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Title:
GM Risk Assessment: pollen carriage from \textit{Brassica napus} to \textit{B. rapa} varies widely between pollinators.

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Abstract

Characterizing insect pollen carriage between closely related plant species is especially challenging where source species possess morphologically identical pollen and share many pollinators in common. Here, we use an SNP-based assay using the plant DNA barcoding locus matK to characterize pollen carriage between cultivated Brassica napus and wild B. rapa in three sites across southern England. The assay differentiated B. napus and B. rapa pollen carried by honey bees (Apis melifera), bumblebees (Bombus spp.), mining bees (Andrena spp.) and hoverflies (Syrphidae) captured on B. napus plants 1-2 m from wild B. rapa, and on B. rapa plants at various distances from the crop. Apis individuals foraging on B. rapa and carrying B. napus pollen were rarely found beyond 10 m from the crop. However, Bombus and Andrena individuals captured on B. rapa occasionally carried crop pollen up to 300 m from the source field. Hoverflies (Syrphidae) carried less pollen overall but featured high proportions of B. napus pollen even at the most distant capture points. We predict that different pollinator species will evoke markedly different patterns of interspecific hybrid formation. We conclude that more exhaustive surveys of this kind will help parameterize future mechanistic models to predict the distribution of hybrids between Genetically Modified B. napus and B. rapa on a landscape scale.

Key index words: Pollinators; GM-Crops; Pyrosequencing; Pollen; Barcode; Brassica
Introduction

Insect-mediated pollen carriage between GM crops and wild relatives is a matter of high controversy. Completion of an environmental risk assessment is a legal requirement for the commercial release of any Genetically Modified (GM) crop in most countries (James 2014) and must give consideration to the potential ecological consequences of transgene flow (Nickson 2008). Of the four crops that currently dominate GM cultivation (James 2014), *Brassica napus* possesses greatest potential for interspecific gene flow and has become the focus of many studies to describe the extent, distribution and consequences of transgene flow (e.g. Hauser, Damgaard & Jørgensen 2003; Ford et al. 2006; Ford et al. 2015). *Brassica rapa* is the species for which there is greatest concern (e.g. Hauser, Damgaard & Jørgensen 2003; Wilkinson et al. 2003). In the UK, wild *B. rapa* grows primarily along the banks of rivers and streams and is distinct from weedy *B. rapa* which at least partly originated as escapes from cultivation (Wilkinson et al. 2003). Transgene movement into the natural riverside *B. rapa* populations carries greatest potential for ecological harm and so is the focus of attention here.

*Brassica* species are naturally entomophilous but fields of the crop are both insect- and wind-pollinated (Mesquida & Renard 1982). Models combining insect- and wind-mediated gene flow components are rare (e.g. Walklate et al. 2004) but nevertheless highly desirable, especially when mechanistic and predictive. Recent modelling initiatives based on observational and/or empirical data have greatly improved our understanding of the relative importance of pollinator identity, pollen carriage and disposition rates per visitation, foraging behaviour, and the spatial separation and relative sizes of *B. napus* fields and recipient *B. rapa* populations (e.g. Rader et al. 2009; Howlett et al. 2011; Chifflet et al. 2011; Rader et al. 2013). Nevertheless, uncertainties remain. There are strong biological grounds for reasoning
that different pollinator groups may vary in the pattern of pollen dispersal from a source field
of *B. napus*. The most important pollinators of this UK crop are thought to be honey bees
(*Apis mellifera*), mining bees (*Andrena* spp.), bumblebees (*Bombus* spp.) and hoverflies
(*Syrphidae*) (Hayter and Cresswell 2006; Woodcock et al. 2013). Crop visitation by each
pollinator species is shaped by their reproductive and nesting strategies. The bee groups will
travel from their nests before foraging in the field and in neighbouring communities (Hayter
and Cresswell 2006) and so pollen dispersal will be constrained by the location and density of
their nesting sites. For honey bees, this feature is further complicated by the anthropomorphic
movement of hives. In contrast, foraging of hoverflies is not restricted by the need to return to
a colonial nest. Thus, it is important to characterise pollen carriage of all components of the
pollinator guild in order to predict patterns of insect-mediated gene flow between cultivated
*B. napus* and *B. rapa*, a wild relative that grows almost exclusively next to riverbanks in the
UK (Wilkinson et al 2003). In this study we use a species-specific Single Nucleotide
Polymorphism (SNP) assay for the *matK* gene to characterize pollen delivery by all major
pollinators between these two close relatives.
Materials and Methods

Field Survey

We surveyed 93 km of five United Kingdom (UK) rivers (Nene, Avon, Wye, Severn and Thames) to identify sites of *B. napus* and *B. rapa* sympathy suitable for the study of insect-mediated pollen delivery (Appendix A Table 1). Three locations were identified near Culham and Appleford (both River Thames; UK Grid Reference SU 52417 94558 and SU 51964 94381 respectively), and Tewkesbury (River Severn; UK Grid Reference SO 88360 34092). The crop and *B. rapa* were only found on the same riverbank and were both in full flower during the time of study. At each site, leaf samples were collected from *B. napus*, *B. rapa* and associated insect-pollinated flowering plants for DNA extraction, and floristic surveys were conducted to identify all pollen sources at the time of pollinator capture (Appendix A Table 2). DNA was extracted from leaves using the DNeasy Plant DNA Extraction Kit (Qiagen, UK).

We also collected reference pollen samples from eight *B. napus* plants in each source field, and eight *B. rapa* plants in each sympatric riparian population and from eight glasshouse grown reference plants of *B. napus* cv. Apex and *B. rapa* (Primrose Hill, Holderness Peninsula, UK Grid Reference: TA 30000 17600).

Pollinator and pollen load collection

Pollinators were captured in plastic vials from *B. napus* flowers at the field margin closest to *B. rapa* (position 0 m), and from *B. rapa* flowers at fixed distances from the field edge (10 m, 50 m, 200 m or 300 m, as determined by the distribution of *B. rapa*; 45 min collection per capture point). Insects were snap-frozen on dry ice, stored at -80° C and identified morphologically using Gibbons (1996), Edwards and Jenner (2009), and Stubbs and Falk (2002). To isolate pollen loads, individual insects were immersed in 1 ml nuclease-free water,
vortexed and centrifuged (5000 rpm for 10 min). The insect body was removed and DNA isolated from the pollen pellet. For this, pollen was suspended in 1 ml nuclease-free water, vortexed briefly and spun (5000 rpm) for 10 min. After decanting the supernatant, the pellet was partially digested using the CelluACE™ XG System (Promega) with a 1:20 v/v mix of CXG Buffer and CXG Enzyme Mix in an incubator-shaker for 6 h at 50 °C. DNA was then isolated using the DNeasy Plant DNA Extraction Kit (Qiagen, UK).

**Screen for crop plastid capture in B. rapa**

Extensive *B. napus*-B. *rapa* mixing or plastid capture by *B. rapa* could confuse estimates of pollen carriage between the species. We therefore screened leaf samples of *B. rapa* plants (Appendix B Table 1) for the presence of *B. napus*-specific plastid markers using the method described by Allainguillaume et al. (2009). At least two of the following chloroplast-specific markers per sample were used to differentiate between *B. napus* and *B. rapa* plastids: Chloro 39, Chloro P and Chloro O (Allender et al. 2007), Chloro H, CAPS1, SNP1 and SNP2 (Allainguillaume et al 2009).

**Brassica-specific real-time PCR Assay**

The gene *matK* is variable (Ford et al. 2009) but prone to failed high stringency amplification (e.g. Bafeel et al. 2011), and so is good for species-specific amplification. We recovered *matK* reference sequences for all co-flowering insect-pollinated species at all sites (Appendix A Table 2) from the BOLD Systems database ([www.boldsystems.org](http://www.boldsystems.org)) and made alignments using Clustal Omega (Sievers et al. 2011). Nested primers were hand-designed to target regions with (near) perfect matches for *Brassica* species but extensive mismatches for other co-flowering species (Appendix A Table 3). Primer specificity was tested on a RotorGene 6000 (Corbett Life Science) using a modification of the nested MT-PCR method described by
Stanley and Szewczuk (2005). PCRs were performed in 20 µl volumes containing 1.0 ng template DNA, 2× Sensimix (Quantace, UK), 200 nM of each primer and 1.5 µM of SYTO9 (Invitrogen). First round PCR was performed with the outer \textit{mat}K primers (Forward1 5’ TAATTTAGAATTTCTGGGTTATCTA 3’; Reverse1 5’ GTCCAGGTCGCTTTACTAATC 3’) comprising: 94° C for 2 min, followed by 15 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 40 s, with a final elongation of 72 °C for 10 min. Second round PCR used 2.5 µl of a 1:12.5 dilution of the first round amplification product as template, the internal primers (Forward2 5’ ATCTATCAAGTTTGCGACTAAAC 3’; Reverse2 5’ CAAAATTTATCTTATGCCAACGAC 3’) and 35 cycles of the thermocycling conditions described above. Amplicons were subjected to High Resolution Melting (HRM) analysis (70 to 90 °C using 0.1 °C step increases every 2 s) to verify identity.

\textbf{Brassica pollen detection limits}

A dilution series was created using reference pollen to calibrate pollen abundance from DNA by RT-PCR and define detection limits. Reference pollen samples from eight \textit{B. napus} plants and eight associated wild \textit{B. rapa} plants from each site were suspended in 400 µl water. The aqueous samples were mixed and sub- aliquots of 1µl transferred to a 1/400 mm$^2$ grid slide (Neber, UK). Pollen grain abundance was counted in triplicate at x400 magnification and a dilution series created containing approximately 20000, 10000, 2000, 1000, 200 and 100 pollen grains. Low-end concentrations (10, 20 grains) were created by hand using an eyelash on a toothpick. Samples were pelleted by centrifugation (5000 rpm for 10 min), DNA extracted and MT-PCR performed as described above.

\textbf{Characterizing Pollen mixtures}
The matK SNP used to quantify *B. napus* pollen comprised one allele (C) present only in *B. napus*, and the alternate allele (A) is seemingly fixed in *B. rapa* and all co-flowering species (Appendix A Table 3). Nested RT-PCR was performed with the incorporation of an M13 biotinylated forward inner primer during the second round PCR (Royo, Hidalgo & Ruiz 2007). Pyrosequencing was performed with a PSQ96MA instrument (Biotage AB, Sweden) with PyroMark Gold Q96 Reagent (Qiagen, UK). The pyrosequencing primer (5’ ATTTCTAATAGATAATGT 3’) was designed using the PSQ96 software to bind one base upstream of the variable SNP position (Appendix A Table 3). Pyrosequencing order for the diagnostic sequence T[A/C]GTAA was as follows: T (both species); A (*B. rapa*-specific or nucleotide with no match for *B. napus*); C (*B. napus*-specific; no match for *B. rapa*); G (both species); T (absent from both species, included as a negative control); A (AA in common for both species). Sequencing then continued for a further 10 bases using nucleotides common to both species to confirm amplicon identity.

Pyrosequencing was used to confirm ‘C’ allele presence in *B. napus* source fields and to describe the relationship between allele peak heights at the diagnostic SNP site and the proportion of *B. napus* in pollen mixtures. Peak height ratios were calculated by dividing the value of the peak height of the diagnostic SNP by that of the shared AA peak two bases downstream. Calculations were performed separately for *B. napus* and *B. rapa* using eight replicates for each species (Appendix A Table 4). In this way a correction constant of 1.11 was derived by dividing mean peak height ratio for *B. rapa* (0.52) by that obtained for *B. napus* (0.47) (Appendix A Table 4). Normalization of *B. napus* allele (C) peak heights in mixed pollen samples was achieved by multiplication with this correction constant.
We first tested the assay using a DNA titration series of the two *Brassica* species. DNA from each species was quantified on a NanoDrop (Fisher Scientific, UK) and adjusted to the same concentration. RT-PCR was performed with template DNA for comprising of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% *B. napus* in mixes of both species, with eight replicates per mix. Resultant amplicons were subjected to pyrosequencing. Regression analysis was conducted using Statistical Package for Social Sciences (SPSS) software (IBM Corp.). We repeated the calibration using DNA isolated from pollen mixes. Here, calibrated pollen mixes (0:100, 20:80, 40:60, 50:50 *B. napus*: *B. rapa* and reciprocal) were adjusted a total of 20,000 grains in 300 µl nuclease-free water.

**Characterization of pollen carriage**

The proportion of *B. napus* pollen carried by each insect (relative to all pollen types) was inferred using the normalized pyrosequencing peak height. Comparisons were then made between pollen loads and various contributing factors (site, collection date, pollinator species, pollinator taxonomic group) by ANOVA and Tukey’s HSD (Honestly Significant Difference) tests performed using the Vegan package in R (Oksanen 2013).
Results

Field survey

*B. rapa* populations at Culham formed discontinuous stands from adjacent to the field margin (1-2 m) to 300 m distance in both up- and downstream directions. At the other sites, *B. rapa* was crop-adjacent but only extended upstream for 300 m at Appleford and downstream for 200 m at Tewkesbury. Floral surveys identified 24 additional insect-pollinated plant species flowering during the collection periods (Appendix A, Table 1).

Screen for crop plastid escape

All 567 *B. rapa* plants screened contained chloroplast haplotypes consistent with *B. rapa* rather than cultivated *B. napus* (Appendix C Table 1), indicating a lack of extensive hybridization or of a significant presence of feral *B. napus*.

Taxon-specific Real-Time PCR

Real-time PCR consistently failed to amplify products from any of the 24 co-located flowering plant species belonging to genera other than *Brassica* (Appendix D Fig. 1). We next determined whether the *Brassica*-specific PCR would be able to detect a reliable signal from the small numbers of pollen grains likely to be recovered from pollinator bodies. Strong, consistent PCR amplification was repeatedly achieved for all samples throughout the pollen dilution series, including those containing just ten pollen grains (Appendix E Fig. 1). There was as little variation between *B. napus* and *B. rapa* pollen samples as there was between technical replicates (repeated DNA extractions) across all dilutions in the series (e.g. Appendix E Figure 2), suggesting that each species has roughly equal numbers of proplastid genomes per pollen grain.
We then characterized the relationship between PCR amplification and pollen abundance for the two *Brassica* species across a dilution series. We found a strong linear relationship between Critical threshold (Ct) values of amplification and pollen grain abundance for both *B. napus* \( (r^2 = 0.621, \text{ data not shown}) \) and *B. rapa* \( (r^2 = 0.885, \text{ data not shown}) \).

**Pollen pyrosequencing assay**

For the pyrosequencing assay to measure relative abundance of *B. napus* in pollen mixtures a robust relationship must exist between normalized pyrosequencing peak height at the diagnostic SNP and the proportion of *B. napus* present. There was a very strong linear relationship between relative abundance of *B. napus* DNA and normalized peak height of the ‘C’ allele (Fig. 1A, \( r^2 = 0.991 \)). The pollen calibration series revealed a similarly strong linear relationship (Fig. 1B, \( r^2 = 0.957 \)). Thus, normalized peak height could be used to estimate abundance of *B. napus* pollen mixtures from a pollinator.

**Pollinator survey**

The 271 pollinators collected from Culham and Appleford (River Thames) included species from three genera of bee (*Apis*, *Bombus*, *Andrena*). Honey bees (*Apis mellifera*) were most abundant at both sites (Table 1). The most common bumblebees belonged to the sister species *Bombus terrestris* and *B. lucorum* (Appleford 24; Culham 29), *Andrena haemorrhhoa* was the most abundant mining bee (Appleford 14; Culham 13) (Table 1). The three genera differed in capture rates with distance from cultivated *B. napus* (Appleford \( \chi^2 = 29.6, p<0.001 \); Culham \( \chi^2 = 32.4, p<0.001 \)). *Apis mellifera* was least likely to be captured away from the crop, with 71% (47/66) from Appleford and 95% (40/42) from Culham being caught <10 m from the field (Table 1). This compares with 43% (29/67) and 59% (33/56) for *Bombus* individuals from Appleford and Culham respectively (Table 1). Specimens of *Andrena* were captured at
low frequency <10 m of the Culham field (41%, 7/17) but not at Appleford (78%, 18/23). By comparison, relatively few pollinators were captured at Tewkesbury. None were bees and all 42 specimens collected belonged to three species of hoverfly (*Rhingia campestris*, *Helophilus pendulus* and *H. trivittatus*; Table 1). There were insufficient captures to make meaningful comparisons between species but when considered collectively at this location, there was a similar proportion of hoverflies caught >10 m from the field margin as for the bees (40%, 17/42, Table 1).

**Pollen carriage**

Considered collectively, the highest proportions of *B. napus* pollen were recovered at field margins from all pollinators (Table 2). Neither collection date nor location influenced the proportion of *B. napus* pollen carried (ANOVA, F = 0.096, p>0.05 and F = 1.165 p>0.05 respectively, ns), but a strong effect was imposed by distance from the *B. napus* field (ANOVA, F=147.5, p<0.001) (Table 2). *B. napus* pollen carriage was significantly higher at the field margin than that at any other distance (Tukey’s HSD test) (Table 2). The bee genera differed in the pattern of *B. napus* pollen carriage with distance (ANOVA F= 20.47, p<0.001). There was a marked difference between *B. napus* pollen carriage by *Apis mellifera* and carriage by *Bombus* spp. and *Andrena* spp. (Tukey’s HSD), but not between *Bombus* and *Andrena*.

All except one individual of bee captured on the field margin carried both pollen species (one *Apis* sample lacked *B. rapa*), with overall averages falling in the range 44-54% *B. napus*. This value declined sharply with distance from the crop and by 10 m had already fallen to between 3% for *Bombus* and to 7-8% for *Apis* and *Andrena* respectively. At the most distant site, carriage by *Apis* declined to below detectable levels, and to around 2% for *Andrena* but
remained constant at approximately 3% for *Bombus* (Table 2). In comparison, hoverflies at Tewkesbury carried a markedly higher percentage of *B. napus* pollen at the field margin (64%) and this proportion declined only relatively slowly; remaining > 30% across all distances (Table 2).

Long-range pollen delivery is more likely if the pollinator has few interim floral visits and so retain much of their crop pollen. The five (of 35) bees captured beyond 200 m that carried detectable levels of *B. napus* pollen included two with >20% *B. napus* pollen (Table 3). These individuals had probably engaged in limited interim foraging before being captured but the majority (94%, 33/35) had either not visited the source field or apparently lost most of their *B. napus* pollen *en route*. Conversely, the hoverflies at Tewkesbury carried *B. napus* pollen significant distances from the field margin. The majority (75%) carried detectable levels of *B. napus* pollen at all distances, with five of eight individuals carrying >20% *B. napus* even at 200 m (Table 3).
Discussion

There is great interest in characterizing insect-mediated pollen movement from cultivated *Brassica napus* to its wild relatives (e.g. Ford et al. 2009, Woodcock et al. 2013, Chifflet et al 2011). *Brassica napus* is visited by a wide variety of pollinating insects, with honey bees (*Apis mellifera*), mining bees (*Andrena* spp.), bumblebees (*Bombus* spp.) and hoverflies (*Syrphidae*) all being important in the UK and elsewhere in northern Europe (Hayter and Cresswell 2006; Woodcock et al. 2013). Several workers have shown that these pollinators have divergent foraging ranges (e.g. Pasquet et al. 2008). However, whilst such works illustrate capacity to disperse pollen, they do not demonstrate pollen carriage over these distances. Rader and colleagues (2011) overcame this limitation by distinguishing the *Brassica* pollen carried by insects from unrelated species on the basis of pollen morphology. They found that although hoverflies carried less pollen than bees, they were more likely to disperse *B. napus* pollen up to 400 m from the isolated source field. However, this approach is unable to distinguish between *Brassica* species and so has limited value for predicting *B. napus* to *B. rapa* delivery. Chifflet et al. (2011) used pollen recovered from captured bees to artificially pollinate bait plants and revealed that larger bodied taxa such as *Bombus* carry viable pollen loads as much as 1,100 m from the source field (Chifflet et al. 2011). By definition, the work also provided tacit evidence of pollen viability and is an attractive option for the characterization of conspecific gene flow mediated by insect vectors. However, for cases of interspecific gene flow there is an additional issue of determining which of the two pollen species gave rise to each seed produced (requiring genetic characterization) and also of discounting bias due to interspecific incompatibility or competition between pollen grains. Our use of species-specific DNA barcoding to track the dispersal of *B. napus* pollen into *B. rapa* allowed us to directly compare how the pollen carriage profiles of different vectors changed with distance from a source *B. napus* field into native populations of the wild
relative. Our findings broadly support previous studies suggesting that several insect vectors can carry *B. napus* pollen over substantial distances (Chifflet et al. 2011; Rader et al. 2011) but since these insects were captured on *B. rapa* flowers, we are also able to demonstrate that these vectors are carrying crop pollen to the wild relative at these distances. The vast majority of individuals representing every pollinator type carried pollen from both *Brassica* species when captured on *B. napus* plants at the field margin close to *B. rapa* (1-2 m). This implies that the pollinators are moving freely between the two species when both are in close proximity; a behaviour that we observed in the field, and implies that they show little or no discrimination between the species.

Perhaps more significantly from the perspective of assessing the risk of pollen carriage from a GM crop, the RT-PCR/pyrosequencing assay uncovered marked differences between vectors in their propensity to carry *B. napus* pollen to *B. rapa* over large distances. We suggest that the pattern of interspecific gene flow to *B. rapa* is therefore also likely to be dependent upon the identity of the vector. We found that honey bees (*Apis mellifera*) are highly effective pollinators of both species but have a strong tendency to remain close to the source field. To some extent this can be explained by lack of hives within close proximity of the source field (none observed within 400 m) and the relatively large size of the food resource represented by the source fields when compared with surrounding semi-natural plant communities. In consequence, we reason that visiting honey bees have travelled relatively large distances to reach the source field and have probably completed foraging activities before returning to the hive. Given *B. napus* is a common break crop in the UK but is not ubiquitous (Wilkinson et al. 2003), we suggest that this is scenario is likely to be more common than situations where the hive is adjacent to the field. Presuming that the hive-source field isolation distance in this study is reasonably representative of the general
situation, we infer that honey bees should generate large numbers of hybrids among
neighbouring stands of sympatric *B. rapa* but yield relatively few medium or long-range
hybrids. The large quantities of *B. napus* pollen carried by these bees imply that pods in these
locally-crossed *B. rapa* plants will have arisen without intermediate visits to other species and
so should often contain several hybrid seeds (something that could be tested empirically).
However, we cannot discount occasional/rare long-range dispersal events by other means
such as infrequent intermediate foraging on *B. rapa* during the return trip to the hive or
through inadvertent transfer of *B. napus* pollen between individuals in the hive.

In contrast to honey bees, *Bombus* and *Andrena* species featured occasional individuals that
carry *B. napus* pollen onto *B. rapa* over distances of at least 300 m from the crop. We
therefore expect a pattern of gene flow from these species to be similar to *Apis* near to the
crop but with occasional pods containing one to several hybrid seeds that decrease in
frequency with distance from the field. The underlying reason behind this apparent increased
propensity to carry crop pollen over larger distances warrants further study but may well
relate to the smaller colony sizes and the absence of communication between colony
members on the location of food sources as occurs in honey bees (Couvillon et al 2014)).
Hoverflies (Syrphidae) are often more numerous and collectively carry substantial quantities
of the pollen from the crop but have a strong tendency to move between plants and to deposit
little pollen on each visit (Gomez & Zamora 1999). In this study, we found that this group of
pollinators have a high tendency to carry crop pollen to wild *B. rapa* flowers >100m from the
field. It is likely that this pattern of dispersal is made possible by the absence of a residential
nest from which foraging is anchored (as it is in bees). We therefore postulate that these
pollinators will migrate across the landscape and typically produce occasional hybrid seed in
pods that otherwise contain conspecific seeds and that hybrid-containing pods would only
slowly reduce in frequency with distance from the crop. Thus, the study has highlighted the
importance of hoverflies for medium to long-range gene flow for the risk assessment of GM
B. napus. Looking more broadly, we suggest the approach adopted here to study pollen
carriage by vectors may be easily adapted to study movement of conspecific pollen variants
and so may ultimately provide a useful tool to help address other ecological problems
associated with pollen delivery.

Conclusion

The study of pollen delivery between these closely related species is complicated by their
indistinguishable pollen and the tendency of B. napus pollinators to visit almost all co-
flowering species (Stanley, Gunning & Stout 2013; Stanley & Stout 2014). Use here of a
targeted PCR amplification-pyrosequencing strategy successfully precluded amplification of
non-Brassica species and allowed us to provide the first full characterization of insect-
mediated pollen delivery from cultivated B. napus to B. rapa. We find that honey bees are
likely to be most important in mediating short-range gene flow but other groups, particularly
the hoverflies, are likely to be more important over larger distances. More generally, we feel
that our use of species-specific SNPs to detect interspecific pollen transfer between close
plant relatives may have broader utility for ecological studies far removed from GM risk
assessment.

Acknowledgements

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References


Table 1. Numbers of pollinating insects captured on *Brassica napus* and *B. rapa* flowers at the three sites. *Collection from *B. napus* flowers on the margin of the cultivated field (0 m from field margin, 1-2 m from the nearest *B. rapa* plant). Collection from *B. rapa* flowers in riparian populations 10 m, 50 m, 200 m or 300 m from the *B. napus* field margin.

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<td><em>Apis mellifera</em></td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Andrena cineraria</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. haemorrhoea</em></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. nitida</em></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Bee totals</td>
<td></td>
<td>89</td>
<td>85</td>
</tr>
<tr>
<td>Tewkesbury</td>
<td>Hoverfly</td>
<td><em>Rhingia campestris</em></td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Helophilus pendulus</em></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. trivittatus</em></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Hoverfly totals</td>
<td>6</td>
<td>19</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>-----------------</td>
<td>---</td>
<td>----</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>511</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Percentage of *Brassica napus* pollen (out of all Brassica pollen) carried by pollinators captured at increasing distance from the field margin.

<table>
<thead>
<tr>
<th>Site</th>
<th>Insect group</th>
<th>Distance from field margin (m.)</th>
<th>0</th>
<th>10</th>
<th>50</th>
<th>200</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bumblebees</td>
<td></td>
<td>45.3</td>
<td>6.3</td>
<td>5.9</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Appleford</td>
<td>Honey bees</td>
<td></td>
<td>59.7</td>
<td>12.8</td>
<td>7.4</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Mining bees</td>
<td></td>
<td>46.0</td>
<td>6.8</td>
<td>2.6</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Bumblebees</td>
<td></td>
<td>44.5</td>
<td>1.0</td>
<td>2.0</td>
<td>-</td>
<td>9.0</td>
</tr>
<tr>
<td>Culham</td>
<td>Honey bees</td>
<td></td>
<td>57.5</td>
<td>0.8</td>
<td>0.0</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Mining bees</td>
<td></td>
<td>4.1</td>
<td>4.1</td>
<td>1.9</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>Tewkesbury</td>
<td>Hoverflies</td>
<td></td>
<td>63.6</td>
<td>35.9</td>
<td>34.4</td>
<td>40.4</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3. Pollinators captured on *Brassica rapa* flowers at or beyond 200 m from the *B. napus* field margin, and the proportion of those individuals carrying >20% rapeseed pollen as inferred by pyrosequencing.

<table>
<thead>
<tr>
<th>Insect group</th>
<th>Species</th>
<th>Individuals captured</th>
<th>Individuals captured carrying <em>B. napus</em> pollen</th>
<th>Individuals captured carrying &gt;20% <em>B. napus</em> pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bumblebee</td>
<td><em>Bombus pascuorum</em></td>
<td>12</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>B. lapidarius</em></td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>B. ruderatus/hortorum</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>B. terrestris/lucorum</em></td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>B. pratorum</em></td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Honey bee</td>
<td><em>Apis mellifera</em></td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mining bee</td>
<td><em>Andrena cineraria</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>A. haemorrhhoa</em></td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>A. nitida</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hoverfly</td>
<td><em>Rhingia campestris</em></td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Helophilus pendulus</em></td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>H. trivittatus</em></td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 1. Linear relationship between pyrosequencing peak height of the *Brassica napus* diagnostic C allele and the reference AA allele for control *B. napus: B. rapa* mixes of (A)
DNA, $r^2 = 0.991$ and (B) pollen, $r^2 = 0.957$. Error bars represent 2x standard deviation.