Genome Analysis and Genetic Mapping of Restorer Loci in *Raphanus*

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Abstract

Genetic variation exists in *Raphanus* that could be of use to *Brassica* breeders. Of particular interest is the Ogura system of cytoplasmic male sterility (CMS) which has been worked on extensively in a *Brassica napus* background. Problems have been experienced in *B. napus* restorer lines due to the inheritance of a large segment of *Raphanus* chromosome containing the fertility restoring locus. This restorer introgression is located on the *Brassica* C genome making it only of use for *B. napus* and not for *B. rapa* or *B. juncea*. This thesis describes the development of the materials necessary for the introgression into the *Brassica* A genome of a defined segment of *Raphanus* chromosome containing a restorer locus.

Defined genetic stocks of *Raphanus* were developed that contained specific loci controlling restoration of Ogura CMS. This material was used to develop populations segregating for specific restorer loci. Extensive RFLP maps of three *Raphanus* populations were developed and aligned, resulting in a robust consensus map of the entire *Raphanus* genome. Three restorer loci were accurately mapped on three separate linkage groups. Segregation data in one population suggested the presence of a fourth restorer locus yet to be mapped. The segment of *Raphanus* that is implicated in the restoration of Ogura CMS in a *B. napus* restorer line developed by INRA was identified and it did not correspond to any of the regions containing the three mapped restorer loci, suggesting the presence of more restorer loci in *Raphanus*.

Comparative mapping between the *Raphanus* genetic map and previously generated *Brassica* A genome RFLP maps demonstrated large regions of collinearity between segments of chromosomes of the two species. Preliminary examination of the two genome maps suggest they contain essentially the same overall genetic content but with large segments of the genomes rearranged with respect to each other. Likely sites of *Raphanus* restorer introgression into the *Brassica* A genome were predicted.

Trigenomic tetraploids were developed in which pairing and recombination between homoeologous segments of *Raphanus* and *Brassica* A chromosomes should result. Progeny of these individuals will allow an assessment of the pattern and extent of recombination that occurs between the chromosomes of the *Raphanus* and *Brassica* A

genomes and should lead to the development of *B. napus* lines carrying Ogura CMS restorer alleles from *Raphanus*.

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Abbreviations

AAFC	Agriculture and Agri-Food Canada
cpDNA	chloroplast DNA
cM	centimorgan
CMS	cytoplasmic male sterility
INRA	Institut National de la Recherche Agronomique (France)
mf	male fertile
ms	male sterile
mtDNA	mitochondrial DNA
pmf	partial male fertile
PCR	polymerase chain reaction
PMC	pollen mother cell
RFLP	restriction fragment length polymorphism

Chapter 1. Introduction

Studies have shown that there is sufficient heterosis in canola (*Brassica napus* and *B. rapa*) to warrant the development of hybrid varieties (Grant and Beversdorf 1985; Falk *et al.* 1994). Currently, two methods of pollination control are being used to produce commercial hybrids in Canada: cytoplasmic male sterility (CMS) and genic male sterility. The CMS system that has received the most attention from canola breeders has been the Ogura CMS system developed extensively in *B. napus* by researchers at INRA (Rennes, France; Pelletier *et al.* 1983).

Male sterile *B. napus* cybrids have been produced via protoplast fusion with Ogura CMS radish (*Raphanus sativus*) (Pelletier *et al.* 1983). A segment of the *R. sativus* genome was then introgressed into the *B. napus* cybrids to produce lines which were male fertile (Pelletier *et al.* 1987). Unfortunately the restorer introgression was quite large and was associated with high levels of glucosinolates. Repeated backcrossing to low glucosinolate *B. napus* lines to break the linkage between restoration and high glucosinolate levels has met with limited success. A further factor limiting the use of the INRA restorer in Canadian breeding programmes is that the introgression is on the *Brassica* C genome (Delourme *et al.* 1998) which limits its use to *B. napus*. For *B. rapa* and *B. juncea* hybrids, it would be more effective to have the restorer on the *Brassica* A genome.

Studies into the nature of fertility restoration in Ogura CMS radish have been complicated by the lack of defined genetic stocks that allow researchers to compare restorers and maintainers in specific lines. This research has been further restricted by the lack of molecular markers assigning restorer alleles to specific loci in the radish genome.

New methods in genome analysis have been developed since the development of the INRA *B. napus* Ogura CMS restorer lines. Recent advances in the understanding of

Brassica genomes through comparative RFLP mapping along with marker-assisted selection and a better understanding of the control of chromosome pairing will make interspecific gene transfer more efficient (Lydiate *et al.* 1993). A research project was therefore proposed whereby restorer genes would be identified and mapped in *Raphanus* using molecular markers. Using the results of this restorer mapping together with comparative maps of the *Raphanus* and the *Brassica* A genomes and marker-assisted selection, a small segment of *Raphanus* chromosome carrying the necessary restorer gene would be introgressed into the *Brassica* A genome to allow the future development of restorer lines for Ogura CMS in *B. napus*, *B. rapa* and *B. juncea*.

This thesis describes work conducted to 1) identify and map Ogura CMS restorer genes segregating in radish populations; 2) develop defined genetic stocks of *Raphanus* that are restorers or maintainers for specific loci controlling Ogura CMS in radish; 3) map the entire *Raphanus* genome; 4) chart regions of collinearity in the *Raphanus* and *Brassica* A genomes and, through comparative mapping, assess the potential for pairing and homoeologous recombination between chromosomes; and 5) develop interspecies hybrids in which recombination between the homoeologous chromosome segments of chromosomes from *Raphanus* and the *Brassica* A genome is promoted.

Chapter 2. Literature Review

2.1 Canola

Canola is the oilseed crop of *Brassica napus* or *B. rapa* with less than 2% erucic acid in the oil and less than 30 micromoles per gram of glucosinolates in the meal (Canola Council of Canada 2001). Canola was derived from rapeseed, the traditional *B. napus* and *B. rapa* oilseed crop, through genetic selection for lower erucic acid and glucosinolate levels. In 2000, 4.9 million hectares of canola were planted in Canada, 2.4 million hectares of which were in Saskatchewan. The total production of canola in Canada in 2000 was 7.1 million tonnes (Canadian Grains Industry 2000).

Brassica juncea is typically grown as a mustard crop but recent breeding efforts to modify fatty acid composition and lower glucosinolate levels in Canada by Agriculture and Agri-Food Canada and the Saskatchewan Wheat Pool have resulted in the development of a canola-type *B. juncea*. *Brassica juncea* canola is expected to be approved for commercialization in 2001.

2.2 Hybrids

Heterosis is the increased vigour of an F_1 hybrid over its parents. If this increased vigour results in commercially valuable characteristics such as yield in a grain crop, the development of hybrid varieties for commercial production is of interest to plant breeders and producers. High parent heterosis for grain yield has been shown to be approximately 50% in winter oilseed rape (Shiga 1976) and 13-70% in spring oilseed rape (*B. napus:* Sernyk and Stefansson 1983; Grant and Beversdorf 1985; Brandle and McVetty 1989; *B. rapa*: Falk *et al.* 1994) making it practical to develop hybrids for commercial production. Heterosis has also been observed for total dry matter and harvest index in spring oilseed rape hybrids (McVetty *et al.* 1990; Sernyk and Stefansson 1983). Seed size of hybrids also tends to be larger perhaps contributing to their rapid emergence in the spring which allows them to out-compete weeds and survive despite

fleabeetle damage (R. Elliot pers. comm.).

Hybrids of homozygous inbred lines have the added benefit of being uniform. This is of particular importance for quality characteristics as well as for ease of harvest. Hybrid seed production is also of interest to seed companies for the protection of income and specialty traits since the farmer must acquire new hybrid seed every year instead of using farm-saved seed.

The commercial production of hybrid seed requires the use of a pollination control system. Hand emasculation is only practical in a few crops where there is a high number of seeds produced per pollination, labour is cheap and the seed is valuable, such as pepper and tomato. Self-incompatibility (SI) has been investigated in many species but production of pure inbred parental lines is challenging (McCubbin and Dickinson 1997). Genic male sterility is also not commonly used for hybrid seed production due to the difficulty of producing inbred lines (Sawhney 1997). A genetically engineered genic system that uses herbicide tolerance for selection against male fertile siblings in the female line has been developed in canola and maize (De Block *et al.* 1987; Mariani *et al.* 1990) and is currently being used successfully for commercial hybrid canola production in Canada. Cytoplasmic male sterility (CMS) has been exploited in several crop species and is one of the systems currently being used for the commercial production of hybrid canola seed in Canada.

Production of F_1 hybrids using CMS requires three breeding lines: the A-, B- and R-lines. The A-line has CMS cytoplasm and no fertility restoring alleles and is, therefore, male sterile. The B-line, also know as the maintainer line, has normal cytoplasm, no restorer alleles and is used as the pollen donor to increase seed of the A-line (Fig. 2.1). The R-line is male fertile, homozygous for all necessary restorer alleles and is genetically distinct from the A- and B- lines. F_1 hybrids are produced in the field by planting alternating strips of A-line and R-line plants and encouraging cross pollination, often with the help of bees or mechanical pollination. Following flowering, the R-line is removed to prevent contamination of the F_1 seed by selfed R-line seed and F_1 seed is harvested from the A-line plants (Fig. 2.1). Commercial F_1 hybrids have been produced using CMS for several major crop species including corn, sunflower and

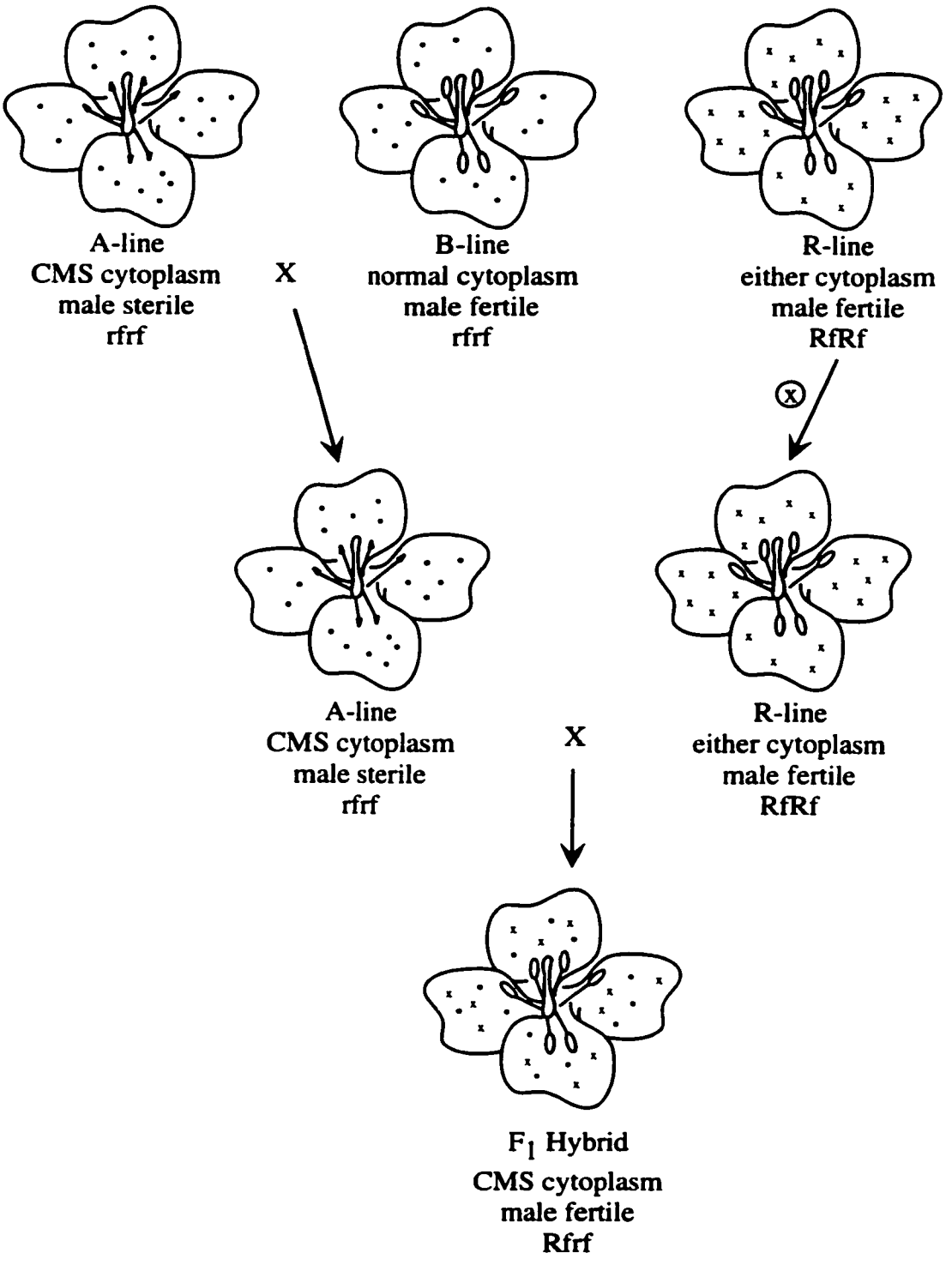


Figure 2.1. Outline of the production of hybrid seed using CMS.

canola.

2.3 Cytoplasmic male sterility

CMS is a maternally inherited condition in which a plant is unable to produce pollen but is capable of full seed set when pollinated by a male fertile plant. CMS typically involves the expression of a mitochondrial gene not found in normal, fertile plants (see Schnable and Wise 1998 for a comprehensive review). Plants carrying mitochondrial genes for male sterility are unable to undergo complete male gametogenesis and therefore cannot produce viable pollen. These genes have often been shown to arise from recombination events within the mitochondrial genome and appear to be co-transcribed with another mitochondrial gene that is not involved in CMS (Budar 1995). CMS can arise spontaneously in breeding lines or from wide or interspecific crosses resulting in the exchange of nuclear and cytoplasmic genomes.

Morphological changes associated with CMS manifest themselves at various stages of pollen development depending on the species and CMS system in question. In many cases pre-mature degradation of the tapetum is involved in CMS (Budar 1995; Schnable and Wise 1998). The tapetum is the layer of cells surrounding the sporogenous tissue and it plays an important role in providing nutrients to the developing pollen grains so that abnormal tapetal function generally leads to pollen abortion (Shivanna *et al.* 1997). Warmke and Lee (1978) observed that mitochodrial replication is rapid in maize tapetal cells between the pre-callose and tetrad stages resulting in a 40-fold increase in the number of mitochondria per cell during this stage of pollen development. Alterations in mitochondrial function would likely have a greater effect on the tapetum than on other tissues with less energy demand.

Expression of CMS can often be overcome by one or more nuclear restorer genes and plants carrying these genes can produce pollen even if their mitochondria carry the sterility-inducing gene (maize: Laughnan and Gabay-Laughnan 1983; radish: Krishnasamy and Makaroff 1994; Koizuka *et al.* 2000). Analysis of male sterile and restored plants can be used to investigate mechanisms of pollen development and mitochondrial function and so help to elucidate nuclear-mitochondrial interactions

(Budar 1995; Schnable and Wise 1998).

2.3.1 T-CMS in Maize

Although found in many plant species, CMS has been studied most extensively in maize, particularly the Texas (T)-CMS system. This system has fallen out of favour for hybrid seed production due to its association with susceptibility to Southern corn leaf blight (*Bipolaris maydis*) but is still being studied as a model for nuclear-mitochondrial associations (Levings 1990). T-CMS is characterised by a failure of the anthers to protrude and by pollen abortion. T-CMS carries a mitochondrial gene, *T-urf13*, that encodes a 13 kDa mitochondrial pore-forming polypeptide, URF13 (Dewey *et al.* 1987). It is found in a mitochondrial DNA (mtDNA) sequence that arose through mitochondrial recombination resulting in a mtDNA sequence that contains two open reading frames, one coding for T-*urf*13 and the other for *orf221* (Dewey *et al.* 1986). Co-incidently, T-*urf*13 has also been implicated in the sensitivity of T-CMS maize to toxins from *Bipolaris maydis* and *Phylosticta maydis* (Dewey *et al.* 1986).

Two complementary dominant restorer alleles, *Rf1* and *Rf2*, are required for restoration of pollen fertility in T-CMS maize (Snyder and Duvik 1969). *Rf1* has been shown to alter transcription of T-*urf*13 and decrease the amount of URF13 by approximately 80% (Dewey *et al.* 1987). *Rf2*, which, unlike *Rf1*, is present in most maize inbreds, does not appear to influence the expression of T-*urf*13 (Dewey *et al.* 1987) and its actual function is still unknown. Since it is present in most maize inbreds, regardless of cytoplasm type, it may have a significant function independent of restoration (Schnable and Wise 1994). Recent work in maize T-CMS has shown that besides the two well characterized *Rf1* and *Rf2* restorer loci, there are an additional two partial restorer loci (*Rf8* and *Rf**) that are environmentally sensitive and have a similar function to that of *Rf1* (Dill *et al.* 1997; Wise *et al.* 1999).

2.4 Ogura CMS

2.4.1 Ogura CMS in radish

In the late 1960s, Ogura described a male sterile Japanese radish (*Raphanus*

sativus) found in the Kagoshima prefecture of Japan (Ogura 1968). This CMS system has since become one of the most studied CMS systems. Cytological studies carried out by Ogura (1968) indicated that the pollen mother cells (PMCs) of these plants developed normally until the microspore stage at which point they began to degenerate rapidly. This degeneration coincided with the early, abnormal degeneration of the tapetum that probably caused a disruption of the nutritional supply to the developing pollen grains. This breakdown of the tapetum also coincided with changes in morphology of the anthers which became thin and white (Ogura 1968). A DNA sequence has been identified that is specific to the mtDNA in Ogura CMS (Bonhomme *et al.* 1992; Krishnasamy and Makaroff 1993) and markers for this sequence have been used to demonstrate the presence of this sterility-inducing cytoplasm in many other radishes, both wild and cultivated (Yamagishi and Terachi 1994a, b, 1996).

Some male sterile radishes have been shown to carry variations of this Ogura-specific mtDNA sequence. Yamagishi and Terachi (1997) found *R. raphanistrum* plants that had sequences that were identical to the Ogura CMS sequence with the exception of two nucleotides and Iwabuchi *et al.* (1999) found two amino acid substitutions and a 39 bp deletion in the same sequence in the radish cultivar Kosena (kos). Yamagishi and Terachi (1997) found that plants that restored fertility to lines carrying the *R. raphanistrum* CMS-specific mtDNA also restored lines with Ogura CMS-specific mtDNA. Iwabuchi *et al.* (1999) had similar results, finding that lines that restored fertility to plants carrying the kos CMS-specific mtDNA also restored plants carrying the Ogura CMS-specific mtDNA sequence. This suggests that the restorers for these three mitochondrial lesions are the same or at least interchangeable.

Early studies on male sterility in Japanese radish suggested that sterility in Ogura CMS is controlled by a single recessive gene (Ogura 1968; Humaydan and Williams 1976), that is, one dominant restorer gene is required to overcome the male sterility. The results of a series of crosses with the Ogura male sterile line 'msGensuke' demonstrated the presence of restorers for Ogura CMS in a range of wild radishes (*R. sativus* and *R. raphanistrum*), irrespective of the cytoplasm type, as well as in cultivated European

and Chinese radish varieties (*R. sativus*) with wild-type cytoplasm (Yamagishi 1998; Yamagishi and Terachi 1994b and 1997). Crosses with *R. raphanistrum* plants carrying CMS cytoplasm demonstrated that restoration of male sterile *R. raphanistrum* lines required only one dominant restorer in the material investigated (Yamagishi and Terachi 1997).

Some studies have found that while one dominant restorer gene explained most of the observed segregation ratios, occasionally one gene was not sufficient (Bonnet 1975; Yamagishi and Terachi 1994b; Hawlader *et al.*1997). Data from Yamagishi and Terachi's study (1994b) as well as that of Koizuka *et al.* (2000) support a model of two dominant restorer genes. Yamagishi and Terachi (1994b) made crosses between the male sterile lines ms-Gensuke (Msg) and Kushikino (Ksk), both with Ogura type mtDNA, and male fertile cultivars Uchiki-Gensuke and Comet. Uchiki-Gensuke maintained sterility in crosses with both male sterile lines while Comet restored fertility. Segregation in the F_2 of the Ksk x Comet cross resulted in 21 male fertile individuals and 13 male sterile individuals which the authors concluded was not significantly different from the 3:1 expected from segregation at a single dominant restorer locus ($p = 0.07$). This ratio, however, also fits the 9:7 ratio ($p = 0.51$) expected for segregation at two dominant restorer loci. Similarly, the F_2 of the Msg x Comet cross segregated 46 male fertile to 25 male sterile individuals which also fits the ratios for segregation at both one and two dominant restorer loci. A backcross of the (Msg x Comet) F_1 onto Msg did not result in the 1:1 ratio the authors were expecting for one dominant restorer but the resulting 33 male fertile and 70 male sterile BC_1 progeny do fit the 1:3 ratio expected for segregation of two dominant restorer loci.

Bonnet (1975) and Nieuwhof (1990) proposed that up to two dominant and one recessive restorer genes, acting independently, were involved in some of their segregating populations. Much of the discrepancy in the number of genes involved in restoration likely stems from the potential for male sterile and wild radish lines to carry restorer genes that are not revealed until the non-restoring alleles are introduced from another parent in a cross.

On top of the major restorer genes, Nieuwhof (1990) also suggested that there

are minor genes influencing the whole system causing the odd male sterile plant to appear in apparently homozygous male fertile populations. This might explain some of the findings of other researchers where over 90% of the individuals from a cross were male fertile (Bonnet 1975; Yamagishi 1998). Nieuwhof also indicated that minor genes may be influencing temperature sensitivity causing some male fertile genotypes to be male sterile only at low (< 17°C) or high (> 20°C) temperatures.

2.4.2 Ogura CMS in Brassica *species*

In the early 1970s, researchers transferred Ogura CMS from radish to several different *Brassica* species. Bannerot *et al.* (1974) crossed an Ogura CMS radish line with *B. oleracea* and *B. napus* then backcrossed the progeny repeatedly to the *Brassica* parent to produce *Brassica* plants with radish cytoplasm. It was found however that the *Brassica* plants with the sterility-inducing cytoplasm had under-developed nectaries, were subject to chlorosis at temperatures below 15°C and had low levels of chlorophyll even at higher temperatures (Rousselle 1981). Pelletier *et al.* (1983), at INRA, France, solved these problems by developing *R. sativus - B. napus* hybrids through protoplast fusion. These cybrids were selected that were male sterile, had normal nectaries and did not have a problem with chlorosis at low temperature. A comparison of the mtDNA of the CMS cybrid and a fertile revertant revealed an open reading frame, *orf138*, that encodes a 19kDa protein that accumulates in the mitochondrial membrane (Bonhomme *et al.* 1992; Grelon *et al.* 1994). Krishnasamy and Makaroff (1993) sequenced the mitochondrial genomes of a radish cultivar with wild type cytoplasm and a line derived from a rapid cycling Ogura CMS radish accession and found the same *orf138* mitochondrial sequence was related to male sterility in radish itself. *Orf138* and another open reading frame, *orf158* or *orfB*, are co-transcribed as a bicistronic mRNA in radish and *B. napus* lines with Ogura CMS cytoplasm while only *orf158* has been shown to be transcribed in normal mitochondria (Bonhomme *et al.* 1992; Krishnasamy and Makaroff 1993).

Researchers in Japan isolated protoplasts of *R. sativus* cv. Kosena, a CMS line, destroyed the nuclei, and fused them with protoplasts of *B. napus* cv. Westar to produce

kos CMS cybrids. These cybrids carried rearranged mtDNA and one of them was male sterile (Sakai and Imamura 1990). This male sterile cybrid contained an open reading frame, *orf125*, that encodes a 17kDa protein with a sequence homologous to that of *orf138* except for two amino acid substitutions and a 39bp deletion (Iwabuchi *et al.* 1999).

The transfer of restorer alleles from *Raphanus* to *Brassica* started with crosses by Heyn (1976) between Ogura CMS *B. napus* (AACC) and a *Raphanobrassica* (RRAACC) carrying restorer alleles. Genetic analysis of these plants indicated that two or possibly three restorers were involved in restoration of fertility to these CMS *B. napus* plants (Heyn 1976). These restored *B. napus* plants were later crossed to the CMS cybrids created by Pelletier *et al.* (1983) and selected for their ability to restore fertility (Pelletier *et al.* 1987). Certain cybrids appeared to require fewer restorers than others for normal pollen function. Pellan-Delourme and Renard (1988) noted a decrease in seed set in these restorer lines and attributed it to the presence of excessive radish genome around the restorer loci and/or elsewhere in the genome. Further backcrossing to *B. napus* successfully increased female fertility presumably by eliminating some of the excess radish genetic material (Delourme *et al.* 1991). Isozyme studies carried out by Delourme and Eber (1992) on restored and non-restored cybrids, demonstrated that the radish *Pgi-2* allele had replaced the rapeseed *Pgi-2* allele in the *B. napus* restorer line, suggesting that the radish chromosome segment had replaced its *B. napus* homoeologue. Delourme *et al.* (1998) used comparative maps based on RAPD and RFLP markers from a normal *B. napus* map and a map of *B. napus* with the radish introgression to demonstrate that the introgression had replaced ~ 50 cM of normal *B. napus* and that there were *B. napus* markers flanking the introgression.

The production of unacceptably high levels of glucosinolates in the seed was associated with the original radish introgression. A QTL for seed glucosinolate content was mapped to a position corresponding to one end of the radish introgression in *B. napus* (Delourme *et al.* 1998) suggesting that either the introgression occurred adjacent to the *B. napus* glucosinolate allele or that the *B. napus* allele was replaced by its radish

homoeologue along with the restorer allele. After years of crossing and selection, INRA and other breeding institutions have apparently broken this linkage (Burns *et al.* 1996; Delourme *et al.* 1999; Pruvot *et al.* 1999) and restored canola hybrids are finally making their way into commercial production in Europe and Canada. A problem limiting the use of these restorers in Canadian breeding programs is that the introgression with the restorer gene is on the *Brassica* C-genome (Delourme *et al.* 1998) and as such it can only be used in *B. napus* (which contains the *Brassica* A and C genomes) and not in *B. rapa* (*Brassica* A genome) nor in *B. juncea* (which contains the *Brassica* A and B genomes).

Scientists working with kos CMS from *R. sativus* cv. Kosena introduced a radish restorer gene into *B. napus* by X-ray-irradiating protoplasts of a radish restorer line and fusing them with protoplasts of a *B. napus* kos CMS cybrid (Sakai *et al.* 1996). Sakai *et al.* (1996) found that only one restorer gene was necessary to produce male fertile plants in some of their cybrids. Koizuka *et al.* (2000) demonstrated that while two restorers (*Rf1* and *Rf2*) were necessary for fertility restoration in kos CMS radish, only one (*Rf1*) was necessary in certain kos *B. napus* cybrids. They suggested that the restorer was functionally the same as one of those required for restoration in Ogura cybrids but noted that while the kos restorer *Rf1* was effective at restoring Ogura cybrids, it was not sufficient for restoration of *B. napus* with the full Ogura radish cytoplasm. The level of ORF125 protein, the product of the CMS-associated *orf125* gene, has been shown to be tightly correlated with the genotype at *Rf1* but not with the genotype at *Rf2* in radish (Koizuka *et al.* 2000). The function of *Rf2* is still not known.

All published markers for Ogura CMS restorer alleles were developed after the restorer had been introgressed into *B. napus*. Delourme and Eber (1992) demonstrated co-segregation of a radish isozyme marker, *Pgi-2,* with a segment of radish DNA carrying a restorer gene within *B. napus*. They claimed a tight linkage with a recombination fraction of 0.25% although this linkage is likely due to the radish DNA being inherited as an intact segment or not at all, with recombination within the introgression being very rare. Therefore, any marker loci on the introgression will be

tightly linked to the restorer genetically although it might be physically separated by half a chromosome. It is not known how closely linked this marker is to the restorer gene in radish itself. Several RAPD markers have also been shown to be tightly linked to the restorer gene in restored *Brassica* lines (Delourme *et al.* 1994; Hansen *et al.* 1997; Delourme *et al.* 1998) but their location on the radish genome is also not known.

2.5 *Brassica* and Allied Species

2.5.1 Brassica species

The genus *Brassica* belongs to the tribe *Brassiceae* and is comprised of 20 cytodemes (Prakash *et al.* 1999). *Brassica* species are grown as a wide range of oilseed, vegetable, fodder, condiment and medicinal crops. Of the many different *Brassiceae* in existence, six are considered crop species and have thus been studied extensively. *Brassica napus, B. juncea, and B. carinata* are grown primarily as oilseed crops. Many different forms of *B. rapa* and *B. oleracea* are important vegetable crops and *B. nigra* and *B. juncea* are grown as condiment crops. Morinaga's work in the 1920's and 30's suggested that three of these crop species (*B. rapa, B. nigra,* and *B. oleracea*) are diploid, monogenomic species while the other three (*B. napus, B. juncea,* and *B. carinata*) are digenomic species which evolved from pairwise hybridizations among these diploids (Prakash *et al.* 1999). U (1935) developed evidence for this theory through extensive cytogenetic analysis and presented his findings in a diagram commonly referred to as U's triangle (Fig. 2.2).

By crossing resynthesised *B. napus* (*B. rapa* x *B. oleracea*) with 'natural' *B. napus*, Parkin *et al.* (1995) were able to confirm the presence of both the A and C genomes in 'natural' *B. napus*. In the hybrid between these two lines, the A genome chromosomes from the resynthesised *B. napus* line paired and recombined in a disomic fashion with *B. napus* chromosomes N1 to N10 while the C genome chromosomes did the same with chromosomes N11 to N19 suggesting the two diploid genomes have remained essentially intact since the evolution of *B. napus* (Parkin *et al.* 1995). Robust maps of *B. oleracea* (Bohuon *et al.* 1996) and *B. rapa* (Salava *et al.* 2001) created using the same set of RFLP probes used in the above *B. napus* map (Parkin *et al.* 1995) and a

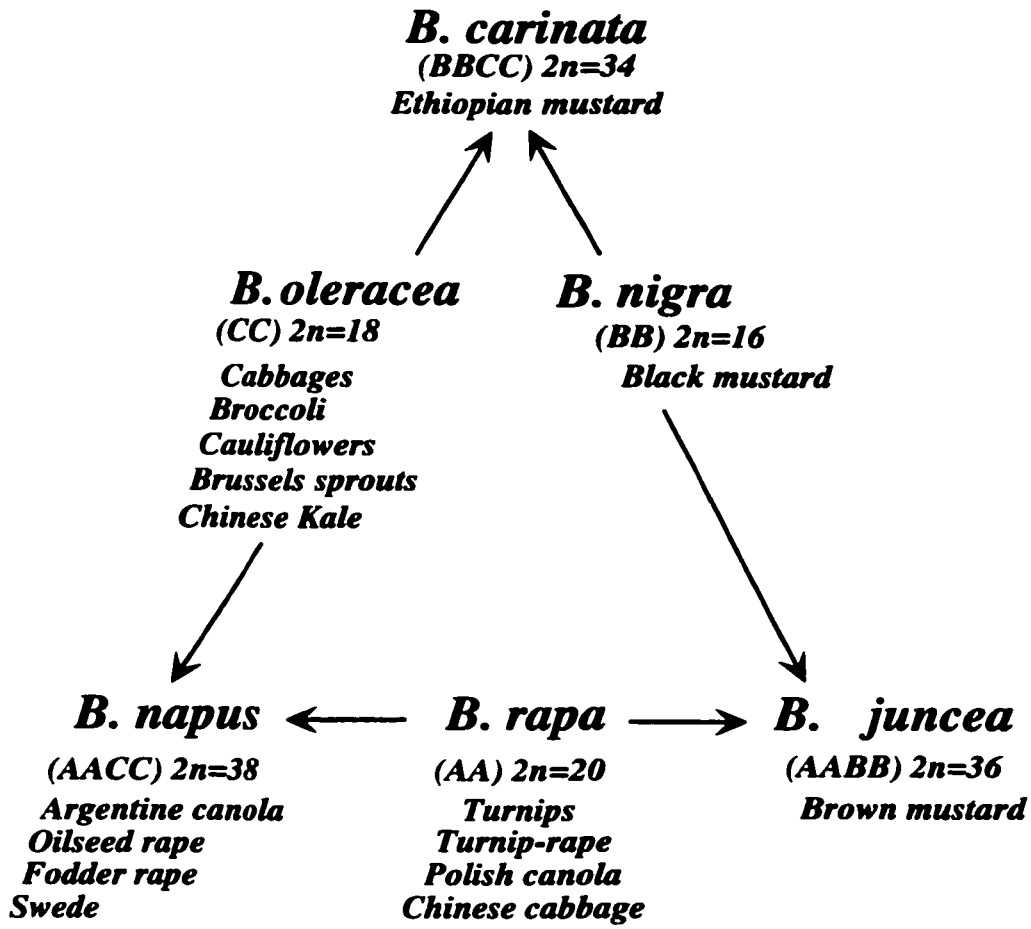


Figure 2.2. Cytogenetic relationships of *Brassica* crops with their genome designations (A, B and C) (redrawn from U, 1935)

related *B. napus* map (Sharpe *et al.* 1995) enabled extensive comparative mapping among the species confirming the presence of intact A and C genomes in *B. napus*. Axelsson *et al.* (2000) used the same strategy to show that *B. rapa* chromosomes paired and recombined exclusively with their A genome homologues in *B. juncea* (J1 to J10) and the *B. nigra* chromosomes similarly paired and recombined exclusively with their B genome homologues (J11-J18).

The ability to resynthesise the amphidiploid species offers new germplasm resources to *B. napus*, *B. juncea* and *B. carinata* breeders and has been used for many different purposes including the development of more productive *B. juncea* (Olsson 1960) and *B. carinata* (Prakash *et al.* 1984) as well as the introgression of genes for specific traits such as yellow seed colour in *B. napus* (Chen *et al.* 1988). Several successful *B. napus* varieties have been developed through crosses with resynthesised *B. napus* and released by Svalof (Olsson 1986).

Synthetic amphidiploids are obtained through sexual hybridisation but the direction of the cross is critical to its success. For example, resynthesised *B. juncea* with *B. nigra* cytoplasm and resynthesised *B. napus* with *B. oleracea* cytoplasm are very difficult to obtain (Prakash *et al.* 1999). The reasons for low levels of success include inhibition of pollen tube growth and embryo abortion (Prakash and Chopra 1991). Ovule culture and embryo rescue are often employed to increase the number of hybrids obtained from interspecies crosses. Meiosis in the resynthesised lines is generally disturbed due to homoeologous pairing between the genomes, resulting in reduced pollen fertility and reduced seed set. Multivalents and univalents have been observed in resynthesised *B. carinata* and resynthesised *B. napus* but are not so prevalent in resynthesised *B. juncea* (Prakash *et al.* 1999 and references therein).

Studies of nuclear and chloroplast DNA (cpDNA) have led to the division of *Brassica* species and their closest allies into two separate phylogenies: the rapa/oleracea lineage and the nigra lineage (Song *et al.* 1990; Warwick and Black 1991 and 1993) (Fig. 2.3). Members of the rapa/oleracea lineage are much more closely related to each other than they are to the members of the nigra lineage as had been suspected based on

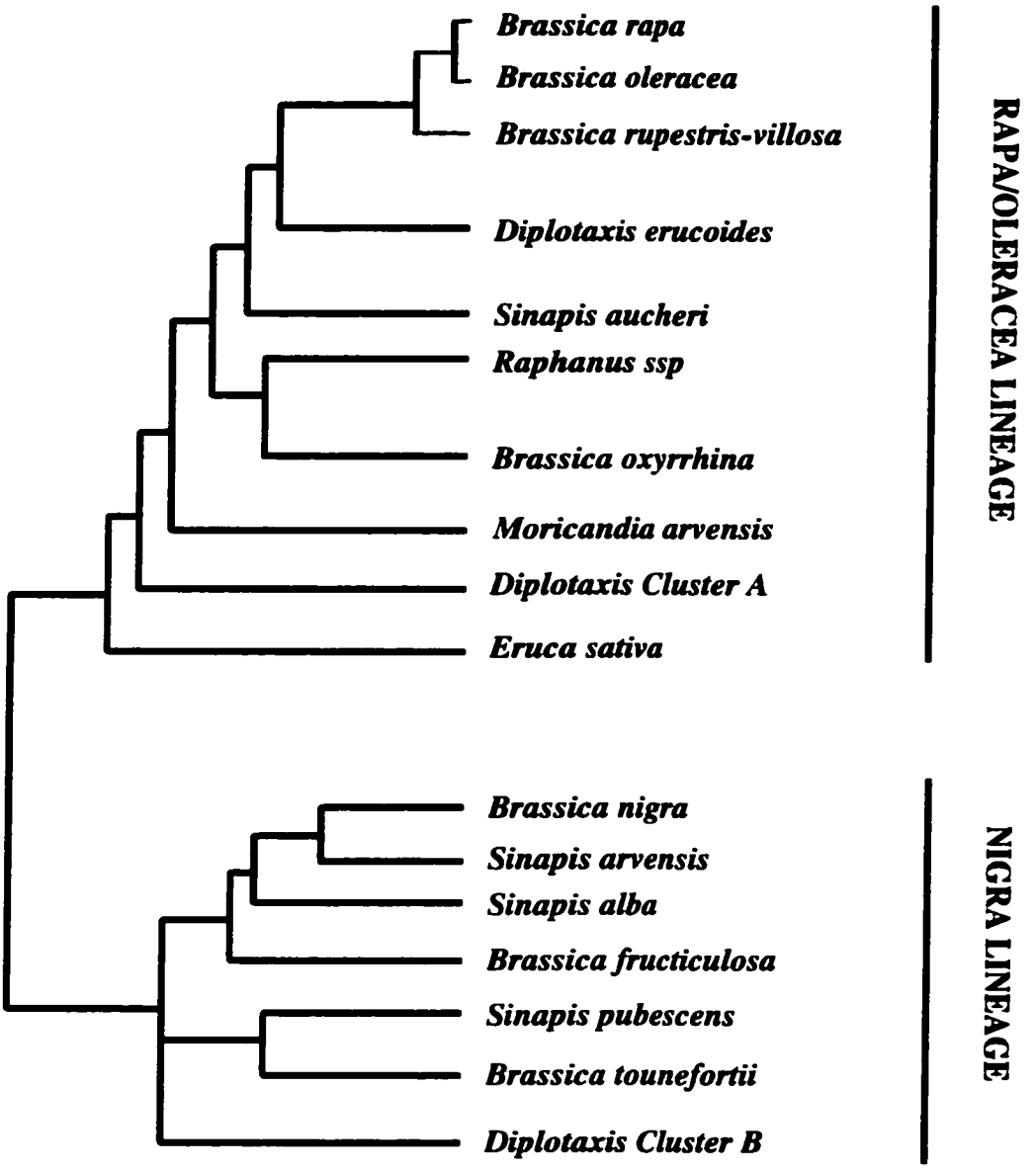


Figure 2.3. Phylogenetic relationships among the diploid species of the Brassiceae tribe as determined by chloroplast genome polymorphisms (redrawn from Warwick and Black, 1991, 1993).

earlier chromosome pairing studies (Prakash *et al.* 1999 and references therein).

2.5.2 Raphanus

Radish (*Raphanus sativus*) is a major vegetable crop in east Asia and a minor crop worldwide. It is grown primarily for its edible root which comes in a range of shapes, sizes and colours. *Raphanus sativus* var. *caudatus* is also grown for its long edible pod in some parts of Asia. *Raphanus sativus* is predominantly an out-crossing species and breeding for hybrids is increasing in popularity to offset inbreeding depression observed with the repeated selfing that has been used to produce a more uniform crop.

The genus *Raphanus* belongs to the tribe *Brassiceae* and is traditionally split into two species: *R. sativus* and *R. raphanistrum*, both with nine chromosome pairs (Warwick and Anderson 1993). *Raphanus sativus* is not known in the wild state although the cultivated form has become a weed in many regions. *Raphanus raphanistrum* is a wild form of radish. The principle difference used by taxonomists to separated the two species is pod morphology (de Candolle 1886). In *R. sativus* the silique is continuous and forms a single cavity. *Raphanus raphanistrum*, in contrast, has siliques that are articulated with a single seed in each division. The seeds are dispersed when the divisions break apart. The two species are known to cross-hybridize, and weedy radishes in California have been shown to be *R. sativus* that has been 'converted' from crop plant to weed through the inheritance of weedy traits from *R. raphanistrum* (Panetsos and Baker 1967). Snow *et al.* (2001) demonstrated that crop genes could be transferred to *R. raphanistrum* and concluded that the resulting hybrid weeds would be likely to persist in the environment. Isozyme studies have demonstrated that *R. sativus* is closely related to *R. raphanistrum* (Lewis-Jones *et al.* 1982). Several cpDNA restriction site maps of these species indicated that their chloroplast DNA differ by only four mutations (Warwick and Black 1991). It is believed that *R. sativus* probably originated from *R. raphanistrum* (de Candolle 1886; Lewis-Jones *et al.* 1982 and references therein).

Aside from its value as a vegetable crop, *Raphanus* carries many traits of interest

to *Brassica* breeders, including resistance to turnip mosaic virus (Fujisawa 1990), resistance to clubroot (Hagimori *et al.* 1992), resistance to beet cyst nematode (Lelivelt *et al.* 1992, 1993), and cytoplasmic male sterility (Ogura 1968).

Raphanus belongs to the rapa/oleracea lineage (Fig 2.3; Warwick and Black 1991), sharing a common ancestor with *B. rapa* and *B. oleracea* after the separation of the rapa/oleracea and nigra lineages. Cytological studies of interspecies hybrids between *R. sativus* and *B. rapa* and between *R. sativus* and *B. oleracea* has demonstrated close chromosome homology as indicated by the presence of up to six bivalents in the amphihaploid produced from each cross (Richharia 1973).

2.6 Genetic mapping

Plant breeders have long used phenotype as a basis for selection for improved cultivars with varying degrees of success. Phenotype, however, is often affected by not only the genetic background of the plant but also the environment in which it is grown. As a result, selection often involves testing in multiple environments and observation of multiple generations adding to the time and expense of developing new and improved cultivars. Sax (1923) first proposed the use of simply inherited genes with clear, easily assessed phenotypes as markers for more complex traits. Unfortunately these morphological markers are few and far between and often have a direct negative effect on fitness or other pleiotropic effects that make them undesirable in breeding programmes. With the development of DNA technologies, the door opened for the development of phenotypically neutral markers that might assist in early-generation selection in a single environment for genes controlling complex traits.

2.6.1 DNA-based marker systems

Restriction fragment length polymorphism (RFLP) markers are based on Southern hybridisation using cDNA or small genomic clones as probes and using polymorphisms in enzyme restriction sites or in the length of sequences separating restriction sites to provide allelic variation. Polymorphisms among individuals are

revealed when radioactively labelled DNA probes are hybridised to homologous restriction fragments after separation by gel electrophoresis. RFLP markers are typically co-dominant allowing heterozygotes to be identified, and they detect relatively large numbers of naturally occurring alleles (Helentjaris *et al.* 1985). The high degree of conservation of gene sequences during evolution makes possible the use of RFLP markers derived from one species in the genetic mapping of related species.

The first linkage maps in plants based on DNA markers were RFLP maps of tomato (Bernatzky and Tanksley 1986) and maize (Helentjaris *et al.* 1986). Figdore *et al.* (1988) assessed the degree of polymorphism detected by RFLP markers in various *Brassica* species and concluded that the level was high enough to warrant their use in *Brassica* genome mapping. Since then, RFLP-based maps of *B. rapa* (Song *et al.* 1990; Chyi *et al.* 1992; Salava *et al.* 2001*), *B. napus* (Landry *et al.* 1991; Ferreira *et al.* 1994; Parkin *et al.* 1995*; Sharpe *et al.* 1995*; Uzonova *et al.* 1995), *B. oleracea* (Slocum *et al.* 1990; Landry *et al.* 1992; Kinian and Quiros 1992; Bohuon *et al.* 1996*), *B. nigra* (Lagercrantz and Lydiate 1995*) and *B. juncea* (Cheung *et al.* 1997b; Axelsson *et al.* 2000*) have been published. RFLP markers that were used to produce some of these *Brassica* maps (*) have also been used in the development of maps of related crucifers: *Arabidopsis* (I. Parkin, pers.comm.), *Crambe* (Ford 2000), *Moricandia* (Beschorner *et al.* 1999), and *Sinapis* (Nelson 2000).

The amplification of specific DNA sequences made possible by polymerase chain reaction (PCR) (Saiki *et al.* 1988) has led to the development of several DNA-based marker systems. PCR-based markers such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and microsatellite markers have gained in popularity compared to RFLP markers as they are less expensive, give quicker results, need not involve radioisotopes and require smaller amounts of DNA.

RAPD markers are generated by amplifying fragments of genomic DNA using a single random oligonucleotide primer. These PCR products are separated by gel electrophoresis and visualised using ethidium bromide stain (Williams *et al.* 1990). Polymorphisms result from differences in and between primer annealing sites. Many

marker loci can be generated rapidly but RAPD markers are generally dominant and their sensitivity to reaction conditions decreases their reproducibility between labs (Penner *et al.* 1993).

The AFLP technique (Vos *et al.* 1995) is based on the selective amplification of sub-populations of restriction fragments of digested genomic DNA. These fragments are separated by high-resolution gel electrophoresis allowing a large number of amplified fragments to be resolved. Many polymorphic loci are usually revealed making AFLPs useful for bulked segregant analysis and high-resolution mapping. As with RAPD markers, however, AFLPs are usually dominant and evolutionarily unrelated to each other making them unsuitable for comparative genome analysis.

Microsatellites or simple sequence repeats offer the advantages of PCR-based markers while overcoming the problem of dominance. These markers are based on polymorphisms in the number of di-, tri-, or tetranucleotide repeats and are detected by PCR using pairs of primers specific to the DNA flanking the repeat (Powell *et al.* 1996). Unlike with RAPD and AFLP markers, generally only one locus is identified by each primer pair, however it is possible to multiplex primer pairs in the same PCR amplification to assay multiple loci per reaction. Microsatellites are amenable to automation and are beginning to play an important role in mapping in several important species (*Arabidopsis*: Bell and Ecker 1994; soybean: Powell *et al.* 1996; wheat: Röder *et al.* 1998). One drawback to microsatellites is that, unlike RFLPs, they often do not detect homoeologous loci in related genomes since they require that the sequences match those of the template precisely. Therefore, despite being time-consuming and requiring large amounts of DNA, RFLP markers continue to be useful for comparative mapping.

2.6.2 Comparative mapping

Loci are often detected by a particular RFLP probe in a wide range of species because the target DNA sequence has retained sufficient DNA sequence similarity to the probe to allow efficient Southern hybridisation. The loci detected in different species are evolutionarily related to one another and their comparative mapping provides insights into genome evolution. By comparing maps of related species generated from a common

set of RFLP probes, the extent and nature of chromosome rearrangement since the species diverged can be assessed. Establishing the relationship between the chromosome segments of different species allows for the transfer of information and resources from well studied species, such as *Arabidopsis* and rice, to related species. The sequence of the whole *Arabidopsis* genome was published in 2000 (The Arabidopsis Genome Initiative 2000) and the sequence of the entire rice genome is expected to be published within a few years (Sasaki and Burr 2000), giving *Brassica* and cereal geneticists a rich source of new information that could be used for mapping in these related crops. Knowing which regions of two genomes are homoeologous can also be used to predict and track pairing and recombination in interspecies hybrids, thereby allowing for the selection of hybrid progeny carrying the desired recombinant genotype.

The first set of comparative maps for plants were in the Solanaceae (Bonnierbale *et al.* 1988; Tanksley *et al.* 1992; Prince *et al.* 1993) where the genomes of tomato, potato and pepper were compared. These three species each have n = 12 pairs of chromosomes but differ in DNA content by two to four fold (Livingstone *et al.* 1999). RFLP analysis indicated that the genomes of tomato and potato are highly conserved with only five paracentric inversions required to explain the differences (Tanksley *et al.* 1992; Bonnierbale *et al.* 1988). Tomato and pepper have more breaks in collinearity, mostly at the ends of highly conserved homoeologous linkage blocks, and involve both inversions and translocations (Prince *et al.* 1993; Livingstone *et al.* 1999).

Comparative mapping in the cereals began with hexaploid wheat (Chao *et al.* 1989). Analysis demonstrated that the three wheat genomes (ABD) along with those of rye and barley, showed extensive collinearity spanning entire linkage groups (Devos *et al.* 1993). Once rice and other cereals were mapped it became evident that there were remarkable similarities among all *Poaceae* (Moore *et al.* 1995; Gale and Devos 1998). In fact, detailed mapping projects involving the major cereal species have indicated that all cereal genomes mapped to date consist of 19 conserved linkage segments represented in the rice genome and found distributed amongst n = 5 to n = 12 haploid chromosomes in different species (Moore *et al.* 1995; Gale and Devos 1998). The large differences in DNA content found between different *Poaceae* species have been attributed to variaton

in repetitive DNA elements in non-coding regions and not differences in actual gene content (Keller and Feuillet 2000). Helentjaris *et al.* (1988) found extensive duplication of RFLP loci when mapping the maize genome suggesting that maize is in fact an ancient polyploid. The theory that the maize genome is a tetraploid has been reinforced by the finding that each segment of the rice genome can be found on two different maize chromosomes (Moore *et al.* 1995).

The majority of comparative mapping studies in *Brassica* have focussed on *B. rapa* (A genome), *B. nigra* (B genome) and *B. oleracea* (C genome) and their relationships to each other and to *B. napus* and *B. juncea*. The amount of nuclear DNA in *B. nigra*, *B. oleracea* and *B. rapa* is similar (Arumuganthan and Earle 1991) but it is packaged into eight, nine and ten chromosome pairs, respectively. Polyploid origins for the diploid *Brassica* species were proposed by Prakash and Hinata (1980) and mapping in the individual genomes has revealed extensive duplication of chromosome segments in *B. rapa* (Song *et al.* 1991; Chyi *et al.* 1992), *B. nigra* (Lagercrantz and Lydiate 1996) and *B. oleracea* (Slocum *et al.* 1990). The extent of duplication has been consistently underestimated, however, due to the presence in all crosses of monomorphic loci that cannot be mapped. For example, Lagercrantz and Lydiate (1996) detected on average 1.9 loci per probe in their *B. nigra* mapping population however, once the residual monomorphism was taken into account, they estimated 2.6 loci were actually detected per probe. A cross between a winter *B. napus* cultivar and a spring *B. napus* cultivar resulted in 1.7 informative loci being detected per RFLP probe (Sharpe *et al.* 1995) while a cross between a resynthesised *B. napus* line and the same winter cultivar resulted in 2.4 informative loci being detected per probe (Parkin *et al.* 1995). RFLP analysis of the resynthesised by cultivar cross enabled additional loci, monomorphic in the cultivar by cultivar cross, to be mapped.

Using a detailed map of *B. nigra*, Lagercrantz and Lydiate (1996) demonstrated that the whole B genome could be divided into eight distinct sets of chromosome segments each of which is found three times across the genome. Comparisons of this B genome map with A and C genome maps constructed with the same RFLP probes

(Parkin *et al.* 1995; Sharpe *et al.* 1995; Bohuon *et al.* 1996) demonstrated the essentially triplicated nature of all three genomes (Lagercrantz and Lydiate 1996). A comparison of the three genome maps revealed a high degree of genome conservation, suggesting they all descended from a common hexaploid ancestor (Lagercrantz and Lydiate 1996). Since divergence, rearrangements including chromosome fusion and fission have probably led to differences in chromosome number.

Initial comparative mapping studies in *Brassica* (Teutonico and Osborn 1994) indicated large regions of homology between the *Brassica* A and C genomes but the limited number of common loci made it difficult to appreciate the full extent of the collinearity. Truco *et al.* (1996) compared the A, B and C genomes and found conserved regions among the three maps. The lowest level of homology was between the A and B genomes followed by the B and C genomes, while the A and C genomes showed the highest level of homology which is as expected from phylogenies based on variation in chloroplast genomes (Warwick and Black 1991). Again, the full extent of collinearity was not elucidated as there were only 85 loci mapped across the three genomes of which only 33-38 were segregating in both members of a pair of genomes and only 25 were common to all three genomes.

Cheung *et al.* (1997a) compared *B. oleracea* to *B. napus* and found that each *B. oleracea* linkage group was homoeologous with between two and three *B. napus* linkage groups. Since probes that hybridise to the C genome can also hybridise to the A genome and there is internal duplication within the C (and A) genome, it is difficult to determine which linkage groups are homologous and which are homoeologous making this comparison difficult. To reduce this confusion, Parkin *et al.* (1995) developed a map from a highly polymorphic cross between resynthesised and normal *B. napus* such that the A and C genomes could be identified based on matching alleles with those of the diploid *B. rapa* and *B. oleracea* parents. From the resulting map, it was possible to determine which linkage groups belonged to the A genome and which belonged to the C genome and demonstrate extensive homoeology between the two genomes. In some cases entire linkage groups were collinear (e.g. N1 with N11) while in others, rearrangements had occurred (e.g. N4 and N5 with N14).

A comparison of a B genome map (Lagercrantz and Lydiate 1996) with A and C genome maps (Parkin *et al.* 1995; Sharpe *et al.* 1995; Bohuon *et al.* 1996) indicated a high degree of conservation of genome content but also demonstrated that the collinear segments are arranged differently within each genome. Several large inversions differentiated the B genome from the A and C genomes and there were instances where the ends of A and C linkage groups had been brought together in internal regions of B linkage groups, or vice versa (Lagercrantz and Lydiate 1996).

Few comparisons have yet been made between the A, B and C genomes and the genomes of species outside of U's triangle. The model plant *Arabidopsis thaliana* also belongs to the family *Brassiceae* and is therefore related to the *Brassica* species. In a comparative mapping study of *A. thaliana* with *B. oleraea*, Kowalski *et al.* (1994) found extensively rearranged collinear segments of 3.7 to 49.6 cM. They also found a rearragement that is present in *B. oleracea* homoeologues showing common marker order, suggesting the rearrangement occurred prior to the polyploidization that led to the *B. oleracea* genome. Lagercrantz (1998) compared *A. thaliana* with *B. nigra* and found that ~90 rearrangements have occurred since they diverged 10-35 million years ago. It also appeared that the *A. thaliana* genome is similar in structure to the diploid progenitor of the hypothesised hexaploid that led to the *Brassica* genomes but that extensive rearrangements have occurred since the polyploidization that led to the modern *B. nigra* genome.

From the various studies across many different plant families, it is apparent that locus order among families is often highly conserved within homoeologous linkage blocks but the blocks themselves are highly rearranged with respect to each other. Breaks between blocks are often associated with telomeric and centromeric regions (cereals: Moore *et al.* 1997; Solanaceae: Livingstone *et al.* 1999; crucifers: Lagercrantz and Lydiate 1996) and cryptic polyploidy is common.

2.6.3 Marker-assisted backcross breeding

Backcross breeding is a method of introducing the allele or alleles controlling a

desirable trait from one genotype (donor) into the genome of another, otherwise desirable, genotype (recurrent parent) without excessively altering the genetic make-up of the recurrent parent (Briggs 1938). Often the donor is only distantly related to the desired genotype and the likelihood that it carries alleles controlling undesirable traits at dispersed loci increases the more exotic the donor germplasm used. Originally, donor germplasm was chosen from closely related material to reduce sterility problems associated with wide crosses. Tissue culture techniques such as embryo rescue (Laibach 1929) have allowed breeders to move further afield in their search for alleles controlling desirable traits for introgression into crop plants and breeders now use wild relatives in crosses. Backcrossing to the recurrent parent is carried out to eliminate the excess donor genetic material thus reducing or eliminating undesirable side effects of introducing donor genetic material.

Prior to the advent of molecular markers, breeders could only speculate which segments of donor genome had been introgressed into hybrid plants and unwanted regions of the donor genome were eliminated through repeated backcrossing combined with selection for plants that appeared to most resemble the recurrent parent. With molecular markers it is possible to determine which regions of the donor genome are present and select for those individuals with the desired segment of donor genome prior to further backcrossing. Tanksley *et al.* (1989) used computer simulations of a tomato backcrossing program and estimated that by using RFLP-based selection across the whole genome, they could effectively reconstruct the recurrent parent genotype in only three generations instead of the more than six generations that would be required using traditional backcross breeding. Marker accelerated backcrossing has made the use of wide crosses more attractive to breeders by improving the speed and reliability with which undesirable portions of the donor genotype can be eliminated.

Entire donor chromosomes and regions unlinked to the gene of interest are relatively easy to eliminate through repeated backcrossing compared to regions of the donor genome linked to the gene of interest which are much more difficult to eliminate (Hanson 1959; Stam and Zeven 1981). Even with 20 backcrosses there can easily still be a 10 cM piece of donor genome linked to the gene of interest (Stam and Zeven 1981).

Retrospective analysis of *Lycopersicon peruvianum* chromosome segments retained around the *Tm-2* locus following introgression into tomato (*L. esculentum*) has demonstrated the efficiency of using markers and high density maps to select individuals with recombination events 1 cM from the gene of interest, thus reducing the size of donor introgression from 20 cM to 2 cM in just two generations (Young and Tanksley 1989).

By using DNA-based markers, it is also possible to detect and select individuals carrying multiple genes of interest at the seedling stage and thereby select for multiple traits including those not expressed early enough for detection and selection prior to the end of flowering and allowing for more efficient backcrossing (Tanksley *et al.* 1989). Good examples of simultaneous selection for multiple traits are fruit weight, pH and soluble solids in tomato (Osborn *et al.* 1987; Paterson *et al.* 1988; Tanksley and Hewitt 1988). Molecular markers are also useful for selecting for recessive alleles during backcrossing since they eliminate the need to self or testcross to detect the presence of the allele prior to backcrossing. In a similar way, molecular markers make testing for homozygous dominant alleles at a given locus simple.

2.7 Interspecific gene transfer

Continued improvement in crop cultivars relies on sources of genetic variability. This is most easily accessed from primary gene pools however, the gene or alleles for a trait of interest are sometimes simply not available in the primary or secondary pool and breeders must look to tertiary gene pools. Interspecies gene transfer has been carried out for over a century in an attempt to transfer valuable genes controlling traits from different wild species into related crop species. Originally crosses were limited to combinations where sexual crossing was successful without human intervention but with the advent of tissue culture techniques designed to negate incompatible embryo-ovule relationships, wider crosses became possible.

For introgression of a segment of donor chromosome into the crop genome recombination must occur. If the donor originates from a primary or secondary gene pool and has at least one genome in common with the recipient, homologous

recombination can occur readily and gene transfer is relatively straight forward. If, however, the donor is from a tertiary gene pool, and as a result has no chromosomes truly homologous to those of the recipient, gene transfer can only occur if there is sufficient homoeology between chromosome segments in the donor and recipient genomes (Fedak 1999).

Pairing between homoeologous chromosomes can be complicated by the presence of pairing control factors such as the *Ph* locus of wheat (Riley *et al.* 1960). Although such a pairing control locus has yet to be identified in *Brassica* species, there is some evidence for it. RFLP marker analysis of crosses between *B. napus* cultivars show only a low level of homoeologous recombination - a frequency of approximately 0.3% that of homologous recombination (Sharpe *et al.* 1995), while resynthesised *B. napus* has been shown to have a level of homoeologous recombination that constitutes approximately 10% of the total recombination events (U. Lagercrantz and D.J. Lydiate, pers. comm.).

Breeders and geneticists have being working with interspecies crosses between *Raphanus* and various *Brassica* species since the early 1900's. The amphidiploid *Raphanobrassica* (RRCC), resulting from pollinations of *R. sativus* (R) with *B. oleracea* (C), was first obtained by Karpechenko (1924). It arose through spontaneous chromosome doubling and had 18 bivalents at meiosis. The reciprocal *B. oleracea* x *R. sativus* cross, however, was not successful (Karpechenko 1924). Sarashima *et al.* (1980) used embryo rescue to make a CC x RR cross, however they made almost ten thousand pollinations and only obtained 12 hybrids. McNaughton (1973) used diploid (CC) and tetraploid (CCCC) *B. oleracea* plants as pollinators in crosses with *R. sativus* and found that crosses with the tetraploid were more successful. Pairing in the resulting RRCC hybrids was primarily bivalent but occasionally 17 bivalents plus two univalents were observed. A number of the RRCC plants were male sterile and many were female sterile with embryo abortion occurring at various stages. Embryological studies have shown that the failure to produce seeds from RRCC hybrids is related to a failure of fertilization and to embryo abortion rather than irregular meiosis (Ellerström and Zagorsheva 1977).

Cytological studies on haploid RC hybrids generated by Richharia (1973) revealed up to six bivalents and multivalents and secondary associations were found in the amphidiploids (RRCC) of this cross. McNaughton (1973) observed a ring of four in one cell and a trivalent in two cells of a haploid RC hybrid, a sign of pairing between R and C chromosomes. Sterility problems appear to lessen with repeated inbreeding and/or backcrossing combined with selection for productive individuals. Forage varieties of *Raphanobrassica* (RRCC) have been developed for commercial production (McNaughton and Ross 1978; Olsson and Ellerström 1980).

Hybrids between *R. sativus* (R) and *B. rapa* (A) are reportedly much more difficult to produce although a few have been developed (U *et al.* 1937; Ellerström and Sjödin 1973). Ellerström and Sjödin (1973) found that most seeds produced from an *R. sativus* by *B. rapa* cross were matromorphic and fertility in the hybrids was low decreasing to complete infertility within five generations. McNaughton and Ross (1978) were unable to produce amphidiploid RRAA individuals despite several thousand pollinations between tetraploid *R. sativus* (RRRR) and tetraploid *B. rapa* (AAAA). Matsuzawa *et al.* (2000) also performed thousands of pollinations and used embryo rescue but still only obtained 18 *R. sativus* x *B. rapa* hybrids. The embryos from these self-pollinated F_1 plants also required rescuing to produce F_2 seeds. The use of *B. rapa* as the female in crosses with *R. sativus* has been used to develop *Brassicoraphanus* (AARR) amphidiploids (Tokumasu 1976). The F_2 and F_3 plants of this material had meiotic irregularities including multivalents, suggesting pairing occurs between A and R chromosomes.

Hybrids between *R. sativus* (R) and *B. napus* (AACC) are also considered very difficult to produce even with embryo rescue techniques and the seeds obtained from this combination are often matromorphic (McNaughton and Ross 1978; Takeshita *et al.* 1980; Lelivelt *et al.* 1993). The genotypes used as parents may have an effect on the success of *R. sativus* by *B. napus*, and reciprocal, crosses. Kamala (1983) performed 100 pollinations with *R. raphanistrum* pollen onto *B. napus* plants, and obtained 43 seeds. Of these, 14 germinated and were true hybrids. Cytological observations of meiosis in these hybrids showed up to 12 bivalents indicating some degree of pairing between the R

genome and the A and/or C genomes. Paulmann and Robbelen (1988) crossed radish lines carrying CMS cytoplasm with pollen from *B. napus*, doubled the chromosomes with colchicine and then crossed the resulting allohexaploid (AACCRR) to *B. rapa* or *B. oleracea* with the intention of encouraging introgression of segments of the radish genome into the C and A genomes respectively. The resulting RCAA and RACC hybrids were repeatedly selected for 2n = 38 chromosomes and backcrossed to *B. napus*. Only through embryo rescue were the interspecific and first backcross progeny successfully produced. Paulmann and Robbelen (1988) observed that pollination with *B. oleracea* was much less successful than with *B. rapa* and seed set in the backcross was much lower than in the F_1. Hybrids derived from a radish carrying a restorer allele were all male sterile until the second backcross generation when a few male fertile plants were identified. Thierfelder *et al.* (1991) used the same strategy in an attempt to transfer nematode resistance from *R. sativus* to *B. napus*. They found that when *B. napus* was the female parent, 9% of cultured ovules germinated while only 2.8% germinated when *R. sativus* was the female.

The use of *Raphanobrassica* and *Brassicoraphanus* hybrids as bridge parents in crosses with *B. napus* has been much more successful than simple *R. sativus* by *B. napus* crosses (Rousselle and Dosba 1985; Agnihotri *et al.*1990; Lelivelt *et al.* 1993). Using *Brassicoraphanus* and/or *Raphanobrassica* also allows for the encouragement of pairing between R and A or R and C chromosomes and discourages pairing between A and C chromosomes. Heyn (1978) and Rousselle and Dosba (1985) used *Raphanobrassica* (RRCC) lines carrying (a) restorer gene(s) for Ogura CMS in crosses with male sterile *B. napus* plants to develop restorer lines for Ogura CMS *B. napus*. In both cases, few F_1 seeds were produced but enough pollen was produced on these plants that backcrossing onto male sterile *B. napus* plants was successful.

Agnihotri *et al.* (1990) successfully used ovary culture followed by embryo rescue to obtain plantlets from a cross between *B. napus* and *Raphanobrassica*. They found that the pods on the *B. napus* plant started to wither 10-15 days after pollination with *Raphanobrassica* pollen and only 20% of the pods cultured *in vitro* had enlarged ovules. In their experiment, 102 ovules were cultured, 24 produced enlarged ovules and

19 embryos were successfully grown into plantlets. The RACC plants that they developed were all male sterile and PMCs in the hybrid had varying numbers of univalents and bivalents but always more than nine bivalents suggesting homoeologous pairing between the R and A genomes was occurring. Backcrosses with *B. napus* pollen resulted in few seeds being produced and only one BC_1 plant produced seed.

Lelivelt *et al.* (1993) also found that using *Brassicoraphanus* (AARR) as a bridge in crosses with *B. napus* was much more successful than simple *R. sativus* by *B. napus* crosses. Cytological examination of PMCs of their F_1 hybrids (RCAA) showed that the majority had the expected 38 chromosomes but that there was a higher level of chromosome association than expected from pairing between just the A genome chromosomes, suggesting the R and C chromosomes were also pairing. They observed low pollen fertility in the F_1 but it improved with repeated backcrossing to *B. napus*.

From this review of the pertinent literature, it is evident that the necessary technologies exist for the characterisation of Ogura CMS restorer genes in radish and the efficient transfer of specific radish restorer alleles to the *Brassica* A genome.

Chapter 3. Methods and Materials

3.1 Plant Methods

3.1.1 Original plant material

The plant materials, along with the most pertinent attributes, the origin and associated references, are listed in Table 3.1.

MST1 is a population of *Raphanus sativus* plants with Ogura cytoplasm and segregating for male fertility restorer alleles (Crucifer Genetics Co-op Catalogue: Wisconsin Fastplants, Madison, WI; Table 3.1). It is maintained by allowing male fertile plants to pollinate male sterile plants as well as other male fertile plants. A small population of MST1 individuals was grown and three male fertile plants were selfed (Sandra Kelly, Advanta Seeds, Winnipeg: pers. comm.) to produce populations R7, R8, and R9. Single plants from HRI Accessions 1047, 1020 and 4597 (Table 3.1) were self pollinated to produce populations R10, R11 and R12 respectively.

3.1.2 Growing conditions

Plants that were to be scored for fertility and interspecific hybrids requiring isolation were grown in growth cabinets with a 16 hour light period at 18°C and an eight hour dark period at 12°C. All other plants were grown in the greenhouse under a 16h photoperiod and a 20/17°C day/night temperature regime.

3.1.3 Phenotypic scoring

CMS phenotypes were scored by examining flowers on all racemes for pollen production over the course of several weeks. Plants that never produced pollen were scored as male sterile, those that did produce pollen were scored as male fertile. Plants that produced pollen on some but not all racemes were scored as partially male fertile (pmf). The pmf plants typically had anthers of intermediate size and only small amounts of pollen were found on those anthers that did shed pollen.

Table 3.1 Original plant materials involved in this study and the pertinent attributes.

Line	Species	Source [a]	Pertinent Attributes	References
MST1	*Raphanus sativus*	CrGC	- Ogura CMS cytoplasm - population segregation for restoring and non-restoring alleles	Crucifer Genetics Co-op Catalogue http://fastplants.cals.wisc.edu/crgc/crgc.html
Accession 1047	*R. raphanistrum*	HRI	- wild radish var. Munra	
Accession 1020	*R. sativus* spp. *caudatus*	HRI	- wild radish var. Magri	
Accession 4597	*R. sativus* spp. *caudatus*	HRI	- wild radish	
R33	*Brassica rapa*	AAFC -1	- canola-quality, self-compatible, doubled haploid breeding line	
A12	*B. oleracea spp.* alboglabra	AAFC -2	- self-compatible, doubled haploid line - rapid flowering	Bohuon *et al.* 1996
DH12075	*B. napus*	AAFC -3	- canola-quality doubled haploid breeding line	
N-o-9	*B. napus*	AAFC -2	- double haploid parent from a *B. napus* mapping population	Parkin *et al.* 1995 Sharpe *et al.* 1995
Rf/noRf	*B. napus*	Advanta Seeds	- pair of isogenic breeding lines - with and without INRA Ogura restorer introgression	

[a] CrGC - Crucifer Genetics Co-op, Madison, WI, USA; HRI - Dave Astley, Genetic Resources Unit, Horticulture Research International, Wellesbourne , U.K. AAFC-1 - Dr. Kevin Falk, AAFC Saskatoon Research Centre, Saskatoon, SK, Canada; AAFC-2 - Dr. D. Lydiate, AAFC Saskatoon Research Centre, Saskatoon, SK, Canada; AAFC-3 - Dr. G. Rakow, AAFC Saskatoon Research Centre, Saskatoon, SK, Canada; Advanta Seeds - Greg Buzza, Advanta Seeds, Winnipeg, MB, Canada.

3.2 Tissue Culture Procedures

3.2.1 Pod culture

Pods were harvested six days after pollination, sterilised in a 2.5% bleach solution for two minutes, and rinsed in sterile water. After removing the pedicel, pods were inserted into sterile Monnier's medium (Monnier 1973) and placed in a culture

room (16h photoperiod; 22°C constant temperature).

3.2.2 Ovule culture

Pods were sterilised in a 2.5% bleach solution for two minutes and rinsed in sterile water. Pods were opened under sterile conditions and the ovules were removed and cultured on Nitsch and Nitsch media (Nitsch and Nitsch 1969; Sigma) supplemented with 0.3g/L caesin hydrolysate, 1.5 % glucose and 1.5% sucrose and placed in the culture room. Pods derived from the AA x CC crosses were removed from the Monnier's medium (Monnier 1973) after approximately ten days, and ovules were removed by dissection and cultured on MS medium (Murashige and Skoog 1962; Sigma) supplemented with 1% sucrose.

3.2.3 Embryo culture

Ovule cultures were examined weekly and any germinating embryos were transferred to fresh MS media (Murashige and Skoog 1962; Sigma) supplemented with 1% sucrose.

3.2.4 Chromosome doubling

The roots of two-week-old haploid seedlings and the roots of haploid plantlets from culture were soaked in a 0.3% colchicine solution for eight hours and rinsed. The seedlings and plantlets were then planted in soilless potting mix in the greenhouse and covered for one week to maintain high levels of humidity during acclimation. Flow cytometry was carried out on excised leaf segments as described by Kudo and Kimura (2001) to confirm chromosome doubling.

3.3 Marker technology

3.3.1 Molecular test for Ogura cytoplasm

Extraction of genomic DNA was carried out as described by Sharpe *et al.* (1995). PCR (polymerase chain reaction) amplification of *Raphanus* total DNA was carried out using primers for the Ogura-specific *orf138* sequence (5' GCC ACG TGT

AGC CCT GTA T 3' and 5' AAT CCC TCC AGA CAG CTT CA 3') and primers for the control *orf158* sequence (5' CTG TCT GGA GGG AAT CAT 3' and 5' CCC CCG AAT CTT ACT CA 3') to test for the presence or absence of Ogura cytoplasm (Bonhomme *et al.* 1992; Krishnasamy and Makaroff 1993). The amplification reactions were performed in 25 μl reaction mixes containing 5 pmol of each ORF138 primer, 10 pmol of each ORF158 primer, 30 ng DNA, 1x PCR buffer (PE Biosystems), 1.5 mM MgCl, 0.2 mM dNTP and 0.5 units of AmpliTaq Gold (PE Biosystems). The amplification consisted of 10 min at 94°C followed by 30 cycles of 45s 95°C, 45s at 53°C and 45s at 72°C, followed by 10 min at 72°C. The products were run out on a 1% agarose gel with a 1 kb ladder, stained with ethidium bromide and visualised using a UV transilluminator.

3.3.2 RFLP markers

Raphanus restriction fragment polymorphism (RFLP) markers were based on *Brassica* RFLP clones used to probe Southern hybridisation filters carrying *Raphanus* genomic DNA digested with *Eco*RI. DNA extraction and Southern hybridization were carried out as described by Sharpe *et al.* (1995) except that all filter washes were carried out at the lower stringency (2X SSC, 0.1% SDS). All *Brassica* RFLP clones were derived from four libraries of genomic DNA fragments: pN, pO, pR and pW (Sharpe *et al.* 1995; Thormann *et al.* 1994) or a library of *B. napus* etiolated seedling cDNA: cA probes (A. Sharpe, pers. comm.). A list of clones used can be found in Appendix 1.1.

3.3.3 Microsatellite markers

The extraction of genomic DNA was carried out as described by Sharpe *et al.* (1995). Flourescently-labelled primers for *B. napus* microsatellites were obtained from A.G. Sharpe and D.J. Lydiate, AAFC, Saskatoon. (The primers were labelled with one of three dyes, hex, 6-fam or tet, which fluoresce at distinct wavelengths and are visualised using Filter Set C on a PE Biosystems ABI 377). Amplification reactions were performed in 10 μl reaction mixes containing 1 μM of forward primer and 1 μM of reverse primer, 40 ng DNA, 1x PCR buffer (PE Biosystems), 2.5 mM MgCl, 0.8 mM

dNTP and 0.5 units of AmpliTaq Gold (PE Biosystems). The amplification conditions were 10 min at 95°C followed by 8 cycles of 15s 95°C, 15s at 50°C and 30s at 72°C followed by 27 cycles of 15s 89°C, 55s at 55 °C and 30s at 72°C followed by 10 min at 72°C. 2.5 μl of the 6-fam-labelled reaction, 2.5 μl of the tet-labelled reaction and 10 μl of the hex-labelled reaction were pooled with 15 μl of water so that the amplification products derived from the three primer pairs could be run in each lane of the gel. Samples were run out on polyacrylamide gels on an ABI 377 using standard conditions including an internal lane standard (Genescan Reference Guide 1997).

3.4 Genetic map construction and analyses

3.4.1 Linkage analysis

The initial genetic linkage analysis was carried out using MAPMAKER version 3.0 (Lander *et al.* 1987) with an initial minimum LOD score of 4.0 to group markers and three-point and multi-point analyses to establish the most-probable locus orders. After careful proof-reading, the final locus order was established by minimising the number of double cross-overs flanking single loci. Recombination frequencies were converted to map distances using Kosambi's mapping function (Kosambi 1944).

3.4.2 Marker distribution

To test whether markers were distributed randomly across the genome, the observed frequency distribution of intervals with varying numbers of crossovers separating adjacent markers was compared with the expected distribution based on a random distribution of both markers and crossovers and calculated according to Dietrich *et al.* (1992). The observed and expected distributions were compared using the Kolmogorov-Smirnov statistic. This non-parametric test is concerned with the mean, standard deviation, and shape of two distributions. It measures the maximum numerical difference between the cumulative distribution functions of the samples and tests against the null hypothesis that they are identical (Steel and Torrie 1980).

3.4.3 Segregation distortion

To test for segregation distortion across the genome, the allele ratio was assessed at each locus and tested against the expected 1:2:1 ratio in F_2 populations and the expected 1:1 ratio in the BC_1 population.

Chapter 4. Developing defined parental material for the identification of loci involved in the restoration of Ogura CMS in radish

Prior to mapping the loci controlling the restoration of Ogura CMS in radish, it was necessary to identify and develop materials with defined interactions and genotypes and to develop populations segregating for male fertile/sterile phenotype and polymorphic at numerous marker loci. Several wild radishes were investigated for their ability to restore or maintain CMS and for their usefulness in developing populations suitable for mapping restorer loci.

4.1 Results

4.1.1 Testing for Ogura cytoplasm

Individuals from all three wild radish accessions and from the MST1 population were tested with the PCR primers specific for Ogura CMS to confirm cytoplasm type. As expected, all of the MST1 plants tested carried the Ogura cytoplasm as demonstrated by the presence of the 1039 bp PCR product characteristic of the ORF138 of Ogura CMS (Fig. 4.1). None of the wild radish plants tested carried the ORF138 band confirming they do not have Ogura cytoplasm (Fig. 4.1).

4.1.2 Assessing MST1 phenotypes

MST1 is a population of early maturing *R. sativus* carrying the Ogura cytoplasm and segregating for alleles that restore male fertility (Table 3.1). To establish the genetics of the restoration of male fertility in MST1, three populations of selfed seed (R7, R8 and R9) each derived from a single male fertile plant were grown and assessed for male fertility (Fig. 4.2). A sample of 34 individuals from the R7 population, 117 individuals from the R8 population and 20 individuals from the R9 population were grown and the phenotype of each plant was noted. These families were expected to segregate for fertility and sterility in accordance with the number of restorer genes that

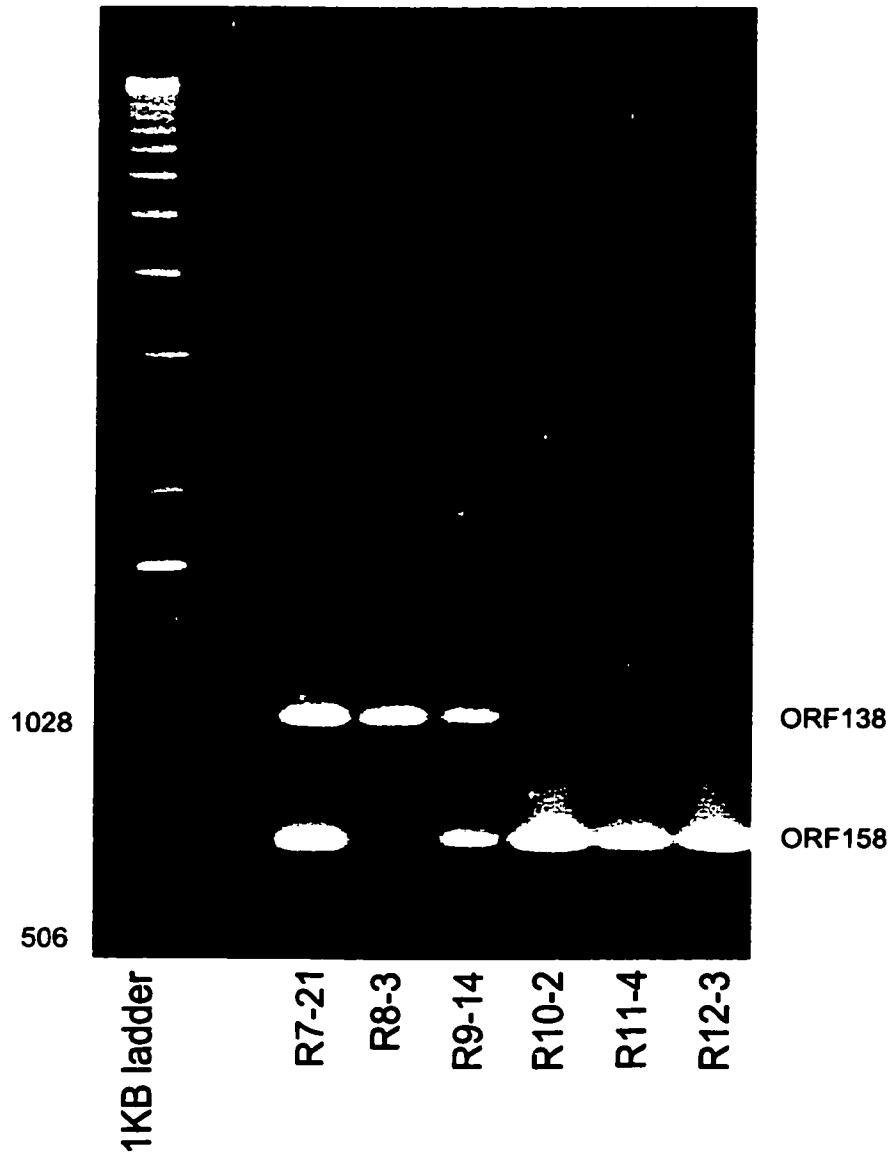


Figure 4.1. Image of an agarose gel containing amplified DNA fragments separated by electrophoresis. 1 kb ladder, size standard; R7-21, R8-3, R9-14, R10-2, R11-4, R12-3, the individual *Raphanus* plants tested; ORF138, the 1039 bp DNA fragment amplified specifically from the genome of Ogura CMS mitochondria; ORF158, the 820 bp DNA fragment amplified from the genomes of both Ogura CMS and wild-type mitochondria.

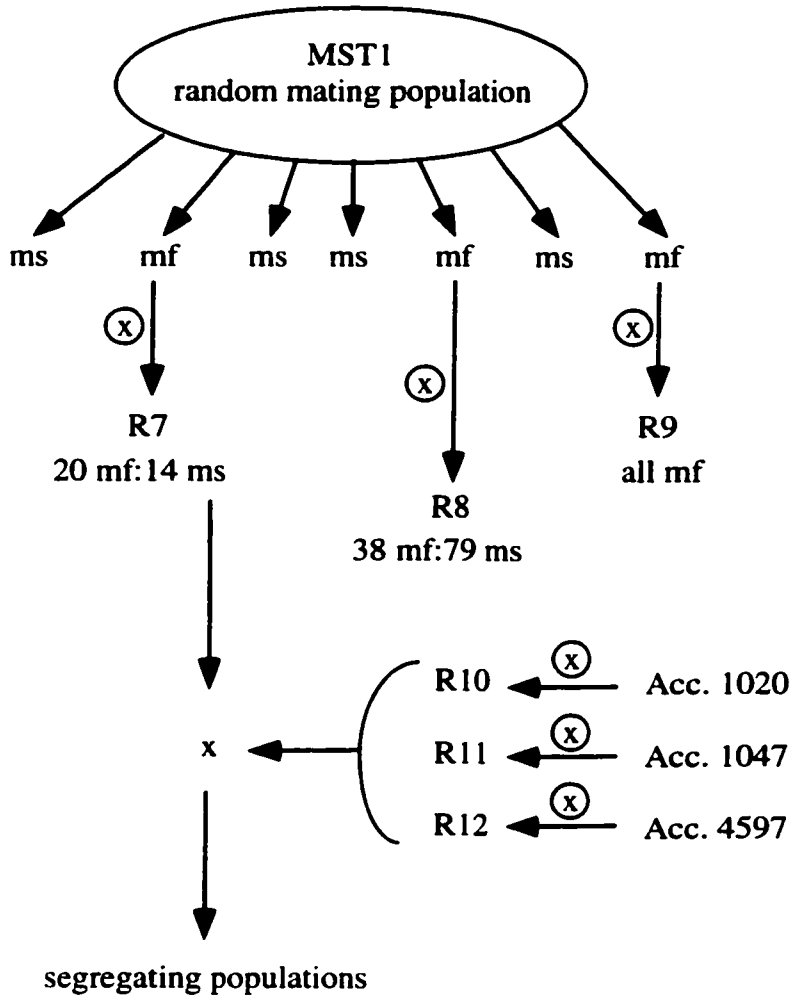


Figure 4.2. Populations developed from the MST1 stock population and used to investigate the genetics of fertility restoration in Ogura CMS in radish.

each of the parent plants carried in a heterozygous state. Family R7 segregated 20 male fertile plants to 14 male sterile plants which is a close fit to the 9:7 ratio expected when two genes with dominant restorer alleles are segregating ($p = 0.76$; Table 4.1). The R8 family had considerably more male sterile individuals (38 male fertile :79 male sterile) which is a close fit to the ratio expected from four dominant restorers segregating ($p = 0.84$; Table 4.1). Family R9 did not segregate and all individuals were male fertile (Table 4.1). Three of the R9 male fertile individuals were self pollinated and their progeny were also uniformly male fertile (Table 4.2), suggesting that the R9 parent plant carried all restorer alleles in the homozygous state.

Table 4.1. Estimating the number of restorer loci based on segregation for male fertility/sterility in families from selfed male fertile individuals from the MST1 population.

MST1 family	n [a]	mf [b]	ms [b]	3:1 [c]	9:7	27:37	81:175
R7	34	20	14	$p = 9.3 \times 10^{-4}$	$p = 0.76$	$p = 0.05$	$p = 6.5 \times 10^{-4}$
R8	117	38	79	$p = 1.8 \times 10^{-13}$	$p = 2.2 \times 10^{-7}$	$p = 0.03$	$p = 0.84$
R9	20	20	0	$p = 1 \times 10^{-4}$	no segregation for male sterility		

[a]: n, the number of individuals tested
[b]: mf, male fertile; ms, male sterile
[c]: p, probability of dominant restorer alleles segregating at one (3 mf:1 ms); two (9:7), three (27:37) and four (81:175) loci, based on chi-square tests.

Table 4.2. Fertility/sterility phenotypes of small selfed progenies derived from three R9 individuals.

R9 family	n [a]	mf [b]	ms [b]
R9-8 ⊗	10	10	0
R9-13 ⊗	9	9	0
R9-14 ⊗	9	9	0

[a]: n, the number of individuals tested
[b]: mf, male fertile; ms, male sterile

4.1.3 Test crosses between male sterile lines and wild radish accessions

Several different male sterile plants from the R7 population were used as females in controlled crosses with individuals from the wild radish lines R10, R11 and R12 and the progeny were grown and assessed for male fertility to test for the presence of restorer genes in radishes with normal cytoplasm (Fig. 4.2; Table 4.3). Six pairwise crosses between individual R7 plants and individual R12 plants produced only male fertile progeny regardless of the male sterile parent pollinated (Table 4.3), suggesting that R12 carried, in a homozygous state, all the restorer alleles necessary for restoring fertility to R7 individuals. Three pairwise crosses were made between R7 and R10 individuals. The progeny of two crosses segregated for fertility and sterility, the third cross only produced two viable seeds both of which gave rise to male fertile plants (Table 4.3). There were too few crosses and progeny to establish the genotype of R10 but since two crosses resulted in segregation for male fertility it suggests that R10 parent had at least one restorer allele. Progeny from four pairwise crosses between R7 and R11 individuals exhibited a range of segregation patterns (Table 4.3). The progeny of one cross (R7-21 x R11-4) were all fertile, while the progeny of another cross (R7-14 x R11-2) were all sterile. The progeny of two crosses (R7-30 x R11-4 and R7-25 x R11-4) segregated for fertility and sterility.

Assuming a two gene model, sterility in R7 individuals would be caused by homozygosity for the non-restoring allele at one ($rf_1rf_1Rf_2$-), the other (Rf_1-rf_2rf_2) or both ($rf_1rf_1rf_2rf_2$) restorer loci. By crossing wild radishes to a range of these R7 plants it was possible to demonstrate that the wild radishes carried restoring alleles at one or both loci (Table 4.3). As all progeny from crosses with R12 plants were male fertile, regardless of the male sterile parent, R12 must have carried restoring alleles at both loci, most likely in the homozygous state. Crosses with R11 plants produced all male fertile, all male sterile and segregating progeny depending on the male sterile R7 parent suggesting R11 carried restoring alleles for only one of the two restorer loci. For example, R11-2 appears to be homozygous for a non-restoring allele also homozygous in R7-14 resulting in all progeny being male sterile. In this case the parental genotypes could have both been $Rf_1Rf_1rf_2rf_2$. In contrast, a cross between R7-21 and R11-4 produced all male fertile

progeny suggesting the parents each carry alleles for different restorers. In this case the R7-21 genotype was probably $rf_1rf_1Rf_2Rf_2$ while the R11-4 parent was $Rf_1Rf_1rf_2rf_2$.

Table 4.3. Segregation for male fertility/sterility in progeny of crosses between male sterile plants and plants from three different wild radish accessions.

ms parent ♀	wild radish parent ♂	n [a]	mf [b]	ms [b]
R7-14	R12-3	9	9	0
R7-19	R12-3	10	10	0
R7-10	R12-3	2	2	0
R7-21	R12-4	10	10	0
R7-25	R12-4	9	9	0
R7-30	R12-4	10	10	0
R7-10	R10-2	2	2	0
R7-14	R10-2	11	3 (3 pmf)	8
R7-19	R10-2	18	17 (5 pmf)	1
R7-14	R11-2	9	0	9
R7-25	R11-4	10	1	9
R7-21	R11-4	8	8	0
R7-30	R11-4	10	2	8

[a]: n, the number of individuals tested
[b]: mf, male fertile; ms, male sterile; pmf, partial male fertile

Not all plants that produced pollen did so on all racemes. Plants carrying a mixture of male sterile anthers and anthers shedding a small amount of pollen were classified as partially male fertile (pmf). Observations of pollen grains from anthers of recently opened flowers, stained with acetocarmine, indicated a preponderance of shrunken, unstained pollen grains in the anthers from male sterile flowers. Pollen grains from the shedding anthers of flowers on the same plant were stained indicating intact cell membranes which is an indication of viability. There were too few pmf plants in these populations to determine the genetics of this trait.

4.2 Discussion

The radish population MST1 is maintained by allowing male fertile plants to pollinate male sterile as well as other male fertile plants. Assuming simple dominant inheritance and balanced segregation ratios, a family from a selfed male fertile plant of this population should behave like an F_2 population therefore the number of genes segregating within the family can be determined based on F_2 segregation ratios. If there are two genes with dominant restorer alleles involved in the restoration of Ogura CMS in radish, as expected based on recent reports (Nieuwhof 1990; Koizuka *et al.* 2000), progeny of selfed male fertile plants should segregate at zero (both homozygous), one (only one heterozygous the other homozygous) and two (both heterozygous) restorer loci. Family R9 did not segregate, suggesting that it had all necessary restorers in the homozygous state. Family R7 appeared to be segregating for dominant restoring alleles at two loci (Table 4.1). Family R8, however, segregated in a manner suggestive of four dominant restorer loci (Table 4.1), more than suggested by previous studies.

Maintaining non-restoring alleles in plants with Ogura cytoplasm is challenging as the plants are male sterile and therefore cannot be selfed. The contrasting segregation patterns for male fertile/male sterile progeny observed in the test crosses described in this chapter as well as work described by Yamagishi *et al.* (1993) and Yamagishi and Terachi (1994b) suggest that male fertile lines exist that maintain sterility at specific restorer loci and carry restoring alleles at other loci. When these are crossed to male sterile plants carrying complementary restoring alleles, male fertile progeny result. When they are crossed to male sterile plants carrying the same non-restoring allele, male sterile progeny result. These male fertile plants with non-Ogura cytoplasm can be used to perpetuate specific restoring or non-restoring alleles in male sterile plants with Ogura cytoplasm. Analysis of segregation for fertility/sterility phenotype within progeny of crosses between male sterile R7 plants and wild radish plants (Table 4.3) revealed the presence of restoring and maintaining alleles in the wild radish germplasm examined. For example, wild radish plant R11-4 appears to be homozygous for the restoring allele at *Rf1* and homozygous for the non-restoring allele at *Rf2* ($Rf_1Rf_1rf_2rf_2$) which makes it

useful for perpetuating restoration at *Rf1* and maintaining sterility at *rf2*, that is, R11-4 is a maintainer of sterility for the *rf2* locus. None of the three wild radish lines tested would be a suitable maintainer for the non-restoring allele *rf1*. To maintain the non-restoring allele *rf1*, other radish lines could be tested through crosses to male sterile plants homozygous for the non-restoring alleles at *rf1* but homozygous for restoring alleles at all other restorer loci. Alternatively, maintainer lines could be developed from existing material, for example, from an $Rf_1rf_1Rf_2rf_2$ individual. This testing and development could be much simplified by the use of co-dominant markers for each of the restorer loci.

Male fertile lines with non-CMS cytoplasm have been identified that always result in male sterile progeny when used as pollinators in crosses with male sterile lines (Yamagishi *et al.* 1993; Yamagishi and Terachi 1994b). It has been suggested that these lines might constitute universal maintainers although the range of male sterile material so far tested is probably too narrow to realistically make this claim. Yamagishi (1998) concluded that DNA markers for restorer loci would clarify the relationships among the restorers identified thus far.

From the material examined here, the cross R7-21 x R11-4 would be appropriate for the mapping of two restorer loci (Chapter 6) and the population R8 would be appropriate for the mapping of one or two additional restorer loci (Chapter 7). Family R9 was chosen as the source of the donor parent for interspecies gene transfer as it appeared to be homozygous for all necessary restoring alleles - see Chapter 10.

The cross R7-19 x R10-2 had only one male sterile individual out of a population of 18 individuals (Table 4.3). Other researchers have noticed rare male sterile plants in populations otherwise fully male fertile (Bonnet 1975; Nieuwhof 1990). Ogura (1968) noted that male sterile plants were always male sterile but male fertile plants could abort pollen under unfavourable environmental conditions such as high temperatures during flowering. Nieuwhof (1990) proposed that the spurious male sterile plants were caused by minor genes influenced by the environment and suggested that plants be grown at 17-20°C to ensure all male fertile phenotypes are expressed. The plants in this experiment

were all grown in growth cabinets under strict temperatures of 18°C days and 12°C nights; perhaps the cool nights influenced full expression of the male fertile phenotype in some plants.

The presence of restoring alleles in radishes with normal cytoplasm was not unexpected. Crosses between several different types of radish (*R. sativus* and *R. raphanistrum*) from Europe, China and Japan have shown that a range of radishes, both *R. sativus* and *R. raphanistrum*, have Ogura CMS restoring alleles regardless of cytoplasm type (Yamagishi and Terachi 1997; Yamagishi 1998) and cultivated radishes from Europe have long been known to carry restorer alleles (Bonnet 1975). As can be seen from these results, it is necessary to test against a range of male sterile plants to ensure all genotypes are revealed. It is not surprising early researchers believed only one gene was necessary for complete restoration as, given the right genotypic combination, it is easy to create populations segregating for only one restorer.

At least two studies (Bonnet 1975; Nieuwhof 1990) suggested there may be a recessive restorer gene for Ogura CMS. It is not possible to test for this in the MST1 populations as the initial parent plants were male fertile and, therefore, they and their selfed progeny would have to be homozygous for this putative recessive restorer. If the wild radishes were carrying the dominant non-restoring allele, there would not have been any male fertile progeny in crosses to R7 individuals. As this was not the case, it is still not clear if there are loci with recessive restoring alleles.

Chapter 5. *Raphanus* genetic linkage map

A genome-wide genetic linkage map of *Raphanus* was constructed in order to develop a genetic framework on which the loci controlling restoration of Ogura CMS could be positioned.

5.1 Results

5.1.1 Mapping populations and cytology

The *Raphanus* mapping populations were developed from a wide cross between lines of *R. sativus* and *R. raphanistrum* in order to maximise polymorphism and the number of informative markers. A single male sterile *R. sativus* plant of the R7 population, R7-21, was pollinated by a single *R. raphanistrum* plant of the R11 population, R11-4 (Fig. 5.1). Ten F_1 plants were grown and all exhibited the male fertile phenotype. A single F_1 plant was self pollinated to produce an F_2 population of 253 seeds. At the same time, the F_1 plant was pollinated by an inbred individual derived from R11-4 to produce a BC_1 population of 82 seeds (Fig. 5.1).

Dr Anne-Marie Chèvre (INRA, Rennes, France) kindly observed meiotic nuclei from three sister F_1 plants derived from the R7-19 x R11-1 cross and found an overwhelming predominance of bivalent pairing between chromosomes at meiosis (Fig 5.2). Of the 50 nuclei observed in two of the three plants, all demonstrated bivalent pairing. In the third plant 33 nuclei demonstrated bivalent pairing while two appeared to have seven bivalents and one quadrivalent and one appeared to have eight bivalents and two univalents (Table 5.1).

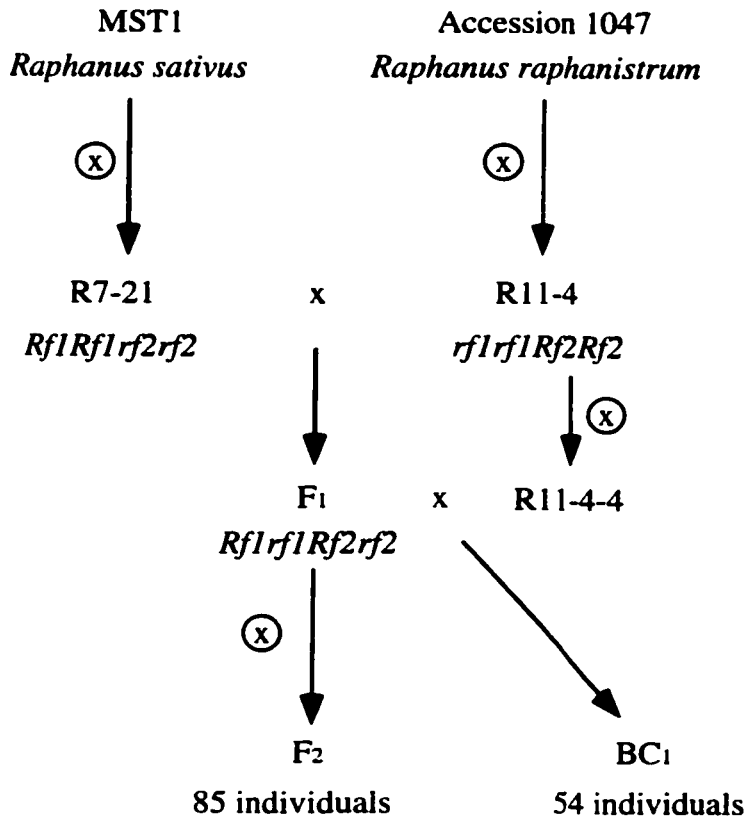


Figure 5.1. Crossing scheme used to develop the *Raphanus* mapping populations.

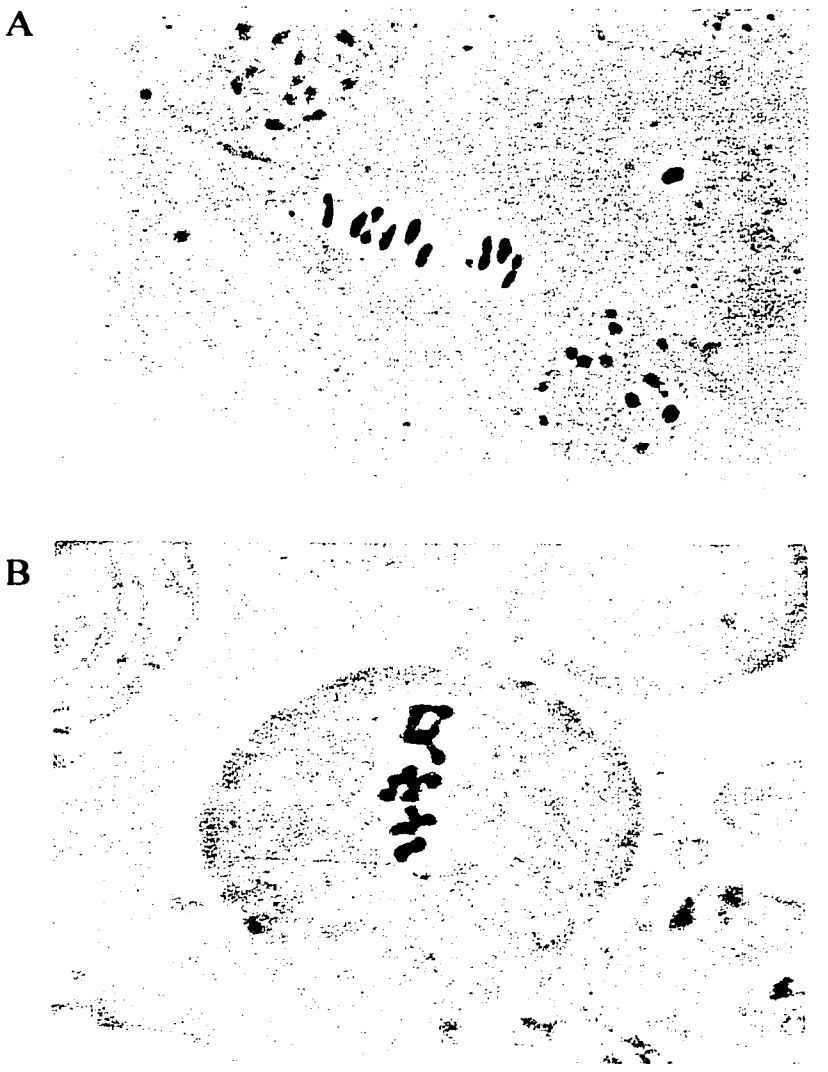


Figure 5.2. Meiotic nuclei from F_1 plants derived from a cross between *R. sativus* and *R. raphanistrum*. A) nine bivalent pairs of chromosomes; B) seven bivalents and one quadrivalent set of chromosomes. Photographs kindly provided by Dr. A.-M. Chevre, INRA France.

Table 5.1. Pairing in meiotic nuclei of three plants of a cross between *R. sativus* and *R. raphanistrum*.

Plant	# nuclei observed	9 II	8 II + 2 I	7 II + 1 IV
1	30	30	-	-
2	36	33	1	2
3	20	20	-	-

Dr Anne-Marie Chèvre (INRA, Rennes, France)

5.1.2 Genetic marker analysis and linkage mapping

DNA samples extracted from 54 individuals of the BC_1 population and 85 individuals of the F_2 population were subjected to RFLP analysis using a total of 171 *Brassica* RFLP probes (Appendix 1.1). Of the 171 *Brassica* probes tested: 144 (84%) detected informative patterns in the BC_1 and/or F_2 populations (133 in the BC_1 and 138 in the F_2); 17 (10%) produced monomorphic patterns; seven (4%) hybridised to mildly repetitive sequences and produced banding patterns that were not possible to score accurately and three (2%) failed to hybridize to the *Raphanus* DNA.

RFLP analysis of the BC_1 population resulted in the expected BC_1 segregation patterns indicating that all individuals were derived from the controlled cross. The markers detected segregation patterns resulting from a combination of segregation at meiosis in the F_1 parent and segregation at meiosis in the heterozygous portions of the recurrent parent (R11-4-4, Fig. 5.1). Those RFLP alleles segregating in the BC_1 population that could be positively identified in the non-recurrent parent (R7-21, Fig. 5.1) were scored first in order to develop a genetic map of meiosis in the F_1. Using this criterion, 133 probes detected 210 polymorphic loci. An additional six loci could be scored based on the segregation of dominant markers in the recurrent parent (R11-4, Fig. 5.1) which were linked in repulsion to alleles from the non-recurrent parent at adjacent loci (Fig. 5.3). Five more loci could be scored in the BC_1 population based on intensity of the bands. Segregation data for all 221 loci described above can be found in Appendix 1.2.

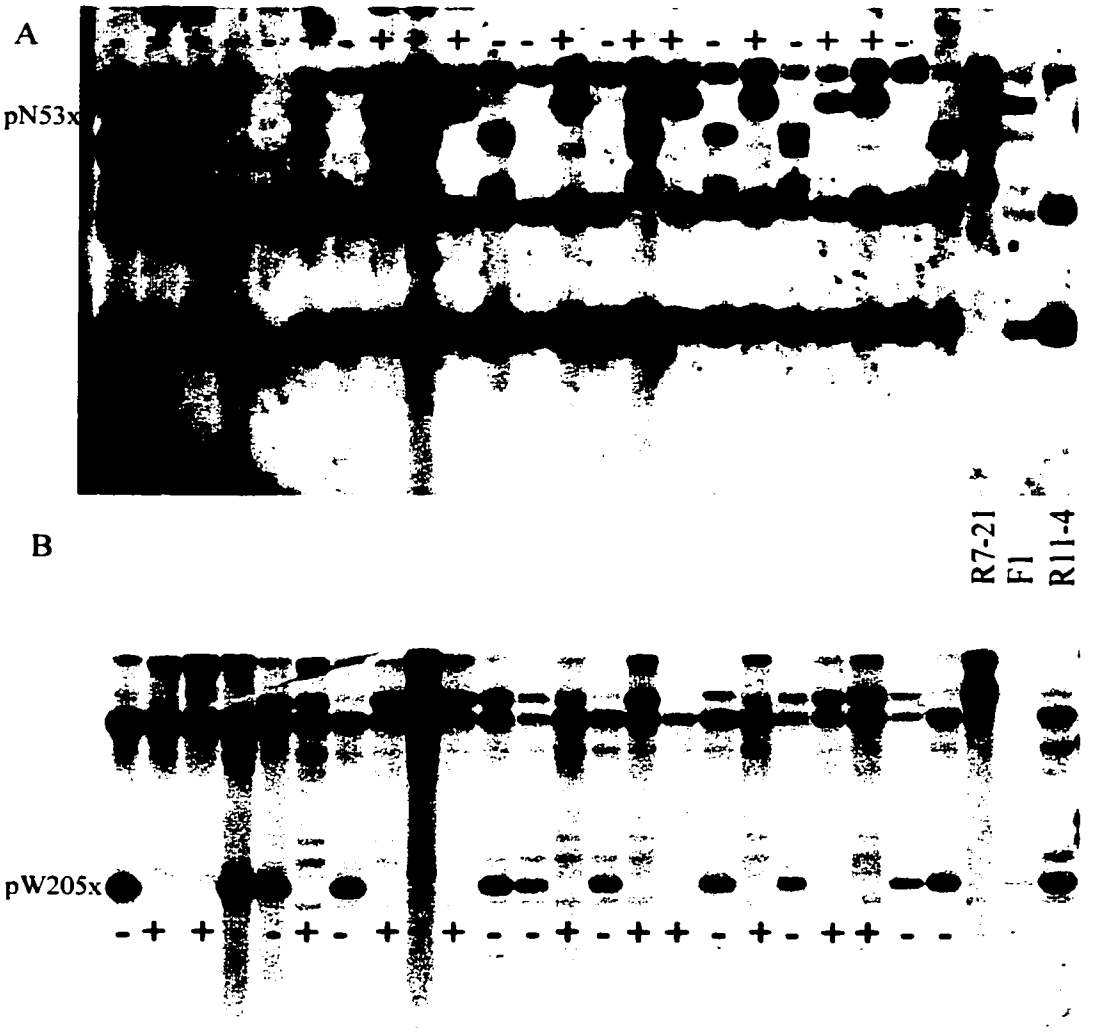


Figure 5.3. BC_1 segregation patterns at a pair of linked RFLP loci. Autoradiographs of 23 BC_1 individuals (lanes 1-23), the non-recurrent parent (R7-21, lane 24), the F_1 (lane 25) and the recurrent parent (R11-4, lane 26), probed with A) pN53 and B) pW205. The segregation for the recurrent parent RFLP allele at pW205x (-, autoradiograph B) is linked in repulsion to segregation for the non-recurrent parent allele at pN53x (+,autoradiograph A). All lanes contain genomic DNA digested with *Eco RI*. -, individual homozygous for R11-4 allele; +, heterozygous individual.

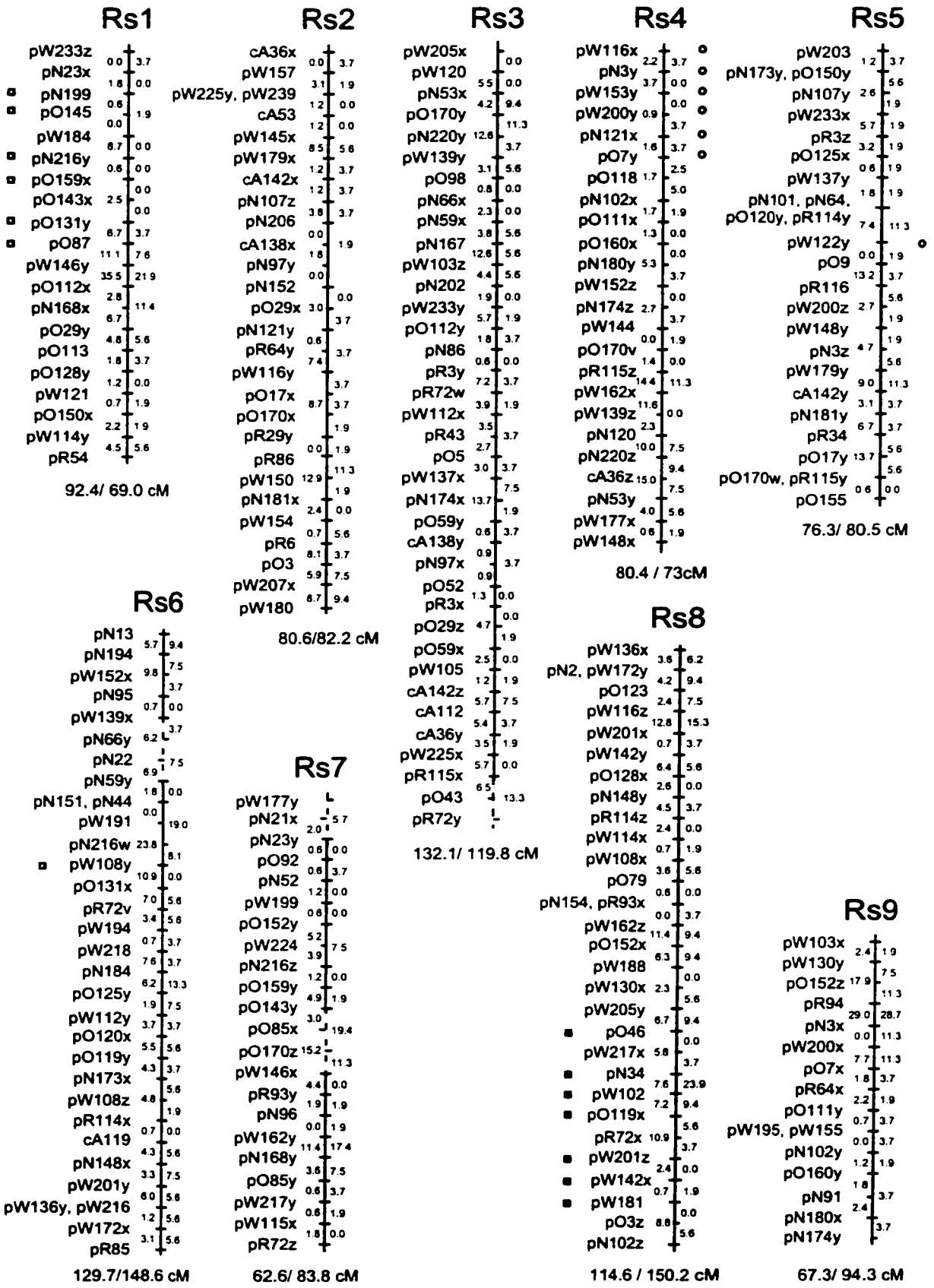
Linkage analysis of the 221 loci produced a genetic map with 12 extensive linkage groups at LOD 4.0. Three pairs of linkage groups exhibited linkage at lower LOD scores, with one pair joining together at LOD 3.8, one pair at LOD 3.4 and the final pair joining at LOD 2.8. The resulting nine linkage groups spanned 915 cM. The genetic linkage map derived from the BC_1 mapping population is presented in Fig. 5.4.

RFLP analysis of the F_2 population yielded the expected F_2 segregation patterns and indicated that the F_2 was a uniform population: no individuals in the F_2 population exhibited RFLP alleles not observed in one or other of the parents. The 138 informative RFLP probes detected 194 distinct co-dominant markers and an additional ten dominant markers for which co-dominant segregation patterns could be deduced based on band intensity (Fig. 5.5). Segregation data for the 204 loci can be found in Appendix 1.3. Initial linkage analysis of the 204 loci produced a genetic map with ten extensive linkage groups at LOD 4.0. Two of the groups joined together at LOD 1.98 to produce a map with nine linkage groups covering 844 cM (Fig. 5.4).

The BC_1 and F_2 maps were aligned using 179 loci common to both maps (Fig. 5.4). This process confirmed the reproducibility of the nine extensive linkage groups. There were four regions of the map where the precise locus order could not be determined because adjacent pairs of loci could not be mapped in the same population. These four regions are indicated by broken lines in Figure 5.4. The linkage groups were arbitrarily assigned the designations Rs1 to Rs9. The aligned map includes a total of 236 marker loci.

Of the 144 informative probes used to develop the combined map, 64 detected only one polymorphic locus, 54 detected two informative loci, 23 detected three informative loci and two identified five informative loci, giving an average of 1.74 informative loci per probe.

Figure 5.4. Aligned RFLP maps of *Raphanus* based on segregating F_2 and BC_1 populations. Vertical lines represent linkage groups with RFLP markers listed on the left. Map distances (cM) for the F_2 map are located to the left of the vertical lines and distances for the BC_1 map are to the right. Total map lengths are shown at the bottom of the individual linkage groups. Open squares (□) to the left of specific loci indicate segregation distortion in the F_2 towards alleles of parent R7-21. Closed squares (■) to the left of specific loci indicate segregation distortion in the F_2 towards alleles of parent R11-4. Open circles (○) to the right of specific loci indicate significant segregation distortion in the BC_1 population towards R7-21 alleles.


Rs1
pW233z
pN23x
pN199
pO145
pW184
pN216y
pO159x
pO143x
pO131y
pO87
pW146y
pO112x
pN168x
pO29y
pO113
pO128y
pW121
pO150x
pW114y
pR54
92.4/ 69.0 cM
Rs2
cA36x
pW157
pW225y, pW239
cA53
pW145x
pW179x
cA142x
pN107z
pN206
cA138x
pN97y
pN152
pO29x
pN121y
pR64y
pW116y
pO17x
pO170x
pR29y
pR86
pW150
pN181x
pW154
pR6
pO3
pW207x
pW180
80.6/82.2 cM
Rs3
pW205x
pW120
pN53x
pO170y
pN220y
pW139y
pO98
pN66x
pN59x
pN167
pW103z
pN202
pW233y
pO112y
pN86
pR3y
pR72w
pW112x
pR43
pO5
pW137x
pN174x
pO59y
cA138y
pN97x
pO52
pR3x
pO29z
pO59x
pW105
cA142z
cA112
cA36y
pW225x
pR115x
pO43
pR72y
132.1/ 119.8 cM
Rs4
pW116x
pN3y
pW153y
pW200y
pN121x
pO7y
pO118
pN102x
pO111x
pO160x
pN180y
pW152z
pN174z
pW144
pO170v
pR115z
pW162x
pW139z
pN120
pN220z
cA36z
pN53y
pW177x
pW148x
80.4 / 73cM
Rs5
pW203
pN173y, pO150y
pN107y
pW233x
pR3z
pO125x
pW137y
pN101, pN64,
pO120y, pR114y
pW122y
pO9
pR116
pW200z
pW148y
pN3z
pW179y
cA142y
pN181y
pR34
pO17y
pO170w, pR115y
pO155
76.3/ 80.5 cM
Rs6
pN13
pN194
pW152x
pN95
pW139x
pN66y
pN22
pN59y
pN151, pN44
pW191
pN216w
pW108y
pO131x
pR72v
pW194
pW218
pN184
pO125y
pW112y
pO120x
pO119y
pN173x
pW108z
pR114x
cA119
pN148x
pW201y
pW136y, pW216
pW172x
pR85
129.7/148.6 cM
Rs7
pW177y
pN21x
pN23y
pO92
pN52
pW199
pO152y
pW224
pN216z
pO159y
pO143y
pO85x
pO170z
pW146x
pR93y
pN96
pW162y
pN168y
pO85y
pW217y
pW115x
pR72z
62.6/ 83.8 cM
Rs8
pW136x
pN2, pW172y
pO123
pW116z
pW201x
pW142y
pO128x
pN148y
pR114z
pW114x
pW108x
pO79
pN154, pR93x
pW162z
pO152x
pW188
pW130x
pW205y
pO46
pW217x
pN34
pW102
pO119x
pR72x
pW201z
pW142x
pW181
pO3z
pN102z
114.6 / 150.2 cM
Rs9
pW103x
pW130y
pO152z
pR94
pN3x
pW200x
pO7x
pR64x
pO111y
pW195, pW155
pN102y
pO160y
pN91
pN180x
pN174y
67.3/ 94.3 cM

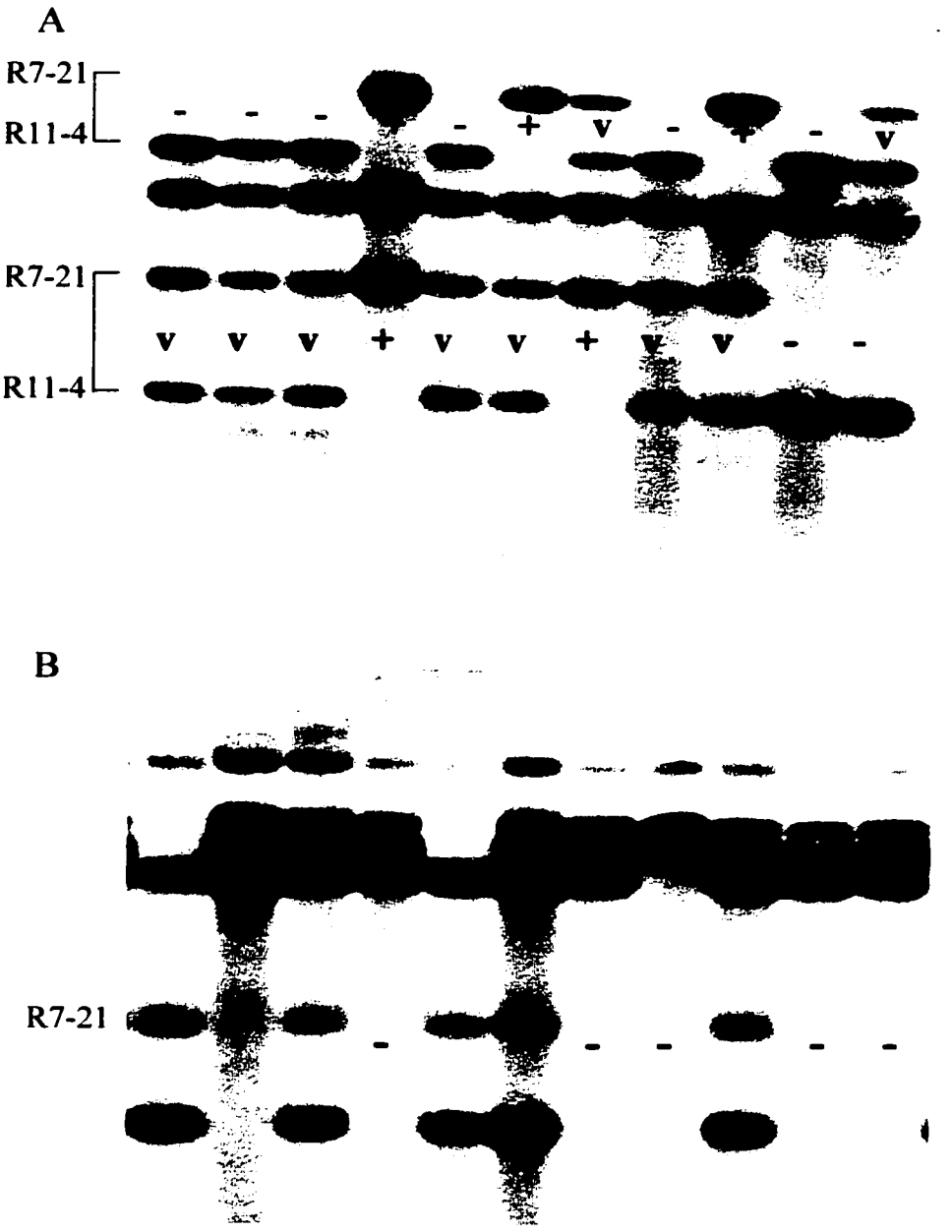


Figure 5.5. Examples of A) co-dominant and B) dominant F_2 segregation patterns at RFLP loci. Autoradiographs of 11 F_2 individuals detected by probes A) pW172 and B) pR115. All lanes contain genomic DNA digested with *Eco RI*. Parental alleles are labelled R7-21 and R11-4. +, individual homozygous for R7-21 allele; -, individual homozygous for R11-4 allele, v, heterozygous individual.

5.1.3 Marker distribution

In the BC_1 map there were 12 clusters of three or more co-incident loci and only six gaps of more than 15 cM (Fig. 5.4). Given the probability of finding two co-incident loci in this population is 0.3, none of the clusters were greater than that expected by chance. In the F_2 map, there were four clusters of three or more co-incident loci and four gaps greater than 15 cM (Fig. 5.4). Given the probability of finding two co-incident loci in this population is 0.15, none of the clusters was unexpected. The distribution of markers did not deviate significantly from a random distribution in the BC_1 map given an average spacing of 4.3 cM and an average of 52 informative gametes ($p = 0.06$; Fig. 5.6) similarly, the distribution of markers in the F_2 map suggested that both the marker loci and the crossovers observed in the population were randomly distributed given an average spacing of 4.4 cM and an average of 164 informative gametes ($p = 0.76$; Fig. 5.7).

5.1.4 Segregation distortion

The segregation ratios at marker loci assayed in the BC_1 population were investigated. Figure 5.8 shows the frequency distribution of the allele frequencies of R7-21 alleles in the F_1 gametes that contributed to the BC_1 population and the distribution of allele frequencies expected from random distortions to an underlying allele frequency of 0.5 at 204 unlinked loci in a population of 54 gametes. Overall, R7-21 alleles were favoured with 92 loci exhibiting an excess of the R7-21 allele and only 60 loci exhibiting an excess of the R11-4 allele ($\chi^2 = 6.7$; $p=0.009$).

The significance of segregation distortion at individual loci was estimated using a chi-square test and all loci with segregation ratios with $p < 0.01$ are indicated on the BC_1 map (Fig. 5.4). The marker locus pW116x had the most distorted segregation ratio ($p = 0.001$) and was one of a cluster of loci on Rs4 that favoured their R7-21 alleles.

Figure 5.9 shows segregation of parental alleles for each locus in the F_2 population. The entire graph appears to be shifted in favour of R11-4 alleles; 142 loci favoured R11-4 alleles in the homozygous state while only 58 favoured R7-21 alleles in the homozygous state ($\chi^2 = 35.3$; $p = 2.9 \times 10^{-9}$). A diagonal line running from point 0.5, 0.0 to 0.0, 0.5 on the graph represents loci where 50% of F_2 individuals are

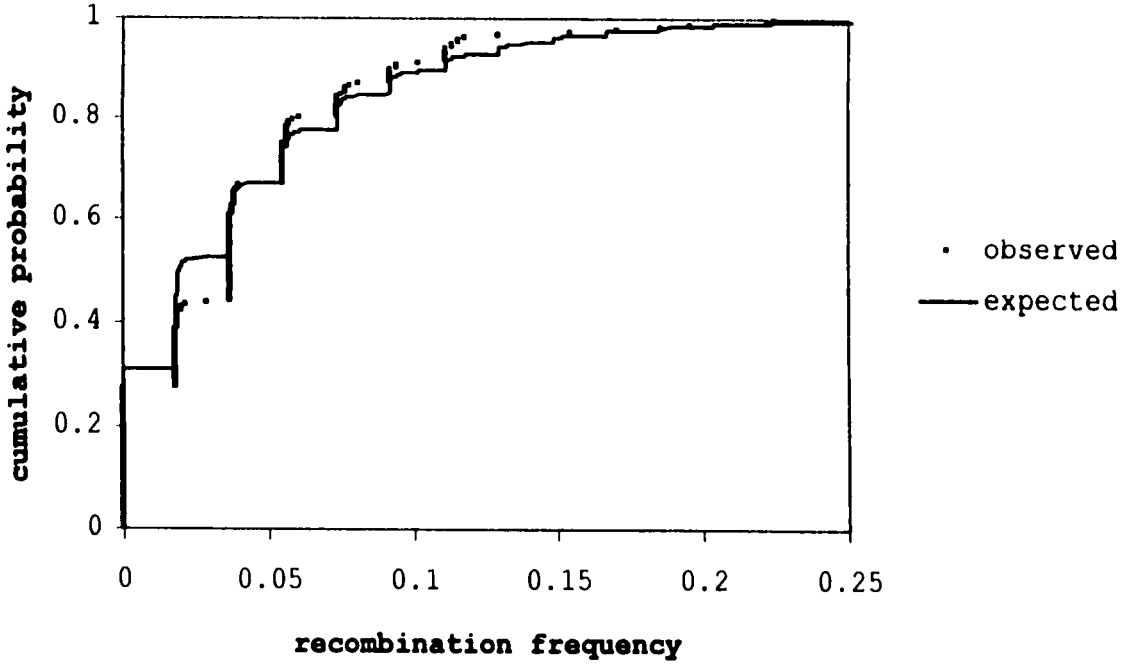


Figure 5.6. Observed and expected distributions of marker intervals in the BC_1 map. The expected distribution was derived based on the assumption that the distribution of marker loci and crossovers are both random. Kolmogorov- Smirnov test statistic = 0.09, p = 0.06.

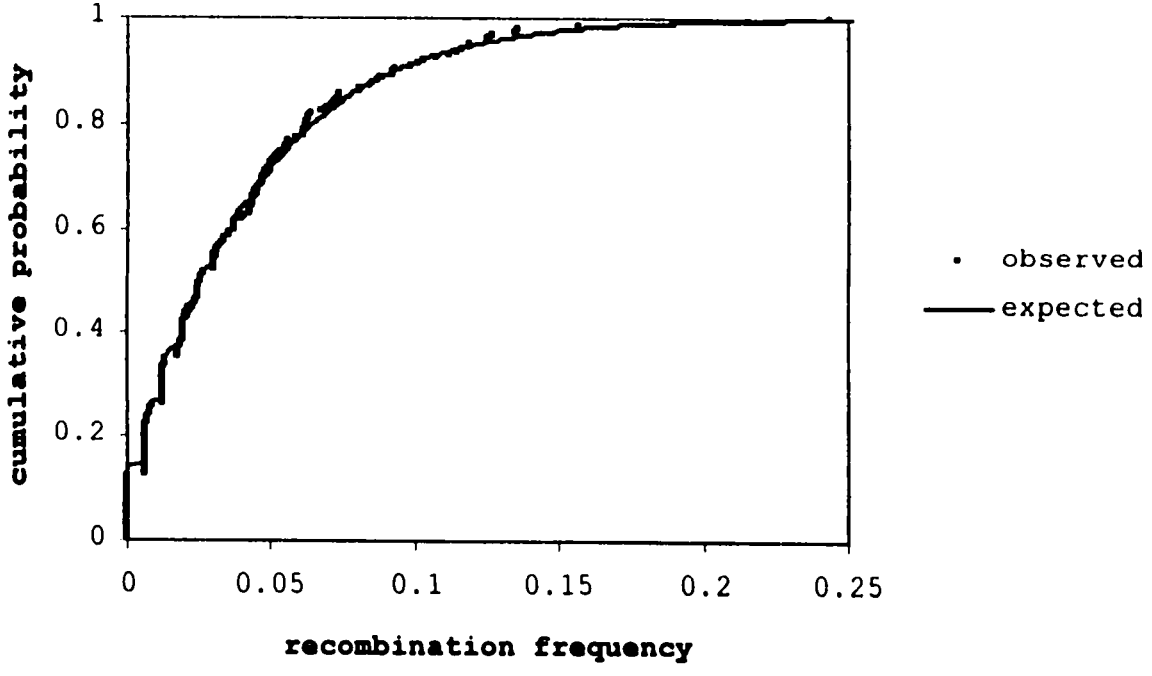


Figure 5.7. Observed and expected distributions of marker intervals in the F_2 map. The expected distribution was derived based on the assumption that the distribution of marker loci and crossovers are both random. Kolmogorov- Smirnov test statistic = 0.05, p= 0.77.

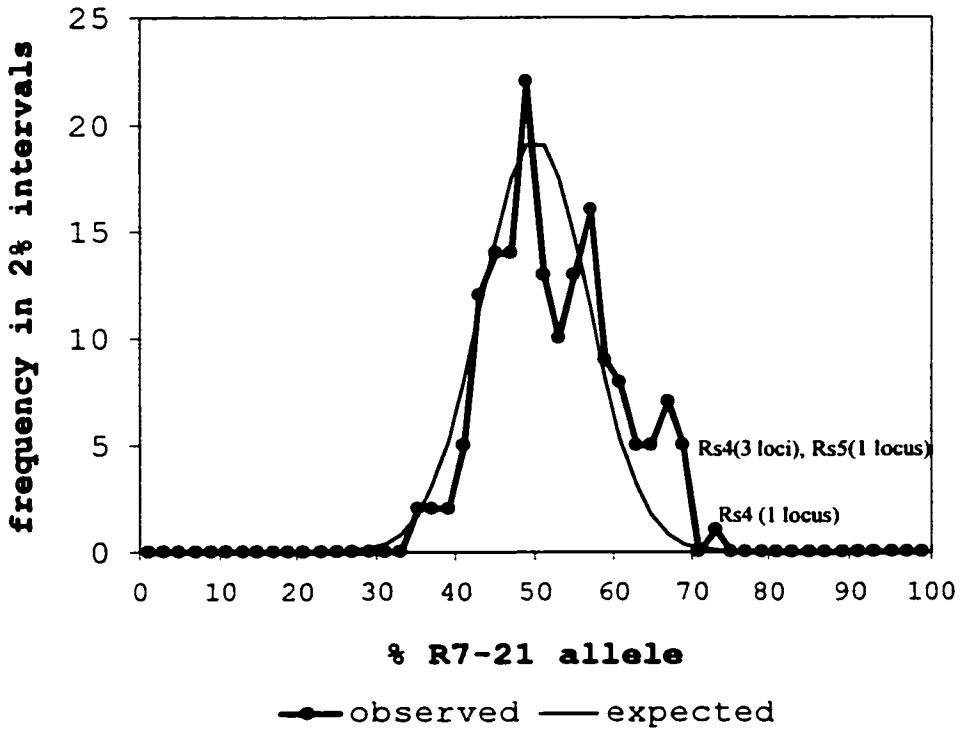


Figure 5.8. Distribution of allele ratios for loci in the BC_1 population. The graph represents 2% windows taken at 2% intervals. Numbers beside points are the linkage groups on which the significantly distorted loci are found.

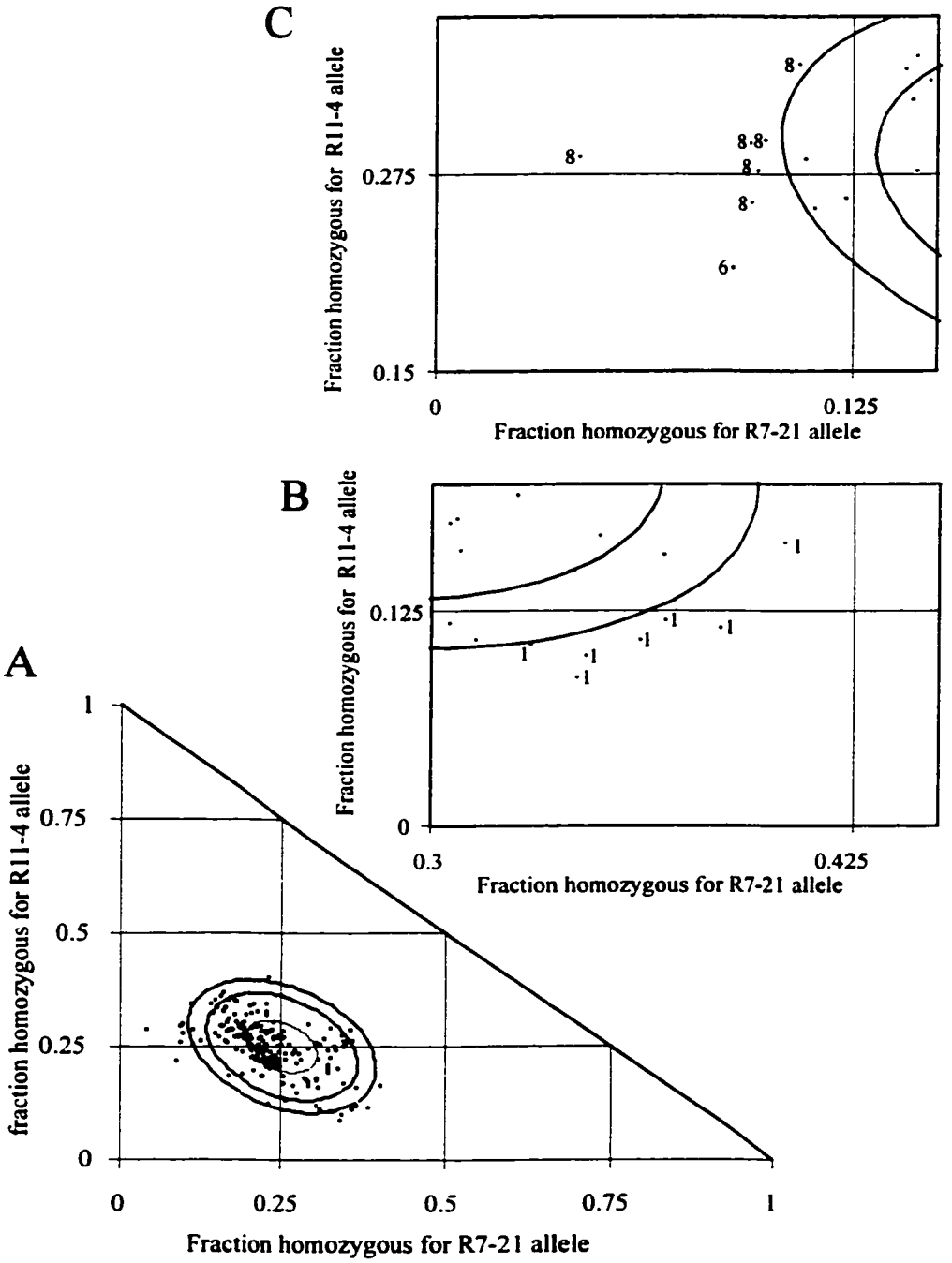


Figure 5.9. A: The distribution of allele ratios for loci in the F_2 map. Inner, middle and outer ellipses represent expected values based on a transmission frequency of 0.5 for each parental allele at p = 0.50, 0.95, and 0.99 respectively. B & C: Close-ups of the regions with rare segregation ratios. The numbers beside observed points identify the linkage group to which the particular loci belong.

heterozygous, and a shift of the data below or above the line indicates a shift in favour of or against the heterozygous state, respectively. In this case, there were more data points below the line (139 loci) than above (61 loci) indicating a selection for heterozygotes (χ^2 = 30.4; p = 3.5×10^{-8}).

The ellipses on the graph represented in Fig 5.9 are composed of points of segregation ratios with expected probabilities of p= 0.5, 0.95 and 0.99 for the inner, middle and outer rings, respectively. All but one of the points where R11-4 alleles are under-represented and R7-21 alleles are favoured were linked to each other on Rs1. All but one of the points that show selection against R7-21 alleles were linked to each other on Rs8 (Figs. 5.4, 5.9).

5.1.5 Recombination

The number of crossovers per gamete was calculated for the BC_1 population and compared to a Poisson distribution (Fig. 5.10A). There were two obvious outliers which corresponded to two individuals that had a very large number of crossovers: individual #32 had 22 crossovers and individual #36 had 19 crossovers. Individual #32 had nine double crossovers (each pair flanking a single locus), seven of which were identified with RFLP markers detected using the same filter which suggests that this filter may not contain DNA from individual #32 in the designated lane. Individual #36 had two double crossovers which upon closer examination of the autoradiographs revealed banding patterns that were not clear. The data for these two individuals at these nine loci were reclassified as missing data and the distribution was recalculated and did not deviate significantly from the expected, Poisson, distribution (Fig. 5.10B; p = 0.33).

The distribution of the number of crossovers per individual in the F_2 population deviated significantly from the expected Poisson distribution (Fig. 5.11A; p = 0.03). There was a large peak at the mean and small peaks at the ends which probably account for this deviation. The two individuals that had only four crossovers per pair of gametes across the whole genome were unique in that they were absent from several filter sets and as a result were not always probed. This resulted in a significant number of missing data points (see scoring data Appendix 1.2) which could lead to the appearance of no crossovers where one or more actually existed. Although there were no obvious outliers

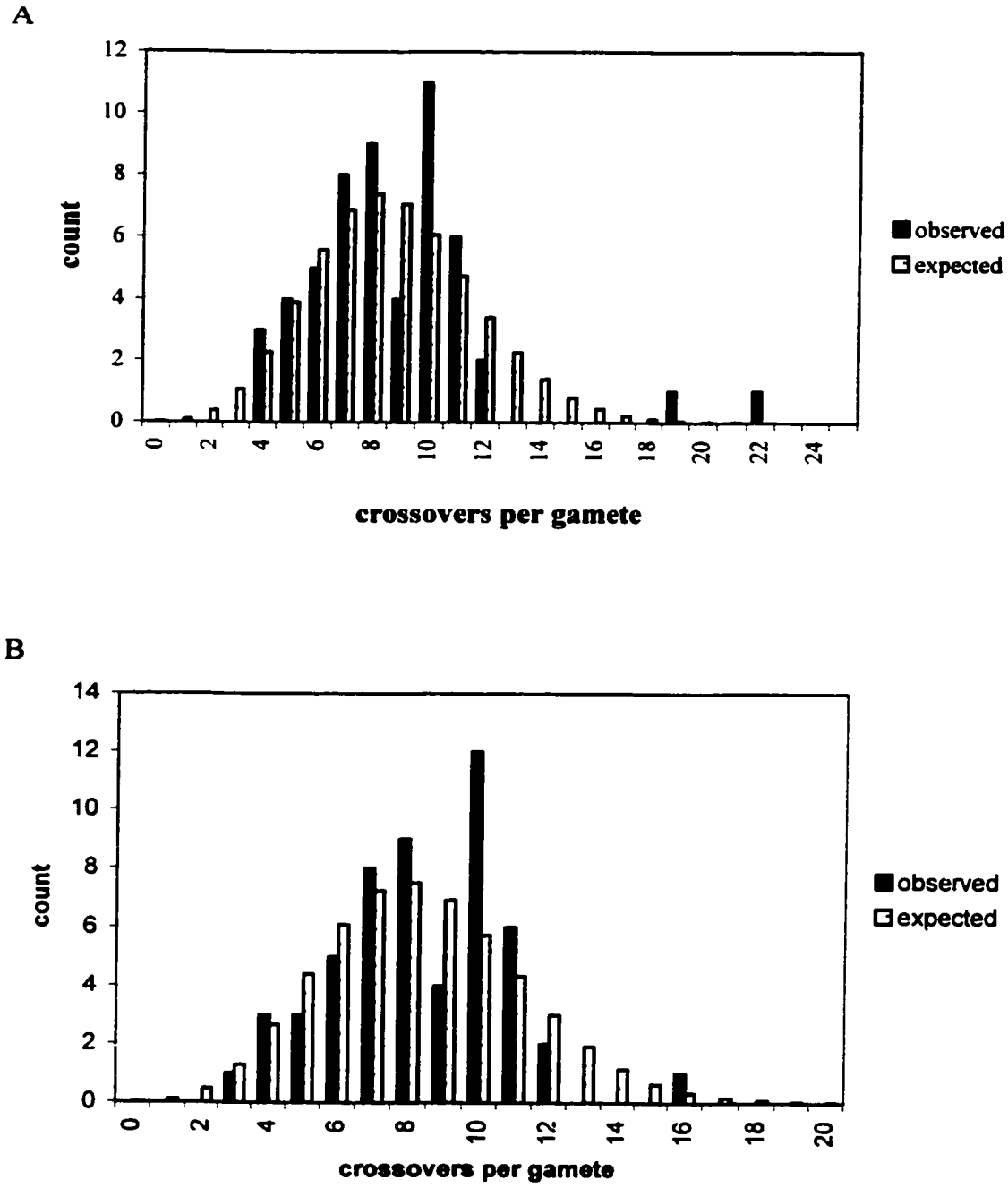


Figure 5.10. Observed and expected distributions of crossovers per gamete in F_1 gametes of the BC_1 population. Expected distribution is a Poisson distribution with mean equal to 8.6 in the uncorrected data set (A) and 8.3 in the data set corrected for obvious errors (B).

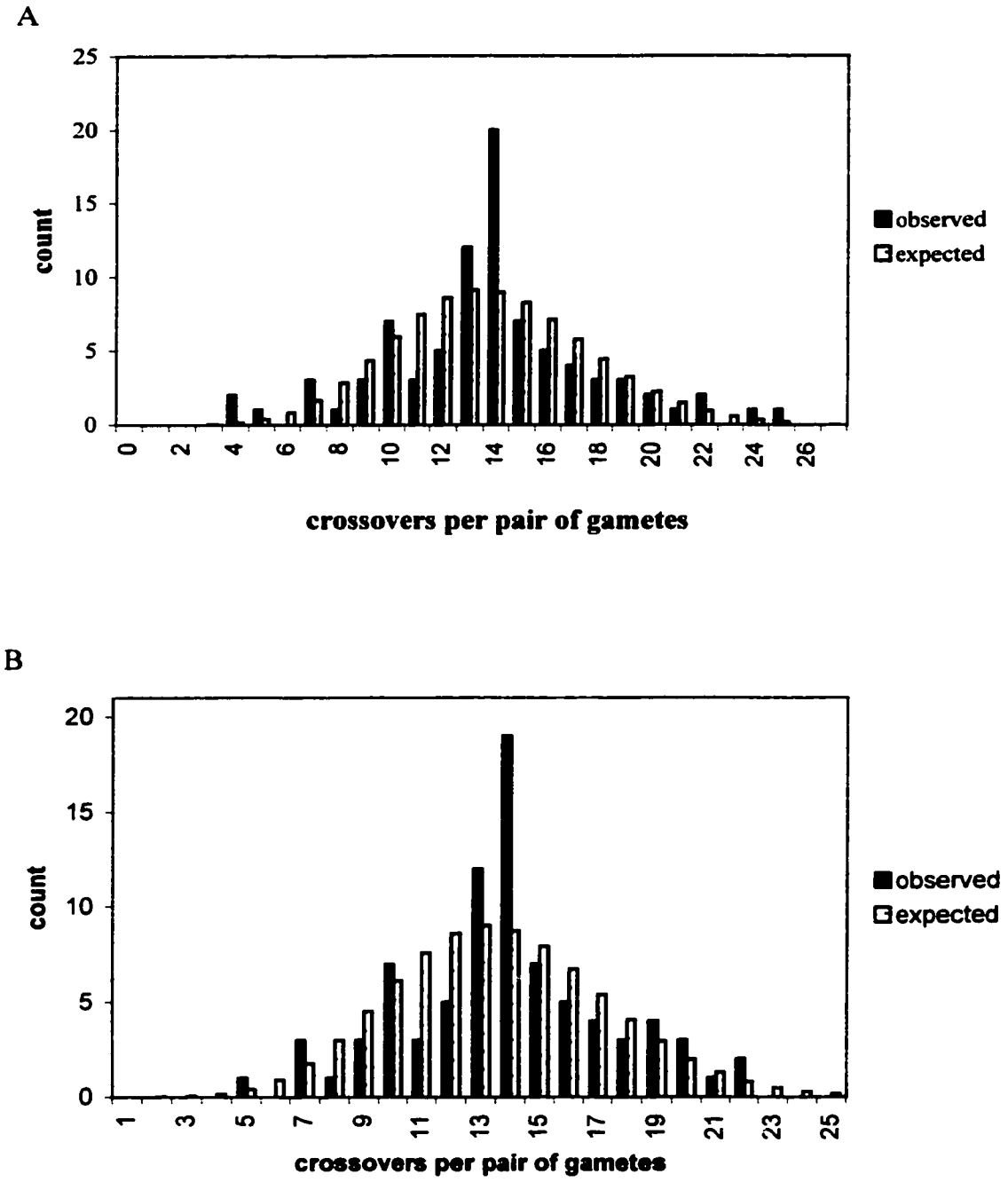


Figure 5.11. Observed and expected distributions of crossovers per gamete inpairs of F_1 gametes of the F_2 population. Expected distribution is a Poisson distribution with mean equal to 13.8 in the uncorrected data set (A) and 13.6 in the data set corrected for obvious errors (B).

in the F_2 population, closer examination of the individuals with the largest number of crossovers brought to light several errors in scoring due to improper classification.

5.2 Discussion

84% of the *Brassica* RFLP probes were informative in the *Raphanus* mapping populations used. There were relatively few probes that did not hybridise to the *Raphanus* DNA indicating close homology between *Brassica* and *Raphanus*. The ability to use *Brassica* probes in genetic mapping of other crucifer species dramatically reduces the cost of RFLP mapping in these species as the probes have already been developed. This is important in species of lesser commercial value where funding for the development of markers is less readily available. Comparative mapping is also facilitated by the availability of a large collection of markers that can be mapped in related species.

Scoring of segregation patterns in the BC_1 mapping population was restricted to those that included an allele that could be positively identified as coming from the non-recurrent parent (R7-21). There were rare F_2 segregation patterns produced when both the F_1 and recurrent parent were heterozygous for the same alleles and rarer BC_1 segregation patterns for loci that were homozygous in the F_1 but heterozygous in the recurrent parent thereby representing segregation produced from meiosis in the recurrent parent rather than in the F_1.

Both maps yielded the same nine linkage groups. The relative ordering of loci scored in both populations was identical in both maps. Although the largest intervals in each map had fairly low LOD scores with two-point analysis, no interval had a LOD score less than 5.4 when using combined data from both populations.

The total map length of approximately 900cM is in keeping with that of other diploid crucifers, e.g., *B. rapa* (Salava *et al.* 2001), *B. oleracea* (Bohuon *et al.* 1996), and *B. nigra* (Lagercrantz and Lydiate 1995). This suggests the map covers most of the genome which, along with the randomness of marker intervals, will allow for the selection of a subset of markers representing the whole genome for use in marker-assisted backcrossing programs using these lines. It will also allow for the alignment of other *Raphanus* maps developed using the same RFLP probes.

The marker spacing across the genome showed no detectable deviations from random behaviour, i.e. no excessively large map intervals and no excess of large map intervals in either map. There were fewer co-incident loci in the F_2 due to the larger population size (178 gametes assayed in the F_2 compared to 54 gametes in the BC_1 population) which allowed for more chance of finding an individual with a crossover between two adjacent loci. There were no significant clusters of marker loci in either map. This suggests that the markers and recombination events are randomly distributed across the genome. Clustering was less than that found in *B. napus* (Parkin and Lydiate 1997; Sharpe *et al.* 1995) and *B. nigra* (Lagercrantz and Lydiate 1995) where significant clustering occurred on many linkage groups. This is in contrast to many other crop species such as tomato (Tanksley *et al.* 1992), oat (O'Donoughue *et al.* 1992), and wheat (Chao *et al.* 1989; Devos *et al.* 1992) where pronounced clustering of marker loci occur, often in regions associated with the centromere.

Segregation distortion is the deviation of the allele frequency away from the 0.5 transmission frequency expected in non-selective conditions, such distortions can be caused by gametes carrying particular alleles having less viability or less success. It can be attributed to selection at any stage of plant growth and development up to tissue collection for DNA extraction, including meiosis and the formation of gametes, pollination, embryo development, seed germination and seedling growth. Segregation distortion at molecular marker loci can occur when they are linked to the loci at which selection occurs and the more tightly linked they are, and the more extreme the selection at the linked locus, the more extreme is the distortion. Skewed markers can therefore appear in clusters with distortion easing as the markers fall further away from the locus at which selection occurs. Significant segregation distortion ($p < 0.005$) at pW116x in the BC_1 population and at pW181 and pO87 in the F_2 population, resulted in groups of loci closely linked to these points being distorted although to a lesser extent.

There appeared to be an overall selection for F_2 individuals homozygous for R11-4 alleles as well as heterozygotes. As the germination efficiency of the F_2 population was high (90%) and only four individuals were eliminated due to inadequate quantities of DNA, it is unlikely that seed or seedling vigour were a factor. Fitness of

the gametes and zygotes could explain the excess of homozygotes and heterozygotes.

Segregation distortion is important when mapping traits. Variation in traits controlled by genes exhibiting appreciable segregation distortion will not segregate in the expected Mendelian ratio. If the desired phenotype is carried by the parent that is under-represented in the marker segregation data, population size must be increased accordingly. Segregation distortion will also complicate estimates of the number of genes controlling a trait by altering segregation ratios.

Mather (1936) first postulated that the frequency of chiasma in a nucleus is under genetic control and that there is an upper limit to the number of chiasma per nucleus. Since then many studies have shown this to be true (e.g., Jones 1974; Lelley 1978). Clearly not enough individuals were observed in this study to show control of crossover frequency in these two populations. There is some indication of it in the BC_1 population as can be seen from the shortfall of observations in the upper portion of the graph (Fig. 5.10B) and in the F_2 population by an excess of individuals having the mean number of crossovers (Fig. 5.11B).

One of the best uses of these graphs is for detecting errors in the data set by identifying the outlying individuals. Excessive numbers of crossovers can be readily detected and corrected as demonstrated by the discovery of the one erroneously loaded lane on one BC_1 filter.

Since the loci of both the F_2 and BC_1 maps segregated into nine distinct and extensive linkage groups with no unlinked loci, little clustering and no large gaps, these linkage groups probably correspond to the nine chromosome pairs of the *Raphanus* genome. Cytological studies at INRA on three sister plants of the F_1 used to generate these maps indicated normal pairing between the two species. This is better pairing than had been previously observed for the *R. sativus* x *R. raphanistrum* cross combination (Panetsos and Baker 1967; Eber *et al.* 1994 and 1998). In view of these mapping and cytology results, these two radishes obviously form highly fertile hybrids and have very similar genome structures, and they should probably be considered different sub-species of the same species. This conclusion had been drawn earlier based on isozyme analysis

of various radish types (Lewis-Jones *et al.* 1982).

This is the first reported map of the *Raphanus* genome and should provide a framework for the mapping of important traits. Mapping and associated marker-assisted selection will allow for more efficient transfer of traits from donor plants while maintaining the background genotype of the recipient parent. Since this map was developed using RFLP probes already mapped in various crucifers, eg., various *Brassica* species (Axelsson *et al.* 2000; Bohuon *et al.* 1996; Lagercrantz and Lydiate 1995; Parkin *et al.* 1995) and *Arabidopsis* (I. Parkin, pers. comm.), comparative mapping will be possible. Comparative mapping will allow for the elucidation of evolutionary relationships amongst crucifers. It will also improve the efficiency of interspecific transfer of interesting traits from *Raphanus* to other crucifers. These themes will be expanded further in Chapters 9 and 10 of this thesis.

Chapter 6. Mapping of *Raphanus* CMS restorer genes segregating in the BC_1 and F_2 populations

Restorer alleles at one or more loci were segregating in the populations used to generate the genome maps of *Raphanus*. This segregation allowed restorer loci to be positioned on the genetic maps.

6.1 Results

6.1.1 Mapping the first restorer locus in the backcross population

The 54 BC_1 progeny segregated 27 male fertile : 27 male sterile individuals, which is consistent with the 1:1 ratio expected when one dominant restoring allele is segregating. The restoring allele segregating in this population (*Rf1*) came from the non-recurrent parent R7-21 (Fig. 5.1). Male fertile individuals were, therefore, carrying the R7-21 allele at the *Rf1* locus. By scoring the inheritance of male fertility from R7-21, it was possible to map this restorer locus near the top of Rs1 (Figure 6.1). With the exception of one individual, segregation for the phenotype was identical to segregation for markers pN23x and pN199. Chi-square analysis on the data for locus pN23x demonstrated that the presence of only one individual homozygous for the R11-4 allele among the male fertile plants and the absence of any individuals heterozygous at pN23x among the male sterile plants was extremely unlikely to have happened by chance (χ^2 = 50, p = 7.4 x 10^{-11}) (Table 6.1).

```
        fsfssssfffffssffsffsffssffsssffffsfssfssfssfsfsssffssf
pW233z  --++---+++++--++-++-++--++---++++-+--+--+--+-+--+++--+
Rf1     +-+----+++++--++-++-++--++---++++-+--+--+--+-+---++--+
pN23x   +-+----+++++--++-++-++--++---++++-+--+--+--+-+--+++--+
pN199   +-+----+++++--++-++-++--++---++++-+--+--+--+-+--+++--+
pN216y  +-+----+++++--++-++-++--++---++++-++-+--+--+-+--+++--+
```

Figure 6.1. BC_1 mapping data for *Rf1* and adjacent RFLP marker loci on Rs1. f, male fertile individual; s, male sterile individual; +, presence of allele from non-recurrent parent R7-21; -, absence of this allele.

Table 6.1. Chi-square tests for marker segregation at locus pN23x in the BC_1 population being unlinked to segregation of male fertile (mf) and male sterile (ms) individuals.

pN23x	observed		expected		$\chi 2$	p[a]	df[a]
	R7-21	R11-4	R7-21	R11-4			
all individuals	28	26	27	27	0.07	0.78	1
mf individuals	27	0	14	13			
ms individuals	1	26	14	13	50	7.4×10^{-11}	1

[a] : p, probability; df, degrees of freedom.

6.1.2 Mapping the second restorer locus in the F_2 population

Eighty-nine individuals from the F_2 mapping population were rated for their male fertile/male sterile phenotype, although marker data was only available for 85 of these plants. Of the 89 F_2 plants, 50 were male fertile, four were partially male fertile (pmf) and 35 were male sterile. The pmf individuals were excluded from the initial genetic analysis of loci controlling restoration. The 50 male fertile : 35 male sterile segregation ratio is a close fit to the ratio expected if dominant restorer alleles were segregating at two restorer loci ($\chi^2 = 0.23$, $p = 0.63$).

As previously described in Chapter 4, the restorer allele at *Rf1* was derived from the R7-21 parent, while the restorer allele at *Rf2* was derived from the R11-4 parent. The male fertile plants were expected to carry restoring alleles at both *Rf1* and *Rf2* in a homozygous or heterozygous state. Male fertile individuals, therefore, could not be homozygous for the non-restoring R7-21 allele at the *Rf2* locus (*rf2rf2*). To map *Rf2*, marker data for the 47 male fertile F_2 individuals was sorted according to the number of individuals homozygous for the R7-21 allele at each marker locus. Two adjacent loci on Rs2, cA142x and pN107z, were not homozygous for the R7-21 allele in any male fertile individuals. The flanking loci only had one or two male fertile individuals homozygous for the R7-21 allele (Figure 6.2). Chi-square analysis on the data for cA142x demonstrated that the scarcity of individuals homozygous for the R7-21 allele among male fertile plants and the overrepresentation of individuals homozygous for the R7-21 allele among the male sterile plants was highly unlikely to have happened by chance ($\chi^2 = 32.6$, $p = 3.0 \times 10^{-7}$). *Rf2* was consequently positioned on Rs2, between or immediately

flanking cA142x and pN107z.

```
A) Rs2:
          fffffffffffffffffffffffffffffffffffffffffffffffff
pW225     vvvvv-vv+v0v+-vv0-v0+0vvvvvvvv-vvvvvvvvvv0000vv
cA53      vvvvv-vv+vvv0-vvv-v-+vvvvvvvvvvvvvvvvvvvvvv--vvv
pW145     vvv0v-vv+vvv+-vvv-v-+vvvvvvvvvvvvvvvvvvvvvv--vvv
pW179     vv-vv-vv+v--0vv0--v-vvvv-vvvvvvvvvvvv-vvvv--vvv
cA142     vv-vv-vvv---0vvv--v-vvvv-vvvvvvvvvvvv-vvvv--vvv
pN107     vv-vv-vvv---00vv--v-vvvv-vvvvvvvvvvvv-vvvv--vvv
pN206     vv-vvvvvv-v-0vvv-vv-vvvv-vvvvvvvvvvvv-vvv+--vvv
cA138     vv-vvvvvv-v-vvvv-vv-vvvv-vvvvvvvvvvvv-vvv+--vvv
pN152     vv-vvvvvv-v-v+vv-vv-vvvv-vvvvvv-vvvvv-vvv+--vvv

B) Rs1:
          fffffffffffffffffffffffffffffffffffffffffffffffff
pW233x    +00vv+vv+vv000000vvv+vvv00000v++v++vv+v+v00000v
pN23x     +++vv+vv+vv+vvvvvvvv+vvv+vv++v++v++vv+v+v+vv+vv
pN199     +++vv+vv+vv+vvvvvvvv+vvv+vv++0++v+0vv0++v+vv+vv
pO145     +++vv+vv+vv+vvvvvvvv+vvv+vv++v++v++vv+++v000000
pN216y    +++v++v-+vv+0vvvvvvv+vv++vv++v++v++vv+++v+v-+vv
```

Figure 6.2. F_2 mapping data for RFLP markers A) on Rs2 with the smallest number of male fertile individuals homozygous for the R7-21 allele and B) on Rs1 with smallest number of male fertile individuals homozygous for the R11-4 allele. f, fertile; +, homozygous for the R7-21 allele; -, homozygous for the R11-4 allele; v, heterozygote (carrying both alleles); 0, missing data. A box is drawn around the two loci with no individuals homozygous for the R7-21 allele, and therefore, the predicted position of the *Rf2* locus and a second box is drawn around the four loci with no individuals homozygous for the R11-4 allele, therefore the predicted position of the *Rf1* locus.

Table 6.2. Chi-square tests for marker segregation at loci cA142x and pN23x in the F_2 population being unlinked to the segregation of male fertile (mf) and male sterile (ms) individuals.

cA142x	observed			expected			$\chi 2$	p [a]	df [a]
	R7-21[a]	H[a] + R11-4[a]		R7-21	H + R11-4				
all individuals	17	41	24	20.5	41	20.5	1.2	0.55	2
mf individuals	0	(32+14) 46		9.5	36.5		32.6	3×10^{-7}	1
ms individuals	17	(7+8) 15		6.6	25.4				
pN23x	observed			expected			$\chi 2$	p	df
	R7-21 + H		R11-4	R7-21 + H		R11-4			
all individuals	26	49	10	21.25	42.5	21.25	8.0	0.02	2
mf individuals	(18+29) 47		0	41.4		5.5	10.8	0.01	1
ms individuals	(7+19) 26		8	30		4			

[a] :R7-21, homozygous for R7-21 parent allele; R11-4, homozygous for the R11-4 parent allele; H, heterozygous (both parental alleles); p, probability the observed segregation did not differ from the expected based on a $\chi 2$ test; df, degrees of freedom for this test.

The placement of *Rf1* at the top of Rs1, near pN23x, was confirmed using the same reasoning, i.e., male fertile individuals could not be carrying the non-restoring allele in the homozygous state (Fig. 6.2). Chi-square analysis on the data for pN23x shows that the scarcity of individuals homozygous for the R11-4 allele among the male fertile plants and the overrepresentation of individuals homozygous for the R11-4 allele among the male sterile plants was highly unlikely to have happened by chance ($\chi^2 = 10.8$, $p = 0.01$) (Table 6.2). Marker pN23x had a distorted segregation ratio ($p = 0.02$; Table 6.2) which significantly reduced the expected number of individuals homozygous for the R11-4 allele.

The predicted genotypes for *Rf1* and *Rf2*, based on the genotypes at marker loci pN23x and cA142x, respectively, were used to predict phenotypes for the F_2 individuals. Of the 47 male fertile individuals, all were predicted to be male fertile. Of the four pmf individuals, all but one were predicted to be male fertile. Of the 34 male sterile

individuals, 11 had marker genotypes expected for male fertile individuals (Figure 6.3). Of these 11 plants, ten were predicted to be heterozygous at *Rf1* based on the marker pN23x and had the restoring allele at *Rf2* in a heterozygous or homozygous state based on markers cA142x and pN107z. There were 29 male fertile individuals that were also heterozygous at *Rf1* and carried the restoring allele at *Rf2* in a heterozygous or homozygous state. It is possible a third restorer locus with a dominant restoring allele was segregating in the F_2 population. This putative locus might interact with *Rf1* such that *Rf1* is epistatic to this third locus when homozygous for the restoring allele but not when the restorer locus at *Rf1* is in the heterozygous state.

Fertile individuals:

```
        fffffffffffffffffffffffffffffffffffffffffffffffffff
p.Rf1   +++vv+vv+vv+vvvvvvvv+vvv+vv++v++v++vv+v+v+vv+vv
p.Rf2   vv-v-v-vvv--0vvv--v-vv-v-v-vvvvvvvvvvv-vvv--vvv
```

Sterile individuals:

```
        sssssssssssssssssssssssssssssssss
p.Rf1   v+vv-+v+v++v-+-vvvv--vvvvv+--vvv-v
p.Rf2   -+-++v-+++++++0+++-vvv++-v+-++vv-v
        X X  XX            X  X  XX    XX X
```

Partial male fertile individuals:

```
p.Rf1   +v-v
p.Rf2   --vv
```

Figure 6.3. Predicted *Rf1* and *Rf2* genotypes (*p.Rf1*, *p.Rf2*), based on marker data for pN23x and cA142x, and their actual phenotypes (f, fertile; s, sterile). +, homozygous for the R7-21 allele; -, homozygous for the R11-4 allele; v, heterozygote; X, individuals where the predicted genotype did not match the phenotype.

The subset of the data for individuals where the expected genotype was heterozygous at *Rf1* was examined for a region of the genome in which homozygotes of one parent or the other was significantly underrepresented among the male fertile plants. There was one region on Rs8, between pO152x and pN34, where only one male fertile individual was homozygous for the R7-21 allele but, due to significant segregation distortion in this region ($p < 0.01$; Chapter 5), this result could have happened by chance

($p > 0.05$). An interval on Rs7 bounded by the loci pO159y and pN216z had only two male fertile plants that were homozygous for the R11-4 allele and none of the male sterile plants were homozygous for the R7-21 allele. This is significantly different from the expected values ($\chi^2 = 10.6$, $p = 0.005$) but still doesn't account for all observed phenotypes. Even taking the regions on Rs7 and Rs8 together did not explain all phenotypes. It was therefore not possible to construct a genetic model that would explain the phenotypes of all the F_2 individuals.

6.2 Discussion

Two restorer loci were mapped with confidence based on the BC_1 and F_2 mapping populations; one near the top of Rs1 and the other on Rs2. There was one individual in the BC_1 population that apparently exhibited a male sterile phenotype while exhibiting the marker genotype expected of a male fertile plant. It is possible that the phenotype of the plant was incorrectly scored or that the wrong DNA sample was analysed. The F_2 segregating population confirmed the placement of *Rf1* near the top of Rs1 although it was not possible to map it as accurately as in the BC_1.

Rf2 could only be mapped in the F_2 population because all the BC_1 individuals carried at least one copy of the restoring allele at *Rf2*. No BC_1 population segregating for *Rf1* was produced from the F_1 because the male sterile plant, R7-21, was not available for backcrossing.

The presence of two dominant restorer loci for Ogura CMS in radish is in keeping with recently published reports (Yamagishi and Terachi 1994b, Koizuka *et al.* 2000). The idea that there might be other loci involved is supported by other research (Bonnet 1975; Niewhof 1990). The ability to map the restorer loci demonstrates that restoration in this population was not as simple as the two gene model suggested by the phenotypic segregation data.

The need for two or more unlinked restorer loci will make the development of inbred lines for hybrid production with these restorers more difficult. The presence of restorers in a wide range of radish germplasm, both wild and cultivated, will also make the development of inbred lines more challenging. Having markers for the restorers

should make it more practical to identify germplasm carrying specific restorer genotypes and to then breed for the presence or absence of these restorer alleles as required for the development of male and female parental lines.

These are the first published markers for Ogura CMS restorer loci in radish. Other markers for an Ogura CMS restorer have been reported (Delourme and Eber 1992; Delourme *et al.* 1994; Hansen *et al.* 1997) but they were all identified by comparing restored *B. napus* cybrids containing a radish introgression of unknown size with cybrids not carrying the introgression. Finding markers linked to the introgression is not very meaningful for the mapping of restorer loci as a large introgressed segment of radish chromosome tends to be either inherited or not and recombination within the introgression is rare. Therefore, any marker loci along the introgression will appear to be very tightly linked to the restorer when they could be a large physical distance away.

The identification of RFLP marker loci co-segregating with *Rf1* and *Rf2* will facilitate the identification of lines homozygous or heterozygous for one, the other or both of these restorers and make it easier to develop populations segregating at specific restorer loci for fine mapping of these loci as well as the mapping of additional restorers (Chapter 7). Markers for restorer loci will also facilitate the selection of interspecies hybrids carrying specific restorers (Chapter 9).

Chapter 7. Mapping the *Raphanus* genes for the restoration of Ogura CMS that were segregating in the R8 population

The R8 mapping population introduced in Chapter 4 appeared to be segregating at a larger number of restorer loci than the F_2 population previously used to map restorer genes. Consequently, the R8 population was analysed with RFLP markers and scored for male sterile/male fertile phenotypes in a similar manner to the F_2 and BC_1 populations (described in Chapters 5 and 6) in order to identify and map the restorer loci segregating in this additional population.

7.1 Results:

7.1.1. Genetic marker analysis and linkage mapping

The R8 population was grown from selfed seed of a male fertile plant from the *R. sativus* MST1 random mating population (Fig. 4.2). DNA samples extracted from the 117 individuals of the R8 population were subjected to RFLP analysis using 99 *Brassica* RFLP probes previously used to identify marker loci segregating in the F_2 and BC_1 populations (Chapter 5) and an additional 12 *Brassica* RFLP probes (Appendix 1.1). Probes were initially selected on the basis of identifying loci in the regions containing the restorer loci *Rf1* and *Rf2*, already mapped in the F_2 and BC_1 populations (Chapter 6), and from the top of linkage group Rs5 (INRA *Rfo* - see Chapter 8). The remaining probes used were chosen at random. The segregation patterns of the RFLP loci assayed in the R8 population were those expected of F_2 populations. Of the 111 probes used on this population, 20 (18%) produced solely monomorphic patterns and the remaining 91 (82%) were informative, each having between one and three scorable segregation patterns. Five of the probes that yielded only monomorphic patterns in the *R. sativus* x *R. raphanistrum* cross were used on the R8 population and of these, four again yielded only monomorphic patterns while the fifth, pO12, produced two segregation patterns.

A total of 23 dominant and 100 co-dominant segregation patterns were scored in

the R8 population. The parental origin of alleles could not be identified because no parental material existed as a reference. Once a core map was established, marker scores were adjusted to ensure that alleles linked in coupling were identified as having the same parental origin. The segregation data for the 123 informative marker loci that were scored can be found in Appendix 1.4.

Of the 123 loci, 57 loci exhibited at least one RFLP allele that was indistinguishable from an allele scored in the BC_1 and/or F_2 populations (the loci marked with an asterisk in Fig 7.1). Of these 57 loci, 32 exhibited alleles indistinguishable from those in R7-21 (*R. sativus*), 11 exhibited alleles indistinguishable from those in R11-4 (*R. raphanistrum*) and 14 exhibited alleles indistinguishable from both the R7-21 and the R11-4 alleles. These 57 loci provided a core aligned map to which additional loci could be added (Fig. 7.1).

Of the 123 loci, all except pO150 could be assigned to linkage groups corresponding to the nine linkage groups previously mapped in the F_2 and BC_1 populations (Chapter 5). The bottom regions of linkage groups Rs1 and Rs2 were absent from the R8 map, in addition there were three gaps of greater than 50 cM on Rs3 and one gap greater than 50 cM on Rs8.

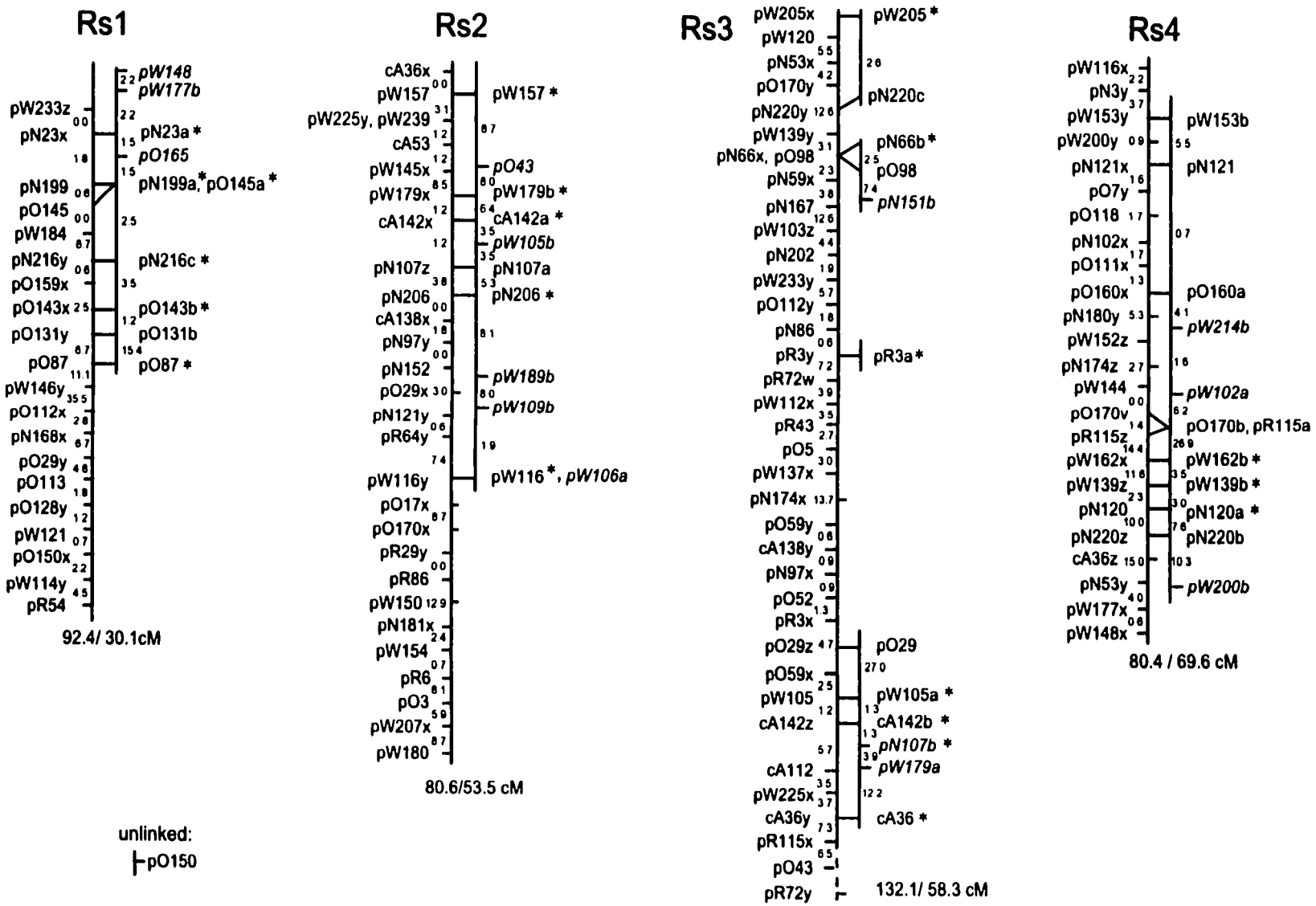
The R8 segregation data added 40 new loci to the overall *Raphanus* map (Fig. 7.1- italicised markers). Ten loci were added to Rs9, the linkage group with the smallest number of loci in the original map. Between two and six loci were added to each of the other linkage groups.

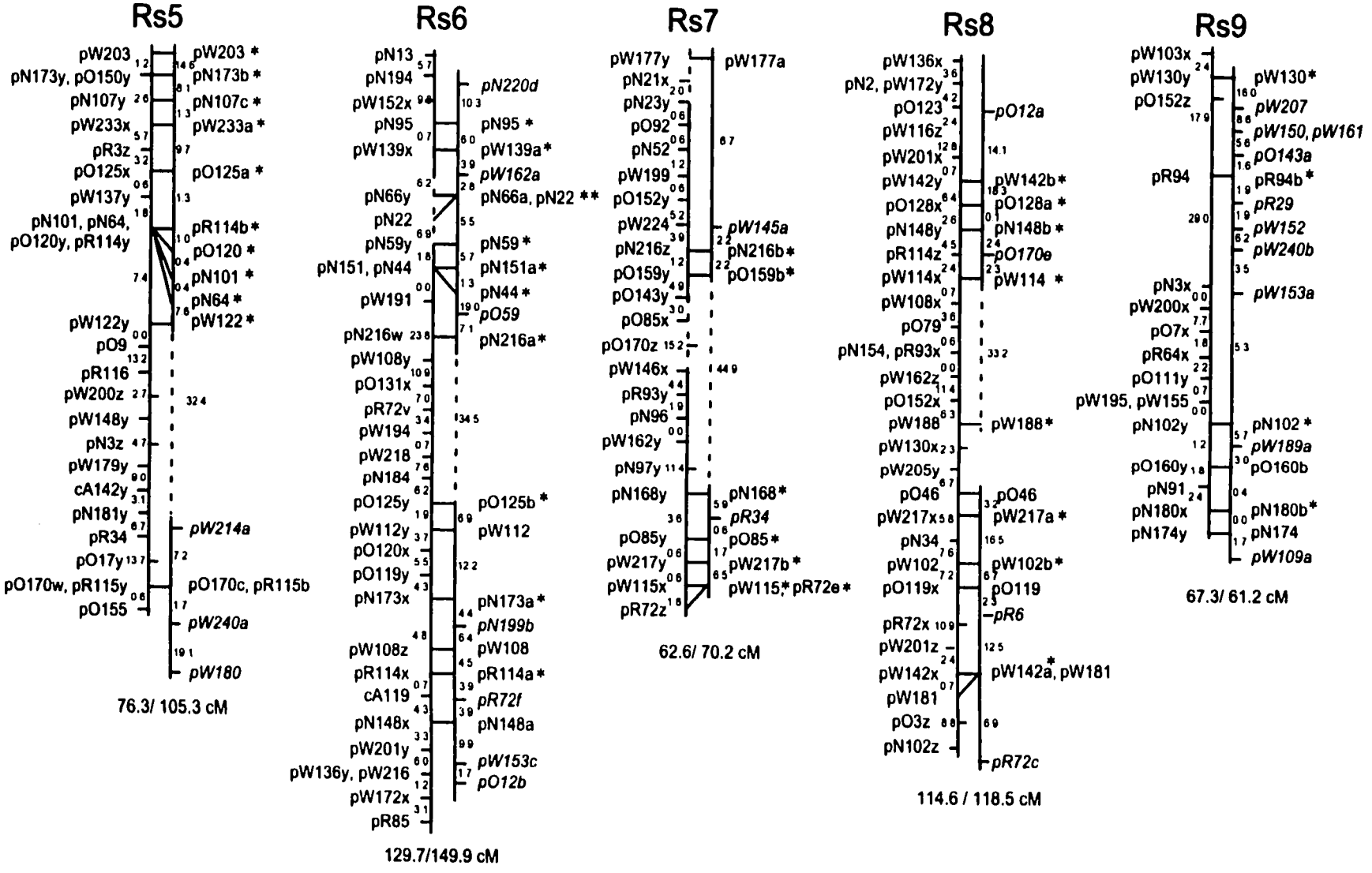
There was no significant segregation distortion at any of the loci surveyed in the R8 population (all $p > 0.01$).

7.1.2. Restorer mapping

R8 was a highly heterozygous male fertile plant from the MST1 population that was selfed to produce an F_2 population that segregated for male fertility (Chapter 4). Progeny were rated for their male fertile/male sterile phenotype. Of the 117 plants scored, 36 were male fertile, three were pmf and 78 were male sterile. The pmf individuals were excluded from the initial genetic analysis to identify loci controlling restoration. The 36 male fertile : 78 male sterile ratio is a close fit to the ratio expected

Figure 7.1 (p. 77 & 78). Alignment of the R8 RFLP map with the original *Raphanus* map from Chapter 5. Vertical lines represent linkage groups with RFLP marker loci listed on the left for the original map and on the right for the R8 map (loci mapped only in the R8 population are italicised). Breaks in the vertical line of the R8 linkage groups (right line) indicate gaps of over 50cM while dashed regions of the lines represent gaps of 30-50 cM. Dashed regions of the lines in the original linkage groups (left line) indicate regions where precise locus order could not be determined. *, loci with bands indistinguishable in size from bands in the original linkage groups and therefore considered allelic. The order of some loci relative to the loci of the original linkage groups could not always be determined due to the lack of aligned flanking loci. Genetic distances listed to the left of the lines are for the F_2 population and those to the right are for the R8 population. Overall length of the linkage group is listed at the bottom (F_2 / R8).


Rs1
pW148
pW177b
pW233z
pN23x
pN23a *
pO165
pN199
pN199a,*pO145a *
pO145
pW184
pN216y
pN216c *
pO159x
pO143x
pO143b *
pO131y
pO131b
pO87
pO87 *
pW146y
pO112x
pN168x
pO29y
pO113
pO128y
pW121
pO150x
pW114y
pR54
92.4/ 30.1cM
Rs2
cA36x
pW157
pW157 *
pW225y, pW239
cA53
pW145x
pO43
pW179x
pW179b *
cA142x
cA142a *
pW105b
pN107z
pN107a
pN206
pN206 *
cA138x
pN97y
pN152
pW189b
pO29x
pN121y
pW109b
pR64y
pW116y
pW116 *, pW106a
pO17x
pO170x
pR29y
pR86
pW150
pN181x
pW154
pR6
pO3
pW207x
pW180
80.6/53.5 cM
Rs3
pW205x
pW205 *
pW120
pN53x
pO170y
pN220c
pN220y
pW139y
pN66b *
pN66x, pO98
pO98
pN59x
pN151b
pN167
pW103z
pN202
pW233y
pO112y
pN86
pR3y
pR3a *
pR72w
pW112x
pR43
pO5
pW137x
pN174x
pO59y
cA138y
pN97x
pO52
pR3x
pO29z
pO29
pO59x
pW105
pW105a *
cA142z
cA142b *
pN107b *
pW179a
cA112
pW225x
cA36y
cA36 *
pR115x
pO43
pR72y
132.1/ 58.3 cM
Rs4
pW116x
pN3y
pW153y
pW153b
pW200y
pN121x
pN121
pO7y
pO118
pN102x
pO111x
pO160x
pO160a
pN180y
pW214b
pW152z
pN174z
pW144
pW102a
pO170v
pO170b, pR115a
pR115z
pW162x
pW162b *
pW139z
pW139b *
pN120
pN120a *
pN220z
pN220b
cA36z
pN53y
pW200b
pW177x
pW148x
80.4 / 69.6 cM
unlinked:
pO150


Rs5
pW203
pN173y, pO150y
pN107y
pW233x
pR3z
pO125x
pW137y
pN101, pN64, pO120y, pR114y
pW122y
pO9
pR116
pW200z
pW148y
pN3z
pW179y
cA142y
pN181y
pR34
pO17y
pO170w, pR115y
pO155
pW203 *
pN173b *
pN107c *
pW233a *
pO125a *
pR114b *
pO120 *
pN101 *
pN64 *
pW122 *
pW214a
pO170c, pR115b
pW240a
pW180
76.3/ 105.3 cM
Rs6
pN13
pN194
pW152x
pN95
pW139x
pN66y
pN22
pN59y
pN151, pN44
pW191
pN216w
pW108y
pO131x
pR72v
pW194
pW218
pN184
pO125y
pW112y
pO120x
pO119y
pN173x
pW108z
pR114x
cA119
pN148x
pW201y
pW136y, pW216
pW172x
pR85
pN220d
pN95 *
pW139a*
pW162a
pN66a, pN22 **
pN59 *
pN151a*
pN44 *
pO59
pN216a*
pO125b *
pW112
pN173a*
pN199b
pW108
pR114a*
pR72f
pN148a
pW153c
pO12b
129.7/149.9 cM
Rs7
pW177y
pN21x
pN23y
pO92
pN52
pW199
pO152y
pW224
pN216z
pO159y
pO143y
pO85x
pO170z
pW146x
pR93y
pN96
pW162y
pN97y
pN168y
pO85y
pW217y
pW115x
pR72z
pW177a
pW145a
pN216b*
pO159b*
pN168*
pR34
pO85*
pW217b*
pW115* pR72e*
62.6/ 70.2 cM
Rs8
pW136x
pN2, pW172y
pO123
pW116z
pW201x
pW142y
pO128x
pN148y
pR114z
pW114x
pW108x
pO79
pN154, pR93x
pW162z
pO152x
pW188
pW130x
pW205y
pO46
pW217x
pN34
pW102
pO119x
pR72x
pW201z
pW142x
pW181
pO3z
pN102z
pO12a
pW142b*
pO128a *
pN148b *
pO170e
pW114 *
pW188*
pO46
pW217a *
pW102b*
pO119
pR6
pW142a*, pW181
pR72c
114.6 / 118.5 cM
Rs9
pW103x
pW130y
pO152z
pR94
pN3x
pW200x
pO7x
pR64x
pO111y
pW195, pW155
pN102y
pO160y
pN91
pN180x
pN174y
pW130*
pW207
pW150, pW161
pO143a
pR94b*
pR29
pW152
pW240b
pW153a
pN102 *
pW189a
pO160b
pN180b*
pN174
pW109a
67.3/ 61.2 cM

if dominant restorers were segregating at four unlinked restorer loci ($\chi^2 = 2x10^{-4}$; $p = 0.99$).

Assuming the restorers are dominant, all male fertile individuals were expected to be homozygous or heterozygous for the restoring allele at each locus and therefore not be homozygous for the non-restoring allele at any of the restorer loci. All loci were surveyed for the absence of homozygous alleles of one parent or the other in the male fertile individuals. Two regions fitting this criterion perfectly were identified: one at the top of Rs1 and the other on Rs2 (Fig. 7.2). These regions contain the *Rf1* and *Rf2* restorer loci identified in the original mapping populations (Chapter 6).

The region of Rs1 where no male fertile individuals were homozygous for one of the parental alleles covers 7.4 cM and spans five loci (Figs 7.2 and 7.1). The RFLP allele linked in coupling to the restorer allele at the locus most closely linked to *Rf1* in the F_2 and BC_1 populations (namely the "+" allele at pN23x: Fig. 6.2) was indistinguishable from the allele linked in coupling to the restorer allele at the corresponding pN23a locus in the R8 population. Chi-square analysis on the data for pN23a shows that the scarcity of individuals homozygous for the non-restoring parent allele among the male fertile plants and the overrepresentation of individuals homozygous for the non-restoring parent allele among the male sterile plants was highly unlikely to have happened by chance ($\chi^2 = 16.4$, $p = 1.0x10^{-3}$; Table 7.1). It is, therefore, most likely that pN23a is linked to one of the fertility restorers segregating in this population.

There were two loci on Rs2, separated by 6.4 cM, where again no male fertile individuals were homozygous for one of the parental alleles (Figs 7.1 and 7.2). One of these loci, cA142a, was the same as the locus shown to be closely linked to *Rf2* in the F_2 and BC_1 populations (cA142x: Fig. 6.2). Chi-square analysis on the data for cA142a demonstrated that the scarcity of individuals homozygous for the non-restoring parent allele among male fertile plants and the overrepresentation of individuals homozygous for the non-restoring parent allele among the male sterile plants was highly unlikely to have happened by chance ($\chi^2 = 25.3$, $p = 1.3x10^{-5}$; Table 7.1).

A

Rs1	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f
pW148	+	v	+	+	v	+	v	v	v	v	v	+	v	v	+	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	v
pW177b	+	v	+	+	v	+	+	v	v	v	v	+	v	v	+	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	v
pN23a	+	v	+	+	v	+	+	v	v	v	v	+	v	v	+	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	v
pN199a	+	v	+	+	v	+	+	v	v	v	v	+	v	v	+	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	v
pO145a	+	v	+	+	v	+	+	v	v	v	v	+	v	v	+	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	v
pN216c	+	v	+	+	v	v	+	v	v	v	v	+	v	v	+	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	-
pO143b	+	0	+	+	v	v	v	v	v	v	v	+	v	v	v	v	+	+	+	v	+	+	v	v	+	+	+	v	v	+	v	v	+	+	+	-
pO131b	+	v	+	+	v	v	v	v	v	v	v	+	v	v	v	v	+	+	+	v	+	0	0	0	0	0	0	0	0	0	0	0	+	+	-
pO87	+	-	v	+	v	v	v	v	v	+	v	+	v	v	v	v	v	v	+	v	v	v	v	v	v	+	+	v	+	v	v	+	+	+	-	-

B

Rs2	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f
pW157	0	0	0	0	0	0	0	0	0	0	0	0	v	v	+	v	v	v	v	-	v	v	+	v	v	v	v	v	v	+	+	+	v	-	v	v
pO43	v	+	+	v	+	+	v	+	+	+	v	+	v	v	+	v	v	v	v	-	v	v	+	+	v	v	v	v	v	+	+	v	v	v	v	+
pW179b	v	+	+	v	+	+	v	+	+	v	v	v	v	v	+	v	+	+	v	v	v	v	+	+	v	v	v	v	v	+	v	v	+	v	v	+
cA142a	v	+	+	+	+	+	v	+	+	+	v	v	v	v	+	v	+	+	v	v	v	v	+	+	v	v	v	v	v	+	v	v	+	v	v	+
pW105b	v	0	0	0	0	0	0	0	0	0	0	0	v	-	+	v	+	+	v	v	v	v	v	+	v	v	v	v	v	+	v	v	+	v	v	+
pN107a	v	+	+	+	+	+	v	+	+	v	v	v	v	0	+	v	+	+	v	v	v	v	v	+	-	v	v	v	v	+	v	v	+	v	v	+
pN206	+	+	+	+	+	+	v	+	+	+	v	v	v	-	+	v	+	+	v	v	v	v	v	+	-	v	v	v	v	+	v	v	+	v	v	+
pW189b	0	0	0	0	0	0	0	0	0	0	0	0	-	-	+	v	+	+	v	v	v	v	v	+	-	v	v	v	v	+	v	v	+	v	v	+
pW109b	v	0	+	+	v	+	v	+	+	+	v	v	-	-	+	v	+	+	v	v	v	+	-	+	-	v	v	v	-	+	v	v	+	v	v	+
pW116	-	v	+	+	v	+	v	+	+	+	v	v	-	-	+	v	+	+	v	v	v	+	-	+	-	v	v	v	-	+	v	v	+	v	v	+

Figure 7.2. RFLP marker mapping data for Rs1 (A) and Rs2 (B) for the male fertile (f) individuals from the R8 population. +, homozygous for one parental allele; -, homozygous for the other parental allele; v, heterozygous (both alleles); 0, missing data. Boxes are drawn around the loci where no individuals were homozygous for the allele from the parent represented by '-', and therefore the predicted location of the restorer allele(s).

Table 7.1. Chi-square tests for marker segregation at loci pN23a, cA142a and pO159b in the R8 population being unlinked to the segregation of male fertile (mf) and male sterile (ms) individuals.

pN23a	observed			expected			χ^2	p[b]	df[b]
	RP[a] + H[a]		NRP[a]	RP[a] + H[a]		NRP[a]			
all individuals	31	46	37	28.5	59	28.5	4.9	0.09	2
mf individuals	(19+17) 36		0	24.3		11.7	25.3	1.3×10^{-5}	1
ms individuals	(12+29) 41		37	52.7		25.3			

cA142a	observed			expected			χ^2	p	df
	RP + H		NRP	RP + H		NRP			
all individuals	29	58	27	28.5	59	28.5	0.1	0.94	2
mf individuals	(16+20) 36		0	27.5		8.5	16.4	1.0×10^{-3}	1
ms individuals	(13+38) 51		27	59.5		18.4			

pO159b	observed			expected			χ^2	p	df
	RP + H		NRP	RP + H		NRP			
all individuals	24	66	23	28.25	56.5	28.25	3.2	0.2	2
mf individuals	(7+28) 35		1	28.7		7.3	10.5	1.8×10^{-2}	1
ms individuals	(17+38) 55		22	62.1		15.9			

[a] :RP, homozygous for the allele from the restoring parent; NRP, homozygous for the allele from the non-restoring parent; H, heterozygous (both parental alleles)

[b] :p, probability the observed segregation did not differ from the expected based on a χ^2 test; df, degrees of freedom for this test.

Assuming a dominant gene model, individuals would be male sterile if one, the other or both of these restorers on Rs1 and Rs2 were homozygous for their non-restoring allele. The homozygous state of the non-restoring allele at the restorer locus on Rs1, as indicated by locus pN23a, accounted for 37 of the male sterile individuals. The homozygous state of the non-restoring allele at the restorer locus on Rs2, as indicated by locus cA142a, accounted for an additional 17 male sterile individuals. However, there were 24 individuals carrying restorer alleles for the mapped restorer loci on both Rs1 and Rs2 (based on pN23a and cA142a) that were still male sterile.

The scoring data for the male fertile plants was re-examined for other regions grossly underrepresented by homozygous alleles of one of the parents. Loci pN216b and pO159b on Rs7 were identified as having only one male fertile individual homozygous for the "-" allele (Fig. 7.3). A chi-square test on the data for locus pO159b demonstrated that the scarcity of individuals homozygous for the "-" allele among the male fertile individuals was unlikely to have happened by chance ($\chi^2 = 10.5$; $p = 0.017$; Table 7.1) and therefore it is likely that this locus is linked to a third fertility restorer in the R8 population.

There were still 12 individuals that were male sterile despite having alleles linked in coupling to all three restoring alleles identified. All 12 were heterozygous at the predicted third restorer locus. There were no other regions where there were significant shortages of homozygotes of one parent or the other so a fourth restorer locus could not be identified in this manner.

All three pmf individuals were homozygous or heterozygous for all three restorers as identified by loci pN23a, cA142a and pO159b.

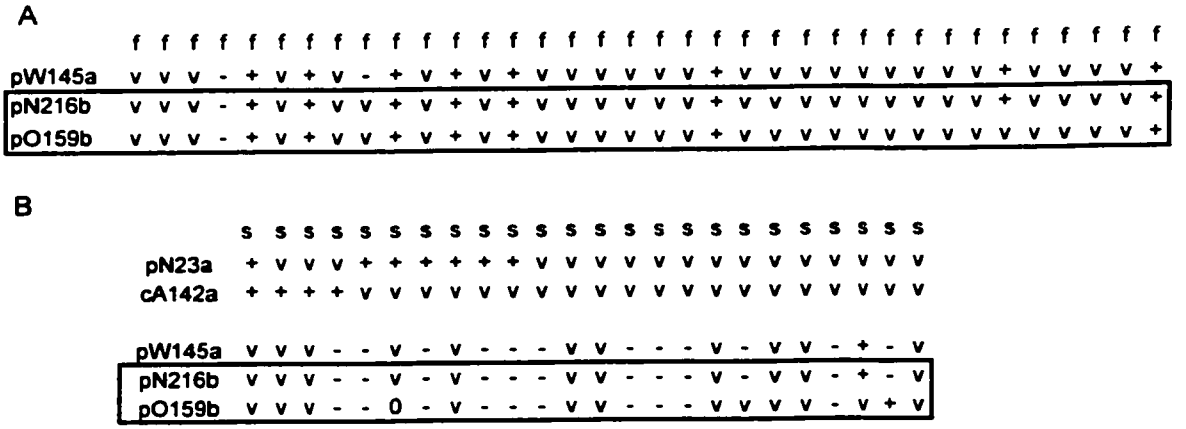


Figure 7.3. RFLP marker mapping data from the R8 population for the region on Rs7 bounded by pW145a and pO159b for A) all mf individuals and B) ms individuals that were carrying restoring alleles on Rs1 and Rs2 as indicated by pN23a and cA142a. +, homozygous for the restoring parent allele; -, homozygous for the non-restoring parent allele; v, heterozygous (both alleles); 0, missing data.

7.2 Discussion

7.2.1. Linkage mapping

The R8 population is the result of a *R. sativus* x *R. sativus* cross but the percentage of probes that resulted in at least one polymorphism was similar to the *R. sativus* x *R. raphanistrum* cross (82% vs 84%, respectively). The number of polymorphic loci detected per probe however was fewer, 1.1 in the R8 population vs 1.7 in the F_2, resulting in a less dense map.

Due to the robustness of the combined F_2 and BC_1 map (Chapter 5) and the methodical process of matching alleles, it was relatively straightforward to align the linkage groups of the R8 map with those of the consensus map (Fig. 7.1) and avoid the potential problems caused by genome duplication. This was especially important in Rs3 where three separate linkage groups plus one lone locus were brought together only because of matching alleles (Fig. 7.1).

The bottom regions of linkage groups Rs1 and Rs2 as well as large portions of Rs3, Rs5, Rs6 and Rs7 were absent from the R8 map. This was likely due to the lower level of polymorphism in the R8 population combined with the reduced number of probes used to develop the map. Most of the loci positioned in these gaps in the other maps were monomorphic in the R8 population. The unlinked locus pO150 may reside on the bottom of Rs1 but since the alleles were not the same size as those scored in other populations it is not possible to be certain. These large monomorphic regions make mapping an entire genome difficult but are not so important when a robust map has already been developed. Several early maps of *Brassica* species were based on limited number of loci due in part to inadequate numbers of probes being used but also due to the relatively low polymorphism of the cross. This resulted in maps with many more linkage groups than chromosome pairs and multiple unlinked loci (e.g., Landry *et al.* 1991; Kinian and Quiros 1992) and makes aligning maps difficult (Hu *et al.* 1998).

Data from the R8 population allowed a total of 40 loci to be added to the combined *Raphanus* map. Only 13 of these 40 new loci were detected by the 12 new RFLP probes used on the R8 population but not on the BC_1 or F_2 populations. The other 27 loci represented loci that were monomorphic or otherwise unmappable (e.g., bands

co-incident with other bands making patterns too difficult to score) in the F_2 and BC_1 populations. Only six of the additional loci mapped to the ends of linkage groups and thus extended them, the rest of the loci were scattered across the genome.

Ten loci were added to linkage group Rs9, four of these appeared to map to a large 29cM interval in the original *Raphanus* map adding useful markers to the map of Rs9 and confirming that the gap was indeed a map interval and was not caused by a spurious association between two small linkage groups. This large interval in the F_2 and BC_1 maps could represent a fragment of the genome conserved between the two parent lines, perhaps suggesting a common ancestry for this region. That it was polymorphic in the ostensibly more closely related *R. sativus* x *R. sativus* R8 population is curious. One explanation might be that *R. sativus* and *R. raphanistrum* have intercrossed relatively recently. Recent reports (Snow *et al.* 2001) have demonstrated that crop radish (*R. sativus*) genes can persist in weedy populations of *R. raphanistrum*. Morphological observations on flower colour and pod structure have also been offered as evidence for past hybridizations between the two species (Kercher and Conner 1996; Lee and Snow 1998).

The ability to align this new map with the original map has resulted in a still more comprehensive overall map of the *Raphanus* genome which will enhance the ability to make comparisons with *Brassica* genomes as well as offer additional markers for future mapping in *Raphanus*.

7.2.2. Restorer mapping

Three restorer loci were mapped with confidence in the R8 population and a fourth was implicated in restoration but not mapped. Two of the three restorers mapped in this population mapped to the same regions as those mapped in the F_2 population. The third mapped to a region that might be implicated in restoration in the F_2 population.

The restorer locus on Rs1 in the R8 population mapped to the same interval as the restorer locus on Rs1 in the BC_1 and F_2 populations, suggesting the same restorer locus had been mapped in all three populations. The restoring alleles linked to the pN23 locus on Rs1 in the F_2 and R8 populations originated from the MST1 *R. sativus* population therefore, it is quite possible that they are the same restorer locus.

The second restorer segregating in the R8 population mapped to the same region of the *Raphanus* genome as did *Rf2* in the F_2 population. The restoring allele in the R8 population came from *R. sativus* while the restoring allele in the F_2 population originated in *R. raphanistrum*. It is most likely that they are the same restorer but it is also possible that they are two different but closely linked ones. It would require cloning the restoring alleles and comparing them to demonstrate with absolute certainty that the restorers are indeed the same. If they are the same restorer, it either evolved prior to the two species diverging or it was introgressed from one species to the other. Yamagishi and Terachi (1997) and Yamagishi (1998) found that *R. raphanistrum* wild radishes posses Ogura cytoplasm and were also male fertile and capable of restoring fertility to a male sterile *R. sativus* radish.

As expected from the phenotypic segregation data, two dominant restorer loci were not sufficient to explain the observed phenotypes. A third restorer was shown to map to an interval on Rs7, in a region where only three loci were mapped, surrounded by large monomorphic stretches of the linkage group. It is in the same region of Rs7 that is possibly involved in determining the phenotype of individuals heterozygous at *Rf1* in the F_2 population. It was not possible to map the fourth putative restorer locus segregating in this population. It is possible that this fourth locus is in a region not yet mapped in the R8 population.

Chapter 8. Identifying the INRA restorer introgression

The *B. napus* Ogura CMS restorer line developed by INRA has a radish introgression of unknown size which behaves genetically as a single restorer (Pelletier *et al.* 1987). This INRA restorer introgression was investigated to determine the region of the radish genome that was transferred to *B. napus* and to ascertain whether it includes one of the restorer loci mapped in Chapters 6 and 7.

8.1 Results:

8.1.1 Population development

Ten plants of each of two near-isogenic lines of *B. napus*, one with the INRA restorer introgression (Rf) and the other without the restorer introgression (no-Rf) were grown and two leaves of each plant were harvested to create bulk tissue samples of each line. A single Rf plant was pollinated with pollen from a single no-Rf plant to produce F_1 seed (Fig. 8.1). Extra tissue was collected for DNA extraction from the two parent plants. Ten F_1 plants were grown and all were male fertile. One F_1 plant was selfed and forty of the resulting F_2 individuals were grown and assayed for male sterile/male fertile phenotypes at flowering (Fig. 8.1). The F_2 plants segregated 33 male fertile : 7 male sterile which is consistent with the 3:1 ratio expected from the segregation of one dominant restorer ($\chi^2 = 1.2$, $p = 0.55$). Tissue was collected from each of the F_2 plants prior to flowering and DNA was extracted for RFLP analysis.

8.1.2 Polymorphism screening of parental bulks

RFLP analysis using the same *Brassica* RFLP probes as had previously been used on the BC_1 population was carried out on the two parental bulks. Of the 139 RFLP probes screened, only 26 (19%) were polymorphic. Since the majority of probes detected several loci in both *B. napus* and *Raphanus*, there were several possibilities for the origin of the polymorphism (Table 8.1). However, 18 of the 26 informative probes

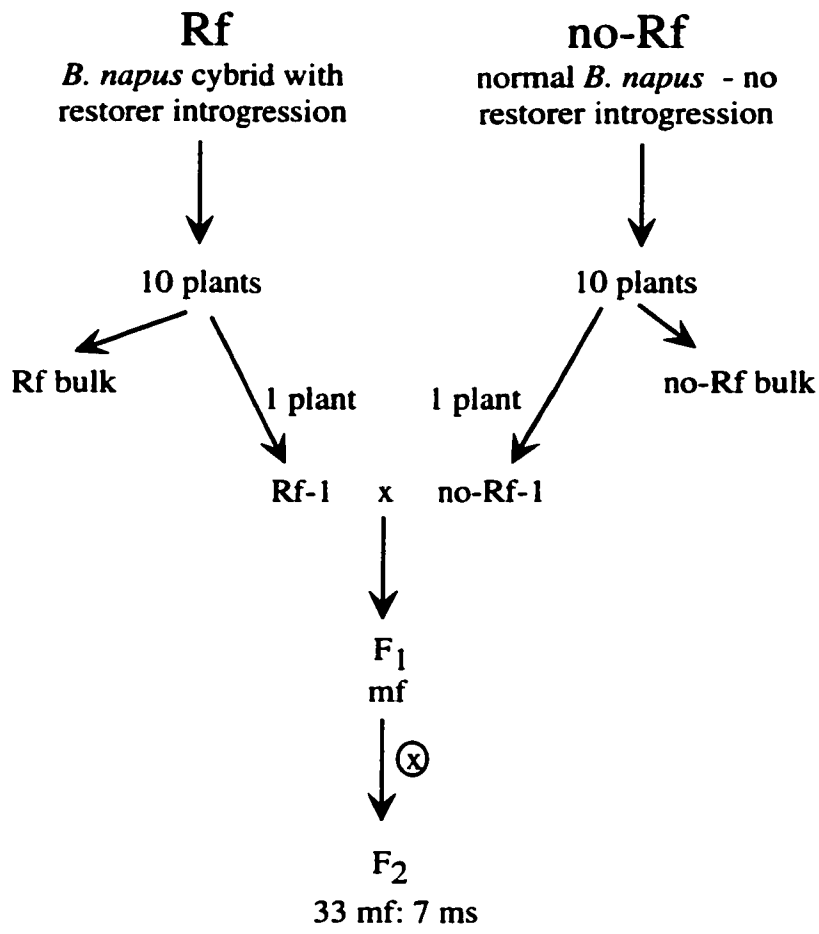


Figure 8.1. Crossing scheme used to develop an F_2 population segregating for the INRA restorer introgression.

Table 8.1. RFLP probes that detected a polymorphism between a *B. napus* line carrying the INRA restorer introgression and a near-isogenic *B. napus* line without the restorer introgression and the locations on the *Raphanus* genome of all mapped loci detected by these informative probes. Polymorphisms linked (L) and unlinked (U) to restoration of fertility based on a segregating F_2 population are indicated in the last column. *, allele indistinguishable from a corresponding *Raphanus* allele at a mapped locus.

Probe	Possible Linkage Groups									linked (L)/ unlinked (U) to restoration
	Rs1	Rs2	Rs3	Rs4	Rs5	Rs6	Rs7	Rs8	Rs9	
cA36		X	X	X						U
pN64					X*					L
pN101					X					L
pN107		X	X		X*					U
pN173					X	X				L
pN180				X					X	U
pN184					X	X				L
pN220			X	X		X				L
pO9					X					L
pO119						X		X		L
pO120					X	X				L
pO125					X	X				L
pO150	X				X					L
pR3			XX		X*					L
pR116				X	X					L
pW116		X		X				X		U
pW122					X					L
pW137			X		X*					L
pW144				X						U
pW148	X			X	X					L
pW177	X			X			X			U
pW179		X	X		X*					L
pW200				X	X				X	L
pW203					X*					L
pW225		X	X							U
pW233	X		X		X					L

detected loci on Rs5 while the *Raphanus* linkage group with the next largest number of candidate loci was Rs4 with only nine such loci (Table 8.1). This result was a strong indication that the restorer introgression originated from Rs5. The 18 markers on Rs5 all mapped together in the upper region of the linkage group (Fig 8.2) covering approximately 50 cM. Seven of the polymorphic alleles were indistinguishable from radish alleles identified in the BC_1 mapping population, supporting the hypothesis that they were in fact *Raphanus* alleles. Six of the seven were alleles of loci mapped on Rs5 (asterisks Table 8.1) and the remaining allele, detected by pN184, was monomorphic in the BC_1 and F_2 populations. Probe pN184 detected a locus that mapped to Rs5 in the R8 population (Fig. 8.2).

8.1.3 Segregation of trait and loci in the F_2

DNA samples from the parents (Rf and no-Rf), all seven male sterile F_2 plants and 23 of the male fertile F_2 plants were probed with the 26 RFLP probes that showed a polymorphism between the Rf and no-Rf parent bulks. Of the eight probes that did not have loci previously mapping to Rs5, six had segregation patterns that did not co-segregate with fertility suggesting they were from other regions of the *B. napus* genome. Presumably these regions were polymorphic between the near-isogenic lines but not in the region including or linked to the restorer introgression. Segregation patterns detected by probes pO119 and pN220 did co-segregate with fertility but represented loci not previously mapped to Rs5. It is possible that the polymorphic alleles detected by these two probes do map to Rs5 but were monomorphic in the *Raphanus* mapping populations used so far. All 18 of the loci that were identified as possibly mapping on Rs5 also co-segregated with fertility restoration.

RFLP probes pW167 and pN3 had not been used on the parental bulks but did map to the region of interest on Rs5 in the F_2 and BC_1 populations. Each of these probes detected a locus that segregated with the male fertile phenotype. Probe cA142 detected a locus that mapped immediately below the region identified by the parental bulks (Fig. 8.2). It too was tested on the segregating population and it did not yield any segregating loci, confirming it was not part of the *Raphanus* introgression.

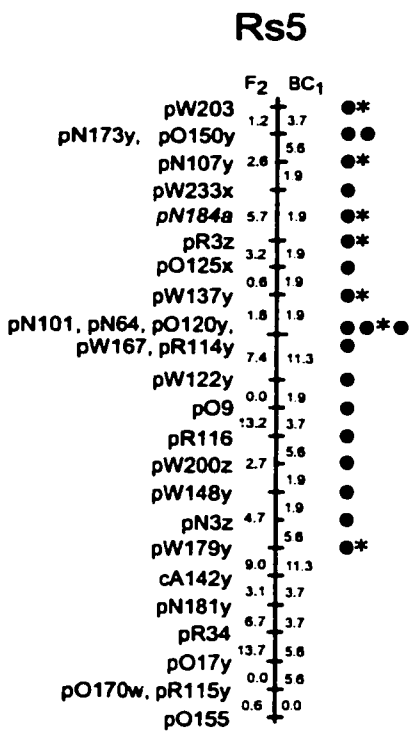


Figure 8.2 *Raphanus* linkage group 5 with markers detected by RFLP probes that also detect polymorphism between the *B. napus* line carrying the INRA restorer introgression and the near isogenic *B. napus* line without the restorer introgression. ●, *Raphanus* loci detected by probes that exhibited a polymorphism between the near-isogenic lines; *, *Raphanus* loci sharing common alleles with loci on the INRA restorer introgression.

8.2 Discussion

By comparing RFLP loci previously mapped in *Raphanus* with those segregating with the restorer introgression in *B. napus*, it was possible to determine that the INRA restorer introgression was a large segment of *Raphanus* linkage group Rs5. Markers developed for the INRA restorer by other researchers (Delourme and Eber 1992; Delourme *et al.* 1994; Hansen *et al.* 1997) have been based on segregation in *B. napus* cybrids, and not in radish. This has resulted in a set of markers that are linked to the introgression but that have not been positioned relative to each other or to the restorer locus. Delourme and Eber (1992) demonstrated tight linkage between radish isozyme marker *Pgi-2* and the restorer with a recombination fraction of 0.25% while Delourme *et al.* (1994) found four RAPD markers completely linked to the restorer introgression. All of these markers were linked to the restorer introgression but their locations relative to the restorer gene in the radish genome are unknown. Recently, several companies have reportedly reduced the size of the introgression as witnessed by the loss of several of the markers associated with the introgression and a reduction in the associated glucosinolate levels (Burns *et al.* 1996; Delourme *et al.* 1999; Pruvot *et al.* 1999). These new lines could be tested with probes detecting loci on Rs5 to more accurately determine the location of the restorer on Rs5 and quantify the proportion of the introgression eliminated in the different events.

The precise location of the restorer on the segment of radish linkage group Rs5 is still unknown as no *Raphanus* mapping population segregating for fertility restoration in this region of the genome has yet been reported. It is believed that only one restorer is needed to restore fertility in the INRA cybrid material (Pelletier *et al.* 1987) and identifying the putative restorer gene in radish would allow for further characterisation of the restorer.

Knowing the approximate location of the restorer gene will allow the more rapid identification of radish populations segregating for the restorer and enable the genetics of this restorer to be studied in its natural genome. None of the *Raphanus* populations investigated so far (Chapters 6 and 7) appear to be segregating for fertility restoration in this region. This indicates the presence of yet another restorer gene in *Raphanus* and suggests that it may be homozygous in the parental material investigated so far. In

maize, one of the restorers required to restore fertility to T-CMS is found in almost all inbred lines and its presence was only discovered when allelism tests were carried out between many different restorer lines (Duvik 1956). Another, more interesting possibility is that the restoring allele on the Rs5 linkage group acts independently of the *Rf1*, *Rf2* and *Rf3* restorers previously identified (Chapters 6 and 7) and that the restoring allele is not present in the populations studied thus far. Still another possibility is that the restorer found on this introgression may not function as a restorer in Ogura-CMS *Raphanus* but acts to restore fertility to the cybrid CMS by correcting a deficiency specific to its interaction with the *Brassica* genome.

Chapter 9: Developing interspecies hybrids to promote intergenomic recombination and facilitate gene transfer between the *Raphanus* and the *Brassica* A genomes.

Specific interspecies hybrids were developed to promote recombination between the *Raphanus* genome and the A genome of *Brassica*. The ultimate intention of this research is to develop *Brassica* genotypes carrying defined segments of *Raphanus* chromosome at homoeologous sites within the *Brassica* A genome. These genotypes will be developed through the process of marker-assisted selection across several generations.

9.1 Results

9.1.1 Crossing scheme

The crossing scheme chosen for the introgression of segments of *Raphanus* chromosome into the *Brassica* A genome is outlined in Figure 9.1. This scheme was chosen for the following reasons:

1. Crosses between *R. sativus* (♀) and *B. oleracea* (♂) do not usually require embryo rescue (Karpechenko 1924). The *R. sativus* plant used in the cross carried Ogura CMS cytoplasm and would test for the presence of restorers.
2. Chromosome doubling of amphihaploids should increase the fertility of the hybrid gametes.
3. The use of resynthesised *B. napus* was expected to avoid the chromosome pairing control found in domesticated *B. napus* and thus promote intergenomic chromosome pairing in the RACC hybrids.
4. The two sets of C genome chromosomes should pair and segregate normally providing the gametes of the RACC hybrid with a complete set of C genome chromosomes and thereby increasing gamete viability. This

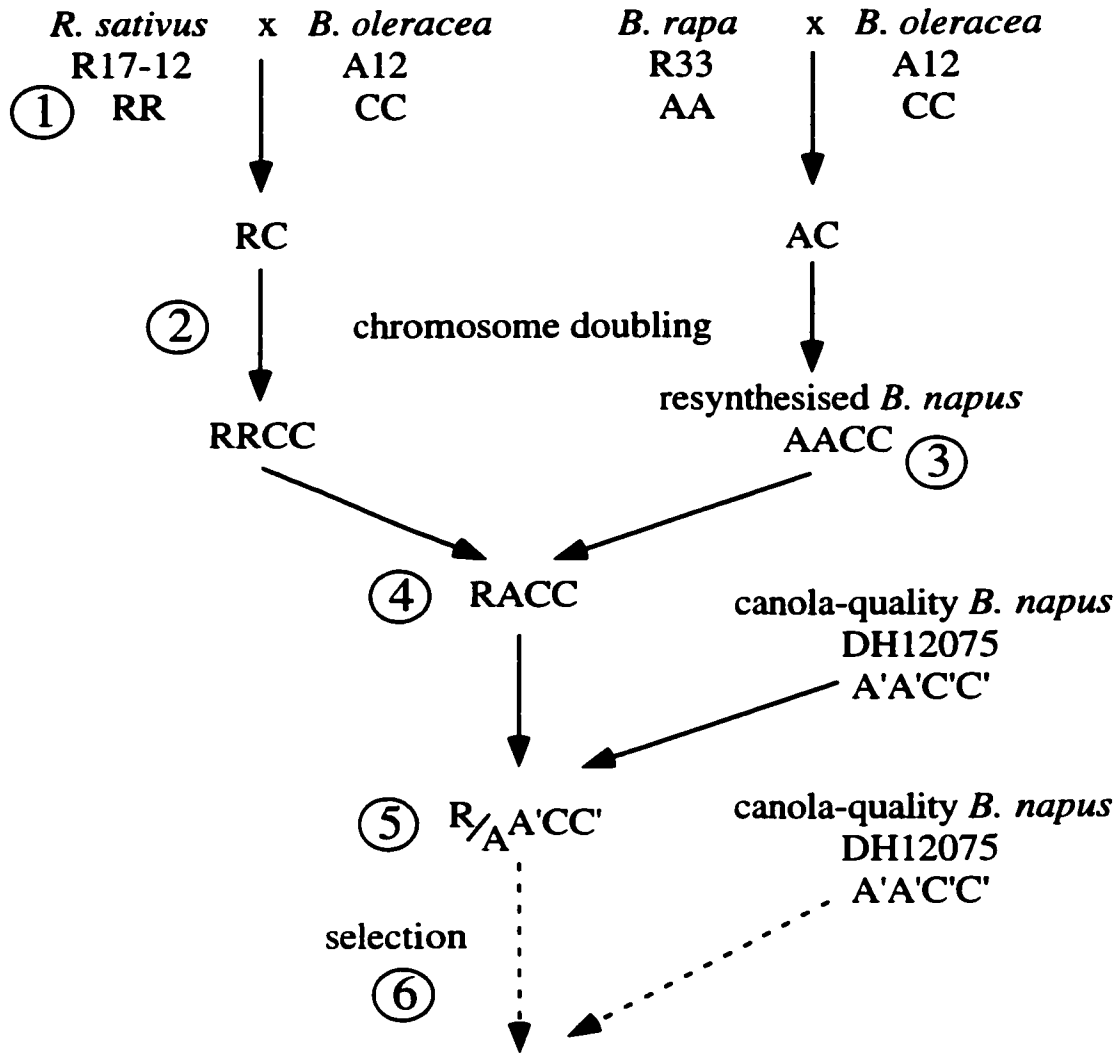


Figure 9.1. Crossing strategy used to develop plants where recombination between the *Raphanus* genome and the *Brassica* A genome would be promoted and selection for individuals carrying R/A hybrid chromosomes would be possible. Numbers refer to comments in section 9.1.1 of the text. R, haploid copy of *R. sativus* chromosomes; A, haploid copy of *Brassica* A genome chromosomes; C, haploid copy of *Brassica* C genome chromosomes, R/A, the reassorted and recombined meiotic products resulting from interaction between the R and A genomes.

is likely to promote the recovery of gametes carrying chromosomes that are the products of homoeologous recombination between R and A genome chromosomes.

5. The products of meiosis in the RACC individuals can be assayed in $^{R}/_{A}$ACC individuals allowing for the detection of hybrid $^{R}/_{A}$ chromosomes derived from intergenomic recombination.
6. Selection of hybrid chromosomes with *Raphanus* segments carrying restorer alleles and overall *B. rapa* chromosome structure derived from homoeologous recombination will allow for the selection of the most appropriate individuals for further backcrossing to *B. napus*.

9.1.2 Development of RRCC and AACC individuals

Population R9 (Figure 4.2) was chosen as the source of the *R. sativus* material for interspecies gene transfer as it appeared to be homozygous for restoring alleles at a full complement of restorer loci (Chapter 4). Plant R9-14 was selfed to produce the male fertile plant R17-12. R17-12 was used as the female in a cross with plants from the doubled haploid *B. oleracea* line, A12 (Table 3.1). Mature seeds were harvested from the R17-12 plant. Two of the resulting amphihaploid RC seeds were germinated and chromosome doubled to produce amphidiploid RRCC plants. Plants derived from the RR by CC crosses had white flowers like the paternal parent A12 and the pod morphology was intermediate between the two parents suggesting that they were indeed hybrids. The colchicine treated hybrids (RRCC) did not produce pollen but were considered doubled due to their larger flower, bud and stem morphology than those of the RC amphihaploids, combined with flow cytometry results which indicated that twice as much DNA was present in cells of the colchicine treated plantlets as in cells of untreated plantlets.

A resynthesised *B. napus* plant, AC2-1, was developed by crossing a plant of the *B. rapa* line R33 (Table 3.1) with pollen from an A12 plant (Table 3.1). These AA by CC crosses were pod cultured six days after pollination, followed by ovule culture ten days later and germinating embryos were rescued as they appeared. One plantlet was chromosome doubled to generate an AACC plantlet which was used in all subsequent

intergeneric crosses. This plant had cream-coloured flowers, intermediate between the yellow and white flower colours of the two parents, suggesting that it was a hybrid. The colchicine treated AACC hybrid was larger and produced considerably more pollen than the untreated, amphihaploid AC plant, indicating that its chromosomes had probably doubled. Flow cytometry results confirmed the dihaploid state of the colchicine treated plantlets as their cells had twice as much DNA as cells of the untreated plantlets.

Cuttings of both the AACC and RRCC plants were made continuously to ensure flowers were available when required for further crosses.

9.1.3 Development of RACC individuals

Since the RRCC hybrids did not produce pollen, the male fertile AACC individual, AC2-1, was used as the pollen donor in the second interspecific cross to produce RACC individuals. Approximately 4.7 ovules were produced per pollination. Ovules from these crosses were rescued and cultured two weeks after pollination and germinating embryos were rescued as they appeared. Of the 733 ovules that were cultured, 43 produced embryos that were subsequently rescued. The first 11 embryos to develop into plantlets were used for further crossing.

9.1.4 RFLP and microsatellite tests for genome composition

Eleven RACC individuals were screened with RFLP probes to confirm their hybrid status. Southern hybridisation filters carrying Eco RI-digested DNA from the original RR (R17-12), AA (R33) and CC (A12) parents and all putative RRCC, AACC and RACC hybrids were produced. Filters were probed with three randomly chosen *Brassica* RFLP probes (pR29, pW233 and pN23) and all RRCC, AACC and RACC individuals were confirmed to contain DNA of the expected A, C and R parental genomes (Fig. 9.2).

The process of testing for hybrid status using RFLP technology is time-consuming and requires large-scale DNA extractions to produce sufficient DNA for a hybridization filter. The availability of a PCR-based test to confirm the genetic composition of the intergeneric hybrids would speed up the testing process, allowing for earlier identification of the desired genotypes. *Brassica* microsatellite markers were

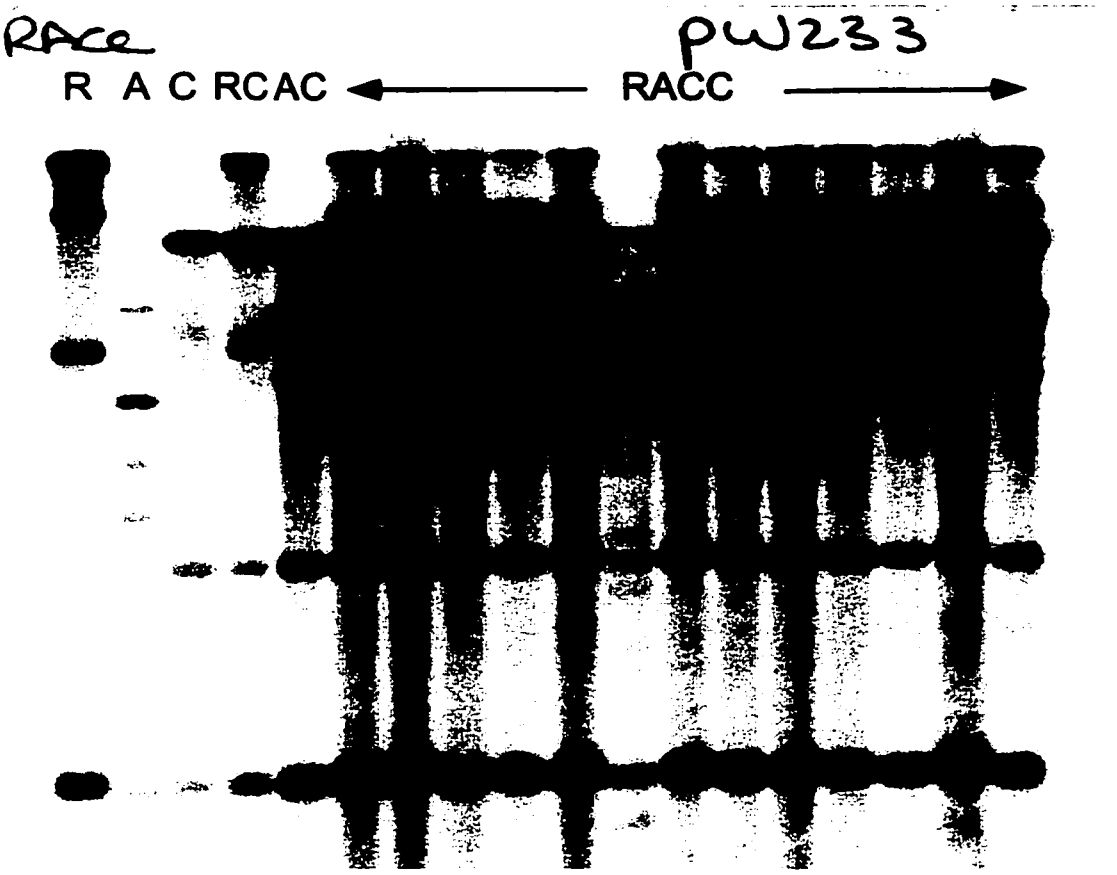


Figure 9.2. Autoradiograph of RFLP alleles from *R. sativus* (R: lane 1), *B. rapa* (A: lane 2), *B. oleracea* (C: lane 3), *R. sativus* x *B. oleracea* (RC: lane 4), *B. rapa* x *B. oleracea* (AC: lane 5), and 13 progeny of the RRCC x AACC cross (RACC: lanes 6-18) detected by probe pW233. All lanes contain genomic DNA digested with *Eco RI.* DNA in lanes 2 and 11 was partially degraded.

tested for efficacy in hybrid analysis in the progeny of intergeneric crosses. The first step was to identify *Brassica* microsatellite primer combinations that also detected microsatellites in *Raphanus*. DNA from nine individuals of the *Raphanus* BC_1 mapping population (Chapter 5), the parents of the mapping population and *B. napus* line N-o-9 (Table 3.1) were screened with a sample of 24 *Brassica* microsatellite primers, selected for their ability to amplify microsatellites in both A and C genome DNA (A. Sharpe, pers. comm.). Eleven of the 24 primer pairs (46%) successfully amplified the *Raphanus* DNA. Eight of the primer pairs that amplified *Raphanus* DNA were used to amplify DNA of the 11 RACC individuals along with the diploid parents. *Brassica* microsatellite primer pair snrf22 detected polymorphisms between the three parental diploid genomes making up the RACC individuals confirming that all RACC individuals tested carried DNA from all three parental genomes (Fig. 9.3). The other seven primer pairs did not detect informative polymorphisms between all three parental lines.

9.1.5 Backcrossing to B. napus

None of the RACC individuals produced pollen necessitating their use solely as females in crosses with *B. napus* line DH12075. Recently opened flowers of RACC plants were mass pollinated with DH12075 three times per week in an isolated growth cabinet to prevent contamination with foreign pollen. Pods that contained enlarged ovules were harvested approximately two weeks after pollination and the ovules were cultured. Cuttings of the RACC plants were made continuously and seeds of DH12075 were planted bi-weekly to ensure flowers of both parents were available when required for crossing. Very few pods produced enlarged ovules and very few of these ovules contained viable embryos. After seven months of 500-1000 pollinations per week followed by the culturing of 30-70 ovules per week, only 30 embryos had been rescued. These embryos are being encouraged to develop into plantlets which will be examined for recombination between the R and A genomes in the future.

9.1.6 Development of CCRR individuals

There was a concern that the sterility of the RRCC and RACC individuals was

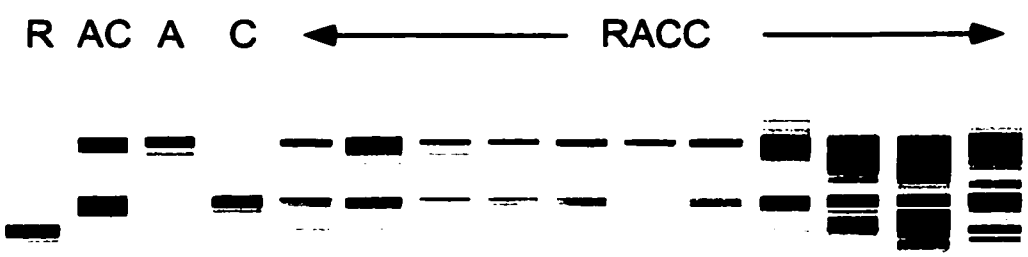


Figure 9.3. Gel image of amplified fragments of DNA from *R. sativus* (R: lane 1), *B. rapa* x *B. oleracea* (AC: lane 2), *B. rapa* (A: lane 3), *B. oleracea* (C: lane 4), and 11 progeny of the RRCC x AACC cross (RACC: lanes 5-15) produced using *Brassica* microsatellite primer pair snrf22.

not simply due to a genetic imbalance between the parental genomes but also due to the inability of R17-12 (*R. sativus*) to donate genetic material that will restore *B. napus* to fertility in the presence of Ogura CMS cytoplasm. As a result, new crosses were initiated with A12 (*B. oleracea*) as the female and R17-21(*R. sativus*, a sib of R17-12), as the pollen donor, in order to develop hybrid plants with *B. oleracea* cytoplasm rather than Ogura cytoplasm. This hybrid combination required ovule and embryo rescue and two plantlets were generated. These plantlets were chromosome doubled and the anthers of the resulting CCRR hybrids shed viable pollen. The two CCRR plants were crossed with pollen from the resynthesised *B. napus* plant AC2-1. A total of 128 ovules from this cross were cultured and three germinating embryos have been rescued. The resulting plantlets will be assayed with microsatellite markers to verify that all three parental genomes are present. The anticipated CCRA plants will be crossed to *B. napus* line DH12075 in the near future.

9.2 Discussion

The crossing strategy outlined above (Fig. 9.1) has resulted in the development of plants containing chromosomes from the A, C and R genomes. At meiosis in the RACC individuals, the diploid set of C genome chromosomes should preferentially pair with their homologues leaving the haploid sets of A and R genome chromosomes to pair with their closest homoeologues. It is expected that the resulting gametes would have a full haploid set of C genome chromosomes and an assortment of complete A genome chromosomes, complete R genome chromosomes and hybrid $^{R}/_{A}$ chromosomes resulting from recombination between the A and R genome chromosomes. Kelly (1996) found that *Brassica* gametes carrying a complete haploid complement of chromosomes from at least one genome, can tolerate a wide range of additional chromosomes from an additional genome, without compromising the viability of the gametes. This would suggest that gametes from RACC plants should have a greater chance of survival than gametes from a RAC plant such as would occur from a cross between *R. sativus* and *B. napus* without the *Raphanobrassica* (RRCC) intermediate. Recent breeding efforts aimed at introgressing traits such as shattering resistance (Agnihotri *et al.* 1990) and resistance to beet cyst nematode (Lelivelt *et al.* 1993) from *Raphanus* to *B. napus* have

relied on crosses with RRCC or RRAA intermediates since crosses between *Raphanus* and *B. napus* were not a viable source of intergeneric progeny.

Progeny have now been developed from the RACC by AACC cross and these plants are expected to contain the products of recombination between chromosomes of the A and R genomes. These plants will be analysed with RFLP markers selected on the basis of genome coverage using existing maps of the R and A genomes (R genome: Chapters 5 and 7; A genome: Parkin *et al.* 1995; Sharpe *et al.* 1995; Salava *et al.* 2001). This marker analysis will assess the pattern and extent of recombination that has occurred at meiosis in the RACC parent. Markers for the restorers identified in Chapters 6 and 7 will be used in concert with the genome maps to select for individuals carrying small segments of *Raphanus* chromosomes containing the restorers but little other *R. sativus* genetic material.

With the exception of the initial *R. sativus* by *B. oleracea* cross, all intergeneric crosses required embryo rescue. Fertilized ovules left on the RRCC and RACC plants for longer than approximately two weeks aborted, possibly due to incompatible interactions between the maternal tissue and the hybrid embryo. Obtaining progeny from the RACC plants proved the most difficult. Very few enlarged ovules were produced and very few of these yielded viable embryos. The reason for the low yield of embryos is not clear but it could have been due to a genetic imbalance between the parental genomes and/or due to meiotic irregularities caused by homoeologous pairing and recombination. The RACC by AACC cross was much less successful than a cross conducted by Agnihotri *et al.* (1990) where *B. napus* was the female and RACC was the male. This would have led to a situation where the female gametes were normal and only the male gametes would have had meiotic irregularities. The sheer number of pollen grains would have made up for the reduced viability of the pollen, and any ovule that did become fertilized would likely have a good chance of survival. There is also a small possibility that an increased level of homoeologous pairing might have occurred as a result of using resynthesised *B. napus* rather than natural *B. napus* as Agnihotri *et al.* (1990) did, thereby leading to increased meiotic irregularities and fewer viable gametes.

The male sterility observed in the intergeneric progeny could have stemmed from the production of genetically imbalanced, and therefore non-viable, gametes. It could

also have stemmed from the presence of Ogura CMS cytoplasm. Roussell and Dosba (1985) made crosses between a *B. napus* (AACC) line with Ogura CMS cytoplasm and a *Raphanobrassica* (RRCC) line carrying Ogura CMS restorers and found that the pollen viability in the RACC progeny ranged from 3 to 82%. They concluded that the observed variability was probably due to differences in expression of the restorer genes as well as to genetic imbalance. It is possible that the *R. sativus* parent R17-12 had all the necessary restorers for Ogura CMS plants with the R nuclear genome alone but these restorers were not sufficient when combined with the C and A nuclear genomes.

A second approach to the development of plants with the RACC genomic make-up was pursued due to the male sterility problems in the intergenomic individuals with Ogura cytoplasm. By developing hybrids with *B. oleracea* cytoplasm, any ill effects caused by the presence of the Ogura CMS cytoplasm would be avoided. Not having the sterility-inducing cytoplasm, however, means that progeny carrying a full complement of Ogura CMS restorers cannot be selected based on phenotype. As genetic markers linked to the restorer loci segregating in *Raphanus* have been developed (Chapters 6 and 7), it should be possible to use genetic markers to select progeny with hybrid chromosomes carrying the regions of the R genome containing these restorers.

When the original crosses were initiated, it was thought that there were only one or two restorer genes needed to restore fertility to plants with Ogura CMS cytoplasm. With only one or two restorers, it would require the introgression of only one, or two segments of the R genome into the A genome. In light of the fact that it might require more than two separate restorers, it is probably more prudent to work in non-CMS cytoplasm and select for the introgression of individual segments containing single restorers. Plants carrying small, homozygous introgressions of segments carrying individual restorers could then be intercrossed to pyramid the restorers in a *B. napus* background. Test crosses of hybrid plants carrying specific restorers, or combinations of restorers, to Ogura *B. napus* cybrids will also allow for investigations into the possibility that only one restorer is necessary for full restoration of fertility in certain cybrids as noted by Koizuka *et al.* (2000).

Although the *Brassica* microsatellites primers did not work as well as the

Brassica RFLP probes in *Raphanus*, it was possible to select one pair that could be used for the purpose of testing for the presence of parental A, C and R genomes. Unlike RFLP clones which are large (1-2kb), microsatellite primers are quite small (~20bp) and therefore less tolerant of mutations in the DNA segments to which they bind. As a result, the RFLP probes bind to homologous segments of DNA in related species more readily than microsatellite primers. Apparently there is still sufficient homology between the primer binding sites surrounding some microsatellites in both *Brassica* and *Raphanus* to allow some of the *Brassica* microsatellites to be used in *Raphanus*. Having PCR-based markers such as these microsatellites will allow for the early testing of plantlets and the identification of hybrids while they are still in culture and before a considerable amount of time and effort is expended on non-hybrid progeny.

Chapter 10. Comparative mapping between the *Raphanus* and *Brassica* A genomes.

The *Raphanus* consensus map generated from the aligned genetic maps developed from the F_2, BC_1 and R8 mapping populations was compared to a *Brassica* A genome consensus map generated from aligned maps developed by other researchers. This comparison identified regions of collinearity and suggested the potential for intergenomic homoeologous recombination.

10.1 Results

10.1.1 Intragenomic duplication in Raphanus

The *Raphanus* map used for comparative mapping was developed by combining the BC_1, F_2 and R8 *Raphanus* maps described in Chapters 5 and 7 (Fig. 7.1). The aligned maps presented no inconsistencies but the precise order of some of the loci could not be determined with confidence because adjacent pairs of loci were not mapped in the same population. Such loci were placed in the most likely position on the map based on relative map distances.

The 155 informative *Brassica* RFLP probes used to develop the combined *Raphanus* map each detected between one and six polymorphic loci (Table 10.1) with an average of 1.8 informative loci per probe. The individual *Brassica* probes and the loci identified in at least one of the three populations are listed in Appendix 1.1. Many probes also detected loci that were monomorphic and were therefore impossible to map. This caused an underestimation in the actual level of locus duplication in the *Raphanus* genome.

Table 10.1. The number of *Brassica* RFLP clones that detected one, two, three, four, five or six polymorphic loci within the *Raphanus* genome, based on data from three mapping populations (BC_1, F_2 and R8).

Number of polymorphic loci	Number of clones
1	69
2	56
3	28
4	1
5	1
6	1

An estimate of the percentage of monomorphic loci in these *Raphanus* populations was calculated based on the number of polymorphic loci detected in both the F_2 and R8 mapping populations versus the number of extra polymorphic loci detected in one population but monomorphic in the other. The ratio of the number of loci polymorphic in both populations to the number of loci polymorphic in only the R8 population would be equivalent to the ratio of the number of loci polymorphic in only the F_2 population to the number of loci monomorphic in both populations if the population of loci is uniform with all loci having an equal chance of being polymorphic in any particular population. There were 98 probes that were used in both the F_2 and R8 mapping populations. These 98 probes detected 71 loci that were polymorphic in both populations, 80 loci that were polymorphic in the F_2 population but monomorphic in the R8 population and 26 loci that were polymorphic in the R8 population but monomorphic in the F_2 population (Table 10.2; Fig.7.1). Therefore, the estimated number of loci monomorphic in both populations was $X = 80*26/71 = 29$. Between the two mapping populations, the 98 probes detected a total of 177 polymorphic loci plus approximately 29 monomorphic loci for an average of 2.1 loci/probe. These 29 monomorphic loci represent 14% of all loci and are important to consider when characterising duplication across the genome.

Table 10.2. Numbers of loci polymorphic in one, the other or both populations identified by a common set of 98 RFLP probes in the F_2 and R8 populations and used to estimate the number of loci homologous to the probe but monomorphic in both populations (X).

numbers of loci:	polymorphic in R8	monomorphic in R8
polymorphic in F_2	71	80
monomorphic in F_2	26	X

The loci in the *Raphanus* genome that are known to be duplicated, based on the combined map (derived from the BC_1, F_2 and R8 populations), are displayed in Table 10.3. The duplicate loci were not scattered randomly across the genome but rather they were found in syntenous clusters distributed across the genome. The size of the observed duplicated segments ranged from one locus to eleven adjacent loci.

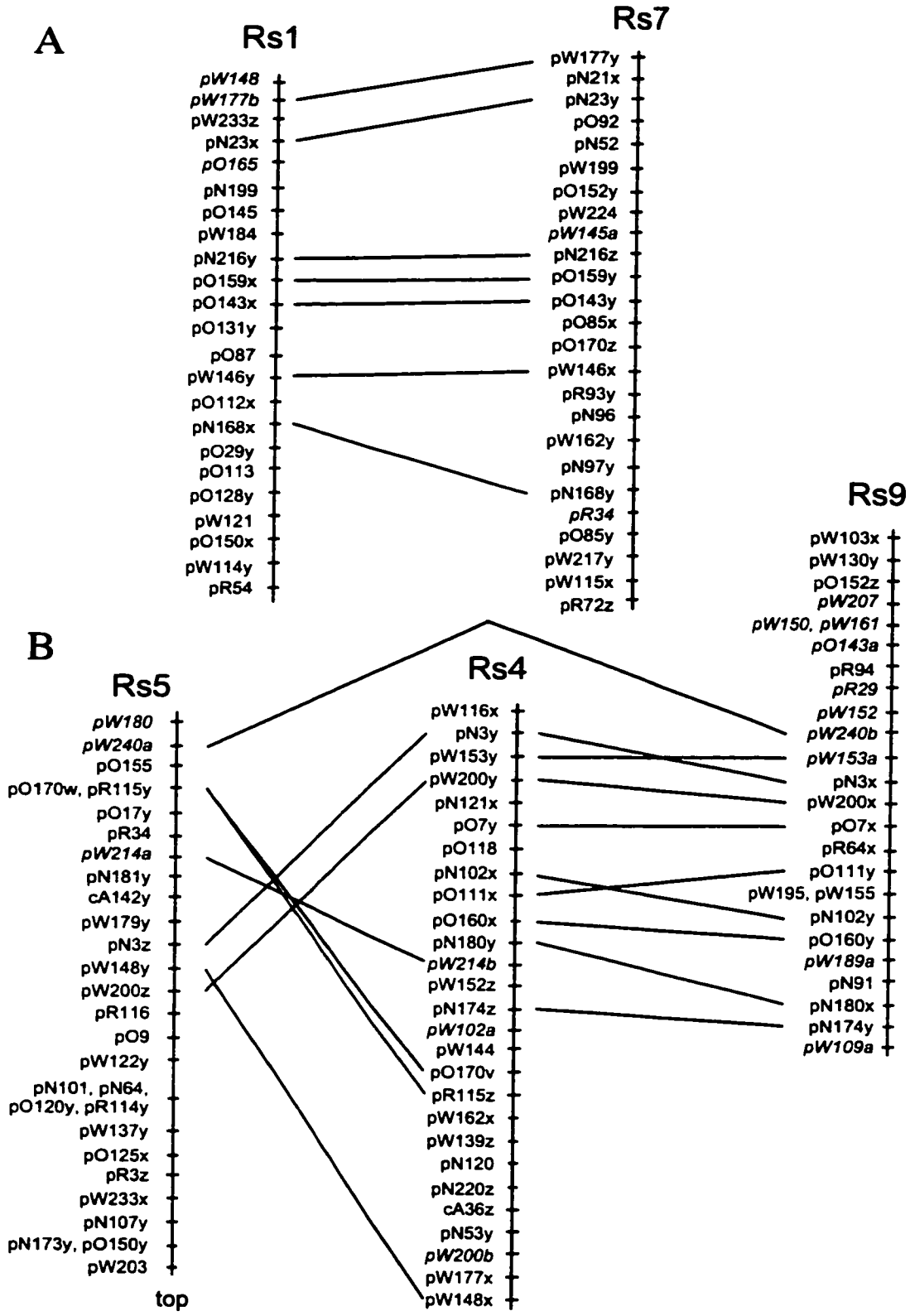
The majority of duplicated regions fell on separate linkage groups. The internal duplications on Rs3, Rs6, and Rs8 each consisted of two duplicated loci while that on Rs7 was a single duplicated locus. Although no whole linkage groups were found to be duplicated, there were a few examples of duplicated loci spanning a large portion of a linkage group. Linkage groups Rs1 and Rs7 are clearly collinear along most of their length, with conserved marker order along all but the bottom ~10cM of each (Fig. 10.1A; Fig. 7.1). Linkage groups Rs4 and Rs9 show conserved marker order along approximately half their lengths with only two sets of two inverted loci (Fig. 10.1B). There was an inversion evident at one end of the region that was collinear between Rs2 and Rs3 and another one between Rs3 and Rs5 (Fig. 10.1C). Other regions of duplication were more scattered as can be seen for linkage groups Rs4 and Rs5 (Fig.10.1B) and Rs3 and Rs5 (Fig. 10.1C).

Table 10.3. A summary of duplicated RFLP loci detected within the *Raphanus* genome. Loci duplicated within a linkage group are in bold.

	Rs2	Rs3	Rs4	Rs5	Rs6	Rs7	Rs8	Rs9
Rs1	pO29	pW233 pO112 pO29	pW148	pO150 pW233 pW148	pO131 pN199 pN216	pW177 pN23 pN216 pO159 pO143 pW146 pN168	pO128 pW114	
Rs2		pO170 cA138 pN97 pO29 pN107 pW105 cA142 pW179 cA36 pW225 pO43	pW116 pN121 pO170 cA36	pN107 pW179 cA142 pN181 pO17 pO170		pO170 pN97	pW116 pO3	pR29 pR64 pW189 pW109 pW207 pW150
Rs3		**pR3** **pO59**	pN174 pO170 pR115 pW139 pN220 cA36 pN53	pN107 pW233 pR3 pW137 pW179 cA142 pR115 pO170	pW139 pN66 pN59 pO59 pN151 pR72v pW112 pR72f	pO170 pN97 pR72	pW205 pR72 pR72	pW103 pN174
Rs4				pW200 pW148 pN3 pR115 pO170 pW214	pN220d pW152 pW139	pW177 pO170 pW162	pW116 pW162 pW102	pW153 pN3 pW200 pO7 pO111 pN102 pO160 pN180 pN174
Rs 5					pO125 pO120 pN173 pR114	pO170	pR114	pW240b pN3 pW200

	Rs2	Rs3	Rs4	Rs5	Rs6	Rs7	Rs8	Rs9
Rs 6					**pW108** **pR72**	pN216 pW162 pR72	pW136 pW172 pO12 pW201 pN148 pW108 pW162 pO119 pR72 pW201 pR72	pW153
Rs7						**pO85**	pR93 pW162 pO152 pW217 pR72 pR72	pO152
Rs8							**pW142** **pW201**	pW130 pO152 pN102

Fig. 10.1 (p. 111-112). Examples of intragenomic duplication within the *Raphanus* genome. Loci mapped in the R8 population only are in italics. ➔indicates an inversion in locus order between groups.


A
Rs1
pW148
pW177b
pW233z
pN23x
pO165
pN199
pO145
pW184
pN216y
pO159x
pO143x
pO131y
pO87
pW146y
pO112x
pN168x
pO29y
pO113
pO128y
pW121
pO150x
pW114y
pR54
Rs7
pW177y
pN21x
pN23y
pO92
pN52
pW199
pO152y
pW224
pW145a
pN216z
pO159y
pO143y
pO85x
pO170z
pW146x
pR93y
pN96
pW162y
pN97y
pN168y
pR34
pO85y
pW217y
pW115x
pR72z
B
Rs5
pW180
pW240a
pO155
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pO17y
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pW122y
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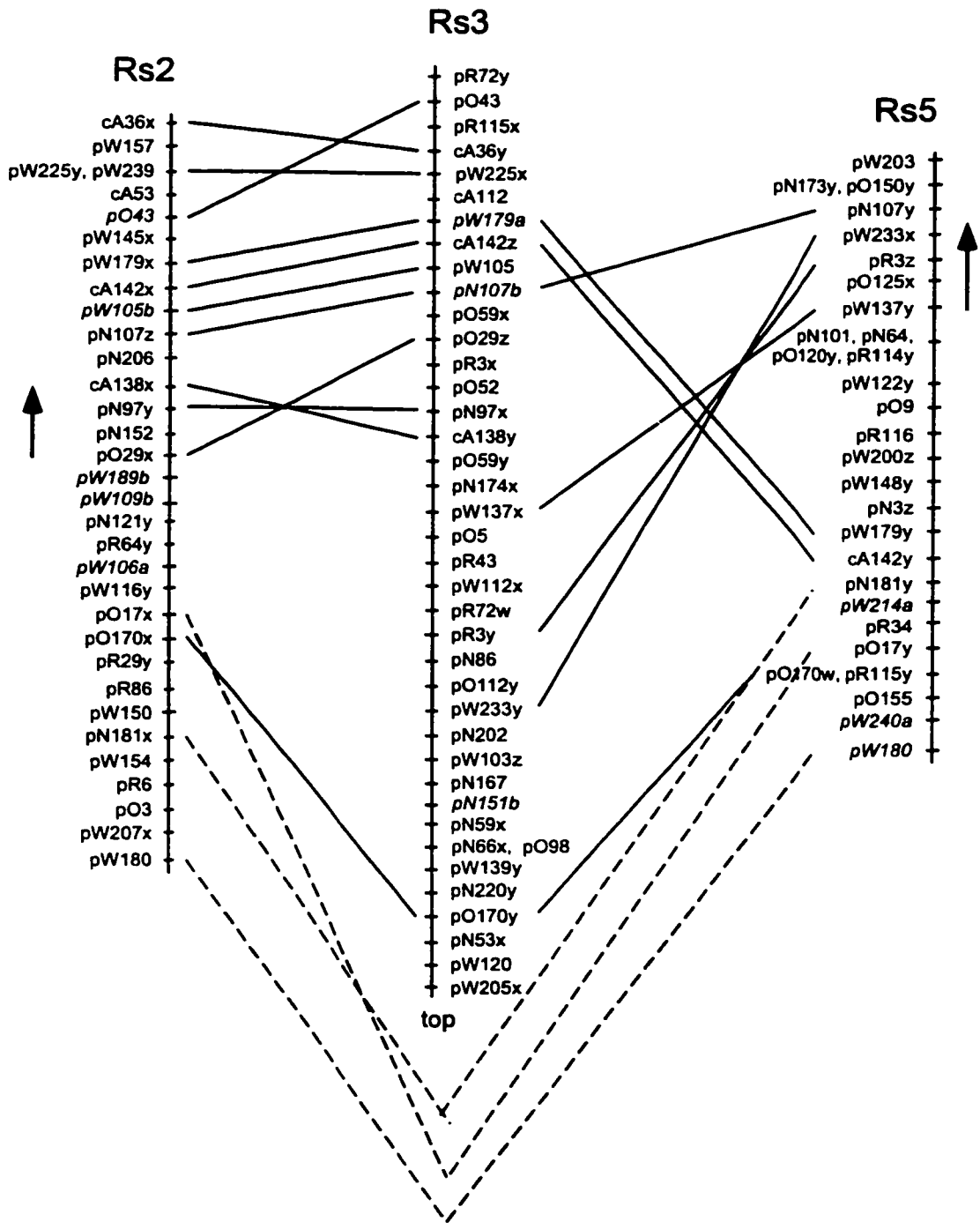
pW137y
pO125x
pR3z
pW233x
pN107y
pN173y, pO150y
pW203
top
Rs4
pW116x
pN3y
pW153y
pW200y
pN121x
pO7y
pO118
pN102x
pO111x
pO160x
pN180y
pW214b
pW152z
pN174z
pW102a
pW144
pO170v
pR115z
pW162x
pW139z
pN120
pN220z
cA36z
pN53y
pW200b
pW177x
pW148x
Rs9
pW103x
pW130y
pO152z
pW207
pW150, pW161
pO143a
pR94
pR29
pW152
pW240b
pW153a
pN3x
pW200x
pO7x
pR64x
pO111y
pW195, pW155
pN102y
pO160y
pW189a
pN91
pN180x
pN174y
pW109a

C


Rs2
cA36x
pW157
pW225y, pW239
cA53
pO43
pW145x
pW179x
cA142x
pW105b
pN107z
pN206
cA138x
pN97y
pN152
pO29x
pW189b
pW109b
pN121y
pR64y
pW106a
pW116y
pO17x
pO170x
pR29y
pR86
pW150
pN181x
pW154
pR6
pO3
pW207x
pW180
Rs3
pR72y
pO43
pR115x
cA36y
pW225x
cA112
pW179a
cA142z
pW105
pN107b
pO59x
pO29z
pR3x
pO52
pN97x
cA138y
pO59y
pN174x
pW137x
pO5
pR43
pW112x
pR72w
pR3y
pN86
pO112y
pW233y
pN202
pW103z
pN167
pN151b
pN59x
pN66x, pO98
pW139y
pN220y
pO170y
pN53x
pW120
pW205x
top
Rs5
pW203
pN173y, pO150y
pN107y
pW233x
pR3z
pO125x
pW137y
pN101, pN64,
pO120y, pR114y
pW122y
pO9
pR116
pW200z
pW148y
pN3z
pW179y
cA142y
pN181y
pW214a
pR34
pO17y
pO170w, pR115y
pO155
pW240a
pW180

There was evidence of genome triplication but it was often obscured by rearrangements in one of the sets of loci. For example, alleles of loci pW179, cA142 and pN107 are all found on Rs2, Rs3 and Rs5 (Fig. 10.1C). With the exception of pN107 on Rs5, these loci were in the same order in each linkage group. Locus pN107y on Rs5 appeared to map quite a distance from the other two loci in this group and was separated from the others by a rearrangement. This rearrangement also confounded evidence of triplication amongst Rs5, Rs4 and Rs9 (Fig. 10.1B).

10.1.2 Intergenomic comparisons

The *Brassica* A genome map used in this comparative mapping exercise was developed from several previously generated *B. napus* and *B. rapa* maps (Parkin *et al.* 1995; Sharpe *et al.* 1995; Salava *et al.* 2001). The *B. rapa* linkage groups R1 to R10 are equivalent to the *B. napus* linkage groups N1 to N10 (Parkin 1995). There were 132 informative *Brassica* RFLP clones used to generate both the consensus A genome map and the *Raphanus* map. Only loci identified by these shared 132 clones were used for comparative mapping.

Table 10.4 shows all possible regions of homoeology between the *Raphanus* genome and the *Brassica* A genome. The number of homoeologous loci per pair of linkage groups ranged from one to 16 and were found across the *B. rapa* genome. Like the *Raphanus* genome, the *Brassica* A genome has duplicated regions made up of one to 15 adjacent loci (Table 10.5). This resulted in each linkage group from *Raphanus* sharing homoeology with several *Brassica* A genome linkage groups. The level of collinearity varied considerably however, and it was possible to align many of the linkage groups based on long stretches of collinearity. Figure 10.2 displays the regions of substantial collinearity between the two genomes for each *Raphanus* linkage group.

Table 10.4. A summary of homoeology between *Raphanus* linkage groups (Rs1 - Rs9) and the linkage groups of the *Brassica* A genome (R1 - R10). Each row represents a *Raphanus* linkage group and each column an A genome linkage group.

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
Rs1	pN199 pO29 pN168		pW152 pW177 pO87 pN168 pO29		pW114	pO165 pN199 pN216 pO159 pO143 pO112		pR54 pN168 pO113 pO29 pO159 pN216 pN199 pO165 pW177	pO150 pW233 pW146 pN216 pO165 pN23	pN23 pN199 pW152
Rs2	cA36 pW157 pW239 pW225 cA53 pW145 cA142 pN206 pN107 pW105 pW179 pO17 cA138 pO29 pN152 pN97	pW109 pN121 pR64 pR29 pW150 pW154 pO3 pW207 pW180 pN181	pN121 pW189 pN97 pO29 cA142 pN107 pN181 pW105 pW179 pO43 pW225 cA53 pW145	pN97	pO17	pW145 pW225 pR6 pR43	pW150 pO3 pW154	pO29 pW225 cA36	pW180 pN181 pW145 cA142	pW189
Rs3	cA36 pW225 cA112 cA142 pO52 pN107 pW105 pW179 cA138 pO29 pN97 pW203	pR115 pW112 pR72	pN167 pO59 pN97 pO29 cA142 pO52 pN107 pW105 pW179 pO43 pW225	pW205 pN202 pN167 pN151 pN59 pO98 pW139 pN97	pN174 pW137 pN66 pN53	pW225 pW137 pR43 pR3 pN86 pO112	pN151 pN59	pO29 pW225 cA36	pW203 pW233 pR3 pO59 pW205 pR43 cA142	pN53 pR115
Rs4	cA36 pN180	pN121 pN102 pN180 pW102 pR115	pN3 pW153 pN121 pW189 pO7 pN102 pO160 pW152 pN120 pW177 pW144	pW139	pN174 pN120 pN53	pN180 pW102	pW162	pW177 cA36		pN53 pW152 pW102 pR115 pN180 pO160 pN102 pO118 pO7 pW200 pN3

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
Rs5	cA142 pN107 pW179 pO17 pN173 pW203 pR114	pR115 pW180 pN181 pO120 pW167	pN3 pW214 pO155 pN107 pN181 pW179 cA142		pO17 pW137	pO9 pN101 pO120 pW167 pW137 pR3			pN184 pW167 pN101 pW122 pW203 pO150 pN173 pW180 pW233 pN184 pN181 pR3 cA142	pR34 pR115 pO155 pW240 pW200 pN3
Rs6	pN199 pN173 cA119 pW108 pN148 pO12 pW136 pR114 pW172 pN13 pR85	pW112 pW218 pW108 pR72 pO120 pO119 pW191	pW153 pW152 pN22 pO12 pN148 pO59	pN44 pN151 pN59 pW139 pN13	pN13 pN194 pN95 pN66 pN148 pW172	pN199 pN216 pO119 pO120 pW218	pW194 pW162 pW108 pW191 pN44 pN151 pN59	pN216 pN199	pN173 pN184 pW191 pO59 pN216	pO119 pN199 pW152
Rs7	pW145 pN168	pO85 pR72	pW177 pW181 pN168 pW145		pW217 pW115 pN2	pW115 pW217 pN52 pO152 pW199 pW224 pN216 pO159 pW145 pO143	pW162	pN168 pN96 pO159 pN216 pW177	pN96 pW146 pN52 pN216 pW145 pO152 pN23	pN21 pN23 pO92
Rs8	pW108 pN148 pO12 pW136 pR114 pW142 pW172	pN102 pW102 pO3 pW108 pR72 pO119	pN102 pW102 pO79 pO12 pW142 pN148 pW181 pW188 pN154	pW205 pW130	pW217 pO46 pW114 pN148 pO123 pW172 pN2	pW217 pO46 pO152 pO119 pW102 pR6	pO79 pW162 pW108 pW130 pO3	pN34	pW130 pW205 pN34 pO152	pO119 pW102 pN102
Rs9	pN180	pW109 pR64 pN102 pN180 pR29 pW150 pW161 pW207	pN3 pW153 pW189 pO7 pN102 pO160 pW152 pN180	pW130	pN174	pO152 pO143 pN180	pW130 pR94 pW150		pW130 pO152	pW152 pW240 pN180 pN91 pO160 pW189 pN102 pO7 pW200 pN3

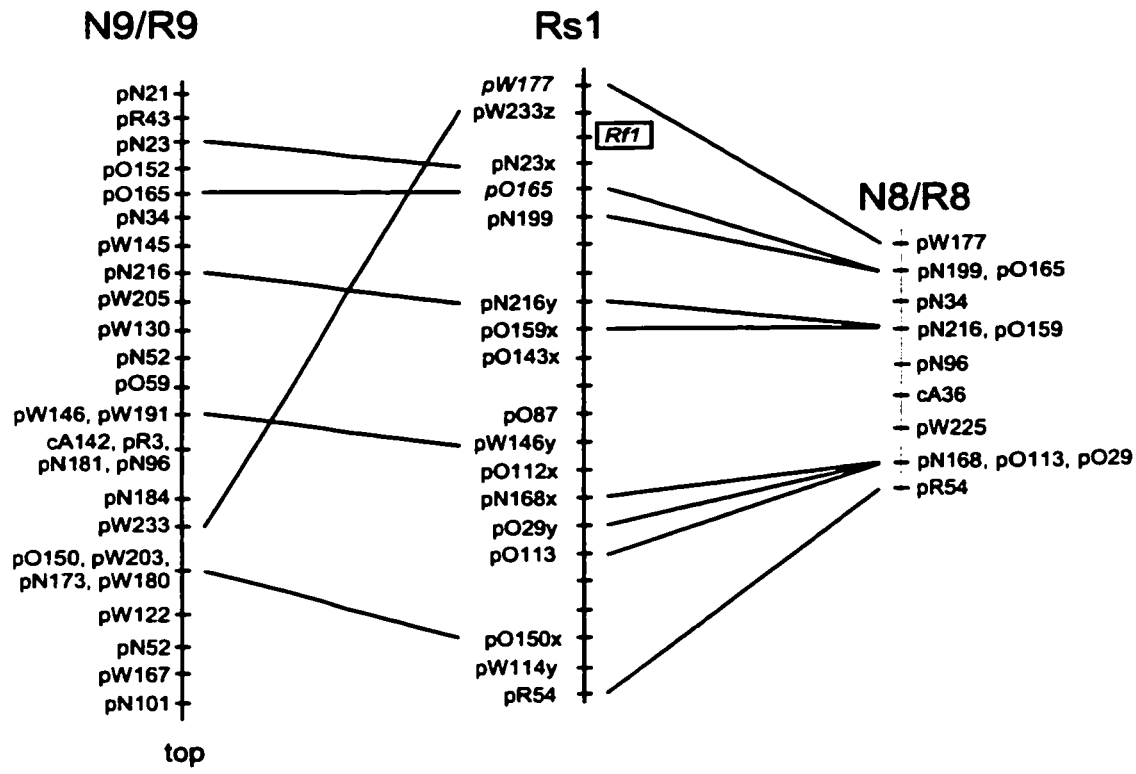
Table 10.5. The distribution of duplicated regions of the *Brassica* A genome across linkage groups.

	R2	R3	R4	R5	R6	R7	R8	R9	R10
R1	2[a]	15	2	3	5	1	5	4	2
R2		4	0	0	6	5	0	2	5
R3		**1**[b]	2	2	4	1	4	5	9
R4				1	0	2	0	2	0
R5					4	0	0	0	1
R6					**3**	0	4	7	4
R7							0	2	0
R8								4	1
R9								**1**	1

[a] The number of duplicated loci detected in each pairwise combination of A genome linkage groups. All loci were detected by the set of 132 *Brassica* RFLP clones used for comparative mapping with the *Raphanus* genome. [b] Duplications within a linkage group are indicated in bold.

Fig. 10.2 (p.118 - 126). The regions of substantial homology between the linkage groups of the *Raphanus* genome (Rs1 - Rs9) and the *Brassica* A genome (R1 - R10). Lines have been drawn between homoeologous loci. ➜ indicates an inversion in locus order between groups. Missing loci in the *Raphanus* map, indicated by a gap in locus order, are those that were derived from RFLP probes not mapped in the A genome map and therefore not relevant to this comparative mapping exercise. Circles on the A genome chromosomes represent centromeres as mapped by Kelly (1996). ⁓ indicates a break in an A-genome chromosome.

A


N9/R9
Rs1
N8/R8
pN21
pR43
pN23
pO152
pO165
pN34
pW145
pN216
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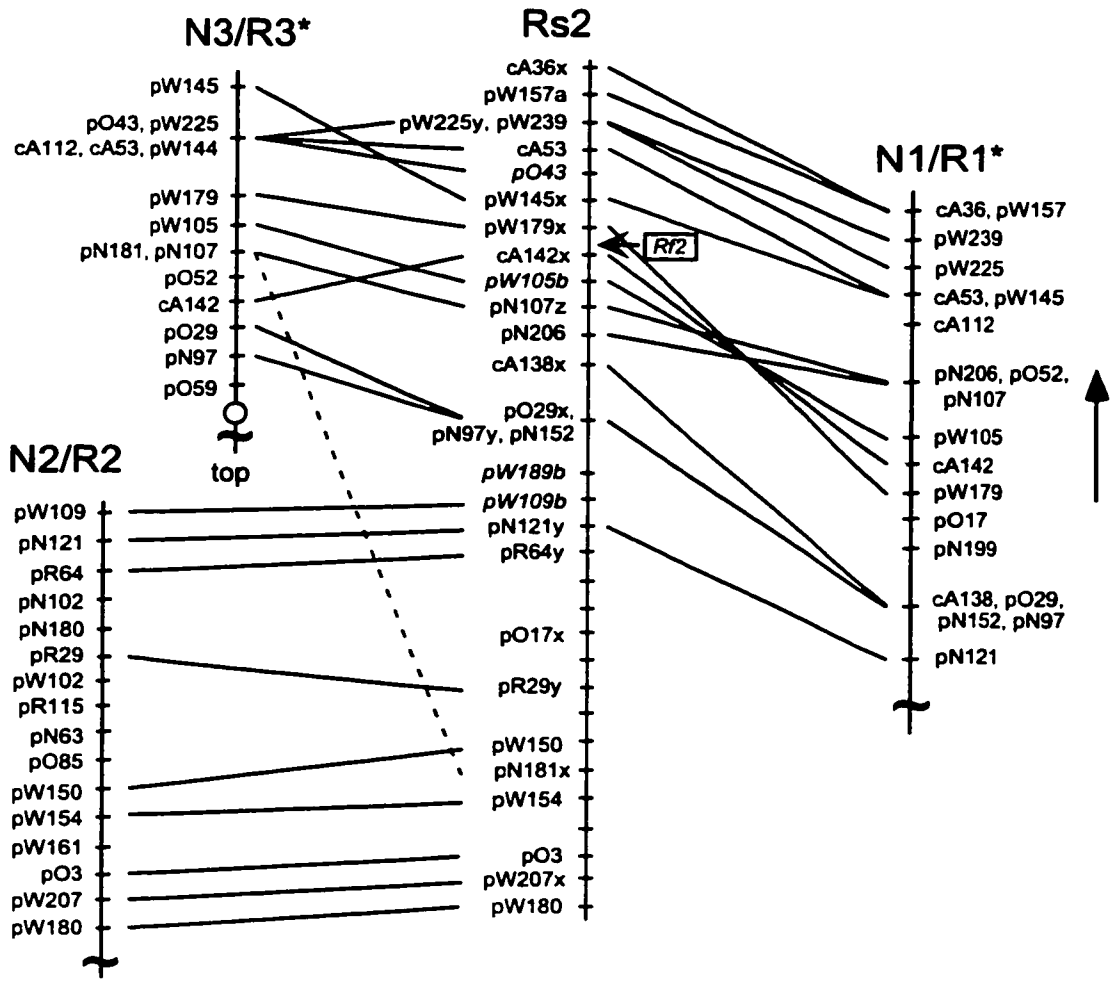
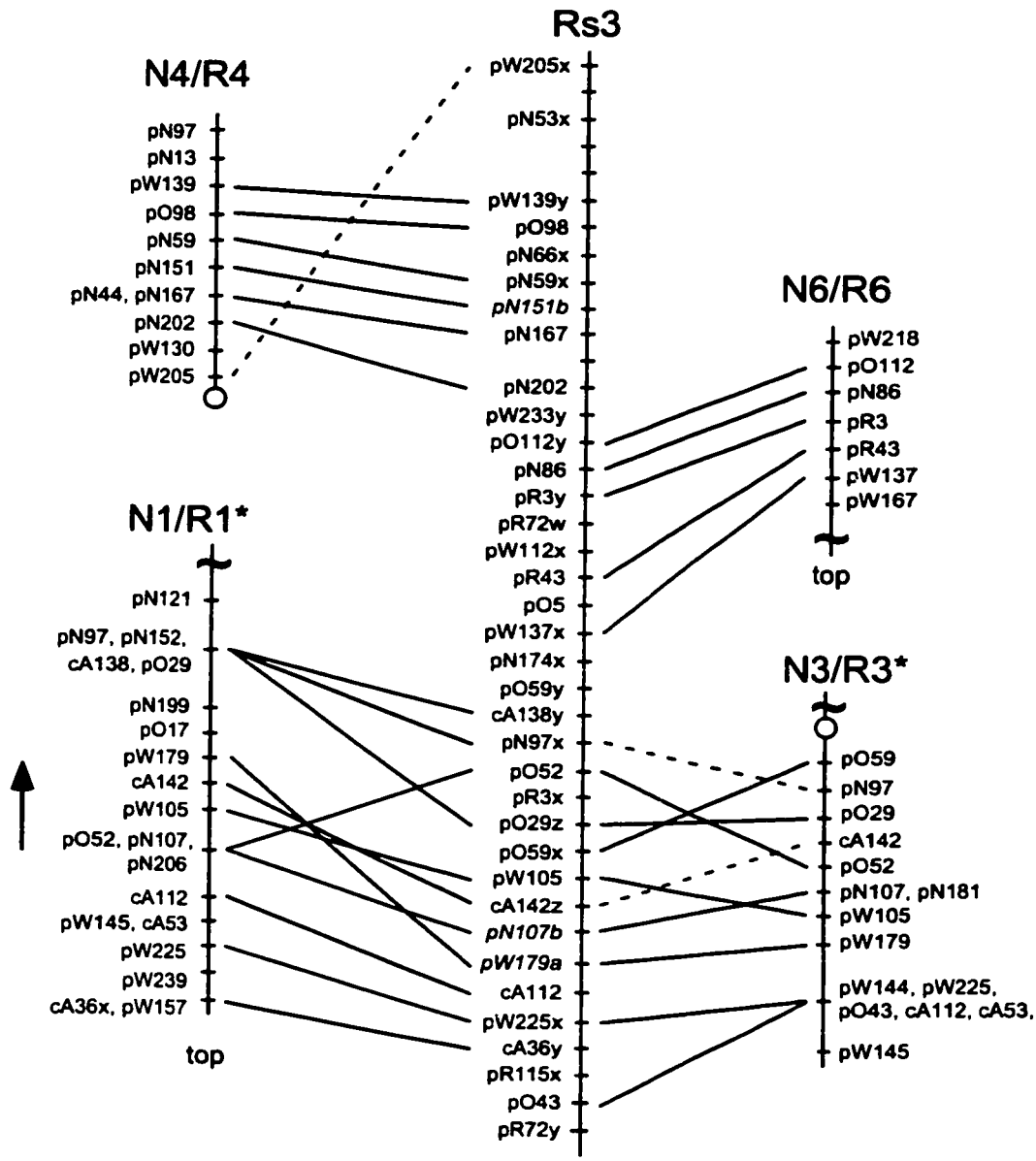
pW130
pN52
pO59
pW146, pW191
cA142, pR3, pN181, pN96
pN184
pW233
pO150, pW203, pN173, pW180
pW122
pN52
pW167
pN101
top
pW177
pW233z
Rf1
pN23x
pO165
pN199
pN216y
pO159x
pO143x
pO87
pW146y
pO112x
pN168x
pO29y
pO113
pO150x
pW114y
pR54
pW177
pN199, pO165
pN34
pN216, pO159
pN96
cA36
pW225
pN168, pO113, pO29
pR54

B



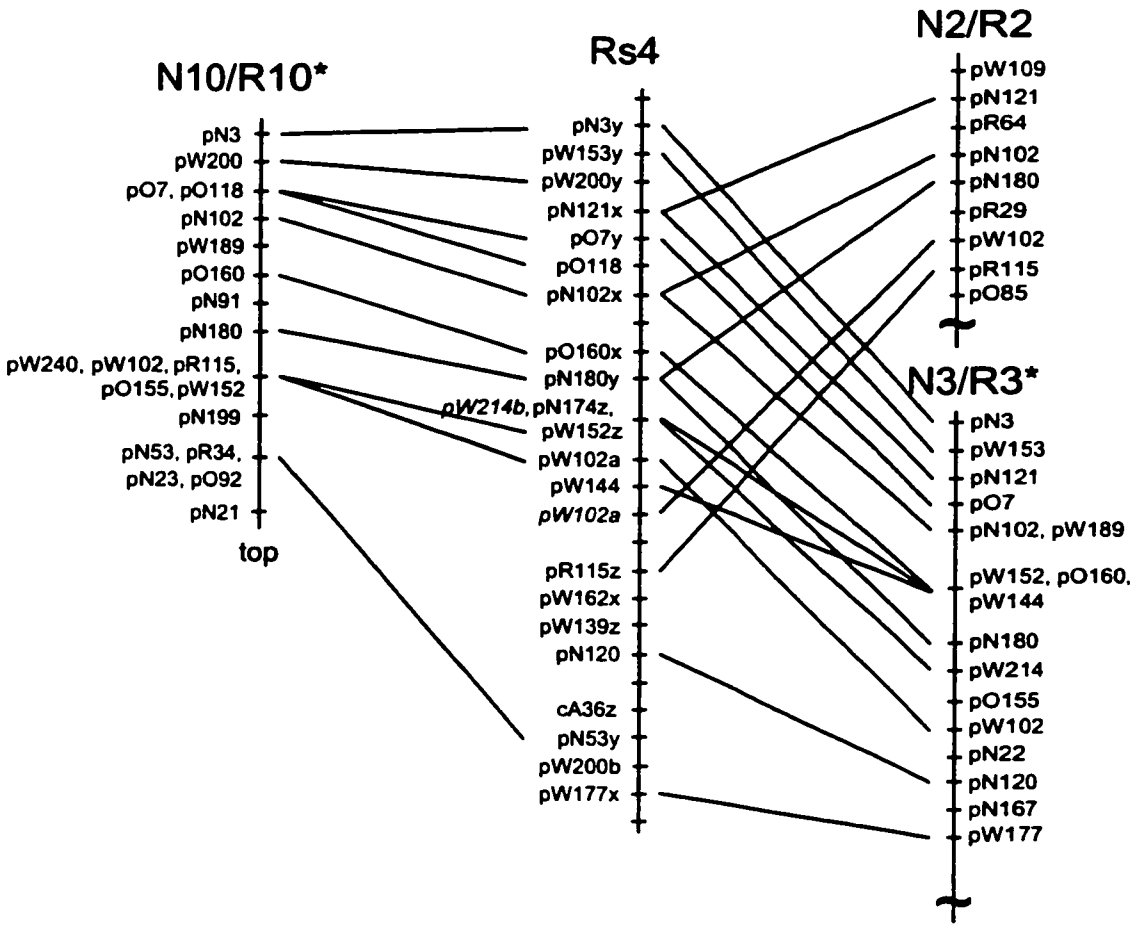
* large segments of N1/R1 and N3/R3 are homoeologous (Parkin 1995)

C



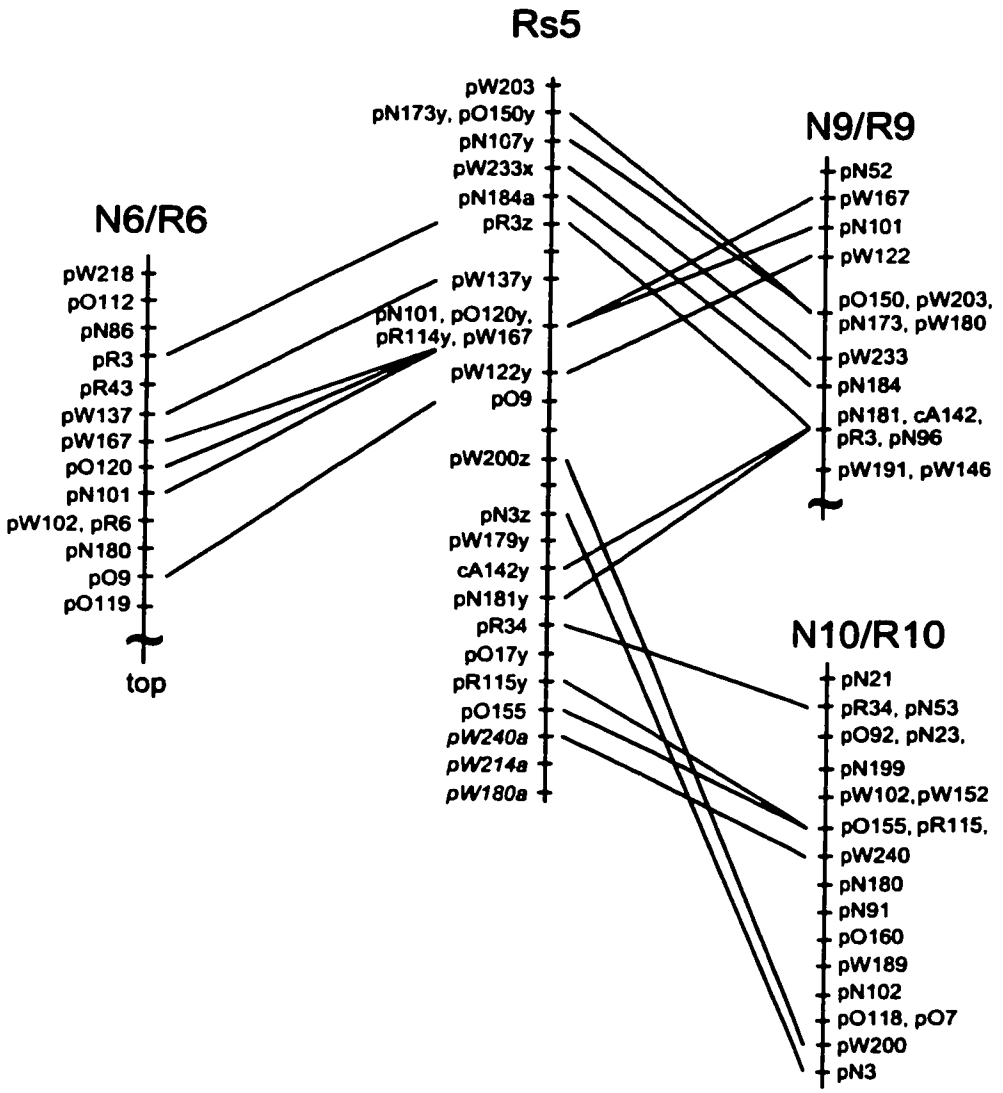
* large segments of N1/R1 and N3/R3 are homoeologous (Parkin 1995)

D



* large segments of N10/R10 and N3/R3 are homoeologous (Parkin 1995)

E


Rs5
N6/R6
pW218
pO112
pN86
pR3
pR43
pW137
pW167
pO120
pN101
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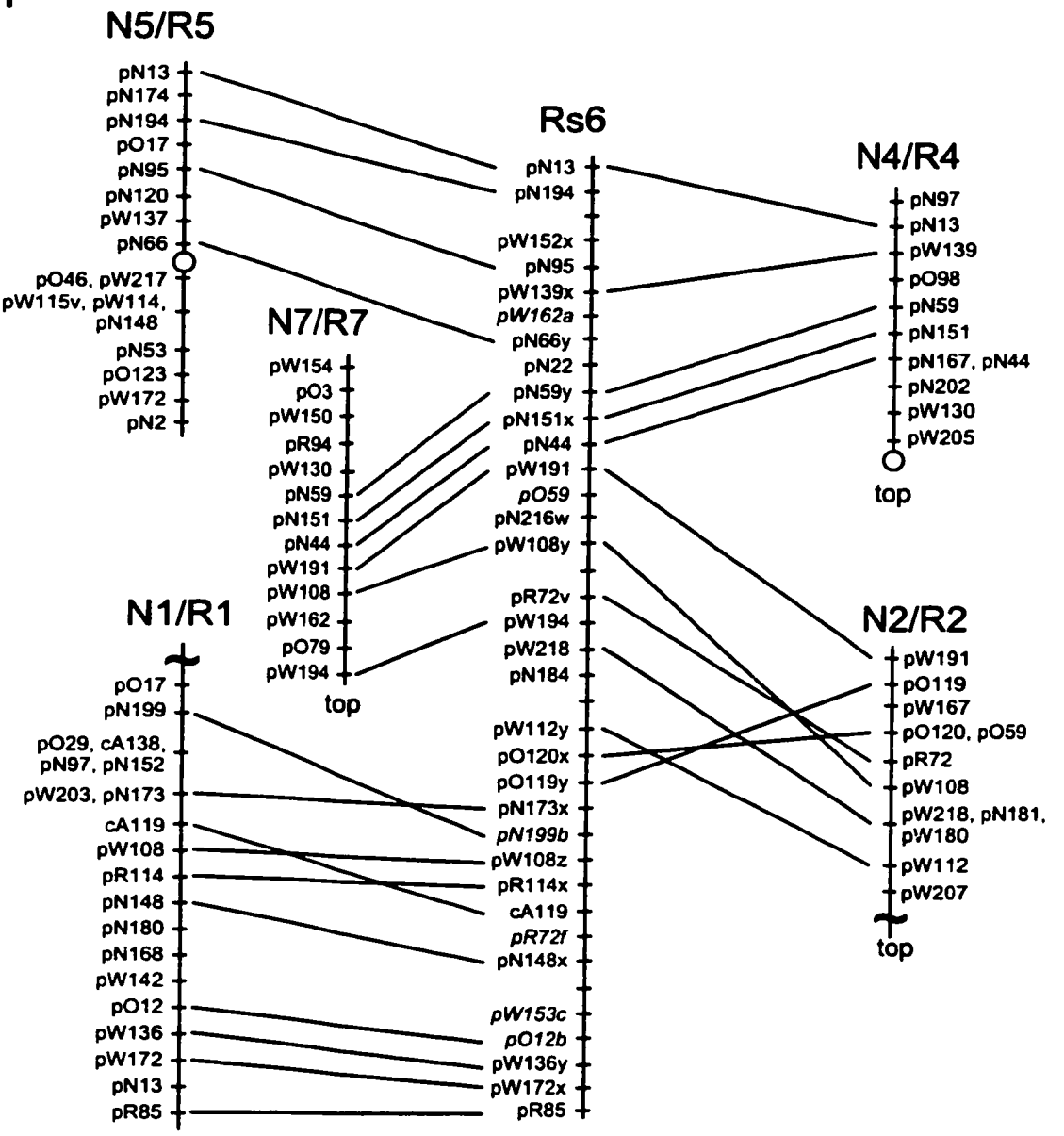
pN180
pO9
pO119
top
pW203
pN173y, pO150y
pN107y
pW233x
pN184a
pR3z
pW137y
pN101, pO120y,
pR114y, pW167
pW122y
pO9
pW200z
pN3z
pW179y
cA142y
pN181y
pR34
pO17y
pR115y
pO155
pW240a
pW214a
pW180a
N9/R9
pN52
pW167
pN101
pW122
pO150, pW203,
pN173, pW180
pW233
pN184
pN181, cA142,
pR3, pN96
pW191, pW146
N10/R10
pN21
pR34, pN53
pO92, pN23,
pN199
pW102,pW152
pO155, pR115,
pW240
pN180
pN91
pO160
pW189
pN102
pO118, pO7
pW200
pN3

F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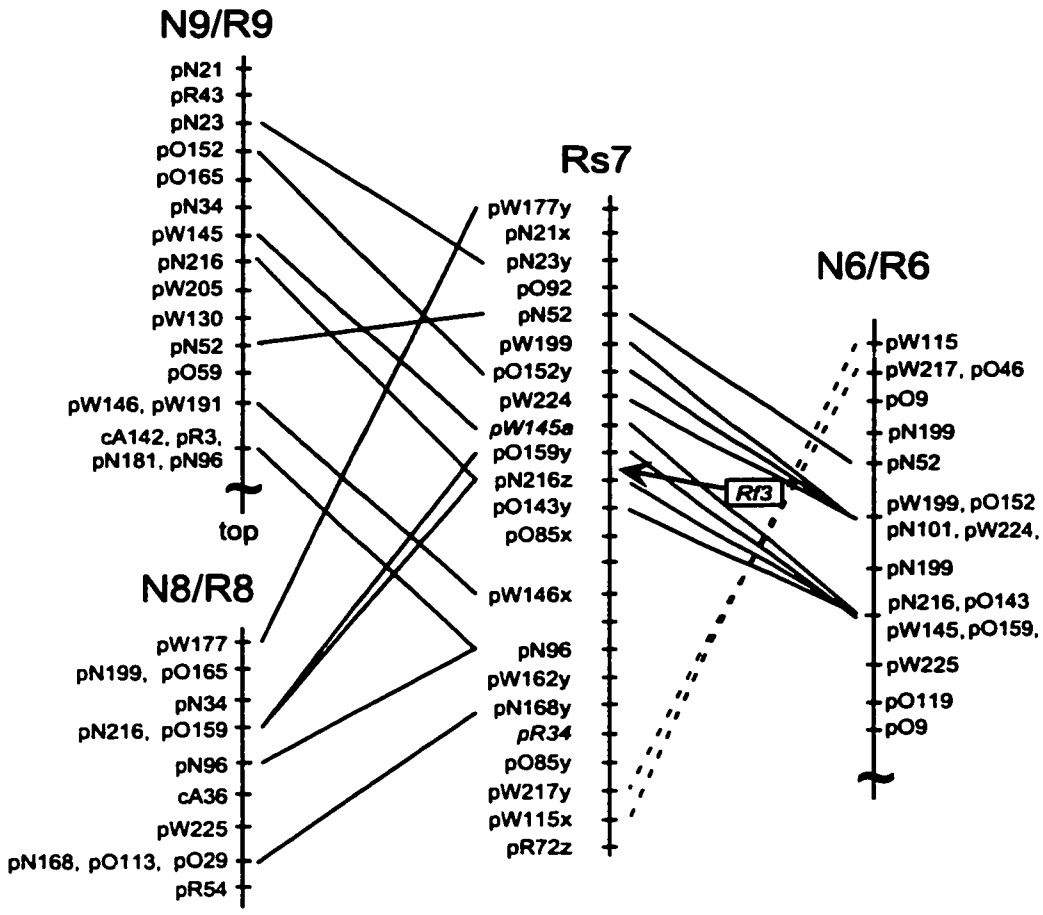
pN194
pO17
pN95
pN120
pW137
pN66
pO46, pW217
pW115v, pW114,
pN148
pN53
pO123
pW172
pN2
Rs6
pN13
pN194
pW152x
pN95
pW139x
pW162a
pN66y
pN22
pN59y
pN151x
pN44
pW191
pO59
pN216w
pW108y
pR72v
pW194
pW218
pN184
pW112y
pO120x
pO119y
pN173x
pN199b
pW108z
pR114x
cA119
pR72f
pN148x
pW153c
pO12b
pW136y
pW172x
pR85
N7/R7
pW154
pO3
pW150
pR94
pW130
pN59
pN151
pN44
pW191
pW108
pW162
pO79
pW194
top
N4/R4
pN97
pN13
pW139
pO98
pN59
pN151
pN167, pN44
pN202
pW130
pW205
top
N1/R1
pO17
pN199
pO29, cA138,
pN97, pN152
pW203, pN173
cA119
pW108
pR114
pN148
pN180
pN168
pW142
pO12
pW136
pW172
pN13
pR85
N2/R2
pW191
pO119
pW167
pO120, pO59
pR72
pW108
pW218, pN181,
pW180
pW112
pW207
top

G


N9/R9
pN21
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pN23
pO152
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pO59
pW146, pW191
cA142, pR3,
pN181, pN96
top
N8/R8
pW177
pN199, pO165
pN34
pN216, pO159
pN96
cA36
pW225
pN168, pO113, pO29
pR54
Rs7
pW177y
pN21x
pN23y
pO92
pN52
pW199
pO152y
pW224
pW145a
pO159y
pN216z
pO143y
pO85x
pW146x
pN96
pW162y
pN168y
pR34
pO85y
pW217y
pW115x
pR72z
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pN199
pN52
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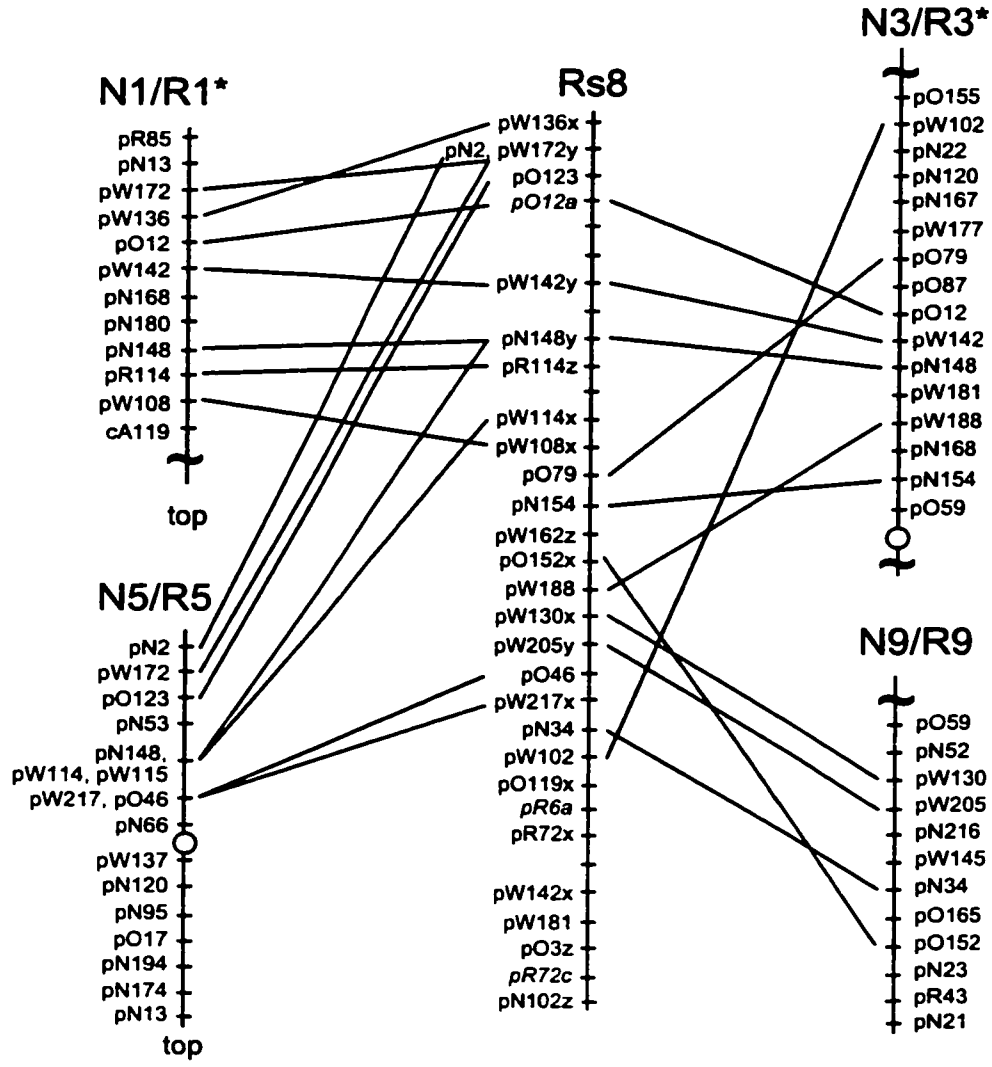
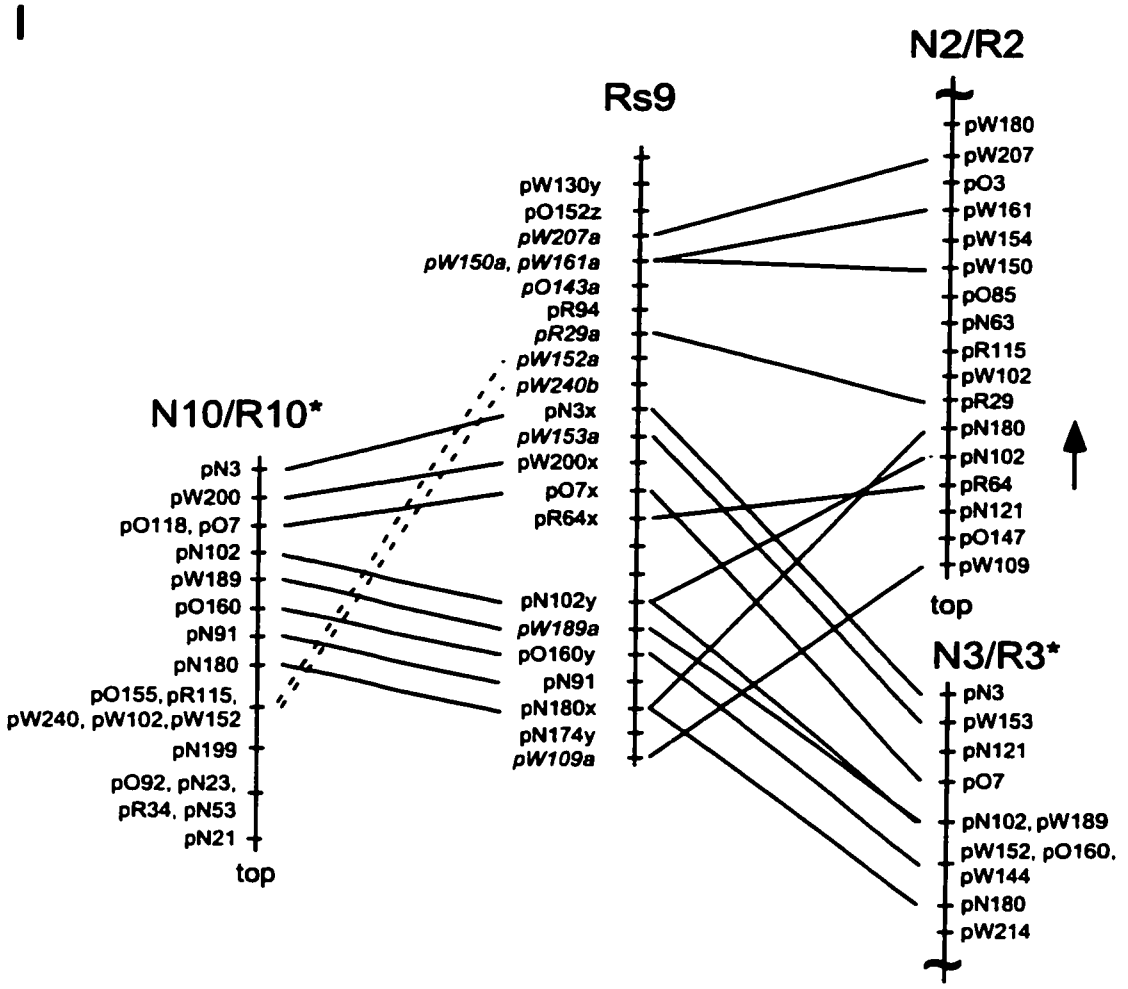
pW225
pO119
pO9

H



* large segments of N1/R1 and N3/R3 are homoeologous (Parkin 1995)

I



* large segments of N10/R10 and N3/R3 are homoeologous (Parkin 1995)

In only one case was an entire *Raphanus* linkage group substantially collinear with a whole linkage group of the *Brassica* A genome, i.e., Rs1 and R8 (Fig. 10.2A). More often, there were segments of two distinct *Brassica* A genome linkage groups substantially collinear with one *Raphanus* linkage group, for example, the top of Rs2 with the top of R1 or its homoeologue R3, and the bottom of Rs2 with the top of R2 (Fig. 10.2B). The bottom of R1 was collinear with the bottom of Rs6, while the bottom of R2 was collinear with the middle of Rs6 (Fig. 10.2F). Centromeres have been mapped for R3, R4 and R5 (Kelly 1996) and appear to coincide with breakpoints in collinearity with the *Raphanus* linkage groups. A good example of this can be seen in Fig. 10.2C. In this instance, the whole of the telocentric chromosome R4 was collinear with the top third of Rs3, and the whole lower arm of R3 was collinear with the bottom of Rs3.

In some instances, duplication in the A genome resulted in two or three regions each equally collinear with a single *Raphanus* linkage group. For example, Rs4 and Rs9 were both collinear with A genome linkage groups R2, R3 and R10 (Fig. 10.2D and 10.2I) and the same segment of R2 was also collinear with Rs2 (Fig. 10.2B). R1 and R3 carried large homoeologous segments (Parkin 1995) and there were two segments of *Raphanus* linkage groups that were substantially collinear with both of these A genome linkage groups, i.e., the bottom of Rs3 (Fig. 10.2C) and the top of Rs2 (Fig. 10.2B). Due to these large intragenomic duplications, direct experimental evidence will be necessary to determine which *Raphanus* chromosome will pair preferentially with which A genome chromosome. Analysis of the progeny of the RACC by AACC cross will likely yield such data.

Translocations and inversions were evident in several of the collinear regions. Rs5 was substantially collinear with R9 except for a translocation that would likely make pairing between these two chromosomes difficult (Fig. 10.2E). There also appeared to be a translocation in the Rs5 region that was collinear with R10 (Fig. 10.2E). There were large inversions, relative to the *Raphanus* chromosomes, on R1 (compared with Rs2 and Rs3: Figs. 10.2B and 10.2C), on R6 (compared with Rs7: Fig. 10.2G) and on R2 (compared with Rs9: Fig.10.2I).

10.2 Discussion

10.2.1 Intragenomic duplication

The level of duplication observed in loci detected by RFLP probes depends on the level of polymorphism in a particular mapping cross and the experimental conditions used to visualise the segregating RFLP alleles. The levels of duplication reported for crucifers has been underestimated in many cases due to the presence of large numbers of monomorphic loci (Song *et al.* 1991; Slocum *et al.* 1990; Chyi *et al.* 1992; Landry *et al.* 1991). Lagercrantz and Lydiate (1996) estimated the number of monomorphic loci in a *B. nigra* mapping population by assuming one or more monomorphic bands detected by a particular probe represented a single additional locus. This assumes all monomorphic banding patterns are from the same locus, potentially underestimating the actual number, and assumes that all monomorphic bands represent loci distinct from those loci with scorable polymorphisms potentially overestimating the actual number of loci. An alternative method of estimating the number of monomorphic loci detected by a particular set of probes was used on the *Raphanus* data available from the F_2 and R8 mapping populations. Assuming the two populations were not related and that the loci were randomly distributed across the genome, the ratio of the number of additional loci mapped in one or other population compared to the number of loci polymorphic in both populations can give some insight into the number of loci monomorphic in both populations and therefore missing from any analysis of genome duplication (Table 10.2). Data from these two mapping populations suggest that 14% of the *Raphanus* loci homologous to the RFLP probes were monomorphic in both populations and therefore not mapped.

Analysis of the highly polymorphic maps of the A, B and C genomes has led to the hypothesis that the *Brassica* diploid species are descended from a common hexaploid ancestor and that relatively large triplicated segments of the ancestral genome have been rearranged through chromosome fusions and fissions during the divergence of the various species (Lagercrantz and Lydiate 1996; Parkin 2000). *Raphanus* is related to *Brassica* species and is part of the rapa/oleracea lineage (Warwick and Black 1991; Fig. 2.3). *Raphanus* is therefore expected to have a genome organisation similar to those of *B. rapa* and *B. olerecea.* While wholesale triplication could not be demonstrated

possibly due to residual monomorphism and chromosomal rearrangements, it was evident that much of the *Raphanus* genome conforms to this hypothesis.

10.2.2 Intergenomic comparisons

Comparative analysis of the *Brassica* A, B and C genomes (Lagercrantz and Lydiate 1996) has suggested that all three of these species have inherited complete but rearranged copies of a highly duplicated ancestral genome. This comparative analysis of *Raphanus* with the *Brassica* A genome supports the hypothesis of gross chromosomal rearrangement during species divergence.

Breakpoints in collinearity between the *Raphanus* and *Brassica* A genomes often appeared to correspond to the breakpoints in *Brassica* A-B-C genome collinearity (Parkin *et al.* 1995; Lagercrantz and Lydiate 1996) and it has been speculated that many breakpoints occur at centromeres (Lagercrantz and Lydiate 1995; Lagercrantz and Lydiate 1996; Kelly 1996). For example, the A genome linkage group R5 and the B genome linkage group G1 were collinear along their entire lengths but a translocation occurred during the divergence of A and C such that the top arm of R5 was collinear with the top arm of C genome linkage group O4 and the bottom arm of R5 was collinear with the bottom arm of O5 (Lagercrantz and Lydiate 1996; Fig. 10.3A). When R5 was compared to the *Raphanus* genome, there was a break in collinearity with each arm being collinear with different *Raphanus* linkage groups (Fig. 10.3A). Similarly, the top of Rs2 was collinear with the top of R1 and the bottom of Rs2 was collinear with the top of R2. Interestingly, the B genome linkage group G5 was also collinear with the tops of R1 and R2 (Lagercrantz and Lydiate 1996; Fig. 10.3B) and was collinear along the whole length of Rs2 suggesting the translocation in the A and C genomes occurred after *Raphanus* diverged from the A and C genomes and suggesting a possible location for the centromere of Rs2.

There was one inversion on R1 relative to Rs2 which was also found in C genome linkage group O1 but not in homoeologous linkage groups of *Arabidopsis*, *Sinapis alba*, *B. nigra* or *Moricandia* (Parkin 2000) therefore this inversion must have occurred after the A and C genomes diverged from the others but before they themselves diverged.

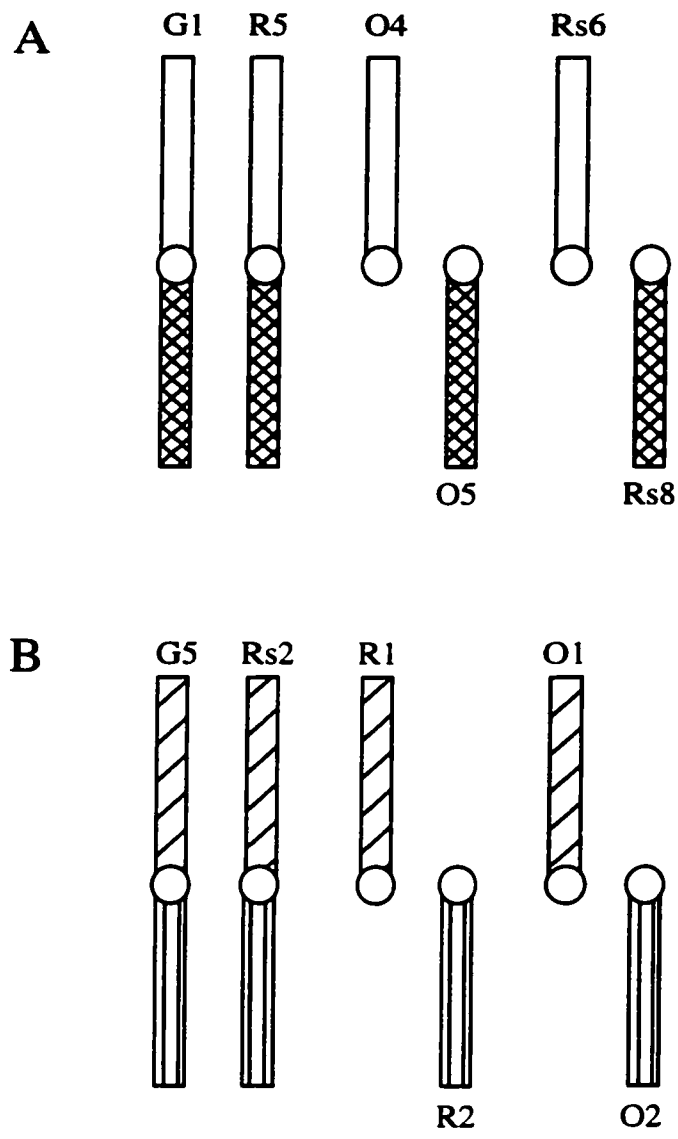


Figure 10.3. Examples of regions of homoeology between the *Brassica* A, B, and C genomes and the *Raphanus* genome. G, B genome linkage groups; R, A genome linkage groups; O, C genome linkage groups; Rs, *Raphanus* linkage groups.

Comparative genome mapping between *Raphanus* and related species will help to identify markers for fine mapping of specific loci in *Raphanus*. Selection of probes known to identify loci in segments of *B. rapa* linkage groups homoeologous to regions of particular interest in the *Raphanus* genome could be used as a means of increasing marker densities in such regions. Other markers, such as microsatellite markers, that have been mapped in the A genome could potentially be exploited for fine mapping in *Raphanus*. The *Arabidopsis* genome has recently been sequenced and will provide sequence information that could be of significant use in mapping of related crucifers including *Raphanus*.

10.2.3 Potential for transfer of restorer alleles from Raphanus *to the* Brassica *A genome*

The restorer locus *Rf1* has been mapped to Rs1 between pW233z and pN23x (Chapters 6 and 7; Fig. 10.2A). Rs1 is substantially collinear with R8 and R9 and no substantial chromosomal rearrangements were detected that could cause non-viable gametes should recombination occur. It is not evident which of these two A genome chromosomes will pair preferentially with Rs1, however, either would be suitable for the introgression of *Rf1*. Since the *Rf1* restorer is near the top of the linkage group, a single recombination event below pN23x would suffice for the transfer of this restorer to the A genome.

The restorer locus *Rf2* has been mapped to Rs2, between pW179x and cA142x (Chapters 6 and 7; Fig. 10.2B). This region of Rs2 is collinear with R1 and R3 although the collinearity is more appreciable between Rs2 and R1. If Rs2 and R1 were to pair and recombine, two recombination events, one on either side of the inversion (Fig. 10.2B) would be required to transfer the region surrounding *Rf2* to R1. Due to the inversion of the region carrying *Rf2* in *Raphanus* compared to the collinear region of R1, it would probably be difficult to reduce the size of an introgression to less than the size of the inversion. If Rs2 and R3 should pair and recombination on the centromere side of cA142 or ideally, on the centromere side and distal to the restorer locus, introgression of a segment of *Raphanus* DNA containing *Rf2* into R3 should be possible assuming homoeologous pairing and recombination occur in the interspecies hybrids.

The restorer locus *Rf3* has been mapped to a region on Rs7 near pO159y and

pN216z (Chapter 7; Fig. 10.2G). This region is collinear with segments of three different A genome linkage groups, namely R6, R8 and R9.

The segment of *Raphanus* chromosome that carries the INRA restorer, and has already been introgressed into *B. napus*, maps somewhere between the top of Rs5 and pW179y (Chapter 8; Fig. 8.2). This is a large segment of *Raphanus* chromosome and the precise location of the restorer locus within this segment is not known. Rs5 is collinear along one half its length with a segment of R9 and along the other half with R10 but there is a translocation within each of these collinear segments (Fig. 10.2E). It is likely that these translocations will make it difficult for the *Raphanus* and A genome linkage groups to pair and recombine in these regions.

It was not possible to determine which of the duplicated collinear regions detected in the *Raphanus* and *Brassica* A genomes would be likely to pair and recombine in an interspecies hybrid. RFLP analysis of the $^{R}/_{A}$ACC progeny generated in Chapter 9 are expected to give a clearer indication of which segments of the *Raphanus* and A genome chromosomes are primary homoeologues and the extent to which pairing and recombination actually occurs.

Given the high degree of collinearity between the genomes of *Raphanus* and *Brassica* in the regions containing the three restorer loci, it should be possible to transfer these restorers from *Raphanus* to the *Brassica* A genome. The use of markers will allow for the selection of individuals with chromosomes with recombination events close to the restorer loci. Ideally the size of the introgression would be reduced to include as little excess *Raphanus* DNA as possible.

Inversions and translocations that are smaller than a chromosome arm have the potential to make interspecific gene transfer difficult. The region containing the restorer gene located in the INRA restorer introgression will be particularly challenging to transfer. The INRA restorer introgression is believed to be on the C genome in *B. napus* restorer lines (Delourme *et al.* 1998) and a comparison of the introgressed region of Rs5 (Chapter 8) with C genome linkage groups previously mapped (Parkin *et al.* 1995) demonstrated the complex relationship between the chromosomes of the two genomes (Fig. 10.4). Given the translocations evident in this region, it is not surprising that there

has been little success in reducing the size of the introgressed segment through backcrossing to *B. napus*.

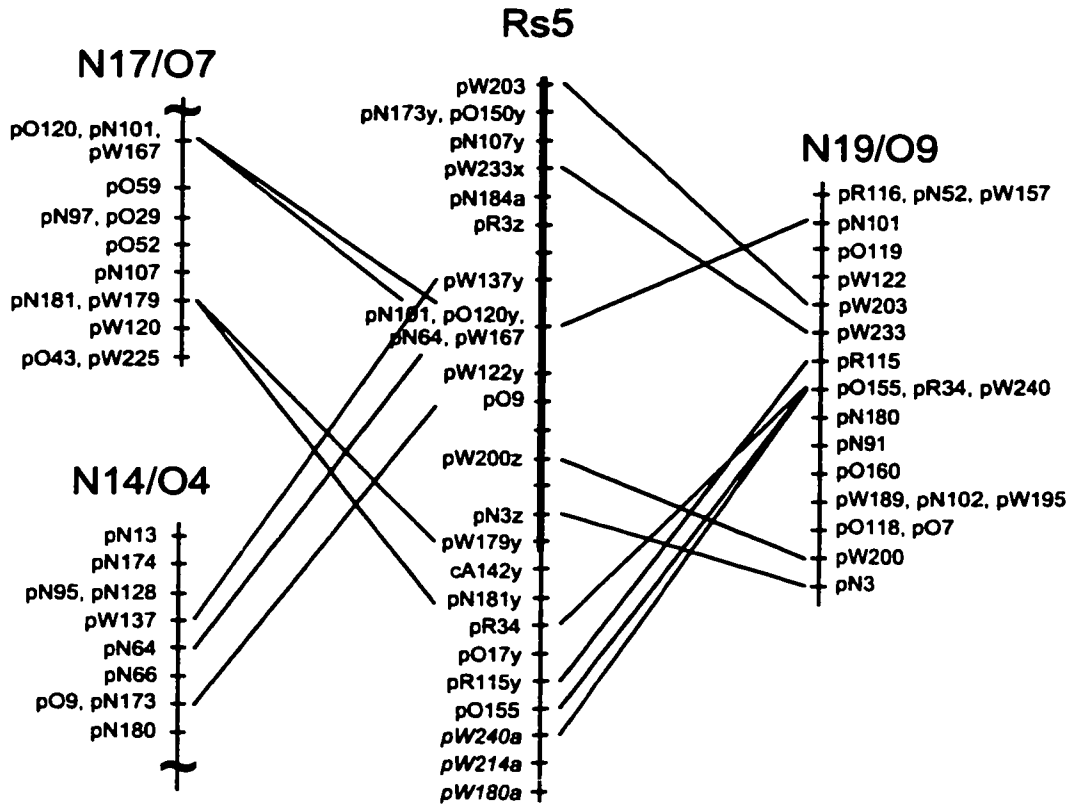


Figure 10.4. *Raphanus* linkage Rs5 with collinear *Brassica* C genome linkage groups. Thickened vertical line on Rs5 indicates segment represented in the INRA restorer introgression (Chapter 8).

Chapter 11. General Discussion

11.1. The *Raphanus* genome

The genetic maps developed in this study are the first published maps of the *Raphanus* genome. The consensus map developed from the individual *Raphanus* maps is robust and covers most of the genome. Cross-overs and RFLP markers are randomly distributed across almost the entire genome. It is comprised of nine linkage groups which probably correspond to the nine chromosomes pairs of the *Raphanus* genome. The consensus map offers an excellent illustration of the structure of the *Raphanus* genome and allows its relationship to other crucifers to be elucidated through comparative mapping.

The *Raphanus* genome appears to be extensively duplicated with evidence of triplicated segments each spanning multiple loci. Unfortunately, residual monomorphism prevents an accurate assessment of the full extent of genome triplication in *Raphanus*. As further *Raphanus* maps are developed and integrated with the consensus map, more light should be shed on this aspect of *Raphanus* genome organisation.

The *Raphanus* genome appears to have very little clustering of marker loci, even less than that of related *Brassica* species (Lagercrantz and Lydiate 1995; Parkin and Lydiate 1997). Comparative mapping of *Raphanus* with the *Brassica* A genome indicated that loci in clustered regions of the *Brassica* A genome were often separated by small map intervals in the collinear *Raphanus* segments (Fig. 10.2). The heterochromatic regions observed around the centromeres of *B. nigra* chromosomes are larger than those observed in of *B. oleracea* and *B. rapa* (Röbbelen 1960). Lagercrantz and Lydiate (1995) proposed that the increased clustering evident in *B. nigra* as compared to *B. oleracea* and *B. rapa* may be due to increased suppression of recombination in these heterochromatic regions. Perhaps *Raphanus* chromosomes have even smaller heterochromatic regions surrounding the centromeres than do *B. oleracea*

and *B. rapa* chromosomes, leading to even less suppression of recombination in these regions and resulting in a closer relationship between the genetic and physical distances.

The comparative genome analysis carried out for this thesis has shown large segments of collinearity between segments of *Raphanus* and *Brassica* A genome linkage groups covering the whole *Raphanus* genome. Due to genome duplication in both species, it was not possible to accurately predict the regions of primary homoeology for many segments. Analysis of the progeny from the RACC by AACC interspecies crosses should demonstrate more clearly which segments of *Raphanus* chromosomes are pairing and recombining with specific segments of *Brassica* A genome chromosomes, giving a better indication of the relationship between the chromosomes of these two species. The consensus map of the *Raphanus* genome along with the existing maps of the *Brassica* A genome, developed using the same RFLP probes (Parkin *et al.* 1995; Sharpe *et al.* 1995; Salava *et al.* 2001), will allow the genetic marker analysis of parental contributions to these progeny to proceed in an efficient manner.

From the preliminary examination of the *Raphanus* and *Brassica* A genome RFLP maps, it is evident that they contain essentially the same overall genetic content but with large segments of the genomes rearranged with respect to each other, in much the same way as the *Brassica* A, B and C genomes are rearranged with respect to each other (Lagercrantz and Lydiate 1996). Once the primary homoeologues have been determined, it will be possible to develop a better understanding of these gross chromosomal rearrangements, leading to an increased understanding of the evolution of *Raphanus* relative to *Brassica* species.

Ongoing efforts in comparative mapping between *Arabidopsis thaliana* and *Brassica* species (Lan *et al.* 2000; Parkin 2000), coupled with the knowledge of the relationship between *Raphanus* and the *Brassica* A genome will help elucidate evolutionary relationships between *Raphanus* and its other genetically well characterised relatives. Being able to access genetic information from these relatives will allow for tremendous gains in radish genetic research at a fraction of the cost of working in radish alone. These relatives could also provide additional markers and related DNA sequences that would facilitate map-based gene cloning, candidate gene identification and marker-

assisted selection in radish.

Although this study focussed on the mapping of Ogura CMS restorer loci in radish, the consensus map can also be used for the mapping of loci controlling other traits in radish for both radish and *Brassica* breeding programmes. Radish breeders now have an overall map of the genome onto which they can map loci controlling traits of importance to their programmes such as shape, colour and size of the root, nutritional value, development time, and self incompatibility. This information will be very useful for marker-accelerated backcrossing in radish breeding programmes. *Brassica* breeders can make use of the *Raphanus* map to map loci controlling traits segregating in radish that are of potential interest in *Brassica* crops, such as resistance to beet cyst nematode (Lelivelt *et al.* 1992), and resistance to diseases such as turnip mosaic virus (Fujisawa 1990), clubroot (Hagimori *et al.* 1992) and blackleg (Chen and Seguin-Swartz 1999). Marker-assisted introgression of these loci could then be carried out in much the same way as proposed in this thesis for the introgression of CMS restorer loci.

11.2 Ogura CMS restorer genetics

Several Ogura CMS radish populations that were segregating for male fertility at defined restorer loci have been developed making it possible to identify and map with confidence three unique restorer loci each with a dominant restorer allele. The presence of the non-restoring allele in the homozygous state at any of the three loci results in a male sterile phenotype. Segregation data from one of the populations investigated for this thesis suggested that there might be a fourth restorer locus but it was not possible to map this locus based on the individuals investigated.

Many reports on the genetics of restoration of Ogura CMS in radish suggest that there is one dominant gene involved (see Chapter 2). This is likely due to the use of genetically undefined parents in crosses used to develop segregating populations. For example, a plant will be male sterile if at least one restorer locus is homozygous for the non-restoring allele (e.g., *rf1rf1Rf2Rf2Rf3Rf3*), it is therefore possible to create populations that are segregating at only one restorer locus (e.g. only at *Rf1*). This could lead to the conclusion that the male fertile parent of a segregating population had only

one restorer when in fact it could have had several (e.g., *Rf1Rf1Rf2Rf2Rf3Rf3*). It is therefore vitally important that the genotype of the male sterile tester line be known when assessing for the presence of restorers in other material.

Another challenge to the determination of the number of genes involved in restoration is that radish is an out-crossing species and as such, plants tend to be heterozygous. The populations can also be heterogeneous. Plants of non-CMS lines can carry both restoring and non-restoring alleles in either the heterozygous or homozygous state with no obvious effect on their own fertility phenotype but with a profound effect on the phenotype of any progeny from a cross with a male sterile plant.

The development of genotypes homozygous for specific restoring alleles, alone and in various combinations, will facilitate the identification of restorers present in other radish material and allow the integration of information from other groups working on the genetics of restoration of Ogura CMS. The genetic study carried out for this thesis involved a limited number of genotypes and it is entirely possible that there are more restorer loci present in the radish gene pool.

One of these additional restorer loci is the one found in the INRA *B. napus* restorer line. The region of the radish genome identified as the segment introgressed into *B. napus* does not contain any of the three restorer loci mapped for this thesis. There are several possible explanations for this: 1) all lines investigated so far are homozygous for the restoring allele found in this region and therefore not segregating in the mapping populations; 2) this restoring allele is independent of the other three restorers and is not present in the populations studied so far; or 3) the restorer on this segment of the radish genome does not function in radish with Ogura CMS cytoplasm, but rather restores fertility to *B. napus* lines with the INRA cybrid cytoplasm. Maize T-CMS restorer *Rf2* is found in most maize inbreds (Snyder and Duvik 1969). Similarly, the restorer found on the INRA introgression may be present in most radish lines including the ones used to develop the mapping populations used to map the three restorer loci described in this thesis. The process of finding a radish line homozygous or at least heterozygous for the non-restoring allele at this putative restorer locus might require test crossing a wide range of radish lines with tester lines of known genotype, combined with RFLP analysis using probes that map to the region of the radish genome

implicated in the INRA restorer introgression. Alternatively, if the restorer on the INRA introgression is one that acts independently of the three currently identified restorer loci in radish, it should be possible to screen for its presence by developing segregating populations from crosses between male sterile lines homozygous for the non-restoring alleles at the three known restorer loci (i.e. *rf1rf1rf2rf2rf3rf3*) and a wide range of male fertile radish lines.

The kos CMS system developed in Japan (Sakai *et al.* 1996) is essentially the same as the Ogura CMS system. Both appear to be mediated by homologous mitochondrial genes and the presence of restorer genes has been shown to reduce the accumulation of the proteins associated with these genes (Krishnasamy and Makaroff 1994; Koizuka *et al.* 2000). Two restorers have been identified that restore fertility to kos CMS radish, only one of which is required to restorer fertility to selected kos *B. napus* cybrids (Koizuka *et al.* 2000). This same restorer also restores fertility to the male sterile INRA *B. napus* cybrid (Koizuka *et al.* 2000). It would be interesting to see if this kos restorer maps to the same segment of the *Raphanus* genome as does the INRA restorer or any of the other three restorers mapped for this thesis. It would be very interesting if it is not on the same segment as the INRA restorer as it would offer an alternative introgression event to select for in the progeny of the interspecies crosses.

11.3 Intergeneric populations

A series of interspecific crosses was successfully carried out resulting in trigenomic tetraploids with the genome constitution of RACC (R - *Raphanus* genome; A - *Brassica* A genome; C - *Brassica* C genome). Meiosis in these plants should result in pairing and recombination between pairs of homologous C genome chromosomes leaving the haploid complements of R and A genome chromosomes to pair with their closest homoeologues. RFLP analysis of the progeny of these plants will allow an assessment of the pattern and extent of recombination that occurred between the chromosomes of the A and R genomes, giving clear evidence of which chromosome segments are primary homoeologues and indicating the potential for interspecific gene transfer between these two species.

The maternal parent of the initial interspecies cross was a *Raphanus* plant with

Ogura CMS cytoplasm, and carrying all the restorer genes necessary for restoration of this cytoplasm in radish. It was thought that by using the CMS cytoplasm, it would be possible to screen for the presence of the restorer alleles at each generation by assessing for the male fertile phenotype. All progeny from the initial *R. sativus* x *B. oleracea* interspecies cross, and the progeny of subsequent crosses through to and including the progeny of the RACC plants, were male sterile. All doubled haploid progeny of the reciprocal *B. oleracea* x *R. sativus* cross, i.e., CCRR plants with *B. oleracea* cytoplasm, shed pollen, indicating that the Ogura CMS cytoplasm is causing the male sterility of the doubled haploid progeny of the initial population derived from the reciprocal cross.

Since the locations of some of the restorer loci on the *Raphanus* genome are now known, it is no longer necessary to rely on phenotype to determine if the restorer alleles are present in the interspecies hybrids. Instead, RFLP analysis could be carried out on the non-CMS hybrid progeny to identify and select individuals carrying hybrid chromosomes with the appropriate *Raphanus* segment. This approach will also allow for the use of normal *B. napus* as the female and the interspecific individual as the male in crosses. Such a cross was carried out by Agnihotri *et al.* (1990) and yielded many viable embryos, perhaps because of the increased chance of having a viable interspecific pollen grain in a given pollination event compared to the chance of having a viable ovule in a single ovary simply due to the sheer number of pollen grains per pollination. Their success could have also been due to the parental genotypes.

It would appear that the interspecific individuals with CMS cytoplasm that have been generated so far will be of use primarily for the insight they will give into recombination between the A and R genomes. Their use for interspecies transfer of restorer genes and generation of male fertile *B. napus* restorer lines will depend on whether they begin to produce viable pollen or not once the current genetic imbalance has been corrected through repeated backcrossing to *B. napus*. The interspecific individuals generated from the reciprocal cross (*B. oleracea* x *R. sativus*) may well be much more useful for the transfer of the restorer alleles from *Raphanus* to the *Brassica* A genome. Ideally, several individuals will be developed that each carry a single restorer from *Raphanus* in an A genome background. These could then be pyramided to develop lines carrying all or specific combinations of restorers. This would enable

testing to be carried out on the INRA cybrids to determine if only one restorer or a specific combination of restorers are necessary for full fertility restoration.

Assuming a complete system can be developed in *B. napus*, it would then be reasonably straightforward to backcross the restorer or restorers into *B. rapa* and *B. juncea*, offering breeders the opportunity to develop hybrids in all three types of canola.

11.4 Conclusion

The work presented in this thesis is a large first step towards the comprehensive identification and mapping of restorer genes for Ogura CMS in *Raphanus* and the transfer of restorers to the *Brassica* A genome. The target is a complete CMS system for *B. napus*, *B. rapa* and *B. juncea*. While the restorers have yet to be transferred successfully, we now have a much better understanding of the genetics of restoration in *Raphanus*, we know specifically where at least three of the restorer loci map on the *Raphanus* genome and, based on comparative mapping between *Raphanus* and the *Brassica* A genome, we can predict where the restorers might be introgressed into the A genome following the appropriate interspecific crosses and intergenomic recombination events.

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Appendix 1. Mapping Data

A1.1. *Brassica* RFLP clones used to develop BC_1, F_2 and R8 maps, the number of polymorphic loci detected by each probe and the linkage group to which they map.

	Mapping Population					
probe	BC_1	F_2	R8	Map Locations		
cA36	3 [a]	2	1	Rs2	Rs3	Rs4
cA53	1	1	-	Rs2		
cA112	1	1	-	Rs3		
cA119	1	1	-	Rs6		
cA138	1	2	-	Rs2	Rs3	
cA142	3	3	2	Rs2	Rs3	Rs5
pN2	1	1	-	Rs8		
pN3	3	2	mono [b]	Rs4	Rs5	Rs9
pN13	1	1	-	Rs6		
pN21	0	1	mono	Rs7		
pN22	-	1	1	Rs6		
pN23	2	2	1	Rs1	Rs7	
pN34	1	1	-	Rs8		
pN44	1	1	1	Rs6		
pN52	1	1	-	Rs7		
pN53	2	2	-	Rs3	Rs4	
pN59	2	2	1	Rs3	Rs6	
pN63	mono	mono	-			
pN64	1	1	1	Rs5		
pN66	2	1	2	Rs3	Rs6	
pN67	mono	mono	-			
pN86	1	1	-	Rs3		
pN87	repetitive [b]	repetitive	-			
pN91	-	1	-	Rs9		
pN95	1	1	1	Rs6		
pN96	1	1	mono	Rs7		
pN97	-	2	mono	Rs2	Rs3	
pN99	repetitive	repetitive	-			
pN101	1	1	1	Rs5		
pN102	3	3	1	Rs4	Rs8	Rs9
pN105	mono	mono	mono			
pN107	2	1	3	Rs2	Rs3	Rs5
pN113	mono	mono	mono			
pN120	1	1	1	Rs4		
pN121	2	2	1	Rs2	Rs4	

probe	Mapping Population			Map Locations		
	BC_1	F_2	R8			
pN123	mono	mono	mono			
pN129	no hybridization		-			
pN148	2	2	2	Rs6	Rs8	
pN151	1	1	2	Rs3	Rs6	
pN152	1	1	-	Rs2		
pN154	1	1	mono	Rs8		
pN167	1	1	-	Rs3		
pN168	1	2	1	Rs1	Rs7	
pN170	mono	mono	-			
pN173	2	2	2	Rs5	Rs6	
pN174	3	-	1	Rs3	Rs4	Rs9
pN180	2	1	2	Rs4	Rs9	
pN181	2	2	mono	Rs2	Rs5	
pN184	1	1	-	Rs6		
pN186	mono	mono	-			
pN194	1	1	-	Rs6		
pN199	1	1	2	Rs1	Rs6	
pN202	1	1	mono	Rs3		
pN206	1	1	1	Rs2		
pN207	repetitive	repetitive	-			
pN213	mono	mono	-			
pN215	repetitive	repetitive	-			
pN216	3	2	3	Rs1	Rs6	Rs7
pN220	2	1	3	Rs3	Rs4	Rs6
pO3	2	1	-	Rs2	Rs8	
pO5	1	1	-	Rs3		
pO7	2	2	-	Rs4	Rs9	
pO9	1	1	mono	Rs5		
pO10	mono	mono	-			
pO12	mono	mono	2	Rs6	Rs8	
pO17	2	-	-	Rs2	Rs5	
pO29	3	1	1	Rs1	Rs2	Rs3
pO43	0	1	1	Rs2	Rs3	
pO46	1	1	1	Rs8		
pO52	1	1	-	Rs3		
pO59	2	2	1	Rs3	Rs3	Rs6
pO70	no hyridization		-			
pO79	1	1	-	Rs8		
pO85	1	2	1	Rs7	Rs7	
pO86	mono	mono	-			
pO87	1	1	1	Rs1		
pO92	1	1	mono	Rs7		
pO98	1	1	1	Rs3		
pO105	mono	mono	mono			
pO111	2	2	mono	Rs4	Rs9	
pO112	2	2	mono	Rs1	Rs3	

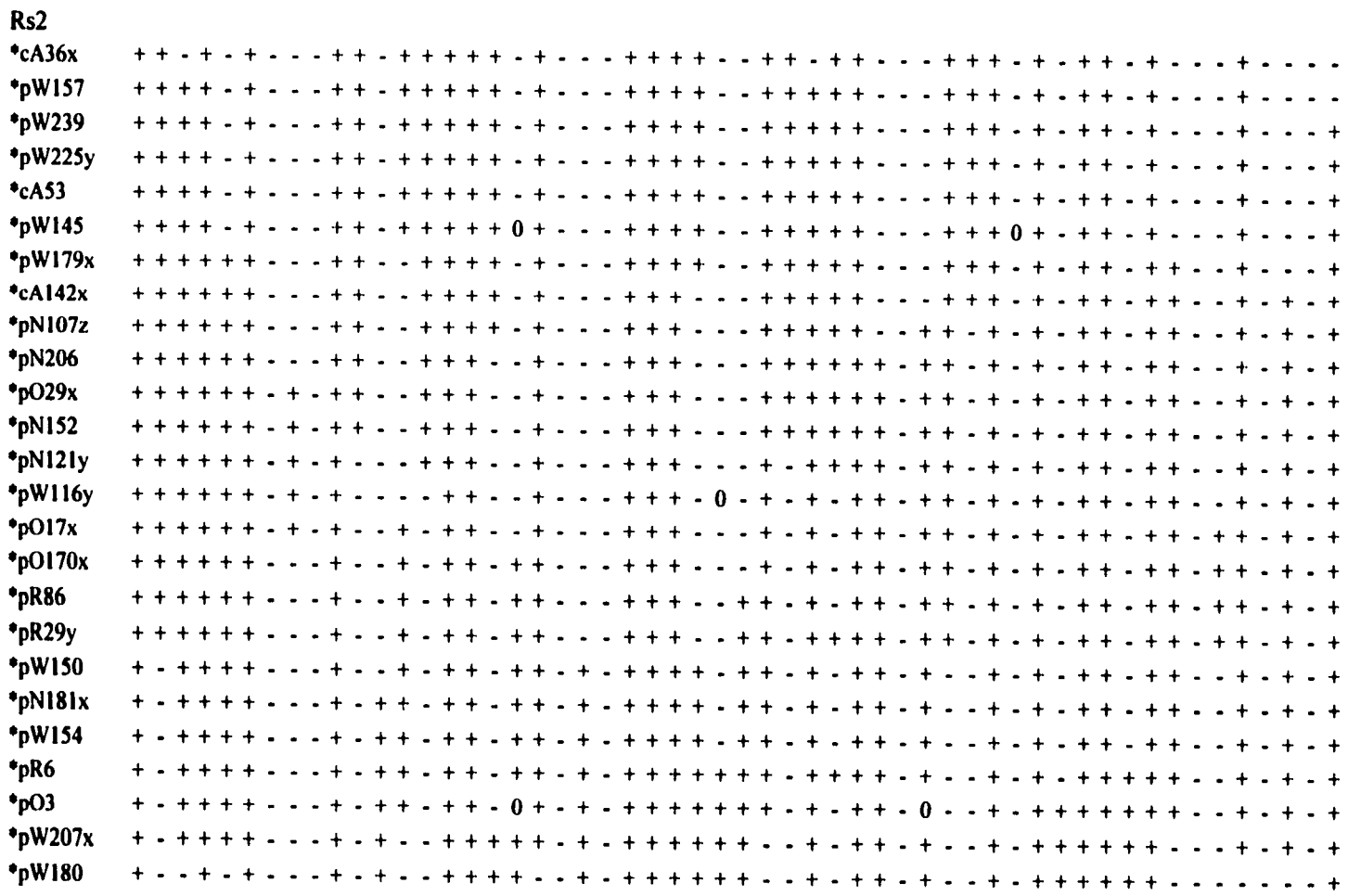
	Mapping Population			Map Locations					
probe	BC_1	F_2	R8						
pO113	1	1	mono	Rs1					
pO118	1	-	-	Rs4					
pO119	2	2	1	Rs6	Rs8				
pO120	2	2	1	Rs5	Rs6				
pO123	1	1	-	Rs8					
pO125	2	2	2	Rs5	Rs6				
pO128	2	2	1	Rs1	Rs8				
pO131	2	2	1	Rs1	Rs6				
pO142	mono	mono	-						
pO143	2	1	2	Rs1	Rs7	Rs9			
pO145	-	-	1	Rs1					
pO147	mono	mono	-						
pO150	2	2	1	Rs1	Rs5				
pO152	3	2	1	Rs7	Rs8	Rs9			
pO155	1	1	-	Rs5					
pO159	2	2	1	Rs1	Rs7				
pO160	2	2	2	Rs4	Rs9				
pO165	-	-	1	Rs1					
pO170	5	2	3	Rs2	Rs3	Rs4	Rs5	Rs7	Rs8
pO171	repetitive	repetitive	-						
pO173	mono	mono	-						
pR3	3	3	1	Rs3	Rs3	Rs5			
pR4	mono	mono	mono						
pR6	1	1	1	Rs2	Rs8				
pR29	1	1	1	Rs2	Rs9				
pR30	repetitive	repetitive	-						
pR34	1	1	1	Rs5	Rs7				
pR43	-	1	-	Rs3					
pR54	1	1	-	Rs1					
pR64	1	2	-	Rs2	Rs9				
pR72	5	3	3	Rs3	Rs3	Rs6	Rs7	Rs8	
pR85	1	1	-	Rs6					
pR86	1	1	-	Rs2					
pR93	2	2	mono	Rs7	Rs8				
pR94	1	1	2	Rs9					
pR97	mono	mono	-						
pR113	no hybridization		-						
pR114	3	3	2	Rs5	Rs6	Rs8			
pR115	3	3	2	Rs3	Rs4	Rs5			
pR116	1	1	-	Rs5					
pW102	1	1	2	Rs4	Rs8				
pW103	2	2	-	Rs3	Rs9				
pW105	1	1	2	Rs2	Rs3				
pW106	-	-	1	Rs2					
pW108	3	2	1	Rs6	Rs6	Rs8			
pW109	repetitive	repetitive	2	Rs2	Rs9				

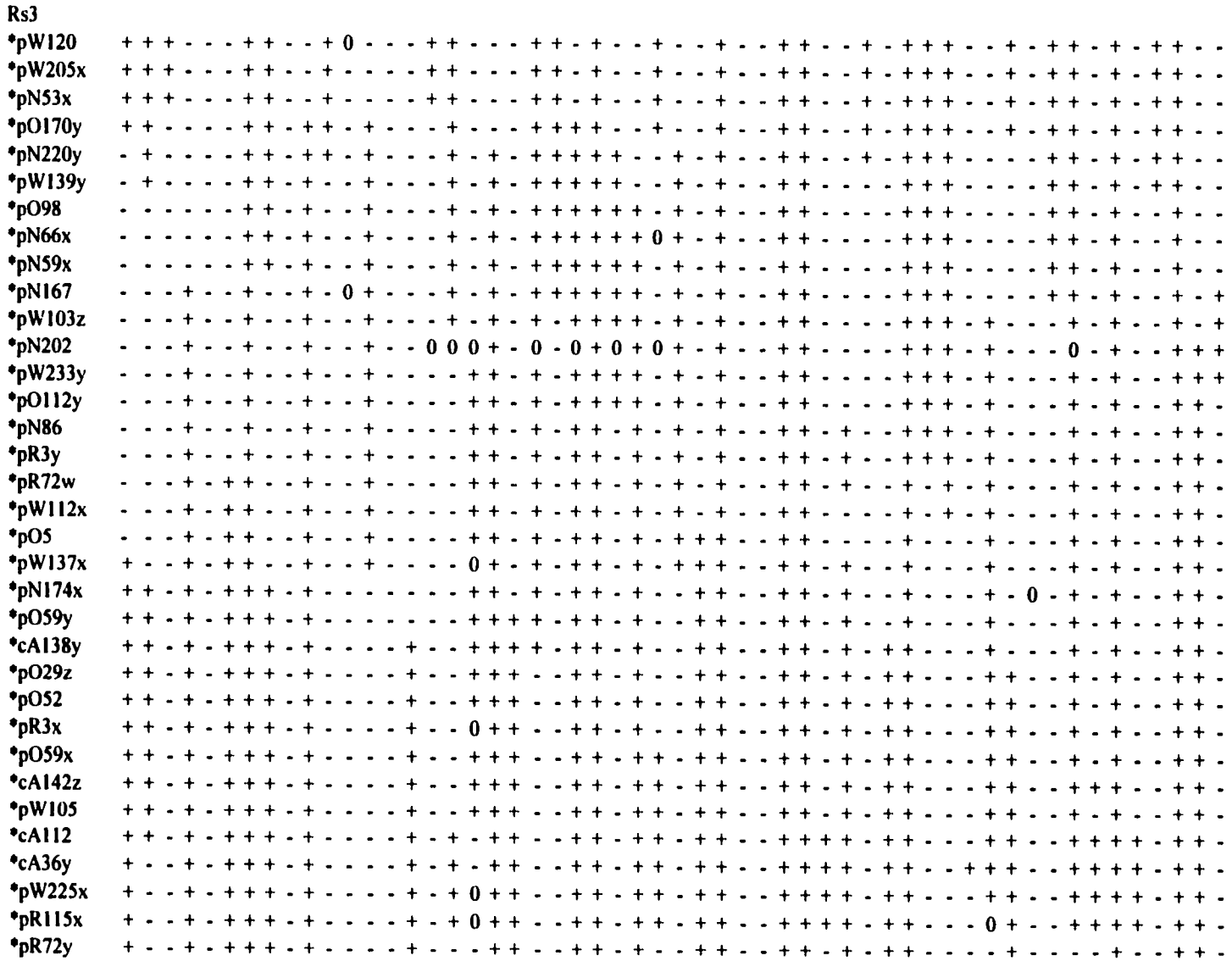
probe	Mapping Population			Map Locations			
	BC_1	F_2	R8				
pW112	2	2	1	Rs3	Rs6		
pW114	2	2	1	Rs1	Rs8		
pW115	1	1	1	Rs7			
pW116	3	3	1	Rs2	Rs4	Rs8	
pW120	1	1	-	Rs3			
pW121	1	1	-	Rs1			
pW122	1	1	1	Rs5			
pW130	2	1	1	Rs8	Rs9		
pW136	2	2	-	Rs6	Rs8		
pW137	2	2	mono	Rs3	Rs5		
pW139	2	3	2	Rs3	Rs4	Rs6	
pW142	2	2	2	Rs8	Rs8		
pW143	-	-	1	Rs9			
pW144	1	1	-	Rs4			
pW145	1	1	1	Rs2	Rs7		
pW146	2	2	mono	Rs1	Rs7		
pW148	2	2	1	Rs1	Rs4	Rs5	
pW150	1	-	1	Rs2	Rs9		
pW152	2	1	1	Rs4	Rs6	Rs9	
pW153	1	1	3	Rs4	Rs6	Rs9	
pW154	1	1	-	Rs2			
pW155	1	1	-	Rs9			
pW157	1	1	1	Rs2			
pW161	-	-	1	Rs9			
pW162	3	3	2	Rs4	Rs6	Rs7	Rs8
pW167	1(COMP)	mono	mono	Rs5(COMP)			
pW172	2	2	-	Rs6	Rs8		
pW176	-	-	mono				
pW177	2	1	2	Rs1	Rs4	Rs7	
pW179	2	2	2	Rs2	Rs3	Rs5	
pW180	1	1	1	Rs2	Rs5		
pW181	1	1	1	Rs8			
pW184	1	1	-	Rs1			
pW186	mono	mono	-				
pW188	1	1	1	Rs8			
pW189	-	-	2	Rs2	Rs9		
pW191	-	1	-	Rs6			
pW194	1	1	-	Rs6			
pW195	1	1	-	Rs9			
pW199	1	1	-	Rs7			
pW200	3	1	1	Rs4	Rs5	Rs9	
pW201	3	3	-	Rs6	Rs8	Rs8	
pW203	1	1	1	Rs5			
pW205	2	1	1	Rs3	Rs8		
pW207	1	1	1	Rs2	Rs9		
pW214	-	-	2	Rs4	Rs5		

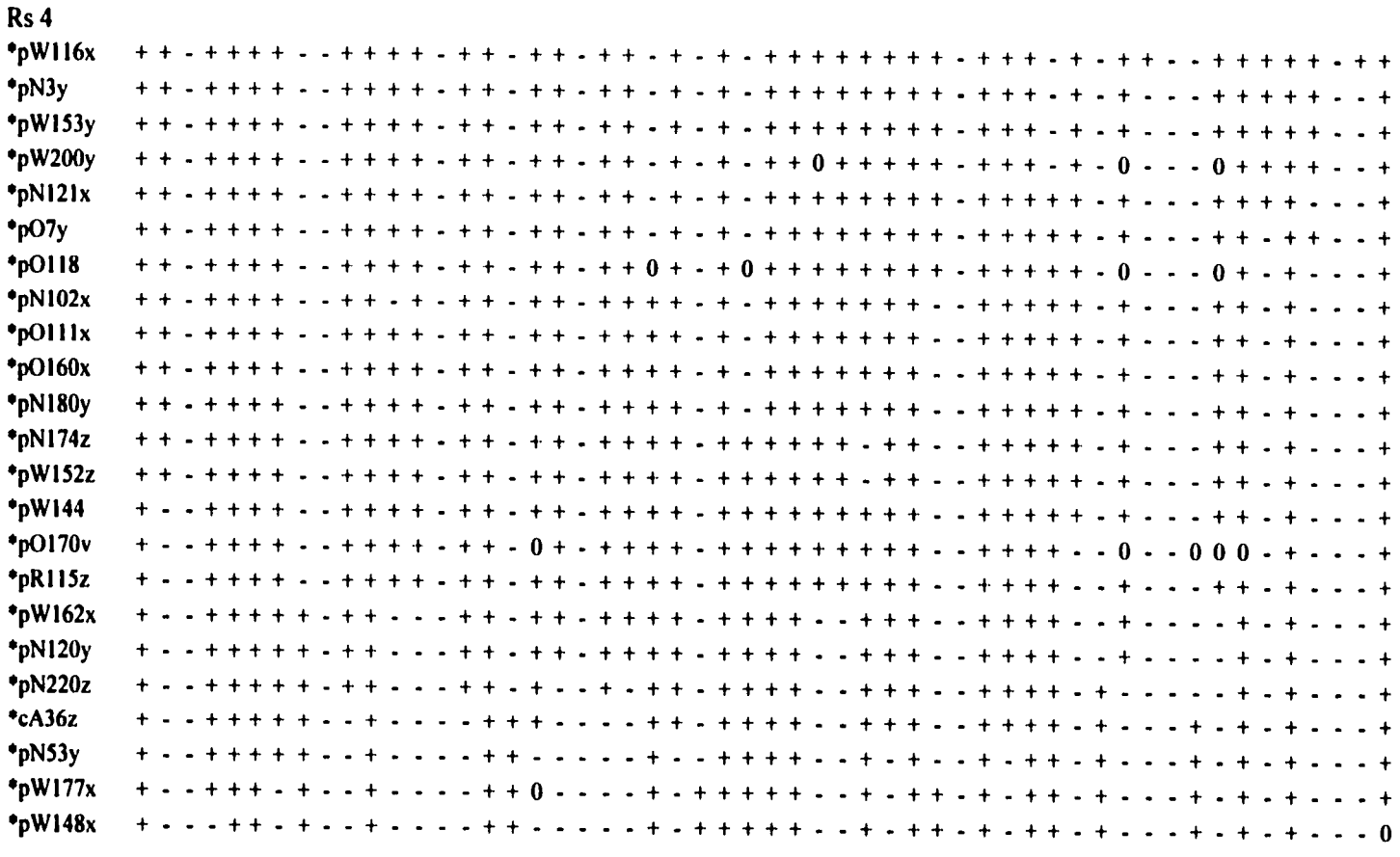
probe	Mapping Population			Map Locations		
	BC_1	F_2	R8			
pW216	1	1	-	Rs6		
pW217	2	1	2	Rs7	Rs8	Rs9
pW218	1	1	-	Rs6		
pW224	-	1	-	Rs7		
pW225	2	2	-	Rs2	Rs3	
pW233	3	3	1	Rs1	Rs3	Rs5
pW239	1	1	-	Rs2		
pW240	-	-	2	Rs5	Rs9	

A1.2. The data for all the RFLP defined loci scored in the BC_1 population. The rows represent loci and the columns BC_1 individuals. Male fertile (f) or sterile (s) phenotype of each individual is indicated at the top of the data set. Within the scoring matrix: +, the presence of the non-recurrent, R7-21, parental allele; -, the absence of this parental allele; 0, missing data point.

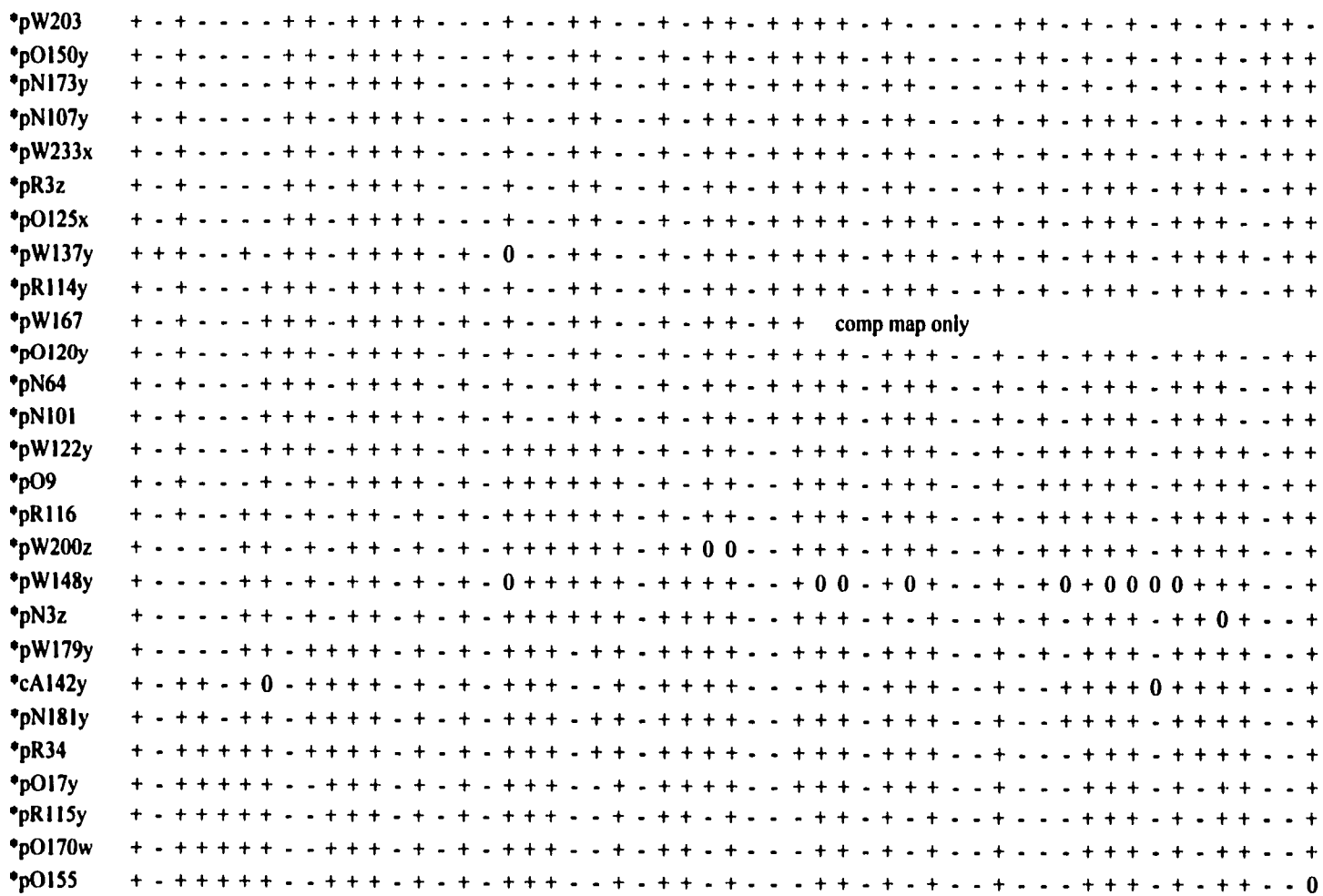
phenotype:	f	s	f	s	s	s	s	f	f	f	f	f	s	s	f	f	s	f	f	s	f	f	s	f	f	s	s	f	f	f	f	s	f	s	s	f	s	s	f	s	f	s	f	s	s	s	f	f	s	s	f
Rs1																																																				
*pW233z	-	-	+	+	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pN23x	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pN199	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pW184	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pN216y	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pO143x	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pO159x	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pO131y	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pO87	+	-	+	-	-	-	-	+	+	+	+	+	-	-	+	-	-	+	+	-	+	+	-	-	-	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
*pW146y	+	-	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	+	-	+	0	-	+	-	+	+	-	+	-	-	+	0	0	-	-	+
*pO112x	+	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	0	0	-	+	+	-	+	+	-	+	-	-	-	+	-	+	-	+	+	+	+	-	+
*pO29y	+	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	0	+	+	+	-	+	+	-	+	+	-	+	+	-	-	-	+	-	+	-	+	+	+	-	-	-
*pO113	+	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-	+	+	+	-	+	+	-	+	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	-	-
*pO128y	+	0	+	-	+	+	+	+	+	-	-	-	+	+	-	0	-	+	+	-	0	+	-	-	0	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
*pW121	+	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
*pO150x	+	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
*pW114y	+	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	0	-	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
*pR54	+	-	+	-	+	+	-	+	+	-	-	-	+	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+	+	-

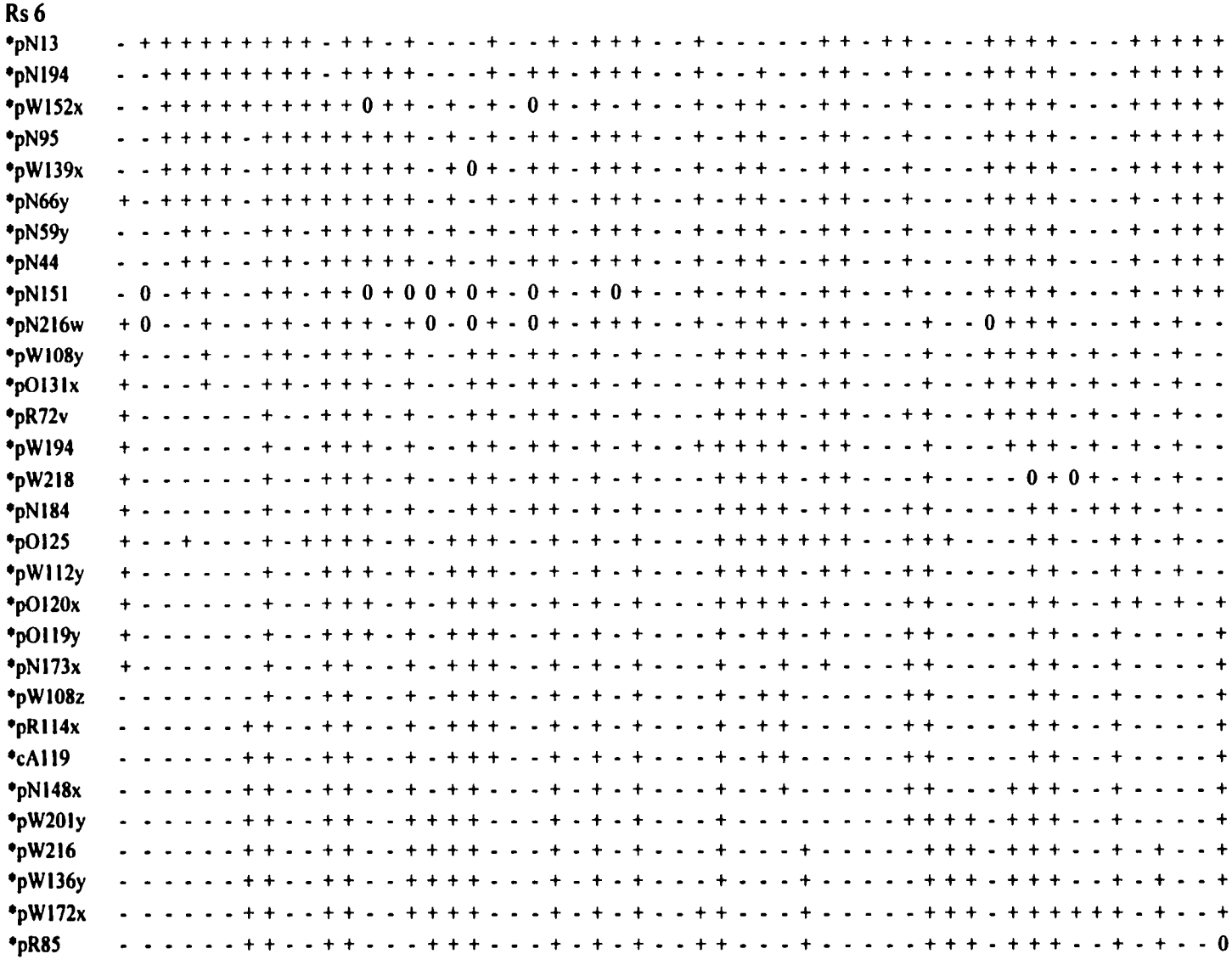

Rs2
*cA36x
*pW157
*pW239
*pW225y
*cA53
*pW145
*pW179x
*cA142x
*pN107z
*pN206
*pO29x
*pN152
*pN121y
*pW116y
*pO17x
*pO170x
*pR86
*pR29y
*pW150
*pN181x
*pW154
*pR6
*pO3
*pW207x
*pW180

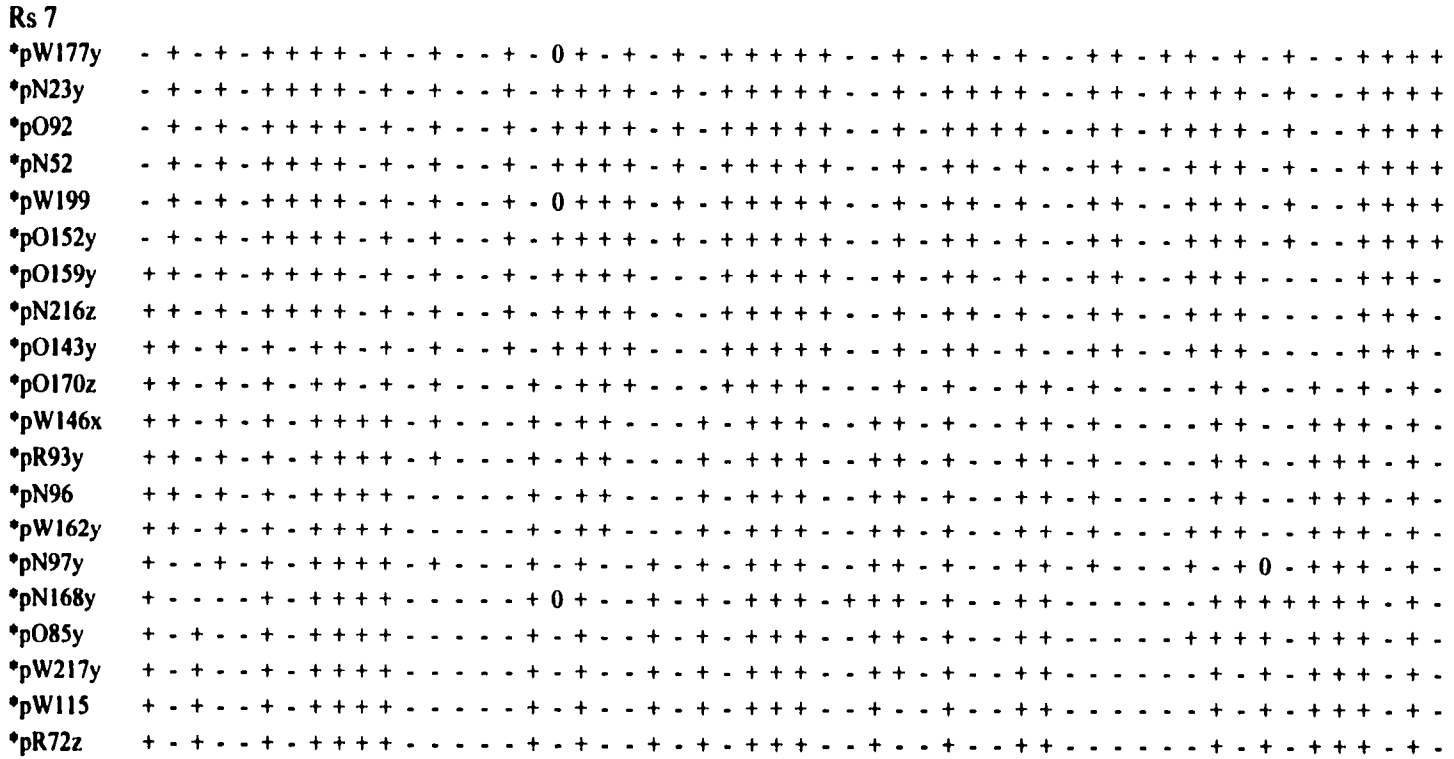

Rs3
*pW120
*pW205x
*pN53x
*pO170y
*pN220y
*pW139y
*pO98
*pN66x
*pN59x
*pN167
*pW103z
*pN202
*pW233y
*pO112y
*pN86
*pR3y
*pR72w
*pW112x
*pO5
*pW137x
*pN174x
*pO59y
*cA138y
*pO29z
*pO52
*pR3x
*pO59x
*cA142z
*pW105
*cA112
*cA36y
*pW225x
*pR115x
*pR72y

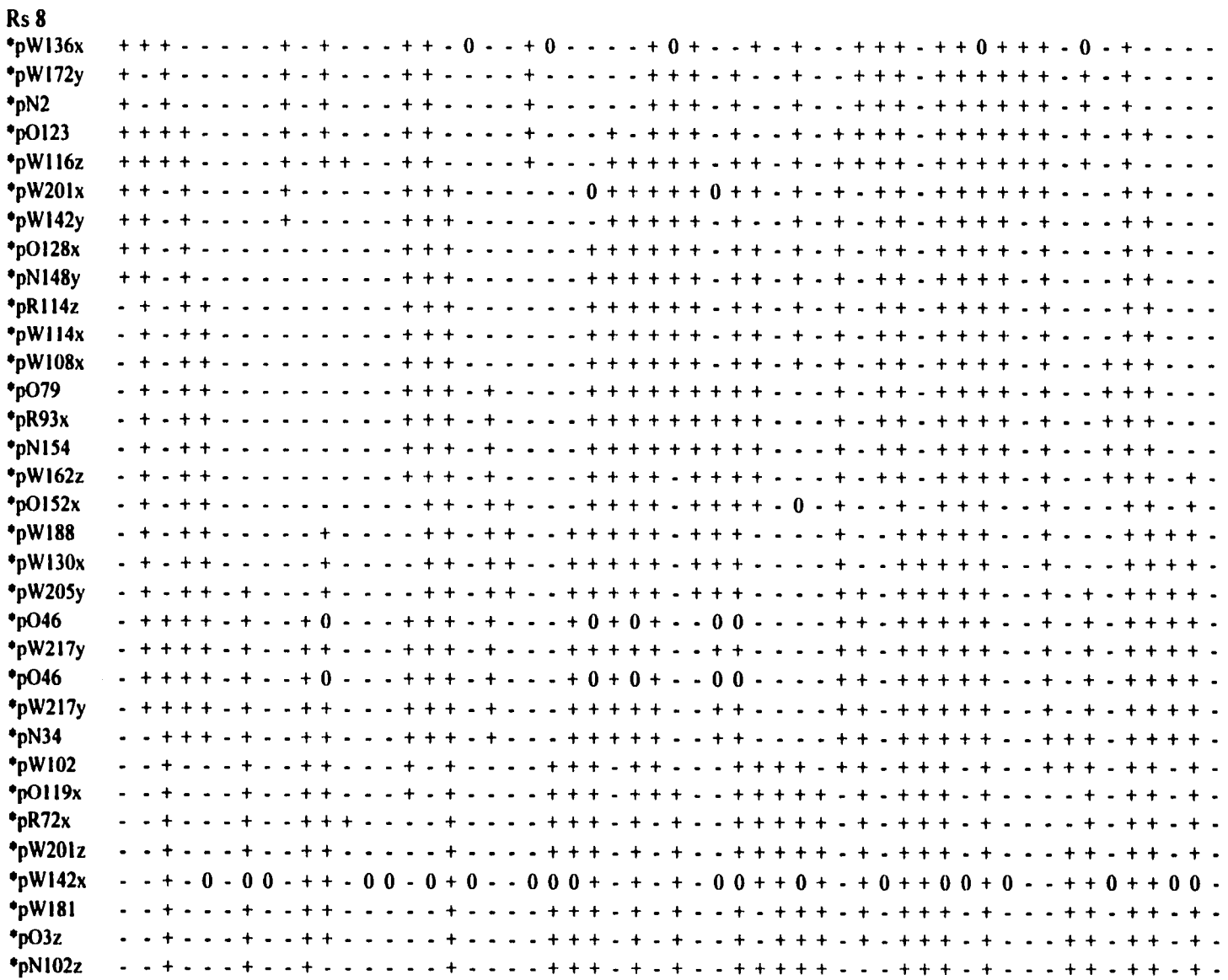

Rs 4
*pW116x + + - + + + - - + + + + - + + - + + - + + - + - + - + + + + + + + + - + + + - + - + + - - + + + + + - + +
*pN3y + + - + + + - - + + + + - + + - + + - + + - + - + - + + + + + + + + - + + + - + - + - - - + + + + + - - +
*pW153y + + - + + + - - + + + + - + + - + + - + + - + + - + - + - + + + + + + + + - + + + - + - + - - - + + + + + - - +
*pW200y + + - + + + - - + + + + - + + - + + - + + - + + - + - + - + 0 + + + + + - + + + - + - 0 - - - 0 + + + + - - +
*pN121x + + - + + + - - + + + + - + + - + + - + + - + + - + - + - + + + + + + + + - + + + + + - + - - - + + + + - - +
*pO7y + + - + + + - - + + + + - + + - + + - + + - + + - + - + - + + + + + + + + - + + + + + - + - - - + + - + + - - +
*pO118 + + - + + + + - - + + + + - + + - + + - + + 0 + - + 0 + + + + + + + + - + + + + + - 0 - - - 0 + - + - - - +
*pN102x + + - + + + + - - + + - + - + + - + + - + + + + - + - + + + + + + + + - - + + + + + - + - - - + + - + - - - +
*pO111x + + - + + + + - - + + + + - + + - + + - + + + + - + - + + + + + + + + - - + + + + + - + - - - + + - + - - - +
*pO160x + + - + + + + - - + + + + - + + - + + - + + + + - + - + + + + + + + + - - + + + + + - + - - - + + - + - - - +
*pN180y + + - + + + + - - + + + + - + + - + + - + + + + - + - + + + + + + + + - - + + + + + - + - - - + + - + - - - +
*pN174z + + - + + + + - - + + + + - + + - + + - + + + + - + + + + + + + + - + + - - + + + + + - + - - - + + - + - - - +
*pW152z + + - + + + + - - + + + + - + + - + + - + + + + - + + + + + + + + - + + - - + + + + + - + - - - + + - + - - - +
*pW144 + - - + + + + - - + + + + - + + - + + - + + + + - + + + + + + + + + - - + + + + + - + - - - + + - + - - - +
*pO170v + - - + + + + - - + + + + - + + - 0 + - + + + + - + + + + + + + + + - - + + + + - - 0 - - 0 0 0 - + - - - +
*pR115z + - - + + + + - - + + + + - + + - + + - + + + + - + + + + + + + + + - - + + + + - - + - - - + + - + - - - +
*pW162x + - - + + + + + - + + - - - + + - + + - + + + + - + + + + - - + + + - - + + + + - - + - - - - + - + - - - +
*pN120y + - - + + + + + - + + - - - + + - + + - + + + + - + + + + - - + + + - - + + + + - - + - - - - + - + - - - +
*pN220z + - - + + + + + - + + - - - + + - + - - + - + + - + + + + - - + + + - - + + + + - + - - - - - + - + - - - +
*cA36z + - - + + + + + - - + - - - - - + + + - - - - + + - + + + + - - + + + - - + + + + - + - - - + - + - + - - - +
*pN53y + - - + + + + + - - + - - - - - + + - - - - - + - - + + + + - - + - + - - + - + + - + - - - + - + - + - - - +
*pW177x + - - + + + - + - - + - - - - - + + 0 - - - - + - + + + + + - - + - + + - + - + + - + - - - + - + - + - - - +
*pW148x + - - - + + - + - - + - - - - - + + - - - - - + - + + + + + - - + - + + - + - + + - + - - - + - + - + - - - 0

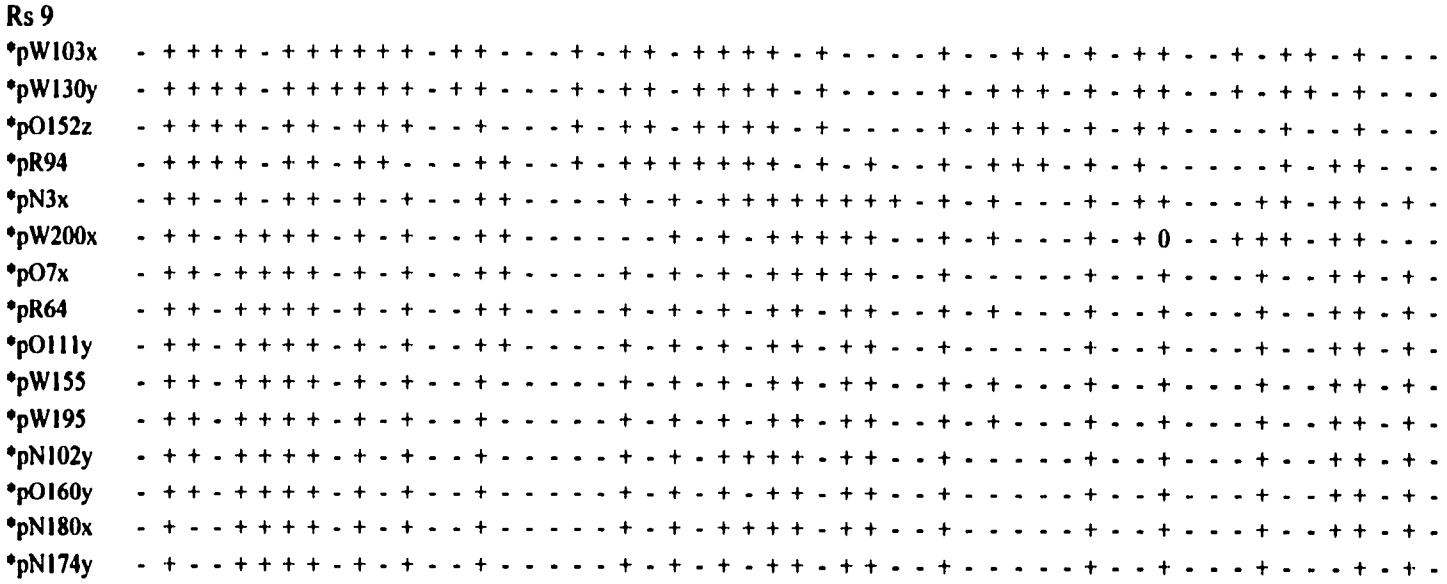
Rs 5


*pW203
*pO150y
*pN173y
*pN107y
*pW233x
*pR3z
*pO125x
*pW137y
*pR114y
*pW167
comp map only
*pO120y
*pN64
*pN101
*pW122y
*pO9
*pR116
*pW200z
*pW148y
*pN3z
*pW179y
*cA142y
*pN181y
*pR34
*pO17y
*pR115y
*pO170w
*pO155

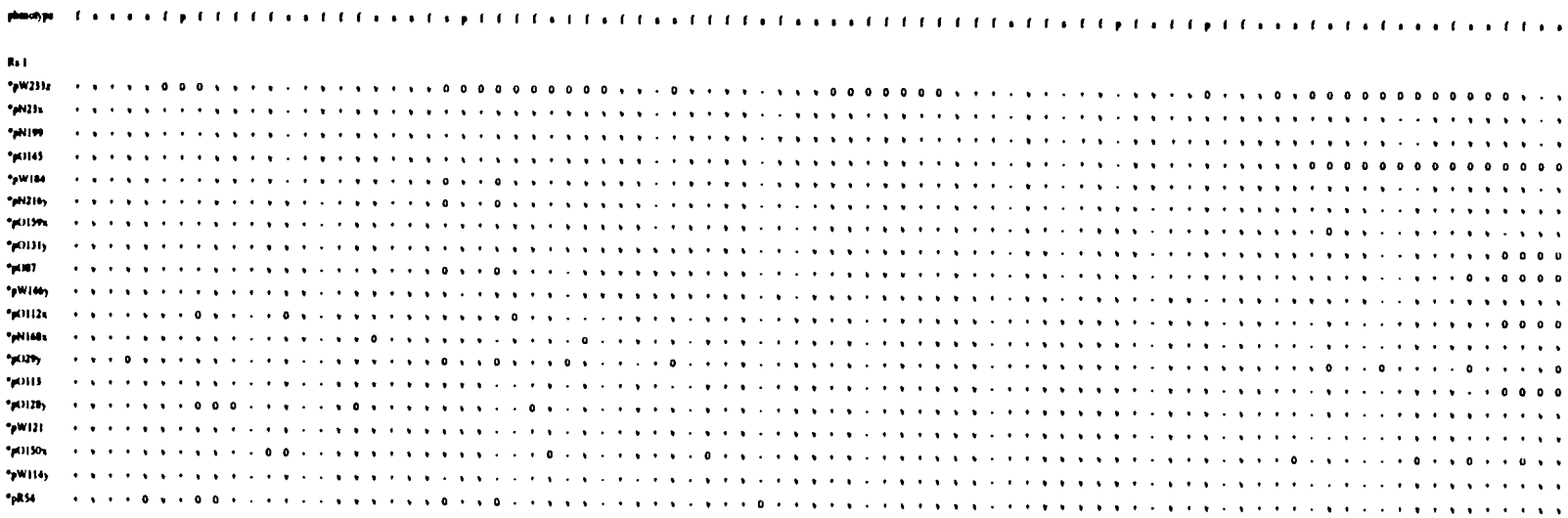

Rs 6
*pN13
*pN194
*pW152x
*pN95
*pW139x
*pN66y
*pN59y
*pN44
*pN151
*pN216w
*pW108y
*pO131x
*pR72v
*pW194
*pW218
*pN184
*pO125
*pW112y
*pO120x
*pO119y
*pN173x
*pW108z
*pR114x
*cA119
*pN148x
*pW201y
*pW216
*pW136y
*pW172x
*pR85

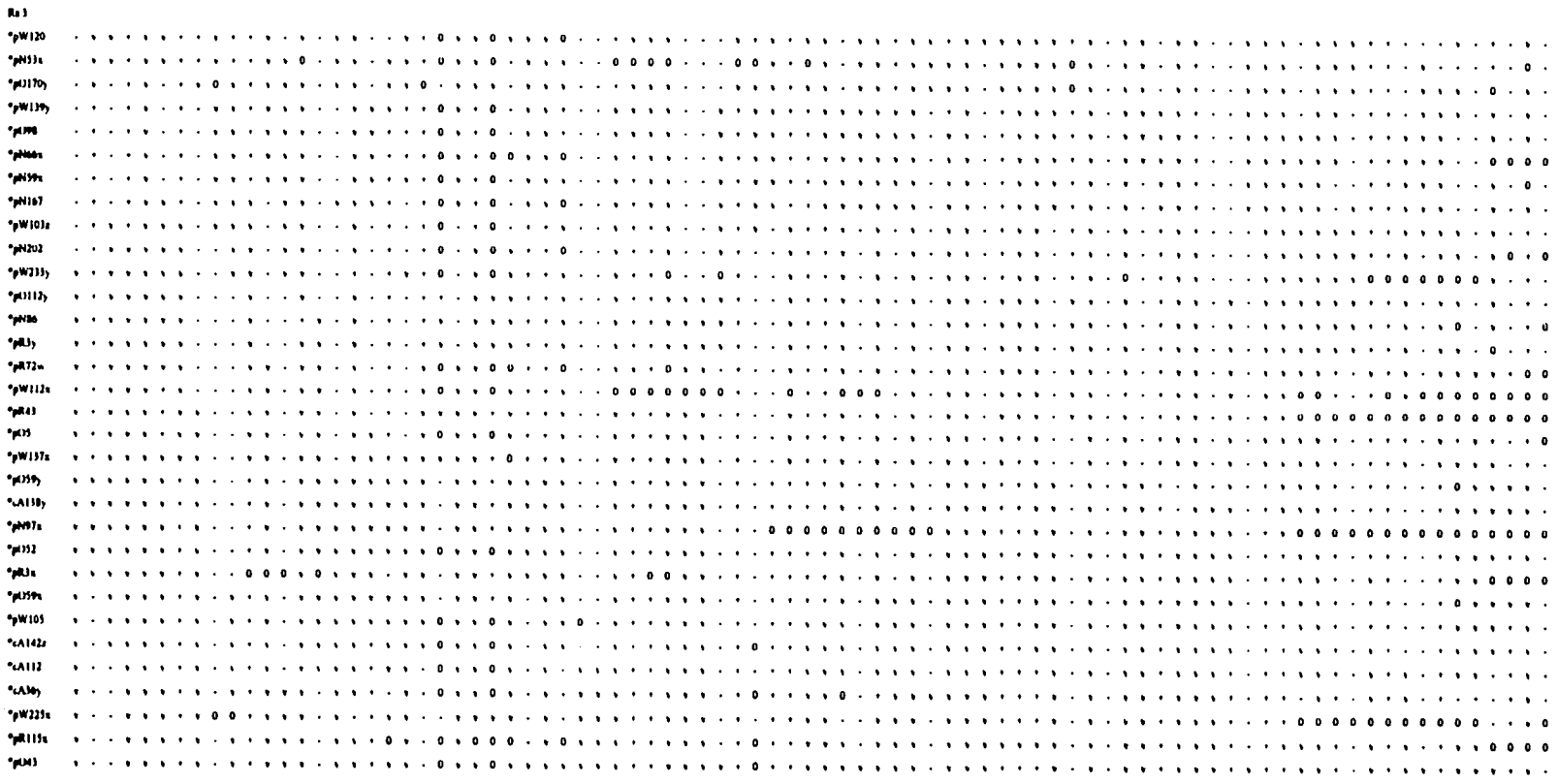

Rs 7
*pW177y
*pN23y
*pO92
*pN52
*pW199
*pO152y
*pO159y
*pN216z
*pO143y
*pO170z
*pW146x
*pR93y
*pN96
*pW162y
*pN97y
*pN168y
*pO85y
*pW217y
*pW115
*pR72z

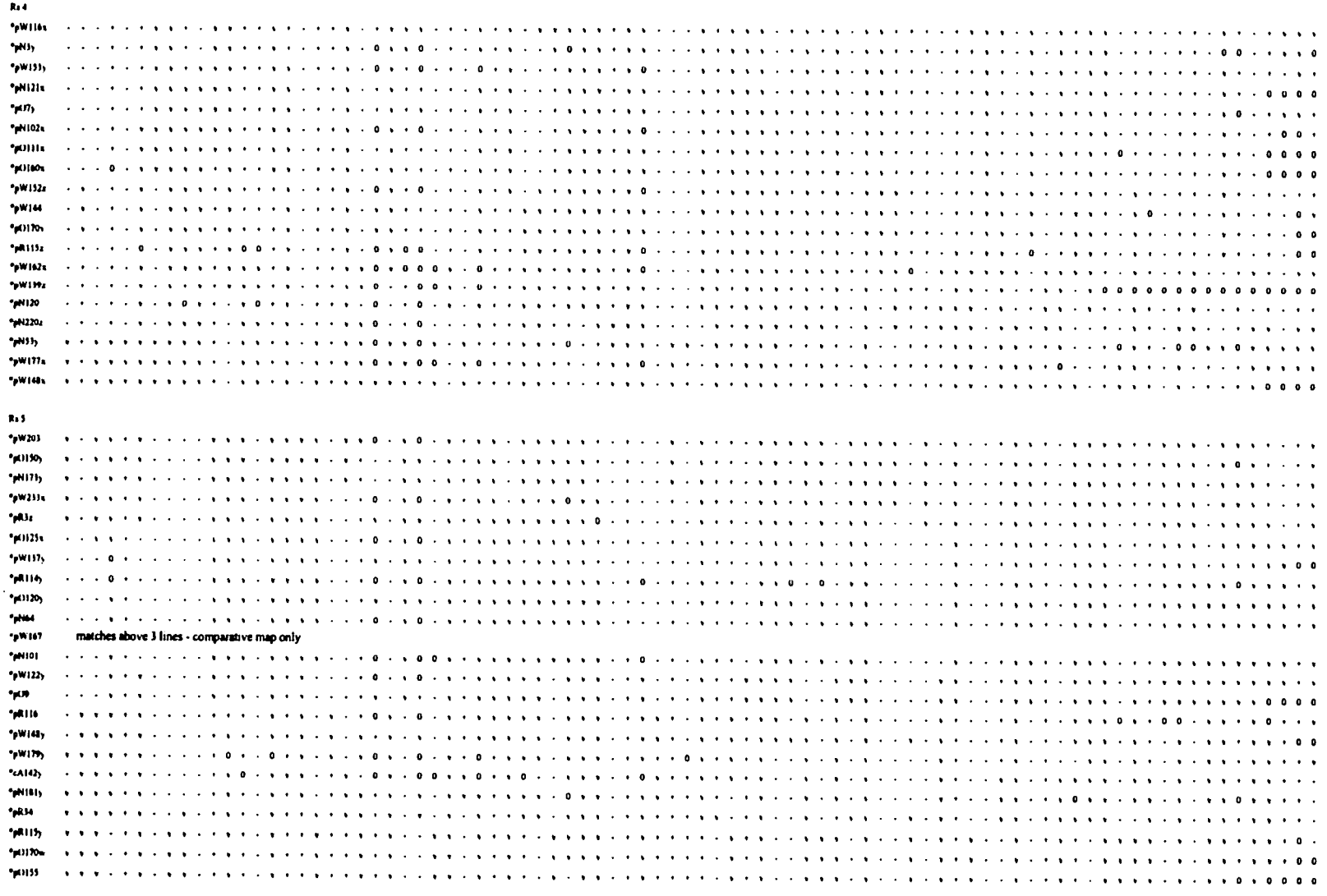

Rs 8
*pW136x
*pW172y
*pN2
*pO123
*pW116z
*pW201x
*pW142y
*pO128x
*pN148y
*pR114z
*pW114x
*pW108x
*pO79
*pR93x
*pN154
*pW162z
*pO152x
*pW188
*pW130x
*pW205y
*pO46
*pW217y
*pO46
*pW217y
*pN34
*pW102
*pO119x
*pR72x
*pW201z
*pW142x
*pW181
*pO3z
*pN102z


Rs 9
*pW103x
*pW130y
*pO152z
*pR94
*pN3x
*pW200x
*pO7x
*pR64
*pO111y
*pW155
*pW195
*pN102y
*pO160y
*pN180x
*pN174y

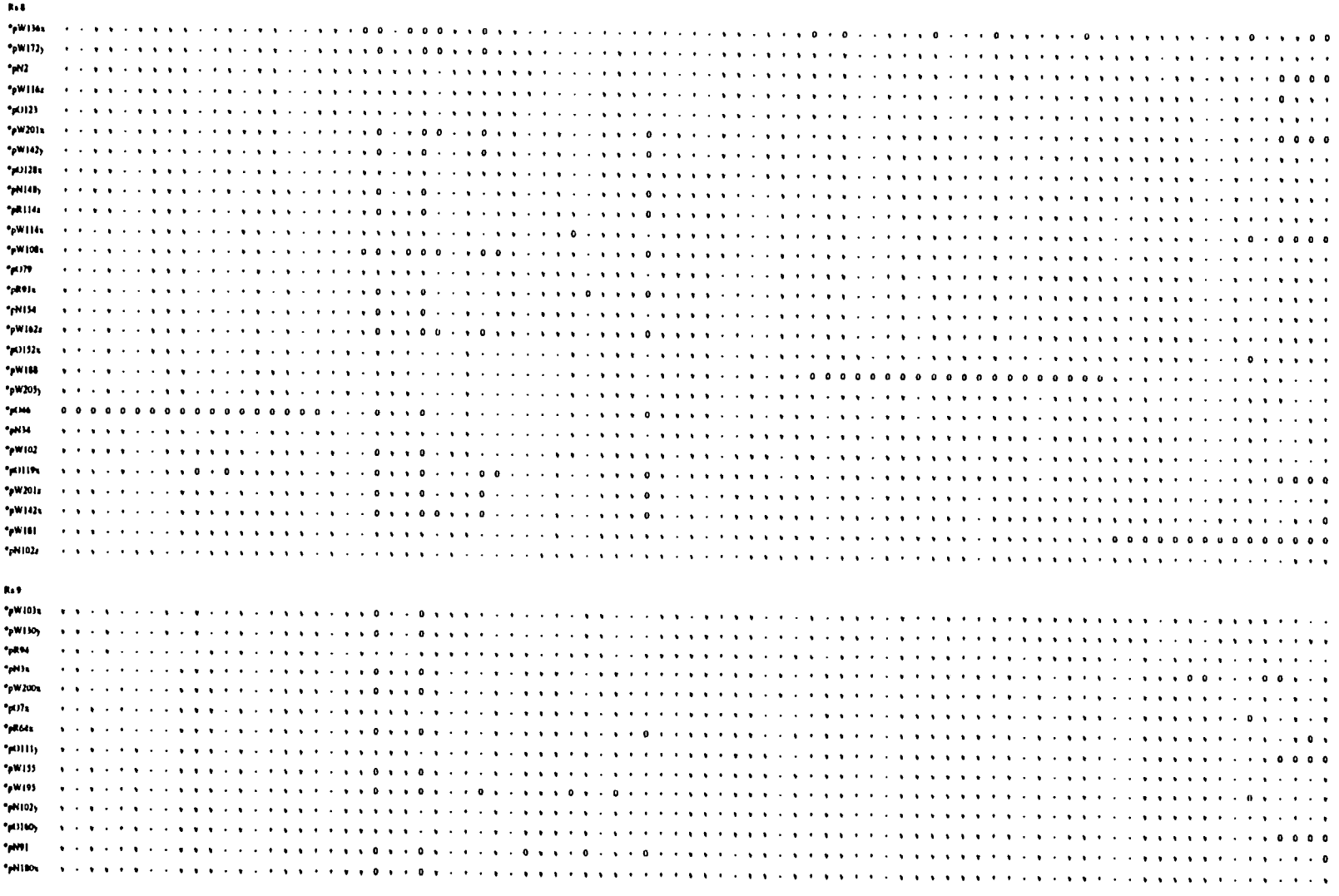
A1.3. The data for the RFLP defined loci scored in the F_2 population. The rows represent the loci and the columns F_2 individuals. Male fertility (f), partial male fertility (p) or sterility (S) phenotype is indicated at the top of the data set. Within the scoring matrix: +, homozygous for the R7-21 parental allele; -, homozygous for the R11-4 parental allele; v, heterozygous (both parental alleles); 0, missing data point.




Rx 3
*pW120
*pN53x
*pU170y
*pW139y
*pU98
*pN66x
*pN59x
*pN167
*pW103z
*pN202
*pW233y
*pU112y
*pN86
*pR3y
*pR72w
*pW112x
*pR43
*pU5
*pW137x
*pU59y
*cA138y
*pN97x
*pU52
*pR3x
*pU59w
*pW105
*cA142z
*cA112
*cA36y
*pW225x
*pR115x
*pU43


Rs 4
*pW116
*pN3
*pW153
*pN121
*pI37
*pN102
*pI3131
*pI3180
*pW152
*pW144
*pI3170
*pR115
*pW162
*pW139
*pN120
*pN220
*pN53
*pW177
*pW148
Rs 5
*pW203
*pI3150
*pN171
*pW233
*pR3
*pI3125
*pW137
*pR116
*pI3120
*pN64
*pW167
matches above 3 lines - comparative map only
*pN101
*pW122
*pI39
*pR116
*pW168
*pW179
*cA142
*pN181
*pR34
*pR115
*pI3170
*pI3155

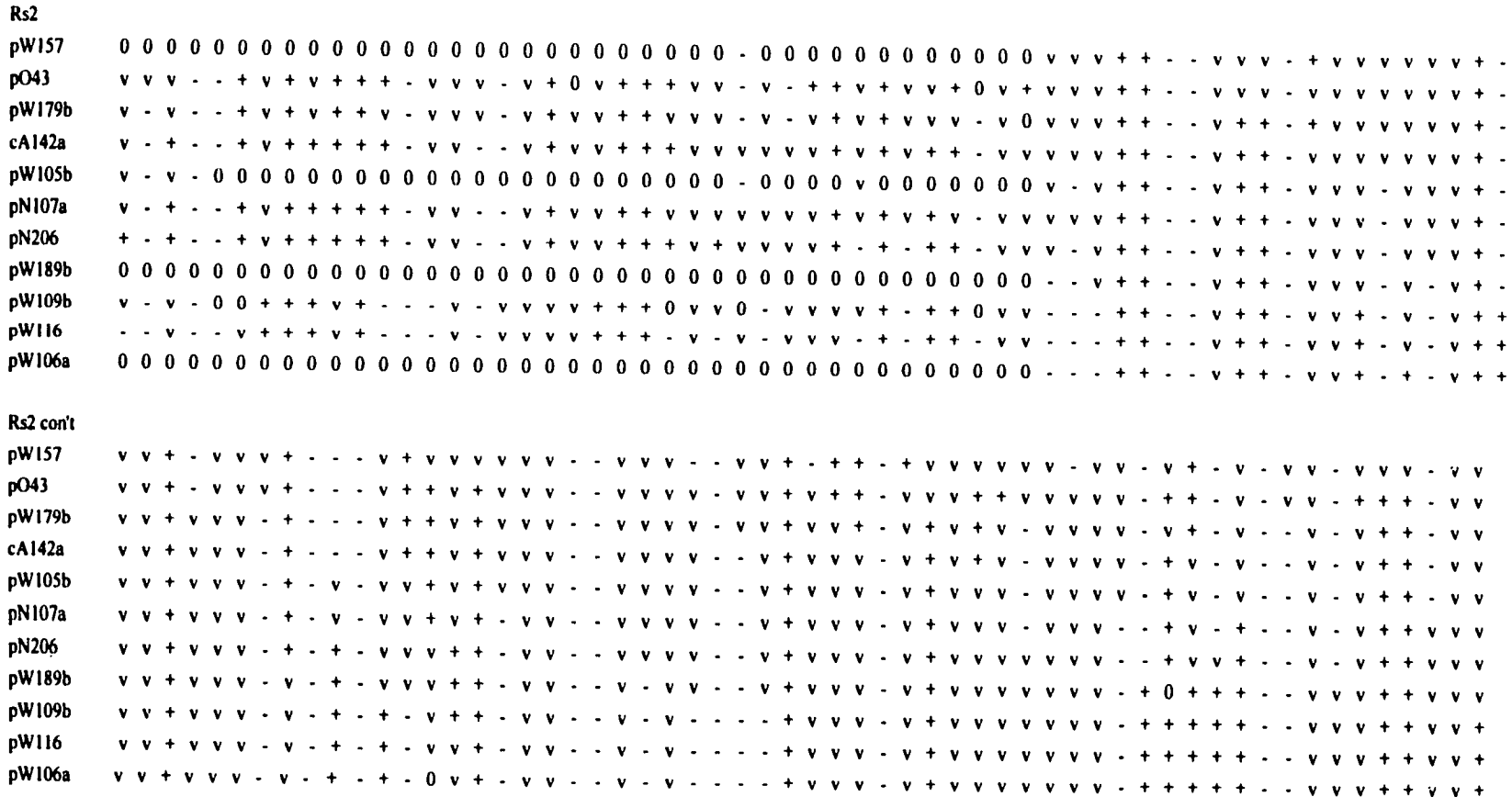
Rs 7	
*pN21x	. , . , . , , , , , , , , 0 , , , . , , 0 0 , , 0 , , , , , , , , , . , . , . . , , , . . , , . 0 , , 0 . . , , , , , . . , , . , , . . , . , , , , , . , , , , , , . 0 0
*pN23y	, . , . , , , , , , , , . , , , . , , , , , , , , , , , , , , . , . , . , . . , , , . . , , . . , , , . . , , , , , . . , , . , , . . , . , , , , , . , , , , , , . . .
*pUJ92	, , . , . , , , , , , , , . , , , . , , , 0 , , 0 , , , , , , , . , . , . . , , , . . , , . . , , , . . , , , , , . , , , . , , . . , . , , , , , . , , , , , , . . .
*pN52x	, , . , . , , , , , , , , . , , , . , , , 0 , , 0 , , , , , , , . , . , . . , , , . . , , . . , , , . . , , , , , . , , , . , , . . , . , , , , , . , , , , , , . . .
*pW199	, , . 0 . , , , , , , , , . , , , . , , , 0 , , 0 0 , , , , , , , , . , . , . 0 , , , . . , , . . , , , . . , , , , , . , , , . , , . . , . , , , , , . , , , , , , . . .
*pUJ152y	, , . , . , , , , , , , , . , , , . , , , , , , , , , , , , , , , , . , . , . . , , , . . , , . . , , , . . , , , , , . , , , . , , . . , . , , , , , . , , , , , , . . .
*pW224	, , . , . , , , , , , , , . , , , . , , , 0 . , 0 , , , , , , , , , . , . , . . , , , . . , , . . , , , . . , , , , , . , , , . , , . . , . , , , , , . , , , , . 0 0 0 0
*pUJ159y	, , . , . , , , , , , , , . , , , . , , , , . , , , , , , , 0 . , , . , . , , . , , , . . , , . . , , , . 0 0 , , , , . , , , , , , . . , . , , , , , . , , , , . , , . .
*pN216x	, , . , . , , , , , , , , . , , , . , , , 0 . , 0 , , , , , , . , , . , . , , . , , , . . , , . . , , , . . , , , , , . , , , , , , . , , . , , , , , . , , , , . , , . .
*pUJ163y	, , . , . , , , , , , , , . , , , . , , , 0 . , 0 . . , , , , . , , . , . , , . , , , . . , , . . , , , . . , , , , , . , , , , , , . , , . , , , , , . , , , , , , , . ,
*pUJ85x	, , . , . , , , , , , , , . , , , . , , , , . , . . , , , , , . , , . , . , . , , , , . , , , . . , , , . . . , , , , , , , , , , , . , , . , , , , , . , , , , , , , , ,
*pW146x	, , . , , , , , , , , , , . , , , , , , , 0 . , 0 0 . , 0 , , . , , . , . , , . , , . . , , , . . , , , , , , , , , , , , , , , , , . , , . , , . , , , , , , , , , , , ,
*pR93y	, , . , , , , , , , , , , . , , , , , , , 0 . , 0 , . , , , , . , , . , . , , 0 , , . . , , , , , , , , . . . , , , , , , , , , , , . , , . , , . , , , , , , , , , , , ,
*pN96	, , . , , , , , 0 0 , , , . , , , , , , , , . , , , . , , 0 0 . , , . , . , , . , , . . , , , , , , , , . . . , , , , , , , , , , , . , , . , . . , , 0 0 , , 0 0 , , , ,
*pW162y	, , . , , , , , , , , , , . , , , , , 0 0 0 . , 0 0 . , 0 , , . , , . , . , , 0 , , . . , 0 , , , , , , . . . , , , , , , , , , , , . , , . , . . , , , , , , 0 , , , , ,
*pN168y	, , . , , , , , , , , , , . , , , , , , , , , , , , . . , , , , , , . , . , . . , , . , , , , , , , , , . . . , , , , , , , , , , , , , , . , . . , , , , , , , , , , , ,
*pUJ85y	, , . , , , , , , , , , , . , , , , , , , , , , , , . . , . , , , , . . , , , , , . . , , , , , , , , , . . . , , , , , , , , , , , , , , . , . . , , , , , , , , , , , ,
*pW217y	, , . , , , , , , , , , , . , , , , , , , 0 , , 0 , . . , . , , , , . , , , , . , , . , , , , , , , , , . . . , , , , , , , , , , , , , , . , . . , , , , , , , , , , , .
*pW115x	, , . , , , , , , , , , , . , , , , , , , , , , , , . . , . , , , , . . , , , , , , . , , , , , , , , , . . . , , , , , , , , , , , , , , . , . . , , , , , , , , , , , .
*pR72x	, , . , , , , , , , , , , . , , , , , , , 0 , , 0 , . . , . , , , , . . , , , , , . . , , , , , , , , , . . . , , , , , , , , , , , , , , . , . . , . , , , , , , , , , .

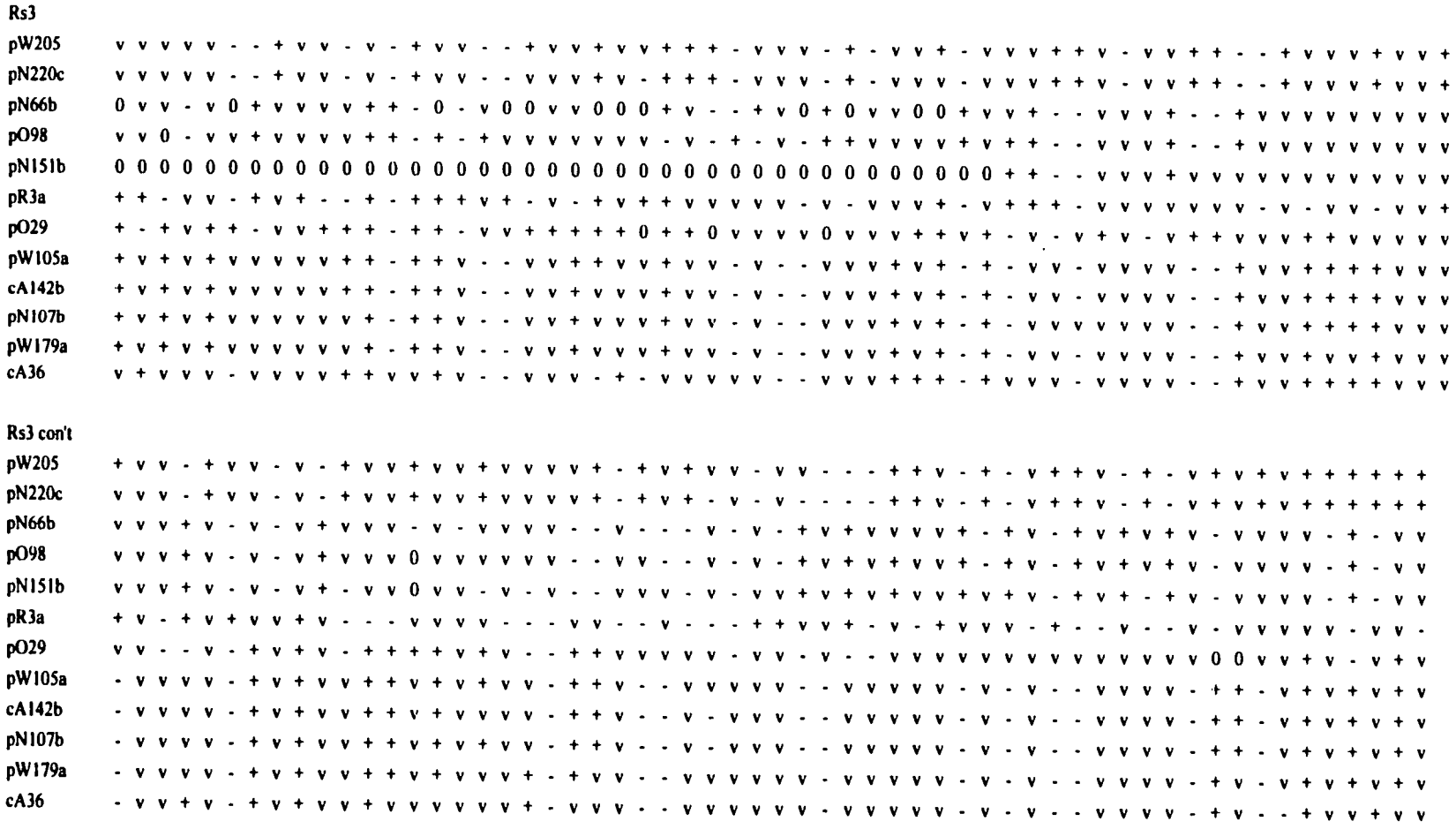

Rs 8
*pW136x
*pW172y
*pN2
*pW116z
*pU123
*pW201x
*pW142y
*pU128x
*pN148y
*pR114x
*pW114x
*pW108x
*pU79
*pR91x
*pW154
*pW162z
*pU152x
*pW188
*pW205y
*pM46
*pN34
*pW102
*pU119x
*pW201z
*pW142x
*pW181
*pN102z
Rs 9
*pW103x
*pW130y
*pR94
*pN3x
*pW200x
*pU7x
*pR64x
*pU111y
*pW155
*pW195
*pN102y
*pU116Oy
*pN91
*pN180x

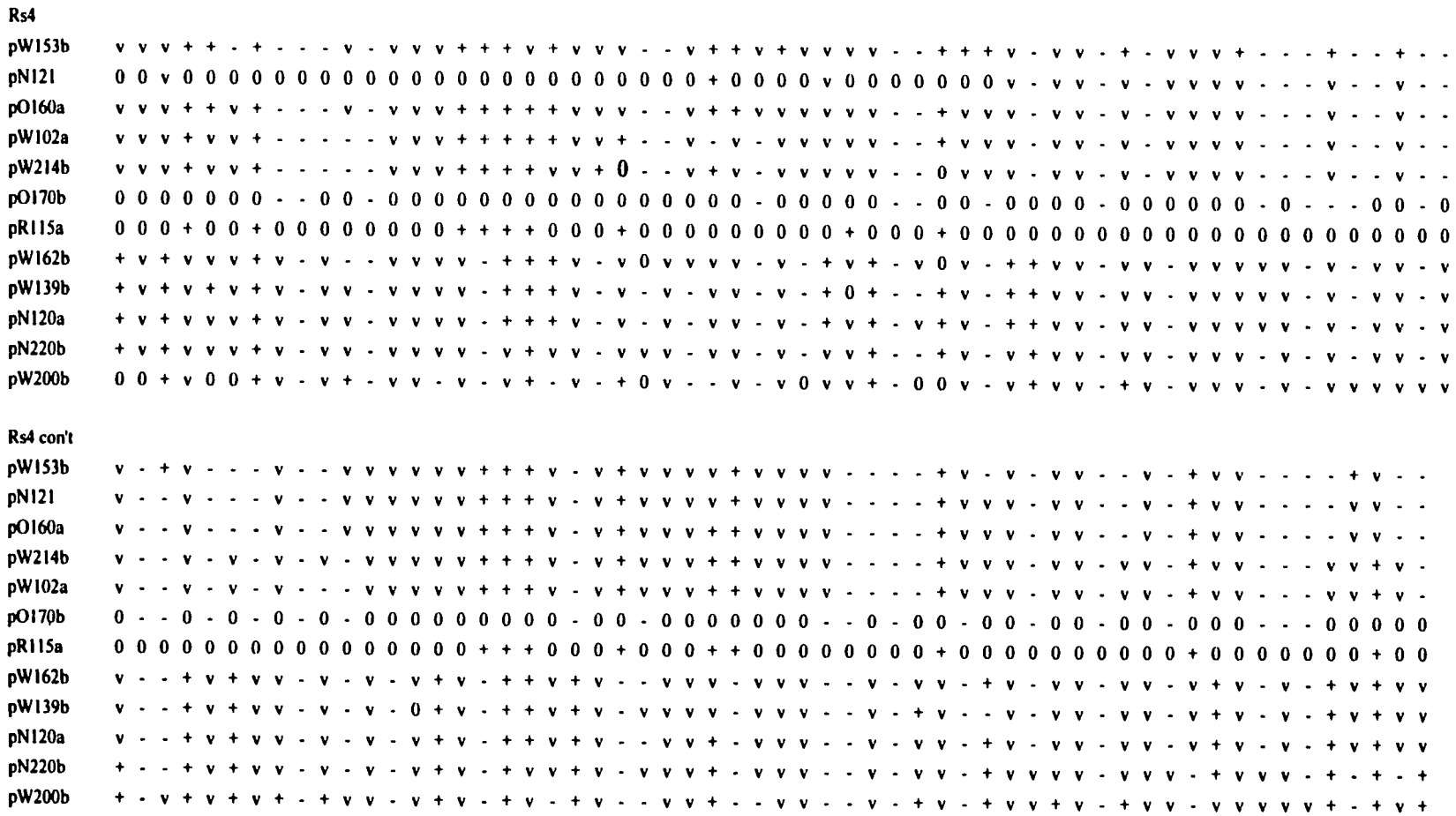
A1.4. The data for the RFLP defined loci scored in the R8 population. The rows represent the loci and the columns R8 individuals. Male fertility (f), partial male fertility (p) or sterility (S) phenotype is indicated at the top of the data set.
Within the scoring matrix: +, homozygous for one parental allele within the likage group; -, homozygous for the other parental allele within the linkage group; v, heterozygous (both parental alleles); 0, missing data point.

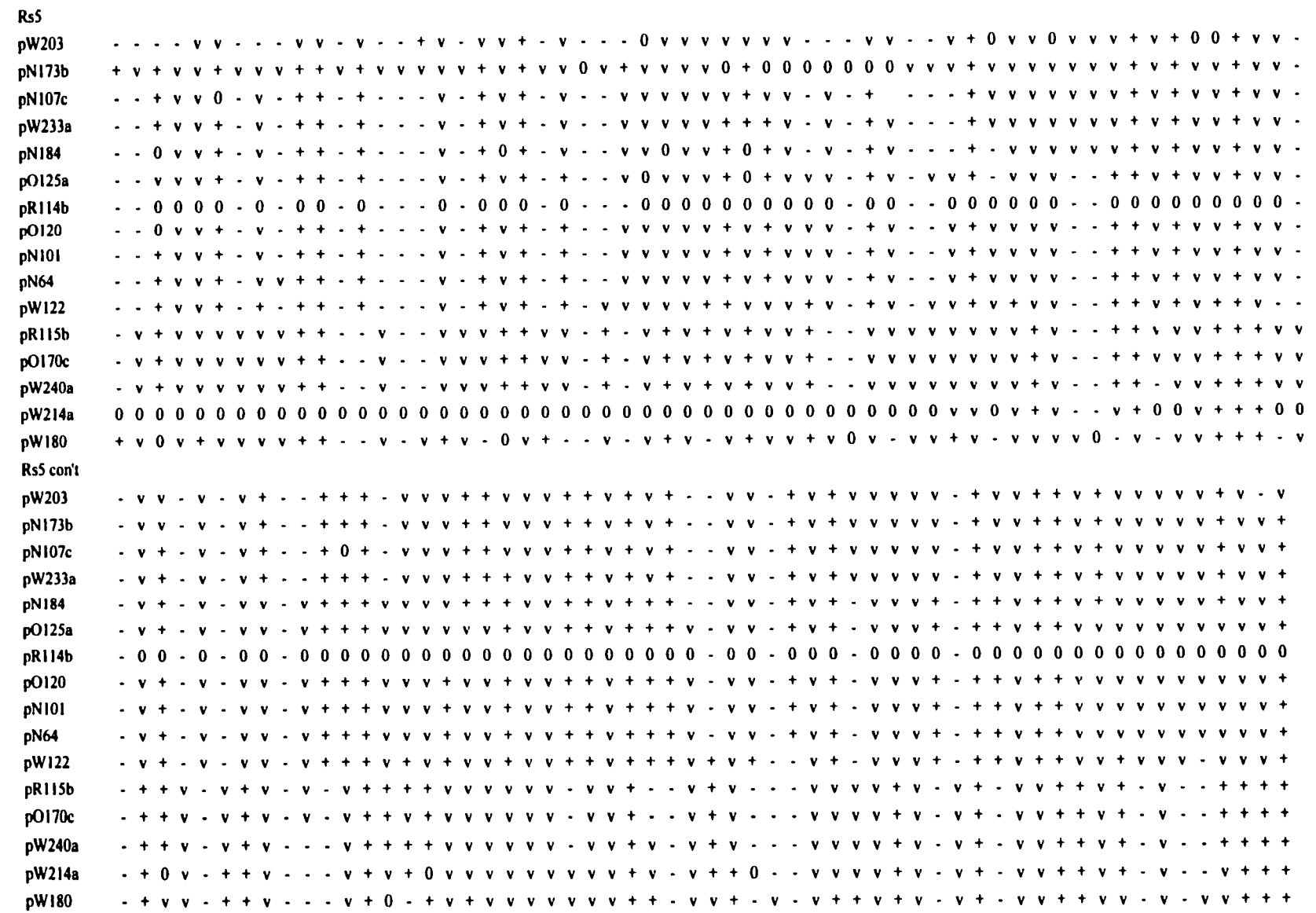
phenotype	f s s s s f s f f f f s s s f s s s s s s s f s s s s s s s s s f s s f s f f f f s p f s s f f f s s p s s s s s s s
Rs1	
pW148	+ v v v + v - + + v + v + + v v - v + v - - v - - - + - + - - v v + v v 0 v + v v - + + v - v + + - - v v - - - v - +
pW177b	+ v v v + v - + + v + v v + + v - v + v - - v - - v + - + - - v v + v v + v + v v - + + v - v + + - - v v v - v v - +
pN23a	+ v v v + v - + + v + - + + + v - v + v - - v - - v + - + - - v v + v v + v + v v - + + v - v + + - - v v v - v v - +
pO165	+ v v v + v - + + v + - + + + v - v + + - - v - - v + - + - - v v + v v + v + v v - v + v - v + + - - v v v - v v - +
pN199a	+ v v v + v - + + v + - + + + v - v + v - - v - - v + - + - - v v + v v + v + v v - v + v - v + + - - v v v - v v - +
pO145a	+ v v v + v - + + v + - + + + v - v + v - - v - - v + - + - - v v + v v + v + v v - v + v - v + + - - v v v - v v - +
pN216c	+ v v v + v - + + v v - + + + v - v + v - - v 0 - + + - + - - v v + v v + v + v v - v + v - v + + - - v v v - v v - +
pO143b	+ v v v + 0 - + + v v - + + v v - v - v - - v - - + + - + - - v v + v v + v + v v - v v - - v + + - - v v v - v v - +
pO131b	+ v v v + v - + + v v - + + v v - v - v - - v - - v + v + - - v v + v v + v + v v - v v - - v + + - - v v v - v v - +
pO87	+ v v v - - - v + v v - v + v + - v v v - - v v v + + v v v - v v + v + v v + v v - v v - - v v v - - v - v - v v - +

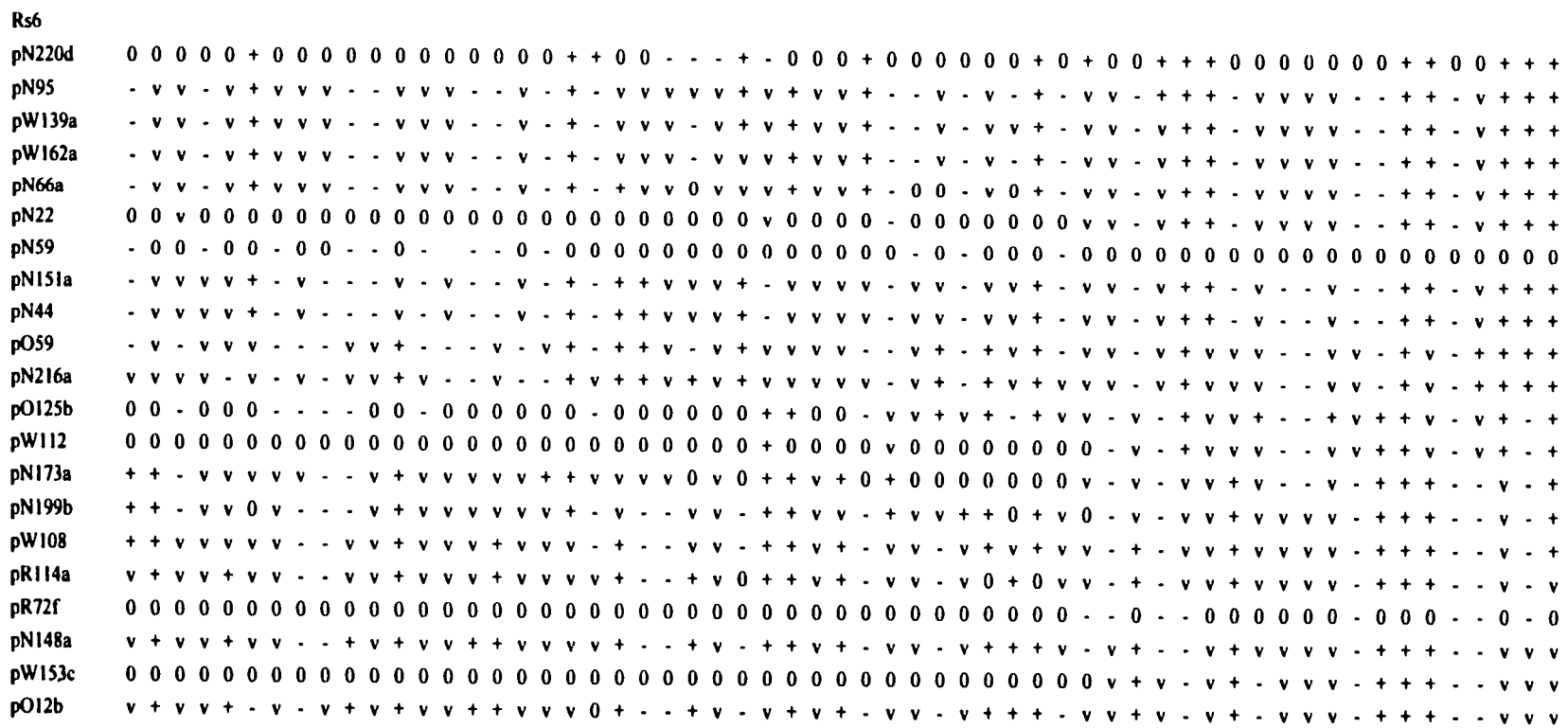
	f s s f f s s s s s s f f p s f f f s s s f s f s s s f f s f s s f f s s s s s f f s s s s s s s s s s s f s s s s
Rs1 con't	
pW148	+ + - v + + - - - v v + v v v v + + - v - + v v - v - v + v v - - v + v - - v - + + - v - + - - - + v - - v - v - -
pW177b	+ + - v + + - - - v v + v v v v + + - v - + v v - v - v + v v - - v + v - - v - + + - v - + - - - + v - - v - v - -
pN23a	+ + - v + v - - - v v + v v v v + + - v - + v v - v v v + v v - - v + v - - v - + + - v - + - - - + v - - v v v - -
pO165	+ + - v + v - - - v v + v v v v + + - v - + v v - v v v + v v - - v + v - - v - + + - v v + - - - + v - - v v v - -
pN199a	+ + - v + - - - - v v + v v v v + + - v - + v v - v - v + v v - - v + v - - v - + + - v - + - - - + v - - v v v - -
pO145a	+ + - v + - - - - v v + v v v v + + - v - + v v - v - v + v v - - v + v - - v - + + - v - + - - - + v - - v v v - -
pN216c	+ + - v + - - - v v v + v v v v + + - v - + v v - v v v + v v - - v + v - - v - + + - v - + - - - + v - v - v v - -
pO143b	+ + - v + - v - v v v + v v v v + + - + - + v v - v v v + v v - - v + v - - v - + + - v - + - - - + v - - - v v - -
pO131b	+ + - v + - v - v v 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 0 0 - 0 - + + - v - + - - - + v - - - v v - -
pO87	+ + - v v - v - + v v v v v v v v + - v v + v v v v v + v v v - - + + - - - v - + - - v - + - v v + v - - - v + - -

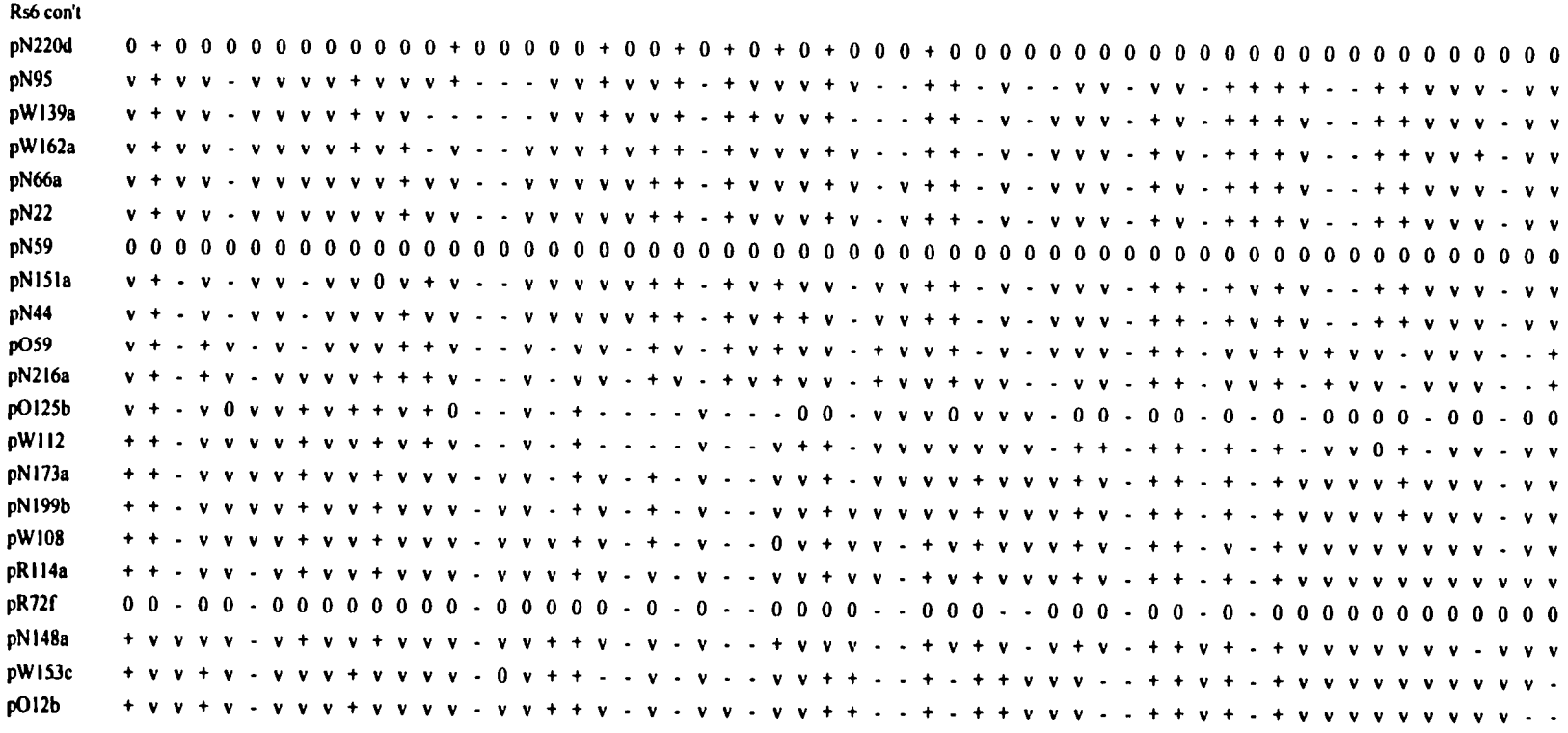

Rs2
pW157
pO43
pW179b
cA142a
pW105b
pN107a
pN206
pW189b
pW109b
pW116
pW106a
Rs2 con't
pW157
pO43
pW179b
cA142a
pW105b
pN107a
pN206
pW189b
pW109b
pW116
pW106a


Rs3
pW205
pN220c
pN66b
pO98
pN151b
pR3a
pO29
pW105a
cA142b
pN107b
pW179a
cA36
Rs3 con't
pW205
pN220c
pN66b
pO98
pN151b
pR3a
pO29
pW105a
cA142b
pN107b
pW179a
cA36

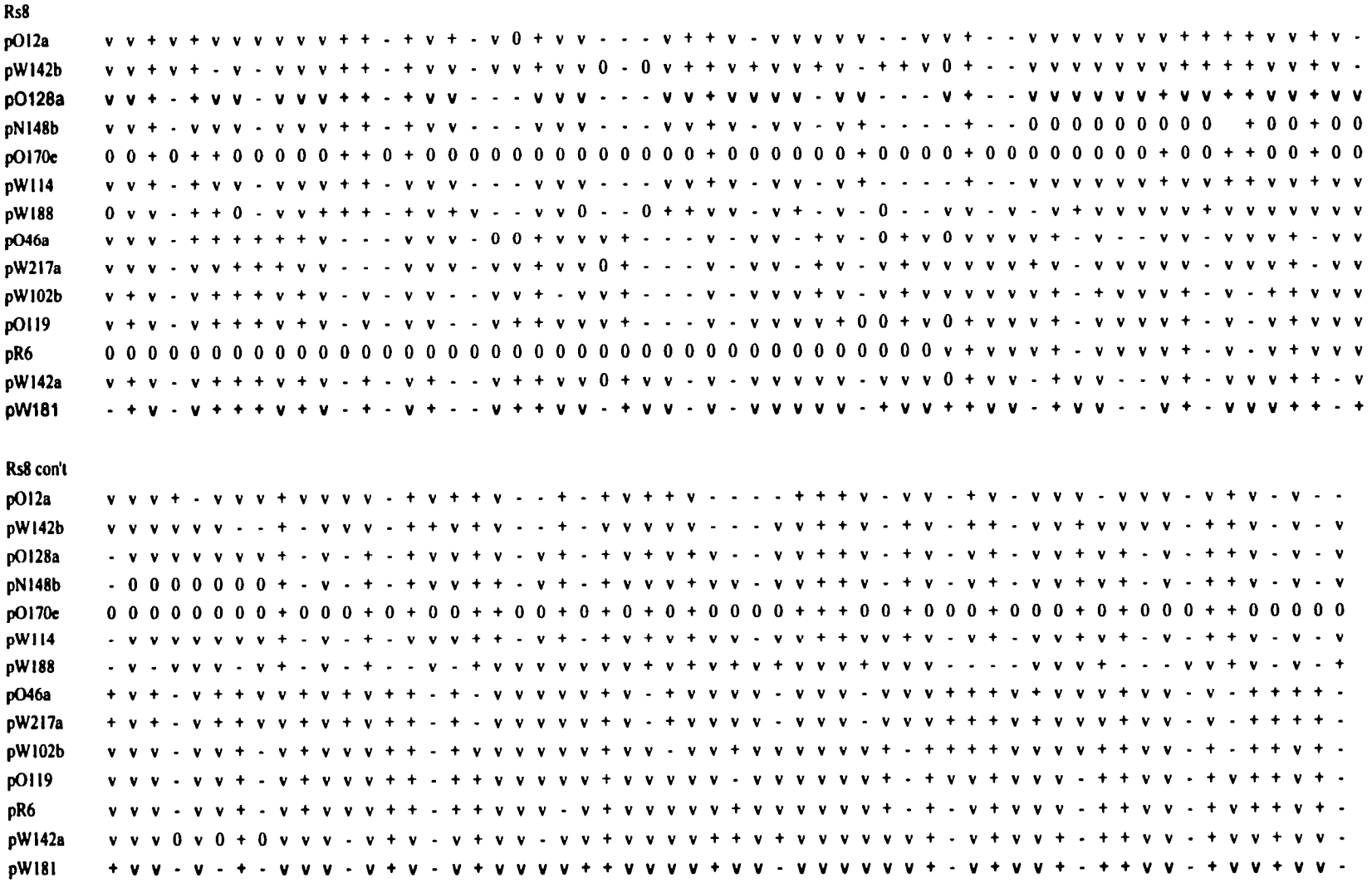

Rs4
pW153b
pN121
pO160a
pW102a
pW214b
pO170b
pR115a
pW162b
pW139b
pN120a
pN220b
pW200b
Rs4 con't
pW153b
pN121
pO160a
pW214b
pW102a
pO170b
pR115a
pW162b
pW139b
pN120a
pN220b
pW200b

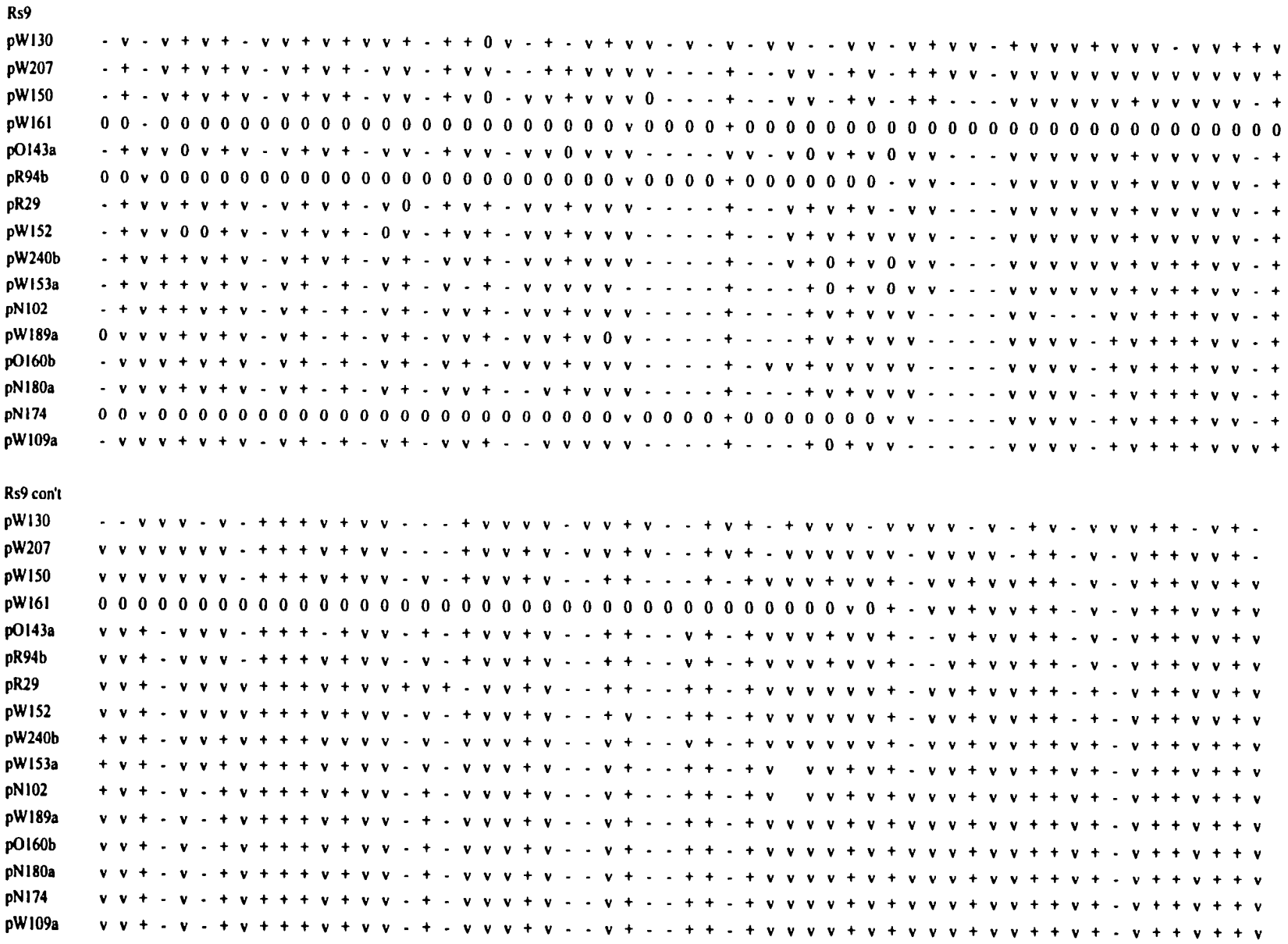

Rs5
pW203
pN173b
pN107c
pW233a
pN184
pO125a
pR114b
pO120
pN101
pN64
pW122
pR115b
pO170c
pW240a
pW214a
pW180
Rs5 con't
pW203
pN173b
pN107c
pW233a
pN184
pO125a
pR114b
pO120
pN101
pN64
pW122
pR115b
pO170c
pW240a
pW214a
pW180


Rs6
pN220d
pN95
pW139a
pW162a
pN66a
pN22
pN59
pN151a
pN44
pO59
pN216a
pO125b
pW112
pN173a
pN199b
pW108
pR114a
pR72f
pN148a
pW153c
pO12b


Rs6 con't
pN220d
pN95
pW139a
pW162a
pN66a
pN22
pN59
pN151a
pN44
pO59
pN216a
pO125b
pW112
pN173a
pN199b
pW108
pR114a
pR72f
pN148a
pW153c
pO12b

Rs7	
pW177a	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 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 v v v + v v v v v + v v + + - v + v v
pW145a	v + v v + v + v + - v + v + - v v + v v + + v - + v v v + v - + + v v - + v - v - - v v v v v v v + v v + + v v + v +
pN216b	v + v v v v v v + - v + v + - v v + v v + + v - + v v v + v - + v v v - v v - v - - v v v v v v v + v v + + v v + v +
pO159b	v + v v v v v v + - v + v + - v v + v v + + v - + v 0 v + v - + v v v - v v - v - - v v v v v v v + v v + + v v v v +
pN168	0 0 v 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 v 0 0 0 0 + 0 0 0 0 0 0 0 v - - + - v v v v v + v - + v v v - - +
pR34	0 0 v 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 v 0 0 0 0 + 0 0 0 0 0 0 0 v - - + - v v v v v + v - + v v v - - v
pO85	v + v v + + v - + - + + - - - v - v v - - + v v v - - v v - - + + v + + v v v v - - + - v v v v v + v - + v v v - - v
pW217b	v + v v v + v - + - + + - - - v v v v - - + v v v - - v v - - + + v + + v v v v - - + - v v v v v + v - + v v v - - v
pW115	0 0 v 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 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 0 0 0 0
pR72e	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 0 - 0 0 0 0 + 0 0 0 0 0 0 0 v - - v - v v v v v + v - v v v v - - v
Rs7 con't	
pW177a	v + - v - v - - v v v v v v v v v v v - - v + v v v v v v - - - + v v + v v 0 v v v v + v + + + + - v - + - + v - v
pW145a	v + - v - v - v + v v v v v v v v v v - - v + v - v v v v - - - + v v + v v v - v v - v v + + + v - v - + - + - - v
pN216b	v + - v - v - v + v v v v v v v v v v - - v + v - v - v v - - - + v v + v v v - v v - v v + + + v - v - + - + - - v
pO159b	v + - v - v - v + v v v v v v v v v v - - v + v - v - v v v v - + v v - v v v - v v - v v + + + v - v - + - + - - v
pN168	v - + v v + - + v + 0 0 - - 0 - 0 0 0 - - + 0 - - 0 + 0 0 + - 0 + 0 - + + v + v v + v v v v v - + v v v + v v - v +
pR34	v - v v v v - + v + v + - - - 0 v v v - - + + - - v v v v + - v v v - + + v + v + + v v v + v - v v v v + v v - v +
pO85	v - v v v v - + v + v + - - - - v v v - - + + - - v v v v + - v v v - + + v + v + + v v v + v - v v v v + v v - v +
pW217b	v - v v v v - + v + v + - - - - v v v - - + + - - v + v v + - v v v - + + v + v + + v v v + v - v v v v + v v v v +
pW115	0 0 0 0 0 0 0 0 0 0 v + - - - - v v v - - + + - - v v v v v - v v - - v + v + + + + v v v + v - v v v v + v v v v +
pR72e	v - + v v v - + v + v + - - - - v v v - - + + - - v v v v v - v v - - v + v + + + + v v v + v - v v v v v v - v v +


Rs8
pO12a
pW142b
pO128a
pN148b
pO170e
pW114
pW188
pO46a
pW217a
pW102b
pO119
pR6
pW142a
pW181
Rs8 con't
pO12a
pW142b
pO128a
pN148b
pO170e
pW114
pW188
pO46a
pW217a
pW102b
pO119
pR6
pW142a
pW181


Rs9
pW130
pW207
pW150
pW161
pO143a
pR94b
pR29
pW152
pW240b
pW153a
pN102
pW189a
pO160b
pN180a
pN174
pW109a
Rs9 con't
pW130
pW207
pW150
pW161
pO143a
pR94b
pR29
pW152
pW240b
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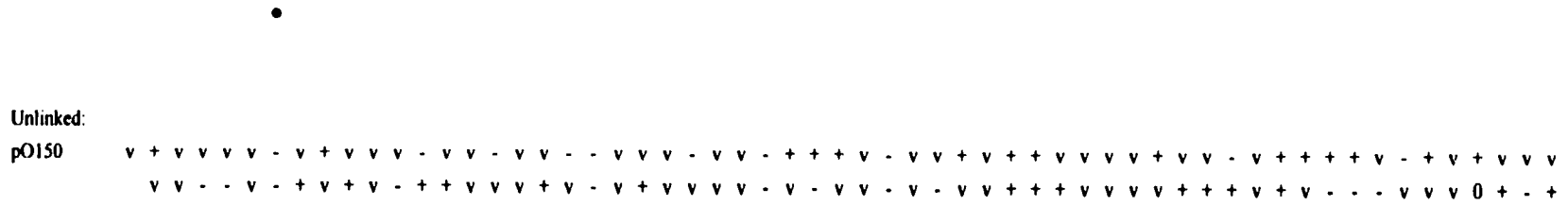
pN102
pW189a
pO160b
pN180a
pN174
pW109a


Unlinked:
pO150
v + v v v v - v + v v v - v v - v v - - v v v - v v - + + + v - v v + v + + v v v v + v v - v + + + + v - + v + v v v
v v - - v - + v + v - + + v v v + v - v + v v v v - v - v v - v - v v + + + v v v v + + + v + v - - - v v v 0 + - +