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MINI REVIEW

## Cytogenetic insights into *Festulolium*

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### Abstract

Climate change calls for new methods and plant materials to breed crops adapted to new environmental conditions. Sustainable forage and amenity grass production during periods of severe drought and heat waves during summer, and unequal distribution of precipitation over the year will require drought-tolerant genotypes. However, high-yielding ryegrasses (*Lolium* spp.), which are the most commonly used grass species, suffer during abiotic stresses. Introgression of drought and heat tolerance from closely related fescues (*Festuca* spp.) offers an opportunity to develop superior hybrid cultivars to mitigate the negative impact of climate change. Intergeneric cross-hybridization and the development of *Festulolium* (*Festuca* × *Lolium*) hybrids was initiated 100 years ago and resulted in registration of almost one hundred cultivars. For a long time, their genome composition was not known and was debated by breeders and geneticists. In the last three decades, molecular cytogenetic and genomic approaches have enabled their detailed characterization. These studies revealed a gradual replacement of *Festuca* chromosomes by those of *Lolium* in consecutive generations leading to an almost complete elimination of *Festuca* chromatin in the introgression forms. On the other hand, amphiploid cultivars seem to be more stable with the optimal proportions of the *Lolium* to *Festuca* genomes at about 2:1. In this mini review, we discuss recent advances in the analysis of the genome composition of *Festulolium* hybrids with a specific focus on genome (in)stability.

*Additional key words:* chromosome pairing, *Festuca* spp., genome balance, genome composition, *Lolium* spp.

### Introduction

Grasslands are one of the largest ecosystems in the world covering about one-third of the Earth's terrestrial surface and comprising 80 % of the agriculturally productive land (Boval and Dixon 2012). Grasses (*Poaceae*), a family of monocotyledonous plants, encompass cereals, turf, and pasture grasses, and other economical species such as bamboos and energy grass *Miscanthus*. They are adapted to a full range of environmental conditions, spanning from hot equatorial areas to the coldest regions towards the poles, and from deserts to aquatic habitats. They play an essential role in agriculture, being a major staple for animal diets, and also provide valuable ecosystem services. Grasses reduce water runoff (Macleod *et al.* 2007), stabilize the soil profile and prevent soil erosion. They also have an aesthetic role and serve as ground cover for cultural and

recreational needs such as sport and amenity lawns.

Recently, cultivation of grasses is heavily impacted by the climate change. The last four years were the hottest on record and severe drought periods are more frequent (Trnka *et al.* 2013, Zahradnický *et al.* 2015). Models of future climate predict continuing change in the next few years with a higher frequency of dry and hot periods during summers and more uneven distribution of precipitation over the year (UN Climate Action Summit 2019). Climate change affects humans globally and in response, new market requirements have emerged, especially in agriculture. To mitigate the impact of climate change and to meet new demands, it is necessary to incorporate new strategies into plant breeding to develop new cultivars suitable for future use.

The gene pool of each species has its limits of genetic variation, and this variation is regularly narrowed down

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*Abbreviations:* GISH - genomic *in situ* hybridization.

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even further during cultivar development. Thus, widening of the gene pool and introgression of genes underlying beneficial traits seem a prerequisite for breeding progress and development of cultivars with the ability to withstand future climatic conditions. One of the approaches already applied with considerable success is interspecific (wide) hybridization.

### Interspecific hybridization

Interspecific hybridization, frequently accompanied by whole genome duplication (process called allopolyploidization) refers to mating of individuals from different species. Allopolyploidization is widespread in nature and has been an important force in plant evolution and speciation (Soltis and Soltis 2009). Many economically important species are allopolyploids, including wheat, rapeseed, cotton, and banana (Morgan *et al.* 2011). Besides these ancient allopolyploids, which have originated thousands of years ago, recent and ongoing natural interspecific hybridization and allopolyploidization events are known, including *Tragopogon* and *Senecio* (Hegarty and Hiscock 2009).

Interspecific hybridization has been used in plant breeding with various aims: 1) merging of entire genomes of parental species to combine their desirable traits (amphiploid forms), 2) merging of entire genomes of parental species to broaden the gene pools, and 3) introgressions of desirable trait/traits from one (frequently wild) species into elite cultivars of another species (introgression forms). One of the most successful interspecific hybrids synthetically developed is *Triticale*, a hybrid of wheat (*Triticum*) and rye (*Secale*). It combines high yielding property of wheat with the ability to grow in harsher conditions due to abiotic stress tolerance inherited from rye (Arseniuk 2015). Similarly, several agronomically important traits have been transferred from wild relatives into cultivars by wide hybridization such as the soft grain endosperm structure from *Aegilops speltoides* into bread wheat (Pshenichnikova *et al.* 2010), black rot resistance from *Brassica carinata* into cauliflower (Sharma *et al.* 2017), and leaf rust resistance from *Aegilops tauschii* to triticale (Majka *et al.* 2018).

### *Festulolium* cultivars

The wide hybridization approach has also been used in the forage grass breeding. *Festulolium* hybrids obtained by crossing *Festuca* ssp. (fescues) with *Lolium* spp. (ryegrasses), combine complementary agronomic attributes of the parental species. Ryegrasses are important fodder crops with high seed yield, excel in digestibility and palatability and show highly desirable rapid and intensive spring growth. Fescues, on the other hand, provide traits associated with abiotic and biotic stress tolerance, such as drought tolerance, freezing tolerance, winter hardiness, and resistance to fungal diseases (Thomas and Humphreys 1991, Jauhar 1993, Plažek *et al.* 2018). To date, 78 *Festulolium*

cultivars have been released worldwide – 33 developed by the amphiploid breeding and 45 resulting from the introgression breeding (Humphreys and Zwierzykowski 2020). Amphiploid cultivars are developed by intermating of F<sub>1</sub> hybrids followed by selection of hybrids with about equal proportions of parental chromatin. On the other hand, introgression cultivars originate from an interspecific F<sub>1</sub> hybrid (frequently created from parents of different ploidy), followed by one or more backcrosses to one of the parents. This results in the forms morphologically close to recurrent parent, but with one or a few improved characteristics introgressed from the other species.

*Festulolium* cultivars are produced from diverse intergeneric hybrids. The most common cross combinations are *L. multiflorum* × *F. pratensis* (amphiploid forms) and *L. multiflorum* × *F. arundinacea* (introgression forms; Humphreys and Zwierzykowski 2020). However, a recent need for drought tolerance in new cultivars calls for the introduction of different species into the mating schemes. According to Černoch and Kopecký (2020), *F. glaucescens* and *F. mairei* are the most promising fescue species for initial hybridization with ryegrasses and the development of resilient *Festulolium* cultivars. Mating in such combinations has already been initiated at IBERS (Institute of Biological, Environmental and Rural Sciences, Aberystwyth) and DLF Seeds & Science (Humphreys and Zwierzykowski 2020, V. Černoch, personal communication).

For a long time, the genetic constitution of many *Festulolium* hybrids remained unknown. Only the availability of molecular methods provided tools for comprehensive investigation of the genome composition of hybrids at either chromosomal, or DNA levels (Thomas *et al.* 1994, Kopecký *et al.* 2011). *Festulolium* hybrids show a unique combination of two features: 1) the similarity between parental *Festuca* and *Lolium* chromosomes at the DNA sequence allows for their pairing in meiosis (homoeologous pairing), and, on the other hand, 2) repetitive elements of the two parental species diversified enough for unambiguous identification of the *Festuca* and *Lolium* chromosomes in hybrids by the genomic *in situ* hybridization (GISH) (Fig. 1). Combination of GISH with high-throughput genotyping, e.g., using diversity arrays technology and single nucleotide polymorphism platforms, provides an invaluable tool for tracking genome structure and evolution during the development of new cultivars and for the protection of existing cultivars.

### Homoeologous chromosome pairing and recombination

It is a general feature of wide hybrids that chromosomes from the parental species (homoeologues) do not pair and recombine in meiosis. The absence of homoeologous chromosome pairing can be a consequence of the DNA sequence dissimilarity or the presence of a genetic system(s) that prevent such pairing. Several meiotic regulators have been identified in allopolyploids including *Ph1* (pairing homoeologous 1) in wheat (Riley and Chapman 1958,

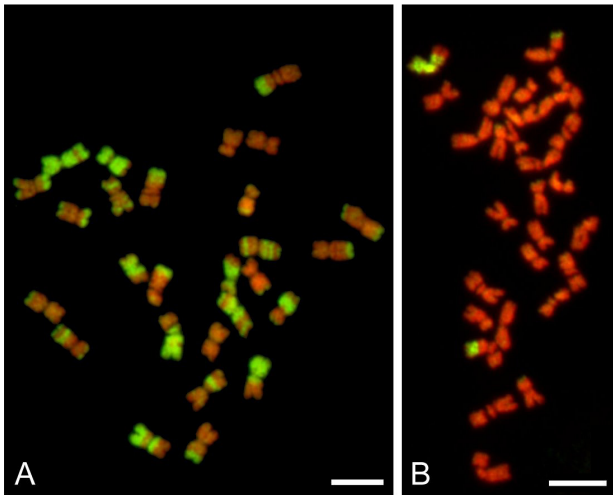


Fig. 1. The genomic constitution of the amphiploid form (cv. Paulita, A) and introgression form (cv. Lofa, B) of *Festulolium*. The genomic DNA of *Festuca* (green fluorescence), chromatin of *Lolium* was counterstained with propidium iodide (red fluorescence). Scale bars are 5  $\mu\text{m}$ . (Photo: D. Kopecký).

Sears and Okamoto 1958) and *PrBn* (pairing regulator in *Brassica napus*) in rapeseed (Jenczewski *et al.* 2003). There are exceptions to the no-pairing rule in wide hybrids, several such hybrids have been identified where homoeologous chromosome pairing does occur, such as *Allium* and *Lilium* hybrids (Van Heusden *et al.* 2000, Khan *et al.* 2009). Similarly, promiscuous pairing of the *Festuca* and *Lolium* chromosomes has been observed in *Festulolium* hybrids, indicating high pairing affinity and interchangeability of homoeologous chromosomes from these two genera, and the absence of a meiotic regulator (Humphreys and Pasakinskiene 1996, Zwierzykowski *et al.* 1998). This feature does lead to extensive meiotic recombination and enormous genetic variability among the progeny, but also to their genomic instability.

Compared to other wide hybrids in plants, the frequency of homoeologous chromosome pairing and recombination in *Festulolium* is extraordinarily high and in some combinations almost reaches the level of homologous pairing. The actual frequency differs among hybrid combinations. Zwierzykowski *et al.* (2008) observed 9:1 ratio of homologous *vs.* homoeologous pairing in tetraploid *F. pratensis*  $\times$  *L. perenne* hybrids. In *L. multiflorum* (Lm)  $\times$  *F. glaucescens* (Fg) hybrids, 8.54 paired Lm-Lm arms, 7.83 Fg-Fg arms and 4.09 associations between homoeologous Lm-Fg arms were observed (Kopecký *et al.* 2009), similarly to the results of Morgan *et al.* (2001), who reported the 5:1 ratio. Detailed analysis of tetraploid F<sub>1</sub> hybrid of *L. multiflorum*  $\times$  *F. arundinacea* indicated a possible association between the phylogenetic distance separating the parents and the frequency of homoeologous chromosome pairing (Kopecký *et al.* 2009).

The extent of homoeologous chromosome pairing seems to be affected by the proportion of the total numbers of chromosomes from both parents and by competition for pairing partners. Kopecký *et al.* (2008) studied the

frequency of homoeologous chromosome pairing in monosomic and disomic substitution lines of *F. pratensis* chromosome(s) in tetraploid *L. multiflorum* and found that the observed frequency of homoeologous pairing was slightly lower than random in disomic lines (where two *F. pratensis* and two *L. multiflorum* chromosomes were present in each orthologous group) suggesting a slight preference for homologous pairing. On the other hand, perfectly normal pairing of homoeologues was observed in monosomic lines (where only a single *F. pratensis* chromosome was present).

Frequent homoeologous chromosome pairing in *Festulolium* leads to extensive recombination of the parental genomes *via* intergeneric chromosome translocations (Thomas *et al.* 1994, Pasakinskiene *et al.* 1997, Humphreys *et al.* 1998). The translocation breakpoints in the recombined *Festuca-Lolium* chromosomes are fairly evenly distributed along the chromosome arms, from the centromere to the telomeres, but with infrequent recombination at the most distal and proximal regions (Zwierzykowski *et al.* 1998, 1999). Accordingly, Kopecký *et al.* (2010) reported the highest frequency of homoeologous recombination in the interstitial to distal parts of the chromosomes. Recombination cold spots (regions with decreased frequency of recombination) were found in the pericentromeric and centromeric regions, and also in the subtelomeric and telomeric regions (the most distal segments representing 10 % of the chromosome length) (Kopecký *et al.* 2010). It is worth noting that both parental species of the analyzed hybrids, *i.e.*, *L. multiflorum*, *F. arundinacea*, and *F. pratensis* are known to have localized distal chiasmata (Rees and Dale 1974, Karp and Jones 1983) so in hybrids, the patterns of crossing over change quite drastically.

A high level of homoeologous pairing may suggest that a meiotic regulator such as the wheat *Ph1* is not present in *Festulolium* hybrids. This may not be entirely true. Jauhar (1975) concluded that *F. arundinacea* possesses a diploid-like pairing regulator. Subsequently, this regulator was identified in all broad-leaved fescues of the *Schedonorus* subgenus (Jauhar 1975, 1993, Kopecky *et al.* 2009). The system, however, differs from the *Ph1*, it is hemizygous-ineffective, or haplo-insufficient (Jauhar 1993). As hemizygous-ineffective it is non-functional in the F<sub>1</sub> hybrids, such as *Lolium*  $\times$  *F. arundinacea*. This haplo-insufficiency hampers its direct utilization in stabilization of hybrid genomes and one may expect reactivation of its function only after the locus doubling. The origin of this system remains unknown. However, Kopecký *et al.* (2009) showed that *F. arundinacea* inherited it from *F. glaucescens*, one of its progenitor, rather than from *F. pratensis*, the other progenitor.

## Genome balance and stability of hybrids

In general, the fertility of allopolyploids relies on regular meiosis, and meiotic irregularities, such as multivalent formation, reduce fertility and may lead to complete sterility. This is not an issue in *Festulolium*. Despite the

formation of quadrivalents and meiotic irregularities leading to frequent aneuploidy (up to 80 % in cvs. Hostýn and Perseus), fertility is high and seed set is sufficient for commercial production (Ghesquiere *et al.* 2010, Kopecký *et al.* 2017).

Another issue affecting the extent of use of *Festulolium* in the grass business is the stability of hybrid genomes and the potential for the elimination of chromatin of one of the species in consecutive generations of sexual reproduction. It has been observed that the *Lolium* chromatin prevails in the *Festulolium* hybrids (Zwierzykowski *et al.* 1998, 2006, 2011, Kopecký *et al.* 2006, 2019, Książczyk *et al.* 2015, Majka *et al.* 2018a, 2019). Zwierzykowski *et al.* (2006, 2011) observed slow and gradual replacement of the *Festuca* chromosomes by those of *Lolium* in consecutive generations of *F. pratensis* × *L. perenne* hybrids (Fig. 2). As this was observed in breeding materials, it was not entirely clear if the gradual elimination of the *Festuca* was a natural or a selection-induced process. A comparison of unselected and selected F<sub>2</sub> - F<sub>4</sub> progenies of *F. pratensis* × *L. perenne* verified process as natural and independent of selection, suggesting the dominance of the *Lolium* chromatin over that of *Festuca* (Zwierzykowski *et al.* 2012). Similarly, J. Majka *et al.* (2018) observed a decreasing number of *Festuca* chromosomes across the F<sub>2</sub> - F<sub>9</sub> generations of *F. pratensis* (4x) × *L. perenne* (4x) together with increasing proportion of rearranged *Festuca* chromosomes (with translocated *Lolium* segment). Interestingly, Majka *et al.* (2019) observed no preference in the transmission of either *Festuca* or *Lolium* alleles to the subsequent generations of *F. pratensis* × *L. perenne* hybrid using inter-simple sequence repeat (ISSR) markers. However, that results might have been compromised by the dominant character of the ISSR markers. Similarly, all amphiploid *Festulolium* cultivars analyzed to date show a higher number of chromosomes of *Lolium* origin compared to those from *Festuca* (Zwierzykowski *et al.* 1998, Kopecký *et al.* 2005, 2006).

Genome composition of *Festulolium* seems to reflect the phylogenetic relationship of the parental species. Thus,

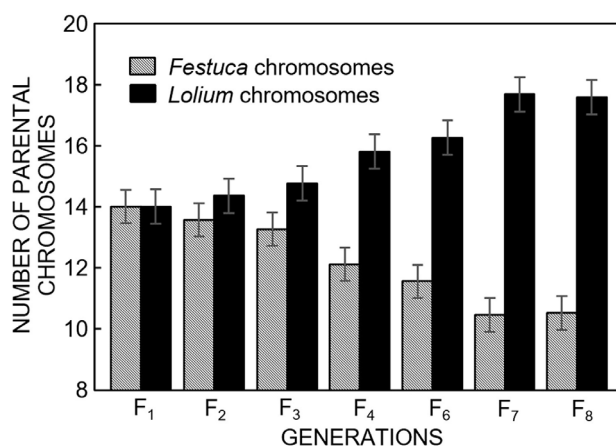


Fig. 2. Genome dominance in subsequent generations of *Festuca pratensis* × *Lolium perenne*. Means ± SEs (adopted from Zwierzykowski *et al.* 2006, 2011).

cv. Lueur developed from a *L. multiflorum* × *F. glaucescens* hybrid had fewer recombinant chromosomes than cultivars developed from *L. multiflorum* × *F. pratensis* hybrids (Kopecký *et al.* 2006). One of the possible explanations is that of a closer relationship of *L. multiflorum* to *F. pratensis* than to *F. glaucescens*. Differences in the genome composition can also mirror different breeding strategies. Closely related cvs. Perun and Perseus (*L. multiflorum* × *F. pratensis*) differ in their genome compositions, which is consistent with their breeding history. Perun is the oldest Czech *L. multiflorum* × *F. pratensis* cultivar with more equal proportion of the parental genomes. On the contrary, Perseus was developed by selection of plants from Perun, primarily for use in Western Europe and with high seed yield, late heading date, and high dry-matter yield. Thus, its genome composition with a higher proportion of the *Lolium* chromatin can be expected (Kopecký *et al.* 2017). Cv. Spring Green has the lowest proportion of the *Festuca* chromatin among all amphiploid cultivars of *L. multiflorum* × *F. pratensis*. It was developed from intercross of cvs. Kemal, Elmet, Prior, and Tandem, and the small amount of *Festuca* chromatin could be a consequence of the fact that cv. Kemal does not carry any *Festuca* chromatin detectable by GISH (Kopecký *et al.* 2006).

Genome stability differs between amphiploid and introgression cultivars of *Festulolium* (Kopecký *et al.* 2019). While amphiploids display relatively stable genome composition and no further shifts towards *Lolium* genome, elimination of introgressed segment(s) has been observed in the introgression forms. Stabilization of the hybrid genome in amphiploid cultivars is observed at about 2:1 proportion of *Lolium* vs. *Festuca* chromosomes (Kopecký *et al.* 2017). This ratio probably creates optimal combinations of parental alleles and the highest heterosis. On the other hand, introgression cultivars appear to be highly unstable and a reversion to the parental (*Lolium*) forms may be completed even within four generations of multiplication, unless the introgressed segment confers a strong selective advantage (Kopecký *et al.* 2019). In some cultivars, the *Festuca* chromatin was not detected in any individual analyzed (Kopecký *et al.* 2006).

Importantly, the rate of elimination of *Festuca* chromatin is different for different chromosomes. King *et al.* (2013) estimated the transmission rates of parental chromosomes in consecutive generations of seven monosomic substitution lines developed from hybrids between *Lolium perenne* and *Festuca pratensis*. They found a much higher transmission of complete *Lolium* chromosomes compared to complete *Festuca* chromosomes in the successive generation after the backcross of diploid monosomic substitution lines of *L. perenne* × *F. pratensis* (13L+1F, the proportion was 89:11, instead of expected 1:1) to *Lolium* parent. Unfortunately, the authors did not indicate the parental origin of the translocated chromosomes and therefore, the overall *Festuca* chromatin elimination could not be assessed from their study. However, chromosomes 2F, 4F, 5F, and 6F were eliminated more frequently than the remaining three *Festuca* chromosomes. Similarly, Kopecký *et al.* (2019) found that chromosome 5F

was eliminated more frequently than other *Festuca* chromosomes in tetraploid monosomic *L. multiflorum* × *F. pratensis* substitution lines.

From the breeder's point of view, the mode of operation of the mechanisms causing shift in genome composition is far from a trivial detail, as it determines the ease of transfer of a chromosome segment with the trait of interest to the progeny and its proper transmission to subsequent generations.

## Conclusions

Growing number of new *Festulolium* cultivars as well as the interest among growers demonstrate that *Festulolium* has become an integral part of the grass industry. *Festulolium* cultivars are grown throughout the world and their role and diversification of their uses will likely keep expanding along with the global climate change impacts. We envisage three main tasks for the future *Festulolium* research and breeding: 1) development of a platform for high-throughput and cheap determination of genome composition, cultivar identification, and legal protection to replace the time-consuming and labourious GISH method, and the costly (per plant, not per datapoint) DArTseq method, 2) finding appropriate combinations of parental species and proper genome balance between *Festuca* and *Lolium* for different climatic conditions to increase the potential of *Festulolium*, and 3) identification of the chromosome pairing regulator in polyploid fescues as a prerequisite to develop new and stable *Festulolium* cultivars with fescue-derived tolerance to abiotic stresses. A close collaboration between the research and breeding should secure a rapid integration of the new knowledge into breeding programs to mitigate the impact of climate change by developing resilient and high-yielding *Festulolium* cultivars.

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