Facilitating co-existence by tracking gene dispersal in conventional potato systems with microsatellite markers

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Based on international findings, Irish co-existence guidelines for the cultivation of GM potato stipulate that an isolation distance of 20 m is required to minimize the spread of transgenic pollen in accordance with required labeling thresholds. As potato tolerant to *Phytophthora infestans* is the most applicable GM crop from an Irish context, we tested the efficacy of this isolation distance under Irish environmental conditions using the conventional variety Désirée as a pollen donor and the male-sterile variety British Queen as a pollen receptor. Gene flow was determined by scoring for berry presence on receptor plants and confirmed using a microsatellite marker system designed to assess paternity in F_1 seedlings. 99.1% of seedlings recovered were identified as having Désirée paternity. Whereas 19.9% (140/708) of total berries formed on receptor plants occurred at a distance of 21 m from the pollen source, only 4 of these berries bore viable true potato seed (TPS), from which 23 TPS germinated. TPS-bearing berry formation was negatively correlated with distance from the pollen source, and although overall distribution of berries and seeds was non-random across the plot, no significant correlation was evident with respect to wind direction. Microsatellite markers were also used to confirm that the foraging beetle *Meligethes aeneus* is a vector for the transmission of potato pollen, but a more detailed statistical analysis of this dataset was limited by inclement weather during the trial. To conclude, we recommend that a two-tiered system be established in regard to establishing isolation distances for the experimental trial and commercial cultivation of GM potato in Ireland, and that responsible crop management be adopted to minimize the establishment of TPS-derived volunteers, which we have noted will emerge through a rotation as a result of pollen-mediated gene flow.

Keywords: *Solanum tuberosum* / potato / microsatellite / gene flow / co-existence

INTRODUCTION

Though gene flow has always been a feature of crop agroecology, the significance of the phenomenon has increased with the development and commercialization of genetically modified (GM) crops. At a European level, the subject of crop-derived gene flow is of particular relevance in regard to the debate on how GM crops are to co-exist with their conventional and/or organic equivalents ('co-existence'). Establishing effective cropping regimes for co-existence that minimize both the potential and subsequent outcome of a gene flow event is a critical step to preserving the genetic integrity of existing crop systems and affording tillage farmers the opportunity to choose the tillage system (organic, conventional, GM) they wish to employ.

A crop's potential for gene flow can be quantified by assessing the four primary modes of gene dispersal: pollen-mediated crop-to-wild relative, pollen-mediated crop-to-crop, seed-mediated crop-to-volunteer and seed-mediated crop-to-feral (Flannery et al., 2005). The imposition of separation distances between GM and related non-GM crops is an accepted measure to offset the challenge pollen-mediated gene flow poses to co-existence (ACRE, 2004; Eastham and Sweet, 2002; McGill et al., 2005; Tolstrup et al., 2003). In contrast, seed-mediated gene flow presents an equal if not greater challenge to the effective management of GM crops (Flannery et al., 2005; McHughen, 2006), but measures to minimize the potential for seed-mediated gene flow are less specific, due to the current deficit in research-based support for regulators (McHughen, 2006).

We have previously published a gene flow index (GFI) model that describes the quantification of a crop's potential for both pollen and seed-mediated gene flow (Flannery et al., 2005). From this model, the composite gene flow potential of potato was calculated to be 11/27.

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Considered to be high for a clonal crop, the value was weighted due to the potential for pollen-mediated crop-to-crop gene flow, which can manifest during the production of true potato seed (TPS). Dependent upon the variety, the entry of TPS into the seed bank will have implications for rotational hygiene (Lawson, 1983), which in turn could have implications for crop quality and seed purity (Eastham and Sweet, 2002).

Previous studies have examined the dispersal of pollen from field-grown transgenic potatoes, and concluded that transgene dispersal is effectively limited (99.98%) to within 10 m of the transgenic population (Conner and Dale, 1996; McPartlan and Dale, 1994; Tynan et al., 1990). On the contrary, a separate study reported the potential dispersal of transgenes up to 1000 m from the donor potato population (Skogsmyr, 1994), with an inference that the pollen beetle (*Meligethes aeneus*) was instrumental in facilitating gene transmission. A critical assessment of this result disputed the efficacy of the described methodology, and concluded that an isolation distance of 20 m was adequate to control pollen-mediated transgene escape from GM potato (Conner and Dale, 1996). This conclusion was supported upon review (Eastham and Sweet, 2002) and has subsequently been adopted as a guideline to facilitate the cultivation of GM potato (Tolstrup et al., 2003).

During the compilation of the GFI model (Flannery et al., 2005) we were conscious of our reliance on international reports, due to a scarcity of Irish-specific research. Though this was especially the case for potato, we concluded that the datasets described in the peer-reviewed reports (Conner and Dale, 1996; McPartlan and Dale, 1994; Tynan et al., 1990) were applicable to Irish agronomic systems. However, the absence of national-specific datasets highlighted a need to address the issue of potato-relevant gene flow.

In the potato-based gene flow studies completed to date, the methodology employed to screen for pollen-mediated gene dispersal has included screening for GUS activity (Conner, 1993; Tynan et al., 1990), NPTII enzyme assay (Conner, 1993), Southern analysis (McPartlan and Dale, 1994) and PCR (McPartlan and Dale, 1994; Skogsmyr, 1994). Co-dominant and highly polymorphic microsatellites or simple sequence repeats (SSRs) are informative genetic markers that can be employed to accurately measure pollen flow through parentage analysis (Slavov et al., 2005). As the use of SSRs circumvents the regulatory constraints associated with the cultivation of GM crops for field testing, SSRs have been used to estimate pollen-mediated gene flow for oilseed rape (Wilkinson et al., 2000), sugar beet (Andersen et al., 2005; Desplanque et al., 1999), perennial ryegrass (Ryan et al., 2007) and rice (Chen et al., 2004).

The objective of our study was to (i) calculate the frequency of pollen-mediated hybridization between two conventional potato varieties, (ii) ascertain whether the pollen beetle *M. aeneus* is a vector of pollen-mediated gene dispersal in potato and (iii) draw comparisons between our data and the existing literature. To this end we adopted a 'worst case scenario' approach that utilized the highly male-fertile *Solanum tuberosum* var. Désirée and the male-sterile *Solanum tuberosum* var. British Queen as pollen donor and receptor populations, respectively, in an experimental field trial, and screened a variety of SSR loci for their utility in tracking pollination between the two varieties.

RESULTS

Flowering periods of pollen receptors and donors

The selection of British Queen and Désirée as appropriate pollen receptor and donor populations, respectively, was based on information supplied through the European Cultivated Potato Database (see: http://www.europotato.org) and on preliminary hybridizations completed in the glasshouse. This was subsequently confirmed in the field where we conducted 45 British Queen × British Queen crosses to confirm British Queen's inability to self-fertilize and control crosses (Désirée × British Queen) to confirm the ability of Désirée to pollinate British Queen (data not shown).

In the 2005 trial, the first open flowers appeared in the British Queen sub-plots 54 days post sowing (dps). The maximum number of flowering inflorescences peaked 7 to 14 days later, and flowering continued for approximately 20 further days (88 dps). Comparatively, the appearance of flowering inflorescences on Désirée commenced at 59 dps, and peaked between 76 and 83 dps, before concluding at 114 dps (Fig. 1). As a result, synchronous flowering between the two varieties was recorded for over 30 days, providing adequate time to facilitate pollen-mediated hybridization should it occur. For British Queen, the total number of flowering inflorescences recorded North, South, East and West of Désirée was 2272, 2024, 1979 and 1767, respectively, with the number of Désirée flowering inflorescences estimated at 12 292 for the duration of the trial.

In the 2006 trial, the flowering of the pollen receptor sub-plots mirrored that of the 2005 trial (data not shown). However, due to the late sowing dates, the flowering of both the pollen receptor and donor plots coincided with unseasonably dry conditions (> 30 °C for first two weeks of August). This resulted in severe damage to the maturing flower heads of the pollen donor, and as a result, the number of donor inflorescences tagged during the trial was only 2.9% (n = 359) of that estimated in 2005.
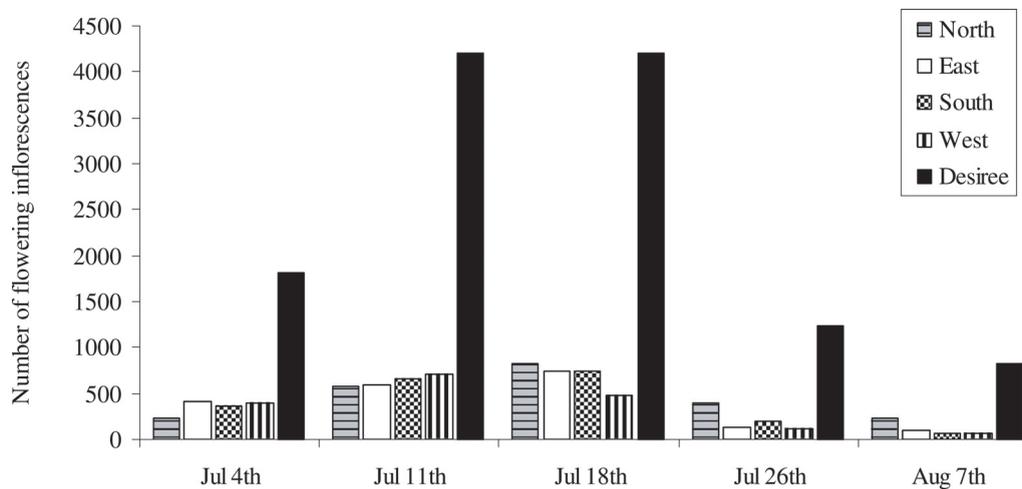
Figure 1. Flowering periods of pollen receptor (var. British Queen) sub-plots situated due North, South, East and West of the pollen donor plot (var. Désirée) during the 2005 trial.

Berry formation and TPS collection

Berry formation was recorded on the pollen receptor sub-plots at each separation distance (5 m, 6 m, 10 m, 11 m, 20 m and 21 m) across the four orientations of the 2005 trial (Fig. 2A). In total, 708 berries were collected across the trial, with 194 berries collected due North, 145 due South, 251 due East and 118 due West of the pollen donor population. During the flowering period, the prevailing winds predominated from West to South (Fig. 2B), and while this may have contributed to the increased frequency of berries collected at the North and East (62.85%, n = 445) versus the South and West (37.14%, n = 263) orientations, no significant correlation was evident between wind direction and berry formation.

Only 9.52% of the berries collected across the trial bore TPS (North 2.57%, South 14.48%, East 15.13% and West 5.93%). Similar to the spatial distribution of berries overall on the field plot, TPS-bearing berries showed a non-random distribution with respect to compass orientation from the pollen source that was not correlated with wind direction. However, when the frequency of TPS-bearing berries was pooled for each distance across the trial, a significant negative association was observed with increased distance from the perimeter of the pollen source ($R^2 = 0.749$, $P < 0.05$).

Of the 1288 TPS that were recovered from the trial, only 774 (60.1%) grew sufficiently to provide adequate tissue for DNA extraction and subsequent genotyping. Regression analysis confirmed negative associations between both the recovery ($R^2 = 0.67$, $P < 0.05$) and germination of TPS ($R^2 = 0.722$, $P < 0.05$) with respect to increased distance from the donor population; however

TPS occurrence and germination were sporadic within each orientation (Tab. 1).

As a consequence of the reduced incidence of flowering in the 2006 trial, berry formation and subsequent TPS recovery was not anticipated. However, 25 berries were collected from the pollen receptor sub-plots. Comparable to 2005, 8% (n = 2, one from 20 m East and one from 5 m West) bore TPS, from which 38 TPS were harvested. 11 seeds subsequently germinated and provided adequate tissue for genotyping.

Genotyping the F_1 populations

Of the 24 SSRs initially tested, only the polymorphic St5130, St5136, St5148 and St0019 loci presented variety-specific alleles (two from St5130 and one each from St5136, St5148 and St0019) that could effectively distinguish between the Désirée and British Queen genotypes (Tab. 2).

Application of these four SSR markers to genotype the F_1 population (n = 774) identified 99.1% (n = 767) of the population as possessing Désirée-specific alleles (Fig. 3). Seventy-six hybrids (9.8%) of the F_1 population possessed a single diagnostic allele, 200 (25.8%) displayed two alleles, 282 (36.4%) possessed three alleles, 167 (21.5%) recorded four alleles and 42 (5.4%) possessed all five alleles. The remaining 0.9% of the hybrid population corresponded to seven F_1 individuals for which the SSR system was not adequately diagnostic to effectively genotype. SSR-based analysis of the hybrid population (n = 11) derived from the 2006 trial confirmed their paternity, as each had at least one of the five available Désirée marker alleles.
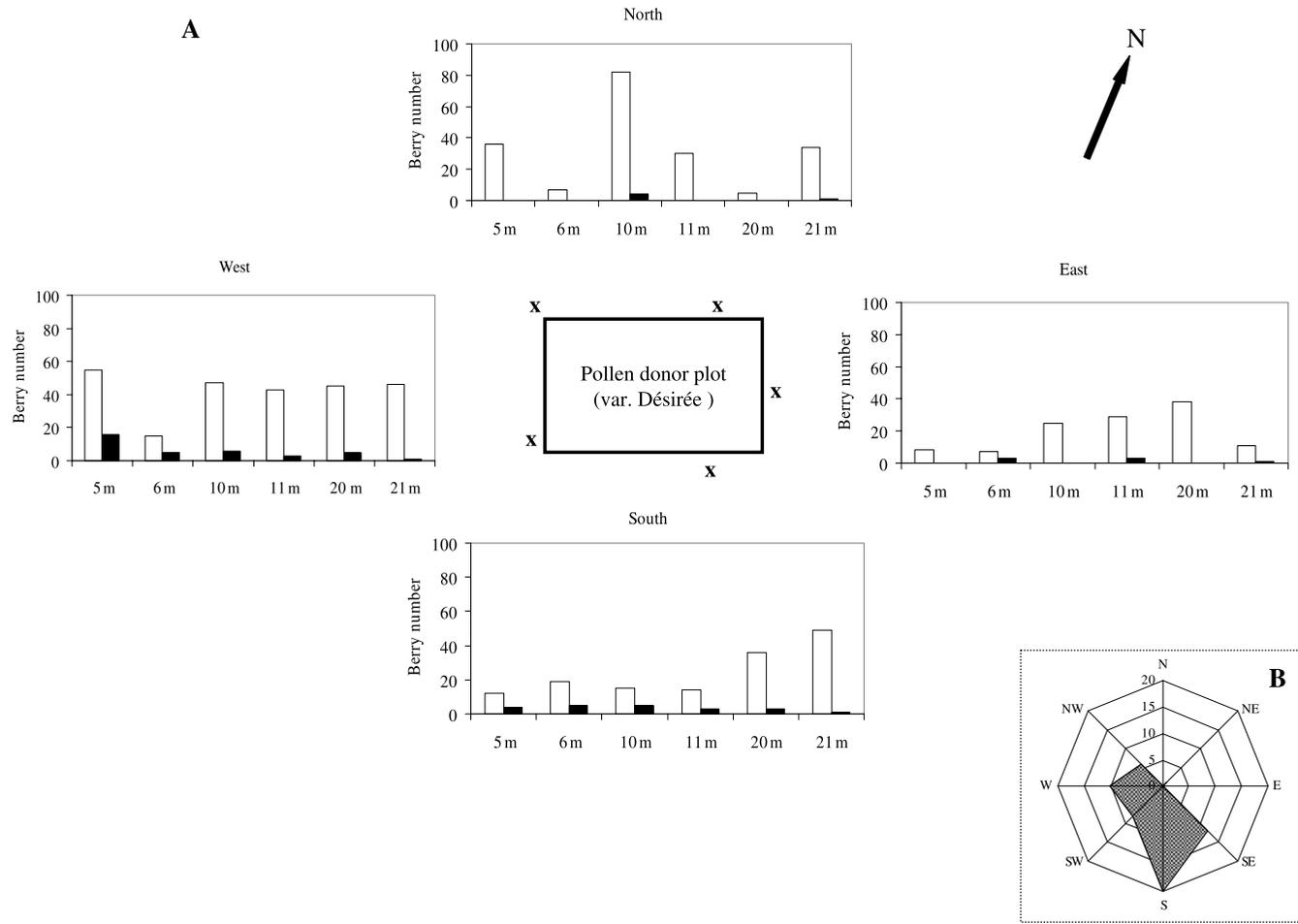
Figure 2. A. Distribution of berries (□) and berry-bearing true potato seed (■, TPS) across pollen receptor sub-plots (var. British Queen) at distances of 5 m, 6 m, 10 m, 11 m, 20 m and 21 m at the North, South, East and West orientation of the pollen donor plot (var. Désirée). In 2006, the footprint of each original receptor sub-plot was surveyed for TPS-derived volunteers, in addition to 5 locations (×) around the pollen donor plot. **B.** Prevailing wind direction (shaded) in days (0–20) during synchronous flowering period (July 4th–August 7th, 2005).

Table 1. Number of true potato seed (TPS) that were recovered (recov.) and subsequently germinated (germ.). TPS was collected from berries that formed on pollen receptors (var. British Queen) at separation distances of 5 m, 6 m, 10 m, 11 m, 20 m and 21 m for each of the four orientations, North, South, East and West of the pollen donor (var. Désirée) central plot. % germination presented in brackets.

	Separation distance											
	5 m		6 m		10 m		11 m		20 m		21 m	
	TPS recov.	TPS germ.	TPS recov.	TPS germ.	TPS recov.	TPS germ.	TPS recov.	TPS germ.	TPS recov.	TPS germ.	TPS recov.	TPS germ.
North	0	0	0	0	28	16	0	0	0	0	22	0
South	110	73	74	47	57	52	77	62	123	66	13	12
East	0	0	45	34	0	0	73	59	0	0	23	6
West	260	109	142	84	126	86	34	25	76	57	5	5
Total	370	182	261	165	211	154	184	146	199	123	63	23
		(49.1%)		(63.2%)		(72.98%)		(79.34%)		(61.8%)		(36.5%)

Table 2. Details of *S. tuberosum* SSR loci with the ability to distinguish between Désirée and British Queen genotypes.

Locus name	Primer sequence (5' – 3')	Annealing temp. (°C)	Average size of alleles (bp)	Allele size (bp)
STM0019	F: AAT AGG TGT ACT GAC TCT CAA TG	55	200	194.21, 203.05, 229.86 (BQ)
	R: TTG AAG TAA AAG TCC TAG TAT GTG			188.38, 203.32, 229.91 (Des)
STM5130	F: AAA GTA CAG CGA AGA TGA CGA C	55	260	254.73, 257.42 (BQ)
	R: TTA CCT TTG CAA CCT TGC C			248.50, 254.82, 257.47, 260.73 (Des)
STM5136	F: GGG AAA AGG AAA AGC TCA A	60	240	215.89, 224.43, 245.04 (BQ)
	R: TAT ATG AAC CAC CTC AGG CAC			224.44, 227.54, 245.00 (Des)
STM5148	F: TCT TCT TGA TGA CAG CTT CG	60	450	443.57, 449.04, 469.07 (BQ)
	R: ACC TCA GAT AGT TGC CAT GTC A			414.83, 443.62, 449.02 (Des)

Morphological analysis of F₁ populations

The value of utilizing the heritable morphological trait of tuber color as a dominant genetic marker for gene flow in potatoes has already been highlighted (Conner and Dale, 1996). To complement the SSR-based data sets collated from the 2005 trial, the F_1 plantlets were allowed to senesce and tuberize in the glasshouse. Of the 74 hybrids, 715 individuals formed tubers. Within this population the segregation of tuber color (yellow:red) for hybrids derived from each separation distance (and pooled within each orientation) was recorded as 95:74 at 5 m, 70:78 at 6 m, 76:57 at 10 m, 52:73 at 11 m, 57:61 at 20 m and 8:14 at 21 m. This equated to the expected 1:1 segregation ratio, with 357 progeny possessing red-skinned tubers and 358 retaining the British Queen-associated yellow skin. Notably, the seven unidentified hybrids that did not amplify specific SSR profiles (see above) did form tubers with red skins, hence confirming their hybrid status and Désirée parentage.

Survey of *M. aeneus* populations

A previous report has provided anecdotal evidence to suggest that the foraging beetle *M. aeneus* can serve as a pollinator for potato (Skogsmyr, 1994). We considered this in the context of the sporadic nature of hybridization observed in the 2005 trial, and hypothesized that a proportion of the gene dispersal recorded was mediated *via M. aeneus*. To this end, we surveyed for the presence of *M. aeneus* on the pollen-receptor flowers of each distance (per orientation) of the 2006 trial. Of the 106 pooled beetle samples (taken over the 28-day sampling period), 23 tested positive for the presence of Désirée-specific alleles (Fig. 4A, SSR analysis in Fig. 3), confirming that the beetle had indeed assisted in the transmission of pollen from the pollen donor to the pollen receptor sub-plots. Though bumblebee activity was observed across the plot at flowering, it was infrequent, and observations noted that the bees resided principally within the Désirée plot and tended to avoid the British Queen plants.

Across the 23 positive pooled beetle samples, a median of three beetles per pooled sample with a maximum of 10 and minimum of one beetle was recorded. The distribution of these gene dispersal events across the trial site was neither orientation nor distance-specific (Fig. 4B) and there was no significant association between detection and distance from the pollen source across the trial (P > 0.05). While the result confirmed the role of *M. aeneus* as a vector for gene dispersal in potato, it was noteworthy that no correlation was observed between the number of pooled beetles collected and the occurrence of
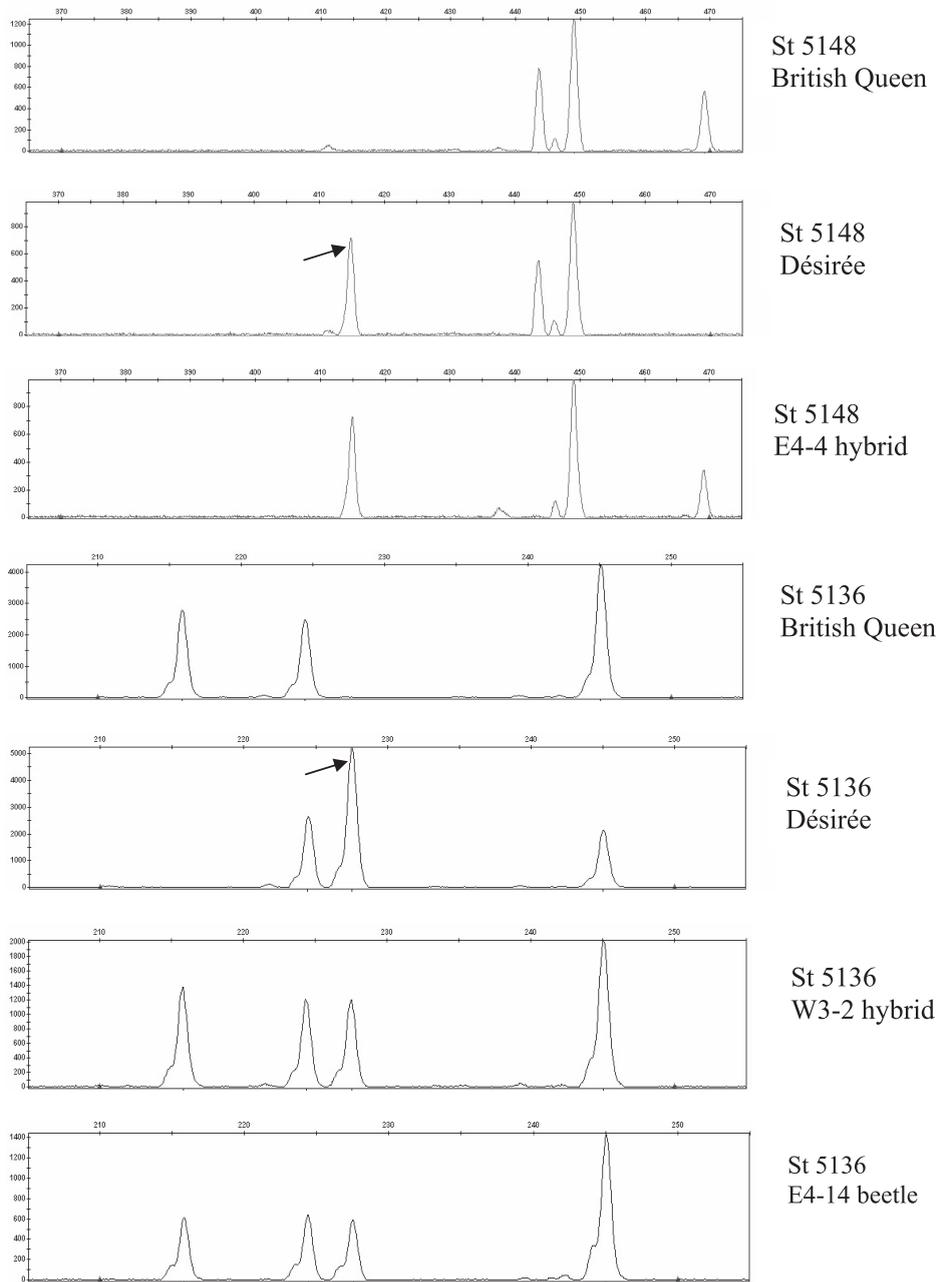
Figure 3. SSR allele profiles in British Queen (pollen receptor), Désirée (pollen donor), their associated hybrids (E4-4, W3-2, N4-34 and S3-13) and a pooled *M. aeneus* beetle sample (E4-14), at the St5148, St5136, St5130 and St0019 loci. Only the St5136 and St5130 loci were assayed in regard to the pooled beetle sample. Arrows indicate the variety-specific alleles diagnostic for the pollen donor.

Figure 3. Continued.

A

B

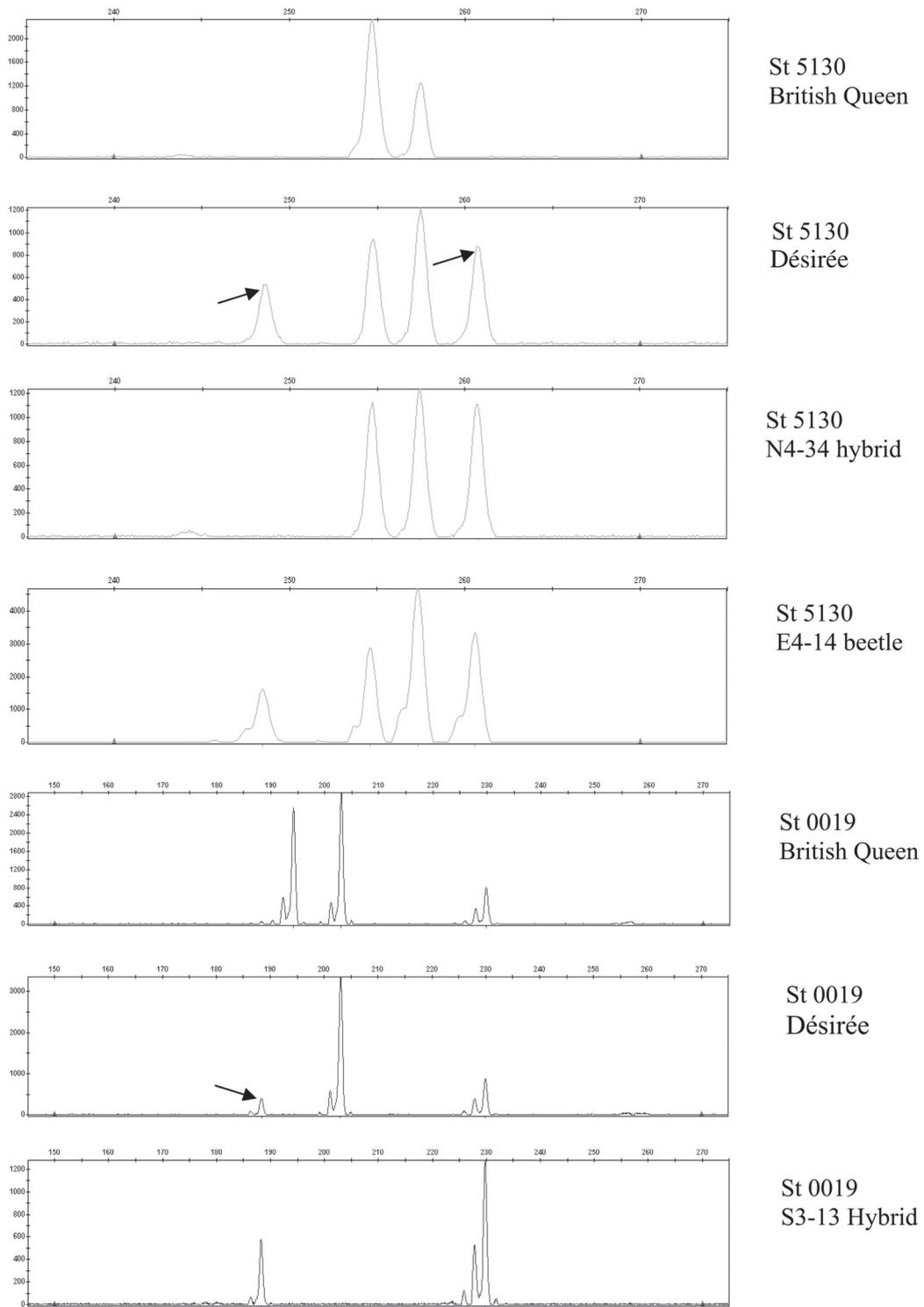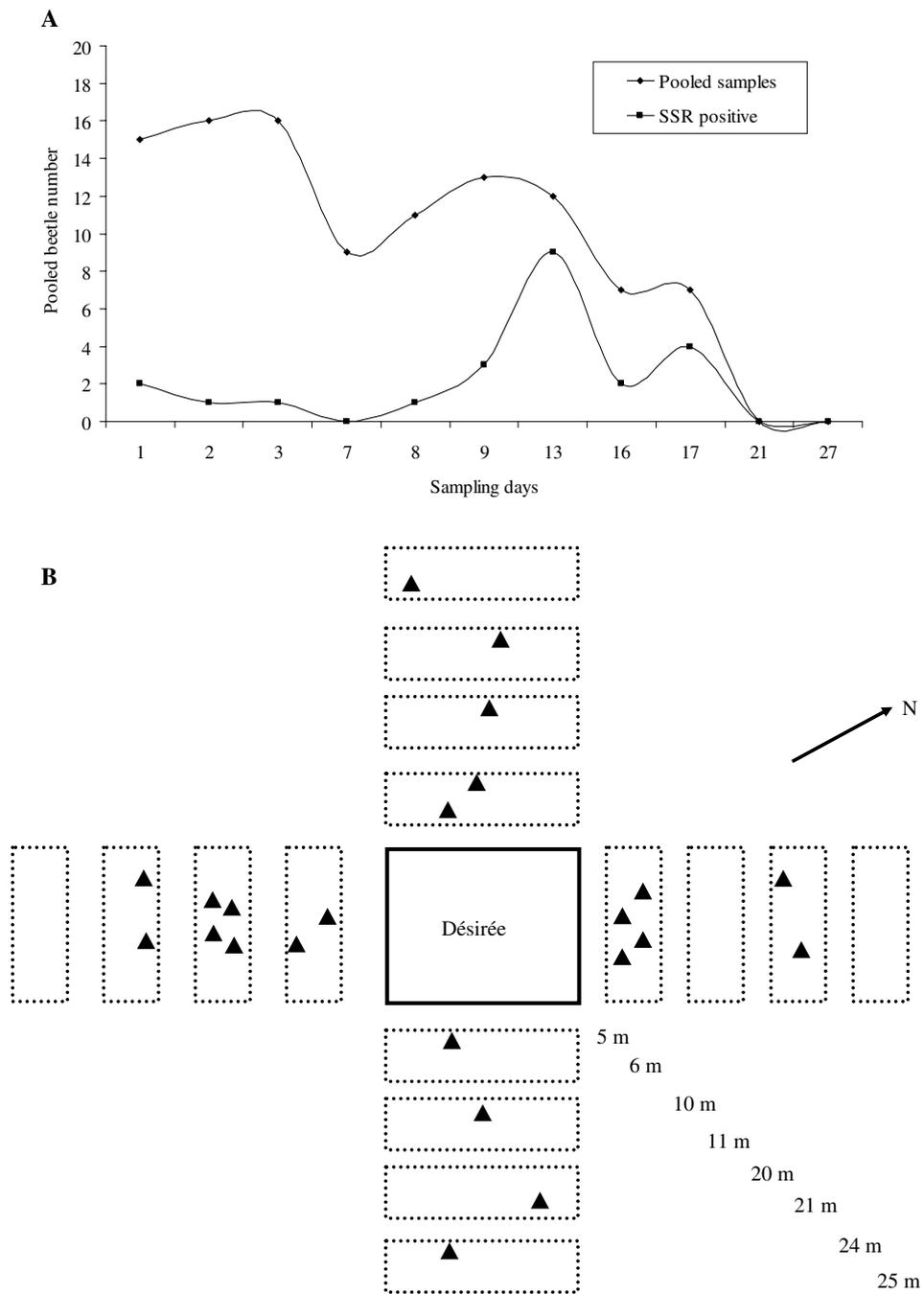**Figure 4. A.** Incidence of pooled *M. aeneus* samples collected from pollen receptor (var. British Queen) flowers that tested positive for Désirée-specific alleles (SSR positive) *via* SSR analysis relative to the number of pooled samples collected over the sampling period. **B.** *M. aeneus*-mediated distribution of Désirée pollen across the pollen receptor (var. British Queen) sub-plots of the 2006 trial site. Each (▲) represents the location of a receptor flower from which a pooled beetle sample was collected and tested positive for Désirée-specific alleles.

pooled beetles possessing Désirée-specific alleles (P > 0.05) during the 4-week sampling period.

Monitoring TPS-derived volunteers

During the summer of 2006, the 2005 trial site was revisited and a survey for TPS-derived volunteers was completed. As expected, a high number of TPS-derived volunteers (average of 324 per m^2) were recorded around the original pollen donor plot (Fig. 1) due to the high fertility of Désirée. In contrast, 30 TPS-derived volunteers were located around the combined footprints of the original pollen receptor (cv. British Queen) sub-plots. SSR analysis confirmed that 90% (n = 27) of this volunteer population possessed a Désirée genotype, highlighting the potential for machinery-based transfer of potato seed. The remaining three volunteers were hybrids between British Queen and Désirée.

DISCUSSION

In 2005 we established a work program to clarify several biosafety issues in regard to the predicted cultivation of GM potatoes in Ireland. In light of the strong evidence presented in previous reports on pollen-mediated gene flow our objective was to:

1. verify the effectiveness of existing separation distances to minimize pollen-mediated gene flow in a field environment;
2. ascertain the potential of *M. aeneus* as a vector for gene transmission in potato.

Our system was similar in design to a previously published field trial (McPartlan and Dale, 1994) but we utilized two conventional potato varieties, and relied on an SSR-based system to score for successful gene flow events between the pollen donor and receptor sub-plots. Adopting a 'worst case scenario' system, our results indicate both that pollen-mediated gene flow in potato can extend upwards of 21 m from the pollen donor population and that *M. aeneus* is a carrier of potato pollen. Adverse weather conditions undermined the 2006 trial and thus our gene flow data (berry number and TPS occurrence) are primarily based on results from the 2005 experiment. While we acknowledge that this truncated dataset is far from ideal, on review of the results we believe that important findings are evident from the existing data.

To facilitate hybridization between the male-sterile receptor (cv. British Queen) and pollen donor (cv. Désirée) plots, we staggered the sowing of both populations, which led to a period of synchronous flowering in excess of 30 days. The SSR-based detection system that we have described, and substantiated by screening for tuber color in the F_1 population, proved to be an effective tool with which to trace field-based hybridization between two potato varieties (Fig. 3). While AFLPs have been employed to study gene flow between potato species (Celis et al., 2004), to our knowledge this is the first report of using SSRs for this purpose in potato. Furthermore, employing the same system to trace insect-mediated pollen transfer between the donor and receptor individuals confirms the versatility of microsatellite markers in recovering gene flow data that may prove important for addressing biosafety issues.

While the thematic novelty of our experiment remains in the re-evaluation of the separation distances and the demonstration that SSRs are a very effective technique with which to monitor both wind- and insect-mediated gene flow in potato, it is critical that the interpretation of these findings remains in context in order to assist future co-existence strategies. By using male-sterile receptors we intentionally biased our experiment in order to maximize the possibility for hybridization, hence adopting a 'worst case scenario' approach that does have a tendency to over-estimate the level of gene flow. However, even under these circumstances, while we achieved substantial berry formation (n = 140) at distances in excess of 20 m, only 2.8% (n = 4) of these berries contained seed of which only 36% (n = 23) germinated.

In the context of co-existence management it is important to consider whether these individuals are likely to survive in the field. Our results clearly indicate that TPS-based volunteers will emerge through a rotation over time. In addition, our results highlight the potential for the machinery-based transfer of TPS within the field. However, it is important to note that the 2005 trial site remained fallow over the winter to purposefully encourage volunteer emergence. So while we concur with previous reports (Lawson, 1983) that highlight the potential of TPS-derived volunteers to challenge efficient crop management (as manifested in our trial with the emergence of 30 TPS-based volunteers), we believe the issue can be negated through vigilant crop management and appropriate rotations. As such the emergence of TPS-based volunteers should pose no threat to the effective co-existence of GM and non-GM potato crops, assuming appropriate crop stewardship has been adopted.

Applying the SSR assay to *M. aeneus* individuals collected from pollen receptor flowers provided an insight into an alternative mode of gene transmission in potato and presented a possible explanation for the irregular pattern of hybridization observed in the 2005 field evaluation. While our research indicates the possibility that individual hybridization events could occur over greater distances as previously suggested (Skogsmyr, 1994). We believe that *M. aeneus*-mediated gene dispersal will not present a consistent challenge to co-existence based on

our survey results (Figs. 4A and 4B), but rather will account for haphazard pollination events that will be dependent upon the travel circumference of the beetle. If a higher degree of containment were required for a specific transgenic potato, the application of insecticide at flowering and/or the planting of bait crops around the periphery of the transgenic potato crop could be considered.

Current guidelines on co-existence relate to maintaining the degree of GM admixture in non-GM material below the 0.9% threshold (Recommendation 2003/556/EC) and it is based on this level that we make our conclusions. In their consideration of previously published data on pollen dispersal from field trials of transgenic potato, Conner and Dale (1996) concluded that 20 m was adequate for initial field evaluations. Based on our experience and taking into account (i) our intentional use of male-sterile receptors, (ii) the isolation of our trial from other sites of potato cultivation, (iii) the clonal nature of potato and significantly (iv) the fact that the release of GM potatoes will involve plants hemizygous for the transgene which implies a 1:1 segregation of pollen with and without the transgene, we advocate that a two-tiered system be established for any future cultivation of transgenic potato in Ireland. For the cultivation of male-fertile GM potato under a Class B license (pre-commercialization trial), we recommend that to minimize the potential and outcome of pollen-mediated gene flow, the trial should be:

- isolated from adjacent sites of potato cultivation by a minimum of 30 m;
- rotated through ryegrass to facilitate the recording of TPS-derived volunteer emergence and establishment prior to burning off with herbicide;
- and that both male-sterile and male-fertile bait plants are established at larger distances (*e.g.* 200–300 m) from the trial to gauge the degree of insect-mediated transfer of transgenic pollen from the site.

Pending results from the Class B trials and additional risk analysis as in accordance with Directive 2001/18 (EC), we recommend that for the commercial cultivation of GM potato and its co-existence with non-GM tillage systems:

- an isolation distance of 20 m is adequate to limit gene dispersal in accordance with EU labeling thresholds;
- responsible crop management is practiced in regard to the control of TPS-derived volunteers and groundkeepers through the rotation.

We acknowledge that our recommendation of 30 m may seem excessive, based on previous literature and the data presented here, and as such could be perceived to be more policy-driven than science-based. However, we believe it is a responsible approach in light of the current negativity

towards GM trials, and while conservative it is not excessive, and should be adopted to alleviate societal concern in regard to the research-based (Class B license) trials of GM potato. Significantly, by recommending unique isolation distances based on the Class of license, an opportunity is still provided to lower the distance if the environmental risk assessments conducted during the Class B cultivation support such an approach. Quite simply, a 30 m separation distance should be seen as the best estimate for the pre-commercialization testing of GM potatoes in Ireland.

A condition of the recent license granted for the Class B cultivation of transgenic blight-resistant potato in Ireland, related to the removal of berries formed during the course of the trial (http://www.epa.ie/Licensing/GMOLicensing/). It is important to note that it is only TPS germination and ultimately the establishment of volunteers within the rotation that will pose an issue for co-existence and not simply the occurrence of berries. This distinction between the potential for gene flow and the outcome of a gene flow event is often overlooked and can generate an unnecessary workload when standard tillage operations (*e.g.* minimum tillage and application of systemic herbicide) would otherwise adequately control TPS-derived volunteers.

To date, 256 notifications for the Class B (pre-commercial) release of transgenic potato have been submitted to the various competent authorities of the European Union's member states (http://biotech.jrc.it/deliberate/dbplants.asp). Eight of these submissions relate to the experimental release of transgenic potato with improved resistance to potato blight (*P. infestans*), which is a much desired agronomic trait of significant economic potential in Ireland (Flannery et al., 2005). As the potential economic benefit of cultivating a blight-resistant potato line becomes more apparent to potato growers, it is reasonable to assume that it will be the larger producers who will initially decide to adopt the technology (Fernandez-Cornejo et al., 2001) due to the reduced cost base, but also because of the increased convenience factor associated with GM crop cultivation, which could appeal greatly to Ireland's part-time farmers (~48%, http://www.teagasc.ie/). If this scenario is to occur, preserving the genetic identity of non-GM cropping systems will be crucial. Central to this will be the establishment of effective co-existence measures through the completion of region-specific research studies. From an applied perspective, we believe that the research outlined here will clarify several issues that have been raised by regulators in regard to the cultivation of GM potato in Ireland. From a scientific perspective, our study confirms that both wind- and insect-mediated gene flow in potato can be assessed using microsatellite markers.

MATERIALS AND METHODS

Plant material and field trial design

The criteria required for selecting the appropriate varieties for the trial included: (i) a high propensity for flowering for both pollen donor and receptor, and (ii) high male fertility for the donor and corresponding male sterility in the receptor. Based on agronomic data collated through the European Cultivated Potato Database (see: http://www.europotato.org), the potato varieties Désirée and British Queen were adopted as the pollen donor and receptor, respectively. Non-transgenic seed tubers of both varieties were obtained from the Teagasc Potato Breeding program at Oak Park, and preliminary hybridizations were conducted under glasshouse conditions to confirm the compatibility of the Désirée male-fertile and British Queen male-sterile genotypes. In 2005, these varieties were incorporated into a field plot design comparable in design to a previously published experiment (McPartlan and Dale, 1994).

Désirée tubers were sown into 20 drills (approximately 50 tubers/drill) in the central plot (17 m × 17 m) with 85 cm separation distance between each drill, and approximately 35 cm between each tuber within a drill. Pollen receptor drills were orientated North, South, East and West of the central plot and at distances of 5 m, 6 m, 10 m, 11 m, 20 m and 21 m from the nearest edge of the Désirée population. Sown with British Queen tubers (approximately 30 tubers/drill), each drill was 10 m in length.

To ensure synchronous flowering of both varieties, Désirée was sown two weeks (April 26th, 2005) prior to the early maturing British Queen (May 11th, 2005). The plot was isolated by at least 300 m from any other site of potato cultivation, and the trial crop was treated as a commercial potato crop in regard to the application of fertilizer and fungicide to offset *P. infestans*.

Arising from the results obtained from the 2005 trial, the trial was repeated in 2006 on a separate site. Again, pollen receptor beds were orientated North, South, East and West of the central plot at distances 5 m, 6 m, 10 m, 11 m, 20 m and 21 m from the nearest edge of the Désirée population, with additional drills sown at 24 m and 25 m for each orientation (Fig. 4B). Due to excessively wet conditions during April and May, Désirée and British Queen were not sown until the first and second week of June respectively. Similar to the 2005 trial, the site was managed as a commercial crop and remained isolated (> 800 m) from other sites of potato cultivation.

Field data collection and true potato seed treatment

The frequency of flowering on the pollen receptor subplots was recorded at weekly intervals by noting the number of inflorescences that had at least one fully opened flower. To prevent duplication, each inflorescence was subsequently tagged. Due to the density of the Désirée crop canopy, the frequency of flowering was conservatively estimated by recording an average of the number of inflorescences appearing on the two external drills (West and East side) of the Désirée plot.

Berry collection from the British Queen drills was conducted at 2-day intervals. For each distance (at each orientation) mature berries were harvested, and the TPS that was collected from each berry was washed, dried and stored at 4 °C. After 12 weeks, TPS were surface-sterilized and sown on MS basal media (Murashige and Skoog, 1962). Seedlings were transferred to compartmentalized trays containing potting compost, and cultivated in the glasshouse at 22 °C using a 16 h light and 8 h dark cycle, before tissue was collected for DNA extraction.

The incidence of the pollinator *M. aeneus* across the 2006 trial site was recorded over a 28-day period. For each sampling date, pollen receptor (var. British Queen) flowers were surveyed, and collected beetles were pooled for each distance (per orientation). In total, 106 pooled samples were processed for subsequent DNA extraction and SSR analysis.

Plant and insect DNA extraction and SSR analysis

Mechanical tissue disruption was completed on freeze-dried leaves or flash frozen (in liquid N_2) pooled beetles using a mixer mill (Retsch MM300). DNA extraction was completed using a modified CTAB method that was adapted for 2 ml eppendorf tubes. Modifications included increasing the final concentration of NaCl (2 M) and extending the duration of the chloroform:isoamyl alcohol (24:1) extraction to 60 min, with the final precipitation completed at room temperature.

To identify the paternity of each F_1 hybrid, we developed an SSR-based genotyping system. SSR-polymerase chain reaction (SSR-PCR) was completed in 20 μL reactions containing: 40 ng TPS DNA and to a final concentration, dNTPs (200 μM), $MgCl_2$ (2.5 μM), BSA (50 μg), forward and reverse primers (250 nM). In total, 24 SSR primer pairs (details not shown) were selected from the literature (Ghislain et al., 2004; Milbourne et al., 1998) and tested for their ability to distinguish between Désirée and British Queen genotypes. For genotyping, the forward primer of each pair was labeled with a florescent dye (Vic, Ned, Pet or 6-FAM, Applied BioSystems, California, USA). For the SSR-PCR, conditions included 95 °C for 10 min, 9 cycles of 94 °C for 60 s, 65 °C for 30 s decreasing 1 °C /cycle, 72 °C for 60 s, 24 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, completed with 72 °C for 10 min. SSR-PCR amplicons were genotyped

on an ABI3100 platform (Applied Biosystems, California USA), with allele analysis completed using ABI Gene Mapper v3.7.

Morphological assessment of hybrid population

The value of utilizing the heritable morphological trait of tuber color as a dominant genetic marker for gene flow in potatoes has already been highlighted (Conner and Dale, 1996). To complement the SSR-based data sets collated from the 2005 trial, the F_1 plantlets were allowed to senesce and tuberize in the glasshouse. From the initial F_1 population (n = 774), 715 individuals developed tubers (after 8–10 weeks) and these were scored for the recessive yellow skin color (aaaa) associated with British Queen, or for the single dominant allele genotype (Aaaa) that is characteristic of Désirée.

Survey of TPS-derived volunteers

After leaving the 2005 trial site fallow over the winter, the establishment of TPS-derived volunteers was studied in June 2006 by surveying the footprint of each of the original pollen receptor sub-plots, in addition to five locations around the original pollen donor plot (Fig. 1).

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