Food and Energy Security
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The potential of novel *Festulolium* (2n = 4x = 28) hybrids as productive, nutrient-use-efficient fodder for ruminants

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Keywords
Festuca arundinacea var. *glaucescens*, *Festuca mairei*, *Festulolium*, field performance, plant-mediated proteolysisplant-mediated proteolysis

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Funding Information
We thank HCC and EBLEX for funding Sally O'Donovan's PhD program at IBERS some of whose research outcomes on use of *Festulolium* hybrids for improved ruminant nutrition are presented here. We also acknowledge BBSRC for funding the research inputs from Alison Kingston-Smith, Mike Humphreys and Alan Gay. The IBERS field trials and seed multiplication were funded through an industrial DefraLINK Consortium (DefraLink Programme LK0688) led by Mike Humphreys and undertaken by Markku Farrell.

Received: 27 September 2014; Revised: 3 November 2014; Accepted: 10 November 2014

***Food and Energy Security* 2014; 3(2): 98–110**

doi: 10.1002/fes3.50

Abstract

The field performance and potential future use of F_1 *Lolium multiflorum* and *Lolium perenne* × *Festuca arundinacea var. glaucescens* and *Festuca mairei* hybrids (2n = 4x = 28) are described. Foliar trait expression in the hybrids was largely determined by the *Lolium* rather than their *Festuca* parent ensuring maintenance of high-forage quality. All four *Festulolium* populations comprised high-yielding genotypes, but the *L. multiflorum* populations were particularly erect and tall, while the *L. perenne* populations had significantly higher numbers of tillers and were prostrate. Forage yields of the *Festulolium* populations assessed in field plot trials were either not significantly different from, or were superior to leading *L. multiflorum* and *L. perenne* cultivars used as controls. Endogenous plant proteases contribute to excessive proteolysis in the rumen which causes environmental N pollution. Protein degradation due to plant-mediated proteolysis was assessed by in vitro exposure of leaves to the environmental conditions of the rumen (39°C, anaerobic) and calculated based on the time taken for protein levels to be reduced to half their original levels ($t^{½}$). Leaf proteins were significantly more stable in *L. multiflorum* × *F. arundinacea var. glaucescens* and *L. perenne* × *F. arundinacea var. glaucescens* F_1 hybrids ($t^{½}$ 18–21 h) than in their respective *Lolium* parental genotypes ($t^{½}$ 4–5 h), and there was a highly significant genome interaction. The $t^{½}$ in the majority of the *L. multiflorum* × *F. arundinacea var. glaucescens* F_1 hybrids studied often exceeded 24 h, whereas $t^{½}$ of their *Lolium* and *Festuca* parents was consistently <14 h. Although inferior to the F_1, *F. arundinacea var. glaucescens* genotypes tested had significantly greater $t^{½}$ than *L. perenne* under rumen-simulated conditions. Significant variation in protein stability was apparent within the F_1 and their respective parent species' groups. The initial protein content of the F_1 hybrids was lower than their respective parents, but following 24-h exposure to anoxia at 39°C, the protein content of both parent and hybrid genotypes was similar. The differences in protein stability between parental and hybrid genotypes was due to the greater rate of protein decline observed in the *Lolium* genotypes. Hence, uptake of these *Festulolium* hybrids as forage crops has potential to directly mitigate environmental impact of livestock farming without affecting production capacity.

Introduction

In mild temperate climates such as that found in the UK ryegrass (*Lolium* spp.) is often the forage grass of choice due to its high yield and nutritious value. However, recently *Festulolium* varieties are increasingly gaining interest as sources of reliable, productive, and nutritive fodder for use in livestock agriculture and for their potential for ecosystem services (MacLeod et al. 2013). Importantly, *Festulolium* also has a higher tolerance to

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stresses such as drought or cold than perennial ryegrass (Ghesquière et al. 2010). *Festulolium* is the result of conventional hybridization of either *Lolium perenne* (perennial ryegrass) or *Lolium multiflorum* (Italian ryegrass) with any related *Festuca* (fescue) species, and may as such be marketed under its own grass category throughout Europe. *Festulolium* varieties may be either amphiploids with combined genome sets of ryegrass and fescue chromosomes, or they may be introgressive forms. In the latter, a limited number of donor gene sequences, most frequently derived from a fescue species, are incorporated into the recipient (ryegrass) genome through a backcross breeding program (Humphreys et al. 2003; Ghesquière et al. 2010). The IBERS-bred variety AberNiche, the first *Festulolium* to gain entry onto the UK National Recommended List is an example of an introgression form and is around 75% Italian ryegrass (*L. multiflorum*) and 25% meadow fescue (*Festuca pratensis*) (Cernoch and Kopecky, pers. comm.) while the French variety Lueur is an example of the amphiploid type with a more balanced ryegrass: fescue genome complement (Ghesquière et al. 2010). The variety Lueur derives from the hybridization of *L. multiflorum* (2n = 4x = 28) with *F. arundinacea* var. *glaucescens* (2n = 4x = 28), a species gaining interest due in particular to its drought and heat tolerance derived from its Mediterranean origin. The introgression breeding approach (Humphreys et al. 2005) has also been used to combine the complementary attributes of high-yielding ryegrass with the drought-tolerance of *F. arundinacea* var. *glaucescens*. This has been achieved through targeted marker-assisted transfers of a single small genome sequence of *F. arundinacea* var. *glaucescens* onto a terminal location of chromosome 3 (Humphreys et al. 2005). This fescue sequence has subsequently been transferred into breeders' lines of both Italian and perennial ryegrass (Humphreys et al. 2012).

Lolium multiflorum × *F. arundinacea* var. *glaucescens* (2n = 4x = 28) hybrid populations have been reported to be highly palatable with voluntary intake, in vivo digestibility of organic matter (DOM), and net energy expressed in fodder units for milk, similar to some of most palatable Italian ryegrass available at that time (Ghesquière et al. 1996). The drought resistance of the variety Lueur derives, at least in part, from its large deep root system and its ability to extract water from depth in the soil profile (Durand et al. 2007). *Festuca mairei* (Atlas fescue), which is related closely to *F. arundinacea* var. *glaucescens* and is indigenous to North Africa, is an alternative source of fescue genes for drought and heat tolerance and has been used (Wang and Bughrara 2005) as a source of novel genome variation for ryegrass through an introgression breeding approach similar to that described by Humphreys et al. (2005).

Farmers are increasingly seeking new grass varieties that provide resilience to climatic stresses, especially if these are accompanied by enhanced opportunities for environmentally sustainable livestock management. Described herein, for the first time is a comparison of the agronomic potential of four amphiploid *Festulolium* populations: *L. multiflorum* × *F. arundinacea* var. *glaucescens*, *L. perenne* × *F. arundinacea* var. *glaucescens* (2n = 4x = 28), and *L. multiflorum* × *F. mairei* and *L. perenne* × *F. mairei* hybrids (all 2n = 4x = 28). They were assessed initially over a 1-year field trial as spaced plants from which plants were selected to provide seed for a subsequent small replicated 1-year field plot trial where their performance was compared with that of elite ryegrass varieties.

In addition to measures of their field performance, the *L. multiflorum* × *F. arundinacea* var. *glaucescens*, *L. perenne* × *F. arundinacea* var. *glaucescens* (2n = 4x = 28), hybrid combinations were investigated for their potential, compared to ryegrass, to improve the efficiency of ruminant nutrition. Ruminant feeds are notorious for their inefficient use due to the poor conversion of ingested protein to milk and meat product and as a consequence, contribute to the high greenhouse gas emissions of nitrous oxide and environmental pollution with ammonia (Ripple et al. 2014). In fresh forage feeding situations, rapid postingestion rates of endogenous (plant) proteolysis can contribute to rates of protein breakdown in excess of that used by microbes (Zhu et al. 1999; Wallace et al. 2001; Kingston-Smith et al. 2010). This occurs during autolysis and cell wall breakdown (Edwards et al. 2008), and through an imbalance between protein supply and energy availability for microbial growth (Johnson 1976). Various plant breeding initiatives have attempted to reduce gaseous emissions by livestock either by encouraging increased rates of microbial N conversion using high sugar ryegrasses (Wilkins and Humphreys 2003), or by delaying the degradation of plant protein in ingested feed (e.g., by stimulating polyphenol oxidase (PPO) expression, Lee et al. 2004), to allow more time for N assimilation by the animal. Shaw (2006) reported that *F. arundinacea* var. *glaucescens* protein was more slowly degraded under rumen conditions and had far greater protein retention than either *L. perenne* or *L. multiflorum.* Shaw (2006) speculated that this may be due to the presence of protein protective mechanisms such as heat-shock proteins that had evolved in the fescue species sufficient to safeguard its adaptation to stresses encountered in Mediterranean conditions. Shaw (2006) also demonstrated slower breakdown of protein than its ryegrass parent in a F_1 *L. multiflorum* × *F. arundinacea* var. *glaucescens* (2n = 4x = 28) hybrid in rumen-simulated conditions. In order to verify and expand the findings of the pilot experiments undertaken by Shaw (2006), both the same and alternative

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Lolium spp. × *F. arundinacea* var. *glaucescens* parental genotypes and their respective hybrids were grown under summer-like conditions in a controlled environment (CE), with plant-mediated proteolysis under the temperature and low oxygen conditions of the rumen assessed in vitro as described previously (Kingston-Smith et al., 2010). Shaw (2006) reported the protein retention of *F. mairei* to be inferior to that of *F. arundinacea* var. *glaucescens* when exposed to these conditions. For this reason, the *Lolium* spp. × *F. mairei* F1 hybrids were excluded from the protein stability assessment.

Materials and Methods

Production of *Festulolium* populations

The *L. multiflorum* × *Festuca arundinacea* var. *glaucescens* (2n = 4x = 28) hybrids were produced by hybridizing either autotetraploid *L. multiflorum* cvs Danergo, Gemini, and Roberta, and a Breeders' Line Bb2534 (all 4n = 4x = 28) with genotypes of *F. arundinacea* var. *glaucescens* (2n = 4x = 28) selected from a natural accession Bn354. The fescue derived from an original INRA, Plantes Fourrageres, Rouen, France collection extracted from an Alpine field location 700 m a.s.l. in the Hautes-Alpes, France.

The *L. perenne* × *F. arundinacea* var. *glaucescens* (2n = 4x = 28) hybrids were produced by hybridizing autotetraploid *L. perenne* cvs AberDell and Dunluce (both 4n = 4x = 28) with genotypes of *F. arundinacea* var. *glaucescens* (2n = 4x = 28) also selected from the natural accession Bn354.

The *L. multiflorum* × *F. mairei* (2n = 4x = 28) hybrids were produced by hybridizing either autotetraploid *L. multiflorum* cv Gemini or Bb2534 (an IBERS Breeders' line derived from a chromosome-doubled variety AberEpic) (both 4n = 4x = 28) with genotypes of *F. mairei* (2n = 4x = 28) derived from an IBERS Accession Bs3065 donated by CSIRO Canberra, Australia and collected from the Atlas Mountains in Morocco.

The *L. perenne* × *F. mairei* (2n = 4x = 28) hybrids were produced by hybridizing autotetraploid *L. perenne* cvs AberDell and Dunluce (both 4n = 4x = 28) with genotypes of *F. mairei* (2n = 4x = 28) also selected from the natural accession Bs3065.

For brevity species names and species' hybrids will be described throughout by the following nomenclature: *L. multiflorum* (4x) = Lm; *L. perenne* (4x) = Lp; *F. arundinacea* var. *glaucescens* (4x) = Fg; *L. multiflorum* × *F. arundinacea* var. *glaucescens* (F_1)(4x) = LmFg; *L. perenne* × *F. arundinacea* var. *glaucescens* (F_1)(4x) = LpFg. *F. mairei* (4x) = Fm; *L. multiflorum* × *F. mairei* (F_1)(4x) = LmFm; *L. perenne* × *F. mairei* (F_1)(4x) = LpFm.

Embryo rescue incorporating use of modified Gamborg and Miller B5 Medium (as described in Humphreys et al. 2005) was employed throughout to generate all the LmFg, LpFg, LmFm, and LpFm F_1 hybrids. The LmFg populations used in the field study derived from seven parental Lm genotypes of variety Danergo, six genotypes of both variety Gemini and Bb2534, and one genotype of Roberta. The Lm genotypes were hybridized onto six alternative Bn354 Fg genotypes to generate the LmFg populations. Four of these Fg genotypes were also used as parents with three genotypes of Lp variety Dunluce, and one genotype of Ba14076 and of AberDell to generate all the LpFg populations. The LmFm populations derived from three parental Lm genotypes of Bb2534, and two of variety Gemini which were hybridized onto four alternative genotypes of Fm Accession Bs3065. One of these Fm plants together with an alternative Fm genotype from Accession Bs3065 were used with two Lp genotypes of variety AberDell and two Lp cv Dunluce genotypes (also used to produce LpFg) to generate all the LpFm populations.

Genotypes of LmFg, LpFg, LmFm, and LpFm following embryo rescue were transferred to pots filled with potting compost and established to maturity in a frost-free glasshouse. Following growing conditions sufficient for vernalization and inflorescence induction (>10 weeks of short days and temperatures of circa 4–10°C) and prior to ear emergence 50 F_1 LmFg genotypes, 34 F_1 LpFg genotypes, 23 F_1 LmFm genotypes, and 27 LpFm F_1 genotypes were transferred in their four groups to separate pollen-proof glasshouses to interpollinate. Seed from each polycross was germinated to produce populations for field assessment as spaced plants.

Field assessments

Spaced-plant field trial

For the field study, four populations each of 300 genotypes of LmFg, LpFg, LmFm, and LpFm hybrids were established as individual spaced plants in a field at IBERS, Aberystwyth University in mid-Wales, UK. Heading date was recorded in accordance with IBERS field assessment protocols as number of days between 1st April and ear emergence. Aftermath heading was scored as 1 = no additional inflorescence to 9 many secondary inflorescences. Other phenotype measures were growth habit (based on a scale of 1 = erect; 5 = prostrate), plant height (cm) at ear emergence, leaf width (based on scale 1 = narrow; 9 = wide), plant width at ear emergence (cm), tiller density (based on a scale of low tiller density = 1; high tiller density = 9), disease score (based on the presence of any rust [*Puccinia* spp.]) infection (score 1 = highly infected; 9 = no infection), and on their comparative plant size

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recorded at 5 time points throughout the growing season (score 1 = small; 9 = large). The first cut was taken on 27 July 2011 with the second on the 1 September 2011.

Fifty high-yielding plants were selected from each population based on average fresh weight over the two harvests, and forage quality of this subset of plants was determined. Forage quality was measured as %water soluble carbohydrate (WSC), %dry organic matter digestibility (DOMD), and %nitrogen and total protein content.

Small-plot field trial

The subsets of 50 plants were then extracted from the field, repotted in potting compost and transferred to pollen-proof isolation houses for seed multiplication. Seed from each LmFg, LpFg, LmFm, and LpFm polycross was sown in a second field trial as three replicate field plots 1 × 3 m at IBERS. Three replicate plots of control varieties Lm cv Danergo, and Gemini (4n = 4x = 28) and Lp cv AberGlyn and AstonEnergy (4n = 4x = 28) were also sown with all plots randomized. Seed of all tetraploid varieties and populations was sown at 3.3 g/m^2 (in accordance with standardized NIAB and IBERS field protocols). The harvests of dry matter forage yield (DMY) were compared over six cuts taken in 2013. Forage quality: (%WSC, %dry matter digestibility (DMD) and %N and crude protein were assessed at Cuts 1, 2, and 4 using NIR technologies and in complete accordance with the standard IBERS protocols. The percentage ground cover at the end of the growing season for all grasses was compared. Detailed meteorological records for IBERS field trials in 2011 and 2013 are provided in: https://share.aber.ac.uk/dept/ibers/intranet/research/weather/default.aspx.

All field data were analyzed according to standard procedures with the menu-driven options within Genstat 13.2 for Windows (VSN International Ltd., http://www.vsni.co.uk) software. For the spaced-plant field trial, least significant differences (LSD) between LpFg, LpFm, LmFg, and LmFm population means ($P < 0.05$) were calculated for heading date, growth habit, plant height, leaf width, plant width, tiller density, disease score, and for plant size. For the small-plot field trial, LSD ($P < 0.05$) was calculated for forage yield during each of 6 cuts, and also for forage quality (%WSC, %DMD, %N) at cuts 1, 2, and 4 between the LpFg, LpFm, LmFg, and LmFm populations and their respective tetraploid *Lolium* control cultivars Lm cv Danergo and Gemini and Lp cv AstonEnergy and AberGlyn.

In vitro determination of plant-mediated proteolysis

The plants selected for protein analysis comprised clonal replicates of the same genotypes of Fg (4x), Lm (4x), and Lp (4x) and their respective LmFg and LpFg F_1 hybrids (all 4x) used as parents for progeny assessed in the field study. They are listed in Table 1. All plants were maintained in 6″ pots in Levington's multipurpose compost under identical conditions in a frost-free glasshouse at IBERS under natural illumination and watered, and when required, fertilized, cut, and repotted to encourage active and consistent plant growth throughout. Plants for protein analysis were maintained to achieve an equivalent ontogeny and with no indication of inflorescence induction to minimize potential interactions due to age difference. To further enhance consistency, all plants were transferred into a CE facility (Gallenkamp PLC Monarch Way, Belton Park, Loughborough, UK and Skye Instruments Lighting, Ddole Enterprise Park, Llandrindod Wells, Powys, UK) and acclimated to constant UK summer conditions for a minimum of 6 weeks under 18 h light at 600 μmol.m^{-2}.sec^{-1} at 22°C, and with 6 h darkness at 14°C, all at 72% humidity.

Mature nonsenescent leaves at equivalent developmental stage were selected from each plant genotype, removed and incubated over three concurrent days producing three replicate results. Replicate groups of parent plants and their F_1 were incubated on separate weeks due to the large number of samples. The leaves of each plant genotype were cut 5 cm above the soil into 1 cm lengths and weighed to provide equal 0.1 g fresh-weight samples for 4 incubation time points; 0, 2, 6, and 24 h. These time points were selected based on previous results (as in Shaw 2006). Leaf sections were transferred into Hungate tubes and filled with 5 mL of anaerobic buffer warmed to 39°C (Van Soest 1967) and, except for those at 0 h, capped quickly under a stream of CO_2, and placed into a water bath heated to 39°C. The tubes containing incubated plant

Table 1. Genotypes of *Lolium multiflorum* (Lm), and *L. perenne* (Lp) varieties hybridized onto *Festulolium glaucescens* var. *arundinacea* (Fg) (all 4x = 28) to produce allotetraploid F1 LmFg and F1 LpFg hybrids (2n = 4x = 28).

Lolium spp. parent variety/plant no.	*Festuca glaucescens* parent accession/ plant no.	LmFg and LpFg (2n = 4x = 28)
Lm cv Roberta/1[1]	X Bn354/4[1]	= LmFg1[1]
Lm cv Gemini/2	X Bn354/8	= LmFg2
Lm cv Gemini/2	X Bn354/8	= LmFg3
Lm cv Gemini/5	X Bn354/8	= LmFg4
Lm cv Danergo/6	X Bn354/35	= LmFg5
Lm cv Danergo/9	X Bn354/35	= LmFg6
Lp cv AberDell/5	X Bn354/15	= LpFg1
Lp cv AberDell/5	X Bn354/8	= LpFg2
Lp cv Dunluce/5	X Bn354/17	= LpFg3
Lp cv Dunluce/5	X Bn354/17	= LpFg4
Lp cv Dunluce/6	X Bn354/8	= LpFg5

[1]Also used previously by Shaw (2006).

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material were removed from the water bath after 2, 6, and 24 h, respectively. Plant material was recovered under vacuum filtration and rinsed with deionized water. Samples were then placed into microcentrifuge tubes, flash frozen in liquid N, and stored at −80°C.

Procedures for protein extraction and measurement

Batches of circa 30 incubated samples in microcentrifuge tubes were removed from the −80°C freezer, placed on ice, and transferred into racks in a freeze dryer for 24 h. The samples were then stored in the dark prior to milling. For milling, freeze-dried samples with two tungsten beads added were placed into 2 × 24 sample milling boxes and milled in a Retsch (GmbH Haan Germany) MM 300 mill at a frequency of 30 sec for 1 min on each side following rotation of the boxes. Where necessary supplementary hand grinding was applied to ensure that the samples were completely homogeneous and ground fully and uniformly. Processed samples were subsequently stored in a cool, dark, and dry place in preparation for protein extraction.

Protein in the ground residue was extracted by grinding in a mortar and pestle with prechilled (4°C) extraction buffer (0.1 mol/L HEPES, pH 7.5 containing 1 mmol/L EDTA, 0.1% (v/v) Triton-X 100 and 0.5% protease inhibitor cocktail (Sigma UK Ltd, Gillingham, UK) and 2 mmol/L dithiothreitol) added to the samples at a ratio of 40 μL/g dry weight. The sample homogenate was transferred into individual 2 mL microcentrifuge tubes, flash frozen in liquid N, and stored at −80°C, until protein analysis.

Homogenate samples were thawed on ice and samples centrifuged at 13,000*g* at 4°C for 10 min and protein content of supernatant was determined with the Bio-Rad Protein Assay kit (Bio-Rad UK Ltd., Hemel Hempstead, UK) against a BSA calibration curve (working range 0–5 μg). Sample volumes between 1 and 10 μL were used to ensure results remained within range of the standard curve and water and reagent controls in a total volume of 200 μL were included to determine background absorbance. The absorbance was read at 595 nm on a BioTek ElX 808 microtiter plate reader (Bio Tek (Bedfordshire, UK), Fisher Scientific) after an incubation time of 15–20 min at room temperature.

The protein degradation time courses were fitted with exponential decay curves of the form

$$c = br^t \quad (1)$$

where c is protein content (mg g^{-1} dry weight), t is time from start of incubation, (hours), b is a fitted parameter describing protein content at time zero, and r is a fitted parameter describing the rapidity of the decay of protein.

This equation was rearranged to allow calculation of the time taken in hours for protein to decay to half its value at time 0 ($t^{1/2}$).

$$t^{1/2} = \frac{log_e 0.5}{log_e r} \quad (2)$$

This parameter was chosen as the greater the $t^{1/2}$, the greater the resistance of the plant proteins to degradation. Nonlinear curve fitting was performed using a Maximum Likelihood Program (Ross 1987) and parallel curve analysis (Ross 1990) was used to determine significant differences between fitted curves, and to estimate standard errors of fitted parameters and $t^{1/2}$.

Results

Fertility in the LmFg, LpFg, LmFm, and LpFm F_1 genotypes employed as parents in their respective polycross combinations was high and more than sufficient to provide seed for the four plant populations used in the initial field study. The second seed production program all used 50 plants/population selected from the first field trial and as such provided an accurate comparison of population seed-set. Overall differences in seed production between the LpFm, LmFm, and LmFg populations were insignificant (total seed produced 277 g (LpFm), 279 g (LmFm), 271 g (LmFg), but total seed production amongst the LpFg was significantly lower (seed produced = 200 g; $\chi^2 = 17.29$, $P < 0.001$).

Spaced-plant field trial

The mean values for the plant traits scored in the spaced-plant field trial and any significant difference ($P < 0.05$) found between the four populations are shown in Table 2. While variation for heading date was evident within each amphiploid hybrid combination, overall no significant difference was observed between the early-heading populations LpFm and LmFg (both population mean date for heading: day 37). However, populations LmFm and LpFg were both significantly later heading ($P < 0.05$, population mean dates for heading: day 38 and day 43, respectively). Aftermath heading was low and was not significantly different in either of the Lp-based populations (LpFg and LpFm), but was significantly greater ($P < 0.05$) in both the Lm-based populations, especially in LmFg.

Although there were significant differences ($P < 0.05$) between all four populations, overall growth habit was distinct and different between the very erect Lm-based and the far more prostrate Lp-based *Festulolium* populations. LmFg genotypes were the most erect and were significantly more erect than LmFm ($P < 0.05$). Conversely, LpFg was more prostrate than LpFm ($P < 0.05$) indicating that growth habit was determined more by the *Lolium*

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Table 2. Mean plant traits scored in a 1 year spaced-plant field trial of 300 plant *Festulolium* populations LmFg, LpFg, LmFm, and LpFm; all 2n = 4x = 28.

Grass hybrid	Mean heading date (day no.)	Aftermath heading score = 1–9	Growth habit score = 1–5	Leaf width score = 1–9	Plant height (cm)	Tiller number score = 1–9	Plant width (cm)	Disease resistance score = 1–9
LmFg (4x)	37 a	6.62c	1.57a	7.23 c	62.20 c	6.41 b	43.58 b	5.65 a
LpFg (4x)	43 c	1.85 a	4.21 d	3.18 a	22.00 a	6.10 ab	56.81 d	6.12 b
LmFm (4x)	38 b	5.92 b	1.77 b	6.54 b	51.30 b	5.46 a	38.56 a	6.37 b
LpFm (4x)	37 a	1.80 a	3.87 c	3.28 a	22.20 a	5.76 a	53.27 c	6.22 b
LSD (P < 0.05)	0.89	0.27	0.14	0.22	1.80	0.30	2.12	0.31
SED	0.45	0.14	0.07	0.11	0.91	0.15	1.07	0.16

For each value within a column, populations having $P < 0.05$ difference are indicated by an alternative letter.

than the *Festuca* parent. LmFg was taller than LmFm ($P < 0.05$). The Lm-based populations were taller than the more prostrate LpFg and LpFm populations.

There was considerable within population variation in plant size but species' effects were evident. While the Lm-based *Festulolium* populations were taller, the overall plant width of the Lp-based populations was greater. The mean plant width of LpFg was greater than that found in LpFm ($P < 0.05$), while width of the Lm-based populations LmFg was larger than LmFm ($P < 0.05$). While differences in plant habit, height, and breadth related to their *Lolium* parent, differences in plant mean tiller number in the *Festulolium* populations corresponded more to their *Festuca* species parent. Significantly higher tiller numbers ($P < 0.05$) were observed in the Fg-based populations LmFg and the LpFg, which were themselves not significantly different. The Fm-based populations LmFm and LpFm with a lower tiller frequency were not significantly different.

Leaf width was determined more by the *Lolium* parent than by the *Festuca* parent, with Lm-based hybrid leaves being significantly broader than those involving Lp. The mean leaf width for LmFg was significantly greater than for LmFm ($P < 0.05$), but both Lm-based populations had significantly wider leaves ($P < 0.01$) than LpFg and LpFm, which were not significantly different.

Rust (predominantly *Puccinia coronata*) infection was compared amongst the four amphiploid hybrid populations. Variation in disease susceptibility was evident throughout, but overall, infection was low with 90% of all plants in the field trial either with low or no infection (scores 6–9). However, LmFg was more susceptible than the other three *Festulolium* populations and had significantly higher frequencies of rust infection ($P < 0.05$).

Plants in all four plant populations continued to grow and increased in plant size throughout the growing season with the 50 largest genotypes from each population selected for seed multiplication (Table 3). The *Festulolium* populations containing Fg (LmFg and LpFg) comprised larger plants than the corresponding populations with Fm (LmFm and LpFm, respectively), but the influence of their *Lolium* parent was also evident. The LmFg commenced growth and developed more extensively during the spring as compared to the other three populations, but it was the LpFg population that demonstrated most growth later in the growing season.

The dry matter yields and forage quality measures for the 50 plant LmFg, LmFm, LpFg, and LpFm selections are presented in Table 4. The mean dry weight for cut 1 (27 July 2011) for the 50 selected plants from LmFg was significantly higher ($P < 0.05$) than for LmFm which in turn was significantly higher ($P < 0.05$) than for LpFm and LpFg. The mean dry weights from each population at cut 2 (1 September 2011) were more comparable, with LmFg and LmFm not significantly different, but both were superior to LpFm and LpFg ($P < 0.05$).

The %WSC of the Lp-based populations, LpFg and LpFm was not significantly different at cut 1 while LmFg and LmFm were significantly ($P < 0.05$) lower. The superior %WSC of the Lp-based populations was maintained at cut 2 ($P < 0.05$), but between the Lm-based populations, LmFm was significantly higher in %WSC than LmFg. The %DMD of the Lp-based populations were not significantly different and also were significantly higher ($P < 0.05$) than those involving Lm both at cut 1 and cut 2. The %N and total protein were also higher in the Lp-based populations over both cut 1 and cut 2

Table 3. Mean ranking of plant size (1 = small – 9 = large) in diverse *Festulolium* populations during the course of a growing season.

Score date	LmFg	LmFm	LpFg	LpFm	LSD $P < 0.05$ (SED)
14 march	5.62 a	4.82 b	4.21 c	3.78 d	0.338 (0.17)
20 April	6.52 a	5.49 c	5.97 b	5.83 b	0.325 (0.16)
14 July	6.62 a	5.95 b	6.67 a	6.06 b	0.274 (0.14)
23 August	6.45 b	5.86 c	6.9 a	6.38 b	0.291 (0.15)

Significant differences ($P < 0.05$) in plant size between populations within each row on each date are indicated by alternative letters.

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Table 4. Mean dry matter yield (DMY), %water soluble carbohydrate (WSC), %dry matter digestibility (DMD), and %nitrogen (N) [and total protein*] of 50 genotypes of *Festulolium* populations LmFg, LpFg, LmFm, and LpFm over two consecutive harvests during summer and autumn 2011; (SE).

Harvest date	Population	Yield DMY(g) (SE)	%WSC (SE)	%DMD (SE)	%N [+total protein*] (SE)
27 July 2011 Cut 1	LmFg (4x)	112.8 a (25.6)	19 b (2.5)	65 b (2.0)	1.5 c (0.3) [9.38]
	LpFg (4x)	48.3 g c (14.0)	23 a (3.4)	79 a (2.0)	2.4 a (0.3) [15]
	LmFm (4x)	93.9 g b (28.1)	19 b (2.2)	66 b (2.4)	1.7 b (0.2) [10.63]
	LpFm (4x)	53.3 g c (12.2)	23 a (4.1)	80 a (2.3)	2.5 a (0.3) [15.63]
LSD ($P < 0.05$)	Cut 1	8.15	1.24	0.86	0.10
1 September 2011 Cut 2	LmFg (4x)	73.2 g a (18.8)	22 c (2.5)	69 b (2.7)	1.6 b (0.2) [10]
	LpFg (4x)	58 g b (13.3)	25 a (3.4)	77 a (2.1)	2.1 a (0.3) [13.13]
	LmFm (4x)	68.1 g a (18.4)	24 b (2.6)	70 b (3.3)	1.6 b (0.3) [10]
	LpFm (4x)	60.9 g b (11.4)	26 a (3.7)	77 a (2.6)	2.0 a (0.2) [12.5]
LSD ($P < 0.05$)	Cut 2	5.95	1.24	1.10	0.10

For each harvest, within column values with the same letter were not significantly different at $P < 0.05$.

($P < 0.05$). The %N and total protein of LmFg and LmFm at cut 2 was the same but at cut 1 was lower in LmFg ($P < 0.05$).

Small plot field trial

Spring 2013 had prolonged low temperatures delaying growth with the consequence that the first harvest was delayed until 24th May. Five further cuts were made with the final cut taken on 24th October. Throughout the growing season, the performance of all four *Festulolium* populations compared well with that of the control varieties (Fig 1). DMY for LmFg and LmFm and the Lm control varieties early in the year (cuts 1 and 2) did not differ significantly. Likewise LpFg and LpFm although lower in yield than their Lm-based counterpart populations ($P < 0.05$) did not differ for DMY from their Lp control varieties. By cut 3 LmFg had a higher yield than LmFm ($P < 0.05$) but neither was significantly different from the Lm control varieties. The DMY of LpFm and LpFg at cut 3 did not differ significantly. The *Festulolium* populations did not differ in yield from the highest yielding Lp control variety (Aston Energy). At cut 4 the DMY of LmFg and LmFm and the highest yielding Lm control (variety Gemini) did not differ significantly. Likewise there was no significant difference in DMY between either LpFg and LpFm and the Lp control varieties. By cut 5 and 6, the forage yield of all varieties (except the inferior yielding control Lp cv AberGlyn in cut 5) irrespective of whether they were Lm- or Lp-based did not differ significantly.

The forage quality of the *Festulolium* populations harvested on Cut 1 (24th May), Cut 2 (25th June), and Cut 4 (3rd August) was consistently equivalent to or better than their respective Lm and Lp (4x) controls. The %DMD of LpFg although the highest in the field trial was not significantly superior to LpFm and Lp cv Aston Energy. All were superior to the Lp control AberGlyn ($P < 0.05$). LmFg and LmFm had similar %DMD to the tetraploid control varieties Lm cv Danergo and cv Gemini. The %WSC of LmFm and LmFg was equivalent to Lm control cvs Danergo and Gemini. LpFm and LpFg had the same %WSC content of Lp control variety Aston Energy with all superior ($P < 0.05$) to Lp cv AberGlyn. There was no significant difference ($P > 0.05$) in %N content and total crude protein between LpFg, LpFm, and the two Lp tetraploid cultivars Aston Energy and AberGlyn. Similarly, there was no significant difference ($P > 0.05$) in %N and total crude protein content between LmFg, LmFm, and the Lm controls Danergo and Gemini.

The %ground cover of the Fg-based populations LpFg and LmFg was superior to all grasses used in the field trial and was significantly greater than their respective controls Lp cv Aston Energy and AberGlyn and Lm cv Gemini ($P < 0.05$).

Protein losses through plant-mediated proteolysis

Figure 2 shows that the initial protein content of Lm and Fg was higher than in their F_1 hybrid progeny and differed significantly ($P < 0.001$). Similarly, the initial protein content of Lp and Fg was higher ($P < 0.001$ for Lp, $P < 0.01$ for Fg), than their respective F_1 progeny. Within both the *Lolium* and the *Festuca* species' groups the initial protein content of the constituent genotypes also differed significantly ($P < 0.001$). There was significant variation in the initial protein content between plants within the LpFg ($P < 0.05$) and LmFg ($P < 0.001$) F_1 hybrid groups. Despite their initial difference in protein content, following 24 h exposure to the temperature and oxygen conditions of the rumen, there remained no significant difference in protein content between *Lolium* and *Festuca* parent and their respective hybrid genotypes.

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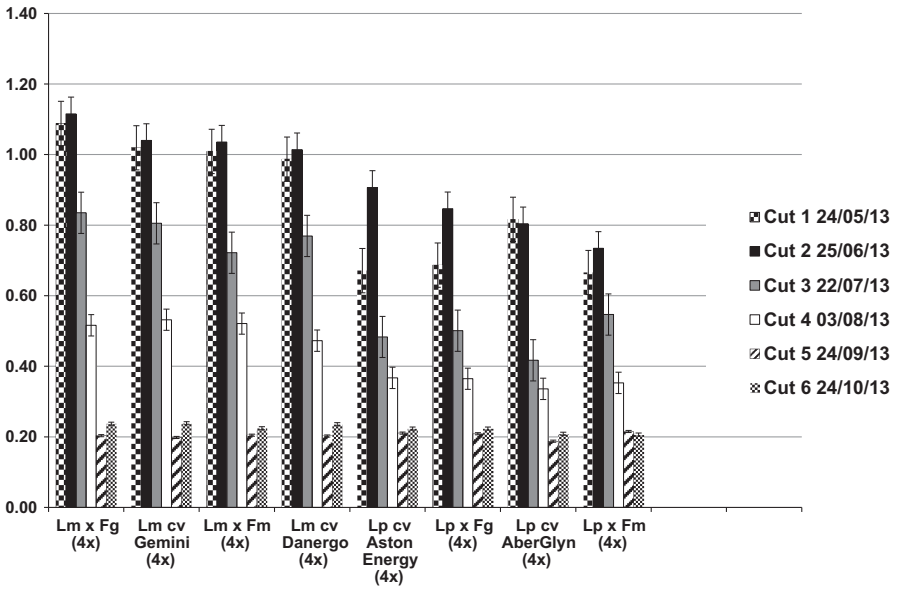


Figure 1. Dry matter yield (DMY kg) of field plot trials of *Festulolium* populations LmFg, LmFm, LpFg, and LpFm (2n = 4x = 28) compared with tetraploid *Lolium multiflorum* (Lm) and *L. perenne* (Lp) cultivar controls. Ranked by total yields (DMY kg).

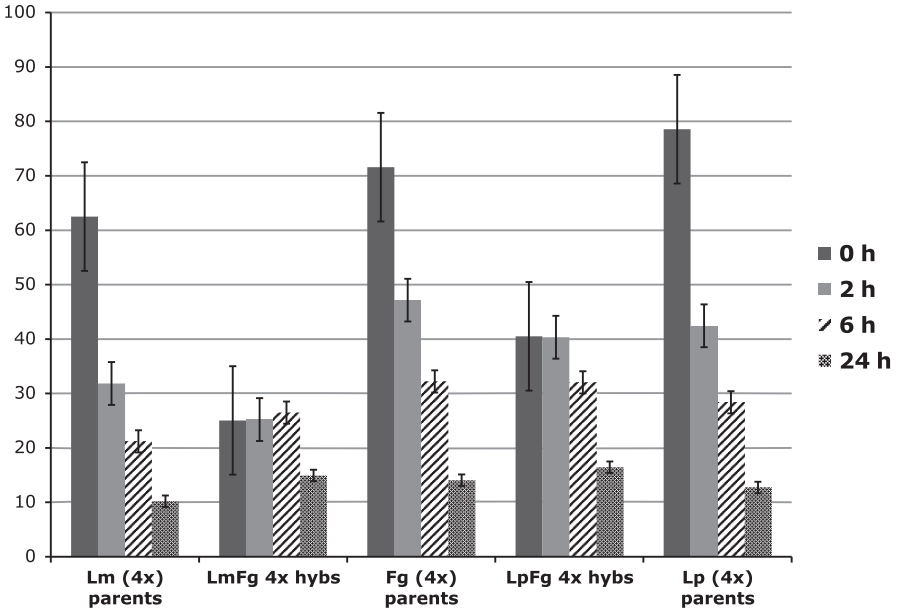


Figure 2. Protein content (mg/g DW) of *Lolium multiflorum, L. perenne, Festulolium arundinacea* var. *glaucescens* parent genotypes, and their *L. multiflorum* × *F. arundinacea* var. *glaucescens* and *L. perenne* × *F. arundinacea* var. *glaucescens* F_1 hybrid progeny when exposed in vitro to 0, 2, 6, and 24 h at 39°C under anoxia.

A comparison of $t^{½}$ of each Lm, Fg parent genotype, and their respective F1 progeny, and of Lp, Fg, and their respective F1 progeny is presented in Table 5. In addition, and using the combined data for all time points, replicates, and genotypes for each parent and hybrid, the total $t^{½}$ values were fitted (Fig. 3). Overall, the $t^{½}$ of the Fg parents was significantly higher ($P < 0.05$) than the Lp (4*x*) genotypes (4.5 h) used to produce the LpFg F_1 hybrids. Similarly, $t^{½}$ of the Fg parents were higher than Lm but in this case, the difference was not significant ($P < 0.3$). However, $t^{1/2}$ for both F_1 hybrid groups (21 h and 18 h LmFg and LpFg, respectively) were significantly greater than their

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Table 5. Protein half-lives ($t^{½}$) (SE) of (i) *Lolium multiflorum* (Lm), *Festulolium arundinacea* var. *glaucescens* (Fg), and their F1 progeny (all 2n = 4x = 28), and (ii) *L. perenne* (4x), *F. arundinacea* var. *glaucescens* (Fg), and their F1 progeny (all 2n = 4x = 28) when exposed in vitro to 24 h of anoxia at 39°C.

L. multiflorum (Lm) (4x)	Lm half-life ($t^{½}$) (SE)	X *F. glaucescens* (Fg) (4x)	Fg half-life ($t^{½}$) (SE)	= F1 LmFg (4x)	LmFg half-life ($t^{½}$) (SE)
Lm cv Roberta/1[1]	3.23 h (1.53)	*X Fg Bn354/4*[1]	8.12 h (2.41)	= LmFg1[1]	9.20 h (2.15)
Lm cv Gemini/2	8.70 h (3.26)	*X Fg Bn354/8*	13.70 (3.57)	= LmFg2	44.1 h ([2])
Lm cv Gemini/2	8.70 h (3.26)	*X Fg Bn354/8*	13.70 (3.57)	= LmFg3	21.15 h (10.19)
Lm cv Gemini/5	18.55 h (18.93)	*X Fg Bn354/8*	13.70 h (3.57)	= LmFg4	25.29 h (10.86)
Lm cv Danergo/6	3.06 h (0.82)	*X Fg Bn354/35*	4.68 h (0.92)	= LmFg5	37.99 h ([2])
Lm cv Danergo/9	1.53 h (0.40)	*X Fg Bn354/35*	4.68 h (0.92)	= LmFg6	61.65 h ([2])
L. perenne (Lp) (4x)	Lp half-life ($t^{½}$) (SE)	X *F. glaucescens* (Fg) (4x)	Fg half-life($t^{½}$) (SE)	= F1 LpFg (4x)	LpFg half-life ($t^{½}$) (SE)
Lp cv AberDell/5	6.04 h (1.65)	*X Fg Bn354/15*	7.40 h (3.77)	= LpFg1	16.96 h (5.91)
Lp cv AberDell/5	6.04 h (1.65)	*X Fg Bn354/8*	13.70 h (3.57)	= LpFg2	21.94 h (13.26)
Lp cv Dunluce/5	9.15 h (3.35)	*X Fg Bn354/17*	3.71 h (1.02)	= LpFg3	26.63 h (12.19)
Lp cv Dunluce/5	9.15 h (3.35)	*X Fg Bn354/17*	3.71 h (1.02)	= LpFg4	20.63 h (7.52)
Lp cv Dunluce/6	1.96 h (0.41)	*X Fg Bn354/8*	13.70 h (3.57)	= LpFg5	9.91 h (2.46)

[1]Lm, Fg, and LmFg genotypes used previously in Shaw (2005).

[2]high SE due to protein $t^{½}$h values that far exceed the 0–24 h sample time points for protein measures.

respective *Lolium* parent groups (Lm, 4.1 h $P < 0.001$ and Lp 4.5 h, $P < 0.01$). LpFg hybrids had significantly greater $t^{½}$ than their Fg parent genotypes ($P < 0.05$) but the differences in $t^{½}$ between LmFg and Fg were not significant ($P < 0.15$). There were significant differences in $t^{½}$ within the Lm ($P < 0.001$), and the Lp ($P < 0.001$), but not between the Fg ($P < 0.25$) genotypes (Table 5). There were also significant differences in $t^{½}$ within both the LmFg ($P < 0.001$) and LpFg ($P < 0.05$) hybrid groups. While collectively Fg genotypes had greater $t^{½}$ than Lp ($P < 0.05$), they did not have greater $t^{½}$ than the Lm group ($P < 0.3$) Within the species' groups, individual genotypes of Lm and Lp were identified (Lm cv Gemini/5 and Lp cv Dunluce/5) as having superior $t^{½}$ compared to certain Fg genotypes (Bn354/17; Bn354/35).While the overall $t^{½}$ for LmFg F_1 was 21 h, genotypes with considerably higher $t^{½}$ values were identified (Table 5). All data on protein content were confined to time points within 0–24 h exposures to the in vitro stress conditions and the observation in three LmFg F_1 genotypes of $t^{½}$ calculations that extended well beyond the 24 h data set led to high SE for the genotypes concerned (Table 5) which carried forward when all LmFg F_1 were combined (Fig 3).

Discussion

Breeding for *Festulolium* varieties has over recent years gained increased importance as an aide to combat climate change and to achieve more sustainable grassland agriculture. This is because it is possible to capture in one variety the agronomic value of ryegrass and the resilience, water, and nutrient-use-efficiency found in different fescue species (Ghesquière et al. 2010). Initially, most advances and cultivars marketed involved Italian ryegrass (*L. multiflorum*) and meadow fescue (*F. pratensis*), a species combination known taxonomically as *Festulolium braunii.* However, in recent years especially at IBERS attention has moved more to employing alternative fescue species such as *F. arundinacea* var. *glaucescens* and more recently *F. mairei* (Humphreys et al. 2013). These have provided novel sources of genes for improved drought resistance, water-use-efficiency, and deep rooting for ryegrass (Humphreys et al. 2005, 2013). The plant–soil interactions generated by certain deep rooting *Festulolium* species combinations including those described herein and others such as *L. perenne* × *F. pratensis* (*Festulolium loliaceum)* can provide other benefits including ecosystem services such as flood mitigation (Humphreys et al. 2013; MacLeod et al. 2013) and have potential to increase soil organic carbon capture by grassland (Kell 2011).

While the potential of *Festulolium* to improve grassland persistency has long been recognized, for reasons such as genome instability (Canter et al. 1999), high costs of seed production compared to ryegrass, and inferior forage quality (Ghesquière et al. 2010) *Festulolium* has not yet been widely used. For these reasons, doubts surrounding its commercial development remain and these limitations must be overcome before *Festulolium* varieties become marketed widely and their full benefits realized. However, recent advances in *Festulolium* breeding technologies combined with an increased awareness of the need to find alternatives to ryegrass to better combat climate change and to

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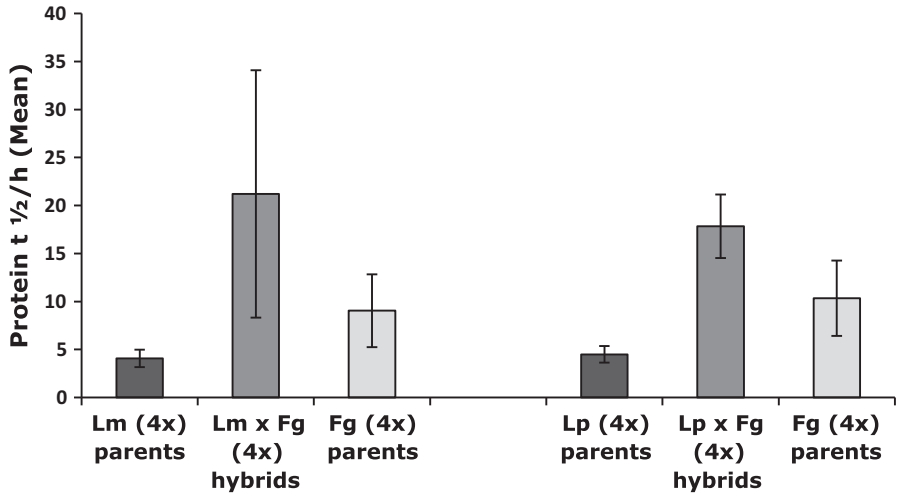


Figure 3. Comparisons of protein stability between tetraploid *Lolium perenne* (Lp), *L. multiflorum* (Lm), and *Festulolium arundinacea* var. *glaucescens* (Fg), and their respective F1 progeny LpFg and LmFg (2n = 4x = 28) when exposed in vitro to 24 h at 39°C under anoxia.

achieve more sustainable grassland systems have encouraged *Festulolium* development and led to the marketing of the cultivar AberNiche in the UK, an expected forerunner for others under development and trial. AberNiche is a synthetic form of *Festulolium braunii* comprising genome combinations of *L. multiflorum* and *F. pratensis*. It was exluded from the current study as in earlier work (Shaw, 2005), *F. pratensis* demonstrated a similar plant-mediated proteolysis to that recorded for *Lolium* spp., and was significantly inferior to the protein retention found in *F. arundinacea* var. *glaucescens* when exposed to the stress conditions applied in the current study. In an alternative field trial at IBERS AberNiche, *L. multiflorum* × *F. arundinacea* var. *glaucescens* (LmFg), and *L. multiflorum* × *F. mairei* (LmFm), all Lm-based tetraploid *Festulolium* hybrids, produced an equivalent forage yield throughout their first year harvests (Humphreys, unpubl.). The current work represents a comprehensive field and laboratory study of four new amphiploid *Festulolium* hybrid combinations, *L. multiflorum* × *F. arundinacea* var. *glaucescens* (LmFg), *L. perenne* × *F. arundinacea* var. *glaucescens* (LpFg), *L. multiflorum* × *F. mairei* (LmFm), and *L. perenne* × *F. mairei* (LpFm), and provides evidence to support their commercial development both in terms of their agronomic performance and their potential to improve ruminant nutrient-use-efficiency and thereby to reduce adverse environmental emissions by livestock. One of the species' combinations has already been developed for commercial use in France. A *L. multiflorum* × *F. arundinacea* var. *glaucescens* variety Lueur equivalent to the LmFg population used here has been developed commercially by INRA (Ghesquière et al. 1996, 2010) but in trials at IBERS has inferior yield to LmFg (Humphreys, unpubl.).

Two possible strategies for increasing efficiency of conversion of forage N to microbial N have been used to improve ruminant nutrition and decrease emissions of greenhouse gases by livestock and both have received considerable recent attention at IBERS over recent years. The first strategy aims at increasing the amount of readily accessible energy during the early part of the fermentation in the rumen. The second strategy aims to increase the protection of forage proteins, and thereby reducing the rate at which their breakdown products are made available to the colonizing microbial population. The high sugar grasses (HSG) are examples of the former where increased WSC has been shown to have a positive impact on meat yields (Lee et al. 2001) and milk production (Miller et al. 2001). The incorporation in legumes of protein protection methods such as increased PPO expression is an example of the second approach as applied to conserved forage (Lee et al. 2004) while increasing the $t^{1/2}$ for protein degradation in the rumen would increase N use efficiency by grazing ruminants, as discussed below.

Extensive nitrogen loss to the environment due to poor incorporation of dietary N by ruminants causes both pollution of ground water and contributes to nitrous oxide production. This is due to excessive proteolysis in the rumen, for which plant-mediated proteolysis is a contributory factor (Zhu et al. 1999; Wallace et al. 2001; Kingston-Smith et al. 2005) and hence current efforts to mitigate impact of ruminant farming through selection of improved forage genotypes. Shaw (2006) demonstrated that Fg was significantly more stable under rumen-like conditions than *Lolium* species. She also demonstrated that Fg was more stable than Fm when exposed to equivalent rumen-simulated trials. Although Shaw (2006) reported benefits

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in terms of improved protein stability in F_1 hybrids compared with Lm, these were lost during the course of a backcross breeding program identical to that described in Morgan et al. (2001). The current work sought to extend and verify the study described in Shaw (2006) by exploring the variation within Lp, Lm, and Fg for protein stability under rumen-like conditions and to determine the extent at which this trait is expressed in *Festulolium* F1 hybrids. Evidence is presented here to show (i) significant variation for protein stability within Lm, Lp, and Fg tetraploid genotypes, (ii) that the range of protein stability in Lm, Lp, and Fg genotypes has significant overlap, (iii) significant *Lolium-Festuca* genome interactions and transgressive segregation in F_1 hybrids between Fg and both Lm and Lp that gave rise to significantly higher protein stability than that expressed by either *Lolium* parent genomes.

Shaw (2006) proposed that protein-protection mechanisms that had evolved in Fg to combat the high temperatures experienced in Mediterranean locations were providing equivalent benefits when grass was exposed to the stresses encountered in the rumen. The current work indicates that the genetic control for protein stability is complex and demonstrates significant variation within and overlapping protein stability between Lm, Lp, and Fg populations. Heterosis between the *Lolium* and *Festuca* genomes in their F_1 hybrid forms provided improved protein stability compared with the *Lolium* and Fg parents. The initial protein content of the Lp, Lm, and Fg parent genotypes was consistently higher than that observed in their progeny. However, following exposure to 24 h of rumen-simulated conditions, despite significant difference in their protein half-lives, there was little or no actual difference in residual protein content between parent and hybrid genotypes. A similar relationship between initial low protein content and slow rates of protein degradation was seen previously with white clover (Kingston-Smith et al. 2006). While the outcome in terms of protein content in both *Lolium* and the *Festulolium* hybrid genotypes at 24 h was similar, the rate of protein decline was significantly greater in the *Lolium* genotypes. Protein degradation in the early time period following ingestion of forage is considered to be important. Despite the availability of peptide and amino acid substrates for microbial growth at this stage, the availability of energy will be determined (and possibly limited) by the extent of microbial colonization and cell wall degradation (Johnson 1976; Edwards et al. 2008). Hence, decreasing plant-mediated proteolysis has the potential to improve delivery of protein and energy by the feed. As protein building blocks are nonlimiting the main consequences of decreasing plant-mediated proteolysis would be predicted to be decreased activity of HAP bacteria and, on a whole animal level, increased N partitioning to product and away from urine. It will be important to now extend the current work by employing animal studies to fully assess the benefits of LmFg and LpFg hybrids as feed both in terms of their potential for improved ruminant nitrogen-use-efficiency and livestock gain and also for environmental gain by limiting N losses into the environment.

In field trials, genotypes of four *Festulolium* populations; LmFg, LpFg, LmFm, and LpFm were selected for seed multiplication; seed set from these and subsequent selections was far in excess of that required for the initial spaced plant trial and subsequent plot trial experiments. The two Lm-based populations were large and erect and contrasted with the two Lp-based populations which were large and high tillering and consistently prostrate. For the majority of foliar traits, it was the *Lolium* (Lm or Lp) rather than the *Festuca* (Fg or Fm) parent that was the determining factor in trait expression as reported previously in a root phenotype study (Humphreys et al. 2013). In the earlier work, root ontogeny was found to be more dependent on the presence of an Lm or an Lp genome rather than whether the accompanying genome complement was Fg or Fm. In the field plot trial, all four *Festulolium* populations compared favorably and were not significantly different in yield or forage quality from their respective Lm and Lp ($4x$) control varieties demonstrating the absence of any suggestion of transfers of deleterious forage characters from their fescue parent.

The combined field-based and in vitro proteolysis study provides evidence that both LpFg and LmFg amphiploid hybrids offer considerable potential for sustainable grassland agriculture. The LmFm and LpFm populations provided similar benefits and would be expected to be particularly drought and heat tolerant (Wang and Bughrara 2005). The root systems of all four populations combined the high growth rate and branching of *Lolium* with the root strength and depth of Fg and Fm (data not shown, Humphreys et al. 2013). The impact of these *Festulolium* hybrids in terms of plant–soil interactions is being assessed currently and will be compared with earlier research (MacLeod et al. 2013) for potential ecosystem service benefits. Taken together, the outcomes of the current research provide compelling evidence for benefits both for agriculture and the environment for future use of *Festulolium* hybrids.

Acknowledgments

We thank HCC and EBLEX for funding Sally O'Donovan's PhD program at IBERS some of whose research outcomes on use of *Festulolium* hybrids for improved ruminant

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nutrition are presented here. We also acknowledge BBSRC for funding the research inputs from Alison Kingston-Smith, Mike Humphreys, and Alan Gay. The IBERS field trials and seed multiplication were funded through an industrial DefraLINK Consortium (DefraLink Programme LK0688) led by Mike Humphreys and undertaken by Markku Farrell.

Conflict of Interest

None declared.

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