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Spontaneous gene flow from rapeseed (*Brassica napus*) to wild *Brassica oleracea*

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Research on the environmental risks of gene flow from genetically modified (GM) crops to wild relatives has traditionally emphasized recipients yielding most hybrids. For GM rapeseed (*Brassica napus*), interest has centred on the 'frequently hybridizing' *Brassica rapa* over relatives such as *Brassica oleracea*, where spontaneous hybrids are unreported in the wild. In two sites, where rapeseed and wild *B. oleracea* grow together, we used flow cytometry and crop-specific microsatellite markers to identify one triploid F_1 hybrid, together with nine diploid and two near triploid introgressants. Given the newly discovered capacity for spontaneous introgression into *B. oleracea*, we then surveyed associated flora and fauna to evaluate the capacity of both recipients to harm cohabitant species with acknowledged conservational importance. Only *B. oleracea* occupies rich communities containing species afforded legislative protection; these include one rare micromoth species that feeds on *B. oleracea* and warrants further assessment. We conclude that increased attention should now focus on *B. oleracea* and similar species that yield few crop-hybrids, but possess scope to affect rare or endangered associates.

Keywords: genetically modified crops; gene flow; transgene; *Brassica napus*; *Brassica oleracea*; associated flora and fauna

1. INTRODUCTION

Commercial genetically modified (GM) crops are dominated by four species (maize, soybean, cotton and rapeseed) and two transgene types (insect resistance and herbicide tolerance) (James 2005). However, recent trends towards GM lines containing multiple inserts (Wilkinson *et al.* 2003a) and new traits (Dunwell 2002) mean that regulation will need to become increasingly sophisticated to accommodate the new hazards that diversification brings (Raybould & Wilkinson 2005). The main risks posed by the large-scale cultivation of GM crops are associated with unwanted ecological change. For instance, while transgenic herbicide tolerant and insect-resistant crops are clearly intended to reduce the abundance of weeds and invertebrates and are beneficial to the farmer, such change may also reduce on-farm biodiversity, either directly through the intended action of the transgene or indirectly through altered farm practice (e.g. Firbank & Forcella 2000). There are also concerns regarding toxicity and allergenicity of expressed transgene products in food; an issue made more acute with the proposed use of GM crops expressing pharmaceutical products (Dunwell 2002).

The movement of transgenes from crop plants into wild populations via gene flow causes concern on several levels, but is unusual because the ecological consequences can occur within or distant from the agricultural setting. For instance, gene flow of transgenes conferring selective advantage to compatible relatives may enhance invasiveness, exacerbate a weed problem or may negatively perturb the interactions with associated fauna or flora in natural communities.

Crude ranking of cross-compatible recipient species according to hybridization frequency applies generally to a crop and has traditionally provided a powerful basis to target research priorities. Problems arise when hybridization is unreported in the wild, but the contact between the crop and potential wild recipients is extensive. This is particularly germane for common weedy or widespread wild relatives, where even extremely low hybridization frequencies could still yield many hybrids. For instance, while 1 in 100 pollinations produces hybrids between rapeseed and the very common weed, *Sinapis arvensis* using ovule culture (Lefol *et al.* 1996), extensive screening of millions of naturally set seeds by several groups has yet to uncover a single confirmed hybrid (Bing *et al.* 1996; Lefol *et al.* 1996; Moyes *et al.* 2002; Chèvre *et al.* 2003), although one putative transgenic hybrid containing resistance to glufosinate ammonium has recently been reported (Daniels *et al.* 2005). In such cases, regulators must either assume gene flow occurs and assess risk on the basis of consequence(s) or disregard transgene movement unless proved otherwise. Clearly, the production of even one fertile spontaneous hybrid could radically alter the way in which such a potential recipient is viewed.

Rapeseed (*Brassica napus*) has 16 cross-compatible relatives growing in the UK that can be ordered according to various criteria (table 1). However, *Brassica rapa* is

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Table 1. Wild relatives of *Brassica napus* in the UK. (Species are ranked in three ways: according to their ease of hybridization (Scheffler & Dale 1994); by the number of associated species with conservational status (Rodwell 1991; Cheffings & Farrell 2005); and by combining the above two to give an overall rank based on the capacity to inflict harm to species of conservational importance. This combined ranking was performed first on the basis of conservational importance (scarcity) of the recipient itself (although none of the species is scarce), second on the number of known associates with conservational status and third on ease of hybridization with the crop.)

recipient species	hybridization ranking[a]	associate ranking	combined ranking
Brassica oleracea[b]	3	1	1
Diplotaxis muralis[a]	7	2	2
Raphanus raphanistrum[a]	6	3	3
Sinapis arvensis[a]	8	3	4
Brassica nigra[b]	5	4	5
Brassica rapa[b]	1	5	6
Brassica juncea[a]	2	5	7
Brassica carinata[a]	4	5	8
Diplotaxis erucoides[a]	7	5	9
Sinapis alba[b]	8	5	10
Brassica tournefortii[a]	9	5	11
Diplotaxis tenuifolia[a]	9	5	11
Eruca vesicaria[a]	9	5	11
Raphanus sativus[a]	9	5	11
Erucastrum gallicum[c]	—	5	12
Hirschfeldia incana[b]	—	5	12

[a] Scheffler & Dale 1994.
[b] Raybould & Gray 1993.
[c] Warwick *et al.* 2003.

consistently ranked of greatest concern (Scheffler & Dale 1994), largely because it readily forms semi-fertile F_1 hybrids (Jørgensen & Andersen 1994; Jørgensen *et al.* 1996; Wilkinson *et al.* 2000; Hansen *et al.* 2001; Warwick *et al.* 2003). Indeed, 32 000 spontaneous F_1 hybrids are predicted annually between these two species in the UK alone (Wilkinson *et al.* 2003b). *Brassica oleracea* receives little attention, principally because hybrid progeny are typically difficult to obtain artificially (19 hybrids from 12 401 pollinations (Chiang *et al.* 1977); one F_1 seed from 'hundreds' of crosses (Honma & Summers 1976)) and no spontaneous hybrids are reported from the wild (Stace 1975; Scheffler & Dale 1994; Wilkinson *et al.* 2000). It nevertheless seems entirely plausible that such hybrids do occur but at rather low frequencies. The objectives of this study were therefore to screen wild *B. oleracea* for the F_1 hybrids and/or introgressants with rapeseed in wild populations adjacent to arable fields that feature rapeseed in the rotation and then to make a preliminary assessment of the scope for ecological harm.

2. MATERIAL AND METHODS
(a) *Plant material*
Foot surveys for coastal *B. oleracea* populations adjacent to arable land were conducted in Kent between UK grid references TR 330 418 and TR 380 484 in June 2004. In addition, reference to remote sensing data covering 15 000 km² of SE England (Wilkinson *et al.* 2000) identified cliff-side arable fields in the area that incorporate rapeseed within the rotation. From these surveys, two sites were

identified in the White Cliffs area of Kent (Hope Point TR 378 461 to TR 373 452 and Langdon Hole TR 349 426 to TR 345 425), where rapeseed is occasionally grown next to natural, cliff-top *B. oleracea* populations. The two fields adjacent to the Hope Point population contained a crop of rapeseed and wheat at the time of sampling. The Langdon Hole population was adjacent to two fields containing wheat at the time of sampling, although rapeseed had been grown there in the previous year (S. Ovenden, National Trust White Cliffs Visitor Centre 2004, personal communication). Leaf samples were collected from all accessible *B. oleracea* plants positioned 1–25 m from arable field margins (842 samples), with the approximate distance of each sample from the field margin being recorded for each sample.

Control 'allopatric' *B. oleracea* populations isolated from arable fields greater than 1 km were identified in three areas: 307 plants near the White Cliffs National Trust Visitor Centre, Kent (TR 334 421), December 2004; 91 samples collected from all accessible plants on the cliff top and beach at Durdle Door, Dorset (SY 809 802); and 36 plants at the base of the cliff face at Kimmeridge Bay, Dorset (SY 900 790), August 2004.

(b) *Screening for hybrids and introgressants*
Brassica napus is an allotetraploid ($2n=4x=38$, AACC) originating from an ancient hybridization between the diploids *B. rapa* ($2n=2x=20$, AA) and *B. oleracea* ($2n=2x=18$, CC). Therefore, the crosses *B. napus*× *B. rapa* and *B. napus*×*B. oleracea* yield triploid hybrids ($2n=3x=29$, ACC and $2n=3x=28$, AAC, respectively). Hybrids between *B. napus* and *B. oleracea* can be detected using a combination of flow cytometry analysis to determine approximate chromosome compliment (i.e. triploidy) and molecular analysis to determine genome constitution.

(i) *Flow cytometry analysis*
All samples from sympatric populations were first screened by flow cytometry to identify triploids. For this, fresh leaf samples (1 cm²) were sent to Plant Cytometry Services (Schijndel, The Netherlands) and subjected to flow cytometry analysis as described by Wilkinson *et al.* (2000).

(ii) *DNA extraction*
DNA of all genotypes was extracted from freshly collected leaf material using the Qiagen DNeasy 96 Plant Kit according to the manufacturer's instructions.

(iii) *Genome assignment*
Plants classified as triploids by flow cytometry were deemed possible F_1 hybrids and hence subjected to molecular characterization. Introgressants could also be present in sympatric and allopatric sites (through gene flow) and expected to be primarily diploid (through backcrossing to *B. oleracea*). Thus, the search for introgressants was entirely based on the molecular screen. Both molecular screens were performed using A-genome-specific amplicons generated by the following microsatellite markers: BRMS043 (Suwabe *et al.* 2002), Na10-A08, Na12-D04 and Ol10-D08 (Lowe *et al.* 2004).

PCRs were performed using Qiagen Multiplex PCR kit according to the manufacturer's instructions on a PTC-100 MJ Research thermocycler, under the following conditions: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 56°C for 90 s and 72°C for 60 s, followed by 60°C for 30 min.

Table 2. Results of PCR analyses showing the occurrence of A-genome-specific markers from DNA extracted from leaf samples of diploid and triploid[*] wild *B. oleracea*, and a *B. oleracea* × *B. napus* F_1 hybrid[**], growing in the White Cliffs area of Kent. (HP, Hope Point; LH, Langdon Hole.)

sample	location (UK grid references)	microsatellite markers			
		BRMS043	Na10-A08	Na12-D04	Ol10-D08
Kent 195[*]	HP (TR 376 457)	−	−	−	−
Kent 201	HP (TR 376 457)	−	+	−	−
Kent 317	HP (TR 375 454)	−	+	−	−
Kent 390[**]	HP (TR 377 460)	+	+	+	+
Kent 460	LH (TR 348 426)	−	+	−	−
Kent 579	LH (TR 348 426)	−	−	+	−
Kent 582	LH (TR 347 425)	−	−	+	−
Kent 704	LH (TR 345 425)	−	−	+	+
Kent 711	LH (TR 345 425)	−	+	−	−
Kent 734	LH (TR 345 425)	−	−	+	+
Kent 767	LH (TR 345 425)	−	+	−	−
Kent 776[*]	LH (TR 345 425)	−	+	−	−
Kent 780[*]	LH (TR 345 425)	−	+	−	−

Amplicon mixes were fractionated on an ABI 3100 genetic analyser and characterized using GENOTYPER v. 3.7NT.

Two markers (BRMS043 and Na12-D04) generated 1–2 amplicons of 275–300 bp when applied to 96 reference *B. rapa* genotypes (see electronic supplementary material 1) and 81 reference rapeseed lines (see electronic supplementary material 2), but failed to amplify products when used on 434 wild genotypes of *B. oleracea* from allopatric populations. The remaining two markers (Na10-A08 and Ol10-D08) generated size-specific amplicons from the A and C genomes. Marker Na10-A08 yielded a single product of 138–146 bases from the *B. rapa* reference samples and two products of size 138–160 and 166–174 bases from *B. napus*. For marker Ol10-D08, the reference *B. rapa* set invariably produced amplicons of 170 bp, whereas the *B. napus* genotypes produced two products: one matching the 170 bp of *B. rapa* and another of 174–182 bp. The allopatric *B. oleracea* populations invariably generated 1–2 amplicons within the size range of 166–216 bp for Na10-A08 and 174–202 bp for Ol10-D08. Further evidence indicating A-genome specificity of these markers derives from linkage maps of *B. napus* (see http://brassica.bbsrc.ac.uk/IMSORB) and *B. rapa* (J. Allainguillaume & M. J. Wilkinson 2006, unpublished data). These markers were applied to all *B. oleracea* leaf samples.

3. RESULTS AND DISCUSSION
Flow cytometry analysis identified four triploids among the samples analysed. Typical results showed discrete, non-overlapping G_1 peaks, with diploid samples positioned around 207 ± 5 CV, compared with 353 ± 4 CV and 307 ± 4 CV for the tetraploids and triploids, respectively. Only one of the triploid plants identified possessed all four A-genome crop-specific markers and thus was confirmed as an F_1 hybrid (table 2). The hybrid probably has genome constitution ACC because all four A-genome markers appeared in its profile and two markers (Na10-A08 and Ol10-D08) generated three amplicons. It was not possible to assess hybrid fertility before the completion of flowering.

One triploid lacking all 'A-genome' markers was presumed to be an autotriploid *B. oleracea* (CCC). The two triploids containing only one such marker were more problematic. One possibility is that these plants are later generation progeny (e.g. F_2 or F_3), in which self-pollination has ensured chromosome number remains approximately triploid, with marker loss occurring by independent assortment. Alternatively, these plants may be autotriploids derived from the fusion of reduced and unreduced gametes from *B. oleracea* parents, one of which contained an introgressed marker.

While the presence of one F_1 hybrid provides insufficient statistical power to provide an accurate estimate of hybridization frequency *per se*, the single *B. napus* × *B. oleracea* hybrid from 842 samples is nevertheless significantly below that reported previously between rapeseed and *B. rapa* (47/3230; Wilkinson *et al.* 2003*b*; $\chi^2 = 10.2$, $p < 0.01$). While the frequency of F_1 hybrids is clearly lower in *B. oleracea* than *B. rapa*, it should be remembered that *B. oleracea* is a polycarpic perennial and thus, a single hybrid produces second generation introgressants over many years. This contrasts with *B. rapa* F_1 hybrids which flower only once. We therefore suggest that the consideration of hybridization frequencies alone probably underestimates the contribution of F_1 hybrids to the introgression of *B. oleracea* populations.

When the same microsatellite markers were applied to the 838 diploid samples, nine individuals contained one or more crop-specific markers, with all markers represented in at least one *B. oleracea* plant (table 2). These plants were tentatively identified as introgressed genotypes. The overall proportion of individuals containing crop-specific markers in sympatric sites was therefore 12 (three triploids and nine diploids) out of 842 (1.4%). No such plants were found among 434 plants from allopatric sites. This is significantly below expectations ($\chi^2 = 6.2$, $p < 0.025$) if these markers simply represent rare *B. oleracea* alleles occurring at the same frequency away from the crop, although such differences could be explained by founder events or genetic drift. However, more plants sampled within 2 m of the field margins were introgressants (seven introgressants among 200 samples, 3.5%) than were noted at greater distances in the same sympatric populations (five introgressants from 642 samples, 0.8% $\chi^2 = 8.0$, $p < 0.01$). This disparity is more difficult to explain by genetic drift or founder effects. These findings, when coupled with the apparent specificity of the markers to the

Table 3. Species present at the White Cliffs SSSI, Kent protected under the 1981 Wildlife and Countryside Act.

common name	species	class order	UK legal protection
peregrine falcon	*Falco peregrinus*	Aves Falconiformes	schedule 1
Mediterranean gull	*Larus melanocephalus*	Aves Charadriformes	schedule 1
slow worm	*Anguis fragilis*	Reptilia Squamata	schedule 5
small blue	*Cupido minimus*	Insecta Lepidoptera	schedule 5
silver-spotted skipper	*Hesperia comma*	Insecta Lepidoptera	schedule 5
Adonis blue	*Lysandra bellargus*	Insecta Lepidoptera	schedule 5
chalkhill blue	*Lysandra coridon*	Insecta Lepidoptera	schedule 5
meadow clary	*Salvia pratensis*	Angiospermopsida Lamiales	schedule 8
oxtongue broomrape	*Orobanche picridis*	Angiospermopsida Scrophulariales	schedule 8
lizard orchid	*Himantoglossum hircinum*	Angiospermopsida Orchidales	schedule 8
early spider orchid	*Ophrys sphegodes*	Angiospermopsida Orchidales	schedule 8

A genome, as suggested by screening two reference sets of 96 and 81 diverse samples of cultivated or wild *B. rapa* and *B. napus*, respectively (see electronic supplementary materials 1 and 2), strongly suggest that introgression has occurred from rapeseed into wild *B. oleracea* within this locality.

We then turned our attention to the scope for ecological harm arising from gene flow. Ecological hazards associated with gene flow can be broadly divided into unwanted changes to the recipient (e.g. enhanced invasiveness) and associated flora and fauna (e.g. local extinctions caused by interactions with the transgene recipient) and undesirable changes to community structure. Given that spontaneous gene flow and introgression occur in *B. rapa* and *B. oleracea*, the relative scope of each recipient to cause any of these outcomes is partly dependent upon the species with which each interacts. We therefore surveyed flora and fauna in the communities containing *B. rapa* and *B. oleracea* for associated species with conservational importance.

Brassica oleracea communities in the White Cliffs area of Kent contained 321 plant species, 53 bird species and 27 butterflies (see electronic supplementary material 3). This land is a site of special scientific interest (SSSI) and contains several species of national conservational importance, protected under the UK Wildlife and Countryside Act 1981 (table 3). The area also supports 12 species of red-listed birds (Gregory *et al.* 2002) and four plants categorized as 'endangered', three as 'vulnerable' and six as 'near threatened' according to IUCN threat categories (Cheffings & Farrell 2005). Co-location of 'red-listed' species and a potential transgene recipient is not a problem in itself, but provides a useful basis for hazard identification. In such instances, further work would be required to assess exposure and risk. There is one relationship that may require further evaluation in this regard; the provisionally red-data listed micromoth species *Selania leplastriana* (Kent County Council 2000) uses *B. oleracea* as a larval food source. Acquisition of Lepidoptera-specific *Cry1A Bt* transgene into *B. oleracea* plants used by these moths clearly has a potential to cause local decline in moth numbers and thus warrants examination.

For comparison, we surveyed 19 *B. rapa* riverside communities along the rivers Thames, Avon and Nene, and found 110 plant species (see electronic supplementary material 4), none of which is protected by law or has recognized conservational status (Cheffings & Farrell 2005). While the diversity and scarcity of associates do not relate directly to ecosystem function and do not consider the social importance of some species, it is nevertheless a valuable perspective from which to compare recipients of gene flow. Viewed in this context, GM *B. oleracea* introgressants apparently possess greater scope to harm species of conservational significance than the introgressants of *B. rapa* (table 1) and for this reason *B. oleracea* probably deserves greater attention than it has received until today.

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