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Title:

GM Risk Assessment: pollen carriage from *Brassica napus* to *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 *mat*K 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

68 that different pollinator groups may vary in the pattern of pollen dispersal from a source field
69 of *B. napus*. The most important pollinators of this UK crop are thought to be honey bees
70 (*Apis mellifera*), mining bees (*Andrena* spp.), bumblebees (*Bombus* spp.) and hoverflies
71 (*Syrphidae*) (Hayter and Cresswell 2006; Woodcock et al. 2013). Crop visitation by each
72 pollinator species is shaped by their reproductive and nesting strategies. The bee groups will
73 travel from their nests before foraging in the field and in neighbouring communities (Hayter
74 and Cresswell 2006) and so pollen dispersal will be constrained by the location and density of
75 their nesting sites. For honey bees, this feature is further complicated by the anthropomorphic
76 movement of hives. In contrast, foraging of hoverflies is not restricted by the need to return to
77 a colonial nest. Thus, it is important to characterise pollen carriage of all components of the
78 pollinator guild in order to predict patterns of insect-mediated gene flow between cultivated
79 *B. napus* and *B. rapa.* a wild relative that grows almost exclusively next to riverbanks in the
80 UK (Wilkinson et al 2003). In this study we use a species-specific Single Nucleotide
81 Polymorphism (SNP) assay for the *mat*K gene to characterize pollen delivery by all major
82 pollinators between these two close relatives.
83

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* sympatry 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 *mat*K 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 *mat*K reference sequences for all co-flowering insect-pollinated species at all sites (Appendix A Table 2) from the BOLD Systems database (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 *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' CAAAATTTATCTTTAGCCAACGAC 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.

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 *B. napus* plants and eight associated wild *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.

Characterizing Pollen mixtures

158 The *mat*K SNP used to quantify *B. napus* pollen comprised one allele (C) present only in *B.*
159 *napus*, and the alternate allele (A) is seemingly fixed in *B. rapa* and all co-flowering species
160 (Appendix A Table 3). Nested RT-PCR was performed with the incorporation of an M13
161 biotinylated forward inner primer during the second round PCR (Royo, Hidalgo & Ruiz
162 2007). Pyrosequencing was performed with a PSQ96MA instrument (Biotage AB, Sweden)
163 with PyroMark Gold Q96 Reagent (Qiagen, UK). The pyrosequencing primer (5'
164 ATTTCTAATAGATAATGT 3') was designed using the PSQ96 software to bind one base
165 upstream of the variable SNP position (Appendix A Table 3). Pyrosequencing order for the
166 diagnostic sequence T[A/C]GTAA was as follows: T (both species); A (*B. rapa*-specific or
167 nucleotide with no match for *B. napus*); C (*B. napus*-specific; no match for *B. rapa*); G (both
168 species); T (absent from both species, included as a negative control); A (AA in common for
169 both species). Sequencing then continued for a further 10 bases using nucleotides common to
170 both species to confirm amplicon identity.
171
172 Pyrosequencing was used to confirm 'C' allele presence in *B. napus* source fields and to
173 describe the relationship between allele peak heights at the diagnostic SNP site and the
174 proportion of *B. napus* in pollen mixtures. Peak height ratios were calculated by dividing the
175 value of the peak height of the diagnostic SNP by that of the shared AA peak two bases
176 downstream. Calculations were performed separately for *B. napus* and *B. rapa* using eight
177 replicates for each species (Appendix A Table 4). In this way a correction constant of 1.11
178 was derived by dividing mean peak height ratio for *B. rapa* (0.52) by that obtained for *B.*
179 *napus* (0.47) (Appendix A Table 4). Normalization of *B. napus* allele (C) peak heights in
180 mixed pollen samples was achieved by multiplication with this correction constant.
181

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* ($r^2 = 0.621$, data not shown) and *B. rapa* ($r^2 = 0.885$, 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 haemorrhoa* 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).

288 **Discussion**

289 There is great interest in characterizing insect-mediated pollen movement from cultivated
290 *Brassica napus* to its wild relatives (e.g. Ford et al. 2009, Woodcock et al. 2013, Chifflet et al
291 2011). *Brassica napus* is visited by a wide variety of pollinating insects, with honey bees
292 (*Apis mellifera*), mining bees (*Andrena* spp.), bumblebees (*Bombus* spp.) and hoverflies
293 (*Syrphidae*) all being important in the UK and elsewhere in northern Europe (Hayter and
294 Cresswell 2006; Woodcock et al. 2013). Several workers have shown that these pollinators
295 have divergent foraging ranges (e.g. Pasquet et al. 2008). However, whilst such works
296 illustrate capacity to disperse pollen, they do not demonstrate pollen carriage over these
297 distances. Rader and colleagues (2011) overcame this limitation by distinguishing the
298 *Brassica* pollen carried by insects from unrelated species on the basis of pollen morphology.
299 They found that although hoverflies carried less pollen than bees, they were more likely to
300 disperse *B. napus* pollen up to 400 m from the isolated source field. However, this approach
301 is unable to distinguish between *Brassica* species and so has limited value for predicting *B.*
302 *napus* to *B. rapa* delivery. Chifflet et al. (2011) used pollen recovered from captured bees to
303 artificially pollinate bait plants and revealed that larger bodied taxa such as *Bombus* carry
304 viable pollen loads as much as 1,100 m from the source field (Chifflet et al. 2011). By
305 definition, the work also provided tacit evidence of pollen viability and is an attractive option
306 for the characterization of conspecific gene flow mediated by insect vectors. However, for
307 cases of interspecific gene flow there is an additional issue of determining which of the two
308 pollen species gave rise to each seed produced (requiring genetic characterization) and also of
309 discounting bias due to interspecific incompatibility or competition between pollen grains.
310 Our use of species-specific DNA barcoding to track the dispersal of *B. napus* pollen into *B.*
311 *rapa* allowed us to directly compare how the pollen carriage profiles of different vectors
312 changed with distance from a source *B. napus* field into native populations of the wild

313 relative. Our findings broadly support previous studies suggesting that several insect vectors
314 can carry *B. napus* pollen over substantial distances (Chifflet et al. 2011; Rader et al. 2011)
315 but since these insects were captured on *B. rapa* flowers, we are also able to demonstrate that
316 these vectors are carrying crop pollen to the wild relative at these distances. The vast majority
317 of individuals representing every pollinator type carried pollen from both *Brassica* species
318 when captured on *B. napus* plants at the field margin close to *B. rapa* (1-2 m). This implies
319 that the pollinators are moving freely between the two species when both are in close
320 proximity; a behaviour that we observed in the field, and implies that they show little or no
321 discrimination between the species.
322
323 Perhaps more significantly from the perspective of assessing the risk of pollen carriage from
324 a GM crop, the RT-PCR/pyrosequencing assay uncovered marked differences between
325 vectors in their propensity to carry *B. napus* pollen to *B. rapa* over large distances. We
326 suggest that the pattern of interspecific gene flow to *B. rapa* is therefore also likely to be
327 dependent upon the identity of the vector. We found that honey bees (*Apis mellifera*) are
328 highly effective pollinators of both species but have a strong tendency to remain close to the
329 source field. To some extent this can be explained by lack of hives within close proximity of
330 the source field (none observed within 400m) and the relatively large size of the food
331 resource represented by the source fields when compared with surrounding semi-natural plant
332 communities. In consequence, we reason that visiting honey bees have travelled relatively
333 large distances to reach the source field and have probably completed foraging activities
334 before returning to the hive. Given *B. napus* is a common break crop in the UK but is not
335 ubiquitous (Wilkinson et al. 2003), we suggest that this is scenario is likely to be more
336 common than situations where the hive is adjacent to the field. Presuming that the hive-
337 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

363 importance of hoverflies for medium to long-range gene flow for the risk assessment of GM
364 *B. napus*. Looking more broadly, we suggest the approach adopted here to study pollen
365 carriage by vectors may be easily adapted to study movement of conspecific pollen variants
366 and so may ultimately provide a useful tool to help address other ecological problems
367 associated with pollen delivery.
368
369
370 **Conclusion**
371 The study of pollen delivery between these closely related species is complicated by their
372 indistinguishable pollen and the tendency of *B. napus* pollinators to visit almost all co-
373 flowering species (Stanley, Gunning & Stout 2013; Stanley & Stout 2014). Use here of a
374 targeted PCR amplification-pyrosequencing strategy successfully precluded amplification of
375 non-*Brassica* species and allowed us to provide the first full characterization of insect-
376 mediated pollen delivery from cultivated *B. napus* to *B. rapa*. We find that honey bees are
377 likely to be most important in mediating short-range gene flow but other groups, particularly
378 the hoverflies, are likely to be more important over larger distances. More generally, we feel
379 that our use of species-specific SNPs to detect interspecific pollen transfer between close
380 plant relatives may have broader utility for ecological studies far removed from GM risk
381 assessment.
382
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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.

Site	Insect group	Species	Distance from field margin (m.)					Total
			0*	10	50	200	300	
Appleford	Bumblebee	*Bombus pascuorum*	3	4	6	-	10	**23**
		B. lapidarius	5	0	2	-	3	**10**
		B. ruderatus/hortorum	2	0	1	-	0	**3**
		B. terrestris/lucorum	1	9	8	-	6	**24**
		B. pratorum	2	3	0	-	2	**7**
	Honey bee	*Apis mellifera*	29	18	16	-	3	**66**
	Mining bee	*Andrena cineraria*	1	0	0	-	0	**1**
		A. haemorrhoa	3	7	4	-	0	**14**
		A. nitida	3	4	1	-	0	**8**
Culham	Bumblebee	*Bombus pascuorum*	0	1	1	-	2	**4**
		B. lapidarius	2	4	6	-	4	**16**
		B. ruderatus/hortorum	3	0	0	-	0	**3**
		B. terrestris/lucorum	7	14	8	-	0	**29**
		B. pratorum	2	0	0	-	2	**4**
	Honey bee	*Apis mellifera*	26	14	1	-	1	**42**
	Mining bee	*Andrena cineraria*	0	0	0	-	0	**0**
		A. haemorrhoa	0	5	5	-	3	**13**
		A. nitida	0	2	1	-	1	**4**
Bee totals			**89**	**85**	**60**	**-**	**37**	**271**
Tewkesbury	Hoverfly	*Rhingia campestris*	3	11	2	2	-	**18**
		Helophilus pendulus	3	3	0	3	-	**9**
		H. trivittatus	0	5	7	3	-	**15**

Hoverfly totals	**6**	**19**	**9**	**8**	**-**	**42**

511

512 **Table 2.** Percentage of *Brassica napus* pollen (out of all Brassica pollen) carried by

513 pollinators captured at increasing distance from the field margin.

514

Site	Insect group	Distance from field margin (m.)				
		0	**10**	**50**	**200**	**300**
Appleford	Bumblebees	45.3	6.3	5.9	-	0.1
	Honey bees	59.7	12.8	7.4	-	0.0
	Mining bees	46.0	6.8	2.6	-	0.0
Culham	Bumblebees	44.5	1.0	2.0	-	9.0
	Honey bees	57.5	0.8	0.0	-	0.0
	Mining bees	-	4.1	1.9	-	0.3
Tewkesbury	Hoverflies	63.6	35.9	34.4	40.4	-

515

516

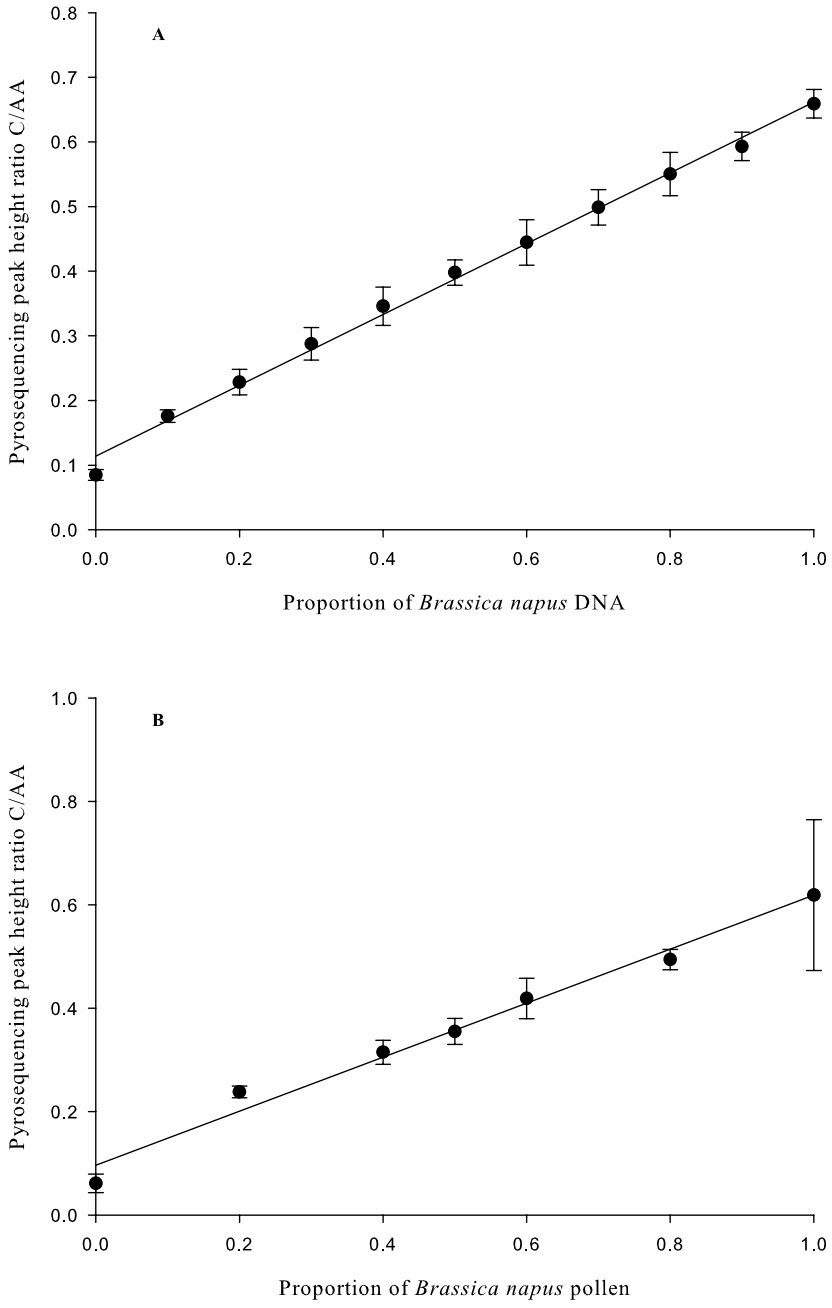
517 **Table 3.** Pollinators captured on *Brassica rapa* flowers at or beyond 200 m from the *B. napus*
518 field margin, and the proportion of those individuals carrying >20% rapeseed pollen as
519 inferred by pyrosequencing.
520

Insect group	Species	Individuals captured	Individuals captured carrying *B. napus* pollen	Individuals captured carrying >20% *B. napus* pollen
Bumblebee	*Bombus pascuorum*	12	2	1
	B. lapidarius	7	1	0
	B. ruderatus/hortorum	0	0	0
	B. terrestris/lucorum	6	0	0
	B. pratorum	4	1	1
Honey bee	*Apis mellifera*	4	0	0
Mining bee	*Andrena cineraria*	0	0	0
	A. haemorrhoa	3	1	0
	A. nitida	1	0	0
Hoverfly	*Rhingia campestris*	2	1	0
	Helophilus pendulus	3	2	2
	H. trivittatus	3	3	3

521

522 **Fig. 1.** Linear relationship between pyrosequencing peak height of the *Brassica napus*
523 diagnostic C allele and the reference AA allele for control *B. napus: B. rapa* mixes of (A)

524 DNA, r^2 = 0.991 and (B) pollen, r^2 = 0.957. Error bars represent 2x standard deviation.



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