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Spontaneous natural formation of interspecific hybrids within the *Festuca-Lolium* complex

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Abstract

Interspecific and intergeneric hybridization within the *Festuca-Lolium* complex is frequently used in forage plant breeding. However, little is known about the natural occurrence and competitiveness of such hybrids. We collected naturally formed hybrids between *Festuca apennina*, *Festuca pratensis,* and *Lolium perenne* in different habitats of Switzerland and the British Isles and studied their origin, the ease of their spontaneous formation, and their competitiveness with parental species. A special attention was paid to the largely sterile triploid forms and their rare sexual progeny. The triploid hybrid *F. apennina* × *F. pratensis* proved to be widespread and often highly competitive in Swiss permanent pastures. The majority of these hybrids originated from *F. apennina* as the seed parent although little or no *F. apennina* grew nearby. In an experimental setting with ample *F. pratensis* pollen provided by neighbouring plants, up to 20 % of seeds from open pollinated *F. apennina* plants were interspecific hybrids; among seeds collected in natural habitats, only 0.35 % were hybrids. At an experimental site at 1 000 m altitude, these triploid hybrids grew much more vigorously than corresponding tetraploid pure *F. apennina*, confirming their great competitiveness at such altitudes in permanent grasslands. The triploid hybrids were only marginally fertile suggesting that vegetative propagation by rhizomes is the cause of their competitive success in grassland. Moreover, triploid progeny retained the chromosome constitution of their mother plants indicating the possibility of apomixis. Natural triploid *F. pratensis* × *L. perenne* hybrids were partially female fertile (a seed set of 0.1 % or less) whereas diploid hybrids did not produce any viable seeds. Progenies of these triploids showed considerable chromosome alterations, such as loss of a genome or recombination due to homoeologous pairing, and only rarely the chromosome constitution of the triploid mother plant was retained. It was concluded that natural triploid interspecific hybrids could expand the range of their progenitor species and might function as bridges transferring genes between them.

Additional key words: *Festuca apennina, Festuca pratensis, Lolium perenne,* triploid hybrid.

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Abbreviations: *Fape* - *Festuca apennina*; FCM - flow cytometry; *Fp* - *Festuca* pratensis; GISH - genomic *in situ* hybridization.

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Introduction

Contrary to animals, plant species frequently engage in hybridization with representatives of different species, or even genera. Rieseberg (1997) reported that about 11 % of plant species arose from interspecific hybridization, ranging from 22 % for British flora to 5.8 % for the mountain flora of North America. This would account for 27 500 hybrids among the 250 000 described plant species. Interspecific hybridization is often accompanied by whole genome duplication in the process called allopolyploidization, one of the key mechanisms of plant speciation. Allopolyploids represent a source of genetic, biochemical, and evolutionary novelty (Soltis and Soltis 1993). Wide hybridization may produce considerable heterosis, providing competitive advantage for the hybrids over their diploid progenitors (Comai 2005), masking deleterious recessive alleles leading to increased mutational robustness (Madlung 2013), and conveying increased stress tolerance through such mechanisms as delayed reproduction, fostering slower development, longer life span, and increased defense against herbivores and pathogens (Lohaus and Van de Peer 2016). In general, allopolyploids display broader adaptation to novel environmental niches compared to their progenitor species, allowing them to expand their range beyond those of the parental species (Te Beest *et al.* 2012). A well-known example within the *Festuca-Lolium* complex is the hexaploid grass species tall fescue (*Festuca arundinacea* Schreb.), which originated by a hybridization event involving winter hardy *F. pratensis* Huds. with drought-resistant *F. glaucescens* Boiss. (Humphreys *et al.* 1995) and was subsequently capable of colonizing climatically diverse European grasslands.

Besides the key role that allopolyploidy plays in plant evolution and diversification, these processes are key to the origin of many crops including wheat, banana, rapeseed, and cotton (Wendel 2015 and references therein). Interspecific hybridization is also frequently used in plant breeding. Targeted merging of two genomes into a single organism offers an opportunity to combine agriculturally important characteristics of two species, to introgress one or a few traits from a contrasting cultivated or a wild relative into elite cultivars of crops, or to widen the gene pools of a crop. It seems that some plant families are more prone to interspecific hybridization and allopolyploidization than others. It has been shown that *Poaceae* species are among those with a high proportion of allopolyploids (Ellstrand 1996). Interspecific hybridization was used to introgress agriculturally beneficial traits from wild or cultivated relatives to wheat, one of the three key crops for human’s food consumption (Matsuoka 2011).

The *Festuca-Lolium* complex is represented by ryegrasses (*Lolium* spp.) and broad-leaved fescues (*Festuca* spp.) from subgenus *Schedonorus*. Close phylogenetic relationship of the species enables numerous hybridization combinations within the complex, giving rise to hybrids found in nature (Banfi *et al.* 2017). The most frequent among such natural hybrids known so far are *L. perenne* × *F. pratensis* found in the British Isles and Northwestern Europe (Farragher 1975, Lewis 1975). These hybrids occur in mature meadows and are often found on waterlogged soils (Humphreys and Harper 2008). As they occur in soils prone to episodic flooding in lowland areas in old grassland and water meadows throughout the UK (Stace 1975), they are thought to offer breeding opportunities for adaptation to waterlogged soils. This may become an important plant breeder′s objective, given increased occurrences of localized or widespread flooding exacerbated by climate change (Humphreys and Harper 2008).

In 2015, we conducted an expedition in the Swiss Alps to collect specimens of *F. apennina* De Not. This allotetraploid arose from hybridization of *F. pratensis* with a so-far unidentified fescue species and thus, it is closely related to *F. pratensis* (Kopecký *et al.* 2016). Due to the morphological similarity between *F. apennina* and *F. pratensis*, some authors (*e.g.* Conert and Hegi 1998) describe *F. apennina* as a subspecies of *F. pratensis*. However, cytological evidence conclusively shows it as a distinct allopolyploid species (Kopecký *et al.* 2016) and it should be referred to as *F. apennina* De Not., paying tribute to the original description by De Notaris (1844). Surprisingly, in our 2015 collection, we found many triploid plants along with tetraploid *F. apennina* and diploid *F. pratensis* (it is difficult to distinguish all three cytotypes from each other in their vegetative stage). Our cytogenetic and molecular analysis revealed that triploids are hybrids of the two, and originated from reciprocal crosses between F. *apennina* and *F. pratensis* where both could be the maternal species, though *F. apennina* is the maternal parent more frequently. The presence of these three cytotypes is altitude dependent, with tetraploid *F. apennina* growing over 1 200 m a.s.l. and diploid *F. pratensis* below 1 800 m a.s.l. Triploid hybrids occur in the overlapping zone, often in sympatry with at least one, or both parents. However, in some locations, triploids were the only cytotype identified indicating their high competitiveness (Kopecký *et al.* 2018). Besides these two, other hybrids were sporadically found in nature with various taxonomical treatment (Holub 1998, Banfi *et al.* 2017).

It is believed that naturally formed hybrids of the *Festuca-Lolium* complex are generally sterile (at least male sterile) and thus, will not lead to the establishment of a new (hybrid) species (Jauhar 1993). However, they may persist by vegetative reproduction. We observed that triploid hybrids of *F. apennina* × *F. pratensis* readily propagate vegetatively *via* rhizomes, which may distribute an individual over long distances. Our analysis revealed clones of an individual dispersed up to 14.4 m in Swiss swards (Kopecký *et al.* 2018). Another means of transmission of the hybrid genome into subsequent generations might be *via* backcrosses of a partially female-fertile hybrid to one of the parental species. Natural hybrids of *L. perenne* × *F. pratensis* in British Isles and France were found to be diploids and triploids formed by fusion of reduced and unreduced gametes (with both combinations LLF and LFF) (Peto 1933, Wit 1964, Essad 1966, Gymer and Whittington 1973, Humphreys and Harper 2008). Although fertility of the hybrids was low, normal viable pollen grains were occasionally recovered from both diploid and triploid hybrids (Humphreys and Harper 2008). This indicates that gene flow is likely between natural populations of the hybrids and their progenitors.

This paper aimed to provide an update on interspecific and intergeneric hybridization within the *Festuca-Lolium* complex. Special attention was paid to the origin of hybrids, their establishment in natural stands, and competitiveness over the parental species.

Materials and methods

Survey of altitudinal distribution of broad-leaved *Festuca* species: The altitudinal distributions of diploid *F. pratensis*, tetraploid *F. apennina,* and their triploid hybrids were studied by sampling plants in six natural grassland areas representing four different Swiss cantons (Table 1 Suppl.). A majority of the sampling sites were used as pasture for cattle grazing. However, at low altitudes, some grasslands were used for hay or silage making and had to be included where no grazed areas were available. At highest altitudes, some scrubby zones with little grazing were included. For each of the grassland areas, one or two contiguous pastures of at least 2 000 m^2 at mid-altitude (1 350 to 1 550 m a.s.l.) were chosen. A 20 × 20 m grid was pre-defined by a geographic information system (GIS) in each pasture. Starting with a random square of 20 × 20 m within this grid, the broad-leaved *Festuca* plant closest to the centre of the square was sampled. This was continued with adjacent squares until at least 50 plants were sampled. Squares with no *Festuca* present were skipped. Starting from the 2 000 m^2 pastures, an altitudinal gradient up and down the slope was followed. Additional sampling was carried out in horizontal transects every 50 m of altitude. Six plants were sampled at each altitude (+/- 10 m) with a distance of about 15 m (20 steps) between the plants. For each of the six grassland areas, a total of 18 to 21 such horizontal transects, covering altitudes of 850 to 1950 m a.s.l. were included (Fig. 1 Suppl.). The highest and the lowest horizontal transects at each locality were from 2.7 to 4.9 km far from each other. From each plant, a ramet was detached and planted into a *Quickpot* tray. Ramets were then allowed to develop in a greenhouse.

Ploidy was determined by flow cytometry (FCM) in two laboratories, Olomouc, Czech Republic and Gatersleben, Germany. At Olomouc, each ramet was measured separately as described by Doležel *et al.* (2007). Nuclear suspensions were prepared from 50 mg leaf tissues of each sample with *Pisum sativum* cv. Ctirad used as the reference standard having 2C = 9.09 pg (Doležel *et al.* 1998). At Gatersleben, nuclear suspensions were prepared from equal amounts of 50 mg leaf tissues of each of one to three ramets together with *Vicia faba*, cv. Tinova (IPK gene bank accession number: FAB 602) as internal reference standard using the *CyStain PI Absolute P* reagent kit (*Sysmex*, Norderstedt, Germany) according to manufacturer's instructions. When ploidy deviations were observed within the pools of up to three ramets, each ramet was measured individually, together with *V. faba*. Diploid plants were considered as *F. pratensis*, tetraploid plants as *F. apennina*, and triploid plants as hybrids of the two. In each locality, 12 triploid plants were selected for determination of maternity. They were selected to best cover the altitudinal range of triploid plant occurrence at each locality. Maternity was determined by analysis of two cpDNA loci. Firstly, a 5-bp indel in the *trn*L-*trn*F intergenic spacer differentiated between *F. pratensis* and *F. apennina* haplotypes (Kopecký *et al.* 2018), but it does not differentiate between *F. pratensis* and *L. perenne* (another potential mating partner) haplotypes. Therefore, a second marker, the gene coding for maturase K (*matK*), was used to differentiate between *F. pratensis* and *L. perenne* haplotypes. Analysis of Genbank records showed that the *matK* region contained three differentiating positions (based on accession HM453060): 514 (C/A), 612 (T/A), and 884 (T/C). The analysis of *trn*L-*trn*F intergenic spacer was done as described in our previous report (Kopecký *et al.* 2018). *matK* region was amplified using primers F318poales and R1460poales (De Vere *et al*. 2012) as follows: reaction volumes of 25 mm^3 contained 12.5 mm^3 of *Combi PPP MasterMix* (*TopBio*, Vestec, Czech Republic), 9.5 mm^3 of ddH_2O, 0.5 µM of each primer, and 5 - 10 ng of genomic DNA. The thermocycling profile was as follows: 95 °C/5 min, 35 × (95 °C/30 s, 53 °C/30 s, 72°C/1 min), 72 °C/10 min. PCR products were purified using the *QIAquick* PCR purification kit (*Qiagen*, Hilden, Germany) and sequenced (*GATC Biotech*, Cologne, Germany) using the PCR primers.

Formation of triploid *F. apennina* × *F. pratensis* hybrids under controlled open pollination: For the assessment of the formation of triploid hybrids under controlled pollination, we used *F. apennina* (*Fape*) and *F. pratensis* (*Fp*) plants as well as their triploid hybrids which ploidy was confirmed by FCM as described above. The plants originated from a small collection in different European countries carried out in 2017 and were cultivated in an experimental field of Agroscope in Reckenholz. Broad-leaved *Festuca* was collected in Austria (Arlberg region), Bulgaria (Rila mountains), Italy (Cortina d'Ampezzo region), Liechtenstein (Alp Malbun region), Romania (Transylvanian Alps), and Switzerland (Glaubenbielen and Steiner Berg regions, see Table 1 Suppl.). All plants were collected from permanent grassland at altitudes between 1 310 and 2 000 m a.s.l. They were planted at random at 0.5 × 0.25 m distances in an 11 × 2.5 m field plot at Zürich-Reckenholz in August 2017. Plants were trimmed to 5 cm stubble height in autumn 2017. During the spring growth in 2018, inflorescence emergence was observed visually at 3- to 4-d intervals and the date of the inflorescence emergence interpolated. Flowering synchrony between a pair of plants was estimated based on the date of inflorescence emergence, assuming an equal delay between inflorescence emergence and flowering. After completion of inflorescence emergence, 29 *Fape*, 34 *Fp*, and 25 triploid hybrid plants were selected so that each country of origin was represented by at least two genotypes of *Fape*. Selected plants were allowed to flower and cross-pollinate openly; all remaining plants were trimmed to 5 cm stubble height. No feral *Festuca* plants flowered closer than 200 m from the selected plants. Seeds were harvested individually from each plant at the start of shedding. Further investigation was initiated with the seeds obtained from *Fape* and triploid hybrid plants. About 200 seeds for each of the *Fape* mother plant were germinated at room temperature. However, the germination rate was low (2.7 %). Therefore, remaining seeds were subjected to consecutive cycles of cold treatment (4 ºC for 14 d) followed by two weeks at room temperature. Up to three cycles were applied to increase the germination rate which reached 73.7 %, on average. All seeds collected from triploid hybrids (0 to 5 seeds per plant, 1.5 on average) were subjected to cold treatment (4 ºC for 21 d) and then allowed to germinate at room temperature. Ploidy of seedlings was determined by FCM as described above. We analyzed all seedlings from *Fape* mother plants germinating without cold treatment and a sample of seedlings germinating with one, two, or three cycles of cold treatments. All seedlings obtained from triploid mothers, 8 in total, were analyzed.

Formation of triploid hybrid progeny *in situ* and their performance: Seeds were collected individually from a total of 49 *F. apennina* plants at 11 natural grassland localities in Switzerland in 2017, at the altitudes of 1 525 to 1 850 m a.s.l. Five localities were in the grassland areas of Glaubenbielen (two localities), Kamor, Steiner Berg and Selamatt (see Table 1). Six further localities were in the municipalities of Trimmis and Sedrun (ct. Graubünden), Boltigen and Guttannen (ct. Bern), Bedretto (ct. Ticino) and Ulrichen (ct. Wallis). The ploidy of each plant (and thus, their assignment to *Fape*) was determined from single leaves by FCM. Ploidy of at least 35 individuals randomly sampled in the surrounding area of the plants was determined by FCM using the pooling approach of up to three ramets, as described above. At all sites except Guttannen and Sedrun, triploid hybrids were observed in the neighborhood of the *Fape* plants from which the seeds were collected. Depending on availability, up to 50 seeds per plant were subjected to two cycles of cold treatment (4 ºC for 14 d) and seedlings were planted to *Quickpot* boxes in the greenhouse. At least three seedlings were obtained from each of 46 *Fape* mother plants (out of 49 plants; three plants did not produce any seeds). In total, 860 seedlings reached the stage (at least two leaves) for ploidy determination. Ploidy of all seedlings was determined by FCM as described above. Plants with ploidy other than tetraploid were studied for chromosome constitution by genomic *in situ* hybridization (GISH). Mitotic metaphase spreads prepared from root tips and GISH were performed according to Masoudi-Nejad *et al.* (2002). The allotetraploid *F. apennina* consists of two genomes, one of *F. pratensis* (*Fp*) and the other one close to current *F. glaucescens* (*Fg*-like; Kopecký *et al.* 2016). In order to determine genome composition, total genomic DNAs of *F. pratensis* and *F. glaucescens* were used as probes labelled with digoxigenin and tetramethyl-rhodamine-5-dUTP, respectively, using *Nick Translation* kits according to manufacturer's recommendation (*Roche Applied Science*, Penzberg, Germany). 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. The digoxigenin labelled probe was detected by the Anti-DIG-FITC (*Roche Applied Science*). 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 an *Olympus AX70* epifluorescent microscope equipped with a *SensiCam B/W* camera (*Olympus*, Tokyo, Japan). *ScionImage*, and *Adobe Photoshop* software were used for processing and pseudo-colouring of the images.

Field performance of triploid, tetraploid, and hexaploid progeny obtained from *in situ* pollination was tested at two sites, Reckenholz (440 m a.s.l.) and Früehbüehl (1 000 m a.s.l.). Each of three 3*x* and four 6*x* genotypes were compared to five 4*x* genotypes to which they were half-sibs. Since each of the 3*x* and 6*x* genotypes was from a different *Fape* mother, 5 × (3+4) = 35 tetraploid genotypes were used. Ten genotypes of the autotetraploid *F. pratensis* cv. Tetrax were used as a control. The 52 (three triploids, four hexaploids, 35 tetraploids, and 10 autotetraploid *Fp*) genotypes were clonally propagated to 6 ramets each. Field experiments were in spring 2018 in a completely randomized block design (0.5 × 0.3 m). At each site, three replications (blocks) were used with separate randomization of the 52 genotypes in each block. Performance of the genotypes was estimated by vigor scores on a 9 (very vigorous) to 1 (plant dead) scale. Vigor was scored three times at Reckenholz and twice at Früehbüehl in the planting year A0 (2018), and five times at Reckenholz and four times at Früehbüehl in the first full harvest year H1 (2019).

Fertility and gene flow in natural *L. perenne* × *F. pratensis* hybrids: Natural hybrids were collected from waterlogged soils in the Thames valley near Oxford based on their inflorescence morphology (Humphreys and Harper 2008). The plants were checked for ploidy using *Cyflow* space flow cytometer (see above). Three plants were found to be mixoploids with diploid and triploid tillers; these plants were divided into individual tillers and each tiller was analyzed using flow cytometer before the plants were put into isolation units. Chromosome counts and GISH (see above) were used to confirm the chromosome numbers and reveal the genomic constitution of the plants. The natural hybrids were subsequently classified into three groups: diploid FpLp, triploids FpFpLp and LpLpFp.

Pollen fertility of the hybrids and their progeny was estimated by acetocarmine staining (Shreiber 1954). To investigate the possibility of production of new hybrids and the potential gene flow between species, controlled pollination experiments in pollen-proof isolation units were initiated. The isolation units had positive displacement of air through filters to ensure no rogue pollen could enter the unit. The natural hybrids were cloned, and two clonal ramets of each hybrid genotype were put into each isolation unit with different combinations of diploid and tetraploid *L. perenne* and *F. pratensis* (each genus represented by at least 10 different genotypes). In isolation units 5 and 6, a single genotype of *L. perenne* or *F. pratensis* was separated into 10 clonal ramets.

Six different isolation units involved these genotypes:
1) natural hybrids (2*x* and 3*x*) and *F. pratensis* and *L. perenne* (both species 2*x* and 4*x*)
2) natural hybrids only (2*x* and 3*x*)
3) natural hybrids (2*x* and 3*x*) and *L. perenne* (2*x*)
4) natural hybrids (2*x* and 3*x*) and *F. pratensis* (2*x*)
5) natural hybrids (2*x* and 3*x*) and a single genotype of diploid *L. perenne*
6) natural hybrids (2*x* and 3*x*) and a single genotype of diploid *F. pratensis.*

After flowering, the plants were removed from the isolation units and the number of florets and seed were counted for an estimate of seed yield and hence fertility of the natural hybrids in different isolation combinations. A sample of the progeny produced from these isolation units was established and analyzed for chromosome number, genomic constitution using GISH and fertility.

Results

A general altitudinal pattern of the distribution of diploid *Festuca pratensis* (2*x Fp*), *Festuca apennina* (4*x Fape*), and their triploid (3*x*) hybrids was found in all six sampled grassland areas in the Swiss Alps (Fig. 1). Diploid *Fp* was the sole species at the lowest altitudes, and tetraploid *Fape* was the sole species at the highest altitudes, except for the grassland area Reichenbachtal. Triploid hybrids prevailed at mid-altitudes at variable frequencies but were often the most abundant and sometimes even the sole cytotype. However, the six grassland areas differed in the altitudinal levels at which dominance of a cytotype changed. For example, 3*x* hybrids were strictly restricted to a zone between 1 150 and 1 350 m a.s.l. in the grassland area Oberseetal. Conversely, triploids were found between 1 000 and 1 950 m a.s.l. in Reichenbachtal. *Fp* and *Fape* did not co-occur in balanced abundance frequently. In just one out of 8 pastures of over 2 000 m^2 each with at least 47 specimens, at locality Passhöhe in grassland area Glaubenbielen, the less frequent species of 2*x Fp* and 4*x Fape* occurred with more than 4 % (Table 1). Triploid hybrids and just one of the parents were present in three 2 000 m^2 pastures, and only triploid hybrids were present in one pasture. In the remaining three pastures, both *Fp* and *Fape* were present, but the less frequent of the two was present with only 2 to 3.5 %. Similarly, among the 108 additional horizontal transects shown in Fig. 1 with six plants sampled, at only 5 transects both *Fp* and *Fape* were found. At 42 transects 3*x* hybrids and only one of the parent species were found, and at 10 transects all samples were 3*x* hybrids. At the remaining 51 transects, either *Fp* or *Fape* were the sole species found.

A great majority (82 %) of randomly selected 72 triploid hybrids among the plants represented in Fig. 1 was derived from *Fape* as the seed parent (Table 2). Just 17 % of triploids were derived from a *Fp* mother. One of the 72 plants (1 %) was derived from a *Lolium* parent and was identified by GISH to be a *Festulolium* hybrid *Lolium perenne* × *F. apennina*. The analysis of cpDNA revealed *Lolium* as the maternal parent of this hybrid. The altitudinal distribution of maternity of the hybrids was not related to the relative frequency of the parental species at respective altitudes (Table 2). One might expect that at lower altitudes, relatively more hybrids would be derived from *Fp* mother, and less so at higher altitudes where *Fp* are less frequent compared to *Fape*. However, even at altitudes below 1 225 m a.s.l. where no *Fape* plants were found in any of the grassland areas investigated, over 80 % of triploid hybrids were derived from a *Fape* mother.

At least some seeds were obtained from 27 out of 29 *Fape* plants, with each of the seven countries of origin (Austria, Bulgaria, Italy, Romania, Liechtenstein, and Switzerland) represented, when exposed to open pollination of the set of 29 plants of *Fape*, 34 plants of *Fp*, and 25 plants of triploid hybrid in an isolated experimental field plot of about 30 m^2. However, triploid hybrids did not shed any pollen. Out of 116 seedlings which germinated without cold treatment 113 (97 %) were 3*x* hybrids; the remaining three seedlings were tetraploid *Fape*. From the seedlings germinating after one, two, or three cycles of cold treatment, only 14, 8, and 4 % were 3*x* hybrids, respectively, and the rest were tetraploids. When tetraploid progeny of a given mother plant required several cycles of cold treatment for germination, about half of the triploid progeny of that plant also required cold for germination. Although a single cycle of cold treatment was sufficient for germination of the majority of the tetraploid progeny of a mother plant, a large majority of its triploid hybrid progeny germinated without cold treatment. Based on this observation, we calculated a linear regression of the percentage of triploid progeny germinating without cold treatment on the number of cold treatment cycles required for germination of the remaining, predominantly tetraploid, progeny of the same tetraploid mother plant. This regression was used to estimate the total number of triploid progeny for those mother plants where only the number of triploid progeny germinating without cold treatment and the cold requirement for germination of the remaining seeds was determined.

Formation of 3*x* progeny was significantly ($r^2 = 0.55$, $n = 29$) correlated with relative chances of pollination by *Fp* plants based on the distance and flowering synchrony of neighboring *Fape* and *Fp* plants in the field (Fig. 2). The chances were calculated assuming a drop in the pollination chance within the square of the metric distance between two plants, and with a 10 % drop per day of flowering asynchrony. According to the linear regression shown in Fig. 2, the receptivity of a 4*x Fape* stigma was about six times less for pollen of *Fp* than for pollen of *Fape*. However, there were differences among regions of origin and plants within a region of origin. Both *Fape* plants from Italy, three out of four from Romania and two out of eight from Bulgaria were clearly less prone to form 3*x* hybrids than those from other regions. Three *Fape* individuals, one each from Austria, Liechtenstein, and Switzerland, produced more than double the number of 3*x* hybrid progeny than would be expected from the respective pollination probability by a neighboring *Fp* plant.

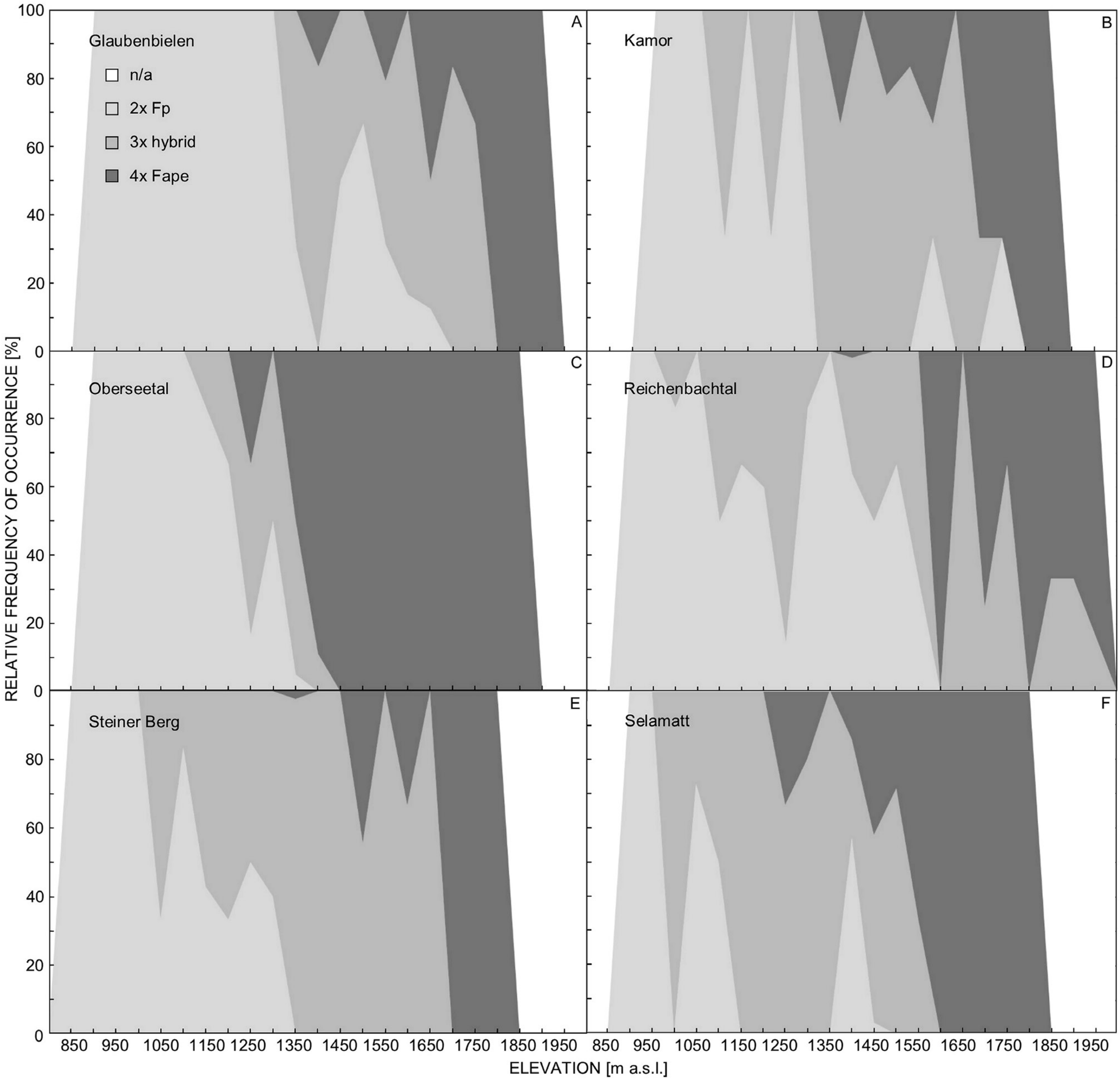

Fig. 1. The distribution of ploidy of broad-leaved *Festuca* individuals sampled in six permanent grassland areas at different altitudes scaled by 50 m. *A* - Glaubenbielen, *B* - Kamor, *C* - Oberseetal, *D* - Reichenbachtal, *E* - Steiner Berg, and *F* - Selamatt. *Light grey* - diploid *Festuca pratensis*, *dark grey* - tetraploid *F. apennina*, *grey* - triploid hybrids.

Out of 860 seedlings obtained from 49 *Fape* plants collected at 11 localities in Switzerland, just three seedlings (0.35 %) were triploid hybrids (Table 2 Suppl.). GISH confirmed that they originated from *in situ* pollination with a *Fp* pollen. Each of these three seedlings was from a different *Fape* mother plant, but two of them grew in the same locality, Schribersboden (grassland area Selamatt). The third one was collected in the locality Küeschte (grassland area Kamor). Both these grassland areas were also the object of an extensive survey of the occurrence of *Fp, Fape*, and their 3*x* hybrids reported above (see Fig. 1 Suppl.). At Küeschte, several potential *Fp* pollen donors were found in the vicinity of the *Fape* plant, the closest one at a distance of 47 m. However, no *Fp* was found among 51 individuals sampled in the pasture around the *Fape* mothers at Schribersboden. The nearest *Fp* plant in that survey was at a distance of about 1 400 m from the *Fape* plants which were mothers of those two triploid seedlings. The rare occurrence of triploids among the progeny of *Fape* was in contrast with the abundance of triploid plants in these localities. The frequency of triploid seed emergence from *Fape* plants at the localities Schribersboden and Küeschte was 0.63 % (two out of 317 seedlings) and 0.68 % (one out of 147 seedlings), however, 49 % (25 out of 51 plants), and 33 % (19 out of 57) plants found in nearby pastures, respectively, were

Table 1. The distribution of ploidy among at least 47 randomly sampled broad-leaved *Festuca* species in 8 pastures of at least 2 000 m^2 in the Swiss Alps with a different mean elevation. *Fp* - *Festuca pratensis*, *Fape* - *Festuca apennina*.

Elevation [m a.s.l.]	Locality	Grassland area	Number of samples	2*x Fp*	3*x* hybrids	4*x Fape*
1364	Mörlialp	Glaubenbielen	59	30.5	69.5	0.0
1566	Passhöhe	Glaubenbielen	48	31.3	47.9	20.8
1406	Loch	Kamor	48	0.0	100.0	0.0
1372	Saaftenboden	Oberseetal	47	2.1	25.5	72.3
1398	Chaltenbrunnen	Reichenbachtal	50	64.0	34.0	2.0
1385	Ahorn	Steiner Berg	70	0.0	98.6	1.4
1445	Zinggen	Selamatt	57	3.5	54.4	42.1
1526	Schribersboden	Selamatt	51	0.0	49.0	51.0

Table 2. The maternity of 3*x* hybrids sampled in six Swiss grassland areas at different altitudes compared to the relative occurrence of their parent species, *Festuca pratensis* and *F. apennina* ([1] - plus one plant from a *Lolium perenne* mother; [2] - two plants from less than 1 025 m a.s.l., both from *F. apennina* mother). *Fp* - *Festuca pratensis*, *Fape* - *Festuca apennina*.

Altitudinal range [m a.s.l.]	Distribution of ploidy of randomly sampled Festuca plants			Maternity of selected 3x hybrids	
	2*x Fp*	3*x* hybrids	4*x Fape*	*Fp* [%]	*Fape* [%]
< 1225	76.1	23.9	0	2 (18.2)	9 (81.8)[2]
1225 to 1325	45.7	47.1	7.1	3 (33.3)	6 (66.7)
1325 to 1425	20.1	66.8	13.2	3 (13.6)	19 (86.4)
1425 to 1525	11.9	54.5	33.6	3 (27.3)[1]	7 (63.6)
1525 to 1625	13.8	42.8	43.4	1 (7.7)	12 (92.3)
>1625	1.9	21.4	76.7	0 (0.0)	6 (100.0)

triploids. This was only slightly higher than the frequency of triploids identified among 540 broad-leaved *Festuca* plants collected over all 11 localities (26 %).

However, not all of the remaining seedlings (out of 860) had the expected 4*x* ploidy. Five of them were hexaploid. The GISH revealed that they resulted from fertilization involving one reduced and one unreduced gamete of *Fape*. All three triploid and four of the five hexaploid seedlings developed sufficiently to assess their vigor in a field experiment (see below).

We investigated the vigour of the three triploid and four hexaploid seedlings compared to their tetraploid half-siblings and ten genotypes of tetraploid *Fp* cv. Tetrax. For each of the triploid and hexaploid seedlings, five randomly selected tetraploid half-siblings were chosen. All three triploid hybrid genotypes outperformed their tetraploid *Fape* half-siblings at both experimental locations, Früehbüehl (1000 m a.s.l) and Reckenholz (450 m a.s.l.) (Table 3). The difference increased from the planting year (A0) to the first full harvest year (H1). At Früehbüehl, 3*x* hybrids were also significantly more vigorous than the tetraploid *Fp* cv. Tetrax. At Reckenholz, there was no significant difference between the performance of 3*x* hybrids and Tetrax. Tetraploid *Fape* performed similarly to Tetrax at Früehbüehl but much poorer at Reckenholz. Hexaploid plants were always less vigorous compared to the tetraploid siblings. The difference was significant for all years and locations, except for Reckenholz in H1. Similar as 4*x Fape,* 6*x Fape* plants performed much better at Früehbüehl (1 000 m a.s.l.) when compared to Reckenholz (450 m a.s.l).

Indehiscent anthers were observed in all 3*x* hybrids at the flowering stage, indicating complete male sterility (Fig. 2 Suppl.). This characteristic allows visual identification of triploids in the field. However, a certain, though an extremely low female fertility was observed. Out of 25 triploid hybrids allowed to reach the seed ripening stage in a field at Reckenholz grown in the presence of 29 *Fape* and 34 *Fp* plants, 15 plants produced a few (one to five) seeds, 28 in total. The germination rate of these seeds was only 25 % (seven out of 28), compared to 74 % of *Fape* and 90 % of *Fp.* Those seven germinating seeds originated from four 3*x* mother plants (Table 3 Suppl.). Compared to *Fape* and *Fp* plants of similar vigour, this corresponds to 0.02 and 0.004 % viable seed set of these cytotypes, respectively.

Of the 7 progenies of triploid hybrids, 4 were tetraploid and 3 were triploid. Tetraploids were less vigorous than triploids and only one tetraploid plant was still alive when GISH analysis was carried out. Chromosome constitution of this 4*x* progeny plant was 21*Fp* + 7*Fg*-like chromosomes, suggesting a scenario of an unreduced egg cell of the

Table 3. Annual averages of vigor scores (scale 1 to 9, 9 is best) of three 3*x* hybrid and four 6*x* *Festuca apennina* genotypes compared to the average of 5 regular 4*x* *F. apennina* progeny of the corresponding *F. apennina* mother plant, or 10 genotypes of 4*x* *F. pratensis* cv. Tetrax. Means within a row not followed by the same letter are significantly different ($P < 0.05$) (^{1} - comparisons 3*x* *vs.* 4*x* and 4*x* *vs.* 6*x*: the *F*-test for the factor "ploidy" in an analysis of variance with a model of y = ploidy mother replication; ^{2} - comparisons with 4*x* *F. pratensis* Tetrax: Duncan's multiple range test, model y = ploidy replication). *Fp* - *Festuca pratensis*, *Fape* - *Festuca apennina*.

Trial site (altitude)	Year	Comparison	3*x* hybrid^{1}	4*x* *Fape*^{1}	6*x* *Fape*^{1}	4*x* *Fp* Tetrax^{2}
Früehbüehl (1000 m a.s.l.)	A0	3*x* *vs.* 4*x*	8.83^{a}	7.26^{b}		6.76^{b}
		4*x* *vs.* 6*x*		6.67^{b}	5.63^{c}	
	H1	3*x* *vs.* 4*x*	8.94^{a}	6.45^{b}		6.59^{b}
		4*x* *vs.* 6*x*		6.20^{b}	4.69^{c}	
Reckenholz (450 m a.s.l.)	A0	3*x* *vs.* 4*x*	7.85^{a}	3.51^{b}		6.99^{a}
		4*x* *vs.* 6*x*		3.22^{b}	2.27^{c}	
	H1	3*x* *vs.* 4*x*	6.49^{a}	1.93^{b}		7.29^{a}
		4*x* *vs.* 6*x*		1.37^{b}	1.00^{b}	

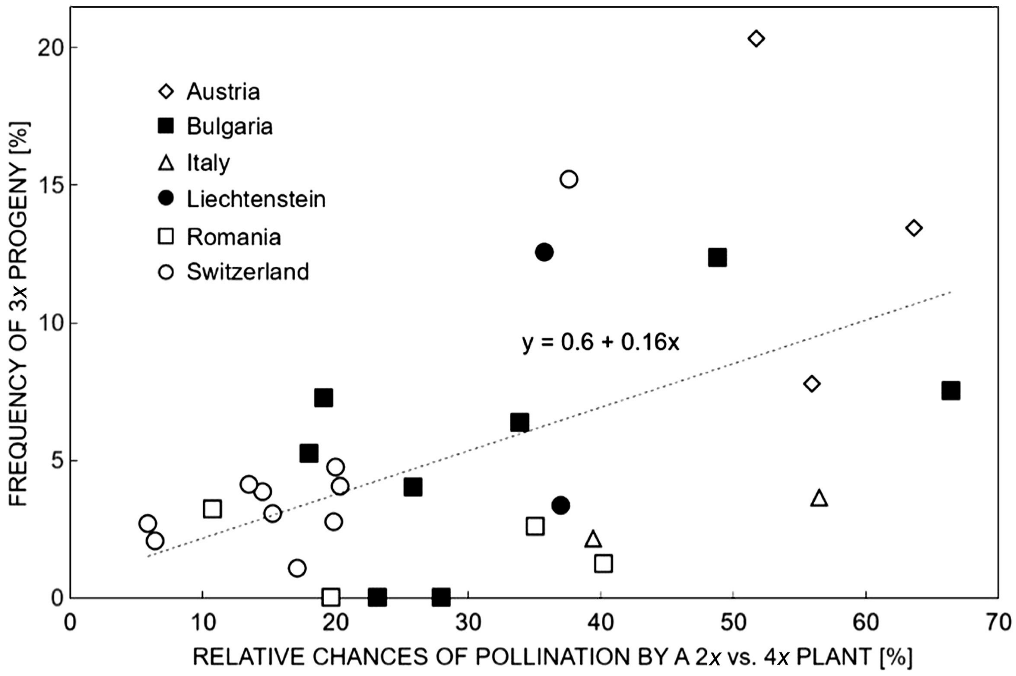

Fig. 2. The occurrence of triploid (3*x*) seedlings in progenies of 28 individual *Festuca apennina* (4*x*) plants originating from 6 European countries plotted against the relative chance of each *F. apennina* to be pollinated by a 2*x* *F. pratensis* plant and thus yielding 3*x* progeny. Individual *Festuca apennina* plants were exposed to open pollination by a fixed number of randomly distributed 2*x* *F. pratensis* and 4*x* *F. apennina* plants. Relative chances were calculated on the basis of distances to neighboring plants and flower synchrony with them. The regression coefficient $r^2 = 0.297$.

triploid hybrid (14*Fp* + 7*Fg*-like) pollinated with a haploid pollen of *Fp* (7*Fp*). All three triploid progeny plants had the same chromosome constitution (14*Fp* + 7*Fg*-like) as their mother plant. Different scenarios are possible in this case, such as a haploid 7*Fp* egg cell after complete loss of the *Fg*-like genome during meiosis, pollinated by a diploid pollen of 4*x* *Fape* (7*Fp* and 7*Fg*-like) or apomictic propagation from an unreduced egg cell of the triploid hybrid.

Pollen fertility, seed set, and seed germination from the isolation units, of natural hybrids of *L. perenne* × *F. pratensis* is shown in Table 4 Suppl. The pollen fertility was somewhat lower in the diploid hybrids with 3.9 % (*FpLp*), compared to 7.8 % in *LpLpFp* triploid and 8.1 % in *FpFpLp* triploid. In diploid hybrids, seed set was 0.5 % but the seed did not germinate, and no viable plants were produced. Seed set in the triploids *LpLpFp* and *FpFpLp* was 0.5 and 0.8 % with the germination rate of 22.0 % (13 plants produced) and 2.1 % (14 plants produced), representing the fertility rate of 0.10 and 0.02 %, respectively.

The 13 progenies from maternal triploids *LpLpFp* came from isolation units 2 and 3, and 14 progenies

Table 4. The genome composition and pollen fertility of the progeny of the triploid hybrids.

Mother plant	Progeny	Isolation unit	Genome composition	Pollen fertility [%]
LpLpFp	1	2	2n=28; 21*Lp* + 7*Fp*	62
	2	3	2n=21; 16*Lp* + 5*Fp*	2
	3	3	2n=20; 14*Lp* + 6*Fp*	36
	4	3	2n=21; 14*Lp* + 7*Fp*	4
FpFpLp	1	1	2n=21; 14*Lp* + 7*Fp*	27
	2	1	2n=21; 14*Lp* + 7*Fp*	29.7
	3	2	2n=21; 14*Lp* + 7*Fp*	30.7
	4	2	2n=21; 14*Lp* + 7*Fp* + recombinations	17.7

from maternal *FpFpLp* came from isolation units 1, 2, 3, and 6. The progenies produced from triploid hybrids (either with each other or with parental species) varied in genome composition (Table 4). Eight progeny plants were studied in detail. Seven of these plants retained the triploid chromosome constitution (including triploidy with one missing chromosome). The combination of parental gametes involved in the hybridization events could not be unambiguously determined, however, *FpFpLp* hybrids, hybridized with each other or with parental species (isolation units 2 and 1) produced progeny (four plants analyzed) with the genome constitution *LpLpFp* (one plant with evidence of homoeologous recombination). This means, that one genome of *F. pratensis* was replaced by one genome of *L. perenne*. The reciprocal of this was not observed, *L. perenne* genome has never been replaced by the genome of *F. pratensis*; the progeny of *LpLpFp* hybrids either possessed the same genomic composition as their mother plant (one plant), had substitutions of two *Fp* chromosomes by two *Lp* chromosomes (one plant) or showed elimination of one *Fp* chromosome. The only tetraploid progeny plant with genome constitution 21*Lp* + 7*Fp* chromosomes (thus, being *LpLpLpFp*) probably arose from the merge of unreduced gamete of the triploid and a normal gamete of diploid *L. perenne*.

The progeny of a *LpLpFp* mother plant differed in pollen fertility. While tetraploid plant (No. 1) had pollen fertility at 62 %, only 2 % pollen fertility was observed in the triploid plant (No. 2) with genome constitution 16*Lp* + 5*Fp*. Unexpectedly, aneuploid plant (No. 3) had high pollen fertility (36 %), much above that of all euploid triploids. On the contrary, all four triploid progeny plants from mother plant *FpFpLp* had similar pollen fertility ranging from 17.7 to 30.7 %.

In natural hybrids, aerial tillering and vivipary were noted. These traits were also observed in a number of the progeny from these plants.

Discussion

Triploid hybrids between diploid *F. pratensis* and tetraploid *F. apennina* occurred frequently and with a clear altitudinal pattern in all six investigated grassland areas of the Swiss Alps. This confirms and expands the previous results (Kopecký *et al.* 2018) where triploids were found much more frequently at about 1 350 m a.s.l. than at 1 550 and 1 750 m a.s.l. The sympatric appearance of both parental species does not seem to be necessary for the widespread distribution of their triploid hybrids. Triploid hybrids were found at lower altitudes down to 996 m a.s.l. whereas no *F. apennina* was found in grassland below 1 236 m a.s.l. (Fig. 1). Similarly, triploid hybrids occurred up to 1 943 m a.s.l. while no *F. pratensis* was found above 1 702 m a.s.l. Moreover, the maternity of the triploid hybrids sampled at different altitudes did not reflect the altitudinal distribution of their parental species. Even at the lowest altitudes (below 1 225 m a.s.l.) where no *F. apennina* was found, most triploid hybrids were derived from a *F. apennina* mother (Table 2). Therefore, wind pollination of locally growing *F. pratensis* by more distant *F. apennina* plants growing at higher altitudes and subsequent shedding of hybrid seeds in the vicinity of the *F. pratensis* was not primarily responsible for the downward expansion of the triploid hybrids. Long-distance seed dispersal is likely to play a more important role for the spread of the triploid hybrids far beyond zones of co-occurrence of the two parent species than pollen dispersal by wind. Haymaking at high altitudes is not common in the grassland areas studied, thus human-mediated seed dispersal by fodder transport probably plays only a minor role. However, grazing cattle are often moved over long distances in the summer grazing systems prevailing at the regions of study. Near the end of the summer period when seeds are ripe on *Fape*, cattle are transferred from higher to lower altitudes to graze the regrowth, and are good candidates as vectors for the downward seed dispersal. “Bristly” grass seeds such as *Nardus stricta* or *Festuca ovina,* to which we may rightly also assign *Festuca apennina* seeds with their awns remain attached to coats of grazing animals when moving through grassland (Mouissie *et al.* 2005), more on sheep but also on cattle coats. Seeds are retained sufficiently long on sheep wool to be transported beyond 1 km distance but not as long on cattle coats. A more likely mode of long-distance dispersal of *F. apennina* seeds is endozoochory, a passage through the digestion system and deposition with faeces. Cosyns *et al.* (2005) studied the emergence of seedlings from cattle dung after the animals had grazed on extensively used grassland. Grasses of the genera *Poa, Holcus*, and *Agrostis* were among the most frequent species emerging. *Festuca rubra* seedlings also emerged, though at lower frequencies. Different *Gramineae* were also among the species that emerged from white-tailed deer pellets in the study of Myers *et al.* (2004). The frequent occurrence of both 4*x* *F. apennina* and 3*x* hybrids in places were grazing cattle like to rest (so-called "Läger"), as observed in our previous study (Kopecký *et al.* 2018), supports the hypothesis that cattle faeces may be the main vector of the long-distance seed dispersal of *F. apennina* seed. Additionally, dung deposits boost the availability of nutrients and thus help to create an optimal environment for the establishment of the high nutrient demanding *F. apennina* and 3*x* hybrids.

Triploid hybrids formed spontaneously in an experimental setting with open pollination among *Fape* and *Fp* (Fig. 2). In this field trial, we investigated the formation of hybrids in the progeny of *Fape* mothers only because most hybrids in nature are derived from *Fape* mothers. Moreover, hybrid progenies are much more easily detected when *Fape* is the seed parent due to the differential germination behavior of tetraploid and triploid seeds. While tetraploid *Fape* seeds require cold treatment for germination (Tyler *et al.* 1978), a seed that develops into a triploid hybrid has a much smaller requirement for cold treatment (Boller *et al.* 2018). This finding was confirmed in the study presented here. Almost all (97 %) of seedlings germinating without cold treatment were triploid hybrids, whereas a great majority of seedlings germinating after one or more cold treatment cycles were tetraploid (86, 92, and 96 % for one, two, or three cycles of the cold treatment, respectively). Analysis of the frequency of hybrid formation identified the metric distance and flowering synchrony to pollinating *Fp* or *Fape* plants growing nearby as a major source of variation. Assuming no self-pollination, the formation of triploid hybrids was only about six times less likely than would be expected from the vicinity to pollinating *Fp* vs. *Fape* plants. Thus, the compatibility of *Fp* pollen on a *Fape* stigma was at least 16 % of the compatibility of *Fape* pollen. If a certain rate of self-pollination of *Fape* is assumed, the relative fertilization success of *Fp* pollen on *Fape* was even higher. The easy cross-fertilization of *Fape* pollinated with *Fp* contrasts with the cumbersome production of interspecific and intergeneric hybrids observed earlier in breeding programs; the seed parent is usually emasculated to avoid self-fertilization, but even then, the cross-fertilization rate is low. For example, Jenkin (1933) obtained only 12 F1 hybrids from 8 000 manually emasculated florets of diploid *L. perenne* pollinated by diploid *F. pratensis*. Gröber *et al.* (1974) obtained less than 1 % seed per emasculated florets of *F. pratensis* when pollinated with *Lolium* species. In their study, the combination *F. pratensis* × *F. arundinacea* yielded the most viable seeds, 5.9 % seeds per emasculated floret when diploid *F. pratensis* was the seed parent, and even 14.6 % when *F. arundinacea* was the seed parent pollinated with autotetraploid *F. pratensis*. In our present study, some *Fape* plants produced much more hybrid progeny than expected from the vicinity to *Fp* pollinators. The highest rate of triploidy among progeny was 20.32 % for a plant which had a 51.7 % chance to be pollinated by 2*x* *Fp*. Therefore, on this particular plant, fertilization success of pollen of 2*x* *Fp* was (20.32/51.7)×100 = 40.01 %. Four more plants had the fertilization success above 32 % (double of the average 16 %) of *Fp* pollen. This may suggest a genetic variation for the tendency to produce interspecific hybrids. Genetic systems controlling interspecific crossability are known in other plant species including *Kr* system in wheat (Krolow 1970). However, the heritability of this character has not been studied in this study.

Despite a relatively good chance of obtaining triploid hybrids in an experimental setting, hybrid seedlings were rare when the seed was collected on *Fape* plants in natural grassland (Table 2 Suppl.). The low number of hybrid seedlings contrasted with the high number of triploid hybrids occurring nearby. At the two localities where triploid seedlings were identified among the progeny of open-pollinated *Fape,* the abundance of triploid hybrids in nearby grassland was 49 and 21 %, but the abundance of triploid progeny from *Fape* was only 0.53 and 0.66 %. Thus, the abundance of triploid hybrids in the grassland was 32 to 92-times higher than the proportion of triploid progeny among seedlings. There are several possible explanations for this discrepancy.

1) Triploid hybrids may have a better chance of establishment under the specific conditions of these mid-altitude sites due to their reduced cold requirement for germination. The cold requirement is a defense mechanism to avoid winter kill of young seedlings germinating in the autumn. However, direct germination after seed shedding may be more advantageous at lower altitudes with milder winters, as providing competitiveness to well-established seedlings in the spring. As climate change accelerates, triploid seed may be able to germinate and establish at higher altitudes and where there is a reduced cold period, and tetraploid seed may increasingly fail to germinate increasing the advantage of triploid seed.

2) Triploid hybrids exhibit extraordinary heterosis (hybrid vigor). Mid-parent heterosis of triploid hybrids over their *Fp* and *Fape* parents of up to 600 % was observed by Boller *et al.* (submitted). All three triploid hybrids raised from open-pollinated *Fape* plants listed in Table 3 were markedly more vigorous than their corresponding tetraploid *Fape* half-sibs. A difference in vigor score of 2.5 units (8.94 *vs*. 6.45), as reported for the 1 000 m a.s.l. site in the year after planting, H1, translated into a 3-fold higher dry mass yield according to the exponential regressions reported by Boller *et al.* (submitted). Thus, triploid hybrids are expected to be much more competitive in the pasture than tetraploid *Fape.* However, hexaploid seedlings (formed from the fusion of reduced and unreduced gametes of *Fape*) were significantly less vigorous than their tetraploid *Fape* half-sibs at high altitudes (Table 3), indicating their low competitiveness in the pastures. In these seedlings, no hybridization but polyploidization is involved, which did not show any benefits to plants studied here. Indeed, in the survey of the ploidy levels in six grassland areas reported here, none of the 1044 *Festuca* plants was hexaploid *Fape*.

3) The rates of triploid hybrids among seeds of open-pollinated *Fape* may have been much higher in the past when their parental species were more frequently co-occurring than today. This possibility is illustrated by pastures composed of triploid hybrids in the absence of both parental species (Table 1 and Fig. 1). Indeed, in the pasture "Loch" in the region Kamor with all 48 *Festuca* plants being triploid hybrids, these hybrids were also very competitive in the grassland biotopes. Over the 48 individual sampling points, we estimated that on average they contributed 32 % (s.d. 21) to the yield of all grassland species present. Similarly, we did not find any other cytotype but triploids in several Swiss alpine pastures during our collection expedition in 2015 (Kopecký *et al.* 2016).

4) Many triploid hybrid seeds may have been brought to the pastures through long-distance seed dispersal from sites of more balanced co-occurrence of *Fp* and *Fape*, rather than having been formed and spread by seed shedding at the sites themselves.

Triploid hybrids were mostly sterile, but a small number of seeds were recovered from such plants exposed to pollen of *Fp* and *Fape*. However, their viability was low with just 0.29 of viable seeds per plant compared to *Fape* with 1 572 of seeds per plant. They produced seedlings that were either triploid like their mother, or tetraploid. Unreduced eggs of a triploid *Fape* × *Fp* mother were likely involved in the formation of tetraploid progeny. Triploid seedlings may have arisen *via* apomixis. Gröber *et al.* (1974) created interspecific and intergeneric hybrids of various species of *Festuca*, *Lolium*, and *Bromus*. They found that hybrids between parents of different ploidy were largely sterile, but particularly vigorous, and suggested that apomictic propagation of otherwise sterile hybrids might be a promising way to exploit their high yield potential in breeding. Apomictic propagation might contribute, in addition to rhizomatous expansion, to the large horizontal spread (up to 14.4 m) of individual triploid hybrid genotypes observed by Kopecký *et al.* (2018). However, this contribution is probably small. The establishment of a triploid seedling originating from a new hybridization event between *Fape* and *Fp* seems more likely than the establishment of a triploid seedling from one of the very rare seeds of a triploid hybrid plant. In their natural habitats, open pollinated *Fape* plants form about 0.35 % of hybrid seeds (Table 2 Suppl.), but in an experimental setting, triploid hybrid plants formed only 0.02 % of viable seeds compared to *Fape* plants (Table 3 Suppl.). Thus, seed propagation of triploid hybrids may be a significant way of spreading only when none or very little 4*x* *Fape* is present in the vicinity. Indeed, at the locality Ahorn, with 99 % triploid hybrids among all *Festuca* sampled, Kopecký *et al.* (2018) found all 18 randomly sampled individuals of triploid hybrids to be genetically different, thus arisen from different hybridization events.

Diploid and triploid *L. perenne* × *F. pratensis* hybrids, commonly known as *Festulolium loliaceum,* are found throughout the UK and Western Europe. This suggests that these hybrids can originate wherever the progenitor species grow sympatrically. These diploid and triploid hybrids differed: the diploids had a somewhat lower pollen fertility (3.9 %) and produced no viable plants from the 476 seeds which were allowed to germinate while triploids had higher pollen fertility (7.8 % in *LpLpFp* and 8.1 % in *FpFpLp*) with relatively high germination rates in triploid *LpLpFp* hybrids (~22 %) and lower, but still some germination in triploid *FpFpLp* hybrids (~2 %). The reason why triploids outperform diploids in the germination rate and, to a lesser extent, pollen fertility remains unknown. One may expect that homoploid (*LpFp*) will show reduced (or completely absent) pairing of homoeologous chromosomes during meiosis leading to unbalanced and non-functional gametes. However, chromosomes of *Festuca* and *Lolium* display promiscuous pairing and recombination in *Festulolium* hybrids, including diploid homoploid of *L. perenne* × *F. pratensis* (Kopecký *et al.* 2008; for visualization of meiosis in diploid *L. perenne* × *F. pratensis* hybrids, see https://olomouc.ueb.cas.cz/getattachment/Research-groups/Kopecky-group/Meiosis-With-Labeled-Parental-Genomes.pdf.aspx?lang=en-US).

The fact that several germinating seeds of triploid mother plants were obtained from the isolation unit consisting exclusively of hybrids (unit 2), suggests that triploids represent the evolutionarily successful type. This is in line with the hypothesis of Husband (2004) that a 'triploid bridge' is expected to be a more frequent strategy for the establishment of hybrid genomes than *via* polyploidization of homoploid hybrids due to the low probability of fusion of two unreduced gametes in natural populations.

In some cases, pollen fertility increased considerably in the subsequent generation of these hybrids (up to 62 %.), which further increases the chances of their establishment in native grasslands. Besides increased pollen fertility, they also developed alternative strategies to persist in different environments: vivipary and aerial tillering. This mode of reproduction is not unexpected, as these hybrids frequently occur in water meadows subjected to seasonal flooding. The offspring of these hybrids, once backcrossed to *L. perenne*, retains the aerial tillering and vivipary, suggesting a genetic mechanism behind these traits, not triggered by the environment alone. These traits and increased fertility lead to increased competitiveness of *Fl. loliaceum*. Gymer and Whittington (1976) and Kulik (2011) reported that *Fl. loliaceum* can compete with other grass species in a sward under disturbed conditions. This agrees with the observations on other allopolyploids. In general, they display broader adaptation to novel environmental niches compared to their progenitor species, hence a greater ability to colonize disturbed and harsher habitats (Te Beest *et al.* 2012), leading to increased invasiveness (Pandit *et al.* 2011). The natural *L. perenne* × *F. pratensis* hybrids used in this experiment were collected from water meadows and their ability to survive waterlogging for at least a part of the year might be assumed to be an adaptive advantage. Similarly, Gymer and Whittington (1976) and Graiss (2011) studied the performance of *Festulolium* hybrids in plot experiments with *L. perenne* and *F. pratensis* under different experimental regimes and found that hybrids increased in proportion over time in most of the conditions. This may trigger potential utilization of natural

Festulolium hybrids in breeding programs, as sources of novel gene combinations for areas prone to flooding.

The hybrid genomes were modified over generations. In two out of four progeny plants of *LpLpFp*, a reduction of *F. pratensis* chromosomes was seen. Similarly, in all four progeny plants of *FpFpLp*, one entire genome of *Fp* was replaced by that of *Lp*, giving the genome composition of progeny *LpLpFp*. The predominance of *Lolium* genome over that of *Festuca* has been observed in other studies on the genome constitution of *Festulolium* hybrids. All commercial cultivars of *L. multiflorum* × *F. pratensis* and *L. perenne* × *F. pratensis* exhibit the prevalence of *Lolium* chromosomes over those of *Festuca* (Kopecký *et al.* 2006). Zwierzykowski *et al.* (2006, 2011) conducted a study over eight successive generations of such hybrids and observed a slow but consistent replacement of the *Festuca* by *Lolium* chromosomes.

In seven out of eight progeny plants, triploidy of the mother plants persisted. How these plants retain triploidy is an intriguing question. Some species of water frogs can reproduce *via* hybridogenesis. This means that during gametogenesis of triploids (ABB), they discard the genome of one of the parental species (A) and produce diploid gametes of the other parental species (BB). The hybrid triploidy (ABB) is restored by fertilization of these gametes with haploid gametes from the parental species whose genome was eliminated (A). Thus, the population is always at the pseudo-F1 generation (Christiansen and Reyer 2009). However, this requires absence of homoeologous chromosome pairing and the chromosomes of *Lolium* and *Festuca* pair regularly. Alternatively, retention of triploidy might be triggered by apomixis. This appears as an unlikely general scenario, as only one out of six triploid progenies with 21 chromosomes had the same genome composition as the maternal plant. Thus, the retention of triploidy in our hybrids remains a mystery.

Conclusions

This paper demonstrates that even rare interspecific hybridization events may allow a new hybrid not only to survive but thrive and extend beyond the range of its progenitor species. As we move into an era of climate change with extremes of weather becoming the norm, new hybrids within the *Lolium-Festuca* complex may find new niches to colonize (Ghesquiere *et al*., 2010). In some cases, they might even outcompete and replace their progenitors: the triploid *F. apennina* × *F. pratensis* hybrids reported here which do not require cold to germinate, and have superior vigour, are replacing the tetraploid parent *F. apennina* which needs a cold treatment to germinate. With milder winters at higher altitudes becoming the norm, tetraploid *F. apennina* may become less competitive with other species and even its hybrids, which germinate in the autumn and thus, have a head start over the parental species in the spring. As with natural hybrids between *L. perenne* and *F. pratensis*, which have found a niche in flooded areas where their parental species cannot compete, they offer possibilities of genetic combinations that can be utilized in breeding programs.

These hybrids can be the bridging species in transferring genes between the progenitor species. There is a certain degree of fertility in the hybrids allowing them to transfer their genetic material in hybridization events with their progenitor species creating unlimited new intra- and interspecific combinations. The frequent occurrence of easily detectable triploid hybrids lets us speculate that the formation of hybrids in nature may be far more common than generally believed.

A natural hybrid has several options to survive: *1*) through apomixis or clonal reproduction *via* rhizomes in diploid or triploid hybrids; *2*) through limited pollen fertility that allows mating with other hybrids or progenitor species; and *3*) through unreduced gametes creating new allopolyploid combinations with increased fertility by restoration of diploid-like chromosome pairing behavior during meiosis.

We envisage that interspecific hybrids enabling the transfer of agriculturally beneficial genes and traits between species will play a crucial role in future breeding to mitigate the impacts of climate change.

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