

Article

Selective Elimination of Parental Chromatin from Introgression Cultivars of xFestulolium (*Festuca* × *Lolium*)

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Abstract: Alien chromosome introgressions can be used to introduce beneficial traits from one species into another. However, exploitation of the introgressions in breeding requires proper transmission of introgressed segments to consecutive generations. In xFestulolium hybrids chromosomes of *Festuca* and *Lolium* readily pair and recombine. This opens a way for introgression of traits (e.g., abiotic and biotic stress resistance) from *Festuca* into elite *Lolium* cultivars. However, retention of *Festuca* chromatin in xFestulolium is uncertain as several studies indicated its gradual elimination over generations of sexual reproduction. Here we investigated genome composition in two subsequent generations of four introgression xFestulolium (*F. pratensis* × *L. multiflorum*) cultivars using genomic *in situ* hybridization. We observed about 27–32% elimination of *Festuca* chromatin in a single round of multiplication. At this pace, *Festuca* chromatin would be completely eliminated in about four generations of seed multiplication. On the other hand, we observed that it is possible to increase the proportion of *Festuca* chromatin in the cultivars by proper selection of mating plants. Nevertheless, once selection is relaxed, the first round of the seed multiplication reverts the genome composition back to the *Lolium* type. Thus, it seems that amphiploid forms of xFestulolium with relatively stable hybrid genomes may be more promising material for future breeding than introgression lines.

Keywords: genome dominance; ryegrass; fescue; hybrid; grass; recombination

1. Introduction

Interspecific or even intergeneric hybridization followed by whole genome duplication has been one of the main mechanisms of plant speciation, including many crop progenitors [1]. Besides ancient allopolyploids, such as wheat and cotton, new interspecific hybrids are produced in breeding programs to widen the gene pool of the crop species, to introgress beneficial traits from wild relatives to elite cultivars, or to combine agriculturally important traits from two different species (or genera). Merging the genomes from different species may disturb their organization and cooperation in the hybrid nucleus. In a majority of allopolyploids, the two parental genomes retain their integrity and their chromosomes (homoeologues) do not recombine via homoeologous chromosome pairing. This is usually controlled by specific genes or loci. Among them the *Ph1* (*Pairing homoeologous 1*) system of wheat is the best known. Only plants with mutated, deleted or suppressed forms of meiotic pairing control genes enable incorporation of chromosome segments via homoeologous recombination [2,3]. However, in hybrids between some species, pairing of homoeologous chromosomes does take place and may be quite frequent. Van der Meer and De Vries reported successful hybridization between

bulb onion (*Allium cepa* L.) and its wild relative *A. roylei* Stearn with the aim to introgress downy mildew resistance into elite cultivars of the bulb onion [4]. Similarly, chromosomes of ryegrass and fescue pair with each other in xFestulolium (*Festuca* × *Lolium* hybrids) [5]. The pairing results in extensive interspecies homoeologous recombination and chromosome reshuffling, and combined with reproduction by outcrossing, brings about enormous genetic diversity where each plant of a population has a different combination of segments of parental genomes. This creates an opportunity for breeders to introgress potentially any chromosomal segment from one species into the other. In this fashion many forms of xFestulolium were developed, including chromosome substitution and recombination lines [6]. Barnes et al. developed diploid *L. perenne* with introgression of *F. pratensis* chromatin in the distal part of the short arm of chromosome 3. Plants with this introgression were extremely drought resistant [7]. Similarly, Kosmala et al. reported introgression of freezing tolerance from *F. pratensis* into diploid *L. multiflorum* [8] and drought resistance was introduced from fescues into ryegrasses in several other breeding programs [9–11]. In general, the crossability of species from both genera enables the combination of high yield, palatability, digestibility and nutrition from ryegrasses and stress related traits (drought and freezing tolerance, winter hardiness, crown rust resistance) from fescues in xFestulolium hybrids [6].

On the other hand, promiscuous pairing of homoeologous chromosomes enables potential elimination of one of the parental genomes in successive sexual generations. In our previous study, we found that *Lolium* chromosomes were more abundant than those of *Festuca* in all screened amphiploid xFestulolium cultivars [12]. In some cultivars, the *Festuca* chromatin was completely eliminated. Zwierzykowski et al. observed gradual replacement of *Festuca* chromosomes by those of *Lolium* between F1 and F6 generations of *L. multiflorum* × *F. pratensis* hybrids [13]. One may speculate that the shift was due to the selection of ‘*Lolium*-type’ plants. Italian ryegrass, in comparison to meadow fescue, has a higher germination rate, faster seed germination and more rapid plant establishment from seed. Therefore, the same authors repeated the analysis on consecutive generations of *L. multiflorum* × *F. pratensis* hybrids with random selection of progenies. Interestingly, they observed a similar trend of *Lolium* dominance, even though the shift was somewhat meager [14]. Genome dominance in interspecific hybrids is not unique to xFestulolium. Van Heusden et al. showed that in a hybrid of onion, one of the genomes (*A. roylei*) also appeared to be predominating: The frequency of *roylei* alleles in the F2 population was on average 56% (vs. 44% of *cepa* alleles) [15].

The mechanism(s) underlying genome dominance at the chromosomal level is so far unknown, and several scenarios have been hypothesized including gametic competition, pollination effects, selection for seed yield and germination, and meiotic drive in females [16]. Besides these, other aspects such as asynchrony of the meiotic rhythms may play a role, as evident in *Brachiaria ruziziensis* × *B. brizantha* hybrids [17]. The continuity of *Festuca*-chromatin elimination in advanced breeding material is questionable. In our previous work, we analyzed three consecutive generations of three registered amphiploid xFestulolium cultivars [18]. We found that despite high genetic variability within each population, the proportion of parental genomes was relatively stabilized without apparent *Festuca*-chromatin elimination in subsequent generations. Kubota et al. also did not observe a reduction in the proportion of the *Festuca* chromatin in successive generations of Japanese xFestulolium cultivars Icarus and Nakei 1 [19]. Similarly, Zwierzykowski et al. found that a shift in genome composition towards one of the parents appeared to reach a plateau between F7 and F8 generations [20]. The outcrossing mode of reproduction, accompanied by the potential *Lolium*-genome dominance, raised a question of the ability of *Festuca* chromatin to be transmitted into the subsequent generation(s) in the introgression xFestulolium cultivars, recently popular among grass breeders. To study this, we employed genomic *in situ* hybridization (GISH) to assess alien chromatin transmission in four cultivars under different management of progeny selection.

2. Materials and Methods

2.1. Plant Material

We used four chromosome introgression cultivars of *x*Festulolium (labelled FL1–FL4) originating from the cross of *L. multiflorum* × *F. pratensis*. All four were at least in F6 after intergeneric hybridization. To estimate transmission frequencies of individual chromosomes, we used the progeny of tetraploid monosomic substitution lines ($2n=4x=28; 27L+1F$) developed in our previous study [21].

2.2. Crossings and Selection

Seeds were germinated in Petri dishes on wet filter paper and seedlings were planted in 30-mm pots in a greenhouse. After 3–4 weeks, plantlets were transferred to a hydroponic culture with an aerated solution of Hydroponex at 0.9 g/l (Hu-Ben, Čerčany, Czech Republic) for root tip collection. Thereafter, the plantlets were transferred to pots (20 cm) in a greenhouse and inter-crossed under different regimes based on the presence of *Festuca* chromatin in the plants of the particular cultivar.

Cvs. FL1 and FL2 (all plants of the cultivar had at least a small proportion of the *Festuca* chromatin): About 25 plants were inter-pollinated and five seeds from each of the five randomly selected mother plants were germinated and analyzed (25 plants for each cultivar).

Cvs. FL3 and FL4 (only some plants carried *Festuca* chromatin, others were ‘pure’ *Lolium*): *I. Pre-selection*: 25 plants were analyzed and only plants with cytologically detectable *Festuca* chromatin were inter-crossed for each cultivar. Five seeds from each of the five randomly selected mother plants were germinated and analyzed (25 plants for each cultivar). *II. Post-selection*: Another 25 plants for each cultivar were analyzed and inter-crossed. Five seeds from each of ten mother plants (IIa/ five randomly selected plants having *Festuca* chromatin and IIb/ five randomly selected plants without *Festuca* introgression) were germinated and analyzed (25+25 plants for each cultivar). The scheme of crossings and selections is given in Figure 1.

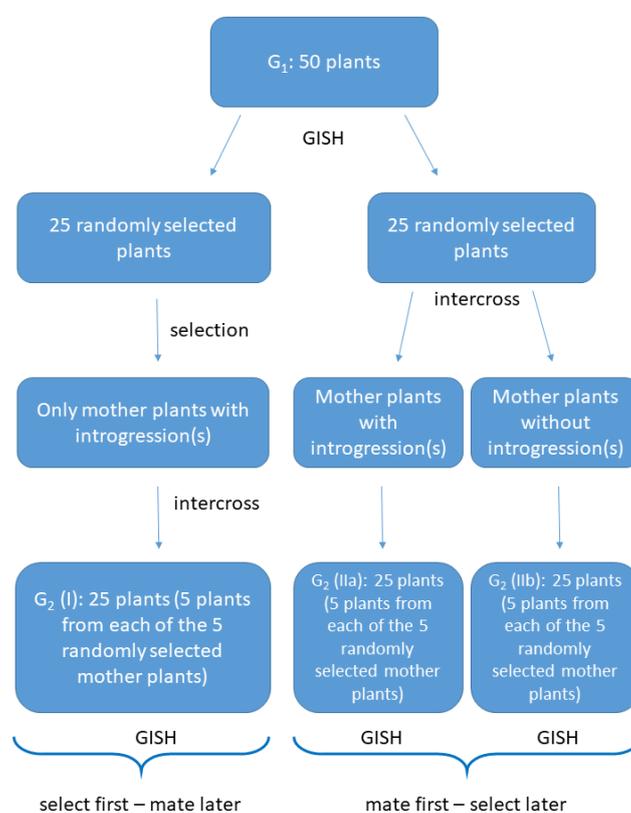


Figure 1. Scheme of crossings of cvs. FL3 and FL4.

2.3. Genomic in Situ Hybridization (GISH) and Microscopy

Mitotic metaphase spreads and GISH were prepared from root tips according to Masoudi-Nejad et al. [22]. Total genomic DNA of *F. pratensis* was labelled with digoxigenin using DIG-Nick Translation Kit according to manufacturer's recommendation (Roche Applied Science, Penzberg, Germany) and used as a probe. Total genomic DNA of *L. multiflorum* was sheared to 200–500-bp fragments by boiling for 45 min and used as blocking DNA. The probe to block ratio was 1:150 with minor variation. Sites of probe hybridization were detected by the Anti-DIG-FITC conjugate (Roche). Chromosomes were counterstained with 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). Slides were evaluated with Olympus AX70 epifluorescent microscope equipped with a SensiCam B/W camera. ScionImage and Adobe Photoshop software were used for processing of color pictures. At least three metaphase spreads were evaluated for each plant. We counted the number of *Festuca* and *Lolium* chromosomes, both with and without homoeologous exchange(s). Centromere identity was used to assign a particular chromosome to one of the two parental genomes. Thus, the chromosome was identified as of *Festuca*-origin if its centromere was labelled with *F. pratensis* probe even if the majority of the chromosome consisted of the *Lolium* chromatin.

The parental generation was designated G₁ and the following generation after intercross of G₁ was designated G₂. In cultivars FL3 and FL4, three types of G₂ were analyzed: G₂/I, G₂/IIa and G₂/IIb (Figure 1).

2.4. Data Analysis

Rates of alien chromatin elimination in introgression forms were compared between generations G₁ and G₂ using the following procedure. Genome composition was represented as a number of *Lolium* (L) and *Festuca* (F) chromosomes, classified into four groups: L or F complete and L or F recombined. In the first step, we calculated the expected (theoretical) genome composition of offspring from each mother plant as arithmetic means of genome composition of respective mother and averaged genome composition of males used in cross pollination. This approach was chosen because the plant origin of pollen was not known. Subsequently, the expected genome composition was compared with the actual genome composition determined using GISH, separately for each group (L or F complete and L or F translocated) and cultivar. For statistical evaluation, the two-sided paired t-test was used in NCSS 9 software (NCSS, LLC., Kaysville, Utah, USA, ncss.com/software/ncss). Each mother plant represented a block with paired values: Expected and observed chromosome numbers. We tested H₀ that both expected and observed chromosome numbers were equal (that is, there were no changes of genome composition between two consecutive generations). We also calculated the mean percentage change in proportional representation of each group of chromosomes (L or F complete and L or F translocated) in the total genome composition between consecutive generations in each cultivar (G₂-G₁) (Tables 1–3). Positive/negative values represent increase/decrease in proportional representation of respective genome groups in the total genome composition.

3. Results

All four cultivars (FL1–FL4) showed high levels of aneuploidy. Chromosome numbers ranged from 25 to 30, and only 35% of plants were euploid (2n=4x=28). The most frequent karyotype was 2n=27 (observed in 39% of plants). Interestingly, the frequency of aneuploidy decreased in the subsequent generation in three cultivars: from 77.3 to 70.8% in FL2, from 64.3 to 56.8% in FL3 and from 70.0 to 66.7% in FL4. In cv. FL1, aneuploidy was stable over the generations (60%).

3.1. Cultivars with Higher Proportion of *Festuca* chromatin (FL1 and FL2)

All G₁ plants of cultivars FL1 and FL2 carried some *Festuca* chromatin (Figure 2). We detected 4.35 and 3.14 chromosomes with homoeologous recombination event(s) and 0.50 and 0.36 complete *Festuca* chromosomes in FL1 and FL2, respectively. Among the recombined chromosomes, 3.55 and 2.32 were of *Lolium*-origin (with centromeres from the *Lolium* genome), whereas only 0.80 and 0.82

were of *Festuca*-origin. In both cultivars, we found a replacement of *Festuca*-origin chromosomes by those of *Lolium*-origin in the G2 generation). While all plants of the G1 generation had at least some *Festuca* chromatin, we found 4 and 3 'pure' *Lolium* plants (out of 25) without detectable *Festuca* chromatin in the G2 generation of FL1 and FL2, respectively. The genome composition changed from 26.05 L + 1.30 F (where L designates *Lolium*-origin chromosomes and F *Festuca*-origin chromosomes) to 26.4 L + 0.84 F in FL1 and from 26.0 L + 1.18 F to 26.67 L + 0.58 F in FL2 (Table 1). The number of translocated chromosomes dropped from 4.35 to 3.20 in FL1 and from 3.14 to 2.42 in FL2. The most dramatic (and statistically significant) was the elimination of complete *Festuca* chromosomes: From 0.5 to 0.16 and from 0.36 to 0.04 in FL1 and FL2, respectively. Thus, the overall reduction of *Festuca* chromatin from generation to generation was about 27% in FL1 and 32% in FL2 (Figure 3).

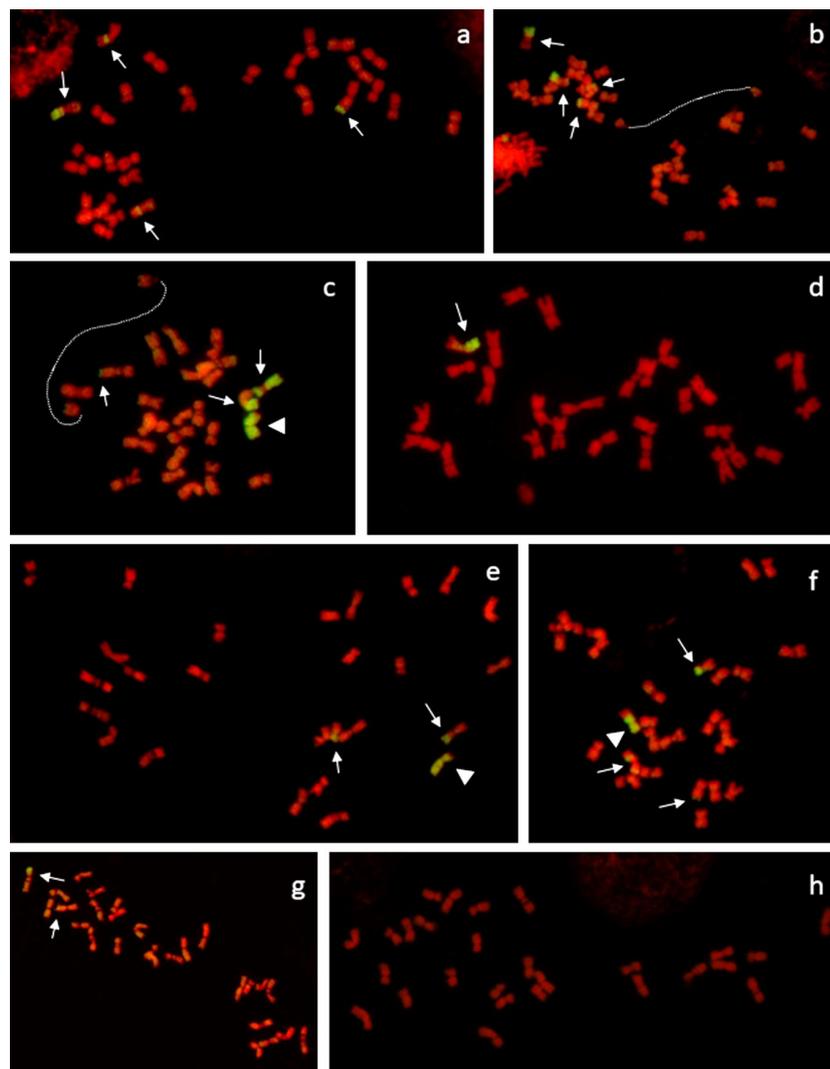


Figure 2. The analysis of genome constitution in four xFestulolium cultivars by *in situ* hybridization. GISH was done on mitotic metaphase spreads of *L. multiflorum* × *F. pratensis* cultivars FL1 (a–b), FL2 (c–d), FL3 (e–f), and FL4 (g–h) over two generations (G₁: a, c, e, g and G₂: b, d, f, h). Total genomic DNA of *F. pratensis* was labeled with FITC and used as a probe (yellow-green color); unlabeled genomic DNA of *L. multiflorum* was used to block hybridization of sequences common to both species. Chromosomes were counterstained by DAPI (red pseudocolor). Chromosomes with homoeologous translocation(s) are arrowed (of *Lolium*-origin: With centromeres from *Lolium*) while arrowheads point to *Festuca*-origin chromosomes (that is those with centromeres from *Festuca*). Dotted lines connect parts of single chromosomes broken in primary (centromere) or secondary (NOR) constriction.

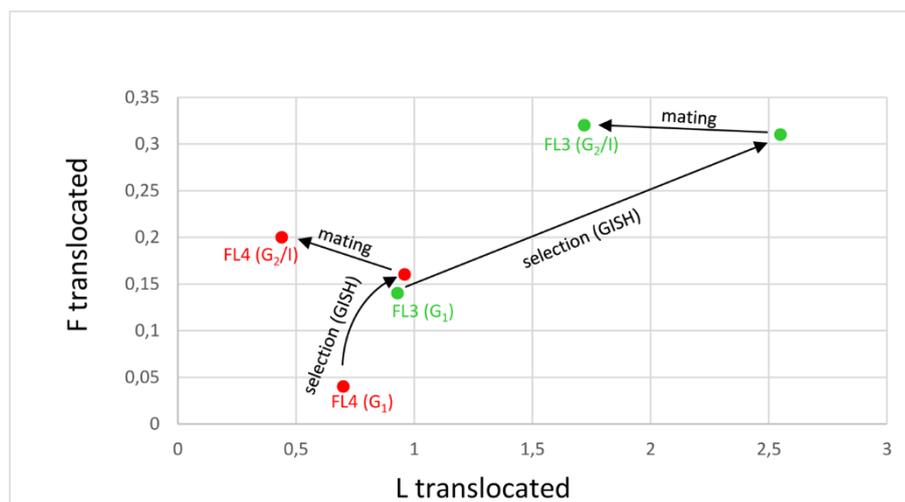


Figure 3. The shift in genome composition in xFestulolium cultivars FL3 and FL4. Plants of G₁ generation were analyzed by GISH and only those with a translocation(s) were used for mating. Note that it is possible to manipulate genome composition of the cultivar and increase the proportion of *Festuca* chromatin (either in the form of *Lolium* chromosomes with translocated *Festuca* segments, or as *Festuca* chromosomes with translocated *Lolium* segments) by selection of mating plants (using GISH). However, the first round of seed multiplication shifts the genome composition back towards the *Lolium* genome. x and y axes: F translocated refers to the number of *Festuca* chromosomes with *Lolium* translocation(s) and L translocated refers to number of *Lolium* chromosomes with *Festuca* translocation(s).

Table 1. Genome constitution in xFestulolium cultivars FL1 and FL2. All 25 G₁ plants from each cultivar were evaluated by GISH (G₁ male). Five randomly selected mother plants (G₁ female) were used for seed collection (five seeds from each mother plant). The 25 plants of the G₂ generation for each cultivar were screened by GISH. Numbers in the table represent the number of *Lolium*-origin (L) or *Festuca*-origin (F) chromosomes. Differences in genome composition between G₁ and G₂ generations were tested separately for each L and F chromosome group by the paired t-test. Significant differences are marked by asterisks. Arrows denote direction of change in genome composition between real and expected genome composition of G₂ plants.

| Cultivar | L complete | L translocated | F translocated | F complete |
|---|-------------------|----------------|----------------|------------------|
| FL1 G ₁ (male) | 22.50 ± 2.97 | 3.55 ± 2.33 | 0.80 ± 0.98 | 0.50 ± 0.87 |
| FL1 G ₁ female | 22.80 ± 2.14 | 3.20 ± 1.17 | 0.80 ± 1.30 | 0.40 ± 0.89 |
| FL1 G ₂ | 23.77 ± 2.23 **↑ | 2.52 ± 2.16 *↓ | 0.68 ± 0.74 | 0.16 ± 0.47 *↓ |
| Change in proportion G ₂ -G ₁ (%) | 4.59 | -3.10 | -0.43 | -1.06 |
| FL2 G ₁ (male) | 23.68 ± 1.61 | 2.32 ± 1.06 | 0.82 ± 0.98 | 0.36 ± 0.64 |
| FL2 G ₁ female | 23.75 ± 2.06 | 2.25 ± 1.25 | 1.25 ± 1.89 | 0.25 ± 0.50 |
| FL2 G ₂ | 24.83 ± 1.31 (*)↑ | 1.88 ± 1.48 | 0.54 ± 0.72 *↓ | 0.04 ± 0.20 ***↓ |
| Change in proportion G ₂ -G ₁ (%) | 4.29 | -1.48 | -1.82 | -0.99 |

L = *Lolium* chromosome;

F = *Festuca* chromosome;

translocated = chromosome bearing homoeologous translocation(s);

(*) $P \leq 0.10$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

3.2. Cultivars with Lower Proportions of *Festuca* chromatin (FL3 and FL4)

In cultivars FL3 and FL4, only some plants had at least a single chromosome segment from *F. pratensis* (19 out of 50 in FL3 and 29 out of 80 in FL4), while other plants had a pure *L. multiflorum* genome. There was not a single plant carrying a complete *Festuca* chromosome in these cultivars. The number of chromosomes with evidence of homoeologous recombination was low: 0.78 and 0.74 in

FL3 and FL4, respectively. Among those, the majority were of *Lolium*-origin (0.64 vs. 0.14 in FL3 and 0.70 vs. 0.04 in FL4).

Two different ways of multiplication were used: First selection—second intercross (G_2/I) and first intercross – second selection (G_2/IIa and G_2/IIb). In the first one, plants were phenotyped by GISH and only plants having homoeologous translocation(s) were used for mating. This altered the pattern of parental genomes from 26.68 L + 0.88 T ($0.64^L + 0.14^F$) to 25.00 L + 2.72 T ($2.43^L + 0.29^F$) in FG1 and from 26.34 L + 0.74 T ($0.70^L + 0.04^F$) to 25.78 L + 1.22 T ($1.11^L + 0.11^F$) in FL4. However, the intercross of selected plants with homoeologous translocation(s) did not ensure stabilization of the translocation(s). We found that only 21 (84%) and 12 (48%) out of 25 progeny plants (G_2/I) carried translocation(s) in FL3 and FL4, respectively. The overall level of *Festuca* chromatin was reduced to 25.58 L + 25.04 T ($1.72^L + 0.32^F$) in FL3 and to 26.36 L + 0.64 T ($0.44^L + 0.20^F$) in FL4 (Table 2). The major contributor to the elimination was the replacement of *Lolium* chromosomes with translocation(s) to complete *Lolium* chromosomes (statistically significant in both cultivars). The number of *Festuca* chromosomes with translocation(s) was relatively stable over the two generations.

In the second regime of multiplication, we first intercrossed all 25 plants and after mating, we collected seeds from five mother plants carrying homoeologous translocation(s), and from five mother plants without any such translocation. There was no statistically significant change in the number of *Lolium* and *Festuca* chromosomes in progeny of plants with translocation(s) (G_2/IIa), even though a slight increase of *Festuca* chromatin was observed in both cultivars (Table 3). However, only 16 and 19 out of 25 plants had translocation(s) in FL3 and FL4, respectively. Among the progenies of mother plants without translocation(s), there were only one and two out of 25 plants with translocation(s) in FL3 and FL4, respectively. In both cultivars, there was a statistically significant difference between the expected and observed number of translocated chromosomes (always of *Lolium*-origin). It appears that mother plants tended to be pollinated by pollen from the same group in both cultivars: Plants with translocation(s) were more frequently pollinated with the plants having translocation(s) and the ‘pure’ *Lolium* plant was preferentially pollinated with other ‘pure’ *Lolium* plants.

Table 2. Genome constitution in xFestulolium cultivars FL3 and FL4 with first selection—second intercross management. The plants were first genotyped by GISH and only plants with *Festuca* chromatin (G_1 male) were used for mating. Five randomly selected mother plants (G_1 female) were used for collection of seeds (five seeds from each mother plant). The 25 plants of G_2 generation (G_2/I) for each cultivar were screened by GISH. The numbers in the table represent either the number of *Lolium*-origin (L) or *Festuca*-origin (F) chromosomes. Differences in genome composition between the G_1 and G_2 generations were tested separately for each L and F chromosome group by the paired t-test. Significant differences are marked by asterisks. Arrows denote direction of change in genome composition between real and expected genome composition of G_2 plants. Note that no complete *Festuca* chromosome was detected in the cultivars FL3 and FL4.

| Cultivar | L complete | L translocated | F translocated |
|------------------------------------|------------------|------------------|----------------|
| FL3 G_1 male for G_2/I | 25.00 ± 2.39 | 2.43 ± 1.99 | 0.29 ± 0.70 |
| FL3 G_1 female for G_2/I | 24.67 ± 2.66 | 2.67 ± 2.25 | 0.33 ± 0.81 |
| FL3 G_2/I | 25.58 ± 1.28 **↑ | 1.72 ± 1.14 ***↓ | 0.32 ± 0.62 |
| Change in proportion G_2-G_1 (%) | 2.94 | -2.98 | 0.04 |
| FL4 G_1 male for G_2/I | 25.78 ± 0.92 | 1.11 ± 0.74 | 0.11 ± 0.31 |
| FL4 G_1 female for G_2/I | 26.40 ± 0.55 | 0.80 ± 0.45 | 0.20 ± 0.44 |
| FL4 G_2/I | 26.36 ± 1.22 | 0.44 ± 0.77 **↓ | 0.20 ± 0.41 |
| Change in proportion G_2-G_1 (%) | 1.75 | -1.90 | 0.15 |

L = *Lolium* chromosome;

F = *Festuca* chromosome;

translocated = chromosome bearing homoeologous translocation(s);

** $P \leq 0.01$, *** $P \leq 0.001$.

Table 3. Genome constitution in xFestulolium cultivars FL3 and FL4 with first intercross—second selection management. Plants of G₁ generation were genotyped by GISH and intercrossed. The G₁ plants were thereafter split into two groups: Those with translocations and those without translocation(s). Five randomly selected mother plants from each group (female for G₂/IIa and female for G₂/IIb) were used for seed collection (five seeds from each mother plant). The plants of G₂ generation (25 for G₂/IIa and 25 for G₂/IIb) were screened by GISH. The numbers in the table represent either the number of *Lolium*-origin (L) or *Festuca*-origin (F) chromosomes. Differences in the genome composition between G₁ and G₂ generations were tested separately for each L and F chromosome group by the paired t-test. Significant differences are marked by asterisks. Arrows denote direction of change in genome composition between real and expected genome composition of G₂ plants.

| Cultivar | L complete | L translocated | F translocated |
|---|-------------------|------------------|----------------|
| FL3 G ₁ male for G ₂ /II | 26.86 ± 1.52 | 0.43 ± 0.79 | 0.10 ± 0.29 |
| FL3 G ₁ female for G ₂ /IIa | 25.60 ± 1.94 | 1.60 ± 0.89 | 0.40 ± 0.54 |
| FL3 G ₂ /IIa | 25.79 ± 2.06 | 1.21 ± 1.14 | 0.33 ± 0.48 |
| Change in proportion G ₂ -G ₁ (%) | -1.02 | 0.72 | 0.30 |
| FL3 G ₁ female for G ₂ /IIb | 28.40 ± 1.52 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| FL3 G ₂ /IIb | 27.64 ± 1.18 | 0.04 ± 0.20 ***↓ | 0.00 ± 0.00 |
| Change in proportion G ₂ -G ₁ (%) | 0.82 | -0.64 | -0.18 |
| FL4 G ₁ male for G ₂ /II | 26.46 ± 0.94 | 0.61 ± 0.66 | 0.02 ± 0.15 |
| FL4 G ₁ female for G ₂ /IIa | 26.20 ± 0.44 | 1.20 ± 0.44 | 0.00 ± 0.00 |
| FL4 G ₂ /IIa | 25.96 ± 1.06 (*)↑ | 1.36 ± 0.99 *↑ | 0.00 ± 0.00 |
| Change in proportion G ₂ -G ₁ (%) | -1.60 | 1.64 | -0.04 |
| FL4 G ₁ female for G ₂ /IIb | 27.00 ± 0.71 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| FL4 G ₂ /IIb | 26.76 ± 1.09 | 0.08 ± 0.28 ***↓ | 0.00 ± 0.00 |
| Change in proportion G ₂ -G ₁ (%) | 0.89 | -0.85 | -0.04 |

L = *Lolium* chromosome;

F = *Festuca* chromosome;

(*) P ≤ 0.10, * P ≤ 0.05, *** P ≤ 0.001.

3.3. Transmission of Individual *F. pratensis* chromosomes in Consecutive Generation

Using tetraploid monosomic substitution lines, where one *Lolium* chromosome was replaced by its *Festuca* homoeologue, we analyzed the transmission frequency of individual chromosomes. There was a clear variation in the transmission rate among different chromosomes: Chromosome 5F was transmitted to the progeny at only 34% frequency (significantly less than expected 50%), while other chromosomes were transmitted almost regularly (Table 4). However, not a single *F. pratensis* chromosome was transmitted to the progeny with frequency above the expected 50%. So, besides the more frequently eliminated chromosome 5F, all *Festuca* chromosomes contribute to the general gradual elimination of the *Festuca* chromatin from the hybrid genome.

Table 4. Transmission of individual chromosomes in monosomic substitution lines (2n=4x=28; 27L+1F).

| Chromosome | Number of plants | Transmission (%) |
|------------|------------------|------------------|
| 1F | 111 | 46 n.s. |
| 2F | 96 | 49 n.s. |
| 3F | 114 | 44 n.s. |
| 4F | 88 | 47 n.s. |
| 5F | 88 | 34 ** |
| 6F | 131 | 48 n.s. |
| 7F | 136 | 48 n.s. |

n.s. non-significant;

** significant deviation from the expected transmission frequency (0.50) at P ≤ 0.01 (Student's t-test).

4. Discussion

Wide hybridization offers a possibility to transfer chromosomes or chromosome segments with beneficial alleles from one species to another. However, for further exploitation of the introgression lines in breeding programs, they must be fertile and chromosomally stable. Homoeologous chromosomes generally do not pair and recombine in interspecific hybrids. This theoretically prevents alien chromosomes, when disomic, from being eliminated from the host genome. However, this is not always the case. Orellana et al. [23] found reduced metaphase I pairing of rye chromosomes and significantly higher numbers of rye univalents compared to wheat chromosomes in disomic additions and substitutions of rye chromosomes in wheat. This led to the elimination of rye chromosomes. This was most probably a consequence of disturbed migration of rye telomeres into the leptotene bouquet [24] as an extension of their erratic behavior in somatic tissues [25].

The absence of a chromosome pairing regulator such as *Ph1* in wheat, and sufficient sequence homology between homoeologues, opens the way for direct competition of homoeologues in hybrids. Such a rivalry frequently (if not always) leads to rapid or gradual elimination (or replacement) of the chromosomal complement from one of the parents. If a “submissive” parental genome is the source of chromosomes to be introgressed, as is the case in xFestulolium hybrids, the risk of its elimination in subsequent generations seems to be enormous. Pickering [26] reported elimination of *Hordeum bulbosum* chromosomes in monosomic substitution lines of *H. bulbosum* × *H. vulgare*. The frequency of the elimination in the hybrids seemed to be tissue-specific and temperature-dependent [27]. Moreover, the preferential elimination of some chromosomes and higher transmission of others was observed also in *Gossypium hirsutum* × *G. australe* and *G. hirsutum* × *G. sturtianum* hybrids [28]. We found a similar phenomenon in this study: Chromosome 5F was transmitted to only 34% of the progeny, while chromosomes 2F, 6F and 7F were transmitted almost regularly (48–49%). This may further hamper the stable incorporation of the traits located on the preferentially-eliminated chromosomes.

Based on the elimination rate from G_1 (parental generation) to G_2 (subsequent generation after intercross of G_1) in the xFestulolium cultivars investigated in this study, we predict that without a selection, and with the same elimination rate, *Festuca* chromatin will be completely eliminated after four generations of multiplication. The reversion of the hybrid genome to the parental (“pure” *Lolium*) form has already taken place in several xFestulolium cultivars, where no *Festuca* chromatin was observed after GISH [12]. Similarly, King et al. [29] found much higher transmission of complete *Lolium* chromosomes compare to complete *Festuca* chromosomes in the successive generation after backcross of diploid monosomic substitution lines of *L. perenne* × *F. pratensis* (13L+1F, the proportion was 89:11, instead of expected 1:1). Unfortunately, authors did not indicate the parental origin of the translocated chromosomes. Thus, the overall *Festuca* chromatin elimination could not be assessed from their study. However, the complete elimination may not always be so rapid in all xFestulolium hybrids. It was evidenced earlier that different combinations of parental species used for interspecific hybridization display different frequency of homoeologous chromosome pairing. We found that *L. multiflorum* × *F. glaucescens* hybrids displayed much less homoeologous recombination events than those of *L. multiflorum* × *F. pratensis* [12]. Moreover, the chromosome segment may retain and persist within populations of the subsequent generations because of the selective advantage. The cultivar AberNile with a *F. pratensis* introgression on chromosome 5 bearing stay-green mutation admittedly with the recessive gene present in disomic form and drought tolerant cultivar AberLink with a single *F. glaucescens* introgression on chromosome 3 display genetic stability over many generations (Prof. Mike Humphreys, personal communication).

Interestingly, it seems that mother plants were preferentially pollinated by pollen from the same group in both cultivars: Plants with translocation(s) were more frequently pollinated with the plants having translocation(s) and ‘pure’ *Lolium* plant was preferentially pollinated with another ‘pure’ *Lolium* plant. We can only speculate on the mechanisms underlying this phenomenon. One may hypothesize that it is caused by the asynchrony in the flowering time of the plants with and without homoeologous translocation(s) or by the (probably meager) action of the potential genetic barrier(s) between both

groups of plants. This preferential mating may also play a role in the stabilized transmission of the *Festuca* segment(s) to successive generations.

Instability of *Festuca* introgressions contrasts with the relative stability of amphiploid xFestulolium cultivars. In our previous work, we confirmed that amphiploid *L. multiflorum* × *F. pratensis* cultivars were relatively stable over three consecutive generations. Despite a large variability within the populations, the genomic composition did not change significantly, and the *Festuca* chromatin was not eliminated any further [18]. Similarly, Zwierzykowski et al. reported that chromosomes of *F. pratensis* were gradually replaced by those of *L. multiflorum* in six consecutive generations of hybrids but became stabilized in the F7-F8 generations without any additional elimination of *Festuca* chromatin [20]. Interestingly, genome composition was stabilized in the proportion of about 65:35 in favor of the *Lolium* genome, the proportion very similar to that of stabilized amphiploid xFestulolium cultivars [12]. Based on the present evidence, we are not able to accurately distinguish between the cause and the effect: If this is a naturally stable proportion of parental genomes, or if the 65:35 proportion was reached as a consequence of 6–8 rounds of multiplication (and potential selection) during which the hybrid genome was stabilized.

To conclude, our study shows a rapid elimination of the *Festuca* chromatin in introgression cultivars of xFestulolium (*L. multiflorum* × *F. pratensis*). Without any selection and if the introgressed segment does not bring a strong selective advantage, such cultivars will most likely revert to the ‘pure’ *Lolium* constitution within about four generations of multiplication. This may prevent their further utilization in grass breeding. From the practical viewpoint, amphiploids with relatively stable hybrid genome constitutions appear to be a more promising material for future breeding. From the evolutionary viewpoint, it would be interesting to investigate the reverse situation: Transmission of *Lolium* chromosome segments in introgression forms of xFestulolium, where the *Festuca* genome predominates (where one or more backcrosses of F1 hybrids to *Festuca* were used). As the *Lolium* genome is the dominant one in hybrids, one may speculate on higher stability of *Lolium* introgressions as compared to those (*Festuca* introgressions) used in this study.

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