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Environmental risk assessment of genetically modified crops: The use of molecular markers to trace insect and wind dispersal of *Brassica napus* pollen

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SUMMARY

This project aimed to develop a better understanding of the potential risks of gene flow and to generate isolation distances required for genetically modified (GM) OSR. The study examined biotic (insect) and abiotic (wind) pollen dispersal over two seasons. A considerable portion of work was devoted to the development of molecular markers, to differentiate *Brassica napus* varieties from each other to distinguish them from their wild relatives.

The project broadly aimed to study gene flow, via pollen movement, from OSR but specifically aimed to:

- Characterise *B. napus* cv. Marinka using molecular markers.
- Elucidate the distance travelled by OSR pollen by biotic dispersal.
- Elucidate the distance travelled by OSR pollen by abiotic dispersal.
- Elucidate pollination/seed set at various distances from a source crop using male sterile bait plants.
- Develop risk assessment/containment strategies.

A risk assessment was carried out to help determine the extent of pollen movement from a crop of oilseed rape (OSR, *Brassica napus*). The project aimed to develop a better understanding of the potential risks of gene flow and to generate isolation distances required for genetically modified (GM) OSR. The study aimed to examine biotic (insect) and abiotic (wind) pollen dispersal over two seasons. A considerable portion of work was devoted to the development of molecular markers, to differentiate *Brassica napus* varieties from each other and to distinguish them from their wild relatives.

Abiotic pollen dispersal results demonstrate that airborne *B. napus* pollen travelled at least 200 m from the source crop.

Seed set in male sterila *B. napus* plants confirms the potential for hybridisation to occur with feral or volunteer populations at crop margins or in the general vicinity of a GM crop. Results from abiotic dispersal experiments suggest that airborne pollen is travelling sufficiently far to effect fertilisation at considerable distances from the crop, however it has been suggested that *B. napus* flowers are ineffective at capturing airborne pollen. This implies that insects may be the primary vectors in *B. napus* pollen dispersal.

The presence of *B. napus* cv. Marinka pollen in bee pellets taken from beehives at 1.6 km from the field indicates that pollen is moving over large distances. Therefore, with foraging up to 1.6 km in all directions from a hive, some pollen transfer and fertilisation up to 3.2 km can be expected. Theoretically, there is potential for pollen to be transferred to distances of at least 10 km by the mixing of bees foraging in different directions from the same hive.

Plastid SSRs provide a new opportunity for high-resolution analysis of inter- and intra-specific variation in the Brassicaceae and for the differentiation of its species. Combining data generated from nuclear and plastic SSRs enables the study of seed and pollen movement, and assists in the assessment of gene flow from GM plants through hybridisation studies.

The detection of low levels of *B. napus* pollen at outer distances tested has implications for transgene movement. This combined with the tendency of *Brassica* to hybridise with its wild relatives, points towards transgene establishment in the wild, particularly if the transgene provides a selective advantage. Gene flow can occur between volunteer and feral populations, which can act as gene pools facilitating the contamination of subsequent rape crops. Hybridisation and introgression between oilseed rape varieties and wild relatives are therefore likely to occur. It is important to establish whether the transgene would confer a direct selective advantage in the feral environment as the rate of introgression and its chance of persistence will be dependent on this.

INTRODUCTION

The use of transgenic plants in breeding may enable the utilisation of a wide variety of novel genes from unrelated organisms that would otherwise be unavailable. Because of the diversity of the genes now accessible for modifying crop plants it is internationally accepted that risk assessments should be performed before modified plants are grown outside the laboratory. This is not because the transgenic plants are intrinsically hazardous, but because the choice of genes available is no longer restricted by sexual incompatibility (Dale and Irwin, 1995).

Pollen transfer and seed distribution are the main routes through which gene flow may occur and, therefore, the roles of each are fundamental to the environmental risk assessment of GM plants (Flannery *et al.*, 2002). Gene flow through pollen depends on factors such as the amount of pollen produced, longevity of the pollen, abiotic or biotic dispersal, dormancy of pollen, plant or weed density in the vicinity, distance between crop and weed, and receptivity of the weed to the pollen. Dispersal of the seed may occur during harvest, transportation, planting and subsequent harvests. If germination of GM seed was to occur, a risk of interbreeding with compatible weedy species would present itself. Consequently, introgressive hybridisation could result in new weeds emerging, which have acquired the GM trait (Daniell, 2002).

Microscopic examination is inadequate for pollen identification to the species or variety level due to the similarity of different grains. In many cases, including the Brassicaceae, pollen cannot be identified below the family level. Molecular analyses represent a viable alternative.

Comparative studies of nuclear and plastid microsatellite (simple sequence repeat; SSR) markers can assist in the analysis of seed and pollen movement and thus help reveal the genetic structure of a population. Nuclear SSRs have been detected in many plant genomes including *Brassica* (Kresovich *et al.*, 1995; Szewc-McFadden *et al.*, 1996; Westman and Kresovich, 1998; Uzunova and Ecke, 1999; Westman and Kresovich, 1999; Plieske and Struss, 2001; Saal *et al.*, 2001; Lowe *et al.*, 2002; Suwabe *et al.*, 2002). In 1996, 17 primers detecting inter- and intra-species variation in *B. napus, B. oleracea* and *B. rapa* were published (Szewc-McFadden *et al.*, 1996). Since then there has been a concentrated effort in the production of *Brassica* SSR primers and approximately 404 *Brassica* nuclear microsatellite primers are currently available through the *Brassica* DB database on the UK CropNet website (http://ukcrop.net).

Plastid (chloroplast) microsatellite markers can be developed from sequence data from closely related species, as plastid primers regularly cross-amplify in related species and have shown widespread intra-specific microsatellite polymorphism. For species that have previously been sequenced to a considerable extent, database searching can discover microsatellite repeats (Provan *et al.*, 2001). Plastid SSRs have been developed in this way for the weed *Arabidopsis thaliana* (Provan, 2000), however to date none have been published specifically for *Brassica*.

MATERIALS, METHODS AND RESULTS

1. Molecular markers for identification of oilseed rape varieties

1.1 Methods

All test species were analysed using PCR restriction fragment length polymorphism (PCR-RFLP) analysis and SSR markers. Nuclear SSRs were selected from CropNet; however, development of specific plastid SSR primers was required. For this reason, DNA sequencing was carried out on eight species for 5 gene regions. These included the plastid *atpB-rbcL* spacer region, *trnL* intron and *trnL-F* intergenic spacer, *rps16*, *rpl16* and the nuclear ribosomal ITS gene region. Nucleotide sequences of three-chloroplast regions A (*atpA* to *rps4*

incorporating *rpoB*; 36,129bps), B (*ndhC* to *cemA* incorporating *rbcL*; 11,002bps) and C (*ndhB* to *23S* incorporating *16S*; 9,419bps) derived from *Brassica napus* cv. Licosmos and comprising approximately one third of the *Brassica* plastid genome were used (Coyne, 2002), and further DNA sequences obtained from Genbank (NCBI, 2003). Homologous sequences were aligned and areas of conserved sequence flanking the SSR were then selected for primer design. Primers were tested on a broad range of Brassicaceae plant material. Statistical analysis was performed by developing presence-absence data for SSR peaks and a Neighbour Joining analysis was then carried out in PAUP 4.0 using the Nei-Li distances (Nei and Li, 1979).

1.2 Results

PCR-RFLP analysis proved adequate for inter-generic differentiation of the Brassicaceae but was unable to distinguish inter-specific differences. Interpretation of banding patterns produced by nuclear SSR analysis is difficult in Brassicaceae as many species are polyploid and numerous SSR peaks are more difficult to interpret.

A total of 215 plastid SSRs were identified with repeat motifs greater than ten. Adenine and thymine mononucleotide repeats such as $(A)_n$ and $(T)_n$ were the most common. Nearly all of the SSRs detected were mononucleotide but some di-, tri- and tetra-nucleotide repeats were detected. Some regions included in primer design do not appear in the tables as the SSRs were interrupted, shorter or absent in some of the species included in the study. Several SSRs of satisfactory length were excluded due to lack of variation (between individuals) in the SSR detected. Ten SSR primers pairs were developed and tested. One primer was selected for each *rpl16*, *rps16* and C regions, two for the *trnL-F* region and three for both the *atpB-rbcL* spacer region (region B) and region A. All primers except *rps16* amplified well and detected inter-generic variation. Polymorphism within *Brassica* was detected using primers developed from the following regions *rpl16*, *atpB-rbcL*, A and C.

Phylogenetic trees were produced for nuclear (Fig. 1) and plastid (Fig. 2) SSR markers. The results were then used to create a combined tree (Fig. 3). Plastid SSR markers successfully distinguished between *Arabidopsis thaliana*, *B. napus*, *B. nigra*, *Brassica oleracea*, *B. rapa*, *Camelina sativus*, *Raphanus sativus* and also within species differences were detected for *B. napus* (Fig. 2).

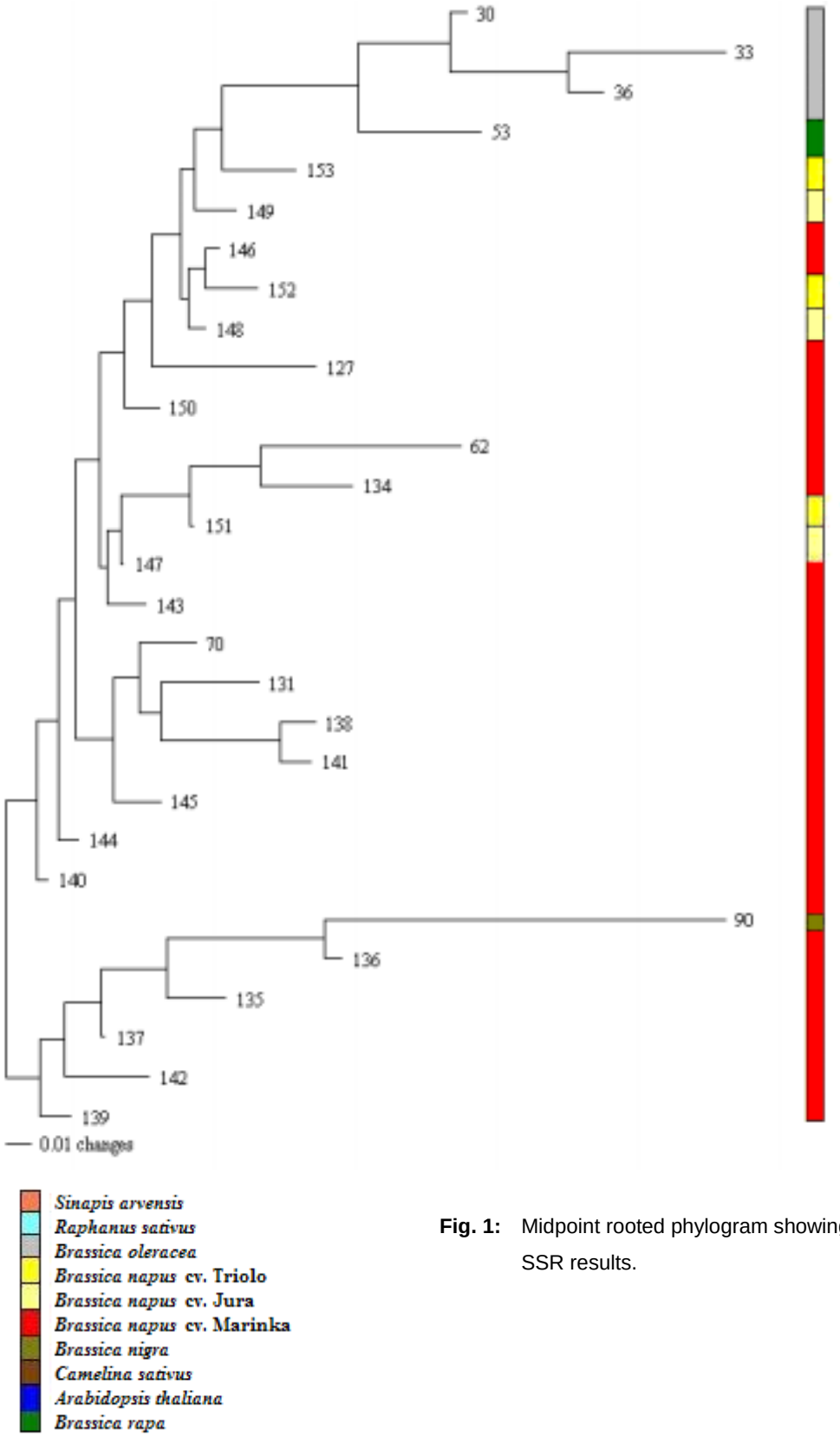


Fig. 1: Midpoint rooted phylogram showing nuclear SSR results.

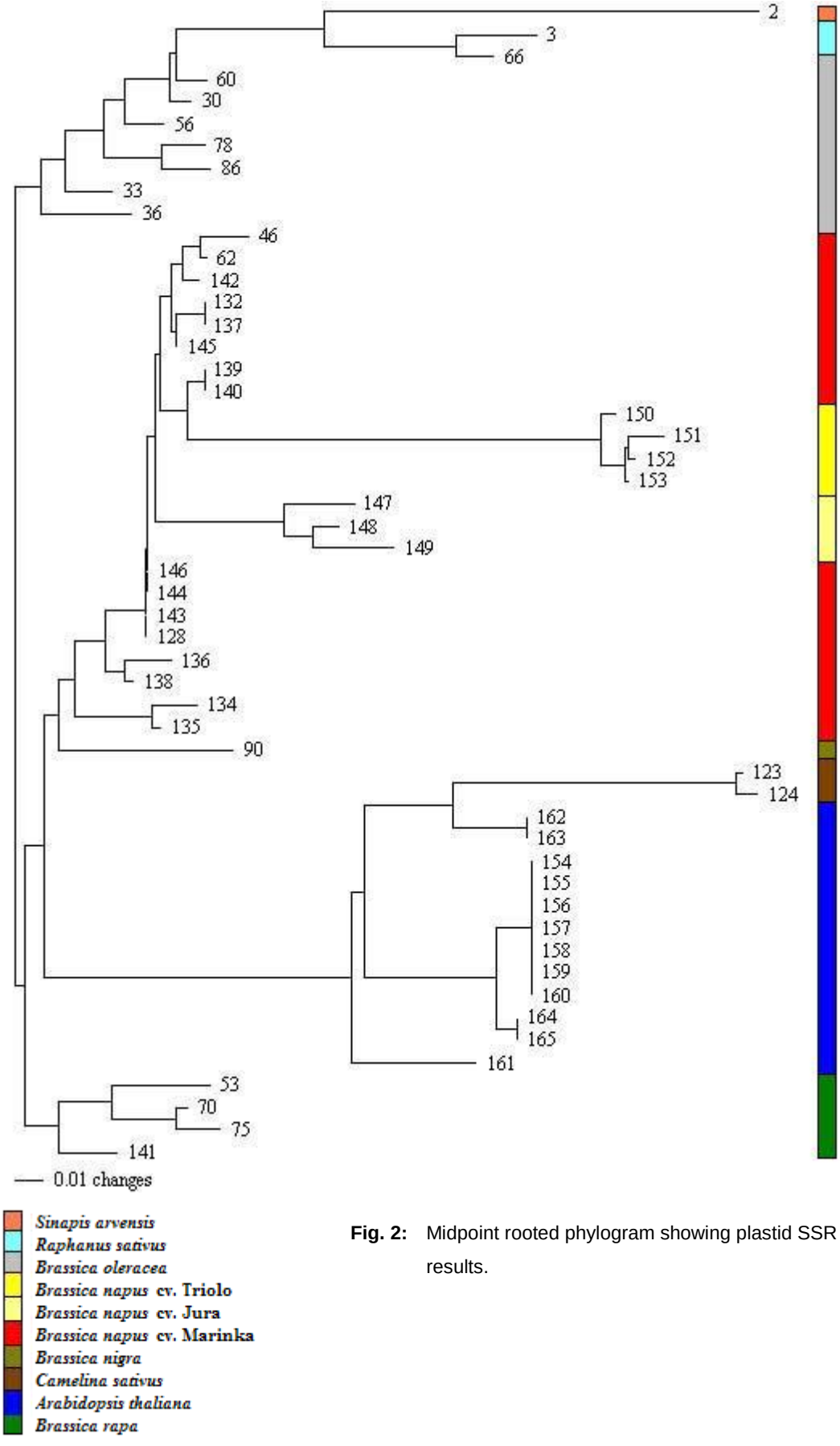
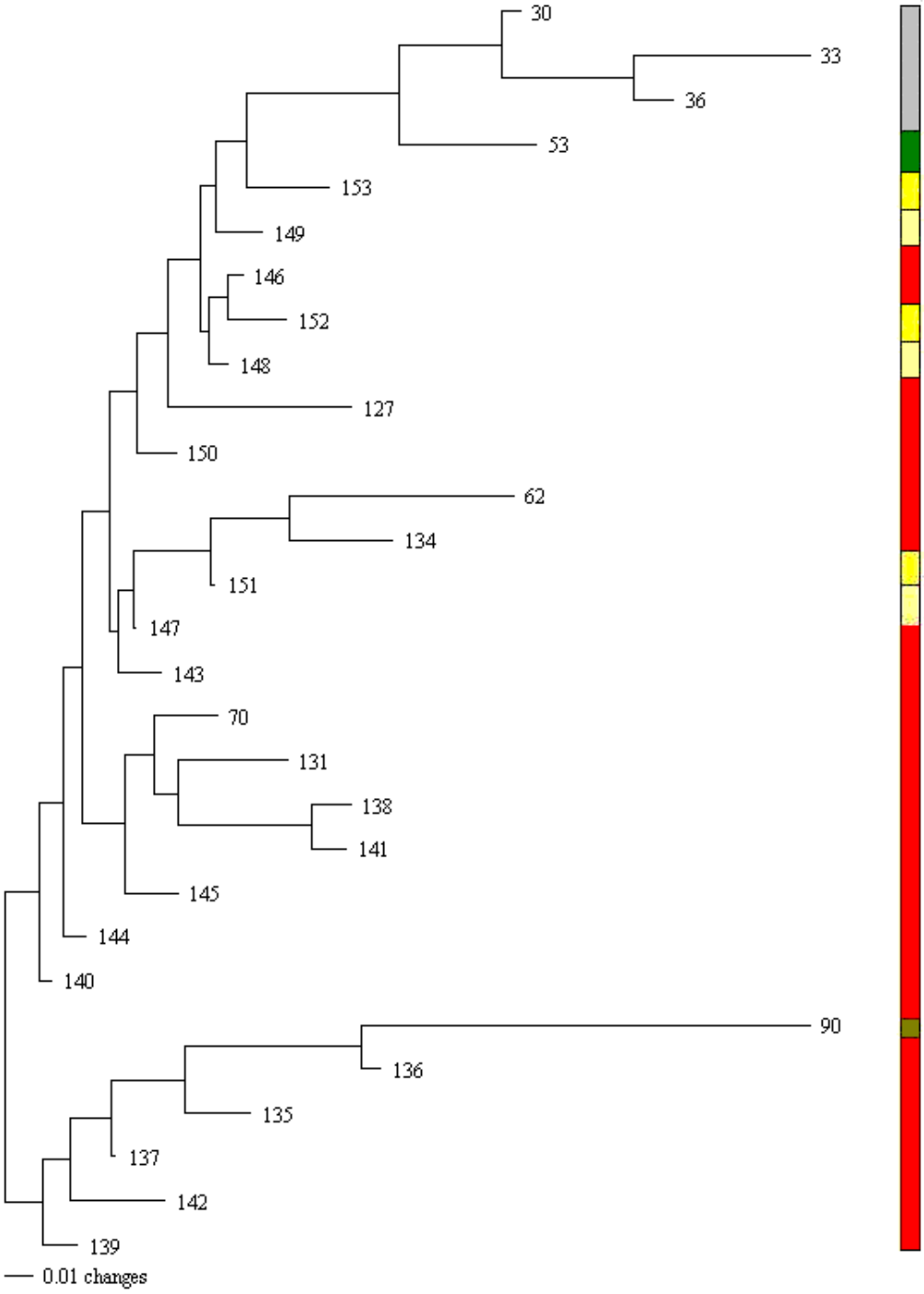


Fig. 2: Midpoint rooted phylogram showing plastid SSR results.



Sinapis arvensis
Raphanus sativus
Brassica oleracea
Brassica napus **cv. Triolo**
Brassica napus **cv. Jura**
Brassica napus **cv. Marinka**
Brassica nigra
Camelina sativus
Arabidopsis thaliana
Brassica rapa

Fig. 3: Midpoint rooted phylogram showing nuclear and plastid SSR results combined.

2. Abiotic pollen dispersal

2.1 Methods

A combination of both volumetric spore traps and passive traps (Fig. 4) were used for detecting airborne pollen. In general, volumetric spore traps generate superior data, as a fixed volume of air is sucked into the trap, which permits the calculation of pollen and spore concentrations. Widespread and replicate use of this type of trap is restricted because a power supply is required and the traps are expensive. The passive trap offers a viable alternative, as it is simple and inexpensive to construct and use, it is portable and does not require any power supply. A modified version of the passive trap developed by Pierre Cour was used for this experiment (Cour, 1974).

Passive traps were used to try to quantify the extent of pollen flux by wind from the *B. napus* cv. Marinka crop. To enable analysis of pollen flow a variety of distances and directions were used. Eight compass directions were used - N, NE, E, SE, S, SW, W and NW. Four distances were used in 2001- 0m, 50m, 100m and 200m. In 2002, the traps were brought closer to the field to analyse in more detail the decrease in pollen flow between 0m and 50m (0m, 12.5m, 25m and 50m in 2002). Since 32 traps were needed, materials for their construction were chosen on a low cost basis. Pollen fluxes were measured and converted into concentration by comparing the results of each type of trap and adjusting the passive trap results accordingly.

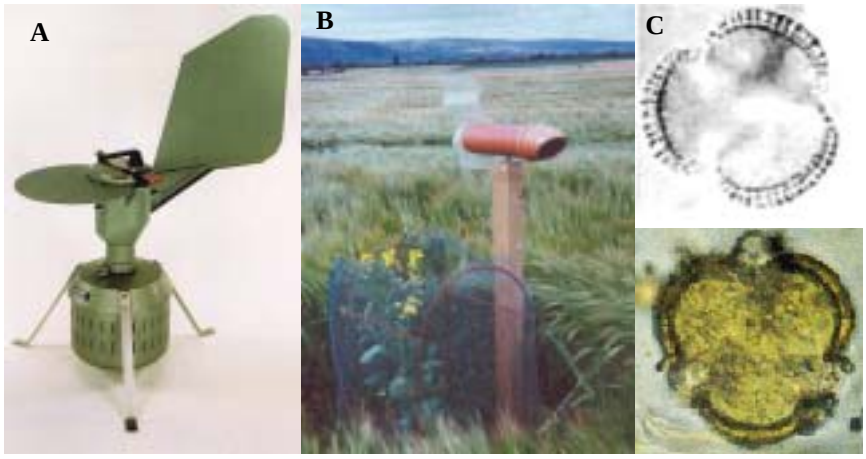


Fig. 4: A= Volumetric spore trap (Burkard, 2001), B= passive trap, C) *B. napus* pollen grain (diameter = 30µm) (Reille, 1992) and D) *B. napus* pollen grain (x400).

2.2 Results

SSR analysis was not performed on pollen grains to confirm their origin was the source crop due to time constraints, however an effort was made to rid the surrounding area of wild relatives, so for this part of the study it was therefore assumed that the Brassicaceae pollen detected originated from the crop and was *B. napus* cv. Marinka. Arcview GIS 3.2 (Environmental Systems Research Institute Inc.) was used to generate charts for presentation purposes. Fig. 5A represents the actual layout of the traps in the field in Oak Park taking the source crop as the centre point, and shows the exponential increase in distance between passive traps. However, to ease data manipulation in Arcview GIS, the chart in Fig. 5B was used for display purposes. Fig. 6 shows Brassicaceae pollen collected in 2001, while Fig. 7 shows 2002 data. Brassicaceae pollen was detected in the outermost traps (200m) to the north-east of the source, which is consistent with prevailing winds.

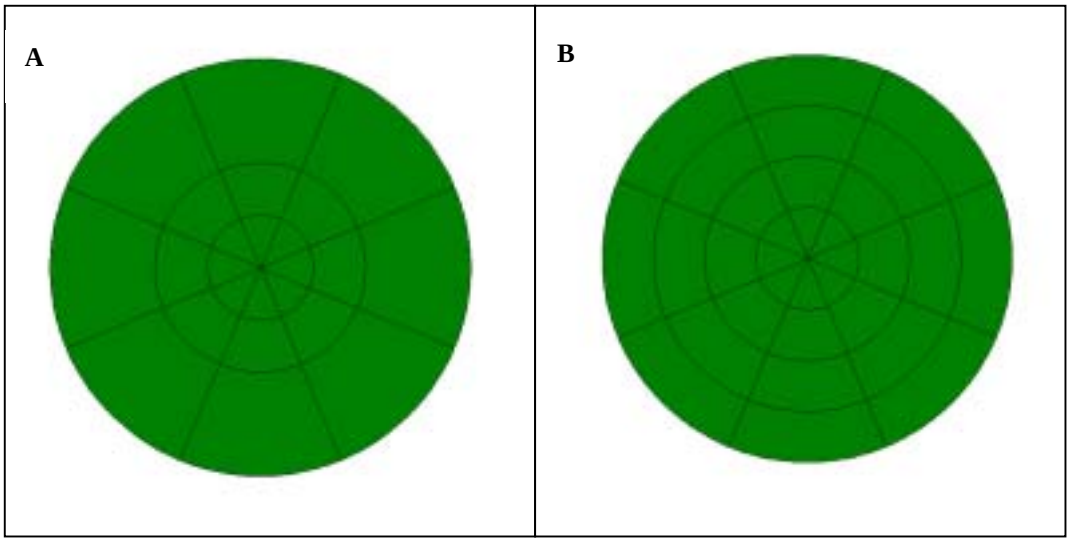


Fig. 5: Arcview GIS charts (A) actual layout and (B) layout used for display purposes.

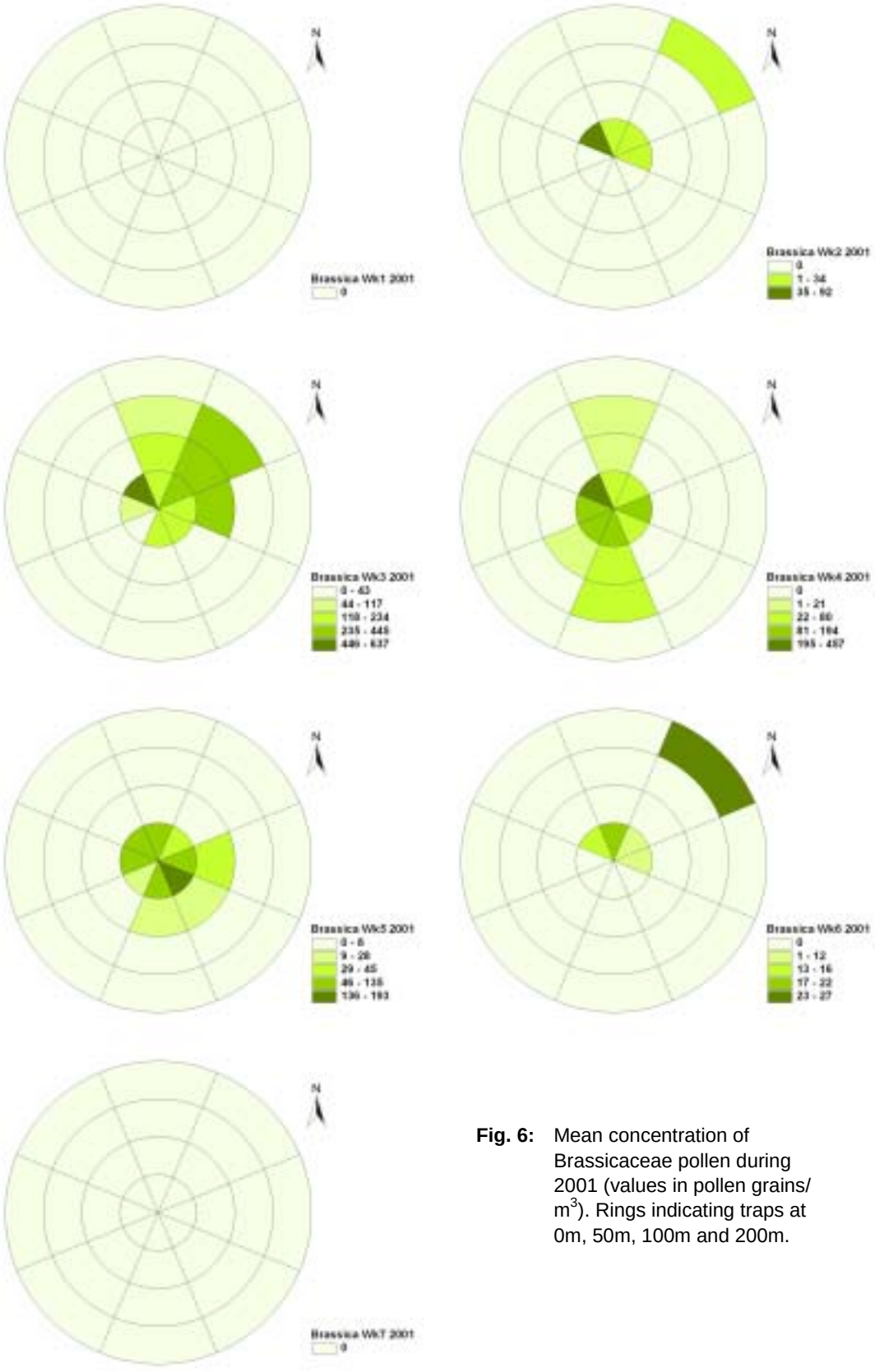


Fig. 6: Mean concentration of Brassicaceae pollen during 2001 (values in pollen grains/ m^3). Rings indicating traps at 0m, 50m, 100m and 200m.

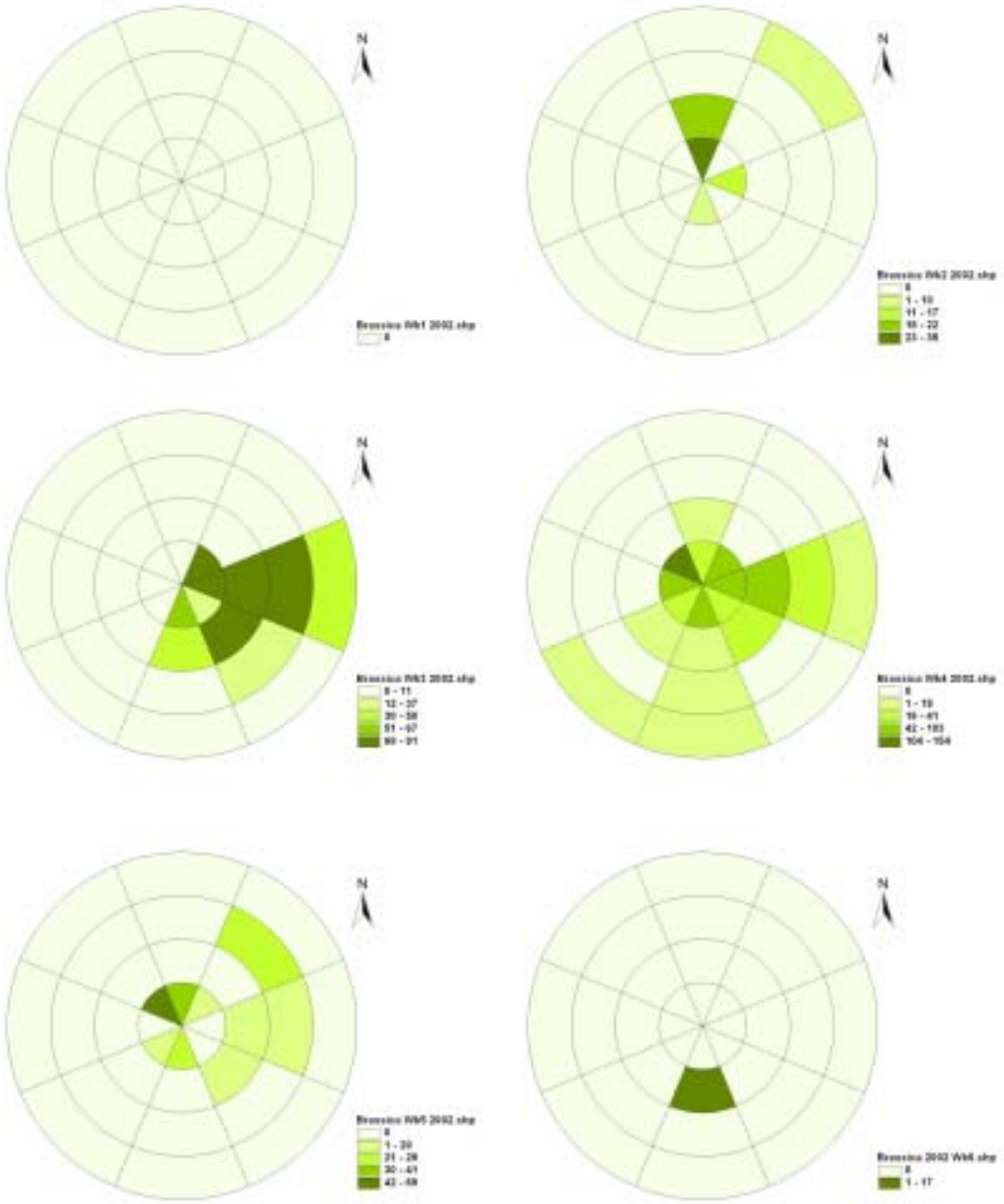


Fig. 7: Mean concentration of Brassicaceae pollen during 2002 (values in pollen grains/m^3). Rings indicating traps at 0m, 12.5m, 25m and 50m.

3. Biotic pollen dispersal

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3.1 Methods

Insects, particularly honeybees (*Apis mellifera*) and bumblebees (*Bombus* species) are believed to play a major role in the transfer of pollen over long distances. Observations of honeybee colonies determined that these insects switch from one forage type to another and that bees carrying many viable oilseed rape pollen grains could be found emerging from a hive (Ramsay *et al.*, 1999). Therefore, to examine the distance over which pollen may be transferred by bees, traps which remove pollen pellets from the bee's legs as it enters the hive were placed on five hives at distances of 100m, 200m, 400m, 800m and 1600m from the source crop.

3.2 Results

In 2001, 35 pollen pellet samples were collected, i.e. five hives for seven weeks and in 2002, 30 pollen pellet samples were collected, i.e. 5 hives for six weeks. The pollen pellet samples were dried in silica gel. The colour and size of pollen loads are useful characters in pollen species identification, therefore microscopic analysis of the pellets was performed to identify the pollen and so determine colour variations associated with silica gel dried Brassicaceae pollen pellets. One hundred pollen pellets were selected at random from each sample. Therefore a sub-sample of 6500 pellets was taken and pellets were separated according to colour (Fig. 8). DNA was extracted using a Sigma REDextract plant DNA extraction kit (Sigma Aldrich) and SSR analysis was performed on 5% of the sub-samples to eliminate non-*Brassica napus* cv. Marinka pellets.

Fig. 8: Bee pellets separated into jars according to colour.

High concentrations of *Brassica napus* cv. Marinka pollen were found in all of the samples tested. SSR analysis however implied that the pellets were not entirely pure, as they contained markers consistent with *B. napus* cv. Marinka and also either *Arabidopsis thaliana* or *Camelina sativus*.

Fig. 9 and Fig. 10 show the mean weight of pollen pellets collected from each hive. A regression line has been added to each graph to demonstrate the distance required to isolate a *B. napus* crop from biotic pollen dispersal.

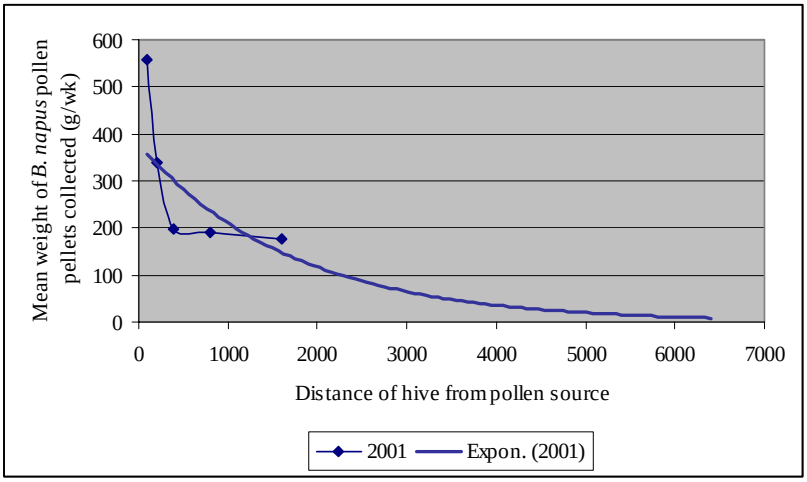

Fig. 9: Graph of distance travelled by insect dispersed pollen in 2001.

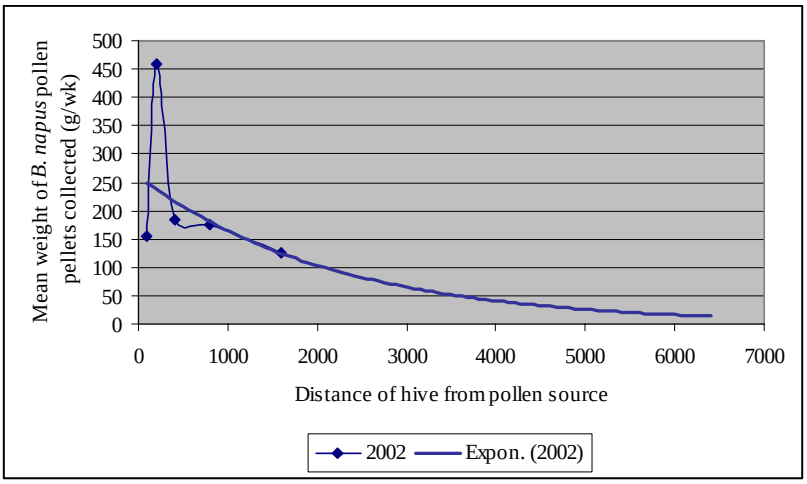

Fig. 10: Graph of distance travelled by insect dispersed pollen in 2002.

4. Biotic/abiotic pollen dispersal

4.1 Methods

In 2001 and 2002, six male sterile bait plants were planted beside each passive trap, enclosed in a frame and chicken wire to prevent pest damage. At the end of the flowering season, the MS plants were harvested and dried, and the F1 seed produced was collected and weighed. Sub-samples of 20 seeds were selected from each F1 plant to be germinated and grown out in five-inch pots in the Trinity College Botanic Garden, Dartry, Dublin. Seedlings were re-potted after 3 weeks and harvested after a further 3 weeks. DNA was extracted using a Sigma REDextract plant DNA extraction kit (Sigma Aldrich) and SSR analysis was performed.

4.2 Results

Thirty-two samples were collected in 2001 and 16 in 2002 (seed from different individuals was kept separate).

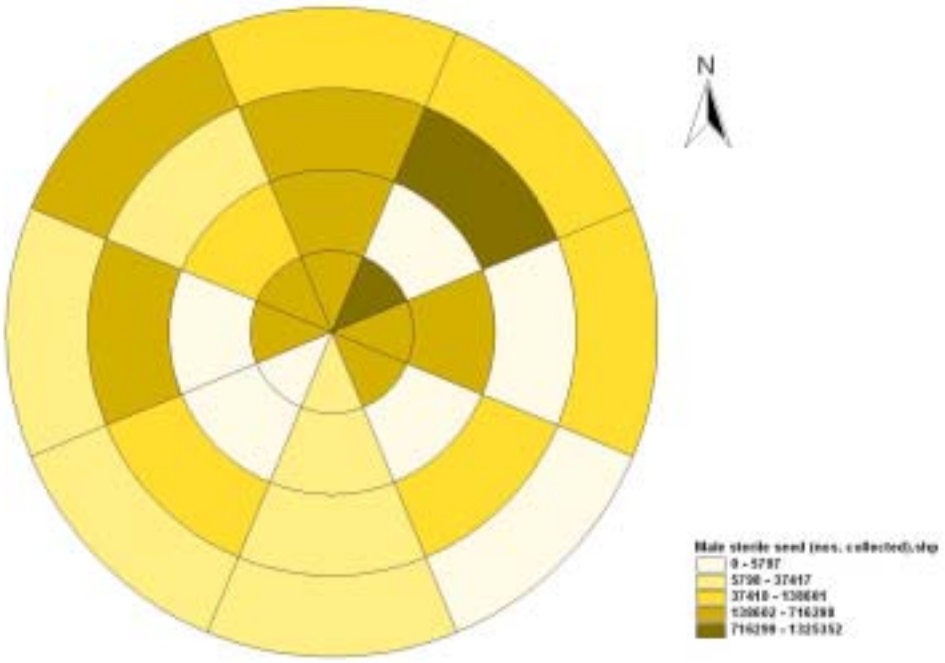

Fig. 11: Number of seed collected from MS *Brassica napus* cv. Triolo (2001).

No pattern between distance and direction from the source crop was detected from the amount of seed collected (Fig. 11). SSR analysis was then performed using the 12A primer (Szewc-McFadden *et al.*, 1996) as a band present at 318bps in *B. napus* cv. Marinka was absent in *B. napus* cv. Triolo MS (MS) parent plants (Fig. 12).

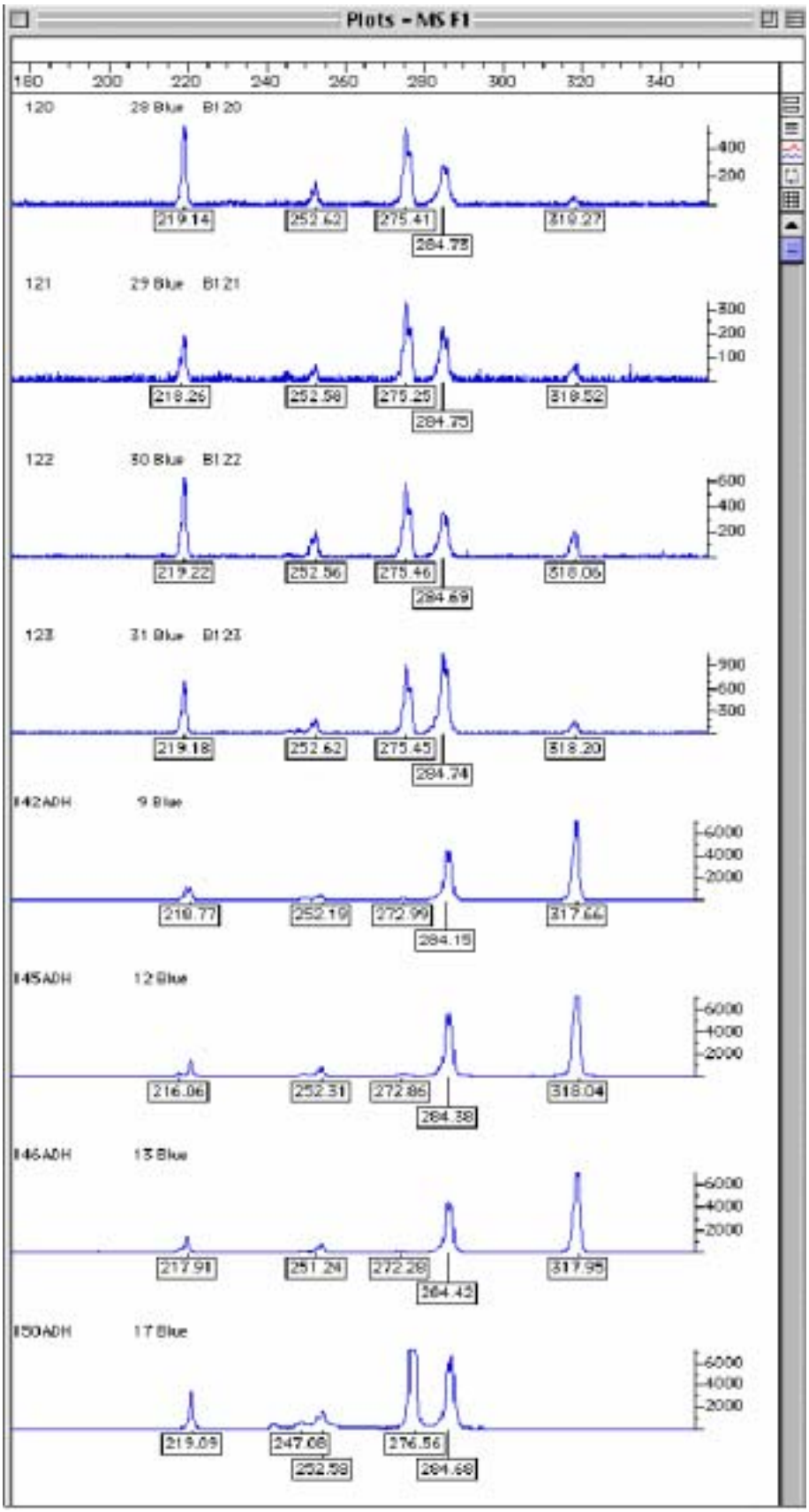

Fig. 12: Banding patterns obtained using primer 12A. 120-124 are bee pollen pellets; 142, 145 and 146 are *B. napus* cv. Marinka and 150 is *B. napus* cv. Triolo.

This band was also found in many of the F1 and therefore when present the progeny were considered to be *B. napus* cv. Triolo x *B. napus* cv. Marinka hybrids. Therefore, from a total of 188 F1 seeds tested, 49 samples shared the same banding pattern as the *B. napus* cv. Triolo MS, while the remaining 139 shared the same banding pattern as the *B. napus* cv. Marinka.

DISCUSSION

Abiotic pollen dispersal results demonstrate that airborne *B. napus* pollen is travelling at least 200m from the source crop. Although this may only be in small quantities there is nevertheless reason for concern. This is especially significant when the results from the male sterile bait plants are taken into account, as set seed was observed at all distances tested, indicating the potential for gene flow.

Seed set in male sterile *B. napus* plants confirms the potential for hybridisation to occur with feral or volunteer populations at crop margins or in the general vicinity of a GM crop. The method of pollination should also be considered. Results from abiotic dispersal experiments suggest that airborne pollen is travelling sufficiently far to effect fertilisation at considerable distances from the crop, however it has been suggested that *B. napus* flowers are ineffective at capturing airborne pollen (Cresswell *et al.*, unpublished). This implies that insects may be the primary vectors in *B. napus* pollen dispersal.

The presence of *B. napus* cv. Marinka pollen in bee pellets taken from beehives at 1.6km from the field indicates that pollen is moving over large distances. This is consistent with observations made by Ramsay *et al.* (1999) that bees carrying many viable oilseed rape pollen grains could be found emerging from a hive. Therefore with foraging up to 1.6km in all directions from a hive, some pollen transfer and fertilisation up to 3.2km can be expected. Similar studies have shown pollen transfer and fertilisation up to 4km could be expected and that bees in a colony in Scotland have been reported to have flown to a crop 5km away (Ramsay *et al.*, 1999), so theoretically there is potential for pollen to be transferred to distances of at least 10km by the mixing of bees foraging in different directions from the same hive.

CONCLUSIONS

Plastid SSRs provide a new opportunity for high-resolution analysis of inter- and intra-specific variation in the Brassicaceae and for the differentiation of its species. Combining data generated from nuclear and plastid SSRs enables the study of seed and pollen movement, and assists in the assessment of gene flow from GM plants through hybridisation studies.

The detection of low levels of *B. napus* pollen at outer distances tested has implications for transgene movement. This combined with the tendency of *Brassica* to hybridise with its wild relatives, points towards transgene establishment in the wild, particularly if the transgene provides a selective advantage. Gene flow can occur between volunteer and feral populations, which can act as gene pools facilitating the contamination of subsequent rape crops (Eastman and Sweet, 2002). Hybridisation and introgression between oilseed rape varieties and wild relatives is therefore likely to occur. It is therefore important to establish whether the

transgene would confer a direct selective advantage in the feral environment as the rate of introgression and its chance of persistence will be dependent on this.

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