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Risk assessment of genetically modified crops in Ireland: Investigations of pollen dispersal from oilseed rape (*Brassica napus* L.) using molecular and pollen trapping techniques

Thesis submitted in fulfilment for the degree of PhD to
University of Dublin, Trinity College
2004

Marie-Louise Flannery BA Sc, MSc, Dip Stat

Declaration

The research was conducted in the Department of Botany, Faculty of Science, University of Dublin, Trinity College; under the supervision of Dr. Fraser Mitchell, Dr. Trevor Hodkinson, Dr. Paul Dowding and Dr. Jimmy Burke of Teagasc, Oak Park, Carlow.

This thesis has not been submitted as an exercise for a degree at any other university. Except where stated, the work described therein was carried out by me alone.

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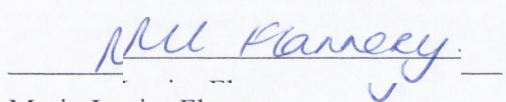

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Finally, to my family for their support and understanding, the girls for their belief in me and Martin for always being there….

Abbreviations

AFLP	Amplified Fragment Length Polymorphism
approx.	Approximately
ATP	Adenine triphosphate
atpB	Beta subunit of chloroplast ATP synthase gene
atpB-rbcL spacer	Non-coding spacer region lying between *atpB* and *rbcL*
bp	Base pairs or number of nucleotides
CI	24:1 Chloroform: isoamyl alcohol
conc.	Concentrated
cpDNA	Chloroplast DNA
CTAB	Cationic Hexadecyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTPs	2'-Deoxynucleotide 5'-triphosphates i.e. dATP, dCTP, dGTP, dTTP
EDTA	Ethylene Diamine Tetra Acetic acid
et al.	and others
FOV	Field of view
H2SO4	Sulphuric acid
in silico	By computer
ITS	Internal transcribed spacer (1 or 2) of 5.8S rDNA
KOH	Potassium hydroxide
Loading dye	0.25% Bromophenol blue and 40% sucrose at pH 8
$MgCl_2$	Magnesium Chloride
Mins	Minutes
PCR	Polymerase Chain Reaction
rbcL	Large subunit of ribulose-1, 5-bisphosphate carboxylase
RFLP	Restriction Fragment Length Polymorphism
SSR	Simple Sequence Repeat
SUW	Sterile Ultrapure Water
TBA	Tertiary butyl alcohol
TBE	Tris Boric acid EDTA: Trisma base, Boric acid, Sodium Ethylene diamine tetra acetic acid
tDNA	Total DNA

TE	Tris HCl and Ethylenediaminetetra-Acetic acid
Tectum	Outer layer of tectate pollen grains
Temp	Temperature
trnF	tRNA-Phe gene
trnL	tRNA-Leu gene
trnT	tRNA-Thr gene
UPW	Ultrapure water
VST	Volumetric spore trap
°C	Degrees Centigrade
10xBuffer	Thermophillic buffer: 10mM Tris-HCl pH 9, 500Mm KCl, 1.0% Triton X-100.
18S rDNA	Nuclear ribosomal gene coding for the 18S subunit
5.8S rDNA	Nuclear ribosomal gene coding for the 5.8S subunit

Abstract

A risk assessment was carried out to help determine the extent of pollen flow from a crop of oilseed rape (OSR, *Brassica napus* L.). The project aimed to develop a better understanding of the potential risks of gene flow from GM *Brassica* in Ireland by examining biotic (insect) and abiotic (wind) pollen dispersal over two seasons. A non-GM cultivar was used for this purpose and was assumed to have a similar pollen dispersal potential to GM cultivars

Microscopic examination of pollen is inadequate for identification of *Brassica* pollen to the species or intraspecific level due to the similarity of different grains. Therefore, molecular markers were developed to differentiate cultivated *B. napus* types (subspecies, varieties and cultivars) from each other and from their wild relatives. Approximately 404 *Brassica* nuclear microsatellite (SSR) primers are currently publicly available, however prior to this thesis no plastid SSR (chloroplast SSR) primers were available specifically for *Brassica*. As the chloroplast genome is uniparentally inherited, different variation patterns to those exhibited by nuclear markers are detected. This is useful when analysing polyploid Brassicaceae, as nuclear SSR fingerprints are more difficult to interpret than haploid plastid fingerprints. Ten plastid SSR primer sets were therefore developed from sequence data of *B. napus* and closely related species, nine of which were found to detect inter- and intraspecific polymorphism when tested on a broad range of Brassicaceae material. The successful development of plastid SSRs provides 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 will assist in the assessment of gene flow from GM plants through hybridisation studies.

Abiotic pollen dispersal was measured using a combination of a single volumetric spore trap and multiple passive traps. Passive traps based on the Cour trap (Cour, 1974) were successfully developed and tested. These incorporated modifications to allow DNA extraction of the pollen collected and protection from the rain. In addition, protocols were devised to remove pollen from the trapping surface and to extract DNA. The utility of these were demonstrated for pollen dispersal studies and for successful DNA extraction and amplification.

To enable analysis of pollen flux by wind from a *B. napus* cv. Marinka crop, traps at a variety of distances (0m, 50m, 100m and 200m in 2001 and 0m, 12.5m,

25m and 50m in 2002) and directions (N, NE, E, SE, S, SW, W and NW) were used. Low concentrations of *B. napus* cv. Marinka pollen were detected at all distances. However, results demonstrate that airborne *B. napus* pollen travelled at least 200m from the source crop. Although this may only be in small quantities there is nevertheless reason for concern.

Meteorological data were also gathered from an automatic weather station on the site. These data were analysed with the biotic pollen data and demonstrated that biotic pollen dispersal coincides with weather conditions including warm temperatures, sunshine, light to medium winds and, most importantly, no rain.

Biotic pollen dispersal was measured by collecting pollen from beehives along a transect at distances of 100m, 200m, 400m, 800m and 1600m from the source crop. As with the passive trap material, a DNA extraction protocol for pollen pellets was devised and successfully applied. The presence of *B. napus* cv. Marinka pollen, identified by a combination of microscope and DNA analyses, in bee pellets taken from beehives at 1.6km from the field indicates that pollen moved over large distances. With foraging up to 1.6km in all directions from a hive, and by taking the hive as a centre point, the diameter of the risk area becomes 3.2km. In addition, extrapolated distances (from regression curves) imply some pollen transfer and fertilisation could occur up to 6.5km.

Male sterile bait plants were planted beside each passive trap for each growing season to detect pollen flow via seed set. The F1 progeny were tested using nuclear SSR analysis. Sixty-four percent of these showed a proportion of markers from the source crop, thus indicating *B. napus* inter-varietal hybridisation and confirming the potential for hybridisation to occur with feral or volunteer populations at crop margins or in the general vicinity of a GM crop. Seed was set in all MS bait traps, the outer one of which was at 200 metres. Gene flow was therefore implied over this distance from the source crop.

In conclusion, the detection of low levels of *B. napus* pollen at outer distances tested, by both wind and insect pollen traps, has implications for transgene movement. The study has shown that pollen dispersal over 3.2km is likely and that it could be extrapolated to 6.5km. Gene flow detected by male sterile bait plants occurs over distances of at least 200m. It is therefore important to establish whether the transgenes, moving these distances will pollinate plants and if gene flow occurs

whether this 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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Chapter 1
Introduction

1.1 Introduction to genetically modified plants and risk assessment

1.1.1 Genetically modified plants

For centuries humans have prioritised the selection, sowing and harvesting of seeds that produce food, in order to sustain them. Global food demand has increased the need for improved crops and as a result plant breeders have exploited germplasms available to them through selective breeding and hybridisation. Genetic modification is an extension of traditional plant breeding. However unlike traditional plant breeding where hundreds of thousands of genes are crossed (Stewart and Wheaton, 2003), this technology allows for the transfer of one or more desirable genes i.e. the genetic composition of a plant is altered through the addition of a transgene. This enables the transfer of a greater variety of genetic information from a broad range of sources in a more controlled, precise way. In other words, by allowing breeders to alter one characteristic at a time, the long periods needed in the past to develop the best yielding, hardiest, best-tasting crop are often no longer required. Genetic modification therefore offers technology capable of producing more nutritious, better tasting, higher yielding, crops that are not only protected from disease and insect attack, but are drought and frost tolerant, and need fewer agrochemical inputs (Land and Farquhar, 1998).

There are a large number of genetically modified crops currently in the research and development phase for the modification of a range of traits (Table 1.1). Traits have been categorised as output, input and marker traits by Lheureux *et al.* (2003). Output traits include resistance to herbicide, insect and other plant pathogens, along with abiotic stress tolerance and increased yield. Input genes describe traits conferring modified ingredient or nutrient composition e.g. increased oil content of oilseed rape seeds, or those enabling the production of industrial or health related compounds e.g. edible vaccines. Others involve gene markers and research for new modification methods. There are a variety of crops that these traits are being applied to (Figure 1.1), in particular model plants such as *Arabidopsis thaliana*, tobacco, potato and tomato (Lheureux *et al.*, 2003). The global area of oilseed rape in 2002 was 25 million hectares (mha), ranking it behind maize (140mha), soybean (72mha) and cotton (34mha) (James, 2003).

Table 1.1: The main traits used for genetic modification (Lheureux *et al.*, 2003)

Trait	Examples of characteristics conferred
Herbicide tolerance	Asulam, bromoxynil, dalapon, gluphosinate, glyphosate, isoxa-flutole, sulphonylurea tolerance
Insect resistance	Bt-derived insect resistance, chymotrypsin protease inhibitor synthesis, potato trypsin protease inhibitor synthesis
Pathogen resistance	
Fungi resistance	Chitinase synthesis, *Fusarium* spp. resistance *Phythophthora infestans* resistance
Bacteria resistance	Expression of anti-microbial proteins, oligogalacturonase lyase synthesis
Nematode resistance	Nematode resistance
Virus resistance	Alfalfa mosaic virus resistance, cucumber mosaic virus resistance, lettuce mosaic virus resistance, maize dwarf mosaic virus resistance
Abiotic stress/yield	
Resistance to abiotic stress	Bruising resistance, drought tolerance
Yield influencing factors	Improved rooting ability, increased yield
Male sterility	Cytoplasmic male sterility
Modified nutrients/ingredients	
Enhancement of nutritional value	Alteration of forage quality, improvement of digestibility, reduction of phosphate pollution
Fatty acid metabolism	Alteration of oil metabolism, high sterate content.
Protein metabolism	Alteration of amino-acid metabolism
Oligosaccharide metabolism	Down-regulation of fructose-6-phosphatase
Starch metabolism	ADP glucose pyrophosphorylase synthesis, alteration of carbohydrate metabolism
Industrial use	
Food processing	High molecular weight glutenin synthesis, improvement of baking quality, improvement of malting quality
Non-food applications	Alteration of lignin biosynthesis, high erucic acid composition
Enzyme production	Alpha-amylase secretion, expression of yeast lipase gene
Health related compounds	
Monoclonal antibodies Phytopharmacies Pharmaceuticals Edible vaccines	Albumin synthesis, antibody synthesis, collagen synthesis, human lactoferrin synthesis, putrescine synthesis
Other output traits	
Modification of colour/form	Alteration of leaf morphology, alteration of pigment production
Modification of ripening	ACC synthase synthesis, auxin synthesis, controlled cell division
Marker traits	Ac/Ds two component transposon system, chlorsulphuron tolerance
Other traits	Cytoplastic exclusion protein synthesis, gene stability testing, gene tagging, increase endogenous hormone level

During the seven-year period 1996 to 2002, there was a 35-fold increase in the global area of transgenic crops, from 1.7mha in 1996 to 58.7mha in 2002 (CropBiotech, 2003). This reflects the highest rate of adoption of a new crop technology to date. During the same period, the number of countries growing transgenic crops more than doubled, increasing from six in 1996 to nine in 1998, to twelve countries in 1999 and to eighteen in 2002, including Argentina, Australia, Bulgaria, Canada, China, Colombia, France, Germany, Honduras, Indonesia, Mexico, Portugal, Romania, South Africa, Spain, Ukraine, Uruguay, and USA (CropBiotech, 2003).

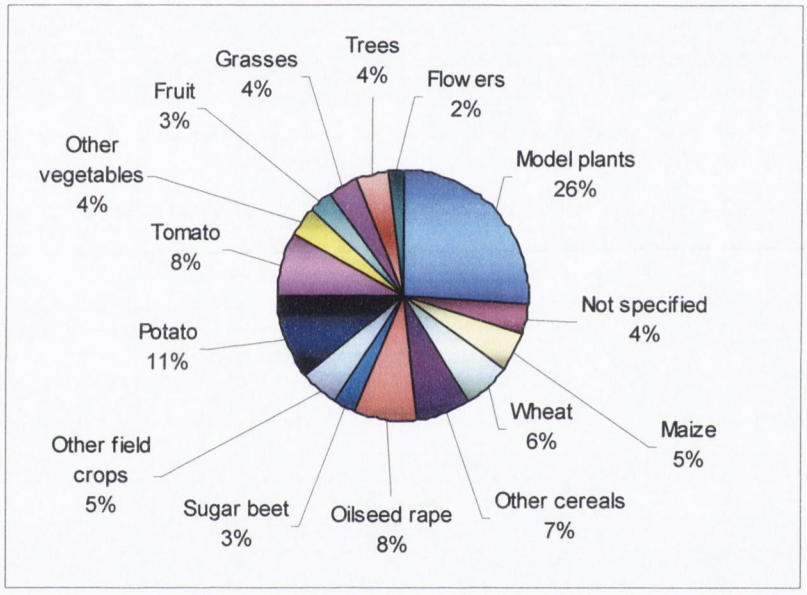

Figure 1.1: Type of GM crops currently in R&D phase in Europe (total number of projects represented = 269) (Lheureux *et al.*, 2003)

GM herbicide tolerant oilseed rape (*Brassica napus*, OSR) is currently the third most commonly cultivated GM crop, with 2.7mha planted globally. In Ireland, GM OSR is not grown commercially. Prior to October 1998, four GM OSR varieties had been approved in the EU under Directive 90/220/EEC (Table 1.2), which must now be renewed under the new Directive 2001/18/EC (section 1.4.2) to avoid discrepancies between consents granted under each Directive (Devos, 2004). A further four are pending approval for environmental release under Directive 2001/18/EC (Table 1.2).

Table 1.2: Oilseed rape (*Brassica napus*) environment and commercial release notifications under Directive 90/220/EEC and Directive 2001/18/EC (European Commission Joint Research Centre, 2004)

Notification number/ State	Publication	Name of the Institutes or Companies	Project title
B/SE/03/6347 Sweden	13/01/2004	BASF Plant Science GmbH	Application for the deliberate release of oilseed rape lines genetically modified for altered oil content in seed.
B/SE/03/6391 Sweden	13/01/2004	Svalöf Weibull AB	Breeding of spring oilseed rape cultivars tolerant to Roundup.
B/GB/03/R38/1 UK	30/07/2003	Bayer CropScience Ltd.	Consent application for a 3 year programme of work to release winter oilseed rape genetically modified for pollen infertility, fertility restoration and herbicide tolerance
B/DE/02/147 Germany	10/03/2003	Bundesanstalt für Züchtungs-forschung an Kulturpflanzen	Production of oilseed rape with genetically modified fatty-acid composition of the storage lipids
C/DE/98/6 Germany	17/02/2003	Bayer CropScience	Glufosinate tolerant Oilseed Rape Liberator pHoe6/Ac
C/DE/96/5 Germany	14/02/2003	Bayer CropScience	Glufosinate tolerant Oilseed Rape Falcon, GS40/90pHoe6/Ac
C/GB/99/M5/2 UK	10/02/2003	Bayer CropScience	Glufosinate Tolerant oilseed rape T45
C/BE/96/01 Belgium	25/02/2004	Bayer BioScience	Oilseed rape Ms8xRf3
C/NL/98/11 Netherlands	22/01/2003	Monsanto	Roundup Ready (glyphosate tolerant) oilseed rape, event GT73

In Europe, there have been 365 releases of GM OSR; 50 in Belgium, 39 in Germany, four in Denmark, three in Spain, two in Finland, 116 in France, 106 in the U.K., four in Italy, 15 in the Netherlands and 26 in Sweden (European Commission Joint Research Centre, 2004).

1.1.2 Introduction to risk assessment of GMOs

'Risk' can be defined as the consequences of an event multiplied by the likelihood of that event happening (*risk = f (hazard, exposure*) (GM Science Review Panel, 2003; Wilkinson *et al.*, 2003) i.e. types of hazard should be defined and quantified according to threat level, while exposure methods should be categorised. Risk is also used as a way of defining the likelihood of something going wrong (Robinson, 2003). A risk assessment, therefore, comprises hazard identification, hazard characterisation, exposure assessment and risk characterisation (Scientific Committees on Plants Food and Animal Nutrition, 2003).

Awareness of the deleterious effects of human activity has historically forced societies to use risk assessment as a means of improving the environments in which they live by allocating scarce resources carefully. Formal risk assessments were first practiced as a means of improving public health through sewer construction (Brown, 2003) and chlorination of drinking water, and expanded to encompass workplace health and safety, food and drug regulation and consumer product safety and has most recently been expanded to adopt environmental protection programs. Therefore, ecological risk assessment is not a new phenomenon and draws on much of the experience and analytical methods developed for addressing earlier risk-related issues. Ecological risk assessment has inherited a range of techniques developed specifically for describing the environment and/or the consequences of human action on it including, descriptive field ecology, natural resource management methods, environmental impact assessment techniques, disease epidemiology, and environmental economics and engineering (Power and Mc Carty, 2002).

The use of transgenic plants in breeding programs may enable the utilisation of a wide variety of novel genes from unrelated organisms that would otherwise be unavailable. Due to 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). Undesirable phenotypes are occasionally produced in conventional plant breeding e.g. high glycoalkaloids in potatoes and high glucosinolates in *Brassica*; and

procedures have been developed in breeding programmes to eliminate variants of this kind (Dale and Irwin, 1995). In the same way, transgenic plants are initially evaluated in the laboratory, growth room and contained glasshouse to examine the plant phenotype, and molecular analysis is also carried out to analyse the copy number and integrity of the transgene inserted (Lheureux *et al.*, 2003). However, measurement of gene flow is not possible in the laboratory as it is a complex process and is dependent on many factors including environmental conditions, plant variety, insect behaviour and plant density. Studies on pollen movement have indicated that laboratory and small-scale experiments may not necessarily predict pollination under commercial conditions (Rieger *et al.*, 2002). Comparative analysis in the field is therefore necessary (European Commission, 2003).

There are a number of potential risks of GM crops. In terms of human health, the development of allergens in both food products and pollen (hay-fever) are among the risks, along with the movement of viral resistance to new host organisms (Dale and Irwin, 1995). Alternatively, the escape of genes which could confer a selective advantage including greater persistence or invasiveness under field conditions; improved tolerance to environmental stress e.g. drought tolerance, salt tolerance, high temperature, heavy metal resistance. The Advisory Committee on Releases to the Environment (1998) have put together specific risks of e.g. herbicide, pesticide or insecticide tolerance including the risk that:

- tolerance could confer a competitive advantage to the GM crop that may increase potential weediness in agricultural environments as a volunteer, and invasiveness in semi-natural habitats as feral populations;
- the insertion of a tolerance gene could result in pleiotropic effects in the genome which results in a phenotype with more vigorous growth and reproduction, resulting in increased weediness or invasiveness;
- tolerance in the crop could compromise the use of the herbicide if it is tolerant to the active ingredient is an important component of a weed control strategy for volunteers in following crops;
- gene transfer from the tolerant crop to sexually compatible weed species could occur, resulting in hybrids that then become more difficult to control in agriculture or semi-natural habitats;
- gene transfer could occur between sexually compatible GM crops with tolerance to different e.g. herbicides resulting in hybrids with tolerance

to more than one herbicide – 'multiple tolerance' - which could be more difficult to control than plants with tolerance to one herbicide;

- seeds of GM crops with tolerance to different herbicides may be sown in the same field in subsequent years, resulting in the build up of a seed bank with tolerance to more than one herbicide (Advisory Committee on Releases to the Environment, 1998).

Risk assessment aims to encompass the following:

- **Molecular characterisation**
  - Information on the donor and recipient organisms
  - Method used for the genetic modification
  - Information on the DNA used in transformation
  - Information on the sequences actually inserted/deleted
  - Information on the expression of the insert
  - Information on inheritance and stability
- **Comparative analysis**
  - Choice of the comparator
  - Field trials
  - Selection of compounds for analysis
  - Agronomic traits
- **Environmental risk assessment**
  - Geographical relevance of data
  - Impact on wild plants
  - Impact on non-modified crops
  - Impact on organisms and ecological processes
- **Nutritional assessment of GM food/feed**

GM *Brassica* varieties represent approx. 8% of GM crops currently in the research and development stage (Lheureux *et al.*, 2003). Their biology and taxonomy are discussed in section 1.4.

1.2 Regulation of GMOs

1.2.1 EU regulation of GMOs

The Scientific Committees on Plants, Food and Animal Nutrition (2003) developed a platform of levels starting with performance and quality evaluations and continuing through to metabolic components for identifying possible unintended effects, and thus assess risk, of GMOs. In each case, key macro- and micro-nutrients, toxicants, anti-nutritional compounds, and other constituents (including moisture and total ash) should be determined. Key nutrients are the major components of human and domesticated animals' diets, i.e. proteins, carbohydrates, lipids/fats, fibre, vitamins and minerals (Scientific Committees on Plants Food and Animal Nutrition, 2003). The specific analyses required depend on the plant species examined, but include a detailed assessment appropriate to the intention of the genetic modification, the considered nutritional value and use of the plant. For example, a fatty acid profile would be included for oil-rich plants (main individual saturated, mono-unsaturated and poly-unsaturated fatty acids) and an amino acid profile (individual protein amino acids and main non-protein amino acids) for plants used as important protein sources. Key toxicants are those compounds, inherently present, whose toxic potency and levels may harm human/animal health (Scientific Committees on Plants Food and Animal Nutrition, 2003). Compositional analyses therefore, represent key components of the risk assessment process, however agronomic trait analyses are also very important. This is because the manifestation of unintended effects may only occur subsequent to deliberate release e.g. altered susceptibility to important pests and diseases, morphological and developmental changes, or even through modified responses to agronomic and crop management regimes (Scientific Committees on Plants Food and Animal Nutrition, 2003).

In the EU, there are extensive regulations that apply to the safety and use of GM crops and their products. Detailed proposals to release GM crops into the environment or to use them for human food or animal feed are considered by statutory advisory committees (Table 1.3). EU directives on the use of GM crops require a detailed science-based risk assessment (Figure 1.2), and therefore each proposal is considered on an individual, case-by-case basis (GM Science Review Panel, 2003).

The GM Science Review Panel (2003) explains that limited duration consents are issued under specific conditions that may also include monitoring

requirements. Consents can be withdrawn if regulatory examination of subsequent evidence finds the product unsafe or insufficiently safe. Conditions are attached to the consent and the consent holder is expected to adhere to good practice and regulatory compliance.

1.2.2 Regulation of GMOs in Ireland

The regulatory process is dynamic, continuous (in that no approval is absolute, it is always under review to take account of advances in science and technology and prevailing knowledge), subject to critical challenge and continuously subject to improvement (Joint Regulatory Authority, 2003). There are currently four laws controlling the release of GM plants and products into the environment (Table 1.3).

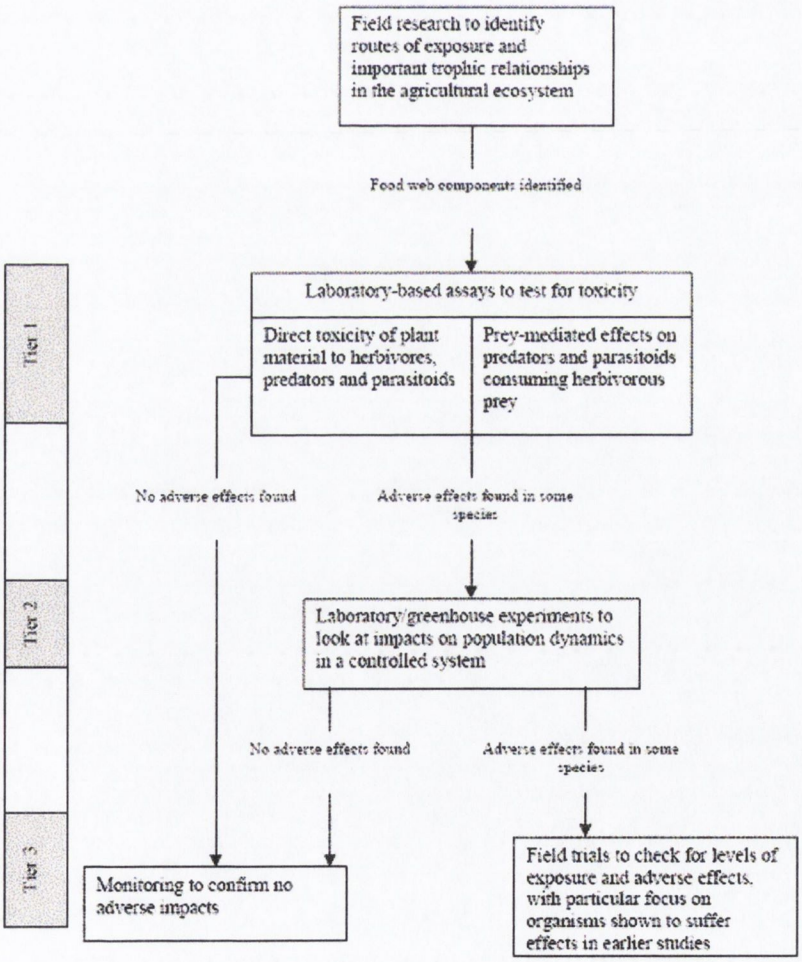

Figure 1.2: Example of a three tiered risk-assessment procedure (GM Science Review Panel, 2003)

Table 1.3: EU laws pertaining to GMOs

Name	Function	Responsible department
Directive 2001/18/EC	Deliberate release of GMOs into the environment	Dept. Environment and Local Government
EC Regulation 1829/2003	GM Food and Feed	Food-Dept. Health and Children Feed-Dept. Agriculture and Food
EC Regulation 1830/2003	Labelling and Traceability of GM products	Dept. Environment and Local Government
EC Regulation 1946/2003	Transboundary movement of GMOs	Dept. Environment and Local Government

Directive 2001/18/EC covers the deliberate release of GMOs into the environment. It also requires that GMOs placed on the market in any form be labelled. Directive 2001/18/EC replaced EC Regulation 90/220/EEC (The Council of The European Communities, 1990) as it introduces a number of standards not previously covered, in particular:

- Principles governing environmental risk assessment
- Mandatory post-market monitoring, including monitoring of possible long-term effects on the environment
- A requirement to communicate information to the public
- A requirement for Member States to ensure labelling and traceability at all stages of marketing
- A requirement that initial approvals of GMOs be limited to a maximum of ten years
- Obligatory consultation of the Scientific Committee(s)
- An obligation to consult the European Parliament on decisions to authorise the release of GMOs (Joint Regulatory Authority, 2003).

Therefore a detailed report, accompanied by a risk assessment (in relation to health and the environment) and a monitoring plan (pre-, during, and post-release) must be submitted to the Environmental Protection Agency (EPA). In Ireland, the EPA then evaluates the report and assesses any risks in relation to health and the environment. All other member states are notified and their views are put together. If there are no objections the EPA grants approval and informs the Commission and

all Member States. Alternatively, if there is an objection the application is referred to the European Food Safety Authority (EFSA) for risk assessment. If the EFSA returns a positive risk assessment report, the application is submitted to the Regulatory Committee, who will vote on the application. An official from the Department of Environment and Local Government represents Ireland on the Regulatory Committee.

In the EU, there is currently a five-year moratorium in operation on the approval of new GM crops and GM foods. However, to date the general position taken by Ireland's government departments on GMOs is: 'positive but precautionary' (Report of the Interdepartmental Group on Modern Biotechnology, Oct. 2000). The Department of Environment and Local Government has the lead role on policy issues but other Government Departments and Agencies are also involved (Table 1.4).

Table 1.4: Other government departments and agencies involved in GMO regulation

Responsible Department	Activity
Department of Environment and Local Government (DELG), through the EPA.	Evaluation of new GMOs and their release for R&D purposes and placing on the market in this country.
Department of Agriculture and Food (DAF) - following approval by DELG.	Regulation of the marketing of GM plant varieties in Ireland.
Department of Agriculture and Food based on EU Commission guidelines.	Rules governing the co-existence of GM crops with non GM crops and wild relatives to avoid cross contamination.
Department of Health and Children - through the Food Safety Authority of Ireland.	Rules on labelling and production of GM food.
Department of Agriculture and Food.	Regulation of the labelling of animal feed containing GM material.

1.3 Risk assessment of genetically modified crops

1.3.1 Risk of gene flow within and between genetically modified crops

A relatively new outlook among biologists is that gene flow is not as highly restricted as some plant evolutionists would perceive it, or extensive as some forest geneticists would, but is idiosyncratic, ranging from very low to very high, varying among species, populations, individual plants and even over a season (Ellstrand, 1992). The extent of gene flow to or from GM varieties is particularly dependent on the species involved, the location, the presence of outcrossing with natural relatives,

the nature of the introduced trait and the environmental consequences of neutral traits (Daniell, 2002). Transgene flow frequencies are also influenced by the size of the pollen source and recipient populations; therefore separation distances should be case specific and tailored to each trial (De Marchis *et al.*, 2003). Dispersal of viable pollen and dissemination in seed are the two main means of gene movement among crops and related species. 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 (Eastman and Sweet, 2002). Dispersal of the seed may occur during harvest, transportation, planting and subsequent harvests (Eastman and Sweet, 2002).

In oilseed rape, seed losses occur due to natural shedding and crop disturbance during harvesting. Adverse weather conditions can bring about losses of up to 50%, while harvesting losses of 20-25% are unexceptional. In ideal conditions losses of 2-5% may be experienced (BioMat.net, 2003). These seed losses may result in difficult to control volunteer populations, which may cause contamination in subsequent crops, field margins, roadsides and soil dumps. Feral populations, mainly *B. napus*, are less common. However, those that do not die out quickly can persist for up to ten years. Therefore if germination of the GM seeds was to occur there is a risk of interbreeding with compatible weedy species.

Introgressive hybridisation could result in new weeds emerging, which have acquired the GM trait e.g. transferring herbicide or insecticide resistance to these species making them difficult to control (Daniell, 2002; Mooney and Gerard, 2003). Therefore, performing environmental risk assessments i.e. field trials, is necessary before modified plants can be grown commercially. There are four approaches used to estimate gene flow resulting from pollen dispersal:

1. Measurement of pollen dispersal from point and/or block sources;
2. Measurement of gene dispersal from point and block sources;
3. Paternity analysis of progeny in sink populations;
4. Inference of gene flow from natural population genetic structure of non-gm varieties (Ellstrand, 1992).

1.3.2 Introduction to gene flow in oilseed rape: weedy potential

People have very different ideas about what a weed is, and consequently there is no definition of a weed that will satisfy most people. However in the broadest sense a weed is any plant growing in the wrong place. Agriculturalists see many weeds whereas conservationists see few (Williamson, 1992). Many species of *Brassica* and related mustards are weeds or have weedy tendencies. *Brassica napus* has been referred to as a minor weed (Williamson, 1992), an occasional weed, escape, or volunteer in cultivated fields (Bailey, 1949; Muenscher, 1980). *Brassica juncea*, *B. nigra, B. rapa* and *Sinapis arvensis* are to some degree agricultural weeds, sometimes serious, in much of the United States (Gleason, 1952; Muenscher, 1980).

Brassica napus has the potential to hybridise with other *Brassicas*. Since the Irish and British countryside contains a taxonomically difficult mixture of *Brassicas* of various ploidy and relatedness, it is unknown why oilseed rape is not a more detrimental and widely established weed (Williamson, 1992). Nevertheless in NW Europe, *B. napus* weeds are very common and widely established, and have rapidly increased in occurrence over the last 50 years (Bing *et al.*, 1991) and may continue to increase in the future.

There is clearly potential for gene flow to occur from genetically modified *B. napus* to other rape varieties and related species. As described in sections 1.4 and 1.5, partial sexual compatibility exists between some related *Brassica* species and other closely related species outside the genus. Crosses between *B. napus* and other species occur with varying degrees of difficulty, and depend greatly on the direction of the cross (Eastman and Sweet, 2002). Oilseed rape pollen is thought to remain viable for between 24 hours and one week according to Mesquida & Renard (1982), and therefore an ever-increasing number of pollen dispersal and gene flow experiments are being conducted (Figure 1.4 and Table 1.6: p19-23) to try to estimate gene flow levels and in turn to estimate the risk of transgene escape.

1.4 The morphology of *Brassica napus*

1.4.1 Brassicaceae

Cruciferae, now also known as Brassicaceae, derived their name from their cruciform (resembling a cross) flower shape. The family consists of approximately 376 genera and 3200 species, which are mainly herbaceous and include crops, condiments, ornamentals, and many weeds (Willis, 1973; Zomlefer, 1994). Many familiar vegetables, such as cabbage, cauliflower and turnip (*Brassica*), radish (*Raphanus*), yellow mustard (*Sinapis*), cress (*Lepidium*) and horse-radish (*Amoracia*) belong to this family along with ornamentals such as, wallflower (*Cheiranthus*), stock (*Matthiola*), honesty (*Lunaria*), candytuft (*Iberius*), *Aubretia* and *Alyssum*. The genus of greatest economic importance is *Brassica* (Langer and Hill, 1982).

1.4.2 *Brassica*

There are about 100 species of *Brassica* (Bailey, 1949), most of which are annual or biennial and several of which are grown extensively in cool, temperate regions. Economically important plants belonging to *Brassica* include broccoli, Brussels sprouts, cabbage, cauliflower, Chinese cabbage, kale, kohlrabi, oilseed rape, turnip and black mustard (Lackey, 2003). *Brassica* species usually have taproots and there is a tendency for storage organs to be developed. The stems are erect and the leaves are alternate, lobed and glabrous (covered with simple hairs). The inflorescence is a raceme bearing numerous, conspicuous flowers that are usually yellow in colour. There are four sepals, arranged as an inner and an outer pair, and four petals each consisting of a narrow, erect claw at the base with a broad, spreading limb above, which together form the pattern of a cross. There are six stamens represented by a relatively short outer pair and four longer inner ones. The ovary is superior and consists of two united carpels divided by a false, membranous septum and a single lobed stigma (Langer and Hill, 1982).

Pollination is usually by insects and there are nectaries at the base of the anther filaments. The fruit is a capsule, referred to as a siliqua when it is longer than it is broad, or a silicula when it is broader than it is long. There are numerous ovules, which are arranged in rows and remain temporally attached to the false septum. On maturity the valves of the fruit dry up and retract. The seeds have no

endosperm and consist mainly of two cotyledons, which act as reserve organs rich in oil (Langer and Hill, 1982).

One of the distinctive properties of the genus is the presence of sulphur containing compounds including glucosides, glucosinolates, thiocyanates and isothiocyanates, which present a protective function as they impart a bitter taste. In cultivated plants they are responsible for the distinctive flavours with which different cultivars are associated (Langer and Hill, 1982).

1.4.3 *Brassica napus*

Brassica napus L., is known as oilseed rape, rape, rapeseed, oil seed rape, and in some countries, including Canada, as canola. It is a mustard crop grown primarily for its seed, which yields about forty percent oil and a high-protein animal feed (Lackey, 2003). Recent interest in the crop has centred on cultivars that have low erucic acid content as oil production, e.g. salad oil, requires a high percentage of oleic and linoleic acids, and no erucic acid. Traditional and other uses have been for lamp oils, soap making, high-temperature and tenacious high-erucic acid lubricating oils and plastic manufacturing (Röbbelen *et al.*, 1989).

Brassica napus produces an inflorescence of yellow, nectar-bearing flowers. The entomophilous flowers are capable of both self- and cross-pollination. Honeybees are the primary pollinators. In cultivated fields, cross-pollination has been reported at about 35% (Lackey, 2003) but varies depending on the availability of insect pollinators, cultivar, and weather. *Brassica napus* belongs to a group of six closely related species (Table 1.5) (Röbbelen *et al.*, 1989).

1.5 Gene flow and hybridisation between Brassicaceae species

Reproductive isolating mechanisms between species are not always effective and as a result gene flow may occur. Hybridisation can be defined as mating between unrelated individuals, however it is often used to describe mating between species i.e. interspecific hybridisation (Judd *et al.*, 1999). This is important in terms of plant evolution, as it is a source of novel gene combinations and a mechanism of speciation. Hybridisation has also been important in plant breeding for the movement of desirable traits from wild species to cultivated species or from one cultivated species to another, as this can result in the production of new cultivars of agronomic and horticultural importance (Judd *et al.*, 1999).

Table 1.5: Closely related *Brassica* species (Lackey, 2003)

Species/ Common Name	Ploidy	Origin
B. napus L. Oilseed rape	Allo-tetraploid (n=19)	Derived from ancient crosses between *B. oleracea* and *B. rapa*. Grown for its oil, but also found as an occasional weed or volunteer in cultivated fields.
B. nigra L. Koch Black mustard	Diploid (n=8)	Originally spread across Old World by trade but is now spread as a weed throughout Europe, Asia, northern India, northern Africa and most of the United States.
B. oleracea L. (=*B. campestris* L.) Cabbage Broccoli Brussels sprouts Cauliflower Kale	Diploid (n=9)	Probably originated in Mediterranean but now more widespread. Its native range includes southern England (Preston et al. 2003)
B. rapa L. (*B. campestris* L.) Turnip Turnip rape Field mustard Bird rape	Diploid (n=10)	Originally spread throughout much of Europe, Asia, northern India, and northern Africa, and is now either grown as a vegetable or oil crop. Occasional weed in much of the United States.
B. carinata A. Braun Abyssinian mustard Ethiopian mustard	Allo-tetraploid (n=17)	Derived from *B. nigra* and *B. oleracea*, presumed to come from an ancient cross or crosses in northeast Africa and occasionally grown in the United States.
B. juncea L. Indian mustard Brown mustard Mustard greens	Allo-tetraploid (n=18)	Derived from Old World crosses of *B. nigra* and *B. rapa*. Now grown for its leaves, or spread as an occasional weed in crops or waste places.

Brassica plants are promiscuous and therefore hybridisation is prevalent. There are ~70,000 naturally occurring interspecific hybrids in the world (Stace, 1984). Intergeneric hybridisation is more unusual, but occurs naturally in a number of genera of Rosaceae including *Amelanchier*, *Crataegus*, apple (*Malus*), pear (*Pyrus*), *Cotoneaster* and *Sorbus* (Judd *et al.*, 1999). Also intergeneric hybrids have been reported in the grass family (Poaceae) such as the cross between wheat and rye that produced the commercially successful *Triticale* (FTBP, 2004). Introgression occurs through backcrossing of hybrids with one or other of the parental species. A hybrid swarm may be produced when gene flow is limited to one or a few sites. Alternatively, hybrid vigour or heterosis may also be induced, which occurs when the hybrid produced has a higher growth rate and an enhanced fitness level over either of the two parental species and may be better adapted for new habitats. Although the importance of hybrid vigour may not be clear in the wild, it has been

demonstrated in many high yielding cultivated varieties such as corn, tomatoes and cabbage (Judd *et al.*, 1999).

Allopolyploids are the result of the union of two or more divergent genomes. Allopolyploid speciation is involved in the evolution of many crops including cotton, wheat and *Brassica*. In 1935, the scientist 'U' proposed that three *Brassica* species *B. nigra* (black mustard), *B. oleracea* (species from which Brussels sprouts, broccoli, cabbage, cauliflower, kale and kohlrabi have been developed) and *B. rapa* (*B. campestris*, turnip) hybridised to produce three other species. These relationships have been confirmed by many studies and have become known as the "Triangle of U" (Figure 1.3) (U, 1935; Song *et al.*, 1988; Soltis and Soltis, 1993).

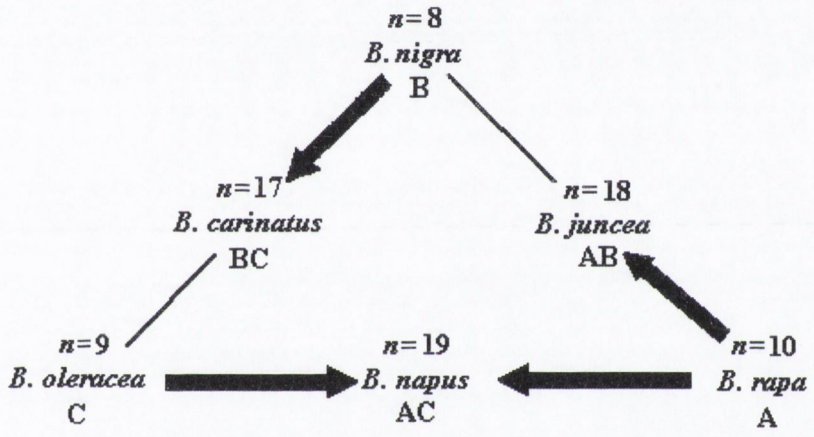

Figure 1.3: The "Triangle of U" (Soltis and Soltis, 1993). The diploid parent plants are located at the points of the triangle, while the tetraploids are at the midpoints of lines between parents. The haploid chromosome number (*n*) and genome constitution (A, B or C) are given for each taxon. Both *B. rapa* and *B. oleracea* have served as the maternal parent of *B. napus* (Soltis and Soltis, 1993)

A large number of gene flow studies have been carried out to date (Stringam and Downey, 1982; Manasse and Kareiva, 1991; Scheffler *et al.*, 1993; Morris *et al.*, 1994; Scheffler *et al.*, 1995; Wilkinson *et al.*, 1995; Méssean, 1997; Pallutt and Hommel, 1998; Pfeilstetter *et al.*, 1998; von Ernst *et al.*, 1998; Champolivier *et al.*, 1999a; Champolivier *et al.*, 1999b; Downey, 1999a; 1999b; Simpson *et al.*, 1999; Sweet *et al.*, 1999; CETIOM, 2000; Götz and Ammer, 2000; Kamler, 2000; Staniland *et al.*, 2000; Beckie *et al.*, 2001; Méssean, 2001; Norris and Sweet, 2002) Figure 1.4 and Table 1.6).

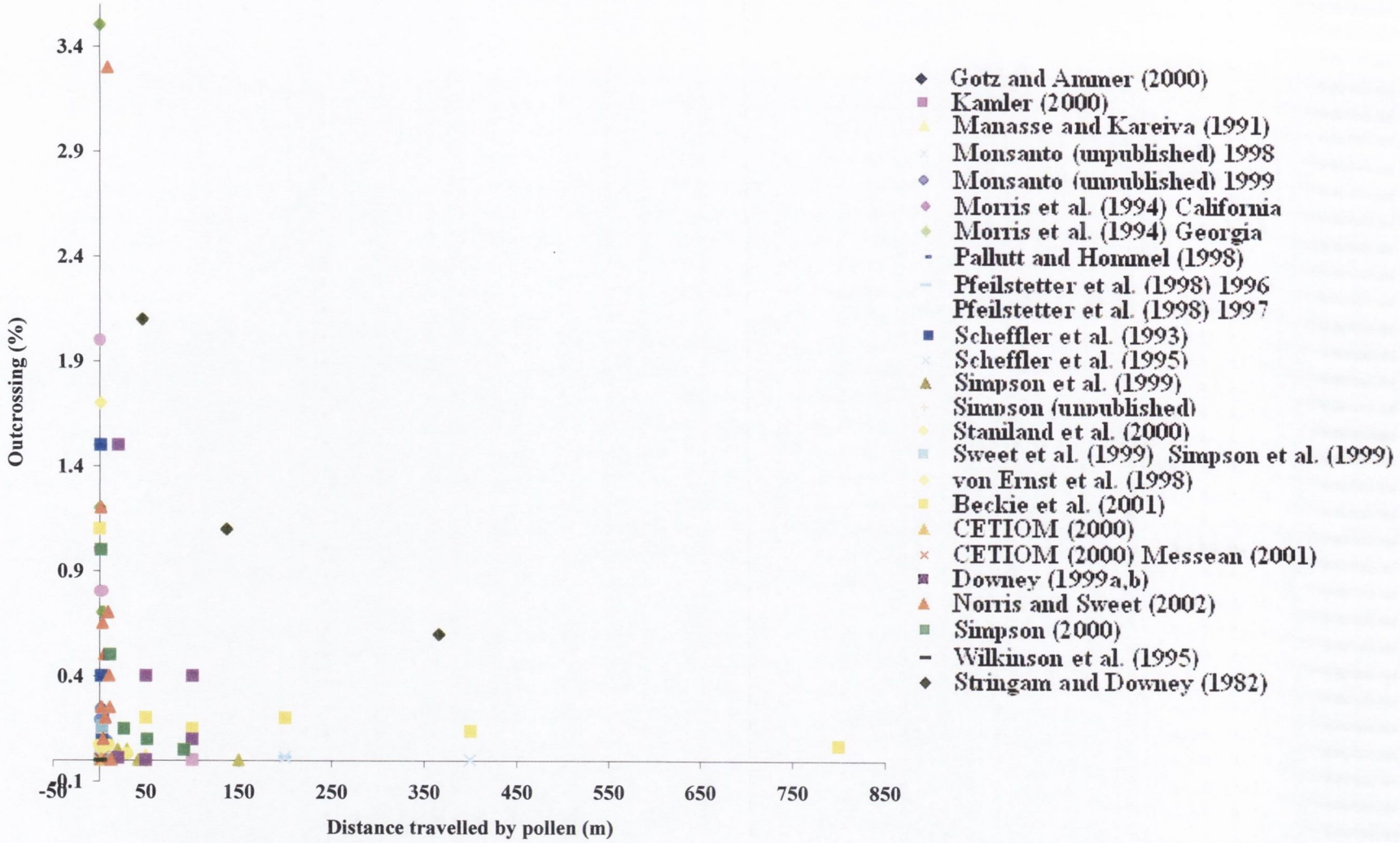

Figure 1.4: Studies on the effect of distance from pollen source on the level of outcrossing in oilseed rape (*B. napus*) (Salisbury, 2002)

Table 1.6: Studies on the effect of distance from pollen source on the level of outcrossing in oilseed rape (*B. napus*) (Salisbury, 2002)

Authors	Experimental Design & Details	Results	
		Distance from Pollen Source (m)	Outcrossing (%)
Beckie et al. (2001)	Large adjacent fields (>32ha) of different HT types – average of 11 sites (Saskatchewan, Canada).	0 50 100 200 400 800	1.1 0.2 0.15 0.2 0.14 0.07
CETIOM (2000)	Different HT types in adjacent fields (France).	30 120 >400	<1 <0.5 0-0.1
CETIOM (2000) Messean (2001)	Surveyed commercial fields 400-1000m from 23 GM source plots (France).	>400	0-0.1
Champolivier et al. (1999a,b) Messean (1997)	Different HT types in adjacent fields at 3 sites (France).	1 20 65	2 0.2 <0.01
Downey (1999a,b)	Large adjacent fields (>16ha) of GM and non-GM at 2 sites (Canada).	Field 1 20 50 100	 1.5 0.4 0.1
		Field 2 20 50 100	 0.01 0 0.40
Gotz and Ammer (2000)	GM source plots ($18m^2$) to non-GM canola (Germany).	0.5	0.43-2.32
Kamler (2000)	GM source plots ($240m^2$) and $72m^2$ non-GM recipient plots (Germany).	100 150	0.05-0.07 0
Manasse and Kareiva (1991)	Unknown.	50 100	0.022 0.01

Table 1.6: continued

Authors	Experimental Design & Details	Results	
		Distance from Pollen Source (m)	Outcrossing (%)
Monsanto (unpublished)	Plots ($16m^2$) of non-GM canola planted at different distances and in different directions from GM experimental area over two years (Canada).	1998 50W 100W 100E 180W 400E	 0.02 0.01 0.01 0 0
Monsanto (unpublished)	Plots ($16m^2$) of non-GM canola planted at different distances and in different directions from GM experimental area over two years (Canada).	1999 50E 50W 50N 100E 100W 100N 175E 200W 225N	 0.09 0.19 0.24 0 0.21 0.09 0.09 0.09 0.09
Morris et al. (1994)	Trial area surrounded by continuous border of up to 4.6 m, at 2 sites (USA).	California 0 0.3 0.6 3 4.6	 2.0 1.0 0.8 0.8 0.4
		Georgia 0 0.3 0.6 3 4.6	 3.5 1.5 1.2 0.7 0.7
Norris (unpublished, cited by Eastham and Sweet, 2002)	Large adjacent fields (10 ha) of GM and non-GM (UK).	Site A 5 25 40 50 100 200	 1.2 0.25 0.65 0.10 0.5 0.2

Table 1.6: continued

Authors	Experimental Design & Details	Results	
		Distance from Pollen Source (m)	Outcrossing (%)
Norris (unpublished, cited by Eastham and Sweet, 2002)	Large adjacent fields (10 ha) of GM and non-GM (UK).	Site B 5 25 50 100 200 250	 3.3 0.7 0.4 0.25 <0.11 <0.1
Pallutt and Hommel (1998)	Gene flow between $360m^2$ neighbouring trial plots (Germany).	-	<1%
Pfeilstetter et al. (1998)	Fertile bait plants at 200m distance (Germany).	1996 200	 0.017
		1997 200	 0.06
Scheffler et al. (1993)	9m diameter circle of GM canola in centre, surrounded by non-GM canola for at least 47m in all directions (UK, Belgium and France).	UK study 1 3 6 12 24 36 47 70	 1.5 0.4 0.11 0.016 0.0041 0.0011 0.00034 0
		Belgian study >32	 0
Scheffler et al. (1995)	$400m^2$ source plot of GM canola and $400m^2$ recipient plots of non-GM canola separated by 200m and 400m (UK).	200 400	0.0156 0.0038

Table 1.6: continued

Authors	Experimental Design & Details	Results	
		Distance from Pollen Source (m)	Outcrossing (%)
Simpson et al. (1999)	Gene flow between National List trial plots ($40m^2$) at 2 sites (UK).	Cockle Park, UK 6 30 42 50	Cultivar range 0.05 0.05 0.05-0.33 0.05-0.16
Simpson et al. (1999)	Gene flow between National List trial plots ($40m^2$) at 2 sites (UK).	Bridgets, UK 10 20 150	 0.05-0.44 0.05 0.11-0.22
Simpson (unpublished, cited by Eastham and Sweet, 2002)	Adjacent blocks (0.8 ha) of GM HT and non-GM (UK).	1.5 11.5 26.5 51.5 91.5	1.0 0.5 0.15 0.1 0.05
Simpson (unpublished, cited by Eastham and Sweet, 2002)	Large source plot (9 ha) to fertile bait plants 100-400m away in all 4 directions (UK).	100S 100E 100W 200S 200E 200W 400S	0.03 0.01 0.13 0.025 0.02 0.03 0.06
Staniland et al. (2000)	30m x 60m GM source plot, surrounded by 15-30m wide non-GM borders – 2 sites over 2 years, 4 directions (Canada).	0 10 30	0.07 0.07 0.03
Stringam and Downey (1982)	Large source field to small ($46m^2$) recipient plots (Canada).	46 137 366	2.1 1.1 0.6
Sweet et al. (1999) Simpson et al. (1999)	Gene flow within National List trial plots ($46m^2$) (Caxton, UK).	 4 8 20 34 50-56	Cultivar range 0.16-2 0.05-0.33 0.16 0.05-0.16 0.05-0.11

Table 1.6: continued

Authors	Experimental Design & Details	Results	
		Distance from Pollen Source (m)	Outcrossing (%)
von Ernst et al. (1998)	Outcrossing to fertile bait plants (Germany).	5-40 >40	1.7 0.05
Wilkinson et al. (1995)	Adjacent fields of spring and autumn-sown canola (Scotland).	>32	0.03-0.05

1.5.1 Genetically modified *Brassica napus* and conventional varieties

Few studies have assessed the risk of hybridisation between GM and conventional varieties of OSR. However, Rieger *et al.* (2002) carried out a large-scale study in Australia to examine the movement of herbicide tolerance genes (resistance to acetolactate synthase (ALS) inhibiting herbicides) from GM *B. napus* crops to conventional *B. napus* crops growing nearby. Source and sink fields were similar in size (25-100 hectares) and seedlings were screened by spraying with an ALS-inhibiting herbicide. They detected gene flow in all fields tested, but concluded that even adjacent commercial *B. napus* fields had much less than 1% gene flow (Rieger *et al.*, 2002).

Between 1999-2002, gene flow experiments have also been conducted by Monsanto scientists Rosenbaum *et al.* (2003) and Pester *et al.* (2001) to estimate mean gene flow rates from a 33-hectare Round-up Ready OSR (resistant to glyphosate) field (source) to a 33-hectare non Round-up Ready OSR field (sink). Source and sink fields adjacent to each other were sampled along transects through the non-Round-up Ready OSR at 10m, 20m, 50, 200m and 400m. Percentage gene flow was determined by examining plants in the sink field and dividing the number of Round-up Ready positive seedlings by the number of seeds germinated and multiplying by 100. Gene flow at 10m, 20m, 50m, 200m and 400m was found to be 0.75%, 0.65%, 0.33%, 0.22% and 0.14% respectively; and mean gene flow decreased exponentially at greater distances from the source (Pester *et al.*, 2001; Rosenbaum *et al.*, 2003).

The examination of commercially sized fields makes this study different to earlier studies where small areas of GM crops were planted e.g. assessment of buffer zones of non-GM crops surrounding small regions of GM crops (Paul *et al.*, 1995).

The presence of a barren region between crops is common in agricultural systems and therefore studies have been done to examine the movement of pollen across this gap and into neighbouring crops (Reboud, 2003). Examination of pollen flow from the field border up to 7m into the crop revealed a steeper decline in cross contamination where intervening plants were present rather than a gap. The same levels of GM progeny were produced by a non-GM crop when separated by 1m of crop as a 3-4m gap. This implies that gaps between crops would require three times the field surface area of crops to achieve the same containment levels (Reboud, 2003).

1.5.2 *Brassica napus* and *Brassica rapa* crosses

Crop to weed gene flow may have important practical and economic consequences (Ellstrand *et al.*, 1999). Therefore, the examination of hybridisation between domesticated plants and wild relatives, more specifically, the flow of genes from domesticated to wild relatives is necessary (Landbo and Jørgensen, 1997). Wild *B. rapa* plants growing within a *B. napus* crop can produce 9-93% hybrid seed. Hybrids spontaneously backcross with their wild parent (*B. rapa*) and are often significantly more fit under field conditions than *B. rapa* (Hauser *et al.*, 1998a). However, backcrossed progeny have reduced fitness compared to wild *B. rapa* (Hauser *et al.*, 1998a; Hauser *et al.*, 1998b). In addition, a high proportion of *B. rapa* seeds from natural populations exhibit dormancy and require a variety of treatments to induce germination (Landbo and Jørgensen, 1997). Hauser *et al.* (2003) have stated that the composition of the pollen cloud (a pollen-laden parcel of air) is likely to be responsible for the variation observed in hybrid seed production rather than planting density alone.

Reproductive interactions also have a role to play when conspecific and heterospecific zygotes are present in a pod. *B. rapa* x *B. napus* zygotes have a 50% survival rate, but *B. napus* x *B. rapa* zygotes have a 15% survival rate, which explains why fewer seeds are produced by various parental plants in mixed plots (Hauser *et al.*, 2003). The transfer of genes from F_1 plants to *B. rapa* via pollen is

only likely where F_1 plants are present in higher frequency than *B. rapa*, however the transfer of genes via seed is likely to occur where F_1 plants grow at low frequency among *B. rapa* plants (Hauser *et al.*, 2003). Canadian studies have shown a mean hybridisation rate between a crop of *B. napus* and a wild population of *B. rapa* to be 13% and therefore state that in terms of risk where *B. rapa* and *B. napus* co-occur there will be hybridisation between them, the degree of which depends on the density and spatial distribution of the weed species relative to *B. napus* (Warwick *et al.*, 2003).

Further studies analysed hybridisation rates when *B. rapa* and *B. napus* were mixed at a 1:1 ratio. Results showed that *B. napus* produced 9% hybrid seed while *B. rapa* produced 13% (Jørgensen and Andersen, 1994).

Brassica napus x *B. rapa* hybrids can be backcrossed under field conditions to produce *B. rapa* like plants carrying a transgene from oilseed rape as early as in the first generation. This suggests the possibility of rapid spread of genes from oilseed rape to *B. rapa* weedy relatives (Mikkelsen *et al.*, 1996).

1.5.3 *Brassica napus* and *Brassica juncea* (brown mustard) crosses

Crosses between a *B. napus* crop intermixed with wild *B. juncea* (brown mustard) showed hybrid seed production of 3% in wild *B. juncea*. Hybrids produced were fertile and backcrossed successfully with their parental species (Jørgensen *et al.*, 1996).

1.5.4 *Brassica napus* and *Hirschfeldia incana* (wild hoary mustard) crosses

Crosses between *B. napus* and *Hirschfeldia incana* (wild hoary mustard) have also been carried out. For example, approximately 2% of the progeny produced from herbicide resistant *B. napus* (crop) interplanted with *H. incana* were hybrids. These hybrid progeny produced very few pollen grains and less than one seed per plant (Lefol *et al.*, 1996b). This very low rate of hybridisation indicates that gene flow between *B. napus* and *H. incana* populations is unlikely. However other studies, carried out by Lefol *et al.* (1996b), indicated that where hybrid *B. napus* x *H. incana* plants were formed, hybrids would be more competitive in a *H. incana* population (Lefol *et al.*, 1995).

1.5.5 *Brassica napus* and *Raphanus raphanistrum* crosses

Chévre *et al.* (1998) planted *Raphanus raphanistrum* (wild radish) within a herbicide resistant *B. napus* (crop) with at a ratio of 1:600; and low rates of hybridisation were detected (0.05%). Although these hybrids have low fertility (less then 1 seed per plant), fertility was recovered through repeated backcrossing with the wild parent (Chévre *et al.*, 1998). Guéritaine *et al.* (2003) confirmed this and reported that hybrids are less likely than either parental species to emerge and survive under agronomic or natural environmental conditions. Opportunities for backcrossing may be limited as a low frequency of these hybrids reach flowering stage, however some do establish and set seed. The restoration of fertility on backcrossing demonstrates the ability for gene flow, albeit at a low rate (Chévre *et al.*, 1998; Guéritaine *et al.*, 2003).

Although low levels of gene flow may occur, the flow of transgenes from *Brassica* species into *R. raphanistrum* is considered likely to be much lower than the flow of transgenes between members of the *Brassica* genus (Guéritaine *et al.*, 2002).

1.5.6 *Brassica napus* and *Sinapis arvensis* crosses

In other studies, *B. napus* (crop) was interplanted with *Sinapis arvensis* (charlock), and although large quantities of seed were tested, no hybrid seed was detected. Moyles *et al.* (2002) examined the introgression of herbicide tolerance genes into wild *S. arvensis* populations using hand pollination and found that when *B. napus* is the maternal parent hybridisation rates were undetectable to 0.0049% of the total seed potential (Moyles *et al.*, 2002). No *S. arvensis* wild population or *B. napus* cultivar was detected, which hybridised readily and rates were too low to detect in the field. It was therefore concluded that risk of gene transfer from *B. napus* to *S. arvensis* is minimal (Moyles *et al.*, 2002). Lefol *et al.* (1996) used bud pollination to cross Canadian spring OSR cv. Westar containing the *bar* gene conferring resistance to the herbicide gluphosinate with *S. arvensis*. Among 2.9 million seeds, no hybrids were found and thus it was concluded that a flower of these two species has a probability smaller than 10^{-10} of having interspecific hybrid progeny (Lefol *et al.*, 1996a).

1.6 Thesis layout and aims

1.6.1 Aims

This study was stimulated by the need for information on pollen-mediated dispersal of transgenes via cross fertilisation. It is the first of its kind to be conducted in Ireland and aimed to test the *risk = f (hazard, exposure*) hypothesis (GM Science Review Panel, 2003; Wilkinson *et al.*, 2003) through *Brassica napus* pollen dispersal, pollination and seed set studies, which were followed by a comparison of the results with published data. In this case, by the movement of pollen over long distances represents exposure, while the hazard is classified as the potential for seed set to occur. Risk assessment studies are numerous in various EU member states however none have assessed wind pollination, insect pollination and seed set in male sterile bait plants simultaneously. Furthermore, all conclusions must be drawn on a case-by-case basis (The European Parliament and the Council of The European Union, 2001) and therefore results from field trails conducted in other member states would not be equivalent or transferable to the Irish countryside. These broad aims were achieved by the application of the following techniques:

- To quantify biotic and abiotic dispersal of pollen from a *Brassica napus* crop a field trial was carried out.
- To determine an appropriate abiotic pollen collection system for these analyses two different pollen detection systems were used. A Burkard volumetric spore trap, equivalent to that used in studies by Solomon *et al.* (1980), Rogers and Levetin (1998) and Sterling *et al.* (1999) was used along with a modified version of the Cour passive trap (Cour, 1974). Passive traps were designed and positioned along transects from the source crop at distances equivalent to those used by others (Pester *et al.*, 2001; Rosenbaum *et al.*, 2003).
- To examine cross fertilisation, seed set and resulting inter-varietal hybrid production in *B. napus*, male sterile bait plants were employed to establish 'worst case scenario' cross-pollination levels.
- To investigate biotic pollen dispersal from *B. napus* source, pollen concentrations were examined by collecting and analysing bee pellets from beehives placed along a transect up to 1600m.

- To identify pollen contained in pollen pellets beyond the family level molecular methods were explored to attempt the isolation and amplification of DNA from bee pollen pellets.

1.6.2 Thesis layout

This thesis is laid out in sections according to the various methods used. A brief introduction to each section gives details of research to date in the particular area; an analysis is then presented and discussed on its own. A final chapter discusses the overall findings of the study, conclusions and consequences.

Chapter 2

Molecular characterisation of Brassicaceae

2.1 Introduction

When doing risk assessment and gene flow studies on members of Brassicaceae it is important to be able to distinguish between its different genera, species and intraspecific taxa. The introduction or identification of a plant in a population with a unique genetic marker is often used to follow the appearance of that marker in the next generation (Eastman and Sweet, 2002). Molecular markers, in particular microsatellite markers, are capable of detecting genetic differences between closely related plants and are therefore useful for this purpose. An extensive list of Brassicaceae nuclear microsatellites is publicly accessible (UKCropNet, 2003). Generally, chloroplast DNA exhibits low inter-specific variation (Palmer, 1987), as has been shown in PCR-RFLP studies where insufficient resolution was produced to differentiate between plant species (Provan *et al.*, 2001; Panda *et al.*, 2003). In spite of this, informative *Arabidopsis thaliana* plastid microsatellite markers have recently become available (Provan and Campanella, 2003). As haploid chloroplast and mitochondrial genomes are uniparentally inherited, they show different variation patterns to those exhibited by nuclear markers. This is useful when analysing polyploid Brassicaceae, as nuclear SSR fingerprints are more difficult to interpret than haploid plastid fingerprints. As there is still an absence of plastid microsatellite markers for *Brassica* species, this chapter of the thesis focuses on the development of molecular markers, namely plastid microsatellite markers, to distinguish between individuals of Brassicaceae at inter-generic, inter-specific and intra-specific levels. These plastid primers could then be used in conjunction with existing nuclear primers for risk assessment, gene flow studies and also wider applications in plant biology such as systematics and plant breeding.

2.1.1 Molecular markers

A number of molecular marker systems have been developed for Brassicaceae species including isozymes (Raybould *et al.*, 1999), RAPD; random amplified polymorphic DNA (Quiros *et al.*, 1995; Grandclement and Thomas, 1996;

Mikkelsen *et al.*, 1996), SRAPs; sequence related amplified polymorphism (Li and Quiros, 2001; Riaz *et al.*, 2001), DNA sequencing, RFLP; restriction fragment length polymorphism (Pradhan *et al.*, 1992), ISSRs; inter simple sequence repeats (Charters *et al.*, 1996), SSRs; microsatellite marker analysis (Powell *et al.*, 1996) and AFLPs; amplified fragment length polymorphism (Negi *et al.*, 2000; Hansen *et al.*, 2003). The methods used in this thesis are discussed below.

2.1.2 PCR - Restriction Fragment Length Polymorphism (PCR-RFLP)

In PCR restriction fragment length polymorphism (PCR-RFLP) analysis, PCR products of a particular DNA region are digested with a specific restriction enzyme, the resultant fragments are separated by gel electrophoresis and visualised via various detection methods including ethidium bromide staining, silver staining, radioactive or fluorescent labelling.

PCR-RFLP is a quick and reliable method of indirectly obtaining sequence information and is most informative when the restriction sites are mapped. However, only sequencing of the fragment will resolve all the differences between samples (Karp *et al.*, 1996).

2.1.3 DNA sequencing

DNA sequencing capabilities are fundamental to systematic studies. Sequences derived from protein coding genes are usually straightforward to align and the interpretation of the data poses few problems. Non-coding intron and spacer regions show higher variation and hence potential for population level studies. The PCR approach can be applied to very small samples e.g. a single pollen grain or even to herbarium specimens or fossils (Karp *et al.*, 1996).

Techniques for large-scale DNA sequencing became available in the 1970s, starting with the Maxam and Gilbert and the Sanger techniques. These techniques relied on radio-isotopically labelled fragments, which were resolved according to their lengths by gel electrophoresis and the resulting auto-radiographic pattern was used to obtain the DNA sequence (Prober *et al.*, 1987b).

Automated sequencing is now widely used and employs fluorescently labelled fragments instead of radio-labelled fragments. Fluorescent tags are attached to chain terminating nucleotides. Each of the four chain-terminator nucleotides

carries a spectrally distinct fluorophore (such as ROX, JOE, FAM and TAMRA) (Prober *et al.*, 1987a). The tag is incorporated into the DNA molecule by the DNA polymerase and causes termination of synthesis and attachment of the fluorophore to the end of the molecule. There are two advantages to this technique, firstly, conventional primers may be used and secondly four enzymatic reactions may be carried out in a single tube as each termination event attaches specifically to one of the four fluorochromes. The DNA bands are detected as they electrophorese past a fluorescence detector. DNA sequence data can then be analysed *in silico* (Old and Primrose, 1994).

2.1.4 Microsatellites (SSRs)

Microsatellites, or simple sequence repeats (SSRs), consist of tandemly repeated multiple copies of mono-, di-, tri- and tetra-nucleotide repeat motifs. Their length polymorphism makes them highly informative markers providing an efficient, accurate means of testing genetic variation in any organism from which DNA can be extracted (Powell *et al.*, 1996). SSR markers are routinely used to investigate the genetic structure of natural populations as the knowledge of how variation is partitioned among populations may have important implications for both evolutionary biology and ecology (Balloux and Lugon-Moulin, 2002). SSRs also have huge potential for characterisation, conservation and utilisation of crop diversity (Szewc-McFadden *et al.*, 1996).

The development of SSRs is often a demanding process involving the detection, isolation and sequencing of SSR loci from genomic DNA libraries, followed by the design and synthesis of primers (Zane *et al.*, 2002; Squirrell *et al.*, 2003). The isolation and cloning of plant microsatellites was first performed with tropical tree species (Conduit and Hubbell, 1991). It has since been estimated that on average every 33kb a microsatellite repeat greater than 20bp in length occurs in plant nuclear genomes compared with every 6kb in mammals (Powell *et al.*, 1996).

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.*, 2002b; 2002a; Tommasini *et al.*, 2003). 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 there are now approximately 404 *Brassica* nuclear microsatellite primers readily available through the *Brassica* DB database on the UK CropNet website (http://ukcrop.net). These include 36 isolated and characterised for *B. rapa* (Suwabe *et al.*, 2002b), twelve isolated for *B. napus, B. oleracea, B. rapa* and *B. nigra*, six of which were specific to either the A, B or C genome and used to identify hybrids (Lowe *et al.*, 2002). Large scale development of 398 *Brassica* SSR markers has also been carried out (Lowe *et al.*, 2004). (Lowe *et al.*, 2004)

2.1.5 Plastid DNA

Plastids are double membrane organelles containing circular, double stranded DNA molecules found in plants, many bacterial strains and some fungi (Lurquin, 2001). Chloroplasts are considered to have derived from the incorporation of eubacteria into other cells forming eukaryotic cells (Margulis, 1970) and therefore it is not surprising that their DNA content is similar in size to a bacterial cell and ranges between 110 and 160kbp (~156kbp in *Arabidopsis*). Plastids often code for functions outside those necessary for the general survival of the organism including antibiotic and heavy metal resistance (Lurquin, 2001).

Plastids are stably inherited in an extra-chromosomal state and can be considered a single, non-recombining unit of inheritance (Palmer, 1991) whose sequence is highly conserved with most variation occurring within non-coding intron and spacer regions. They are uniparentally inherited in most species of angiosperm although are biparentally inherited in species such as *Pelargonium* (Gillham, 1991). Since most angiosperms plastids are inherited maternally (Vendramin *et al.*, 1996) it is possible to elucidate the relative contributions of seed and pollen flow to the genetic structure of natural populations by comparing nuclear and chloroplast markers (Provan and Campanella, 2003).

The *Arabidopsis thaliana* chloroplast genome is 154,500bps and has been fully sequenced (Sato *et al.*, 1999). Comparisons and inferences can therefore be made with the *Brassica* genome.

2.1.6 Plastid microsatellite markers

The chloroplast genome has mainly been used in the study of plant populations and evolutionary genetics (Palmer, 1991). Until recently, the inability to detect sufficient levels of polymorphism due to low substitution rates was the main limitation associated with the chloroplast genome. The discovery of polymorphic mononucleotide repeats in chloroplast genomes analogous to nuclear SSRs has provided a new approach to detecting cytoplasmic variation that had previously gone undetected (Provan *et al.*, 2001).

Unlike the regular approach to nuclear SSR development, the nature of plastid SSRs (usually <15 mononucleotide repeats) and the need for genome specificity means that the screening of libraries for these motifs is redundant. Microsatellite markers can be obtained through sequence data from closely related species as chloroplast primers regularly cross-amplify in related species and have shown widespread intra-specific microsatellite polymorphism. Alternatively for species that have previously been sequenced, database searching can discover microsatellite repeats (Provan and Campanella, 2003).

Chloroplast SSRs were developed in this way for the weedy *A. thaliana* (Provan *et al.*, 2001). Intra-population variation was detected using seven mononucleotide repeat loci, resulting in 11 haplotypes from 22 individuals. It is unlikely that these levels of variation would be detected using standard chloroplast RFLP methodology, thus indicating the high resolution of the chloroplast SSR technique (Provan *et al.*, 2001). Prior to this *trnL* chloroplast primers *c* and *f* (Taberlet *et al.*, 1991) were used to distinguish *Brassica napus* from *B. rapa* (Scott and Wilkinson, 1999).

2.1.7 Primer design for amplification of microsatellites

Universal primers amplify a given section of DNA in a wide range of taxa and hence have been extremely useful in molecular genetics. The primary reason being that they allow DNA amplification in a species, which has never before undergone molecular analysis. The most straightforward method of designing primers is to align homologous sequences from as many different taxa as possible. Areas of conserved sequence around an area of interest can be then selected for primer design. This was the approach used in this thesis.

Primers can be 13-80bp long, however in most cases 18-24bps are sufficient (Palumbi, 1996). The longer the primer, the higher the annealing temperature and the greater the specificity. A disadvantage of making long primers is that more non-specific primer products are present in the primer mixture and as a result primer artefacts (primer dimers) and non-specific amplification may occur. Primers may be any sequence, though the ideal primer has equal numbers of each nucleotide without internal repeats or self-similarity. This avoids self-priming and primer-dimer products. Guanine-cytosine (GC) rich primers withstand higher annealing temperatures but can be prone to self-annealing (Kidd and Ruano, 1995).

Although specificity is obtained by maximising primer template similarity, amplification products may still be obtained even when the similarity is not perfect. Single internal mismatches have little effect on PCR product yield when there are 6-10 matched bases on either side of the mismatch. However single mismatches at or near the 3' end significantly reduce amplification. Thymines (Ts) appear to be able to base pair with all three other bases fairly well and so may be used more freely where template sequence is unknown. Alternatively, degenerate primers may be developed. These primers are complex mixes of oligonucleotides of different sequences. The advantage of this is that theoretically there will be an exact match of a target sequence to something in the primer mix (Palumbi, 1996).

The biggest assumption made about PCR is that the product produced is the product desired. Random priming is reduced by using long primers with reasonably high annealing temperatures, and by using pairs of primers known to be in a given orientation and a given distance apart. In this case the most direct indication that the PCR product is the correct one is its size i.e. if the product is the predicted size then it is likely to be the one for which the primers were designed. However, indisputable confirmation may only be obtained by sequencing the PCR product and aligning it to the original matrix (Kidd and Ruano, 1995).

2.1.8 Aims

This part of the thesis aimed to create inter-generic, inter-specific and intraspecific markers to distinguish between Brassicaceae. More specific objectives were

1. To detect variation using PCR-RFLP analysis of specific gene regions.

2. To detect inter-specific variation in *Brassica* using DNA sequencing.
3. To test a selection of nuclear SSR primers from the UKCropNet (UKCropNet, 2003) for polymorphism and for their capacity to differentiate various members of Brassicaceae.
4. To design chloroplast SSRs for *Brassica* and its wild relatives.
    - To carry out DNA sequencing on a range of Brassicaceae collected in the field and grown from seed.
    - To align with sequences from other sources e.g. formerly published on Genbank and scan for SSR repeats.
    - Use SSRs detected to design primers and test on a broad range of crop and wild genera, species, subspecies and intraspecific taxa of Brassicaceae.
5. Use new primers to elucidate the degree of variation between accessions and determine the ability of the markers to distinguish between or identify oilseed rape varieties/taxa.

2.2 Methods

2.2.1 Isolation of DNA from leaf material

DNA was extracted using a modified version of the protocol by Doyle and Doyle (1987) (Gawel and Jarret, 1991). A 30ml chloroform resistant tube containing 10ml of cationic hexadecyl trimethyl ammonium bromide (CTAB) buffer and 40µl mercaptoethanol, as well as a pestle and mortar were preheated to 65°C in a water bath. The CTAB buffer comprised of 100mM Tris-HCl at pH 8.0, 1.4M NaCl, 20mM ethylene diamine tetra acetic acid (EDTA) and 2% CTAB. EDTA is usually included to inhibit metal dependent enzymes by chelating such divalents as Mg^{2+} and Ca^{2+}; mercaptoethanol is included to protect DNA from quinines, disulfides, peroxidases and phenoloxides (Hokinson, pers. comm. 1999).

A 0.15–0.2g sample of silica gel dried leaf material was ground with the preheated pestle and mortar, and the buffer gradually added. The homogenised slurry was then transferred to a centrifuge tube and incubated for 10mins at 65°C. The homogenised material was extracted once with 24:1 chloroform: isoamyl alcohol and placed in a horizontal position on a shaker for 30mins. The sample was centrifuged at 6.440rcf (6 x *g*) for 10mins. The aqueous phase containing the DNA was removed and transferred into a fresh centrifuge tube using a transfer pipette. An equal volume of isopropanol was added and the tube inverted to precipitate the DNA. The sample was then placed into the –20°C freezer overnight (or up to a week) to further precipitate the DNA.

The sample was centrifuged at 3,220rcf (3 x *g*) for 10mins to pellet the DNA and the supernatant poured off. A 3ml aliquot of ethanol (70%) was added and the solution mixed gently. The sample was centrifuged again at 3,220rcf (3 x *g*) for 5mins to pellet the DNA. The supernatant was poured off and the tube inverted on a paper towel for 5mins to let the excess wash buffer drain away. The tube was then turned right way up and the pellet allowed to dry for 20mins to remove all traces of ethanol.

The pellet was re-suspended in 500µl TE buffer. The tubes were labelled with an identification number, species, date and origin and the DNA was stored in the freezer at –20°C or –80°C until use.

2.2.2 Estimation of fragment size

An 8µl measure of total DNA (tDNA) was mixed with 1µl loading dye and pipetted into a well of a 1.2% agarose gel (1.2% w/v agarose/ 1xTBE) stained with 1µl of 10mg/ml ethidium bromide stain. 1µl 1kb ladder (Gibco BRL) mixed with 1µl loading dye (0.25% bromophenol blue and 40% sucrose solution) was loaded into the first and last lanes of the gel. Following electrophoresis at 118V for 30-45mins the gel was viewed on a UV lightbox. UV light was used to visualise the stained DNA and determine the position of the fragments, which appeared as bands. DNA fragments separate according to size and therefore migrate at different rates. The ethidium bromide stain intercalates between the base pairs of DNA and therefore the intensity of bands visualised demonstrates the fragment concentration. Gels were recorded as digital images using the Kodak Electrophoresis Documentation and Analysis System 120 (Kodak digital science).

2.2.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) reactions were performed in a Perkin Elmer™ DNA Thermal Cycler (Geneamp® PCR System 9700). Approximately 100ng (2µl) of template DNA was placed in the bottom of a 200µl micro-centrifuge tube. A master mix was set up for the required number of samples containing the appropriate PCR reagents. Reagents for a single reaction are given in Table 2.1.

Table 2.1: PCR reagent concentrations for a 25µl reaction

Reagent	Quantity (Conc.)
Diluted template DNA	2µl (±100ng)
Sterile ultrapure water (SUW)	6.75µl
10xBuffer* (Promega)	5µl
dNTPs (Invitrogen)	1µl (0.2mM)
Primer 1	3µl (50ng)
Primer 2	3µl (50ng)
$MgCl_2$ (Promega)	4µl (2mM)
Taq polymerase (Promega)	0.25µl (1.5 units)
Total	**25µl**

*10xBuffer - Thermophillic buffer: 10mM Tris-HCl pH 9, 500mM KCl, 1.0% Triton X-100.

Taq polymerase was added last and then the complete master mix was mixed gently. The required volume (either 23µl for a 25µl reaction or 46µl for a 50µl reaction) of master mix was added to each tube of template DNA. Care was taken to prevent cross contamination of template. The tubes were centrifuged for 5 seconds to spin down the reagents to the bottom of the tube. All samples were kept on ice, which serves to slow enzyme activity throughout the procedure until samples were loaded into the Thermal Cycler (Table 2.2). PCR products were visualised and recorded in the same way as for total DNA (tDNA).

Table 2.2: PCR thermocycler conditions for a standard reaction

PCR Step	Temperature	Time (secs)	No. of cycles
Denature	97°C	60	32
Anneal	48°C	60	
Extension	72°C	180	
Final ext.	72°C	420	
Soak	4°C	∞	

Six different gene regions were successfully isolated. Five were chloroplast regions (Table 2.3 and Figure 2.1) – *trn*L intron, *trnL-F* intergenic spacer (Taberlet *et al.*, 1991), *atpB-rbcL* intergenic spacer (Samuel *et al.*, 1997), *rpl16* (Jordan *et al.*, 1996), *rps16* (Oxelmann *et al.*, 1997), and one was of nuclear origin – the internal transcribed spacer (ITS) region.

Table 2.3: Primer sequence, target region and genetic origin

Genetic origin	Target region	Primer Sequence
Chloroplast DNA	*trnL-F* (Taberlet *et al.*, 1991)	Fwd: c: CGAAATCGGTAGACGCTACG Rev: f: ATTGAACTGGTGACACGAG
Nuclear DNA	ITS-1, ITS-2 (Sun *et al.*, 1994)	Fwd: AB101: CGAATTCATGGTCCCGTGAAGTGTTC Rev: AB102: AGAATTCCCCGGTTCGCTCGCCGTT
Chloroplast DNA	*rps16* (Oxelmann *et al.*, 1997)	Fwd: 16F: GTGGTAGAAAGCAACGTGCGACTT Rev: 2R: TCGGGATCGAACATCAATTGCAAC
Chloroplast DNA	*rpl16* (Jordan *et al.*, 1996)	Fwd: F71: GCTATGCTTAGTGTGTGACTCGTTG Rev: R1661: CGTACCCATATTTTTCCACCACGAC
Chloroplast DNA	*atpB-rbcL* (Samuel *et al.*, 1997)	Fwd: 1R: GTTTCTGTTTGTGGTGACAT Rev: 2R: GAAGTAGTAGGATTGATTCTC

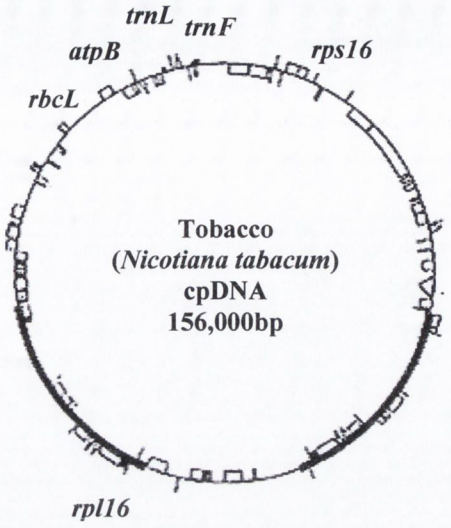

Figure 2.1: Structure of chloroplast DNA in *Nicotiana tabacum* L. (Solanaceae). The positions of the 5' *trnL* exon (UAA), the *trnL/F* intergenic spacer and the *trn*F exon (GAA) (Taberlet *et al.*, 1991), *atpB-rbcL* intergenic spacer (Samuel *et al.*, 1997), *rpl16* (Jordan *et al.*, 1996) and *rps16* (Oxelmann *et al.*, 1997)

2.2.4 PCR-RFLP

For each restriction digestion of PCR product, a 20µl reaction was prepared with 10µl of PCR product, 2µl 10x restriction buffer and 7µl of SUW. In principle, 1 unit of restriction endonuclease completely digests 1µl of purified DNA in 1 hour using the recommended assay conditions, however extra units (2.5 units) were added to ensure complete digestion. The reaction was incubated at 37°C for 2 hours. The reaction was stopped by the addition of 1µl of 0.25M EDTA. 10µl of the restriction digestion product was loaded onto an 8% non-denaturing polyacrylamide gel (Table 2.4), which separates fragments of 60 to 400bps and run for 4hrs at 120V on a Gibco BRL® vertical gel rig Model V15.17. Gels visualised and recorded in the same way as for tDNA.

Table 2.4: Polyacrylamide gel electrophoresis

Reagent	Quantity (Conc.)
Sterile ultrapure water (SUW)	34.48mls
Accugel 29:1 Acrylamide: Bisacrylamide solution. National Diagnostics™	10mls
10xTBE	5mls
Ammonium persulfate (APS, 10%)	0.5mls
Tetra methyl ethylene diamine (TEMED)	10µl

2.2.5 Nuclear microsatellite markers

Twelve primer pairs were selected from published primers (Table 2.5) according to three specific criteria firstly genome specificity, secondly, that they had been identified as polymorphic within a mapping population and thirdly that they had been used for mapping and the locus in question had been useful in analyses by Lowe *et al.*, (Unpublished). Reverse primers were labelled at the 5' end with FAM, JOE or TAMRA fluorescent dyes to enable detection on the automated genetic analyser.

SSR PCRs were carried out using reagents as in Table 2.1 and conditions as in Table 2.2 for a 25µl reaction and run out on 1.2% agarose gels as with tDNA to check for successful amplification.

Amplified SSR reactions were run on the automated genetic analyser (ABI Prism™ 310; Applied Biosystems) using the following protocol. 24µl Hi-Di formamide (Applied Biosystems) and 0.25µl ROX were added to a 0.5ml microcentrifuge tube. Samples were diluted by 1:40 and 0.5µl of each FAM, JOE or TAMRA was then added to the formamide solution. The samples were vortexed to ensure adequate mixing. The tubes were centrifuged for 5 seconds to spin down the reagents to the bottom of the tube and were then heated to 95°C for a minimum of 5mins on a heated block (Grant). The samples were cooled on ice. The tubes were centrifuged again, the lids removed and replaced with septa. The samples were run using module GS STR POP4 (1ml) A for 28mins. When the run was completed samples were processed using Genescan® version 3.2.1 (Applied Biosystems) using GSROX500A size standard and the SSR matrix FJTR A. Samples were then imported into Genotyper® version 2.5 (Applied Biosystems) to further compare banding patterns.

2.2.6 Amplification of DNA fragments for DNA sequencing

PCR reactions to amplify specific gene regions were carried out in the same way as in section 2.2.3 using the primers listed in Table 2.3.

2.2.6.1 Spin column purification of PCR products for DNA sequencing

An aliquot of TE buffer was heated to 65-70°C. 400µl binding solution (Gibco BRL) was added to amplification reactions (50µl) and mixed thoroughly. A spin cartridge was placed in a 2ml wash tube and the sample was loaded into the spin cartridge. The mixture was centrifuged in a microcentrifuge at 12,000 × *g* for 1min. The flow through was discarded. The spin cartridge was placed back into the 2ml wash tube and 700µl wash buffer (Gibco BRL) was added. The mixture was centrifuged at 12,000 × *g* for 1min. The flow through was discarded. The mixture was centrifuged again at 12,000 × *g* for 2mins to remove all residual wash buffer. The spin cartridge was placed into a 1.5ml recovery tube and 50µl warm TE buffer was added directly to the centre of the spin cartridge. The samples were then incubated at room temperature for 1min and centrifuged at 12,000 × *g* for 2mins so that the purified DNA was eluted into the recovery tube.

2.2.6.2 Cycle sequencing

The sequencing reaction was performed by adding 1µl Big Dye Terminator Cycle Sequencing mix (Applied Biosystems), 1.8µl sterile ultrapure water (SUW), 3.5ml sequencing buffer (200mM Tris, 5mM $MgCl_2$, pH 9), 0.7µl primer ($5ng\mu l^{-1}$) and 3µl PCR product to 200µl tubes. The tubes were centrifuged in a microcentrifuge for 5 seconds to spin down the reagents to the bottom of the tube. Samples were kept on ice until they were loaded into the Thermal Cycler (Geneamp® PCR System 9700) and run according to conditions in Table 2.5.

Table 2.5: Cycle sequencing conditions

Cycle	Temperature (°C)	Duration
Denaturating	96	10sec
Annealing	50	5sec
Extension	60	4mins

2.2.6.3 Clean up of cycle sequencing reactions

A master mix sufficient for the total number of samples was made up using 50µl 100% ethanol and 2µl 3M sodium acetate per sample. 52µl of this master mix was then added to the required number of 0.5ml tubes. The cycle sequencing reaction mixture was then added and following mixing was left at room temperature for 5mins. The samples were placed on ice for 5-10mins. The samples were then centrifuged at 12,000rcf (12 × *g*) for 25mins. The ethanol solution was drained off and 300µl 70% ethanol was added and centrifuged at 12,000rcf (12 × *g*) for 15mins to wash the sample. The ethanol solution was drained off and 300µl 70% ethanol was added for a second time. Following centrifugation at 12,000rcf (12 × *g*) the ethanol was drained off. Samples were placed between two layers of tissue and left overnight to ensure evaporation of all ethanol.

25µl of template suppressant reagent (TSR; Applied Biosystems) was added to the lypholised samples to denature the DNA. The samples were vortexed to ensure adequate mixing. The tubes were centrifuged for 5 seconds to spin down the reagents to the bottom of the tube and were then heated to 95°C for ≥2mins on a heated block (Grant). The samples were cooled on ice. The tubes were centrifuged again, the lids removed and replaced with a septa.

Following denaturation, the samples were run on a 310 Genetic Analyser using ABI Prism™ 310 module SEQ POP6 (1.0ml) E for 105mins for a long run or using module SEQ POP6 Rapid (1.0ml) E for 55mins for a short run.

2.2.6.4 DNA sequence analysis

Sequenced DNAs were processed using DNA Sequence Analysis™ 3.4.1 (Applied Biosystems). Forward and reverse strands of sequenced DNA were assembled in Sequencher™ 3.1 (Gene Codes Corporation) or Autoassembler 2.1™ (Applied Biosystems). A consensus sequence was then created and saved in Fasta format. Sequences were also imported into PAUP and aligned by eye.

Nucleotide sequences of three-chloroplast regions A (*atpA* to *rps4* incorporating *rpoB*; 36,129bps; Figure 2.7), B (*ndhC* to *cemA* incorporating *rbcL*; 11,002bps; Figure 2.8) and C (*ndhB* to *23S* incorporating *16S*; 9,419bps; Figure 2.9) derived from *Brassica napus* cv. Licosmos and spanning approximately one third of

the *Brassica* plastid genome were used (Coyne, 2002). The *B. napus* cv. Licosomos sequences were developed by Coyne and Kavanagh (2002) via the construction of a library i.e. a cloned set of cpDNA restriction endonuclease fragments covering the entire genome. A continuous sequence was then developed by employing a 'primer walking' strategy i.e. designing primers from the end of existing sequences. The sequences were then assembled using GeneJockey II from Biosoft®. Further DNA sequences obtained from Genbank were also used (NCBI, 2003)(Table 2.14).

All plastid DNA sequences were then aligned and screened for regions containing microsatellite repeats for primer development. A Perl script (Thiel, 2003) modified by Dr. Nicolas Salamin (Trinity College, Dublin) was used for this purpose. The script was made up of two components, the first defined the type of SSR detected in terms of unit size i.e. mononucleotide, di-nucleotide, tri-nucleotide etc. and the number of times this unit was repeated. The second component took into account the possibility of an interruption in the SSR. This produced extensive data. The number of sequences examined, total size of examined sequences, total number of identified SSRs, number of SSR containing sequences, number of sequences containing more than one SSR and number of SSRs present in compound formation were all investigated.

The sequences were put into Fasta format, imported into Clustal W (Higgins *et al.*, 1994) and aligned using a Blosum, fast algorithm for multiple sequence alignment. The resulting matrix was then used along with the SSR information to search for regions suitable for plastid primer development.

2.2.7 Plastid microsatellite primer design

Following the alignment of homologous sequences of four chloroplast regions (Figure 2.1 and Table 2.3): *trnL* intron, *trnL-F* intergenic spacer (Taberlet *et al.*, 1991), *atpB-rbcL* intergenic spacer (Samuel *et al.*, 1997), *rpl16* (Jordan *et al.*, 1996), *rps16* (Oxelmann *et al.*, 1997), from a range of Brassicaceae, primers were designed according to the positioning of microsatellite (SSR) regions. Areas of conserved sequence flanking the SSR were then selected for primer design.

When a suitable region was discovered, primers of approximately 20bp in size were designed from conserved regions either side of the microsatellite, keeping the GC content high. To develop forward primers the 5' to 3' sequence of target

DNA was used, while the reverse and complement of the target (forward strand) was used for the reverse primer. For each primer pair one of the primers was selected for fluorescent labelling. Wherever possible one of the amplification primers for the whole locus was used as one of the primers. In most cases fragment lengths of 100-300bps were aimed for. Multiplexing on the Genetic Analyser was enabled by using different dye-labels, thus allowing three loci to be screened in each injection of the capillary. Where possible a number of constraints were applied to the primers during design:

1. To prevent self-annealing non-complementary primers were chosen.
2. None of the primers had a 3' thymine (T), as T is the least discriminatory nucleotide (primers with a 3' T have greater mismatch tolerance).
3. At least one adenine or T was incorporated in the 3' most triplet to prevent mismatch tolerance of primers with consecutive cytosine's or guanine's.
4. The predicted dissociation temperatures of the primers in each pair were kept roughly the same (Kidd and Ruano, 1995).

The following formula was used to calculate the melting temperature (T_m) of the primer (MWG, 2003):

$$T_m = 69.3 + (0.41 \times GC\%) - \frac{650}{\text{Seq length}}$$

$$\text{where } GC\% = \frac{G + C}{\text{Seq length}} \times 100$$

Primers were tested on a broad range of plant material using PCR conditions as in Table 2.2. PCR products were treated in the same way as nuclear SSRs except that a 1:80 dilution was necessary before loading them onto the automated genetic analyser.

2.2.8 Statistical analysis of SSR markers

Microsatellite fragments are not easily interpreted in polyploid species such as *Brassica napus*. Furthermore some non-specific amplification products were detected. For this reason the fingerprints were interpreted as presence-absence data

of particular fragments. A matrix of pairwise-distance measures was created in PAUP 4.0 (Swofford, 1999). This matrix used the similarity measure *S* developed by Dice (1945) and applied to molecular data by (Nei and Li, 1979)). The equation is as follows:

$$S = \frac{2N_{AB}}{(N_A + N_B)}$$

where N_A is the number of bands in individual A, N_B is the number of bands in individual B and N_{AB} is the number of bands they have in common.

This measure varies from 0 when samples compared have no bands in common to 1 when they are identical (Cole and Kuchenreuther, 2001). This method works best if each marker locus produces a single band (or at least the same number of bands) for every allele; that is, there are few or no "null" alleles that don't produce any band. Ideally, each gel lane should assay a single genetic locus. The distance matrix was subjected to Neighbour Joining analysis and the results were displayed using phylograms produced in PAUP 4.0 (Swofford, 1999). Phylograms are phylogenetic trees that indicate the relationship between the taxa and also convey a sense of time or rate of evolution via branch length.

2.3 Results

2.3.1 DNA extractions

164 DNA extractions were carried out from 82 individuals of verified specimens i.e. of known origin and identification (Appendix 2.1-2.4). All DNAs were column cleaned (Gibco BRL) prior to further analysis to produce molecular markers.

In an effort to produce markers for species and cultivar identification in *Brassica* and wild relatives the efficiency of PCR-RFLP was tested first, followed by nuclear and plastid microsatellite marker analysis.

2.3.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Four restriction enzymes were tested on five amplified gene regions: the plastid *atpB-rbcL* spacer region, *trnL* intron and *trnL-F* intergenic spacer; *rps16*, *rpl16* and the nuclear ITS gene region. The restriction enzyme *Eco*RI was uninformative and no restriction occurred using *Bam*HI. *Mse*I and *Hinf*I were found to be the most useful for generating polymorphic fingerprints (Figure 2.2). Figure 2.2 (A) shows *atpB-rbcL, rpl16* and *rps16* digested with *Mse*I, while Figure 2.2 (B) shows *atpB-rbcL, rpl16* and *rps16* digested with *Hinf*I.

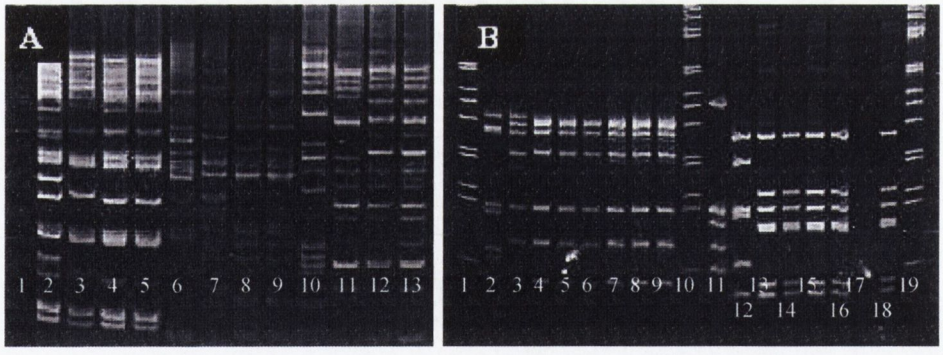

Figure 2.2: PCR-RFLP: (A) PCR products of *atpB-rbcL* (2-5), *rpl16* (6-9) and *rps16* (10-13) digested with *Mse*I. *Sinapis arvensis* (2, 6 and 10), *Raphanus sativus* (3, 7 and 11), *Brassica oleracea* ssp. *capitata* (4, 8 and 12) and *B. oleracea* ssp. *gemmifera* (5, 9 and 13). (B) PCR products of *atpB-rbcL* (2-9) and *trnL-F* (11-18) digested with *Hinf*I. 1, 10 and 19 are molecular weight ladders (1kb). *S. arvensis* (2 and 11), *R. sativus* (3 and 12), *B. oleracea* ssp. *capitata* (4, 5, 13 and 14), *B. oleracea* ssp. *gemmifera* (6, 7, 15 and 16) and *B. oleracea* ssp. *italica* (8, 9, 17 and 18). Products are visualised on an 8% non-denaturing polyacrylamide gel

*Mse*I successfully differentiated between *Sinapis arvensis*, *Raphanus sativus* and *Brassica oleracea* species. However, it was unable to distinguish differences between *B. oleracea* subspecies. Although *Hinf*I generates fewer fragments than *Mse*I, it also enables differentiation between *Sinapis arvensis*, *Raphanus sativus* and *Brassica oleracea*. However, this enzyme was also unable to distinguish differences between *B. oleracea* subspecies.

2.3.3 Nuclear SSRs

Twelve nuclear SSRs were tested for polymorphism among *Brassica* species (Tables 2.6-2.13). The 12A marker distinguishes *Arabidopsis thaliana* spp., *B. napus* cv. Triolo and *Sinapis arvensis.* However there is no consistent distinction between *B. napus* cv. Marinka, *B. napus* ssp. *napobrassica, B. nigra, B. oleracea, B. rapa* or *Raphanus sativus.* The 35D marker distinguished *B. oleracea* individuals from *B. rapa* individuals however the *B. napus* individuals were split up and grouped with various other *Brassicas.*

The 38A marker separated the *B. napus* individuals into *B. oleracea* and *B. rapa* type individuals. The 59A1 marker separated out most *B. napus* individuals tested but grouped some *B. napus* individuals with *B. oleracea, B. nigra* and *B. napus* ssp. *napobrassica* individuals and some with *B. rapa* type individuals. The 72A and 83/1 markers distinguished the *B. oleracea* species from other species tested. The nga129, Na10-E08, Na12-A01 and Na12-D03 markers failed to distinguish between species. The 68/1, Na10-B04, Na10-E02, Na12-E06a markers did not amplify consistently across species.

The results of a Neighbour Joining analysis using all bands obtained from nuclear SSR analysis (Table 2.7 and 2.8) are shown in two ways: an unrooted-phylogram (Figure 2.3) and a midpoint-rooted phylogram (Figure 2.4). The unrooted-phylogram demonstrates the distance or differences between species by branch lengths and also by the orientation of the branches. The midpoint-rooted phylogram (roots the tree at a point of maximum separation of two groups) is included so that branches that are unclear on the unrooted tree may be easily distinguished. Most of the *Brassica* species tested separate into their taxonomic groups. *B. napus* ssp. *napobrassica* (62) and *B. nigra* (90) do not; they group with *B. napus* cv. Marinka individuals.

Table 2.6: Nuclear microsatellite primer sets used

SSR	Repeat motif	Forward primer sequence	Reverse primer sequence	Expected size range	No of alleles	Reference
12A	$(GA)_{11}$ $(AAG)_4$	5' GCCGTTCTAGGGTTTGTGGAA	5' GAGGAAGTGAGAGCGGGAAATCA	250-325	21	(Szewc-McFadden *et al.*, 1996)
35D	$(GA)_{13}$	5' GCAGAAGGAGGAGAAGAGTTGG	5' TTGAGCCGTAAAGTTGTCACCT	210-270	11	
38A	$(TG)_{11}$	5' TGGTAACTGGTAACCGACGAAAATC	5' ACGCTGTCTTCAGGTCCCACTC	150-200	14	
59A1	$(CA)_{11}$	5' TGGCTCGAATCAACGGAC	5' TTGCACCAACAAGTCACTAAAGTT	425-480	4	
68/1	$(GA)_{34}(ACACA)_2$	5' TCGCATGCTCCTCTAGACTCG	5' TTTAGCACGGGAATGTCAGG	200-400		
72A	$(TAA)_5(GA)_9$	5' GCCCACCCACCTTCTTGTCCT	5' CCCTTCATCCAAACTCCTCCTCGT	240-300	12	
83/1	$(GA)_{11}$	5' GCCTTTCTTCACAACTGATAGCTAA	5' TCAGGTGCCTCGTTGAGTTC	190-240	3	
nga129	$(AG)_{20}$	5' TCAGGAGGAACTAAAGTGAGGG	5' CACACTGAAGATGGTCTTGAGG	169-188	6	(Westman and Kresovich, 1999)
Na10-B04		5' GCGTCGAGAGAGATCGAGAG	5' CTCACCGTCACTGCTTCATC	140-160		(Lowe *et al.*, 2002)
Na10-E08		5' TCGCGCATGTAATCAAAATC	5' TGTGACGCATCCGATCATAC	120-160		
Na12-D03		5' GGTAAGCCAAAAACCCTTCC	5' GAAACCGGTAACAAAGTCGG	320-350		
Na12-E06a		5' TTGGGTTGACTACTCGGTCC	5' CCGTTGATTTGGCTAAGACC	100-110		

Table 2.7: Presence-absence matrix of all nuclear SSR markers

Species id no.	B213	B215	B218	B221	B223	B225	B230	B234	B235	B254	B266	C126	C129	C130	C131	C133	C135	C138	C139	C150	C151	C154	C155	C156	C158	C168	D438	D446	D448	D449
30	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
36	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
53	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
62	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	0	1	1	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0
90	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0
127	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0
131	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1
134	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
135	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
136	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
137	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
138	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0
139	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
140	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0
141	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
142	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
143	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
144	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
145	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
146	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0
147	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0
148	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0
149	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0
150	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	0	0	1	0	0
151	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
152	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	1	0	0	0	1	0	0	0
153	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0

Table 2.7: Presence-absence matrix of all nuclear SSR markers

Species id no.	F244	F248	F250	F251	F256	F259	F272	F279	F282	F283	F286	F298	G190	G191	G194	H169	H174	H175	H176	H184	H185
30	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0
33	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0
36	0	1	0	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0
53	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0
62	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0
70	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
90	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
127	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0
131	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
134	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0
135	1	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0
136	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
137	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0
138	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0
139	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
140	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	0
141	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
142	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
143	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0
144	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	0
145	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1
146	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
147	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0
148	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0
149	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
150	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	0
151	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	0
152	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0
153	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0

Key for Table 2.7: (see Appendix 1 for further information)

Species identification numbers:

30 *B. oleracea* ssp. *capitata*

33 *B. oleracea* ssp. *gemmifera*

36 *B. oleracea* ssp. *italica*

46 and 53 *B. rapa*

62 *B. napus* ssp. *napobrassica*

70 *B. rapa* ssp. *rapifera*

90 *B. nigra*

131 *B. napus* ssp. Sheila

127 134-146 *B. napus* ssp. Marinka

147-149 *B. napus* ssp. Jura (MS)

150-153 *B. napus* ssp. Triolo (MS).

Primers used are indicated by a letter, while each number indicates the peak size (Table 2.5)

B: 35D

C: 38A

D: 59A

E: 72A

F: 83/1

G: *nga*129

H: Na10-E08

Six out of the twelve primer sets tested consistently produced informative polymorphic bands (Tables 2.8-2.13). The 12A primer (Szewc-McFadden *et al.*, 1996) produced the most informative results since a single peak at 318bps was found to be present in *B. napus* cv. Marinka parent plants and was absent in *B. napus* cv. Triolo male sterile parent plants (a male sterile *Brassica* used as a bait plant in later experiments).

The combination of all nuclear SSR markers tested in a Neighbour Joining analysis (Figures 2.3 and 2.4) failed to differentiate between different varieties of *Brassica napus* however, it did succeed in interspecific separation of *Brassica napus*, *B. oleracea* and *B. rapa*. *Brassica nigra* falls out between *B. napus* individuals however the long arm indicates that it is quite separate from the other groups as can be seen more clearly on the unrooted tree (Figure 2.4).

Table 2.8: Cross amplification in *Brassica* species using nuclear SSR marker 35D

B: 35D	*B. oleracea*	*B. rapa*	*B. nigra*	*B. napus*
B. oleracea	√ (221, 223, 230)			
B. rapa	√ (221, 223, 230)	√ (221, 254)		
B. nigra	√ (221, 225, 266)	√ (215, 218, 221, 225, 254, 266)	?	
B. napus	√ (213, 215, 218, 221, 223, 225, 230, 234, 235, 254, 266)	√ (213, 215, 218, 221, 223, 225, 230, 234, 235, 254, 266)	√ (213, 215, 218, 221, 223, 225, 230, 234, 235, 254, 266)	√ (213, 215, 218, 221, 223, 225, 230, 234, 235, 254, 266)

Table 2.9: Cross amplification in *Brassica* species using nuclear SSR marker 38A

C: 38A	*B. oleracea*	*B. rapa*	*B. nigra*	*B. napus*
B. oleracea	√ (129, 130, 150)			
B. rapa	√ (129, 151)	√		
B. nigra	√ (131, 133, 135, 154, 156)	√ (129,131, 135, 151, 154)	?	
B. napus	√ (129, 133, 139, 150,151, 155, 156)	√ (129, 133, 139, 150,151, 155, 156)	√ (131, 133, 135, 154, 156)	√ (129, 133, 139, 150,151, 155, 156)

Table 2.10: Summary of cross amplification in *Brassica* species using nuclear SSR marker 59A

D: 59A	*B. oleracea*	*B. rapa*	*B. nigra*	*B. napus*
B. oleracea	X			
B. rapa	√ (446)	√ (446)		
B. nigra	X	√ (446)	?	
B. napus	√ (438, 446, 448, 449)	√ (438, 446, 448, 449)	√ (438, 446, 448, 449)	√ (438, 446, 448, 449)

Table 2.11: Summary of cross amplification in *Brassica* species using nuclear SSR marker 83/1

F: 83/1	*B. oleracea*	*B. rapa*	*B. nigra*	*B. napus*
B. oleracea	√ (248, 259)			
B. rapa	√ (251, 259, 283)	√ (256, 298)		
B. nigra	√ (256, 298)	√ (256, 298)	?	
B. napus	√ (248, 272, 282, 286)	√ (248, 251, 283, 286)	√ (256, 298)	√ (244, 248, 250, 259, 279, 282, 283, 286)

Table 2.12: Summary of cross amplification in *Brassica* species using nuclear SSR marker *nga*129

G: nga129	*B. oleracea*	*B. rapa*	*B. nigra*	*B. napus*
B. oleracea	√ (191, 194)			
B. rapa	√ (191, 194)	√ (194)		
B. nigra	√ (191, 194)	X	?	
B. napus	√ (190, 191, 194)	√ (190, 191, 194)	√ (190, 191, 194)	√ (190, 194)

Table 2.13: Summary of cross amplification in *Brassica* species using nuclear SSR marker Na10-E08

H: Na10-E08	*B. oleracea*	*B. rapa*	*B. nigra*	*B. napus*
B. oleracea	X			
B. rapa	√ (174, 175)	√ (174, 175)		
B. nigra	√(175)	√ (174, 175)	?	
B. napus	√ (175, 176)	√ (175, 176)	√ (174, 176)	√ (169, 174, 175, 176, 184, 185)

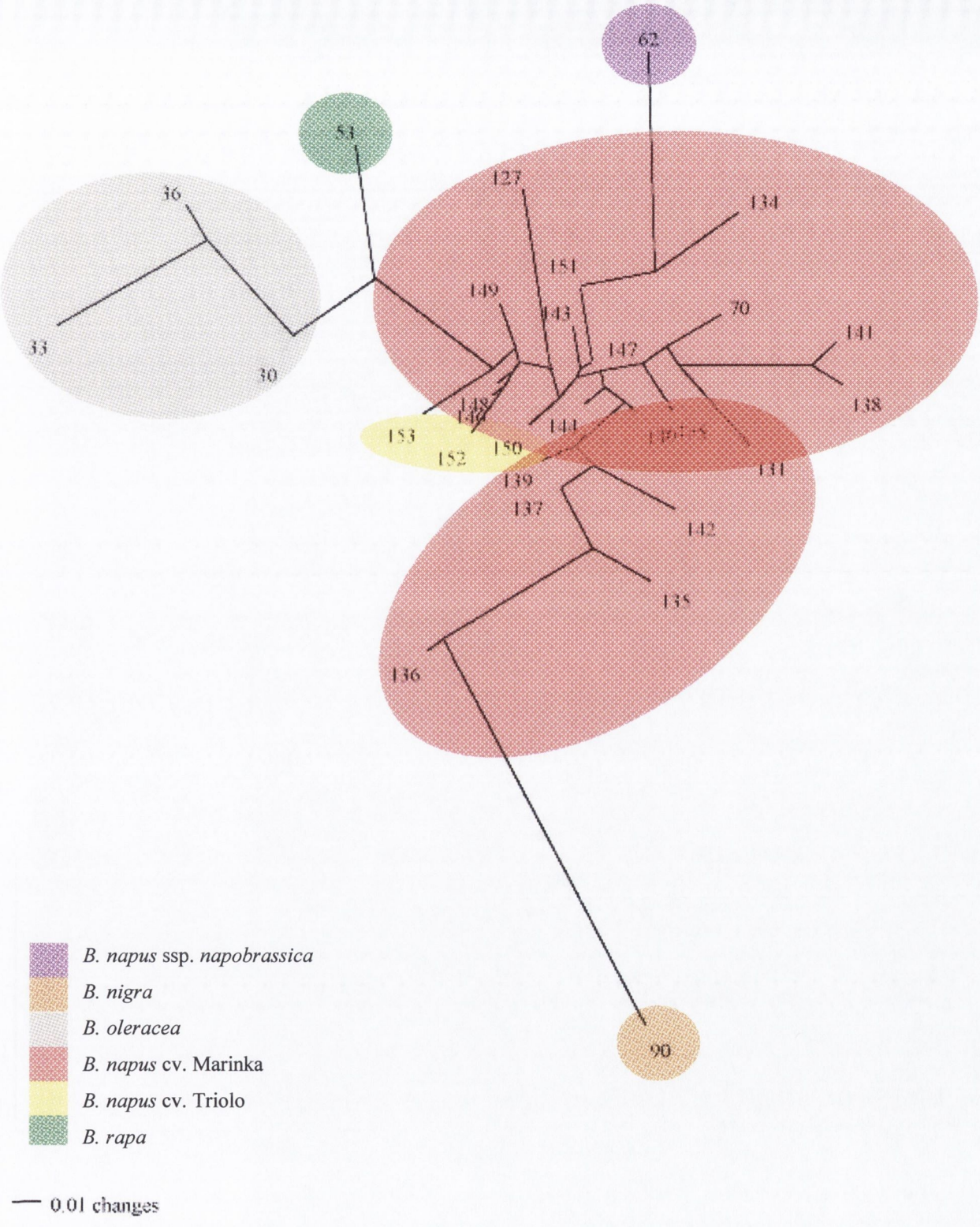

Figure 2.3: An unrooted-phylogram showing the results of a Neighbour Joining analysis using all bands obtained from a nuclear SSR analysis

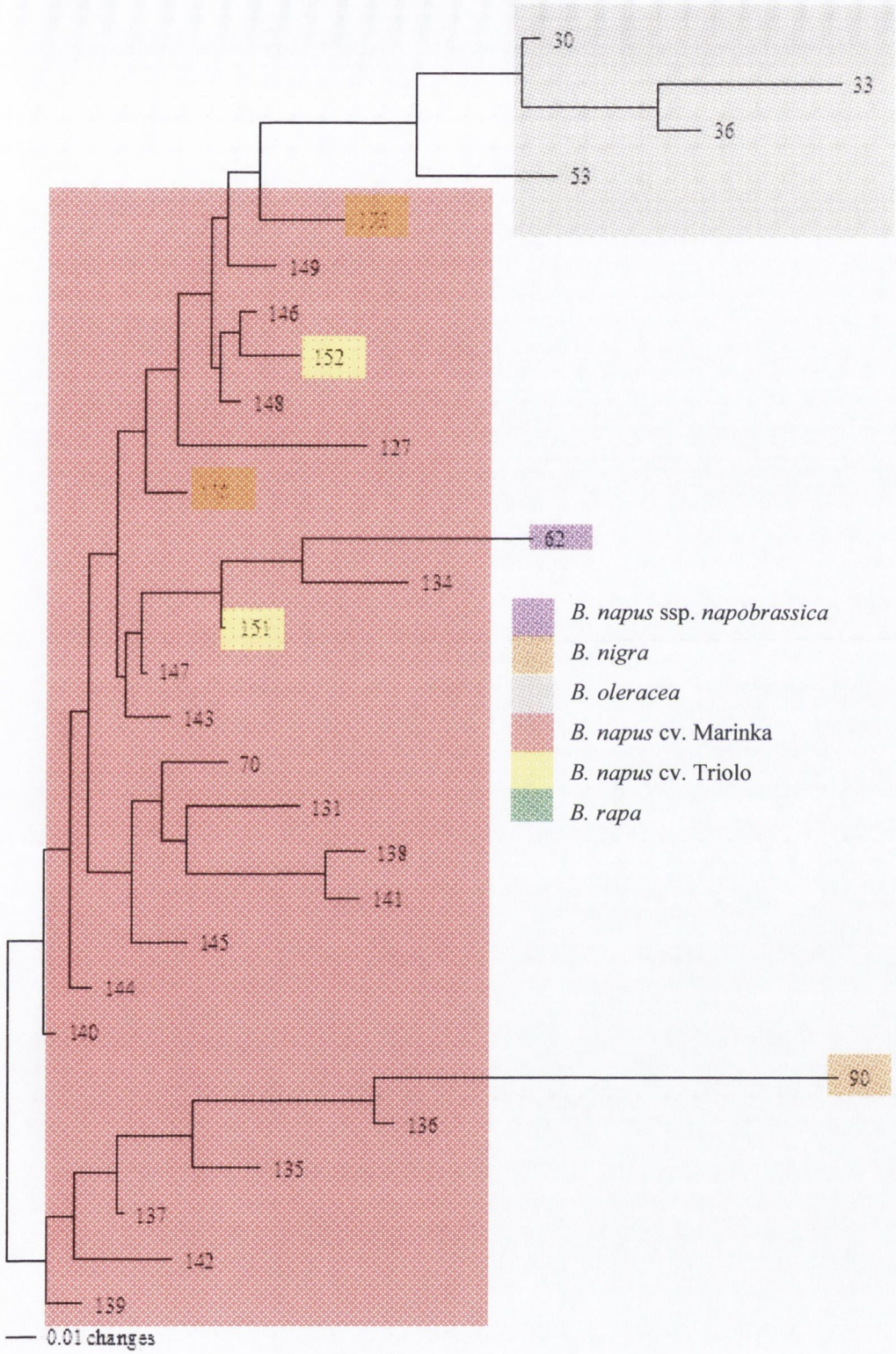

Figure 2.4: A midpoint-rooted phylogram showing the results of a Neighbour Joining analysis using all bands obtained from a nuclear SSR analysis

2.3.4 DNA sequencing

Eight species were successfully sequenced for five gene regions (the plastid *atpB-rbcL* spacer region (Appendix 2.1), *rpl16* (Appendix 2.2), *rps16* (Appendix 2.3), *trnL* intron and *trnL-F* intergenic spacer (Appendix 2.4), and the nuclear ITS gene region (Figure 2.5 and 2.6).

The ITS gene region is shown as a chromatogram of *Brassica napus* cv. Marinka in Figure 2.5 and as a matrix of seven species in Figure 2.6. Despite multiple attempts *Camilina sativus* (123) ITS sequencing failed probably due to copy type heterogeneity. The first row of the matrix shows intergeneric, interspecific and inter-varietal variation in the ITS1 region among the *Brassica* species tested.

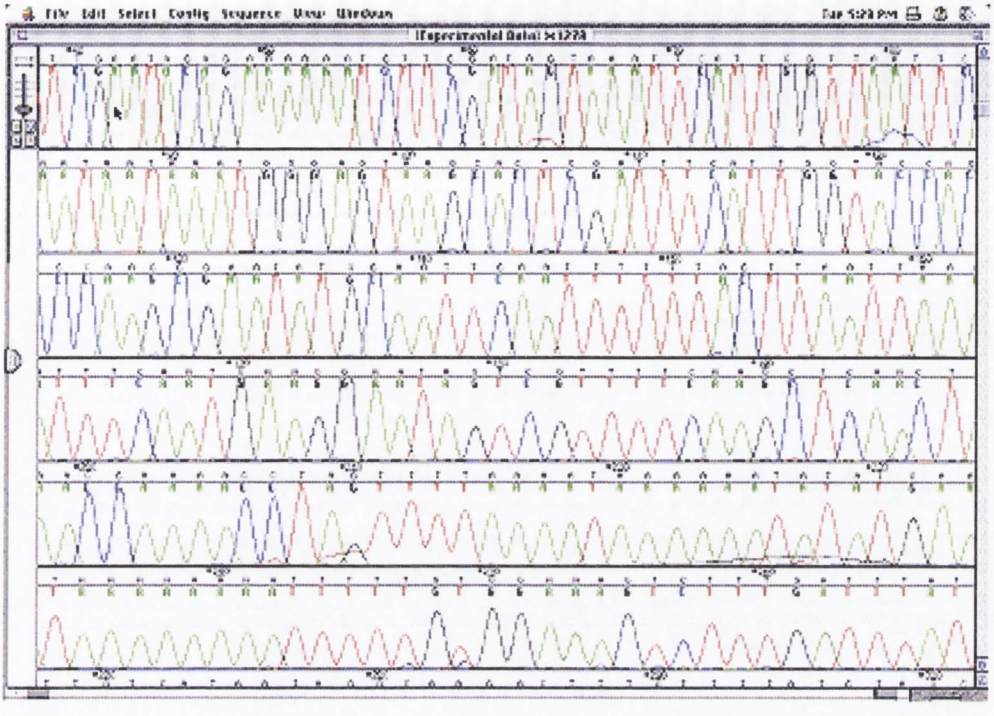

Figure 2.5: A chromatogram of an ITS DNA sequence of *B. napus* cv. Marinka viewed in Sequencher

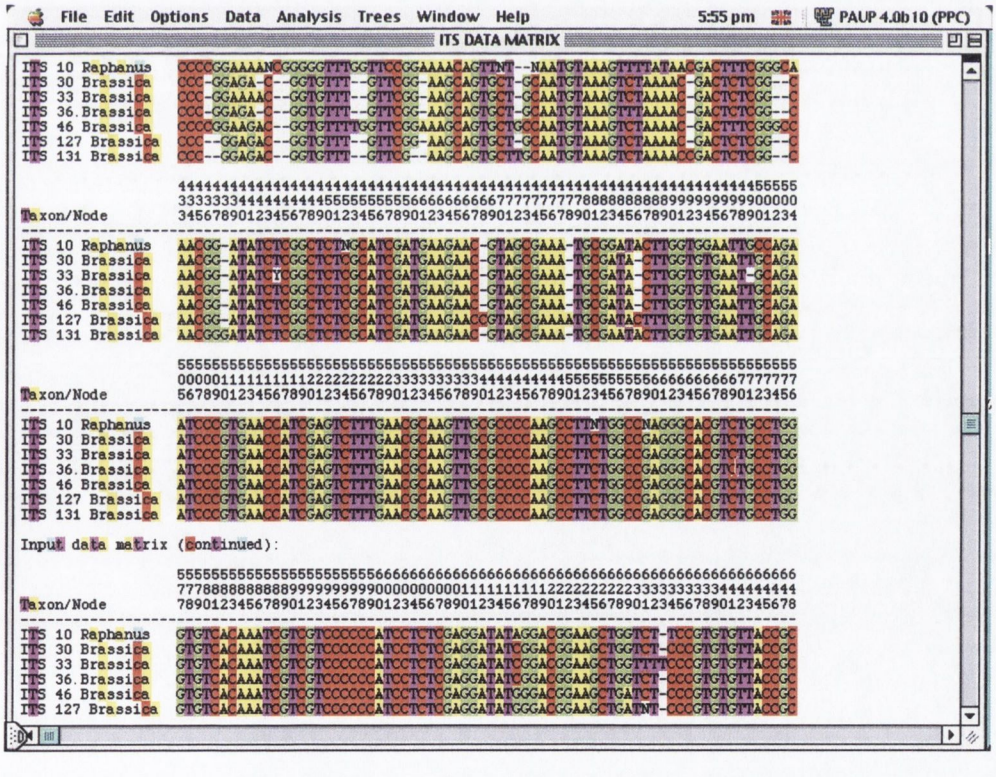

Figure 2.6: An ITS data matrix viewed in PAUP 4.0. 10: *Raphanus sativus*; 30: *B. oleracea* ssp. *capitata*; 33: *B. oleracea* ssp. *gemmifera*; 36: *B. oleracea* ssp. *italica*; 46: *B. rapa*; 127: *B. napus* cv. Marinka; 131: *B. napus* cv. Sheila

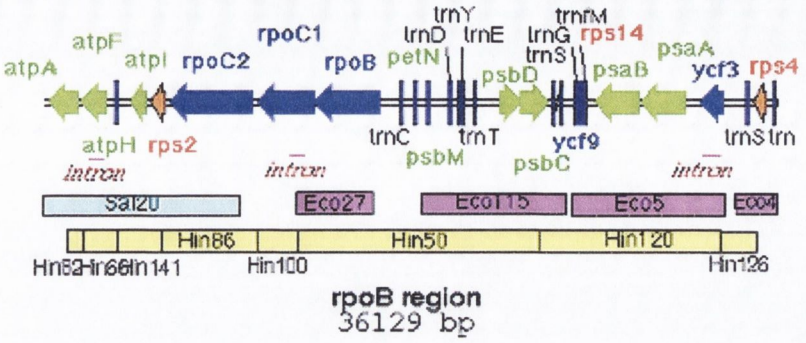

Figure 2.7: The structure of sequenced region A

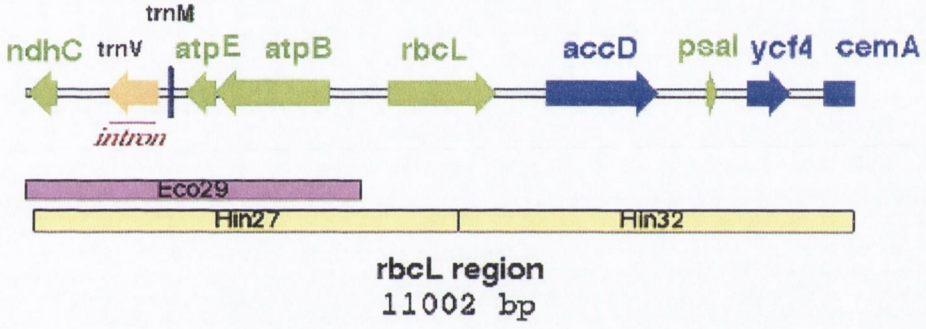

Figure 2.8: The structure of sequenced region B

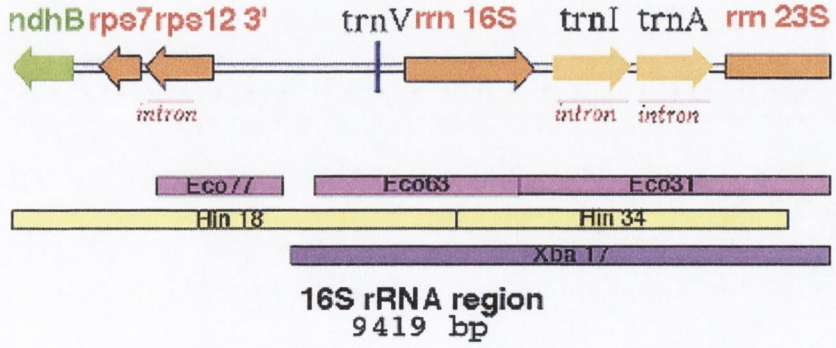

Figure 2.9: The structure of sequenced region C

Table 2.14: DNA sequences obtained from Genbank

Accession	Source of cp DNA sequence	Size (bp)	Definition
NC_000932	*Arabidopsis thaliana* Columbus	154478	Complete chloroplast genome (Sato *et al.*, 1999).
AF264734	*Brassica napus*	849	*Brassica napus* chloroplast *atpB-rbcL* intergenic spacer region (Zhang, 2000)
AF451571	*Brassica rapa* ssp. *chinensis*	1655	tRNA-Thr (*trnT*) gene, partial sequence; tRNA-Leu (*trnL*) gene, complete sequence; and tRNA-Phe (*trnF*) gene, partial sequence; chloroplast genes for chloroplast products (Yang *et al.*, 2002).
AF451572	*Brassica rapa* ssp. *pekinensis*	1655	
AF451573	*Brassica rapa* ssp. *rapa*	1655	
AF451574	*Brassica oleracea* var. *capitata*	1650	
AF451575	*Brassica juncea*	1655	
AF451576	*Raphanus sativus*	1654	
AF451577	*Raphanus sativus*	1655	
AF451578	*Brassica nigra*	1660	
AF451579	*Brassica nigra*	1660	
AF451580	*Sinapis alba*	1656	
AF451581	*Sinapis alba*	1657	
AF451582	*Lepidium virginicum*	2023	
Y15350	*Brassica cretica*	370	Intergenic region between *trnL* and *trnF* (Lanner).
Y15351	*Brassica cretica*	370	
Y15353	*Brassica hilarionis*	370	
Y15355	*Brassica insularis*	370	
Y15357	*Brassica montana*	370	
Y15358	*Brassica montana*	370	
Y15359	*Brassica oleracea*	370	
Y15360	*Brassica rupestris*	370	
Y15361	*Brassica villosa*	370	

2.3.5 Plastid microsatellite primer design

215 plastid SSRs were detected from a total of 57 sequences examined (equivalent to 163,720bps) (Table 2.14). Adenine and thymine mononucleotide repeats i.e. $(A)_n$ and $(T)_n$ are more common than cytosine and guanine repeats $(C)_n$ and $(G)_n$. Nearly all of the SSRs detected were mononucleotide but some di-, tri- and tetra-nucleotide repeats were recorded. Each SSR region was then examined for primer design. SSRs which were interrupted, shorter or absent in some of the sequences species were excluded. Several SSRs of satisfactory length were removed

due to lack of variation between species. Other SSRs were eliminated as the region surrounding the SSR was unsuitable for primer design e.g. insufficient G's and C's repeated A and T regions. For *trnL-F* and *rps16* no SSRs with 10 or more repeats were detected so the search parameters were altered to detect SSRs with 7 or more repeats (Table 2.15).

Table 2.15: Overall results of SSR search

Sequence	Total number of sequences examined	Total size of examined sequences (bp)	Total number of identified SSRs[1]	Number of SSR containing sequences	Average number of SSRs per sequence	Number of sequences containing more than 1 SSR	Number of SSRs present in compound formation
rps16	8	6884	39	8	4.9	8	2
rpl16	4	6039	8	4	2	4	0
trnL-F	30	34779	110	30	3. 7	15	3
Region A	2	63750	33	2	16.5	2	0
Region B	11	27669	18	10	1.8	3	0
Region C	2	24599	7	2	3.5	2	1
Total	57	163720	215	56	3.9	34	6

[1] 7 or more repeats for *trnL-F*; 10 or more repeats for all other sequences

[2] See Figures 2.8, 2.9 and 2.10 for more information on Regions A, B and C

Ten sets of SSR primers were designed and tested (Table 2.16). One primer set was developed for each of the regions C, *rpl16, rps16* and *trnL-F*, three for the region B and four for region A (and were named appropriately). Highlighted regions in Figures 2.10-2.19 show the sequences used for primer design, in each case a variable SSR region is present with conserved regions either side.

Primers were tested using the conditions outlined in Table 2.2. Amplification was successful with all primers, except the MF_RPS1 set. Primers set MF_A3 was monomorpic i.e. contained only one allele, while all others tested detected were polymporphic and detected a minimum of five alleles (Table 2.16).

Table 2.16: Plastid microsatellite primer sets

SSR	Forward primer sequence	Reverse primer sequence	Annealing temp. (°C)	Expected Size	No of alleles
MF_TRN1	5' TCAATTGCACATTCTAGAATTCTAAG	5' CAATTCAATATGGTTATATATTAGAG	55	160-180	5
MF_RPL1	5' GGTTCCGTCGTTCCCATCGC	5' CATAATAATTAGATAAATCTGTTCC	55	160-190	7
MF_RPS1	5' GTGGTAGAAAGCAACGTGCGACTT	5' ACTTGAGTTACGAGAGTACGAATG	50	170-180	0
MF_A1	5' AATGGTATGACTAGCTTATAAGG	5' CTTAACAATGAGATGAGGCAATC	55	270-300	9
MF_A2	5' CGGATCTATTATGACATATCC	5' GAAATATGAATACACTAGATTAGG	55	125-150	10
MF_A3	5' CCTGGCGGTATCAAGATGCCACT	5' GCCATAATGGTACAGAACTAT	55	120-200	1
MF_B1	5' GAAGGAATAGTCGTTTTCAAG	5' CATAAATAGAGTTCCATTTCGG	55	150-160	5
MF_B2	5' CGGCAGGAGTCATTGGTTCAAA	5' GATTTTGTAACTAGCTGACG	55	140-175	14
MF_B3	5' CTTATATTCATAAGCGAAGAAC	5' AATAACAATAGATGAATAGTCA	55	230-240	6
MF_C1	5' GGGCCGTTATGCTCATTACG	5' TCCTATTCATGGGGATTCCG	55	300-310	8

The PCR/sequencing forward primer was used in conjunction with a designed primer for the *rps16* gene region. Three SSR repeats are contained in this region, $(C)_{7\text{-}9}$ (blue block), $(A)_{7\text{-}8}$ (yellow block) and $(T)_{7\text{-}8}$ (red block) (Figure 2.10), however amplification was not successful for all species.

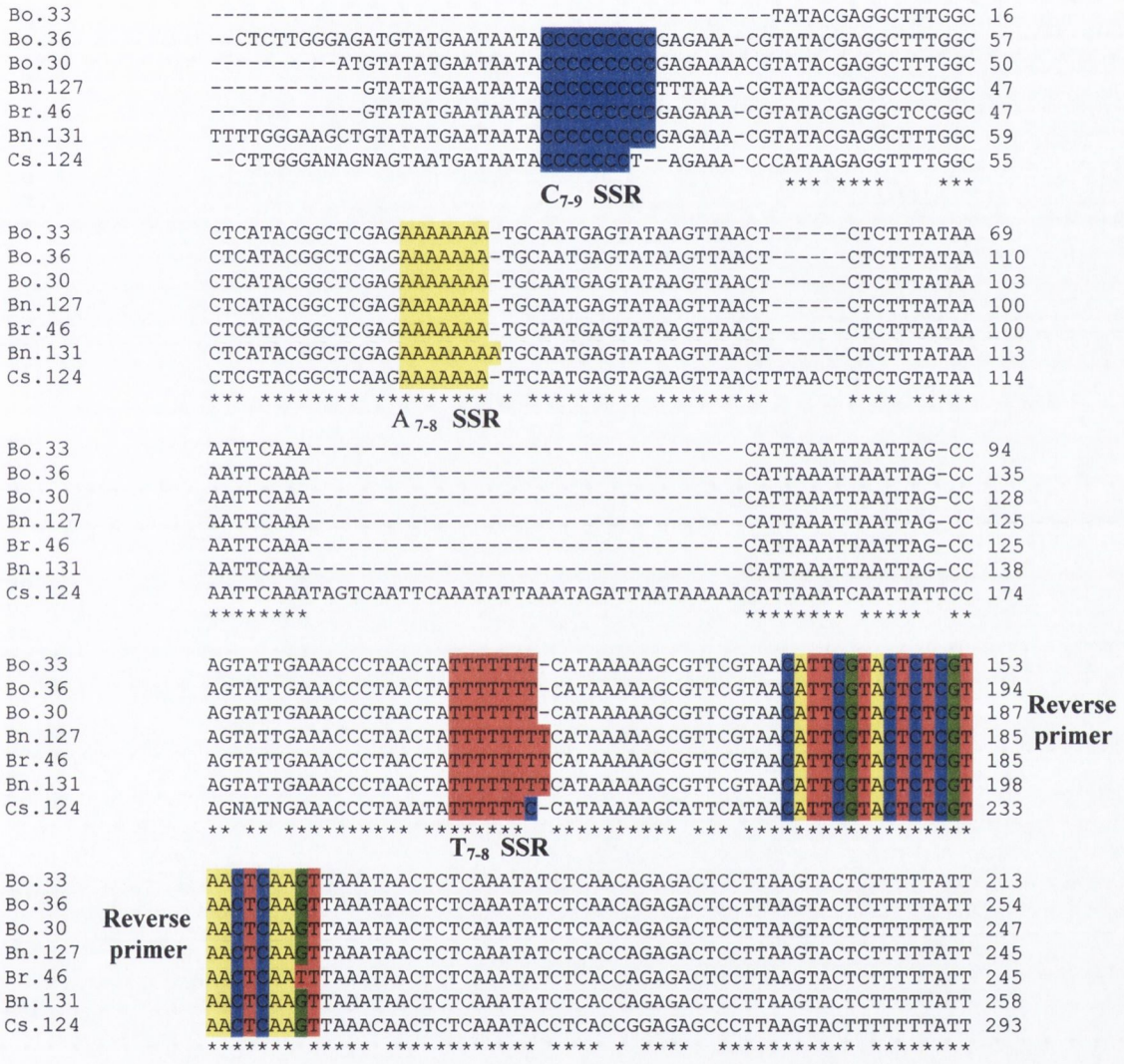

Figure 2.10: Clustal W multiple sequence alignment of *rps16* primer set 1. Bn. 30: *B. oleracea* ssp. *capitata*; Bn.33: *B. oleracea* ssp. *gemmifera*; Bn.36: *B. oleracea* ssp. *italica*; Ar.000932: *Arabidopsis thailiana*; 46: *B. rapa*; 123: *Camelina sativus*; 127: *B. napus* cv. Marinka and 131: *B. napus* cv. Sheila

A $(T)_{13}$ SSR repeat was found in the *rpl16* region and so primers were designed from conserved sequences either side of this region (Figure 2.11). This primer separated *B. napus* cv. Triolo, *S. arvensis, R. sativus, C. sativus, A. thaliana* and *B. rapa* from *B. oleracea* ssp. *capitata, B. oleracea* ssp. *gemmifera, B. oleracea* ssp. *italica, B. napus* ssp. *napobrassica* and *B. napus napus.* However this primer set failed to distinguish the various *B. oleracea* spp. and *B. napus* spp. from each other.

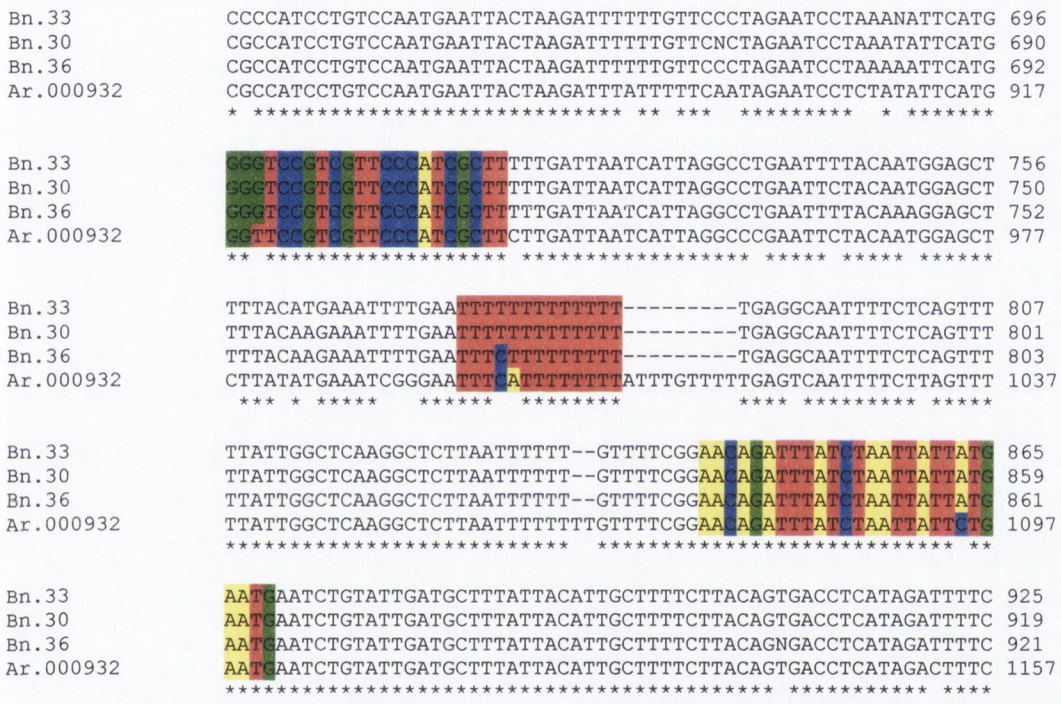

Figure 2.11: Clustal W multiple sequence alignment of *rpl16* primer set 1. Ar.000932: *A. thailiana*; Bn. 30: *B. oleracea* ssp. *capitata*; Bn.33: *B. oleracea* ssp. *gemmifera* and Bn.36: *B. oleracea* ssp. *italica*

For the *trnL-F* gene region, the SSR primer was based on an $(A)_{7\text{-}10}$ SSR repeat (Figure 2.12). This primer separated *S. arvensis* and *B. napus* cv. Triolo from all other *Brassica* species tested, however failed to differentiate between *R. sativus, B. rapa, B. oleracea* ssp. *capitata, B. oleracea* ssp. *gemmifera, B. oleracea* ssp. *italica, B. napus* ssp. *napobrassica* and *B. napus napus.* Although it distinguished *C. sativus* and *A. thaliana* from all other *Brassica* species tested, it failed to distinguish them from each other.

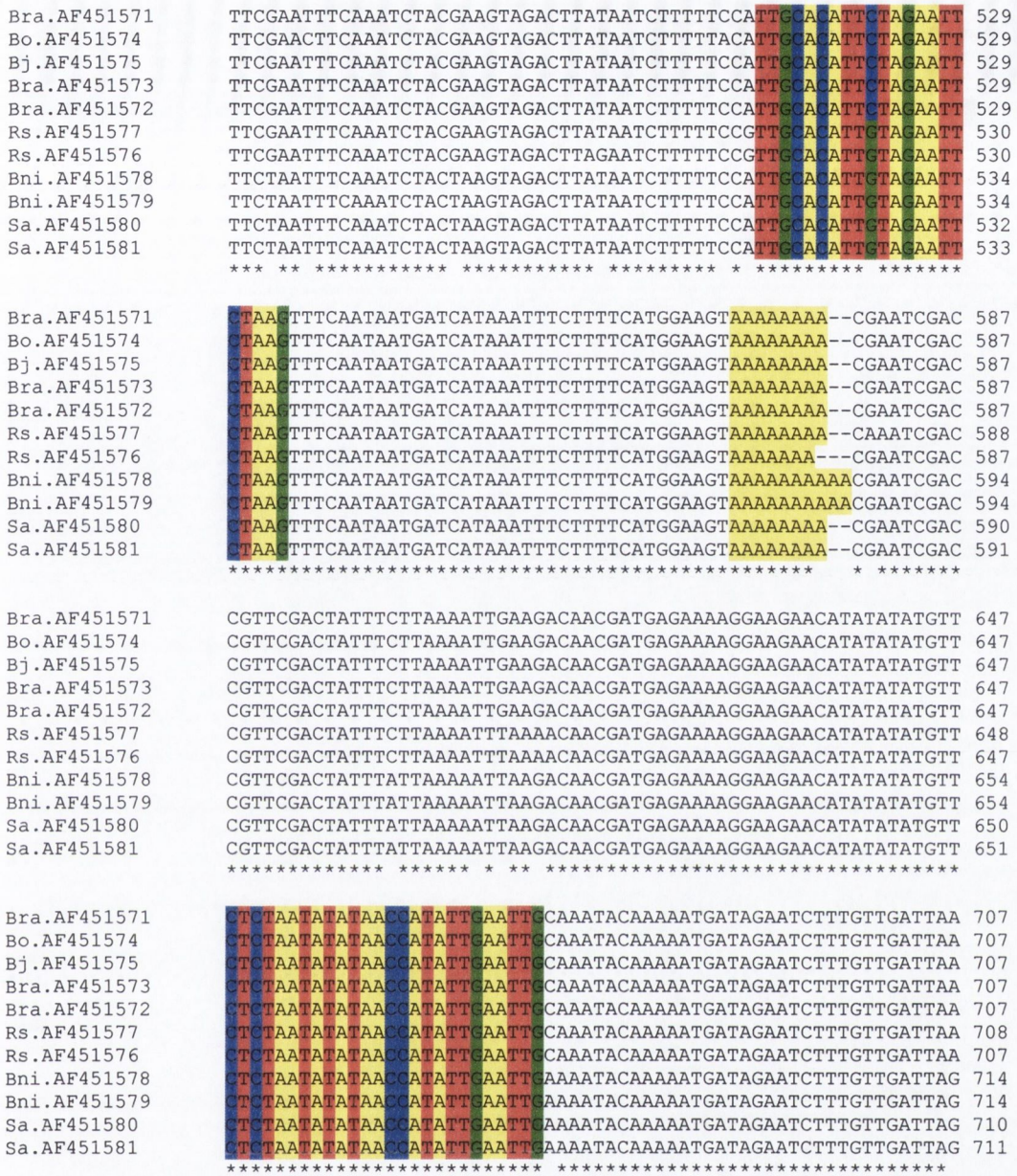

Figure 2.12: Clustal W multiple sequence alignment of *trnL-F* primer set 1. Bj.AF451575: *B. juncea*; Bn.AF264734: *B. napus*; Bni.AF451578: *B. nigra*; Bni.AF451579: *B. nigra*; Bo.AF451574: *B. oleracea* var. *capitata*; Bra.AF451571: *B. rapa* subsp. *chinensis*; Bra.AF451572: *B. rapa* subsp. *pekinensis*; Bra.AF451573: *B. rapa* subsp. *rapa*; Rs.AF451576: *R.sativus*; Rs.AF451577: *R. sativus*; Sa.AF451580: *S. alba* and Sa.AF451581: *S. alba*

Three sets of SSR primers were designed for region A. Primer set 1 was based on a $(T)_{10}$ SSR repeat (Figure 2.13). This primer separated *B. napus* cv. Triolo, *S.arvensis, R. sativus, C. sativus A. thaliana* from all other *Brassica's* tested.

```
Ar.000932       AAAGAGAGATGTCCTGAACCACTAGACGATGGGGGCATACTTGCTCAACCGCCATCATAC 19272
Bn. Lico        AAAGAGAGATGTCCTGAACCACTAGACGATGGGGGCATACTTGCTCAACCGCCATCATAC 20716
                ************************************************************

Ar.000932       TATGATCATAGTATGATCAGTTTTTTTAAATTGTCAATATAATGAAATGAAATGGTATGA 19332
Bn. Lico        TATGATCATAGTATGATCAGTTTTTTAAAATTGTCAATATAATCAAATG-----GTATGA 20771
                ************************** *************** *****      ******

Ar.000932       CTAGCTTATAAGGTTTTTT-CTTTTTTCTATAGAATTCTATATCATTTTTTTTTT--ACA 19389
Bn. Lico        CTAGCTTATAAGATTTTTTATTTTTTTCTATAGCATTCTATATCATTTTTTTATTTTACA 20831
                ************ ******  ************ ****************** **  ***

Ar.000932       TTTGTATTCATATTCTAATCATAATTCTATAACAAATA--AATATACTTTCTTTTTATAT 19447
Bn. Lico        TTTATATTCATATTCTAATCACAATTCTATAAAAAAAAATCGATATATTTTCTTTTTATAT 20891
                *** ***************** ********** *** *   ***** *************

Ar.000932       TTGAAGTGGAAATATTAAAATTAAAAAATAAATAAAAAAAAAATCTAAAAATCGTCTAGTA 19507
Bn. Lico        TTGAAGTGGAAATATGAAAAAAAAAAAAAAAAACGAAAAAAA----------GTCTAGTA 20940
                *************** ****  ****** ***  *******           ********

Ar.000932       CGGAATCTTTTCAAGAAGTGATTGGTCTAACATAAAAAAAAAAAAGAAAAAAAGAGGGTT 19567
Bn. Lico        TAAAATCTTTTAAAGAAGTGATTGGTCTGACAGAAAAAAAATAAA-----AAAGAGGGTT 20995
                   ******** *************** *** ******** ***     **********

Ar.000932       AAGTTTCGTTTTTTTTTTACTTTCCTTGATAGATTGCCTCATCTCATTGTTAAGAAATAG 19627
Bn. Lico        AAGTTTCGTTTTTTT--TACTTTACTTAATAGATTGCCTCATCTCATTGTTAAGAAATAG 21053
                ***************  ****** *** ********************************

Ar.000932       TAGTATCCCTGTCTAACACTAATCCAAGAAAGTCAGCCAGAATCCATCTT-CTAATTAGG 19686
Bn. Lico        TAGTGTCCCTATCTAACACTAACCCAAGAAAGTCAGACAGAATCCATCTTTCTTCCCTAA 21113
                **** ***** *********** ************* ************* **
```

Figure 2.13: Clustal W multiple sequence alignment of region A primer set 1. Ar.000932: *A. thailiana* and Bn.Lico: *B. napus* cv. Licosomos

Primer set 2 was based on two SSR repeats - $(C)_8(T)_{12}$ and $(T)_9$ (Figure 2.14). Although this primer does not differentiate between *B. napus* spp., it is a diagnostic marker in the separation of *S. arvensis* from *B. rapa* spp. from the various *B.oleracea* spp.. However, it cannot distinguish between *B. oleracea* and *R. sativus*.

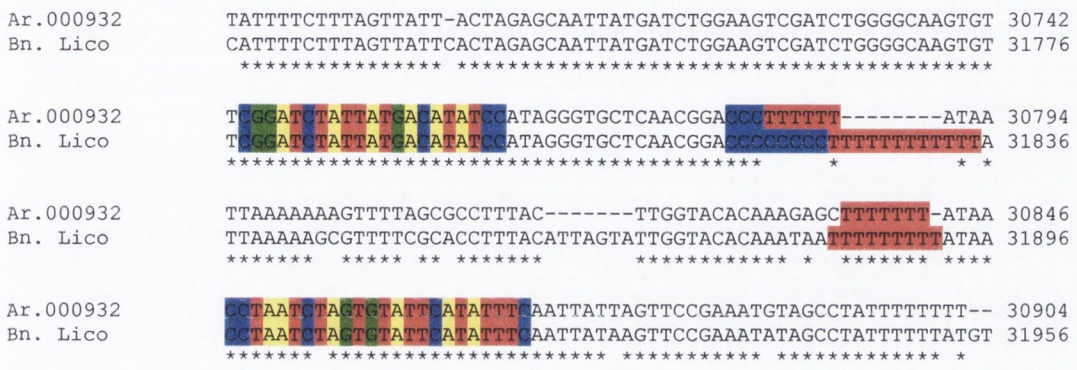

Figure 2.14: Clustal W multiple sequence alignment of region A primer set 2. Ar.000932: *A. thailiana* and Bn.Lico: *B. napus* cv. Licosomos

Primer set 3 was based on a $(T)_{11\text{-}13}$ SSR region (Figure 2.15). No polymorphism was detected by this primer set.

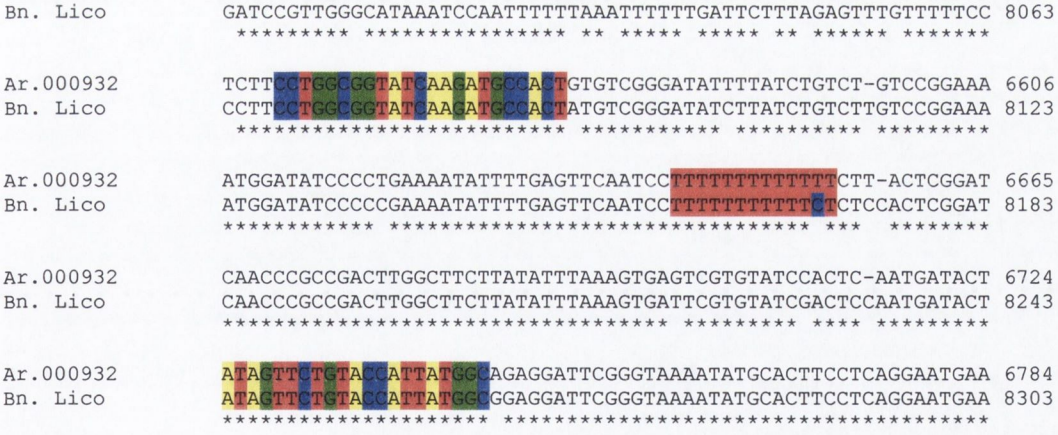

Figure 2.15: Clustal W multiple sequence alignment of region A primer set 3. Ar.000932: *A. thailiana* and Bn.Lico: *B. napus* cv. Licosomos

Three sets of SSR primers were designed for the region B (based on the *atpB-rbcL* intergenic spacer region). Primer set 1 was based on a $(A)_{7\text{-}8}(T)_{5\text{-}6}$ SSR repeat (Figure 2.16). This primer separated *S. arvensis, R. sativus* and *B. napus* cv. Triolo from all other *Brassica*'s tested, however failed to differentiate between all other *Brassica* species. Although it distinguished *C. sativus* and *R. sativus* from other *Brassica* species it failed to discriminate them from each other.

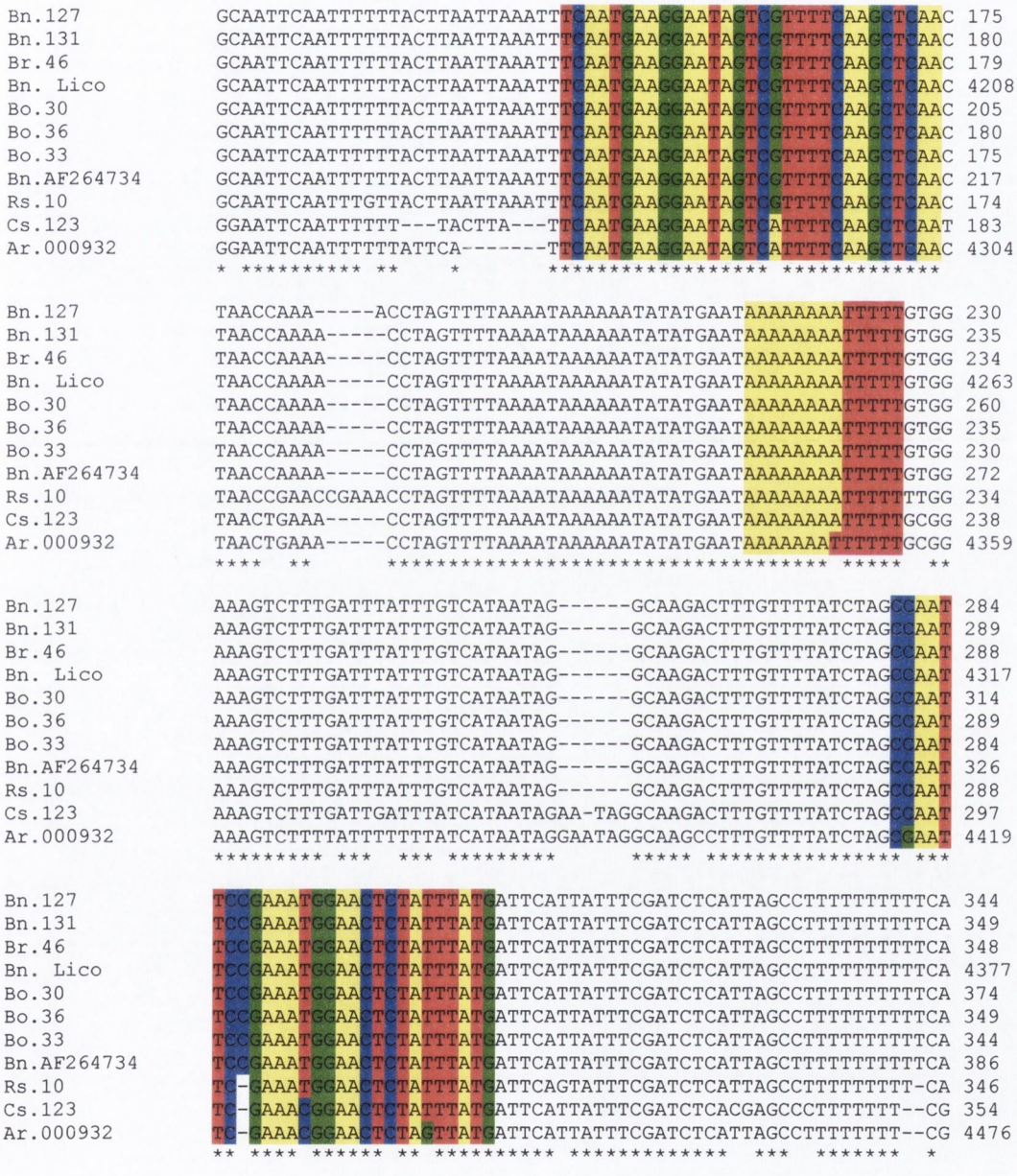

Figure 2.16: Clustal W multiple sequence alignment of region B primer set 1. Ar.000932: *A. thailiana*; Bn.10: *R. sativus*; Bn.30: *B. oleracea* ssp. *capitata*; Bn.33: *B. oleracea* ssp. *gemmifera*; Bn.36: *B. oleracea* ssp. *italica*; 46: *B. rapa*; 123: *Camelina sativus*; 127: *B. napus* cv. Marinka; 131: *B. napus* cv. Sheila and Bn.AF264734: *B. napus*

Primer set 2 was based on a $(T)_{16}$ SSR repeat (Figure 2.17). This primer may be used as a diagnostic marker to separate *C. sativus A. thaliana, B. rapa* ssp. *rapifera* and *B. nigra* from all other *Brassica* species tested. However, it failed to differentiate between *B. oleracea* ssp., *B. napus* ssp. *napobrassica* and *B. napus* ssp. *napus.*

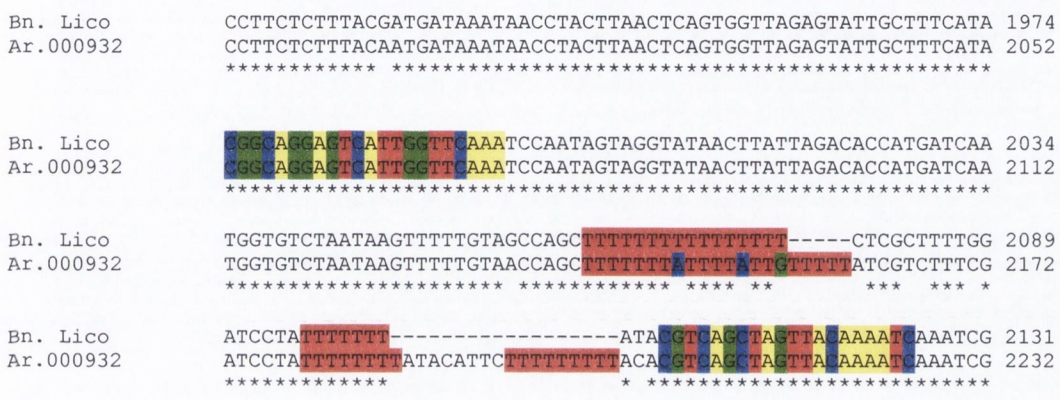

Figure 2.17: Clustal W multiple sequence alignment of region B primer set 2. Ar.000932: *A. thailiana* and Bn.Lico: *B. napus* cv. Licosomos

Primer set 3 was based on a $(T)_{8\text{-}10}$ SSR repeat (Figure 2.18). This primer separated *B. oleracea* ssp. *capitata, R. sativus, S. arvensis, B. napus* ssp. *napobrassica* and *A. thaliana* however failed to differentiate between the remaining *Brassica* species.

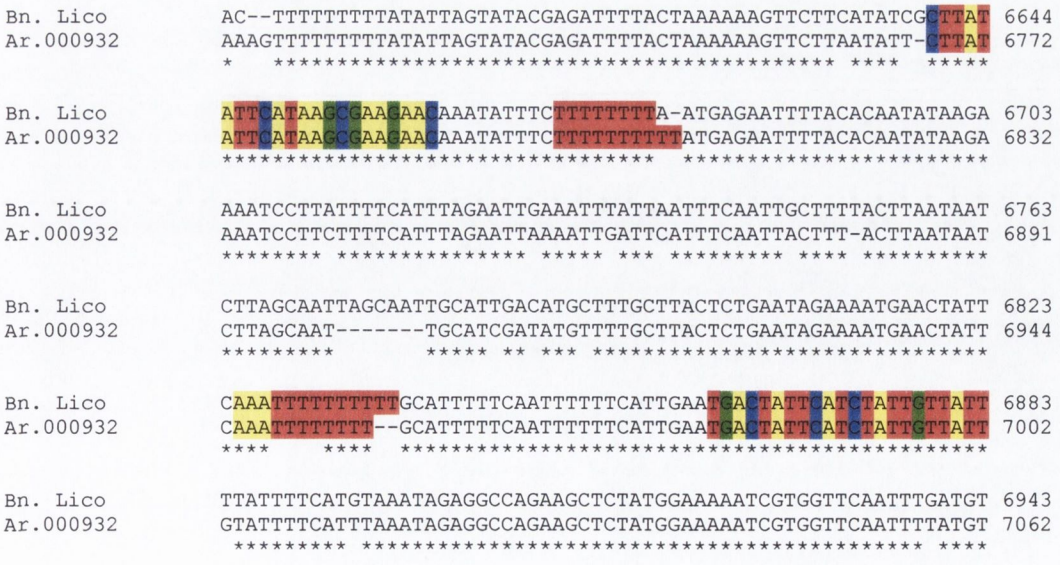

Figure 2.18: Clustal W multiple sequence alignment of region B primer set 3. Ar.000932: *A. thailiana* and Bn.Lico: *B. napus* cv. Licosomos

One set of primers based on a $(T)_{13}$ SSR repeat was designed for region C (Figure 2.19). This primer detected polymorphism and differentiated *B. napus* cv. Triolo, *B. nigra* and *R. sativus* from the other species tested. However *A. thaliana* spp. could not be distinguished from some *B. napus* individuals, which in turn could not be distinguished from *B. oleracea, B. rapa* or *C. sativus.*

```
Bn. Lico        TGAAAAGCTAATCATAGGTTCTTCTCTCCATCGGAACAATAGGGCCGTTATGCTCATTAC 494
Ar.000932       TGAAAAGCTAATCATAGGTTCTTCTCTCCATCGGAACAATAGGGCCGTTATGCTCATTAC 2040
                ************************************************************

Bn. Lico        GAAACTTGTTGACGAGATGAAATATAACCAAGGTATATCTTTTTGATCAGAGGTTGAATC 554
Ar.000932       GAAACTTGTTGAAGAGATGAAATATAACCAAGGTATATCTTTTTGATCAGAGGTTGAATC 2100
                ************ ********************************************

Bn. Lico        GATCATCAGAAGAAGGATTAGGCCAAAAATTAGGATACATTCTGGGAAAATAAAACTTCC 614
Ar.000932       GATCATCAGAAGAAGAATTAGGCCAAAAATTAGGATACATTCTGGGAAAATAAAACTTCC 2160
                ************** ********************************************

Bn. Lico        ATCGAAGAGAAGCAAATGAAAGGCTTTCATAAAAATTCTCGTAGAATCGAGAATGAAATT 674
Ar.000932       ATCGAAGAGAAGCAAATGAAAGGCTTTCATAAAAATTCTCGTAGAATCGAGAATGAAATT 2220
                ************************************************************

Bn. Lico        TTCATTCTGTACATGCCAGATCATGAATTAGTAACTGCATCCAATCTCCAAAAAAAAACC 734
Ar.000932       TTCATTCTGTACATGCCAGATCATGAATTAGTAACTGCATCCAATCTCCAAAAAAAAACC 2280
                ************************************************************

Bn. Lico        AATTTTTTTTTTTTTGAATGGAATATTTACGGAATCCCCATGAATAGGTTAAAACCTTAT 794
Ar.000932       AATTTTTGATTTTTGGAATGGAATATTTACGGAATCCCCATGAATAGGATAAAACCTTAT 2340
                ******* ***** ********************************** ***********
```

Figure 2.19: Clustal W multiple sequence alignment of region C primer set 1. Ar.000932: *A. thailiana* and Bn.Lico: *B. napus* cv. Licosomos

Table 2.17: Presence-absence matrix of all plastid SSR markers

Id	T1 157	T1 164	T1 165	T1 179	T1 185	R1 157	R1 168	R1 169	R1 171	R1 175	R1 179	R1 185	R.C 283	R.C 284	R.C 300	R.C 301	R.C 307	R.C 308	R.C 309	R.C 311	R.B1 155
2	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
3	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
30	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
33	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
36	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
46	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
53	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
56	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
60	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
62	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
66	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
70	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
75	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
78	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
86	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
90	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
123	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
124	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
128	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
132	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
134	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
135	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
136	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
137	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
138	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
139	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
140	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
141	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
142	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
143	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
144	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
145	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
146	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
147	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
148	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
149	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
150	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
151	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
152	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
153	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
154	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
155	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
156	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
157	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
158	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
159	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
160	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
161	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
162	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
163	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
164	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
165	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0

Table 2.17: Presence-absence matrix of all plastid SSR markers (continued)

Id	R.B1 157	R.B1 161	R.B1 162	R.B1 164	R.B2 140	R.B2 142	R.B2 144	R.B2 145	R.B2 146	R.B2 147	R.B2 148	R.B2 151	R.B2 152	R.B2 153	R.B2 155	R.B2 157	R.B2 170	R.B2 171	R.B3 233	R.B3 239	R.B3 240
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
30	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
33	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
36	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
46	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
53	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
56	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
60	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
62	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
66	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
70	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
75	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
78	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
86	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
90	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
123	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
124	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
128	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
132	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
134	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
135	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
136	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
137	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
138	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
139	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
140	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
141	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
142	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
143	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
144	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
145	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
146	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
147	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
148	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
149	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
150	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
151	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
152	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
153	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
154	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
155	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
156	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
157	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
158	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
159	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
160	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
161	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
162	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
163	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
164	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
165	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Table 2.17: Presence-absence matrix of all plastid SSR markers (continued)

Id	R.B3 241	R.B3 242	R.B3 243	B.A1 273	B.A1 279	B.A1 280	B.A1 283	B.A1 284	B.A1 296	B.A1 308	B.A1 309	B.A1 310	B.A2 127	B.A2 136	B.A2 138	B.A2 139	B.A2 141	B.A2 142	B.A2 144	B.A2 145	B.A2 150	B.A2 155	B.A3 196
2	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
3	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
30	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
33	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
36	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
46	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
53	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
56	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
60	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
62	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
66	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
70	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
75	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
78	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
86	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
90	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
123	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
124	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
128	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
132	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
134	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
135	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
136	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
137	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
138	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
139	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
140	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
141	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
142	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
143	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
144	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
145	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
146	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
147	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1
148	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1
149	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1
150	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
151	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
152	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
153	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
154	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
155	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
156	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
157	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
158	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
159	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
160	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
161	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1
162	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
163	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
164	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
165	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1

Key for Table 2.17:

Species identification numbers (see Appendix 1 for further information):

2: *Sinapis arvensis*

3: *Raphanus sativus*

30: *B. oleracea* ssp. *capitata*

33: *B. oleracea* ssp. *gemmifera*

36: *B. oleracea* ssp. *italica*

46 and 53: *B. rapa*

56: *B. oleracea* ssp. *gemmifera*

60: *B. oleracea* ssp. *gemmifera*

62: *B. napus* ssp. *napobrassica*

66: *Raphanus sativus*

70 and 75: *B. rapa* ssp. *rapifera*

78 and 86: *B. oleracea* ssp. *capitata*

90: *B. nigra*

123 and 124: *Camelina sativus*

128 132 134-146 *B. napus* ssp. Marinka

147-149 *B. napus* ssp. Jura (MS)

150-153 *B. napus* ssp. Triolo (MS)

154-165 *Arabidopsis thaliana*

Primers used are indicated by name with a number indicating the peak size (Table 2.16)

T1=MF_TRN1

R1= MF_RPL1

RA1= MF_A1

RA2= MF_A2

RA3= MF_A3

RB1= MF_B1

RB2= MF_B2

RB3= MF_B3

RC1= MF_C1

The combination of all plastid SSR markers tested (Table 2.17) in a Neighbour Joining analysis produced an unrooted tree (Figure 2.20) and midpoint-rooted tree (Figure 2.21), which successfully differentiated *Arabidopsis thaliana, Brassica napus* cv. Marinka, *B. napus* cv. Triolo, *B. oleracea, B. nigra*, *B. rapa, Camelina sativus, Raphanus sativus,* and *Sinapis arvensis* individuals into species groups. This primer combination failed to separate *B. rapa* (46) and *B. napus* ssp. *napobrassica* (62) from *B. napus* cv. Marinka individuals.

The combination of all nuclear and plastid SSR markers tested in a Neighbour Joining analysis produced an unrooted tree (Figure 2.22) and midpoint-rooted tree (Figure 2.23), which did not differentiate the groups as successfully as the plastid marker analysis. Although *A. thaliana, B. napus* cv. Triolo, *C. sativus, R. sativus,* and *S. arvensis* individuals separated into species groups, this analysis

failed to separate *B. oleracea, B. nigra*, *B. napus* ssp. *napobrassica*, *B. rapa* and from *B. napus* cv. Marinka individuals.

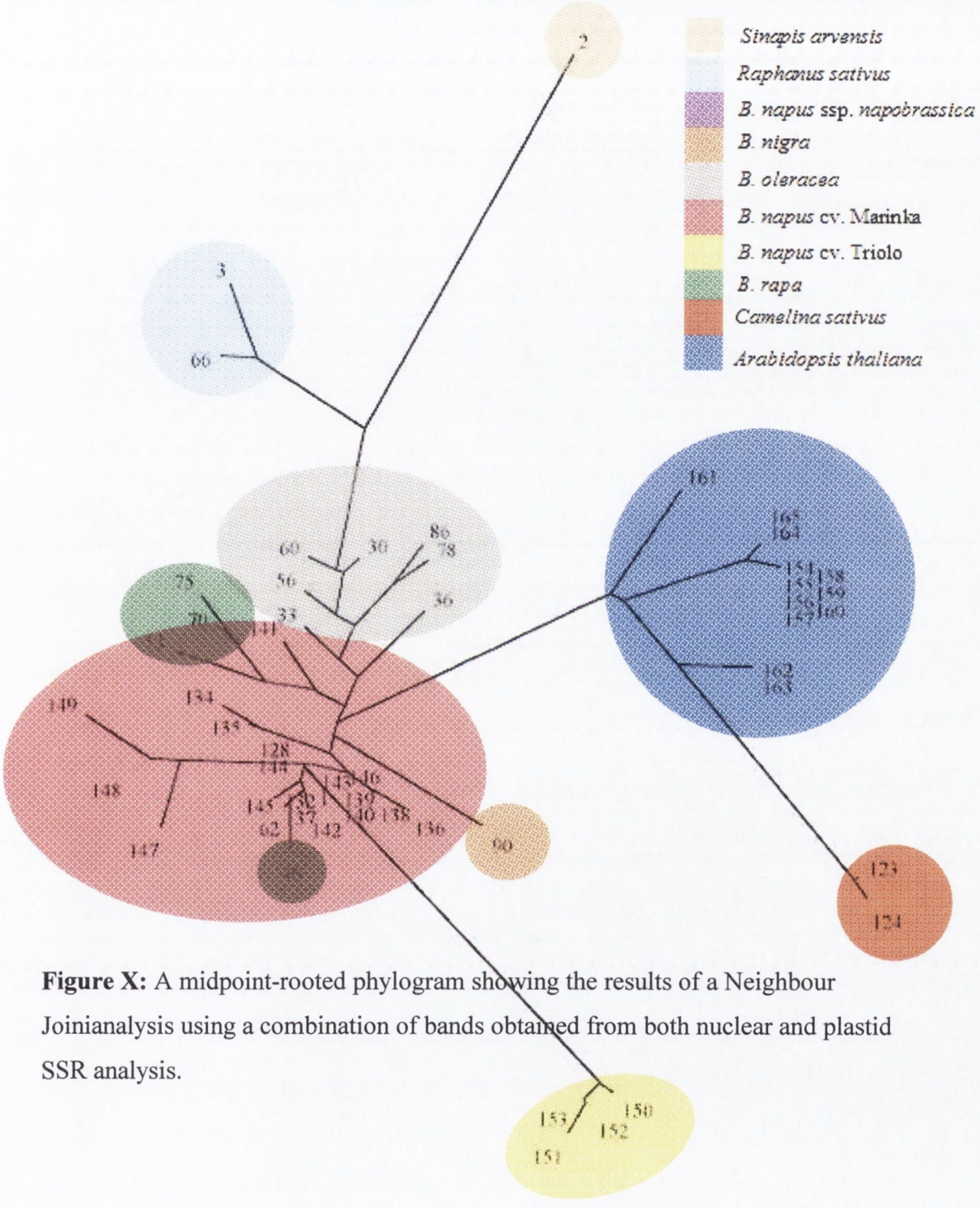

Figure X: A midpoint-rooted phylogram showing the results of a Neighbour Joinianalysis using a combination of bands obtained from both nuclear and plastid SSR analysis.

Figure 2.20: An unrooted-phylogram showing the results of a Neighbour Joining analysis using all bands obtained from a plastid SSR analysis

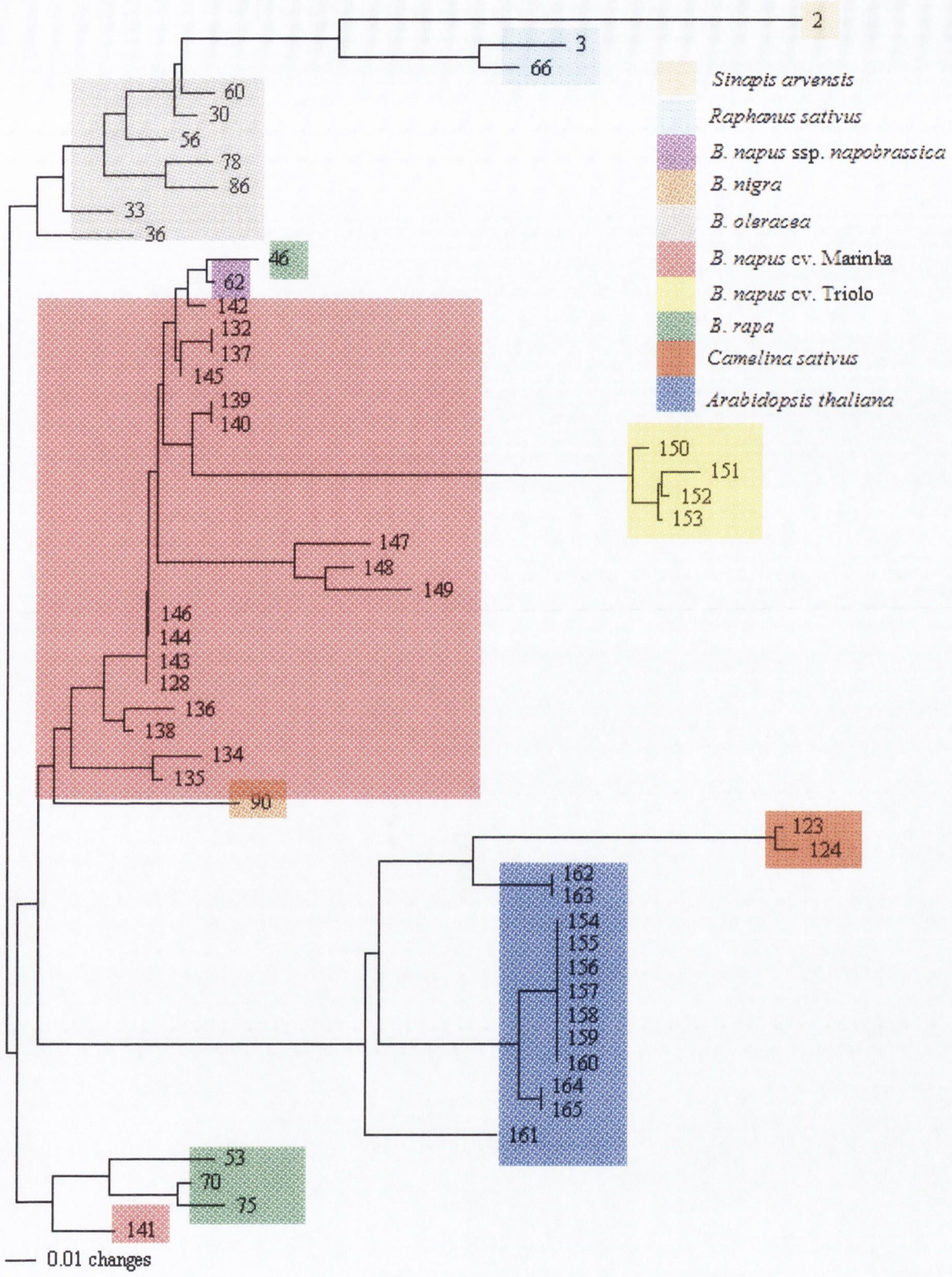

Figure 2.21: A midpoint-rooted phylogram showing the results of a Neighbour Joining analysis using all bands obtained from a plastid SSR analysis

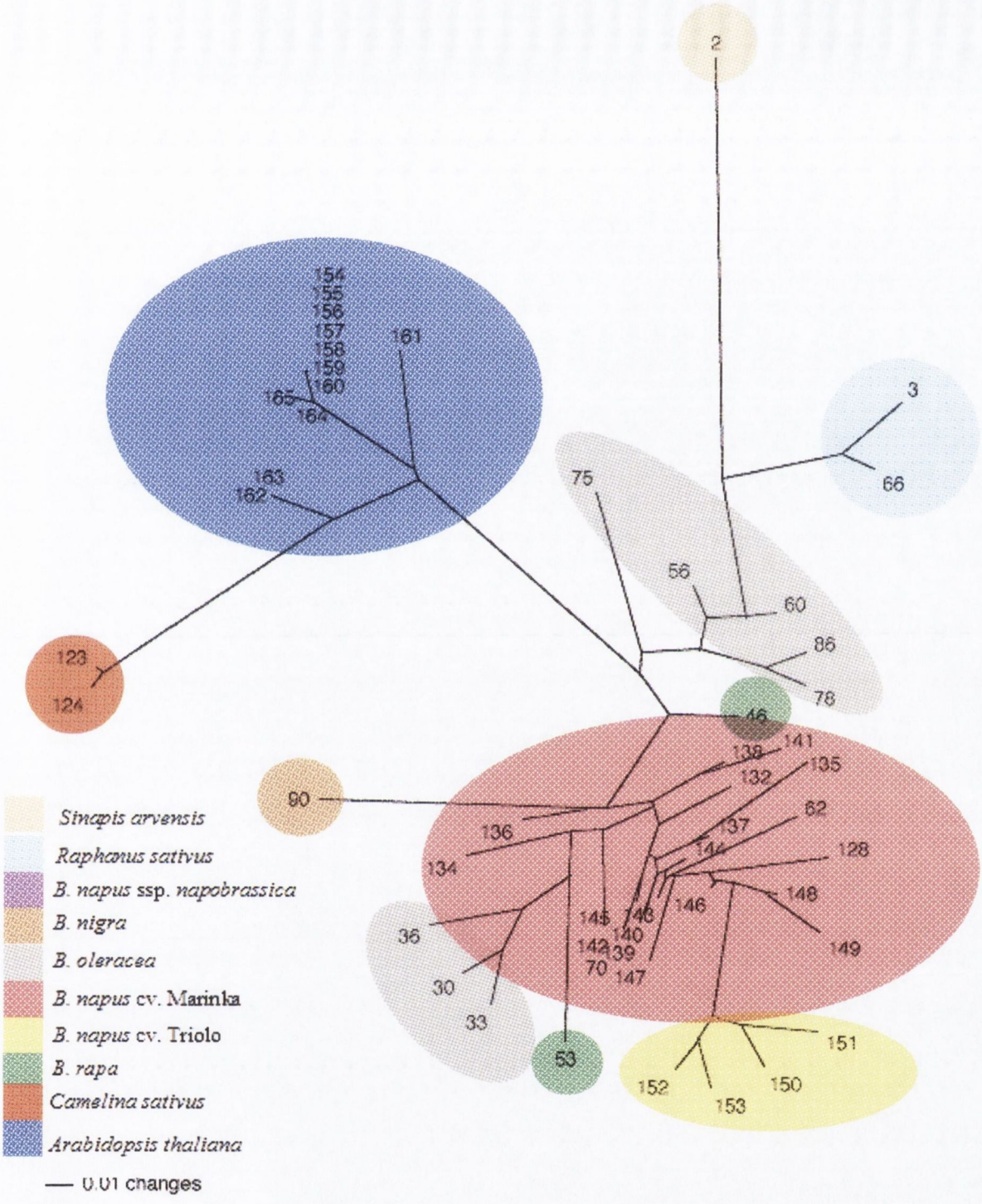

Figure 2.22: An unrooted-phylogram showing the results of a Neighbour Joining analysis using a combination of bands obtained from both nuclear and plastid SSR analysis

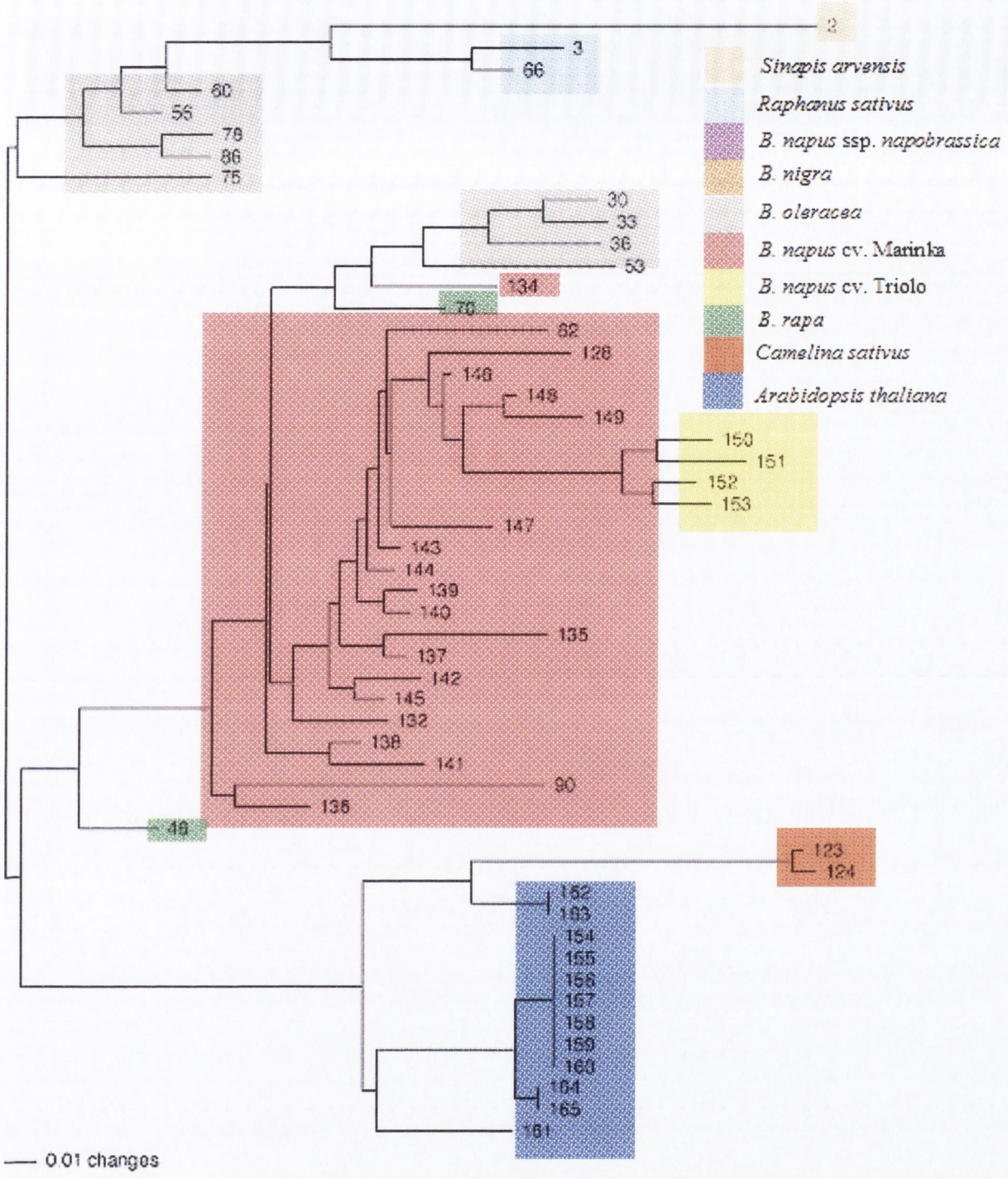

Figure 2.23: A midpoint-rooted phylogram showing the results of a Neighbour Joining analysis using a combination of bands obtained from both nuclear and plastid SSR analysis

2.4 Discussion

A range of techniques was used to test verified specimens of Brassicaceae (i.e. of known origin and identification).

2.4.1 PCR-RFLP

PCR-RFLP, tested on four chloroplast regions and one nuclear gene region, was successful for intergeneric differentiation of Brassicaceae species, but failed to reveal intraspecific variation. For example, it could differentiate *Sinapis arvensis*, *Raphanus sativus* and *Brassica oleracea* from each but was unable to distinguish differences between *B. oleracea* subspecies. It is not known what sequence variation accounts for the polymorphisms recorded due to the complexity of the fingerprints but a large proportion of this may be accounted for by variable microsatellite repeats. These have been assayed separately with the plastid SSR marker system (section 2.4.4).

The most informative markers were produced using restriction enzymes *Mse*I and *Hinf*I, as polymorphic fingerprints were generated when tested on five different gene regions (the plastid *atpB-rbcL* spacer region, *trnL* intron and *trnL-F* intergenic spacer, *rps16*, *rpl16* and the nuclear ITS gene region) (Table 2.3 and Figure 2.1). This technique may be useful as a quick assay for intergeneric studies on Brassicaceae especially for those laboratories without sequencing facilities or who are restricted by cost.

2.4.2 Nuclear SSRs

Nuclear SSRs can be assumed to be ubiquitous in all angiosperms and have been detected in *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.*, 2002b). Twelve of these nuclear SSR primer sets were selected and tested for polymorphism among *Brassica* species (Table 2.5) in this chapter. As SSR fragments are not easily interpreted in polyploid species, the fingerprints obtained were interpreted as presence-absence data of particular fragments. In this way they were treated very much like a multilocus DNA fingerprint where fragments are not assigned to individual loci for analysis (a dominant marker system) and homozygotes can't be distinguished from heterozygotes (Hodkinson *et al.*, 2002b).

A Neighbour Joining analysis based on a matrix of pairwise-distance measures (Nei and Li, 1979) was created in PAUP 4.0 and carried out using all bands obtained from six of the primers. A combination of midpoint-rooted and unrooted phylograms was found to be the most informative means of displaying and interpreting the data. The analysis succeeded in interspecific separation of *Brassica napus*, *B. oleracea* and *B. rapa*, however two species did not separate into their taxonomic groups, *B. napus* ssp. *napobrassica* and *B. nigra* grouped with *B. napus* cv. Marinka individuals. As *B. napus* ssp. *napobrassica* is a subspecies of *B. napus* and therefore very closely related it is not surprising that the analysis was not strong enough to differentiate them. With regard to the odd grouping of *B. nigra* with *B. napus* cv. Marinka individuals, the long-branch separating *B. nigra* indicates that the species are highly different from each other despite their sister group status in this analysis.

Therefore the results indicate that the nuclear microsatellite markers tested here provide a high-resolution technique for the detection of variation among Brassicaceae. Although the production of microsatellite markers is considered to be a laborious and demanding process there are a large number of *Brassica* primer sets available through the *Brassica* DB database on the UK CropNet website (http://ukcrop.net) that makes this inconvenience redundant. The combination of primers used here could be applied to various applications. For example, they can be used to investigate the genetic structure of natural populations as the knowledge of how variation is partitioned among populations may have important implications for both evolutionary biology and ecology (Balloux and Lugon-Moulin, 2002). SSRs also have huge potential for characterisation, conservation and utilisation of crop diversity (Szewc-McFadden *et al.*, 1996), and may therefore help to conserve and use Brassicaceae genetic resources more sensibly (Westman and Kresovich, 1999).

2.4.3 DNA sequencing

Five gene regions (the plastid *atpB-rbcL* spacer region, *trnL* intron and *trnL-F* intergenic spacer, *rps16*, *rpl16* and the nuclear ITS gene region) were successfully sequenced for eight Brassicaceae individuals. The ITS region has proven to be a valuable tool for intergeneric studies in many organisms, however botanists often experience difficulties in detecting sequence variations below the species level. This

has not been the case with *Brassica* as can be seen in ITS1 in Figure 2.6. ITS1 shows intergeneric, interspecific and inter-varietal variation among the *Brassica* species tested. Difficulties are not uncommon when attempting to sequence the ITS gene region of plant species. Despite multiple attempts none were successful in sequencing *Camilina sativus*. This may be due to sequence heterogeneity at ITS loci and concerted evolution, which results in either a homogenous or heterogenous ITS gene sequences amplified by PCR. Sequencing via PCR amplification is possible where a homogenous region is present as all ITS repeats are the same. However, where a gene region is heterogenous sequencing is impaired due to multiple copies of the ITS gene region. Cloning using a bacterial plasmid of *Escherichia coli* may be carried out to separate ITS copy types (Hodkinson *et al.*, 2002b). The ITS gene region was not utilised further in this analysis because of this technical complication (high throughput marker systems were needed), however the sequence information gained has potential for use in population and systematic studies.

The four plastid regions (*atpB-rbcL* spacer region, *trnL* intron and *trnL-F* intergenic spacer, *rps16* and *rpl16*) sequenced also revealed polymorphism. Although the resolution gained from DNA sequencing could be used to identify different species and varieties of *Brassica*, sequencing in not a high throughput technique and it is labour-intensive and expensive. Therefore, these sequences were used in conjunction with others acquired from Genbank (NCBI, 2003) and Coyne and Kavanagh (2002) to develop plastid microsatellite primers.

DNA sequencing capabilities are considered fundamental to systematic studies and the ability to apply the PCR approach to very small samples e.g. a single pollen grain or even to herbarium specimens or fossils makes it even more valuable (Karp *et al.*, 1996). This analysis has shown that Brassicaceae are available for DNA sequencing analysis.

2.4.4 Plastid SSRs

Studies by Provan (2000) showed that plastid SSRs could be developed from sequence information in Brassicaceae and used to detect variation among natural populations of *Arabidopsis*, however no such studies had been carried out for *Brassica*.

The development of a plastid SSR system in *Brassica* in this chapter involved the identification of 215 SSRs from information gathered from 57

sequences (163,720bps) from a total of six plastid gene regions examined (Table 2.14). From this ten plastid SSR primer sets were successfully developed, nine of which were found to detect polymorphism when tested on a broad range of Brassicaceae material (Table 2.16). Primer set two for region A was found to be diagnostic for the separation of *Sinapis arvensis* from *Brassica rapa* spp. from the various *B. oleracea* spp., Primer set two for region B was also found to be diagnostic for the separation of *Arabidopsis thaliana, B. rapa* ssp. *rapifera*, *B. nigra* and *Camelina sativus* from all other *Brassica* species tested. However, it failed to differentiate between *B. oleracea* ssp., *B. napus* ssp. *napobrassica* and *B. napus* ssp. *napus.* The combination of all plastid SSR markers tested in a Neighbour Joining analysis produced phylograms (Figures 2.21 and 2.22), which successfully differentiated the species tested into their taxonomic groupings. This primer combination failed to separate *B. rapa* (46) and *B. napus* ssp. *napobrassica* (62) from *B. napus* cv. Marinka individuals.

B. napus (AC genome) is a polyploid resulting from the hybridisation of the diploids *B. oleracea* (C genome) and *B. rapa* (A genome) (U, 1935). It is therefore possible that the *B. napus* individuals that *B. rapa* (46) is grouping with share the same chloroplast DNA i.e. *B. rapa* was the maternal parent of the cross. Alternatively it may be necessary to speculate that since the individual was collected in the field and cytogenetic analysis has not been done, it may be the result of a mis-identification and is in fact a feral *B. napus* individual.

The difficulties experienced in separation of *B. napus* from *B. oleracea* and *B. rapa* individuals may be due to complex hybridisation patterns in the genus (the triangle of 'U'), that is, either parent *B. oleracea* or *B. rapa* can be the maternal parent of the cross i.e. the chloroplast donor. Therefore, the result is *B. napus* plants with the nuclear genome of *B. oleracea* (C genome) and a chloroplast genome *B. rapa*; or alternatively *B. napus* plants with the nuclear genome of *B. rapa* (A genome) and the chloroplast genome of *B. oleracea* (U, 1935). This would explain why some *B. napus* individuals group with *B. oleracea* and some group with *B. rapa.*

2.4.5 Combination of nuclear and plastid SSRs

The combination of all plastid SSR markers tested in a Neighbour Joining analysis produced phylograms (Figures 2.21 and 2.22), which successfully

differentiated *A. thaliana, Brassica napus* cv. Marinka, *B. napus* cv. Triolo, *B. oleracea, B. nigra, B. rapa, C. sativus, R. sativus,* and *S. arvensis* individuals into species groups. However, this marker combination also failed to separate *B. rapa* (46) and *B. napus* ssp. *napobrassica* (62) from *B. napus* cv. Marinka individuals.

In addition, some *B. rapa* (53; A nuclear genome) and *B. oleracea* (30, 33, 36; C nuclear genome) individuals grouped with *B. napus* cv. Marinka (AC nuclear genome) individuals, and not with other individuals of their species as can be clearly seen in Figure 2.23 and 2.24. In this case it is likely that *B. napus* cv. Marinka is exhibiting plastid genome capture i.e. contains the plastid genome of *B. rapa* but also contains the nuclear genome of *B. oleracea* (C nuclear genome). This phenomenon of plastid capture has been seen in other species (Hodkinson *et al.*, 2002b; Cronn and Wendel, 2004). For example, the polyploid *Miscanthus* × *giganteus* (Poaceae) has the plastid DNA of *M. sacchariflorus* and not *M. sinensis* its other parental genome (Hodkinson *et al.*, 2002b). In this case with evidence from the plastid DNA alone the plants could be mistaken to be *B. rapa* with no evidence of the hybrid origin. The opposite is the case for *B. oleracea* (30, 33, 36; C nuclear genome) individuals grouped with *B. napus* cv. Marinka. It is likely that *B. napus* cv. Marinka is exhibiting plastid genome capture i.e. contains the plastid genome of *B. oleracea* but also predominantly contains the nuclear genome of *B. rapa* (A nuclear genome).

2.5 Conclusion

The successful development of plastid SSR markers is important in the area of plant molecular biology, as they can be used to distinguish between individuals of Brassicaceae at inter-generic, inter-specific and intra-specific levels. It is well known that the evolutionary history of natural plant populations can be gained through analysis of chloroplast variation (Provan and Campanella, 2003). In addition, they are very significant in terms of risk assessment, gene flow studies and also wider applications in plant biology such as systematics and plant breeding. It is therefore anticipated that once these primers are published they will have wide utility to the research community.

The 12A nuclear marker and plastid SSR markers (Region A2 and Region B2) developed and tested in this chapter, were then applied to pollen extracted DNA in chapters 4 and 5.

Chapter 3

Abiotic dispersal: pollen sampling methods and identification

3.1 Introduction

Cross pollination is the main natural means of gene transfer in flowering plants so any assessment of risk e.g. in GM crops, requires information on the potential for dispersal of the pollen grains. Wind can be an efficient means of dispersing airborne particles (McCartney and Lacey, 1991) but oilseed rape pollen grains are believed to be predominantly dispersed by insects and are relatively large (20-30μm in diameter). However they are comparable in size with many other pollen grains e.g. grass pollens and fungal spores, which are primarily dispersed by wind (Raynor *et al.*, 1974) and therefore it seems possible that large numbers of oilseed rape pollen may be released into the air each year during flowering (McCartney and Lacey, 1991). In addition, it has been suggested that through foraging for nectar and pollen, bees facilitate the release of pollen into the air (Vaissiére *et al.*, 1994).

3.1.1 Quantifying wind dispersed pollen

The likelihood of an individual wind dispersed pollen grain reaching its target is so remote that large numbers of pollen grains must be produced to effect pollination. In particular, anemophilous plants must produce considerably more pollen than entomophilous, which can exploit the more predictable behaviour of insects (Moore *et al.*, 1991). Palynologists, evolutionary geneticists, taxonomists, medical geneticists and ecologists rely on this excess of material either in fresh or fossil state, to determine which species have been around for millions of years, which have gone extinct or are ancestors of present day plants or which cause asthma and other illnesses.

Dispersal by wind is determined by a series of physical processes related to the physical characteristics of the particle and environmental factors; particle morphology and wind strength being the two most important (McCartney, 1997). Clouds of pollen grains remain airborne following release from the anther and can be transported over considerable distances through air turbulence and by upward motion in rising convection currents (Mullins and Emberlin, 1997). Grass pollen has been recorded at heights of 2000m (Dowding, 1987). Ultimately these pollen grains

will be removed from the air by impaction or sedimentation. Removal of pollen grains from the air will occur in accordance with Stoke's law (Gregory, 1973), in which the rate of deposition in still air is proportional to the square of the radius of the pollen grains. Sunshine, light winds, lack of rain and warm air temperatures have been described as conducive to high pollen dispersal rates (Dowding, pers. comm.).

3.1.2 Distance travelled by OSR pollen

In spite of the attractive entomophilous flower of OSR, good seed yields may occur in the absence of an insect pollinator (Free and Nuttall, 1968). This is thought to be due to the shaking of the plants during windy conditions as was shown in a study by Williams (1978) where greenhouse grown plants produced more seeds per pod on plants that were shaken than those that were grown in still air (Jenkinson and Glynne-Jones, 1953; Williams, 1978). Therefore the movement of plants during windy conditions increases self-pollination (Williams, 1984).

There is a great deal of variation in the published data on the extent of airborne pollen present in the vicinity of oilseed rape crops. The deposition of airborne pollen on sticky surfaces has been measured at 32m (Mesquida and Renard, 1981) and 40m (Olsson, 1955). Suction traps have been used to quantify the level of airborne pollen above OSR fields. Rotorod samplers detected 48 grains per m^3 (Langridge and Goodman, 1982) while volumetric spore traps detected 80-115 grains per m^3 (Williams, 1984). Other studies have found a reduction to 2-11% of source emissions at 100m from the crop (McCartney and Lacey, 1991), while others have reported the much higher level of 27-69% at 100m, with 10-11% at 360m (Timmons *et al.*, 1995). Also under dispute are gene flow levels ranging from 1.2% at 1.5km and 0.08% at 2.5km (Timmons *et al.*, 1995). The main reason for both of these levels of variation appears to be attributable to the size of the pollen source (Timmons *et al.*, 1995).

As conflicting measurements have been reported regarding the distance travelled by oilseed rape pollen, there are therefore considerable differences in the isolation distances deemed necessary for a GM OSR crop (Treu and Emberlin, 2000; Eastman and Sweet, 2002). The separation distance between two conventional varieties of OSR to give less than 1% cross pollination is 1.5m (Agricultural Biotechnology in Europe, 2002). Others have reported 0.07% of plants

were pollinated but a plateau of approximately 0.2% was measured between 50 and 400m (Coghlan, 2001). However, low levels have also been reported at 1.5-2.5km from the source, with densities of 10-12% of field margin levels at 360m (Timmons *et al.*, 1996). On the whole, it is agreed that most cross-pollination occurs over short distances and successful pollination seems to decline exponentially with distance from the pollen source (Eastman and Sweet, 2002). Figure 1.4 and Table 1.6 (section 1.5: p18-23) gives detail of distances recorded for oilseed rape (Salisbury, 2002).

3.1.3 Trapping airborne pollen

Many different methods of monitoring the pollen content of the atmosphere are currently in use, nevertheless the basis of any method of pollen sampling is to collect grains so that they may be examined microscopically (Rapiejko, 1996). Generally speaking, to be effective, a pollen sampler should trap all particles in the range of 5-75μm efficiently, irrespective of wind velocity and should be capable of yielding a continuous volumetric record of atmospheric pollen count (Hyde, 1972). There are a number of samplers available including cylinder traps, sedimentation traps, whirling arm samplers, impactors (e.g. seven-day volumetric spore trap) and filtration samplers (Mullins and Emberlin, 1997). For this study a seven-day volumetric spore trap (VST, suction sampler) and a modified version of a filtration sampler were utilised (Cour, 1974).

3.1.4 Suction samplers

There are numerous types of sampling devices available for collection of bio-aerosol materials. Each device has its advantages and disadvantages depending on the particular environment in which it is used and the type of sample to be collected. The most commonly used devices include rotating impactors, suction trap devices, liquid impingers and dry cyclonic collectors (Solomon *et al.*, 1980; Cage *et al.*, 1996; Frenz and Lince, 1997; Chakraborty *et al.*, 1998; Rogers and Levetin, 1998; Sterling *et al.*, 1999). The advantages of the first two are that they are volumetric, require little maintenance and can create a permanent slide, which can be archived. However, counting of particles is labour intensive and requires skilled personnel for particle identification. Liquid impingers are generally used for bio-

aerosol collection for immunochemical analysis for marker presence or absence (Cage *et al.*, 1996). The seven-day volumetric spore trap VST (Burkard) is the most commonly used suction sampler in pollen and spore dispersal studies (Berman, 2003). Suction samplers extract pollen grains from measured volumes of aspirated air by providing a sticky surface to which grains can adhere for later examination (Burkard, 2001). These devices offer particular advantages for the collection of relatively small particles, as the intake velocity can be designed to approximate to the mean wind speed (Burkard, 2001; Berman, 2003).

The VST is fitted with a drum, around which is placed a sticky transparent strip. The drum is moved at a rate of two millimetres per hour via a clockwork mechanism thus the concentration of airborne particles can be calculated hourly. The drum takes seven days to complete a revolution (Figure 3.1).

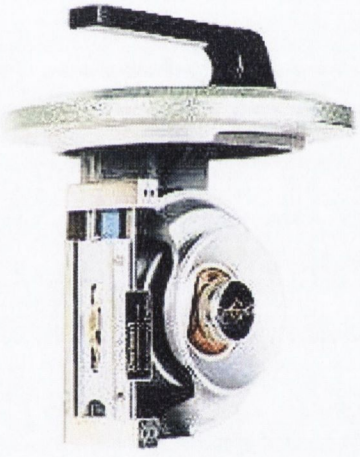

Figure 3.1: The drum of a seven-day volumetric spore trap (VST)

Air is sucked through a narrow slit (2x14mm) in the casing, which encloses the drum, by means of a vacuum pump at a constant rate of ten litres per minute. This air is delivered at 5.8m/s to the trapping surface, depositing pollen grains, fungal spores and other particles less than 1μm by impaction on the sticky strip. Each hour particles in $0.6m^3$ of air are deposited on $28mm^2$ of tape. The volume represented by each field of view is calculated from this relationship. The resulting pollen count is expressed as a number of grains per cubic metre of air (Burkard, 2001; Berman, 2003). This method is often used by aerobiologists to provide a pollen forecast, which is distributed to people suffering from pollen and spore allergies including asthma, via the radio, TV, internet etc (Palynological Laboratory,

2001). Disadvantages of this trap include its need for a power supply and its cost, which precludes the use of multiple traps.

There is no strict standard for the height or position of suction samplers for airborne pollen analysis. Traps have been positioned at 10-30m where the local tree canopy is high (10-30 metres) (Zawisza *et al.*, 1993) or in urban settings (12m) (Rogers and Levetin, 1998) as this level is considered high enough to eliminate local disturbance factors. Under field conditions, elevations consistent with meteorological stations 0.5m (Chakraborty *et al.*, 1998) and 1.5m (Rogers and Levetin, 1998) have been used. In other studies analysing pollen concentrations within oilseed rape crops, VSTs were placed at the centre of plots at an elevation of 40cm (McCartney and Lacey, 1991). Therefore, for farmland locations, an elevation of two metres is sufficient for airborne pollen analysis, as this elevation is greater than the maximum height of the crops, which may be considered the local disturbance factor.

3.1.5 Passive traps

Deposition samplers are the simplest and cheapest method of collecting airborne pollen grains, by merely allowing them to settle or impact on a transparent sticky surface, which can then be examined microscopically. Microscope slides or plastic tapes usually serve as sticky surfaces (e.g. slides coated with Vaseline or glycerine jelly) and are exposed for short periods of time (Rapiejko, 1996). Most samplers of this type are also simple to construct and use, are portable, do not require any power supply and are inexpensive. Therefore, the advantage is that many traps can be deployed. The device is usually placed 150-180cm above the ground level or at the roof level (Rapiejko, 1996). Unfortunately, in the natural environment the catch is affected by windspeed, turbulence and the orientation of a slide in respect to wind direction. For example, with increased wind velocity there is increased efficiency in the capture of smaller particles. In some conditions the usage of bigger sticky surface is advised such as Petri dishes, beakers and cuvettes, which may offer advantages during wet periods (Rapiejko, 1996).

Passive filtration/impaction samplers were developed by Cour (1974). The original design consisted of a 400cm^2-exposed screen, which has a tail/rudder to enable the trap to rotate to face continually into the wind (Cour, 1974). As air passes through the screen, pollen is impacted onto it (Figure 3.2A). Once the pollen grains

are counted and the data combined with a continuous record of wind run, a semi-quantitative indication of the pollen content in the atmosphere can be made. A modified version of a filtration sampler was utilised for this study with an important modification to protect the filter from rain (Figure 3.2B, see Section 3.2.4). Advantages of this trap include its ease of use and construction, lack of requirement for a power supply and inexpensive cost.

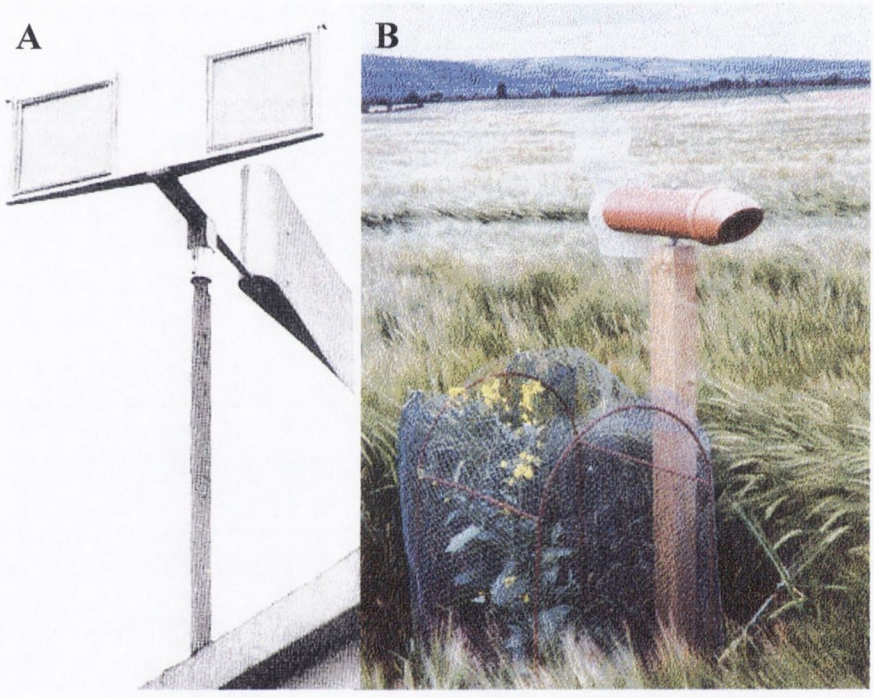

Figure 3.2: (A) Cour trap (Cour, 1974). (B) Passive trap

3.1.6 Pollen identification

Angiosperm pollen grains are made up of a cellulose inner layer called the intine and an outer layer of sporopollenin called the exine. Sporopollenin is the substance, which facilitates pollen grains to survive fossilisation and whose surface is sculptured to generate morphological characters used to identify pollen grains (Moore *et al.*, 1991). Although it is not possible to distinguish *Brassica* pollen at or below the species level using light microscopy because of the similarity of grains between species, identification to genus is feasible using light microscopy (at x400 magnification). The eyepiece of the microscope is usually fitted with a graticule so that pollen grains may be measured according to the calibrations. Features of pollen grains used in identification are surface sculpturing, size and shape; the presence

and distribution of pores, furrows, spines, grooves and reticulations over the exine; along with exine and intine thickness of the pollen grain are valuable in classification. Whether the pollen occurs as a simple or compound grain is also important. Grains are measured according to their polar and transverse axes (Moore *et al.*, 1991; Burkard, 2001).

Brassicaceae pollen are typically 20-30μm in diameter (Moore *et al.*, 1991). Their shape is described as round/irregularly round or triangular depending on the individual grain and the orientation in which it is viewed (Moore *et al.*, 1991). This variation between round and triangular is due to the presence of three furrows, at the edge of which the tectum (outer surface) narrows (Figure 3.3). The surface is described as a net or pitted. The net is a lace like pattern and may be either beaded (appearing as a string of beads) or pitted (appears covered with depressions rather than perforations) (Moore *et al.*, 1991). The exine exhibits medium thickness. When stained, two layers are visible with a row of regularly spaced rods forming pillars (collumellae) between them. The outer layer (tectum) is incomplete leaving the surface with a beaded appearance (Figure 3.3).

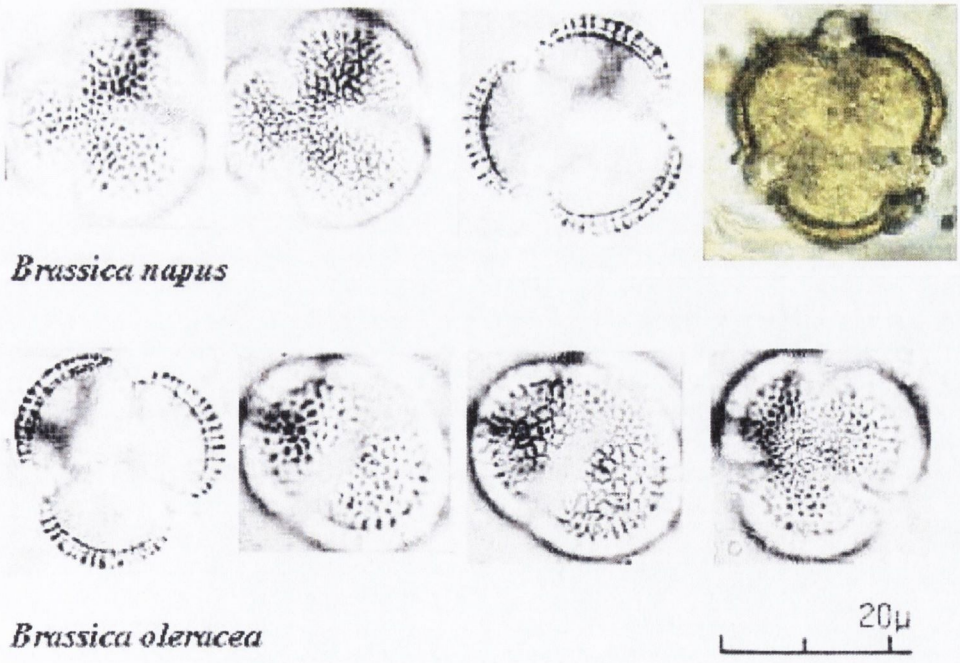

Figure 3.3: *Brassica napus* and *B. oleracea* pollen. Black and white pictures by Reille (1992), colour picture at x400; 20μ = 20μm

Conventional methods for the identification of pollen have been limited to the observation of pollen with optical or scanning electron microscopes and comparison with reference collections. The problems associated with the examination of the risks of wind pollination between *Brassica* species have been described, and it has been emphasised that pollen detected on VSTs may come from an entirely different species of *Brassica* with morphologically identical pollen (Timmons *et al.*, 1995). For this reason alternative identification methods are needed and molecular markers offer considerable potential in this respect. Therefore markers have been used in this thesis for species differentiation of pollen.

3.1.7 Preparation of pollen grains

Pollen grains are prepared in various ways to assist in their identification. Unprepared grains taken directly from the flower or from the air may be in a dry and shrunken condition, with their furrows and pores having sometimes disappeared from view. These may be prepared by 'swelling' the grains (re-hydrate by suspending in a drop of water) and visualised under the microscope (Hodges, 1974). However, this method may not be adequate, as fresh pollen grains still possess their intine, which obscures the surface sculpture of the pollen grain.

An alternative, more efficient method known as aceteolysis involves the removal of cellulose by acid-hydrolysis (Moore *et al.*, 1991). This serves to remove the intine, which allows light to pass through the pollen grain and so reveals previously hidden surface patterns under light microscopy.

3.1.8 Aims

This chapter aimed to provide details of the methods employed in the collection and identification of abiotically dispersed pollen. The results were used to evaluate risk assessment in terms of gene flow as a result of wind-dispersed pollen. Specific objectives were:

- To develop and employ an efficient method for the collection and identification of oilseed rape (*Brassica napus*) pollen
- To test the ability of a Burkard volumetric spore trap for oilseed rape pollen collection
- To develop a passive trap based on the Cour trap (Cour, 1974)
- To test the low cost passive pollen trap as an alternative to the VST

- To evaluate methods to efficiently remove pollen from the trapping surface of the passive pollen trap for microscopic analysis and DNA extraction.
- To examine pollen movement by wind from a *B. napus* source crop in terms of flux.
- To compare results from the passive traps with the VST to determine the efficiency of each in pollen collection.

3.2 Methods

3.2.1 Site selection and experimental design

A crop of *Brassica napus* cv. Marinka was planted at Teagasc, Oak Park, Co. Carlow (52°52'N, 07°55'W) to monitor pollen movement in 2001 and again in 2002. In 2001, the 'pump field' was used and measured approximately four hectares. In 2002, the 'Sawmill field' measuring approximately three hectares was used (Figures 3.4 and 3.5; p94 & 95). The sites were selected on the basis that there were no other crop *Brassica* species within a radius of 2km. Local ground surveys were conducted before and during the *Brassica napus* cultivar's flowering time. Wild individuals of any member of the Brassicaceae were removed, their presence recorded and a DNA extraction from leaf material was then carried out. Therefore a record of Brassicaceae species found at Oak Park was kept. These samples were included in the molecular analysis and could therefore be eliminated from the results serving to minimise the extent of interference of external pollen with the target pollen. The experiment was also designed so that multiple pollen samples at specific distances and directions from the crop could be taken. A detailed analysis of the site was carried out (Table 3.1) and a description of the soil was provided by Michael Conroy (Oak Park, Teagasc). Figures 3.6 and 3.7 (p96 & 97) show the layout of other crops in the surrounding area.

Table 3.1: Description of both sites used located at Teagasc, Oak Park, Carlow

Parameter	Units	Description
Slope	Vertical	Undulating
Exposure	From N	Not exposed
Elevation	m	56m
Latitude Longitude	°N °E	52°52'N 07°55'W
Mean annual temperature	°C	9.4 (1951-80 Kilkenny)
Mean annual rainfall	mm	824 (1951-80 Kilkenny)
Mean annual windspeed	ms^{-1}	3.44
Mean annual wind direction		SSE

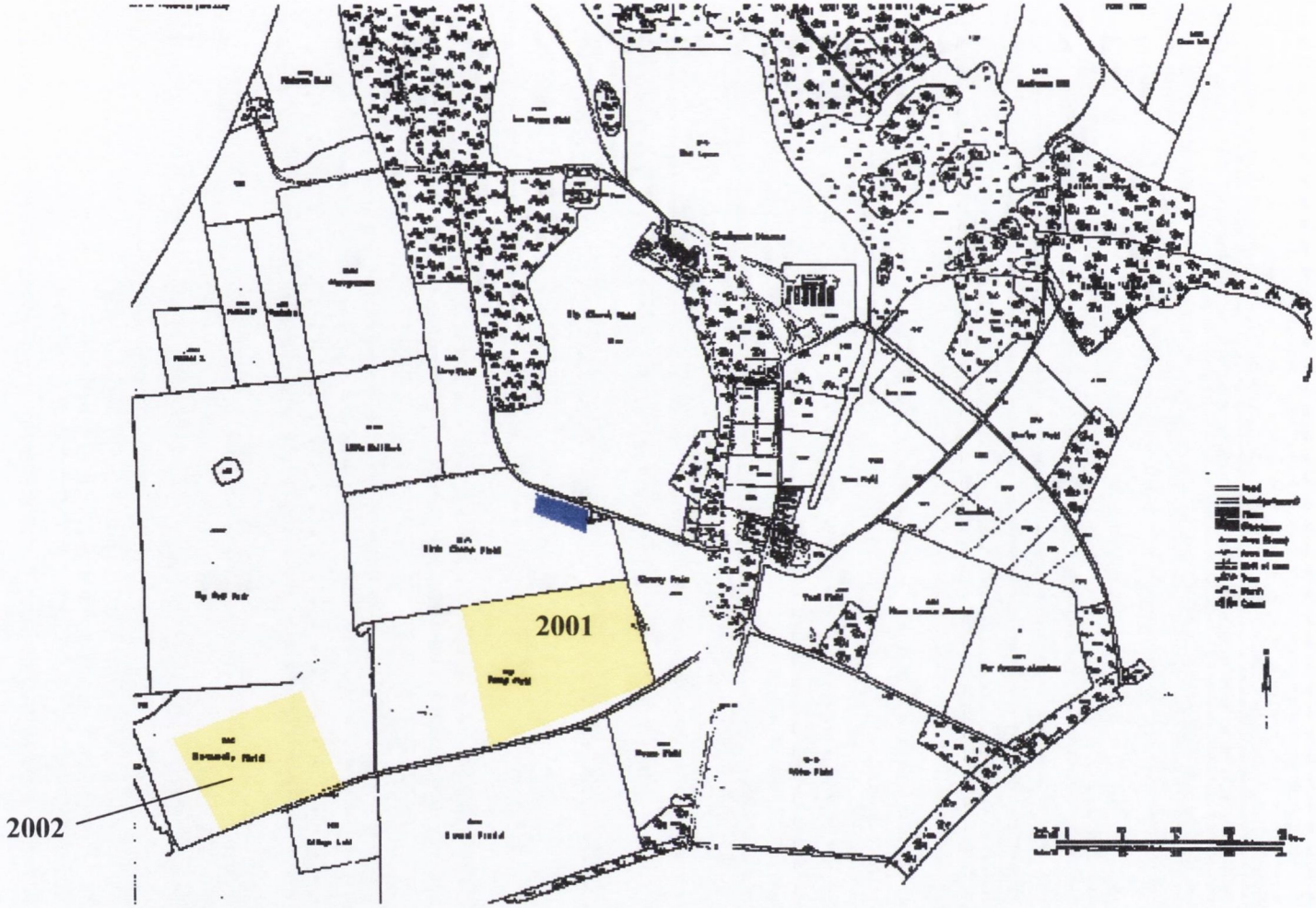

Figure 3.4: Site map of Teagasc, Oak Park, Carlow. The 2001 and 2002 sites are shown and the position of the meteorological station is marked in blue

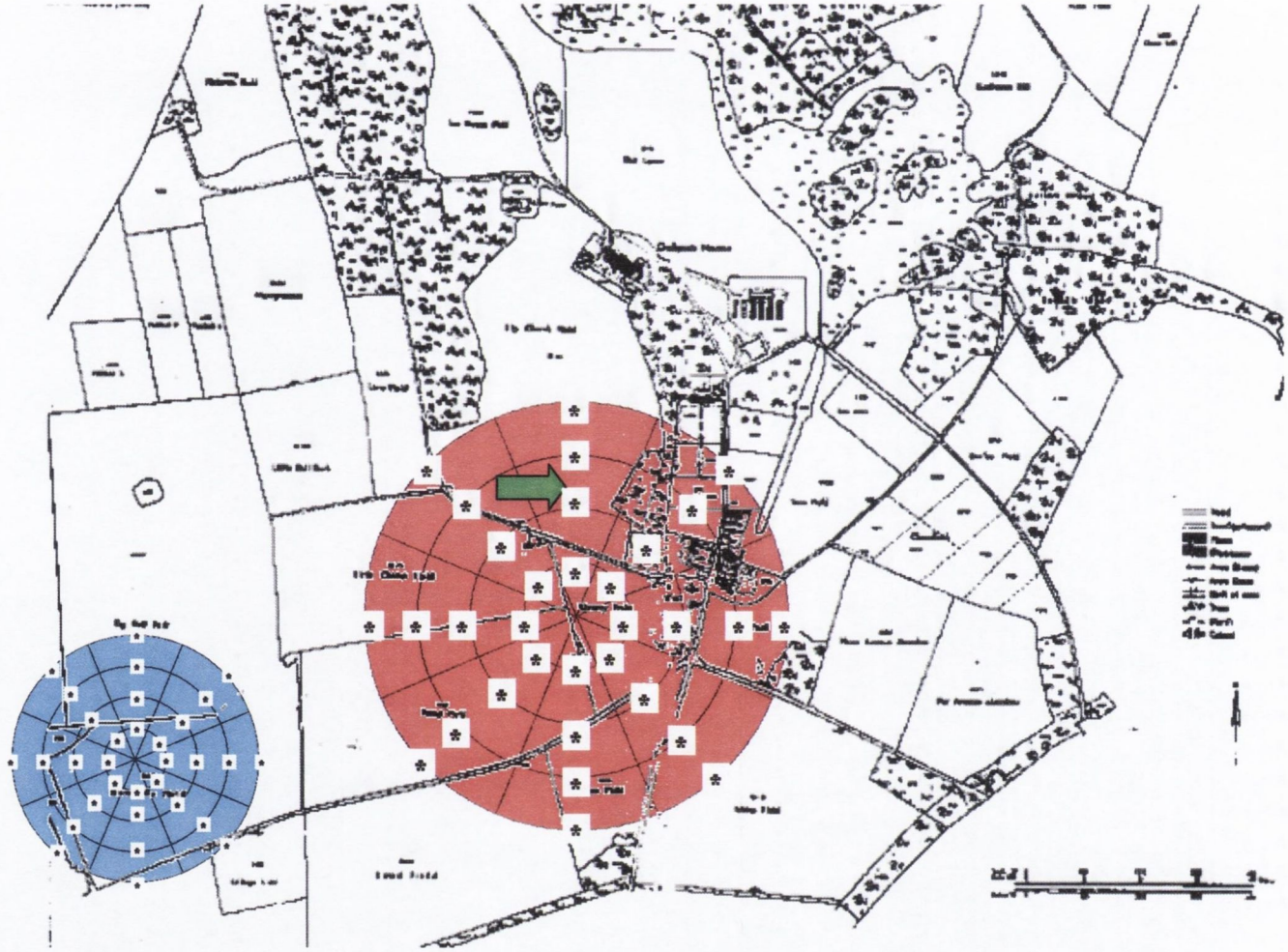

Figure 3.5: Site map of Teagasc, Oak Park, Carlow. The green arrow shows the meteorological station, the position of the volumetric spore trap and the Passive trap in 2001, while the charts show the areas sampled: red indicates 2001, blue indicates 2002. Collection sites are shown as *

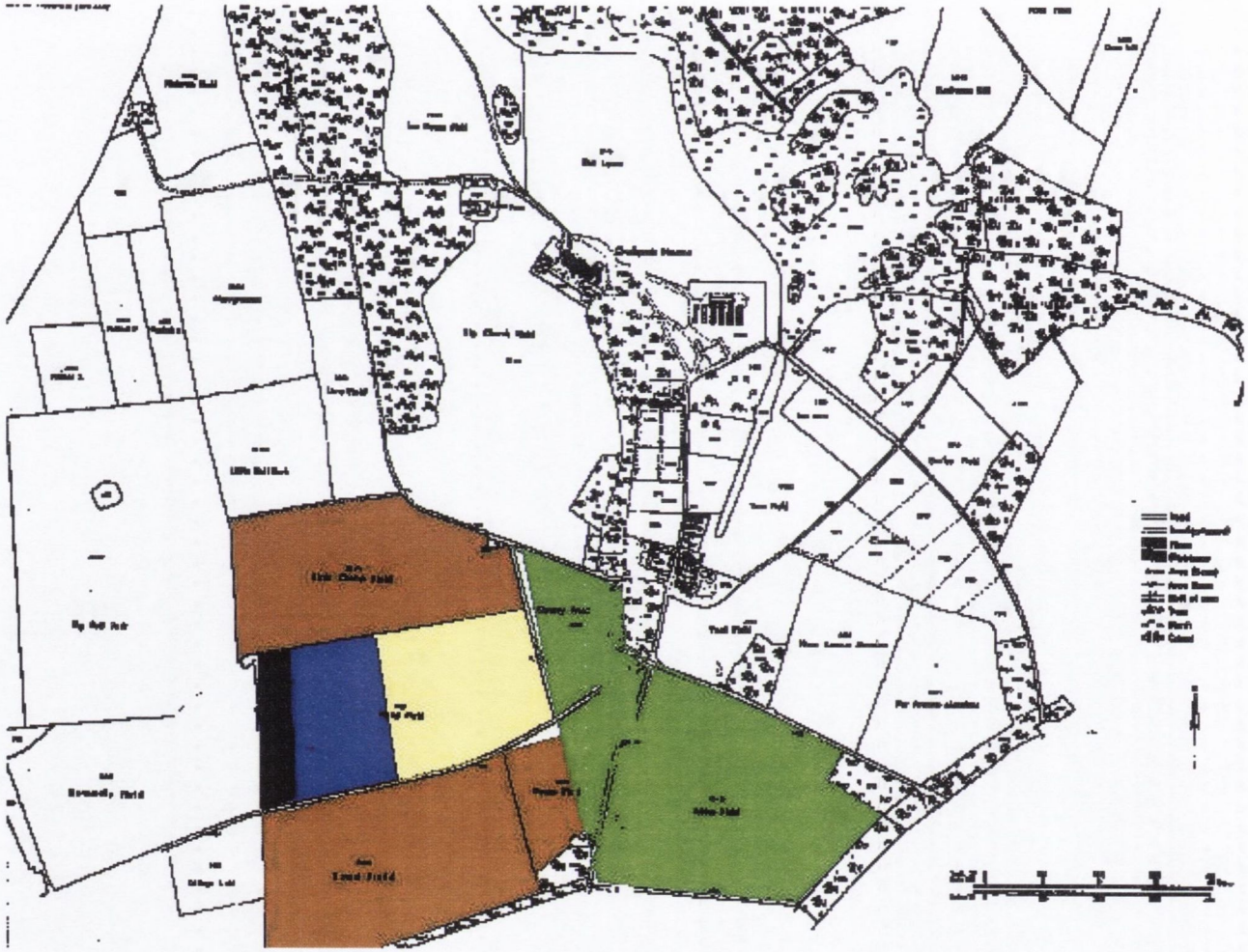

Figure 3.6: Site map of Teagasc Oak Park in 2001. Yellow indicates the position of the *Brassica napus* cv. Marinka crop, green indicates wheat crops, black indicates a *Camelina sativus* crop, brown indicates barley crops and the blue indicates a lupin crop

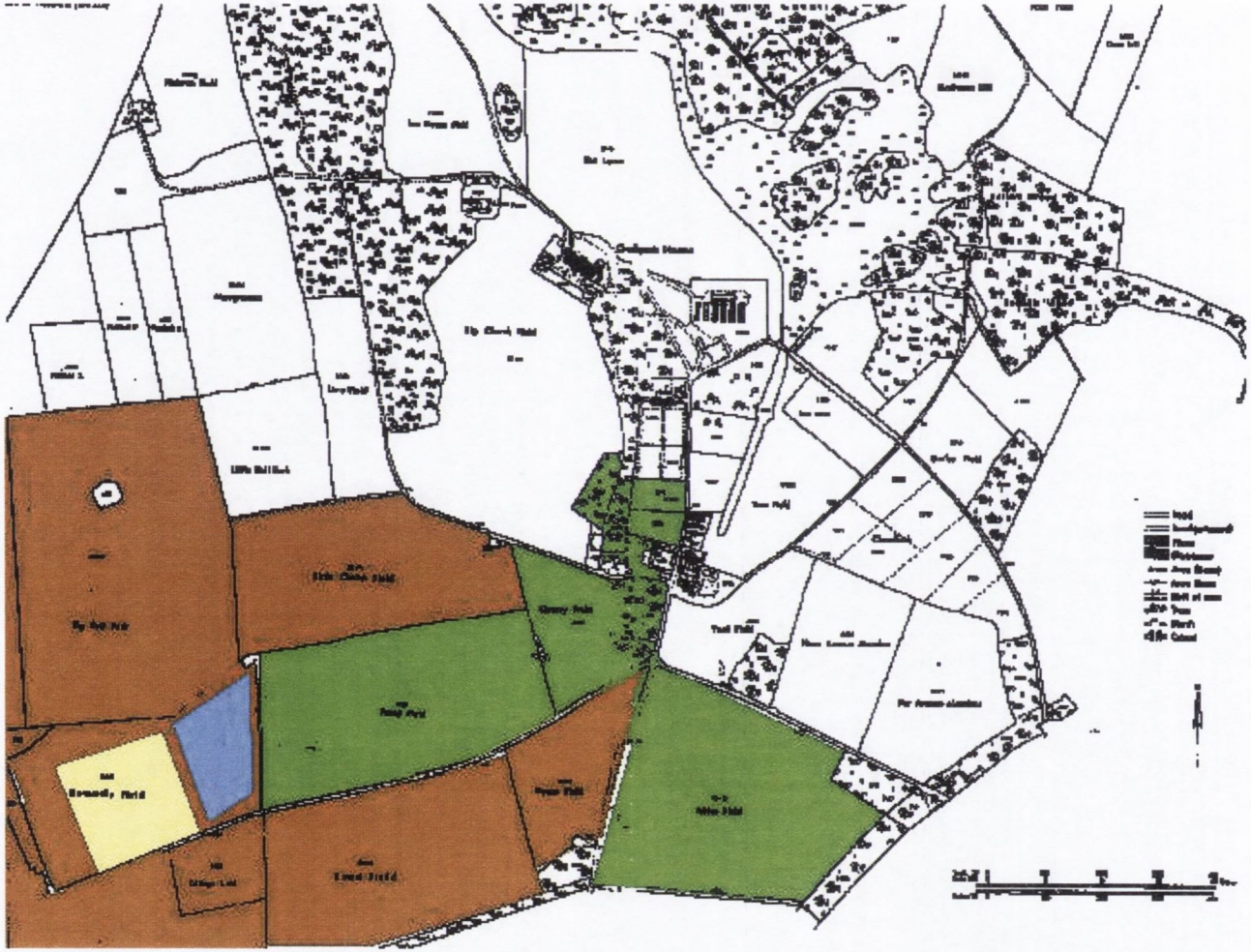

Figure 3.7: Site map of Teagasc Oak Park in 2002. Yellow indicates the position of the *Brassica napus* cv. Marinka crop, green indicates grass and wheat crops, brown indicates barley crops and blue indicates a potato crop

The 'pump field' consists of two soils graduating from heavy to light textured from east to west across the site. Heavy textured soil (loam to clay loam) in this case refers to grey-brown, podzolic, free draining with heavy textured clay brown horizon derived from boulder clay (glacial till). This soil has a high moisture holding capacity of greater than 200mm. The light textured soil, referred to as sandy loam, consists of free-draining, brown earth with a depth of approximately 50-75cm, derived from fluvial glacial gravels, with a low moisture holding capacity.

3.2.2 Pollen traps

A combination of both VSTs and passive traps were used for this analysis. VSTs produce superior data, as a fixed volume of air is sucked into the trap and results are in terms of concentration. A power supply is required for this trap and they are expensive, therefore it is not feasible to carry out such analyses on a large scale. The passive trap produces data in terms of flux; however these values can be converted to concentrations by comparing the results of the VST with the two passive traps (21 and 22) located nearby and then adjusting the passive trap results accordingly.

3.2.3 Seven-day volumetric spore trap (VST)

A seven-day VST was positioned 75m north of the crop in 2001 (Figure 3.8) and approx. 800m NE of the crop in 2002. Melinex® tape (200 gauge, clear) was coated in an even layer of white petroleum jelly (Vaseline®) before placing the drum into the trap (Berman, 2003). Steam was used to melt the Vaseline® and affix the tape to the drum. Melinex® tape is insoluble in water and has a melting point of 50°C so is ideal for this purpose.

The deposit on the trapping surfaces is macroscopically visible and is known as the 'trace'. The Melinex® tape was removed from the drum every seven days and divided into several equal sections of 48mm, each representing a 24-hour period. Three drops of lactophenol (consisting of 10g phenol dissolved in 10ml distilled water, with 10ml glycerine and 10ml lactic acid added) were used to stick each section to a microscope slide. Three drops of an aniline blue/lactophenol (0.4% w/v) were used to stain the trace and a large cover slip (22mm x 50mm) was then placed over the slide. The count was determined by examining eight fields of view every

two millimetres (approximately 1hr) using an objective lens (x40) and ocular lenses (x10). This was done 24 times, which is equivalent to a 24hr period (Berman, 2003).

Figure 3.8: Seven-day VST at an elevation of three metres located 75m (2001) from the *Brassica* crop at Teagasc, Oak Park, Carlow

There are many sampling and counting techniques that can be employed when analyzing aerobiological slides. Bi-hourly, hourly and continuous line transects are the most commonly used. One of the most common problems encountered with the transect technique is that it is not representative of the whole slide and localized 'clumps' of material on the slide may be interspersed among areas that contain no material at all.

Pollen concentrations (pollen grains per m^3 in a given hour) were determined from the counts produced during a transect count using the following formula:

$$C = A \times \beta \times \Lambda$$

C = concentration (pollen grains/m^3 in the given hour)

A = the sum of pollen/spores counted in the eight fields of view in the given hour

β = field of view conversion factor

Λ = volume conversion factor

The field of view conversion factor (β) is calculated as follows. The area of a one hour segment of Melenix® tape ($28mm^2$) is calculated by multiplying the width of the intake orifice by the distance travelled by the drum in an hour i.e. 14mm x 2mm. The number of fields of view in each 1hr segment (176) is calculated by dividing the area of a one-hour segment ($28mm^2$) by the area of a x400 field of view ($0.1591mm^2$). Since eight fields of views are counted per hour, the conversion factor is 22. The volume conversion factor (Λ) is calculated by expressing L/min as m^3 /hr ($x60x10^{-3}$).

3.2.4 Passive trap data

Passive traps were used to quantify the extent of pollen flux by wind from the *B. napus* cv. Marinka crop. To enable analysis of pollen flow the passive traps (modified Cour traps) were placed at a number of distances and directions from the source crop. Eight compass directions from the crop were used: N, NE, E, SE, S, SW, W and NW. Four distances were used in 2001: 0m, 50m, 100m and 200m. In 2002, following consideration of the results, the traps were brought closer to the field to analyse in more detail the observed decrease in pollen flow between 0m and 50m (0m, 12.5m, 25m and 50m). Since 32 traps were needed, materials for their construction were chosen on a low cost basis.

Initial work involved the discovery of a sticky substance to enable the adherence of pollen grains. Traditional adhesives such as Vaseline® and silicone oil could not be used due to interference with subsequent DNA extractions (Hodkinson, personal communication, 2000). Therefore, water-soluble alternatives were investigated by extracting DNA from anthers (whole anthers to ⅛ of an anther) along with small amounts of various adhesives. Glycerol was found to be the most effective adhesive. The effectiveness of different trapping surfaces along with the removal of pollen from it was also evaluated.

Modifications of the Cour trap included the construction of a rain protection device to prevent the dissolution of the adhesive and loss of trapped pollen. Accordingly an enclosed passive trap was developed (Dowding, pers. comm). The barrel of the trap was made by cutting 100mm diameter PVC soil pipes (Wavin®) into 400mm lengths. A rain-hood was made from a transversely bisected 100mm diameter PVC double-socketed swept bend pipe (Wavin®). The muslin gauze

(150mm x150mm) saturated with glycerol was held in place using 50mm lengths of 100mm diameter PVC soil pipes (Wavin®), which had been cut on their side and sprung into place. The pivot of the trap was made from 250mm long 10mm threaded bars. Lock nuts either side of the pipe held these in place. Holes of a 10mm diameter and 100mm depth were drilled in wooden stakes (50mm x 50mm x 2m) into which the threaded bars were inserted. The tail of the trap was made from 150mm x 250mm corrugated plastic (Astralight) and was inserted into 140mm cuts in upper and lower walls of the barrel of the trap.

3.2.5 Pollen extraction following collection from passive traps

The mesh was divided into two segments one for microscopic analysis of pollen and the other for DNA extraction. Each segment was then further divided into eight and added to 25ml sterile ultrapure water (SUW) in a 50ml tube and placed on a shaker for 2hrs at high speed. Following shaking the mesh was removed and the remaining suspension was centrifuged at 6,440rcf (6 x *g*) for 10mins. The supernatant was decanted off. Following centrifugation two different steps were taken. For microscopic analysis the remaining pellet was suspended in 500µl UPW and transferred to a 1.5ml microcentrifuge tube. Two 250µl UPW washings transferred any remaining suspension to the microcentrifuge. The tubes were then stored at 4°C to inhibit microbial growth prior to analysis. For DNA extraction the pellet was resuspended in preheated CTAB and transferred immediately to a 1.5ml microcentrifuge tube (section 3.2.8).

3.2.6 Microscopic analysis

The extracted suspension was viewed by pipetting 10µl onto a microscope slide and covering with a cover slip (22x32mm). The total number of pollen grains was determined by examining five fields of view at x400 magnification for each of five 10µl subsamples (using a Lecia, MPS32). The abundance of Poaceae pollen was examined to determine a continuous background pollen level, which could subsequently be used to aid statistical analysis. Twenty-five measurements of the abundance of Brassicaceae and Poaceae pollen were taken for each sample and recorded. Other observed material including non-Brassicaceae and non-Poaceae pollen grains, fungal spores, soredia, soil particles and other debris were not

recorded. Results were displayed using charts generated in Arcview GIS 3.2 (Environmental Systems Research Institute Inc.).

3.2.7 Pollen DNA extraction and isolation

A 0.5ml 2% CTAB extraction buffer was preheated in a sterile 1.5ml microcentrifuge tube to 65°C in a water bath. Mercaptoethanol (2µl) was added to the CTAB prior to use. Micro-pestles were also preheated to 65°C in a water bath. This solution was used to re-suspend the pellet from section 3.2.5 or anthers collected prior to the field season (divided into four) were analysed. A small amount of extraction buffer (50µl) was added to the ¼ anther and a preheated micro-pestle was used to grind the anthers. The remaining buffer was added and swirled gently to suspend the slurry.

The mixture (pellet and CTAB or anther and CTAB) was incubated at 65°C for 10mins. Chloroform: isoamyl alcohol (24:1; 0.5ml) was added into the microcentrifuge tube and mixed, gas that built up released by loosening the lid briefly. The tubes were positioned horizontally on a shaker for 30mins. Further gas build up was released prior to centrifugation at 12,880rcf (12 x *g*) for 10mins. The tubes were carefully removed from the centrifuge taking care not to disturb the separation. The aqueous upper phase containing the DNA was removed and transferred to a fresh 1.5ml microcentrifuge tube. The remaining organic layer was discarded. An equal volume of isopropanol was added and the tubes inverted gently to precipitate the DNA. The samples were then placed in a -20°C freezer. The DNA was then pelleted and resuspended in TE as in section 2.2.1 (p36).

3.2.8 PCR amplification of the *trnL* intron

PCR analysis of extracted mesh pollen DNA was carried out using conditions in section 2.2.3 (Table 2.1 p37) using primers c and d (Taberlet *et al.*, 1991).

3.2.9 Meteorological data

Synoptic meteorological data measurements were available from the meteorological station at Teagasc, Oak Park, Carlow during both 2001 and 2002. Mean weekly measurements for rainfall, while mean hourly measurements

windspeed, temperature, wind direction, relative humidity and irradiance, were calculated for the flowering period of *B. napus* cv. Marinka in 2001 and 2002 and compared (Appendix 3).

Table 3.2: Meteorological parameters measured and the instruments used to measure them (P.R.T. = Platinum resistance thermometer)

Parameter	Units	Instrument
Relative humidity @1.5m	Percentage	Campbell Scientific MP100A probe
Air temperature @1.5m	°C	Campbell Scientific MP100A probe (P.R.T.)
Wind direction	Degrees	Campbell Scientific wind vane
Irradiance	W/m^2	Kipp and Zonen typecm5 pyranometer
Windspeed	m/s	Campbell Scientific A100R anemometer
Rainfall	mm	Casella 0.1mm tipping bucket
Poaceae temperature	°C	Campbell Scientific PT100 (P.R.T.)
Soil temperature @ 0.1m	°C	Campbell Scientific PT100 (P.R.T.)
Soil temperature @ 0.3m	°C	Campbell Scientific PT100 (P.R.T.)

3.3 Results

3.3.1 Sampling period

Pollen samples were collected weekly from both the VST and passive traps for the duration of and for a week prior to flowering. Figure 3.9 shows the progression of flowering over the seven weeks of the 2002 season, while Table 3.3 shows the date of sowing and beginning and end of flowering for each OSR variety.

Figure 3.9: Progression of *Brassica napus* cv. Marinka crop during weeks 1 to 7 of the 2002 season

Table 3.3: Planting and flowering dates of each variety sown

Species	Site	Date of Sowing	Flowering Start	Flowering End
Brassica napus cv. Marinka	Oak Park	23/04/01	05/06/01	23/07/01
Brassica napus cv. Triolo (Male sterile)	Oak Park	17/05/01	09/07/01	27/08/01
Brassica napus cv. Marinka	Oak Park	17/04/02	17/06/02	27/07/02
Brassica napus cv. Jura (Male sterile)	Oak Park	27/05/02	24/06/02	29/07/02

3.3.2 Meteorological data results

Meteorological data corresponding to the *Brassica napus* cv. Marinka flowering period were analysed (Figures 3.10-3.15). As relative humidity is an associated variable its results are not reported, that is, if irradiance and temperature are high the relative humidity will be low and if it is rainy and cool relative humidity will be high. In 2001, week one had low rainfall, light winds, high irradiance, and a mean temperature of 10°C. Week two had medium rainfall, light winds, low irradiance and a mean temperature of 12°C. Week three had low rainfall, medium windspeed, high irradiance and a mean temperature of 13°C. Week four had light winds and a mean temperature of 16°C; with high irradiance and low rainfall for the beginning of the week; but high rainfall and low temperatures towards the end of the week. Week five had low rainfall, high winds, high irradiance and a mean temperature of 15°C. Week six had high rainfall, high windspeed, high irradiance and a mean temperature of 12°C. Week seven had low rainfall, high windspeed, mixed irradiance and a mean temperature of 13°C.

Therefore in 2001, the overall conditions during weeks one, part of two, three, part of four, five, part of six and seven; were conducive to pollen distribution, while during part of weeks two, four and six; conditions were unfavourable for pollen distribution.

In 2002, week one had medium rainfall, high windspeed, high irradiance, and a mean temperature of 12°C. Week two had low rainfall, high windspeed, high irradiance and a mean temperature of 13°C. Week three had low rainfall, medium windspeed, high irradiance and a mean temperature of 12°C. Week four had medium windspeed and a mean temperature of 12°C, with high and low rainfall coinciding with high and low irradiance on different days. Week five had low rainfall, medium winds, high irradiance and a mean temperature of 13°C. Week six had low rainfall, medium windspeed, high irradiance and a mean temperature of 14°C. Week seven had low rainfall, medium windspeed, low irradiance and a mean temperature of 16°C.

Therefore in 2002, the overall conditions during weeks one, two, three and part of four; were conducive to pollen distribution, while during part of week four, and weeks five, six and seven conditions were not as favourable for pollen distribution.

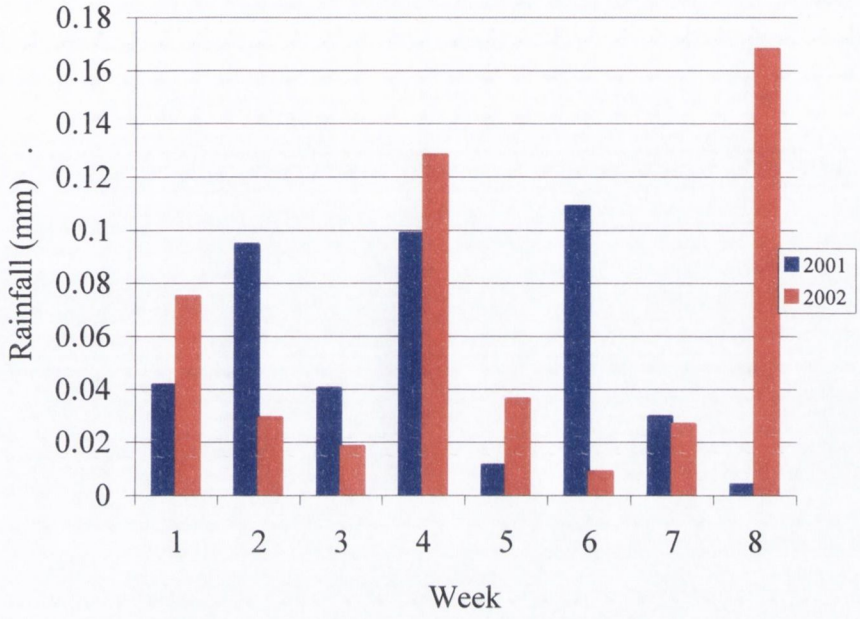

Figure 3.10: Mean hourly rainfall measurements during 2001 and 2002 flowering periods

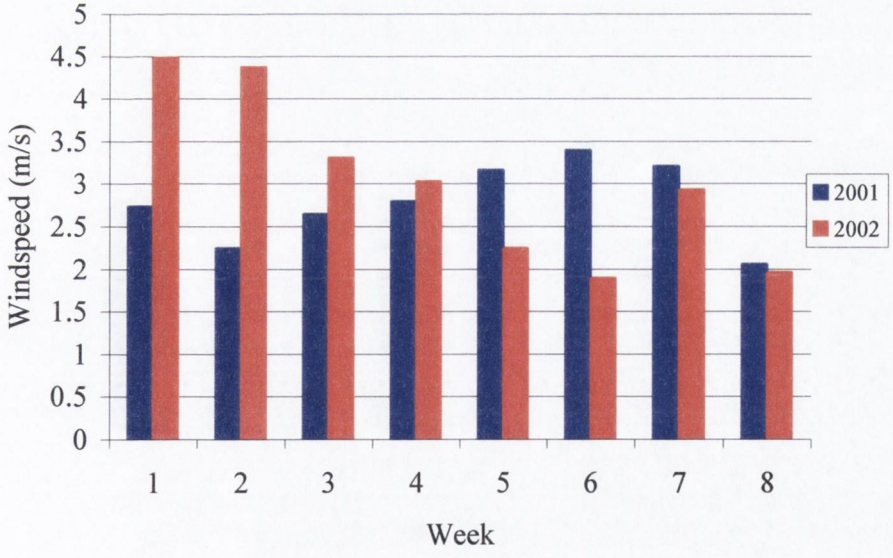

Figure 3.11: Mean weekly windspeed measurements during 2001 and 2002 flowering periods

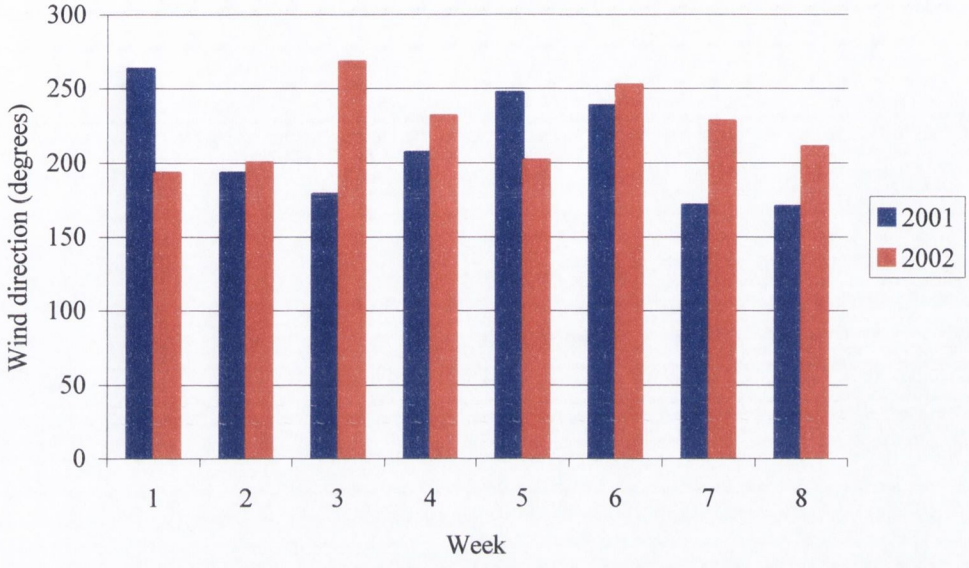

Figure 3.12: Mean hourly wind direction measurements during 2001 and 2002 flowering periods

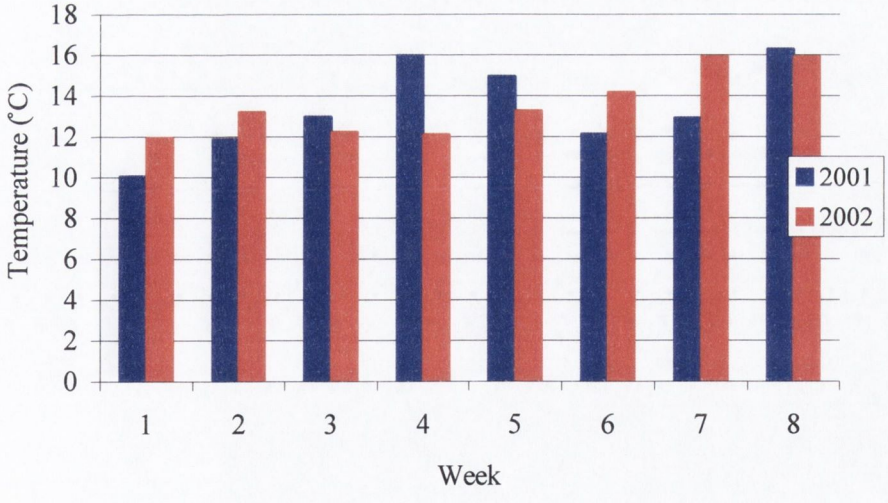

Figure 3.13: Mean hourly temperature measurements during 2001 and 2002 flowering periods

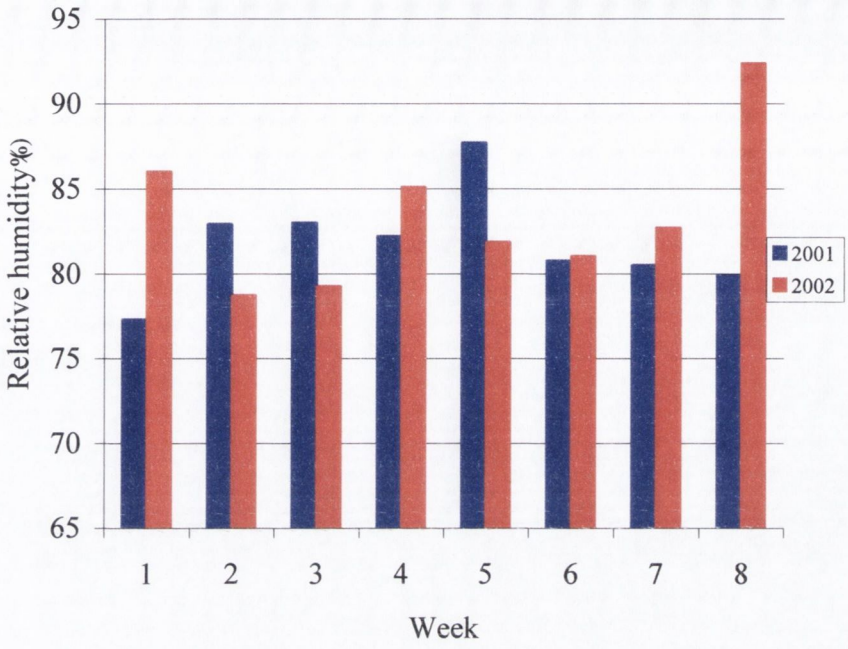

Figure 3.14: Mean hourly relative humidity measurements during 2001 and 2002 flowering periods

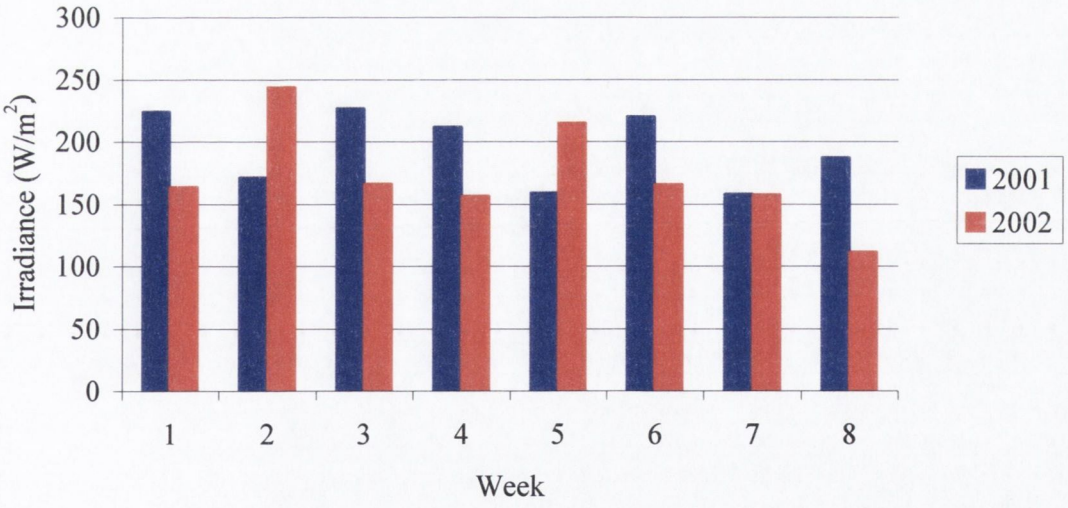

Figure 3.15: Mean hourly irradiance measurements during 2001 and 2002 flowering periods

3.3.3 Passive trap pollen collection

The number of pollen grains detected in each trap was calculated as a percentage of the total pollen collected for the week. Rose diagrams indicate the percentage of total Brassicaceae and Poaceae pollen detected in each trap in each week (Figures 3.13 to 3.15).

Figure 3.16 shows the layout of the site and the numbering system used for the passive traps generated using Arcview GIS 3.2 (Environmental Systems Research Institute Inc.). Figure 3.17A 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 Figure 3.17B was used for display purposes.

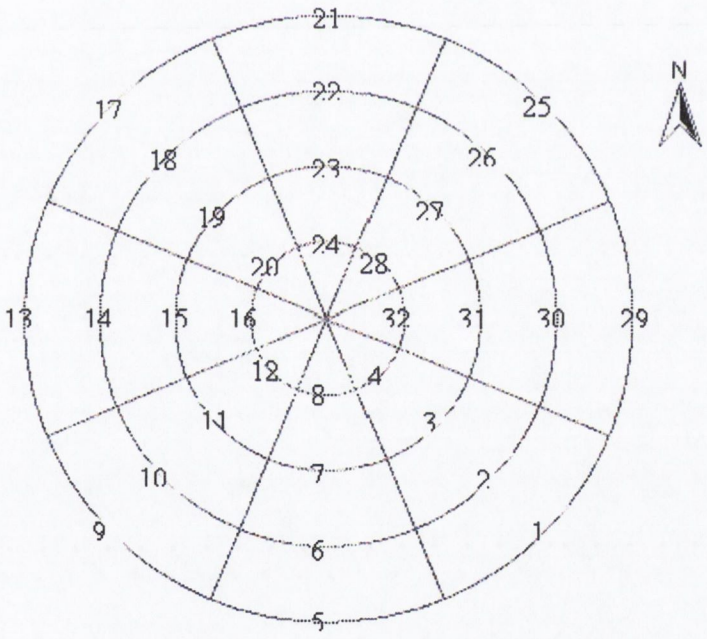

Figure 3.16: Chart representing the actual layout of the site and showing the locations and numbers allocated to each passive traps. Rings indicate distances of 0m, 50m, 100m and 200m in 2001 and 0m, 12.5m, 25m and 50m in 2002

In 2001 (Figure 3.19), no Brassicaceae pollen was collected by the passive traps during week one and week seven as the crop has either not started or finished flowering. In week two, (SSW wind with a speed of 2.25m/s) 25% of the week's total Brassicaceae pollen was detected at 200m NE of the crop. In week three, 3%

and 2% of the week's total pollen were collected at 50m and 100m N, 6% and 8 % at 50m and 100m NE, while 15% was detected at 50m E of the crop. In week four SSW winds of 3.02m/s were experienced, however 6% and 7% of the week's Brassicaceae pollen was detected in traps 50m and 100m S of the crop. In week five WSW winds of 3m/s were experienced and 4% of pollen was detected at 50m E; while 4% was detected at 50m SE and; and 4% was detected at 50m S. In week six WSW winds of 3.39m/s were experienced and 25% of pollen was detected at 200m NE. In 2001, the most pollen movement is occurring during weeks three and four, which correspond to the peak flowering weeks.

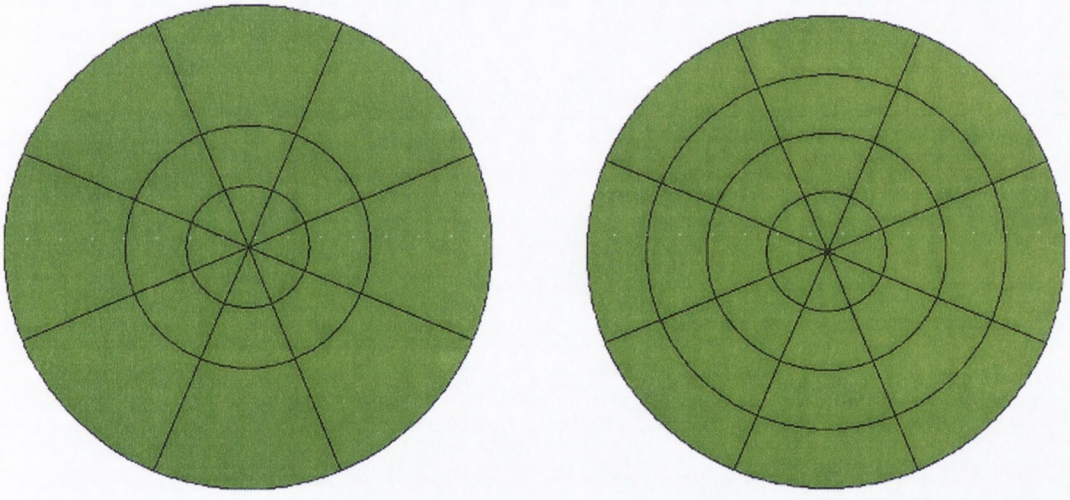

Figure 3.17: (A) Chart representing the actual layout of the passive traps and (B) Chart used for display purposes

In 2002 (Figure 3.19), strong winds were experienced during weeks one, two and three. No Brassicaceae pollen was collected by the passive traps during week one as the crop has either not started flowering. In week two 10% of the weeks total Brassicaceae pollen was detected at 200m NE of the crop, while a further 10% was detected at 50m N. In week three, 13%, 13% and 9% of the week's total pollen were collected at 50m, 100m and 200m E of the crop, while 13% and 4% were detected 50 and 100m SE of the crop; and 7% and 5% were collected at 50m and 200m S of the crop. In week four, SW winds of 3.02m/s were experienced, 2% was detected at 50m N, 9%, 11% and 4% were detected in traps at 50m, 100m and 200m E and 5%

was detected in traps at 50m SE. In week five, lighter winds of 2.24m/s were experienced, 9% of the pollen was detected in traps at 100m NE; 50m and 100m E; and at 50m SE. In week six at the end of flowering, 100% of the pollen was detected at 50m S of the crop. In 2002, the most pollen movement is occurring during week three, which corresponds to the peak flowering week as can be seen in (Figure 3.19). During both 2001 and 2002, Poaceae pollen was also collected (Figures 3.20 and 3.21).

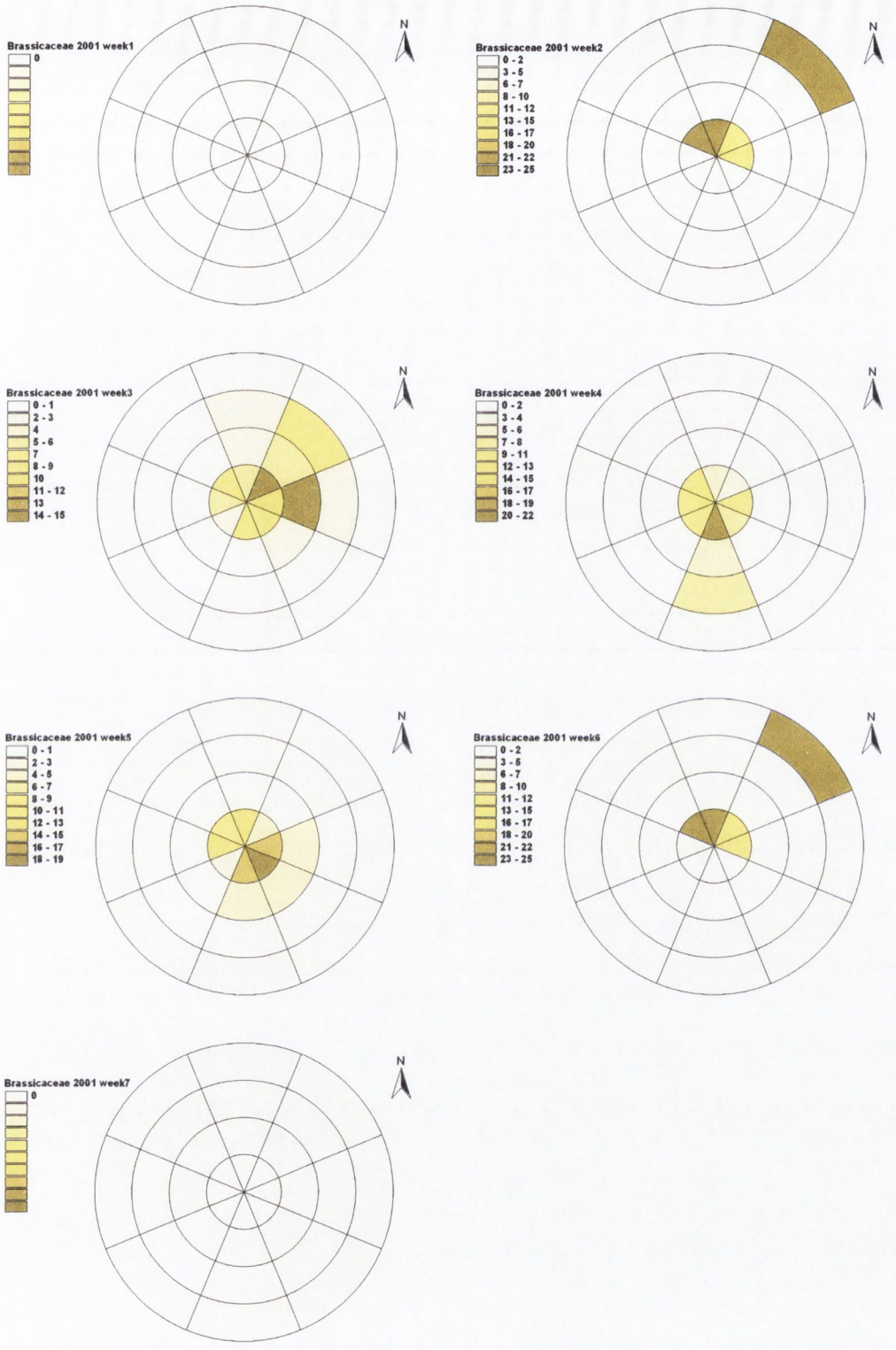

Figure 3.18: Brassicaceae pollen calculated for each sector as a percentage of the total Brassicaceae pollen collected in each week of 2001. Rings indicating traps at 0m, 50m, 100m and 200m

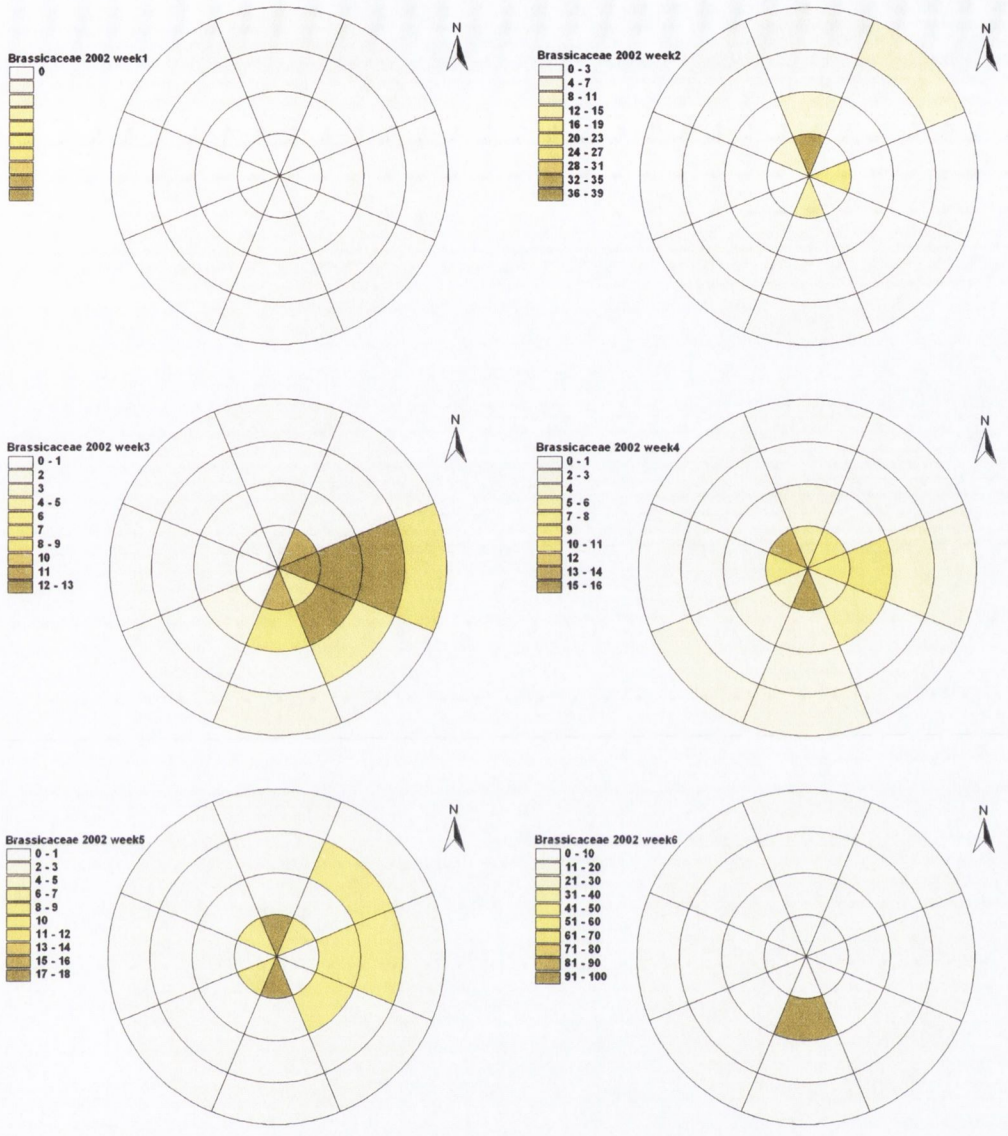

Figure 3.19: Brassicaceae pollen calculated for each sector as a percentage of the total Brassicaceae pollen collected in each week of 2002. Rings indicating traps at 0m, 12.5m, 25m and 50m

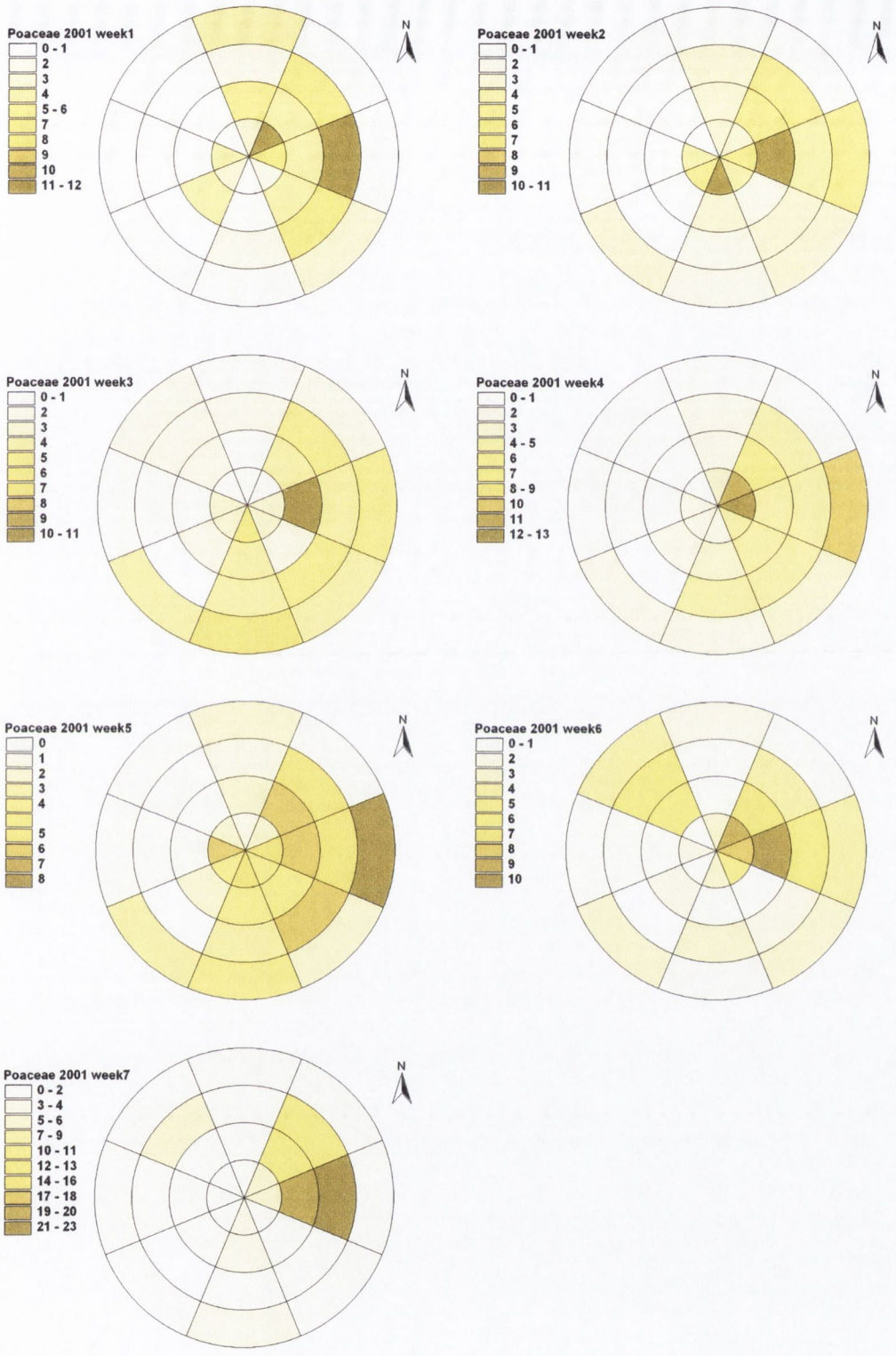

Figure 3.20: Poaceae pollen calculated for each sector as a percentage of the total Poaceae pollen collected in each week of 2001. Rings indicating traps at 0m, 50m, 100m and 200m

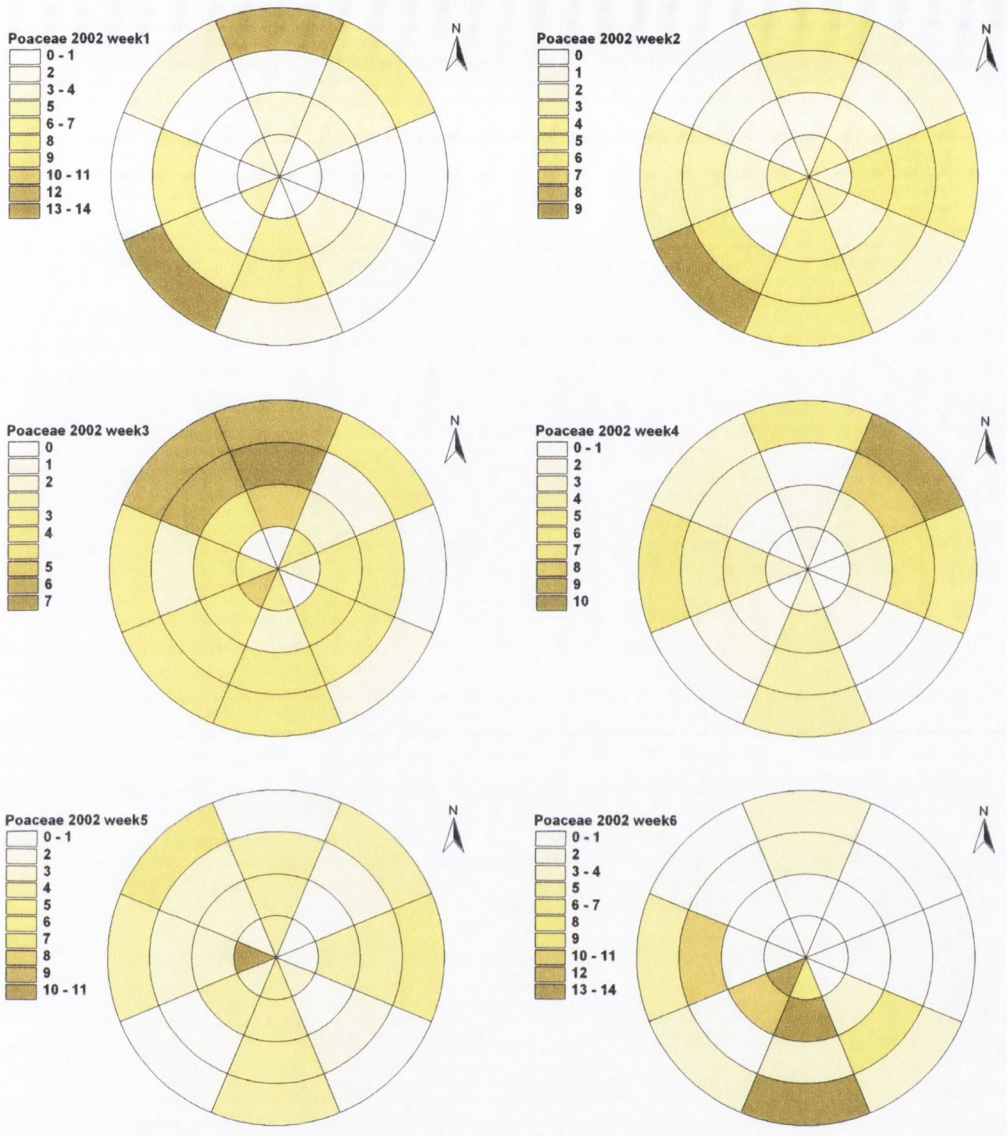

Figure 3.21: Poaceae pollen calculated for each sector as a percentage of the total Poaceae pollen collected in each week of 2002. Rings indicating traps at 0m, 12.5m, 25m and 50m

3.3.4 Passive trap data results and meteorological data

The numbers of pollen grains collected by each passive trap were then analysed for correlation with the meteorological data i.e. windspeed, wind direction, temperature, irradiance, relative humidity and rainfall (section 3.3.2, p105).

In 2001, week one had low rainfall, light winds, high irradiance, and a mean temperature of 10°C. The combination of conditions was conducive to pollen dispersal however no Brassicaceae pollen was detected, as the crop had not started flowering yet. During week two conditions were not favourable for most of the week however, on two days conditions were favourable i.e. the rainfall level was 0mm, irradiance was high and windspeed was medium to low; Brassicaceae pollen reached 200m this week. Week three had low rainfall, medium windspeed, high irradiance and a mean temperature of 13°C. These favourable conditions assisted Brassicaceae pollen movement to 100m. Week four had light winds and a mean temperature of 16°C; with high irradiance and low rainfall for the beginning of the week; but high rainfall and low temperatures towards the end of the week. Although favourable conditions were present towards the beginning of the week Brassicaceae pollen was not dispersed beyond 50m. Week five had low rainfall, high winds, high irradiance and a mean temperature of 15°C and Brassicaceae pollen was detected at 200m. Week six had high rainfall, high windspeed, high irradiance and a mean temperature of 12°C. Despite the rain pollen was distributed to 200m. Week seven had low rainfall, high windspeed, mixed irradiance and a mean temperature of 13°C. However despite these favourable conditions the crop had finished flowering and therefore no Brassicaceae pollen was detected.

In 2002, week one conditions were conducive to pollen dispersal (medium rainfall, high windspeed, high irradiance, and a mean temperature of 12°C), however no Brassicaceae pollen was detected, as the crop had not started flowering yet. In week two conditions were favourable for most of the week; there was low rainfall, high windspeed, high irradiance and a mean temperature of 13°C. Although the crop had only just begun flowering, Brassicaceae pollen reached 200m in week two. Week three had favourable conditions (low rainfall, medium windspeed, high irradiance and a mean temperature of 12°C) and Brassicaceae pollen travelled 200m. Week four had medium windspeed and a mean temperature of 12°C with high and low rainfall coinciding with low and high irradiance on different days. Despite this Brassicaceae pollen was still dispersed to 200m. Week five had low

rainfall, medium winds, high irradiance and a mean temperature of 13°C and Brassicaceae pollen was dispersed to 100m. Week six had low rainfall, medium windspeed, high irradiance and a mean temperature of 14°C and Brassicaceae pollen was detected at 100m. Week seven had low rainfall, medium windspeed, low irradiance and a mean temperature of 16°C and Brassicaceae pollen was detected at 50m. It can therefore be concluded that pollen dispersal is high when conditions include warm temperatures, sunshine, light to medium winds and most importantly no rain.

3.3.5 Statistical analyses of VST data: comparison of Poaceae and Brassicaceae pollen collected

The mean concentrations of Brassicaceae and Poaceae pollen (pollen grains/m^3) detected at 100m from the *Brassica* crop by the VST in 2001 are shown in Figure 3.22. Pollen concentrations of Poaceae were as expected much higher than Brassicaceae as Poaceae pollen is adapted for wind pollination. In 2002, the VST was located 800m from the source crop and no Brassicaceae pollen was detected.

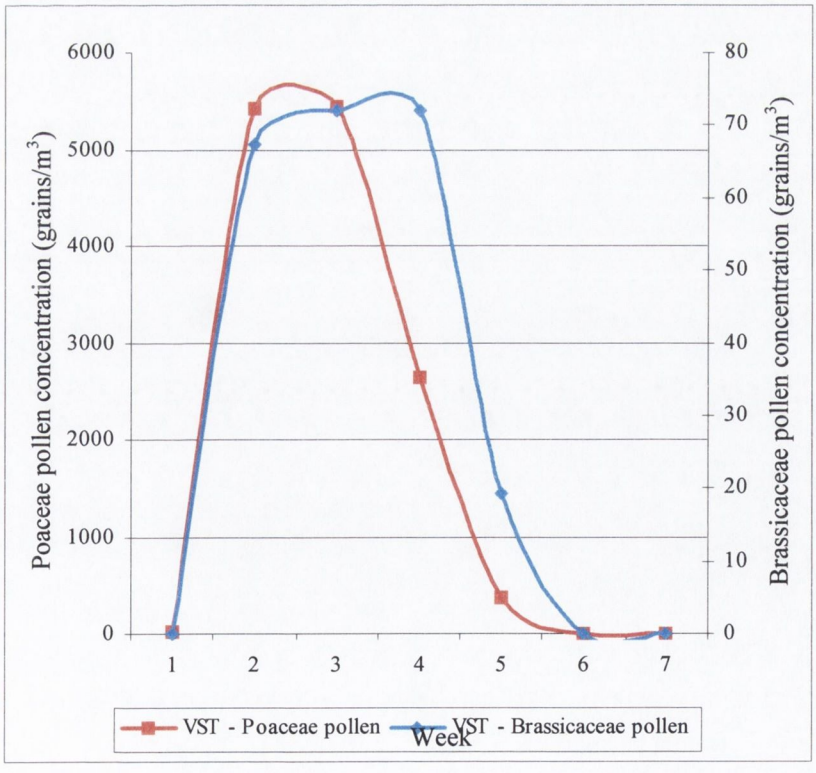

Figure 3.22: Mean concentration of Brassicaceae and Poaceae pollen (pollen grains/m^3) at 100m from *Brassica* crop detected by the VST in 2001

Linear regression results of Brassicaceae and Poaceae pollen concentrations showed an R^2 value of 83% and a regression coefficient of 64.657 with p = 0.004, indicating that the VST collected approximately 65 times more Poaceae pollen than Brassicaceae pollen (Tables 3.4, 3.5 and 3.6, Figure 3.23).

Table 3.4: Model summary of the linear regression of Brassicaceae and Poaceae pollen concentrations collected by the VST

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.912[a]	0.832	0.798	1140.237

[a]Predictors: (Constant), Brassicaceae

Table 3.5: ANOVA[b] of Brassicaceae and Poaceae pollen concentrations concentrations collected by the VST

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	32081797.07	1	32081797.07	24.676	.004[a]
	Residual	6500702.335	5	1300140.467		
	Total	38582499.4	6			

[a]Predictors: (Constant), Brassicaceae, [b]Dependent Variable: Poaceae

Table 3.6: Regression coefficients from linear regression of Brassicaceae and Poaceae pollen concentrations collected by the VST

Model		Unstandardised Coefficients	Standardized Coefficients	t	Sig.	Std. Error	Beta
		Model	B				
1	(Constant)		-141.64	607.78		-0.233	0.825
	VST - Brassicaceae		64.657	13.016	0.912	4.967	0.004

[a]Dependent Variable: Poaceae

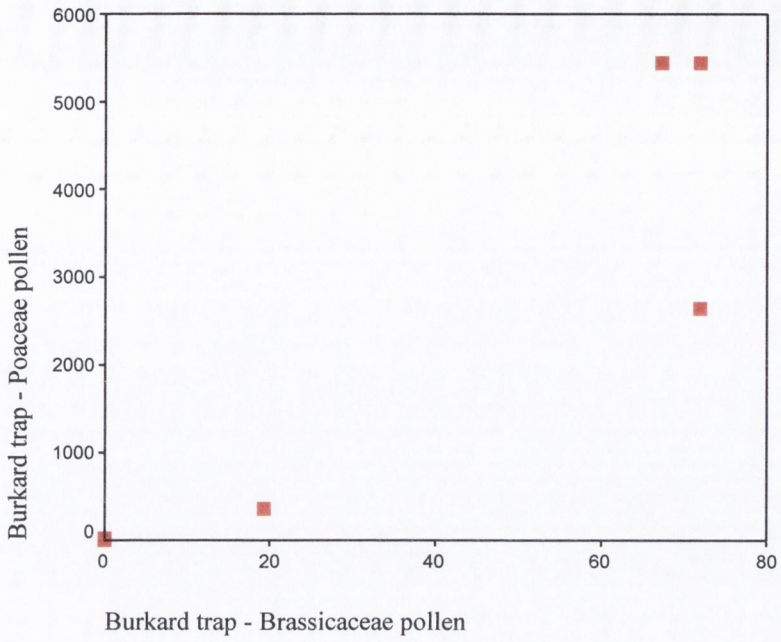

Figure 3.23: Scatterplot of Poaceae and Brassicaceae pollen concentration collected by the VST

3.3.6 Statistical analysis of data collected from passive traps

The median number of both Brassicaceae and Poaceae pollen grains were calculated for the five fields of view for each sub-sample. The mean, standard deviation and standard error were also calculated for each sample. Meteorological data were also included in the analyses.

Wind direction, windspeed, rainfall and daylight hours were considered to be the most important meteorological factors in relation in pollen flow (Dowding, pers. comm.). Therefore in order to remove as many null values as possible night-time hours and hours with rainfall were removed from the analysis. It was also considered that certain wind directions were needed for pollen to flow from the crop into the different traps. For example, if a southerly wind is blowing then traps to the south of the crop would not receive any pollen from the crop. Therefore windspeed and flux were only taken into account where appropriate wind directions were experienced. The pollen flux (pollen grain no./ m^3 in a given week) recorded was determined by taking the raw count i.e. the median of five fields of view (FOVs) and multiplying by the following equation:

$$Flux = \frac{2 \times C \times A}{\alpha \times T}$$

C = median of 5 FOVs

α = area of FOV (m^2)

A = area of mesh (m^2)

T = time (hrs)

This figure takes the area of the coverslip into account rather than the FOV. Since half the mesh was used for pollen analysis the result was multiplied by two. A volume adjustment was made as 10µl of a 1ml solution were used. A time adjustment was also made to incorporate the number of hours in a week (168) into the equation.

3.3.7 Statistical analysis of Poaceae pollen collected by the VST and passive traps

Mean weekly concentrations of Poaceae pollen grains calculated for the VST were compared with the weekly average concentration calculated for the two nearest passive traps (22 and 23; 2001 50m and 100m N of the field). Wind run (m/s^2) was calculated for each trap position, for each week by dividing the total relevant windspeeds (m/s) by the total number of seconds (s). From this a measure of concentration could be determined for the passive trap data (Figure 3.24).

Linear regression of Poaceae pollen concentrations collected by the VST compared with those collected by the passive trap was then carried out to enable comparison of detection levels of Poaceae pollen by the VST versus the passive traps. An R^2 value of 82% and a regression coefficient of 7.909 with $p = 0.005$ was obtained, indicating that the VST is approximately eight times more efficient in Poaceae pollen collection than the passive trap (Tables 3.7, 3.8 and 3.9, and Figure 3.25). Consequently, all pollen concentrations calculated for the passive traps were multiplied by 8 (Figures 3.28 to 3.31, p125-128). It was noted that the levels of Poaceae pollen increased after week one and declined after week four, which coincided with the flowering season of the crop grasses sown on the Teagasc, Oak Park, Carlow site and coincidently with the *Brassica napus* cv. Marinka crop.

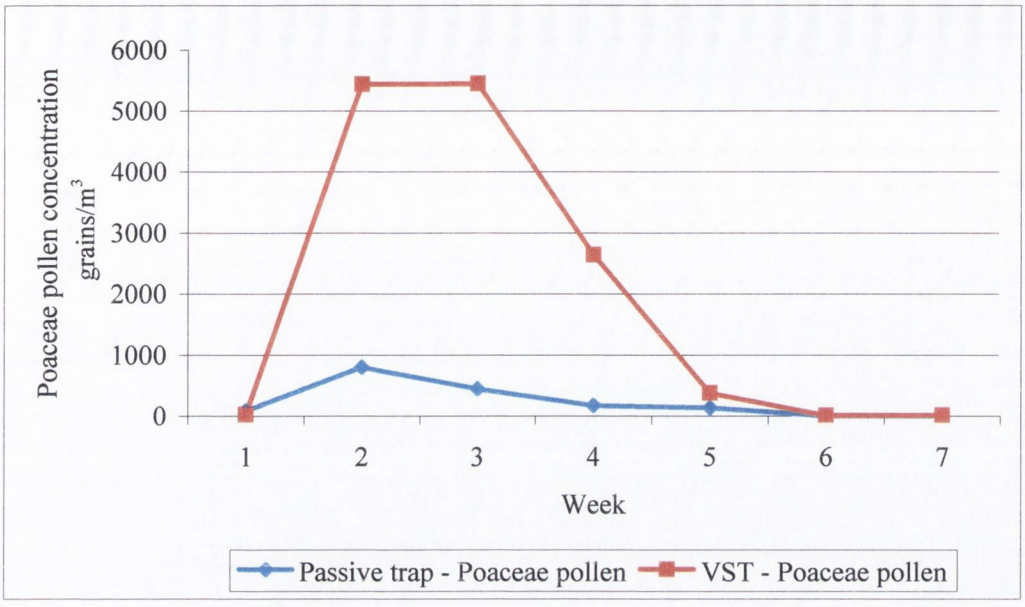

Figure 3.24: Poaceae pollen concentrations collected by the VST compared with those collected by passive traps 22 and 23

Table 3.7: Model summary of the linear regression of Poaceae pollen concentrations collected by the VST and the passive traps

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.905[a]	0.82	0.784	1179.3465

[a]Predictors: (Constant), Passive

Table 3.8: ANOVA[b] of Poaceae pollen concentrations collected by the VST and the passive traps

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	31628209	1	31628209	22.74	.005[a]
	Residual	6954290.3	5	1390858.1		
	Total	38582499	6			

[a]Predictors: (Constant), Passive; [b]Dependent Variable: Burkard

Table 3.9: Regression coefficients[a] from linear regression of Poaceae pollen concentrations collected by the VST and the passive traps

Model		Unstandardised Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	136.374	591.045		0.231	0.827
	Passive trap – Poaceae pollen	7.909	1.659	0.905	4.769	0.005

[a]Dependent Variable: Burkard

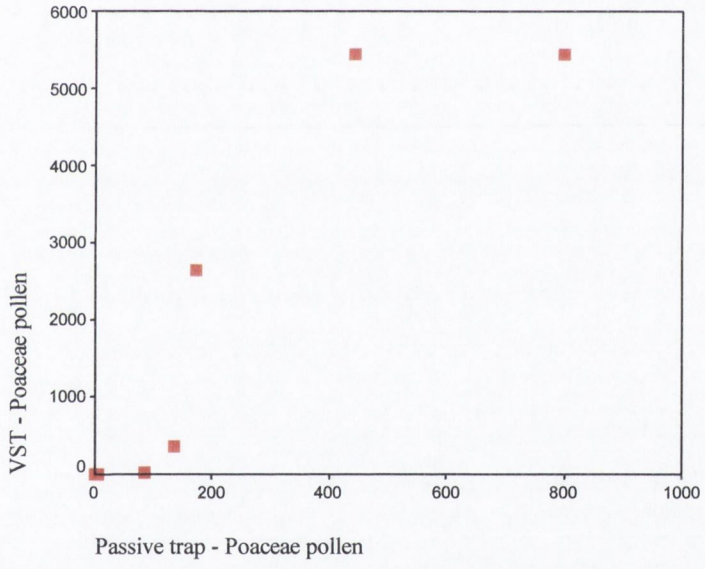

Figure 3.25: Scatterplot of Poaceae pollen collected by the VST and the passive traps

3.3.8: Statistical analysis of Brassicaceae pollen collected by the VST and the passive traps

Mean weekly concentrations of Brassicaceae pollen grains were also calculated for the VST and compared with the weekly average concentration calculated for the two nearest passive traps (22 and 23, Figure 3.26).

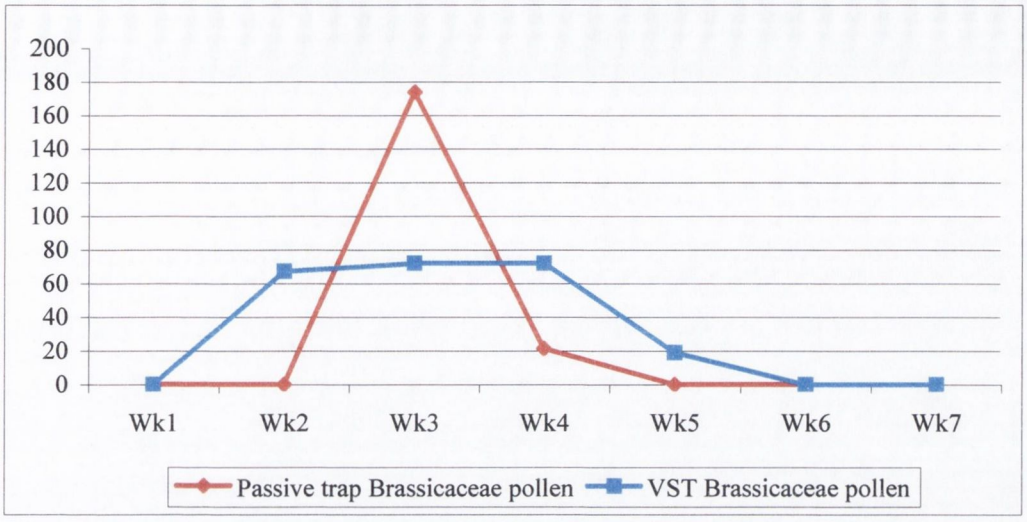

Figure 3.26: Brassicaceae pollen concentrations collected by the VST compared with those collected by passive traps 22 and 23

Linear regression of Brassicaceae pollen concentrations collected by the VST compared with those collected by the passive trap was also carried out to enable comparison of detection levels of Brassicaceae pollen by the VST versus the passive traps. An R^2 value of 30.1% and a regression coefficient of 0.303 with $p = 0.202$ was obtained (Tables 3.10, 3.11 and 3.12; and Figure 3.27). As the relationship was not statistically significant, the relationship derived for the more numerous Poaceae pollen was used for Figures 3.30 and 3.31.

Table 3.10: Model summary of the linear regression of Brassicaceae pollen concentrations collected by the VST and the passive traps

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.549[a]	0.301	0.161	32.7533

[a]Predictors: (Constant), Passive trap - Brassicaceae pollen

Table 3.11: ANOVA[b] of Brassicaceae pollen concentrations collected by the VST and the passive traps

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2310.216	1	2310.216	2.153	.202[a]
	Residual	5363.886	5	1072.777		
	Total	7674.102	6			

[a]Predictors: (Constant), Passive trap - Brassicaceae pollen; [b]Dependent Variable: VST - Brassicaceae pollen

Table 3.12: Coefficients[a] of the linear regression of Brassicaceae pollen concentrations collected by the VST and passive traps

Model		Unstandardised Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	24.484	13.651		1.794	0.133
	Passive trap – Brassicaceae pollen	0.303	0.206	0.549	1.467	0.202

[a]Dependent Variable: VST - Brassicaceae pollen

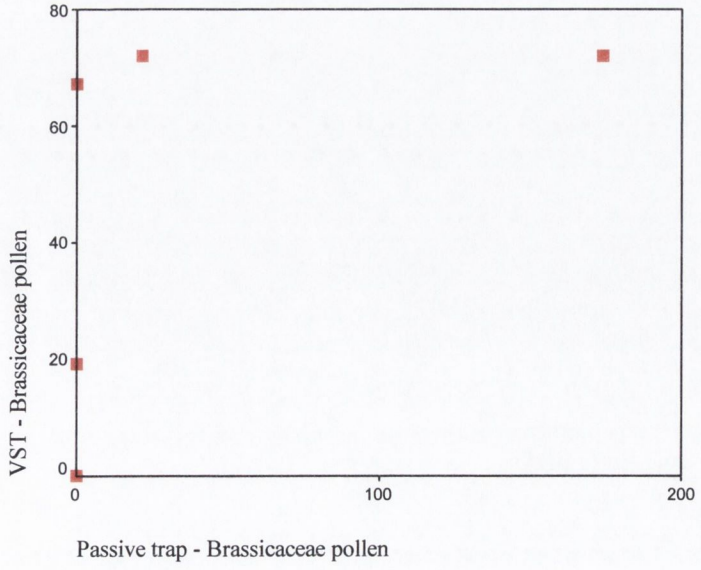

Figure 3.27: Scatterplot of Brassicaceae pollen collected by the VST and passive traps

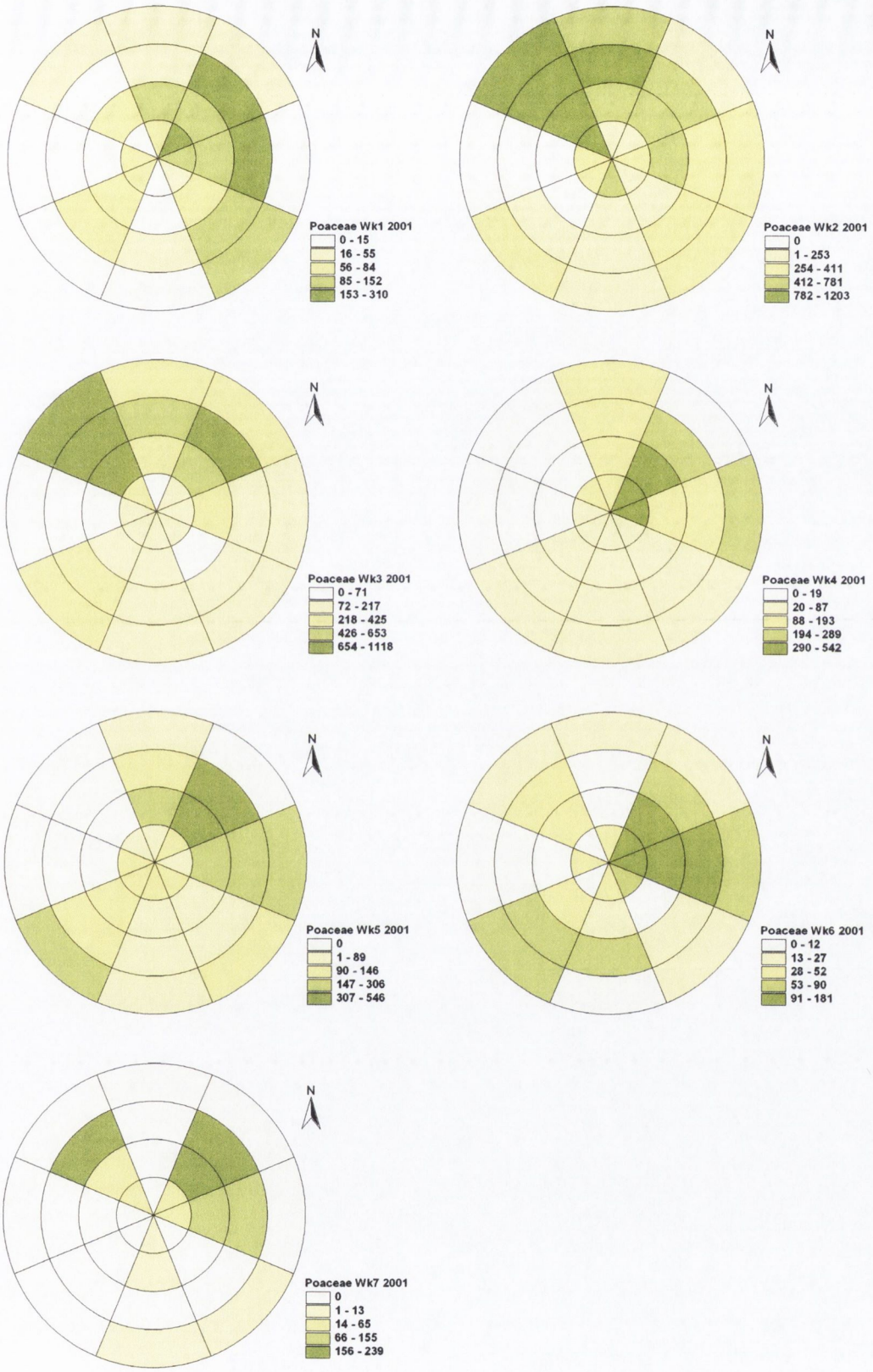

Figure 3.28: Mean concentration of Poaceae pollen during 2001 (values in pollen grains/m^3). Rings indicating traps at 0m, 50m, 100m and 200m. All concentrations were adjusted according to the factors discussed in Section 3.3.5

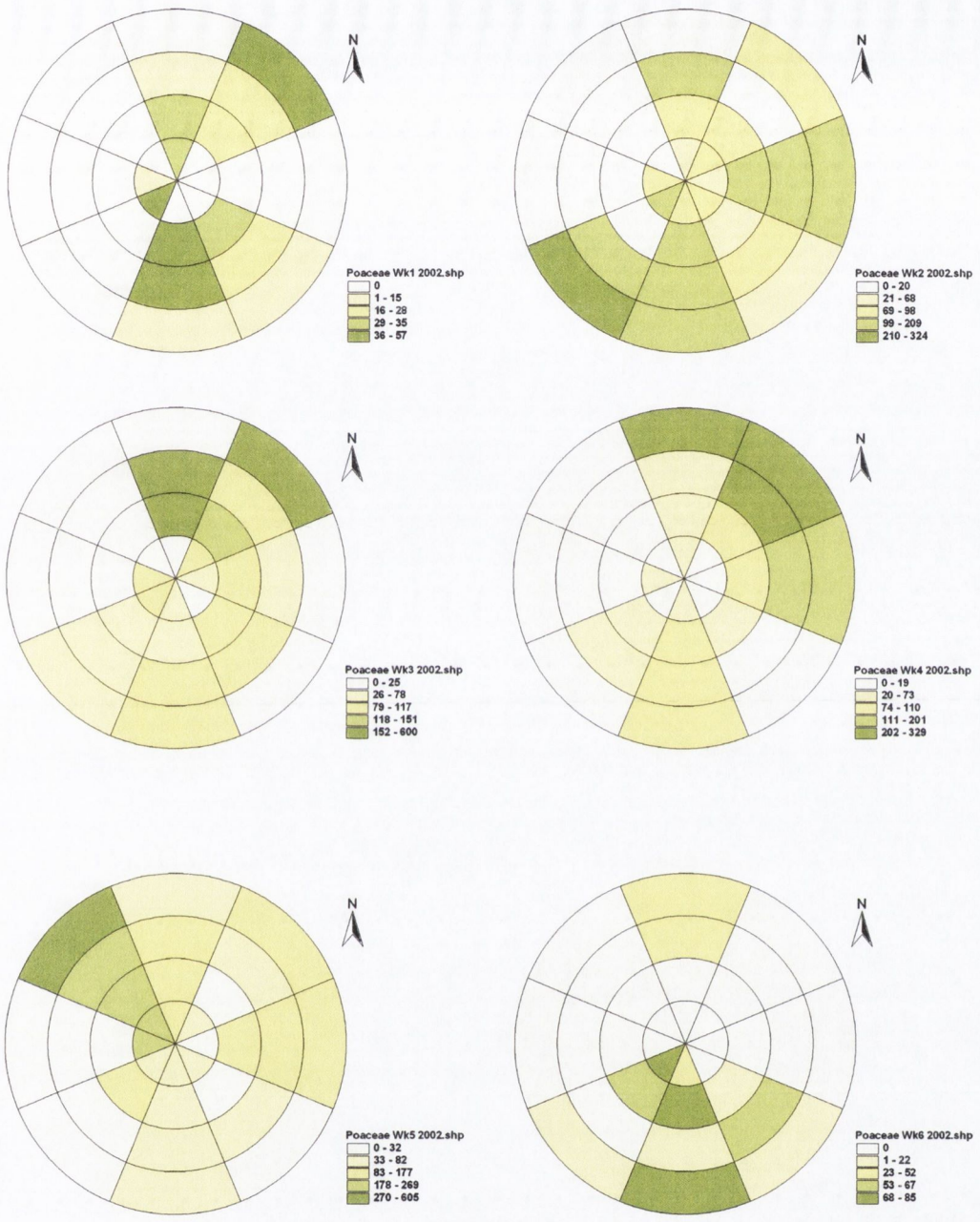

Figure 3.29: Mean concentration of Poaceae pollen during 2002 (values in pollen grains/m^3). Rings indicating traps at 0m, 12.5m, 25m and 50m. All concentrations were adjusted according to the factors discussed in Section 3.3.5

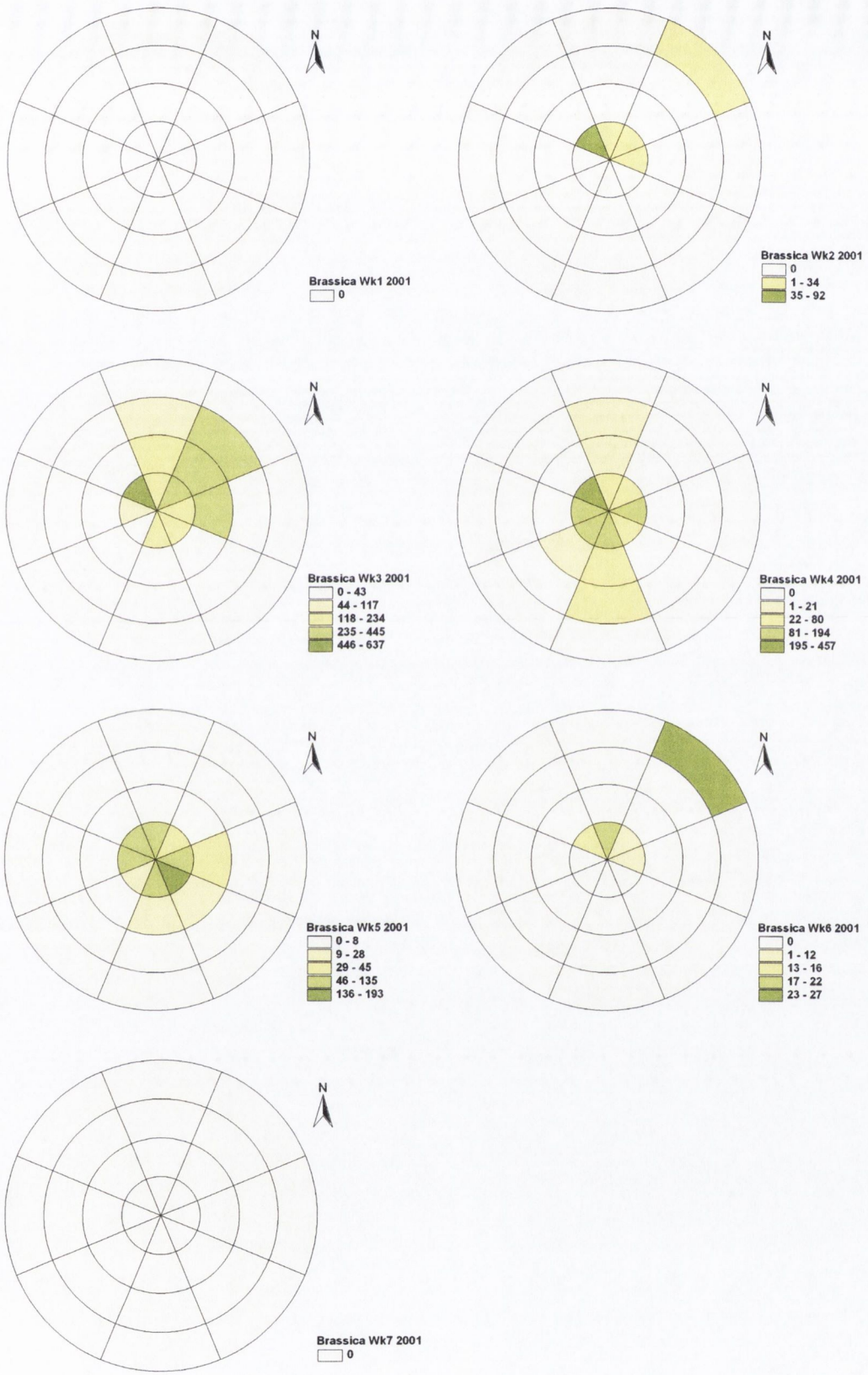

Figure 3.30: Mean concentration of Brassicaceae pollen during 2001 (values in pollen grains/m^3). Rings indicating traps at 0m, 50m, 100m and 200m. All concentrations were adjusted according to the factors discussed in Section 3.3.5

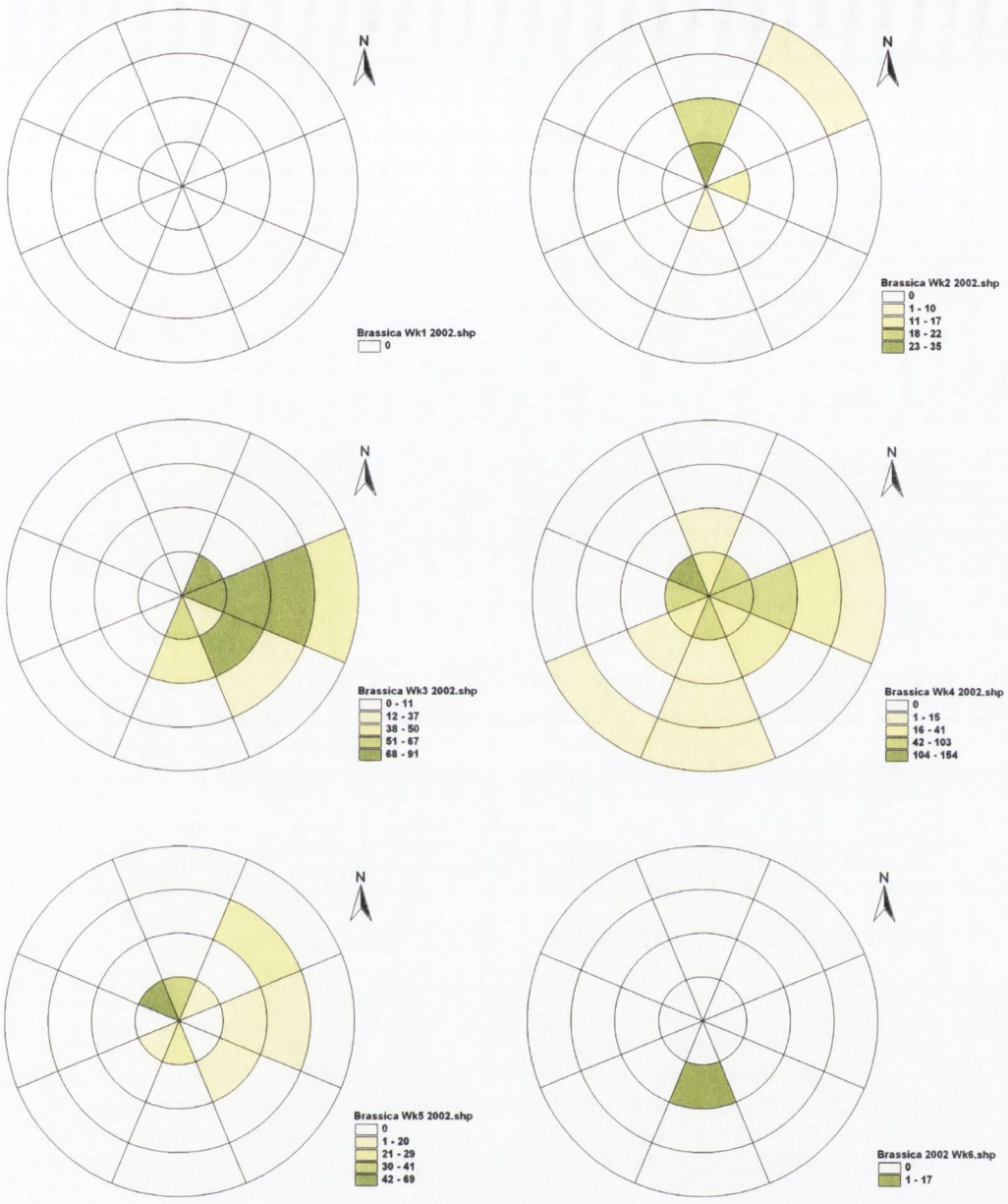

Figure 3.31: Mean concentration of Brassicaceae pollen during 2002 (values in pollen grains/m^3). Rings indicating traps at 0m, 12.5m, 25m and 50m. All concentrations were adjusted according to the factors discussed in Section 3.3.5

All pollen concentration data from the passive traps (Section 3.3.3) were multiplied by a factor of eight to account for the lower efficiency of the passive trap in pollen collection (Section 3.3.5). Pollen concentrations were also modified so that parameters including rainfall hours, night-time hours and a combination of the two

were incorporated, however following statistical analysis the results obtained were not deemed significantly different to those displayed above and so are not shown.

3.3.9 Molecular analysis

PCR analysis for the *trnL* intron using primers c and d (Taberlet *et al.*, 1991), was performed on a selection of samples of DNA extracted from the passive trap's mesh and weak amplification was obtained (Figure 3.32). Samples were amplified using different quantities of template DNA (3µl and 5µl; and 37 cycles) to test amplification. Multiple bands with length variation are evident in the samples, indicating either the presence of multiple genera or multiple species.

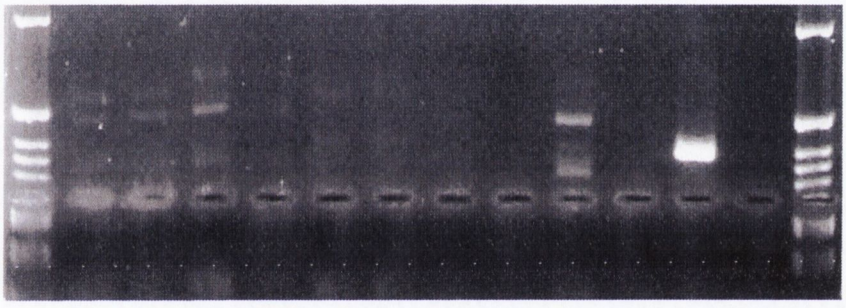

Figure 3.32: Gel of *trnL* intron lanes 1 and 14 are 1kb ladders. Lane 2 and 7 are week 2 trap 19, 3 and 8 are week 4 0m SE, 4 and 9 are week 4 100m S, 5 and 10 are week 4 50m SW. Lane 12 contains a *Sinapis arvensis* positive control, and lane 13 contains a negative control. Weak bands are visible in lanes 2, 3, 4, 5, 6, 7 and 10

3.4 Discussion

3.4.1 Passive trap design

An effective passive trap was successfully developed and tested over two seasons. The trap was based on the Cour trap (Cour, 1974) but required a number of modifications, as the original silicone based adhesives would interfere with subsequent DNA extraction of the pollen collected. The discovery of a suitable trapping surface and adhesive came in the form of muslin and glycerol. A method was then devised for the removal of pollen from the muslin and glycerol to permit DNA extraction. In addition, as glycerol is water-soluble, a protective device was required to prevent dissolution of samples by rain. The muslin was kept dry by a barrel and rain hood made from PVC soil pipes (Wavin®, section 3.2.4).

3.4.2 Volumetric spore trap results

In 2001, the VST results indicated that there was 65 times more Poaceae pollen in the air than Brassicaceae pollen. This large difference may be attributable to the nature of each type of pollen grain. Brassicaceae grains are adapted for insect dispersal, are sticky and have a heavy sculpted surface, while Poaceae grains are designed for airborne dispersal and are smooth, light and not sticky. In addition, Poaceae pollen originated from a widespread diffuse source i.e. there are a number of Poaceae sources all over the Oak Park site, where as Brassicaceae pollen originated at a point source i.e. the test crop. It is therefore expected that a greater concentration of Poaceae pollen would be present in the atmosphere and sampled by the VST.

Data from 2002 are not presented, as the VST was positioned too far away from the *Brassica napus* cv. Marinka crop for pollen collection. However, this demonstrated that even with the assistance of the prevailing wind no airborne pollen was detected at 800m NE for the 2002 crop.

3.4.3 Comparison of the volumetric spore trap and passive traps

The use of both the VST and the passive traps in 2001 was advantageous in that the passive trap could be compared to and calibrated against an existing accepted trap. Both the volumetric spore trap and the passive traps confirmed the hypothesis that, a greater concentration of Poaceae pollen was likely to be present in

the atmosphere, as much greater concentrations of Poaceae pollen than Brassicaceae pollen were detected (Figure 3.24, p121).

Once the passive trap data had been converted from flux to concentration results were compared with the VST. The VST was eight times more efficient at Poaceae pollen collection than the passive trap. This disparity could be attributed firstly, to the active suction mechanism of the VST making it a more efficient pollen trap. Secondly, the rain hood, the barrel or the mesh of the passive trap may have caused the airflow through the passive trap to experience interference i.e. decrease in windspeed. This decrease in windspeed may have resulted in deposition of pollen on the barrel or rain hood and consequently decreased deposition of pollen grains on the mesh.

Analyses of the relationship between Brassicaceae pollen collection by the VST and passive traps were inconclusive. The numerical relationship for Poaceae pollen (eight times) was used to configure the Brassicaceae pollen in terms of concentration, despite the differences in Poaceae and Brassicaceae pollen noted above. In addition, as all other Brassicaceae sources were eliminated from the site, this analysis assumes that the *Brassica napus* cv. Marinka crop is the only source of Brassicaceae pollen. Furthermore, Figures 3.30 to 3.31 (p127 and 128) produced in this way are sufficiently similar to the raw data Figures 3.18 to 3.19 (p112 and 113). to be acceptable. That is, both the 2001 and 2002 seasons, high or low pollen collection is highlighted by the same traps or sectors.

3.4.4 Passive trap data in terms of percentage of total pollen collected, concentration and meteorological conditions

As Poaceae pollen collection was carried out to enable quantification of Brassicaceae pollen and originated from a number of sources i.e. has a diffuse dispersal pattern the Poaceae pollen results will not be discussed further.

In 2001, weeks one and seven yielded no Brassicaceae pollen, despite favourable meteorological conditions (low rainfall, light to medium windspeeds, mixed to high irradiance, and mean temperatures of 10-13°C), as the crop has either not started or finished flowering. During week two, although conditions were not generally favourable, 10% of the total pollen collected for that week, corresponding to 80 Brassicaceae pollen grains per m^3 collected in any given hour, was recorded at 200m NE, while a further 10% (176 grains/m^3) was detected at 50m N. This

dispersal is likely to have occurred on days during the week where no rainfall was combined with high irradiance and a medium windspeed. Favourable meteorological conditions during week three (low rainfall, medium windspeed, high irradiance and a mean temperature of 13˚C), combined with the crop reaching peak flowering assisted greater Brassicaceae pollen movement (13% (725 grains/m^3), 13% (725 grains/m^3) and 9% (396 grains/m^3) of the week's total pollen were collected at 50m, 100m and 200m E of the crop, while 13% (705 grains/m^3) and 4% (201 grains/m^3) were detected 50 and 100m SE of the crop; and 7% (361 grains/m^3) and 5% (90 grains/m^3) were collected at 50m and 200m S of the crop). During week four despite mixed meteorological conditions (light winds and a mean temperature of 16˚C; with high irradiance and low rainfall for the beginning of the week; and high rainfall and low temperatures towards the end of the week) pollen reached 200m (2% (117 grains/m^3) was detected at 50m N, 9% (689 grains/m^3), 11% (230 grains/m^3) and 4% (116 grains/m^3) were detected in traps at 50m, 100m and 200m E and 5% (290 grains/m^3) was detected in traps at 50m SE). In week five, the combination of lighter winds and the winding down of flowering, resulted in lower levels being detected (4% (330, 203 and 225 grains/m^3) was detected at 50m E, SE and S respectively). During week six, although conditions were not generally favourable and the crop was at the end of flowering pollen still travelled 50m S (100%, 136 grains/m^3) of the crop.

In 2002, week one conditions were conducive to pollen dispersal (medium rainfall, high windspeed, high irradiance, and a mean temperature of 12˚C), however no Brassicaceae pollen was detected, as the crop had not started flowering yet. Week two conditions were favourable for most of the week (low rainfall, high windspeed, high irradiance and a mean temperature of 13˚C) and although the crop had only just begun flowering, 25% of the total Brassicaceae pollen collected for that week, (corresponding to 273 and 219 Brassicaceae pollen grains per m^3 collected in any given hour) reached 200m. Favourable meteorological conditions during week three (low rainfall, medium windspeed, high irradiance and a mean temperature of 12˚C) combined with the crop reaching peak flowering assisted greater Brassicaceae pollen movement (3% (1852 grains/m^3) and 2% (926 grains/m^3) of the week's total pollen were collected at 50m and 100m N, 6% (2373 grains/m^3) and 8% (3560 grains/m^3) at 50m and 100m NE, 10% (2860 grains/m^3) and 1% (329 grains/m^3) were detected at 50m and 100m E of the crop, while 1%

(345 grains/m^3) was detected at 50m S of the crop). Although Brassicaceae pollen was not detected at 200m, it is evident that greater quantities of airborne pollen were present. Week four had mixed meteorological conditions (medium windspeed and a mean temperature of 12°C with high and low rainfall coinciding with high and low irradiance on different days), which corresponded with Brassicaceae pollen not travelling beyond 100m (6% (434 grains/m^3) and 7% (521 grains/m^3) were detected at 50m and 100m S of the crop). During week five, despite favourable conditions (low rainfall, medium winds, high irradiance and a mean temperature of 13°C), the decreased flowering of the crop meant lower concentrations of airborne pollen were detected (4% (330, 203 and 225 grains/m^3) at 50m E, SE and S respectively). Reasonably good dispersal conditions during week six (low rainfall, medium windspeed, high irradiance and a mean temperature of 14°C) allowed 25% (219 grains/m^3) to be distributed to 200m NE. This dispersal is likely to have occurred on days during the week where no rainfall.

In 2001 and 2002, the greatest Brassicaceae pollen movement in terms of percentage of total pollen collected occurred in week three, which correlates with the peak flowering period of the *Brassica napus* cv. Marinka crop (Figures 3.9, 3.18 and 3.19; p104, 112 and 113). The greatest distance at which pollen was detected was 200m. As expected the passive trap data (with the exception of week four in 2001) indicates that during both the 2001 and 2002 flowering seasons the distribution of Brassicaceae pollen follows wind direction and windspeed as shown in Figures 3.28 and 3.29 (p125 and 126), while the distribution of Poaceae pollen is relatively random. Therefore, the pattern exhibited by Brassicaceae pollen is confirming that it is originating from a point source, while the scattered dispersal of Poaceae pollen demonstrates that it is originating from multiple diffuse sources (Figures 3.30 and 3.31, p127 and 128).

Seasonal trend of pollen concentrations exhibited in Poaceae and Brassicaceae pollen indicated that flowering was synchronous (Figure 3.22, p117). Although this trend had not been anticipated, it makes sense as the Teagasc, Oak Park, Carlow site is an agricultural research centre and the flowering of many of the spring barley and wheat crops coincided with that of *B. napus* cv. Marinka (Figures 3.6 and 3.7, p96 and 97).

3.4.5 Molecular analysis

Bands obtained from PCR analysis of the plastid *trnL* intron (using primers c and d) indicate that the methods used for pollen removal from the mesh and kit DNA extraction, were successful. As the amplification obtained was weak i.e. bands are not present in all samples tested, further refinement of the technique may be necessary. Nevertheless amplification of SSR markers may prove more successful as the regions are shorter and may therefore amplify more easily than this plastid intron region. In addition, the length variation detected in the amplified intron of different extractions suggests that a range of species may have provided pollen for these extractions and not just *Brassica*. Sequencing of these products is required for species identification.

3.5 Conclusion

The results reported here indicate that the majority of pollen was deposited very close to the source, but that pollen was also dispersed over large distances (200m) at low frequencies. In addition, it has been demonstrated that wind dispersal of Brassicaceae pollen is very dependent on the direction and speed of the wind.

The ability to extract DNA from collected pollen may be a valuable technique for analysis of deposited pollen from wind-pollinated species. This is especially valid as approvals for GM *Lolium*, wheat and barley (European Commission Joint Research Centre, 2004) may be granted in the near future requiring field study of biotic pollen dispersal.

These data are not highly relevant in terms of buffer zones as honeybees are the primary pollinator of Brassicaceae and as insect dispersal will be much greater than wind dispersal they form the basis of proposed buffer zone requirements. In spite of this, this system can be applied to other crops that are wind pollinated. Moreover, from this analysis when considering appropriate sizes for buffer zones of GM crops windspeed and direction must be taken into account. That is, where a site is exposed, greater buffer zones would be needed to the north, northeast and east of the crop, according to the prevailing wind direction. This method therefore has potential for use in risk assessment studies for the analysis of pollen movement from GM wind dispersed crops.

Gene flow from crop sized areas have been studied using non-GM crops (Timmons *et al.*, 1996; Bilsborrow *et al.*, 1998) and GM crops (Champolivier *et al.*,

1999; Downey, 1999; Simpson *et al.*, 1999). The significance of wind pollination of *Brassica napus* pollen was examined using volumetric spore traps (VSTs) in a study by Timmons *et al.* (1995). They reported *Brassica* pollen levels at 360m as 10-11% of those collected at the field margin (0m). The results reported here cannot be compared with those by Timmons *et al.* (1995) as only one VST was used and as it was located at 100m, it is unknown how much Brassicaceae pollen would have been collected at the field margin. At any rate, the VST results may not be comparable, due to the size of the source crop. That is, Timmons *et al.* (1995) showed that pollen concentrations around agricultural scale fields are very much higher and have dispersal characteristics dissimilar to those of experimental scale plots.

The significance of wind dispersed Brassicaceae pollen is questionable in terms of fertilisation and gene flow. It has been reported that oilseed rape pollen remains viable for between 24 hours and one week depending on conditions (Mesquida and Renard, 1982), however in this study as passive trap samples were collected on a weekly basis it is unknown how many pollen grains were viable at the time of deposition. In addition, it has been questioned whether the architecture of *Brassica napus* is suitable to facilitate the reception of airborne pollen (Cresswell *et al.*, Unpublished) as a study by Cresswell *et al.* (Unpublished) showed wind pollination is unlikely to be an effective means of cross-pollination.

A large number of field trials have been carried out for oilseed rape examining pollen and gene flow with apparently contradictory results. Nevertheless the potential for the formation of hybrids exists and therefore it is sensible to focus ecological risk assessment programs on the ecological consequences of the transgene in question (Wilkinson, 2003).

Chapter 4
Biotic pollen dispersal

4.1 Introduction

4.1.1 Bee pollination and foraging distance

Insects, particularly honeybees (*Apis mellifera*) and bumblebees (*Bombus* species) are believed to play a major role in the transfer of pollen between plants over long distances and are considered to be important pollinators of oilseed rape (Ramsay *et al.*, 1999). Studies of foraging bumblebees have used mark-recapture or tracking methods have shown that individual bees tend to be area-constant i.e. they will forage the same area repeatedly for hours or even days (Heinrich, 1976; Dramstad, 1996; Thompson, 1996; Saville *et al.*, 1997; Osborne *et al.*, 1999; Walther-Hellwig and Frankl, 2000; Osborne and Williams, 2001).

Determination of the foraging distances travelled by honeybees and bumblebees has been the goal of a number of studies. In Sheffield, UK, long range foraging of honeybees was examined by decoding 'waggle-dances' (Beekman and Ratnieks, 2000). The median distance foraged was found to be 6.1km, while the mean distance was 5.5km. Only 10% of the honeybees were found to forage within 0.5km of the hive, while 50% travelled more than 6km, 25% more than 7.5km and 10% more than 9.5%. In another study, harmonic radar was used to track individual bumblebees and thus examine foraging distances (Osborne *et al.*, 1999). These studies found that bumblebees regularly forage over 200m (70m-631m) even when plentiful food was available from an OSR source nearby the hive. These results conflict with earlier theories that bees are subject to the pressures of energy economics, and do not forage at great distances from the nest when abundant nectar and pollen sources are close by e.g. agricultural settings (Seeley, 1985).

Low levels of in-hive pollen transfer may lead to cross-pollination (Vaissiére *et al.*, 1994), therefore where a hive is located 2km from the crop some pollen transfer and fertilisation up to 4km can be expected. Bees in a colony in Scotland have been reported to have flown to a crop 5km away, so theoretically there is potential for pollen to be transferred to distances of 10km by the mixing of bees foraging in different directions from the same hive (Ramsay *et al.*, 1999).

The transfer of pollens from bee to bee within the hive means that there is potential for pollen from a transgenic plant to be deposited on a conventional one, thus spreading the transgene. In the case of oilseed rape, this causes concern, as the recipient plant could be a weedy relative or a non-GM crop of a grower trying to maintain non-GM status. Many studies have been carried out on conventional crops to help identify isolation distances to preserve crop purity. Examination of pollen carryover using fluorescent dyes has revealed that pollen deposition by bees on flowers visited after a donor plant quickly declines i.e. the majority is deposited on the next few flowers, however a small amount is carried over and may be deposited much later in a foraging trip (Cresswell *et al.*, 1995; Poppy, 1998). It has also been noted that where several oilseed rape crops exist it is unlikely that honeybees will go further than the first or second field to forage. In spite of this a small proportion of bees may travel long distances to feed even when comparable forage exists nearer to the hive (Osborne *et al.*, 2001).

Although a honeybee colony may collect nectar and pollen from many species and potential foraging flights can be up to 10km (Ramsay *et al.*, 1999), several factors limit the potential for pollen movement. Individual honeybee foragers tend to collect nectar and pollen from a single plant species during a single visit (Hodges, 1974). However, observations of honeybee colonies have also shown that honeybees switch from one forage-type to another and that honeybees may carry many viable pollen grains when they emerge from a hive (Ramsay *et al.*, 1999). Second, given abundant flowers, such as in a cultivated field, individual honeybee foragers tend to collect nectar and pollen from flowers in the same or immediately adjacent plants (Cresswell *et al.*, 1995). Third, honeybees are very sensitive to barometric pressure, and decrease foraging distances in response to impending adverse weather (Hodges, 1974).

4.1.2 Honeybee pollination

The honeybee (*Apis mellifera*) is considered by many to be one of the most important general pollinators (Percival, 1947), but is dependent on pollen for its survival. While nectar provides the carbohydrate portion of a bee's diet, pollen almost exclusively supplies the protein, fat, mineral and vitamin components, therefore adequate pollen stores are essential in the hive at all times (Hodges, 1974).

During a single season, it has been estimated that a weight of 25-40kg of pollen is required by a single colony, therefore large quantities of pollen are carried into the hive (Butler, 1976).

Figure 4.1: A honeybee scrambling along a flower (Durham's Bee Farm, 2000)

Foraging honeybees (Figure 4.1) have evolved to maximise their harvesting of pollen and nectar. The method used for collection of pollen is dependent on the flower structure of the plant concerned. When collecting pollen, worker bees will initially collect nectar from flowers where both pollen and nectar are present (e.g. *Brassica*) (Durham's Bee Farm, 2000). In this case, the bees will arrive with their honey stomach empty and will gather nectar to moisten mix the dry pollen into a paste-like substance, which is suitable for packing pollen loads. Alternatively, when pollen is in short supply honeybees will visit wind-pollinated flowers such as grasses, and will bring honey with them from the hive. The mixing process makes the colour of pollen loads quite different from that seen on the anthers of the flower (Hodges, 1974) and therefore the colour of pollen loads is a useful character in pollen species identification. The colour of a freshly collected oilseed rape honeybee load is yellow (Sawyer, 1981).

4.1.3 Pollen traps

When pollen is collected for human consumption or for research, pollen traps are placed on the hive. These traps remove the pollen granules from the bee's pollen basket and allow it to fall down into a tray for removal by the beekeeper. In a typical trap the honeybee has to crawl up through a hole of ~7mm diameter to

enter the hive and in the process of doing so, the pollen is scrapped harmlessly from the honeybee's leg and it drops down into the tray for collection later. The contents of the tray are then sifted to remove any foreign matter that is frequently found in the hive, i.e. wings and legs. The pollen is then either frozen or rapidly dried. The latter treatment is desirable for DNA work to preserve the DNA and silica gel is an effective desiccation agent for this purpose.

4.1.4 Acetolysis of pollen pellets

As noted in section 3.1.7, fresh pollen is difficult to identify as surface sculpturing is obscured by the intine and so a chemical treatment known as aceteolysis, which removes cellulose by acid-hydrolysis is used. This serves to remove the intine and reveal previously hidden surface patterns. This method is very effectively and is used by palaeopalynologists to separate pollen grains from sediments.

4.1.5 Aims

Since honeybees are the primary pollinators of oilseed rape and are believed to play a major role in the transfer of pollen over long distances, the target of this analysis is to examine pollen transfer by bees along various distances from a *Brassica napus* crop. The distance travelled by OSR pollen via biotic dispersal was elucidated by carrying out the following:

- Measuring biotic pollen dispersal by placing traps in beehives along a transect at distances of 100m, 200m, 400m, 800m and 1,600m from a *Brassica napus* cv. Marinka crop.
- Collecting, weighing and preserving (in silica gel) pollen pellets on a weekly basis.
- Identifying pollen pellets using colour coding and microscopic analysis of acetolysed of pellets.
- Characterising pollen pellets using molecular techniques specifically SSR markers.

4.2 Methods

4.2.1 Collection of pollen pellets

Beehives (2 x 5 hives) were positioned along a transect at 100m, 200m, 400m, 800m and 1,600m from the *Brassica napus* cv. Marinka crop during the 2001 and 2002 seasons. The hives were fitted with a comb, which serves to detach the pollen pellets from the legs of the bees as they enter the hive following foraging (Figs. 4.2A and 4.2B). A collection box (Fig. 4.2C) was placed below the comb and was emptied on a weekly basis. The contents were dried using silica gel and stored for later analysis.

Figure 4.2: Pollen pellet collection system. (A) Beehive no. 5 located at 1,600m from OSR crop. Arrows indicates position of comb. (B) Upper frames of hive removed to make comb visible. (C) Collection box with pollen pellets

4.2.2 Analysis of pollen pellets

A reference collection of pollen pellets was created prior to examination of the selected subsamples. This was done by spreading a representative sample of pellets on a sheet of white paper and separating according to colour. Microscopic

analysis was then performed on a number of pellets to check whether various shades represented pollen from single (monospecific) or multiple species.

In 2001, 35 samples were collected i.e. 5 hives for seven weeks and in 2002, 30 samples were collected i.e. 5 hives for six weeks. 100 pollen pellets were selected at random from each sample. Therefore, a sub-sample of 6,500 pellets was taken. Firstly, pollen pellets were colour coded (Figure 4.3, Table 4.1). The structural design of the surface of pollen is usually used in identification however in this case identity was obscured by the presence of the intine. Therefore as fresh Brassicaceae pollen is yellow in colour, a selection of yellow, golden, light-brown, dark-brown and orange pellets were acetolysed to examine colour variation of silica gel dried Brassicaceae pellets, to assist in the separation of Brassicaceae pollen from all other types. SSR analysis was then performed on 5% of the Brassicaceae pellets using diagnostic markers for *Brassica napus* cv. Marinka to confirm its presence (section 2.4.4).

Figure 4.3: Pollen pellets separated according to colour

4.2.3 Acetolysis of reference pollen pellets

Identification of bee pollen was aided by an initial re-hydrating potassium hydroxide (KOH) wash of the silica gel desiccated pellets, followed by acetolysis. Many of the reagents used in acetolysis are corrosive and react vigorously with

water especially concentrated sulphuric acid and acetic anhydride, therefore all materials with which they come into contact with should be dry.

5ml 10% KOH were added to a test tube containing the pollen pellet to release and remove humic acids. A glass rod was used to break up the pellet. The test tube was heated in a boiling water bath for 5mins, centrifuged at 4,025rcf (4 x *g*) for 5mins. The resultant supernatant was poured off and discarded.

5ml SUW was added and stirred well. The mixture was centrifuged at 4,025rcf (4 x *g*) for 5mins and the supernatant poured off. In a fume hood, 10ml of glacial acetic acid was added to dehydrate the residue. The mixture was stirred, centrifuged at 4,025rcf (4 x *g*) for 5mins and the supernatant poured off. 10ml of acetolysis mixture (acetic anhydride: concentrated H_2SO_4, 9:1) was added carefully, as the mixture is explosive in water. The test tube was heated in a boiling water bath for 2mins serving to break down polysaccharides including cellulose, and was then centrifuged at 4,025rcf (4 x *g*) for 5mins. The supernatant was poured into an acetolysis mixture waste container. 5ml glacial acetic acid was added to dissolve any cellulose acetate produced during acetolysis. The mixture was stirred, centrifuged and the supernatant poured off. 5ml tertiary butyl alcohol (TBA) was added, stirred and the supernatant poured off. The residue was transferred to a glass vial using a Pasteur pipette and 1-2 drops of silicone oil added. The vial was then placed in the oven at 75˚C until all the TBA had evaporated (approx. 6 hrs).

A drop of the acetolysed pollen in silicone oil was placed on a microscope slide and spread as a thin film over an area the size of a cover slip. A cover slip was placed on top and sealed using clear nail varnish. Silicone oil was used as the mounting medium to allow pollen grains to rotate or turn freely once pressure was applied to the cover slip, therefore enabling the view of both equatorial and polar orientations of the pollen grain. The slides were examined using a microscope (Lecia, MPS32) at x400 magnification. Identification was aided by the use of TCD reference slides and reference books (Moore *et al.*, 1991; Reille, 1992).

4.2.4 Pollen pellet kit DNA extraction

DNA was extracted using a REDextract plant DNA extraction kit (Sigma Aldrich). Approximately, one eighth of a pellet was mixed with 100µl extraction solution by vortexing and heated to 95°C for 10mins. 100µl dilution solution was

added and the solution was vortexed again. Samples were stored at –20°C until use.

4.2.5 PCR amplification of DNA from pollen pellets

PCR amplification and nuclear and plastid SSR marker analysis of pollen pellets was carried out by adding 2µl kit extracted DNA, 2µl dilution solution, 10µl REDextract PCR mix and 3µl forward and reverse primer to a 200µl microfuge tube. PCR conditions are outlined in Table 2.3 (page 10) and nuclear SSR primer set 12A, and plastid SSR primer sets: Region A2 and Region B2. Amplified SSR reactions were treated the same way as nuclear SSR reactions in section 2.2.5 (page 12). SSR marker analysis was carried out using the methods outlined in section 2.2.8 (page 16 and 17).

4.3 Results

4.3.1 Results

The mean dry weight of pollen pellets collected from each hive is presented in Figures 4.4 and 4.5. A regression line has been added to each to predict the distance required to isolate a *B. napus* crop according to biotic pollen dispersal.

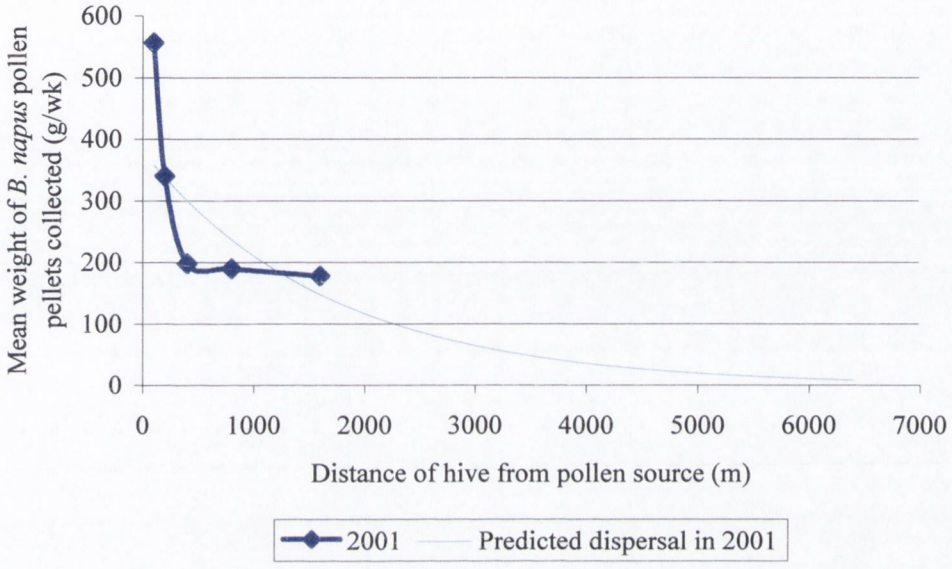

Figure 4.4: Distance travelled by insect dispersed pollen in 2001

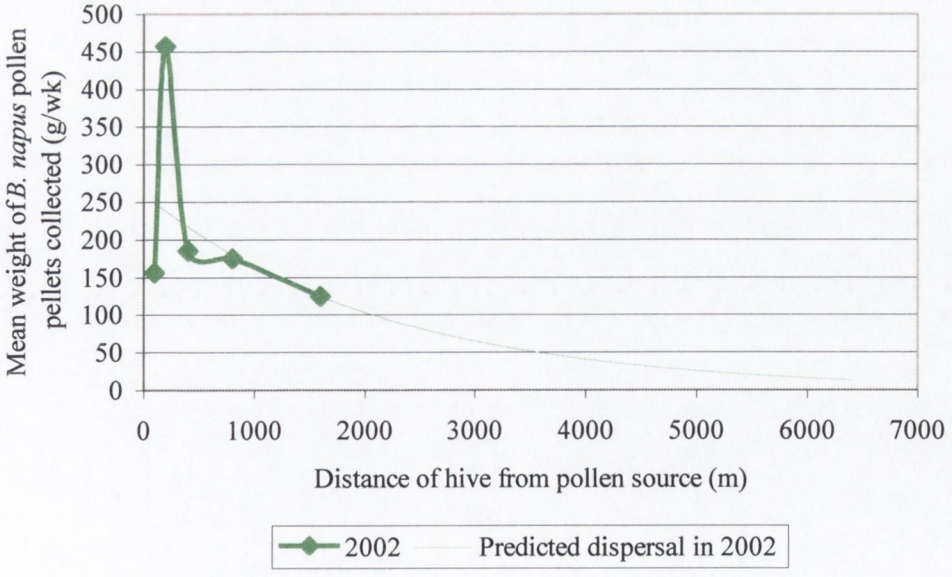

Figure 4.5: Distance travelled by insect dispersed pollen in 2002

The results of colour coding bee pollen pellets are shown in Table 4.1.

Table 4.1: Separation of pollen pellets samples according to colour (number of pellets in each sample of 100)

Sample	Year	Week	Hive	Pale green	Dark pale green	Yellow Green tinge	Greenish yellow	Bright orange	Dull orange/ rust	Brown with yellowish tinge	Legum-inous species	Dark brown	Dark Brown with yellow tinge	Black With yollow tinge	Black with green tinge	Plum	Black	Brassica-ceae
1	2001	1	100m	0	0	0	0	0	0	5	0	0	0	0	0	0	0	95
2	2001	1	200m	0	0	0	0	1	2	0	0	0	0	0	13	0	0	84
3	2001	1	400m	0	0	0	0	17	0	15	0	0	0	0	13	0	0	55
4	2001	1	800m	0	0	0	0	0	0	3	0	0	0	0	0	0	0	97
5	2001	1	1,600m	0	0	0	0	0	0	26	0	0	0	0	10	2	2	61
6	2001	2	100m	1	0	0	0	0	0	33	0	0	20	0	0	0	0	46
7	2001	2	200m	0	0	0	0	0	4	28	0	3	0	0	0	0	0	65
8	2001	2	400m	0	0	0	0	0	0	0	0	9	0	0	0	0	0	91
9	2001	2	800m	3	0	0	0	0	3	23	0	0	0	0	0	0	0	72
10	2001	2	1,600m	0	0	0	0	1	3	50	0	2	0	0	0	0	0	45
11	2001	3	100m	0	0	2	25	0	0	10	0	3	0	0	0	0	0	60
12	2001	3	200m	16	0	0	0	0	0	0	0	0	0	0	0	0	1	83
13	2001	3	400m	3	0	0	0	0	0	33	0	0	0	0	0	0	1	63
14	2001	3	800m	18	0	0	0	1	1	39	0	0	0	0	0	1	1	40
15	2001	3	1,600m	0	0	0	0	0	0	10	10	42	0	0	7	0	0	31
16	2001	4	100m	16	0	0	0	0	0	0	0	29	0	0	0	0	2	53
17	2001	4	200m	5	0	0	0	0	0	0	0	0	18	0	0	2	1	74
18	2001	4	400m	2	0	0	0	0	0	20	0	4	0	0	0	1	0	73
19	2001	4	800m	17	0	0	0	0	0	0	0	42	0	0	7	0	0	34

Table 4.1: Separation of pollen pellets samples according to colour (contd)

Sample	Year	Week	Hive	Pale green	Dark pale green	Yellow Green tinge	Green -ish yellow	Bright orange	Dull orange/ rust	Brown with yellowi sh tinge	Legum -inous species	Dark brown	Dark Brown with yellow tinge	Black With yollow tinge	Black with green tinge	Plum	Black	Brassic -aceae
20	2001	4	1,600m	0	0	0	0	0	1	44	0	0	0	0	20	0	0	35
21	2001	5	100m	17	0	0	0	0	0	21	0	0	0	0	5	0	0	57
22	2001	5	200m	0	5	0	0	0	0	40	0	0	0	0	0	1	4	50
23	2001	5	400m	12	0	0	0	0	0	32	0	0	0	0	9	5	1	41
24	2001	5	800m	11	0	0	0	1	0	31	0	0	0	0	18	4	3	33
25	2001	5	1,600m	0	0	0	0	0	0	35	0	0	0	0	3	1	9	52
36	2002	1	100m	0	0	0	0	5	0	5	0	0	0	2	4	0	0	84
37	2002	1	200m	0	0	0	0	0	0	18	0	0	0	0	0	0	0	82
38	2002	1	400m	9	0	0	5	5	0	0	0	0	7	0	0	2	0	73
39	2002	1	800m	0	0	0	0	0	0	19	0	0	0	0	0	13	0	68
40	2002	1	1,600m	0	0	0	0	0	0	0	0	0	27	0	0	0	0	73
41	2002	2	100m	1	0	0	0	0	0	30	0	0	0	0	0	0	0	69
43	2002	2	400m	0	0	0	0	0	2	0	0	0	0	0	0	0	0	98
46	2002	3	100m	0	0	0	0	0	3	0	0	22	30	0	0	0	0	44
47	2002	3	200m	7	0	0	0	0	0	0	0	0	11	0	0	0	0	82
48	2002	3	400m	1	0	0	0	0	0	27	0	0	0	0	7	0	0	65
49	2002	3	800m	0	0	0	0	0	1	25	0	0	0	0	2	0	0	72
50	2002	3	1,600m	7	0	0	0	3	0	0	0	0	0	9	11	0	0	70
51	2002	4	100m	3	0	0	4	0	19	20	0	0	0	0	4	0	0	51

Table 4.1: Separation of pollen pellets samples according to colour (contd)

Sample	Year	Week	Hive	Pale green	Dark pale green	Yellow Green tinge	Greenish yellow	Bright orange	Dull orange/ rust	Brown with yellowish tinge	Legum - inous species	Dark brown	Dark Brown with yellow tinge	Black with yollow tinge	Black with green tinge	Plum	Black	Brassica-ceae
52	**2002**	**4**	**200m**	0	0	0	0	0	0	38	0	0	4	0	0	0	0	59
53	**2002**	**4**	**400m**	12	0	0	0	0	5	0	0	0	34	0	18	0	0	31
54	**2002**	**4**	**800m**	4	0	0	15	0	0	0	0	8	21	0	3	0	0	50
55	**2002**	**4**	**1,600m**	16	0	0	0	4	0	0	0	15	5	0	0	9	10	41
56	**2002**	**5**	**100m**	12	0	0	0	0	0	0	0	15	0	0	0	0	0	74
58	**2002**	**5**	**400m**	0	0	0	0	0	0	0	0	27	0	0	0	0	0	73
59	**2002**	**5**	**800m**	0	0	0	0	0	0	0	0	34	0	0	0	0	8	58

In 2001, high levels of pollen pellets were collected at 100m, 800m and 1600m (1342g/wk, 490g/wk and 397g/wk) during week one, which rapidly declined until week four (150g/wk, 85g/wk and 127g/wk) before increasing again. Although the high levels during weeks one and two may be explained by the presence of the *C. sativus* crop this does not explain the dip seen in week four when the *B. napus* cv. Marinka crop was in the peak stages of flowering. Therefore it must be considered that the trap may have been working inefficiently.

The trend seen in 2001 at 200m, is what would have been expected for the flowering season, that is a peak in collection during weeks two (420g/wk), three (477g/wk) and four (687g/wk) (Figure 4.7). The trend in 2001 at 400m, is consistent with the 200m findings and shows a peak in collection during weeks two (250g/wk), three 411/wk) and four (289g/wk) (Figure 4.8). This is evident in at 200m in 2002, where the data show a significant decrease between weeks one (712g/wk) and two (264g/wk), however an increase at week three (291g/wk) which peaks at weeks four (458g/wk) and five (434g/wk). This is also evident in at 800m in 2002, where collection peaks during week four (256g/wk). At 1600m, high pollen levels decrease from almost 400 g/wk collected in week one to 138g/wk in week four and increase again after that. In 2002, a similar trend is seen although some missing data makes it more difficult to judge the results.

The weight of *Brassica* pollen collected each week in 2001 and 2002 at each distance were compared (Figures 4.6 to 4.10).

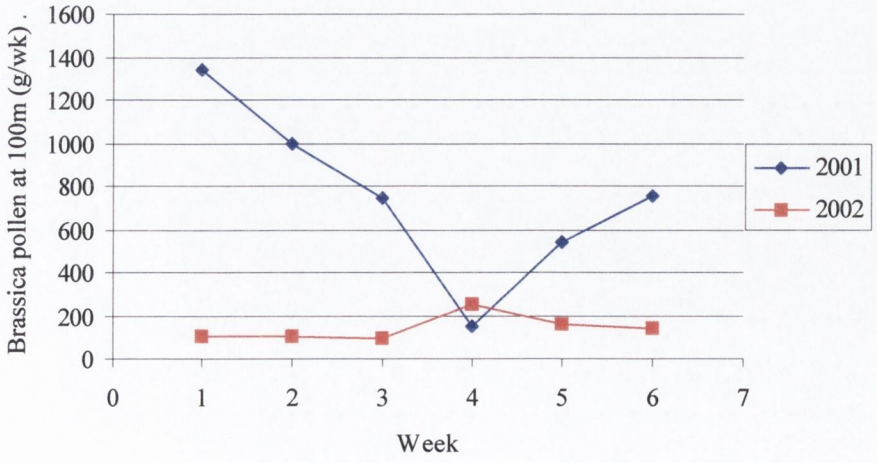

Figure 4.6: Concentration of *Brassica* pollen (g/wk) collected at 100m

The trend for the hive collections at 100m (Figure 4.6), shows a substantial decrease in pollen pellet quantities collected between week one and four, with an increase in weeks five and six. In 2002, a low but consistent level was collected with a slight increase in week four.

In 2001 at 200m, during weeks three and four a peak in collection was experienced (Figure 4.7). The 2002 data shows a decrease between weeks one, two and three, with an increase again after week five.

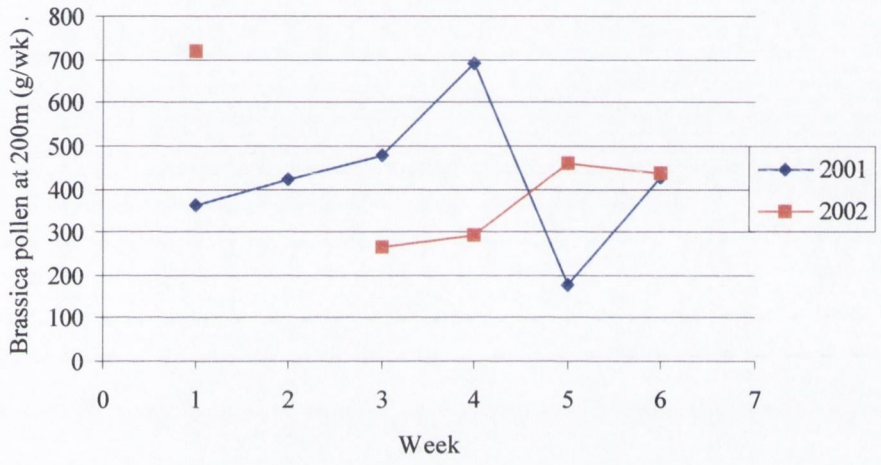

Figure 4.7: Concentration of *Brassica* pollen (g/wk) collected at 200m

The trend for 2001 at 400m, shows a peak in collection during weeks two, three and four (Figure 4.8). The 2002 data shows a decrease between weeks one and two, however a peak at week three is seen.

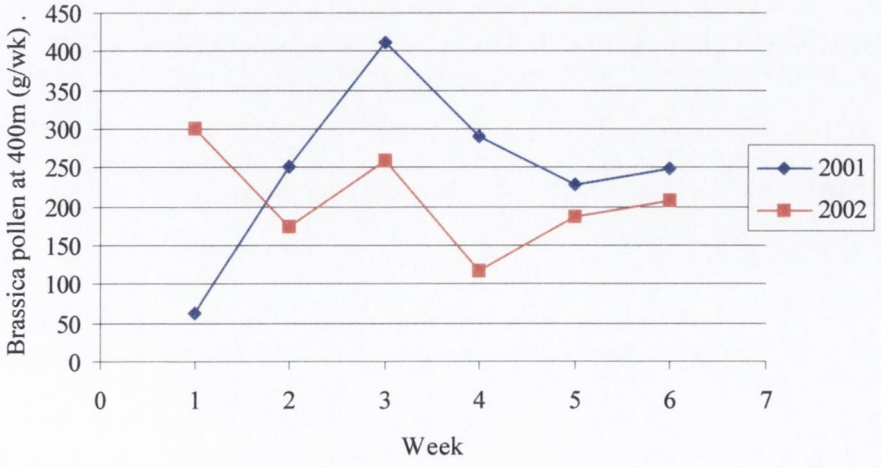

Figure 4.8: Concentration of *Brassica* pollen (g/wk) collected at 400m

In 2001, at 800m the quantity of pollen pellets collected decreased between weeks one and four and increased again until week six (Figure 4.9). In 2002, pollen pellet collections were greatest during week four.

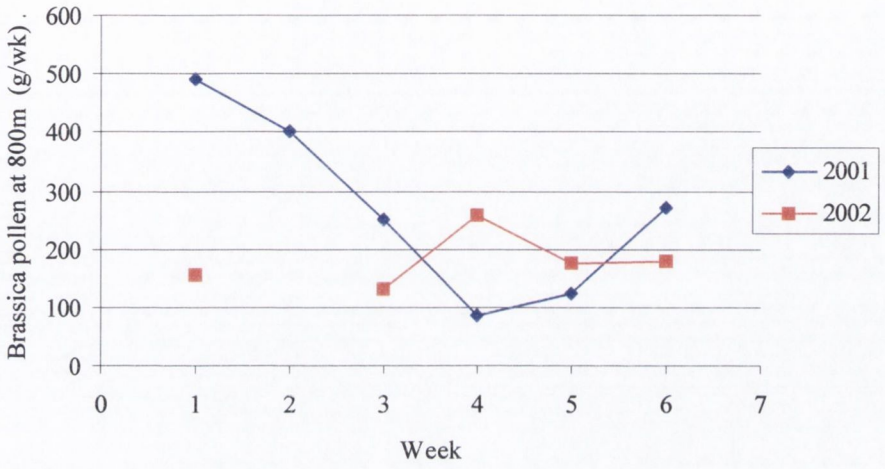

Figure 4.9: Concentration of *Brassica* pollen (g/wk) collected at 800m

In 2001, pollen pellet collection at 1,600m showed a steady decline between weeks one and four, but then increased again. In 2002, a similar decline was seen between weeks three and four.

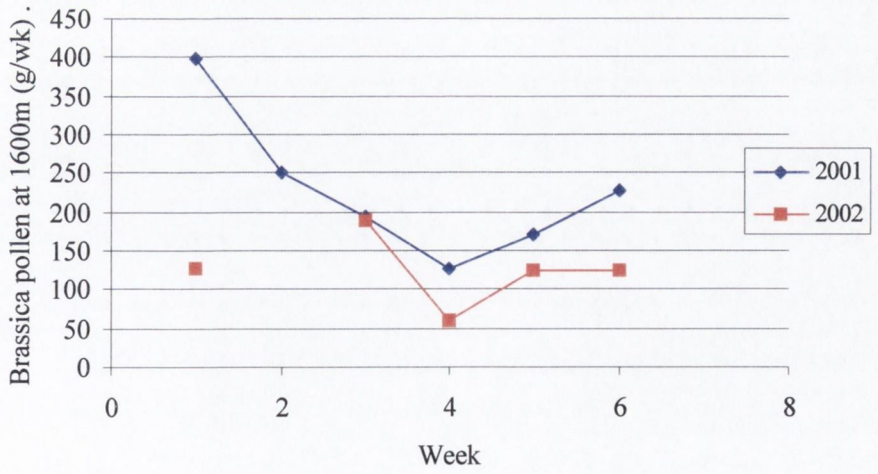

Figure 4.10: Concentration of *Brassica* pollen (g/wk) collected at 1,600m

4.3.2 Molecular analysis of pollen pellets

Amplification of DNA extracted from pollen pellets was successful (Figure 4.11) using nuclear SSR primer set 12A (Figure 4.12), and plastid SSR primer sets: Region A2 (Figure 4.13) and Region B2 (Figure 4.14). High concentrations of *Brassica* pollen were found in all of the samples tested. Results of a statistical analysis on SSR marker findings were difficult to interpret as multiple peaks were seen in some plastid SSR samples (Figures 4.13 and 4.14). These peaks were consistent with *Brassica napus* cv. Marinka and also either *Arabidopsis thaliana* (it is unlikely that bees forage on *A. thaliana*) or *Camelina sativus* (Table 4.2 summarises the data contained in the midpoint rooted and unrooted phylograms, Figures 4.15 and 4.16) and therefore imply that the DNA extract used for the analysis was not pure. This in turn implies that the pollen pellets contained pollen from a number of sources. In spite of this, it can be concluded that *Brassica napus* cv. Marinka pollen was present in beehives located 1,600m from the source.

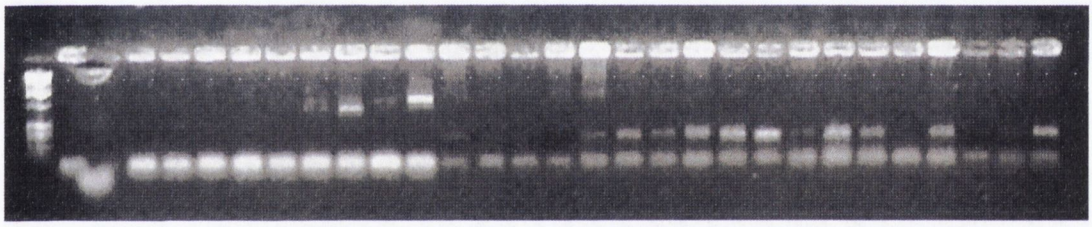

Figure 4.11: Gel of SSR PCR products of bee DNA

A matrix of pairwise-distance measures was created in PAUP 4.0 (Swofford, 1999) using the data obtained from the three markers (12A nuclear SSR marker, and the two plastid markers: Region A2 and Region B2) combined with fingerprints obtained from leaf material of other Brassicaceae (section 2.3.3 and 2.3.5). An unrooted and a midpoint rooted phylogram were produced from the Neighbour Joining analysis (Figures 4.15 and 4.16). Plastid marker analysis revealed that a number of individuals contained multiple alleles consistent with *Brassica napus* cv. Marinka and either *Camelina sativus* or *Arabidopsis thaliana* (Table 4.2). As a result some pollen pellet DNA samples are grouping with *Brassica napus* cv. Marinka and some are grouping with either *Camelina sativus* or *Arabidopsis thaliana* (Figures 4.15 and 4.16).

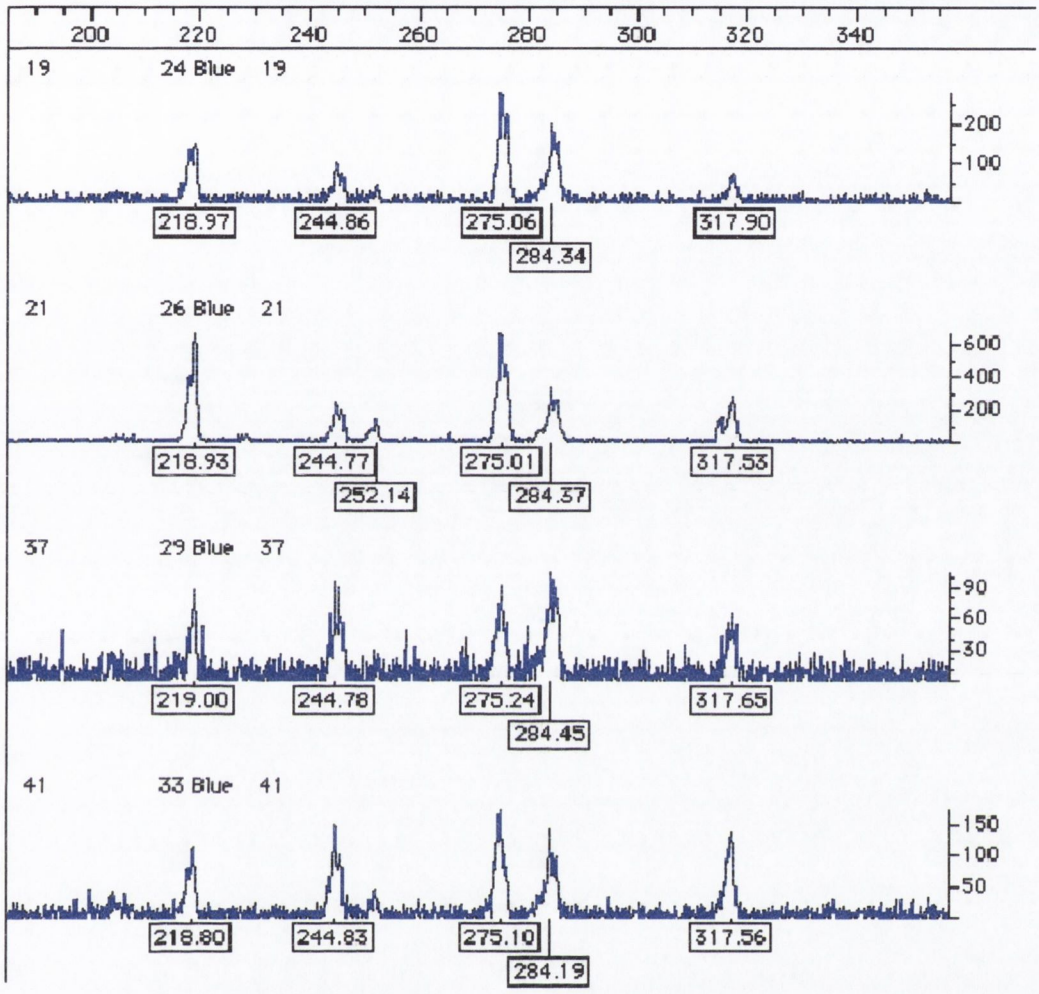

Figure 4.12: Pollen pellets amplified using the nuclear SSR marker 12A marker banding pattern (representative samples shown)

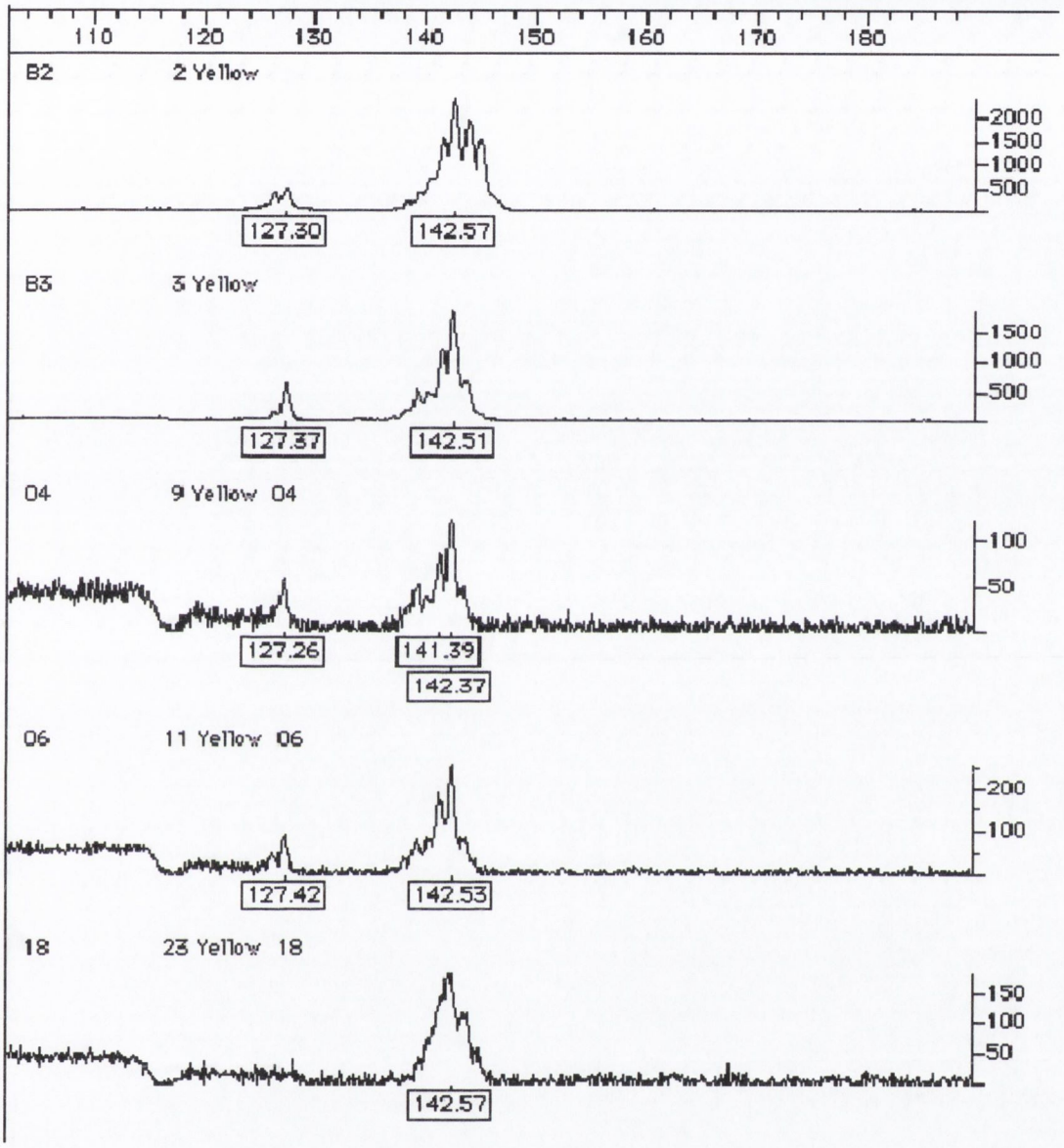

Figure 4.13: Pollen pellets amplified using the plastid SSR markers. MF_RA2 marker banding pattern (representative samples shown)

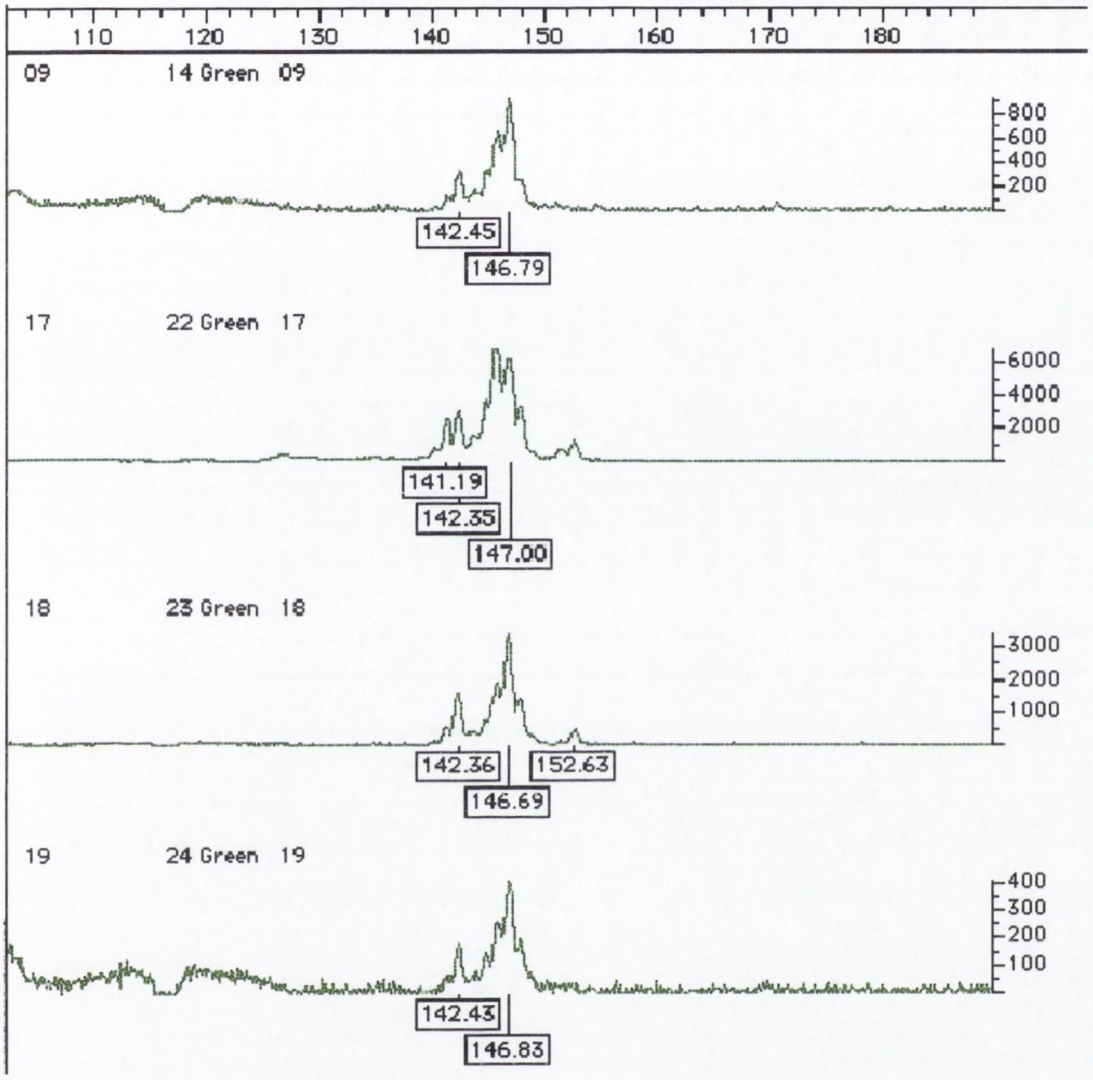

Figure 4.14: Pollen pellets amplified using the plastid SSR markers. MF_RB2 marker banding pattern (representative samples shown)

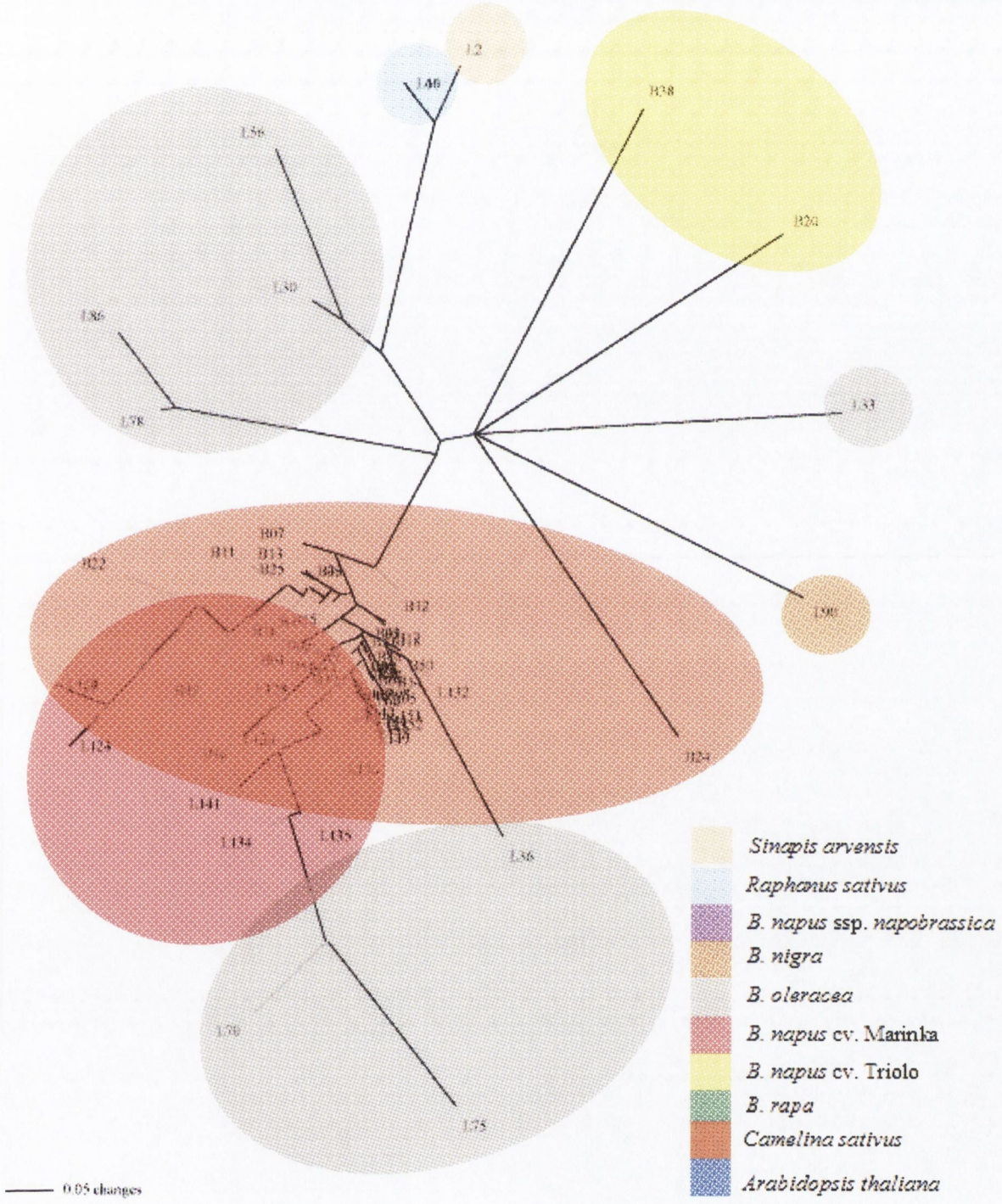

Figure 4.15: An unrooted phylogram showing the results of a Neighbour Joining analysis using all bands obtained from bee pellet SSR analysis

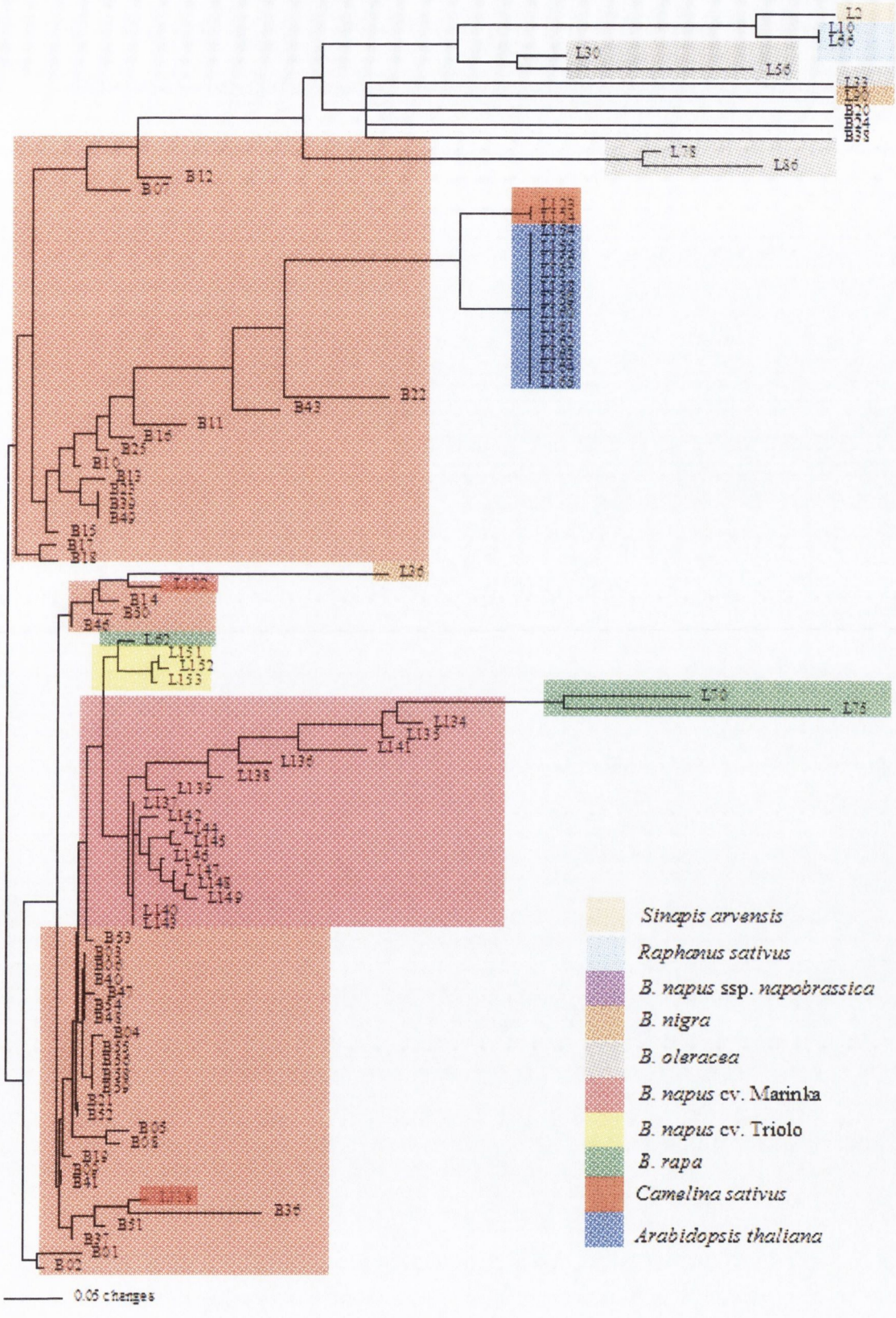

Figure 4.16: A midpoint rooted phylogram showing the results of a Neighbour Joining analysis using all bands obtained from bee pellet SSR analysis

Table 4.2: Groupings obtained from SSR analysis of pollen pellets using one nuclear marker (12A) and two plastid markers (MF_RA2 and MF_RB2)

Year	Groupings displayed in the phylogram					
	Pellets gouping with *B. napus* cv. Marinka		*B. napus* cv. Marinka and *Camelina/Arabidopsis*		Other Brassicaceae*	
2001	1	Week1 100m	10	2001 Week2 1,600m	7	2001 Week2 200m
	2	Week1 200m	11	2001 Week3 100m	12	2001 Week3 200m
	3	Week1 400m	13	2001 Week3 400m		
	4	Week1 800m	14	2001 Week3 1,600m		
	5	Week1 1,600m	15	2001 Week4 100m		
	6	Week2 100m	16	2001 Week4 200m		
	8	Week2 400m	17	2001 Week4 400m		
	9	Week2 800m	18	2001 Week5 200m		
	19	Week4 800m	22	2001 Week5 400m		
	21	Week5 100m	23	2001 Week5 1,600m		
			25	2001 Week3 800m		
2002	36	Week1 100m	39	2002 Week1 800m		
	37	Week1 200m	43	2002 Week2 400m		
	40	Week1 1,600m	49	2002 Week3 800m		
	41	Week2 100m				
	46	Week3 100m				
	47	Week3 200m				
	48	Week3 400m				
	50	Week3 1,600m				
	51	Week4 100m				
	52	Week4 200m				
	53	Week4 400m				
	54	Week4 800m				
	55	Week4 1,600m				
	56	Week5 100m				
	58	Week5 200m				
	59	Week5 800m				

*SSR amplification of B20, B24 and B38 samples failed.

4.4 Discussion

4.4.1 Discussion of sampling results

Analysis of insect dispersed pollen in 2001 reveals the same trend as 2002 (Figures 4.4 and 4.5). Both the 2001 and 2002 collections are showing a dramatic decrease in the overall quantity of pollen pellets collected between hives at 200m and 400m (556g/wk to 185g/wk in 2001 and 457g/wk to 186 g/wk in 2002). However, although the quantities of pollen pellets collected are lower, this trend is not reflected in the percentage of *Brassica* pollen collected, which remains high (Table 4.1). In spite of both curves being different, it is interesting to note that the extrapolated distance is the same for both years (6,500m).

The weight of *Brassica* pollen collected each week in 2001 and 2002, at each distance, was compared (Figures 4.6 to 4.10). In 2002, over the season a much smaller quantity of pollen pellets were collected at 100m (156g/wk). However, this also does not reflect a lower proportion of *Brassica* pollen being collected (Table 4.1). Thus suggesting that the trap may not have been working efficiently i.e. bees were getting into the hive without their pellets being detached from their legs. Alternatively a smaller colony may have been present in one or other of the hives.

The high percentages of Brassicaceae pollen in pollen pellet samples reported suggests that although the foraging strategies of bees are complex they appear to be following the nearest neighbour pollination or flower constancy rule (Levin and Kerster, 1974; Zimmerman, 1981; Rasmussen and Broedsgaard, 1992). This rule states that bees foraging for nectar or pollen move to the nearest neighbouring flower or plant of the same species, if it has proved rewarding i.e. the *B. napus* cv. Marinka crop.

4.4.2 Molecular analysis of pollen pellets

Amplification of DNA extracted from pollen pellets was successful (Figures 4.11 to 4.14). Although high concentrations of *Brassica* pollen were found in all of the samples tested, the findings of analyses of plastid SSR markers were difficult to interpret as multiple peaks were seen in some samples from plastid SSR analysis (Figures 4.13 and 4.14). The presence of multiple alleles in plastid SSR analysis implies that the DNA extract used for the analysis was not pure. Initial hypotheses assumed that the presence of multiple markers was associated with multiple pollen

types in the pollen pellet, and peaks were consistent with *Brassica napus* cv. Marinka and either *Camelina sativus* or *Arabidopsis thaliana* (Figures 4.13 and 4.14). In 2001, a *C. sativus* crop was planted nearby and although it had finished flowering prior to the collection period, there was the possibility that it remained as a pollen source for bees for a number of weeks. However, similar fingerprints were obtained in 2002 when no *Camelina* source was present and therefore it was concluded that either the pollen pellet DNA extraction process should be refined slightly or the PCR conditions be altered for a more stringent reaction (Figures 4.8 and 4.9). In spite of this, markers consistent with *Brassica napus* cv. Marinka were obtained, and it was concluded that *B. napus* cv. Marinka pollen was most likely present in beehives located 1.6km from the source. that is they are not found in any other Brassicaceae studied in this analysis.

4.5 Conclusion

Many apiculturalists have stipulated that bees will forage on individual plant species on any given forage trip, except where abundance of pollen source is limited (Hodges, 1974). The presence of *B. napus* cv. Marinka pollen in pollen pellets taken from hive five at 1.6km from the field indicates that pollen is moving over large distances confirming previous studies (Vaissiére *et al.*, 1994; Timmons *et al.*, 1995; Champolivier *et al.*, 1999; Osborne *et al.*, 1999; Ramsay *et al.*, 1999; Beekman and Ratnieks, 2000). Taking the hive as a centre point then the diameter of the risk area becomes 3.2km. In addition extrapolated distances imply a risk area of 6.5km. This has implications for transgene movement and when combined with the tendency of *Brassica* to hybridise with its wild relatives points towards transgene establishment in the wild.

As Brassicaceae pollen was detected in all hives regardless of the distance of the hive from the source crop, this analysis confirms that bees will forage nearby when abundant nectar and pollen sources are close by, but will also travel considerable distances (1.6km) as is consistent with the literature (Vaissiére *et al.*, 1994; Osborne *et al.*, 1999; Ramsay *et al.*, 1999; Beekman and Ratnieks, 2000). Others have also reported findings that pollen can travel considerable distances from source plots, which are in agreement with those reported here, including that by Thompson *et al.* (1999) where airborne pollen dispersal declined in a leptokurtic

fashion and was recorded up to 3,000m. Similarly, Timmons *et al.* (1995) reported low mean levels (3 grains m^3) of airborne pollen at 2,000m from the source. Therefore from these analyses, it is reported that to prevent insect dispersal, buffer zones needed to contain genetically modified (GM) crops would be a minimum of 3.2km from the data collected here (a maximum was not found as *Brassica napus* cv. Marinka pollen was detected in all hives). Additionally, although extrapolated data are less reliable, a minimum of 6.5km is suggested. This is consistent with studies carried out by Ramsay *et al.* (1999), where bees were reported to have flown to a crop 5km away indicating potential for pollen to be transferred to distances of 10km by the mixing of bees foraging in different directions from the same hive.

In conclusion, alternative strategies could be used to prevent the spread of GM crops. The reduction or elimination of pollen production could be achieved by controlling flower or pollen production or pollen compatibility. On the other hand, the reduction or elimination of cross-pollination resulting from bee visitation could be achieved by inducing apomixis in GM crops (Williams, 2002).

Chapter 5

Examination of pollen dispersal through seed set in male sterile bait plants

5.1 Introduction to seed set in male sterile bait plants

5.1.1 Introduction

Male sterile (MS) bait plants essentially act as pollen detectors as they produce no pollen of their own, and therefore offer an opportunity to establish 'worst case scenario' cross-pollination levels (Agricultural Biotechnology in Europe, 2002). *Brassica napus* is mainly self-pollinating and therefore higher levels of gene flow are detected in MS plants as competition occurs between 'foreign' OSR pollen and 'selfed' OSR pollen in the fertile plants (Salisbury, 2002). Therefore results from fully fertile bait plants show a much lower incidence of cross-pollination occurring than those from male sterile plants, and represent the likely levels of gene flow that may occur from crops to isolated wild or feral rape plants. As a result data from MS bait plants have been found to overestimate cross fertilisation to similar male-fertile populations by about one order of magnitude (Ramsay *et al.*, 2003). Where male sterile OSR plants are used to measure outcrossing, rather than fertile canola plants, outcrossing is detected over longer distances (Salisbury, 2002). The main advantage of MS plants is that they show pollen deposition and enable calculation of fertilisation and gene flow rates.

The significance of both wind and insects as vectors of oilseed rape pollen have been widely researched with contradictory results that are probably influenced not only by varying environmental and topographical conditions, but also by the differences in research methodology used (Champolivier *et al.*, 1999). Experiments using male sterile plants allow estimations to be made of gene flow via pollen from particular source crops and hence provide valuable data on isolation distances required to prevent cross-contamination.

Male sterile or emasculated bait plants have been used to detect outcrossing at 400m (Simpson *et al.*, 1999; Sweet *et al.*, 1999), 600m (Simpson, 2000), 1.5km (Timmons *et al.*, 1995) and 4km (Thompson *et al.*, 1999) from the pollen source. Sweet *et al.* (1999) found up to 7% of male sterile flowers were fertilised at 400m. Thompson *et al.* (1999) reported 88.4% of male sterile flowers were fertilised 1m from the field edge, 13-57% at 500m and 5% at 4km.

Levels of outcrossing have been measured as between 12% and 47% (Becker *et al.*, 1992). Timmons *et al.* (1995) studied bait plants to investigate whether the low levels of pollen detected at long distances were sufficient enough to effect significant levels of gene flow. Emasculated and de-petalled oilseed rape plants were placed at increasing distances from an oilseed rape field, and levels of seed set were recorded. Seeds produced on plants 2km away from the source contained 38 chromosomes and appeared phenotypically normal for *B. napus*, suggesting that there were sufficient viable pollen grains at distances of at least 2km to affect gene flow. However, although the removal of petals may reduce the attractiveness of plants to pollinating insects, the prevention of visiting insects to de-petalled plants is not guaranteed so this gene flow may not have been effected entirely by wind pollination (Timmons *et al.*, 1995).

The relative importance of wind and insect pollination was described by Thompson *et al.* (1999) as being difficult to examine in field conditions. In their study, existing field OSR crops of 55ha were used as pollen donors to measure levels of pollen dispersal. Antibiotic resistance gene screening (germination tests on kanamycin containing media and PCR for the *nptII* marker) was utilised to determine the source of pollen on MS *B. napus* bait plants, situated at various distances from the source. At one of the bait sites, with a pollination rate of 33 %, the majority of the sample (>80 %) was fertilised by pollen from the nearest crop 900m away. Levels of cross-pollination were recorded at a maximum distance of 4km from the nearest known source (Champolivier *et al.*, 1999). The patterns of pollen dispersal recorded in this study suggest that insects played an important part in pollination.

Simpson *et al.* (1999) measured pollen dispersal from a large area (approx. 9ha) of winter oilseed rape using plots of MS bait plants and fully fertile plants. Six bait plants were positioned in linear plots at a range of distances (100m, 200m, 400m) in directions north, south, east and west from the pollen source. During the study it was observed that the incidence of pollinating insects was notably low at flowering time. MS bait plants were pollinated and seed set in all plots (Simpson *et al.*, 1999).

According to EU legislation relating to seed purity of basic and certified seed (Council Directive 69/208/EEC, Commission Decision 95/32/EEC,

98/173/EEC and 99/84/EEC; (Bock *et al.*, 2002)) a minimum separation distance of 100m to any other rape crop has to be acknowledged for certified seed production. In addition, OECD seed schemes require a minimum of five years to have lapsed since *Brassica* was sown on that field. Seed producers in some EU states choose greater distances and time periods (e.g. seven years). EU legislation requires a minimum distance of 300m to any other rape crop for hybrid certified seed production (Bock *et al.*, 2002). Complete containment of a GM trait using isolation distance alone is impractical under commercial production conditions (Downey, 1999). No system of field production for canola can guarantee 100% purity or complete freedom from GM pollen (Moyes and Dale, 1999) given both pollen flow and temporal and spatial seed movement. To ensure successful co-existence of organic, conventional (non-GM) and GM canola crops, industry and growers need to accept similar standards of purity to those currently used for canola seed production crops worldwide, allowing for example, a threshold of up to 1% off-types(Moyes and Dale, 1999).

5.1.2 Aims

This section aims to examine cross-fertilisation, seed set and resulting intraspecific hybrid production in *B. napus* male sterile plants. This offers an opportunity to establish 'worst case scenario' cross-pollination levels. Specific objectives include:

- Complement the passive trap data (Chapter 2), by planting six male sterile plants beside each passive trap i.e. at 0m, 50m, 100m and 200m in 2001; and 0m, 12.5m, 25m and 50m in 2002 and in eight directions (N, NE, E, SE, S, SW, W and NW).
- Collect seed at the end of the flowering season. Record weight to determine the amount of seed set. Germinate a portion under greenhouse conditions. Collect fresh samples for DNA analysis and for drying in silica gel.
- Employ nuclear SSRs to perform paternity analysis on each individual tested.

5.2 Methods

5.2.1 Male sterile plants

Brassica napus cv. Triolo MS seed was used in 2001, while *B. napus* cv. Jura MS seed was used in 2002. Seed was germinated and grown out in seed trays (104 plants per tray) in greenhouses in Oak Park. Six individuals were planted beside each passive trap and enclosed in a frame to prevent pest damage (Figure 3.2B). At the end of the flowering season, all 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 MS plant and were then germinated and grown out in five-inch pots in the Trinity College Botanic Gardens, Dartry, Dublin. Samples of both *B. napus* cv. Triolo and *B. napus* cv. Jura seed were also grown out to examine germination rates of male sterile seed and frequency of haploid genotypes emerging. Compost was made up using a 1:1:1 mixture of organic compost: loam: sand to create a well drained, nourishing soil for the plants. Seedlings were re-potted after 3 weeks and harvested after a further 3 weeks. Samples for DNA extraction were taken by closing the lid of a 1.5ml microcentrifuge tube on a leaf creating a leaf disk with a 10mm diameter. 2-3 leaves were also placed in silica gel for long term storage.

5.2.2 DNA extraction

DNA was extracted using a REDextract plant DNA extraction kit (Sigma Aldrich). Approximately, half the 10mm leaf disc collected was mixed with 100µl extraction solution by vortexing and heated to 95°C for 10mins. 100µl dilution solution was added and the solution was vortexed again. All samples were stored at -20°C until use.

5.2.3 PCR amplification and microsatellite marker analysis

PCR amplification and SSR marker analysis was carried out by adding 4µl kit extracted DNA, 10µl REDextract PCR mix and 3µl forward and reverse primer to a 200µl microfuge tube. PCR conditions are outlined in Table 2.3 and nuclear SSR primer set 12A. Amplified SSR reactions were treated the same way as nuclear SSR reactions in section 2.2.5. Statistical analysis was performed as in section 2.2.8,

however as the results were inconclusive the presence or absence of a single diagnostic marker (318bp) was used to distinguish the hybrid progeny.

5.3 Results

5.3.1 Sampling results

Thirty-two samples were collected in 2001 and 16 in 2002 (seed from different individuals was kept separate). One major deviation from the protocol occurred during the 2002 season. The accidental destruction of 20 of 36 MS plots prevented laboratory analysis being performed. Eight of the twenty removed were at 0m but it is assumed that the rate of fertilisation in these plants would have been comparable to other 0m plots. The remaining 12 plots were at 12.5m, 25m and 50m in the W, NW, N and NE directions (Results not shown due to incomplete data set). In 2002, an additional four plots of MS bait plants were situated along a transect the furthest located at 800m NE of the crop. Seed set occurred in all plots where plants remained until harvest.

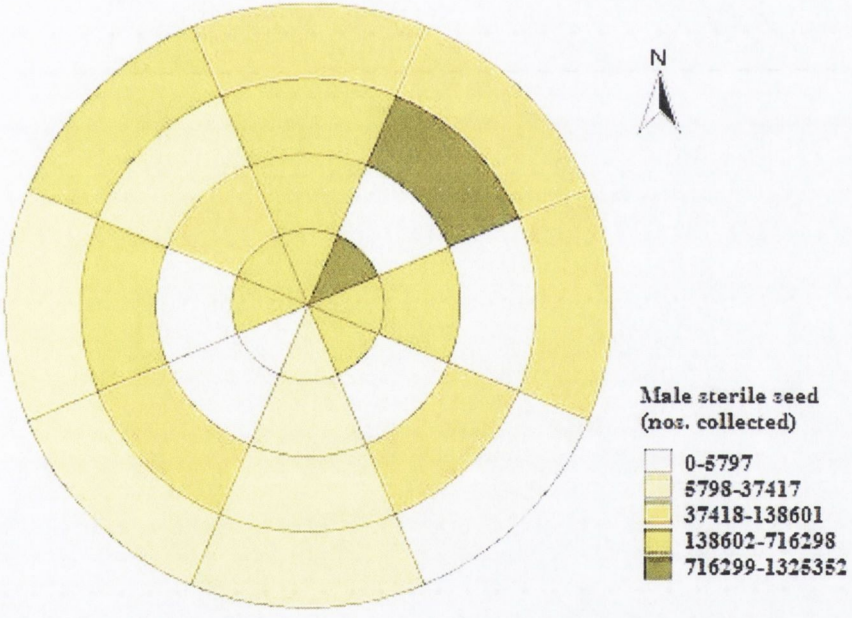

Figure 5.1: Seed collected from MS *Brassica napus* cv. Triolo in 2001. Numbers of seed collected from the six bait plants at each site were divided into 5 exponentially increasing groups, with darker colours representing greater numbers of seed collected. Rings indicate male sterile plants at 0m, 50m, 100m and 200m

In 2001, the effect of direction is clear from the quantity of seed collected from plants to the northeast of the source crop, which is consistent with south-westerly

prevailing winds and the direction of the beehive transect (Figure 5.1 and 5.2). Seed viability was high as all seed tested in the greenhouse germinated and grew. The effect of distance from the crop is not as evident as direction. However, the large quantity of seed was collected from plants situated at 100m NE is apparent, which is also consistent with both the south-westerly prevailing wind and the direction of the beehive transect (Figure 5.1 and 5.3).

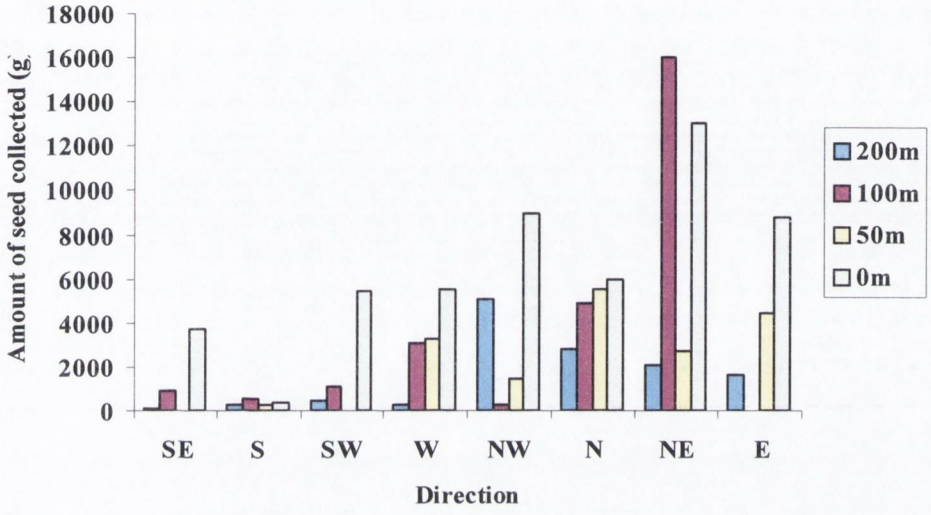

Figure 5.2: The amount of seed (g) collected from MS *Brassica napus* cv. Triolo plants in 2001 according to direction

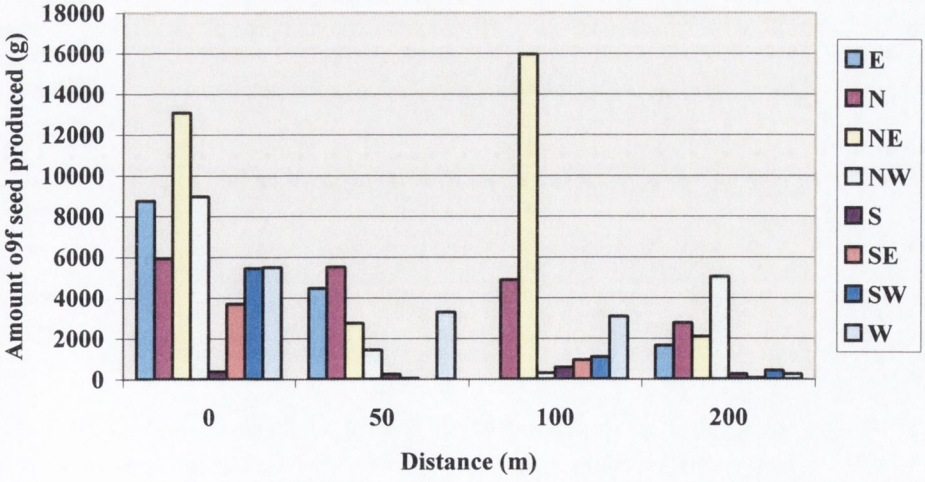

Figure 5.3: The amount of seed (g) collected from MS *Brassica napus* cv. Triolo plants in 2001 according to distance

5.3.2 SSR analysis

The nuclear marker 12A (Szewc-McFadden *et al.*, 1996) was the most informative marker for studying parentage of F1 hybrid seed (Figures 5.4 and 5.5).

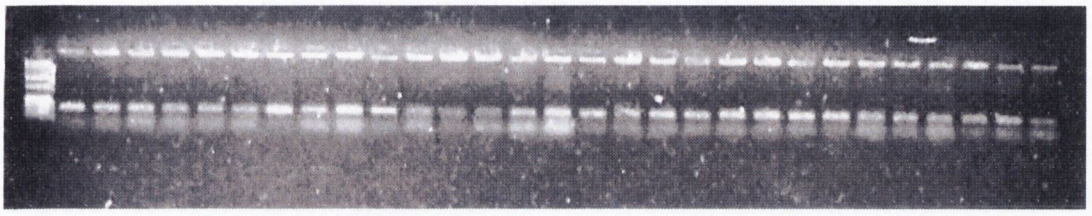

Figure 5.4: 12A SSR amplified in the progeny of the MS plants

The phylograms produced from the Neighbour Joining analysis of all peaks obtained using the 12A nuclear SSR marker, were difficult to interpret. Only *Raphanus sativus* (2 and 66), *Brassica nigra* (90), *B. oleracea* ssp. *capitata* (30 and 86), *B. oleracea* ssp. *gemmifera* (33 and 58) and *B. rapa* ssp. *rapifera* (70) were successfully distinguished (Figures 5.6 and 5.7). The reliability of this type of analysis is hindered by the low number of alleles detected.

Therefore, the presence or absence of a single diagnostic peak was examined (Table 5.1). A band present at 318bps in *B. napus* cv. Marinka was absent in *B. napus* cv. Triolo (MS) parent plants and all other Brassicaceae tested. This band was also found in many of the F1 progeny and therefore when present the progeny were considered to be a *B. napus* cv. Triolo × *B. napus* cv. Marinka hybrid. From a total of 188 F1 seeds tested, 82 (36%) samples lacked the 318bp band and were considered *B. napus* cv. Triolo (MS) (see section 5.4). The remaining 147 shared the *B. napus* cv. Marinka band. Therefore, 64% of the seed tested shared the *B. napus* cv. Marinka marker and were assumed to be a F1 hybrids - *B. napus* cv. Triolo × *B. napus* cv. Marinka (Table 5.1).

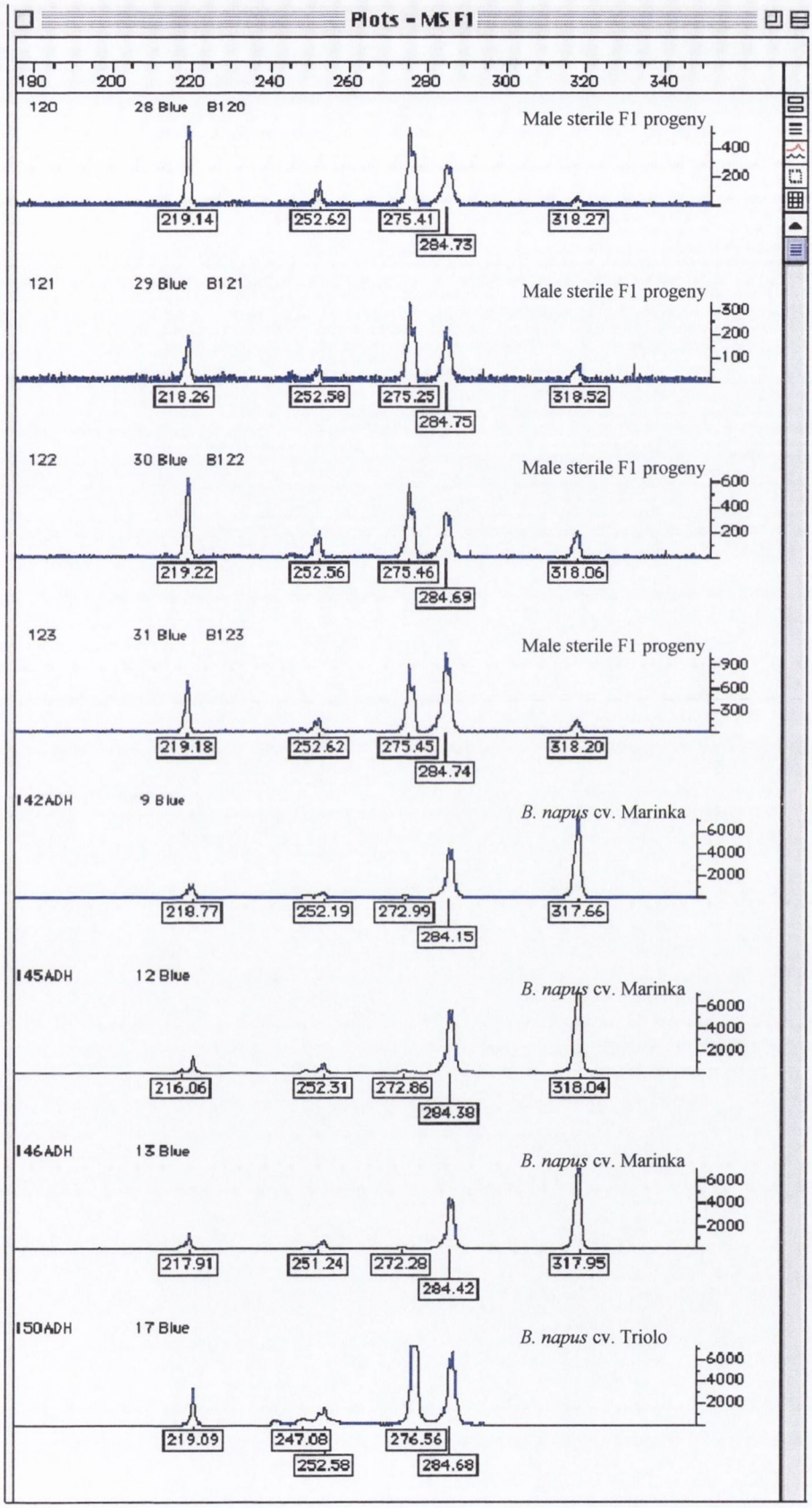

Figure 5.5: Banding patterns obtained using primer 12A. 120-123 are male sterile F1 progeny individuals; 142, 145 and 146 are *B. napus* cv. Marinka and 150 is *B. napus* cv. Triolo (MS)

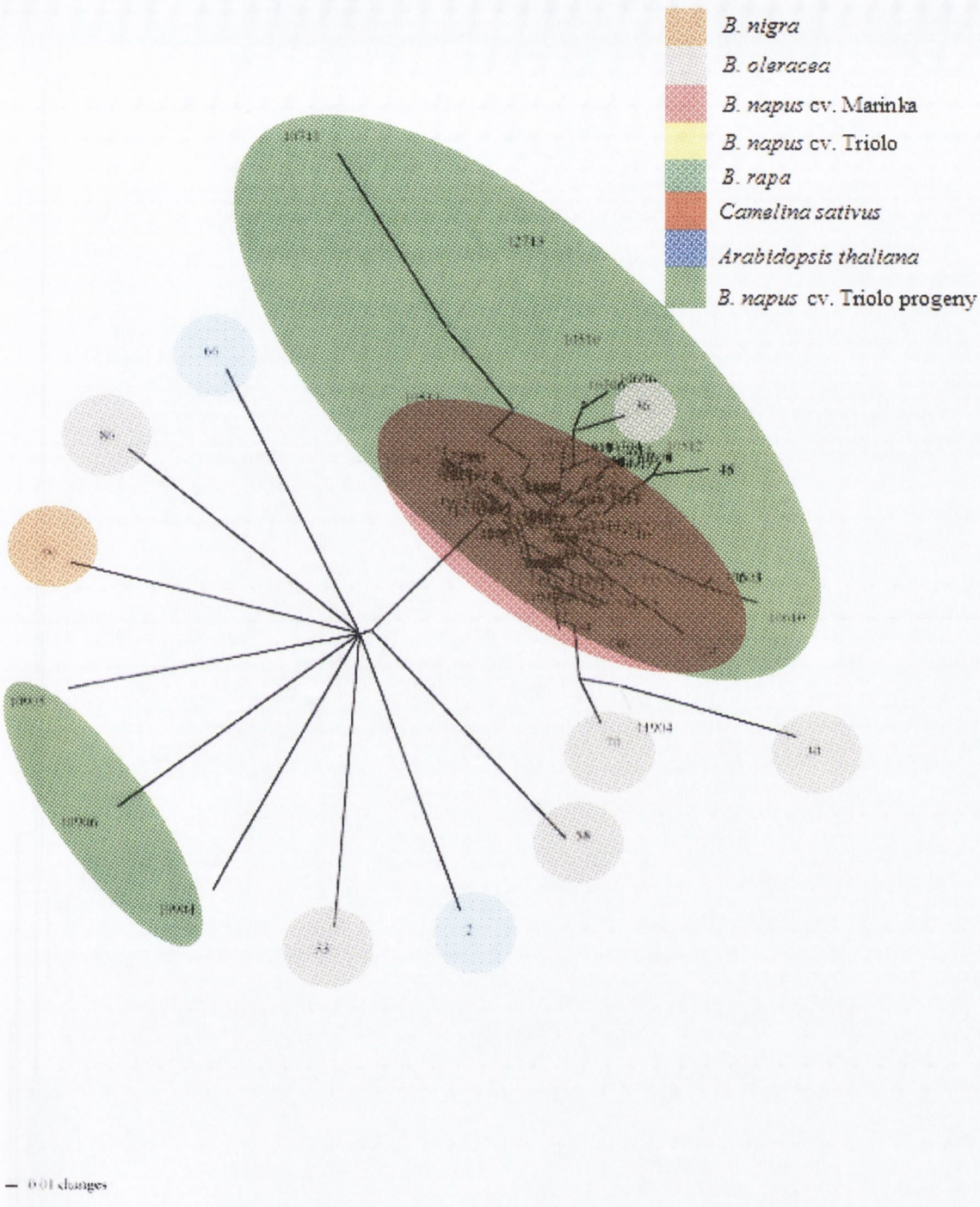

Figure 5.6: An unrooted phylogram showing the results of a Neighbour Joining analysis using all bands obtained from SSR analysis of male sterile progeny

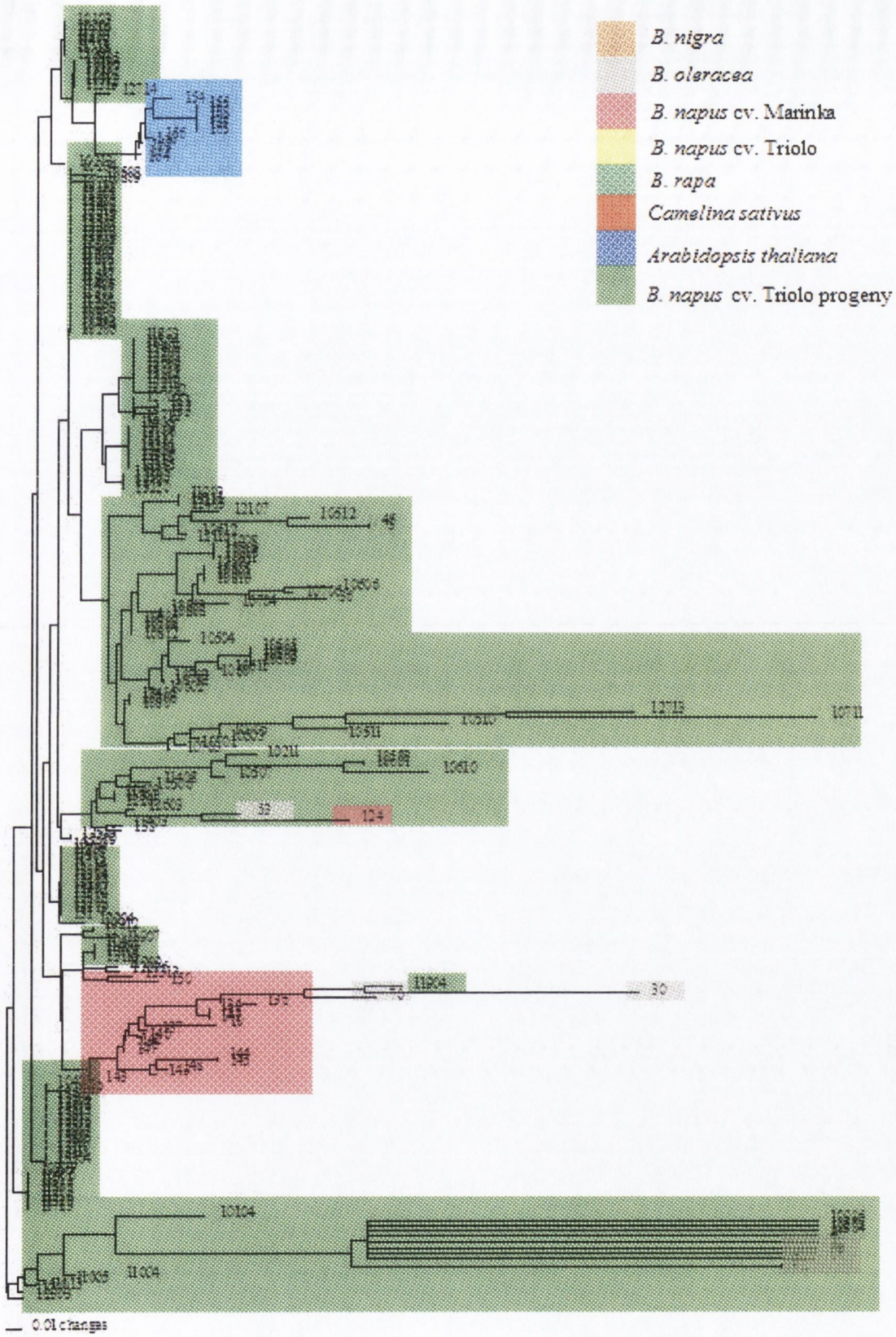

Figure 5.7: A midpoint rooted phylogram showing the results of a Neighbour Joining analysis using all bands obtained from SSR analysis of male sterile progeny

Table 5.1: F1 individuals from seed of *B. napus* Triolo containing the *B. napus* cv. Marinka marker (318bp) and those lacking the marker

B. napus cv. Marinka Band present at 318bp						*B. napus* cv. Triolo Band absent at 318bp		
127	10211	11401	12505	10506	10811	2	162	12707
131	10301	11402	12509	10507	10812	10	163	12712
134	10401	11403	12602	10508		30	164	12713
135	10402	11406	12603	10509		33	165	12714
136	10403	11407	12604	10510		36	10906	12812
137	10404	11408	12605	10511		46	10905	12904
138	10405	11601	12304	10601		53	10904	12906
139	10406	11602	12315	10602		56	11803	13114
140	10907	11715	12314	10603		62	11804	13116
141	10908	11717	12404	10605		66	11903	13205
142	10903	11718	12608	10610		70	11904	13218
143	11004	11712	12811	10701		75	11906	13220
144	11005	11713	12813	10702		78	12009	10505
145	11006	11714	12817	10703		86	12106	10512
146	11011	11805	12818	10704		90	12107	10604
147	11012	11801	12819	10708		123	12108	10606
148	11013	11802	12903	10709		124	12110	10607
149	11207	11905	12905	10710		150	12111	10608
10103	11208	11907	12907	10712		151	12112	10609
10104	11209	11908	12908	10801		152	12201	10611
10105	11201	12002	13110	10802		153	12313	10612
10106	11210	12001	13111	10803		154	12405	10705
10107	11203	12003	13112	10804		155	12409	10706
10108	11301	12008	13115	10805		156	12503	10711
10204	11302	12010	13203	10806		157	12507	
10205	11303	12406	13204	10807		158	12508	
10206	11318	12407	13219	10808		159	12606	
10212	11319	12408	10503	10809		160	12705	
10213	11320	12504	10504	10810		161	12706	

5.4 Discussion

This section focused on seed set of *B. napus* cv. Triolo male sterile bait plants. Seed set occurred in bait plants in all eight directions and at all distances. The distribution patterns of wind-dispersed pollen showed a decrease in concentration with distance from the crop (chapter 3). Seed set in bait plants does not observe this pattern. Figures 5.1 to 5.3 show higher numbers of seed being produced by bait plants to the north and northeast of the source crop. As the transect of beehives was in the north-easterly direction it is unknown whether this seed set was as a result of abiotic or biotic fertilisation. However, it is likely that it is resulting from biotic dispersal as high levels of Brassicaceae pollen were not collected from passive traps at 200m NW, 100m W or 100m SW of the crop, while high levels of seed set are evident. In addition, the ability of *Brassica napus* individuals to be receptive to airborne pollen has been questioned (Cresswell *et al.*, pers com).

Molecular analysis of *B. napus* cv. Triolo × *B. napus* cv. Marinka individuals focused on the variation produced by nuclear microsatellite (SSR) markers as chloroplasts are maternally inherited and therefore would not detect the newly introduced paternal DNA donated by the pollen. The nuclear SSR marker 12A produced a *B. napus* cv. Marinka-specific marker of 318bp in length. This marker was present in all *Brassica napus* cv. Marinka individuals tested. A quantitative analysis was not performed as only twenty seeds per plot of six male sterile plants were germinated and analysed. However, as all other Brassicaceae sources were eliminated from the site, this analysis assumes that the *B. napus* cv. Marinka crop is the only source of Brassicaceae pollen. Sixty-four percent of the seed tested showed the *B. napus* cv. Marinka marker and therefore were considered to be hybrid progeny *B. napus* cv. Triolo × *B. napus* cv. Marinka.

Thirty-six percent of the seed tested did not contain the *B. napus* cv. Marinka marker. It is unlikely that the low level of the *B. napus* cv. Marinka marker obtained indicates that the bait plants were fertilised by another source. It is more probable that the DNA extraction process may require refinement to improve PCR amplification of SSR products. Alternatively, although all the *B. napus* cv. Marinka individuals tested contained the marker they may not have passed it on to their progeny if they were heterozygotes. Therefore it is possible that this marker is

specific (not diagnostic) and as a result the marker would not be expected to be seen in every *B. napus* cv. Triolo × *B. napus* cv. Marinka individual. To clarify this uncertainty further *B. napus* cv. Marinka, *B. napus* cv. Triolo and *B. napus* cv. Triolo × *B. napus* cv. Marinka individuals could be screened.

It may be unremarkable that seed set occurred at 200m in 2001 and 800m in 2002 when it is taken into account that Timmons *et al.* (1995) recorded seed set in bait plants at 2km using emasculated plants as pollen receivers. Thompson *et al.* (1999) carried out a study on a much larger scale using existing field crops of 55ha as pollen donors and although the majority of the sample was fertilised by pollen from the nearest crop 900m away, levels of cross-pollination were recorded at a maximum distance of 4km from the nearest known source (Champolivier *et al.*, 1999).

In spite of this, several analyses have shown a link between source crop size and dispersal distance (Salisbury, 2002). As the source crop used for these analyses was smaller (4 hectares) it follows that lower levels of dispersal and gene flow would be detected. However, as seed set was observed in all MS bait plants a maximum distance was not obtained.

5.5 Conclusion

Although it has been said that MS bait plants represent 'worst case scenario' cross-pollination levels as they produce no pollen of their own (Agricultural Biotechnology in Europe, 2002), the important factor is that even if the pollen is travelling long distances at low frequencies there is still potential for gene flow at long distances. The method of pollination should also be considered. Results from abiotic dispersal experiments (Section 3.3) suggest that airborne pollen does travel sufficiently large distances to effect fertilisation, however it has been suggested that *B. napus* flowers are ineffective at capturing airborne pollen (Cresswell *et al.*, pers com). This implies that insects are the primary vectors in *B. napus* pollen dispersal. If the hazard and exposure model (Wilkinson *et al.*, 2003) is applied then even though exposure may be low the hazard is high. Male sterile varieties and lines will outcross with neighbouring fully fertile GM oilseed rape at high frequencies and at relative large distances.

As seed set occurred in all bait plants, it may be noted that small groups of Brassicaceae plants will attract bees and therefore present an opportunity for gene flow between crop and volunteer or feral populations. These can in turn act as gene pools facilitating the contamination into subsequent rape crops (Eastman and Sweet, 2002). Studies to develop models of pollen spatial dynamics based on individual pollinator behaviour have been developed to explore the consequences of plant spatial arrangement for gene flow in plant populations (Heinrich, 1976; Morris, 1993; Dramstad, 1996; Thompson, 1996; Saville *et al.*, 1997; Osborne *et al.*, 1999; Walther-Hellwig and Frankl, 2000; Osborne and Williams, 2001). They found that the predicted dispersal distance increased with increasing interplant distance. Since all male sterile bait plants were fertilised it is unknown whether bees altered their movement patterns in response to bait plant spacing.

Timmons *et al.* (1995) found a pollination frequency of 0.8% at 2,500m from a commercial scale source crop, using male sterile bait plants. Thompson *et al.* (1999) measured gene flow from oilseed rape within a 70km^2 area, to bait plants at distances 0-4000m from the nearest oilseed rape crop and found that seed was consistently set up to the maximum 4km, where there was a 5% pollination rate. In spite of this, it must be remembered that studies on male sterile bait plants represent the potential for gene flow i.e. due to self-fertilisation the actual pollination levels for male-fertile plants are likely to be much lower (Treu and Emberlin, 2000). The use of existing large-scale oilseed crops by Thompson *et al.* (1995) is considered highly realistic as it shows that large pollen sources such as fields can interact to increase gene flow (Treu and Emberlin, 2000).

Following generation of a hybrid between oilseed rape and a wild relative, gene flow between the two species is not a certainty due to the viability of the hybrid. The success of the F1 and its progeny will be influenced by: fertility, ability to propagate vegetatively, ability to give viable F2 and backcross progeny (Scheffler and Dale, 1994).

In conclusion, as pollen dispersal can be considerable (4km) hybridisation and introgression between oilseed rape varieties and wild relatives are likely to occur. Consequently, it is probable that transgene movement from GM to non-GM crops or feral populations is likely to occur. Therefore, it is important to establish

whether the transgene would confer a direct selective advantage in the feral environment, as the rate of introgression will be dependent on this.

Chapter 6
Discussion

6.1 Methods developed for this study

6.1.1 Passive trap: a collection system for trapping airborne pollen

A passive trap based on the Cour trap (Cour, 1974) was successfully developed and tested during the course of this study. In addition, an appropriate adhesive which enabled subsequent DNA extraction was discovered. Bands obtained from PCR analysis of the plastid *trnL* intron (using primers c and d) indicate that the methods used for pollen removal from the mesh and kit DNA extraction, were successful (Figure 3.32: p129). The amplification fragments obtained were weak and further refinement of the technique may be necessary, however as SSR regions are shorter they may amplify more easily than this plastid intron region. In addition, the length variation of different extractions detected in the *trnL* intron amplified suggests that a range of species may have provided pollen for these extractions and not just *Brassica.* Sequencing of these products is required for species identification. However, this work has nonetheless demonstrated the utility of this pollen capture / DNA extraction procedure for pollen dispersal studies.

Although refinement may be necessary, this technique has potential for use in a number of applications including pollen dispersal and gene flow studies of GM crops, as many of the main commercial crops are wind pollinated including maize, sugar beet, wheat, barley and ryegrass.

6.1.2 Molecular markers for the identification of Brassicaceae pollen

As microscopic analysis of pollen is not a robust enough technique to discern inter- or intraspecific variation among Brassicaceae, an alternative identification system was required. Molecular markers i.e. PCR-RFLP, DNA sequencing, nuclear and plastid microsatellite (SSR) marker techniques were therefore explored.

PCR-RFLP may be useful as a quick assay for intergeneric differentiation of Brassicaceae, however as was the case with Panda *et al.* (2003), PCR-RFLP did not detect intraspecific variation among the *Brassica* species tested. Therefore the technique (with the specific gene regions and restriction enzymes used in this case) is

not robust enough to discern interspecific variation and is inferior in terms of high throughput genotyping.

DNA sequencing revealed variation within the nuclear ITS region, and chloroplast regions *trn*L intron, *trnL-F* intergenic spacer (Taberlet *et al.*, 1991), *atpB-rbcL* intergenic spacer (Samuel *et al.*, 1997), *rpl16* (Jordan *et al.*, 1996), *rps16* (Oxelmann et al., 1997). Therefore DNA sequencing shows high potential for use in systematic studies. However, as DNA sequencing is a demanding and labour intensive task SSR analysis was examined.

Nuclear microsatellite marker analysis provides a high-resolution technique for the detection of variation among Brassicaceae. As there is an abundant supply (404) of *Brassica* primer sets through the *Brassica* DB database on the UK CropNet website (http://ukcrop.net) they are convenient markers. Nuclear SSR markers can be routinely used for a variety of purposes including investigation of the genetic structure of natural populations (Balloux and Lugon-Moulin, 2002) and for the characterisation, conservation and utilisation of crop diversity (Szewc-McFadden *et al.*, 1996). The need for extensive screening makes these markers less exploitable, however twelve were analysed here, of which the following would be recommended to other researchers 35D, 38A, 59A, 72A, 83/1 (Szewc-McFadden *et al.*, 1996), *nga*129 (Westman and Kresovich, 1998) and Na10-E08 (Lowe *et al.*, 2002).

As nuclear SSR markers may be hard to interpret in polyploid species such as *Brassica napus*, DNA sequenced chloroplast gene regions were examined for the presence of SSR repeat regions. Ten plastid SSR primers were then designed (section 2.2.7), nine of which detected polymorphism. In total, 57 different alleles were detected. These primers may now be applied in the detection of variation among individuals of Brassicaceae at inter-generic, inter-specific and intra-specific levels.

Systematic studies analysing evolutionary history of wild *Brassica* plant populations will benefit from these markers along with studies of GM risk assessment, gene flow studies and also wider applications in plant biology such as plant breeding and population genetics.

6.1.3 Extraction and analysis of pollen pellets for counting and DNA analysis

Identification of pollen pellets using microscopy and colour differentiation are adequate techniques to identify pollen to the generic level but DNA methodology is required to gain identify pollen below this taxonomic level.

6.2 Discussion of pollen dispersal results

A maximum range of greater than 200m but less than 800m was determined for wind dispersed Brassicaceae. As expected (Moore *et al.*, 1991), analyses of the meteorological data confirmed that the high levels of abiotic pollen dispersal detected coincided with optimal weather conditions for aerial pollen dispersal, including warm temperatures, sunshine, light to medium winds and most importantly no rain.

Analysis of the pollen pellets collected from the beehives presented a different picture. Only hives at 200m and 400m in 2001 showed similar trends in Brassicaceae pollen collection. The lack of correlation between the flowering progression of the crop and pattern of collection may be due to the abundance of pollen relative to the size of each bee colony. That is, the crop is producing vast quantities of pollen, while the needs of the colony would be much lower, therefore regardless of whether the crop is at the peak flowering or whether only a portion of flowers remain, there will be ample supply of pollen for the bees. At 1600m, high pollen levels decrease from almost 400g/wk collected in week one to 138g/wk in week four and increase again after that. In 2002, a similar trend is seen although some missing data makes it more difficult to interpret the results. The erratic quantities collected by the bees may be attributed to the trap working inefficiently, or alternatively may simply be attributed to the complexity of bee foraging patterns. Alternatively the bees may be foraging on another, more attractive, pollen source.

The high percentages of Brassicaceae pollen in pellet samples reported suggests that although the foraging strategies of bees are complex they appear to be following the nearest neighbour pollination rule (Levin and Kerster, 1974; Zimmerman, 1981; Rasmussen and Broedsgaard, 1992) so foraging will be from the nearest crop and if this is GM then the risk of transgene movement via bees increases.

The results reported here indicate that the majority of pollen was deposited very close to the source, but that pollen was also dispersed over large distances

(200m) at low frequencies. In addition, it has been demonstrated that wind dispersal of Brassicaceae pollen is dependent on the direction and speed of the wind.

Data collected for wind dispersed pollen are not highly relevant in terms of buffer zones for Brassicaceae, as honeybees are the primary pollinator and therefore as insect dispersal will be much greater than wind dispersal they form the basis of proposed buffer zone requirements. In spite of this, the system adopted here can be applied to other crops that are wind pollinated. Moreover, from this analysis when considering appropriate sizes for buffer zones of GM crops windspeed and direction must be taken into account. That is, where a site is exposed, greater buffer zones would be needed to the north, northeast and east of the crop, according to the prevailing wind direction. In practice it would be safer to extend the buffer zone, in all directions, to this distance. This method therefore has potential for use in risk assessment studies for the analysis of pollen movement from GM wind dispersed crops.

The significance of wind dispersed Brassicaceae pollen is questionable in terms of fertilisation and gene flow. Oilseed rape pollen remains viable for between 24 hours and one week depending on conditions (Mesquida and Renard, 1982). However, in this study, passive trap samples were collected on a weekly basis, therefore it is unknown how many pollen grains were viable at the time of deposition. In addition, it has been questioned whether the architecture of *Brassica napus* flowers is suitable to facilitate the reception of airborne pollen (Cresswell *et al.*, Unpublished). A study by Cresswell *et al.* (Unpublished) showed wind pollination as unlikely to be an effective means of cross-pollination.

6.3 Future study

Further analyses could be carried out on the extracted DNA samples from the passive traps to confirm the identity of Brassicaceae pollen. Length variation was detected in the amplified *trnL* intron region but DNA sequencing will be required for species identification, which would help confirm or refute the presence of *B. napus* cv. Marinka pollen. Alternatively, amplification of microsatellite markers may prove more successful for the DNA work from passive traps as the shorter microsatellite regions may amplify more easily than the plastid *trnL* intron used here. Quantitative analyses could be carried out on the progeny of the male sterile bait plants to clarify

the number of hybrid progeny (*B. napus* cv. Triolo × *B. napus* cv. Marinka) present. Likewise quantitative analyses could also be carried out on the bee pollen pellets.

The successful development of plastid SSR markers in this thesis is an important advance. They can be used to distinguish between individuals of Brassicaceae at inter-generic, inter-specific and intra-specific levels and are therefore offer a new opportunity in the study of variation within and between *Brassica* populations. The combination and comparison of nuclear and plastid SSR markers has a significant role to play in evolutionary and ecological studies of *Brassica* and also wider applications in plant biology such as systematics and plant breeding. SSR markers offer high potential for risk assessment and gene flow studies of *Brassica*.

GM maize has recently been approved for commercialisation in the UK (Mason, 2004). Many other wind-pollinated crops including sugar beet, barley and wheat are under investigation to assess the potential risks they may pose to the environment. The likelihood and extent of gene flow to non-GM commercial varieties (Loureiro *et al.*, 2003; Sweet, 2003; Van Acker *et al.*, 2003; Weekes *et al.*, 2003) or in the case of sugar beet to its wild relative *Beta maritime* are under investigation (Pohl-Orf *et al.*, 2000; Arnaud *et al.*, 2003; Bartsch *et al.*, 2003a; Bartsch *et al.*, 2003b). The passive trap designed for this study would be ideal for analysis of wind dispersal of pollen from these types of crops.

The study of bee-plant relationships has wide application and the ability to extract DNA from pollen pellets offers a new method of detecting what species bees have been foraging on. In addition, long range biotic dispersal distances could be elucidated as molecular marker analyses can now be carried out on pollen pellets, which may be a more efficient technique than analyses using pollen incorporating fluorescent dyes or other tracking systems (Osborne *et al.*, 1999; Beekman and Ratnieks, 2000; Williams and Osborne, 2002) or even to analyse whether bees are foraging on nearby sources or travelling to distant sources regardless of the local supply (Heinrich, 1976; Dramstad, 1996; Thompson, 1996; Saville *et al.*, 1997; Osborne *et al.*, 1999; Walther-Hellwig and Frankl, 2000; Osborne and Williams, 2001).

6.4 Conclusion

In conclusion, the detection of low levels of *B. napus* pollen at outer distances tested, by both wind and insect pollen traps, has implications for transgene movement. The study has demonstrated pollen dispersal up to 1.6km and that if within hive pollen transfer occurs then dispersal up to 3.2km is likely. In addition, extrapolated data shows dispersal up to 6.5km. Gene flow detected by male sterile bait plants occurs over distances of at least 200m. Abiotic dispersal shows a maximum range of greater than 200m but less than 800m for Brassicaceae.

From these analyses it is evident that pollen dispersal and gene flow occur at considerable distances from an oilseed rape crop. It is therefore important to look beyond pollen dispersal towards frequency of hybrid formation, the fitness level of hybrids produced and the occurrence of back-crossing. It is important to ascertain whether the fitness levels of backcrossed progeny are lower, equal to or higher than parental species. The impact of a transgene on the surrounding environment is important where hybrids are formed as in the worst case scenario, a direct selective advantage could be conferred, which in turn would impact rate of introgression and persistence in the feral environment.

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No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
1		*Lolium*	*perenne*		Ryegrass		10/10/2000	TCD	leaf	CTAB	Fresh
2		*Sinapis*	*arvensis*		Charlock		13/10/2000	Plant, Grand Canal	leaf	CTAB	Fresh
3		*Raphanus*	*sativus*		Radish		13/10/2000	Plant, Dartry	leaf	CTAB	Fresh
4		*Sinapis*	*arvensis*		Charlock		18/10/2000	Plant, Grand Canal	pollen	CTAB	Fresh
5		*Sinapis*	*arvensis*		Charlock		18/10/2000	Plant, Grand Canal	pollen	CTAB	Fresh
6		*Sinapis*	*arvensis*		Charlock		18/10/2000	Plant, Grand Canal	pollen	CTAB	Fresh
7		*Sinapis*	*arvensis*		Charlock		18/10/2000	Plant, Grand Canal	pollen	CTAB	Fresh
8		*Raphanus*	*sativus*		Radish		18/10/2000	Plant, Dartry	pollen	CTAB	Fresh
9		*Raphanus*	*sativus*		Radish		18/10/2000	Plant, Dartry	pollen	CTAB	Fresh
10		*Raphanus*	*sativus*		Radish		18/10/2000	Plant, Dartry	pollen	CTAB	Fresh
11		*Raphanus*	*sativus*		Radish		18/10/2000	Plant, Dartry	pollen	CTAB	Fresh
12		*Sinapis*	*arvensis*		Charlock		18/10/2000		pollen	CTAB	Fresh
13		*Sinapis*	*arvensis*		Charlock		18/10/2000		pollen	CTAB	Fresh
14		*Sinapis*	*arvensis*		Charlock		18/10/2000		pollen	CTAB	Fresh
15		*Sinapis*	*arvensis*		Charlock		18/10/2000		pollen	CTAB	Fresh
16		*Sinapis*	*arvensis*		Charlock		06/11/2000	Plant, Grand Canal	leaf	Kit	Silica dried
17		*Raphanus*	*sativus*		Radish		06/11/2000	Plant, Dartry	leaf	Kit	Silica dried
18		*Sinapis*	*arvensis*		Charlock		06/11/2000	Plant, Grand Canal	pollen	Kit	Silica dried
19		*Raphanus*	*sativus*		Radish		06/11/2000	Plant, Dartry	pollen	Kit	Silica dried
20		*Sinapis*	*arvensis*		Charlock		08/11/2000	Plant, Grand Canal	leaf	Kit	Fresh
21		*Sinapis*	*arvensis*		Charlock		08/11/2000	Plant, Grand Canal	pollen	Kit	Fresh
22		*Sinapis*	*arvensis*		Charlock		08/11/2000	Plant, Grand Canal	pollen	Kit	Fresh
23		*Sinapis*	*arvensis*		Charlock		08/11/2000	Plant, Grand Canal	pollen	Kit	Fresh
24		*Raphanus*	*sativus*		Radish		08/11/2000	Plant, Dartry	leaf	Kit	Fresh
25		*Raphanus*	*sativus*		Radish		08/11/2000	Plant, Dartry	pollen	Kit	Fresh

No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
26		*Raphanus*	*sativus*		Radish		08/11/2000	Plant, Dartry	pollen	Kit	Fresh
27		*Raphanus*	*sativus*		Radish		08/11/2000	Plant, Dartry	pollen	Kit	Fresh
30		*Brassica*	*oleracea*	*capitata*	Cabbage		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
31		*Brassica*	*oleracea*	*capitata*	Cabbage		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
32		*Brassica*	*oleracea*	*capitata*	Cabbage		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
33		*Brassica*	*oleracea*	*gemmifera*	Sprout		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
34		*Brassica*	*oleracea*	*gemmifera*	Sprout		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
35		*Brassica*	*oleracea*	*gemmifera*	Sprout		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
36		*Brassica*	*oleracea*	*italica*	Broccoli		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
37		*Brassica*	*oleracea*	*italica*	Broccoli		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
38		*Brassica*	*oleracea*	*italica*	Broccoli		31/01/2001	Plant, Dunnes Stores	leaf	CTAB	Fresh
39		*Brassica*	*oleracea*	*italica*	Broccoli		31/01/2001	Plant, Dunnes Stores	floret	CTAB	Fresh
40		*Brassica*	*oleracea*	*italica*	Broccoli		31/01/2001	Plant, Dunnes Stores	floret	CTAB	Fresh
41		*Brassica*	*oleracea*	*italica*	Broccoli		31/01/2001	Plant, Dunnes Stores	floret	CTAB	Fresh
42	TH6	*Brassica*	*oleracea*	*gemmifera*	Sprout		02/04/2001	Plant, Dartry	leaf	CTAB	Fresh
43	TH6	*Brassica*	*oleracea*	*gemmifera*	Sprout		02/04/2001	Plant, Dartry	leaf	CTAB	Fresh
44	TH7	*Brassica*	*oleracea*	*gemmifera*	Sprout		02/04/2001	Plant, Dartry	leaf	CTAB	Fresh
45	TH7	*Brassica*	*oleracea*	*gemmifera*	Sprout		02/04/2001	Plant, Dartry	leaf	CTAB	Fresh
46	TH1	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
47	TH1	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
48	TH2	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
49	TH2	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
50	TH3	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
51	TH3	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
52	TH4	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh

No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
53	TH4	*Brassica*	*rapa*		Wild turnip		02/04/2001	Dodder	leaf	CTAB	Fresh
54	TH8	*Brassica*	*oleracea*	*gemmifera*	Sprout		18/04/2001	Plant, Dartry	leaf	CTAB	Fresh
55	TH8	*Brassica*	*oleracea*	*gemmifera*	Sprout		18/04/2001	Plant, Dartry	leaf	CTAB	Fresh
56	TH9	*Brassica*	*oleracea*	*gemmifera*	Sprout		18/04/2001	Plant, Dartry	leaf	CTAB	Fresh
57	TH9	*Brassica*	*oleracea*	*gemmifera*	Sprout		18/04/2001	Plant, Dartry	leaf	CTAB	Fresh
58	MW1	*Brassica*	*oleracea*	*gemmifera*	Sprout	Wellington	18/04/2001	Commercial seed	leaf	CTAB	Fresh
59	MW1	*Brassica*	*oleracea*	*gemmifera*	Sprout	Wellington	18/04/2001	Commercial seed	leaf	CTAB	Fresh
60	MW2	*Brassica*	*oleracea*	*gemmifera*	Sprout	Wellington	18/04/2001	Commercial seed	leaf	CTAB	Fresh
61	MW2	*Brassica*	*oleracea*	*gemmifera*	Sprout	Wellington	18/04/2001	Commercial seed	leaf	CTAB	Fresh
62	BA1	*Brassica*	*napus*	*napobrassica*	Swede	Best of all	19/04/2001	Commercial seed	leaf	CTAB	Fresh
63	BA1	*Brassica*	*napus*	*napobrassica*	Swede	Best of all	19/04/2001	Commercial seed	leaf	CTAB	Fresh
64	BA2	*Brassica*	*napus*	*napobrassica*	Swede	Best of all	19/04/2001	Commercial seed	leaf	CTAB	Fresh
65	BA2	*Brassica*	*napus*	*napobrassica*	Swede	Best of all	19/04/2001	Commercial seed	leaf	CTAB	Fresh
66	SG1	*Raphanus*	*sativus*		Radish	Scarlet globe	19/04/2001	Commercial seed	leaf	CTAB	Fresh
67	SG1	*Raphanus*	*sativus*		Radish	Scarlet globe	19/04/2001	Commercial seed	leaf	CTAB	Fresh
68	SG2	*Raphanus*	*sativus*		Radish	Scarlet globe	19/04/2001	Commercial seed	leaf	CTAB	Fresh
69	SG2	*Raphanus*	*sativus*		Radish	Scarlet globe	19/04/2001	Commercial seed	leaf	CTAB	Fresh
70	SE1	*Brassica*	*rapa*	*rapifera*	Turnip	Snowball early white stone	19/04/2001	Commercial seed	leaf	CTAB	Fresh
71	SE1	*Brassica*	*rapa*	*rapifera*	Turnip	Snowball early white stone	19/04/2001	Commercial seed	leaf	CTAB	Fresh
72	SE2	*Brassica*	*rapa*	*rapifera*	Turnip	Snowball early white stone	19/04/2001	Commercial seed	leaf	CTAB	Fresh
73	SE2	*Brassica*	*rapa*	*rapifera*	Turnip	Snowball early white stone	19/04/2001	Commercial seed	leaf	CTAB	Fresh
74	PTM1	*Brassica*	*rapa*	*rapifera*	Turnip	Purple top Milan	19/04/2001	Commercial seed	leaf	CTAB	Fresh
75	PTM1	*Brassica*	*rapa*	*rapifera*	Turnip	Purple top Milan	19/04/2001	Commercial seed	leaf	CTAB	Fresh
76	PTM2	*Brassica*	*rapa*	*rapifera*	Turnip	Purple top Milan	19/04/2001	Commercial seed	leaf	CTAB	Fresh
77	PTM2	*Brassica*	*rapa*	*rapifera*	Turnip	Purple top Milan	19/04/2001	Commercial seed	leaf	CTAB	Fresh

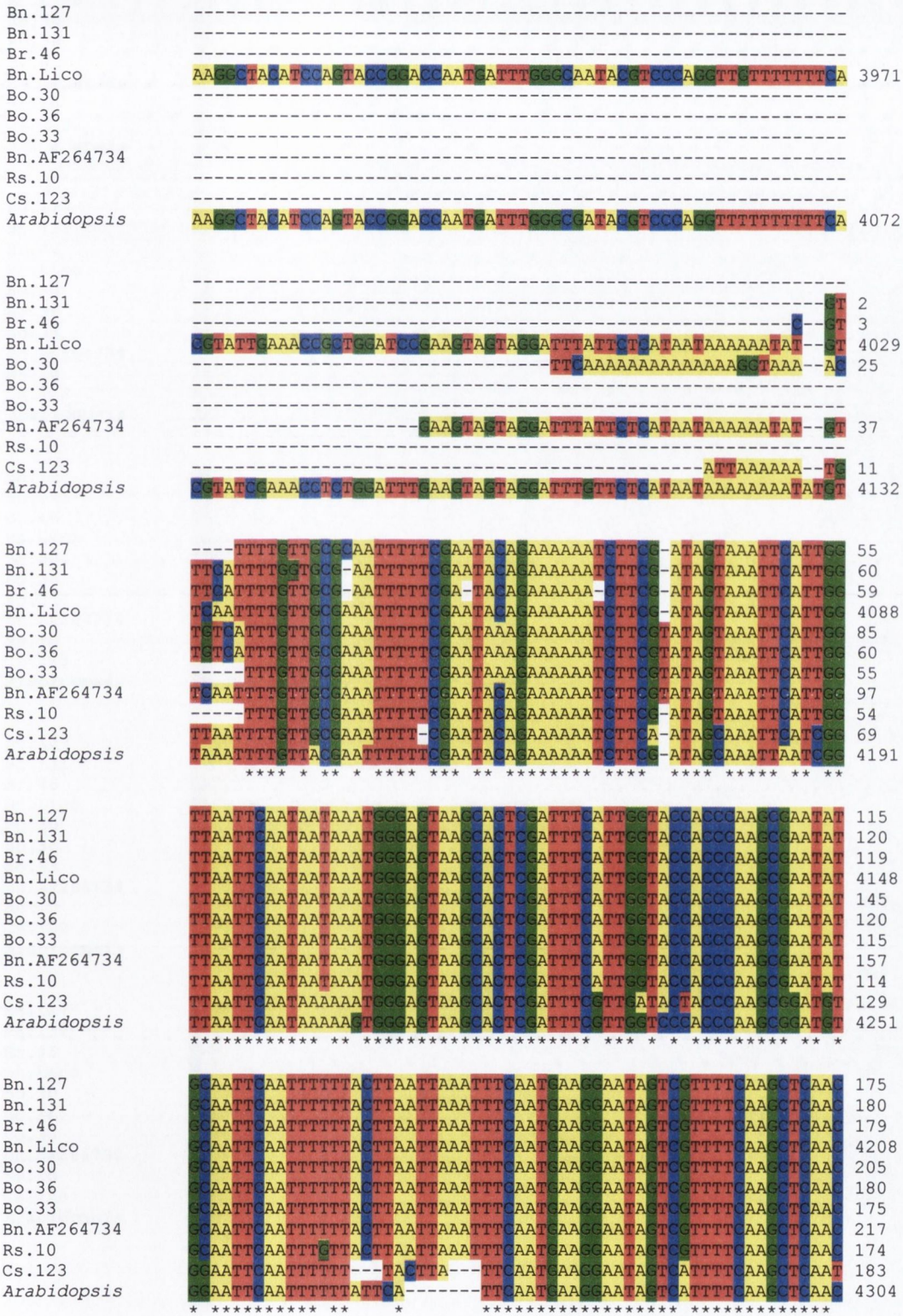
No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
78	OC1	*Brassica*	*oleracea*	*capitata*	Cabbage	Old common cabbage	19/04/2001	Commercial seed	leaf	CTAB	Fresh
79	OC1	*Brassica*	*oleracea*	*capitata*	Cabbage	Old common cabbage	19/04/2001	Commercial seed	leaf	CTAB	Fresh
82	CS1	*Brassica*	*oleracea*	*capitata*	Cabbage	Savoy		Commercial seed	leaf	CTAB	Fresh
83	CS1	*Brassica*	*oleracea*	*capitata*	Cabbage	Savoy		Commercial seed	leaf	CTAB	Fresh
84	CS2	*Brassica*	*oleracea*	*capitata*	Cabbage	Savoy		Commercial seed	leaf	CTAB	Fresh
85	CS2	*Brassica*	*oleracea*	*capitata*	Cabbage	Savoy		Commercial seed	leaf	CTAB	Fresh
86	DE1	*Brassica*	*oleracea*	*capitata*	Cabbage	Durham early	08/05/2001	Commercial seed	leaf	CTAB	Fresh
87	DE1	*Brassica*	*oleracea*	*capitata*	Cabbage	Durham early	08/05/2001	Commercial seed	leaf	CTAB	Fresh
88	DE2	*Brassica*	*oleracea*	*capitata*	Cabbage	Durham early	08/05/2001	Commercial seed	leaf	CTAB	Fresh
89	DE2	*Brassica*	*oleracea*	*capitata*	Cabbage	Durham early	08/05/2001	Commercial seed	leaf	CTAB	Fresh
90	MU1	*Brassica*	*nigra?*		Mustard		08/05/2001	Mustard Seed (Jar)			
91	MU1	*Brassica*	*nigra?*		Mustard		08/05/2001	Mustard Seed (Jar)			
92	MU2	*Brassica*	*nigra?*		Mustard		08/05/2001	Mustard Seed (Jar)			
93		*Sinapis*	*arvensis*		Charlock		21/05/2001	Plant, Oakpark			
94		*Sinapis*	*arvensis*		Charlock		21/05/2001	Plant, Oakpark			
95		*Brassica*	*napus*		Charlock		21/05/2001	Plant, Oakpark			
96		*Brassica*	*napus*		Charlock		21/05/2001	Plant, Oakpark			
97		*Sinapis*	*arvensis*		Charlock		21/05/2001	Plant, Oakpark			
98		*Sinapis*	*arvensis*		Charlock		21/05/2001	Plant, Oakpark			
99		*Sinapis*	*arvensis*		Charlock		21/05/2001	Plant, Knockbeg			
100		*Sinapis*	*arvensis*		Charlock		21/05/2001	Plant, Knockbeg			
101		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	18/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
102		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	19/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
103		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	20/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
104		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	21/06/2002	Plant, Oakpark	leaf	CTAB	Fresh

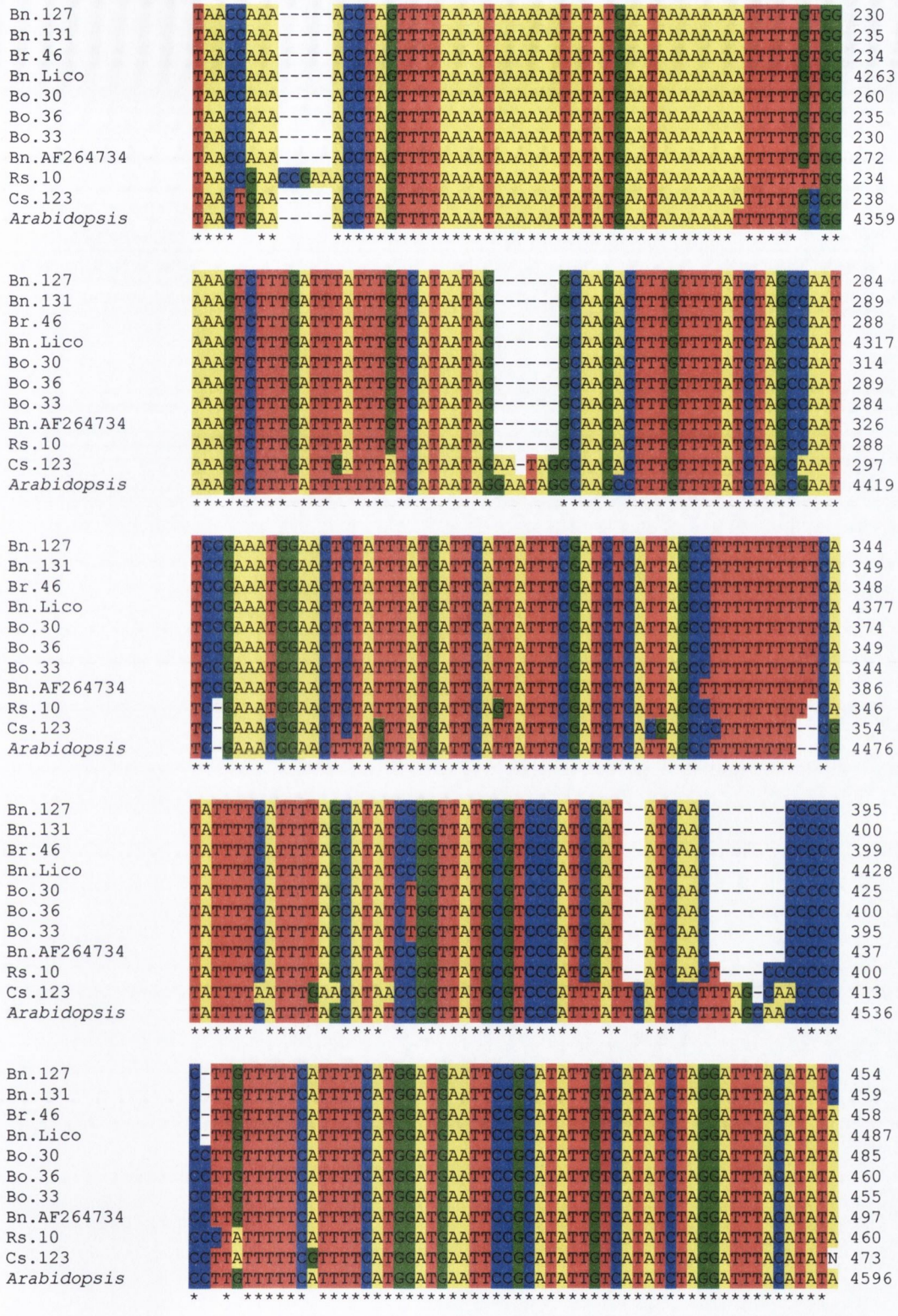
No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
105		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	22/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
106		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	23/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
107		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	24/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
108		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	25/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
109		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	26/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
110		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	27/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
111		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	28/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
112		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	29/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
113		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	30/06/2002	Plant, Oakpark	leaf	CTAB	Fresh
114		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	01/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
115		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	02/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
116		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	03/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
117		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	04/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
118		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	05/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
119		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	06/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
120		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	07/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
121		*Epilobium*	*angustifolium*		Rosebay Willow Herb		19/07/2001	Plant, Oakpark	leaf	CTAB	Fresh
122		*Epilobium*	*angustifolium*		Rosebay Willow Herb		19/07/2001	Plant, Oakpark	leaf	CTAB	Fresh
123		*Camelina*	*sativus*		Camelina		19/07/2001	Plant, Oakpark	leaf	CTAB	Fresh
124		*Camelina*	*sativus*		Camelina		19/07/2001	Plant, Oakpark	leaf	CTAB	Fresh
125		*Filipendula*	*ulmaria*		Meadowsweet		19/07/2001	Plant, Oakpark	leaf	CTAB	Fresh
126		*Filipendula*	*ulmaria*		Meadowsweet		19/07/2001	Plant, Oakpark	leaf	CTAB	Fresh
127		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	23/10/2001	Plant, Oakpark	leaf	CTAB	Fresh
128		*Brassica*	*napus*		Oil seed rape	Marinka	23/10/2001	Plant, Oakpark	leaf	CTAB	Fresh
129		*Phaseolus*			Bean		23/10/2001	Plant, Oakpark	leaf	CTAB	Fresh

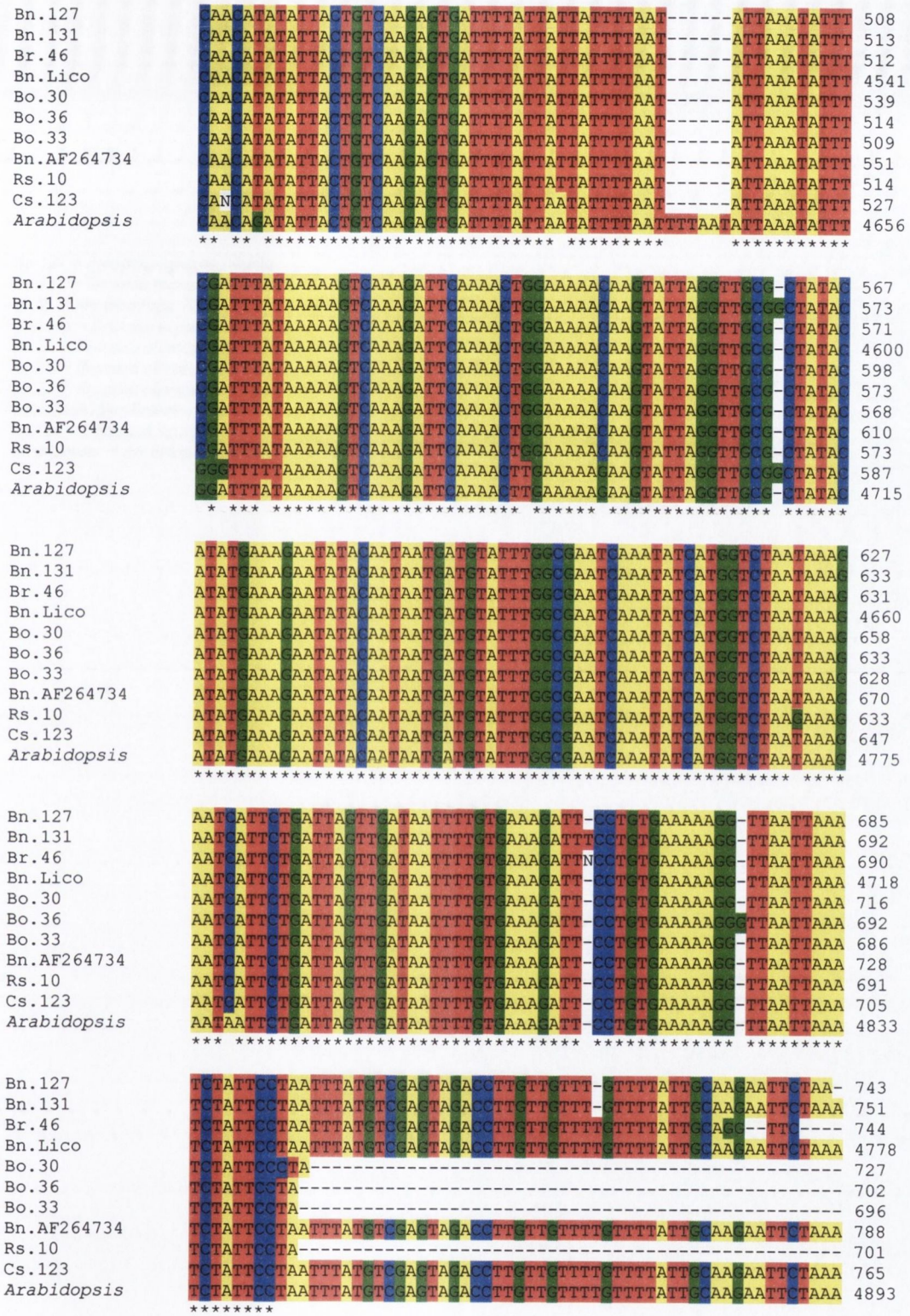
No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
130		*Phaseolus*			Bean		23/10/2001	Plant, Oakpark	leaf	CTAB	Fresh
131		*Brassica*	*napus*	*napus*	Oil seed rape	Shiela	23/10/2001	Plant, Oakpark	leaf	CTAB	Fresh
132		*Brassica*	*napus*	*napus*	Oil seed rape	Shiela	23/10/2001	Plant, Oakpark	leaf	CTAB	Fresh
133		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	20/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
134		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	21/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
135		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	22/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
136		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	23/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
137		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	24/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
138		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	25/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
139		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	26/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
140		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	27/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
141		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	28/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
142		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	29/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
143		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	30/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
144		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	31/07/2002	Plant, Oakpark	leaf	CTAB	Fresh
145		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	01/08/2002	Plant, Oakpark	leaf	CTAB	Fresh
146		*Brassica*	*napus*	*napus*	Oil seed rape	Marinka	02/08/2002	Plant, Oakpark	leaf	CTAB	Fresh
147		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Jura	03/08/2002	CPB-Twyford	seed	CTAB	Fresh
148		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Jura	04/08/2002	CPB-Twyford	seed	CTAB	Fresh
149		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Jura	05/08/2002	CPB-Twyford	seed	CTAB	Fresh
150		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Triolo	06/08/2002	SCRI	seed	CTAB	Fresh
151		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Triolo	07/08/2002	SCRI	seed	CTAB	Fresh
152		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Triolo	08/08/2002	SCRI	seed	CTAB	Fresh
153		*Brassica*	*napus*	*napus*	Oil seed rape	Male sterile, Triolo	09/08/2002	SCRI	seed	CTAB	Fresh
154	AD001	*Arabidopsis*	*thaliana*			Landsberg		Nottingham SC	Rosette Leaf	Kit	Fresh

No.	Plt Id	Genus	Species	Subspecies	Name	Variety/ Cultivar	Date	Origin	Source	Extraction	Material
155	AD002	*Arabidopsis*	*thaliana*			Landsberg		Nottingham SC	Rosette Leaf	Kit	Fresh
156	AD003	*Arabidopsis*	*thaliana*			Landsberg		Nottingham SC	Rosette Leaf	Kit	Fresh
157	AD004	*Arabidopsis*	*thaliana*			Landsberg		Nottingham SC	Rosette Leaf	Kit	Fresh
158	AD005	*Arabidopsis*	*thaliana*			Landsberg erecta		Nottingham SC	Rosette Leaf	Kit	Fresh
159	AD006	*Arabidopsis*	*thaliana*			Landsberg erecta		Nottingham SC	Rosette Leaf	Kit	Fresh
160	AD007	*Arabidopsis*	*thaliana*			Landsberg erecta		Nottingham SC	Rosette Leaf	Kit	Fresh
161	AD008	*Arabidopsis*	*thaliana*			Landsberg erecta		Nottingham SC	Rosette Leaf	Kit	Fresh
162	AD009	*Arabidopsis*	*thaliana*			Wassilewskija		Nottingham SC	Rosette Leaf	Kit	Fresh
163	AD010	*Arabidopsis*	*thaliana*			Wassilewskija		Nottingham SC	Rosette Leaf	Kit	Fresh
164	AD011	*Arabidopsis*	*thaliana*			Wassilewskija		Nottingham SC	Rosette Leaf	Kit	Fresh
165	AD012	*Arabidopsis*	*thaliana*			Wassilewskija		Nottingham SC	Rosette Leaf	Kit	Fresh

Appendix 2.1: ClustalW (1.82) multiple sequence alignment gene region B




Bn.127
Bn.131
Br.46
Bn.Lico
Bo.30
Bo.36
Bo.33
Bn.AF264734
Rs.10
Cs.123
Arabidopsis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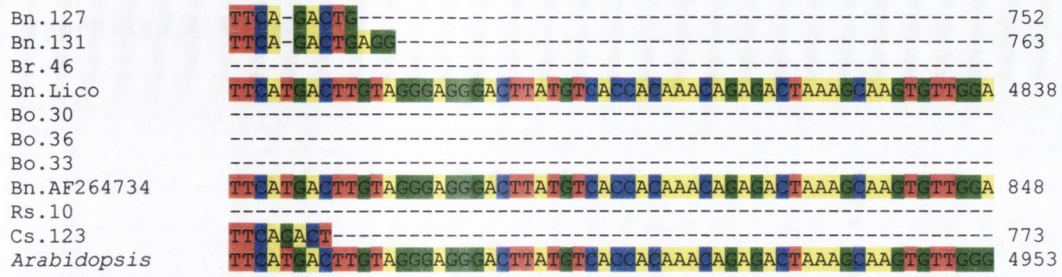
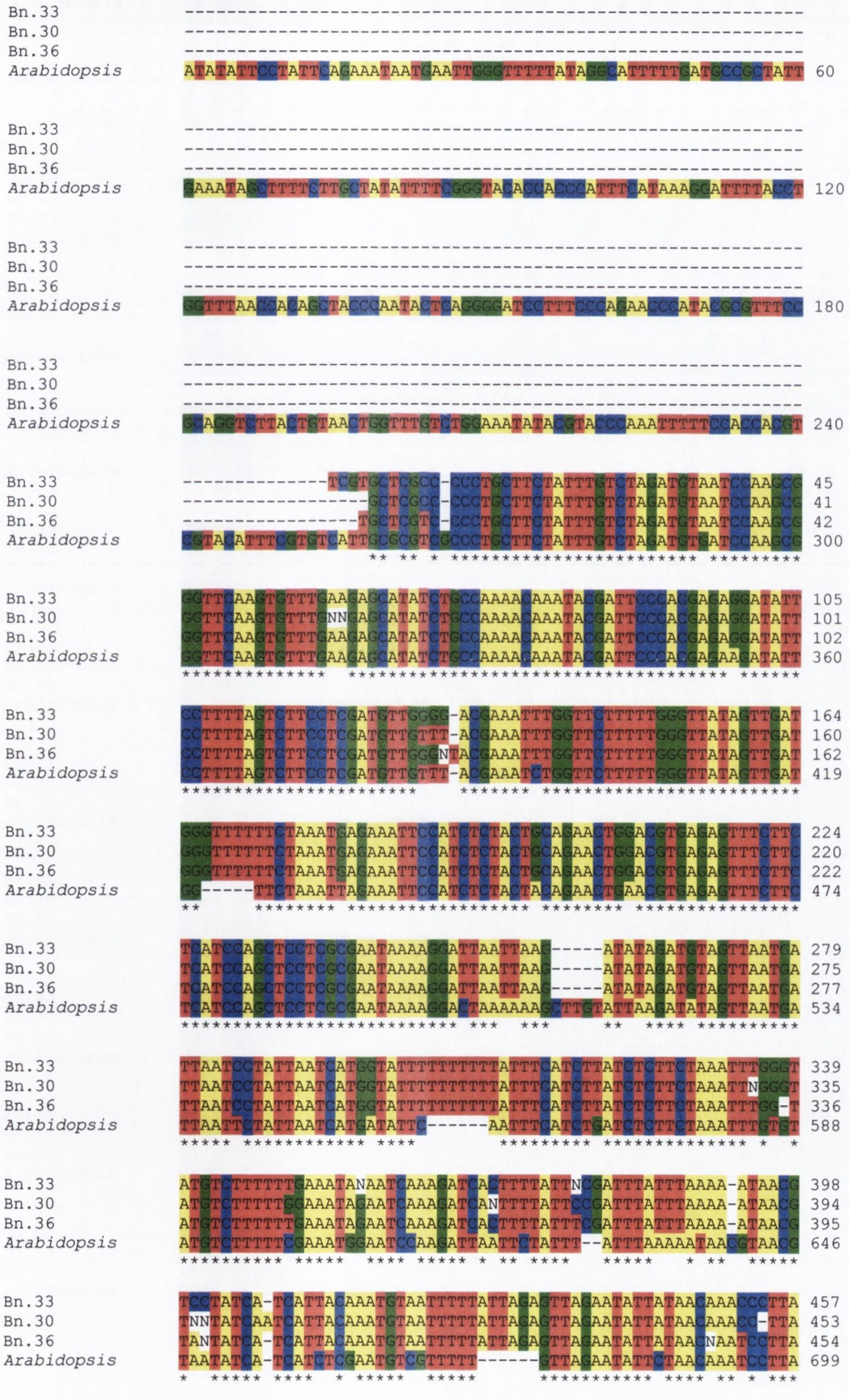
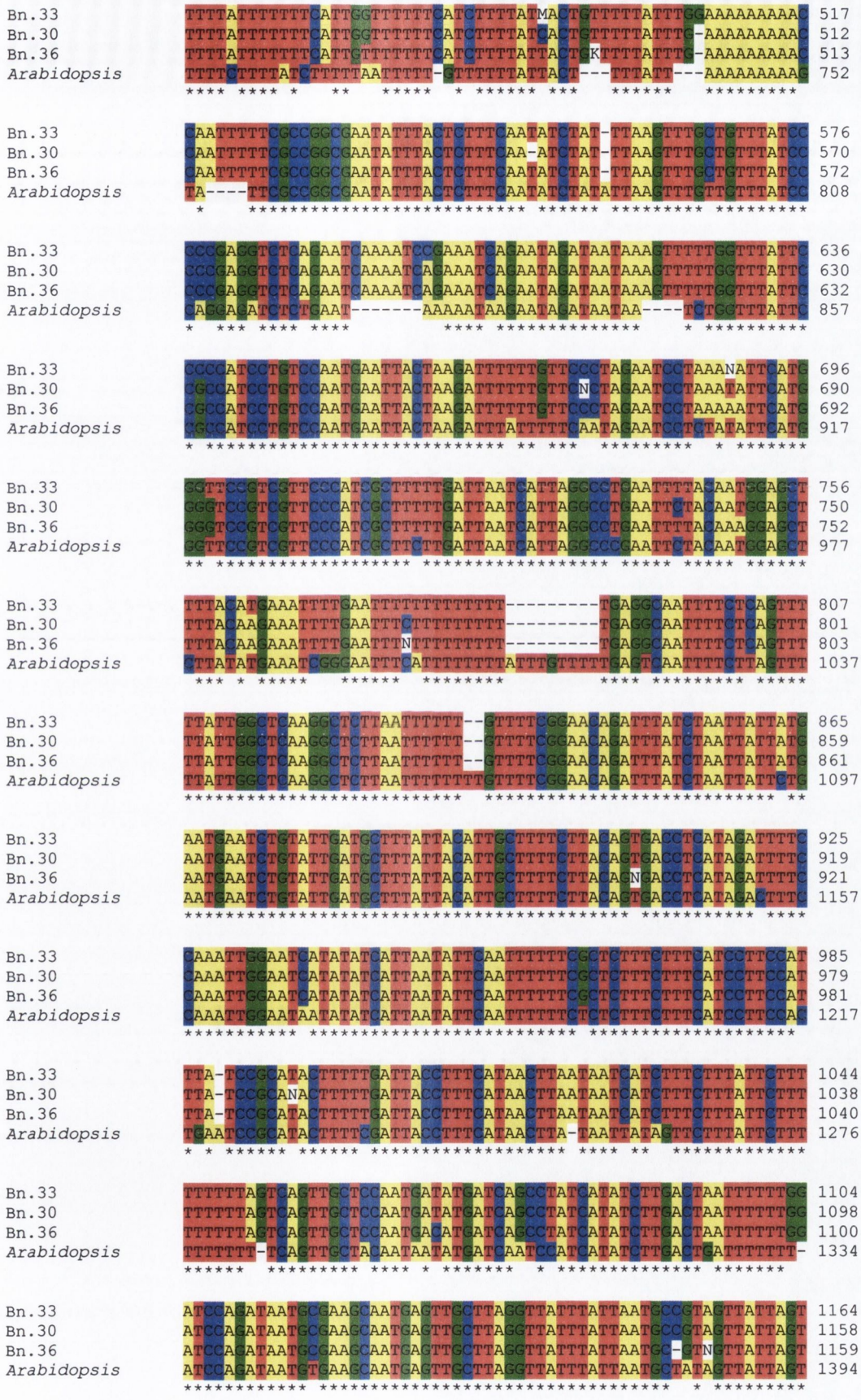
Bn.131
Br.46
Bn.Lico
Bo.30
Bo.36
Bo.33
Bn.AF264734
Rs.10
Cs.123
Arabidopsis

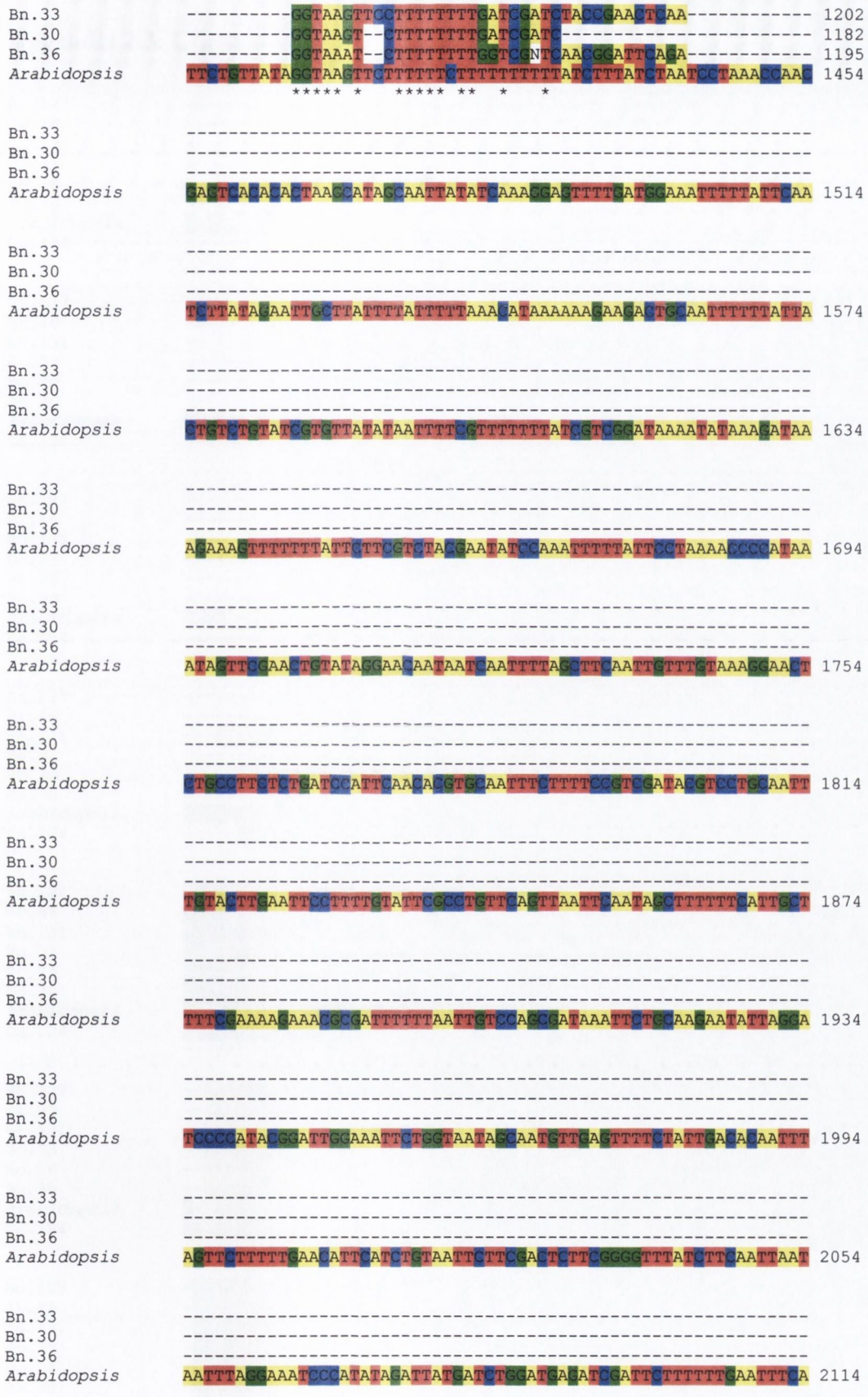


Bn.127 = *Brassica napus* cv. Sheila
Bn.131 = *Brassica napus* cv. Marinka
Br.46 = *Brassica rapa*
Bn.Lico = *Brassica napus* Licosomos
Bo.30 = *Brassica oleracea* ssp. capitata
Bo.36 = *Brassica oleracea* ssp. *italica*
Bo.33 = *Brassica oleracea* ssp. *gemmifera*
Bn.AF264734= *Brassica napus*
Cs.124 = *Camelina sativus*
Arabidopsis = *Arabidopsis thaliana*

Appendix 2.2: ClustalW (1.82) multiple sequence of the *rpl16* gene region



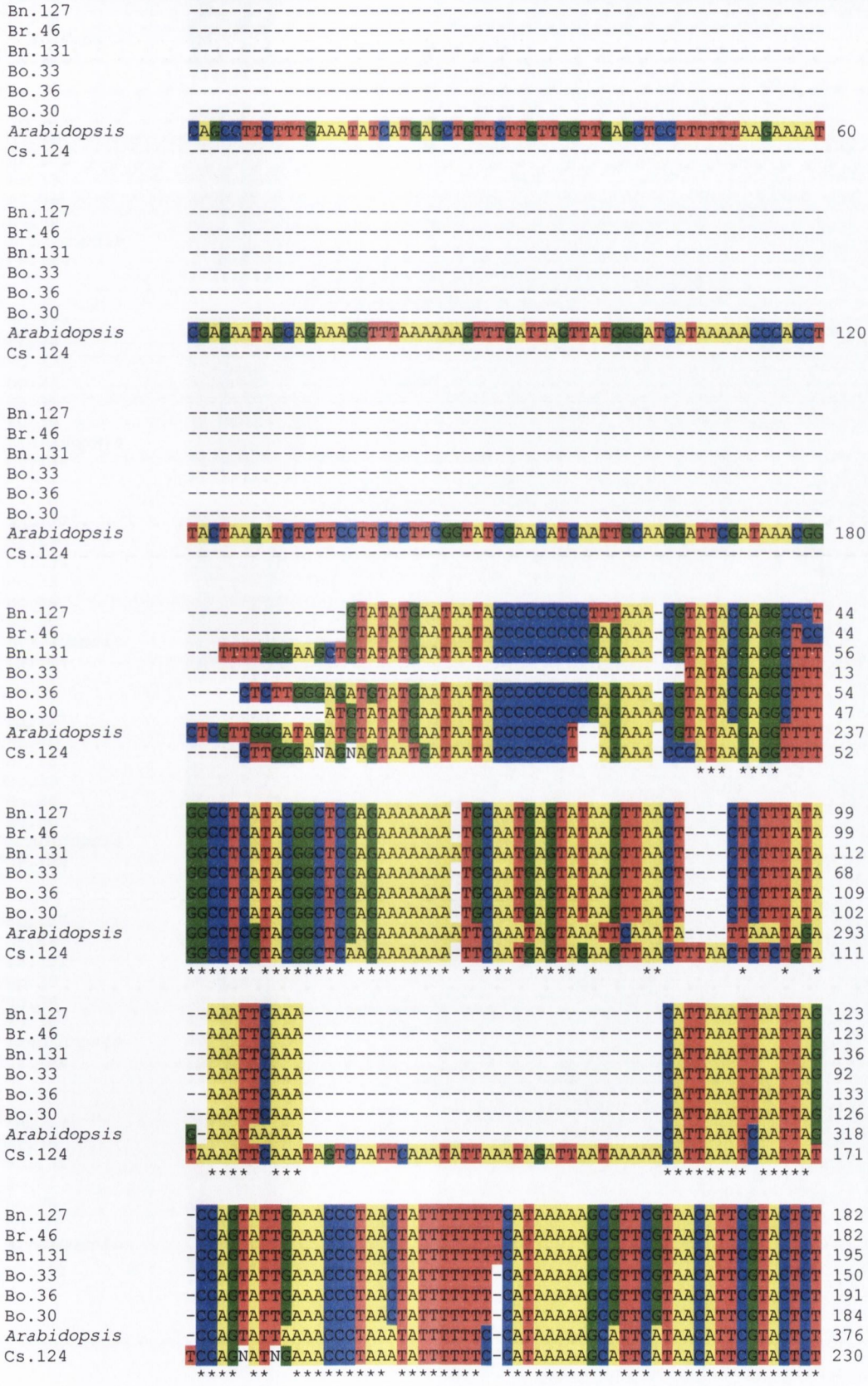

Bn.33
Bn.30
Bn.36
Arabidopsis

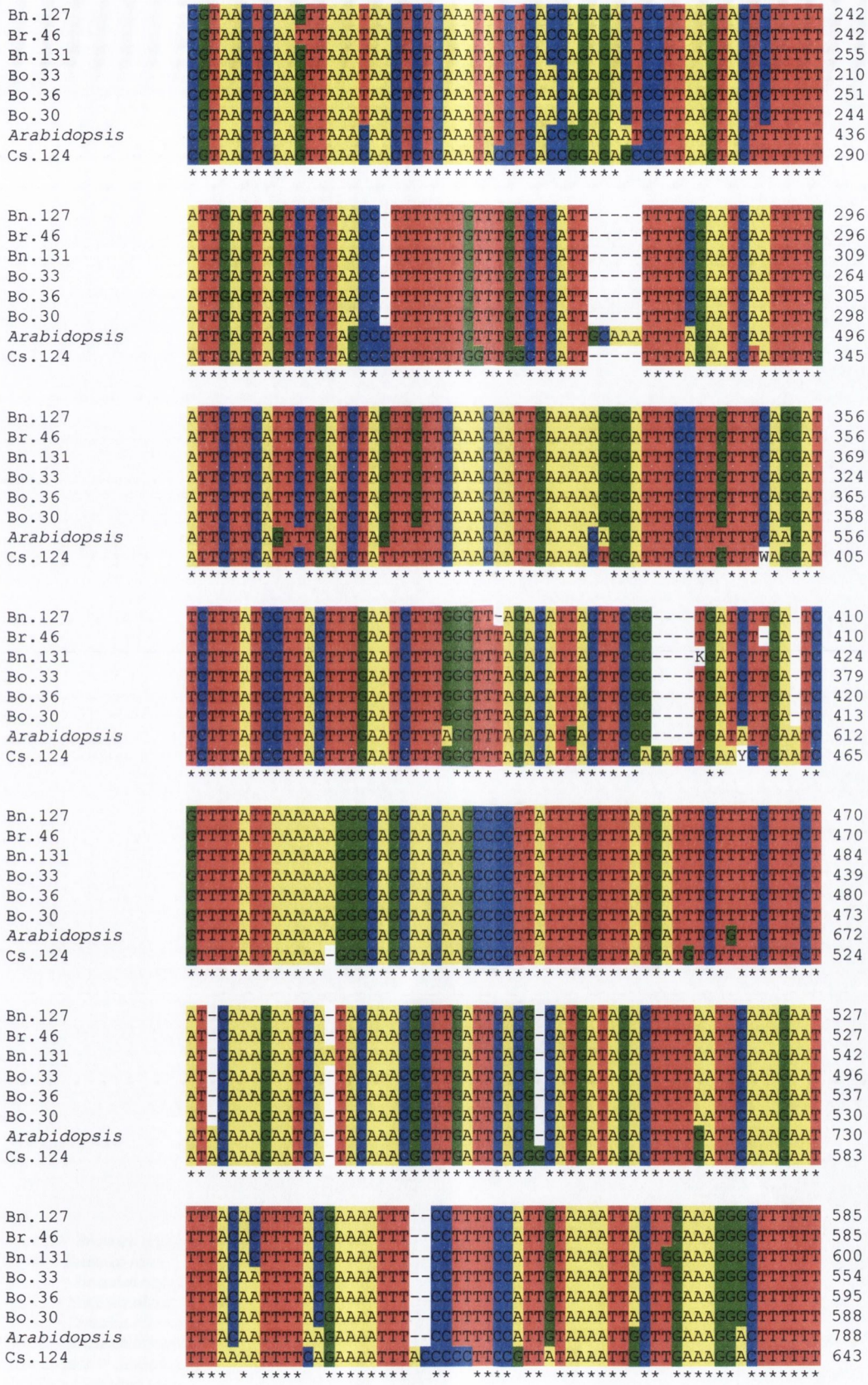


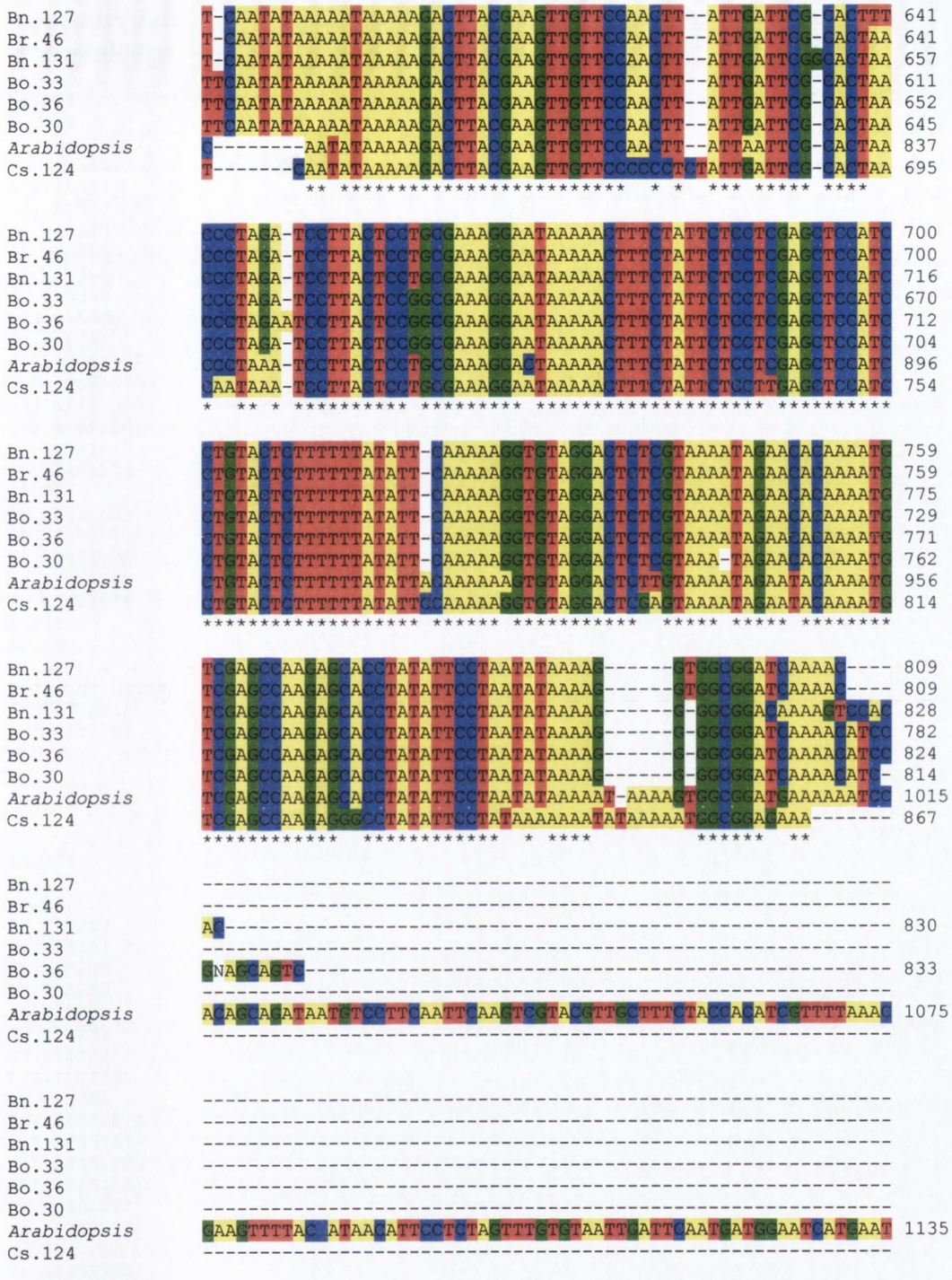
Key

Bo.30 = *Brassica oleracea* ssp. *capitata*
Bo.33 = *Brassica oleracea* ssp. *gemmifera*
Bo.36 = *Brassica oleracea* ssp. *italica*
Arabidopsis = *Arabidopsis thaliana*

Appendix 2.3: ClustalW (1.82) multiple sequence of gene region *rps16*



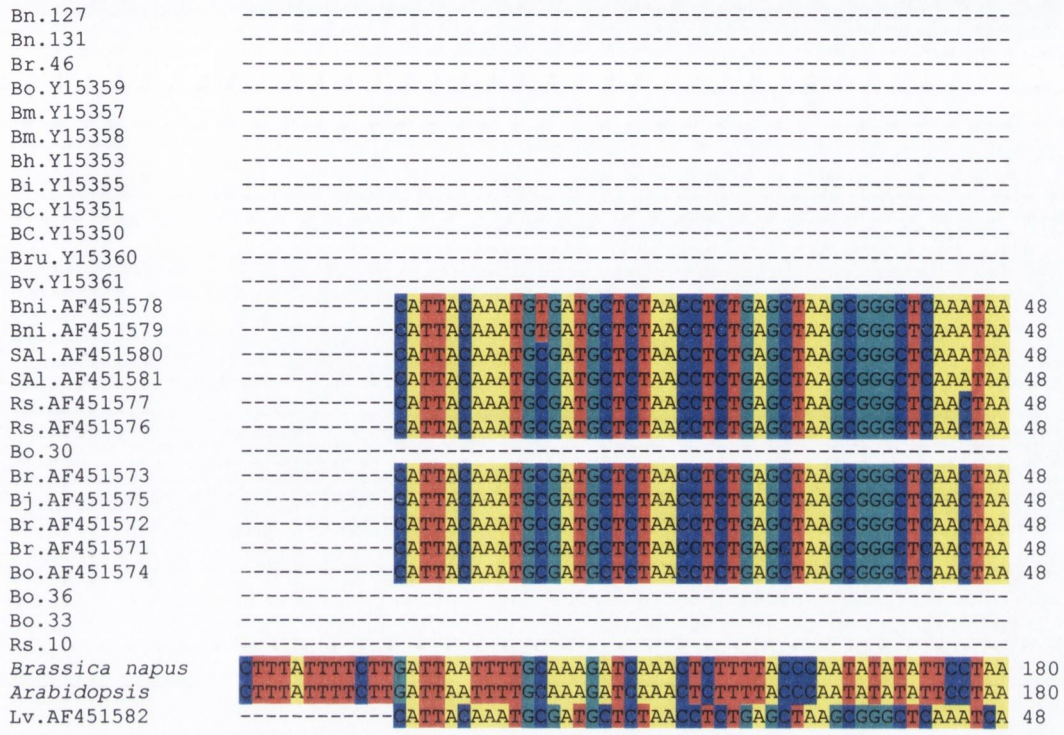

Bn.127
Br.46
Bn.131
Bo.33
Bo.36
Bo.30
Arabidopsis
Cs.124

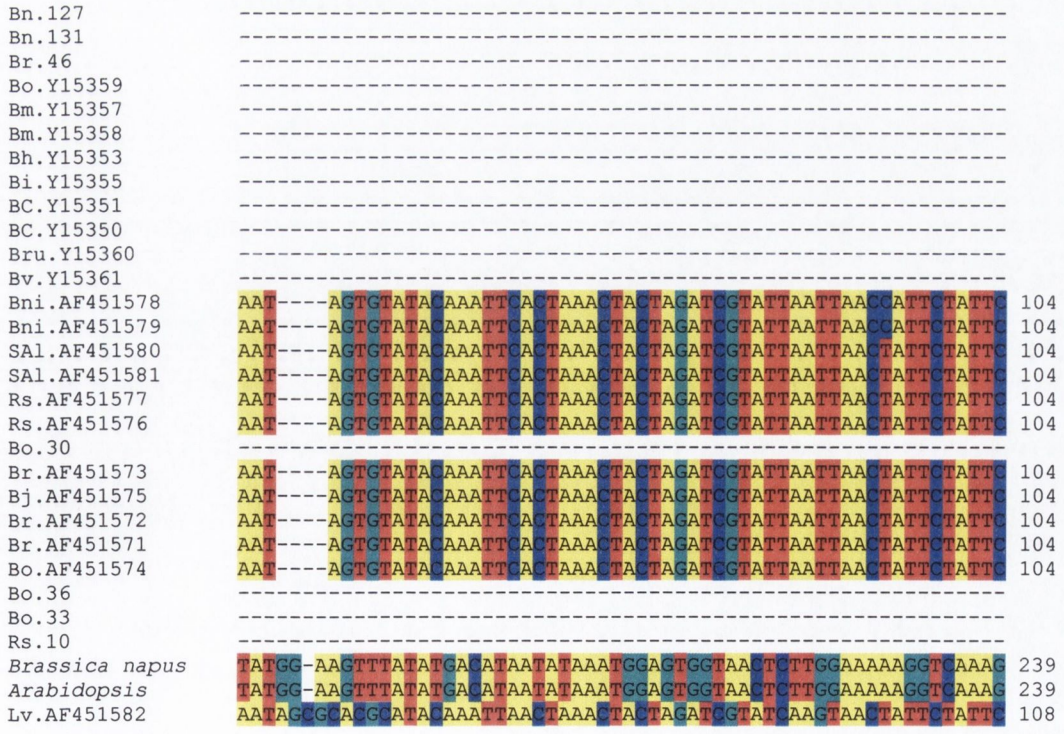


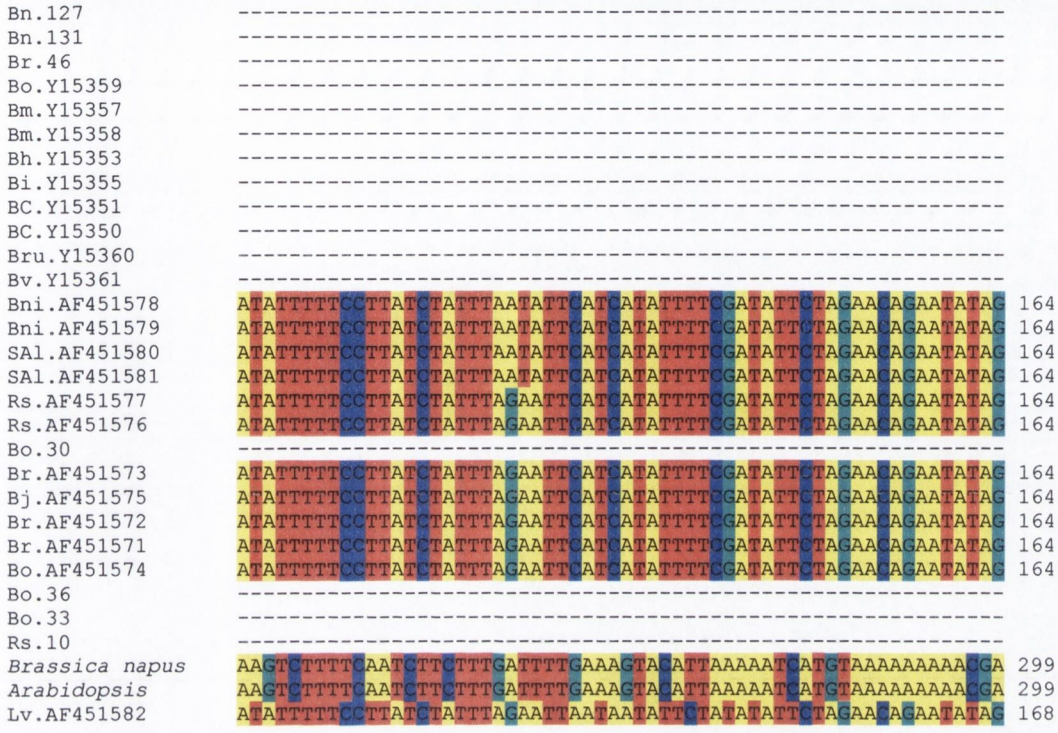
Key

Bn.127 = *Brassica napus* cv. Sheila
Br.46 = *Brassica rapa*
Bn.131 = *Brassica napus* cv. Marinka
Bo.33 = *Brassica oleracea* ssp. *gemmifera*
Bo.36 = *Brassica oleracea* ssp. *italica*
Bo.30 = *Brassica oleracea* ssp. *capitata*
Arabidopsis = *Arabidopsis thaliana*
Cs.124 = *Camelina sativus*

Appendix 2.4: ClustalW (1.82) multiple sequence alignment *trn*L intron, *trnL-F* intergenic spacer regions



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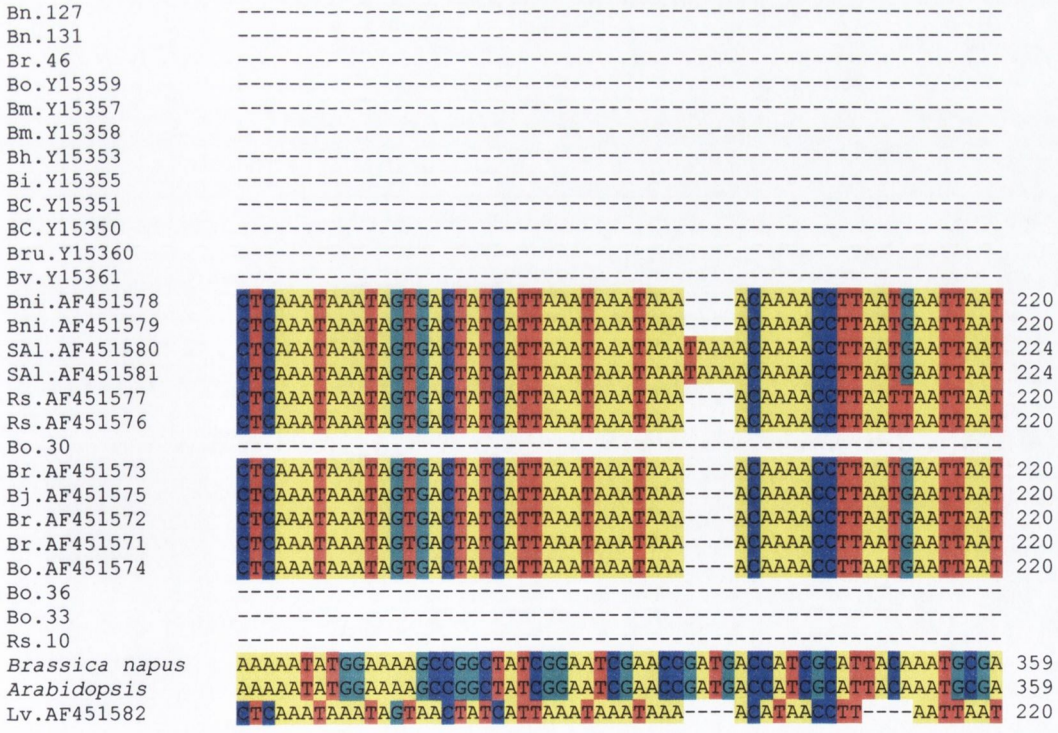
Bm.Y15357
Bm.Y15358
Bh.Y15353
Bi.Y15355
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
Bni.AF451578 164
Bni.AF451579 164
SAl.AF451580 164
SAl.AF451581 164
Rs.AF451577 164
Rs.AF451576 164
Bo.30
Br.AF451573 164
Bj.AF451575 164
Br.AF451572 164
Br.AF451571 164
Bo.AF451574 164
Bo.36
Bo.33
Rs.10
Brassica napus 299
Arabidopsis 299
Lv.AF451582 168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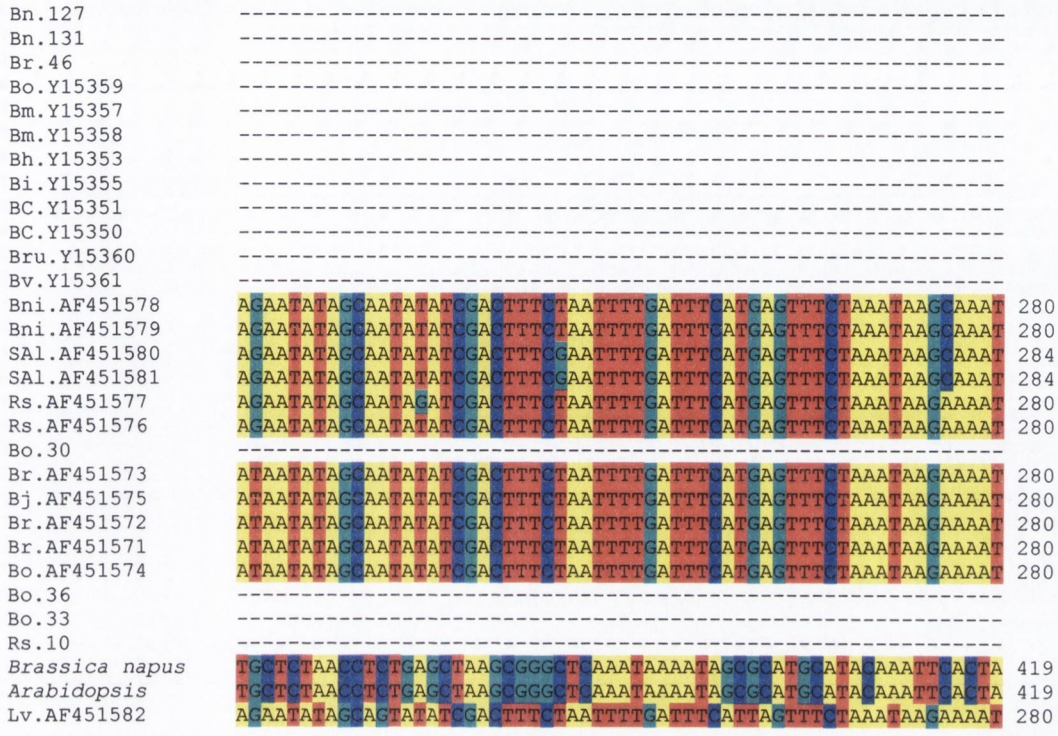
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
Bni.AF451578 220
Bni.AF451579 220
SAl.AF451580 224
SAl.AF451581 224
Rs.AF451577 220
Rs.AF451576 220
Bo.30
Br.AF451573 220
Bj.AF451575 220
Br.AF451572 220
Br.AF451571 220
Bo.AF451574 220
Bo.36
Bo.33
Rs.10
Brassica napus 359
Arabidopsis 359
Lv.AF451582 220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Bh.Y15353
Bi.Y15355
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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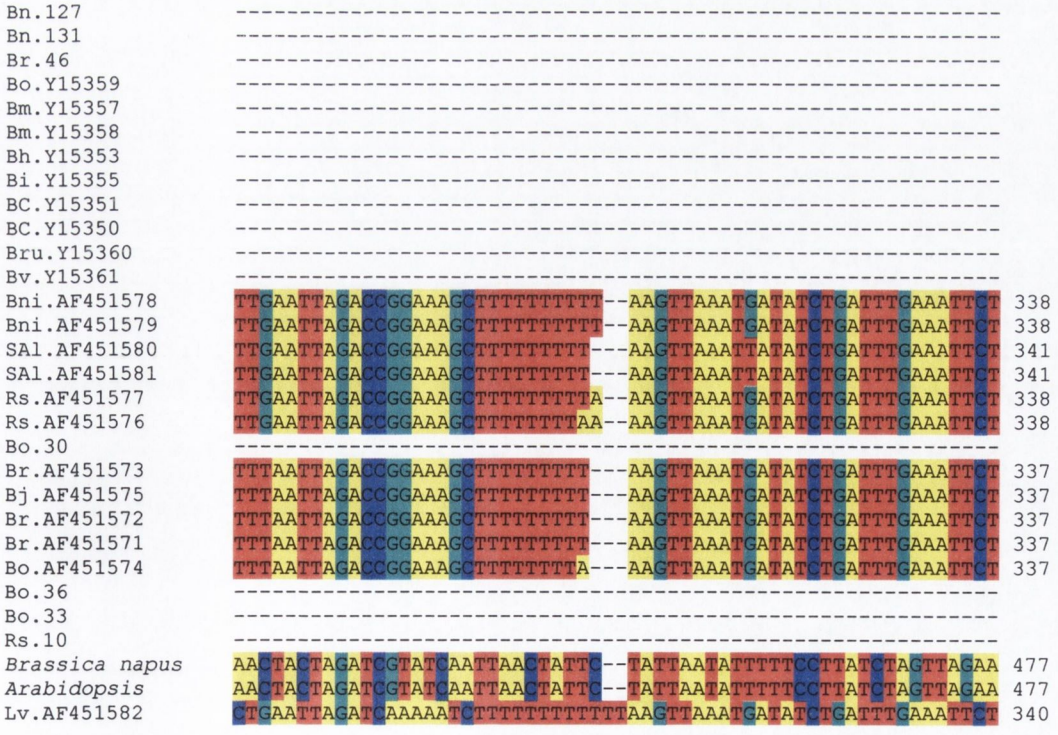
Bni.AF451579 280
SAl.AF451580 284
SAl.AF451581 284
Rs.AF451577 280
Rs.AF451576 280
Bo.30
Br.AF451573 280
Bj.AF451575 280
Br.AF451572 280
Br.AF451571 280
Bo.AF451574 280
Bo.36
Bo.33
Rs.10
Brassica napus 419
Arabidopsis 419
Lv.AF451582 280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Bru.Y15360
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Bni.AF451579 338
SAl.AF451580 341
SAl.AF451581 341
Rs.AF451577 338
Rs.AF451576 338
Bo.30
Br.AF451573 337
Bj.AF451575 337
Br.AF451572 337
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Bo.AF451574 337
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Bo.33
Rs.10
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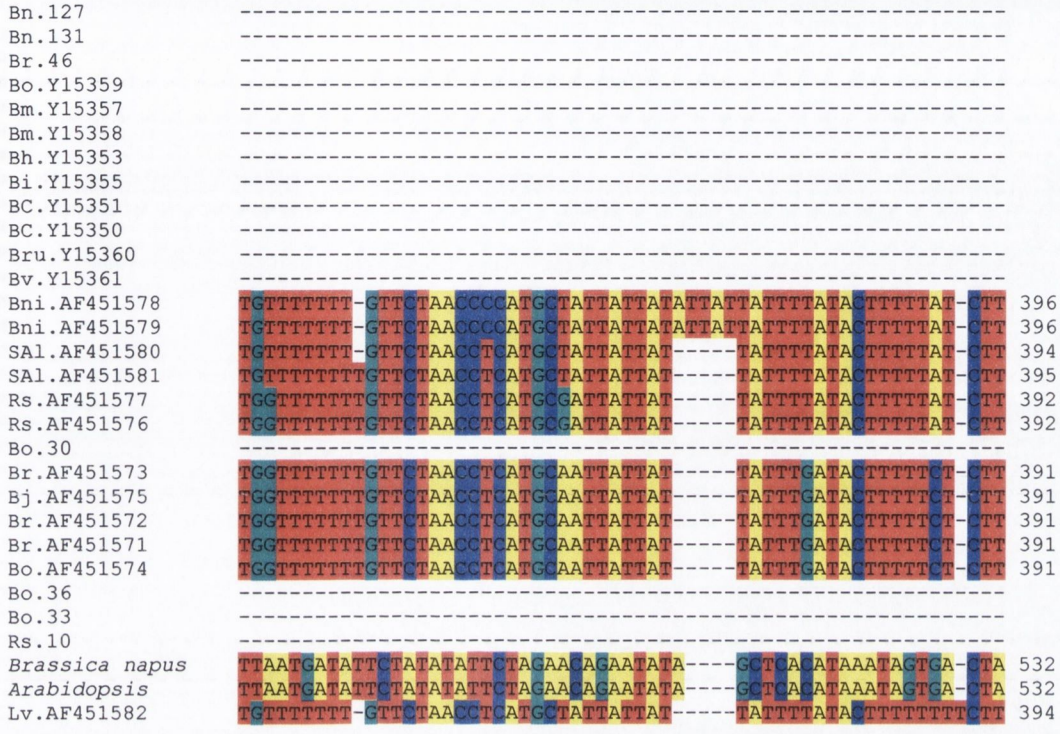
Arabidopsis 477
Lv.AF451582 340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SAl.AF451580
SAl.AF451581
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Rs.AF451576
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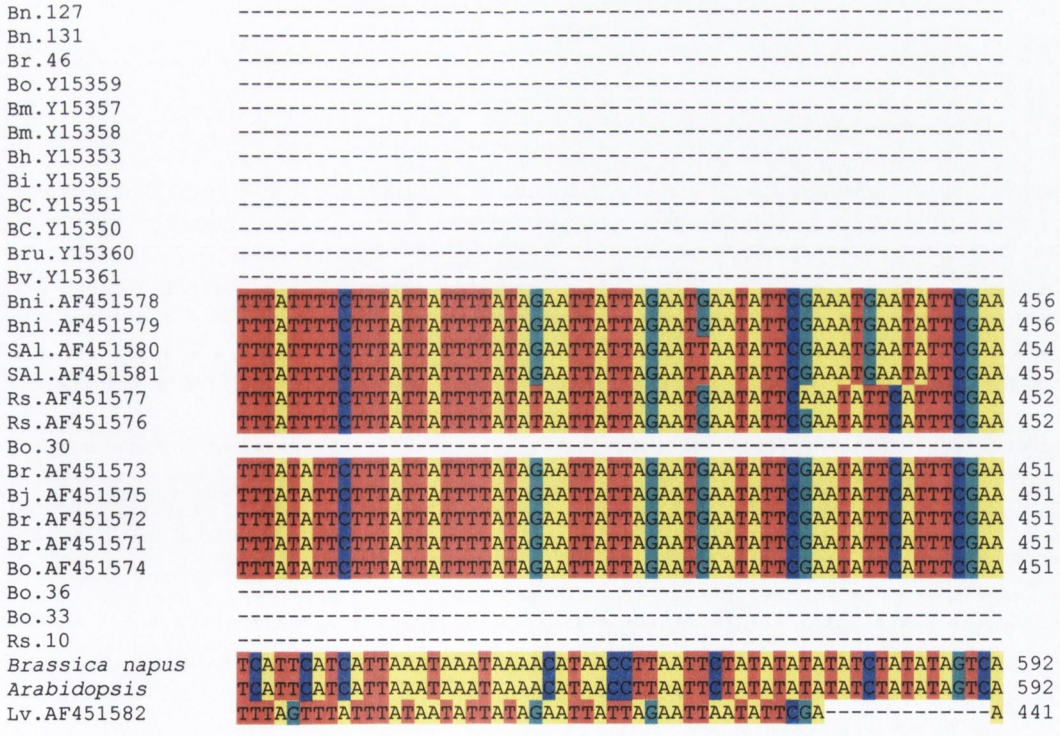
Arabidopsis
Lv.AF451582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Bo.AF451574
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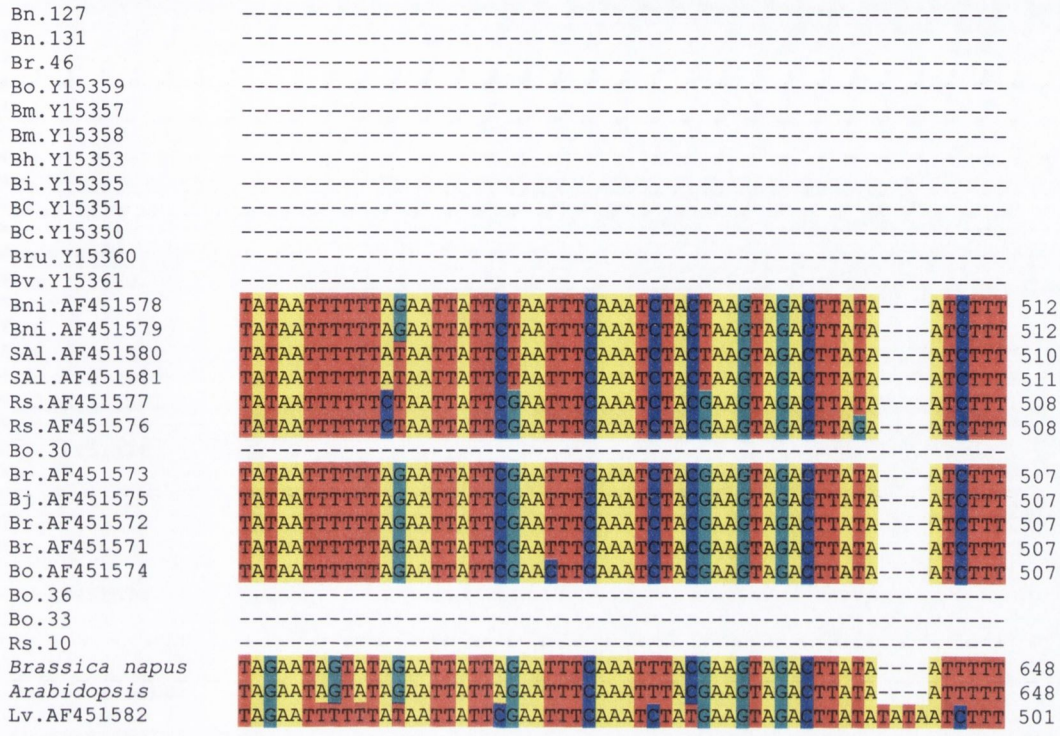
Bo.33
Rs.10
Brassica napus
Arabidopsis
Lv.AF451582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BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
Bni.AF451578 512
Bni.AF451579 512
SAl.AF451580 510
SAl.AF451581 511
Rs.AF451577 508
Rs.AF451576 508
Bo.30
Br.AF451573 507
Bj.AF451575 507
Br.AF451572 507
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Bo.AF451574 507
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Bo.33
Rs.10
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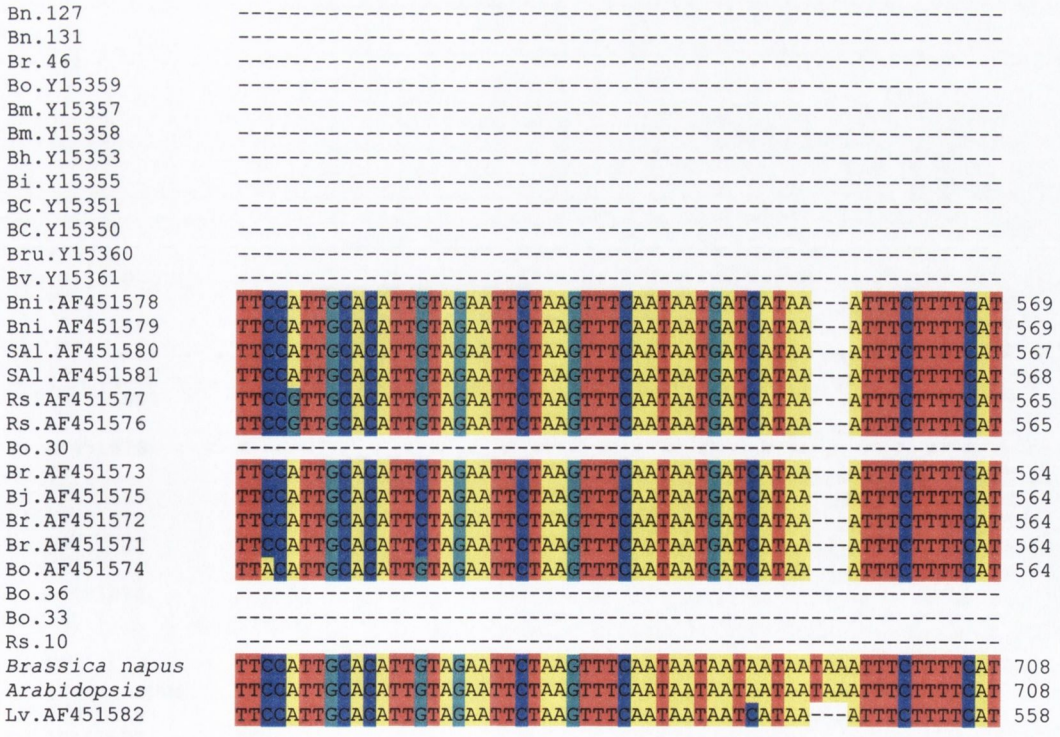
Arabidopsis 648
Lv.AF451582 501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BC.Y15350
Bru.Y15360
Bv.Y15361
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Bni.AF451579 569
SAl.AF451580 567
SAl.AF451581 568
Rs.AF451577 565
Rs.AF451576 565
Bo.30
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Bj.AF451575 564
Br.AF451572 564
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Bo.AF451574 564
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Bo.33
Rs.10
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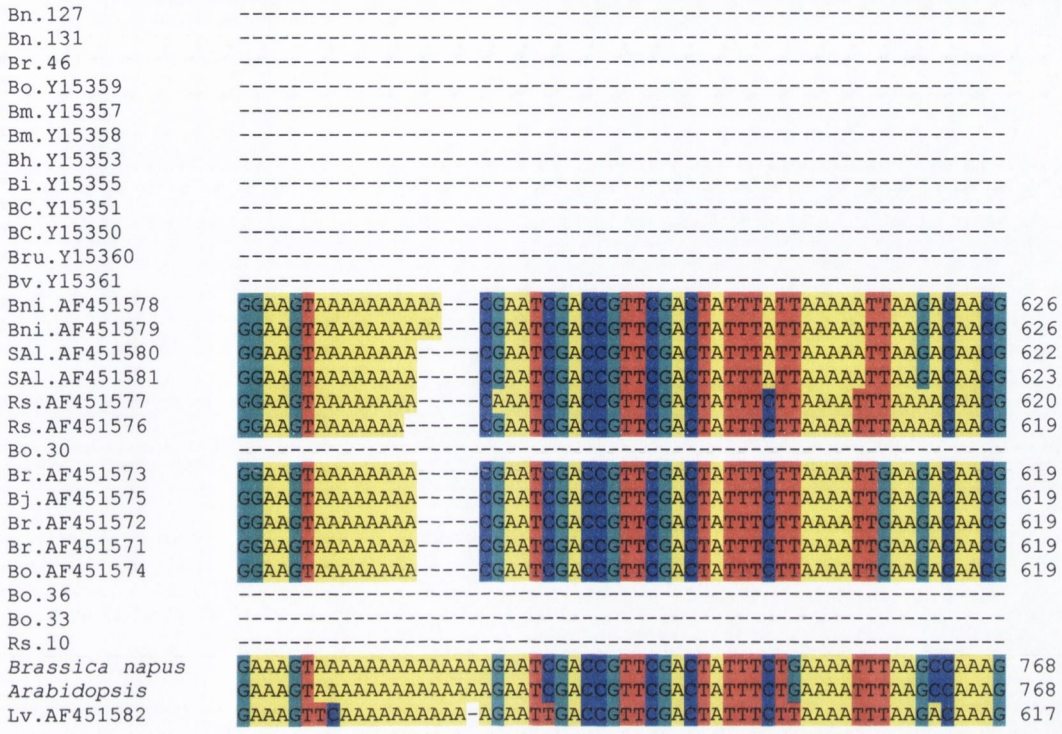
Arabidopsis 708
Lv.AF451582 558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Bi.Y15355
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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Bni.AF451579 626
SAl.AF451580 622
SAl.AF451581 623
Rs.AF451577 620
Rs.AF451576 619
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Bj.AF451575 619
Br.AF451572 619
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Bo.33
Rs.10
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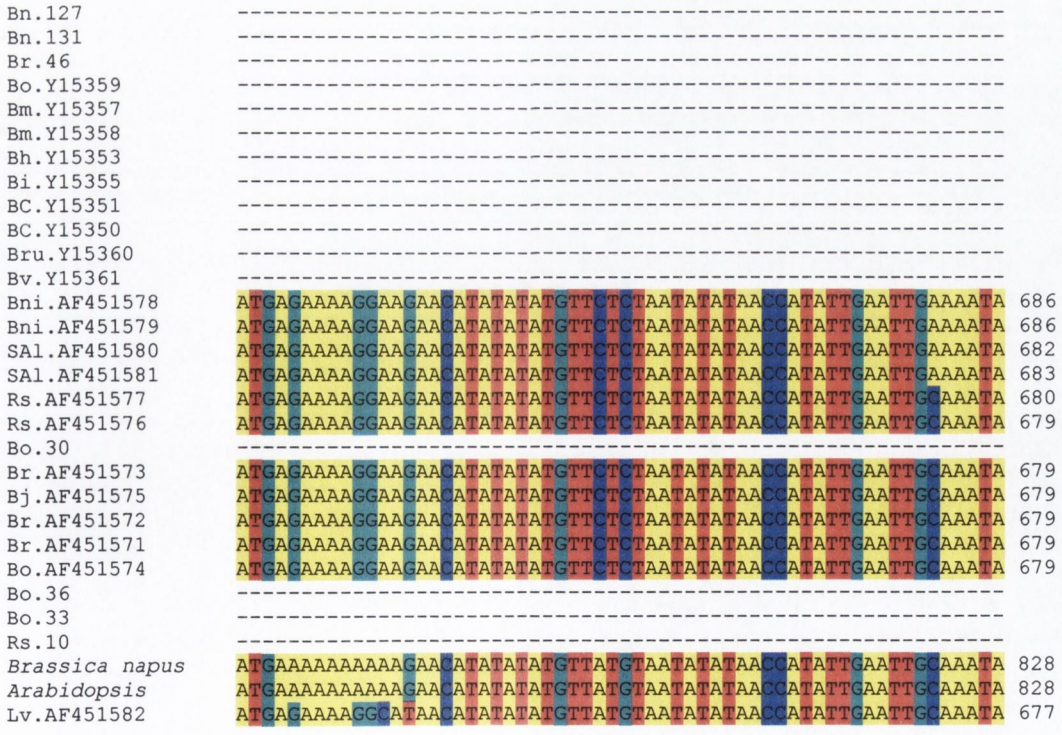
Arabidopsis 768
Lv.AF451582 617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BC.Y15350
Bru.Y15360
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Bni.AF451579 686
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SAl.AF451581 683
Rs.AF451577 680
Rs.AF451576 679
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Bj.AF451575 679
Br.AF451572 679
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Bo.33
Rs.10
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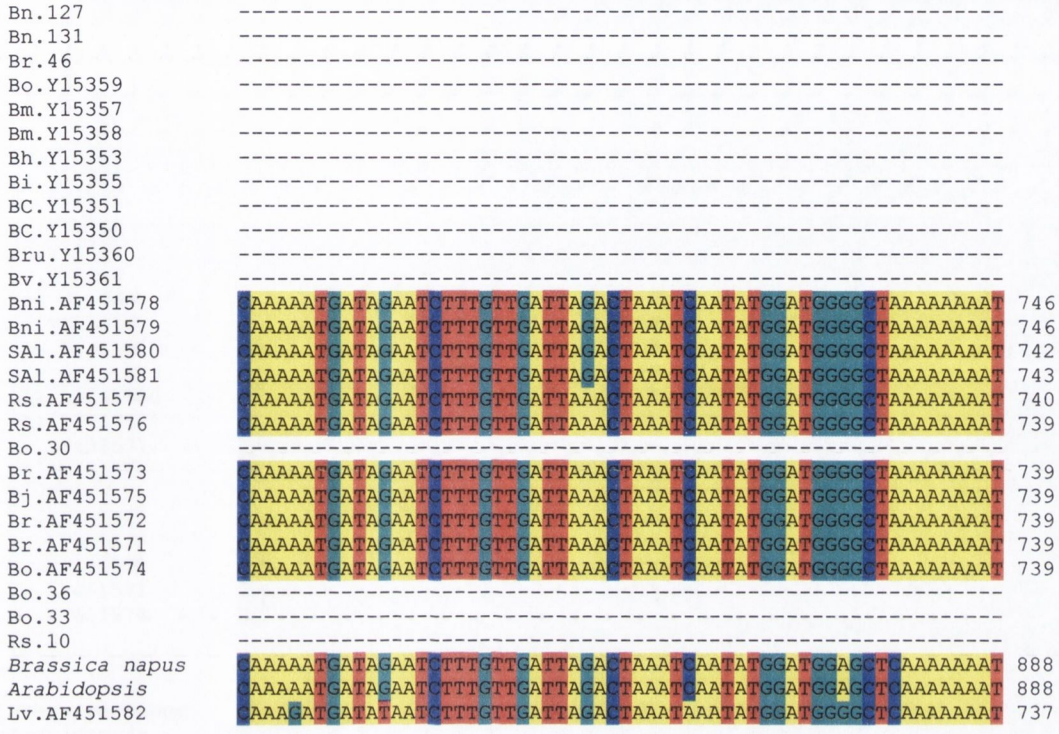
Arabidopsis 828
Lv.AF451582 677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Bm.Y15358
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BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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Bni.AF451579 746
SAl.AF451580 742
SAl.AF451581 743
Rs.AF451577 740
Rs.AF451576 739
Bo.30
Br.AF451573 739
Bj.AF451575 739
Br.AF451572 739
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Bo.36
Bo.33
Rs.10
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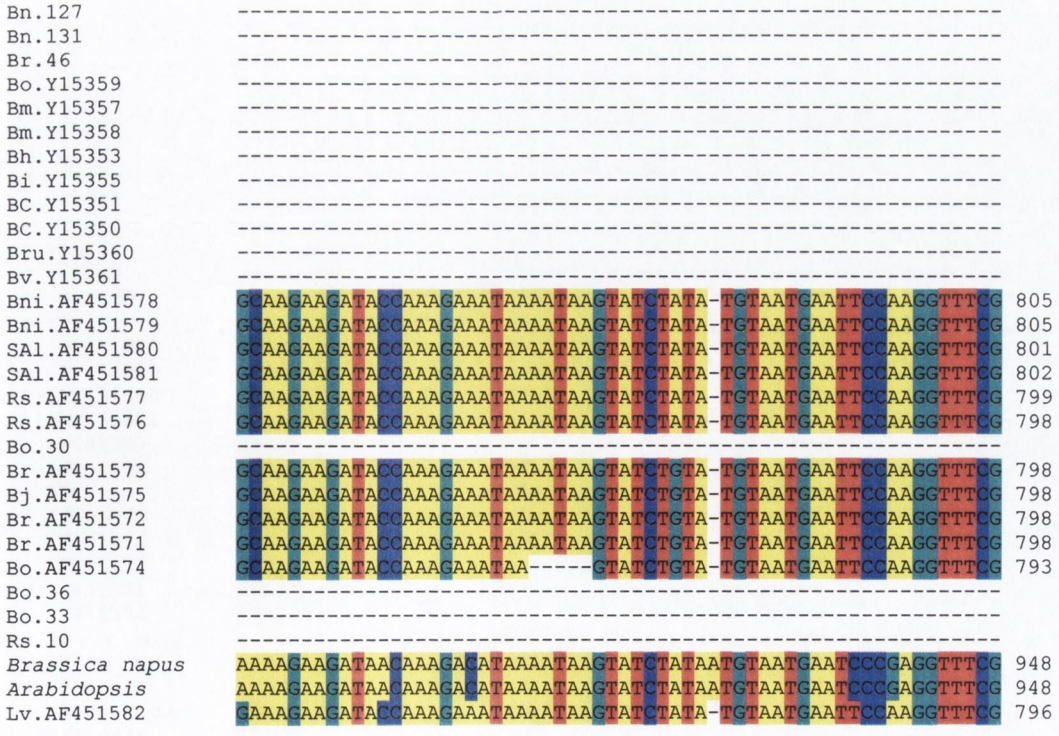
Arabidopsis 888
Lv.AF451582 737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Bn.131
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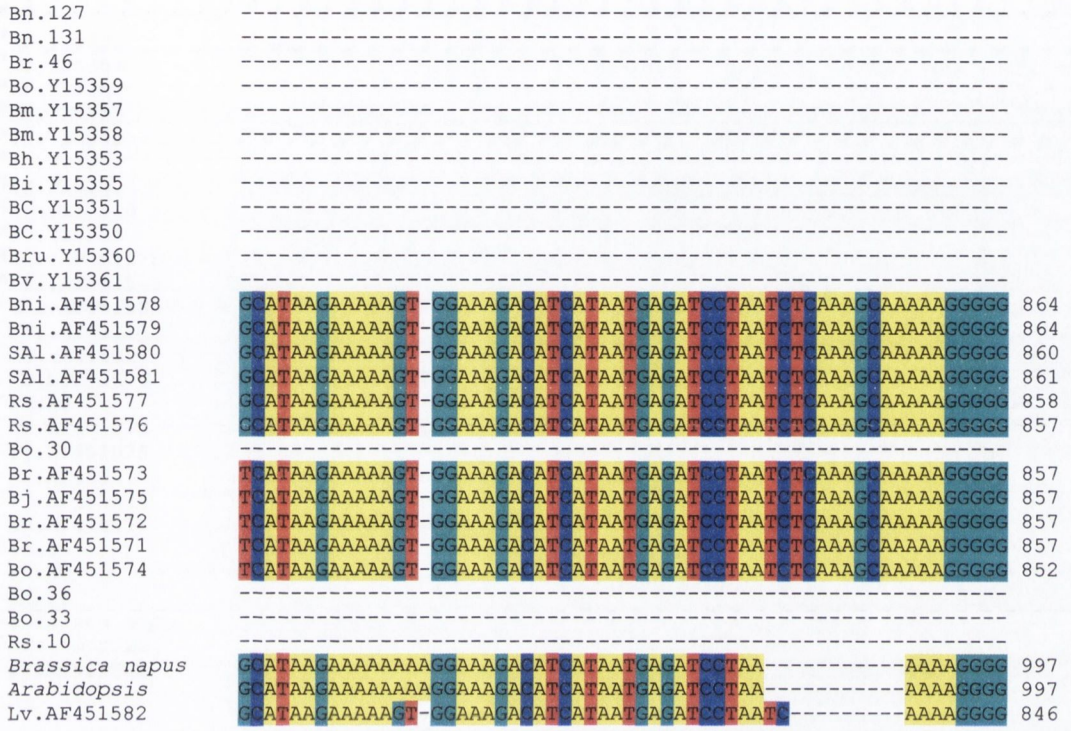
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
Bni.AF451578 805
Bni.AF451579 805
SAl.AF451580 801
SAl.AF451581 802
Rs.AF451577 799
Rs.AF451576 798
Bo.30
Br.AF451573 798
Bj.AF451575 798
Br.AF451572 798
Br.AF451571 798
Bo.AF451574 793
Bo.36
Bo.33
Rs.10
Brassica napus 948
Arabidopsis 948
Lv.AF451582 796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Bn.131
Br.46
Bo.Y15359
Bm.Y15357
Bm.Y15358
Bh.Y15353
Bi.Y15355
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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Rs.AF451577 GCATAAGAAAAAGT-GGAAAGACATCATAATGAGATCCTAATCTCAAAGCAAAAAGGGGG 858
Rs.AF451576 CCATAAGAAAAAGT-GGAAAGACATCATAATGAGATCCTAATCTCAAAGCAAAAAGGGGG 857
Bo.30
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Bo.AF451574 TCATAAGAAAAAGT-GGAAAGACATCATAATGAGATCCTAATCTCAAAGCAAAAAGGGGG 852
Bo.36
Bo.33
Rs.10
Brassica napus GCATAAGAAAAAAAAGGAAAGACATCATAATGAGATCCTAA----------AAAAGGGG 997
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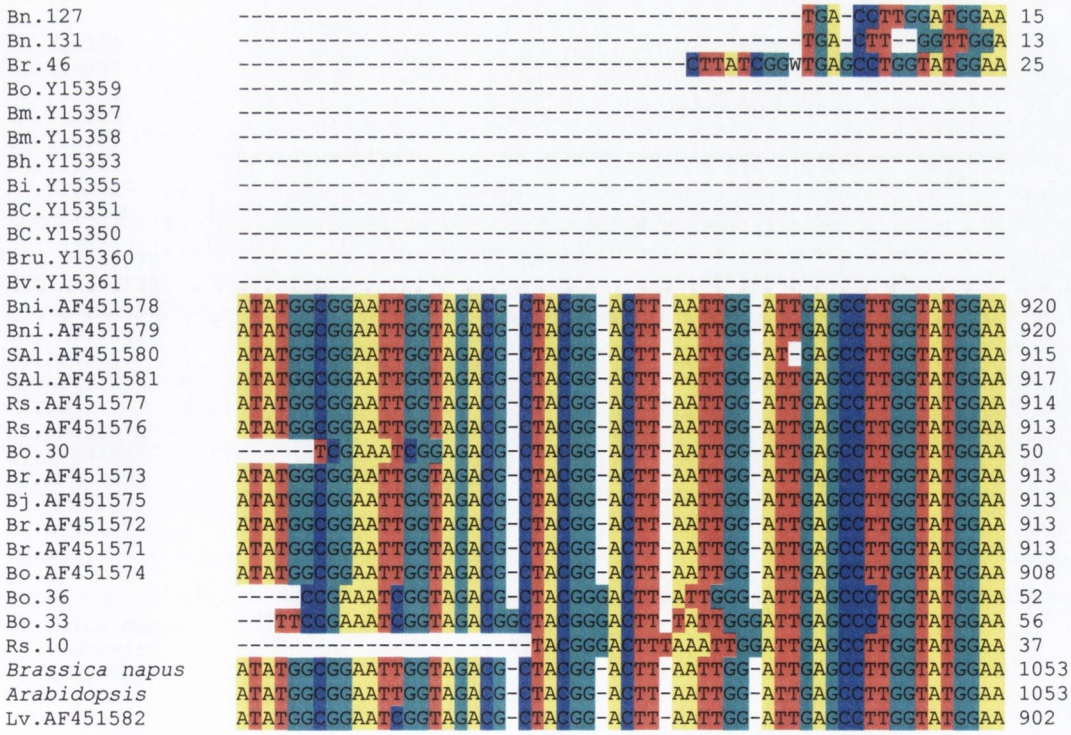
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Bm.Y15358
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Bi.Y15355
BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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Rs.AF451576 ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 913
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Bj.AF451575 ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 913
Br.AF451572 ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 913
Br.AF451571 ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 913
Bo.AF451574 ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 908
Bo.36 -----CCGAAATCGGTAGACG-CTACGGGACTT-ATTGGG-ATTGAGCCCTGGTATGGAA 52
Bo.33 ---TTCCGAAATCGGTAGACGGCTACGGGACTT-TATTGGGATTGAGCCCTGGTATGGAA 56
Rs.10 ----------TACGGGACTTTAAATTGGATTGAGCCTTGGTATGGAA 37
Brassica napus ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 1053
Arabidopsis ATATGGCGGAATTGGTAGACG-CTACGG-ACTT-AATTGG-ATTGAGCCTTGGTATGGAA 1053
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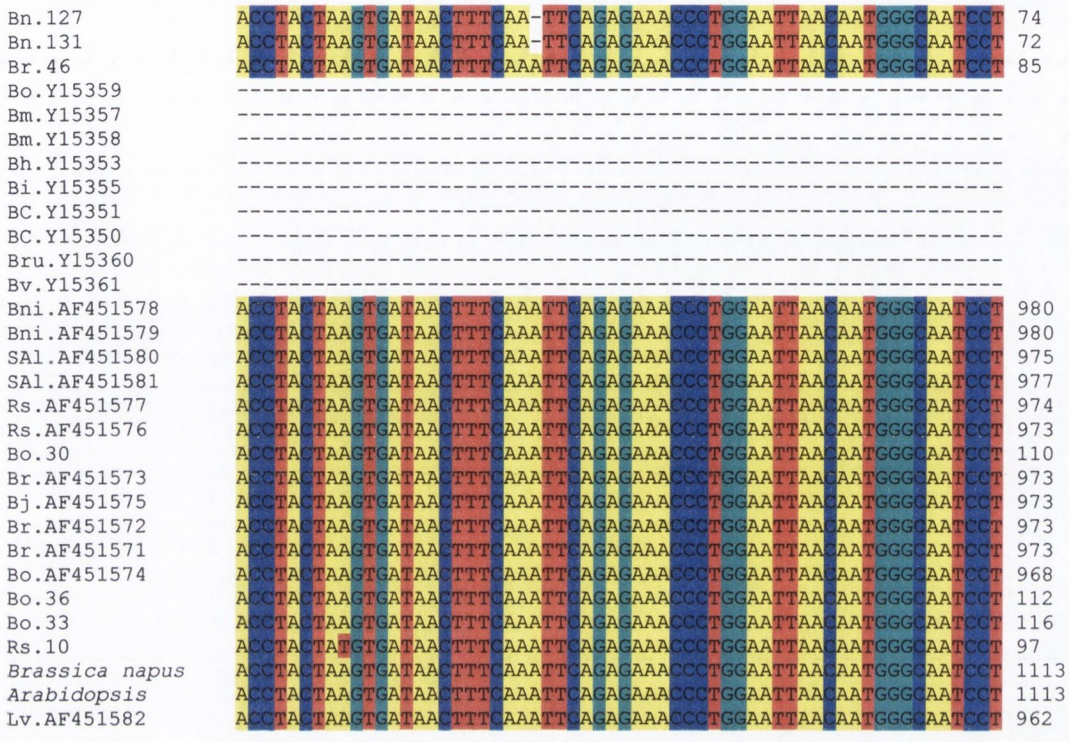
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BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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Bni.AF451579 980
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SAl.AF451581 977
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Rs.AF451576 973
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Br.AF451573 973
Bj.AF451575 973
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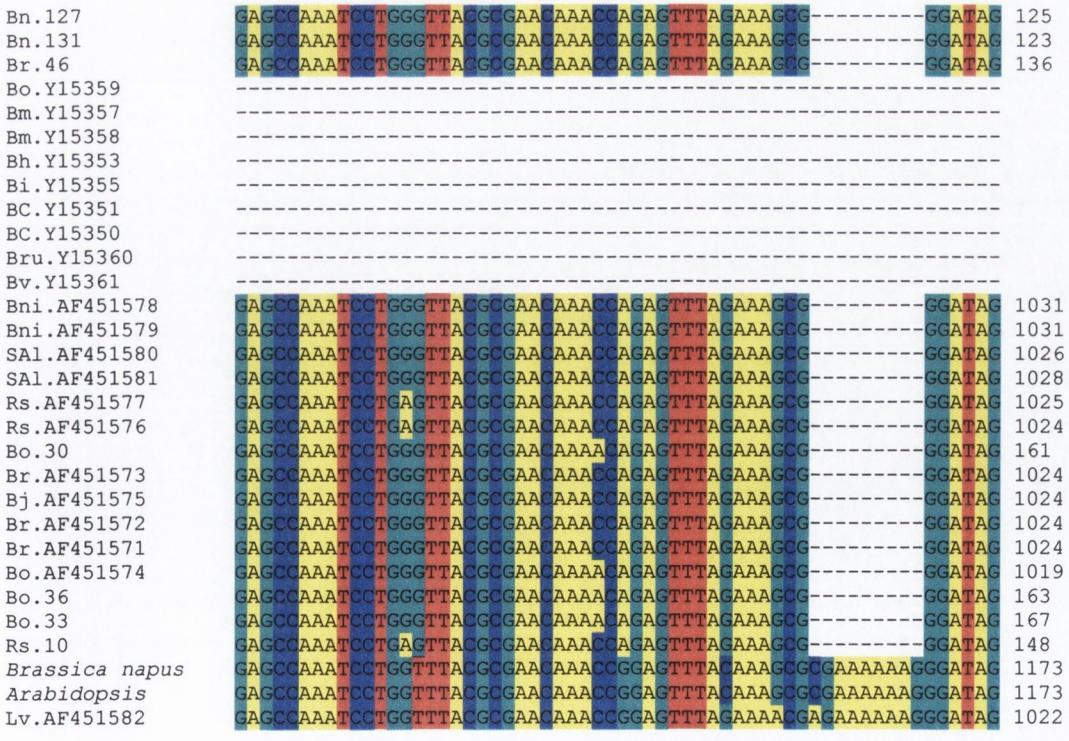
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Bru.Y15360
Bv.Y15361
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Bj.AF451575 1024
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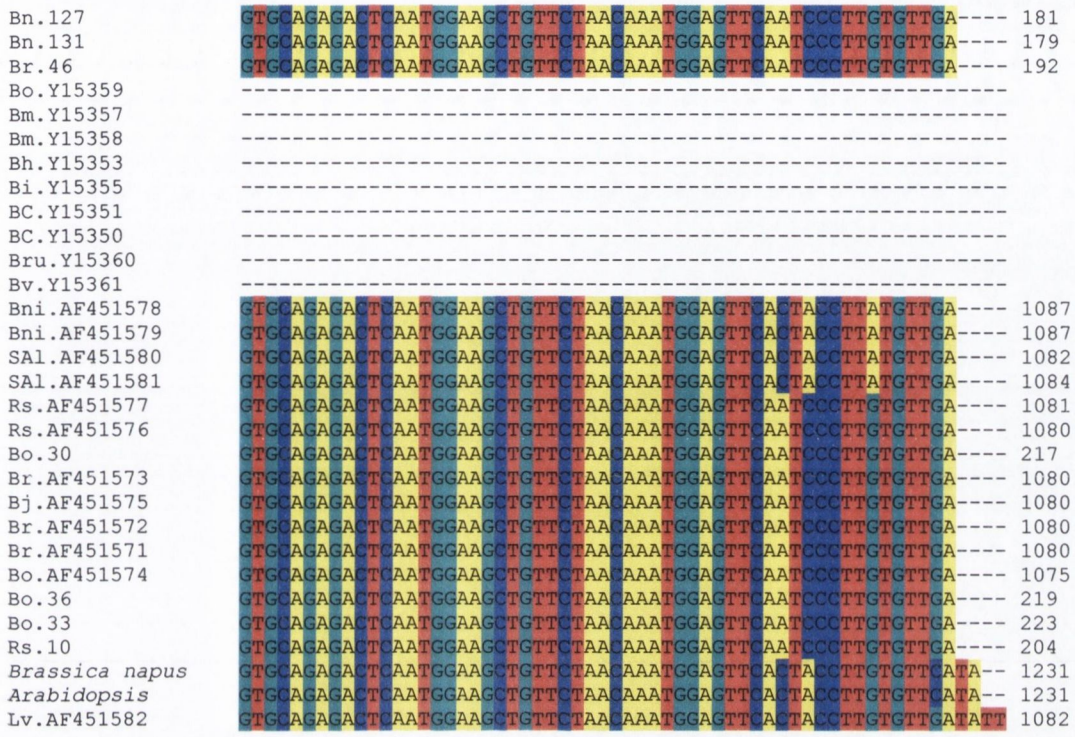
Lv.AF451582 1022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Br.46 GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCAATCCCTTGTGTTGA---- 192
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Bm.Y15358
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BC.Y15350
Bru.Y15360
Bv.Y15361
Bni.AF451578 GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCACTACCTTATGTTGA---- 1087
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SAl.AF451580 GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCACTACCTTATGTTGA---- 1082
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Rs.AF451576 GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCAATCCCTTGTGTTGA---- 1080
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Bo.33 GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCAATCCCTTGTGTTGA---- 223
Rs.10 GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCAATCCCTTGTGTTGA---- 204
Brassica napus GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCACTACCTTGTGTTCATA-- 1231
Arabidopsis GTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCACTACCTTGTGTTCATA-- 1231
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Bni.AF451579
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SAl.AF451581
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Bo.30
Br.AF451573
Bj.AF451575
Br.AF451572
Br.AF451571
Bo.AF451574
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Arabidopsis
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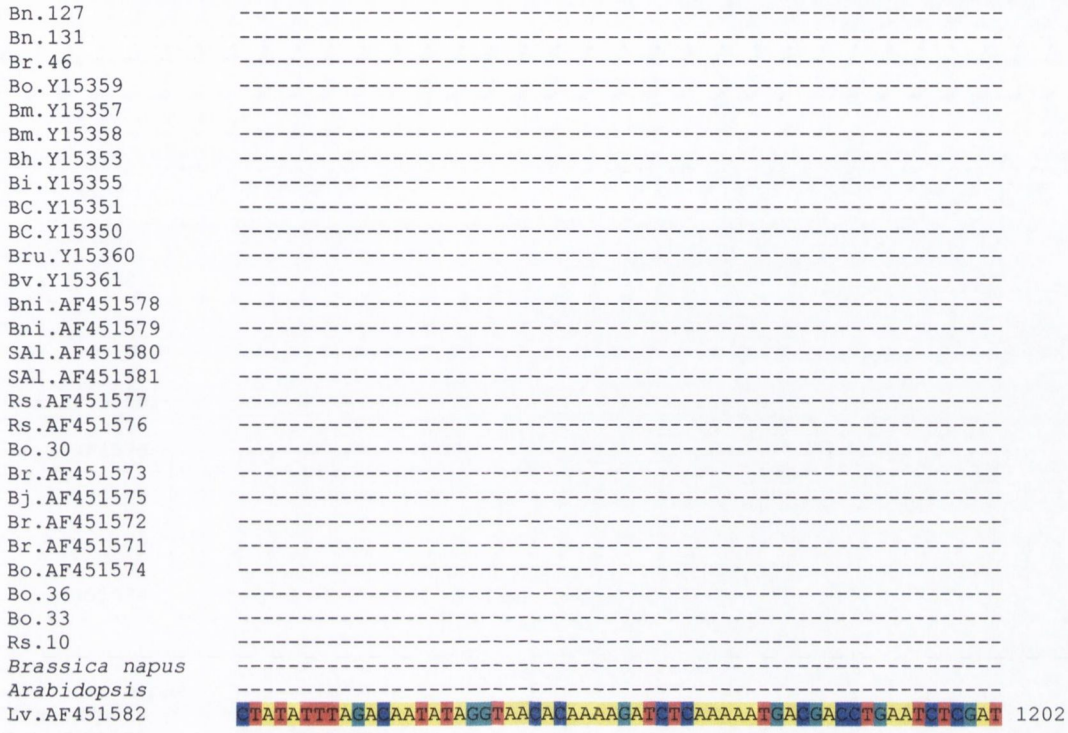
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Bv.Y15361
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Bni.AF451579
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Rs.AF451577
Rs.AF451576
Bo.30
Br.AF451573
Bj.AF451575
Br.AF451572
Br.AF451571
Bo.AF451574
Bo.36
Bo.33
Rs.10
Brassica napus
Arabidopsis
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1202


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Bru.Y15360
Bv.Y15361
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Rs.AF451577
Rs.AF451576
Bo.30
Br.AF451573
Bj.AF451575
Br.AF451572
Br.AF451571
Bo.AF451574
Bo.36
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Brassica napus
Arabidopsis
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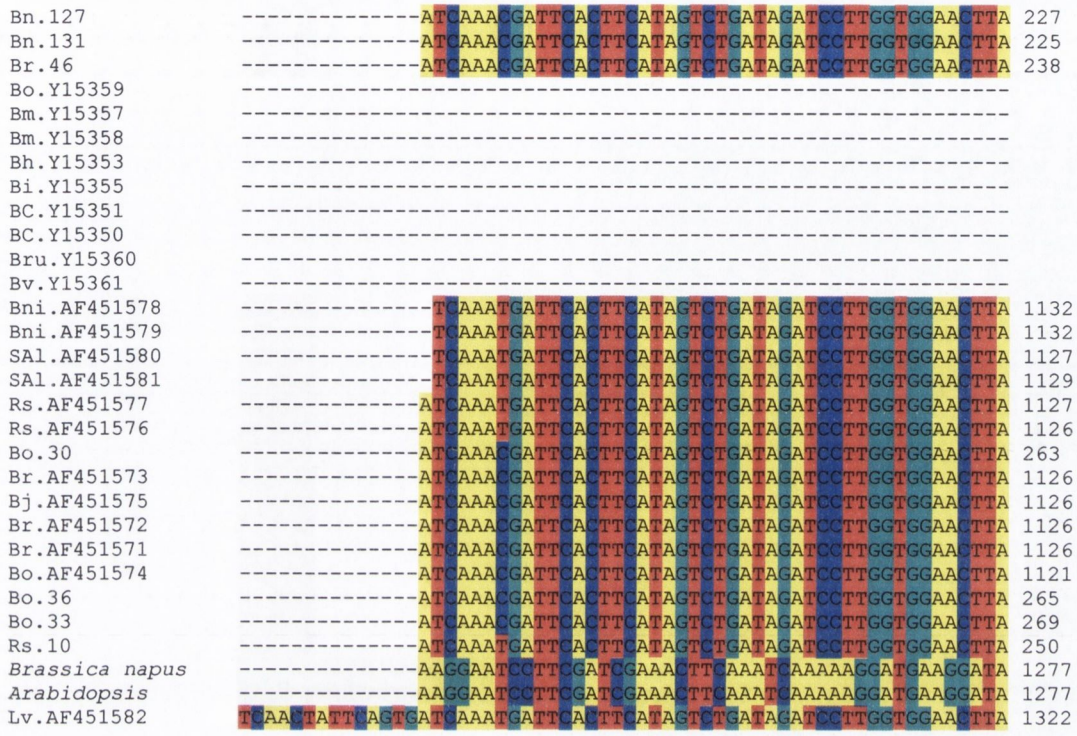
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Bru.Y15360
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Rs.AF451576 1126
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Br.AF451572 1126
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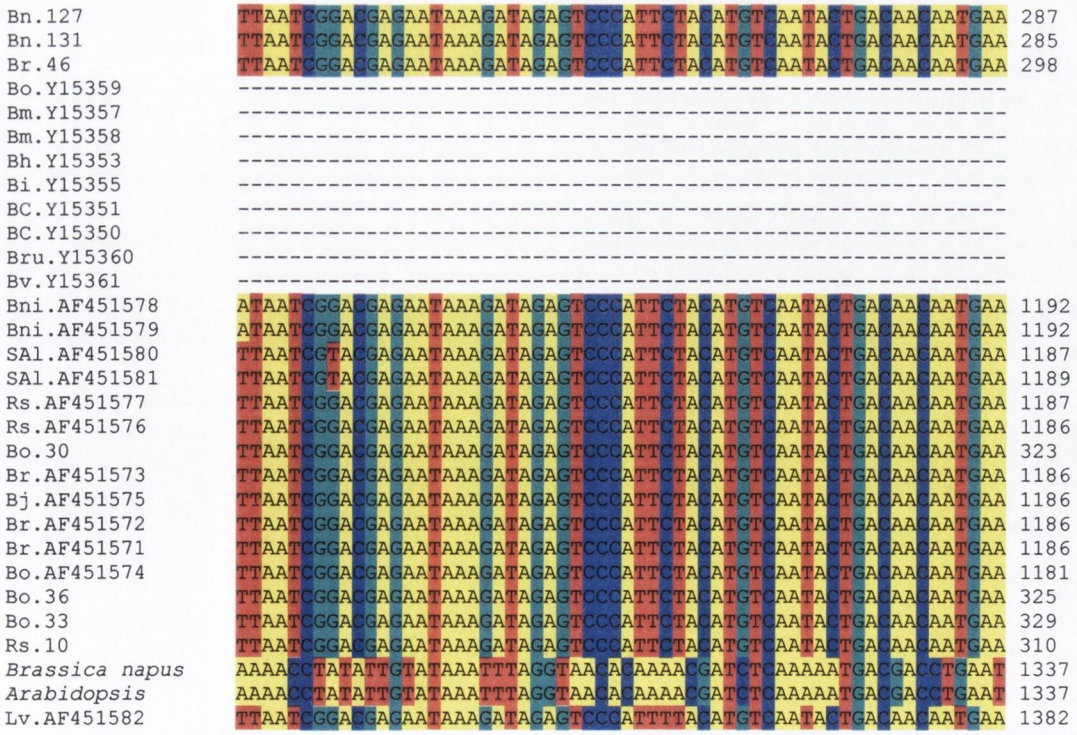
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BC.Y15351
BC.Y15350
Bru.Y15360
Bv.Y15361
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SAl.AF451581 1189
Rs.AF451577 1187
Rs.AF451576 1186
Bo.30 323
Br.AF451573 1186
Bj.AF451575 1186
Br.AF451572 1186
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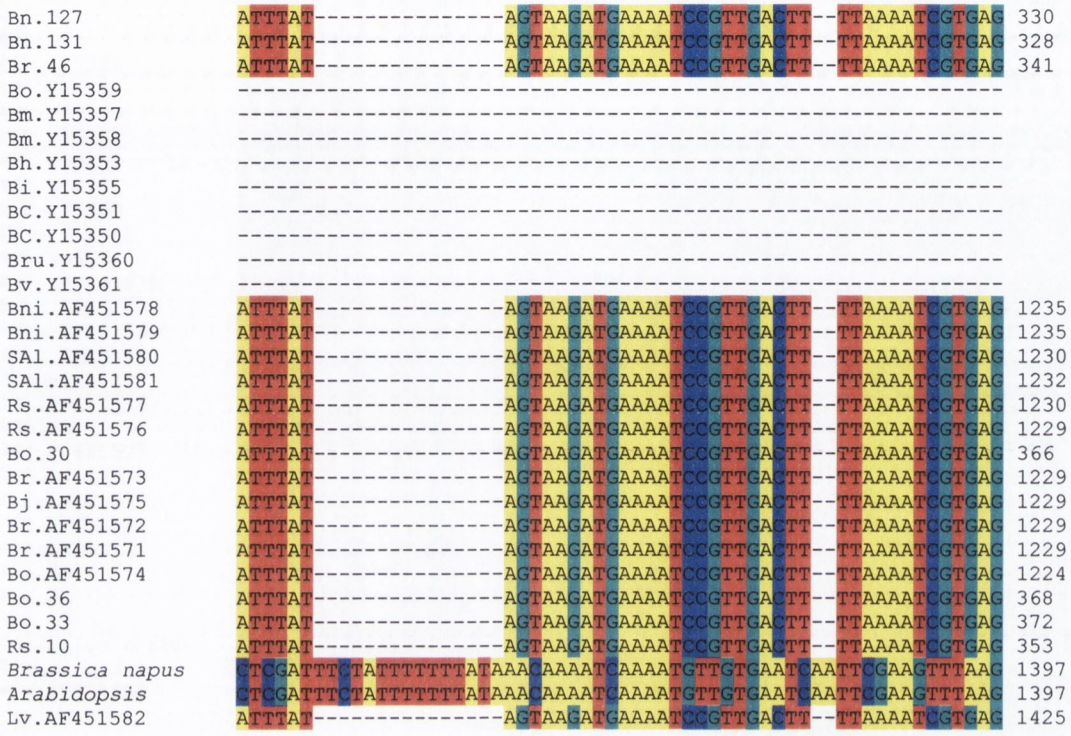
Arabidopsis 1337
Lv.AF451582 1382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Bj.AF451575 1229
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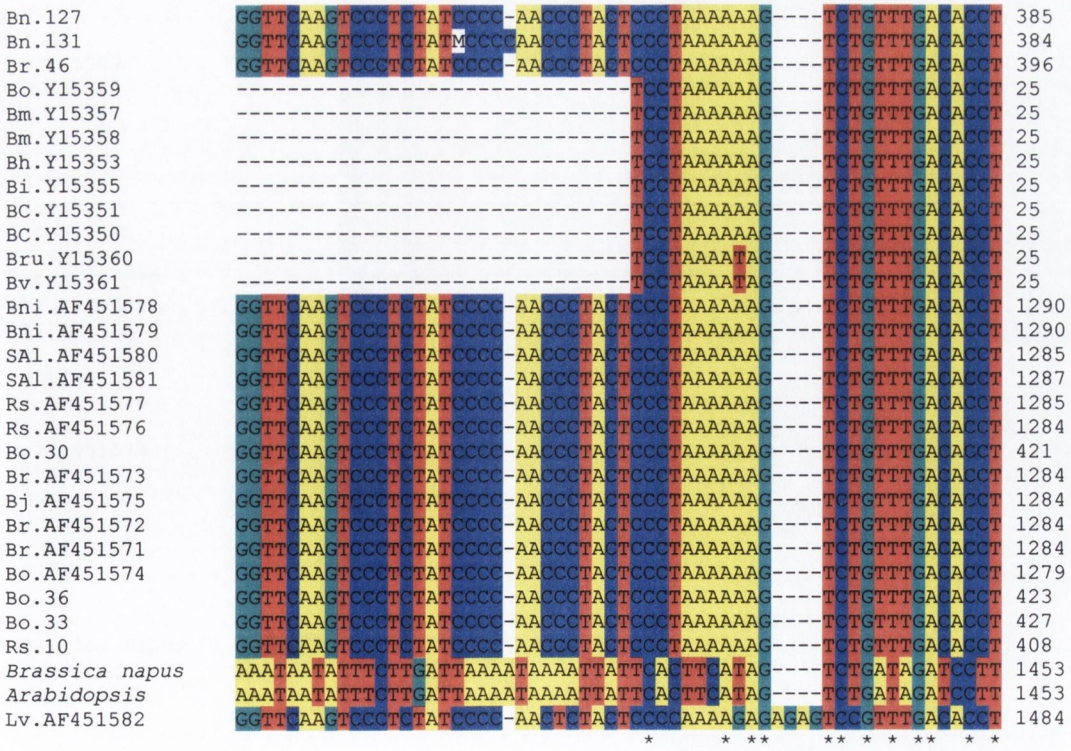
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Bj.AF451575 1284
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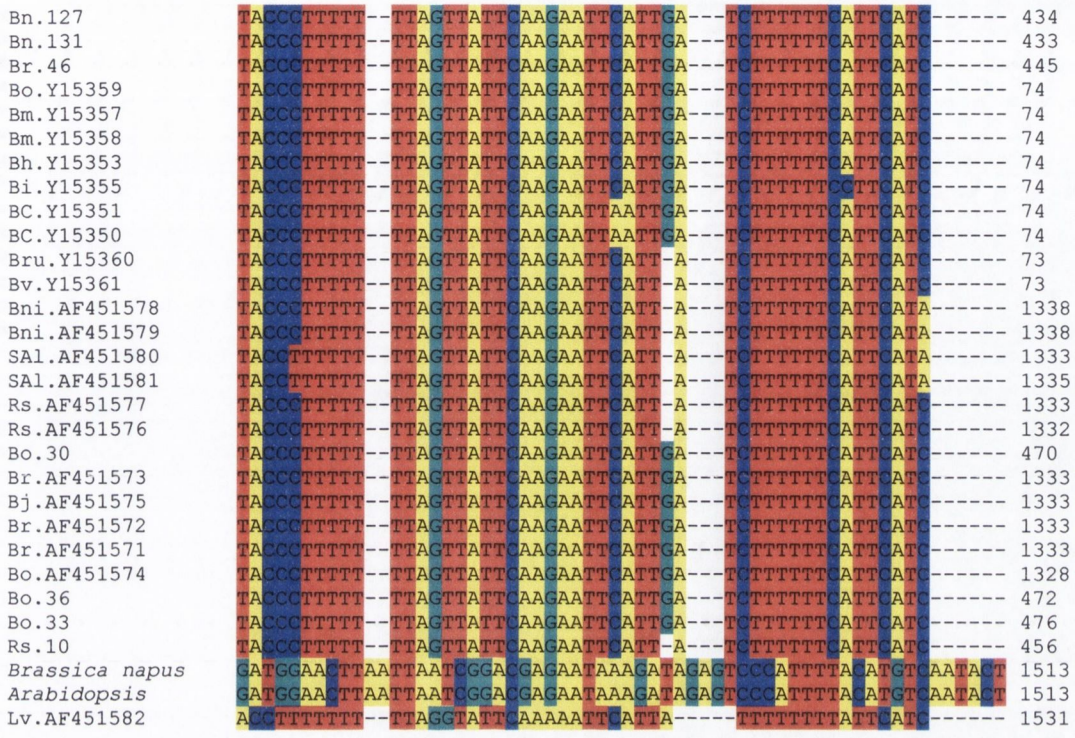
Lv.AF451582 1484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BC.Y15350 74
Bru.Y15360 73
Bv.Y15361 73
Bni.AF451578 1338
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Rs.AF451576 1332
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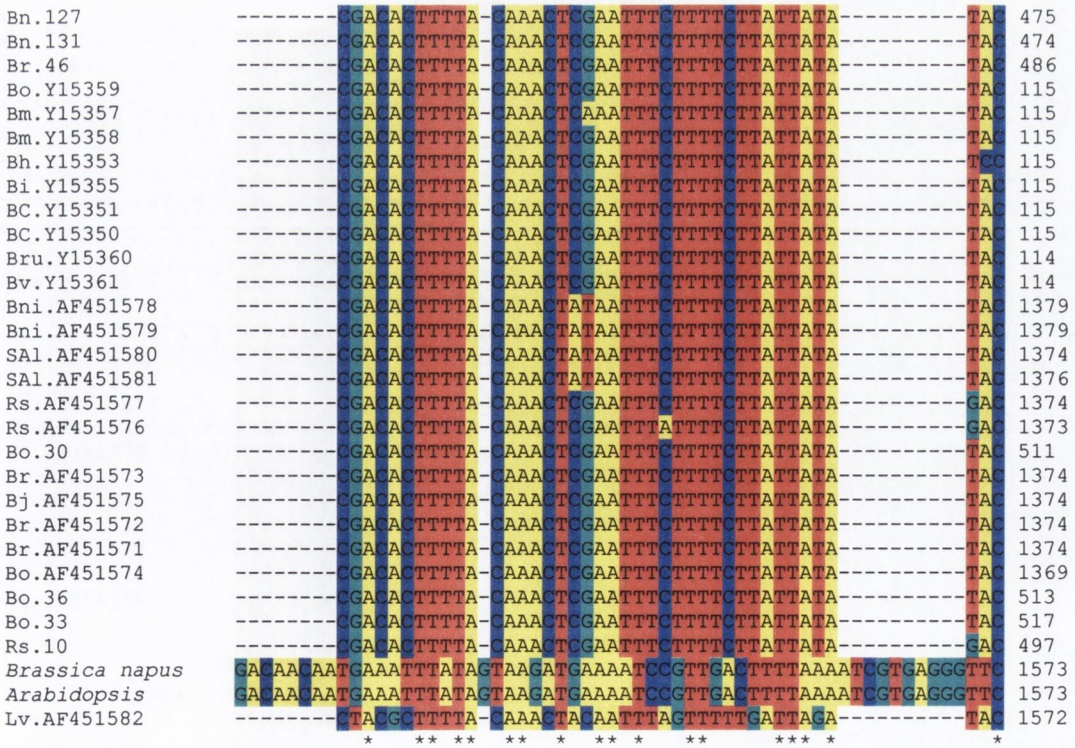
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BC.Y15350 115
Bru.Y15360 114
Bv.Y15361 114
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Rs.AF451576 1373
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Bj.AF451575 1374
Br.AF451572 1374
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Bo.36 513
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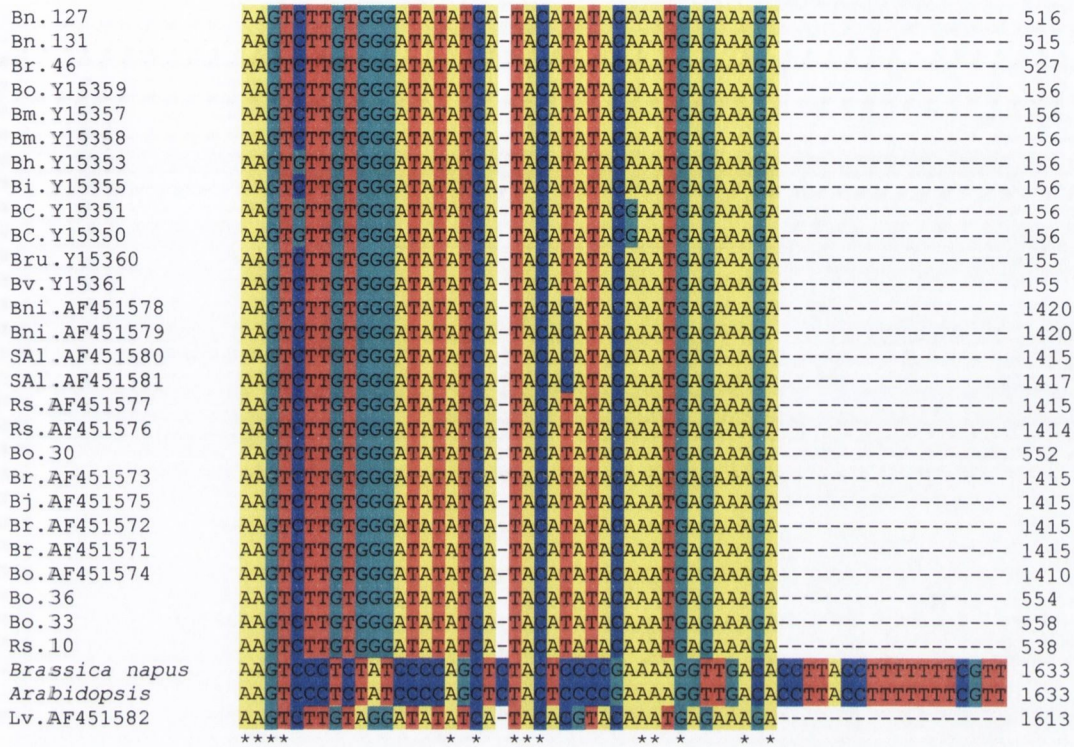
Arabidopsis 1573
Lv.AF451582 1572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BC.Y15351 156
BC.Y15350 156
Bru.Y15360 155
Bv.Y15361 155
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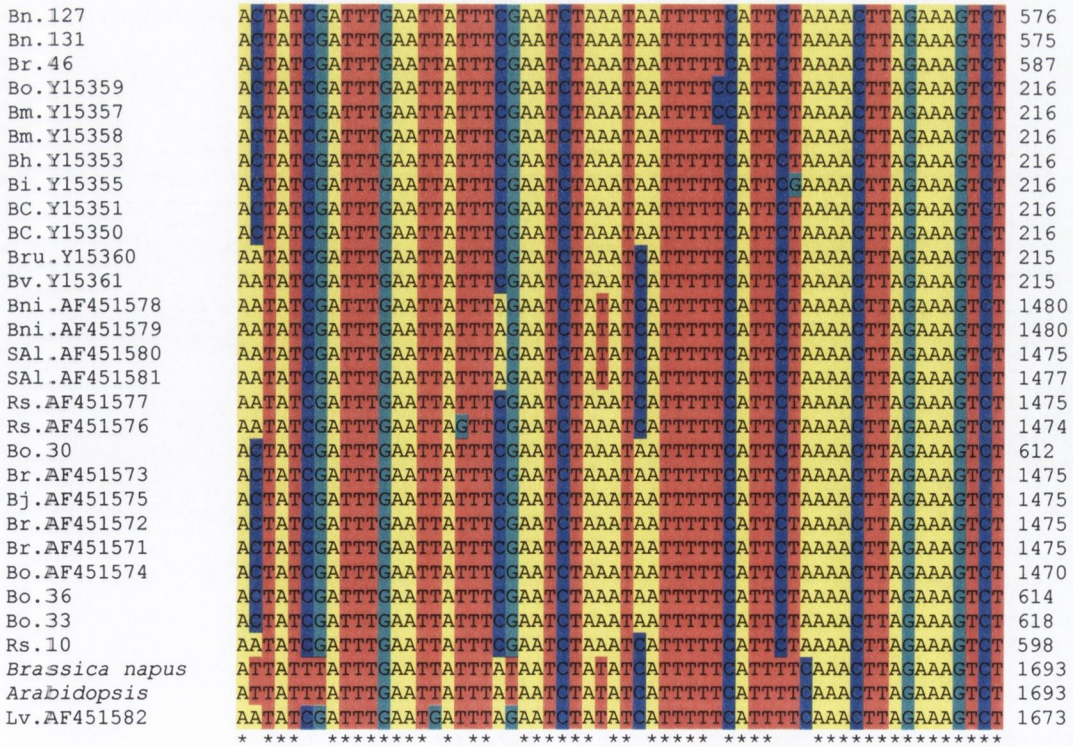
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BC.Y15351 216
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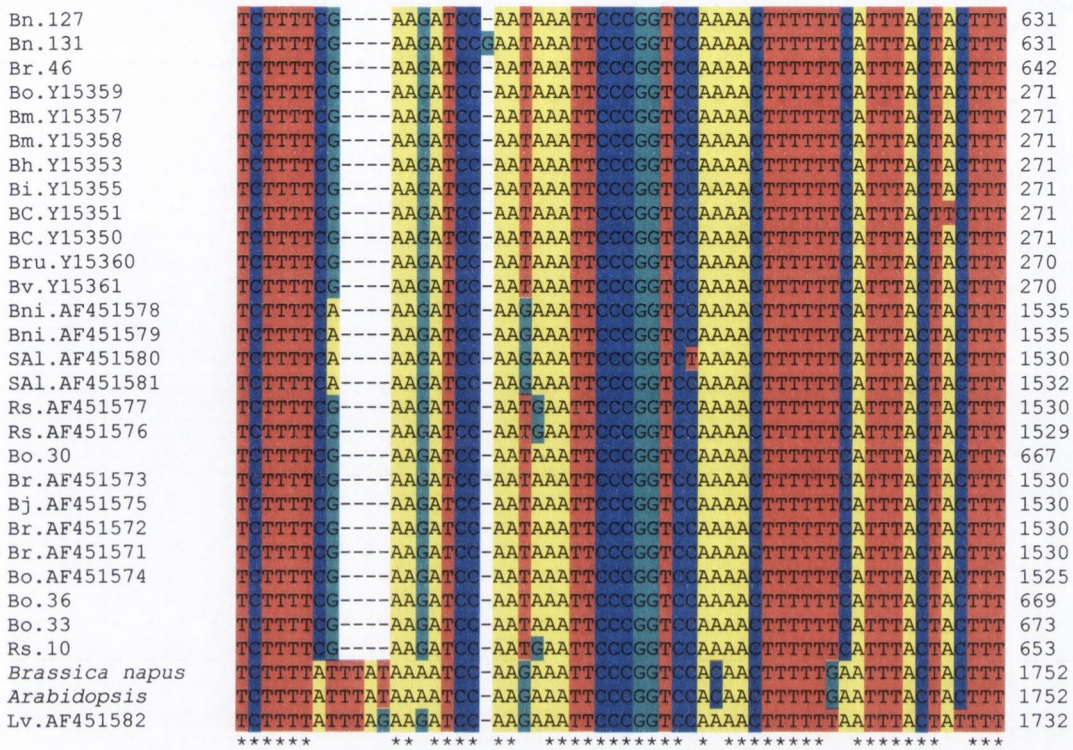
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Bv.Y15361 270
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Rs.AF451576 1529
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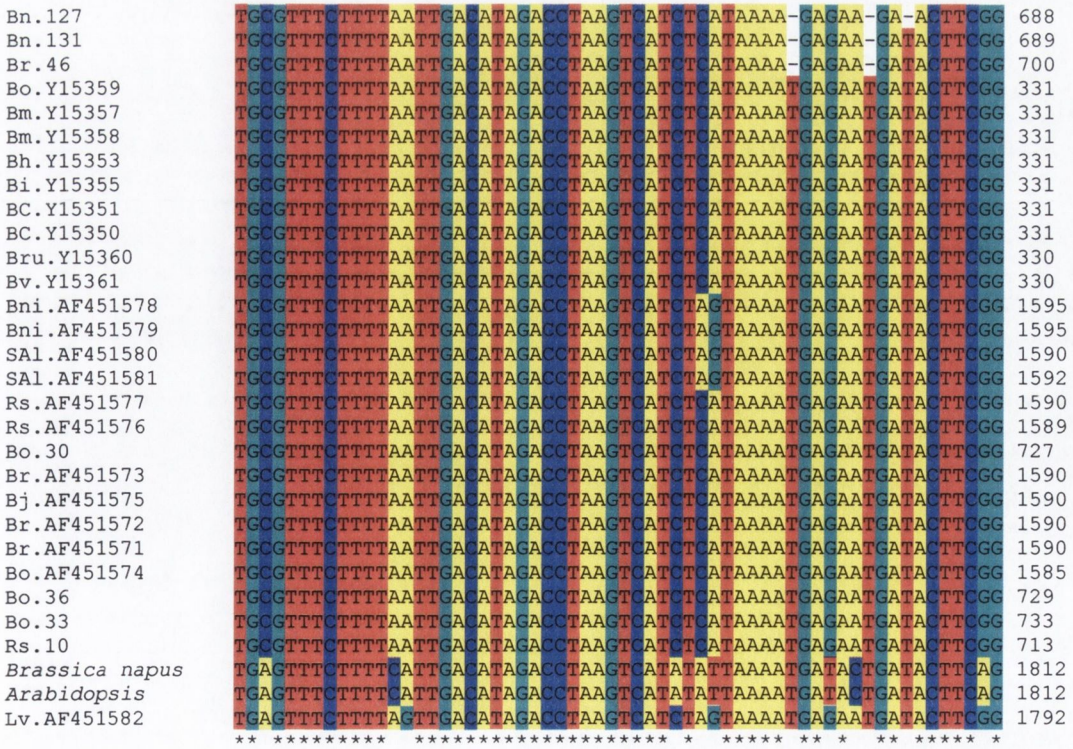
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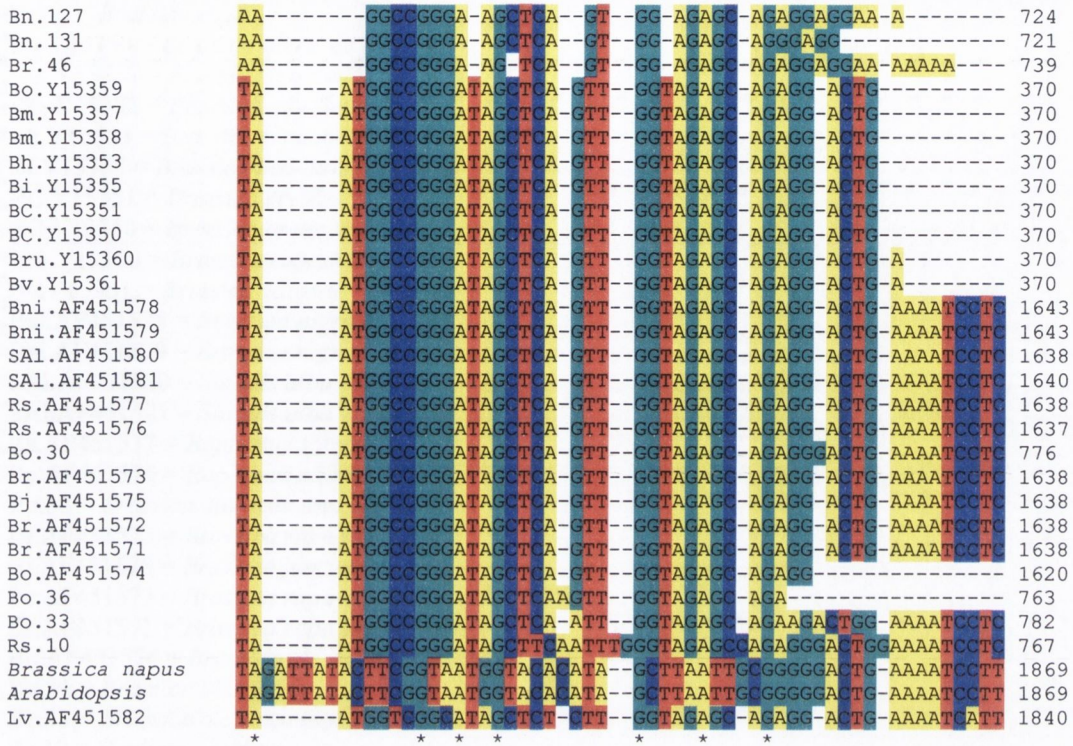
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Br.AF451572 1638
Br.AF451571 1638
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Arabidopsis 1869
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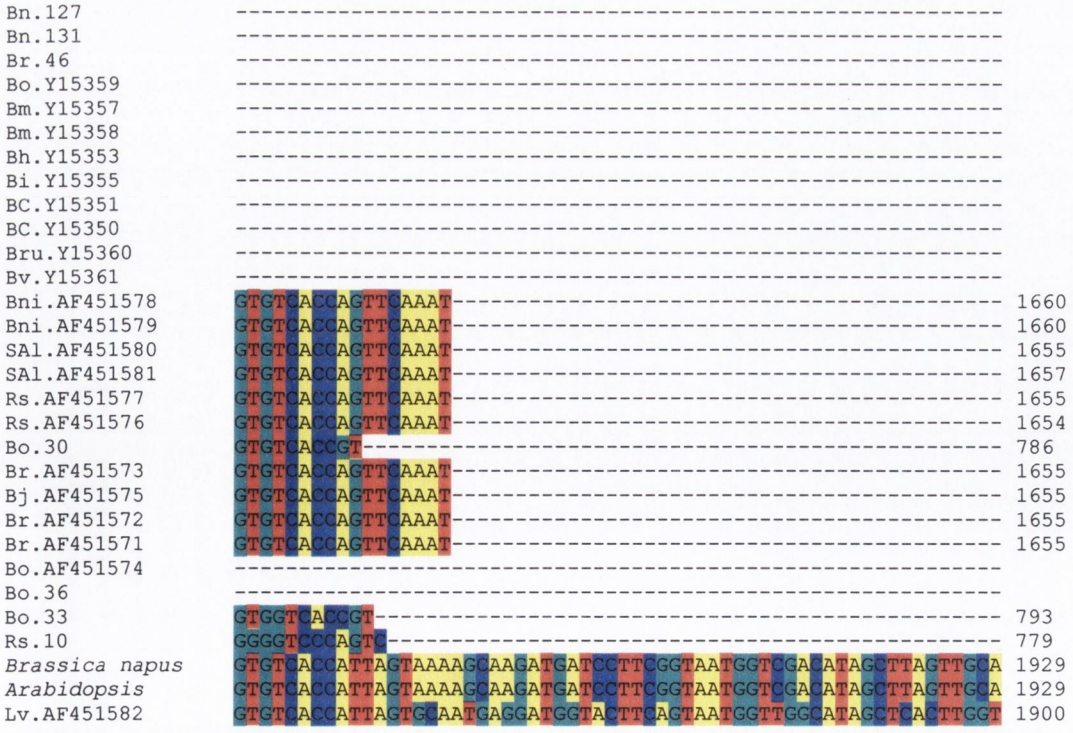
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Bo.AF451574
Bo.36
Bo.33 793
Rs.10 779
Brassica napus 1929
Arabidopsis 1929
Lv.AF451582 1900

Key
Bn.127 = *Brassica napus* cv. Sheila
Bn.131 = *Brassica napus* cv. Marinka
Br.46 = *Brassica rapa*
Bo.Y15359 = *Brassica oleracea*
Bm.Y15357 = *Brassica montana*
Bm.Y15358 = *Brassica montana*
Bh.Y15353 = *Brassica hilarionis*
Bi.Y15355 = *Brassica insularis*
BC.Y15351 = *Brassica cretica*
BC.Y15350 = *Brassica cretica*
Bru.Y15360 = *Brassica rupestris*
Bv.Y15361 = *Brassica villosa*
Bni.AF451578 = *Brassica nigra*
Bni.AF451579 = *Brassica nigra*
SAl.AF451580 = *Sinapis alba*
SAl.AF451581 = *Sinapis alba*
Rs.AF451577 = *Raphanus sativus*
Rs.AF451576 = *Raphanus sativus*
Bo.30 = *Brassica oleracea* ssp. *capitata*
Br.AF451573 = *Brassica rapa* ssp. *rapa*
Bj.AF451575 = *Brassica juncea*
Br.AF451572 = *Brassica rapa* ssp. *pekinensis*
Br.AF451571 = *Brassica rapa* ssp. *chinensis*
Bo.AF451574 = *Brassica oleracea* var. *capitata*
Bo.36 = *Brassica oleracea* ssp. *italica*
Bo.33 = *Brassica oleracea* ssp. *gemmifera*
Rs.10 = *Raphanus sativus*
Bn. Lico = *Brassica napus* cv. Licosomos
Arabidopsis = *Arabidopsis thaliana* Columbus
Lv.AF451582 = *Lepidium virginicum*