DOI: 10.32615/bp.2020.138 BIOLOGIA PLANTARUM 64: 789-797, 2020

Breeding and genetics of two new amphiploid *Festulolium* synthetics with improved yield and digestibility

J. BAERT*, K. VAN LAERE, C. VAN WAES, A. GHESQUIERE, and J. APER

Flanders Research Institute for Agriculture, Fisheries and Food, Plant Sciences Unit, 9090 Melle, Belgium

Abstract

In order to introduce drought tolerance and improved cell wall digestibility from fescue in fodder ryegrasses, we developed two amphiploid *Festulolium* synthetics. One is a synthetic composed of three selected drought tolerant F1 hybrid genotypes from a cross between tetraploid *Lolium multiflorum* and hexaploid *Festuca arundinacea*, further on called LMFA. The other is a synthetic composed of five selected genotypes with soft leaves from a cross between tetraploid *Lolium perenne* and tetraploid *Festuca pratensis*, further on called LPFP. We produced seeds in polycrosses of two generations of both amphiploids, *i.e*., syn1 and syn2, and tested them in plot trials to determine the yield and fodder quality. The syn1 of both *Festulolium* populations had a higher annual dry matter yield than the reference *Lolium* cultivars and *Festulolium* cultivars composed of the same parental species. However, the syn2 of LMFA did not show an improved drought tolerance during a dry growing season compared to other *Festulolium* cultivars, and the seed yield of LMFA syn1 was low and dropped extremely in syn2. The number of chromosomes of LMFA also decreased gradually from F1 to syn2, and there was a clear shift in chromosome composition towards the *Lolium* genome. The LPFP synthetic performed better. Although the sugar content was significantly lower than the sugar content of the perennial ryegrass cultivars, organic matter digestibility (OMD) of LPFP was as high as OMD of the tetraploid perennial ryegrass cultivars. The cell wall digestibility (NDFD) of LPFP was significantly higher than the NDFD of both parental species and higher than the NDFD of all tested *Festulolium* cultivars. The seed yield of LPFP was the same in syn1 and syn2. The chromosome number remained on average the same and no clear shift of the chromosome composition to one of the composing genomes was observed. Overall, chromosome analysis revealed a high number of aneuploidy in syn1 and syn2 generations of both LMFA and LPFP and a lot of variation in number of *Lolium*, *Festuca* and recombinant chromosomes, and in the *Lolium*:*Festuca* genome ratio was observed among different genotypes of the same population. Therefore, selection for genotypes with a more stable genome composition will be a prerequisite for a sufficient seed yield and a broader exploitation of these new *Festulolium* synthetics.

Additional key words: *Festuca arundinacea*, genomic *in situ* hybridization, forage yield, *Lolium multiflorum*, seed yield, GISH.

Introduction

Perennial ryegrass (*Lolium perenne* L.; Lp) and Italian ryegrass (*Lolium multiflorum* Lam.; Lm) are the most common fodder grasses in NW Europe. They have a high yield and a good digestibility. Due to global warming, dry spells during summer will more often occur in this temperate maritime region. Drought periods cause severe reductions in dry matter yield of ryegrass (Aper *et al*. 2013). Tall fescue (*Festuca arundinacea* Schreb.; Fa) is more drought tolerant than *Lolium* because of its higher root biomass (Cougnon *et al*. 2016). However, the digestibility of the organic matter of tall fescue is at least 5 % points lower compared to Lp (Cougnon *et al*. 2013). In recent years the digestibility of perennial ryegrass was improved by breeding for a higher sugar content, though a high sugar content in the grass may cause rumen acidosis. Therefore it might be better to improve the digestibility of the cell wall in order to further improve the organic matter digestibility. Meadow fescue (*Festuca pratensis* Huds., Fp) has a higher cell wall digestibility than diploid perennial ryegrass (Baert *et al*. 2013). Interspecific hybridization of ryegrass with tall fescue or meadow fescue may improve yield, especially under drought conditions, and cell wall digestibility of the ryegrass. It is known that the genome composition in *Festulolium* is not stable. Kopecky *et al.* (2006) performed genomic *in situ* hybridisation (GISH) on over 600 *Festulolium* plants and revealed a large range

Submitted 15 June 2020, *last revision* 17 September 2020, *accepted* 18 September 2020.

Abbreviations: DM - dry matter; Fa - *Festuca arundinacea*; Fp - *Festuca pratensis*; GISH - genomic *in situ* hybridisation; Lm - *Lolium multiflorum*; Lp - *Lolium perenne*; NDFD - neutral detergent fibre digestibility; OMD - organic matter digestibility; WSC - water soluble carbohydrates.

Acknowledgements: We would like to thank Magali Losschaert, Veerle Buyssens, and Katrien Liebaut for their assistance in the GISH analysis.

* Corresponding author; e-mail: joost.baert@ilvo.vlaanderen.be

of variation in *Lolium*:*Festuca* genome composition and intergenomic recombination. The result is a highly variable progeny with every single plant having a unique chromosome constitution (Kopecky *et al*. 2017). Due to genomic instability, the seed yield of amphiploid *Festulolium* cultivars may be low (Ghesquière *et al*. 2010).

Here, we bred two novel amphiploid *Festulolium* populations, one from a Lm × Fa cross and one from a Lp × Fp cross and studied their agricultural value and genomic composition.

Materials and methods

Interspecific crosses, evaluation of F1 progeny plants, and composition of polycrosses: In 2011 we made interspecific hand crosses between *Lolium* and *Festuca*. Tetraploid genotypes of *Lolium perenne* (Lp) cultivars Meltador and Maurizio were crossed with tetraploid genotypes of *Festuca pratensis* (Fp), obtained after tetraploidization of the diploid cultivar Merifest by colchicine treatment. Tetraploid genotypes of *Lolium multiflorum* (Lm) cv. Livictory were crossed with hexaploid genotypes of *Festuca arundinacea* (Fa) cv. Barolex. The F1 plants together with their parents were visually scored for spring and summer growth, crown rust resistance, leaf softness and winter hardiness in the field in 2012 and for drought tolerance in a rain-out shelter in 2013. From the cross Lm × Fa we selected 3 F1 genotypes with a high score for drought tolerance. From the Lp × Fp cross we selected 5 F1 genotypes with a high score for leaf softness.

All selected plants had average to good scores for growth, rust resistance, and winter hardiness (Table 1). With these two groups we carried out two polycrosses in 2013. The synthetic population originating from the Lm × Fa F1 hybrids is called further on LMFA and from the Lp × Fp F1 hybrids LPFP.

Forage yield, feed quality, and seed yield of new amphiploid synthetics: The syn1 seeds of the polycrosses were sown in a plot (8 m²) trial on a sandy loam soil in Belgium, Merelbeke (50°58'55"N, 3°46'18"E) in April 2014 together with the *Festulolium* cv. Festilo, a registered cultivar in Belgium resulting from a cross between tetraploid *Festuca pratensis* and tetraploid *Lolium hybridum* (Lh), and other reference cultivars of Lp cvs. Abermagic and Aberbite, of Lm cvs. Podium and Caballo, of Fp cv. Merifest, of Fa cv. Kora, and of *Festulolium* amphiploids Lp × Fp cvs. Prior and Fabel, of Lm × Fa cvs. Becva and Lofa, and Lm × Fp cvs. Aberniche and Achilles. These reference cultivars were also included in the Eucarpia multisite *Festulolium* trial (Kopecky *et al*. 2018). Three replicates of each entry were arranged in a randomized complete block design. The plots were mown and sampled at 4 cuts in 2015 and 5 cuts in 2016 to determine the dry matter (DM) yield and the quality parameters: organic matter digestibility (OMD), water soluble saccharide content (WSC), and neutral detergent fibre digestibility (NDFD) by near infrared reflectance spectroscopy (NIRS). Therefore, the samples were scanned on a *FOSS XDS* monochromator instrument with *ISIscan v. 2.85.1* software (*Foss*, Hilleroed, Denmark). The calibration development was executed with *WINSI v. 4.9.0.* software using modified partial least squares regression (Shenk and Westerhaus 1991). The reference methods for the NIRS calibrations were: the pepsin cellulase method described by De Boever *et al*. (1988) for OMD, a iodometric titration for WSC (Wiseman *et al*. 1960) and the determination of cell wall digestibility described by Goering and Van Soest (1970) for NDFD. The data were analysed by *ANOVA* using the *Statistica* (v.13.5.0.17, *Tibco*, Palo Alto, CA, USA) software.

At the end of the second harvest year of the plot trial, 1 000 plants of each of the two syn1 populations LMFA and LPFP were randomly sampled in the plots and multiplied

Table 1. Characteristics of parents and selected F1 amphiploid genotypes used in polycrosses (scores 1-9; 9 = best).

Parent/ F1	Species + cultivar	Polyploidy	Headings	Inflorescence	Spring growth	Summer growth	Rust resist.	Winter hard.	Leaf soften.	Drought toler.	Polycross
P	Lm Livictory	4*x*	30 Apr	ear	7	2	4	5	7	2	
	Lp1 Maurizio	4*x*	12 May	ear	6	6	7	5	9	2	
	Lp2 Meltador	4*x*	9 Jun	ear	5	5	4	5	7	4	
	Fa Barolex	6*x*	25 Apr	panicle	6	6	3	7	3	7	
	Fp Merifest 4*x*	4*x*	12 May	panicle	5	7	7	4	7	5	
F1	Lm × Fa	5*x*	28 Apr	panicle	8	8	8	7	7	7	LMFA
	Lm × Fa	5*x*	5 May	panicle	8	8	8	7	7	9	
	Lm × Fa	5*x*	5 May	panicle	8	8	8	5	7	9	
	Lp1 × Fp	4*x*	12 May	ear	8	5	5	7	7	5	LPFP
	Lp1 × Fp	4*x*	12 May	ear	6	6	6	7	9	5	
	Lp1 × Fp	4*x*	8 May	ear	8	6	6	9	9	8	
	Lp1 × Fp	4*x*	22 May	ear	7	8	7	7	9	3	
	Lp2 × Fp	4*x*	22 May	ear	7	7	6	7	9	3	

to syn2 seeds in 2017. Seed yield (kilogram of germinating seeds per square meter) of the synthetics was determined on the isolation plots of the polycrosses and multiplications. The seed yield of tetraploid Lp and Lm synthetics and of the *Festulolium* cv. Festilo was measured on adjacent isolation plots as a reference. In May 2018 the syn2 seeds of the new synthetics LMFA and LPFP were sown in a plot trial following a randomized complete block design on a sandy loam soil in Merelbeke together with *Festulolium* reference cvs. Aberniche, Achilles, and Festilo. The plots were mown and sampled at 5 cuts in 2019 to determine the DM yield and the OMD, WSC, and NDFD, according the methods described above. In 2019 spring and summer were dry in Merelbeke. The rainfall in April/May and July/August was 54 and 79 mm, respectively, compared to 103 mm and 141 mm in ten years before.

Determination of the genome stability of the *Festulolium* synthetics: We performed genomic *in situ* hybridisation (GISH) on at least 7 genotypes of the syn1 and syn2 of the two *Festulolium* synthetics LMFA and LPFP and of the syn3 and syn4 of the cv. Festilo for comparison. For this we made chromosome spreads from young apical root tips according to the "SteamDrop" method (Kirov *et al*. 2014). Root tips were pre-treated with ice-cold water and fixated in a ethanol:acetic acid 3:1 (v/v) solution at room temperature for 45 min. Cell suspensions were made from the fixated root tips after digestion with 0.6 % enzyme mixture [0.6 % (m/v) cellulase *Onozuka RS* (*Duchefa Biochemie*, Haarlem, The Netherlands), and 0.6 % (m/v) pectolyase *Y-23* (*Duchefa Biochemie*] during 105 min at 37 °C. Chromosome spreads were prepared with 1:1 (v/v) ethanol: acetic acid as fixative 1 and 0:1 (v/v) ethanol:acetic acid as fixative 2. Total genomic DNA of Lm, Lp, Fa, and Fp was extracted from 100 mg of fresh young leaves using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987). The genomic DNA was labelled as a probe by biotin-16-dUTP or digoxigenin by nick translation after sonication (40 V, 15 s). Block DNA was obtained by sonication (40 V, 4 min 30 s) of genomic DNA to fragments of 100 - 300 bp. Denaturation, hybridization, and detection was performed as described in Van Laere *et al*. (2010) with a probe:block ratio of 1:20. Stringency wash was done by washing the slides twice in 2× saline sodium citrate (SSC) at room temterature for 15 min, twice in 0.1 × SSC at 50 °C for 7 min and twice in 2 × SSC at 50 °C for 5 min to RT. Biotin and digoxigenin labelled probes were detected with streptavidin-Cy3 (*Sigma-Aldrich*, St. Louis, USA) and anti-dig-FITC (*Roche*, Mannheim, Germany). The chromosomes were counterstained with 0.1 mg cm^{-3} DAPI and mounted in *Vectashield* (1:100; v/v). Chromosome analysis was done with an *AxioImager M2* (*Zeiss*, Zaventem, Belgium) fluorescence microscope equipped with an *Axiocam MRm* camera (*Zeiss*). Images were captured by *ZEN* software (*Zeiss*). Analysis of hybridization signals was carried out on at least 5 well-spread metaphases of each genotype. The number of *Lolium*, *Festuca*, and recombined chromosomes and the proportion of the *Lolium* and *Festuca* genome in the *Festulolium* synthetics were analysed using *DrawID* (Kirov *et al*. 2017). As a control for cross hybridisation between *Festuca* and *Lolium*, GISH was performed on Fp chromosome spreads using the Lm probe and on Lm and Lp chromosome spreads using the Fp probe.

Results

The dry mass yield of syn1 of LMFA was intermediate between the DM yield of both parental species but

Table 2. Total annual dry matter (DM) yield, organic matter digestibility (OMD), sugar content (WSC), and NDF digestibility (NDFD) of two new *Festulolium* synthetics (syn1) compared to cultivars of their parental species and commercial *Festulolium* cultivars measured in 2015 and 2016. Means followed by different letters are significantly different at a 5 % level (Duncan's multiple range test).

Cultivar	Species	Ploidy	DM yield [kg m^{-2} $year^{-1}$]	OMD [%]	WSC [% of DM]	NDFD [%]
Podium	Lm	2*x*	1.56 bc	72.2 fg	15.2 cde	66.2 e
Caballo	Lm	4*x*	1.48 de	72.8 efg	15.6 bcde	65.7 e
Kora	Fa	6*x*	1.75 a	69.9 g	14.4 de	70.1 cd
Becva	Lm × Fa	4*x*	1.34 f	76.1 cd	15.9 bcde	69.7 cd
Lofa	Lm × Fa	4*x*	1.38 f	75.7 cde	14.9 cde	69.3 d
LMFA syn1	Lm × Fa	5*x*	1.59 bc	74.2 def	15.1 cde	70.1 cd
Abermagic	Lp	2*x*	1.52 cd	79.7 ab	20.1 a	70.2 cd
Aberbite	Lp	4*x*	1.43 ef	81.0 a	19.8 a	71.9 bc
Merifest	Fp	2*x*	1.35 f	78.5 abc	13.6 e	73.1 ab
Fabel	Lp × Fp	4*x*	1.43 ef	79.7 ab	17.0 bc	73.0 b
Prior	Lp × Fp	4*x*	1.35 f	79.3 ab	17.7 b	70.4 cd
LPFP syn1	Lp × Fp	4*x*	1.59 bc	80.8 a	15.7 bcde	75.4 a
Aberniche	Lm × Fp	4*x*	1.58 bc	75.4 cde	17.6 b	68.8 d
Achilles	Lm × Fp	4*x*	1.58 bc	77.3 bcd	15.4 bcde	72.2 bc
Festilo	Lh × Fp	4*x*	1.62 b	78.6 abc	16.4 bcd	74.1 ab

Table 3. Total annual dry matter (DM) yield, organic matter digestibility (OMD), sugar content (WSC) and NDF digestibility (NDFD) of two new *Festulolium* synthetics (syn2) compared to commercial *Festulolium* cultivars in the dry year 2019. Means followed by different letters are significantly different at a 5 % level (Duncan's multiple range test).

Cultivar	Hybrid type	DM yield [kg m^{-2} $year^{-1}$]	OMD [%]	WSC [% of DM]	NDFD [%]
Aberniche	Lm × Fp	1.55 a	75.7 b	14.6 a	73.6 b
Achilles	Lm × Fp	1.46 ab	76.1 b	12.4 b	75.7 b
Festilo	Lh × Fp	1.46 ab	78.7 ab	13.6 ab	77.4 ab
LMFA syn2	Lm × Fa	1.48 ab	77.1 b	14.3 ab	74.7 b
LPFP syn2	Lp 3 Fp	1.40 b	83.3 a	13.9 ab	80.4 a

significantly higher than the cvs. Becva and Lofa with the same parental composition (Table 2). LMFA syn1 had a similar DM yield as the *Festulolium* reference cvs. Aberniche, Achilles, and Festilo. The digestibility of LMFA syn1 was slightly superior to cvs. of the parental species because of the combination of a similar WSC content to the *Lolium* parent and a similar NDFD to the *Festuca* component. Although LMFA syn1 was composed of drought tolerant components, the DM yield of the syn2 was not superior to the *Festulolium* reference cultivars in the dry season of 2019 (Table 3). On the contrary, cv. Aberniche outyielded LMFA.

The dry matter yield of the syn1 of LPFP was superior to the DM yield of cvs. of the parental species and significantly higher than the cvs. Fabel and Prior with the same parental composition (Table 2). The DM yield of LPFP syn1 was very similar to the yield of the *Festulolium* reference cvs. Aberniche, Achilles, and Festilo. The digestibility of LPFP was as high as the OMD of the tetraploid perennial ryegrass cultivars but the WSC content was significantly lower than the WSC content of the perennial ryegrass cultivars. The NDF digestibility was significantly higher compared to both parental species and higher than all *Festulolium* cultivars. In 2019, the total DM yield of the syn2 of LPFP was lower than the DM yield of the *Festulolium* reference cultivars but the digestibility was significantly higher due to the significantly higher cell wall digestibility (Table 3).

The seed yield of both new amphiploid synthetics was much lower than the seed yield of the *Lolium* species (Fig. 1). The seed yield of LPFP remained rather stable in the two consecutive generations at about 30 % relative to the perennial ryegrass reference. The seed yield of LMFA dropped extremely from syn1 to syn2, from 18 to 5 % relative to the Italian ryegrass reference. For comparison:

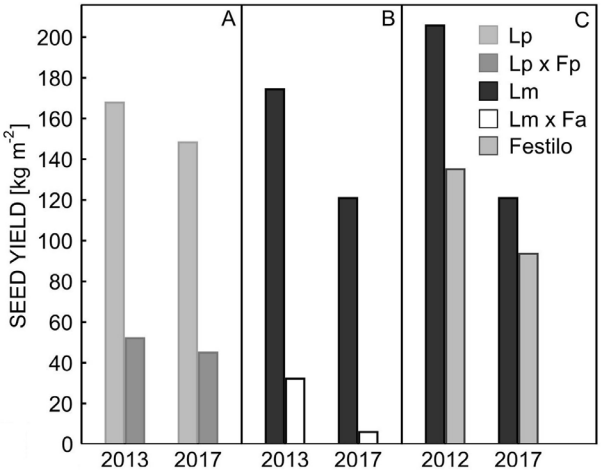

Fig. 1. The seed yield of syn1 (2013) and syn2 (2017) of *Lolium perenne* (Lp) × *Festuca pratensis* (Fp) and *Lolium multiflorum* (Lm) × *Festuca arundinacea* (Fa) amphiploid *Festulolium* compared to the seed yield of tetraploid synthetics of Lp (*A*) and Lm (*B*) in the same harvest years, and syn3 (2012) and syn4 (2017) of *Festulolium* cv. Festilo (*C*) (unreplicated plots on the same fields).

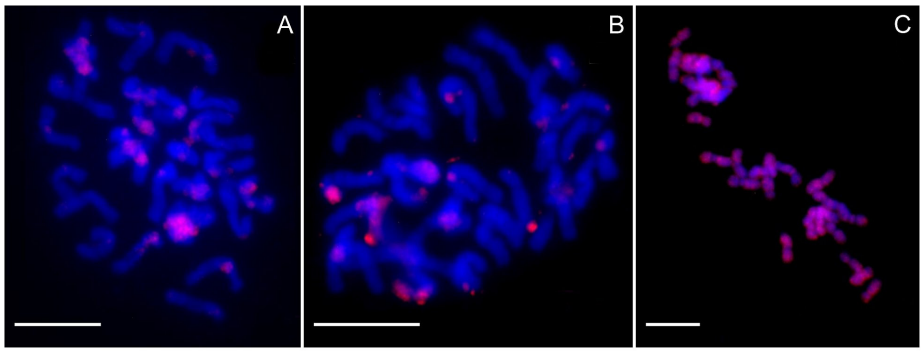

Fig. 2. Genomic *in situ* hybridisation of the parental species: *A* - Fp chromosomes (*blue*) hybridised with an Lp probe (*red*). *B* - Lm chromosomes (*blue*) hybridised with an Fp probe (*red*). *C* - Lp chromosomes (*blue*) hybridised with an Lm probe (*red*). *Bars* are 10 µm.

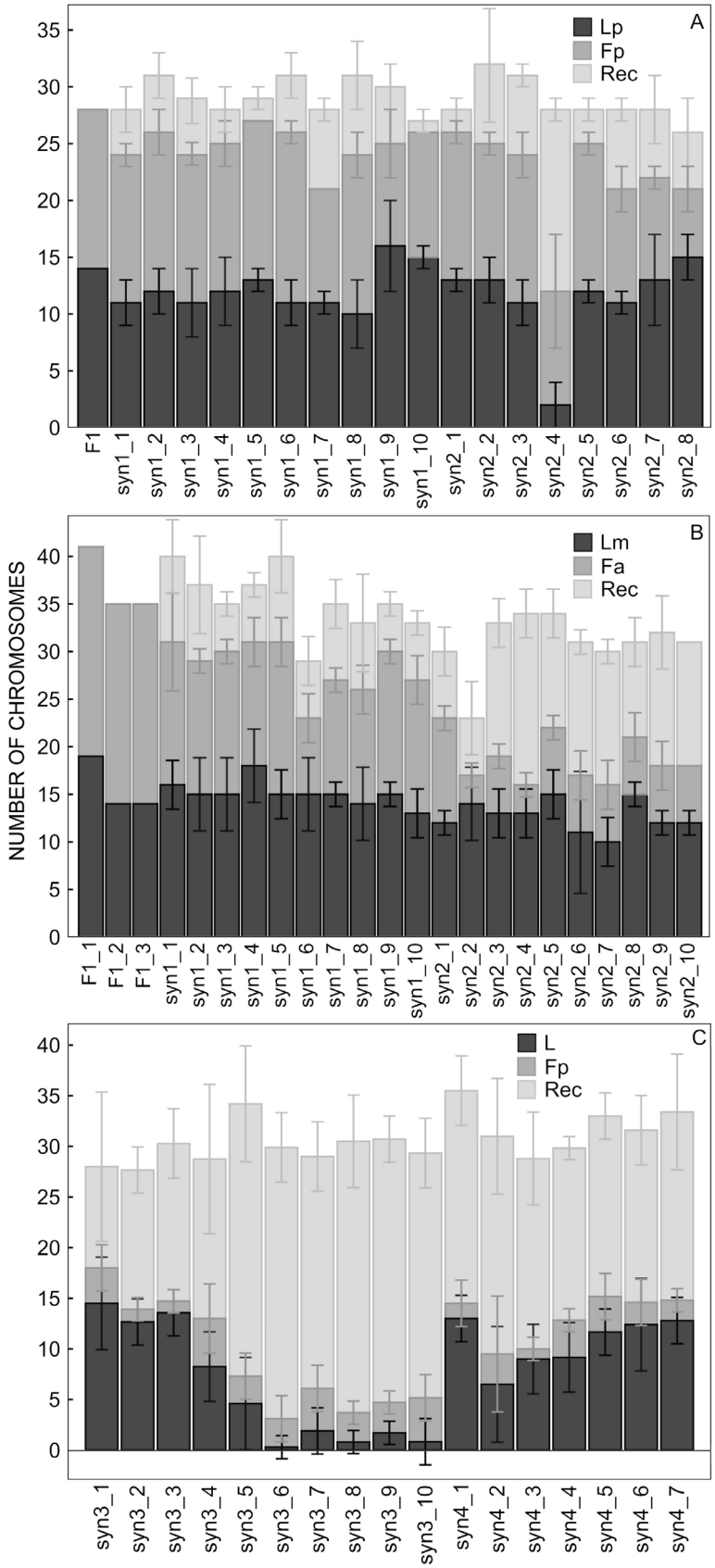

Fig. 3. Chromosome composition of F1, syn1, and syn2 genotypes of *Lolium perenne* (Lp) × *Festuca pratensis* (Fp) hybrids (*A*) and *Lolium multiflorum* (Lm) × *Festuca arundinacea* (Fa) hybrids (*B*), and of syn3 and syn4 genotypes of cv. Festilo (*C*).

the seed yield of the Lh × Fp amphiploid cv. Festilo increased from 66 % in syn3 to 77 % in syn4 relative to the Italian ryegrass reference.

First we confirmed the chromosome number of the parental species: Lp: 2*n*=4*x*=28, Lm: 2*n*=4*x*=28, Fp: 2*n*=4*x*=28, Fa: 2*n*=6*x*=42. Second, we determined the amount of cross hybridisation between *Festuca* and *Lolium* by GISH. Although Kopecky *et al*. (2008) reported no staining of *Festuca* chromosomes by a *Lolium* probe, we could observe that there is little (9.68 ± 8.66 %) cross hybridisation between these 2 parental species (Fig. 2), most probably accounting for conserved repetitive sequences. Lm and Lp share 47.25 ± 9.86 % similarity (Fig. 2). However, overall, we can conclude that it will be possible to distinguish between the *Festuca* and *Lolium* parental genomes in the *Festulolium* amphidiploids using GISH.

For the F1 genotypes of LMFA, we could observe 35 chromosomes, which could be expected based on the chromosome numbers of the parental species. Since this odd number of chromosomes in the F1 generation, we expected chromosome instability in the further generations. The syn1 genotypes that were analysed contained between 28 and 40 chromosomes, of which 13 to 18 Lm chromosomes, 8 to 16 Fa chromosomes, and 5 to 9 recombinant chromosomes. Remarkably, the syn2 genotypes had only left between 23 and 34 chromosomes (Figs. 3*B* and 4*A*,*B*), of which 10 to 15 came from Lm, 3 to 11 from Fa, and 6 to 18 were recombinant. Due to the GISH procedure, we could observe that sometimes chromosomes break down at the centromeres, which makes it not always possible to count the exact number of chromosomes, explaining the chromosome amount variation within one genotype.

The Lm:Fa genome ratio in the F1 genotypes is expected to be 40:60, which is confirmed by our GISH analysis (Table 4). Determination of the Lm:Fa genome ratio revealed for most syn1 genotypes a genome composition showing a slight shift towards the Lm genome (Table 4). In the syn2 generation this shift towards the Lm genome is more clear and present for all tested syn2 genotypes (Table 4). The genotype syn2_2 for example has about 75 % Lm genome and only about 25 % Fa genome left. This genotype also has only 23 chromosomes, so most probably this genotype just lost many of the Fa chromosomes.

The F1 genotype of LPFP contained 28 chromosomes (2*n*=4*x*=28), *i.e.*, 14 Lp and 14 Fp chromosomes. Since both parental species are tetraploids with a similar

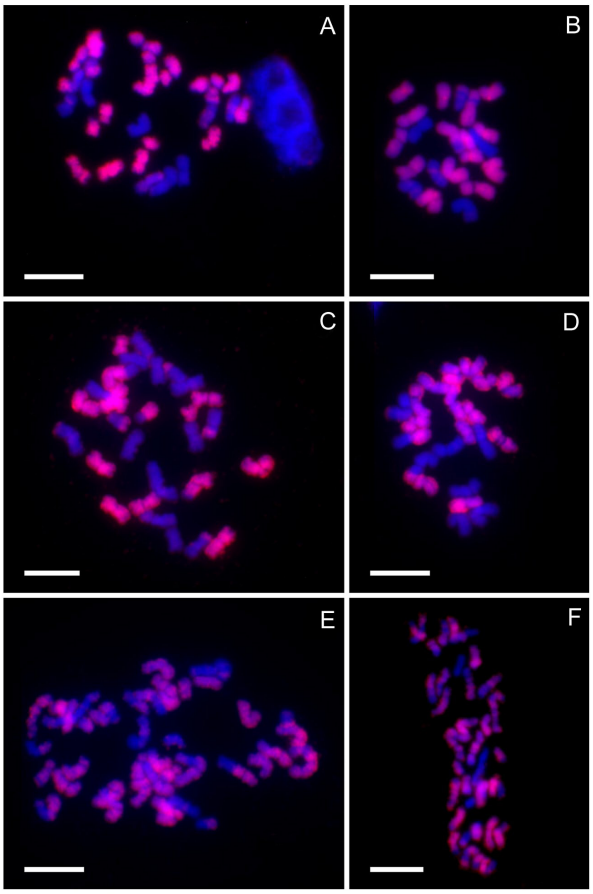

Fig. 4. *A*,*B* - genomic *in situ* hybridisation of syn1 *Lolium multiflorum* (Lm) × *Festuca arundinacea* (Fa) (syn1_6, *A*) and syn2 Lm × Fa (syn2_2, *B*); *red* - Lm probe, *blue* - Fa block. *C,D* - GISH analysis of syn1 *Lolium perenne* (Lp) × *Festuca pratensis* (Fp) (syn1_7, *C*) and syn2 Lp × Fp (syn2_8, *D*); *red* - Lp probe, *blue* - Fp block. *E,F* - GISH analysis of syn3 cv. Festilo (syn3_5, *E*) and syn4 cv. Festilo (syn4_5, *F*); *red* - *Lolium* (L) probe, *blue* - Fp block. *Bars* are 10 μm.

Table 4. The *Lolium multiflorum* (Lm) : *Festuca arundinacea* (Fa) genome ratio in the F1, syn1, and syn2 genotypes of the Lm × Fa hybrid.

Genotype	Lm genome [%]	Fa genome [%]	Genotype	Lm genome [%]	Fa genome [%]
F1_1	41.04 ± 1.04	58.96 ± 1.12	Syn2_1	50.91 ± 1.34	49.09 ± 1.34
F1_2	40.17 ± 1.92	59.83 ± 1.89	Syn2_2	75.33 ± 6.23	24.67 ± 6.23
Syn1_1	45.78 ± 3.82	54.22 ± 3.82	Syn2_3	56.61 ± 9.82	43.40 ± 3.61
Syn1_2	48.07 ± 3.36	51.93 ± 3.36	Syn2_4	63.72 ± 5.24	36.28 ± 5.24
Syn1_3	47.35 ± 4.42	52.65 ± 4.42	Syn2_5	56.34 ± 2.61	43.66 ± 2.61
Syn1_4	54.81 ± 2.91	45.19 ± 2.91	Syn2_6	51.00 ± 6.33	49.01 ± 6.33
Syn1_5	44.70 ± 6.85	55.31 ± 6.85	Syn2_7	56.86 ± 5.09	43.14 ± 5.09
Syn1_6	59.49 ± 10.78	40.51 ± 10.78	Syn2_8	62.26 ± 4.82	37.74 ± 4.82
Syn1_7	50.30 ± 2.51	49.70 ± 2.51	Syn2_9	63.06 ± 3.81	36.95 ± 3.81
Syn1_8	47.11 ± 3.70	52.89 ± 3.70	Syn2_10	58.01 ± 0.47	42.00 ± 0.47
Syn1_9	51.24 ± 1.10	48.76 ± 1.10			
Syn1_10	47.93 ± 4.20	52.07 ± 4.20			

Table 5. The *Lolium perenne* (Lp) : *Festuca pratensis* (Fp) genome ratio in the F1, syn1, and syn2 Lp × Fp amphidiploid genotypes.

Genotype	Lp genome [%]	Fp genome [%]	Genotype	Lp genome [%]	Fp genome [%]
F1	48.99 ± 0.83	51.02 ± 0.83	Syn2_1	47.47 ± 0.92	52.53 ± 0.92
Syn1_1	44.23 ± 4.42	55.77 ± 4.42	Syn2_2	47.63 ± 10.20	52.37 ± 10.20
Syn1_2	43.92 ± 3.87	56.03 ± 3.87	Syn2_3	45.78 ± 4.41	54.22 ± 4.41
Syn1_3	43.78 ± 4.42	56.48 ± 4.20	Syn2_4	27.70 ± 6.94	72.30 ± 6.94
Syn1_4	48.62 ± 7.65	51.35 ± 7.65	Syn2_5	45.27 ± 2.96	54.73 ± 2.96
Syn1_5	47.91 ± 1.03	52.09 ± 1.03	Syn2_6	50.53 ± 3.74	49.47 ± 3.74
Syn1_6	38.70 ± 3.99	61.32 ± 3.95	Syn2_7	56.39 ± 5.99	43.62 ± 5.99
Syn1_7	48.36 ± 0.69	51.64 ± 0.69	Syn2_8	65.37 ± 0.48	34.63 ± 0.48
Syn1_8	41.69 ± 8.56	58.31 ± 8.56			
Syn1_9	60.74 ± 7.96	39.26 ± 7.96			
Syn1_10	55.48 ± 2.89	44.52 ± 2.89			

Table 6. The *Lolium* (L) : *Festuca pratensis* (Fp) genome ratio in the syn3 and syn4 *Festulolium* cv. Festilo.

Genotype	L genome [%]	Fp genome [%]	Genotype	L genome [%]	Fp genome [%]
Syn3_1	71.80 ± 1.29	28.20 ± 1.26	Syn4_1	72.26 ± 8.35	27.74 ± 8.35
Syn3_2	78.12 ± 2.65	21.88 ± 2.35	Syn4_2	66.58 ± 16.42	33.42 ± 16.42
Syn3_3	77.88 ± 4.88	21.70 ± 5.53	Syn4_3	66.48 ± 6.23	33.52 ± 6.23
Syn3_4	56.20 ± 4.90	43.80 ± 4.90	Syn4_4	63.03 ± 4.84	36.97 ± 4.83
Syn3_5	56.38 ± 6.96	43.63 ± 6.96	Syn4_5	68.24 ± 7.23	31.77 ± 7.53
Syn3_6	46.47 ± 6.05	53.53 ± 6.05	Syn4_6	71.35 ± 4.36	28.65 ± 4.36
Syn3_7	49.97 ± 7.84	50.03 ± 7.84	Syn4_7	67.77 ± 4.95	32.23 ± 4.95
Syn3_8	48.91 ± 4.98	51.09 ± 4.98			
Syn3_9	47.12 ± 8.35	52.88 ± 8.35			
Syn3_10	46.92 ± 6.87	53.08 ± 6.87			

chromosome amount, we could expect to observe $2n=4x=28$ chromosomes in further generations. Indeed, half of the 10 syn1 and 8 syn2 genotypes that were analysed by GISH contained 28 chromosomes, but a high number of aneuploidy was also observed (Figs. 3*A* and 4*C*,*D*). Also here it has to be taken into account that due to the breakdown of some chromosomes at the centromeres during the GISH procedure, it is not always possible to count the exact number of chromosomes of each genotype. High frequencies of aneuploidy were also seen in *Festulolium* studies before (Kopecky *et al*. 2017), *e.g.*, in Fp × Lp hybrids (Majka *et al*. 2019). In our syn1 and

syn2 genotypes, there was a large variation of Lp, Fp, and recombinant chromosomes. In the syn1 genotypes between 10 and 16 Lp and between 9 and 15 Fp chromosomes were observed. In addition, between 2 and 7 chromosomes were recombinant (Figs. 3*A* and 4*C*,*D*). The Lp:Fp genome ratio is expected to be 50:50. By calculating the percentage Lp and Fp genome present in the F1, syn1, and syn2 genotypes, we could conclude that this Lp:Fp genome ratio is quite stable, with some exceptions (Table 5). In most genotypes the percentage Lp and Fp is about 50 %. The genotypes syn1_6 and syn2_4 showed a shift towards the Fp genome, while the genotypes syn1_9 and syn2_8 had a shift towards the Lp genome (Table 5).

As a comparison, we also did GISH analysis on syn3 and syn4 genotypes of the commercial tetraploid Lh × Fp *Festulolium* cv. Festilo. As a probe, we used a mix of Lp and Lm labelled genomic DNA. We could observe a high number of recombinant chromosomes both in syn3 and syn4 genotypes. The syn3 genotypes had between 0 and 15 *Lolium* chromosomes, between 1 and 5 Fp chromosomes, and between 10 and 27 recombinant chromosomes (Figs. 3*C* and 4*E*,*F*). Most syn3 genotypes had a rather stable genome composition, only 3 syn3 genotypes showed a clear shift towards the *Lolium* genome (Table 6). Syn4 genotypes had 7 to 13 *Lolium* chromosomes, 1 to 4 Fp chromosomes, and 17 to 22 recombinant chromosomes. However, due to the breakage of some chromosomes during the GISH procedure, the exact number of chromosomes is difficult to determine per genotype. In syn4 generation the shift towards the *Lolium* genome was much more pronounced in all genotypes (Table 6).

Discussion

By crossing Lm and Fa we aimed at breeding a drought tolerant synthetic *Festulolium* cultivar. The F1 genotypes composing the LMFA synthetic population were at least as drought tolerant as the tall fescue parent. Although the syn1 of LMFA was higher yielding than Lm × Fa reference cultivars and had a similar yield as Lm × Fp cultivars, the syn2 showed no DM yield improvement compared to the Lm × Fp *Festulolium* cultivars during the dry growing season of 2019. A possible reason might be the reduction of the Fa genome in LMFA from F1 to syn2 resulting in less drought tolerance. Syn2 seeds were harvested on syn1 plants sampled in plots at the end of two management years and might be affected by a kind of natural selection. In switch grass and timothy natural selection for survivorship increased biomass yield (Casler and Smart 2013, Reheul *et al.* 2003). Here this effect was not observed. On the one hand, the sampling might have caused a shift towards the more persistent component, *i.e.* tall fescue. On the other hand, syn1 genotypes with a larger *Lolium*/*Festuca* ratio might have been advantaged directly after sowing because of their faster establishment. In mixtures with perennial ryegrass the content of Fa was lower than the sown proportion in the first year after sowing but increased over the years and dominated after 3 years (Cougnon *et al.* 2013). Also the seed yield of LMFA was very low. This could be expected when producing seeds from pentaploid F1 genotypes. The seed yield dropped from syn1 to syn2. This coincided with a decrease of the chromosome number and a clear shift of the genome composition towards the *Lolium* genome. We can expect a continued shift towards the *Lolium* genome in further generations of the Lm × Fa amphiploid selection. Kopecky *et al*. (2018) found in cvs. Becva and Lofa, all developed from Lm × Fa hybrids followed by backcrossing onto tetraploid Lm, a large proportion of plants with no evidence for the presence of any *Festuca* chromatin. This indicated the rapid elimination of the *Festuca* chromatin and a preferential transmission of *Lolium* chromosomes or advantage gained by genotypes having predominance of the *Lolium* genome complement.

The breeding goal of the Lp × Fp cross was to develop a highly digestible *Festulolium* cultivar. For that the LPFP synthetic was really successful with a significantly better digestibility than all *Festulolium* cultivars in all growing seasons. It was as digestible as the tetraploid perennial ryegrass cv. Aberbite. The high digestibility of Aberbite was due to its high sugar content. The high digestibility of LPFP was due to its outstanding cell wall digestibility, contributed by the Fp genome. The DM yield of the new LPFP synthetic was significantly better than the DM yield of existing Lp × Fp cultivars. Also the seed yield of syn1 and syn2 of LPFP was stable. The chromosome number remained the same in the successive generations of this Lp × Fp amphiploid and GISH revealed no clear shift to one of the composing genomes. However the seed yield was lower than the seed yield of the Lh × Fp cv. Festilo containing Lm in its genomic composition. This might be due to a closer relationship between Fp and Lm than between Fp and Lp resulting in a more stable genomic composition because of more frequent homoeologous recombination between Fp and Lm than between Fp and Lp (Kopecky *et al*. 2018).

Festilo is a registered cultivar listed in Belgium and commercially available. Yet it shows some instability in genome composition. There is still a shift towards the *Lolium* genome from syn3 to syn4. GISH analysis by Kopecky *et al*. (2017) of 3 consecutive generations (1st generation the one for cultivar registration) of 3 *Festulolium* cultivars revealed that the gradual shift towards *Lolium* is observed in the early generations and reaches a plateau in later generations where the proportions of parental genomes become stabilized. This was also seen by Zwierzykowsky *et al*. (2006, 2011), where a gradual shift towards *Lolium* was observed from F2 to F7 and then a genome stabilisation between F7 and F8. So it will be important to evaluate our 2 selected amphidiploid synthetics over further generations. Stabilization of the genome composition will be a prerequisite for a stable and sufficient seed yield and a broader exploitation of the *Festulolium* synthetics. Backcrossing to *Lolium* may improve seed yield but reduces the *Festuca* genome in the genomic composition of the *Festulolium*. There is a lot of variation in seed yield among single genotypes of the new *Festulolium* synthetics. We will try to improve the seed

yield by selecting single tetraploid genotypes with a high seed yield.

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