INHERITANCE AND LINKAGE OF MORPHOLOGICAL, ISOZYME AND RAPD MARKERS IN GRASSPEA

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of

Doctor of Philosophy

in the

Department of Crop Science and Plant Ecology

University of Saskatchewan

By

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Saskatoon, Saskatchewan, Canada

Spring, 1997

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfilment

of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

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Fall 1996

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INHERITANCE AND LINKAGE OF MORPHOLOGICAL, ISOZYME AND RAPD MARKERS IN GRASSPEA

Experiments were designed to determine the outcrossing rate, to detrmine the inheritance of markers and to establish a basic linkage map in grasspea. The outcrossing rate in grasspea ranged from 1.7 to 2.7% among the eight combinations of planting method and location. The outcrossing rate in this study (2.2±0.6%) suggests that individual lines of grasspea should be maintained in isolation to maintain their genetic integrity. As outcrossing rate may vary considerably among different genotypes, further experiment should be done with different flower colour genotypes, different genotypes of the same flower colour and different loci. The appropriate isolation distance for grasspea should be determined.

The inheritance and linkage was determined for one morphological, 11 isozyme and 72 RAPD markers from five crosses. The inheritance of flower color was monogenic. The isozymes, ACO-1, ACO-2, AAT-1, AAT-2, EST-6, FDH, LAP-1, PGD-2, SKDH and TPI-1, showed codominant expression with monogenic inheritance, though LAP-1 and PGD-2 showed distorted segregation in the crosses PI 426891.1.3 x PI 283564c.3.2 and PI 426891.1 x PI 172930.4, respectively. The distortion was likely due to linkage of these loci to factors affecting viability. The isozyme Est-3 showed monogenic inheritance with dominant expression. All RAPD markers, except $UBC368_{425/655}$ segregated in a 3:1 ratio with band dominant over no band. The RAPD markers $UBC304_{831}$, $UBC304_{964}$, $UBC322_{1432}$, $UBC328_{831}$, $UBC332_{1118}$, $UBC332_{1581}$, $UBC333_{617}$, $UBC349_{752}$, $UBC365_{1013}$ and $UBC388_{459}$ showed distorted segregation. Marker $UBC368_{425/655}$ segregated in a co-dominant fashion.

From four crosses (PI 283564c.3 x PI 426885.2, PI 358601.5 x PI 173714.5, PI 426891.1

x PI 172930.4, PI 283549a.6 x PI 202803a.3), which included only a few (≤6) polymorphic loci, only two, PI 283564c.3 x PI 426885.2 and PI 358601.5 x PI 173714.5, established a linkage between AAT-2 and SKDH. The cross PI 426891.1.3 x PI 283564c.3.2, which included one morphological, three isozyme and 71 RAPD markers established 14 linkage groups including 69 markers (1 morphological, 3 isozyme and 65 RAPD markers). Seven of these linkage groups contained at least 5 markers and are assumed to represent the seven chromosomes of the grasspea genome (n=7). The total genome length covered by 75 markers (69 linked and six unlinked) is about 929 cM and covered about 67% of the grasspea genome.

Considering cost, simplicity and abundance of markers, RAPD is more efficient than isozyme markers in linkage studies. More RAPD markers should have been included in the linkage study in order to develop a saturated linkage map in grasspea. Inclusion of backcross or F3 progenies in the linkage study would help overcome the precision problem associated with dominant markers like RAPD markers.

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ABSTRACT

Experiments were conducted to determine the outcrossing rate, the inheritance of markers and establish a basic linkage map in grasspea, *Lathyrus sativus* L. The outcrossing rate in a white-flowered line of grasspea ranged from 1.7 to 2.7% among eight combinations of gene frequency and location. The outcrossing rate in this study (2.2±0.7%) suggests that individual lines of grasspea should be maintained in isolation to maintain their genetic integrity.

Inheritance and linkage were determined for one morphological, 11 isozyme and 72 RAPD markers in five F_2 populations (all RAPD markers were in one F_2 population). The inheritance of flower colour was monogenic with colour dominant over white. The isozymes, ACO-1, ACO-2, AAT-1, AAT-2, EST-6, FDH, LAP-1, PGD-2, SKDH and TPI-1, were codominantly expressed with monogenic inheritance. The isozymes LAP-1 and PGD-2 segregated in a non-Mendelian ratios in the crosses PI 426891.1.3 x PI 283564c.3.2 and PI 426891.1 x PI 172930.4, respectively. The isozyme EST-3 was monogenically inherited and dominantly expressed. Most RAPD markers segregated in a 3:1 ratio. Marker $UBC368_{425/655}$ segregated in a co-dominant fashion. The RAPD markers $UBC304_{831}$, $UBC304_{964}$, $UBC308_{990}$, $UBC322_{1432}$, $UBC328_{831}$, $UBC332_{1118}$, $UBC332_{1581}$, $UBC333_{617}$, $UBC349_{752}$, $UBC365_{1013}$ and $UBC388_{459}$ showed distorted segregation.

In two F_2 populations, PI 283564c.3 x PI 426885.2 and PI 358601.5 x PI 173714.5, a linkage between AAT-2 and SKDH was reconfirmed. In the cross PI 426891.1.3 x PI 283564c.3.2, one morphological, three isozyme and 71 RAPD markers

were mapped resulting in the delineation of 14 linkage groups including 69 markers (1 morphological, 3 isozyme and 65 RAPD markers). The total genome length covered by these 75 markers (69 linked and six unlinked) was about 864 cM.

Considering cost, simplicity and abundance, RAPD analysis was more efficient than isozyme analysis in developing linkage map.

ACKNOWLEDGEMENTS

First and foremost, I thank my supervisor, Dr. A.E. Slinkard, for the advice, guidance and assistance he offered me during the course of my study. I am grateful to him for offering me a research assistantship to carry out my graduate study. Thanks also go to each of my committee members; Drs. M. Devine, R.J. Baker, G. Scoles and J.D. Mahon for their suggestions and constructive criticisms. I am indebted to the College of Agriculture, University of Saskatchewan, for granting me the O.M. Elviss Postgraduate Scholarship, the L.H. Hantelman Postgraduate Scholarship, and the Rene Vandeveld Postgraduate Scholarship during my graduate program.

I thank Dr. R. Jain and Peter Eckstein for their technical assistance and help in laboratory operations. My thanks are also extended to my fellow colleagues, particularly C.P. Andrahennadi, for their constant support and accompaniment throughout my graduate program.

I am grateful to my wife, Latifa; son, Emran; and daughter, Samiha for their love and support. Last, but not the least, I am grateful to the rest of my family, particularly my parents whose contribution is the most in my life. I love you mom and dad!

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LIST OF ABBREVIATIONS

Abbreviation	Description
6-PGD (=PGD)	6-phosphogluconate dehydrogenase
AAT	aspartate aminotransferase
ACO	aconitase
BSA	bulked segregant analysis
EST	esterase
FDH	formate dehydrogenase
LAP	leucine aminopeptidase
LOD	log of the odds
MAS	marker assisted selection
NIL	near-isogenic line
ODAP (=BOAA)	β-N-oxalyl-L-α,β-diaminopropionic acid
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RI	recombinant inbreds
SCAR	sequence characterized amplified region
SKDH	shikimate dehydrogenase
TPI	triosephosphate isomerase

1. INTRODUCTION

Grasspea (*Lathyrus sativus* L.), also known as chickling pea, chickling vetch or khesari dhal, is a self-pollinated (Zohary and Hopf 1988), annual, herbaceous legume in the tribe Vicieae (Adan.) de Candolle of the family Leguminosae (Fabaceae). Like faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medikus), chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.), it is an Old World cultigen, and perhaps the first crop domesticated in Europe (Kislev 1989). Grasspea is cultivated in the Mediterranean Basin and in temperate, and tropical countries from the Canary Islands in the West through Germany in the North and Ethiopia in the South, to India and Central Asia in the East (Kislev 1989).

Grasspea has great potential, both as a food and a fodder crop, due to its high nutritional value and its capability to fix large amounts of nitrogen and tolerate poor drainage (Purseglove 1974, Smartt 1976). Even though grasspea has been used as a pulse for a long time, its widespread dissemination and utilization as a forage crop has resulted in little evolutionary progress as a grain crop (Yunus and Jackson 1991), probably due to the presence in the seed of the neurotoxin, β-N-oxalyl-L-α,β-diamino-propionic acid (ODAP), which causes lathyrism, a neuroparalytic disease, in humans and animals. Outbreaks of lathyrism have been reported in Spain (Doughty and Walker 1982), India (Rutter and Percy 1984) and Ethiopia (Haimanot et al. 1990). It has been suggested,

however, that adequate variability is present for selection and development of cultivars with low toxicity (Kaul et al. 1986). Use of grasspea as a pulse has been confined mainly to Bangladesh, Ethiopia, India, Nepal and Pakistan. Its tolerance to poor drainage and its ability to fix large amounts of nitrogen in the soil make it a valuable crop for subsistence agriculture (Yunus and Jackson 1991, Campbell et al. 1994). Very little ODAP accumulates in the aerial parts of the grasspea plant during the vegetative phase (Addis and Narayan 1994), offering the possibe safe use of grasspea as a forage crop prior to the onset of pod filling. Seeds of some types of grasspea may be used as a feedstuff for poultry (Low et al. 1990).

ODAP is the most limiting factor in the spread and cultivation of grasspea. The first priority in the improvement of this potential pulse crop is the development of cultivars with low or zero ODAP concentration. The inheritance of ODAP concentration is quantitatively controlled (Quader et al. 1986, Tiwari 1994). However, marker assisted selection (MAS) could be used to develop low or zero ODAP cultivars. A genetic linkage map, consisting of easily scored polymorphic marker loci spaced throughout the genome, is an essential prerequisite to detailed genetic studies in any organism (Lander et al. 1987). A linkage map serves as a reference point for further mapping of new genes of interest. A saturated linkage map, involving easily scorable markers, e.g., isozymes and random amplified polymorphic DNA (RAPD), will facilitate MAS for complex traits, e.g., disease resistance or non-toxic (zero ODAP) lines in grasspea.

RAPD markers are useful for indirect selection and developing linkage maps because of their abundance, speed, simplicity and low cost. The RAPD assay is based on

the use of short, arbitrary-sequence oligonucleotides as primers for the amplification of randomly selected segments of the target genome. RAPD markers have several advantages over restriction fragment length polymorphism (RFLP) markers, as mentioned by Rafalski et al. (1991):

1) RAPD analysis requires a smaller amount of DNA (1%) than RFLP analysis.

2) RAPD analysis does not require digestion with restriction endonucleases.

3) RAPD analysis is free from the risk of radioactive hazard, unlike RFLP analysis.

4) RAPD analysis is simpler than RFLP analysis, thus saving time and money.

Furthermore, the exchange of RAPD information between laboratories is simple, as only the primer DNA sequence and the size of the polymorphic band are required. Thus, RAPD markers provide another useful tool that can be used in plant breeding programmes. However, when planning for an linkage experiment, the dominant inheritance (Rafalski et al. 1991) and the lack of reproducibility (Gu et al. 1995) of RAPD markers has to be taken into consideration.

The outcrossing rate of a species must be considered when making a decision on the most appropriate breeding method for improvement. Knowledge of the outcrossing rate is also useful in designing experiments for genetic and linkage studies, and maintaining genetic purity. Several studies in barley (*Hordeum vulgare* L.), lima bean (*Phaseolus lunatus* L.), wild oat (*Avena fatua* L.), and rose clover (*Trifolium hirtum* All.), all predominantly self-pollinating species, have shown that low outcrossing rates (between 1 and 10 %) had a significant effect on the genetic structure of the populations

(Harding and Tucker 1964, as stated by Jain 1976).

The objectives of the present study were to:

1) Determine the outcrossing rate in grasspea in Saskatchewan,

2) Identify genetic markers in grasspea by determining the genetic basis of several morphological, isozyme and RAPD polymorphisms,

3) Develop a molecular linkage map of grasspea by determining linkages among RAPD markers and genes controlling morphological and isozyme polymorphisms, and

4) Compare the efficiency of isozymes to RAPD markers in the development of a linkage map.

2. LITERATURE REVIEW

2.1 Taxonomy of Grasspea

Lathyrus sativus L., known as grasspea, chickling vetch or khesari dhal, is an ancient Old World cultigen (Kislev 1989). *Lathyrus* is in the tribe Vicieae of the family Leguminosae (Fabaceae). The first post-Linnaean taxonomist to recognize the tribe Vicieae was Adanson who called it a section (Kupicha 1977). As cited by Kupicha (1977), de Candolle recognized Vicieae as a tribe and included the genera *Cicer* L., *Ervum* L. *Faba* L, *Lathyrus*, *Orobus* L., *Pisum* L. and *Vicia* L. within it. Later, Bentham (1865) as cited by Kupicha (1977) recognised six genera in Vicieae: *Cicer*, *Vicia*, *Lens* Miller, *Lathyrus*, *Pisum* and *Abrus* L. Hutchinson (1964) placed *Abrus* in its own tribe Abraceae. In a recent taxonomic treatment of Vicieae, Kupicha (1981) included, in addition to *Lathyrus*, the following genera: *Vicia* L. (Faba bean and vetches), *Pisum* L. (pea), *Lens* Medikus (lentil) and *Vavilovia* A. Fed. She placed *Cicer* into the Tribe Cicereae.

In early classifications eight sections were identified in the genus *Lathyrus* (Senn 1938). Later Kupicha (1983) recognized 13 sections. Allkin et al. (1986) recognized 160 species and 45 subspecies of grasspea. *Lathyrus sativus* had been placed in the section *Cicercula*, but Kupicha (1983) combined this section with the section *Lathyrus* since the species in these two taxa are very similar in general morphology. Currently, *Lathyrus sativus* L. is placed in the section *Lathyrus* along with 33 other species, including the

recently described *L. belinensis* (Maxted and Goyder 1988).

2.2 Cytogenetics of *Lathyrus*

Lathyrus is predominantly a diploid genus with 2n=2x=14 chromosomes. The chromosome number of more than 60 *Lathyrus* species has been reported with only three species having more than 14 somatic chromosomes. Two species, *L. pratensis* and *L. venosus*, are tetraploid with 2n=28 chromosomes and one species, *L. palustris*, is hexaploid with 2n=42 chromosomes (Campbell et al. 1994). A cytological study of *Lathyrus sativus*, using both root tip and embryo cells confirmed a chromosome number of 2n=14 (Kar and Sen 1991). The chromosome length varied from 3.5 to 5.5 μm in root tip cells and from 4.5 to 6.5 μm in embryo cells. Four chromosomes had secondary constrictions. They identified five chromosome types:

Type A: Extremely subterminal to nearly subterminal primary constriction.

Type B: Nearly submedian to submedian primary constriction.

Type C: Nearly median to submedian primary constriction.

Type D: Two constrictions, primary and secondary. Of the three arms, one arm is longer than the other two which are more or less equal in size.

Type E: Two constrictions, primary and secondary. Middle arm is shorter than the other two arms which are unequal in size.

The types of chromosomes in the grasspea genome were $B_8C_2E_4$ for root tip cells and $A_4B_2C_4D_2E_2$ for embryo cells. Despite the fact that chromosome complements are essentially identical in morphology in all organs, their manifestation under the

microscope may differ due to penetration of pretreatment agents and fixatives and the response of the organ to the processing steps involved. Comparative studies of chromosome complements should be based on cells from corresponding tissues.

Experimental interspecific hybridization in the genus was shown in sweet pea (*L. odoratus*) as early as 1916. Burpee (1916) and others tried to introduce yellow flower colour genes into the cultivated species from wild relatives in the same genus. Interspecific hybridization between other species in the genus *Lathyrus* has been attempted by many researchers since the report of the successful crossing of *L. hirsutus* x *L. odoratus* by Baker in 1916, as cited by Campbell et al. (1994). Interspecific crosses in *Lathyrus* have rarely been successful (Senn 1938, Hitchcock 1952, Davies 1957, Yunus and Jackson 1991). Senn (1938) reported no success in 458 attempts at interspecific crossing, although he did not try to cross any of the native western species. Lwin (1956) succeeded in crossing *L. cicera* with *L. sativus*. However, subsequent attempts of this cross have been unsuccessful (Davies 1957, Khawaja 1988). Khawaja (1988) reported that *L. sativus* crosses readily with *L. amphicarpos* when the latter is used as the female parent.

In certain cross combinations fertilization is successful, but embryo abortion occurs during development. The stage of development at which abortion takes place differs with the cross combination. Cytological studies of the F_1 hybrids between *L. amphicarpos* x *L. sativus*, *L. amphicarpos* x *L. cicera*, and *L. odoratus* x *L. chloranthus* were conducted by Khawaja (1988), who showed 50 to 70% chromosome homology and pollen fertility in conformity with meiotic pairing. Recently, Yunus and Jackson (1991)

attempted interspecific hybridization between grasspea and 15 other species in the section *Lathyrus*. Only two species, *L. amphicarpos* and *L. cicera*, produced viable F_1 hybrids with low fertility, when crossed with *L. sativus* as the male. When *L. sativus* was the female, only one cross (*L. gorgoni*) produced an F_1, but the seedlings were inviable. Yunus and Jackson (1991) summarised the *Lathyrus* germplasm resources in terms of the gene pool concept developed by Harlan and De Wet (1971) as follows:

Gene pool	Ordination	Cultigen	*Lathyrus* species
I 1A	(1st order)	Cultigen	*L. sativus*
1B	(2nd order)	Wild counterpart	unknown
II	(3rd order)	Cross compatible species producing more or less fertile hybrids	*L. amphicarpos* *L. cicera*
III	(4th order)	Cross compatible species producing viable, but sterile, hybrids	*L. gorgoni* *L. latifolius*
	(5th order)	Cross compatible species producing inviable hybrids	*L. chloranthus* *L. annus*
	(6th order)	Other related species not producing any hybrids	*L. basalticus* *L. cassius* *L. chrysanthus* *L. hierosolymitanus* *L. hirsutus* *L. marmoratus* *L. odoratus* *L. pseudo-cicera* *L. tingitanus*
	(7th order)	Distantly related species	Other species in *Lathyrus* section *Lathyrus* species in other sections

From the information available on crossing, fertility, and chromosome behaviour of the hybrids, it may be concluded that a breeding strategy involving alien genetic transfer for the improvement of grasspea is possible through the readily crossable species *L.*

amphicarpos and *L. cicera*. It may also be possible to use embryo rescue techniques to obtain plants from interspecific crosses that result in aborted embryos.

2.3 Grasspea and Lathyrism

The term lathyrism was coined by Cantani in 1873 and describes symptoms following the ingestion of seed of *Lathyrus* species (Cohn and Streifler 1983). Two clearly different toxic syndromes are known: a) Osteolathyrisim--- the effects are seen in different animals following ingestion of the seeds of *Lathyrus odoratus*, L. *hirsutus* and *L. pusillus* which contain beta-aminopropionitrile (BAPN). The pathological changes were mainly in the skeleton and connective tissues; b) Neurolathyrism--- observed in humans after the consumption of seeds of *Lathyrus sativus*. The pathological symptom is characterized by an irreversible neurological disorder which results in paralysis of the lower limbs. This results from the presence of the non-protein amino acid, β-*N*-oxalyl-L-α,β -diaminopropionic acid (β-ODAP), in the grasspea seed (Spencer et al. 1986). A second name, β-N-oxalylamino-L-alanine (BOAA), has also been used in the literature. It was suggested that beta-ODAP be used in preference to β-BOAA (Bell and O'Donovan 1974, Abegaz et al. 1994). As cited by Tiwari (1994), the history of lathyrism goes back to 130 to 210 A. D. when Galen reported the disease. Duke George of Wurtemburg banned consumption of grasspea flour in his principality in 1671 because of its "paralysing effects on the legs" (Spencer and Schaumburg 1983). Throughout the 18th, 19th and 20th centuries outbreaks of lathyrism occurred in South Asia, Europe, Northern Africa, the Middle East, and Russia (Stockman 1929, Selye 1957, Barrow et al. 1974, Griffin et al. 1978). During World War II a well documented outbreak of lathyrism

occurred among inmates confined to a labour camp in the Ukraine (Spencer and Schaumberg 1983). Starting in September 1942, their daily food rations consisted of 400 g of grasspea seeds cooked in salt water plus 200 g of bread. In December 1942, a monophasic outbreak of spastic paraparesis involving 800 inmates began, mostly among the malnourished. Some victims were unable to maintain bladder function and urinated 30 to 40 times during the night. In January 1943, grasspea was removed from their diet and no additional cases appeared.

2.3.1 Environmental effects on ODAP

The ODAP concentration of grasspea differs widely among accessions and environments (Dahiya and Jeswani 1975, Ramanujam et al. 1980, Lambein et al. 1994). The absence of overt lathyrism in the coastal areas where *Lathyrus* is produced and consumed, as compared to more inland areas, prompted a study on the effect of salinity on β-ODAP in grasspea. Haque et al. (1993) reported that β-ODAP concentration in seeds and vegetative parts decreased with increased salinity.

2.3.2 Genetic detoxification of ODAP

Selection and breeding for low neurotoxin lines has been effective in reducing ODAP concentration in grasspea seed. Screening of germplasm has resulted in the identification of several lines that have low ODAP concentration, even lower than that obtained by processing (Jeswani et al. 1970, Nerkar 1972, Misra et al. 1979, Ramanujam et al. 1980). Lines are now available with as low as 0.03% ODAP, compared to 0.3% to 1% in lines commonly grown in the Indian subcontinent (Campbell and Briggs 1987). A

breeding program in Canada resulted in the release of germplasm LS 8246 (LS82046) having an ODAP concentration of 0.03% in the seed (Campbell and Briggs 1987). This line is being tested in several countries, including Bangladesh, Ethiopia and Pakistan. Quader et al. (1989) reported ODAP concentration of 0.01% in selected F_2 plants. Roy et al. (1993) developed somaclones from internode explants of grasspea with ODAP concentration of 0.01% in the seed and 0.015% in the leaf.

2.4 Outcrossing in Legumes

Plant breeders have always recognized the importance of breeding system variation in designing optimal procedures for recombination, selection and cultivar development. In a review article, Gorz and Haskins (1971) discussed the role of cross-fertilization rates in forage crop breeding in terms of nonrandomness of outcrossing, fate of hybrids originating from outcrosses, and the maintenance of cultivar purity. Several studies in barley, lima bean, wild oat, and rose clover, all predominantly selfing species, have shown that low outcrossing rates of 1-10% could have a significant effect on the genetic structure of their populations (Harding and Tucker 1964, Jain 1976). Many legumes (e.g., *Lathyrus, Lens, Vicia, Pisum, Glycine, Cicer*, etc.) have a typical papillionate flower with stigma and stamens contained within the keel petal until the flower is tripped, which is usually after anthesis (Hardy and Quesenberry 1984). However, outcrossing apparently occurs in *Lathyrus sativus* and *Vicia faba* at a higher frequency than is acceptable in a truly self-fertilized crop (Smartt 1984, Yunus et al. 1991, Metz et al. 1993, Rahman et al. 1995,). In lentil natural outcrossing varied from 2.2 to 2.9 in Turkish and Greek landraces to 6.6%

among Chilean populations (Erskine and Meuhlbauer 1991). The rate of outcrossing can vary significantly, depending on genotype, as reported in faba bean and grasspea (Metz et al. 1993, Rahman et al.1995, respectively). The outcrossing rate varied for different genotypes from 1 to 55% in faba bean (Metz et. al. 1993) and from 9.8 to 27.8% in grasspea (Rahman et al. 1995). In grasspea the variation in outcrossing rate among different genotypes may have been due to the bright flower colour on some plants attracting more pollinators (Rahman et al. 1995).

2.5 Molecular Markers and Their Uses

Standard breeding procedures utilize the genetic variability present within the available gene pool of crop species to synthesize new cultivars. The characters selected are yield, disease and stress resistance and specific qualities required for food use or processing (Gebhardt and Salamini 1992). Most of these traits are controlled by several genes. The availability of closely linked genetic markers, diagnostic for superior expression of a trait, would greatly facilitate selection. Although easily scorable morphological markers (e.g., flower colour, seed coat colour, etc.) are useful for marker assisted selection (MAS), pleiotropy, epistasis and, above all, the limited abundance of such markers has led to the search for other types of markers.

Isozyme loci are excellent biochemical markers since they are usually codominantly inherited, do not show pleiotropic effects, rarely exhibit epistasis and are not affected by the environment. The history of the application of isozymes in plant breeding goes back to 1966 when Brewbaker (1966) proposed the use of isozyme

phenotypes for cultivar identification. Isozymes have been used very successfully in certain aspects of plant breeding and genetics as nearly neutral genetic markers (Tanksley et al. 1989). Unfortunately, the number of genetic markers provided by isozyme assays is insufficient for most applications in plant breeding. As a result, even with the use of isozymes as biochemical markers, the full potential of genetic mapping in plant breeding has not been achieved (Tanksley 1983).

The recognition of RFLP (restriction fragment length polymorphisms) as phenotypically neutral molecular markers and their use in the construction of highly saturated linkage maps was first demonstrated by Botstein et al. (1980). The first genetic linkage map of the human genome, based on RFLP markers, was published in 1987 (Donis-Keller et al. 1987). RFLP maps have been constructed in recent years for several plant species including lentil. (Tahir et al. 1993); pea (Weeden and Wolko 1990); maize, *Zea mays* L. (Helentjaris et al. 1986, Helentjaris 1987); rice, *Oryza sativa* L. (McCouch et al. 1988); tomato, *Lycopersicon esculentum* L. (Bernatzky and Tanksley 1986, Zamir and Tanksley 1988. Tanksley and Mutschler 1990, Helentjaris et al. 1986); and potato, *Solanum tuberosum* L. (Bonierbale et al. 1988). A highly saturated genetic map facilitates the identification of markers tightly linked with the gene(s) of interest. The linked markers can be used in either (MAS) in conventional breeding or as a starting point for gene isolation and cloning.

A recent modification of the polymerase chain reaction (PCR) (Saiki et al. 1988) has resulted in the availability of a relatively new form of molecular marker, random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990). Unlike RFLP,

RAPD markers 1) require about 1% as much genomic DNA per assay as RFLP markers. 2) do not require digestion with restriction endonuclease and 3) do not require the use of radioactivity (Rafalski et al. 1991). However, the dominant nature of RAPD markers prevents the detection of heterozygotes (Rafalski et al. 1991), thus, reducing the precision of mapping experiments from F_2 populations (Mather 1963). Since their inception, RAPD markers have been used to tag major genes for disease resistance in tomato (Martin et al. 1991); common bean, *Phaseolus vulgaris* L. (Miklas et al. 1993); and lettuce, *Lactuca sativa* L. (Michelmore et al 1991, Paran et al. 1991); and to develop comprehensive genetic maps in several plant species (Williams et al. 1990, Quiros et al. 1991, Reiter et al. 1992). Martin et al. (1991) used RAPD analysis on a pair of near-isogenic lines (NIL) of tomato, differing in resistance to *Pseudomonas syringae* pv. *tomato*, to identify RAPD bands coming from an introgressed region of chromosome 5 containing the *Pto* gene (conferring resistance to *Pseudomonas syringae* pv. *tomato*). They reported that the RAPD assay was more efficient than the standard RFLP approach in identifying markers closely linked to the locus of interest. Rafalski et al. (1991) suggested that, provided a thermocycling instrument is available, start-up costs for the RAPD assay are minimal. Furthermore, the exchange of RAPD information between laboratories is simple, as only the primer DNA sequence and the size of the polymorphic band are required. Thus, RAPD markers provide another useful tool that can be used in plant breeding programmes.

Recently, bulked segregant analysis (BSA) and near isogenic lines (NIL) have been used to detect and map markers tightly linked to disease resistance gene(s) (Martin

et al. 1991, Michelmore et al. 1991). BSA involves comparing two pooled DNA samples from contrasting individuals in a segregating population originating from a single cross. Each pool, or bulk, contains individuals that are identical for a particular trait, but are arbitrary at all unlinked genomic regions. The two pools, contrasting for a specific trait, are analyzed to identify markers that distinguish them. Markers that are polymorphic between the pools will be genetically linked to the gene for the specific trait. An approach that combines both BSA and NIL strategies may also be useful for identifying RAPD markers tightly linked to target loci (Michelmore et al. 1991, Haley et al. 1993). To overcome the problems associated with failure to detect heterozygosity with RAPD markers in mapping experiments, the use of recombinant inbreds (RI) was suggested (Burr et al. 1988). Recombinant inbreds present several advantages over F_2 and backcross populations that are traditionally used for gene mapping. In RI the genotypic frequencies are fixed and are perpetual populations that can be propagated by many investigators and in a variety of environments. Information obtained from mapping in RI populations is cumulative (Burr et al. 1988). Kesseli et al. (1994) suggested that, since a RAPD marker is inherited as a dominant locus, techniques like sequence characterized amplified region (SCAR) or progeny testing can be used in suitable situations to refine the genetic linkage map of a target region.

Cultivar identification has an important role in the protection of plant breeder's rights and for monitoring seed production and marketing. Conventional morphological and pigmentation traits, as well as disease resistance, have been used to distinguish the uniqueness of new soybean cultivars for purposes of plant variety protection (Rongwen et

al. 1995). With an increasing number of cultivars and a finite number of conventional characters, it has become apparent that such traits will not suffice to establish uniqueness. Molecular markers, like RFLP, RAPD, and microsatellites, are currently being used in crop plants to develop a unique DNA profile for each cultivar (Gorg et al. 1992, He et al. 1992, Rongwen et al. 1995).

Molecular markers can be effectively used to study genetic diversity. Genetic diversity is useful for several reasons, e.g., identifying cultivars, analyzing the pattern of gene flow, detecting phylogenetic relationships among closely related species, identifying duplication of accessions, selecting areas for additional plant exploration (Yndgaard and Hoskuldsson 1985) and selecting diverse parents for crossing in a breeding program (Camussi et al. 1983). In plant breeding, genetic distance measures have been used to group inbreds, cultivars, or populations for breeding purposes. Although informative and practical, the use of protein and isozyme markers has often been limited by their low frequency in many crop species (Goodman and Stuber 1980).

Recently, RAPD markers have been used as a tool for measuring genetic relationships. RAPD markers are technically simpler and lower in cost than RFLP markers (Weeden et al. 1992a). Isabel et al. (1995) measured genetic diversity in black spruce (*Picea spp.*) from enzyme and RAPD data and found complete congruence between gene diversity estimates derived from enzyme and RAPD markers. Link et al. (1995) used RAPD markers to study the genetic diversity in European and Mediterranean faba bean germplasm. They identified European small-seeded lines and Mediterranean lines as distinct groups with European large-seeded lines intermediate. They concluded

that RAPD markers are useful for classification of germplasm and identification of divergent heterotic groups in faba bean.

2.6 Polymorphism and Genetics in Vicieae

2.6.1 Grasspea:

Jackson and Yunus (1984) studied 49 accessions of grasspea for morphological variation. They concluded that grasspea is a highly variable species and that flower colour was related to geographical distribution since blue-flowered accessions were mainly found in Southeast Asia, Ethiopia and the Indian subcontinent, whereas accessions with white and mixed coloured flowers had a more westerly distribution. The following polymorphisms in morphological traits of grasspea were reported by Chowdhury (1992).

Flower colour: blue, bicolor, white

Seed shape: angular, globular

Seed coat colour: black, tan, brown, grey, green, white

Seed structure: wrinkled, smooth

Seed coat lusture: dull, shiny

Seed coat pattern: no pattern, dotted, spotted, marbled

Quader et al. (1986) studied the inheritance of flower colour from two white- x blue-flowered crosses. They reported 13:3 and 1:1 ratios in the F_2 and the backcross to the white-flowered parent, respectively, for flower colour in both crosses, with blue flower colour dominant to white. A dominant inhibitor of flower colour was proposed. One of the dominant alleles suppressed or inhibited the expression of the other dominant allele.

The parent with blue flower possessed the gene '*B*' which had no visible effect by itself as both the alleles *B* and *b* gave blue flower colour. They also observed that plants with white-coloured flowers had a higher toxin concentration compared to plants with blue-coloured flowers. A similar finding was observed by Ramanujam et al. (1980).

Kumari et al. (1993) studied the inheritance of flower colour involving three distinct flower colour parents; P505 (blue), P28 (pink) and P27 (white). In the F_1, blue flower colour was dominant to pink or white. Crosses between pink and white-flowered parents produced a blue-flowered F_1, suggesting a gene interaction in colour production. The F_2 of P505 x P27 resulted in four phenotypic classes; blue, pink, blue tinged, and white. The four classes fit a genetic ratio of 36:9:3:16 for blue:pink:blue tinged:white (P=0.30-0.50). Segregation into 295 coloured plants and 81 colourless (white) plants fit 3 coloured :1 colourless ratio. The P505xP28 cross showed a complementary gene ratio of 9 blue:7 pink. At least four genes are postulated to interact with flower colour in grasspea. The suggested genotypes of these three parents were P505:*CCPPBBII*; P28:*CCPPbbii*: and P27:*ccppbbII*. *C* was responsible for flower colour production, *I* produces an inhibitory action to suppress blue colour, *P* combined with *C* and *bb* produces pink flower colour, and P combined with *C* and *B* produces blue colour.

Data from six crosses between parents with high and low ODAP concentrations indicated the importance of additive and nonadditive gene effects for this trait (Quader et al. 1987). Additive gene effects were observed in the cross P5 x P6 and predominantly additive gene effects in cross P2 x P4. In two crosses (P3 x P4 and P5 x P3), nonadditive gene effects predominated with significant additive x additive interaction in cross P5 x

P3. Predominance of fixable additive effects in the majority of crosses indicated the feasibility of early generation selection for ODAP concentration in grasspea.

Tiwari (1994) made all possible crosses (including reciprocals) between five grasspea lines with low to high ODAP concentration. Plants of both parents, the F_1 and the F_2 were evaluated under field conditions and ODAP analyzed by the *ortho*-phthalaldehyde spectrophotometric method. Many of the F_1 progenies of low x low ODAP crosses were low in ODAP concentration, indicating that these lines carried the same genes for low seed ODAP concentration. The F_1 progenies of the low x high ODAP crosses were intermediate in ODAP concentration, indicating incomplete dominance for ODAP concentration. The F_2 progenies segregated over the entire parental range. The continuous variation, together with a near normal distribution of the F_2 populations in both low x low ODAP and low x high ODAP crosses, indicated that ODAP concentration was quantitatively inherited. Reciprocal crosses produced different results in some cases, indicating a maternal effect on ODAP concentration. Broad sense heritability estimates of ODAP concentration varied from 17 to 93%.

Yamamoto et al. (1986) reported interspecific hybridization and isozyme polymorphism in 16 annual species of *Lathyrus*, including grasspea. They observed variation in banding patterns for amylase (AMY), glutamic dehydrogenase (GDH), indophenol oxidase (IPO), malic dehydrogenase (MDH) and peroxidase (PRX). Fifteen bands were detected for AMY, 18 for GDH, five for IPO, seven for MDH and 27 for PRX, but they offered no genetic explanation for the observed bands. Yunus et al. (1991) reported variation in six enzymes, galactose dehydrogenase (GD), glutamate oxaloacetate

transaminase (=aspartate aminotransferase) (AAT), isocitric dehydrogenase (IDH), malic dehydrogenase (MDH), peroxidase (PRX) and 6-phosphogluconate dehydrogenase (6-PGD) in grasspea. Polymorphism was common for PRX and 6-PGD, but little polymorphism occurred for GOT (AAT), with only two accessions showing variation. The level of apparent heterozygosity was higher than expected in this predominantly autogamous species.

Chowdhury (1992) assessed genetic polymorphism in grasspea by analyzing 20 isozyme loci of 13 enzyme systems and eight morphological characters. Out of 20 isozymes, 19 were polymorphic in at least one geographical region. The number of alleles ranged from five in ACO-1, EST-1, EST-2, PGD-1, PGD-2, TPI-1 and TPI-2 to two for ACO-2, ADH, AAT-1, AAT-3, ENP, LAP-1, LAP-2 and PGM. The banding pattern of the heterozygotes suggested a dimeric structure of the isozymes AAT-2, PGD-1, PGD-2, TPI-1 and TPI-2 and a monomeric structure for ACO-1, ACO-2, ENP and EST-1, EST-2, LAP-1, ME-3 and SKDH-2. Observed heterozygosity (1.5%) suggested the presence of natural outcrossing in grasspea.

2.6.2 Other genera

Polymorphisms for over 100 genetic markers, including morphological and molecular markers, have been reported in lentil (Tahir et al. 1993). Variation for most of the morphological traits has been observed within the cultivated species. However, polymorphisms for the isozyme loci have been largely restricted to crosses between *L. culinaris* and its wild relatives (*L. orientalis*, *L. odemensis*, *L. nigricans*, and *L. ervoides*).

Muehlbauer and Slinkard (1981) reviewed the genetics of *Lens* and listed only 10 genes (*Fn, Gh, Gs, P, Pi, Sbv, Scp, W, Yc* [synonym *O*], *Yc-i* [synonym I] at that time. More recently, new morphological loci (*Blsc-1, Blsc-2, Chl, Glp, Grp, Hsc, Pdp, Tan, Tgc, Tnl, Xan*) were identified by Vandenberg (1987), Vandenberg and Slinkard (1987) and Vaillancourt (1989), making a total of 21 morphological loci in lentil. Since then, at least 91 polymorphic isozyme and RFLP markers have been identified in lentil (Tahir et al. 1993). Recently, 63 polymorphic RAPD markers have been reported in lentil (Eujayl et al. 1995). With the exception of W15 and V08b all primers produced dominantly inherited bands. Primer W15 and V08b provided bands that segregated codominantly.

More studies have been done on the genetics of garden pea than on any other pulse crop. Weeden and Marx (1987) reported allozyme polymorphism and monogenic inheritance for 15 loci. Weeden (1988b) listed 50 isozyme loci, for which the inheritance had been reported. Later, polymorphism was reported for 281 genes including 37 isozymes and 244 other loci (Weeden et al. 1991). Recently, polymorphism was reported for 56 RFLP, 2 RAPD and 4 microsatellite loci in pea (Dirlewanger et al. 1994).

Polymorphism was reported for morphological, isozyme, RFLP and RAPD markers in faba bean (Moreno et al. 1981, Ricciardi et al. 1985, Waly and Abdel-Aal 1987, Duc et al. 1990, Torres et al. 1993). Cabrera (1988) reported four loci controlling flower colour in faba bean. Two complementary recessive genes, w_1 and w_2, produced white flowers. Brown and yellow colours were regulated by the *Yf/yf* locus and yellow was recessive. The locus *Sdp/sdp* controlled distribution of pigment on the flower and spotted was dominant to solid pigmentation. In a study of the genetics of tannin

concentration in the seed coat and its relationship with flower colour in faba bean, Cabrera and Martin (1989a) reported the predominance of additive genetic variance in the inheritance of tannin concentration. The genes controlling red testa (*r*), spotted flower (*Sdp*) and yellow pigment of the flower (*yf*) negatively influenced tannin concentration in the testa. Link et al. (1995) reported that 35 informative primers in faba bean yielded 365 RAPD bands, 289 of which were polymorphic with a mean of 8.3 polymorphic bands per primer.

2.7 Linkage

2.7.1 Lentil

Linkage studies in lentil were reported by Zamir and Landizinsky (1984), Tadmor et al. (1987), Havey and Muelhbauer (1989), Muelhbauer et al. (1989), Vaillancourt (1989). The most recent review of genetic linkage between previously reported morphological, isozyme and molecular markers was presented by Tahir et al. (1993). These reported linkages were arranged into seven tentative linkage groups, based on combined linkage data from different authors, and included some regions of homology shared with pea (conserved linkage). The map included 7 morphological, 25 isozyme, 38 RFLP and 6 other loci. Currently, ten linkage groups are recognized, but this should eventually be reduced to seven as mapping continues. Tahir and Muehlbauer (1994) developed RI from interspecific *Lens* hybrids to map isozyme and morphological markers in lentil. F_2 families from eight interspecific (*L. culinaris* X *L. orientalis*) crosses were advanced to the F_6 by single seed descent to develop eight sets of RI lines. Each of the

eight sets of lines was assayed for parental alleles at 2 to 8 isozyme loci, using starch gel electrophoresis and at four morphological trait loci (growth habit, epicotyl colour, pod indehiscence and cotyledon colour). Single locus goodness of fit to the expected 1 : 1 ratio was determined for each of the marker loci, and linkage relationships between the marker loci were determined. Six linkage groups were identified, and included a total of 17 isozyme loci and four morphological trait loci. Previously reported linkages among isozyme loci and morphological trait loci were confirmed (*Gs/Aat-p*, *Pi/Gal1*, *Yc/Pgm-p*, *Aat-c/Me2*, *Aat-mb/Skdh* and *Fk/Pgd-p*), and three additional linkage groups were identified (*Gh/Gal1*, *Gal2/Aat-mb/Skdh* and *Skdh/Aldo/Nag*). The genetic map of lentil was expanded and confirmed based on these linkages, and a database was generated that can be used to add additional markers to the gene map. Recently, a genetic linkage map for lentil, based on RAPD, was published based on an F_2 population comprising 40 individuals (Eujayl et al. 1995). Seventy RAPD, oligonucleotide and morphological loci were segregating in this F_2 population. The map consisted of nine linkage groups including 27 RAPD, three oligonucleotides and one morphological marker covering 173 cM of the genome.

2.7.2 Pea

Pisum sativum is the only species in the tribe Viceae with a detailed map that consists of morphological and physiological mutants, allozyme and other protein variants, RFLP markers, and structural genes identified from DNA clones (Blixt 1974; Weeden and Wolko 1990). Linkage maps have been established for 281 loci including 37 isozyme

loci and 244 other loci (Weeden et al. 1991).

2.7.3 Faba bean

Cabrera and Martin (1989b) analyzed 10 loci and found close genetic linkage (p = 0.19 ± 0.04) between *Sdp/sdp* (solid distribution of pigment in the flower) and *Yg/yg* (yellow seed coat colour). The recombination frequency for *Sc/sc* (brown seed coat) and *N/n* (hilum colour) was 0.44±0.04, indicating possible linkage. *Sc/sc* was epistatic on *N/n*. Independent assortment was found for all other loci; *Yf/yf* (yellow flower) was epistatic to *Sc* (brown seed coat).

Suso et al. (1993) reported on the inheritance and linkage of isozyme loci for lactate dehydrogenase, phosphoglucomutase, formate dehydrogenase, shikimate dehydrogenase, superoxide dismutase and aspartate aminotransferase from seeds of *Vicia faba* and the linkage relationships among these isozyme loci. The allozymes at each locus behaved in a codominant manner and segregated in the expected Mendelian ratio. Linkage tests between these loci showed that they segregated independently.

Torres et al. (1993) reported the linkage relationships of nine isozymes, one RFLP marker and 43 RAPD markers in *Vicia faba*. Two F_2 populations were analyzed. Eleven independent linkage groups were identified in these populations. One of the linkage groups contained the 45S ribosomal RNA array and could be assigned to the large metacentric chromosome I on which the nuclear organizer region is located. This linkage group also contained two isozyme loci, *Est* and *Tpi-p*, suggesting that it may share some homology with chromosome 4 of garden pea on which three similar markers are syntenic.

2.8 Conserved Linkage Groups

Comparative genetic studies shed light on genetic and chromosomal evolution. They also provide information on phylogenetic relationships among distant taxa. To compare the linkage relationships of homologous enzyme-coding genes in divergent species, it is necessary to establish which genes are homologous. Before the introduction of biochemical and molecular techniques in genetics, it was very difficult to determine single gene homologies between widely divergent taxa. Most linkage maps consisted of genes affecting morphological characters such as leaf shape and pigment deficiencies in plants. Since the number of genes affecting these traits is often very large, it has been difficult, if not impossible, to determine which loci are homologous in different species. Until recently, cytological analysis of interspecific hybrids and a survey of naturally occurring chromosomal diversity within populations were the primary means of addressing the mechanisms involved in chromosomal evolution (Stebbins 1971, Jackson 1984, Grant 1987). Our knowledge about chromosome evolution in higher plants is based largely on meiotic analyses and comparisons of karyotypes. The former is necessarily restricted to interfertile species, as the analysis is performed on hybrid plants. The latter, while not limited to crossable species, has poor resolution, since change in karyotype can be brought about by many different processes and often these changes are not distinguishable, based on morphological comparisons of mitotic chromosomes.

Comparative genome mapping adds a powerful new technique for investigating the mode and tempo of chromosome evolution. This approach involves the use of biochemical and molecular markers, such as isozyme, RFLP, and RAPD markers, to map

the genomes of two species for a common set of markers. Comparative gene mapping allows one to determine the extent and nature of chromosomal rearrangements between cross-incompatible species. This method, thus, opens up comparisons among distantly related genomes which are not amenable to analysis by traditional cytogenetic techniques. This approach was pioneered by Tanksley and co-workers using tomato RFLP probes to map the tomato, chili pepper (*Capsicum annum* L.) and potato genomes (Bonierbale et al. 1988). These studies demonstrated that more distantly related species accrue a greater number of rearrangements between their genomes than do closely related species. In the case of tomato and potato, Bonierbale et al. (1988) were able to determine the location of three particular inversions differentiating potato from tomato. Comparative mapping of the tomato and potato genomes revealed essentially complete conservation of linkage groups with only a small number of inversions differentiating these species. In contrast, comparative mapping of the tomato and pepper genomes revealed extensive rearrangements including many interchromosomal translocations. A comparative study of maize and sorghum (*Sorghum bicolor* L.) genomes revealed another pattern of chromosomal evolution of chromosomal segments in which duplications, inversions and translocations apparently were the principal mechanism of chromosomal evolution (Whitkus et al. 1992).

Comparative mapping studies may have utility outside of evolutionary biology. For example, chromosome walking strategies in maize may be more efficiently executed in sorghum, given the conservation of gene order, fewer duplicated loci and the smaller size of the sorghum genome (Whitkus et al. 1992).

A comparative linkage study may also enable the assignment of a new gene to an already existing linkage group. In addition, a comparative linkage study may aid in assigning linkage groups to specific chromosomes. A comparison of the arrangement of loci in tomato and potato helped assign several potato linkage groups to the corresponding chromosomes in tomato. Nine chromosomes apparently were unaltered by any detectable rearrangements. In tomato, more than 400 molecular markers have been located on the genetic map (Bonierbale et al. 1988, Tanksley et al. 1988). Based on the position of the markers already mapped in potato, it was possible to predict the position of an additional 200 markers from tomato. The position of the unmapped isozyme loci in potato can also be predicted, based on their position in the tomato map. The potato and tomato comparative maps have already provided the basis for determining the chromosomal localization of patatin-coding genes in both species. The possibility of producing chromosome substitution lines between these species is suggested by the fact that many chromosome segments are conserved in potato and tomato (Bonierbale et al. 1988).

2.8.1 Conserved linkage groups in pea and lentil

A 560-cM linkage map consisting of 64 morphological, isozyme, and DNA markers in lentil was compared to the previously established pea map (Weeden et al. 1992b). Of the markers mapped in *Lens*, 36 had homologous or possibly homologous counterparts mapped in pea. Most of the genomic clones from lentil did not hybridize to pea DNA under relatively stringent conditions (two washes in 2x SSC at 65°C). Thus, for

RFLP loci only the random cDNA clones and clones containing known genes were useful for comparing chromosomal regions with conserved linkage groups. Isozyme loci also were helpful, although in complex enzyme systems, such as diaphorase, homology between specific pea and lentil isozymes was difficult or impossible to determine. Comparison of this map with that established previously for *Pisum sativum* revealed eight regions in which linkage among marker loci apparently had been conserved since divergence of the two genera. Allotting 10 cM for each terminus of 11 linkage groups and 20 cM for each of the nine unmapped markers, the total genome length coverage approaches to 10-Morgan in lentil. The conserved linkage groups constituted at least 250 cM, or approximately 40% of the anticipated minimum length (10-Morgan) for lentil genome. These two genera represent disparate lineages within the tribe Viceae, indicating that all members of this tribe may possess linkage groups similar to those identified in *Lens* and *Pisum*. The *Pisum* and *Lens* maps differ in the regions surrounding the 45S ribosomal RNA tandem repeats and in the position and distribution of the genes encoding the small subunit of ribulose bis-phosphate carboxylase. Despite rearrangements in gene order and copy number, as well as the presence of numerous inversions and translocations within both *Pisum* and *Lens*, significant portions of the lentil and pea genomes remain co-linear. Many of the linkages conserved between lentil and pea are also likely present in grasspea, faba bean and perhaps other closely related genera (e.g., *Cicer*). Indeed, recent studies on the linkage relationships of isozyme loci in chickpea suggest several conserved linkage groups among the genera *Cicer*, *Pisum*, and *Lens* (Gaur and Slinkard 1990, Kazan et al. 1993).

2.8.2 Conserved linkage groups among faba bean, pea and lentil

The linkage map of faba bean was compared with those of pea and lentil and conserved linkage groups were observed (Torres et al. 1993). The position of homologous loci was compared in order to identify conserved linkage groups. The comparison was limited to isozyme loci and the 45s ribosomal RNA array, because homologous relationships among RAPD markers were difficult to evaluate. Two possible conserved linkage groups were detected, using this small set of loci. One of the 45S ribosomal RNA arrays in pea was also syntenic with an esterase locus and *Tpi-p* (Weeden and Wolko 1990). suggesting that linkages on chromosome 4 in pea may be conserved in faba bean. In pea the 45s ribosomal RNA array is over 50 map units from *Tpi-p,* and in lentil the ribosomal array apparently is linked to different markers. However, the *Rrn-Est-Tpi-p* linkage is conserved within the Viciae. In the second linkage group, the isozyme loci *Prx-1* and *Acp-1* represented a stronger case for a conserved linkage group. In pea *Prx-1* is linked to *Acp-1* and *Nag* (Weeden and Marx 1987). In lentil *Prx-1* is linked to *Nag* (Weeden et al. 1988), indicating that this region is probably conserved between pea and lentil. The identification of linkage between *Prx-1* and *Acp-1* in faba bean suggests that this linkage has been conserved in *Vicia* as well.

2.9 Distorted Segregation

Distorted segregation in linkage analysis is a common phenomenon in plants. The segregation of markers in the F_2 population is usually distorted to some extent (Bonierbale et al. 1988, McCouch et al. 1988, Gebhardt et al. 1989). The degree of

distortion depends on population size as well as the number of heterozygous loci in the F_1 and their ease of classification. Lethality or reduced competitive ability (gametophytic or sporophytic), originating from one or more genes in the vicinity of markers, could be one reason for distorted segregation (recombination is a prerequisite for viability). Another explanation for distorted segregation is the nonhomologous and/or homologous pairing of the chromosomes of non-allelic regions (e.g., pairing of duplicated regions), giving rise to abnormal chromosomes after recombination and eventually lethality (Landry et al. 1991, Ellis et al. 1992). However, this does not preclude the use of such loci from mapping studies (Cai et al. 1994). It also is fairly common to find that markers displaying distorted segregation are clustered on particular linkage groups and that distortion in the whole cluster is toward one of the parental alleles, indicating selection for or against that allele (or closely linked loci) from that parent (Helentjaris et al. 1986, Landry et al. 1992, Prince et al. 1993). Cai et al. (1994) found that more than 40% of the distorted RAPD markers clustered on linkage group I in *Citrus* and most of the remaining markers clustered on linkage groups II, III, IV and VIII. They suggested that most of the distorted loci in the *Citrus* genome may be linked to genes exposed to direct selection. In a number of cases the clustered loci were skewed toward one particular parent (Helentjaris et al. 1986, Havey and Muehlbauer 1989, Doll 1991, Cai et al. 1994, Truco and Quiros 1994). Explanations for distorted segregation in different crops are mentioned below.

- Tomato lines, showing distorted segregation of *hp*, have a gametophytic gene linked with *hp* on chromosome 12; gametes possessing this gene are at a disadvantage with respect to fertilization (Mochizuki and Kamimura 1987).

- In barley distorted segregation was caused by certation between pollen grains of different genotypes, independent of the female genotype. Furthermore, certation was controlled by a newly designated gene, *Ga2*, at a locus linked with the esterase multilocus (Est1, Est2, and Est4) (Konishi et al. 1990).
- In the genus *Beta* distorted segregation ratios apparently indicate linkage of the marker with genetic factors affecting gametogenesis, pollen function and embryo development (Abe 1991).
- In sugar beet (*Beta vulgaris* L.) gametic selection of the linked lethal loci caused distorted segregation (Pillen et al. 1992, 1993).
- In rye (*Secale cereale* L.) the main region for deviating segregation ratios was localized near the self-incompatibility loci (Philipp et al. 1994).

2.10 Planning Linkage Experiments

The amount of information about the recombination fraction provided by any set of data depends upon two factors: the completeness of the classification and the closeness of the linkage. No type of progeny segregation for two pertinent genes gives more information per individual than an F_2 completely classified into the ten possible genotypes, even though complete classification can only rarely be achieved without a progeny test (Allard 1956). With complete classification the amount of information per individual varies strikingly with changes in the magnitude of the recombination fraction (p). At p=0.50 (independent assortment) each individual contributes 8 units of information about p (Table 8, Allard 1956). The amount of information per individual

gradually increases as p becomes smaller until at p=0.01 each individual contributes more than 200 units of information about p, or more than 25 times more information than is obtained with independence.

The magnitude of i_p (information per individual) in the usual backcross is half of the i_p in F_2 with complete classification. This is easily understandable since the value of p in the F_2 with complete classification is influenced by crossing over in both sexes. The value of i_p for a number of the common F_2 ratios is given in Table 8 of Allard (1956). The comparative efficiency of various types of data is readily visualized in a diagram such as Figure 2.1 in which backcross and various types of F_2 data are compared with an F_2 population with complete classification as the standard. In general, the loss of information caused by incomplete classification is greatest in repulsion. This fact should be kept in mind in planning linkage experiments. It should also be kept in mind that Figure 2.1 compares the relative efficiency of various types of progenies to F_2 with complete classification. Given the approximate cross-over value, Table 8 of Allard (1956) makes it possible to rapidly estimate the size of the population required for a given degree of accuracy. Thus, if dominance is complete at both loci, the cross is in repulsion, p is approximately 0.05, and a standard error not larger than 0.05 is required, $n=(i_p\sigma^2)=[(1.006)(0.05)^2]^{-1}=398$ in F_2.

The precision of linkage mapping is low with dominant markers in the F_2 mapping population due to the confounding of heterozygotes with dominant homozygotes. Linkage detection and mapping in F_2 populations is highly inefficient for dominant markers in repulsion (Allard 1956, Mather 1963) and results in ambiguous

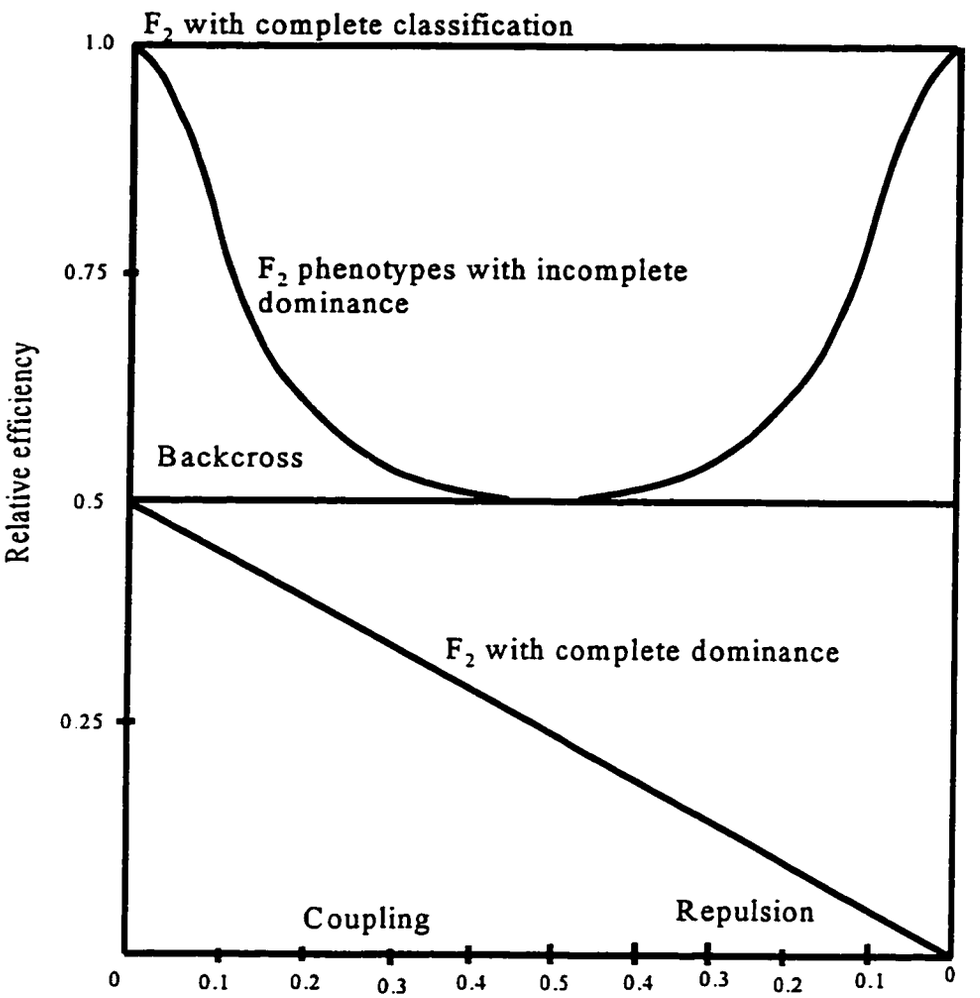


Figure 2.1 Relative efficiency of different types of data for estimation of the recombination fraction (After Allard 1956).

linkage maps (Chaparro et al. 1994). Inefficiency of linkage mapping, associated with repulsion phase, may be overcome by including more markers in a linkage experiment and excluding markers linked in repulsion phase and analysing the data in repulsion phase separately. If RAPD markers are used in a mapping experiment in F_2 populations, it may be useful to generate a small test-cross population in conjunction with the F_2 of interest and check for repulsion linkage. However, it is unlikely that a recessive tester would be readily available for most morphological and RAPD loci (Chaparro et al. 1994). Alternatively, F_2 progeny used to generate the map could be genotyped by performing F_3 progeny tests for the alignment of repulsion-phase linkage groups in existing F_2 populations, where generating a test cross may not be feasible. This would require the generation and genotyping of an F_3 family from each F_2 individual used to generate the original map. Such an effort would be extremely laborious (Chaparro et al. 1994). Another alternative is to develop a linkage map by analyzing RI or doubled haploids, thus, eliminating heterozygous F_2 genotypes and the resulting loss of information for dominant markers.

3. MATERIALS AND METHODS

3.1 Outcrossing Study

3.1.1 Plant materials

Two pure lines of grasspea, PI 426886b and PI 206891a, were selected for this study. Both lines were self pollinated for two years to check for homozygosity. Line PI 426886b was homozygous dominant for blue flower colour and PI 206891a was homozygous recessive for white flower colour.

3.1.2 Planting plans

Three experimental methods were used to estimate outcrossing rates at three locations, Preston Avenue plots, the Kernen Farm and the Goodale Farm.

Method 1:

The lines PI 426886b (blue-flowered) and PI 206891a (white-flowered) were planted in alternate rows in a 4-row block with four replications at each of three locations in 1993. The rows were 3 m long with 1 m between rows and plants were spaced 15 cm apart within each row. The white-flowered and blue-flowered plants were seeded in a 1:1 ratio and allowed to pollinate naturally. Five plants were randomly selected from each of the eight white-flowered (recessive) rows at each location and 20 seeds were collected from each selected plant. These were bulked into a family from each location, space

planted (10 cm apart in rows 30 cm apart) in 1994 and the frequency of blue-flowered heterozygotes recorded for each family.

Method 2

Ten white-flowered plants were arranged in a staggered configuration (McKellar et al. 1991) and were surrounded by 20 blue-flowered plants, all with a 50 cm spacing between plants. The white-flowered and blue-flowered plants were seeded in a 1:2 ratio at each location in 1993. One hundred seeds were sampled from each white-flowered (recessive) plant. Seeds harvested from all 10 white-flowered plants were bulked into a family from each location. Seeds of each family were space planted (10 cm apart in rows 30 cm apart) in 1994 and the frequency of blue-flowered heterozygotes recorded for each family.

Method 3

This method is a modification of the design used by Wells et al. (1988). At each location in 1993 30 plants of the dominant blue-flowered line PI 426886b were space planted in each of 10 rows (300 plants), except that two plants of the recessive white-flowered line PI 206891a were substituted into the five odd-numbered rows (10 plants). Thus, the white-flowered and blue-flowered plants were grown in a ratio of 10:290 or 1:29. The inter- and intra-row spacings were 30 cm and 10 cm, respectively. This method was chosen to minimize the chance of intragenotypic crossing. About 50 seeds were harvested from each white-flowered (recessive) plant (line PI 206891a) and bulked into a

family. Seeds from each family were space planted (10 cm apart in rows 30 cm apart) in 1994 and the frequency of blue-flowered heterozygotes recorded.

These three methods were repeated at three locations 1) Preston Avenue plots, 2) the Kernen Farm, and 3) the Goodale Farm, in order to sample three environmemts. Three families, one from each planting design, were grown from each location for a total of nine families. The outcrossing rate was estimated separately for each of the nine families. A χ^2 test was performed for homogeneity of the outcrossing rates over the nine families (Jain 1979).

3.1.3 Statistical analyses

The outcrossing rate (t) and its variance were estimated as follows (Harding and Tucker 1964):

$$\hat{t} = \frac{a}{\{(a+b)*p\}} \quad \text{.....3.1}$$

$$s_t^2 = \frac{\hat{t}(1-\hat{t}p)}{(a+b)*p} \quad \text{..... 3.2}$$

where, a and b are numbers of Aa (blue-flowered plants) and aa (white-flowered plants). respectively, in the progeny of selected aa individuals; p and q are the frequencies of the A and a allele, respectively, in the population in which outcrossing occurred.

The χ^2 for heterogeneity was calculated for the outcrossing rates among families as follows (Jain 1979):

$$\chi^2 = \sum \frac{(\hat{t}_i - \bar{t})^2}{s_i^2} \quad \text{.....3.3;}$$

$$\text{where } \bar{t} = \frac{\sum\left(\frac{\hat{t}_i}{s_i^2}\right)}{\sum\left(\frac{1}{s_i^2}\right)} \quad \text{.....3.4;}$$

and $\bar{t}$ is the mean outcrossing rate.

3.2 Inheritance and Linkage Studies

3.2.1 Crossing plan

On the basis of existing polymorphisms for isozyme and morphological traits, eight sub-accessions were selected as parents and five crosses between pure lines (PI 283564c.3 x PI 426885.2, PI 358601.5 x PI 173714.5, PI 426891.1 x PI 172930.4, PI 283549a.6 x PI 202803a.3, and PI 426891.1.3 x PI 283564c.3.2) were made. Homozygosity of the markers in the parents was confirmed by excluding any markers segregating in the F_1, based on 7 to 8 F_1 plants. One F_2 population (PI 426891.1.3 x PI 283564c.3.2) was scored for morphological, isozyme, and RAPD markers and the other four F_2 populations were scored for isozyme markers only.

3.2.2 Population size

In the present study 100 F_2 plants from the cross PI 426891.1.3 x PI 283564c.3.2. and 180 F_2 plants each from the four other crosses were analysed.

3.2.3 Isozyme analysis

3.2.3.1 Tissue used

In preliminary isozyme assays on grasspea, tissue from cotyledons of freshly germinated seeds resulted in better resolution of bands than leaf tissue. Therefore, cotyledon tissue was used in all subsequent analyses. Thirty seeds (five seeds each of six accessions or subaccessions), along with a standard (PI 426891), were germinated for each electrophoretic run. Hard seeds were scarified by hand to initiate germination. Individual seeds (Captan treated) were placed in separate holes in a 10 cm-thick Styroblock for germination. The Styroblock was covered with a moist paper towel to prevent desiccation and placed in a plastic container partially filled with water. Care was taken that water did not splash into the Styroblock. The container was covered with perforated Saran Wrap and left in light at room temperature for germination. Forty-eight hours following incubation the cotyledons was dissected and isozymes extracted (Section 3.2.3.4).

3.2.3.2 Gel preparation

Hydrolysed potato starch (Sigma, S-4501) (12%w/v) was added to the appropriate gel buffer (Tables 3.1 and 3.2) for the specific enzyme under investigation. Initially, 13 enzymes were tested in all four gel buffer systems and the gel buffer that produced the most distinct banding for at least one enzyme was selected for subsequent studies. The volume of the gel buffer varied, depending upon the thickness of the gel. Three different sizes of gel molds were used (250, 325 and 400 mL), resulting in three thicknesses of gel.

Table 3.1 Electrode and gel buffer systems used in starch gel electrophoresis.

Electrode buffer	Gel buffer
System I (Selander et al. 1971)	
0.19 M Boric acid (11.75 g/L) adjusted to pH 8.1 with lithium hydroxide	1 part electrode buffer 9 parts tris-citrate buffer [0.05 M Trisma base (pH 6.2 with citric acid)]
System II (Cardy et al. 1980)	
0.065 M L-Histidine (10.1 g/L) adjusted to pH 6.5 with citric acid	1 part electrode buffer 3 parts distilled water
System III (Meizel and Market 1967)	
0.135 M Trisma base (16.35 g/L) adjusted to pH 7.0 with citric acid	1 part electrode buffer 14 parts distilled water
System IV (Clayton and Tretiak 1972)	
0.04 M Citric acid (8.41 g/L) adjusted to pH 6.1 with N-(3-aminopropyl)-morpholine	1 part electrode buffer 19 parts distilled water

Table 3.2 Isozymes and optimal gel buffer system for different enzyme systems used in starch gel electrophoresis studies of grasspea.

Enzyme	Enzyme commission (E.C.) no.	Desig-nation	Polymorphic isozyme	Gel system
Aconitase	4.2.1.3	ACO	ACO-1,-2	II
Aspartate aminotransferase	2.6.1.1	AAT	AAT-1,-2	I
Esterase	3.1.1.-	EST	EST-3	I
			EST-6	III
Formate dehydrogenase	1.2.1.2	FDH	FDH	I
Leucine aminopeptidase	3.4.11.1	LAP	LAP-1	I
6-phosphogluconate dehydrogenase	1.1.1.44	PGD	PGD-2	II
Shikimate dehydrogenase	1.1.1.25	SKDH	SKDH	IV
Triosephosphate isomerase	5.3.1.1	TPI	TPI-1	III

Two slices were obtained from the thin (250 mL), three slices from the medium (325 mL) and four slices from the thick (400 mL) gel, using a nylon thread for slicing and thin plexiglass strips as thickness gauges (Section 3.2.3.6). The suspension was heated in a vacuum flask over a bunsen burner with vigorous shaking until it started boiling and became clear. The gel was then de-gassed with a water aspirator and poured into the gel mold. Any air bubbles or particulate material entrapped in the gel was removed immediately with a spatula. A plastic plate was gently placed over the gel mold to eliminate any air bubbles. After cooling 10 to 15 min. at room temperature, the gel mold was wrapped in Saran Wrap to prevent drying and then left overnight at room temperature.

3.2.3.3 Isozyme assay

Four different electrode and gel buffer systems were used for the separation of various isozymes (Tables 3.1 and 3.2). The electrophoresis apparatus and gel molds used in these studies were manufactured locally, following the designs provided by Weeden and Emmo (1984).

3.2.3.4 Preparation of isozyme extracts

Germinated seeds were removed from the Styroblock with forceps, blotted on filter paper, and the seed coat removed. About 80 mg of one cotyledon was cut off with a scalpel and placed on a plastic weighing boat kept on ice. After adding 8 to 10 drops of extraction buffer to each boat, the tissue was homogenized with a plexiglass rod. The extraction buffer was slightly modified from that described by Gottlieb (1981) and was prepared fresh daily by adding 1.0 g PVP-40, 20 mg $MgCl_2$, 8 mg KCl, 4 mg EDTA, 2 drops Triton X-100 and 0.2 mL β-mercaptoethanol in 10 mL of 0.1 M Tris-HCl, pH 7.4 buffer. The homogenate was absorbed onto filter paper wicks (2 x 10 mm, Whatman # 3) and kept on ice. Wicks were loaded on the gel immediately after homogenization of the last sample.

3.2.3.5 Electrophoresis

The gel was prepared 15 to 20 h before starting electrophoresis. The Saran Wrap and plastic plate were removed gently from the top of the gel. Excess gel material was removed with a scalpel and a slit was cut across the gel 6 cm from one edge. The gel was wrapped and kept inside the refrigerator at about 5°C for 45 to 60 min. In the meantime

the enzyme extract was prepared as described in Section 3.5. When the samples were ready, the wicks were blotted (to remove excess sample) and inserted into the slit, leaving a 1 cm margin at each side of the gel. The wicks were loaded 0.30 cm apart. Thirty-two samples were loaded in each gel. A standard sample (PI 426891) was loaded in each gel in the first and last lanes. To monitor migration of the enzyme, two wicks were soaked in dye (bromophenol blue), blotted, and inserted one at each end of the slit. A layer of Saran Wrap was placed on the surface of the gel tray and the gel tray was placed onto the electrophoretic apparatus filled with appropriate electrode buffer. The cathodal (nearer the slit) and anodal ends of the gel were connected with the electrode buffer by sponge bridges. The electrophoretic apparatus was kept in the refrigerator and connected to the power supply.

A constant energy flux of 250 volts was provided to System I gels, 300 volts to System II gels and 150 volts to System III and System IV gels. In all cases the initial current was not allowed to exceed 50 mA. A lower voltage was set if the initial current exceeded 50 mA. After 10 to 15 minutes the power was switched off and the wicks were removed with forceps and the slit was cleaned with Q-tip (cotton buds) dipped in electrode buffer. The slit was then closed by placing a glass rod between the cathodal edge of the gel and the adjacent mold wall to insure that the two sections of the gel were held tightly and air bubbles, if any, were released from the slit. An ice bag was placed on the top of the gel in order to avoid warming the gel during electrophoresis. The power was turned back on and the gels were run until the tracking dye reached the sponge (usually 3 to 5 hrs, depending upon the thickness of the gel).

3.2.3.6 Staining of enzymes

After the run was complete, the gel was removed from the refrigerator and trimmed at the edges to get a rectangular slab about 8 x 15 cm. A notch was made at the upper left corner of the gel to identify the position of the first sample in the gel. The gel was sliced horizontally into 1 to 2 mm thick slices by pulling a nylon thread through the gel slab over successive layers of plexiglass strips placed along the side of the gel slab. Each slice was then stained for a different enzyme. The recipes that provided the best differential staining for assaying each enzyme are presented in Appendix 1. The gel was scored immediately. When a photograph was needed, it was taken immediately after staining. The gels were sometimes stored by fixing overnight in 50% ethanol.

3.2.3.7 Nomenclature of the isozymes and their loci

Isozymes and their loci were designated following the guidelines of Weeden (1988a). The enzymes were designated by their standard abbreviations in uppercase letters, e.g., LAP, ENP. When an enzyme system (=enzyme) exhibited two or more isozymes (coded by different loci), they were designated by sequential numbering based on their mobility on the gel; the most anodal isozyme was numbered 1 with the numbering proceeding cathodally, e.g., AAT-1, AAT-2. The allozymes (coded by different alleles of a single locus) of an isozyme were identified by a letter suffix immediately following the number. The most anodal allozyme was designated as 'a', the second most anodal 'b' and so on, e.g., AAT-3a, AAT-3b. In order to maintain consistency

in designating allozymes over different gels, a standard sample (PI 426891) was loaded in all gels (Chowdhury 1992).

3.2.4 RAPD protocol

3.2.4.1 Principles

Random amplified polymorphic DNA (RAPD) is a technology, developed by Williams et al. (1990), which results in a new type of molecular marker. The basis for RAPD technology is the polymerase chain reaction (PCR). The PCR is used to amplify a segment of DNA that lies between two regions of a known sequence (Saiki et al. 1988). Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by DNA polymerase. These oligonucleotides typically have different sequences and are complementary to sequences that 1) lie on opposite strands of the template DNA and 2) flank the segment of DNA that is being amplified. The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the four 2',-deoxynucleotide 5'- triphosphates (dNTP). The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. This cycle of denaturation, annealing, and DNA synthesis is then repeated many times. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product. The major product of this exponential reaction is a segment of double-stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose

length is defined by the distance between the primers.

Random amplified polymorphic DNA, distinct from the PCR process, is based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence (Williams et al. 1990). A sequence will be amplified only if the random primer matches the genomic template at the two specific sites, one on each complementary strand, which bracket a short sequence of template DNA. The primer sequences are known, so a successful amplification reaction can be repeated.

3.2.4.2 DNA extraction

A modification of the procedure described by Murray and Thompson (1980) was followed for DNA extraction. About 250 mg of young leaf material (about 6 leaves) was collected in a weighing boat and placed on ice. The leaves were ground into powder with liquid nitrogen in a mortar and pestle. The powder was placed in a 1.5 ml Eppendorf tube. Then, 250 µL of extraction buffer (Tris-HCl 100 mM pH 8.0, EDTA 25 mM, NaCl 1.4 M, CTAB 2%) (preheated to 60°C) was added to the powder which was ground again at room temperature in a mortar and pestle. The pestle was washed with an additional 250 µL of extraction buffer. After a brief vortexing the tube was incubated at 60°C in a water bath for about 40 minutes. Then 500 µL of chloroform:isoamyl alcohol (24:1) was added and vortexed for 5 minutes. After vortexing, the extract was centrifuged for 5 minutes at 19,870 x g. The upper aqueous phase was transferred into a new 1.5 ml Eppendorf tube and 400 µL of isopropanol (cooled to -20°C) was added. The tube was centrifuged for 15 minutes at 19,870 x g. After decanting, the pellet was washed with 500 µL of 80%

ethanol and centrifuged for 5 minutes at 19,870 x g. Decanting, washing and centrifuging were repeated. The pellet was air dried for about one hour and dissolved into 100 µL TE buffer (10 mM Tris, 1 mM EDTA). This method yielded DNA at about 0.11 to 0.15% of the fresh leaf weight. Thus, 250 mg of fresh leaf produced about 275 to 375 µg of DNA.

3.2.4.3 Polymerase chain reaction (PCR)

The PCR reaction condition described by Williams et al. (1990) was used as standard. Starting with the standard protocol an experiment was conducted to determine the optimum concentration of reaction buffer, $MgCl_2$, Taq DNA polymerase, dNTPs, primers and genomic DNA for the 25 µL reaction mixture to produce discrete bands with consistent resolution. The concentration of the components in the standard reaction mixture was 1X buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 0.001% gelatin), 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM primer, 0.5 units of Taq DNA polymerase, and 25 ng of genomic DNA. Mixing of the reaction components was always performed on ice. The mixture in each reaction tube was vortexed briefly before overlaying with paraffin oil. Amplification was performed in a thermocycler (Thermolyne Temp-Tronic Model DB66925) for 40 cycles after initial denaturation for 4 min at 94°C. Each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 37°C and 2 min polymerization at 72°C. The 40 cycles were followed by final extension at 72°C for six min.

3.2.5 Determination of the homology of RAPD markers

In some instances two RAPD markers may arise from the same primer and show tight linkage with each other. These two RAPD markers may represent two different loci or one locus with codominant expression. These two possibilities can be distinguished by testing for homology of the RAPD markers, as follows:

3.2.5.1 Purification of DNA fragments from agarose gels

DNA fragments were excised from the agarose gel and purified following a recommended protocol (QIAGEN Inc. Chatsworth, CA). The two RAPD products each from the primers UBC353 and UBC368 were run on agarose gel electrophoresis to separate the DNA fragments ($UBC353_{395}$ and $UBC353_{964}$; $UBC368_{425}$ and $UBC368_{655}$) for probe preparation, and visualized with long-wave UV light (360nm). The DNA fragments of interest were excised from the gel as a block using a clean razor blade. The DNA fragments were reamplified using 1 µl of the excised gel, containing the DNA fragments of interest, in the reaction mixture. The reamplified DNA fragments were run on an agarose gel, visualized under UV light and excised with a clean sharp razor blade. The excised DNA fragments were purified using QIAquick™ DNA extraction units (QIAGEN Inc., Chatsworth, CA). An agarose gel piece, containing the fragment of interest, was incubated with 3 volumes of buffer QX1 at 50°C for 10 minutes to dissolve the gel. One gel volume of isopropanol was added to the melted DNA solution. Melted agarose solution was then placed in a QIAquick spin column in a 2 ml collection tube and centrifuged for 1 minute at 19,870 x g. The flow-through was discarded and the QIAquick column was placed back in the same collection tube. Then 0.5 ml of buffer QX1 was

added to the QIAquick column and centrifuged for 1 min at 19,870 x g. The column was washed by adding 0.75 ml of buffer PE to the column and centrifuging for 1 min at 19,870 x g. The flow-through was discarded and the column centrifuged for an additional 1 min at 19,870 x g. The column was then placed in a clean 1.5 ml microfuge tube. To elute, 50 µl of 10 mM Tris-HCl, pH 8.5 was added to the centre of the QIAquick column and centrifuged for 1 min at 19,870 x g.

3.2.5.2 Southern transfer

Southern transfer was used to transfer the DNA fragments from the 1.2% agarose gel to the membrane. The Amersham (Amersham International plc, UK) protocol was followed for Southern blotting. RAPD products, obtained using the UBC353 and UBC368 primers, were electrophoresed on a 1.2% agarose gel, visualized under UV-light and photographed. After electrophoresis, the agarose gel was placed in 0.25M HCl until the dye (bromophenol blue) changed colour. The gel was then rinsed in distilled water and placed in denaturation buffer to completely cover the gel. After leaving the gel for 30 min at room temperature with shaking, the gel was rinsed with distilled water and treated with neutralizing buffer for about 15 min at room temperature. The capillary blot was set using 20 x SSC buffer and Hybond-N^+ membrane. After setting up the blot the transfer was allowed to proceed overnight. After blotting, the DNA was fixed on the membrane by baking the membrane in an oven at 80°C for 2 hours.

3.2.5.3 Preparation of the probe

Radioactive probes were prepared using a random primer DNA labelling system (GIBRO/BRL). DNA fragments for labelling were purified as described in Section 3.2.4.1. Twenty five to 30 ng of the DNA (in 20 µl H_2O) was placed in a 1.5 ml screw-capped vial, and denatured by heating in a boiling water bath for 5 min, and cooled immediately on ice. While on ice, the following were added to the reaction mixture: 2 µl each of dATP, dGTP, and dTTP, 15 µl random primers buffer mixture, 5 µl [α-^{32}P]dCTP (10 µCi/µl), and 1 µl Polk (3U). The total volume was adjusted to 50 µl by adding 3 µl of ddH_2O. The reaction mixture was tapped gently, centrifuged briefly at 19,870 x g, and incubated for 1 h at 22°C. Unincorporated nucleotides were removed from the labelled DNA using a Sephadex column unit (Pharmacia). The column was rinsed once with TE buffer. Radioactive probe (50 µl) and 400 µl of TE were added to the column. The filtrate was discarded as it did not contain probe. The column was again filled with 400 µl TE and the filtrate, containing radioactive probe DNA, was collected in a 1.5 ml Eppendorf tube.

3.2.5.4 DNA-DNA hybridization

The protocol recommended by Amersham (Amersham International plc, UK) was followed for DNA-DNA hybridization. The membranes, containing the immobilized DNA, were soaked in water and prehybridized in 10 ml of 5x SSPE, 5 x Denhardt's solution, and 0.5% (w/v) SDS for 1 h at 65°C. The radioactive probe (23 µl) was mixed with 200 µg of salmon sperm DNA and 1 ml of QuickHyb™ solution, heated at 100°C

for 5 min, and then added to the hybridization solution. The membranes were hybridized with the probe in QuickHyb™ rapid hybridization solution (Stratagen) at 65°C for 18 h. Both prehybridization and hybridization were performed in roller bottles in a mini hybridization oven (Bio/Can Scientific). The membranes were washed into plastic storage boxes at 65°C using buffer A (40 mM NaH_2PO_4 (pH 7.2), 1 mM EDTA, 5% SDS, and 0.5% blocking reagent) and buffer B (40 mM NaH_2PO_4 (pH 7.2), 1 mM EDTA, 1% SDS) successively until the blots were free of background signals. The signals was monitored using a hand-held Thyac III Geiger counter (Victoreen).

The wet membranes were wrapped in Saran Wrap and exposed to Kodak X-OMAT™ AR films in cassettes with Cronex Quanta III intensifying screens.

3.2.6 Statistical analyses

3.2.6.1 Inheritance

A test of the agreement of observed with expected single factor inheritance was performed for each marker from different populations separately using Chi-squared goodness-of-fit test (Mather 1963). The phenotypic markers were expected to segregate in a 3:1 ratio, as in most cases they showed complete dominance. The isozymes were expected to segregate either 3:1 (when one parent has the null allele, and the other allele is dominant over the null allele) or 1:2:1 (when the parents have different alleles and both alleles are codominant in expression). The RAPD phenotypes were expected to segregate in a 3:1 ratio, as the bands are either present or absent and presence is usually dominant over absence.

3.2.6.2 Linkage

MAPMAKER (Lander et al. 1987), a computer program, was used to detect linkage. MAPMAKER determines linkage grouping based on two criteria, a) the recombination fraction and b) its LOD (log of the odds) value. LOD is the $\log_{10}$ of the ratio between two likelihoods. The numerator is the likelihood of obtaining the observed data, given the recombination fraction is as estimated, and the denominator is the likelihood of obtaining the observed data, if the recombination fraction is fixed at 50% (not linked). MAPMAKER estimates the linkage group on the basis of two-point linkage test data similar to the procedure described in LINKAGE-1 (Suiter et al. 1983). However, once the linkage group is determined, MAPMAKER uses multipoint analysis to determine the correct order and the map distance. The construction of an n-point linkage map from two-point linkage test data revealed errors in genetic order, so linkage maps were developed using multipoint analysis (Lander et al. 1987). The expected frequency of the observed phenotypes can be calculated theoretically in terms of the recombination fraction. The maximum likelihood method was used to estimate the recombination fraction having the smallest standard error (Mather 1963). The likelihood of obtaining the observed data (P) is given by the expression,

$$P = \frac{n!}{a_1!a_2!....a_t!}(m_1)^{a_1}(m_2)^{a_2}.....(m_t)^{a_t},$$

where n is the number of individuals in the segregating population, $a_1....a_t$ the numbers of individuals observed in the segregating classes, and $m_1.....m_t$ the expected proportions of individuals in the segregating classes. The expected proportions, denoted by m, are known in terms of p, the quantity which is estimated. The method of maximum

likelihood depends on the maximization of this expression, with respect to p. The expression and its logarithm will both be maximum at the same value of p. Hence, we can find the requisite recombination fraction by maximizing the logarithm of the likelihood expression with respect to p. The maximization is done by differentiation of the log of likelihood expression and equating to zero.

$$L = C + a_1 \log m_1 + a_2 \log m_2 + \ldots\ldots\ldots\ldots + a_t \log m_t \quad \ldots\ldots\ldots\ldots 3.5$$

$$\frac{\delta L}{\delta p} = a_1 \frac{\delta \log m_1}{\delta p} + \ldots\ldots\ldots\ldots + a_t \frac{\left(\delta \log m_t\right)}{\delta p} = 0 \quad \ldots\ldots\ldots\ldots 3.6$$

In cases where the same two factors are segregating in more than one F_2 population, a Chi-squared test of homogeneity was performed to check the agreement of linkage over different populations. If the populations were homogeneous, the data were pooled to give an estimate of the recombination fraction.

The recombination fraction between adjacent markers on the map was used to estimate the standard error, using the equation shown by Allard (1956) and Mather (1963).

$$V_p = \left[n \sum \left\{ \frac{1}{m_t} \left(\frac{\delta m_t}{\delta p} \right)^2 \right\} \right]^{-1} \quad \ldots\ldots\ldots\ldots 3.7$$

where, V_p = variance of the estimated recombination p, n = number of individuals in the segregating population, m_t = expected frequency of a particular phenotypic class (expressed as a function of p), $\frac{\delta m_t}{\delta p}$ = differentiation of m_t with respect to p. The map

units were converted to crossover units, then formula from Allard (1956) or Mather (1963) was used to calculate standard error. No back transformation was done because it has little effect on the unit. Although not statistically correct to get standard error for map units, this should give good enough estimate of precision.

Commands in MAPMAKER were executed with 'error detection' and 'use three point' 'on'. When 'error detection' is on, all multipoint analyses allow a small possibility (1% by default) of typing error in each locus for each individual and correct the map order and map distance accordingly. When 'use three point' was on, three-point analyses were used by order finding commands (e.g. compare, try, etc.) to restrict the set of locus orders that must be examined. Any three-point order was excluded whose relative log-likelihood was worse than the best by a threshold. The threshold was 4.0 in the present analyses. Any multiple locus order that contained one or more excluded three-point sub-orders was itself excluded. The linkage groups were detected on the basis of two point linkage test using the 'group' command. The criteria for declaring a linkage were LOD = 3 and recombination fraction = 0.30. For the linkage groups containing not more than six markers, the map order was determined by using 'compare' command. The 'compare' command displays the best 20 map orders with respective log-likelihood values. The best order was set at a log-likelihood value of zero. The rest of the 20 displayed orders had negative log-likelihood values. In the present analyses, any two orders were considered equally likely (ambiguous) if their log likelihood values (absolute value) differed by not more than a LOD of two. If the linkage group included more than six markers, a subset of six markers was searched manually by looking at the 'big lod'

table displayed by the 'big lod' command. The 'big lod' table displayed only those two point linkages for which the LOD value was at least 3. Six markers that were successively linked with a higher LOD value were chosen as a subset. Any markers tightly linked in repulsion phase were excluded from the subset. The ambiguity of the map order of subset was checked by 'compare' command. Once a subset with a definite order was determined the rest of the markers in the linkage group were placed in order with the 'try' command. The 'try' command placed each of the remaining markers in every possible marker interval of the existing order and displayed the log-likelihood values for the placement. Once all the markers within a linkage group were ordered, the order was verified by 'ripple' command. The window size and log-likelihood threshold was set to five and two respectively in the ripple command. Then, map distance between the successive markers was determined by 'map' command. The map distance was set to Haldane's cM (Haldane 1919).

4. RESULTS

4.1 Outcrossing Study

The outcrossing rate in grasspea was estimated from seed collected under nine combinations of gene frequency (3) and location (3). In some instances sample size was less than planned due to either germination failure or lack of sufficient seed. At Goodale an early frost reduced seed yields on the recessive parent plants, resulting in a small sample size for gene frequency 0.5 and 0.33 and no sample for gene frequency 0.033 (Table 4.1). The outcrossing rate was adjusted for the different gene frequencies and ranged from 1.7 to 2.7% among the eight remaining combinations.The average outcrossing rate was 2.2±0.7%. The χ^2 test of homogeneity (χ^2 =1.2, ns) showed that the outcrossing rate was uniform among the eight combinations of gene frequency and location. The different gene frequencies were adjusted for in the estimate of outcrossing.

4.2 Optimization of the RAPD Protocol

Different concentrations of the reaction components in the RAPD protocol were tested for optimization (Table 4.2). The components were tested one at a time, keeping the concentration of the others unchanged. Once the concentration of a component was optimized the optimum concentration was used in succeeding optimization tests. The concentration of the components did not vary from the standard, except for Taq

Table 4.1 Estimates of outcrossing rates (t) for grasspea under nine combinations of gene frequency and location.

Gene frequency-location[y]	(a+b)=N	Blue (a)	White (b)	q	p	t±SE
F1-P	786	9	777	0.500	0.500	0.023±0.008
F1-K	801	8	793	0.500	0.500	0.020±0.007
F1-G	362	3	359	0.500	0.500	0.017±0.009
F2-P	1011	16	995	0.333	0.667	0.024±0.006
F2-K	958	17	941	0.333	0.667	0.027±0.006
F2-G	265	4	261	0.333	0.667	0.023±0.011
F3-P	520	10	510	0.033	0.967	0.020±0.006
F3-K	503	10	493	0.033	0.967	0.021±0.006
F3-G[z]	-	-	-	-	-	-
Average						0.022±0.007

[y]F1-P = gene frequency (q) at Preston, F1-K= gene frequency (q) at Kernen Farm, F1-G = gene frequency (q) at Goodale, F2-P = gene frequency (q) at Preston etc.
[z] -= Missing.

polymerase, Mg^{++}, and dNTPs. The optimum concentration of the components in the reaction mixture was 1X buffer (50 mM KCl; 10 mM Tris-HCl, pH 9.0 at 25°C; 0.1% Triton X-100), 1.5 mM or 4 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM primer, 1.5 units of Taq DNA polymerase, and 25 ng of genomic DNA. The initial experiment was done with two primers (UBC305 and UBC317) for which a reaction mixture with 4 mM $MgCl_2$ was optimum. However, later work showed that 4 mM $MgCl_2$ produced an inconsistent banding pattern for many primers. Promising primers (primers which produced well resolved bands in the initial screening) were tested with both 1.5 mM and 4 mM Mg^{++}. Results indicated that only four primers (UBC305, UBC314, UBC317, UBC349) produced clearer and more consistent bands at 4 mM Mg^{++}, whereas the other 15 primers (Appendix 2) produced clearer and more consistent bands at 1.5 mM Mg^{++}.

Table 4.2 Concentrations of the components in the polymerase chain reaction mixture.

Reaction buffer (X)	$MgCl_2$ (mM)	Taq DNA pol. (units)	Primer (µM)	dNTP (µM)	Genomic DNA (ng)
0.5	1.0	0.25	0.10	100	**25**
1.0[z]	**1.5**	0.50	0.15	150	50
2.0	2.0	1.00	**0.20**	**200**	75
	2.5	**1.50**	0.25	250	100
	2.0	2.00	0.30	300	150
	3.5				
	4.0				
	4.5				
	5.0				

[z] Optimum concentrations are noted in heavy type.

4.3 Polymorphism of the Markers

The morphological and isozyme marker polymorphisms were available from a previous study (Chowdhury 1992). Parents of the cross PI 426891.1.3 x PI 283564C.3.2 were screened for RAPD polymorphism using 100 primers (UBC301 to UBC400). Among the 100 primers tested, 19 primers produced consistently resolvable polymorphic RAPD bands. In the cross PI 283564c.3 x PI 426885.2 isozymes AAT-2, EST-3, LAP-1 and SKDH were polymorphic. In the cross PI 358601.5 x PI 173714.5 isozymes AAT-1, AAT-2, ACO-1, ACO-2 and SKDH were polymorphic. In the cross PI 426891.1 x PI 172930.4 isozymes AAT-1, AAT-2, AC0-2, EST-3, FDH and PGD-2 were polymorphic. In the cross PI 283549a.6 x PI 202803a.3 isozymes EST-6, LAP-1, PGD-2 and TPI-1 were polymorphic. In the cross PI 426891.1.3 x PI 283564C.3.2 flower colour, AAT-2, SKDH, LAP-1 and 72 RAPD markers were polymorphic (Appendix 3). These 72 polymorphic markers were produced by 19 primers.

4.4 Inheritance Study

Inheritance of the markers was studied separately in the different F_2 populations. Most of the markers followed a typical Mendelian segregation.

4.4.1 PI 283564c.3 x PI 426885.2 F_2 population

Isozymes AAT-2, LAP-1 and SKDH had three phenotypic classes in the F_2 of this cross, indicating that these three loci were all co-dominant in expression (Fig 4.1). Thus, AAT-2, LAP-1 and SKDH segregated in a 1:2:1 ratio, the codominant Mendelian monogenic ratio expected for isozymes (Table 4.3). The two-band phenotypes in the heterozygotes of LAP-1 and SKDH suggested a monomeric structure for LAP-1 and SKDH (Figure 4.1). The isozyme EST-3 was expressed in a dominant fashion with the band dominant over no band (null allele) and segregated in a 3:1 ratio (Table 4.3). As the heterozygotes of EST-3 showed only one band (Figure 4.1), the quaternary structure of EST-3 could not be inferred.

4.4.2 PI 358601.5 x PI 173714.5 F_2 population

All five isozymes in this cross were expressed in a codominant fashion with three phenotypic classes in the F_2 and gave a good fit to a 1:2:1 ratio (Table 3). The two-band phenotypes of the ACO-1, ACO-2 and SKDH heterozygotes indicate a monomeric structure and the three-band phenotypes of the AAT-1 and AAT-2 heterozygotes indicate a dimeric structure (Figure 4.1).

Figure 4.1 Typical banding pattern of different isozymes segregating in various F_2 populations of grasspea. a) AAT-1 and AAT-2 segregating in PI 358601.5 x PI 173714.5, b) ACO-1 and ACO-2 segregating in PI 35601.5 x PI 173714.5, c) EST-3 segregating in PI 283564c.3 x PI 426885.2, d) EST-6 segregating in PI 283549a.6 x PI 202803a.3, e) FDH segregating in PI 426891.1 x PI 172930.4, f) LAP-1 segregating in PI 283564c.3 x PI 426885.2, g) PGD-2 segregating in PI 426891.1 x PI 172930.4, h) SKDH segregating in PI 283564c.3 x PI 426885.2, and i) TPI-1 segregating in PI283549a.6 x PI 202803a.3. F=fast, H=heterozygote, S=slow.

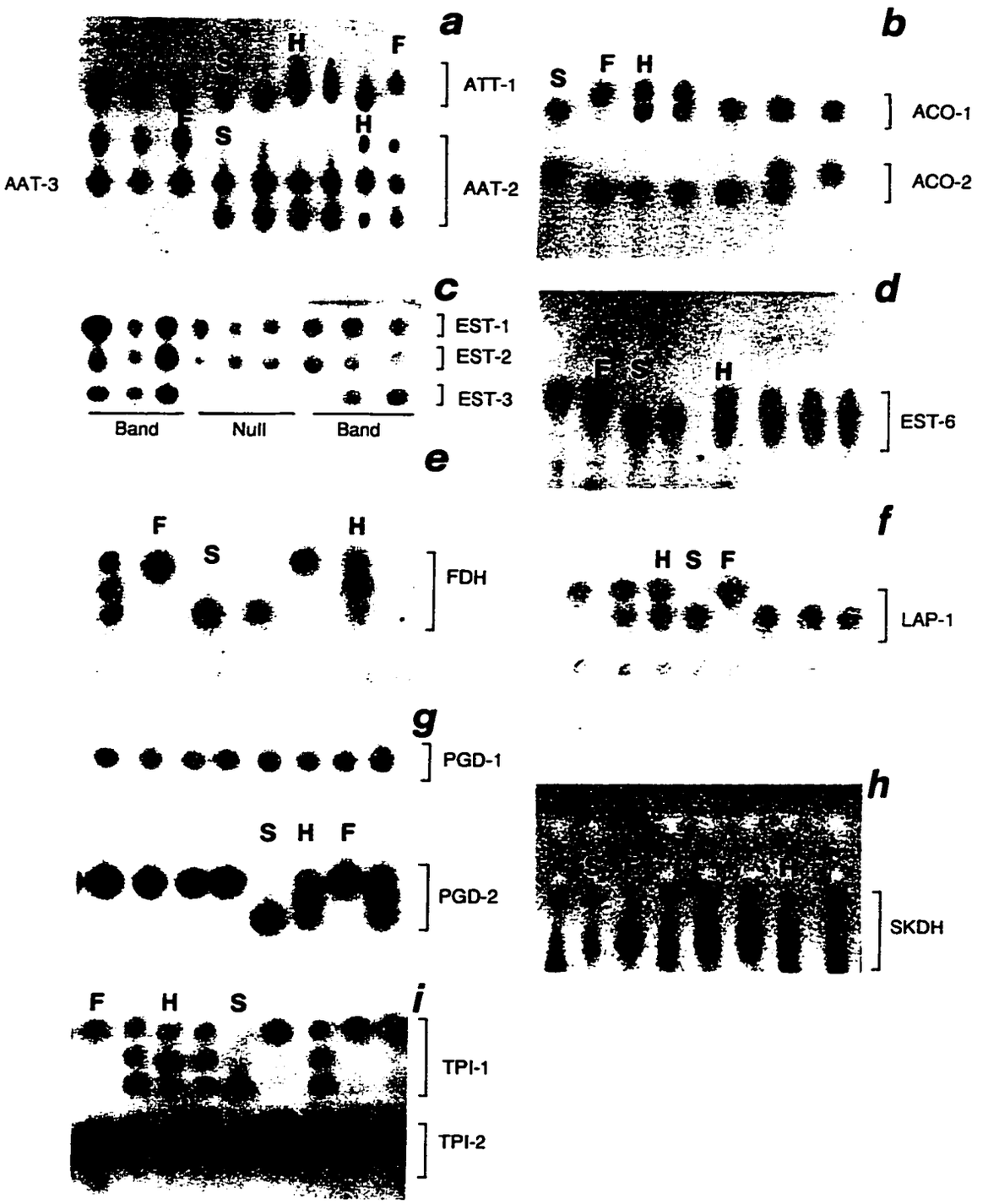

a
S
H
F
ATT-1
F
S
H
AAT-3
AAT-2
b
S
F
H
ACO-1
ACO-2
c
EST-1
EST-2
EST-3
Band
Null
Band
d
F
S
H
EST-6
e
F
S
H
FDH
f
H
S
F
LAP-1
g
PGD-1
S
H
F
PGD-2
h
SKDH
F
H
S
i
TPI-1
TPI-2

Table 4.3 Chi-squared test of goodness-of-fit to a single locus ratio for morphological, isozyme and RAPD loci in five F_2 populations of grasspea.

F_2 population	Locus	N	Observed ratio F:H:S or A- aa	Expected ratio	χ^2
PI 283564c.3 x PI 426885.2	*Aat-2*	180	48:82:50	1:2:1	1.5
	Est-3	180	45:135	1:3	0.3
	Lap-1	180	40:90:50	1:2:1	1.1
	Skdh	180	52:92:36	1:2:1	2.9
PI 358601.5 x PI 173714.5	*Aat-1*	180	40:98:42	1:2:1	1.5
	Aat-2	180	49:100:31	1:2:1	5.7
	Aco-1	180	44:96:40	1:2:1	1.0
	Aco-2	180	42:95:43	1:2:1	0.6
	Skdh	180	33:102:45	1:2:1	4.8
PI 426891.1 x PI 172930.4	*Aat-1*	180	52:88:40	1:2:1	1.7
	Aat-2	180	46:92:42	1:2:1	0.3
	Aco-2	180	44:39:180	1:2:1	1.4
	Est-3	180	34:146	1:3	3.6
	Fdh	180	40:102:38	1:2:1	3.2
	Pgd-2	180	41:106:33	1:2:1	6.4*
PI 283549a.6 x PI 202803a.3	*Est-6*	180	52:82:46	1:2:1	1.8
	Lap-1	180	42:92:46	1:2:1	0.3
	Pgd-2	180	38:92:50	1:2:1	1.7
	Tpi-1	180	38:86:56	1:2:1	4.0
PI 426891.1.3 x PI 283564c.3.2	*Aat-2*	100	31:49:20	1:2:1	2.5
	Lap-1	100	14:65:21	1:2:1	11.0**
	Skdh	100	23:49:28	1:2:1	0.5
	Fl.col.	100	25:75	1:3	0.0
	$UBC304_{791}$	100	28:72	1:3	0.1
	$UBC304_{831}$	100	13:87	1:3	7.7**
	$UBC304_{917}$	100	22:78	1:3	0.5
	$UBC304_{964}$	100	35:65	1:3	5.3*
	$UBC304_{2025}$	100	21:79	1:3	0.9
	$UBC305_{831}$	100	23:77	1:3	0.2
	$UBC305_{917}$	100	22:78	1:3	0.5
	$UBC305_{1432}$	100	20:80	1:3	1.3
	$UBC305_{482}$	100	21:79	1:3	0.9

Continued...

Table 4.3 continued....

F_2 population	Locus	N	Observed ratio	Expected ratio	χ^2
PI 426891.1.3 x PI 283564c.3.2 Continued	$UBC308_{289}$	100	24:76	1:3	0.1
	$UBC308_{630}$	100	21:79	1:3	0.9
	$UBC308_{990}$	100	15:85	1:3	5.3*
	$UBC314_{964}$	100	23:77	1:3	0.2
	$UBC314_{1662}$	100	27:73	1:3	0.2
	$UBC317_{559}$	100	27:73	1:3	0.2
	$UBC317_{617}$	100	31:69	1:3	2.1
	$UBC317_{1175}$	100	22:78	1:3	0.5
	$UBC317_{1363}$	100	20:80	1:3	1.3
	$UBC322_{1432}$	100	40:60	1:3	12.0**
	$UBC322_{1745}$	100	20:80	1:3	1.3
	$UBC322_{1927}$	100	29:71	1:3	0.8
	$UBC328_{682}$	100	21:79	1:3	0.9
	$UBC328_{831}$	100	39:61	1:3	11.0**
	$UBC331_{487}$	100	21:79	1:3	0.9
	$UBC331_{587}$	100	21:79	1:3	0.9
	$UBC331_{716}$	100	19:81	1:3	0.9
	$UBC331_{1175}$	100	21:79	1:3	0.9
	$UBC332_{1118}$	100	39:61	1:3	11.0**
	$UBC332_{1581}$	100	14:86	1:3	6.5*
	$UBC332_{1834}$	100	20:80	1:3	1.3
	$UBC333_{506}$	100	24:76	1:3	0.1
	$UBC333_{617}$	100	40:60	1:3	12.0**
	$UBC333_{682}$	100	17:83	1:3	3.4
	$UBC333_{964}$	100	21:79	1:3	0.9
	$UBC333_{1235}$	100	31:69	1:3	1.9
	$UBC349_{752}$	100	38:62	1:3	9.0**
	$UBC349_{1235}$	100	21:79	1:3	0.9
	$UBC349_{1297}$	100	24:76	1:3	0.1
	$UBC353_{395}$	100	22:78	1:3	0.5
	$UBC353_{873}$	100	26:74	1:3	0.1
	$UBC353_{964}$	100	29:71	1:3	0.8
	$UBC353_{682}$	100	17:83	1:3	3.4
	$UBC360_{424}$	100	24:76	1:3	0.1
	$UBC360_{1118}$	100	21:79	1:3	0.9

Continued....

Table 4.3 *Concluded....*

F_2 population	Locus	N	Observed ratio	Expected ratio	χ^2
PI 426891.1.3 x PI 283564c.3.2 Continued	$UBC360_{1746}$	100	19:81	1:3	1.9
	$UBC362_{1174}$	100	25:75	1:3	0.0
	$UBC362_{1297}$	100	25:75	1:3	0.0
	$UBC364_{559}$	100	17:83	1:3	3.4
	$UBC364_{649}$	100	23:77	1:3	0.2
	$UBC364_{738}$	100	28:72	1:3	0.5
	$UBC364_{1013}$	100	23:77	1:3	0.2
	$UBC364_{1174}$	100	24:76	1:3	0.1
	$UBC364_{1235}$	100	28:72	1:3	0.5
	$UBC364_{1581}$	100	20:80	1:3	1.3
	$UBC364_{1662}$	100	26:74	1:3	0.1
	$UBC365_{341}$	100	33:67	1:3	3.4
	$UBC365_{752}$	100	21:79	1:3	0.9
	$UBC365_{1013}$	100	40:60	1:3	12.0**
	$UBC365_{1928}$	100	29:71	1:3	0.8
	$UBC368_{425}$	100	23:77	1:3	0.2
	$UBC368_{559}$	100	27:73	1:3	0.2
	$UBC368_{655}$	100	21:79	1:3	0.9
	$UBC368_{791}$	100	24:76	1:3	0.1
	$UBC372_{682}$	100	27:73	1:3	0.2
	$UBC372_{1064}$	100	26:74	1:3	0.1
	$UBC372_{1235}$	100	23:77	1:3	0.2
	$UBC388_{459}$	100	12:88	1:3	9.0**
	$UBC388_{532}$	100	22:78	1:3	0.5
	$UBC388_{752}$	100	27:73	1:3	0.2
	$UBC388_{1175}$	100	24:76	1:3	0.1
	$UBC388_{1363}$	100	23:77	1:3	0.2
	$UBC388_{1662}$	100	26:74	1:3	0.1

*,** Significant at P=0.05 and 0.01 level, respectively.

4.4.3 PI 426891.1 x PI 172930.4 F_2 population

Five of the six isozymes in this cross (except EST-3) were expressed in a codominant fashion with three phenotypes in the F_2 and gave a good fit to a 1:2:1 ratio, except for PGD-2 (Table 4.3). PGD-2 showed distorted segregation and was deficient for the female parental type. EST-3 had a null allele and was expressed in a dominant fashion with band dominant over no band (null allele) and segregated in a 3:1 ratio. The banding patterns of the heterozygotes (Figure 4.1) suggested a monomeric structure for ACO-2 (two bands) and a dimeric structure (three bands) for AAT-1, AAT-2, FDH and PGD. As the heterozygotes of EST-3 showed only one band, the quaternary structure of EST-3 could not be inferred from the present experiment.

4.4.4 PI 283549a.6 x PI 202803a.3 F_2 population

All four isozymes in this cross segregated in a codominant fashion with three phenotypic classes in the F_2 and segregated in a 1:2:1 ratio (Table 4.3). The banding pattern of the heterozygotes (Figure 4.1) suggested a monomeric structure for EST-6 and LAP-1 (two bands) and a dimeric structure for PGD-2 and TPI-1 (three bands).

4.4.5 PI 426891.1.3 x PI 283564c.3.2 F_2 population

The three isozymes in this cross segregated in a codominant fashion with three phenotypic classes in the F_2 and segregated in a 1:2:1 ratio except for LAP-1(Table 4.3). LAP-1 showed distorted segregation and was deficient in the male parental type. The banding patterns of the heterozygotes (Figure 4.1) suggested a monomeric structure for

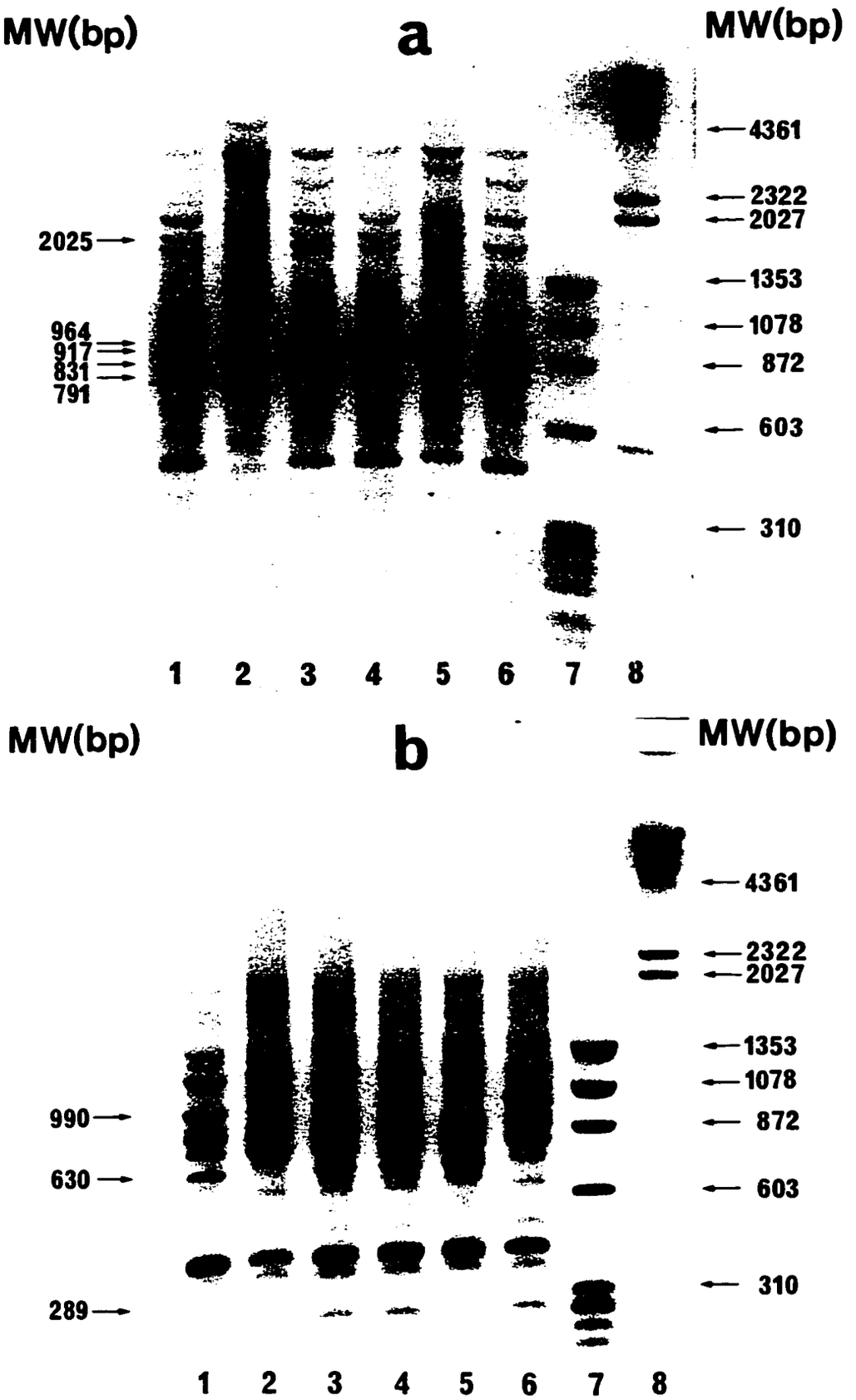
LAP-1 and SKDH (two bands) and a dimeric structure for AAT-2 (three bands). Flower colour showed a 3:1 segregation pattern with blue flower dominant over white (Table 4.3). The 3:1 segregation in F_2 indicates that blue vs. white flower colour in grasspea is controlled by a single gene.

The polymorphic RAPD markers (bands) were scored as either present or absent, as was observed in the parents and F_2. However, individual F_1 plants showed all of the polymorphic bands, indicating dominant inheritance of the markers (Figure 4.2). All the RAPD markers, except $UBC304_{831}$, $UBC304_{964}$, $UBC308_{990}$, $UBC322_{1432}$, $UBC328_{831}$, $UBC332_{1118}$, $UBC332_{1581}$, $UBC333_{617}$, $UBC349_{752}$, $UBC365_{1013}$, and $UBC388_{459}$, followed a 3:1 Mendelian segregation (Table 4.3).

4.5 Linkage Study

Linkage was analyzed separately for each individual F_2 population. Markers showing distorted segregation were also included in the linkage analysis as suggested by Lie et al. (1994). The threshold criteria for the detection of linkage between a pair of markers were a minimum LOD value of 3 and a maximum map distance of 50 cM. A Morgan is defined as the distance along which at least one cross over is expected to occur per gamete per generation. Any pair of markers which had a LOD score of less than 3.0 and a map distance higher than 50 cM was considered unlinked. Haldane's mapping function (Haldane 1919) was used to convert the recombination fraction into map distance.

Figure 4.2 RAPD banding pattern in the cross PI 426891.1.3 x PI 283564c.3.2. a) RAPD markers generated by the primer UBC304. Lane 1, 2 and 3 = individual F_2 plants, lane 4 = F_1, lane 5 = parent PI 283564c.3.2, 6 = parent PI 426891.1.3, lane 7 = ϕ174 DNA/*Hae* III molecular weight markers, lane 8 = λDNA/*Hind* III molecular weight markers. b) RAPD markers generated by the primer UBC308. The lane identification is same as above. Arrows indicate the position of polymorphic markers.


MW(bp)
a
MW(bp)
4361
2322
2027
2025
1353
1078
964
917
831
791
872
603
310
1 2 3 4 5 6 7 8
MW(bp)
b
MW(bp)
4361
2322
2027
1353
1078
990
872
630
603
310
289
1 2 3 4 5 6 7 8

4.5.1 PI 283564c.3 x PI 426885.2 F_2 population

The two isozyme loci *Aat-2* and *Skdh* were linked with a map distance of 24±4.1 cM (Table 4.4). *EST-3* and *LAP-1* were unlinked (independent) loci in this F_2 population.

Table 4.4 Joint segregation of *Aat-2* and *Skdh* loci in F_2 of 283564c.3 x PI 426885.2 and 358601.5 x PI 173714.5 of grasspea (repulsion phase).

	Genotype[y]									Map distance
F_2 population	FF	FH	FS	HF	HH	HS	SF	SH	SS	(cM)
PI 283564c.3 x PI 426885.2	3	20	25	12	60	10	37	12	1	24.0
PI 358601.5 x PI 173714.5	4	19	26	12	72	16	17	11	3	31.0

[y]F=fast, H=heterozygote, S=slow.

4.5.2 PI 358601.5 x PI 173714.5 F_2 population

The two isozymes loci *Aat-2* and *Skdh* were linked with a map distance of 31 ± 5 cM (Table 4.4). *Aat-1*, *Aco-1* and *Aco-2* were unlinked (independent) loci in this F_2 population.

4.5.3 PI 426891.1 x PI 172930.4 F_2 population

No linkage was evident among the isozyme loci (*Aat-1*, *Aat-2*, *Aco-2*, *Est-3*, *Fdh*, *Pgd-2*) in this F_2 population.

4.5.4 PI 283549a.6 x PI 202803a.3 F_2 population

No linkage was evident among the isozyme loci (*Est-6, Lap-1, Pgd-2, Tpi-1*) in this F_2 population.

4.5.5 PI 426891.1.3 x PI 283564c.3 F_2 population

Out of 76 markers (1 morphological, 3 isozyme and 72 RAPD) studied in this F_2 population, 69 markers (1 morphological, 3 isozyme and 65 RAPD) were assigned to 14 linkage groups on the basis of two-point linkage tests (Appendix 4). Six markers were unlinked.

A definite order was suggested for all linkage groups, (Figure 4.3) except for linkage groups IV and VII. The ambiguity in map order of the groups IV and VII was due to the inclusion of several markers tightly linked in repulsion phase. When the markers in repulsion phase were analysed separately a definite map order was observed (Figure 4.3). In linkage group VII, markers $UBC360_{1746}$, $UBC353_{395}$, $UBC308_{289}$, $UBC331_{716}$, and $UBC368_{791}$ could not be aligned in a single order as $UBC360_{1746}$ and $UBC368_{791}$ failed to establish linkage to the rest of the five markers.

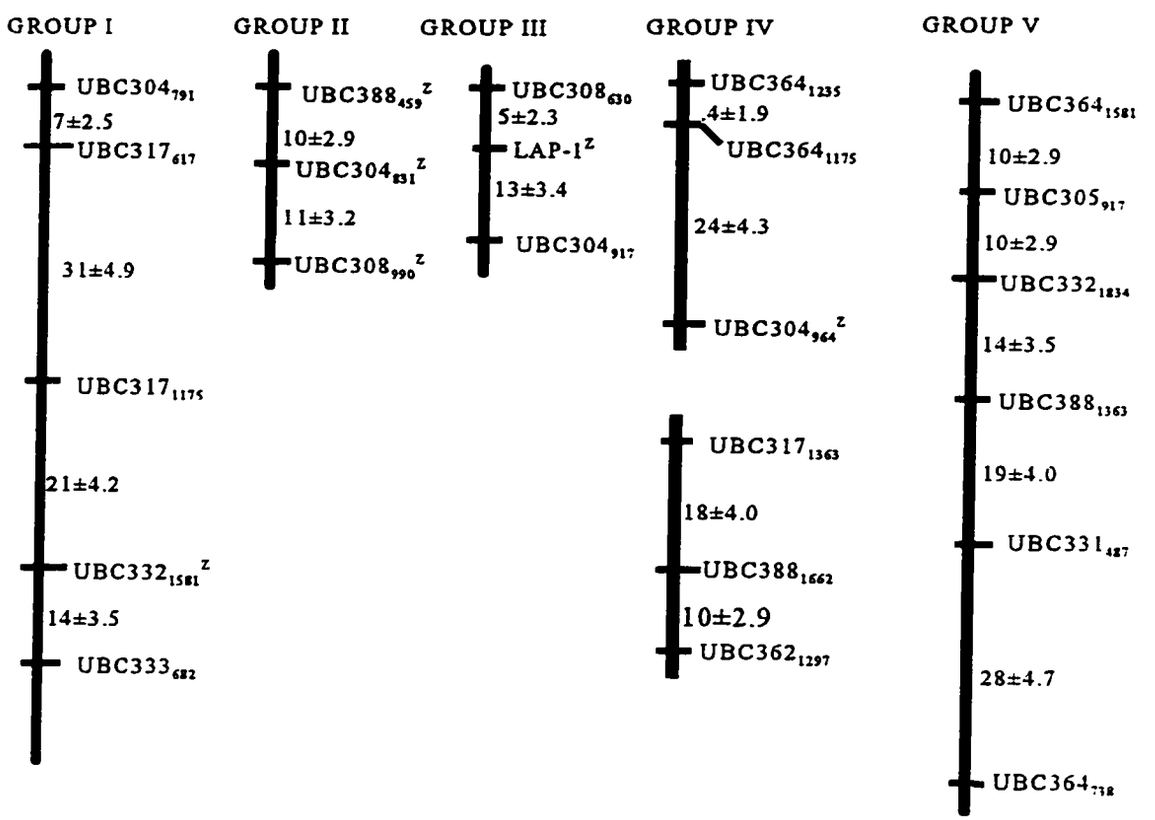


Figure 4.3 Linkage map in grasspea. The solid vertical bars represent linkage groups. The horizontal bars represent marker positions. The alpha-numeric figures at the right of the horizontal bars represent the markers. The numeric figures between two adjacent markers represent map distance in cM ± SE. Due to the ambiguity in overall map order, partial orders are shown for linkage groups IV and VII.

[Z] Represents distorted marker

Figure 4.3 continued.....

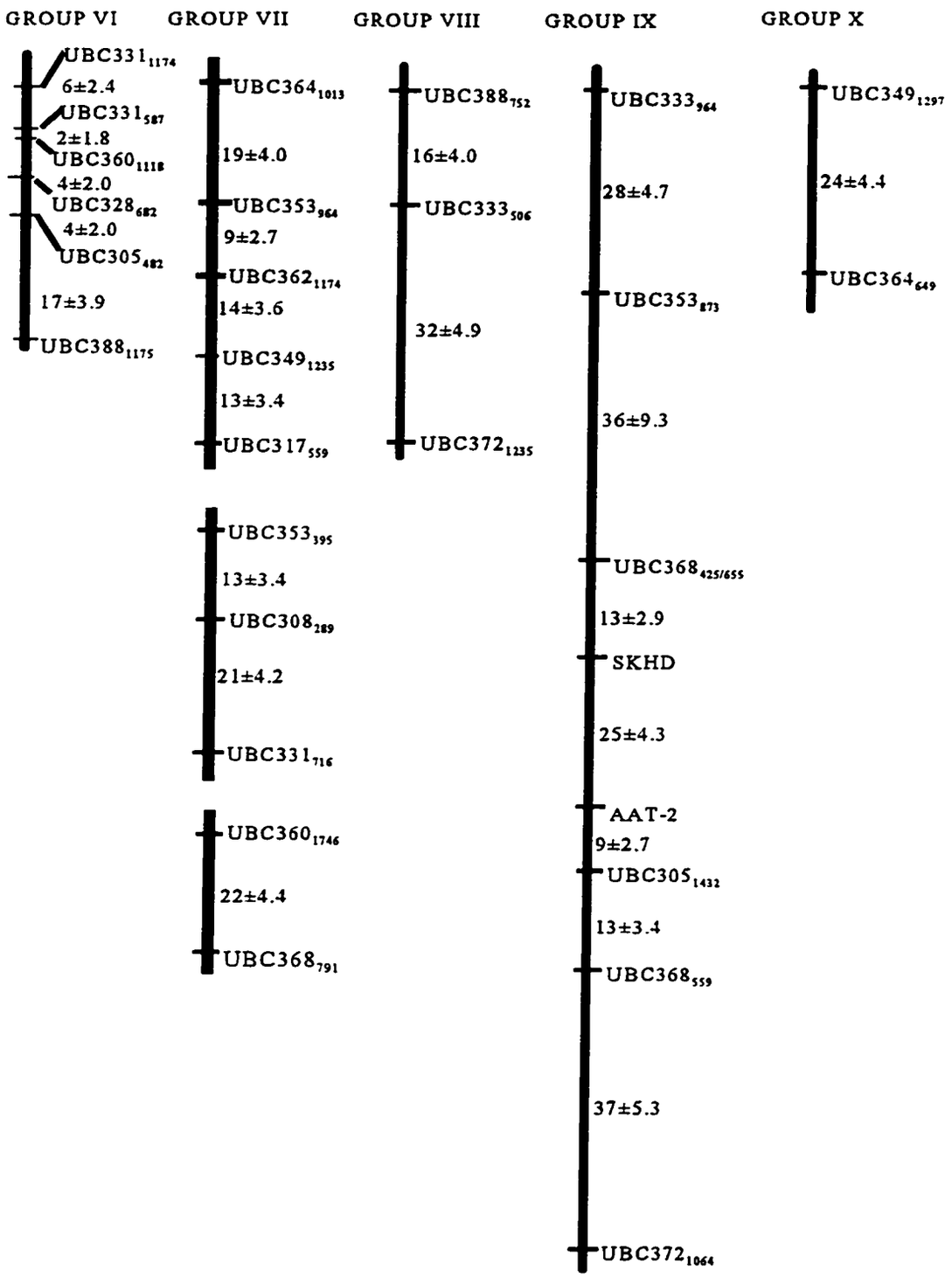
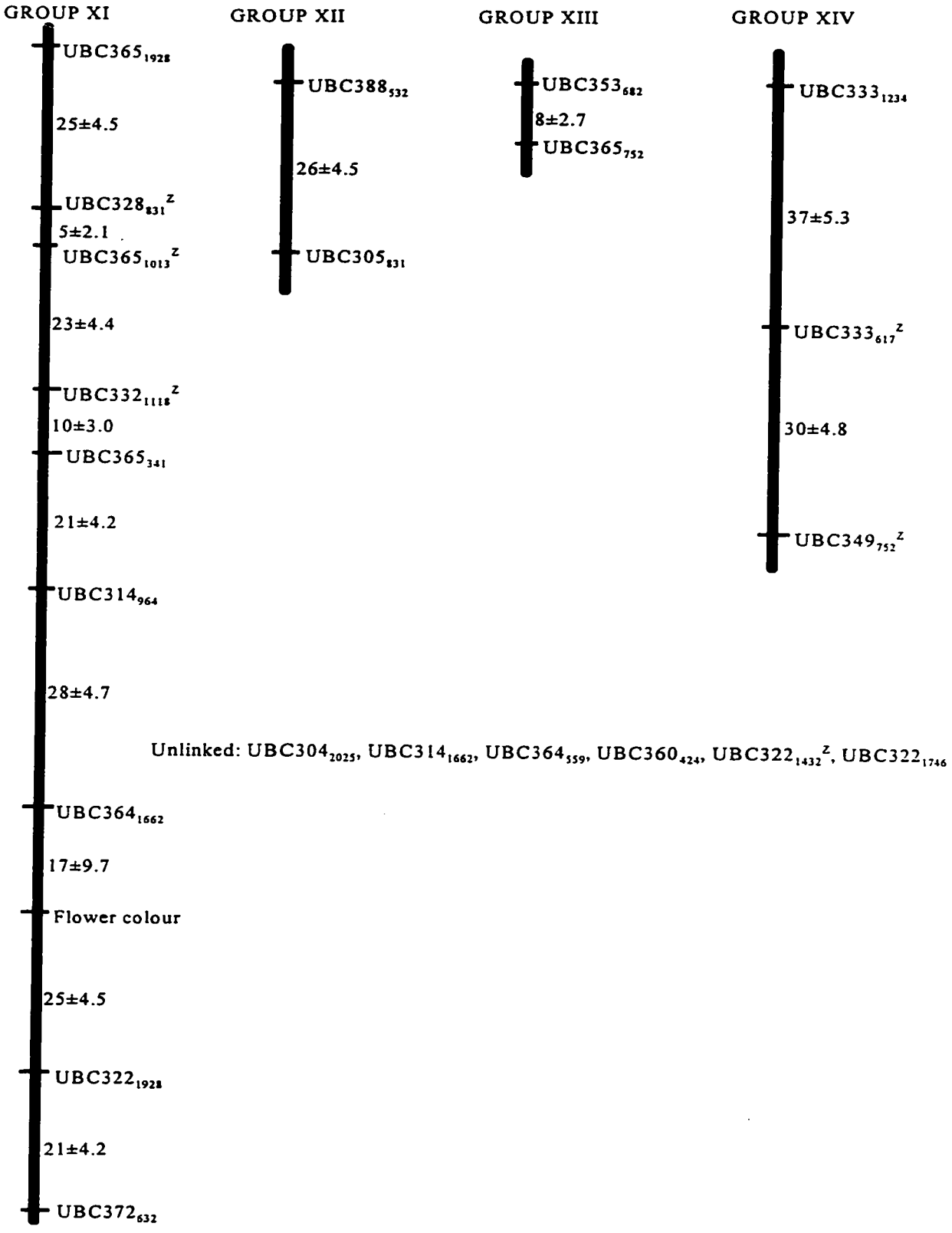


Figure 4.3 concluded.



4.5.6 Homogeneity of recombination between AAT-2 and SKDH

The linkage between AAT-2 and SKDH was consistent over three crosses. In cross PI 283564c.3 x PI 426885.2 the map distance was 24 cM, in cross PI 358601.5 x PI 173714.5 the map distance was 31 cM and in cross PI 426891.1.3 x PI 283564c.3.2 the map distance was 22 cM. A Chi-squared test of homogeneity was performed before pooling the data over the three crosses (Table 4.5).

Table 4.5 Chi-squared test of homogeneity for the joint segregation of AAT-2 and SKDH over three different F_2 populations of grasspea (repulsion phase).

F_2 population	Genotype[y]										
	FF	FH	FS	HF	HH	HS	SF	SH	SS	N[z]	χ^2
PI 283564c.3 x PI 426885.2	3	20	25	12	60	10	37	12	1	180	
PI 358601.5 x PI 173714.5	4	19	26	12	72	16	17	11	3	180	13.7^{NS}
PI 426891.1.3 x PI 283564c.3.2	1	9	21	8	35	6	14	5	1	100	

[y]F=fast, H=heterozygote, S=slow.
[z]N= Sample size.
[NS]Non significantat P=0.05.

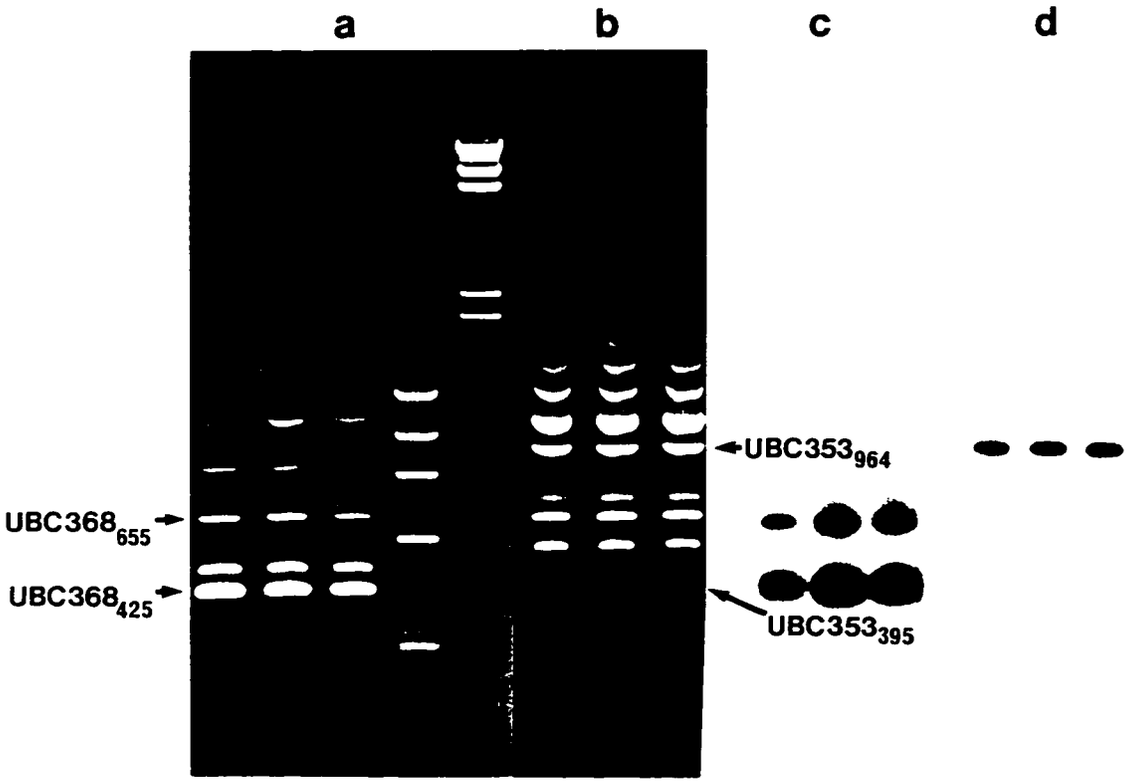
The χ^2 value was nonsignificant, indicating homogeneity of the co-segregating classes over different crosses. The data were pooled over three crosses for AAT-2 and SKDH. A map distance of 25±4.3 cM was obtained from the pooled data.

4.5.7 Codominance in RAPD markers

A few linked markers were amplified by the same primer. Eight such pairs were observed, distributed over seven linkage groups ($UBC317_{617}$ and $UBC317_{1175}$ on group I; $UBC364_{1235}$ and $UBC364_{1175}$ on group IV; $UBC331_{1174}$ and $UBC331_{578}$ on group VI;

$UBC365_{1928}$ and $UBC365_{1013}$ on group XI; $UBC365_{1013}$ and $UBC365_{341}$ on group XI; $UBC333_{1234}$ and $UBC333_{617}$ on group XIV; $UBC353_{964}$ and $UBC353_{395}$ on group VII and $UBC368_{425}$ and $UBC368_{655}$ on linkage group IX). Linkage between pairs of markers originating from a single primer raises the question as to whether the markers in the pair are in fact two different dominant markers or are they two alleles at one locus. For the first six pairs, individuals with no band for both markers (double recessive) were observed in the F_2, providing evidence against codominance. For the last two pairs ($UBC353_{395}$ vs $UBC353_{964}$ and $UBC368_{485}$ vs $UBC368_{655}$) no such individuals were observed. Codominance (homology of the bands) was tested for the pairs $UBC353_{395}$ and $UBC353_{964}$ and $UBC368_{425}$ and $UBC368_{655}$ through probing, Southern blotting and DNA hybridization. When $UBC353_{964}$ was used as probe in Southern blotting, it did not hybridize with $UBC353_{395}$, indicating two separate linked loci. However, when $UBC368_{425}$ was used as a probe in Southern blotting, it hybridized with both $UBC368_{425}$ and $UBC368_{655}$ markers (Figure 4.4). Thus, homology was suggested for the pair $UBC368_{425}$ and $UBC368_{655}$ on linkage group IX. The bands $UBC368_{425}$ and $UBC368_{655}$ were rescored as a single marker with co-dominant expression and a good fit to a 1:2:1 ratio was observed. The data were then reanalysed to produce the linkage map presented in Figure 4.3.

Figure 4.4 Southern blotting for the marker pairs $UBC368_{425}$ vs. $UBC368_{655}$ and $UBC353_{395}$ vs. $UBC353_{964}$ in grasspea. a) Two "linked" polymorphic bands generated by the primer, UBC368. b) Two "linked" polymorphic bands generated by the primer, UBC353. c) Autoradiograph of 'a' when hybridized against the marker $UBC368_{425}$; separate bands indicate homology as the marker hybridized with both bands, d) Autoradiograph of 'b' when hybridized against the marker $UBC353_{964}$; single band indicates two separate linked loci as the marker hybridized with only one band.


a
b
c
d
UBC353964
UBC368655
UBC368425
UBC353395

4.5.8 Genome analysis

Of the 83 markers, 75 (91%) were analysed in a single cross, PI 426891.1.3 x PI 283564c.3.2, and all 14 linkage groups were established from this cross. Accordingly, interpretation of the linkage map will be based on this cross. Of the 75 markers (1 morphological, 3 isozyme and 71 RAPD), 69 markers were assigned to 14 linkage groups, comprising a genome length of 864 cM (Haldane's map distance) (Table 4.6 and Figure 4.3). The number of markers per linkage group ranged from 2 (2.9%) to 10 (14.5%). Within linkage groups the average distance between adjacent markers varied from 5.6 to 33.5 cM. The morphological marker (flower colour) and three isozyme markers were distributed in three linkage groups (III, IX and XI) (Figure 4.3). Flower colour, an easily detectable marker, was mapped near one end of linkage group XI and flanked by two RAPD markers, $UBC364_{1662}$ and $UBC322_{1928}$ with a map distance 17 cM ± 9.7 and 25 cM ± 4.5, respectively, indicating a loose linkage.

Table 4.6 Size distribution of linkage groups in grasspea.

Linkage group	Marker		Length		
	Number	Percent of total markers	cM[z]	Percent of total length	Average distance (cM)
Group 1	5	7.2	74	8.5	18.5
Group 2	3	4.3	21	2.4	10.5
Group 3	3	4.3	19	2.2	9.5
Group 4	6	8.7	28	3.2	5.6
Group 5	6	8.7	85	10.0	17.0
Group 6	6	8.7	32	3.7	6.4
Group 7	10	14.5	100	11.6	11.1
Group 8	3	4.3	48	5.6	24.0
Group 9	8	11.6	157	18.2	22.4
Group 10	2	2.7	24	2.8	24.0
Group 11	10	14.5	175	20.2	19.4
Group 12	2	2.9	26	3.0	26.0
Group 13	2	2.9	8	0.9	8.0
Group 14	3	4.3	67	7.8	33.5
Total	69	100.0	864	100.0	16.9

[z]cM = centiMorgan.

4.5.9 Distribution of the markers showing distorted segregation over linkage groups

Clustering was observed at least for a few of the distorted markers in the PI 426891.1.3 x PI 283564.c.3.2 F_2 (Figure 4.3). Markers, $UBC304_{831}$, $UBC308_{990}$ and $UBC388_{459}$ were distorted and clustered in linkage group II. All three markers were deficient in the recessive (female parental) type (Table 4.3, Appendix 3). Markers $UBC332_{1118}$, $UBC365_{1013}$, and $UBC328_{831}$ were linked in linkage group XI and deficient in the dominant (male parental) type (Table 4.3, Appendix 3).

4.5.10 Efficiency of isozyme and RAPD protocol in the linkage experiment

The efficiency of isozyme and RAPD markers in developing linkage maps was compared in terms of precision, cost, simplicity and the abundance of the markers.

4.5.10.1 Precision

The precision, information, or sensitivity in a linkage mapping experiment is measured as the reciprocal of the variance of the estimated recombination fraction (Allard 1956, Mather 1963). Nine of the 11 isozyme markers showed codominance, whereas 70 of the 71 RAPD markers showed complete dominance and one showed incomplete dominance. As most isozyme markers are codominant, the precision is greater than dominant RAPD markers. As an F_2 population was used in the linkage experiment, the precision of isozyme markers was always higher than for RAPD markers for any recombination value and for any linkage phase (coupling or repulsion). The difference in precision is less with loose linkage and higher with tight linkage. The difference in precision between isozyme and RAPD markers is maximized with tight linkage in repulsion.

4.5.10.2 Cost, simplicity and abundance of markers

The cost per polymorphism/sample for isozyme markers was about 19% higher than for RAPD markers (Table 4.7). The major costs involved in the use of RAPD markers are labour and PCR. The major costs involved in the use of isozyme markers are labour and staining. The RAPD protocol is simpler (less laborious) and requires about

0.06 hr per polymorphism/sample, whereas isozymes requires about 0.07 hr per polymorphism/sample. A major limitation in the use of isozyme markers in gene mapping is the low number of resolvable polymorphic isozyme loci segregating in a particular cross. Equally important is the limited number of resolvable loci in the species. In the PI 426891.1.3 x PI 283564c.3.2 F_2 population 71 RAPD markers were identified, whereas only three isozyme markers were identified.

Table 4.7 Cost comparison for isozyme and RAPD protocol.

Protocol	Steps	$/polymorphism/gel slice[y]	$/polymorphism/sample[z]
RAPD	DNA extraction	0.54	0.03
	PCR	3.83	0.19
	Electrophoresis	1.45	0.07
	Labour	8.33	0.42
Total		14.15	0.71
Isozyme	Tissue extraction	0.03	0.00
	Electrode buffer	0.29	0.00
	Gel buffer	0.03	0.00
	Stain buffer	0.03	0.00
	Staining	2.53	0.13
	Starch	1.48	0.07
	Labour	12.50	0.63
Total		16.89	0.83

[y]An average of one polymorphic marker per gel slice for isozyme markers and three polymorphic markers per gel for RAPD markers with three slices per starch gel.

[z]Twenty samples per gel.

5. DISCUSSION

5.1 Outcrossing Rate in Grasspea

The outcrossing rate of a species must be considered when making a decision on the most appropriate breeding method for improving the species. Knowledge of the outcrossing rate is also useful in designing experiments for genetic and linkage studies, and maintaining genetic purity. In the present experiment the outcrossing rate of grasspea was 2.2±0.7%. This helps to explain the presence of heterozygosity in natural populations of grasspea. Heterozygosity was reported for isozyme loci in natural populations of grasspea (Yunus et al. 1991; Chowdhury 1992). Yunus et al. (1991) reported about 40% heterozygosity for 6-*Pgd*. Chowdhury (1992) reported 1.5% heterozygosity in the germplasm collection of grasspea. Grasspea lines are usually maintained by harvesting seed from lines grown in adjacent rows in the field. However, the outcrossing rate in this study (2.2%) suggests that individual lines of grasspea should be increased in isolation in order to maintain their genetic integrity. Further study should be conducted to determine the isolation distance required to minimize outcrossing in grasspea, as affected by pollinator density.

In the present experiment the outcrossing rate was measured with only one genotype (PI 206891a). However, inter-genotypic differences in outcrossing rate may also occur in grasspea. Rahman et al. (1995) observed significant variation in outcrossing rate

among grasspea genotypes with different flower colour. Genotypes with red and white flower colour had the highest (27.8%) and the lowest (9.8%) outcrossing rate, respectively. It was assumed that these differences were due to the red-coloured flowers attracting more bees than the white-coloured flowers. The lower outcrossing rate in Saskatchewan may be explained by low bee density and activity (no data collected) and the prevalence of low temperatures and strong winds in Saskatchewan during the grasspea flowering season.

Metz et al. (1993) tested 180 faba bean genotypes and obtained outcrossing rates ranging from 1 to 55%. Similar large scale trials should be conducted to provide a more generalized estimate of outcrossing rate in grasspea under Saskatchewan conditions. Moreover, outcrossing rate may be trait dependent, as reported by Yunus et al. (1991) in grasspe, thus, additional loci should be tested.

5.2 Inheritance

Flower colour segregated in the F_2 into a 3 blue:1 white ratio, suggesting that flower colour is controlled by a single locus with blue dominant to white. However, controversy exists regarding the inheritance of flower colour. Kumari et al. (1993) studied the inheritance of flower colour involving three flower colour parents; P505 (blue), P28 (pink) and P27 (white). Crosses of pink-flowered and white-flowered parents yielded blue flowers, suggesting a gene interaction in colour production. They concluded that at least four genes were interacting in the production of flower colour. *C* is responsible for flower colour production, *I* produces an inhibitory action to suppress blue colour, *P* combined

with *C* and *bb* produces pink flower colour, and *P* combined with *C* and *B* produces blue colour. The suggested genotypes of these three parents were P505 (blue) CCPPBBII; P28 (pink) CCPPbbii; and P27 (white) ccppbbII. They reported 3:1 segregation of colour vs. colourless (white) which agrees with the present finding. Quader et al. (1986) studied the inheritance of flower colour in two white flowered x blue flowered crosses and also reported that blue flower colour was dominant to white. They suggested two gene were involved, one with an inhibitory action. They suggested that B is dominant over b and responsible for blue flower colour. However, all the bb genotypes were not white flowered. To make bb genotypes white flowered, a dominant gene W was involved at another locus. If this explanation is true, then selfing of the white-flowered F_2 plants (bbWw) should give rise to white and blue flowered progeny in a 3:1 ratio and if a cross is made between white (bbWW) and blue (bbww), all F_1 plants should be white flowered. In our available grasspea germplasm only blue and white flower-coloured material were available. To unravel the complicted inheritance of flower colour, crosses should be made among parents with different flower colour (red, pink, blue, and white) in all combinations.

Three phenotypes (1:2:1 ratio) were observed for all isozyme markers except EST-3 which showed two phenotypes and segregated into a 3:1 ratio with the presence of the band dominant over its absence (null). In lentil a null allele was observed for the isozyme EST-4 (band dominant over no band) and the F_2 gave a good fit to a 3:1 ratio (Andrahennadi 1994). Among the 10 isozymes, PGD-2 from the cross PI 426891.1 x PI 172930.4 and LAP-1 from the cross PI 426891.1.3 x PI 283564c.3.2 showed disturbed

segregation. All other isozymes segregated in a 1:2:1 ratio. An agreement with either 3:1 or 1:2:1 ratio suggested that the isozymes are monogenic. Three phenotypic classes in the F_2 with codominant expression was also reported for most of the isozymes studied in lentil (Vaillancourt 1989, Andrahennadi 1994) and chickpea (Gaur and Slinkard 1990).

Ten RAPD markers showed disturbed segregation. Distorted segregation in linkage analysis is a common phenomenon. In practice, segregation of markers in the F_2 is usually distorted with certain markers of a specific parent (Bonierbale et al. 1988, McCouch et al. 1988, Gebhardt et al. 1989). In lentil when 20 RFLP, 8 isozyme, and 6 morphological markers were segregating in the F_2, 5 RFLP, 3 isozyme and one morphological marker showed distorted segregation (Havey and Muehlbauer 1989). The distorted segregation in lentil may be due to linkage to some chromosome region conditioning differential fertility or viability.

The common phenomenon in distorted segregation is close linkage of the markers to genes or chromosome regions affecting gametogenesis, fertilization or embryogenesis. In tomato, lines showing distorted segregation for *hp* were characterized by having *hp* linked with a gametophytic gene on chromosome 12; gametes possessing this gametophytic gene were at a disadvantage with respect to fertilization (Mochizuki and Kamimura 1987). In barley distorted segregation was caused by pollen certation in different genotypes, independent of the female genotype (Konishi et al. 1990). Furthermore, pollen certation was controlled by the gene *Ga2* at a locus linked with the esterase multilocus (Est1, Est2, and Est4). In the genus *Beta* distorted segregation arose from linkage of markers with genetic factors affecting gametogenesis, pollen function and embryo development (Abe and Tsuda 1988, Abe 1991). In some such cases

recombination is a prerequisite to viability. In rye (*Secale cereale*) the main region of deviating segregation ratios was localized near the self-incompatibility locus (Philipp et al. 1994). Another explanation for distorted segregation is the nonhomologous and/or homologous pairing of non-allelic regions (e.g., pairing of duplicated regions) in certain chromosomes, giving rise to abnormal chromosomes after recombination and eventually lethality (Landry et al. 1991, Ellis et al. 1992).

However, distorted segregation does not preclude the use of such markers from mapping studies. Computer simulation showed that a lethal gene, which may eliminate a specific haplotype and give rise to distorted segregation of markers linked with it, does not interfere with the recombination estimates (Lie et al. 1994).

5.3 Linkage

AAT-2 and SKDH segregated in three F_2 populations (PI 283564c.3 x PI 426885.2, PI 358601.5 x PI 173714.5, and PI 426901.1.3 x PI 283564c.3.2) and were linked in all three F_2 populations. In lentil, linkage between SKDH and AAT-mb was reported (Muehlbauer et al. 1989; and Tahir and Muehlbauer 1994). Linkage between AAT-m and SKDH was reported in pea (Weeden and Marx 1987) and lentil (Muehlbauer et al. 1989). However, the banding position of AAT-mb in lentil or AAT-m in pea and lentil does not match with the banding position of AAT-2 in grasspea. Information about the subcellular localization of AAT in grasspea may provide a further clue to its possible homology.

In total 14 linkage groups were identified in grasspea (n=7). The number of linkage groups can possibly be reduced by a) increasing the number of markers, b) increasing the

population size, c) progeny testing, or d) using the backcross generations, F_1xP_1 and F_1xP_2, instead of F_2. Inclusion of markers in the repulsion phase may fail to establish linkage due to their high standard error or low LOD values for the recombination fraction. This problem is critical when the markers are in repulsion phase and at the same time tightly linked. The problem could be overcome by including more markers in coupling phase or, alternatively, by increasing population size. If dominance is complete at both loci, the cross is one of repulsion phase, p (recombination fraction)=0.05, and a standard error less than 5% is required, then $n=(i\sigma^2)^{-1} = [(1.006)(0.05)^2]^{-1}= 398$, where n is the population size (Allard 1956). However, in practice the experimenters have a limitation in increasing population size. When using dominant markers in linkage mapping, analyzing more markers and excluding markers linked in tight repulsion may improve the precision of the map and alignment of the markers within the linkage group.

For linkage groups IV and VII a definite marker order could not be suggested. The ambiguity of the order on these two linkage groups might be due to the inclusion of several markers linked tightly in repulsion phase (Appendix 3). The mean information for dominant loci in repulsion phase is essentially zero for F_2's, when loci are tightly linked (Mather 1963). Thus, in the absence of codominance, markers in repulsion phase are difficult or impossible to order unless population sizes are large. Kesseli et al. (1994) reported 2 codominant (RFLP) and 11 dominant RAPD markers in the *Dm13* region (includes the gene for resistance to downy mildew) of the genetic map of lettuce. One RAPD was in repulsion phase to the others and mapped with nearly equal likelihood (LOD<1) to eight possible positions. The RAPD markers in coupling phase also were not

precisely aligned. Thus, the number of potential orders in the region was more than 20. They commented that assembling loci into linkage groups is increasingly easy as the number of loci increases. However, the precise gene order becomes progressively more difficult as the number of loci increases. They suggested that changes in population size make critical differences. Another factor they considered is the mode of inheritance of the markers (3:1 or 1:2:1). For dominant markers tight linkage in repulsion phase can be mapped only with low accuracy. Dominant markers can be turned into codominant markers by using technique like SCAR (sequence characterised amplified region). Automation of the protocol would allow progeny testing for RAPD markers. SCAR is a time demanding and expensive technique, involving sequencing of both ends of the RAPD markers, but it can be used profitably for markers in the vicinity of a gene of interest.

The two-point linkage test results were compared with multipoint linkage test results to check the discrepancy in the map distance between markers from two-point and multipoint linkage tests. It was observed that in most cases at LOD≥3 the two-point linkage test gave similar estimates of map distance between adjacent markers as was given by the multipoint test. However, in some cases (e.g., repulsion phase with tight linkage between dominant markers, small population size, genotyping error, etc.), considerable deviation can occur in map distances between adjacent markers calculated from two point linkage tests and multipoint linkage tests. Thus, it has been suggested that, while linkage groups should be detected by two-point linkage test, gene order and map distance should be calculated using multipoint linkage test (Lander et al. 1987).

It is fairly common to find that markers displaying distorted segregation are clustered on particular linkage groups and that the distortion is toward one of the parental alleles in the entire cluster, indicating selection for or against that allele (and closely linked loci) from that parent (Helentjaris et al. 1986, Landry et al. 1992, Prince et al. 1993). In grasspea distribution of the distorted markers on the linkage group was nonrandom, forming a cluster on a particular region of a linkage group. In linkage group II, distorted segregation occurred for markers $UBC388_{459}$, $UBC304_{831}$ and $UBC308_{990}$ and they were linked. All three markers were deficient in the recessive (female parental) type. In linkage group XI, markers $UBC332_{1118}$, $UBC365_{1013}$, and $UBC328_{831}$ and in linkage group XIV markers $UBC333_{617}$ and $UBC349_{752}$ were linked and all were deficient in the dominant (male parental) type. Cai et al. (1994) found that more than 40% of the distorted RAPD markers clustered on one linkage group and most of the remaining markers clustered on five other linkage groups in *Citrus sp.* However, computer simulation showed that lethal genes, that cause distortion in segregation of the linked markers, do not interfere with the estimate of the recombination fraction (Lie et al. 1994).

The total genome length covered by 75 markers is about 692 cM (in terms of recombination fraction). In lentil seven linkage groups were mapped by 76 markers and covered around 570 cM of the genome (Tahir et al. 1993). In pea 61 markers covered about 650 cM (Weeden et al. 1992b).

The present linkage map will serve as a reference point for further linkage studies in grasspea. Linkage of flower colour with ODAP concentration was reported

earlier (Ramanujam et al. 1980, Quader et al. 1986). ODAP concentration appears to be quantitatively controlled (Quader et al. 1987, Tiwari 1994), but it is possible that only a few major genes control most of the variation. Thus, ODAP concentration could be included in linkage studies to detect tight linkage of molecular markers with major genes for ODAP. Bulked segregant analysis or selective genotyping (Lander and Botstein 1989) could be used for this purpose. As the gene for flower colour has been mapped to linkage group XI, molecular markers from this linkage group along with new markers could be screened for markers linked with one of the major genes for ODAP concentration. Two major limitations of the use of RAPD markers in mapping experiments are 1) inconsistency in the resolution of the bands and 2) dominant type inheritance. Optimizing RAPD protocol, screening the parents with more primers and searching only for consistently resolvable bands (usually faint bands are inconsistently resolvable), increasing the number of polymorphic markers or population size, and progeny testing should overcome these limitations. Automation of the RAPD protocol will facilitate collecting RAPD data for a larger number of F_2 individuals in a mapping experiment and make it feasible to determine F_2 genotype from F_2-derived F_3 ($F_{2:3}$) data.

6. SUMMARY AND FUTURE STUDIES NEEDED

6.1 Summary

- The outcrossing rate in grasspea in this study was 2.2±0.7%.
- Flower colour and the isozyme EST-3 were monogenic with dominant gene expression. Other isozyme markers (AAT-1, AAT-2, ACO-1, ACO-2, EST-6, FDH, LAP-1, PGD-2, SKDH, TPI-1) were monogenic with a co-dominant gene expression. Most of the 72 RAPD markers followed a typical 3:1 Mendelian segregation.
- A linkage map of grasspea was established, consisting of 14 linkage groups. These 14 linkage groups included 69 morphological, isozyme and RAPD markers and included 864 cM total length.
- Considering cost, simplicity and abundance of markers, RAPD markers were more efficient than isozyme markers in linkage studies.

6.2 Future Studies Needed

- The outcrossing rate may vary considerably among different genotypes of a species and different environments. Additional outcrossing experiments should be undertaken, including different flower colour genotypes, different genotypes of thesame flower colour and different marker loci grown under more diverse

environments.

- Experiments should also be undertaken to determine the appropriate isolation distance required to minimize contamination of pure lines of grasspea.
- More markers should be included in the linkage study to obtain a saturated linkage map in grasspea. Along with F_2, backcross or $F_{2:3}$ data should be included to overcome the precision problem associated with dominant markers like RAPD markers.

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8. APPENDICES

Appendix 1. Recipes for staining grasspea enzymes

ACO (Cardy and Beversdorf 1984)	
0.1 M Tris-HCl, pH 8.0	50 mg *
cis-Aconitic acid	100 mg
$MgCl_2$	50 mg
Isocitrate dehydrogenase	20 mg
NADP	10 mg
MTT	10 mg
PMS	10 mg

AAT (Variation of Weeden and Emmo 1984)	
0.1 M Tris-HCl, pH 8.0	50 mL *
Aspartic acid	200 mg
α-Ketoglutaric acid	100 mg
Fast blue BB salt	100 mg
Pyridoxal 5' phosphate	2 mg

EST (Weeden and Emmo 1984)	
0.1 Potassium phosphate, pH 6.0	50 mL
α-Napthyl acetate	50 mg
β-Napthyl acetate (both substrates dissolved together in 3 mL acetone)	50 mg
Fast blue RR salt	50 mg

FDH (Variation of Wendel and Weeden 1989)	
0.05 M Tris-HCl, pH 7.5	50 mL
Formic acid, Na salt	2 g
NADH	10 mg
MTT	10 mg
PMS	2 mg

LAP (Variation of Weeden and Emmo 1984)	
0.1 M Potassium phosphate, pH 6.0	50 mL
$MgCl_2$	100 mg
L-leucyl-β-napthylamide HCl (dissolved in 1 mL N,N-dimethylformamide)	20 mg
Fast black K salt	20 mg

PGD (Wendel and Weeden 1989)	
0.1 M Tris-HCl, pH 8.0	50 mL
6-Phosphogluconic acid	20 mg
$MgCl_2$	50 mg
NADP	10 mg
MTT	10 mg
PMS	10 mg

Continued.....

Appendix 1 continued.....

SKDH		**TPI**	
(Wendel and Weeden 1989)		(Wendel and Weeden 1989)	
0.5 M Tris-HCl, pH 8.5	50 mL	0.5 M Tris-HCl, pH 8.0	50 mL
Shikimate dehydrogenase	50 mg	Arsenic acid, Na salt	75 mg
MTT	10 mg	Dihydroxyacetone phosphate	10 mg
NADP	10 mg	G3PDH	300 units
PMS	2 mg	NAD	10 mg
		MTT	10 mg
		PMS	2 mg

*Mix together and adjust pH to 8.0 with NaOH.

Appendix 2. List of the primers used in the genetic linkage study in grasspea.

Primer #	Primer identification (sequence in 5'→3' direction)
304	AGT CCT CGC C
305	GCT GGT ACC C
308	AGC GGC TAG G
314	ACT TCC TCC A
317	CTA GGG GCT G
322	GCC GCT ACT A
328	ATG GCC TTA C
331	GCC TAG TCA C
332	AAC GCG TAG A
333	GAA TGC GAC G
349	GGA GCC CCC T
353	TGG GCT CGC T
360	CTC TCC AGG C
362	CCG CCT TAC A
364	GGC TCT CGC G
365	TAG ACA GAG G
368	ACT TGT GCG G
372	CCC ACT GAC G
388	CGG TCG CGT C

Appendix 3. Marker phenotype of the grasspea parents in the different crosses.

Cross	Marker[Z]	Female parent	Male parent
PI 283564c.3 x PI 426885.2	AAT-2	F	S
	EST-3	band	no band
	LAP-1	S	F
	SKDH	S	F
PI 358601.5 x PI 173714.5	AAT-1	F	S
	AAT-2	F	S
	ACO-1	F	S
	ACO-2	S	F
	SKDH	S	F
PI 426891.1 x PI 172930.4	AAT-1	S	F
	AAT-2	S	F
	ACO-2	S	F
	EST-3	band	no band
	FDH	F	S
	PGD-2	F	S
PI 283549a.6 x PI 202803a.3	EST-6	F	S
	LAP-1	F	S
	PGD-2	S	F
	TPI-1	S	F
PI 283564c.3.2 x PI 426891.1.3	Flower colour	white	blue
	AAT-2	F	S
	SKDH	S	F
	LAP-1	S	F
	$UBC304_{791}$	band	no band
	$UBC304_{831}$	band	no band
	$UBC304_{917}$	no band	band
	$UBC304_{964}$	band	no band
	$UBC304_{2025}$	no band	band
	$UBC305_{482}$	band	no band
	$UBC305_{831}$	no band	band
	$UBC305_{917}$	band	no band
	$UBC305_{1432}$	band	no band
	$UBC308_{289}$	band	no band

Continued...

Appendix 3. continued....

Cross	Marker[z]	Female parent	Male parent
PI 283564c.3.2 x PI 426891.1.3	$UBC308_{630}$	no band	band
	$UBC308_{990}$	band	no band
	$UBC314_{964}$	band	no band
	$UBC314_{1662}$	no band	band
	$UBC317_{559}$	no band	band
	$UBC317_{617}$	band	no band
	$UBC317_{1175}$	band	no band
	$UBC317_{1363}$	no band	band
	$UBC322_{1432}$	band	no band
	$UBC322_{1746}$	no band	band
	$UBC322_{1927}$	no band	band
	$UBC328_{682}$	band	no band
	$UBC328_{831}$	band	no band
	$UBC331_{487}$	band	no band
	$UBC331_{587}$	band	no band
	$UBC331_{716}$	band	no band
	$UBC331_{1175}$	band	no band
	$UBC332_{1118}$	band	no band
	$UBC332_{1581}$	band	no band
	$UBC332_{1834}$	band	no band
	$UBC333_{506}$	no band	band
	$UBC333_{617}$	no band	band
	$UBC333_{682}$	band	no band
	$UBC333_{964}$	no band	band
	$UBC333_{1235}$	no band	band
	$UBC349_{752}$	no band	band
	$UBC349_{1235}$	no band	band
	$UBC349_{1297}$	no band	band
	$UBC353_{395}$	band	no band
	$UBC353_{682}$	no band	band
	$UBC353_{873}$	no band	band
	$UBC353_{964}$	no band	band
	$UBC360_{424}$	no band	band
	$UBC360_{1118}$	band	no band
	$UBC360_{1746}$	band	no band
	$UBC362_{1174}$	no band	band
	$UBC362_{1297}$	no band	band
	$UBC364_{559}$	no band	band
	$UBC364_{649}$	no band	band

Continued....

Appendix 3. continued.....

Cross	Marker[z]	Female parent	Male parent
PI 283564c.3.2 x PI 426891.1.3	$UBC364_{738}$	band	no band
	$UBC364_{1013}$	no band	band
	$UBC364_{1175}$	band	no band
	$UBC364_{1235}$	band	no band
	$UBC364_{1581}$	band	no band
	$UBC364_{1662}$	band	no band
	$UBC365_{341}$	band	no band
	$UBC365_{752}$	no band	band
	$UBC365_{1013}$	band	no band
	$UBC365_{1928}$	band	no band
	$UBC368_{425}$	no band	band
	$UBC368_{559}$	band	no band
	$UBC368_{655}$	band	no band
	$UBC368_{791}$	band	no band
	$UBC372_{682}$	no band	band
	$UBC372_{1064}$	band	no band
	$UBC372_{1235}$	no band	band
	$UBC388_{459}$	band	no band
	$UBC388_{532}$	no band	band
	$UBC388_{752}$	no band	band
	$UBC388_{1175}$	band	no band
	$UBC388_{1363}$	band	no band
	$UBC388_{1662}$	no band	band

[z] RAPD markers were designated according to the procedure followed by Paran et al. (1991). The first three letters indicate the source of the primers. The three numbers following the letters indicate the primer code and the subscript indicates the molecular weight of the polymorphic bands in the base pair (bp).

Appendix 4. Joint segregation of successive linked markers in F_2 of PI 426891.1.3 x PI 283564.3.2 in grasspea.

Linkage group	Markers	Observed phenotype[z] frequencies A-A- A-aa aaA- aaaa or A-F A-H A-S aaF aaH aaS or FF FH FS HF HH HS SF SH SS	r	Map distance (cM)	LOD
I	$UBC304_{791}$ vs $UBC317_{617}$	67 05 02 26	0.06	7	16
	$UBC317_{617}$ vs $UBC317_{1175}$	63 06 15 16	0.24	33	4
	$UBC317_{1175}$ vs $UBC332_{1581}$	75 03 11 11	0.17	21	5.3
	$UBC332_{1581}$ vs $UBC333_{682}$	78 08 02 12	0.12	14	8
II	$UBC388_{459}$ vs $UBC304_{813}$	84 04 03 09	0.09	10	5.8
	$UBC304_{831}$ vs $UBC308_{990}$	82 05 03 10	0.10	11	5.9
III	$UBC308_{630}$ vs LAP-1	14 64 00 00 00 21	0.05	5	11
	LAP-1 vs. $UBC304_{917}$	14 59 05 00 06 16	0.11	13	6.8
IV	$UBC364_{1235}$ vs $UBC364_{1175}$	72 00 04 24	0.04	4	14.1
	$UBC364_{1175}$ vs $UBC304_{964}$	63 13 02 22	0.15	18	5.4
	$UBC317_{1363}$ vs $UBC388_{1662}$	69 11 02 22	0.18	22	4.8
	$UBC388_{1662}$ vs. $UBC362_{1297}$	70 04 05 21	0.09	10	8.4
V	$UBC364_{1581}$ vs $UBC305_{917}$	75 04 03 17	0.09	10	8.8
	$UBC305_{917}$ vs $UBC332_{1834}$	77 03 05 17	0.09	10	8.3
	$UBC332_{1834}$ vs $UBC388_{1363}$	73 07 04 16	0.12	14	6.2
	$UBC388_{1363}$ vs $UBC331_{487}$	71 06 08 15	0.16	20	4.9
	$UBC331_{487}$ vs. $UBC364_{738}$	65 13 06 15	0.21	27	4.0
VI	$UBC331_{1174}$ vs $UBC331_{587}$	76 03 03 18	0.06	6	11
	$UBC331_{587}$ vs $UBC360_{1118}$	78 01 01 20	0.02	2	15
	$UBC360_{1118}$ vs $UBC328_{682}$	77 02 02 19	0.04	4	12
	$UBC328_{682}$ vs $UBC305_{482}$	77 02 02 19	0.04	17	12
	$UBC305_{482}$ vs. $UBC388_{1175}$	70 09 06 15	0.14	17	6

Continued...

Appendix 4. continued....

VII	$UBC364_{1013}$ vs $UBC353_{964}$	67 10 04 19	0.15	18	6.1
	$UBC353_{964}$ vs $UBC362_{1174}$	69 02 06 23	0.08	09	10.9
	$UBC362_{1174}$ vs $UBC349_{1235}$	72 03 07 08	0.11	12	8.2
	$UBC349_{1235}$ vs $UBC317_{559}$	70 09 03 18	0.13	14	7.79
	$UBC353_{395}$ vs. $UBC308_{289}$	71 07 03 19	0.11	12	8.2
	$UBC308_{289}$ vs. $UBC331_{716}$	69 05 10 16	0.17	21	4.8
	$UBC360_{1746}$ vs $UBC368_{791}$	71 10 05 14	0.18	22	4.5
VIII	$UBC388_{752}$ vs. $UBC333_{506}$	68 05 08 19	0.13	16	7.0
	$UBC333_{506}$ vs. $UBC372_{1235}$	66 10 10 14	0.24	32	3.0
IX	$UBC333_{964}$ vs $UBC353_{873}$	67 07 12 14	0.22	29	3.3
	$UBC353_{873}$ vs $UBC368_{655/425}$	15 38 21 10 15 01	0.26	37	3.4
	$UBC368_{655/425}$ vs. SKDH	17 07 01 06 39 08 00 02 20	0.17	20	13.0
	SKDH vs. AAT-2	01 08 14 09 35 05 21 06 01	0.18	22	8.7
	AAT-2 vs. $UBC305_{1432}$	29 47 03 02 02 17	0.09	10	8.3
	$UBC305_{1432}$ vs $UBC368_{559}$	71 09 02 18	0.12	14	7.6
	$UBC368_{559}$ vs. $UBC372_{1064}$	63 10 11 16	0.25	35	3.7
X	$UBC3491_{297}$ vs. $UBC364_{649}$	68 08 09 15	0.19	24	4.5
XI	$UBC365_{1928}$ vs $UBC328_{831}$	56 16 05 23	0.19	25	5.0
	$UBC328_{831}$ vs $UBC365_{1013}$	58 03 02 37	0.04	5	17
	$UBC365_{1013}$ vs $UBC332_{1118}$	50 10 11 29	0.18	23	7
	$UBC332_{1118}$ vs $UBC365_{341}$	59 02 08 31	0.09	10	12
	$UBC365_{341}$ vs. $UBC314_{964}$	63 04 13 20	0.18	23	05
	$UBC314_{964}$ vs. $UBC364_{1662}$	64 12 09 15	0.24	32	3
	$UBC364_{1662}$ vs Fl. colour	49 24 26 01	0.16	25	5
	Fl. colour vs. $UBC322_{1928}$	64 11 07 18	0.19	25	5
	$UBC322_{1928}$ vs. $UBC372_{682}$	64 07 09 20	0.17	21	6
XII	$UBC388_{532}$ vs. $UBC305_{831}$	69 09 8 14	0.20	26	3.5
XIII	$UBC353_{682}$ vs. $UBC365_{752}$	78 05 01 16	0.07	8	4.2
XIV	$UBC333_{1234}$ vs $UBC333_{617}$	51 18 09 22	0.26	37	3.4
	$UBC333_{617}$ vs. $UBC349_{752}$	49 11 13 27	0.22	30	4.9

[z] F= homozygous for fast allele, H= heterozygous, S=homozygous for slow allele