Studies on self-incompatibility in grasses

Submitted for the degree of Doctor of Philosophy

in the University of Adelaide

by

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March, 1987 .

Photograph of a starch gel illustrating the relative mobilities for GPI of the main five species studied.

Samples 1-3 and 21-24 *P. coerulescens Gpi-2 1-1*

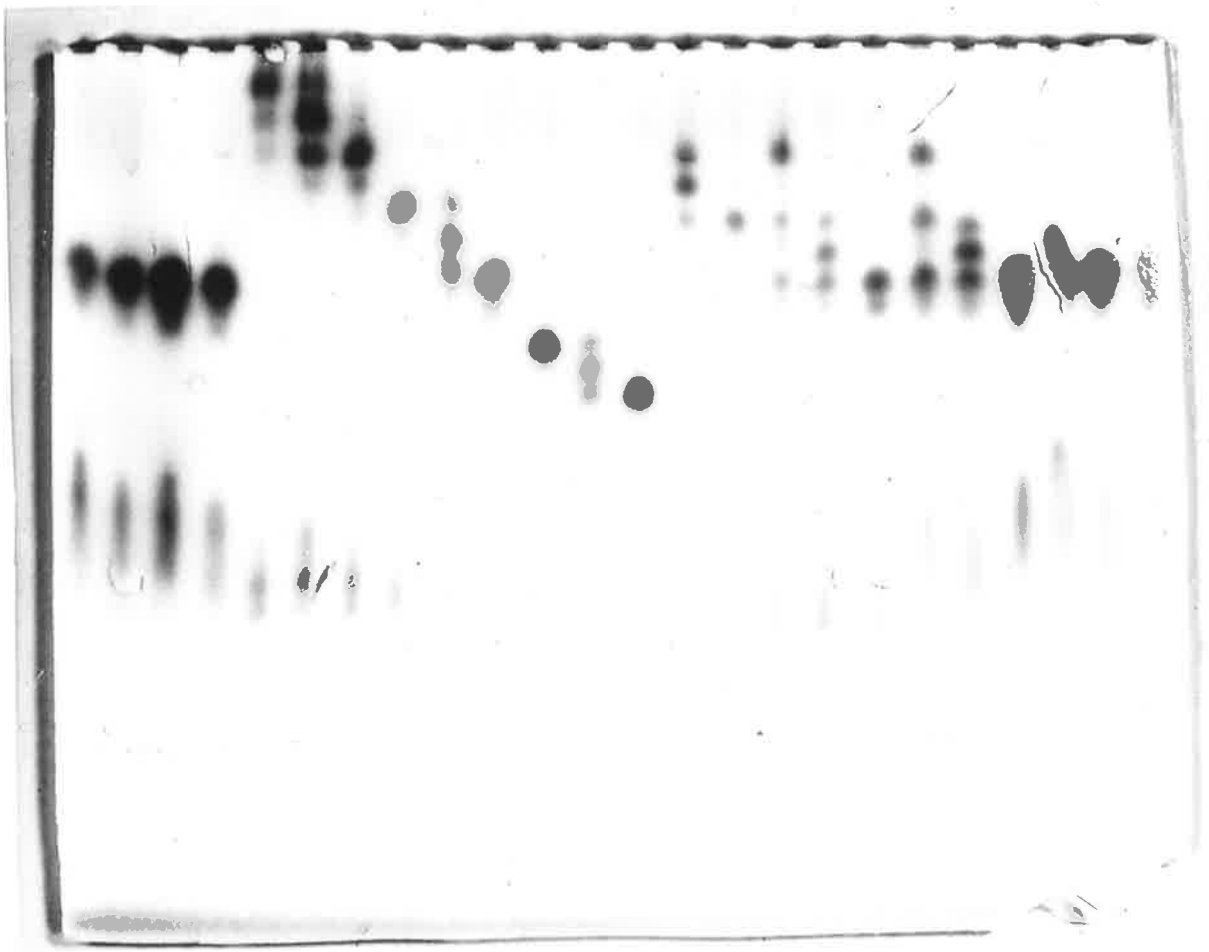
4-6 *P. coerulescens Gpi-2 1-1, 1-2, 1-3*

7-11 *F. pratensis Gpi-2 1-1, 1-2, 1-3, 2-2, 2-3*

12-14 *H. lanatus Gpi-2* 1^1-1^1, 1^1-*1, 1-1*

15-17 *A. myosuroides Gpi-2 1-1, 1-2, 2-2*

18-20 *S. cereale Gpi-2 3-3, 3-4, 4-4*



Contents

Appendix 2

Are there more than two incompatibility loci in the grasses?
Leach, C.R., 1984, *Heredity* **52**:303-305.

Appendix 3

Linkage disequilibrium and gametophytic self-incompatibility.
Leach, C.R., Mayo, O. and Morris, M.M., 1986, *Theor. Appl. Genet.* **73**:102-112.

Appendix 4

The incompatibility loci as indicators of conserved linkage groups in the Poaceae.
Leach, C.R. and Hayman, D.L., 1987, *Heredity* (in press).

Appendix 5

Stability of self-incompatibility systems.
Mayo, O. and Leach, C.R. (submitted for publication).

Appendix 6

Titles of other articles published on work carried out during the period of candidature.

Summary

The operation of gametophytic self-incompatibility systems may lead to differential transmission of different genotypes of pollen grains. Genes which are linked to these incompatibility genes may show disturbed segregation ratios in progenies. Methods are developed for linkage estimation, calculation of appropriate sample sizes and identification of controls needed for accurate analyses of disturbed segregation ratios. These methods are those appropriate for isozyme determining structural genes linked to an incompatibility locus for crosses of various incompatibility status.

These methods are applied in the analysis of progenies from each of five grass species selected from tribes well separated on the evolutionary time scale. Linkage of one of the self-incompatibility loci (*S*) to the locus of the structural gene determining the enzyme glucose phosphoisomerase (*Gpi-2*) has been detected in all species indicating conservation of this linkage association.

A considerable amount of further linkage data is presented for the five species including possible linkage of structural genes for malate dehydrogenase (*Mdh-2*) and 6-phosphogluconate dehydrogenase (*6 Pgd-2*) to the second (*Z*) of the two incompatibility loci present in the grasses. Additional linkage data offer further evidence of conserved linkage associations in the Poaceae.

Conserved linkage associations in the plant and animal kingdoms are discussed. The observation that particular conserved, tightly linked sets of genes are consistently found to be in linkage disequilibrium is argued to be an indication that selection is involved in maintaining these linkage associations rather than their occurrence being random. Theoretical studies presented show that though self-incompatibility systems may delay the attainment of linkage equilibrium they do not shelter lethals.

Declaration

I declare that this dissertation comprises my own work, except where specifically stated to the contrary, and that it is not substantially the same as any dissertation which has already been submitted to any other University.

Carolyn R. Leach

Acknowledgements

I would like to acknowledge my supervisors Dr. D.L. Hayman and Prof. J.H. Bennett and express my particular thanks to Dr. Hayman for his critical advice, his guidance and tolerance in dealing with a postgraduate student of mature years.

I am deeply indebted to Dr. O. Mayo who has been a loyal friend and a tirelessly enthusiastic and encouraging colleague throughout the years of this study.

Mrs. D. Golding, Karen Goodwins, Michele Norton and Jo Richter all helped with watering plants, spraying glasshouses, collecting soil, washing pots and general glasshouse maintenance. To them I offer my thanks. Special thanks are due to Angela Binns for not only helping with all those tasks but also for assistance in pouring gels and for the cheerfulness she brought to every job of work.

I thank Roy Goodwins for extra assistance in maintaining plants while I was absent on sabbatical in 1982.

Clive Chesson's expertise in electrophoretic procedures and gel interpretation is acknowledged and was greatly appreciated, as was that of Margaret Morris in the area of computing.

I thank my sons Nicholas and Richard for help with weekend watering and repotting of plants.

Thanks go to Sue Suter for help in the preparation of this thesis.

Finally, I would like to express my thanks to my husband Brian for his patience and support over the years of this study.

1 Introduction

1.1 The extent and genetic control of self-incompatibility in grasses

Incompatibility systems in angiosperms have been attracting attention in recent years, both because of their importance in practical plant breeding and as a result of the interest in them as examples of cellular interaction and recognition (Heslop-Harrison, 1983).

Over half of the families of flowering plants have members with highly developed self-incompatibility systems. Their manifestations are diverse but the principal effect is to ensure that a plant which is potentially fertile in both sexes is fertilized by another, genetically different, individual of the same species.

Incompatibility systems are classified as heteromorphic or homomorphic according to whether or not they are associated with differences in floral structure. Homomorphic systems are further classified according to the genetic control of pollen phenotype. Systems are described as being sporophytic if pollen behaviour is determined by the genotype of the plant producing it and gametophytic if pollen behaviour is determined by the gene(s) carried by the pollen. The genetic control of gametophytic self-incompatibility is by genes at one, two or, rarely, three or more multi-allelic loci. Matings are incompatible if the allele(s) in the pollen are matched by alleles expressed in the diploid tissue of the pistil. In both compatible and incompatible pollinations, pollen germinates and pollen tubes may grow through the stigma into the transmitting tissue of the style. Tube growth from an incompatible pollination is arrested in the style. The tip of the tube becomes thickened and there is often a deposit of callose in the tip (Heslop-Harrison, 1978).

The grasses, a family of about 700 genera and 10,000 species, display a range of breeding systems from dioecism to autonomous apomixis (Connor, 1979). The first accounts of self-incompatibility in grasses were published in the 19th century and, in the first half of this century, several workers (Beddows, 1931; Troll, 1931; Smith, 1944) reported observations on a large number of grass species showing the extensive nature of this phenomenon. However, it was not until the early 1950's that Lundqvist

(1954) offered a genetic solution to self-incompatibility in *Secale cereale*. This system invokes gametophytic specificity determined by alleles at two loci (*S* and *Z*) which assort independently or are loosely linked. Each locus has a large number of different alleles e.g. in *Festuca pratensis* $S = 14$ and $Z = 13$ (Lundqvist, 1969). Identity between pollen and pistil for the alleles at each locus leads to incompatibility. Thus an $S_{1.2}\ Z_{1.2}$ style rejects all pollen of types S_1Z_1, $S_1\ Z_2$, $S_2\ Z_1$ and $S_2\ Z_2$; specifically the pollen produced by anthers of a plant of that genotype, but will accept pollen differing by at least one *S* or *Z* allele e.g. all pollen from a plant of genotype $S_{3.4}\ Z_{1.2}$. This system has subsequently been found in all genetically analysed self-incompatible grass species (Table 1.1).

Species	Reference
Secale cereale	Lundqvist, 1954, 1956
Festuca pratensis	Lundqvist, 1955, 1961a
Phalaris coerulescens	Hayman, 1956
Hordeum bulbosum	Lundqvist, 1962a
Dactylis aschersoniana	Lundqvist, 1965
Briza media	Murray, 1974
Lolium perenne	Cornish, Hayward and Lawrence, 1979
Lolium multiflorum	Fearon, Hayward and Lawrence, 1983

Table 1.1 Two locus incompatibility systems identified in grass species.

Where gametophytic self-incompatibility is controlled by two loci there may be differences in the compatibility of reciprocal crosses. Further, there are three classes of compatible pollinations (Hayman, 1956). Using these features as diagnostic, Weimark (1968) classified *Briza media* L. (confirmed by Murray, 1974), *Lolium perenne* L. (confirmed by Cornish *et al.*, 1979), *Holcus lanatus* L., *Cynosurus cristatus* L., *Festuca rubra* L., *Arrhenatherum elatius* (L.) J & K Presl., *Deschampsia flexuosa* (L.),

Alopecurus pratensis L., and *Phalaris arundenacea* L. as having a two locus gametophytic system of self-incompatibility.

This list includes both diploid and polyploid species. Preliminary evidence (Lundqvist, 1957), from crosses between autotetraploid sibs of *Secale cereale* and using seed set as the criterion of incompatibility, suggested that one *S-Z* pair in the pollen, when matched in the pistil, was sufficient for incompatibility. Later tests in autotetraploid *Festuca pratensis* supported this suggestion and also showed that the presence of an additional allele in the pollen not matched in the pistil did not eliminate incompatibility (Lundqvist, 1962b). Fearon *et al.* (1984a and b) undertook a thorough investigation into the mode of expression of self-incompatibility in autotetraploid *Lolium perenne*. They determined the incompatibility genotypes of members of two families and were able to show that only one *S-Z* pair in the pollen needs to be matched for incompatibility to occur.

The grasses are classified into 23 tribes, have a basic chromosome number of n=5 or n=7 and fossil records of grass pollen are found in the Paleocene (65 million years) (Muller, 1981). The two locus gametophytic system of self-incompatibility is unique to the grasses and is presumed to have arisen only once. The grasses in which this system has so far been described are temperate species; very little has been reported about the tropical species.

1.2 The use of self-incompatibility systems in genetic analysis

Individual grass flowers consist of three separate structures : the lodicules, succeeded by one, two or even more alternating staminal whorls, and the uniloculate terminal gynoecium. The floral organs are enclosed by the anthoecium, the lemma (bract) and the palea (bracteoli). Collectively, the flowers and the bract are called a 'floret' (Connor, 1979). Only one seed is set per floret but a typical grass inflorescence contains a large number of florets. Since a completely self-incompatible plant sets no seed with its own pollen, crosses between plants of different incompatibility genotypes may be made, easily, by bagging inflorescences together. The operation of this type of

incompatibility system means that it is technically possible to perform genetic analyses in a diverse range of grass species without the tedium and technical problems of hand emasculation of florets. Thus using these features of an incompatibility system considerably extends the grass species which may be investigated beyond the relatively restricted group of cereals and pasture plants of agricultural importance.

It is a feature of gametophytic systems of self-incompatibility that they lead to differential transmission of different genotypes of pollen grains. It follows that genes which are linked to the incompatibility genes may also show disturbed segregation ratios in progenies. Brieger and Mangelsdorf (1926) were the first to argue that

"Since reciprocal crosses give different results so far as the transmission of the sterility factors is concerned, it follows that any factor linked to the sterility factors must show corresponding differences."

They went on to report linkage between flower colour and so called self-sterility factors in *Nicotiana sanderae*. This species has a single locus gametophytic system of self-incompatibility described earlier by East and Mangelsdorf (1925).

Casual observations of linkage to the *S* -locus (single locus gametophytic) have been described by a number of workers and are summarized in Table 1.2.

Species	Nature of linkage association	Reference
Nicotiana langsdorfii *Nicotiana alata*	Genes governing metrical characters	Anderson and De Winton, 1931
Oenothera organensis	S_{20} to a rare pollen lethal Recombination fraction = 0.3%	Emerson, 1941
Nemisia strumosa	Buff and bicolor genes. Percent crossing over = 8 and 30 respectively.	Riley, 1944
Lotus corniculatus	Possible linkage to gene controlling keel colour	Bubar, 1957[1]
Petunia hybrida	Possible linkage to grandiflora gene G.	Bianchi, 1959[1,2]
Trifolium pratense L.	Linkage or identity with flower colour gene R.	Denward, 1963
Brassica oleraceae[3]	Genes governing hypocotyl and stem colour, spotting of anthers and markings of sepals and fruit.	Thompson and Taylor, 1965

Table 1.2 Linkage to the *S*-locus

1. Material from Ph.D. theses cited by de Nettancourt (1977).
2. Report questioned by Reichmann-Philip (1963).
3. Sporophytic system of self-incompatibility.

All these workers were dealing with linked morphological markers. The development and application of protein characterization techniques in genetic studies, primarily in the assay of isozyme variation, has greatly increased the number of characters which may be investigated in linkage studies.

Differential segregation ratios for isozyme loci in reciprocal crosses are being used increasingly to indicate linkage to the gene(s) controlling gametophytic self-incompatibility. A summary of these reports is presented in Table 1.3.

Species	Nature of Report	Reference
Nicotiana alata	Linkage of peroxidase to the *S*-locus. For one locus $r_{S\text{-}PER}$ = 3% and another $r_{S\text{-}PER}$ = 34%	Labroche *et al.*, 1983[1]
Camellia japonica	Distorted segregation ratios for alcohol dehydrogenase. Linkage to *S*-locus offered as an explanation.	Wendel and Parks, 1984
Lycopersicon peruvianum	Linkage of peroxidase to the *S*-locus ($r_{S\text{-}PER}$ = 9.9%)	Tanksley and Loaiza-Figuera, 1985
Lolium multiflorum	Observation of disturbed segregation ratio for peroxidase (52:62:7) when expected was 1:2:1). Not recognised as attributable to linkage to *S*-locus.	Polans and Allard, 1985
Secale cereale L.	Observation of disturbed segregation ratio for phosphoglucose isomerase (54:47:22 *cf* 1:2:1). Not recognised as attributable to linkage to *S*-locus.	Figueiras *et al.*, 1985
Plantago lancolata	Linkage of phosphoglucomutase to *S*-locus (2:227:234 *cf* 1:2:1 leading to $r_{S\text{-}PGM}$ = 0.8%) Linkage of 6-phosphogluconate dehydrogenase to *S*-locus (36:581:525 *cf* 1:2:1 leading to $r_{S\text{-}6PGD}$ = 6.4%)	Van Dijk, 1985
Secale cereale	Linkage of peroxidase to one of the incompatibility loci ($r_{S\text{-}PER}$ = 0-2%)	Wricke and Wehling, 1985

Table 1.3 Linkage associations to S-loci.

1. This claim is discussed later in this thesis.

Simmonds (1966) appears to have been the first to derive theoretical expectations and variances of linkage estimates for a locus linked to the self-incompatibility locus from both backcross and F_2 segregations. He was led to this analysis to explain disturbances in monohybrid segregations in diploid potatoes associated with the observation that siblings of like genotype tended to be inter-compatible. His analysis was restricted to phenotypes determined with complete dominance. Increasing use is being made of the incompatibility system to detect linkage relationships. However, not

all data are being analysed correctly and it is an appropriate time for a full consideration of the types of crosses, methods of linkage estimation, progeny sizes and controls needed for accurate analysis of disturbed segregation ratios. This is presented in Chapter 2.

It should be noted that, as pointed out by Thompson and Taylor (1965), linkage of a marker gene to the *S*-locus in a plant having a sporophytic self-incompatibility system does not lead to disturbed segregation.

1.3 Comparative gene mapping

Before the availability of biochemical and molecular techniques in genetics, it was very difficult to determine single gene homologies among widely divergent taxa. Most of the linkage maps consisted of genes affecting morphological characters such as eye colours in animals or leaf shape and pigment deficiencies in plants. Since the number of genes potentially affecting these characters in a single organism is often large, it has been difficult or impossible to determine which loci are homologous in different species.

Isozymes have a number of properties which make them especially useful in comparing individuals both within and between species and populations. These advantages include the following.

1. Allelic expression is usually co-dominant and free of epistatic and environmental effects.
2. Enzyme specificity allows alleles to be attributed to loci and a comparison of loci in different populations and species.
3. Each allelic difference is detected as a mobility difference. These differences are independent of the function of the enzyme and the overall amount of variation of the enzyme in question.
4. The loci sampled are not influenced by whether or not the genes are variable but rather by tissue expression, suitable extraction procedures and availability of reagents for assay.

5. A large number of loci can be assayed from one individual; in some cases from a single sample of tissue.

The problems of post-translational modification of electrophoretic mobility, differential tissue specificity of some enzymes, duplication of genetic material such as in polyploids should not be overlooked. However, prior to the recent introduction of the use of restriction fragment length polymorphisms isozyme assay has offered the best system for the identification of a range of homologous loci and allowed more reliable comparisons to be made of their number and linear order in divergent species.

Comparative gene mapping studies in mammals have been underway for well over ten years. The development of somatic cell hybridization techniques made it possible to determine the chromosome and sometimes the chromosome arm position of specific enzyme coding genes (Ruddle and Creagan, 1975).

Numerous syntenic groups of loci are reported to be common between Man and mouse through the 80 million years since these species diverged. Linkage conservation has also been reported among mouse, rat, rabbit, dog, sheep, pig, cow and primate genomes (McKusick, 1980; O'Brien and Nash, 1982; Womack, 1983; Echard *et al.*, 1984; Lalley and Diaz, 1984; Roderick *et al.*, 1984; Womack and Moll, 1986).

Comparisons between the fish genera *Xiphophorus* and *Poeciliopsis* indicate no rearrangements of the linkage groups examined (Leslie, 1982; Morizot and Siciliano, 1983).

To date there are only limited data from many species and it is perhaps unwise to speculate about gene conservation among taxonomic groups above the family level. However, Morizot (1983) reports a linkage group assignment of the locus coding for muscle pyruvate kinase in *Xiphophorus* fishes which he claims suggests at least partial homology throughout the vertebrates of a syntenic group of some seven isozyme loci. This suggests stability of a linkage group throughout 400 million years of evolutionary divergence.

For many groups of animals the similarity of genetic maps suggested by chromosome banding patterns have been confirmed by studies of electrophoretic

markers. These banding techniques are not possible in plants. Very little is known about linkage conservation in plants, and that is restricted to a few species of economic importance (see Chapter 1.4 and 1.5). It is possible that, in the future, a well populated linkage map based on DNA sequences may provide information about the evolution of plant genomes. Plant DNA fragments that hybridize to individual probes can be considered truly homologous, and the genomic distribution of duplications and multigene family members can be compared among different genera.

1.4 Comparative mapping of enzyme coding genes in the Poaceae

The chromosomal locations of over 70 isozyme structural gene loci have been identified in wheat (*Tricitum aestivium* 2n = 6x = 42). Inter genomic variation is common among the members of paralogous (duplicated) isozyme structural gene sets in this species and diverse sets of aneuploid derivatives have been used in these studies.

Due to the valuable role that knowledge of the location of isozyme structural genes can play in the isolation, characterization and maintenance of alien chromosomes and chromosome segments in strains of wheat a considerable volume of literature exists on the comparison of *T. aestivum* isozyme gene locations with other members of the Triticeae (Hordeae), in particular, *Secale cereale* and *Hordeum vulgare* and to a lesser extent, *Agropyron* spp. and other *Triticum* species (Barber *et al.*, 1968; Tang and Hart, 1975; Hart *et al.*, 1980; Hart and Tuleen, 1983; Miller, 1984; Salinas *et al.*, 1985). The evidence suggests that gene synteny relationships and chromosomal arm locations are largely conserved in different Triticeae genomes.

Despite this wealth of information on chromosome locations leading to a consistent picture of conserved synteny very little is known of the actual linkage relationships between loci in these chromosome arms. The information available for members of the Triticeae is summarized in Table 1.4.

Species	Loci and Recombination frequency		Chromosome	Reference
Triticum aestivum	*Gpi-D1 - Glu-D1*	36.2 ± 4.5%		Chojecki *et al.*, 1983
	Gpi-D1 - Gli-D1	34.5 ± 4.4%	1q	
	Glu-D1 - Gli-D1	48.3%		
	Gli-B1- centromere	9		Payne *et al.*, 1982
	Glu-B1 - Nor1	22	1q	Payne *et al.*, 1984
	Per-B1, Hk-B1 located between *Nor 1* and centromere, *Hk -B1* proximal		1q	Ainsworth *et al.*, 1984
Secale cereale	*Pgd-2 - Mdh-1*	16 ± 3 cM	2Rq	Figueiras *et al.*, 1985
	Per-3 - Per -4	26 ± 4 cM	2Rp	
	Mdh-2 - Got-3	21 ± 2 cM	3Rq	
	Est-1 - Est-2	0.18 ± 0.042		
	Est-1 - Est-6,-5,-7	0.19 ± 0.019		Wehling and
	Est-2 - Est-6,-5-7	0.27 ± 0.043	6q	Schmidt-Stohn, 1984
	Est-2 - Est-3	0.24 ± 0.027		
	Est-8 - Est-9	0.002		
Hordeum vulgare	*Est-2 Est-1 Est-4* 0.002 0.005		3	Kahler and Allard, 1970
	Adh-1,2 - Pgm	0.2 ± 0.03		
	Pgm - Ndh	0.27 ± 0.04	4	(after)
	Adh1,2 - Ndh	0.45 ± 0.04		Brown, 1983
	Aat-3 - Est 2,1,4	0.44 ± 0.04	3	
	Pgd-2 - Aco-2		5	
Avena barbata	*Est -10 - Pxa-5*	0.23 ± 0.03		Marshall and Allard, 1969
	Pxa-5 - Acp-5	0.04 ± 0.01		
*Avena fatua**	*Est-3 - Amp -1*	0.23 ± 0.09		Miller, 1977

Table 1.4. Established linkage relationships for members of the Triticeae.

*Other conflicting reports of linkage relationships for esterase loci may be found in Clegg and Allard, 1972 and Bending, 1974.

There has been a broad range of studies involving isozyme loci in maize (*Zea mays* L.). A total of 37 structural genes encoding isozymes has been assigned to specific linkage maps. Linkages between specific alleles and agronomically favourable genes have been used to improve plant yield by direct selection for specific allozymes

correlated with yield response (Goodman and Stuber, 1983). Indeed maize is probably one of the best mapped plants in the Poaceae and some evidence of conserved associations can be found in comparing syntenic groups in maize with those in the Triticeae.

The efficiency in making crosses imparted by the incompatibility system may be used in acquiring progenies from which estimates of linkage of isozyme loci to each other can be obtained. These studies can greatly increase the diversity of species from which data may be obtained. The results of such linkage analyses are presented in Chapter 4.3. These linkage relationships can also be compared with established syntenic associations for members of the Triticeae (Chapter 5).

1.5 Comparative gene mapping in plant families other than the Poaceae

There is some evidence of conservation of linkage in the Solanaceae. Qurios and McHale (1985) report that *Prx-2* and *Prx-3* are linked in diploid potato species. The putative homologous loci in tomato are also linked (Rick *et al.*, 1974). The 14 loci mapped in *Capsicum annuum* (Tanksley, 1984) reveal that two linkage blocks *Est-1-Est-7* and *Pgi-1 -Est-4* have remained intact since the divergence of *Capsicum* and *Lycopersicon.*

To extend the comparison across families and consider the Poaceae and the Solanaceae one finds the report of linkage of *Prx-7* (leaf peroxidase with isoelectric point pH 7.0) to one of the incompatibility loci in *S. cereale* (Wricke and Wehling, 1985) and close linkage of *Prx-1* (leaf peroxidase) to the incompatibility locus in *Lycopersicon esculentum* (Tanksley and Loaiza-Figuera, 1985). Indeed Valizadih (1978) reports linkage of *Pox-C* to the sex determining factor in *Ficus carica* L. (Moraceae). Thus it appears that there may be conservation of linkage both within and between the monocotyledons and dicotyledons even though they are believed to have diverged about 65 million years ago (Muller, 1981).

Extending the search for homologies to the gymnosperms which diverged from the angiosperms more than 100 million years ago (Stebbins, 1971) one finds a pair of

esterase loci α *Est* and *F Est* tightly linked (r = 0.02-0.06) (Harry, 1986). Thus this report provides some evidence of tight linkage associations of the esterase loci across the major groups of the plant kingdom (see Table 1.4 and Tanskley (1984) above).

1.6 Conserved linkage associations and linkage disequilibrium

As indicated above linkage associations of esterase loci appear to be highly conserved throughout evolution. The linkage relationships for these loci in *S. cereale* and *H. vulgare* are given in Table 1.4.

However, despite the apparent age of these linkage associations natural populations of wild barley in Israel exhibit strong linkage disequilibrium at three loci (Brown *et al.*, 1977). For example, one population polymorphic for *Est-1*, *-2* and *-4* shows complete asociation between *Est-1* and *Est-4*, 20% of maximum association between *Est-1* and *Est-2* and 40% between *Est-4* and *Est-5*. Associations also occur between esterases and other loci, and in most wild barley populations in Israel. Further evidence of these sorts of associations are also found in polymorphic populations of *Avena barbata* (Allard *et al.*, 1972) in both wild and cultivated accessions of *Lycopersicon esculentum* (Rick *et al.*, 1974) and possibly in rice cultivars (Nakagahra *et al.*, 1975).

The relationship between breeding systems, linkage equilibrium and possible mechanisms for the conservation of linkage will be discussed in this thesis. It has long been suggested that genetical systems in plants determine the amount of heterozygosity found in natural populations of plants (Darlington, 1958). However, Fisher (1935) demonstrated that, by itself, enforced heterozygosis does not shelter lethal mutations in such a way as to explain their accumulation from this cause alone. Heteromorphic self-incompatibility systems only affect the proportion of heterozygotes at loci closely linked to the *S*-locus (Strobeck, 1980) and balanced lethal factors in *Oenothera* do not increase the amount of variation maintained in such populations compared to those without lethals (Ellstrand, 1978).

The main problem of investigating the population dynamics of self-incompatibility systems is that data are few, in particular little is known of their linkage relationships.

The final chapter of this thesis reports a theoretical investigation of linkage disequilibrium and gametophtyic self-incompatibility. A study of the stability of gametophytic self-incompatibility systems is presented in Appendix 5.

2 Detection and estimation of linkage for an isozyme structural gene locus linked to a gametophytic self-incompatibility locus

2.1 Introduction

The operation of a gametophytic self-incompatibility system in appropriate crosses leads to differential transmission of pollen grains of different genotypes. Consequently genes which are linked to self-incompatibility genes may show disturbed segregation ratios.

The theory which follows involves the locus of a structural gene determining isozyme variants linked to an incompatibility locus. One major difference between the analysis presented here and that already given by Simmonds (1966) is that isozyme analysis enables all genotypes at a particular locus to be identified by specific banding patterns on a gel whereas Simmonds' analysis was restricted to phenotypes with complete dominance.

Alleles at the isozyme structural locus are denoted A_1, A_2 etc. The recombination frequency is represented by the symbol r. The female parent is written first in all crosses.

2.2 Gametophytic self-incompatibility controlled by a single multi-allelic *S*-locus

Three types of pollination can be recognised (Table 2.1). There are no differences between reciprocal crosses in compatibility relationships.

Genotype of parents	Type of pollination
S_1S_2 x S_1S_2	Incompatibile
S_1S_2 x S_1S_3	50% compatible
S_1S_2 x S_3S_4	Fully compatible

Table 2.1 Types of pollination recognisable with single locus control of gametophytic self-incompatibility

Consider a cross in which the parents are 50% compatible the male being heterozygous and the female homozygous for isozyme determining genes. The outcome is set out in Table 2.2.

Parent genotypes	$\frac{S_1A_1}{S_2A_1}$		x	$\frac{S_1A_1}{S_3A_2}$			
Gametes	S_1A_1	S_2A_1	x	$S_1A_1{}^*$	S_3A_2	$S_1A_2{}^*$	S_3A_1
Frequency	$\frac{1}{2}$	$\frac{1}{2}$		$\frac{1-r}{2}$	$\frac{1-r}{2}$	$\frac{r}{2}$	$\frac{r}{2}$
Genotypes in progeny	$\frac{S_1A_1}{S_3A_2}$	$\frac{S_1A_1}{S_3A_1}$		$\frac{S_2A_1}{S_3A_2}$	$\frac{S_2A_1}{S_3A_1}$		
Frequency	$\frac{1-r}{2}$	$\frac{r}{2}$		$\frac{1-r}{2}$	$\frac{r}{2}$		
Isozyme genotypes		A_1A_1		A_1A_2			
Frequency		r		1-r			

Table 2.2 Details of a cross with the male parent 50% compatible and heterozygous for an isozyme determining gene for which the female is homozygous.

*Pollen carrying the S_1 gene is not compatible.

Consider now the reciprocal cross (Table 2.3)

Parent genotypes			$\frac{S_1A_1}{S_3A_2}$		x	$\frac{S_1A_1}{S_2A_1}$	
Gametes	S_1A_1	S_3A_2	S_1A_2	S_3A_1	x	S_1A_1*	S_2A_1
Frequency	$\frac{1-r}{2}$	$\frac{1-r}{2}$	$\frac{r}{2}$	$\frac{r}{2}$		$\frac{1}{2}$	$\frac{1}{2}$
Isozyme genotypes in progeny			A_1A_1			A_1A_2	
Frequency			$\frac{1}{2}$			$\frac{1}{2}$	

Table 2.3. Expectations for the reciprocal cross as detailed in Table 2.2

*Pollen carrying S_1 gene is not compatible.

The ratio of $A_1A_1 : A_1A_2$ in the progeny (Table 2.2) will be r: 1-r and not 1:1 as expected if the gene were not linked to one of the incompatibility genes. Thus if it is found that the observed frequencies of A_1A_1 and A_1A_2; say y_1 and y_2, depart significantly from the expected $(y_1 + y_2)/2$ and $(y_1 + y_2)/2$ in this cross but not in the reciprocal (Table 2.3) it may be concluded that the isozyme locus is linked to the incompatibility locus.

The observed proportion of A_1A_1 in the progeny of the first cross $(y_1/(y_1 + y_2))$ gives a direct estimate of r. The variance of this estimate is $V(\hat{f}) = \hat{f}(1-\hat{f})/n$ where $n = y_1 + y_2$.

The sample size to be 95% sure that a particular observed ratio will differ from 1:1, for a given recombination frequency may be calculated from equation 2.1 below. The sample sizes necessary for a range of recombination frequencies are shown in Table 2.4.

$$\frac{r - 0.5}{\sqrt{r(1-r)/n}} = 5\% \text{ level of } t_{n-1} \text{ (for small n) or } 5\% \text{ level of standard normal deviate} \qquad 2.1$$

Recombination frequency (r)	Sample size (n)
0.1	$0.5625\ t_{n-1}$ *
0.2	$1.7778\ t_{n-1}$ *
0.3	21
0.4	93

Table 2.4 Sample size (n) required to be 95% sure of detecting a significant departure from expectation of 1:1 for a given recombination frequency (r) for a locus linked to the incompatibility locus.

* For small values of r the estimates are not very accurate. Substituting $t_1 = 12.71$, as an indicator of the upper limit these values would have, leads to n=7 for r=0.1 and n=23 for r=0.2.

When both parents are heterozygous for the isozyme genes a typical cross would be of the following form:

$$\frac{S_1A_1}{S_2A_2} \times \frac{S_1A_1}{S_3A_2}$$

By following the same procedure as set out in Table 2.2 a set of expected frequencies may be derived. These frequencies and the corresponding observed frequencies are shown in Table 2.5.

Isozyme genotypes	A_1A_1	A_1A_2	A_2A_2	Total
Expected	$\frac{r}{2}$	$\frac{1}{2}$	$\frac{1-r}{2}$	1
Observed	y_1	y_2	y_3	n

Table 2.5 Outcome from a cross where both parents are heterozygous for the isozyme genes.

If the observed numbers differ significantly from the expected ratio of 1:2:1 and control crosses (e.g. Table 2.3) have ruled out differential viability of different isozyme

genotypes it may be deduced that this departure is attributable to linkage and the recombination frequency can be estimated by the method of maximum likelihood (equation 2.2).

The logarithm of the likelihood expression is

$$L = y_1 \ln(r/2) + y_2 \ln(1/2) + y_3 \ln((1-r)/2) \qquad 2.2$$

maximizing by differentiation and equating to zero the equation of estimation becomes:

$$\frac{dL}{dr} = \frac{y_1}{r} - \frac{y_3}{1-r} = 0$$

The solution is $\hat{r} = \frac{y_1}{y_1+y_3}$

The variance of this estimate can be derived as follows:

$$-\frac{1}{V(\hat{r})} = E\left[\frac{d^2L}{dr^2}\right]_{r=\hat{r}}$$

$$\frac{d^2L}{dr^2} = \frac{y_1}{r^2} + \frac{y_3}{(1-r)^2}$$

$$-E\left[\frac{d^2L}{dr^2}\right]_{r=\hat{r}} = \frac{n}{2r} + \frac{n}{2(1-r)}$$

$$\text{Var } \hat{r} = \frac{2\hat{r}(1-\hat{r})}{n} \quad \text{where } n = y_1 + y_2 + y_3$$

The sample sizes required to be 95% sure of detecting a significant departure from expectation of r = 0.5 may be derived from the solution of equation 2.3. They are double those calculated for the corresponding recombination frequency set out in Table 2.4.

$$\frac{r/2 - 0.25}{\sqrt{\frac{2r(1-r)}{4n}}} = \text{5\% level of } t_{n-1} \text{ or 5\% level of S.N.D.} \qquad 2.3$$

There are two appropriate controls:

1. Demonstrating that the cross is 50% compatible using a pollination test. This rules out possible differential transmission due to certation (Heribert Nilsson, 1916).
2. The observation of undisturbed segregation ratios in crosses of equivalent compatibility status. This indicates that disturbances are not likely to be due to viability differences in the progeny associated in some way with isozyme genotypes.

2.3 Gametophytic self-incompatibility controlled by two multi-allelic loci *S* and *Z*

When two loci are involved in determining gametophytic self-incompatibility a number of different features may occur.

1. Four classes of crosses may be detected.

   Fully compatible e.g. $S_{1.2}Z_{1.2}$ x $S_{3.4}Z_{3.4}$

   75% compatible e.g. $S_{1.2}Z_{1.2}$ x $S_{2.3}Z_{2.3}$

   50% compatible e.g. $S_{1.2}Z_{1.2}$ x $S_{1.3}Z_{1.2}$

   Fully incompatible e.g. $S_{1.2}Z_{1.2}$ x $S_{1.2}Z_{1.2}$

2. There may be reciprocal differences in compatibility between the parents

e.g. $S_{1.1}Z_{1.2}$ x $S_{1.2}Z_{1.2}$ 50% compatible

$S_{1.2}Z_{1.2}$ x $S_{1.1}Z_{1.2}$ incompatible

3. The isozyme locus may be linked to either incompatibility locus

e.g. $\frac{S_1A_1}{S_1A_1}Z_{1.2}$ x $\frac{S_1A_1}{S_2A_2}Z_{1.2}$

or $S_{1.1}\frac{Z_1A_1}{Z_2A_1}$ x $S_{1.2}\frac{Z_1A_1}{Z_2A_2}$

4. Different isozyme loci may be linked to each incompatibility locus.

e.g. $\frac{S_1A_1}{S_1A_1}\frac{Z_1B_1}{Z_2B_1}$ x $\frac{S_1A_1}{S_2A_2}\frac{Z_1B_1}{Z_2B_2}$

5. The incompatibility loci may themselves be linked. If this is the case the question arises as to whether it is possible to classify pollinations as 50% and 75% compatible.

Consider a cross in which 50% of the pollen genotypes are also found in the stylar genotype. The outcome of such a cross in the case where the *S* and *Z* loci are linked is set out in Table 2.6.

Genotypes of parents		$\frac{S_1Z_1}{S_2Z_3}$*	x	1) $\frac{S_1Z_1}{S_2Z_2}$	or	2) $\frac{S_1Z_2}{S_2Z_1}$	
Pollen genotypes		S_1Z_1		S_2Z_2	S_1Z_2		S_2Z_1
Frequency in terms of recombination frequency (r)	(1)	$\frac{1-r}{2}$		$\frac{1-r}{2}$	$\frac{r}{2}$		$\frac{r}{2}$
	(2)	$\frac{r}{2}$		$\frac{r}{2}$	$\frac{1-r}{2}$		$\frac{1-r}{2}$
Compatibility of pollen		-		+	+		+

Table 2.6 Outcome of a cross in which pollen and style share 50% of genotypes. *Linkage phase of the female parent does not influence the argument.

Thus irrespective of the linkage phase of the male parent 50% of the pollen is ineffective in fertilization and so 50% compatible pollinations would be expected to be found if the incompatibility loci were linked.

Consider now a cross in which 25% of the pollen genotypes are also found in the stylar genotype. The outcome of such a cross is set out in Table 2.7.

Genotypes of parents		$\frac{S_1Z_1}{S_3Z_4}$*	x 1)	$\frac{S_1Z_1}{S_2Z_2}$	or 2) $\frac{S_1Z_2}{S_2Z_1}$
Pollen genotypes		S_1Z_1	S_2Z_2	S_1Z_2	S_2Z_1
Frequency in terms of recombination frequency (r)	(1)	$\frac{1-r}{2}$	$\frac{1-r}{2}$	$\frac{r}{2}$	$\frac{r}{2}$
	(2)	$\frac{r}{2}$	$\frac{r}{2}$	$\frac{1-r}{2}$	$\frac{1-r}{2}$
Compatibility of pollen		-	+	+	+

Table 2.7 Outcome of a cross in which pollen and style share 25% of genotypes.

*Linkage phase of the female parent does not influence the argument.

Thus the outcome is different for the different linkage phases of the male parent and depends upon the value of the recombination frequency between the loci. The expected percentage of compatible pollen for a range of recombination frequencies is set out in Table 2.8.

Recombination frequency	Linkage phase 1)	Linkage phase 2)
0.05	52.5	97.5
0.1	55	95
0.2	60	90
0.3	65	85
0.4	70	80
0.45	72.5	77.5
0.5	75	75

Table 2.8 Percentage of compatible pollen for a range of recombination frequencies

from a cross of the form $\frac{S_1Z_1}{S_3Z_4}$ x 1) $\frac{S_1Z_1}{S_2Z_2}$ or 2) $\frac{S_1Z_2}{S_2Z_1}$

If the incompatibility loci were tightly linked ($r \leq 0.1$) it would only be possible to distinguish 3 types of pollination. This situation would be indistinguishable from the case where the genetic control of self-incompatibility was by genes at a single locus. For recombination frequencies in the range of 0.2-0.3 a continuity of degree of compatibility would be expected. However, as many workers have classified pollinations as 75% compatible (e.g. see Table 1.1 for references) it is reasonable to deduce that if the loci are linked, this linkage must be loose. The loci could be in the same linkage group though themselves not show a recombination frequency less than 0.5. The arguments which follow for 75% compatible crosses involving linked loci are only true if one of the latter two situations pertains.

In considering a single linked isozyme locus the order of the loci must be considered.

i.e. *S-A-Z, A-S-Z, A-Z-S.*

6. There may be combinations of the situations considered in 2-4.

The discussion which follows includes illustrations of all of the above, though in some cases more than one point is covered in a single example.

2.3.1 Independent assortment of the incompatibility loci

Disturbed segregation ratios of isozyme genotypes are expected only if the male parent is either 50 or 75% compatible with the female and also heterozygous for the isozyme genes. The compatibility status may be determined by pollination tests (3.1.4). Alternatively if the cross is known to be between a parent and progeny it must be 50% compatible.

Male parent 50% compatible with female

The argument is developed for an isozyme locus linked to the *S* locus (Table 2.9). It is exactly the same for the *Z* locus.

Parent genotypes	$\frac{S_1A_1}{S_1A_1}Z_{1.2}$	x	$\frac{S_1A_1}{S_2A_2}Z_{1.2}$			
Gametes	$S_1A_1Z_1$	$S_1A_1Z_2$	x $S_1A_1Z_1{}^*$	$S_1A_1Z_2{}^*$	$S_2A_2Z_1$	$S_2A_2Z_2$
and frequencies	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1-r}{4}$	$\frac{1-r}{4}$	$\frac{1-r}{4}$	$\frac{1-r}{4}$
			$S_1A_2Z_1{}^*$	$S_1A_2Z_2{}^*$	$S_2A_1Z_1$	$S_2A_1Z_2$
			$\frac{r}{4}$	$\frac{r}{4}$	$\frac{r}{4}$	$\frac{r}{4}$

Isozyme genotypes in progeny	A_1A_1	A_1A_2	Total
Expected frequency	r	1-r	1
Observed frequency	y_1	y_2	n

Table 2.9 Derivation of isozyme genotype expectations for a cross where the male parent is 50% compatible.

*Pollen carrying S_1Z_1 or S_1Z_2 is not compatible.

If the observed frequencies differ significantly from the expected ratio of 1:1 and control crosses have ruled out differential viability it may be deduced that the departure is attributable to linkage and the recombination frequency may be estimated directly

$$\hat{r} = \frac{y_1}{y_1+y_2}; \quad V(\hat{r}) = \frac{\hat{r}(1-\hat{r})}{n}$$

In the case where both parents are heterozygous for the isozyme genes e.g.

$$\frac{S_1A_1}{S_1A_2}\ Z_{1.2} \quad x \quad \frac{S_1A_1}{S_2A_2}\ Z_{1.2}$$

the same male gametes (Table 2.9) are transmitted and combine equally frequently with A_1 and A_2 bearing gametes from the female leading to an expectation of

$$\frac{r}{2}(A_1A_1) : \frac{1}{2}(A_1A_2) : \frac{(1-r)}{2}(A_2A_2) \quad .$$

If the observed values $y_1(A_1A_1)$, $y_2(A_1A_2)$, $y_3(A_2A_2)$ differ significantly from 1:2:1 and controls do not, this difference may be attributed to linkage. The recombination frequency may be estimated (equation 2.2) as

$$\hat{r} = \frac{y_1}{y_1+y_2}; \quad V(\hat{r}) = \frac{2\hat{r}(1-\hat{r})}{n}$$

Where both parents are heterozygous for different isozymes e.g. A_1A_2 x A_3A_4 the progeny will fall into four classes as follows:

Parent genotypes	$\frac{S_1A_1}{S_1A_2}Z_{1.2}$	x	$\frac{S_1A_3}{S_2A_4}Z_{1.2}$

Gametes and frequency	$S_1A_1Z_1$	$S_1A_1Z_2$	$S_1A_3Z_1{}^*$	$S_1A_3Z_2{}^*$	$S_2A_4Z_1{}^*$	$S_2A_4Z_2{}^*$
	$\frac{1}{4}$	$\frac{1}{4}$	$\frac{1\text{-}r}{4}$	$\frac{1\text{-}r}{4}$	$\frac{1\text{-}r}{4}$	$\frac{1\text{-}r}{4}$
			X			
	$S_1A_2Z_1$	$S_1A_2Z_2$	$S_1A_4Z_1{}^*$	$S_1A_4Z_2{}^*$	$S_2A_3Z_1$	$S_2A_3Z_2$
	$\frac{1}{4}$	$\frac{1}{4}$	$\frac{r}{4}$	$\frac{r}{4}$	$\frac{r}{4}$	$\frac{r}{4}$

Isozyme genotypes	A_1A_3	A_1A_4	A_2A_3	A_2A_4
Frequency	$\frac{r}{2}$	$\frac{1\text{-}r}{2}$	$\frac{r}{2}$	$\frac{1\text{-}r}{2}$

Table 2.10 Derivation of isozyme genotype expectations for the case where parents are heterozygous for different isozyme genes.

*Pollen incompatible

Thus the relative frequency of progeny produced from pollen carrying A_3 *vs* A_4 is r : 1-r and so it is possible to obtain a direct estimate of r; $V(\hat{f}) = \frac{\hat{f}(1\text{-}\hat{f})}{n}$.

If the male parent is 50% compatible with the female parent and heterozygous for two isozyme loci one of which is linked to *S* and the other to *Z*

$$\text{e.g.} \quad \frac{S_1A_1}{S_1A_1}\frac{Z_1B_1}{Z_2B_1} \quad \text{x} \quad \frac{S_1A_1}{S_2A_2}\frac{Z_1B_1}{Z_2B_2}$$

it follows (as in Table 2.9) that Z_1 and Z_2 bearing pollen grains are selected against equally frequently and so B_1 and B_2 will be selected against equally frequently. Thus there will be no disturbance in the progeny segregation ratio for B_1:B_2. Thus, in this

case, it is only possible to detect and estimate linkage for an isozyme gene locus linked to either the *S* or *Z* locus; not both.

Sample sizes are the same as for the equivalent expectations in the previous section (2.2).

Appropriate controls involve observing undisturbed segregation ratios for the same isozyme in a cross of similar compatibility status. This rules out both certation and diploid viability effects.

Male parent 75% compatible with female

Parent genotypes $\frac{S_1A_1}{S_2A_1}Z_{1.2}$ x $\frac{S_2A_1}{S_3A_2}Z_{2.3}$

Female gametes	Male gametes			
A_1	$S_2A_1Z_2$*	$S_2A_1Z_3$	$S_3A_2Z_2$	$S_3A_2Z_3$
irrespective of the incompatibility genotype	$\frac{1-r}{4}$	$\frac{1-r}{4}$	$\frac{1-r}{4}$	$\frac{1-r}{4}$
	$S_2A_2Z_2$*	$S_2A_2Z_3$	$S_3A_1Z_2$	$S_3A_1Z_3$
	$\frac{r}{4}$	$\frac{r}{4}$	$\frac{r}{4}$	$\frac{r}{4}$
Isozyme genotypes in the progeny	A_1A_1	A_1A_2	Total	
Expected frequency	$\frac{1+r}{3}$	$\frac{2-r}{3}$	1	
Observed frequency	y_1	y_2	n	

Table 2.11 Derivation of isozyme genotype expectations for the case where the male parent is 75% compatible with the female.

*Pollen incompatible.

Thus if the observed frequencies differ significantly from the expected ratio of 1:1 and appropriate control crosses have been scored the recombination frequency may be estimated using the method of maximum likelihood. It follows that:

$$\hat{r} = \frac{2y_1 - y_2}{y_1 + y_2} \; ; \quad V(\hat{r}) = \frac{(1+\hat{r})(2-\hat{r})}{n} \; .$$

The case where both parents are heterozygous for the isozyme genes e.g.

$$\frac{S_1A_1}{S_1A_2} Z_{1.2} \quad x \quad \frac{S_2A_1}{S_3A_2} Z_{2.3}$$

may be summarized as in Table 2.12.

Isozyme genotypes in progeny	A_1A_1	A_1A_2	A_2A_2	Total
Expected frequency	$\frac{1+r}{6}$	$\frac{1}{2}$	$\frac{2-r}{6}$	1
Observed frequency	y_1	y_2	y_3	n

$$\hat{r} = \frac{2y_1 - y_3}{y_1 + y_3} \qquad V(\hat{r}) = \frac{2(1+\hat{r})(2-\hat{r})}{n}$$

Table 2.12 Summary of genotypes, frequencies and linkage estimate for an intercross of isozyme heterozygotes. Male parent 75% compatible.

The sample size to be 95% sure that a particular observed ratio will differ from 1:1 for a given recombination frequency may be calculated from equation 2.4 below. The sample size necessary for a range of recombination frequencies are shown in Table 2.13.

$$\frac{\frac{1+r}{3} - 0.5}{\sqrt{\frac{(1+r)(2-r)}{9n}}} = 1.96 \qquad 2.4$$

Recombination frequency (r)	Sample size (n)
0.1	51
0.2	93
0.3	213
0.4	861

Table 2.13 Sample size (n) required to be 95% sure of detecting a significant departure from expectation of 1:1 for a given recombination frequency (r) for a locus linked to the incompatibility locus.

When both parents are heterozygous for the isozyme genes the sample size for a given recombination frequency will be double that given in Table 2.13.

It is not possible to have an undisturbed segregation for a gene which is linked to one of the incompatibility genes if the male parent is 75% compatible with the female and so an appropriate control must be found in a cross of different compatibility status (i.e. 50% compatible).

In this case it is possible to detect and estimate linkage for two isozyme loci one of which is linked to *S* and the other to *Z*.

$$\text{e.g.} \quad \frac{S_1A_1}{S_2A_1}\,\frac{Z_1B_1}{Z_2B_1} \quad \text{x} \quad \frac{S_2A_1}{S_3A_2}\,\frac{Z_2B_1}{Z_3B_2}$$

Referring back to Table 2.10 it can be seen that some pollen carrying Z_2 is selected against. This pollen will also carry B_1 in 1-r cases compared with B_2 in r cases and so the situation is the same as for a locus linked to *S*. Thus the above cross would lead to:

$$A_1A_1 : A_1A_2 \quad \text{and} \quad B_1B_2 : B_1B_2$$

$$\frac{1+r_1}{3} : \frac{2-r_1}{3} \qquad \frac{1+r_2}{3} : \frac{2-r_2}{3}$$

where r_1 = recombination frequency between *A* and *S*

r_2 = " " " *B* and *Z*.

In the case where both parents are heterozygous for one or both of the isozyme genes expectations of the form summarized in Table 2.11 apply. The joint segregation ratios of *A* and *B* may be obtained as the product of the appropriate ratios above.

2.3.2 Linkage of the incompatibility loci

The arguments which follow assume the absence of interference. The recombination frequency between the first pair of loci is denoted by r_1 and between the second pair by r_2. In all cases only the male gamete genotypes are given in detail as all female gametes will be equally effective in fertilization. Thus the contribution, by the female, to the isozyme genotype of the progeny will be either identical from a homozygote or $\frac{1}{2}A_1$ and $\frac{1}{2}A_2$ from a heterozygote.

The results are set out in tabular form.

Male parent 50% compatible with female

For a cross of the form $\frac{S_1A_1Z_1}{S_1A_1Z_2}$ x $\frac{S_1A_1Z_1}{S_2A_2Z_2}$

the four possible arrangements for the male genotype and the expected gametic frequencies are listed in Table 2.14.

	Arrangement			
	1	2	3	4
Genotype of male parent	$\frac{S_1A_1Z_1}{S_2A_2Z_2}$	$\frac{S_1A_2Z_1}{S_2A_1Z_2}$	$\frac{S_1A_2Z_2}{S_2A_1Z_1}$	$\frac{S_1A_1Z_2}{S_2A_2Z_1}$
Gametes		Frequency		
$S_1A_1Z_1$*	$1-r_1-r_2+r_1r_2$	r_1r_2	$r_1(1-r_2)$	$r_2(1-r_1)$
$S_2A_2Z_2$				
$S_1A_2Z_2$*	$r_1(1-r_2)$	$r_2(1-r_1)$	$1-r_1-r_2+r_1r_2$	r_1r_2
$S_2A_1Z_1$				
$S_1A_1Z_2$*	$r_2(1-r_1)$	$r_1(1-r_2)$	r_1r_2	$1-r_1-r_2+r_1r_2$
$S_2A_2Z_1$				
$S_1A_2Z_1$*	r_1r_2	$1-r_1-r_2+r_1r_2$	$r_2(1-r_1)$	$r_1(1-r_2)$
$S_2A_1Z_2$				

Table 2.14 Expected gamete frequencies from the male in a cross where the incompability loci are linked and the isozyme locus is between them. Male parent 50% compatible with female.

*Pollen incompatible.

One member of each alternative pair of gametes is lost leading to the transmission of A_1:A_2 in a ratio of $r_1 : 1-r_1$ for arrangements 1 and 4 and $1-r_1 : r_1$ for arrangements 1 and 3.

If the cross were of the form $S_{1.2}\,Z_{1.1}$ x $S_{1.2}\,Z_{1.2}$ these ratios of $A_1 : A_2$ would be in terms of r_2.

The same is true for all other possible orders of the loci i.e. *A-S-Z* and *A-Z-S*. Thus for a cross in which the male parent is 50% compatible with the female it is possible to detect linkage of an isozyme locus to only one of the incompatibility loci.

Appropriate sample sizes are the same as in Table 2.4.

The case where both parents are heterozygous for the isozyme genes is also equivalent to the case where the incompatibility loci are unlinked.

Male parent 75% compatible with female

For a cross of the form $\frac{S_1A_1Z_1}{S_2A_1Z_2} \times \frac{S_2A_1Z_2}{S_3A_2Z_3}$

arrangements for the male genotype and the expected gamete frequencies are listed in Table 2.15.

	Arrangement			
	1	2	3	4
Genotype of male parent	$\frac{S_2A_1Z_2}{S_3A_2Z_3}$	$\frac{S_2A_2Z_2}{S_3A_1Z_3}$	$\frac{S_2A_2Z_3}{S_3A_1Z_2}$	$\frac{S_2A_1Z_3}{S_3A_2Z_2}$
Gametes		Frequency		
$S_2A_1Z_2$*, $S_3A_2Z_3$	$1-r_1-r_2+r_1r_2$	r_1r_2	$r_1(1-r_2)$	$r_2(1-r_1)$
$S_2A_2Z_3$*, $S_3A_1Z_2$	$r_1(1-r_2)$	$r_2(1-r_1)$	$1-r_1-r_2+r_1r_2$	r_1r_2
$S_2A_1Z_3$*, $S_3A_2Z_2$	$r_2(1-r_1)$	$r_1(1-r_2)$	r_1r_2	$1-r_1-r_2+r_1r_2$
$S_2A_2Z_2$*, $S_3A_1Z_3$	r_1r_2	$1-r_1-r_2+r_1r_2$	$r_2(1-r_1)$	$r_1(1-r_2)$
Total				
A_1	$\frac{r_1+r_2}{2}$	$1-\frac{r_1+r_2}{2}$	$\frac{1-r_1+r_2}{2}$	$\frac{1+r_1-r_2}{2}$
A_2	$1-\frac{r_1+r_2}{2}$	$\frac{r_1+r_2}{2}$	$\frac{1+r_1-r_2}{2}$	$\frac{1-r_1+r_2}{2}$

Table 2.15. Expected gamete frequencies from the male in cross where the incompatibility loci are linked and the isozyme locus is between them. Male parent 75% compatible with female.

*Pollen incompatible.

The segregation ratio depends on both r_1 and r_2 and so detection and estimation of linkage would not be possible.

The orders *A-S-Z* and *A-Z-S* are entirely symmetrical with each other though different from the above. The expectations for the same genotypes as listed in Table 2.15 but with the order *A-S-Z* are given in Table 2.16.

	Arrangement			
	1	2	3	4
Genotype of male parent	$\frac{A_1S_2Z_2}{A_2S_3Z_3}$	$\frac{A_2S_2Z_2}{A_1S_3Z_3}$	$\frac{A_2S_2Z_3}{A_1S_3Z_2}$	$\frac{A_1S_2Z_3}{A_2S_3Z_2}$
Gametes		Frequency		
$A_1S_2Z_2$*	$1-r_1-r_2+r_1r_2$	$r_1(1-r_2)$	r_1r_2	$r_2(1-r_1)$
$A_2S_3Z_3$				
$A_1S_3Z_3$	$r_1(1-r_2)$	$1-r_1-r_2+r_1r_2$	$r_2(1-r_1)$	r_1r_2
$A_2S_2Z_2$				
$A_1S_2Z_3$	$r_2(1-r_1)$	r_1r_2	$r_1(1-r_2)$	$1-r_1-r_2+r_1r_2$
$A_2S_3Z_2$				
$A_1S_3Z_2$	r_1r_2	$r_2(1-r_1)$	$1-r_1-r_2+r_1r_2$	$r_1(1-r_2)$
$A_2S_2Z_3$				
Total				
A_1	$\frac{2r_1+r_2-2r_1r_2}{2}$	$1-\frac{2r_1+r_2-2r_1r_2}{2}$	$\frac{1+r_2-2r_1r_2}{2}$	$\frac{1-r_2+2r_1r_2}{2}$
A_2	$1-\frac{2r_1+r_2-2r_1r_2}{2}$	$\frac{2r_1+r_2-2r_1r_2}{2}$	$\frac{1-r_2+2r_1r_2}{2}$	$\frac{1+-r_2-2r_1r_2}{2}$

Table 2.16. Expected gamete frequencies from the male parent in a cross where the incompatibility loci are linked and the isozyme locus is distal. Male parent 75% compatible with female.

Once again detection and estimation of linkage would not be possible. A number of examples of the application of these methods of estimation are presented in Chapter 4 of this thesis.

3 Materials and Methods

3.1 Plant material

A total of eight different grass species were used in this study. They are all diploid (2n=14) and self incompatible and are members of different tribes (Darlington and Wylie, 1955) widely separated on the evolutionary time scale (Stebbins, 1971). With one exception (*Secale cereale*), the species chosen were perennials and so crosses could readily be made between parental and progeny plants.

The primary aim of the experimental work was to detect disturbed segregation ratios involving the incompatibility genes and linked isozyme structural gene loci. Hence, crosses between plants of known relative compatibility status were required. Further, for reasons discussed in Chapter 2 of this thesis, the most suitable cross was one in which the male parent was 50% compatible with the female. Such pairs of plants are most readily obtained by backcrossing progeny with parental plants.

In all species the selection of plants was determined by their genotypes at the *Gpi*-2 (glucose phosphoisomerase) locus. All plants were classified as described in 3.2.

The protocol for seed germination, plant management and pollen compatibility testing was common to all species. Other features specific to individual species are discussed under separate headings.

3.1.1 Seed germination

Up to 50 seeds were placed in plastic petri dishes on filter paper kept moist on squares of Wettex soaked in a nutrient solution (Appendix 1.1). These dishes were wrapped in aluminium foil and left at 4°C for up to 5 days. They were then placed at 25°C until it was evident that germination had occurred at which time the petri dishes were placed in a well lit place so that the leaves could photosynthesize adequately.

3.1.2 Plant management

Seedlings were planted out into 2" pots filled with John Innes potting mixture and arranged in boxes in groups of 30 or 56. They were maintained in a temperature controlled glasshouse. When well established they were trimmed back and repotted into 5" or 8" pots with a fertilizer pellet. They were left in pots of this size, watered once or twice daily, fertilized regularly, trimmed occasionally and, in the case of the perennials, repotted when it became clear that they were very pot bound. This was usually once each year.

To induce early flowering plants were removed from the glasshouse and placed outside to expose them to cold night temperatures (1-6°C) in May and early June. On returning to the glasshouse they were given 16 hours of light by providing additional, artificial light from a number of 250 watt globes. With this regime of treatment flowering could generally be induced in mid July and subsequently a second round of natural flowering occurred in mid October.

After cessation of flowering and maturation of seed heads, plants were trimmed back and maintained through the summer by adequate watering.

Second and subsequent seasons of flowering were achieved by cutting plants back severely, breaking them up into small clumps and repotting early in the year, thereby allowing them to become re-established prior to cold treatment.

Many replicas of any given genotype of a perennial species could readily be obtained by division of an original plant at almost any stage of the year.

3.1.3 Crossing procedures

Crosses between plants of suitable compatibility status (3.1.4) were made either by bagging together in a glassine bag one head from each plant or by surrounding two plants with porous cellophane to exclude pollen from outside sources but allow free exchange of pollen between flowering heads. On completion of flowering the plants were separated and individual heads or sometimes groups of heads from a single plant enclosed in a glassine bag. Each head was labelled with a tag recording the species,

parents of the cross and date of crossing. Where individual heads were bagged they were separated prior to drying off to avoid loss of seed which might shed from a brittle dry head.

3.1.4 Pollination tests

Tests to determine the degree of pollen compatibility were carried out using an adaptation of Lundqvist's (1961b) rapid screening technique as follows:
Mature stigmas from heads about to release anthers were removed and placed in an ordered array of up to 16 (4x4) in 25 mm petri dishes containing a layer of sucrose agar (Appendix 1.2). They were either pollinated immediately or within the next 5 days. If not used immediately they were stored at 4°C.

Pollen was collected in glassine bags placed over mature inflorescences in the morning. After allowing a few hours for shedding each bag was removed and the collected pollen sprinkled over the stigmas by cutting a corner from the bag and puffing the pollen out over the stigmas. Care was taken not to apply too much pollen. The petri dishes were incubated at 25°C for 24 hours in the dark.

The proportion of pollen germinated was determined by staining the stigmas with cotton blue (Watkins, 1931) and scoring according to Hayman (1956). Stigmas were placed in a drop of cotton blue stain (Appendix 1.3) on a slide and heated gently for about 30 seconds over a spirit burner. Then a cover slip was placed over the drop of stain and 24 or more hours allowed for complete penetration of stain before scoring. To be 95% sure of distinguishing pollinations which are 75% compatible from those which are 50% compatible at least 58 pollen grains, including at least 37 compatible ones must be scored.

Attempts were made to use aniline blue fluorescence (Martin, 1959) as an index of the compatibility status of a cross. Aniline blue stain (Appendix 1.4) was applied to stigmas and the excess blotted off after a few seconds. Stigmas were mounted in glycerol and viewed by U.V. fluorescence microscopy. Compatible grains fluoresce only in the pollen tube, the grain remaining unstained. Incompatible pollen possesses a

short abortive tube and a bright plug of fluorescing callose inside the grain. This technique was not as successful in the species tested as it is reported to be in *Lolium* (Cornish *et al.*, 1979; Fearon *et al.*, 1983) and cotton blue was used in preference.

3.2 Electrophoresis methods

3.2.1 Preparation of extracts

All extracts used for electrophoresis were prepared immediately before use since a number of methods of storing extracts were tried but none was found to be satisfactory. Freezing and thawing of extracts resulted in changes in protein mobility or a severe degradation of enzyme activity.

Only leaf tissue was used for electrophoresis. Leaf samples were collected into tubes containing a small amount of water and could be stored this way at 4°C for a few hours. For some enzyme systems the age of the plant at the time of testing was important.

Extracts with approximately 50 mg leaf/100 µl extraction buffer (Appendix 1.5) were prepared, at room temperature, by taking a measured length of leaf and crushing between thumb and forefinger prior to macerating with a small teflon pestle in the well of a multiwell perspex tray. The length of leaf taken depended upon the width of the leaf as after a series of width/length/weight measurements it was found that samples produced in this way were sufficiently uniform. The pestle was cleaned on tissue between samples.

Wicks of Whatman 3 MM chromotography paper were placed in each well and allowed to absorb the extract. These wicks were then removed, folded in half and inserted into slots in the gel. The size of the wick depended upon the number of slots in the gel. For 24 slot gels wicks were 3 mm x 10 mm, for 16 slots, 6 mm x 10 mm and for 12 slots, 9 mm x 10 mm.

3.2.2 Electrophoresis

Electrophoresis was carried out horizontally in a cold room at 4°C. Gel trays were 155 mm x 200 mm x 6 mm and each could have 12, 16, or 24 slots allowing that number of sample wicks to be inserted. Electrostarch at a concentration of 12.8% was used.

Gels were prepared the day before use and allowed to cool and set overnight. A number of different gel and tray buffer systems and running conditions were used (Appendix 1.7). Bromophenol blue was included in the gel mixture. Gels with systems 1 and 2 were run under conditions of constant power as follows: Using a Pharmacia, Constant power ECPS 3000/150 power pack set at 400 V, 50 mA and 10W per gel electrophoresis was allowed to proceed for 45 minutes after which time the wicks were removed and slots filled with running buffer. The power was then raised to 12 W and electrophoresis continued until the borate front reached the anodal wicks.

For the continuous buffer system 3, gels were run at a constant voltage of 300 V for 5 hours.

At the completion of the run, gels were removed from the electrophoresis equipment, sliced in half with a fine wire mounted in a hacksaw blade and stained for at least two different enzyme systems (Appendix 1.8).

After completion of staining gels were fixed in 50% methanol and stored in sealed plastic bags at 4°C.

3.2.3 Notation of isozyme systems

Isozymes are referred to in the text by abbreviations such as GPI, glucose phosphoisomerase; also known as PGI, phosphoglucose isomerase. A full list of abbreviations is given in Appendix 1.8 along with the staining systems for the various enzymes.

Genotypes are written in italics, e.g. *Gpi* and the gene responsible for the fastest migrating bands (anodal or cathodal) is given the lowest number.

Gpi-1 1-1, *Gpi-1 2-2* and *Gpi-1 1-2* indicate homozygotes and a heterozygote for *Gpi-1* where the lower numbers indicate faster migrating bands.

For continuing discussion of a particular locus the gene symbol is often omitted and just mobilities indicated.

3.3 Statistical analysis

For single locus segregations the chi-squared method or exact binomial was used to test the significance of departures from expectation.

For contingency tables Fisher's exact test for 2x2 extended to RxC tables (Hancock, 1975) as available on a Vax computer was used for statistical comparison.

For the comparison of a pair of recombination frequencies a standard normal deviate test (S.N.D) was used.

The symbol P is used in all cases to indicate the probability of getting as great or greater deviation of observed from expected due to chance if the hypothesis under test is true.

3.4 Linkage estimation

For the estimation of the recombination frequency between one of the incompatibility loci and an isozyme locus the principles outlined in Chapter 2 were used.

For the estimation of recombination frequencies between pairs of isozyme loci maximum likelihood methods were used. In some cases where the marginal segregations were disturbed account was taken of these disturbances (Mather, 1957). Where the calculation of these estimates involved the solution of polynomials the MATLAB package available on the Vax computer was used (Moler, 1981). In other cases estimation procedures outlined by Lawrence *et al.* (1979) were appropriate.

In cases where no recombinant individual was observed the upper limit for a recombination frequency detectable in a sample of the size scored was calculated from the binomial distribution.

3.5 Programs for individual species

3.5.1 *Phalaris coerulescens* (Phalarideae)

The origin and sources of this material are listed in Table 3.1.

Plant or cross designation	Origin	Source
CPI 14422, 19185	Portugal	Seed provided by D.L. Hayman
CPI 19158,66,68,69,70,71,74,76,78	Algeria	from collections made by J.F. Miles
CPI 19192	Tunisia	and C.A. Neal Smith and crosses
CPI 19193	Greece	between accessions made by D.L.H.
CPI 14415-1,2,3		R. Oram, CSIRO, Plant Industry, Canberra
I x IIA, II x IA		
I x IIB, II x IB		
III x IA	Seed provided by D.L.H. from crosses made	
IV x IIIA	*circa* 1977 (Parent plants not available)	
III x IVB, IV x IIIB		
IV x IIIC	Origin not documented	
DLH 1-1,2,3,4,5	Mature plants maintained in the Department of Genetics, University of Adelaide since 1977 when the seed above were obtained and crosses made	

Table 3.1. Origin and sources of *Phalaris coerulescens* material.

Seeds from all sources and crosses were put into petri dishes and those which germinated were planted out and handled according to 3.1.2. Plants were tested for their GPI genotype (3.2.2) and pairwise compatibility status (3.1.4).

In the following season the progeny from pairs of plants which were reciprocally 50% compatible were raised and typed for their GPI genotypes.

A series of pollination tests was carried out to determine the degree of compatibility of each member of one of these progenies with their parents and with a random sample of progeny pollinators.

In subsequent seasons much larger progenies were raised from one pair of crosses. Plants from the second season of crosses were typed for GOT, PGM, 6PGD, ACP, LAP, SKD, SOD, PER, and DIA with a view to etstablishing linkage relationships between the structural genes determining these isozymes.

3.5.2 *Holcus lanatus* (Aveneae)

The origin and sources of this material are listed in Table 3.2.

Plant designation	Origin	Source
BY514	France	
BY521	Portugal	DSIR Palmerston North,
BY522	DDR	New Zealand
BY527	New Zealand	

Table 3.2. Origin and sources of *Holcus lanatus* seed material.

Seeds from each different source were plated out (3.1.1) and seedlings planted (3.1.2). Plants were tested for their GPI genotypes (3.2) on gels run with *Phalaris coerulescens* samples as controls. Those found to have GPI variants were kept and crossed.

In the second season progeny were raised, typed and backcrossed to parents. In an endeavour to ensure maximum seed set some pairs of plants were taken to a number of isolated locations and allowed to cross naturally.

In the third season three large progenies from these crosses were planted out. Two of these were tested for GOT, PER, ACP, 6PGD, MDH, LAP, PGM, SOD, EST, ACO, ADH and DIA to establish a linkage map for *H. lanatus*.

3.5.3 *Alopecurus myosuroides* (Agrosteae)

The origin and sources of this material are listed in Table 3.3.

Plant designation	Origin	Source
#3	Little Crawley, Buckinghamshire	
#4	Horncastle, Buckinghamshire	B.G. Murray
#5	Chichley, Buckinghamshire	University of Auckland
#6	Welsh Border Counties	New Zealand

Table 3.3. Origin and source of *Alopecurus myosuroides* seed material.

Seeds from each location were plated out (3.1.1), seedlings planted (3.1.2), pollination tests performed (3.1.4) and plants typed for GPI (3.2) on gels run with *P. coerulescens* samples as controls.

Crosses were made between plants of different GPI genotypes. In the second season progenies were raised and plants from these were backcrossed to the parent plants. In an endeavour to ensure maximum seed set some pairs of plants were taken to isolated locations and allowed to cross naturally.

In the following season a number of progenies were raised and in addition to typing for GPI some of these were tested for ACO, ACP, PER, 6PGD, SOD, GOT, LAP, MDH, PGM, CAT, ALD, ADH and DIA to establish a linkage map for *A. myosuroides*.

3.5.4 *Festuca pratensis* (Festuceae)

The origin and source of this material are listed in Table 2.4.

Plant designation	Origin	Source
79281-84	Sweden	A. Lundqvist
SV79,80	Sweden	A. Lundqvist
CPI 68326		CSIRO Plant Introductions

Table 3.4. Origin and source of *Festuca pratensis* seed material.

Seeds from 79281-84 were provided in packages containing individual heads pollinated by a pollen cloud. They must be at least maternal half sibs. Seeds from all acquisitions were handled according to 3.1.1 and 3.1.2 and leaf samples typed for GPI on gels run with *P. coerulescens* controls.

Plants did not flower in the first season and so in the second season they were taken to a location where late autumn and early winter conditions are extremely cold for Australia (1-8°C day and night). They were left there for six weeks prior to returning to the glasshouse and long day treatment.

Pollination tests were carried out between members of 79283 progeny and pairs, 50% compatible, selected and crossed. Progenies were raised in the next season and scored for GPI.

Plants were selected for backcrossing. Unfortunately, despite cold treatment no plants flowered.

In the subsequent season, some plants were subjected to 2 weeks treatment in the cold room (4°C constant) and others cold nights in the glasshouse. Abundant flowering occurred, the original crosses were repeated and backcrosses performed.

The first intercross progenies were tested for 6PGD, PGM, GOT, ACP, DIA and LAP to establish linkage relationships for *F. pratensis* isozyme systems.

3.5.5 *Hordeum bulbosum* (Hordeae)

The origin and sources of this material are listed in Table 3.5.

Plant description	Origin	Source
CPI 18968	Unknown	Waite Agricultural Research Institute
CPI 15012	"	"
CPI 14276	Greece	"
CPI 13889	Cyprus	"
J1 x L6	PBI Cambridge	P. Sharp, PBI Cambridge
J2 x L3	PBI Cambridge	P. Sharp, PBI Cambridge
Cb 2929	Canada	R. Pickering, DSIR, Palmerston North, New Zealand
C6 2951	Tunisia	" " "
2984 x 2929/1	PBI x Canada	" " "

Table 3.5. Origin and source of *Hordeum bulbosum* seed material.

Seeds from all acquisitions were handled as set out in 3.1.1 and 3.1.2 and leaf samples were typed for GPI on gels run with *P. coerulescens* controls.

Crosses were made between plants of appropriate GPI genotypes. Plants were tested for DIA, GOT, PGM, 6PGD, LAP, ACP and PER.

3.5.6 *Dactylis aschersoniana* (Festuceae)

The origin and source of this material are listed in Table 3.6.

Plant designation	Origin	Source
K2324	Wales	DSIR, Palmerston North New Zealand
K2366	Mainz	
K2306	France	
K2372	Italy	

Table 3.6. Origin and source of *Dactylis aschersoniana* material.

Seeds from all acquisitions were handled according to 3.1.1 and 3.1.2 and leaf samples were typed for GPI on gels run with *P. coerulescens* controls.

Plants of appropriate GPI types were crossed and in the following season progeny plants were backcrossed to parents.

Seeds from these crosses were grown and the progeny were typed for GPI.

3.5.7 *Gaudinia fragilis* (Aveneae)

The origin and sources of this material are listed in Table 3.7.

Plant designation	Origin	Source
G86	Kew Gardens	D.L. Hayman
G286 (075-81-00529)	Kew Gardens	D.L. Hayman

Table 3.7. Origin and source of *Gaudinia fragilis* seed material.

This material was available for study for only the last year of the program (1986). *Gaudinia fragilis* is known to be self-incompatible but the genetic control of the system is not established as yet.

Seeds from both sources were plated out (3.1.1), seedlings planted (3.1.2) and leaf samples typed for GPI on gels with *P. coerulescens* as controls.

A diallele of all reciprocal pollination tests (3.1.4) was set up and scored. Crosses were made between two pairs of plants each isolated in separate glasshouses. Parent plants were also tested for DIA, PER, ACP and ACO.

3.5.8 *Secale cereale* (Hordeae)

Secale cereale was the only annual included in this study. Under the conditions in the glasshouses used for the other grasses under study, this species flowered within about six weeks of planting out, produced a limited number of heads maturing over a short period of time and dried off relatively quickly. Up to fifty seeds were often produced in a single head and these could be planted out quite soon after harvesting. Thus up to three generations could be produced in a single year.

These advantages were counterbalanced by the impossibility of backcrossing progeny to parents. Further, the program was confounded by a considerable amount of self-fertility in many of the acquisitions tested. Thus in addition to the analysis of disturbed segregation ratios and linkage, the results of mixed selfing and crossing for this species are included.

The origin and sources of the material tested are listed in Table 3.8.

Plant designation	Origin	Source
S.A. rye	Bulk S.A. rye	C. Driscoll
Koeräng (KO88)	Korea	D.L. Hayman
448, 016 OP	S.A. bulk rye	C. Driscoll
076, 462 ⊕	crosses and selfs	
Rye 55	Transbaikal	D.L. Hayman
OB rye	Aberystwyth	D.L. Hayman

Table 3.8. Origin and source of *Secale cereale* seed material.

Seeds from each location were handled as described in 3.1.1 and 3.1.2 and leaf samples typed for GPI genotyped on gels run with *P. coerulescens* samples as controls.

In the first season plants were allowed to open pollinate. Progeny plants were selfed and crossed and seed from plants which appeared to give no seed on selfing grown.

Segregation ratios in selfed progeny were investigated. A comparison of the relative rates of selfing and crossing in a single inflorescence was made.

Progenies from the intercross of sibs from a family with no evidence of selfing were raised and scored for their GPI genotypes. One of these was also typed for GOT, LAP, ACP, DIA, ACO, PER, 6PGD, PGM and SKD in an attempt to extend the linkage map of *S. cereale*.

4 Results

4.1 The genetic control of self-incompatibility

Phalaris coerulescens, Holcus lanatus, Festuca pratensis, Secale cereale, Dactylis aschersoniana and *Hordeum bulbosum* have all been reported to have a two locus gametophytic system of self-incompatibility (see Chapter 1.1 for references).

Pollination tests were carried out on *Alopecurus myosuroides* and *Gaudinia fragilis* (3.1.4). Both species showed rapid arrest of self pollen. Compatible and incompatible pollen grains were observed on the stigmas of both species after cross pollination indicating that the control of pollen phenotypes is gametophytic. Reciprocal differences in pollen compatibility were found in *G. fragilis* (Table 4.1) but not in *A. myosuroides*.

Plant number	1	2	3	4
1	–	50	75	50
2	50	–	100	50
3	50	100	–	100
4	50	50	100	–

Table 4.1. Percent compatibility in reciprocal pollination tests in *G. fragilis*. (– indicates incompatible).

Thus whilst it is possible to conclude that the genetic control of self-incompatibility in *G. fragilis* is by two loci the control in *A. myosuroides* can only be assumed to be the same as that found in all other gametophytically self-incompatible grass species for which the genetic control has been established (Table 1.1).

4.2 Analysis of disturbed GPI segregation ratios

4.2.1 *Phalaris coerulescens*

A total of 206 plants were raised from the nine crosses listed in Table 3.1 and after typing for GPI, seven of the nine progenies were shown to be homozygous. The remaining two progenies (I x IIB and II x IB) consisted of plants all of which were heterozygous. Plants CPI 14415-2 and 3 were also heterozygous. Progenies from crosses between two

pairs of plants which were reciprocally 50% compatible yielded the results listed in Table 4.3.

Cross	Parents	Parent Genotype	Progeny Genotypes				Total	Germination rate
			Gpi-2 1-1	*Gpi-2 1-3*	*Gpi-2 3-3*			
8205	I x IIB-12 x I x IIB-16	*1-3* x *1-3*	2	12	10		24	24/24
8206	I x IIB-16 x I x IIB-12	*1-3* x *1-3*	2	15	12		29	29/34
		Total	4	27	22		53	
			1-1	*1-2*	*1-3*	*2-3*		
8208	14415-2 x -3	*1-3* x *1-2*	13	21	14	11	59	59/62
8209	14415-3 x -2	*1-2* x *1-3*	10	11	13	15	49	49/54
		Total	23	32	27	26	108	

Table 4.2. GPI typings from the progeny of 50% reciprocally compatible plants.

The results from crosses 8205 and 8206 (Table 4.2) are homogeneous; P=1 (3.3) and the totals do not fit the expected Mendelian segregation ratio of 1:2:1 ($\chi_2^2 = 12.25$; $P < 0.01$). However, the results from 8208 and 8209 which are also homogeneous ($P = 0.35$) fit the expected 1:1:1:1 ratio ($\chi_3^2 = 1.56$; $0.3 < P < 0.5$).

Thus, applying the methods developed in Chapter 2 of this thesis, the results from 8205 and 8206 lead to an estimate; $r = 0.1538 \pm 0.0701$, for the recombination frequency between *Gpi-2* and one of the incompatibility genes.

The results from a series of pollination tests; testing each member of the progenies of 8205 and 8206 with the parents of the cross and a series of sibs as pollinators are presented in Tables 4.3 and 4.4.

Plant	I x IIB-12	I x IIB-16	8205 -1	8205 -22	8205 -24	8205 -5	8205 -14	8205 -4	Incompatibility genotype	GPI genotype
1	H	H	-	-	-	H	X	H	$S_{1.3}Z_{1.2}$	*3-3*
2	X	X	H	H	H	H	H/T	H	$S_{1.2}Z_{1.1/1.2}$	*3-3*
3	H	H	-	-	-	H	T	H	$S_{1.3}Z_{1.2}$	*3-3*
4	T	-	H	H/T	H	H	T	-	$S_{1.2}Z_{1.2}$	*1-1*
5	X	X	H	H/T	H	-	H	H	$S_{1.2}Z_{1.1/2.2}$	*1-3*
6	T	T	H	H	H	H	H	H	$S_{1.3}Z_{1.1/2.2}$	*3-3*
7	X	X	H	T*	H/T	H/T	H*	H	$S_{1.2}Z_{1.2}$	*1-3*
8	T	H/T	T	H*	T	-	H/T	X	$S_{1.2}Z_{1.1/2.2}$	*1-3*
9	X	X	H	H	H	H	H	X	$S_{1.2}Z_{1.2}$	*1-3*
10	H	H	-	-	-	H	H	X	$S_{1.3}Z_{1.2}$	*1-3*
11	X	X	-	-	-	H	H/T	X	$S_{1.3}Z_{1.2}$	*3-3*
12	X	X	H	H	H	H	H/T	X	$S_{1.2}Z_{1.2}$	*1-3*
13	T	H	X	X	X	X	X	X	$S_{1.2}Z_{1.1/2.2}$	*1-1*
14	H	H	H	H	H	H	-	H	$S_{1.3}Z_{1.1/2.2}$	*3-3*
15	T	H	H	H	H	-	H	X	$S_{1.2}Z_{1.1/2.2}$	*1-3*
16	X	X	H	H/T	H	H	H	X	$S_{1.2}Z_{1.2}$	*1-3*
17	T	H	H*	T	H*	X	H	X	$S_{1.2}Z_{1.1/2.2}$	*1-3*
18	X	X	-	-	-	-*	H	X	$S_{1.3}Z_{1.2}$	*3-3*
19	H	H/T	-	-	-	H	H	X	$S_{1.3}Z_{1.2}$	*3-3*
20	X	X	-	-	-	H	H	X	$S_{1.3}Z_{1.2}$	*3-3*
21	X	X	-	-	-	H	H/T	X	$S_{1.3}Z_{1.2}$	*1-3*
22	X	H	-	-	-	-*	-	X	$S_{1.3}Z_{1.2}$	*1-3*
23	X	X	X	X	X	X	X	X	———	*1-3*
24	H	H	-	-	-	-	H	X	$S_{1.3}Z_{1.2}$	*3-3*

Table 4.3. Results of pollination tests of progeny 8205.

– = incompatible; H = 50% compatible; T = 75% compatible; H/T = unable to distinguish between 50 and 75% compatibility (3.1.4); X = not done or not scorable; * indicates result inconsistent with what is expected.

Table 4.4. Results of pollination tests of progeny 8206. (Legend as for Table 4.3)

Plant	I x IIB-12	I x IIB-16	8206 -9	-10	-18	-20	-27	-3	-8	-25	-22	Incompatibility genotype	GPI genotype
1	X	X	X	X	X	X	X	X	X	X	X	–	*3-3*
2	T	T	T	H*	H	X	H	T*	X	X	X	$S_{1.3}Z_{1.1/1.2}$	*3-3*
3	H	H	H	H	H	X	H	–	–	X	X	$S_{1.3}Z_{1.2}$	*3-3*
4	H	H	H	H	H	X	H	–	–	X	X	$S_{1.3}Z_{1.2}$	*1-3*
5	X	X	X	X	X	X	X	X	X	X	X	–	*1-3*
6	X	X	X	X	X	X	X	X	X	X	X	–	*3-3*
7	H	T	T*	H	H	X	X	T	T	X	H	$S_{2.3}Z_{1.1/1.2}$	*1-1*
8	X	X	H	H	H/T	X	H	–	–	X	–	$S_{1.3}Z_{1.2}$	*1-3*
9	X	X	–	–	–	–	X	X	H	H	H	$S_{2.3}Z_{1.2}$	*1-3*
10	X	X	–	–	–	–	–	X	X	H	X	$S_{2.3}Z_{1.2}$	*1-3*
11	H	T	X	X	X	X	X	X	X	X	X	$S_{2.3}Z_{1.1/2.2}$	*1-3*
12	–	H	X	X	X	X	X	X	X	X	X	$S_{2.3}Z_{1.2}$	*1-3*
13	T	T	X	X	X	X	X	X	X	X	X	$S_{1.3}Z_{1.1/2.2}$	*1-3*
14	X	X	X	X	X	X	X	X	X	X	X	–	*1-3*
15	–	H	–	–	–	X	X	H	H	X	H	$S_{2.3}Z_{1.2}$	*1-3*
16	X	X	X	X	X	X	X	X	X	X	X	–	*1-3*
17	H	H	X	X	–	X	X	X	–	X	H	$S_{1.3}Z_{1.2}$	*3-3*
18	–	H	–	–	–	X	–	X	H	X	–	$S_{2.3}Z_{1.2}$	*3-3*
19	H	T	T*	H	H/T	X	H	T	X	X	X	$S_{2.3}Z_{1.1/2.2}$	*1-1*
20	X	X	–	–	–	–	–	X	X	X	X	$S_{2.3}Z_{1.2}$	*1-3*
21	X	X	T*	T*	T	X	H	T	X	X	X	$S_{2.3}Z_{1.1/2.2}$	*1-3*
22	H	H	X	X	H	X	X	X	H	X	–	$S_{1.3}Z_{1.2}$	*3-3*
23	H	H	H	H	H	X	H	–	X	X	X	$S_{1.3}Z_{1.2}$	*3-3*
24	X	X	–	–	–	X	–	H	X	X	X	$S_{2.3}Z_{1.2}$	*1-3*
25	H	H	H	H	H	H	H	–	X	–	X	$S_{1.3}Z_{1.2}$	*3-3*
26	X	X	–	–	–	X	–	H	X	X	X	$S_{2.3}Z_{1.3}$	*3-3*
27	–	H	–	–	–	–	–	X	H	H	–	$S_{2.3}Z_{1.2}$	*3-3*
28	H	H	H	H	H	X	H	–	X	X	X	$S_{1.3}Z_{1.2}$	*3-3*
29	X	X	H	H	H	X	H	T	X	X	X	$S_{2.3}Z_{1.1/2.2}$	*1-3*

As the parents of these progenies were sibs and reciprocally 50% compatible it is possible to allocate them genotypes. Additional information (Hayman, pers. comm.) enables the precise allocation of the genotype $S_{1.2}Z_{1.2}$ to I x IIB-16. Thus I x IIB-12 must have a genotype of the form $S_{2.x}Z_{1.2}$ and, for simplicity, this may be called $S_{2.3}Z_{1.2}$. Hence it is possible to assign genotypes to almost all of the plants tested (Tables 4.3 and 4.4).

The joint classification of the incompatibility genotypes at the *S* locus and the GPI genotypes are summarized (Table 4.5). It is not possible to distinguish all the homozygotes at the *Z* locus. Statistical analysis of the data in Table 4.5 is presented in Table 4.6.

CROSS	8205			8206		
Genotype	$S_{1.3}$	$S_{1.2}$	Total	$S_{1.3}$	$S_{2.3}$	Total
Gpi-2 1-1	0	2	2	0	2	2
Gpi-2 1-3	3	8	11	3	9	12
Gpi-2 3-3	9	1	10	7	3	10
Total	12	11	23	10	12	24

Table 4.5. Summary of S and GPI genotypes of progeny in crosses 8205 and 8206.

CROSS	8205		8206	
	χ_1^2/χ_2^2	Prob.	χ_1^2/χ_2^2	Prob.
Segregation at the *S* locus	0.18	P = 0.7	0.17	P = 0.7
Segregation at the *Gpi* locus	5.61	0.05 < P < 0.1	5.3	0.05 < P < 0.1
Joint segregation *S/Gpi*	-	0.01	-	0.02

Table 4.6. Summary of the statistical analysis of data in Table 4.5.

The data from crosses 8205 and 8206 (Table 4.5) are homogeneous and indicate linkage between *S* and *Gpi-2* The recombination frequency can be calculated according to Lawrence *et al.* (1979) and is $r_{S\text{-}GPI} = 0.1489 \pm 0.0367$. This value is not significantly different from that calculated above (SND = 0.05; P = 0.96). Thus it seems reasonable to

conclude that the estimation of recombination frequencies between the loci of isozyme structural genes and incompatibility genes using disturbed segregation ratios is reliable.

The accumulation of data over subsequent seasons yielded a total of 207 plants. The results were homogeneous (P = 0.64) substantiating the claim that the disturbances in segregation ratio are not due to viability effects and the totals are presented in Table 4.7.

Cross	GPI genotypes *1-1*	*1-3*	*3-3*	Total*
8205	5	61	43	109
8206	6	49	43	98
Total	11	110	86	207

Table 4.7. Pooled data from a number of seasons for the progeny of 50% reciprocally compatible plants.

*Germination ratio in *P. coerulescens* were always similar to those given in Table 4.2.

The results are homogeneous (P = 0.66) and do not fit the expected 1:2:1 segregation ratio ($\chi_2^2 = 64.84$; $P < 0.001$). They lead to an estimate $r_{S\text{-}GPI} = 0.1134 \pm 0.0312$.

4.2.2 *Holcus lanatus*

Plants from the BY514 acquisition were the only ones showing GPI variants. One of these samples had a greater mobility than *P. coerulescens* control samples and was designated *Gpi-2 1*[1].

Progenies raised from the reciprocal crosses of BY514-5 and BY514-6 gave the results listed in Table 4.8.

Parents	GPI genotype of parents	Genotypes of Progeny				
		Gpi-2 1^1-*1*	*Gpi-2 1-1*	Total	χ_1^2(1:1)	Probability
BY514-5 x BY514-6	1^1-*1* x *1-1*	21	15	36	1.00	0.3 < P < 0.5
BY514-6 x BY514-5	*1-1* x 1^1-*1*	24	18	42	0.86	0.3 < P < 0.5

Table 4.8. GPI typings of plants in original crosses of *H. lanatus*.

Seed set from a number of the backcrosses was high and all of the seeds put into petri dishes germinated. Only one or two seedlings were lost before typing was possible. However, only two of the four progenies planted were completely typed as these alone provided adequate information.

The results of typing the backcross progenies are recorded in Table 4.9.

Cross	GPI genotype of parents	GPI genotypes of progeny			
		1^1-1^1	1^1-*1*	*1-1*	Total
BY514-5 x 5/6-3+	1^1-*1* x 1^1-*1*	14	40	15	69
5/6-3 x BY514-5* (a)	1^1-*1* x 1^1-*1*	5	11	7	23
BY514-5 x 5/6-6	1^1-*1* x 1^1-*1*	23	55	54	132
BY514-5 x 5/6-10* (b)	1^1-*1* x 1^1-*1*	2	2	3	7

Table 4.9. GPI typings of backcross progenies of *H. lanatus*.

+5/6-3 indicates plant number 3 in the cross BY514-5 x BY514-6
*progeny not fully typed as (a) reciprocal undisturbed (see below)
(b) BY514-5 x 5/6-3 undisturbed and
BY514-5 x 5/6-6 disturbed (see below)

These crosses are between plants which are reciprocally 50% compatible. The results from BY514-5 x 5/6-3 fit 1:2:1 ($\chi_2^2 = 1.78$; $0.3 < P < 0.5$) but those from BY514-5 x 5/6-6 do not ($\chi_2^2 = 11.55$; $P < 0.01$). Applying the methods set out in Chapter 2 an estimate $r_{S\text{-}GPI} = 0.2987 \pm 0.0563$ of the recombination frequency between *Gpi-2* and *S* is obtained.

4.2.3 *Alopecurus myosuroides*

Plants from all sources were polymorphic for GPI. The GPI mobilities corresponded almost exactly to *P. coerulescens 1-1* and *1-2*.

The results of typing the plants in the first season of crosses are listed in Table 4.10.

Parents	GPI genotypes of parents	Genotypes of progeny: *Gpi-2 1-1*	*Gpi-2 1-2*	*Gpi-2 2-2*	Total	χ_1^2/χ_2^2	P
#4.2 x #584-7	*1-2* x *1-1*	14	15		29	0.03	0.8-0.9
#584-7 x #4-2	*1-1* x *1-2*	34	22		56	2.57	0.1-0.2
#4-2 x #5-2	*1-2* x *1-2*	3	5	2	10	-	0.85
#5-2 x #4-2	*1-2* x *1-2*	8	14	5	28	0.70	0.3-0.5

Table 4.10. GPI typings of plants from the original crosses in *A. myosuroides*.

Severe problems were encountered with the flowering of plants selected for backcrossing. Whereas the progeny plants were extremely vigorous and flowered well, #4-2 was invariably male sterile in the second season and so very few seeds were obtained from crosses with this plant as male parent.

The results of typing the backcross progenies grown are recorded in Table 4.11.

Cross	GPI genotypes of parents	GPI genotypes of progeny: *1-1*	*1-2*	*2-2*	Total	Seed Germination
#4.2 X 4/5-15	*1-2* x *1-2*	23	27	5	55	55/62
#4.2 x 4/5-2	*1-2* x*1-2*	29	30	7	66	66/80
#4.2 x 4/5-10	*1-2* x *1-2*	9	8	2	19	19/20
4/5-10 x #4-2	*1-2* x *1-2*	5	4	1	10	10/10
Total		66	69	15	150	
#584-7 x 4/5-21	*1-1* x *1-2*	33	28		51	51/56
4/5-21 x #584-7	*1-2* x *1-1*	22	22		44	44/45

Table 4.11. GPI typings of backcross progenies of *A. myosuroides*.

These crosses are between plants which are reciprocally 50% compatible. The segregation ratio from the first four crosses listed in Table 4.11 are homogeneous (P = 0.996) and do not fit 1:2:1 ($\chi_2^2 = 35.64$; $P < 0.001$). The last pair of crosses are controls. The first giving an undisturbed transmission ratio (χ_1^2 (1:1) = 0.41; $0.3 < P < 0.5$) and the second showing no influence of isozyme type on viability.

Data from the first four crosses lead to an estimate $r_{S\text{-}GPI} = 0.1852 \pm 0.0449$ of the recombination frequency between *S* and *Gpi-2*.

The fact that *Alopecurus myosuroides* shows the same pattern of disturbed segregation ratios and a similar linkage value to those species so far reported strongly supports the assumption of the two locus incompatibility system operating in this species.

4.2.4 *Festuca pratensis*

This species proved to be highly polymorphic for GPI. Five different mobilities were found, one faster and one slower than the *P. coerulescens* control samples. Pollination tests revealed that 79283-14, 16 and 18 were all reciprocally 50% compatible.

Under the conditions maintained in the glasshouses the inflorescences of *F. pratensis* were extremely fragile. Many were lost due to breaking before seed maturity, in the first flowering season before the introduction of cellophane pollination cages or outdoor isolation of plants for crossing. Thus the seed set in this first season was low though adequate to detect reasonably small recombination frequencies (see Table 2.4).

The results of typing the plants from crosses between these 50% reciprocally compatible parents are listed in Table 4.12.

Parents	GPI genotype of parents	Genotypes of Progeny				Total	Seed Germination
		Gpi-2 1-1	*Gpi-2 1-2*	*Gpi-2 1-3*	*Gpi-2 2-3*		
79283-16 x 79283-18	*1-3* x *1-2*	2	13	5	15	35	22/30 + 13/20
79283-18 x 79283-16	*1-2* x *1-3*	2	2	5	11	20	20/27
		1-1	*1-2*	*2-2*			
79283-14 x 79283-18	*1-2* x *1-2*	6	10	2		18	18/24
79283-18 x 79283-14	*1-2* x *1-2*	6	9	3		18	18/21
		12	19	5		36	

Table 4.12. GPI typings of *F. pratensis* progeny from crosses of reciprocally 50% compatible plants.

The results from the second pair of crosses are homogeneous (P = 1.00) and fit 1:2:1 (χ_2^2 = 2.83; 0.2 < P < 0.3). The results from the first pair of crosses need to be rearranged to take account of the respective contributions of the male and female gametes (Table 4.13).

	male (*1*)		male (not *1*)	
	GPI		GPI	
Parents	*1-1*	*1-3*	*1-2*	*2-3*
16 x 18	2	5	13	15
	1-1	*1-2*	*1-3*	*2-3*
18 x 16	2	2	5	11

Table 4.13. Progeny from Table 4.12 rearranged to take account of gamete contributed by male parent.

Thus these plants, which were known to be maternal half sibs, evidently share the *S-Z* combination associated with *Gpi-2 1*. The data lead to an estimate of the recombination frequency $r_{S\text{-}GPI} = 0.2000 \pm 0.0539$.

The results from backcrossing progeny and parent are listed in Table 4.14.

Cross	GPI genotypes of plants		GPI genotypes of progeny				Total	Seed germination
			Gpi-2 1-2	*Gpi-2 1-3*	*Gpi-2 2-2*	*Gpi-2 2-3*		
18 x 16-1 x 18*	*2-3*	*1-2*	13	12	2	1	28	28/40
18 x 18 x 16-1	*1-2*	*2-3*	0	8	0	28	36	36/40

Table 4.14. GPI typings of *F. pratensis* backcross progeny.

*Seeds from a single flowering head.

The progeny from 18 x 16-1 x 18 do not fit 1:1:1:1 ($\chi_2^2 = 17.43$; $P < 0.001$) and lead to an estimate of $r_{S\text{-}GPI} = 0.107 \pm 0.0585$. This does not differ significantly from the estimate obtained above (SND = 1.67; P = 0.10).

However, the reciprocal progeny of 18 x 18 x 16-1 is extremely difficult to account for. Whilst it is consistent with its reciprocal in that *Gpi-2 2* is the allele expected to be transmitted only by recombination the segregation ratio of 8:28 does not fit the expected 1:1 ($\chi_1^2 = 11.11$; $P < 0.001$) for the contribution by the female parent and so these data have not been included in the linkage analysis.

Pooling the data from the original and backcross progenies ($P_{homog.} = 0.82$) leads to an estimate $r_{S\text{-}GPI} = 0.1687 \pm 0.0411$.

4.2.5 *Secale cereale*

The original material from Bulk S.A. rye included only one plant which was heterozygous *3-4* for GPI; all other plants were *Gpi-2 3-3*. This plant was allowed to open pollinate. All progeny from this open pollination must be maternal half sibs. Plants

which were GPI type *3-4* were selfed and intercrossed. The results of typing three of these intercross progenies for GPI are listed in Table 4.15.

Cross	GPI genotypes of parents	GPI genotypes of progeny				
		Gpi-2 3-3	*Gpi-2 3-4*	*Gpi-2 4-4*	Total	Seed germination
17OP-3 x 17OP-4	*3-4* x *3-4*	8	13	0	21	21/47
17OP-4 x 17OP-3	*3-4* x *3-4*	24	26	7	57	57/68

Table 4.15. GPI genotypes of intercross progeny from the open pollination of the original GPI heterozygote.

The results of the GPI typings from the first pair of crosses are homogeneous (P = 0.23) and lead to an estimate of recombination $r_{S\text{-}GPI} = 0.1795 \pm 0.0615$.

However, pollination tests in *S. cereale* appeared somewhat unreliable due to extremely late arrest of pollen tubes in self pollinations and *S. cereale* is often found to have a reasonably high degree of self fertility. For these reasons crosses were selected because the parents were also segregating for diaphorase (DIA) in such a way that this isozyme would act as an indicator of selfing. Plant 17OP-3 gave no seed in two selfed heads and no seed in three crosses with 17OP-8 and so it was considered reliably self-incompatible. Plant 17OP-4 was heterozygous for DIA whereas 17OP-3 was homozygous. The results of the joint classification of their reciprocal progenies are listed in Table 4.16.

Cross			GPI				Comments
17OP-3 x 17OP-4			*3-3*	*3-4*	*4-4*		
GPI *3-4*	*3-4*	*1-2*	7	7	0	14	*Dia-1 1-2* must be cross progeny
DIA		DIA					
DIA *2-2*	*1-2*	*2-2*	1	6	0	7	Data homogeneous P = 0.17. Deduce all cross progeny.
			8	13	0	21	
17OP-4 x 17OP-3				GPI			
GPI *3-4*	*3-4*		*3-3*	*3-4*	*4-4*		
DIA *1-2*	*2-2*	*1-1*	6	4	1	11	*Dia 1 1-1* must be self progeny. Data homogeneous P = 0.20
		DIA *1-2*	7	15	5	27	
		2-2	11	7	1	19	
			24	26	7	57	

Table 4.16. Joint classification of reciprocal progenies for GPI and DIA.

There is a clear indication that the progeny 17OP-4 x 17OP-3 includes plants produced by both selfing and crossing.

Results from selfing plants which are segregating for only GPI or DIA can lead to undisturbed segregation ratios (Table 4.17).

Parents	GPI *3-3*	GPI *3-4*	GPI *4-4*	DIA *1-1*	DIA *1-2*	DIA *2-2*	Tests of significance
448OP-11 ⊕	3	7	6			16	
448OP-15 ⊕	5	9	3			17	P (homogeneity) = 0.44
	8	16	9			33	χ_2^2 (1:2:1) = 0.09 P > 0.9
016OP-6 ⊕	58			15	26	17	χ_2^2 (1:2:1) = 0.76 0.5 < P < 0.7

Table 4.17. Selfed progenies showing undisturbed segregation ratios.

However, the results for the isozyme typings of 17OP-4 x 17OP-3 are homogeneous. Thus the disturbed segregation ratio is occurring in both self and cross progeny. An explanation for such a result is that 17OP-4 is heterozygous for an allele at the *S* locus (or a locus very close to it) that confers self fertility. Thus 17OP-4 would have a genotype of the form

$$\frac{S_1P_3}{S_1P_4}\ Z_{1.2}$$

which would lead to a disturbed segregation ratio for GPI when this plant self pollinated.

Plant 17OP-3 which shares the unique and common source of the P_4 allele from plant 17 would have a genotype of the form

$$\frac{S_xP_3}{S_1P_4}\ Z_{1.2}\ (x \neq 1)$$

and so when used as pollen parent would also give a disturbed segregation for GPI.

As the results from 17OP-4 x 17OP-3 are homogeneous (P = 0.20) and also homogeneous with the results from the reciprocal cross (P = 0.23) the original estimate of $r_{S\text{-}GPI} = 0.1795 \pm 0.0615$ is appropriate.

Some progeny from 17OP-4 x 17OP-3 were intercrossed. The results of GPI and DIA typings are given in Table 4.18.

	Parents			GPI 3-3	GPI 3-4	GPI 4-4	Total	Comments
	4332 x 4350		*1-1*	4	17	5	26	from selfing $\frac{26}{28} = 93\%$
GPI	*3-4*	*3-4*	DIA *1-2*	1	1	0	2	from crossing
DIA	*1-1*	*2-2*						χ_2^2 (1:2:1) = 2.54
								0.2 < P < 0.3
	4350 x 4332			*3-3*	*3-4*	*4-4*		
GPI	*3-4*	*3-4*	DIA *1-2*	3	0	0	3	from crossing
DIA	*2-2*	*1-1*	*1-2*	6	9	2	17	from selfing $\frac{17}{20} = 85\%$
								χ_2^2 (1:2:1) = 1.94
								0.3 < P < 0.5

Table 4.18. Intercrosses of progeny from 17OP-4 x 17OP-3

Plant 4332 must have been produced from 17OP-4 ⊕ and 4350 could have been produced by selfing or crossing. The results in Table 4.18 indicate that the majority of seeds in the two reciprocal single heads scored were produced by selfing and each of the plants selected (4332 and 4350) is homozygous for the S_f allele

$$\frac{S_f P_2}{S_f P_4} \quad Z_{1.1/1.2/2.2}$$

as each gives an undisturbed GPI segregation ratio.

Thus although these results are consistent they are confounded by selfing. For this reason new material was sought and the results from intercrosses of plants which consistently set no seed on selfing are listed in Table 4.19.

Parents	GPI genotypes of parents	Genotypes of progeny *Gpi-2 3-3*	*Gpi-2 3-4*	*Gpi-2 4-4*	Total	Seed Germination
14 x 2-5 x 14 x 2-1	*3-4* x *3-4*	8	13	9	30	30/30
2 x 14-10 x 2 x 14-2	*3-4* x *3-4*	7	13	8	28	28/28
2 x 14-8 x 14 x 2-9	*3-4* x *3-4*	7	9	5	21	21/22
		22	35	22	79	
				χ_2^2 (1:2:1) = 1.03		0.5 < P < 0.7
2 x 14-3 x 14 x 2-4	*3-4* x *3-4*	1	14	13		28/28
				χ_2^2 (1:2:1) = 10.29		P < 0.1

Table 4.19. GPI typings of sib progenies from plants which did not set seed on selfing.

The first three progenies are homogeneous (P = 0.98) and have an undisturbed segregation ratio but this last one indicates linkage of S and GPI; $r_{S\text{-}GPI} = 0.0714 \pm 0.0688$.

4.3 Linkage mapping analysis

4.3.1 *Phalaris coerulescens*

No variants for GOT, PGM, 6PGD, ACP, LAP, SKD, SOD or PER were found in the material tested. Variants for DIA were found and the majority of the 207 plants classified for GPI were also typed for this enzyme. The joint classification of the material for GPI and DIA is given in Table 4.20.

Cross 8205 = I x IIB-12 x I x IIB-16 GPI (*1-3* x *1-3*) DIA (*1-2* x *2-3*)

Cross 8206 = I x IIB-16 x I x IIB-12 GPI (*1-3* x *1-3*) DIA (*2-3* x *1-2*)

Cross 8205		GPI genotypes			
		1-1	*1-3*	*3-3*	Total
DIA genotypes	*1-2*	0	10	9	19
	1-3	0	9	12	21
	2-2	1	9	4	14
	2-3	3	16	8	27
Total		4	44	33	81
Cross 8206					
DIA genotypes	*1-2*	1	7	9	17
	1-3	0	5	10	15
	2-2	1	14	8	23
	2-3	4	20	12	36
Total		6	46	39	91

Table 4.20. Joint classification of 8205 and 8206 for GPI and DIA.

The DIA typings for plants in cross 8205 fit the expected 1:1:1:1 segregation ratio ($\chi_3^2 = 4.28$; $0.2 < P < 0.3$) but those for 8206 do not ($\chi_3^2 = 11.81$; $P < 0.01$). Applying the methods set out in Chaper 2 an estimate $r_{S\text{-}\cancel{GPI}\,DIA} = 0.3516 \pm 0.0501$ is obtained. Although the data from 8205 fit Mendelian segregation ratios they also fit expectations based on $r = 0.35$ ($\chi_1^2 = 1.17$; $0.2 < P < 0.3$) and so these two crosses are consistent.

The test for independence of the joint classification of GPI and DIA in cross 8206 leads to $P_{indep} = 0.30$ indicating that GPI and DIA must be on opposite sides of the S locus. Applying the method of Lawrence *et al.* (1979) an estimate $r_{GPI\text{-}DIA} = 0.5000 \pm 0.0524$ is obtained from these data. The results of the GPI typings for this cross alone lead to an estimate $r_{S\text{-}GPI} = 0.1219 \pm 0.0485$ and so a linkage map of these loci in *P. coerulescens* can be drawn (Fig. 4.1).

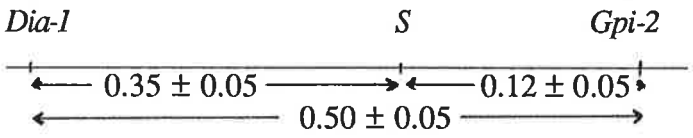


Fig. 4.1. Linkage map for *P. coerulescens* (from cross 8206).

4.3.2 *Holcus lanatus*

Of the twelve further isozyme systems tested in this species GOT, PER, ACP, 6PGD and MDH were variable. The progenies of BY514-5 x 5/6-3 and BY514-5 x 5/6-6 were typed and gave the results listed in Table 4.21.

Both 6PGD and MDH give disturbed segregation ratios in cross BY514-5 x 5/6-3 in which the GPI segregation is not disturbed. As GPI has been shown to be linked to S (4.2.2) these disturbed segregation ratios suggest that 6PGD and MDH are linked to *Z* (Chapter 2). The segregation ratio for MDH in the other progeny (BY514-5 x 5/6-6) is in agreement with the 1:2:1 expected but that for 6PGD is not. This might be due to misclassification or it might be attributable to chance when the large number of χ^2 tests performed in the analysis of these results is considered. Exact probabilities (Hancock, 1979) associated with tests of independence of all segregating loci are listed in Table 4.22.1 and 4.22.2. The probability (P = 0.003) of MDH and 6PGD assorting independently in cross BY516-5 x 5/6-6 supports the view that the departure of the 6PGD segregation ratio from expected is attributable to chance.

Thus recombination frequencies $r_{Z\text{-}6PGD} = 0.0741 \pm 0.0452$ and $r_{Z\text{-}MDH} = 0.0833 \pm 0.0782$ may be derived.

Cross	Parent genotypes	Progeny genotypes			Significance test χ_1^2 (1:1) χ_2^2 (1:2:1)	Probability
		Gpi-2 1[1]-1[1]	*Gpi-2 1[1]-1*	*Gpi-2 1-1*		
BY514-5 x 5/6-3	*1[1]-1* x *1[1]-1*	14	40	15	$\chi_2^2 = 1.78$	$0.3 < P < 0.5$
x 5/6-6	*1[1]-1* x *1[1]-1*	23	55	54	$\chi_2^2 = 11.55$	$P < 0.01$
		Got-1 1-1	*Got-1 1-2*			
BY514-5 x 5/6-3	*1-1* x *1-2*	29	19		$\chi_1^2 = 2.08$	$0.1 < P < 0.2$
x 5/6-6	*1-1* x *1-2*	70	62		$\chi_1^2 = 0.48$	$P = 0.5$
		Per-1 1-2	*Per-1 2-2*			
BY514-5 x 5/6-3	*2-2* x *1-2*	34	34		$\chi_1^2 = 0$	$P = 1$
x 5/6-6	*2-2* x *2-2*	not segregating				
		Acp-1 1-1	*Acp-1 1-2*	*Acp-1 2-2*		
BY514-5 x 5/6-6	*1-2* x *1-2*	5	24	14	$\chi_1^2 = 4.35$	$0.1 < P < 0.2$
		Acp-2 1-1	*Acp-2 1-2*			
B514-5 x 5/6-3	*1-2* x *1-1*	16	29		$\chi_1^2 = 3.75$	$0.05 < P < 0.1$
5/6-6	*1-2* x *1-1*	22	23		$\chi_1^2 = 0.02$	$0.8 < P < 0.9$
		6Pgd-2 1-1	*6Pgd-2 1-2*	*6Pgd-2 2-2*		
BY514-5 x 5/6-3	*1-2* x *1-2*	25	40	2	$\chi_2^2 = 18.31$	$P < 0.001$
x 5/6-6	*1-2* x *1-2*	20	22	5	$\chi_2^2 = 9.77$	$P < 0.01$
		Mdh-2 1-1	*Mdh-2 1-2*	*Mdh-2 2-2*		
*BY514-5 x 5/6-3	*1-2* x *1-2*	1	13	11	$\chi_2^2 = 8.04$	$0.01 < P < 0.02$
x 5/6-6	*1-2* x *1-2*	6	27	9	$\chi_2^2 = 3.86$	$0.1 < P < 0.2$

Table 4.21. Isozyme typings for segregating loci in *Holcus* progenies.
*Isozyme typings were carried out over an extended period of time and in some cases plants were not of a suitable age to resolve all systems.

The probabilities in Tables 4.22.1 and 4.22.2 indicate a number of significant associations between loci. The two way classifications of these segregations and linkage estimates (3.4) are listed in Tables 4.23.1-5.

	GPI (2)	GOT (1)	PER (1)	ACP (2)	6PGD (2)	
GOT (1)	0.3990					Cross
PER (1)	0.0164*	1.0000				BY514-5
ACP (2)	0.0545*	0.3510	0.1055			x 5/6-3
6PGD (2)	0.1289	0.6785	0.5397	0.2552		
MDH (2)	0.8585	–	0.2112	–	0.0953	

Table 4.22.1

	GPI (2)	GOT (1)	ACP (1)	ACP (2)	6PGD (2)	
GOT (1)	0.7582					Cross
ACP (1)	0.5780	0.0173*				BY514-5 x 5/6-6
ACP (2)	0.1865	1.0000	0.8490			
6PGD (2)	0.2055	0.5166	1.0000	0.3725		
MDH (2)	0.2271	0.5785	0.2479	0.1129	0.0030*	

Table 4.22.2

Tables 4.22.1 and 2. Exact probabilities for the independent assortment of pairs of isozyme loci.

* indicates a probability of less than 5%.
– indicates that no plants were jointly classified for these isozymes.

Cross BY514-5 x 5/6-3 Isozyme classifications

Linkage estimates and standard errors

		GPI (2) *1-1*	*1-2*	*2-2*	Total	
	1-2	4	18	12	34	
PER(1)	*2-2*	10	21	3	34	$r_{GPI-PER} = 0.2414 \pm 0.0734$
		14	39	15	68	

Table 4.23.1

		GPI (2) *1-1*	*1-2*	*2-2*		
	1-1	0	11	5	16	
ACP (2)	*1-2*	8	16	5	29	$r_{GPI-ACP(2)} = 0.2778 \pm 0.0668$
		8	27	10	45	

Table 4.23.2

		MDH(2) *1-1*	*1-2*	*2-2*		
	1-1	0	4	7	11	
6PGD(2)	*1-2*	1	9	3	13	$r_{MDH-6PGD} = 0.2174 \pm 0.0842$
	2-2	0	0	0	0	
		1	13	10	24	

Table 4.23.3

		ACP (1) *1-1*	*1-2*	*2-2*		
	1-1	1	16	4	21	$r_{ACP(1)-GOT} = 0.4444 \pm 0.0786$
GOT (1)	*1-2*	4	6	9	19	
		5	19	13	40	

Table 4.23.4

		MDH(2) *1-1*	*1-2*	*2-2*		
	1-1	3	7	4	14	
6PGD(2)	*1-2*	0	13	4	17	$r_{MDH-6PGD} = 0.2256 \pm 0.0717$
	1-3	3	0	0	3	
		6	20	8	34	

Table 4.23.5

Tables 4.23.1-5. Two-way classifications of isozymes in *H. lanatus*.

Thus the following linkage maps may be drawn up (Fig. 4.2).

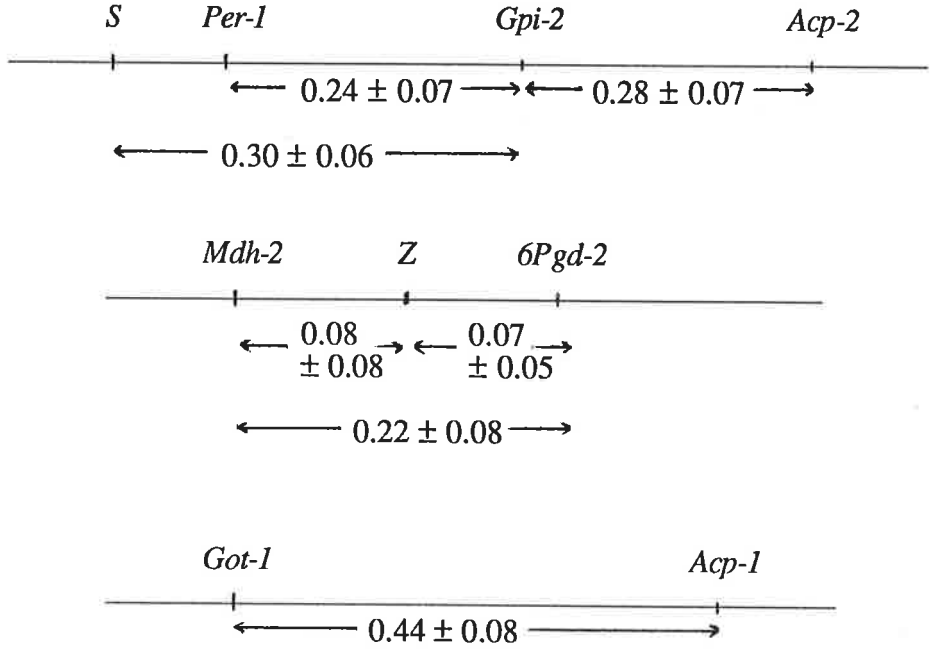


Fig. 4.2. Linkage map for *H. lanatus*.

An alternative explanation for the disturbed segregation ratio for 6PGD in both crosses is that it is showing linkage to S in cross BY514-5 x 5/6-6 ($r_{S\text{-}6PGD} = 0.2000 \pm 0.0412$) and to Z in cross BY514-5 x 5/6-3 ($r = 0.0741 \pm 0.0452$). If this were the case it would mean that S and Z are linked and would lead to the following linkage map (Fig. 4.3).

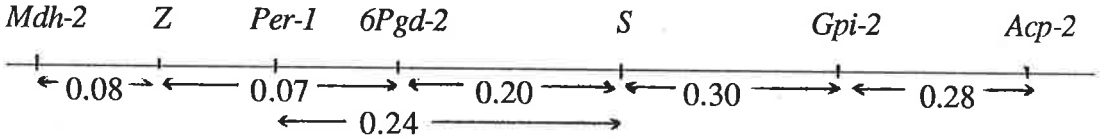


Fig. 4.3. Alternative linkage map for *H. lanatus*.

The following genotypes satisfy the observations (Table 4.24).

Genotypes			Expected outcome on backcrossing to BY514-5
BY514-5	x	BY514-6	
$Z_1D_1S_1P_1$		$Z_1D_2S_3P_2$	
$Z_2D_2S_2P_2$		$Z_3D_1S_1P_2$	
	5/6-3	$Z_1D_2S_2P_1$ / $Z_3D_1S_1P_2$	undisturbed GPI and disturbed 6PGD – linkage to Z
	5/6-6	$Z_1D_2S_1P_1$ / $Z_3D_1S_3P_2$	disturbed GPI and disturbed 6GPD –linkage to S

where D_1 and D_2 are used to represent the alleles of *Pgd-2*
P_1 and P_2 are used to represent the alleles of *Gpi-2*

Table 4.24. Genotypes and expectation in backcrosses if S and Z are linked.

The derivation of these genotypes requires that both 5/6-3 and 5/6-6 include a recombinant gamete : 5/6-3; a double recombination event between Z and 6PGD and also between S and GPI and 5/6-6; between Z and 6PGD. In addition it is expected that either 6PGD and PER should show linkage or if PER were distal to GPI then ACP and PER should show linkage. Neither of these associations is found.

Furthermore, the suggested recombination fraction between the *S* and *Z*-loci (0.27) means that it would not be possible to detect pollinations which were 75% compatible (Chapter 2). Pollinations of this compatibility status were reported in *H. lanatus* by Weimark (1968).

Thus, although this explanation is interesting, in view of the synteny of *Mdh-2 6Pgd-2* and *Gpi-1* (≡ *Gpi-2* here) in *H. vulgare* (Salinas *et al.*, 1985) it does seem the less likely of those offered.

4.3.3 *Alopecurus myosuroides*

Variants were found in four of the thirteen additional isozyme systems tested. The classifications for one of the progenies (4 x 4/5-2) are listed in Table 4.25.

Cross	Parents genotype	Progeny genotypes			Significance test χ_1^2(1:1), χ_2^2(1:2:1)	Probability
		Gpi-2 1-1	*Gpi-2 1-2*	*Gpi-2 2-2*		
4 x 4/5-2	*1-2* x *1-2*	29	30	7	15.21	P < 0.001
		Aco-1 1-1	*Aco-1 1-2*			
4 x 4/5-2	*1-1* x *1-2*	20	34		3.63	0.05 < P < 0.1
		Per-1 1-1	*Per-1 1-2*	*Per-1 2-2*		
4 x 4/5-2	*1-2* x *1-2*	12	3	6	13.91	P < 0.001
		Acp-1 1-1	*Acp-1 1-2*	*Acp-1 2-2*		
4 x 4/5-2	*1-2* x *1-2*	4	12	5	0.56	0.7 < P < 0.8
		Acp-2 1-1	*Acp-2 1-2*			
4 x 4/5-2	*1-2* x *1-1*	20	25		0.56	0.3 < P < 0.5
		6Pgd-31-2	*1-3*	*2-2*	*2-3*	
4 x 4/5-2	*1-2* x *2-3*	11	9	14	11 $\chi_3^2 = 1.13$	0.7 < P < 0.8

Table 4.25. Isozyme typings for segregating loci in *Alopecurus* progenies.

The exact probabilities obtained for tests of independent assortment of these loci considered pairwise is given in Table 4.26. There is one significant (P < 0.05) association: GPI/PER. The two way classification of these segregations and the linkage estimate (3.4) are given Table 4.27.

	GPI (2)	ACO (1)	ACP (1)	ACP (2)	PER (1)
ACO (1)	0.5701				
ACP (1)	0.6196	0.4126			
ACP (2)	0.9066	1.0000	0.0608		
PER (1)	0.0297	1.0000	0.1638	0.1638	
6PGD(3)	0.4199	0.5720	0.4487	0.8126	0.3529

Table 4.26. Exact probabiliites of independence of 2 way classification of segregating loci in *A. myosuroides*.

Cross 4 x 4/5-2

		Isozyme classification GPI (2)			
		1-1	*1-2*	*2-2*	
PER(1)	*1-1*	8	4	0	12
	1-2	0	1	2	3
	2-2	3	1	2	6
		11	6	4	21

Linkage estimate and standard error

$r_{GPI-PER} = 0.2878 \pm 0.0988$

Table 4.27. Two way classification of isozymes in *A. myosuroides*.

The following linkage map for *A. myosuroides* may be drawn (Fig. 4.4).

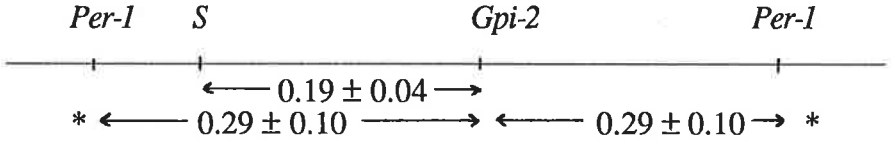


Fig. 4.4. Linkage map of *A. myosuroides*

*The location of *Per* relative to *S* is not known.

It is not possible to determine the location of PER relative to S. However, the distance between PER and GPI (0.27 ± 0.10) is comparable with that found for *H. lanatus* (0.24 ± 0.07) (4.3.2) and other information from *T. aestivum* (Ainsworth *et al.*, 1984) *S. cereale* (Wricke and Wehling, 1985) and *L. multiflorum* (Polans and Allard, 1986, see Chapter 5) indicate the proximal location. The greater recombination frequency between S and GPI in *H. lanatus* compared with the other species studied (see summary Table 5.1) could be explained by a small inversion including S and PER (Fig. 4.5).

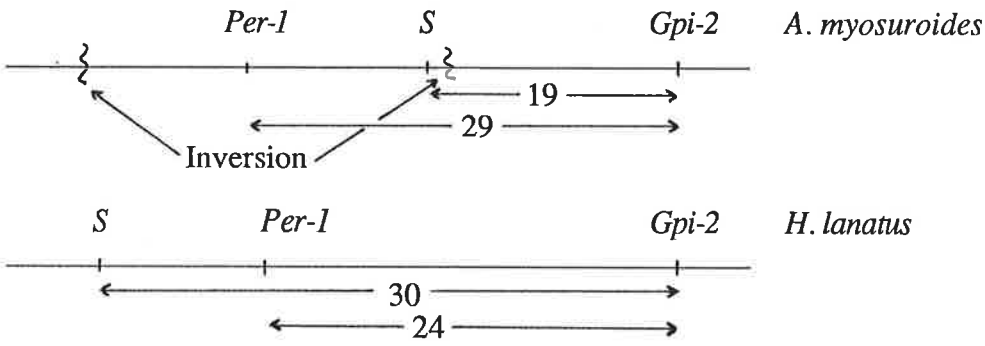


Fig. 4.5. Suggested relative locations of *S* and *PER* in *A. myosuroides* and *H. lanatus*.

4.3.4 *Festuca pratensis*

The original plants were tested for DIA, GOT, 6PGD, PGM, ACP and LAP in addition to GPI. The progeny of 79283-16 x 79283-18 and its reciprocal were typed for PGM and 6PGD with the following results: Table 4.28.

Cross	Parents genotype	Progeny genotypes				Significance test χ_1^2(1:1), χ_3^2(1:1:1:1)	Probability
		Gpi-2 1-1	*Gpi-2 1-2*	*Gpi-2 1-3*	*Gpi-2 2-3*		
79283-16 x 79283-18	*1-3* x *1-2*	2	13	5	15	13.34	P < 0.01
79283-18 x 79283-16	*1-2* x *1-3*	2	2	5	11	10.8	0.01 < P < 0.02
		Pgm-1 1-1	*Pgm-1 1-2*				
16 x 18	*1-2* x *1-1*	5	13			3.56	0.05 < P < 0.1
18 x 16	*2-2* x *1-2*	5	14			4.26	0.02 < P < 0.05
		6Pgd-1 1-2	*6Pgd-1 2-2*				
16 x 18	*1-2* x 2-2	8	11			0.47	0.3 < P < 0.5
18 x 16	*2-2* x *1-2*	10	7			0.53	0.3 < P < 0.5

Table 4.28. Isozyme typings for segregating loci in *F. pratensis*.

The exact probabilities obtained for tests of independence of these loci considered pairwise are given in Table 4.29.1 and 2.

	GPI	PGM
PGM	0.2000	
6PGD(1)	0.7328	1.0000

Table 4.29.1 16 x 18 progeny

	GPI	PGM
PGM	0.6001	
6PGD(1)	0.8704	1.0000

Table 4.29.2 18 x 16 progeny

Tables 4.29. Two way classification of plants for association of isozyme loci.

The segregation ratios for PGM are both only very poorly in agreement with expectation and as the departure is in the same direction in both crosses it is unlikely to be attributable to linkage. No pairs of loci tested gave an indication of linkage.

4.3.5 *Secale cereale*

Three (GOT, LAP, and ACP) of the nine further isozyme systems tested in this species proved to be polymorphic. The results are listed in Table 4.30.

Cross	Parent genotypes	Progeny genotypes			Significance tests $\chi_1^2(1:1)$ $\chi_2^2(1:2:1)$	Probability
12 x 14-3 x 14 x 17-4						
		Gpi-2 3-3	*Gpi-2 3-4*	*Gpi-2 4-4*		
	3-4 x *3-4*	1	14	3	10.29	P < 0.01
		Got-3 1-1	*Got-3 1-2*	*Got-3 2-2*		
	1-2 x *1-2*	5	13	4	0.96	0.5 < P < 0.7
	1-2 x *2-2*	*Lap-1 1-2*	*Lap-1 2-2*			
		16	12		0.57	0.3 < P < 0.5
	1-2 x *2-2*	*Acp-1 1-2*	*Acp-1 2-2*			
		10	17		1.81	0.1 < P < 0.2

Table 4.30. Isozyme typings for segregating loci in *S. cereale*.

The exact probabilities obtained for tests of independence of these loci considered pariwise are given in Table 4.31.

	GPI (2)	GOT (3)	LAP (1)
GOT (3)	0.6714		
LAP (1)	0.5584	0.0088*	
ACP (1)	0.5129	0.7126	0.1241

Table 4.31. Exact probabilities of independence of 2 way classification of segregating loci in *S. cereale*.

There is one significant association : LAP/GOT. The two way classification of these segregations and the linkage estimate (3.4) are given in Table 4.32.

		Isozyme classification GOT (3)				Linkage estimate
		1-1	*1-2*	*2-2*		The upper limit for a
	1-2	5	7	0	12	recombination frequency
LAP(1)	*2-2*	0	6	4	10	detectable in this
		5	13	4	22	progeny is r = 0.2831

Table 4.32. Two way classification of isozymes in *S. cereale.*

The following linkage map for *S. cereale* may be drawn (Fig. 4.6).

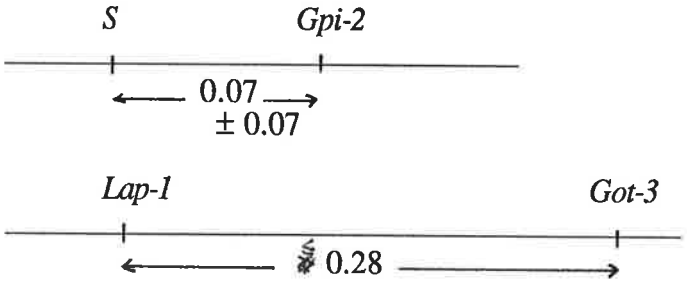


Fig. 4.6. Linkage map for *S. cereale* derived from this study.

Tang and Hart (1975) and Hart and Tuleen (1983) report that *Amp-1* (*Lap-1*) and *Got-1* and *Got-2* are syntenic in *S. cereale.* These workers used samples from 7 day old etiolated seedlings and found three zones each indicating a dimeric protein product. The material used here was approximately 4 weeks old and the three zones staining indicated 2 dimers separated by a monomer. Thus it is highly probable that the enzyme referred to here as *Got-3* is in fact the same as *Got-2* above.

4.4 Other species

4.4.1 *Hordeum bulbosum*

Seeds from the first six acquisitions listed in Table 3.5 yielded only homozygotes for GPI. Material from the last three acquisitions was only obtained in the last season of this study (1986) and included one variant for GPI.

4.4.2 *Dactylis aschersoniana*

Seeds from K2306 and K2366 were the only ones to germinate. Plants appeared to be highly polymorphic for GPI. Seed set was never very high and it was not until the last season of this study (1986) that a reasonably large backcross progeny revealed that the material under study was clearly tetraploid and presumably *D. glomerata.*

4.4.3 *Gaudinia fragilis*

A total of 4 plants was raised; two from each source. They included variants for GPI and a number of other isozyme systems tested.

5 Discussion

Results from the analysis of disturbed segregation ratios given here (4.2), reports from Cornish *et al.* (1980) and Fearon *et al.* (1983) and data in a paper by Figueiras *et al.* (1985) all present a consistent observation of linkage of one of the incompatibility loci, in the grasses, to *Gpi-2*. The results are summarized in Table 5.1.

Species	Recombination frequency	Standard error	Sample size
Lolium perenne (Cornish *et al.*, 1980)	0.1538	0.0252	
Lolium multiflorum (Fearon *et al.*, 1983)	0.2414	0.0795	
Phalaris coerulescens	0.1134	0.0312	207
Holcus lanatus	0.2987	0.0563	132
Alopecurus myosuroides	0.1852	0.0449	150
Festuca pratensis	0.1687	0.0411	83
*Secale cereale**	(1) 0.0714	0.0688	28
	(2) 0.1902		30
	(3) 0.1795	0.0615	78
	(4) 0.2895	0.0578	123

Table 5.1. Estimates of the recombination frequency between the *S* locus and *Gpi-2* locus.

*Estimates (1) + (3) are reported (4.1.5), (2) Appendix 4 and (4) is derived from data presented by Figueiras *et al.* (1985).

The value of this recombination frequency varies. Even for a given species (*S. cereale*) a range of values may be observed in material with different genetic backgrounds. This is consistent with the observation of Jones (1982). *L. perenne* and *L. multiflorum* produce fertile hybrids (Naylor, 1960). Further, *L. perenne* or *L. multiflorum* and *F.pratensis* hybridize naturally and artificially and although these hybrids are sterile (Jenkin, 1933; Wit, 1959; Reusch, 1959; Lewis *et al.*, 1973) there are indications of some homology of genetic material (Osborne *et al.*, 1977). Thus their similarity is to be expected. However, what is shown here is that there is conservation of this linkage association across five different tribes of the Poaceae.

Further evidence of conservation is found in the collation of information relating to one of the peroxidase loci. This isozyme has been given a range of symbols in different species influenced by the electrophoretic methods used for its detection. The results are summarized in Table 5.2. All these reports tend to suggest that *Per* is closer to the *S*-locus than is *Gpi-2*. This close linkage is found in both the Poaceae and the Solanaceae and so involves linkage to one of the two self incompatibility loci in the grasses and the single *S*-locus in the dicotyledons.

Species	Nature of Report	Reference
Holcus lanatus	Linkage of *Gpi-2* to *Per-1* $r_{GPI-PER} = 0.24 \pm 0.07$ (*Per-1* closer to *S*)	This thesis 4.3.2.
Alopecurus myosuroides	Linkage of *Gpi-2* to *Per-1* $r_{GPI-PER} = 0.29 \pm 0.10$	This thesis 4.3.3
Triticum aestivum	Deletion mapping of chromosome 1B showing linkage of *Gpi-1* to *Per-1*	Ainsworth, 1984
Secale cereale	Linkage of *Gpi-1* to *Prx-7* Linkage of *S* and *Prx-7* r_{S-PER} = 0-0.2%	Wricke and Wehling, 1985
Lolium multiflorum[1]	Disturbed segregation of *Per* apparently misinterpreted but leads to an estimate of $r_{S-PER} = 0.12 \pm 0.04$ (2.2)	Polans and Allard, 1985
Lycopersicon esculentum[2]	Linkage of *S* to *Prx-1* (Single locus gametophytic incompatibility system and member of the Solanaceae) r_{S-PER} = 9.9%	Tanskley and Loaiza-Figueroa, 1985
Nicotiana alata[3]	Linkage of S (single locus/Solanaceae) to *Per* For one locus r_{S-PER} = 3% and another r_{S-PER} = 34%	Labroche *et al.*, 1983

Table 5.2. Linkage associations involving peroxidase loci.

Footnotes to Table 5.2.

1. Polans and Allard (1985) report an observed segregation ratio for PER in *L. multiflorum* of 52:62:7 when the expected was 1:2:1. They argue that it must "result from different degrees of compatibility conferred by PER allozymes or loci closely linked to them, on the pollen and stigmas of the parental plants of our test crosses."

   They cited Pandey (1967) as the source of such a suggestion. However, Pandey was looking at stylar tissue in *Nicotiana alata* and these workers were studying leaf tissue. Not only is there marked tissue specificity for peroxidase isozymes but Bredemejer and Blaas (1980) demonstrated that there is no relationship between *S* genotypes and peroxidase zymograms of either style or pollen in *N. alata*.

   The disturbance presumably does result from different compatibility reactions conferred by a locus linked to *Per*, namely the *S* locus. Their data lead to an estimate of $r_{S\text{-}PER} = 0.12 \pm 0.04$.

2. Tanksley and Loaiza Figueroa (1985) in reporting linkage of *Prx* to *S* in *L. esculentum* claim to rule out the possibility that there is a second locus involved in the determination of self-incompatibility in tomato. They state:
   "If an additional locus did exist and were required for the self-incompatibility reaction, then even if *S* were completely linked to *Prx-1*, the *Prx-1* allele, in *cis* with the *S* allele being selected against, would still be transmitted 33% of the time since only one quarter of the gametes would possess both the linked *S* allele and the allele from the independent locus."

   If fact of course, one half of all gametes must possess an allele from any given locus and if the linked locus is the one against which selection is operating the *Prx-1* allele will be transmitted only if there is recombination between it and the *S*-locus, irrespective of the contribution from the other locus (Chapter 2).

3. Labroche *et al.* (1983) report linkage of peroxidase genes to the *S*-locus in *N. alata*. These workers claim that genetic analysis of peroxidase isozymes showed that each of the two cathodic bands was controlled by genes and separate loci. They denote these loci P_1 and P_{11}. At each locus there were two allelic forms; P_1^+ or P_{11}^+ for presence of activity (dominant) and P_1^- or P_{11}^- for absence of activity (recessive). P_1 was apparently closely linked to the *S* locus (3 cM) whereas P_{11} showed a recombination frequency of 34% with the *S* locus. Surprisingly, no individuals with absence of activity for both loci were found though 3000 were tested.

   A sample of 3000 should detect recombination events that occur with frequencies as low as 0.1%. Although Labroche *et al.* (1983) appear to have convincing evidence of null alleles at one of the two anodic peroxidase loci analysed this does not seem to be the case for the cathodic peroxidase variants. It is not possible to reanalyse their data suitably but a satisfactory explanation for the control of the cathodic variants seems to be by a single locus with two co-dominant genes. The fact that, despite testing 3000 plants, they were unable to find a single individual lacking activity at both postulated loci would seem to outweigh the conflicting recombination estimate of 34 cM from one cross.

To date there has been no published report of linkage to the second of the two loci determining self-incompatibility. Scarrot (1981) suggests that there is some evidence, in *L.*

multiflorum, that *Z* is linked to *Got-3*. She also claims that *S* and *Z* may be syntenic. However, Lewis *et al.* (1980) mapped *Gpi-2* to chromosome 6 and *Got-3* to chromosome 2 in *L. perenne* and so this later suggestion seems to be unlikely. The results of the *6Pgd-2* segregations in *H. lanatus* (4.3.2) showed disturbances in two crosses indicating possible linkage to *S* in one case and to *Z* in the other. Only the latter is thought to be correct. This disturbed segregation ratio along with that for *Mdh-2* and the significant association in the joint segregation of *6Pgd-2* and *Mdh-2* indicate that both *6Pgd-2* and *Mdh-2* are linked to *Z* and evidently are flanking.

A slightly different picture emerges if the information is collated as follows:

1. *Got-3* linked to *Z* in *L. multiflorum* (Scarrott, 1981).
2. *Mdh-2* and *Got-3* are linked in *S. cereale* (Figueiras *et al.*, 1985).
3. *Got-3* is located on the homoeologous chromosomes 3 in *S. cereale* and *H. vulgare* (Tang and Hart, 1975; Brown & Munday, 1982).
4. *Mdh-2*is reported as being on chromosome 3 in *H. vulgare* (Brown and Munday, 1982) *cf* chromosome 5 (Benito *et al.*, 1984).

Thus an appropriate assessment of all these data might be that *Got-3*, *Mdh-2* and *Z* are linked. This leads to a problem in interpreting the *6Pgd-2* segregations in *H. lanatus* but this gene is located on non-homoeologous chromosomes in wheat, rye and barley (7Bq in Miller, 1984; 2q Salinas & Benito, 1983; 4 Rao & Rao, 1980; 5 Brown & Munday, 1982) and so its position is probably not highly conserved.

The syntenic and linkage relationships for those species of the Poaceae, with n=7, for which information is available and which have been extended by the material presented in Chapter 4 are summarized in Table 5.3.

The conservation of linkage blocks is clear. The species of the Poaceae studied here are all self-incompatible whereas the ones for which data were previously available are chiefly self-compatible. However, evidence presented in 4.2.5 for *S. cereale* suggests that self-fertility may arise due to a single allelic difference at one of the self-incompatibility loci. The precise nature of this incompatibility breakdown has not been as thoroughly

Loci considered in this study	Information from previous studies							Information contributed by this study			
	T. aestivum[1]	*S. cereale*[2]	*H. vulgare*[3]	*A. intermedium*[13]	*L. perenne*	*L. multiflorum*	*Cynosurus cristatus*[7]	*P. coerulescens*	*H. lanatus*	*A. myosuroides*	*S. cereale*
Acph-1	4q	7p		L1							
-2			4		6[12]	?[15]					
Aco-1	6q										
Amp-1	6	6	6								?[11]
Dia-1								6			
*Gpi-1**	1p	1	5	L3	6[4]	6[5]		6	6	6	1
Got-1	6p	6	6[10]								
-2	6q	6	6[10]			?[15]					
-3	3q	3	3[10]	L2	2[4]	2[4]					?[11]
Mdh-1		2					?[7]				
-2	1q	1q, 3q	5,3	L2			?[7]		?[14]		
Per	1p	1p[6]		L5		6[5b]			6	6	
6Pgd-2	7	2q,4	5						?[14]		
S		1[6]			6[8]	6[5a,b]		6	6	1	
Z						2[9]			?[14]		

Table 5.3.1. Current linkage information for the loci studied in these investigations, including results from this study.

Numbers in the body of the table are chromosome numbers (for homoeologies see Table 5.3.2). p = short arm. q = long arm.
? indicates chromosome number not known.
Superscripts refer to footnotes. See below.

* *Gpi-1* (*T. aestivum, S. cereale, H. vulgare*) *Gpi-2* (all other species listed).

1. See Hart (1986); 2. Figueiras *et al.* (1985); 3. Brown (1987); 4. Lewis *et al.* (1980); 5a. Fearon *et al* (1983); 5b. after Polans and Allard (1985); 6. deduced from Wricke and Wehling (1985); 7. *Mdh-1* - *Mdh-2* linked : Ennos (1986); 8. Cornish *et al.* (1980); 9. Scarrott (1981); 10. Nielsen, pers. comm., 1986; 11. Linked (4.3.5); 12. Fearon, pers. comm. 1985; 13. Figueiras *et al.*, 1986; 14. Linked (4.3.2); 15. Linked Hayward *et al.* (1979).
For species other than *T. aestivum, S. cereale, H. vulgare, A. intermedium* and *L. perenne* the assignment of chromosome number is based on the assumption of homoeology attributable to the *S-GPI* linkage association.

	T. aestivum[1]	*S. cereale*[2]	*H. vulgare*[3]	*Agropyron*[5]
	1	1R	5	L3
	2	2R	2H	L5
	3	3R	3H	L2
Chromosome	4	4R	4H	L4
number	5	5R	7H[4]	*
	6	6R	6H	L7
	7	7R	1H[4]	L1

Table 5.3.2. Homoelogous chromosome relationships in the Poaceae.

1. Miller (1984);
2. Miller (1984);
3. Salinas *et al.*, 1985;
4. Brown, 1983;
5. Figueiras *et al.* (1986)

* Information not available.

investigated as were cases reported by Lundqvist (1958, 1960, 1968) but in these later studies breakdown was found to be little influenced by genes other than *S* and *Z*.

Much of this linkage information relates to genes linked to the incompatibility loci. Theoretical studies (Appendix 3) on the relationship between linkage disequilibrium and gametophytic self-incompatibility indicate that although incompatibility systems may delay the loss or fixation of linked genes they do not shelter lethals. Thus the linkage associations revealed here are not likely to be directly related to the proximity of the isozyme loci to the self-incompatibility loci. Indeed there appears to be a marked lack of conservation of the location of the *6Pgd-2* locus although it is apparently quite tightly linked to one of the self-incompatibility loci in *H. lanatus*.

Many workers argue that there is no structure on a scale larger than the gene family (i.e. a group of closely linked genes with related functions, together with sequences controlling them) that is developmentally relevant. Genes and gene families are tied together in chromosomes as a means of ensuring proper disjunction during cell division, but their large scale arrangement has no developmental relevance (Maynard Smith, 1982). Maynard Smith cites his own work with *Drosophila subobscura*, a species in which all natural populations are polymorphic for large paracentric inversions affecting all autosomal arms, none of which has any morphological effect.

This argument is supported by observations such as those on two species of muntjac. *Muntiacus muntjak* (the Indian muntjak) has a diploid chromosome complement of 2n=6 (female); 7 (male); yet it forms viable but sterile hybrids with the closely related *Muntiacus reevesi* (2n=46) (Wurster and Benirschke, 1970).

By way of contrast long term conservation of chromosome number and G-banding properties have been reported for a number of mammalian groups e.g. Marsupialia over more than 50 million years (Rofe and Hayman, 1985).

Clearly selective forces operate to restrict the sorts of chromosomal changes that may occur in the chromosome complement. In groups such as those listed above the structural changes that are detected are intra- rather than inter-chromosomal.

Thus to argue that conservation of linkage groups will always occur is not valid. However, there are sufficient situations, such as the species in the Poaceae with 2n=14 (x=7), where searching for such conserved units is rewarding.

Nadeau and Taylor (1984) contend that it is probably unnecessary to regard linkage conservation as evidence that certain segments are protected from chromosome rearrangement because of regulatory or functional interaction between the loci involved. They estimate that there have been 178 ± 39 chromosome rearrangments since the divergence of humans and mouse. Further, these arrangements are apparently random so that many chromosome segments are expected to be conserved regardless of the function of the loci within them. Thus conserved segments are relics of ancient linkage groups not yet disrupted by chromosome rearrangements. They say that one should look for particular segments protected from rearrangement.

More than thirty years ago, Dobzhansky developed the concept of co-adaptation to account for differences in properties of chromosomes that have different gene arrangement due to inversion. He noted that the suppression of recombination that is associated with these inversions would protect the group of genes within these inversions from disruption by recombination. He also postulated that inversions combining together favourable gene combinations would be favoured by selection. Subsequently, it has been shown, theoretically, that selection in combination with suppression of recombination due to linkage, inbreeding or restriction of population size can lead to a build-up of non random associations among alleles at different loci so that favourable groups of genes are selected as co-adapted units (reviewed in Lewontin, 1974). However, there is little evidence of the adaptive value (fitness) of particular gametic associations.

Epistatic selection can create non-random associations between alleles and, because of this, linkage disequilibrium has been used in the search for the effects of selection in natural populations (Nevo, 1978; Brown, 1979).

Linkage disequilibrium can also be generated by random drift in finite populations as is reflected in the variance of linkage disequilibrium (Hill, 1975) and data on linkage disequilibrium have been used to estimate population sizes (Hill, 1981).

There are a number of examples of conservation of linkage associations of groups of esterase loci in both animals (Webster, 1973; Baker, 1975) and plants (Kahler and Allard, 1970; Allard *et al.*, 1972; Weir and Cockerham, 1974; Nakagahra *et al.*, 1975; Brown *et al.*, 1977; Tanksley and Rick, 1980; Wehling and Schmidt-Stohn, 1985; Harry, 1986). In the first five of these examples the linkage associations described are from population data and they are found in conjunction with linkage disequilibrium.

In barley, one of the plant species included in these references, there are a total of 27 esterase isozymes at 10 loci (Hvid and Nielsen, 1977). The loci *Est-1,-2* and *-4* are tightly linked (Kahler and Allard, 1970), are located on chromosome 3 and assort independently of *Est-3* (Nielsen and Frydenberg, 1971). The loci *Est-2* and *-9*, *Est-5* and *-9* and *Est-3* and *-10* also assort independently (Hvid and Nielsen, 1977).

Tanksley and Rick (1980) in reporting tight linkage of *Est-1,-5,-6* and *-7* in *Lycopersicon esculentum* compare this with the situation found in barley (Kahler and Allard, 1970) suggesting that it would be remarkable if the nearly identical linkage patterns in barley and tomato had evolved independently. They argue further, that the absence of epistatic interactions in tomato supports the antiquity of the association and suggests that it predates the divergence of the monocots from the dicots.

As mentioned (1.5) Harry (1986) reports tight linkage of two esterase loci on the gymnosperm (*Calocedrus dicurrens* [Torr.] Florin). Thus it is possible that this association is more than 100 million years old.

Tandem duplication is the model generally put forward as the origin of these tightly linked complexes. However, the *Est-1,-2* and *-4* loci in barley which are very closely linked and might thus be postulated to have arisen by gene duplication have widely differing properties. These may be summarized as follows:

1. *Est-4* variants have higher activity than *Est-1*.
2. *Est-4* variants are dimeric whereas *Est-1* and *-2* are monomeric.
3. The inactivation temperatures of *Est-1*, *-2*and *-4* are 50, 60 and 70°C respectively.
4. *Est-2* variants reverse their order on agar compared with starch; *Est-1* does not. *Est-4* variants are not revealed on starch.

Thus it would appear that there is not a close relationship in terms of the origin of these loci but rather that the close association, which is as yet in linkage disequilibrium, has been brought about by selection due to some functional requirement. However, it is worth noting that the three linked esterase loci in *H. vulgare* are in chromosome 3. This is the chromosome in which it has been suggested that *Z* of the *S-Z* pair of loci would be located in *Hordeum*. The rate of approach to linkage equilibrium is affected by the presence of a self-incompatibility system and although *H. vulgare* is self-compatible this linkage disequilibrium might be in part a relic of the effect of the self-incompatibility system. Nevertheless, it seems that the observations of the apparently different origin of the esterase loci in barley and their conserved tight linkage relationships in both plants and animals may provide evidence of the sort necessary to refute the claim, mentioned above, of Nadeau and Taylor (1984) that segments are protected regardless of the function of the loci within them (Ohno (1973) presented a similar argument).

Whilst this set of genes presumably satisfies the concept of a gene family the genes themselves apparently differ from the classic examples such as the haemoglobins, immunoglobins and actins in that they are evidently not produced by gene duplication. They show evidence of selection in maintaining these complexes and thus support the notion that larger blocks of genes might be at particular selective advantages and would reach linkage equilibrium more rapidly.

Further information pertinent to an examination of the population genetic properties of tightly linked polymorphic loci should be provided by RFLP mapping. Fragments that hybridize indvidual probes indicate true homology and linkage disequilibrium patterns may indicate the nature of evolutionary events that have affected genetic regions. The cumulation of such data would thus build up information about more loosely linked genes and help indicate the magnitude of a region which may be a selective unit.

To date the comparative mapping data from plants appear to reveal a high degree of conservation of gene order. This is not always the case in animals. Chromosome banding studies of the Marsupialia (Rofe and Hayman, 1985) show not only evidence of conserved karyotype but also in the Phalangeroidea substantial karyotype repatterning.

All the grass species examined here have n=7. Accordingly, it would be most interesting to see whether in those species within the same genus, which have n=5 the same or different repatterning events have been involved.

6 Concluding Remarks

The importance of self-incompatibility, either as a nuisance or as a benefit to the plant breeder has been detailed in a number of reviews (Lewis, 1956; Duvick, 1966; de Nettancourt, 1972). The determination of *S*-genotypes can be a complex and difficult task and it is desirable to replace the procedures of test pollinations and progeny analyses by direct methods which allow the identification of *S*-alleles present in each individual plant considered. Establishing linkage relationships between the *S*-locus and marker loci which can be scored in seedlings is one method of achieving this. The number of *S*-alleles which can be detected in this way is limited and close linkage is required to avoid classification error. However, it appears that the locus of one of the structural genes determining peroxidase isozymes is closely linked to the *S*-locus in the Solanaceae and this locus as well as that for glucose phosphoisomerase which is also linked to *S* (of the *S*-*Z* pair) in the Poaceae should be valuable in such investigations. Theoretical considerations indicate that the *S* and *Z* loci are at most loosely linked in all the species studied and experimental work suggests that the locus determining malate dehydrogenase is relatively tightly linked to *Z*, providing a marker for the second of the *S*-*Z* pair of incompatibility loci in the Poaceae.

Studies using self-incompatible grass species greatly increase the range of plants from which linkage data may be obtained. Once the map locations of isozyme genes are known they can be used as biochemical markers to map other genes - isozyme, morphological, physiological, phytopathological etc. They may also be used in studying the inheritance of continuous or metric characters.

The evidence of conservation of many linkage relationships across the tribes in the Poaceae suggests that knowledge of linkage relationships derived from one species may facilitate the use of isozyme analysis as a tool for detecting the introgression of genes from wild germ plasm.

Finally the conservation of specific groups of linked genes found to be in linkage disequilibrium in populations lends support to the suggestion that these linkage associations are selectively rather than randomly determined and, though self incompatibility systems may retard the approach to linkage equilibrium they do not shelter lethals.

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8 Appendices

Appendix 1

1.1 Nutrient solution for germinating seeds

1 M	$Ca(NO_3)_2$	1.0 ml/litre
1 M	KNO_3	1.0 ml/litre
1 M	$MgSO_4$	0.5 ml/litre
1 M	NaH_2PO_4	0.5 ml/litre

Micro nutrients (as below) 0.2 ml/litre

H_3BO_4	2.5 gm/litre
$MnCl_2.H_2O$	1.5 gm/litre
$ZnCl_2$	0.1 gm/litre
$CaCl_2.2H_2O$	0.05 gm/litre
$Mo\ O_3$	0.05 gm/litre

1.2 Agar for pollination tests

2% agar

10% sucrose

100 ppm (20 mg/200 ml) boric acid

1.3 Cotton blue stain

Methyl blue	0.1 gm
Phenol crystals	20 gm
Lactic acid	40 ml
Glycerine	20 ml
H_2O	20 ml

1.4 Aniline blue

Aniline blue	0.6 gm
K_3PO_4	6.0 gm
H_2O	300 ml

1.5 Hepes Lysis buffer

20 mM Hepes 100 ml (477mg Hepes in 100ml H_2O; pH to 7.6 with 1M NaOH)

ß-mercaptoethanol 100 µl

Triton X 100 100 µl

NADP 10 mg

NAD 10 mg

Stored at -80°C in 5 ml aliquots.

Add additional 5% ß-mercaptoethanol immediately before using.

1.6 Abbreviations

Tris Tris (hydroxymethyl) amino methane

NAD ß-Nicotinamide-adenine dinucleotide

NADH ß-Nicotinamide-adenine dinucleotide, reduced

NADP ß-Nicotinamide-adenine dinucleotide phosphate

MTT Methyl thiazolyl tetrazolium

PMS Phenazine methosulphate

EDTA Ethylenediaminetetraacetic acid

Hepes N-2 hydroxyethylpiperazine-N'-2 ethane sulphonic acid

1.7 Gel and tray buffer systems

		pH
1.*Gel	1 part lithium borate (1.2 gm LiOH, 11.89 gm H_3BO_3, 1 litre H_2O)	
	and 9 parts Tris citrate (6.2 gm Tris, 1.6 gm citric acid, 1 litre H_2O)	8.3
Tray	The lithium borate buffer only	8.3
2.†Gel	14 mM Tris, 4 mM citric acid	7.4
Tray	0.3 M H_3BO_3, 0.1 M NaOH	8.7
3.†Gel	12.5 mM Tris, 4 mM citric acid	7.0
Tray	37.5 mM Tris, 12.5 mM citric acid	7.0

* Fearon, C.H., pers. comm., 1982

† Østergaard, H. *et al.*, 1985.

1.8 Staining systems

1. *Alcohol dehydrogenase* (ADH) EC 1.1.1.1 System 1 Reference 2

0.1 M Tris/HCl pH 8.0	100 ml
Analar ethanol	3 ml
NAD	30 mg
MTT	25 mg
PMS	2 mg

Incubate in dark at 37°C

2. *Peroxidase* (PER) E.C. 1.1.1.17 System 2 Slots in centre of gel. Ref. 2.

H_2O	100 ml
Benzidine	200 mg dissolved in 1 ml acetone.
Glacial acetic acid	0.5 ml

Stain cathodal region.

Incubate at room temperature for about 15 mins then add

H_2O_2	0.2 ml

3. *Shikimate dehydrogenase* (SKD) E.C. 1.1.1.25 System 2 Ref. 4

0.1 M Tris/HCl pH 8.0	100 ml
1 M $MgCl_2$	0.2 ml
Shikimic acid	100 mg
MTT	15 mg
PMS	4 mg
NADP	10 mg

Incubate in dark at 27-30°C.

4. *Malate dehydrogenase* (MDH) E.C. 1.1.1.37 System 3. Ref. 5.

0.1 M Tris/HCl pH 7.5	90 ml
$Na_2CO_3H_2O$ 18.15 gm in 50 mls H_2O	
L-malic acid 13.4 gm make up to 100 ml	10 ml
NAD	50 mg
MTT	30 mg
PMS	5 mg

Incubate in dark at 37°C.

5. *6-Phosphogluconate dehydrogenase* (6PGD) E.C.1.1.1.44 System 3. Ref. 1.

0.1 M Tris/HCl pH 7.1.	100 ml
Na_3-6-phosphogluconic acid	20 mg
1 M $MgCl_2$	0.5 ml
NADP	5 mg
MTT	15 mg
PMS	2 mg

Incubate in dark at 37°C.

6. *Glucose-6-phosphate dehydrogenase* (G6PD) E.C. 1.1.1.49 System 3. Ref. 2.

0.1 M Tris/HCl pH 8.0	100 ml
Monosodium glucose-6-phosphate	100 mg
MTT	8 mg
PMS	2 mg
NADP	10 mg
1 M $MgCl_2$	1 ml

Incubate in dark at 37°C.

7. *Diaphorase* (DIA) E.C. 1.6.2.2. System 1. Ref. 2

H_2O	90 ml
1 M Phosphate buffer pH 7.0	10 ml
NADH	20 mg
0.1% phenolindo-2,6 dichlorophenol	7 ml
MTT	15 mg

Incubate in dark at 37°C.

8. *Catalase* (CAT) E.C. 1.11.1.6. System 2 Ref. 2.

(1) 0.1 ml H_2O_2 (100 vol) in 100 ml H_2O

Incubate for 15 mins

(2) Pour off H_2O_2. Rinse gel in H_2O and immerse in freshly prepared 50/50 mixture of 2% potassium ferricyanide

2% ferric chloride

9. *Superoxide dismutase* (30) E.C. 1.13.1.1. System 1. Ref. 2

Smithies buffer 0.9 M → 1/9 100 ml

MTT	27 mg
PMS	5 mg

Incubate at 37°C. View under U.V. (3660 + 2537° A).

10. *Ornithine carbomyltransferose* (OCT) E.C. 2.1.3.3. System 2. Ref. 2.

0.5 Tris malate pH 7.2	100 ml
Carbomoyl phosphate	50 mg
L-ornithine hydrochloride	300 mg
2% lead nitrate	5 ml

Incubate gel in this solution for about 10 minutes. Rinse, immerse gel in 1% ammonium sulphide solution. Wash again with H_2O.

N.B. The second part of this procedure must be carried out in a fume hood.

11. *Glutamate oxaloacetate transaminase* (GOT) E.C. 2.6.1.1.
System 1. (Ref. 1. modified).
Also known as *Aspartate amino transferase* (AAT).

0.1 M Tris HCl pH 7.1	50 ml
0.4 M Na_2HPO_4 pH 7.4	50 ml
L-aspartic acid	130 mg
α ketoglutaric acid	50 mg
ETDA-Na_2	7 mg
Pyrodoxyl 5' phosphate	3 mg
Fast blue BB salt	150 mg

keep separate

Incubate in the dark at 37°C.

12. *Phosphoglucomutase* (PGM) E.C. 2.7.5.1. System 3. Ref. 1.

0.1 M Tris HCl pH 7.1	100 ml
1 M $MgCl_2$	0.5 ml
α-d-glucose-1-phosphate	25 mg
NADP	5 mg
MTT	15 mg
PMS	1.2 mg
Glucose-6-phosphate dehydrogenase	20 units

Incubate in the dark at 37°C.

13. *Esterase* (EST) E.C. 3.1.1.1 System 2. Ref. 2.

0.2 M Na acetate pH 5.5	100 ml
α-naphthyl acetate	40 mg
ß-naphthyl acetate	20 mg
Fast blue RR	150 mg

dissolve in 1 ml acetone

14. *Acid phosphatase* (ACP) E.C. 3.1.3.2. System 2. Ref. 3.

0.2 M Na acetate pH 5.0	100 ml
Na-α-naphthyl-acid phosphate	50 mg
1 M $MgCl_2$	0.5 ml
Fast garnet GBC salt	75 mg

Presoak gel in 0.4 M Na acetate (pH 5.0) in fridge for 30 mintues before staining.

Incubate at 37°C in dark.

15. *Leucine amino peptidase* (LAP). E.C. 3.4.11. System 2. Ref. 3.

also known as *Amino peptidase* (AMP)

0.1 M phosphate buffer pH 7.0	100 ml
L-leucine-ß-naphthyl amide	20 mg dissolved in 0.5 ml absolute methanol
Fast Black K salt	25 mg

Incubate at 37°C in dark.

16. *Aldolase* (ALD) E.C. 4.1.2.7 System 1. Ref. 2.

0.05 Tris HCl pH 7.4	100 ml
Fructose 1-6 diphosphoric acid	550 mg
NAD	50 mg
MTT	30 mg
PMS	2 mg

Glyceraldehyde-3-phosphate dehydrogenase 100 units.

Incubate at 37°C in dark.

17. *Aconitase* (ACO) E.C. 4.2.1.3. System 1. Ref. 2 (modified).

0.4 M Tris HCl pH 8.0	100 ml
1 M $MgCl_2$	7 ml
cis Aconitic acid	100 mg
NADP	20 mg
MTT	20 mg
PMS	6 mg
Isocitrate dehydrogenase	70 µl (20 mg/2 ml)

Incubate in dark at 37°C.

18. *Glucose phosphoisomerase* (GPI) E.C. 5.3.1.9. System 1. Ref. 3.

also known as *Phosphoglucose isomerase* (PGI)

0.2 M Tris HCl pH 7.4	100 ml
Fructose-6-phosphate	15 mg
NADP	7 mg
MTT	15 mg
PMS	5 mg
1 M $MgCl_2$	1.0 ml
Glucose 6 phosphate dehydrogenase	20 units

Assay at 37°C in dark.

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Appendix 4

THE INCOMPATIBILITY LOCI AS INDICATORS OF CONSERVED LINKAGE GROUPS IN THE POACEAE

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Gametophytic self-incompatibility controlled by genes at two loci, first described by Lundqvist (1954, 1956) in *Secale cereale* and Hayman (1956) in *Phalaris coerulescens* has subsequently been found in all genetically analysed self-incompatible species belonging to the Poaceae (see Leach, 1983 for references). This sytem is unique to the grasses and is presumed to have arisen only once. The two loci (*S* and *Z*) assort independently and each have a large number of different alleles, e.g. *Festuca pratensis* *S*=14 and *Z*=13 (Lundqvist, 1969). Identity between pollen and pistil for the alleles at each locus leads to incompatibility. Thus an $S_{1.2}Z_{1.2}$ style rejects all pollen of S_1Z_1, S_1Z_2, S_2Z_1, S_2Z_2 genotypes; specifically that produced by anthers of a plant of that genotype but will accept pollen differing by at least one S or Z allele.

e.g. $S_{1.2}Z_{1.2}$ x $S_{1.3}Z_{1.2}$

$\downarrow$

S_1Z_1 S_1Z_2 S_3Z_1 S_3Z_2

i.e. 50% of pollen in such crosses would be compatible. Genotype combinations in which 75% and 100% of pollen is compatible are possible. A completely self-incompatible plant sets no seed with its own pollen and so crosses between plants of different incompatibility genotypes may be made by simply bagging heads together. The compatibility relationship betwen plants can be established from pollination tests.

As a result of the potential for differential transmission of different types of pollen, it follows that genes which are linked to the self-incompatibility genes may have a disturbed rate of transmission. Consider a pair of plants which are reciprocally 50% incompatible and

each of GPI-2 (glucose phosphoisomerase) type 1-3 with *Gpi-2* linked to the *S* locus. These could have genotypes

$$\text{female } \frac{S_2P_1}{S_3P_3} Z_{1.3} \quad \text{x} \quad \text{male } \frac{S_2P_1}{S_1P_3} Z_{1.3}$$

(where P_1 is used to represent *Gpi-2-1* etc.) and when crossed they would give the following gametes:

non-recombinant	recombinant	non-recombinant	recombinant
$S_2P_1Z_1$	$S_2P_3Z_1$	(circled) $S_2P_1Z_1$	(circled) $S_2P_3Z_1$
$S_3P_3Z_1$	$S_3P_1Z_1$	$S_1P_3Z_1$	$S_1P_1Z_1$*
$S_2P_1Z_3$	$S_2P_3Z_3$	(circled) $S_2P_1Z_3$	(circled) $S_2P_3Z_3$
$S_3P_3Z_3$	$S_3P_1Z_3$	$S_1P_3Z_3$	$S_1P_1Z_3$*
freq. of each $\frac{1-r}{4}$	$\frac{r}{4}$	$\frac{1-r}{4}$	$\frac{r}{4}$

where r = recombination frequency between the *S* locus and *Gpi-2* (*P*) locus.

All pollen types S_2Z_1 and S_2Z_3 (circled) are incompatible and so *Gpi-2-1* is transmitted only in recombinant gametes $S_1P_1Z_1$ and $S_1P_1Z_3$ (*). The female gametes carry *Gpi-2-1* and *Gpi-2-3* (indicated as P_1 and P_3) equally frequently leading to an expected proportion of r/2 GPI type 1-1 in the progeny of this cross. GPI types 1-3 and 3-3 have expectations of 1/2 and (1-r)/2 respectively. Other sorts of crosses may also lead to disturbed segregations but this cross is one from which the recombination fraction may be calculated using maximum likelihood estimation. Loci not linked to the incompatibility loci will not give disturbed segregation ratios.

The grasses are classified into 23 tribes, have a basic chromosome number of n=5 or n=7 and fossil records of grass pollen are found in the Paleocene (65 million years) (Muller, 1981). The chromosomes of species with a basic number of 7 are generally metacentric and form on average one chiasma per arm at metaphase. Very few plants are well mapped genetically; and gene mapping techniques are restricted since it is not possible to G band

plant chromosomes in the same way as animal chromosomes or to set up somatic cell lines. However, the behaviour of the incompatibility system means that the linkage groups in which the incompatibility genes are located may be readily identified.

Linkage of *PGI-2* (*Gpi-2*) (E.5.1.3.9) to one of the loci determining gametophytic self-incompatiblity has been reported in *Lolium perenne* (Cornish *et al.*, 1980) and in the closely related *Lolium multiflorum* (Fearon *et al.*, 1983). These observations suggest the question "Is there evidence for the conservation of this linkage in other grasses with the same incompatibility system?".

Five species of grasses were chosen for study (Table 1). The two locus incompatibility system has been demonstrated in three of these, *Secale cereale* (*loc. cit.*), *Phalaris coerulescens* (*loc. cit.*) and *Festuca pratensis* (Lundqvist, 1955, 1961). *Holcus lanatus* is highly self-incompatible, shows gametophytic determination of incompatibility and differences in the compatibility status between reciprocal crosses (Weimark, 1968). *Alopecurus myosuroides* is highly self-incompatible and shows gametophytic determination of incompatibility (Leach, unpublished). Both of these species are likely to have the same incompatibility system as if found in all other grasses. All species chosen were diploid with n=7 (Darlington *et al.*, 1955) and each comes from taxonomically different tribes well separated on an evolutionary time scale (Stebbins, 1971).

With the exception of *S. cereale* all the species are perennials and so the procedure generally adopted to acquire plants of suitable incompatibility relationships has been to backcross progeny plants to parents. Where the original acquisitions were known to be sibs or half sibs pollination tests were carried out to establish pairs of plants suitable for crossing. *S. cereale* is an annual and so only sib crosses are possible. Many of the introductions of this species tested exhibited a degree of self-compatibility and so an independent marker had to be used as an indicator of crossing. Table 1 shows the results of the linkage analyses carried out.

The estimate of the recombination frequency from *H. lanatus* is significantly greater than that for *L. perenne, P. coerulescens* and *S. cereale*. No other pair of these values is significantly different. These estimates are based upon the disturbance in the segregation

ratio. Viability effects for GPI segregation ratios have been found in crosses of similar compatibility status. Thus the disturbed segregations observed are the result of linkage to the incompatibility factors and not due to viability effects. Whilst it is not possible to prove that it is the same self-incompatibility locus linked to *Gpi*, the close linkage values suggest that a unit has been conserved and that the same loci are involved in each species.

Both *Alopecurus myosuroides* and *Holcus lanatus* show the same pattern of disturbed segregation and a similar linkage value. The observations strongly suggest that they also possess the two locus system of incompatibility.

These data should be of interest not only to workers on evolution, but also to plant breeders as it might be possible to extrapolate from the results of linkage studies in one grass species to possible linkage relationships in another. Such limited linkage data as are available certainly support this view (Miller, 1984; Brown, 1983).

A theoretical analysis of the effect, in terms of linkage disequilibrium (Leach *et al.*, 1986), that a single locus gametophytic self-incompatibility system has on genes linked to it reveals that the enforced heterozgosity of the system does not shelter lethal mutations. Thus it is reasonable to assume that genes of economic importance closely linked to the incompatibility loci are neither protected nor less readily available for selection. However, it was found that where an incompatibility system is operating selection intensities (for removing deleterious genes) of less than unity are more effective at lowering gene frequency than is complete selection.

Acknowledgement

We acknowledge financial support from the Australian Research Grants Scheme. Mr. Clive Chesson gave valuable technical advice.

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Species	Recombination frequency	Standard error	Sample size
L. perenne (Cornish *et al.*, 1980)	0.1538	0.0252	
L. multiflorum (Fearon *et al.*, 1983)	0.2414	0.0795	
A. myosuroides	0.1852	0.0449	150
P. coerulescens	0.1134	0.0312	207
F. pratensis	0.2381	0.0933	42
H. lanatus	0.2987	0.0563	132
*S. cereale**	1) 0.0714 2) 0.1902	0.0688	28 30

Table 1. Estimates of the recombination frequency between the *S* locus and *Gpi-2* locus.

*Estimate (1) is from a single head from a full sib cross and (2) is an upper limit based on observing no recombinant in a collection of related full sib progenies where these plants were from *bona fide* crosses.

SUMMARY

Linkage between an incompatibility locus and the locus of the gene encoding the enzyme glucose phosphoisomerase has been demonstrated for the grasses *Alopecurus myosuroides*, *Phalaris coerulescens*, *Festuca pratensis*, *Holcus lanatus* and *Secale cereale* suggesting the conservation of a common chromosomal element in the evolution of the Poaceae.

Appendix 5

STABILITY OF SELF-INCOMPATIBILITY SYSTEMS

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Abstract

Multi-locus self-incompatibility systems offer few obvious adaptive advantages to the species possessing them. However, the gametophytic system's independent gene action allows the separate genes in a two gene system to behave as if they were individually not involved in a systematic disruption of panmixia. Under such circumstances, fixation of one of the two genes is readily obtained if an allele possesses a selective advantage. The resulting single gene system (the classic *Nicotiana* system) is then resistant to disruption, except by genes which allow selfing, which rapidly reach fixation.

Key Words: Gametophytic self-incompatibility· Selfing· Linkage disequilibrium·

1. Introduction.

It is well known (see e.g. Mayo 1966 for discussion) that since the probability of extinction of a gametophytically determined self-incompatibility system is non-zero, long term stability of such systems is problematic. However, they are one of the most widespread outbreeding mechanisms in the Angiosperms, so that the quasi-stability which they exhibit is certainly substantial. Thus, discussion of this quasi-stability is of some interest.

Gametophytic self-incompatibility systems are characterized by large numbers of allelles, and it has long been established that a high mutation rate is necessary to maintain the numbers seen in relatively small populations. This has proven very difficult to explain, since mutations to novel specificities have not been observed, but an explanation may come about if the self-incompatibility mechanism itself is understood, a prospect now in sight (Anderson *et al.* 1986). Maintenance of variability is less dependent on a high mutation rate in tetraploid or multi-locus

systems (Mayo 1971), and may be one of the reasons for the persistence of such systems (Mayo 1978).

Two other ways in which stability may be investigated are through the population dynamics of more than one gene locus, either genes linked to a self-incompatibility locus, or the dynamics of the multi-locus systems themselves; and through the process of attainment of self-fertility through the fixation of genes which override the effects of the incompatibility system.

2. The behaviour of pairs of gene loci.

The main problem of investigating the population dynamics of self-incompatibility systems is that data are few; in particular little is known of their linkage relationships. However, this is changing (Leach and Hayman 1987). Thus, the results of simulation, while interesting, require much more experimental validation.

What has been demonstrated for a locus linked to a single gene self-incompatibility locus is that the linkage disequilibrium is very different from that between any two autosomal genes not associated with a breeding system (Leach, Mayo & Morris 1987). Thus, linkage disequilibrium can either steadily decline to zero, oscillate as decline proceeds, or otherwise depart from a steady decline. Furthermore, linkage equilibrium may be established before genotypic frequencies reach their panmictic expectations.

If one also considers selective differences at the locus linked to the self-incompatibility gene, similar phenomena occur: for example, linkage equilibrium may be established while selection is still bringing about a decline in the frequency of a deleterious gene. Overall, although a gametophytic self-incompatibility system will delay the loss of a deleterious gene or the fixation of an advantageous gene, it will not allow the sheltering of lethals.

When one considers the population genetics of multi-locus systems, the data are even more scanty. Table I shows some of the very limited information on frequencies of numbers of allelles. By the method of Bateman (1947) (see also

Fisher (1947)), estimates of the numbers of alleles in the population are 34.1 for the S and 35.3 for the Z locus. The allelic frequencies at the two loci are homogeneous (p=0.39 by Fisher's " exact" test). They also show the same type of distribution, i.e. very long-tailed, that is seen for the single locus system (see e.g. Mayo 1966) and for simulated populations of the two-locus system (Table 2). (20 alleles have been simulated at each locus as a likely lower limit for the real number, population size in Fearon's and Lawrence's case being unknown.) Fearon's and Lawrence's data do not, however, allow assessment of departures from Hardy-Weinberg equilibrium at either locus, nor allow assessment of gametic association between the S and Z loci. In simulated populations of sizes between 100 and 500, whether with small or large numbers of allelles, we find that gene frequencies change very slowly from generation to generation, that there is Hardy-Weinberg equilibrium at each locus, and that there is no correlation between the numbers of homozygotes at the two loci, either in the same generation or in alternating generations. Thus, in these small populations, there is little evidence of the genotypic association expected in infinite populations (Weber, Wricke and Trang, 1982), apart from the absence of dual homozygotes. This may relate to the fact that the true, long-tailed allele frequency distribution has not been modelled in the deterministic analyses of Weber *et al.* (*loc. cit.*) and Charlesworth (1979).

3. Attainment of self-fertility

Fisher (1941) was probably the first to point out that a gene which allowed self-fertilization in a self-incompatible plant would rapidly reach fixation, unless it was at a severe disadvantage through diminished viability in its carriers. Such genes occur in heteromorphic self-incompatibility systems, and their rate of increase has long been the subject of controversy (see Bodmer 1984 and Piper, Charlesworth and Charlesworth 1985 for references). There is good evidence that in species showing both facultative self-pollination through cleistogamy and facultative cross-

pollination through chasmogamy, plants arising from the latter have much higher viability (Mitchell-Olds and Waller 1985).

Hence, species in which there is almost complete inbreeding or almost complete outbreeding are expected to be much more frequent than species with intermediate frequencies of the two types of fertilization, and this is usually the case (Lande and Schemske 1985, Schemske and Lande 1985, Waller 1986). There are examples of such breeding systems, however, especially in forest trees. For example, Brown, Matheson and Eldridge (1975) found 76% outcrossing in *Eucalyptus obliqua*, and Cheliak, Dancik, Morgan, Yeh and Strobeck (1985) found 88% outcrossing in *Pinus banksiana*. In this latter case, there was a significant decline in the outcrossing rate over a four year period of observation. In neither species was the outbreeding mechanism fully elucidated, so that the high frequency of self-pollination cannot be attributed to the breakdown of a well defined self-incompatibility system. One of us (C.R.L.) has, however, identified apparent genetical breakdown in the two locus gametophytic self-incompatibility system of rye (*Secale cereale*). Lundqvist (1958) reported incompatibility breakdown caused by mutants with lost pollen specificity. This was little influenced by genes other than the *S* and *Z* incompatibility genes (Lundquist 1960, 1968). In the new work cited here, self-fertility appears to arise as a consequence either of a simple mutation at the S locus or at a closely linked locus. (Disturbed segregation ratios for an isozyme locus known to be linked to the *S*-locus were observed at the linked locus in some cases of selfing.)

It is of considerable interest to determine what factors influence the spread of genes which allow selfing or possibly incompatible pollination but not selfing, especially as there is evidence that mixed pollination can allow self-fertilization when pure self-pollination is ineffective (Visser and Marcucci 1984).

Accordingly, we have simulated populations of annual plants having the single locus gametophytic self-incompatibility system (Mayo 1966). We have incorporated the possibility of pollination of a given plant by up to four other plants

as well as itself, and have made the probability of pollination by a given plant decline linearly with distance from the other plant. We have also made fertilization dependent to a modest extent on the proportion of compatible pollen; this makes little difference to the results.

We have then examined the approach to loss or fixation of an unlinked gene where a newly occurring mutant allows incompatible pollination either including selfing (Table 3) or excluding selfing (Table 4). This latter case corresponds either to a long established incompatibility system in which selfing is invariably lethal through homozygous recessives or to a system in which selfing is qualitatively different from incompatible crossing in a way not related to self-incompatibility. This is not realistic, given that selfing is usually possible when the incompatibility barrier can be breached, but represents the limiting case of inbreeding depression.

In Tables 3 and 4, we find that such genes are likely to be lost rapidly unless selfing is possible, i.e., selfing (as Fisher and others have noted) is a greater reproductive advantage than almost anything else. We also note that such genes are more likely to be fixed if the proportion of incompatible pollinations to be expected under random mating is very large. That is, the more allelles there are in such a system, the more protection it has against breakdown of the system. This is to be expected *a priori* , but nonetheless may be part of the explanation of the very large numbers of allelles usually found in such systems. Finally, a result consistently obtained but not shown in the tables, is that genes which allow self-pollination can exist for a hundred or more generations once their frequency rises above a few percent, and in such cases they are almost invariably fixed.

Acknowledgement: We thank Dr. M.J. Lawrence for making available unpublished data.

Table 1. Numbers of genes at the two self-incompatibility loci in a population of *Lolium perenne* (Fearon and Lawrence, 1986).

No. of occurrences		1	2	3	4-5	6	7-9	10-12	No.plants sampled	
No. of alleles	S	12	1	2	0	1	0		1	38
	Z	12	2	0	1	0	2		0	39

Table 2. Allelic frequency distribution in the two-locus system, with 20 alleles at each locus. (Simulation over at least 500 generations.)

	0-	0.025-	0.05-	0.075-	0.1-	0.125-	0.15
Frequency class	0.025	0.05	0.025	0.1	0.125	0.15	0.175
N = 100	0.375	0.225	0.125	0.150	0.075	0.025	0.025
N = 500	0.425	0.088	0.187	0.125	0.112	0.05	0.012

Table 3. Fate of a new mutant which allows incompatible pollination. Selfing is possible.

Population size	Initial No.of s.i.genes	Fixation No. trials	Fixation Time	Fixation No.s.i. genes remaining	Loss No. trials	Loss Time	Loss No.s.i. genes remaining
100	3	11	92.64± 5.27	2.64±0.15	6	3.33±6.17	3.0±0.0
	4	6	101.33±10.18	2.67±0.42	7	4.43±1.25	4.0±0.0
	6	2	82.00±11.00	3.5 ±1.5	10	10.30±4.27	5.9±0.1
500	3	4	156.50±11.75	3.0 ±0.0	8	5.50±1.39	3.0±0.0
	4	4	202.00±12.86	3.75±0.25	11	3.45±0.76	4.0±0.0
	6	5	236.40±7.88	4.20±0.58	7	10.00±5.05	6.0±0.0

Table 4. Fate of a new mutant which allows incompatible pollination. Selfing is lethal.

Population size	Initial No.of s.i.genes	Fixation No. trials	Fixation Time	Fixation No.s.i. genes remaining	Loss No. Trials	Loss Time	Loss No.s.i. genes remaining
100	3	4	121.25±6.49	2.75±0.25	19	6.00±1.58	3.0±0.0
	4	2	218.0 ±0.00	3.0 ±0.0	10	4.30±1.09	3.0±0.0
	6	1	149.0	4.0	11	4.19±1.58	6.0±0.0
500	3	6	270.0 ±52.93	2.83±0.17	6	4.83±1.66	3.0±0.0
	4	3	546.67±233.68	3.67±0.33	12	4.91±1.96	4.0±0.0
	6	1	369.0	4.0	11	20.19±8.45	6.0±0.0

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Appendix 6

Titles of other articles published on work carried out during the period of candidature.

1. Mayo, O. and Leach, C.R. (1985) Models of parent-offspring conflict in small populations. Biomet. J., 27: 483-489.

My part in this work was deriving the theoretical expressions which these models lead to.

Dr. Mayo was the major contributor to this paper.

2. Bennett, J.H., Leach, Carolyn, R. and Goodwins, J.R. (19860 The inheritance of style length in *Oxalis rosea.* Heredity 56: 393-396.

I was responsible for collating and analysing all the data, deriving the model and relating it to previous work in the same species, as well as clarification of much confusing material previously published. I also prepared the first draft of this paper. Professor Bennett assisted in producing the final draft. Mr. Goodwins was responsible for plant management during the course of the experimental work which was carried out some years earlier.