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SUCCESSFUL POLLEN DISPERSAL MODULATES WITH FLOWERING PHASE IN FIELD-HYBRIDIZING GRASSES

Conor Meade, Eimear Ryan, Ewen Mullins and Martin Downes

ABSTRACT

Gene-flow between intensively and extensively farmed grass populations is an ongoing feature of agroecological landscapes, especially in Atlantic northwestern Europe. Adjoining population boundaries and in-field admixture of grass types via winter forage dispersal facilitates both in-field hybridisation and recruitment of immigrant seedlings. Here we examine the paternal hybridisation of one grass species (*Lolium multiflorum*, pollen donor) into the population of a second, fully inter-fertile, grass species (*L. perenne*, pollen receptor) via pollen-mediated gene-flow in an experimental field plot. Using weekly counts of successful pollination in 470 individual receptor plants based on paternity analysis in 4281 germinated F_1 seedlings, we determined the extent of evident hybridisation (hybrid progeny that show some paternal morphology) and silent hybridisation (hybrid progeny that show no paternal morphology) over the course of floral anthesis. Co-dominant morphological traits in F_1 progeny underestimated microsatellite-validated genetic hybridisation by approximately 30%, while background pollen competition dampened the overall rate of successful pollen donor pollination. Overall pollination from the donor plot followed a composite decline model. However over the course of floral anthesis the successful pollination pattern was changeable, and showed varying levels of affinity to three tested decline distribution models.

INTRODUCTION

The vegetation of the Atlantic seaboard of North-western Europe, including Ireland, comprises diverse grassland agroecological habitats maintained by an evolving array of agricultural management strategies (EEA 2008). In intensively managed silage and grazing pastures, as occur in the most productive dairy and animal rearing regions, plant biodiversity within grassland swards is strongly controlled by re-seeding with forage cultivars (mostly *Lolium perenne*) (Rath and Peel 2005; Sullivan *et al.* 2010). In less intensively managed grasslands, including hay meadows, permanent pastures and rough grazing commonage, plant communities are much more biodiverse, controlled by a combination of abiotic environmental factors, grazing intensity and immigration and disturbance history (Sullivan *et al.* 2010).

Intensive grassland cultivation indirectly impacts on these more extensively managed grasslands in a number of ways. For example, where intensive grazing has been abandoned at a field site this allows the development of a more biodiverse and compositionally stable plant community (Reisch *et al.* 2017). In addition, pollen dispersal (from flowering swards) and seed dispersal (for example

through hay deposition for winter feed) also introduces a background 'genetic rain' of elite forage cultivar germplasm into semi-natural grassland populations (Meade and Mullins 2005). These kinds of migration events are an ongoing feature of the agricultural landscape, and in relation both to the increasingly erratic stress regime associated with climate change, and the prospective future introduction of new elite cultivars, recruitment of immigrant genotypes will play a crucial role in determining the fate of semi-natural populations and communities (Ellstrand *et al.* 1999; Norberg *et al.* 2012).

At a global level, the increasing spread of novel plant genotypes into atypical habitats, especially in grassland agroecological landscapes, is seen to have wide-ranging ecological impacts, and frequently imposes new selective pressures on naturalised populations in low management habitats (Ellstrand *et al.* 1996). In many cases, this immigration involves discrete taxa or species with easy-to-identify ecological and genetic footprints (Sakai *et al.* 2001), but in other cases, for example the case of gene-flow between intensive and extensive grass sward populations, the respective populations are inter-fertile, leading to spread not of immigrant plant populations, *per se*, but of novel hybrid genotypes (Currat *et al.* 2006).

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In the widely cultivated grasses, interfertility between species is very common (Ellstrand *et al.* 1996), and the progress and extent of interspecies hybridisation among intermixing populations can be difficult to monitor. However, when incompatibility is not a factor, expansion in hybrid frequency within a population is in principle controlled by the same reproductive, selective and dispersal (seed and pollen) parameters that apply to the expansion of any minority genotype within a species metapopulation (Levin 1981; Meade and Mullins 2005). In terms of dispersal—and expansion of genotype distributions—via pollination, classic models infer in this case a leptokurtic distribution in successful pollination against distance from the pollen donor plant(s), varying from steep inverse-exponential declines to more gradual linear declines, depending on pollination mechanism (Ellstrand 1992; Giddings *et al.* 1997; Rognli *et al.* 2000; Shaw *et al.* 2006).

We hypothesise that this dispersal model holds true in the case of co-located interfertile grass species populations. Here we use a field pollination experiment in an Irish farm setting to examine the dynamics of interspecies hybridisation in grasses, between a donor *Lolium multiflorum* population (Italian ryegrass, Lm) and a receptor *L. perenne* population (perennial ryegrass, Lp) during floral anthesis. These forage species are widely cultivated in Northwestern Europe, and in temperate regions globally, and frequently occur in sympatry across agroecological landscapes. Phylogeographic analysis identifies a common origin for the two species in the Eastern Mediterranean *c.* 3.0 mya (Inda *et al.* 2013) and many extant wild types and commercial cultivars are interfertile, sharing a base diploid state of $2N=14$ (Ryan *et al.* 2006). *Lolium multiflorum* is distinguishable from *L. perenne* in having an annual/ biennial habit and the diagnostic presence of awns on the inflorescence. Hybrid crosses between the two species with *L. multiflorum* as the paternal parent show incomplete, co-dominant inheritance of awns. Importantly, awnless florets are a fixed character among *L. perenne* cultivars, and so inheritance of awns is not prone to false-positive Type II error scoring (Ryan *et al.* 2006). The objectives of this study are to evaluate (i) the extent of 'silent' hybrid frequency among F_1 genotypes; (ii) the spatial dispersal model for successful pollination at peak pollen production, and (iii) the relative consistency of the dispersal curve over the course of floral anthesis.

MATERIALS AND METHODS

A radial donor-receptor plot design comprised of 528 *Lolium multiflorum* 'Trajan' pollen donor plants and 470 *L. perenne* 'Aberdart' pollen receptor plants, was established in an arable field setting at

Oak Park, Co. Carlow, Ireland in 2013 (Figure 1). Seeds were germinated in September/October, viable seedlings were transplanted to growth pots the following March. Mature potted plants were placed in formation in a field of sugarbeet (*Beta vulgaris* L.) at the beginning of April, 4 weeks prior to anticipated heading dates. The donor population was arranged in a hexagonal plot 4 x 4 metres in size at the centre of the field formation, and receptor plants were arranged individually in radial arcs 8, 16, 32, 64, 96, 128, 160, 192 and 224m distant from the donor plot, with each plant on each arc 6.28 metres apart (= circumference/ radius) (Figure 1). Both donor and receptor plants were elevated 30cm above ground level to ensure that inflorescence heading overtopped the sugar beet ground crop, while viability of the experimental population was maintained in the field by daily watering and the addition of wire netting to protect from grazing by wild hares.

Due to an unusually cold and wet spring, floral anthesis began in late May (3 weeks later than expected). Pollination observations began when anthesis was observed on 25% of donor plant individuals, which occurred on 31 May. For the following 21 days, each receptor-plant inflorescence with newly open stigmas was labelled, generating a complete array of pollen receptivity on every receptor plant in the experimental plot over the 3-week flowering period. Mean wind direction over each 24-hour period was recorded at the on-site field weather station, and observations ended on Sunday 20 June when the proportion of actively fertile donor plants dropped below 10% of the total population.

Receptor plants were later collected from the field and returned to an outdoor watered plot, and fertile seed was collected from labelled inflorescences beginning with Week 1 on 30 August. Seed was packaged and dried, and from each inflorescence 25 seeds were planted in potting trays containing an in-house grass potting compost used at the Teagasc Crops Research Centre in Oak Park. Following germination, 12 randomly selected seedlings were each transplanted to separate 4cm pots and grown on to maturity. In May the following year, paternal identity of the entire F_1 *L. perenne* seedling cohort (containing putative x *L. perenne* and x *L. multiflorum* crosses) was validated using morphological diagnostics (the presence/ absence of inflorescence awns and rolled versus folded leaf blades in the culm). To establish the level of silent gene-flow (morphologically indistinct F_1 *L. perenne* seedlings that have *L. multiflorum* paternity) all seedlings recovered from 8–64m were screened using PCR and capillary electrophoresis analysis of *L. perenne* and *L. multiflorum* allele identity at the diagnostic microsatellite locus H01H06, as per the protocol of Ryan *et al.* (2007). For seedlings recovered from arcs 96–224m from the donor plot, only those showing hybrid morphology were microsatellite-validated.

The distribution of successful hybrid pollination events from weeks 1–3 were test-fitted against 3 types of leptokurtic distribution model (after Shaw *et al.* 2006): (i) the Exponential Decline Model (probability of fertilisation $p = \exp^{-r/k}$; where r is radial distance from the donor and k is a distribution constant); (ii) the inverse Power Law Model ($p = [c + r]^{-b}$; where r is radial distance from the pollen donor and b and c are constants for species-specific biology and environment, respectively); and (iii), the composite decline model ($p = [c + r]^{-b} \cdot \exp^{-r/k}$), incorporating both of the above. These three models have been previously applied to capture, respectively, elevated short-distance dispersal frequency distributions, elevated long-distance dispersal frequency distributions and dispersal distributions showing elevated near and distant dispersal frequencies (as described and tested in Shaw *et al.* 2006). Permutation tests were carried out individually on Microsoft Excel, using extreme value starting points and iterative adjustment towards maximum correlation against the observed dataset.

RESULTS

Flowering peaked in week 1 for the donor *L. multiflorum* population and during week 2 in the *L. perenne* receptor population (Table 1, Figure 1). During week 3, when donor *L. multiflorum* fertility dropped from 30 to below 10%, 44% of receptor plants were still receptive (Table 1). In total over 11000 F_1 seeds were harvested from *L. perenne* receptor plants that were fertile during the 3-week observation window, with *c.* 4280 (*c.* 39%) germinating. While donor *L. multiflorum* and receptor *L. perenne* populations were similar in size (528 and 470 individuals, respectively), 96% of all seedlings germinated were Lp x Lp progeny, and 4% Lp x Lm. Based on validation at the H01H06 microsatellite locus of all 1084 F_1 seedlings recovered from rows 8–64 across weeks 1–3, silent hybrids showing Lp x Lp morphology but Lp x Lm microsatellite paternity numbered 34 in total and accounted for *c.* 29% of F_1 hybrid seedlings (Table 1, Figure 2).

In week 1, observed successful fertilisation of *L. perenne* receptor flower ovules by *L. multiflorum* donor flower pollen, recorded as seedlings with awns, followed a composite decline distribution model ($c = 1$, $b = 1$, $k = 50$; Pearson's $R = 0.998$, $p < 0.0001$), with *c.* 62% F_1 Lm paternity on the 8m radial arc, declining to 1–5% paternity between the 96 and 224m arcs (Table 1, Figures 1–4). In week 2, the pollination pattern generated a best fit with an exponential decline model ($k = 10$; Pearson's $R = 0.923$, $p < 0.01$) (Table 1; Figure 3A, 3B). In week 3, the fertilisation pattern for Lm pollen became more stochastic and was not correlated with any of the tested decline distribution models (Table 1).

When the inferred 0.30 frequency of morphologically silent hybrids in arcs 8–64 is added to the total observed counts of morphological hybrids in arcs 96–224 across the dataset, the Composite Decline Model generated the strongest correlation in both weeks 1 and 2 (Table 1, Figure 3A, 3B), however correlation with the Exponential Decline and Inverse Power Law models also changed. Wind direction fluctuated during the observation period (Figure 1), and the spatial distribution of successful Lm pollination was significantly correlated with wind direction only on the inner 8m arc in week 2 (Table 1).

In terms of background Lp paternity across the receptor plot, mean Lp F_1 seedlings per fertile receptor plant per week declined from 5.65 in week 1 to 3.50 in week 3 (Table 1, Figure 2). During week 2 and 3, these paternity levels were consistent between the inner and outer radial arcs across the plot, despite some outlying values associated with small sample size, however during week 1, a difference was evident, corresponding to the period of highest Lm paternity, and lowest Lp paternity, in the inner 8–64m arcs (Table 1, Figure 2). The observed frequency of Lp x Lp fertilisation was not significantly correlated with any of the tested decline distribution models (data not shown), indicating a weak or undetectable background pollen density effect associated with the concentric positioning of receptor plants across the experimental plot.

DISCUSSION

The data presented here indicate that the patterns of pollination between adjacent or sympatric grass populations are likely to be dynamic and changeable over the course of floral anthesis. Successful inter-specific fertilisation of *L. perenne* flowers by *L. multiflorum* pollen followed a decline pattern that varied both temporally (in terms of distance x likelihood x time point, Figure 2 and 3) and spatially (in terms of correlation with observed prevailing wind direction, Figure 1). At peak pollen release during week 1, this fertilisation pattern followed a composite decline pattern, with very high pollination rates adjacent to the donor, and much reduced, but stabilised, pollination rates up to 224m from the donor (Figure 4). As flowering in the donor population declined, the observed pattern of field pollination was altered, characterised by a dramatic reduction in short distance pollination, and greater stochasticity in longer distance pollination, and this resulted in reduced correlation between the observed pollination distribution frequency and all three tested models. Nevertheless, when silent hybrid frequency is included (i.e., the frequency of F_1 individuals with Lm paternity that do not show an awned inflorescence) the

Table 1. Summary data and analysis of pollination patterns in *L. perenne* receptor plants in the Oak Park Field plot, Carlow, Ireland. Week 1, 2 and 3 refers to the three observed weeks of pollination over the period of floral anthesis in the donor (*L. multiflorum*) and receptor (*L. perenne*) plant populations in May and June. Germinated F₁ seedling data are seed recovered from fertile *L. perenne* inflorescences (stigmas opened) during each specified week. See text for discussion.

	Test criteria	Week 1	Week 2	Week 3	Total
Total pollen donor plants		528	528	528	
Fertile donor plants (at week start)		387	317	159	
Proportion of donor plants fertile		0.73	0.60	0.30	
Total pollen receptor plants		472	472	472	
Total fertile receptor plants (at week start)		297	326	207	
Proportion of receptor plants fertile		0.63	0.69	0.44	
Total recovered seed		3264	4536	3312	11112
Total germinated F1 seed†		1790	1759	740	4289
Proportion germinated		0.55	0.39	0.22	0.39
Total F1 Lp x Lp in arcs 8-224		1677	1715	724	4116
Proportion of Lp x Lp in total F1		0.94	0.97	0.98	0.96
Total F1 Lp x Lm Hybrid in arcs 8-224		113	44	16	173
Proportion of Hybrids in total F1		0.06	0.03	0.02	0.04
F1 seedlings screened at H01H06 locus in arcs 8-64		455	499	130	1084
Total F1 Lp x Lm H01H06 Hybrids in arcs 8-64		78	28	11	117
Morphologically Silent Hybrids arcs 8-64		12	15	7	34
Proportion of Silent Hybrids arcs 8-64		0.15	0.54	0.64	0.29
Average Lp F1 per receptor plant		3.55	3.63	1.53	2.90
Average Lp F1 per fertile receptor plant		5.65	5.26	3.50	4.80
Variance between arcs within each week	ANOVA	F 1.99*	F 1.59 ns	F 1.73 ns	
Variance between weeks	ANOVA				F 28.87 ***
Average Hybrid F1 per receptor plant		0.24	0.09	0.03	0.12
Average Hybrid F1 per fertile receptor plant		0.38	0.13	0.08	0.20
Variance between arcs within each week	ANOVA	F 56.70 ***	F 9.61***	F 10.48 ***	
Variance between weeks	ANOVA				F 13.959 ***
Wind Direction/ Hybrid Pollination Correlation	Pearson's R				
Arc 8-64		0.075 ns	0.140 ns	0.017 ns	
Arc 8		0.671 ns	0.743*	0.393 ns	
Hybrid Pollination Model Best Fit‡	Pearson's R				
Exponential Decline Model $p = e^{-r/k}$	$k = 10$	0.998**, 0.997**	0.923*, 0.991*	0.431 ns, 0.427 ns	0.986*, 0.986*
Inverse Power Law Model $p = (c + r)^{-b}$	$b = 1, c = 1$	0.997***, 0.994***	0.907**, 0.991**	0.434 ns, 0.434 ns	0.986**, 0.986**
Composite Decline Model $P = (c + r)^{-b} e^{-r/k}$	$b = 1, c = 1, k = 50$	0.999***, 0.998***	0.916**, 0.992**	0.422 ns, 0.418 ns	0.988**, 0.987**

†Reaching sexual maturity

‡Results for best performing permutation of each model, correlation values for exclusion and inclusion, respectively, of inferred silent hybrid frequency in arcs 96-224

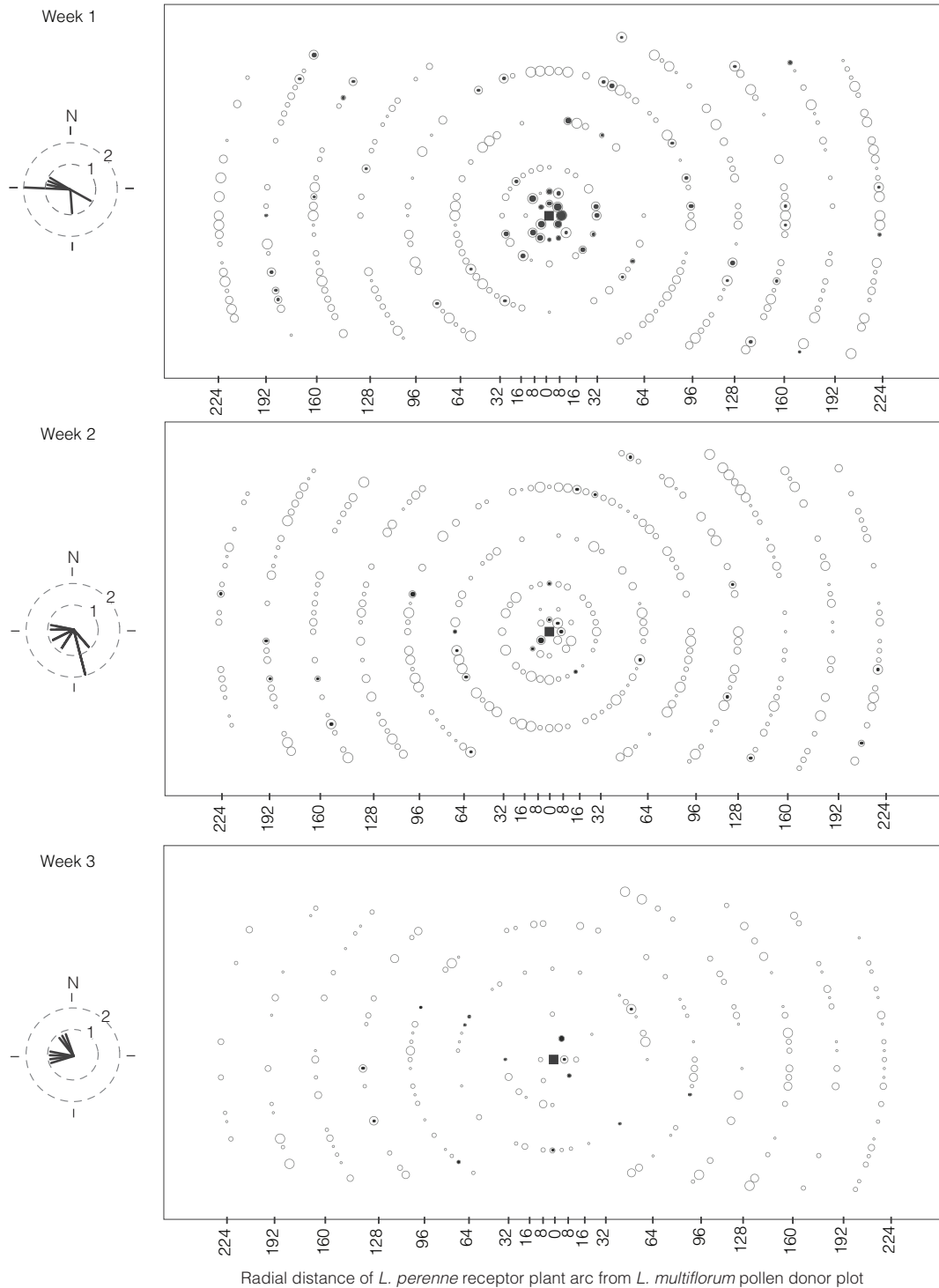


Fig. 1—Spatial distribution of successful pollination of *L. perenne* receptor plants for weeks 1, 2 and 3 at the field pollination plot in Oak Park, Carlow. The black square indicates position of *L. multiflorum* pollen donor plot. Open circles indicate total F_1 progeny recorded per receptor plant (0–12 seedlings), dark inner circles indicate subtotal that show hybrid *L. multiflorum* paternity. For radial arcs 8–64m, paternal identity of all 1083 seedlings validated using microsatellite locus H01 H06, for arcs 96–224, hybrid identity is validated for morphological hybrids only. Wind direction is measured in number of wind days per week.

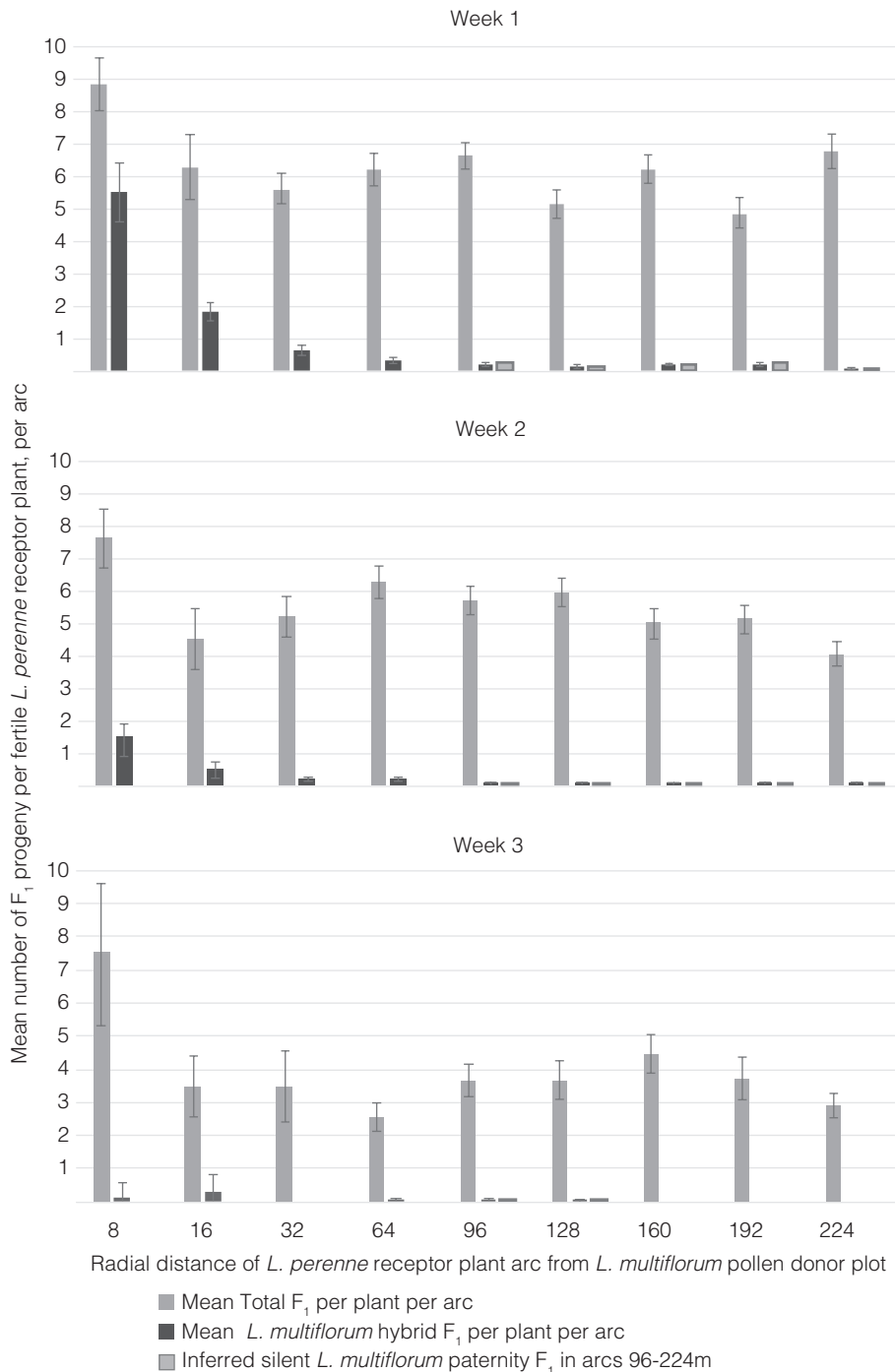


Fig. 2—Mean number of F₁ seedlings, hybrid F₁ seedlings, and inferred proportion of silent hybrid seedlings, per radial arc for weeks 1–3, with standard error (SE) indicated.

composite decline model remained optimal for both weeks 1 and 2, and for the full 3-week data (Table 1). The shifts in affinity for the different dispersal models (Table 1, Figure 3) reflects the slightly higher proportion of long-distance pollination events observed as anthesis progresses, a pattern which the composite model is by design sensitive to (because

it incorporates the inverse power expression, Figure 3B). These and other decline models are evident for many different species, independently, in gross measures of pollen distribution during flowering (Lavigne *et al.* 1998; Ma *et al.* 2004; Shaw *et al.* 2006; Petti *et al.* 2007; Beckie and Hall 2008; Matter *et al.* 2013). In the case of the more detailed time-series

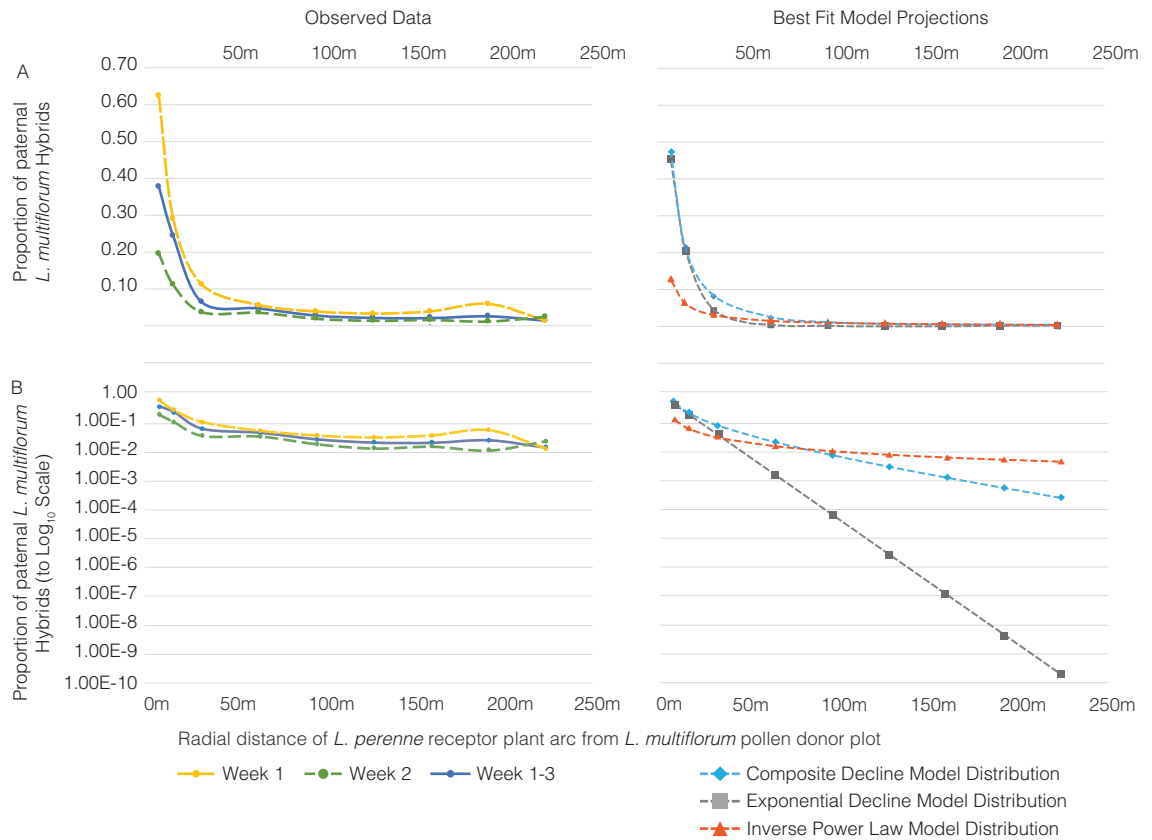


Fig. 3—Proportion of hybrid F_1 seedlings recorded per arc, per week, by distance from pollen donor plot, measured against best fit decline model distributions. Panel A, Scaled by proportion of F_1 seedlings from observed data and best-fit model inference. Panel B, Scaled by proportion of F_1 seedlings from observed data and best-fit model inference to a base of Log₁₀.

data presented here, what is distinct is that the successful pollen distribution pattern changes over the course of flowering maturity in the donor populations, with altering affinity for the different dispersal models as donor pollen production declines.

Due to prevailing climatic conditions at the site, it was expected that wind would emerge as an important factor in pollen distribution in this experiment, but this was not the case (Table 1). The spatial distribution of Lm F_1 seedlings showed some correlation with wind direction in week 1 (Figure 1), however this correlation was significant only in week 2 in arc 8, adjacent to the pollen donor plot. It is likely that pooled weekly pollination counts lacked resolution where day to day wind direction, and pollen direction, became variable (Figure 1), as occurred during this experiment when multiple atmospheric pressure systems passed over the field site. While day to day wind direction was recorded, logistical constraints meant day to day monitoring of receptor flower fertility was not possible.

The difference in peak flowering dates between the donor and receptor populations contributed

to the changing paternity profile of F_1 seed from week 1 through week 3, principally via reduced pollen load in the 8–64m radial arcs as *L. multiflorum* fertility declined (Table 1, Figure 2). While receptor plant density was greater in the 8–32m zone compared to the remainder of the field plot, this did not result in significantly greater Lp paternity in these arcs. Correspondingly, the receptor plant layout appears to have captured the predicted donor Lm pollination distribution without introducing uneven background levels of pollen competition from receptor plant flowers.

However, in terms of the overall frequency of hybrid Lm F_1 progeny, these are underrepresented compared to paternal Lp F_1 progeny when considered against the similar size of the two parental populations. This difference could arise as a result of mating barriers between the two species, or from local pollen competition, which for most mating events in most receptor plants would have involved Lp pollen produced by the two closest adjacent receptor plants, 6.28m distant. Ryan *et al.* (2007) demonstrated that synchronously flowering

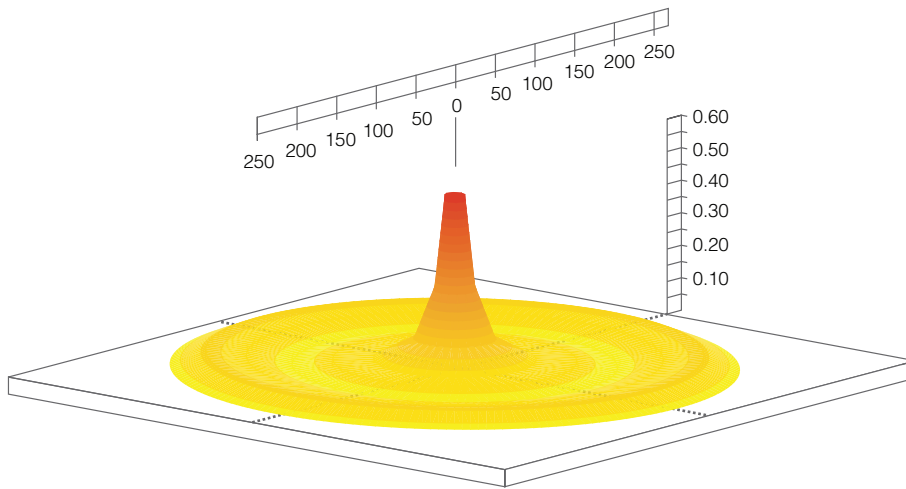


Fig. 4—Spatial pollination distribution curve showing mean proportion of hybrid progeny per fertile *L. perenne* receptor plant by radial distance in metres from *L. multiflorum* pollen donor plot (0–250m), at centre, as recorded at Teagasc Oak Park grassland site.

L. perenne and *L. multiflorum* are fully interfertile in the field, implying that, in this case, local pollen competition is the more likely factor for the reduced hybrid F_1 cohort. Importantly, although the experimental plot was placed in an arable setting to minimise pollen rain from the surrounding landscape, ryegrass swards comprise up to 50% of regional agricultural planting. For this reason background *L. perenne* pollen levels may also have played a role, most likely by reducing overall levels of successful within-plot pollination.

The design and scale of this field analysis presents challenges for replication, and the large sampling regime enforced limited individual sample sizes on seedling germination analysis. Nevertheless, inclusion of 520 pollen donor plants and 478 pollen receptor plants simultaneously flowering over 3 consecutive weeks, in addition to paternity analysis in a cohort of >4289 field-pollinated F_1 seedlings constitutes a robust evaluation of this field pollination environment. These data affirm that hybridisation patterns between interfertile grass species in an agroecological setting can be expected to follow generalised patterns for gene-flow within individual species. As such, it can be anticipated that the closer the proximity, and/or the larger the size of the donor grass sward population, the greater the rate and spatial extent of successful pollination into adjacent grasslands will be (Flannery *et al.* 2005), with follow-on impacts on wider population genetic diversity across the landscape (Munzbergova *et al.* 2013; Reisch *et al.* 2017). In addition, as we have documented here, the pollen dispersal pattern between populations can also be expected to fluctuate with flowering phase, and so in a mosaic field landscape this will generate a shifting profile of more intense and less intense pollen dispersal zones. Based on established cropping and forage management

systems throughout Northwestern Europe, it is reasonable to assume that background gene-flow of this kind is an established feature of these grassland landscapes, and where diagnostic taxonomic traits are co-dominant—as in the present study—in many cases this gene-flow is likely to be silent (and undetected in botanical surveys).

Over large areas of Ireland, but particularly in areas where high natural value extensive pastures dominate in the west and northwest of the island (Matin *et al.* 2016) average field size is considerably smaller than the experimental areas included in the present study (Deverell *et al.* 2009; Zimmerman 2012), indicating that pollen flow between fields is likely to be ubiquitous in these areas, even for small populations of flowering grasses. In terms of the broader impact of these patterns on the biodiversity and ecology of grassland habitats, this is dependent on the extent to which successful pollen flow and the recruitment of F_1 juveniles to the receptor population are correlated.

In the present experiment, F_1 progeny were germinated and grown to sexual maturity for identification purposes. The actual rate of hybrid grass seedling recruitment in grassland habitats is likely to be lower. This is due to the combined effect of two factors in the receptor populations: (i) pollen competition from within the receptor populations; and (ii) grazing, which both thins the canopy of flowering heads across grassland swards, and promotes vegetative tillering, creating very dense groundcover with little exposed ground for seedling emergence (Bullock *et al.* 1995). In this context, stable, biodiverse habitats with dense niche occupation—such as species-rich semi-natural grasslands—display elevated resistance to new immigration (Tilman 1997; Kennedy *et al.* 2002), whereas habitats that experience frequent

or intense disturbance facilitate greater levels of seedling recruitment (Bullock *et al.* 1995), and are much more prone to, for example, alien species invasions (Seastedt and Pyšek 2011). Thus in the absence of any change to established management practice in semi-natural grassland communities (which functions as the controlling disturbance for the habitat), it is likely that actual levels of annual hybrid F_1 recruitment will be much less than the potential inferred background rate of pollination between and among cultivated swards.

At the same time, these background rates of potential immigration and dispersal may facilitate community change over longer periods of time (Ellstrand, Prentice and Hancock 1999; Currat *et al.* 2008). In particular, as we move forward into a more unstable climatic regime (Seastedt and Pyšek 2011) with more pronounced fluctuations in water and temperature stress that introduce potentially significant abiotic disturbance to European grasslands, opportunities may increase for hybrid progeny to become established. The present study confirms that the patterns of dispersal and pollination in these scenarios will be dynamic; impacted by spatial context and planting history, as well as the overall levels of pollen competition across the landscape, and the phenology of floral anthesis.

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