leq 0.01$).

Among the analysed cultivars, one plant of cv. 'Spring Green' (Spring Green 516) had an introgression of a complete Fp chromosome N with the remaining three chromosomes in the quadruplet, with chromosome N being normal, non-recombined chromosomes of *L. multiflorum*. Hence, this plant, as far as this specific homoeologous group is concerned, was a monosomic introgression. Pairing within this quadruplet did not deviate from the 1:1 ratio of homologous to homoeologous pairing expected for random selection of pairing partners. The second plant (Spring Green 483) had an introgression of one arm of the Fp chromosome B with the remaining three chromosomes in the quadruplet being normal chromosomes Lm. For this specific Fp arm, there was no significant deviation from random pairing. The third plant (Spring Green 550) did not have a complete Fp chromosome or chromosome arm and was included in the study because of an unusual behaviour of NOR chromosomes. The plant had 12 chromosomes with 45S rDNA loci, all of *L. multiflorum* origin. Given that the plant was tetraploid, a maximum of three quadrivalents with the probe signal were expected, but frequently the 45S rDNA loci were observed in four distinct configurations, including instances of four quadrivalents (Figure 2h).

One plant of cv. 'Sulino' (Sulino 197) had translocated Fp segments on the quadruplet that included the Fp chromosome B, one on the short arm and the other on a long arm. There was no significant deviation from random MI pairing in this quadruplet. The second plant (Sulino 387) had an introgression of a single chromosome N of Fp. Again, pairing within the quadruplet, which included chromosome N, was random. The last plant (Sulino 401) had 30 chromosomes. In its N-chromosome homoeologous group, there were three complete Fp chromosomes and the fourth chromosome was an Lm-Fp translocation. The observed ratio of homologous vs. homoeologous pairing in this quadruplet did not significantly differ from random.

Homoeologous recombination of individual F. pratensis chromosomes

The frequency of homoeologous recombination was scored in 417 progeny plants obtained either after backcrosses of monosomic single chromosome introgressions as females to *L. multiflorum* (84 plants) or after intermating of these monosomic introgressions to generate disomic introgressions (333 plants). In a great majority of cases, there was a single translocation breakpoint per arm. Given that in an intercross-generated progeny, both gametes can contribute a recombined chromosomes while in the backcross only the egg cell could, the average recombination rate for individual lines ranged from 0.47 exchanges per chromosome per gamete for an intercross of chromosome N monosomics to 0.72 exchanges per chromosome per gamete for the intercross of chromosome F monosomics (Table 2).

The frequency of double exchanges per arm was low, with only 22 such chromosome arms recorded among 707 gametes that contributed to the analysed progeny sample, which corresponds to 3% double exchanges per chromosome per gamete, on average. In three instances, the hybridization pattern of the total genomic probe suggested very strongly that both products of the same double exchange were recovered in the same progeny. Such an event is possible with adjacent segregation from a quadrivalent. If indeed this was the case, the overall frequency of double crossovers was only about 2%. Consequently, the average frequency of double chiasmata per arm in the studied material must have been between 8% and 12%.

Discussion

It is obvious that the MI pairing success of chromosomes does not depend only on adequate DNA sequence homology and that structural chromosome aberration may affect MI pairing. In a well illustrated

case, individual arms of rye chromosomes paired with wheat homoeologous arms with frequencies ranging from 0% to 26% (Naranjo & Fernandez-Rueda 1991). These differences were clearly related to structural rearrangements that differentiate the wheat and rye genomes. Owing to ancient translocation, the terminal segment of the rye chromosome arm 2RS is homoeologous to wheat group-6 chromosomes (Devos *et al*. 1993), and as a consequence the entire 2RS arm practically does not pair with wheat group 2S arms; 2RL is co-linear with wheat and pairs 26% of the time. The same holds true for homoeologues present in wheat, where chromosome 4A is involved in a double translocation and three inversions (Devos *et al*. 1995), which prevent it from pairing with either 4B or 4D. In wheat, long segments of perfect homology, at times covering up to 3/4 of a chromosome arm's length, may be insufficient to support any MI pairing if the terminal segments are structurally different (Curtis *et al*. 1991, Lukaszewski 1997).

Barring structural differences between homologues that can make pairing initiation impossible, the MI pairing frequency in hybrids is a good reflection of genome affinity and hence of species divergence. In this respect, genera *Festuca* and *Lolium* are unique. Despite their phylogenetic distance, their hybrids show almost complete MI (hence chiasmate) pairing of homoeologues. With the standard system of measuring species divergence, based on their chromosome ability to pair, these two genera could be considered very closely related, if not identical. However, as even the most rudimentary methods of in-situ probing with total genomic DNAs attest, the chromosomes of the two genera are indeed distinct.

The use of GISH relies primarily on dispersed repetitive DNA sequences (Schwarzacher & Heslop-Harrison 2000): the greater the difference in the relative proportions of different repeats the better the discrimination between parental genomes in their hybrids. Labelled DNA probing as a tool in the studies of chromosome behaviour in the *Lolium* × *Festuca hybrids* is relatively recent (Thomas *et al*. 1994) and it is yet to produce a substantial body of data. All information on the meiotic chromosome behaviour in these hybrids is derived from scoring general levels of meiotic metaphase I pairing, or of pachytene synapsis across the entire genome. Here the results may be unreliable and interpretations may vary. In particular, pachytene scoring may produce misleading results as the synaptonemal complex (SC) formation is indifferent to homology (Loidl 1990) and capable of extensive formation even in the absence of homology, for example in a haploid (Gillies 1974).

In many diploid *Lolium* × *Festuca* hybrids, bivalent frequency may be similar to that in the parental species themselves, suggesting a high pairing affinity of homoeologues (Jauhar 1975). In tetraploid hybrids, where in the process of selection of a pairing partner each chromosome faces a choice of its homologue or of either of the two homoeologues, the presence of multivalents or heterogeneous bivalents may be used as a direct measure of affinity. However, published reports on the frequencies of quadrivalent associations may produce contrasting results (Ahloowalia 1967, De Roo 1968, Lewis 1980) perhaps because the frequency of bivalent and quadrivalent associations in MI may be affected by several factors. First, this process is likely under genetic control. In *L. perenne*, Simonsen (1973) postulated a multigene system controlling chiasma frequency with each gene having a minor individual effect. The same principle of genetic control of chromosome pairing is apparent in the study of Aung and Evans (1985), who managed to manipulate the level of multivalent formation using appropriate parental genotypes. In this study, there were no significant differences in the overall pairing levels among the introgression lines, suggesting that individual Fp chromosomes did not introduce a genetic system of pairing control. Aung and Evans (1985) also did not observe significant deviations in pairing indices (arms paired per chromosome; frequency of quadrivalents) between autotetraploid *L. multiflorum* (such as the one used as a control in the study) and individual introgression lines, suggesting in their set of materials that chromosomes of *F. pratensis* also do not introduce any major chromosome pairing control factors that would affect tetraploid *L. multiflorum* to any detectable level.

As only chiasmatic MI pairing is of a direct genetic consequence in terms of chromosome reduction and recombination, the configurations formed in the SC stages are of relatively little consequence (see Gillies 1974). However, in the *Lolium–Festuca* complex, the SC stages were frequently studied (Crowley & Rees 1968, De Roo 1968, Jenkins & Rees 1991, Thomas & Thomas 1994). Thus, Thomas (1995) observed preferential homologous pairing of

L. multiflorum chromosomes in tetraploid hybrids of *L. temulentum* × *L. multiflorum*. Morgan (1990) was even able to discriminate the parental chromosomes according to their length in *L. multiflorum* (Lm) with *F. donax* Lowe (Fdo) and *L. multiflorum* with *F. drymeja* Mert. et W. D. J. Koch (Fdr) hybrids. Non-homologous bivalents of large *F. donax* chromosomes were present in 54% of PMCs in an LmLmFdo hybrid. However, in tetraploid hybrids LmLmFdoFdo, mostly symmetrical bivalents were present, suggesting mostly homologous pairing.

These and other studies, based on genome-wide observations, either in MI or in the pachytene, indicate that in the absence of competition for pairing partners, there is little pairing preference for homologues over homoeologues in wide hybrids of *Lolium* × *Festuca*. In this study, we have considerably increased the resolution of the analysis and show that indeed, this principle extends to single chromosomes and even single chromosome arms. Where there is a choice between a homologous and homoeologous pairing partner, there is a low preference in favor of pairing with a homologue. For each individual chromosome of *F. pratensis* that we analyzed, this amounted to only about 15% preference, assuming that zero preference would indicate completely random pairing and 100% preference would limit pairing to strictly homologous. However, when the entire genomes were present, such as in the tetraploid F_1 hybrids FpFpLmLm, this minor preference for individual chromosomes becomes a clearly identifiable factor for the entire genome, producing a highly significant increase in bivalent pairing at the expense of quadrivalents (Table 1). This is the same effect as that observed on the genome-wide basis in the LmLmFdoFdo hybrids described by Morgan (1990).

The absence of any significant variation in the meiotic behavior of individual chromosomes, (we have tested all seven Fp chromosomes), is a strong indication that there are no major structural differences separating the Fp and Lm genomes. This supports earlier observations on this subject, based on the synaptonemal complex formation (Jauhar 1993) and the frequencies of homoeologous recombination in the early generations of intergeneric hybrids (Zwierzykowski *et al*. 1999). Our observations also show that there is no quantitative difference in the meiotic behavior between experimental materials, such as our introgression lines, and commercial cultivars selected for agronomic performance, including high seed production and hence regularity of meiosis and fertility.

The chromosomes present in introgression lines were never completely limited in their choice of pairing partners by the choices made by the other chromosomes present in the quadruplet. Such a limit would be imposed by limiting crossovers (chiasmata) to single ones per arm. Any screening of wide *Lolium* × *Festuca* hybrids clearly shows that multiple crossovers per arm are possible, hence pairing partner exchanges are possible as well. For example, in a monosomic introgression, even a ring bivalent of two homologues need not preclude the third from homologous pairing leaving it to pair only with the Fp homoeologue. A frying pan trivalent can be formed if two chiasmata per arm are permitted. We have attempted to estimate the frequency of such multiple crossovers per arm and arrived at a range of ca. 8 to 12%. The range was based on the frequency of progeny chromosomes with two translocation breakpoints per arm. Confounding was the presence, in the same plants, of pairs of translocated Fp-Lm chromosomes with mirror probe-block patterns. Such pairs very likely represented both products of a crossover event. Given that segregation of chromosomes in anaphase I (AI) was predominantly from quadrivalents, and 'adjacent' orientation in MI directs both chromosomes bound by a chiasma to the same AI pole, presence of such pairs was not unexpected. This raises the issue of single crossovers: if both products of a double crossover end up in the same gamete with a noticeable frequency, both products of single exchanges would be expected to do so more frequently, generating even a greater error in the estimates of homoeologous recombination in these hybrids. We did not attempt to quantify this effect for single crossovers: while the hybridization patterns for double crossovers are unique and easily recognizable, the error for single crossovers would likely be considerable. Unfortunately, we did not study the orientation of quadrivalents with sufficient detail to discuss this issue further but it appears clear that the frequencies of homoeologous recombination in these materials are overestimated by the frequency of adjacent segregation from quadrivalents. Given that the crossover rates as listed in Table 2 must be overestimated, it is surprising that in all instances they are still lower than one half of the observed MI homoeologous pairing rates. It is not

clear why such a discrepancy exists. In *L. perenne* × *F. pratensis* hybrids, King *et al*. (2002) have convincingly demonstrated a 1:1 correspondence between the MI chiasma frequency (equivalent to our measure of 'arms paired per chromosome') and recombination frequency as measured by the translocation breakpoints among progeny.

In summary, this study extends earlier studies on the MI chromosome pairing in the *Lolium* × *Festuca* hybrids and confirms that despite their phylogenetic distance, chromosomes show only a minor preference for homologous pairing when faced with a choice between two options: homologous vs. homoeologous. When such options are not available, pairing is perfectly random. This pattern was observed for all seven chromosomes of *F. pratensis* so it appears to be a genome-wide feature. The reason for the observed behavior is not clear. High homoeologous MI pairing in these hybrids may be due to a very permissive system of chromosome pairing control that overlooks differences between the parental chromosomes. Considering the ease of genome discrimination by GISH in the *Lolium–Festuca* hybrids, their repetitive DNA sequences must differ substantially. On the other hand, it appears just as likely that while the DNA repeats diverged markedly during evolution of the two genera, the sequences involved in chromosome pairing have been conserved enough to facilitate regular pairing partner recognition and crossing over.

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