THE DEVELOPMENT AND UTILIZATION OF GENETIC MARKERS FOR BARLEY

Kenneth James Chalmers

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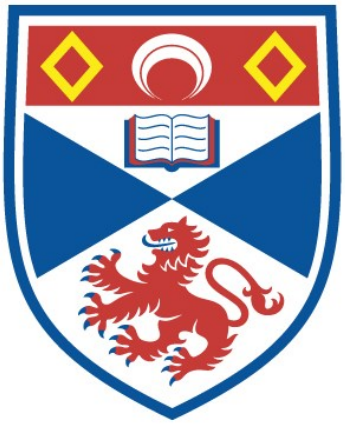

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THE DEVELOPMENT AND UTILIZATION OF GENETIC MARKERS IN BARLEY

Kenneth James Chalmers

Presented for Ph.D.

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University of St. Andrews

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ABSTRACT

The development of new and novel polymorphic assay methods represents one of the most significant developments in plant and animal biology. The exploitation of these genetic markers is of relevance to both applied and fundamental research. Recombinant DNA technology provides the opportunity to develop phenotypically neutral genetic markers in any organism from which DNA can be extracted. The research described in this thesis has focused on the development, evaluation and exploitation of genetic markers in barley.

Both protein and DNA based molecular markers were evaluated as a means of detecting polymorphism in *H. vulgare* and *H. spontaneum*. New methods for detecting polymorphisms based on the Polymerase Chain Reaction (PCR) were assessed and successfully applied to barley. The segregation of alleles at morphological, isozyme and RFLP loci were monitored in doubled haploid (DH) populations of barley. In order to detect molecular variability, both clones of known function and anonymous clones were employed. Clones homologous to the hordein gene complex on chromosome 1H were used in conjunction with DHs to intra-chromosomally map various members of this multi-gene family. Allelic variation at the genetic loci segregating in the DH population was evaluated in relation to the expression of quantitative traits. This study established that several isozymes and RFLP loci were

significantly associated with quantitative trait loci (QTL) of agronomic importance.

Grain isozyme and ribosomal DNA (rDNA) variability was examined in *Hordeum spontaneum* populations sampled from 27 geographical sites in Israel. Considerable phenotypic variability was observed and isozyme and rDNA variants were identified that were not present in the *H. vulgare* gene pool. Furthermore, the variability detected was quantified and correlated with a range of ecogeographical factors. The distribution of grain isozyme and rDNA phenotypes was non-random with particular phenotypes being restricted to specific geographical areas of Israel. Information on the spatial distribution of diversity in *H. spontaneum* is a pre-requisite for the optimization of sampling and germplasm collecting strategies.

Opportunities and limitations to the exploitation of genetic markers in barley improvement are considered.

Declaration

I Kenneth James Chalmers hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree of professional qualification.

Signed Date 10/9/91

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No.12 on 1st October 1987 and as a candidate for the degree of Ph.D. on 1st October 1988.

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CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Requirements appropriate to the degree of Ph.D.

R.J. Abbott,

September 1991.

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ABBREVIATIONS

In addition to the abbreviations listed in the 'Policy of the Journal and Instructions to Authors' Biochemical Journal (1978) **169**:1-27, the following are used.

cDNA	complimentary DNA
cpDNA	chloroplast DNA
CTAB	hexadecyltrimethyl-ammonium bromide
DDT	dithiothreitol
DH	doubled Haploid
DMSO	dimethylsulphoxide
dNTPs	deoxyribonucleotide triphoshates
IEF	isoelectic Focusing
IGS	intergenic spacer region
IPTG	isopropylthiogalactoside
MES	4-morpholineethan-sulphonic acid
DUS	distinctness, uniformity and stability
MOPS	morpholinopropanesulpfonic acid
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue)
CIP	calf intestinal phosphatase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide Gel Electrophoresis
PCR	polymerase Chain Reaction
PMS	phenazine methosulphate
PVPP	polyvinylpyrrolidone

QTL	quantitative trait locus
RAPD	randomly amplified polymorphic DNA markers
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
SDB	spermidine digestion buffer
SDS	sodium dodecyl sulphate
Slv	spacer length variation
SSD	single seed descent
X-gal	5-Bromo-4-chloro-3-indolyl-B-D-galactoside

Chapter One

Introduction.

1.1. Introduction to Barley and Barley breeding

Barley (*Hordeum vulgare* L.) is one of the oldest cultivated cereals and is grown in many regions of the world. In terms of area planted, barley is the fourth most important cereal after rice, maize and wheat. It is adapted to a range of environments and soil types and is utilized in the diets of both man and livestock. *Hordeum spontaneum* Koch is the wild progenitor from which *Hordeum vulgare* is thought to have evolved and the two species are sexually crossable (Zohary, 1963) allowing extensive opportunities for gene transfer. *H. spontaneum* is widely distributed in the Near East and is found in a range of habitats (Nevo *et al.*, 1979) and exhibits resistance to pests and pathogens and is adapted to differing environmental conditions. Thus extensive genetic variation is available for manipulation in a barley breeding programme and a number of germplasm collections are maintained (Plucknett *et al.*, 1983).

In many respects barley is an excellent species for genetic and molecular studies. *H. vulgare* L. is a diploid species (2n = 2x =14) with seven pairs of relatively large (6-8 μm) chromosomes, two of which are satellited (Nilan 1964). Being a naturally self pollinating species the majority of the genetic loci will be in the homozygous condition. A relatively well characterised classical genetic map of barley has been developed (Sogaard and Wettstein-Knowles, 1987) and the

linkage groups have been equated to the seven pairs of homologous barley chromosomes.

The methods used to develop new cultivars of inbreeding species such as barley have been outlined by Poehlman (1979). Essentially, these methods involve the identification of genetic variability for the trait of interest; sexual hybridization to produce a segregation progeny and an efficient means of selection. The result of the crossing phase is a very diverse population which contains all the possible recombinants for characters that differ in the parents. In the early generations the genetic variation is not fixed, so the progeny of any single plant continues to segregate. However, with each generation of selfing, individual genotypes become progressively more inbred. Thus the genetical effect of inbreeding is to lower, generation by generation, the probability of an individual being heterozygous for alleles at any given locus. The breeding of most inbreeding crop plants such as barley is therefore through the development of homozygous lines generated by automatic self pollination according to the methods of pedigree selection. For this breeding method selection begins in the F_2 generation on individual plants and continues in the F_3 and subsequent generations by evaluating ear rows and family plots using a pedigree based procedure. In other words, the pedigree or ancestry of the selections can be traced back to individual F_2 plants.

The pedigree system has been very successful and the majority of new cereal cultivars have resulted from this breeding strategy. Nevertheless, pedigree selection in common with other methods of plant breeding is a long process and is therefore less responsive to change in objectives. However the most recalcitrant problem in the use of the pedigree system is the difficulty of identifying high yielding genotypes in the early generations where only a limited amount of seed is available and this is normally heterozygous. To overcome this problem the breeder may delay intense selection until progenies are approaching homozygosity and sufficient seed is available for evaluation. The technique of single seed descent (SSD) first used in soybean (Brim, 1966) is one method used to achieve rapid generation advancement. Individual families are produced from single unselected seed, retained in each generation from a sample of F_2 individuals. The essential feature of this scheme is that there has been no conscious selection during the production of inbred lines.

An alternative method for rapid generation advancement is based on the production and exploitation of haploids. Haploids are sporophytes which possess the gametic chromosome number (Kimber and Riley, 1963) which in barley is seven ($n = x = 7$). The production of haploids followed by chromosome doubling to produce doubled haploids (DH) allows the creation of completely homozygous material in a single generation from heterozygous parents. DH have been used extensively in barley for cultivar production and genetical

studies (reviewed by Kasha, 1974). Two main methods of haploid production are available for barley: the *H. bulbosum* inter-specific hybridization method (Kasha and Kao, 1970) and the regeneration of plants from microspores via anther culture (Powell, 1990). These two methods differ fundamentally in that the *H. bulbosum* method samples the female gametes and the anther culture system samples the male gametes. Improvements in the efficiency of regeneration from barley microspores (Finnie *et al.*, 1989) indicate that this approach may be an acceptable an useful alternative for the creation of barley DHs.

Both SSD and DH breeding strategies can result in an improvement in the efficiency of selection since there are no dominance related effects and little within family segregation. The objective is therefore to select between inbred lines rather than within a segregating population. Regardless of the breeding strategy adopted a major challenge for the barley breeder is to identify desirable recombinant phenotypes amongst a segregating population, ie. to translate phenotypic into genotypic selection.

1.2. Genetic markers

Polymorphic genetic markers have many applications in plant breeding e.g. varietal and parental identification and the chromosomal location of traits of economic importance. Studies based on the transmission of Mendelian genetic markers provide a powerful analytical tool, allowing many genetical

principles to be tested. The theory exploits the fact that a marker locus identifies a chromosomal segment and enables that segment to be maintained in subsequent generations of selfing or crossing. However, the potential usefulness of this approach is limited by the number of markers available in many important plant species. Plant cultivars generally differ phenotypically with respect to only a small number of morphological markers, the majority of which represent undesirable mutant loci. In addition, alleles at these loci are normally recessive, and are therefore masked in the heterozygous condition.

1.3. Isozymes

The development of electrophoresis and enzyme specific stains has provided many new genetic markers, the variants of which generally have little deleterious effect on phenotype. Isozymes or multiple molecular forms of enzymes, are enzymes that share a common substrate but differ in electrophoretic mobility (Markert and Moller, 1959). The majority of isozymes exhibit codominant expression and relatively high levels of polymorphism (Tanksley, 1983). Isozyme variability has been used extensively for a range of purposes such as, delineating phylogenetic relationships (Ayala, 1975), estimating population genetic parameters e.g. outcrossing rates, migration levels or fitness components (Brown, 1979), producing preliminary linkage maps (Goodman *et al.*, 1980), tagging morphological or physiological characters of interest for convenient screening in

breeding programmes (Tanksley *et al.*, 1982) and characterising germplasm collections (Brown, 1978). Several isozyme loci have been found to be linked to genes confering resistance to pests and used as markers for transferring genes for disease resistance into a range of plant species (McMillin and Allen, 1987). However many plant species, particularly inbreeders, exhibit low levels of isozyme polymorphism (Rick and Forbes, 1975; Nevo *et al.*, 1979). In order to maximise the level of polymorphism detected inter-specific crosses have been evaluated and linkages identified for eventual exploitation (Tanksley and Rick,1980). Furthermore many of these biochemical markers have the disadvantage of being developmentally regulated, with phenotypes expressed only at certain stages of development or only in some specific tissue or organ.

1.4.1. Restriction Fragment Length Polymorphisms

Molecular biology has recently provided methodologies that enable the list of useful markers to be extended considerably. These approaches are based on the ability to detect DNA sequence variation. Restriction fragment length polymorphisms (RFLPs) were first used as a tool for genetic analysis in 1974. Linkage of temperature-sensitive mutations of adenovirus to specific restriction fragment length differences were used to locate the mutations on a physical map of the restriction fragments (Grodzicker *et al.*, 1974). RFLP markers have been shown to have great potential for use in human

genetics, holding out the promise of saturating the human genome with polymorphic markers (Jeffreys, 1979; Murray *et al.*, 1984), and in enabling human genes to be more readily mapped (Botstein *et al.*, 1980). The use of RFLPs as genetic markers for disease was first proposed for the analysis of β-thalassemia (sickle-cell anaemia) (Kan and Dozy, 1978; Phillips *et al.*, 1980; Little *et al.*, 1980). Botstein *et al.*, (1980) described the theoretical basis for mapping genes associated with disease in man and this approach has been extended to plants allowing new procedures to be applied to specific problems which have been previously unamenable to conventional genetic analysis. Tanksley (1983), Tanksley *et al.* (1989) and Beckmann and Soller (1983) have reviewed the potential usefulness of such markers in plant improvement programmes. Many of the proposed uses of RFLPs are similar to those previously proposed for isozymes.

1.4.2. Properties of RFLPs

RFLP inheritance patterns depend on the genomes examined: nuclear RFLPs behave as Mendelian markers, whereas cytoplasmic RFLPs are usually maternally inherited. Nuclear RFLPs are mainly codominant (Petes and Botstein, 1977; Burr *et al.*, 1983; Helenjartis *et al.*, 1985), generally lack obvious phenotypic effects and often generate multiple alleles (Burr *et al.*, 1983; Antonorakis *et al.*, 1985; Polans *et al.*, 1985; Wyman and Whyte, 1980; Jeffreys *et al.*, 1985). An important advantage of RFLPs as compared to morphological and

biochemical markers is that the RFLP profile can be determined from DNA extracted at any stage of the organisms life cycle and from most tissues (Rogers and Bendich, 1985). Table 1.1 summarises the properties of RFLPs as genetic markers.

1.4.3. RFLP methodologies

Restriction endonucleases are enzymes that have the ability to recognize specific nucleic acid sequences (called restriction sites) and cleave the DNA at these or adjacent sites (Zebeau and Roberts, 1979). The number of fragments produced and the sizes of each fragment will be determined by the distribution of the restriction sites in the DNA.

Relatively small DNAs, such as chloroplast DNA, will on average produce about 40 discrete restriction fragments when digested with a typical restriction enzyme recognizing a 6 bp sequence, for example the chloroplast of *Zea mays* will in theory produce 39 bands when cut with such an enzyme (Table 1.2.). The restriction fragments produced by digestion of purified chloroplast DNA can be separated according to size by subjecting the DNA to agarose gel electrophoresis after digestion. After the gel is stained with ethidium bromide, the pattern of restriction fragments can be directly observed in ultraviolet light. Chloroplast DNAs which differ from one another in base sequence, or have been rearranged by insertions, deletions or inversions will produce restriction fragments of different length. These differences in size arising

Table 1.1. Properties of RFLPs as genetic markers.

Numbers	1. Unlimited number of probe x enzyme combinations. 2. Probes not restricted to coding sequences. (i.e. Able to detect silent variations and variation in flanking sequences.
Convenient	1. Detectable in all tissues. 2. Detectable at all stages of development. 3. DNA samples have long shelf life. 4. Informative about kind of variation.
Useful	1. Ubiquitous. 2. Genomic RFLPs show Mendelian inheritance. 3. Organellar RFLPs show maternal inheritance. 4. Stably inherited. 5. Codominant. 6. RFLPs may detect multiple alleles. 7. Devoid of epistatic and pleiotropic effects.
Drawbacks	1. Costly in time and materials. 2. Requires trained personel.

Table 1.2. DNA content of Some Representitive Organisms and Organelles.

Organism	DNA content (per genome)		Theoretical number of fragments produced on restriction digestion	
	picograms	kilobase pairs	4 bp cutter	6 bp cutter
E. coli	0.0047	4.2×10^3	16,000	1,025
Chloroplast (*Zea mays*)	0.0002	1.6×10^2	625	39
Mitochondrion (*Zea mays*)	0.0007	5.7×10^2	2,200	139
Arabidopsis thaliana	0.07	7.0×10^4	2.7×10^5	1.7×10^4
Oryza sativa	0.6	5.8×10^5	2.2×10^6	1.4×10^5
Lycopersicon esulentum	0.7	7.1×10^5	2.7×10^6	1.7×10^5
Hordeum vulgare	4.0	3.9×10^6	1.5×10^7	9.5×10^5
Zea mays	7.5	7.2×10^6	2.8×10^7	1.6×10^6
Homo sapiens	3.2	3.2×10^6	1.2×10^7	7.8×10^5

One picogram of DNA = 0.965×10^6 kb.

from restriction enzyme digestion are called restriction fragment length polymorphisms (RFLPs).

RFLP analysis can be applied to chromosomal DNA also, but is more complex due to the greater complexity of nuclear DNA. Digestion of the DNA from a higher plant with a typical restriction enzyme produces millions of discrete DNA fragments in a continuous range of sizes (Table 1.2). If digested DNA is subjected to gel electrophoresis and stained with ethidium bromide no distinct fragments can be visualized. So many fragments are produced that the DNA appears to run as a continuous smear. However the individual restriction fragments are still well-resolved in the gel and may be visualised by the use of cloned DNA probes and DNA-DNA hybridization techniques. Specific fragments are therefore detected by the use of an appropriate probe consisting of a cloned DNA sequence homologous to a particular DNA fragment or some portion of it.

1.4.4. The Use of 'Southern' Hybridization techniques to Detect RFLPs in Genomic DNA Digests

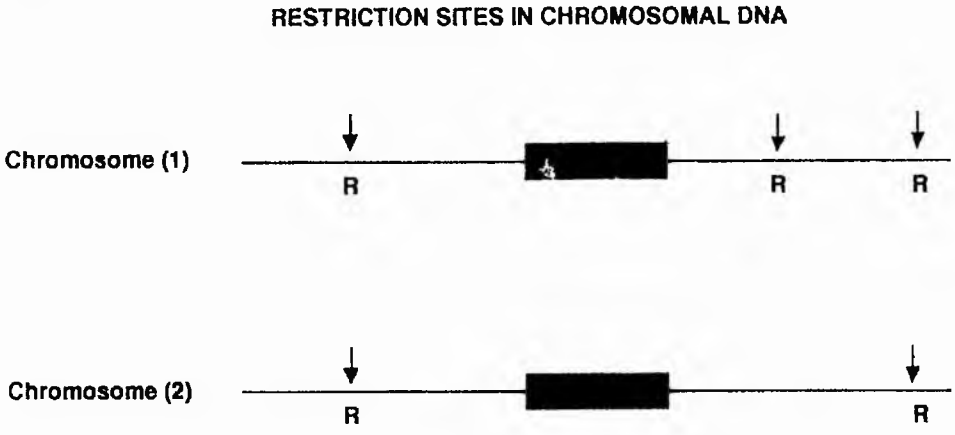
RFLPs are detected in nuclear DNA digests as follows. DNA from the plants to be compared are first digested with a suitable restriction enzyme and then size separated on an agarose gel. In order to use DNA-DNA hybridization to detect specific fragments the probe and the DNA in the gel are first denatured to render them single stranded. The gel is soaked in

NaOH to denature the DNA and then transferred out of the gel on to a solid support such as nitrocellulose (Southern, 1975).

After a filter is prepared by Southern transfer, it can be probed with a specific DNA probe. The probe is first denatured and allowed to hybridize with the filter containing the nuclear DNA under investigation. Under conditions favouring DNA-DNA hybridization, the denatured probe specifically hybridizes to any nuclear restriction fragments on the filter which are homologous to it. In order to visualize this process the DNA probe is generally radioactively labeled with ^{32}P. The labeled probe is allowed to hybridize with the filter, excess unhybridized probe is washed away and the filter placed against photographic film for autoradiographic exposure. Following development of the film, the specific DNA fragments that hybridize to the probe are visualized as bands on the film. A diagrammatic representation of an RFLP is presented in Figure 1.1.

Probes used in RFLP analysis are usually generated by cloning genomic DNA or cDNAs from the same organism. However, the DNA probes used in RFLP detection do not have to be homologous with known genes. Any DNA sequence will suffice as long as it hybridizes with part of one of the DNA fragments produced by the restriction digest.

(a)



(b)

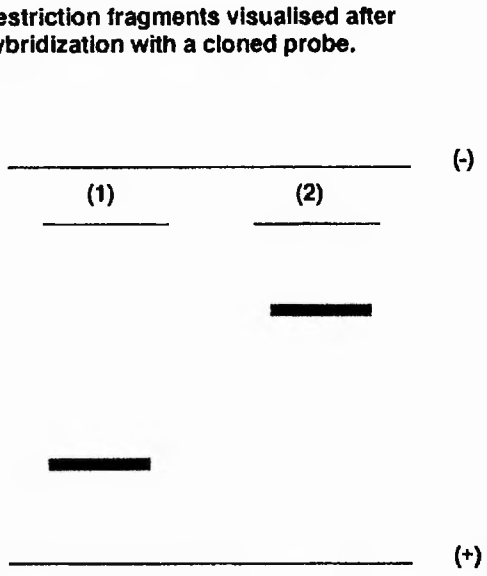


Figure 1.1 (a) A diagramatic representation of a restriction fragment length polymorphism. A homologous region of DNA from two individuals is represented. Both individuals contain a sequence (indicated by the dark box) that has been cloned. The restricion site on the right side of the cloned sequence has been abolished by the alteration of the DNA sequence. A larger DNA fragment is therefore produced when DNA from chromosome 2 is digested with this restriction enzyme. (b) Autoradiographic visualization of the fragments containing the cloned DNA sequence is shown. Adapted from Beckman and Soller 1983.

1.4.5. DNA Sequence Variation Resulting in RFLPs

DNA sequence variation resulting from a point mutation will occasionally result in the gain or loss of a restriction site for a particular restriction endonuclease. DNA alterations involving larger regions such as inversions, deletion/insertion rearrangements or translocations, will alter locally the relative distribution of restriction sites for several restriction endonucleases simultaneously. In all of these instances, the size distribution of the DNA restriction fragments generated by endonuclease digestion of the region affected by the mutational event will differ from that obtained in an unaffected individual, yielding a restriction fragment length polymorphism. This will be generated, in the case of point mutations only by those enzymes whose recognition sites include the mutation. In the case of DNA rearrangements, restriction sites which either span or are included in the mutated region will all detect polymorphisms. RFLPs can therefore be informative as to the nature of the genetic variation detected.

A large variety of restriction enzymes having different recognition specificities are available and RFLPs in both coding and noncoding DNA sequences can be detected with the appropriate probes. Thus RFLPs can be used to monitor a significant portion of the genome, and should be virtually unlimited in their ability to detect large numbers of polymorphisms. Studies carried out in humans (Jeffreys, 1979)

suggested that on average 1% of the human genome may be polymorphic. As a first step in evaluating the feasibility of using RFLPs in a particular organism, several investigators have examined the degree of genetic variability detected by RFLPs in plants. Riven *et al.* (1983); Burr *et al.*, (1983), and Helenjartis *et al.*, (1985) have reported significant variability among different maize inbred lines when either repetitive or single-copy DNA sequences were used as probes for RFLPs. RFLPs have been shown to exist in all species so far examined (reviewed in Beckmann and Soller 1986), but the extent of the polymorphism detected varies from one organism to another.

1.4.6. Source of probes

A large part of most plant genomes consist of highly repeated DNA sequences. In order to be able to resolve distinct hybridization bands, genetic mapping with RFLPs generally requires the use of probes homologous to single or low-copy genes. Alternatively, for uses such as varietal identification, higher copy number clones detecting a complex banding pattern may be more useful. DNA content may vary considerably between species (Table 1.2) and most of the extra DNA between small and large plant genomes can be attributed to repetitive sequences, and the absolute amount of single copy appears to remain relatively constant at 0.7×10^5 to 2×10^5 Kb. Analysis of the barley genome estimates that there is approximately 3.9×10^6 Kb of DNA of which at least 70% consists of repeated sequences (Bennett and Smith, 1976;

Rimpau *et al.*, 1980). According to Rimpau *et al.*, (1980), 10-20% are tandemly arranged repeats while 50-60% are interspersed among one another or among non-repeated sequences which includes those of very low copy number. The latter are divided into two groups: 21% being short (mostly about 700 bp but up to 2500 bp) and the remainder being long (more than 10,000 bp). Consequently, libraries constructed from total genomic DNA are not usually very efficient sources of useful clones for RFLP mapping, and single-copy enrichment procedures are required.

1.4.7. cDNA clones as a source of low copy number probes

Probes consisting of only unique or near-unique sequences can be obtained by utilizing complimentary DNA (cDNA) clones relying on the fact that most mRNAs are transcribed from single-copy sequences (Burr *et al.*, 1983; Helenjartis and Gestland, 1983). As with isozymes, the codominant nature of RFLPs allows the identification of all genotypes (parental and recombinant) in any segregating population. However, unlike isozymes that are limited in number by the presently available enzyme stains, the number of cDNA markers is only limited by the number of expressed genes. The ability to detect variation with cDNA markers is greater than isozymes since the basis of variation of RFLPs is the presence of "neutral" restriction sites and insertion/deletion mutations, and there is a large number of restriction enzymes available for screening. This is in contrast

to the allelic variation of isozymes that is derived from changes in the amino acid sequence of the protein. Variation in DNA also includes silent substitutions in the third base pair of codons and sequence divergence in the noncoding flanking regions. Hence variation detected by isozymes is only a subset of the variation found in DNA sequences. The restriction sites detected with RFLP analysis are often outside of the coding region and, thus, may evolve at a faster rate. However, the use of cDNA clones may result in the nonrandom distribution of markers over the chromosomes, limiting mapping to coding regions of the genome or to the sequences immediately flanking these regions. However, it may be possible to extend the areas covered using unique genomic DNA fragments to detect RFLPs. These loci could potentially be in regions not represented by cDNAs.

1.4.8. Genomic clones as a source of low copy number probes

Clones not containing repetitive sequences can be screened from libraries constructed from total genomic DNA. It has been observed in maize (Burr *et al.*, 1988) and tomato (Tanksley *et al.*, 1987) that when clone libraries are constructed from total genomic DNA digested with methylation sensitive restriction endonucleases such as Pst I, they are enriched for single copy sequences. Burr *et al.* (1988) have proposed that these enzymes cleave the DNA only in undermethylated regions since it is these regions which contain the unmethylated coding sequences of the genome.

Alternatively single or low-copy containing clones can be identified following hybridization to colonies with radioactively labelled total genomic DNA. Low-copy clones are identified by a lack of detectable hybridization, while clones containing repeated sequences hybridize with varying intensities to the total genomic DNA probe.

1.4.9. Factors influencing the levels of Restriction fragment length polymorphism detected

The construction and use of RFLP genetic maps depends on the ability to detect useful levels of polymorphism in the population or cross being examined. In order to have the best possibility of detecting polymorphism it is important to first understand the factors affecting the level of polymorphism detected by a particular probe. Miller and Tanksley (1990a) have attempted to determine under which conditions maximum levels of polymorphism may be detected. Using data from an RFLP study of tomato (Miller and Tanksley, 1990b) they examined the relationship between the level of polymorphism detected and clone size, restriction enzyme, size of hybridizing fragments and the source of the clones used as probes. No relationship was found for clone size and the level of polymorphism, but there was a strong positive relationship between the level of polymorphism detected and the average size of the fragments produced by each restriction enzyme. Similar correlations between increased fragment size and increased polymorphism have been reported previously in

lettuce (Helenjartis *et al.*, 1985) and in rice (McCouch *et al.*, 1988). These results suggest that the selective use of enzymes generating larger fragments can result in the increased detection of polymorphism. This is, however, contrary the prediction of Kreitman and Aguade (1986). By using 4 bp cutter restriction enzymes one theoretically increases the length of sequence scored per probe by increasing the number of restriction sites surveyed. The available data would suggest that in plants, individual base change mutations are not being detected and most of the RFLPs being observed are due to larger rearrangements of DNA. However, further studies with a wider range of plants are required before general conclusions can be made.

In tomato (Miller and Tanksley 1990b), cDNA clones were found to hybridize to larger fragments and to detect significantly more variation than either random genomic clones or PstI genomic clones selected on the basis of hypomethylation. The fact that cDNA clones behave differently may indicate that the PstI clones are not coincidental with coding regions even though coding regions and areas surrounding these regions may be under methylated. This may be attributed to the high level of sequence variability present within introns and also in the areas flanking coding regions (Bird, 1987). The fact that cDNA clones detect significantly larger fragments may reflect different distributions of nucleotide composition and distribution of such sequences

resulting in less frequent restriction sites for particular enzymes.

1.5.1. Polymorphism assay methods based on the Polymerase Chain Reaction.

The exploitation of RFLPs as genetic markers is one of the most successful applications of recombinant DNA techniques. However cost, technical skill required, utilization of radioactive isotopes and the inability to handle large numbers of samples in a reasonable time have inhibited the practical application of RFLPs in many fields of plant genetics and breeding (Landry and Michelmore, 1987). Alternative marker systems based on the *in vitro* amplification of specific DNA sequences offer a rapid and technically straight forward alternative to the use of RFLPs. The most notable of these techniques is based on the use of the polymerase chain reaction (PCR). As with RFLPs, PCR based markers enable variation at the DNA sequence level to be identified. PCR is being widely used for efficient amplification of specific sequences of genomic DNA, notably in the field of early diagnosis of human genetic disorders (Vosberg, 1989).

1.5.2. PCR Methodologies

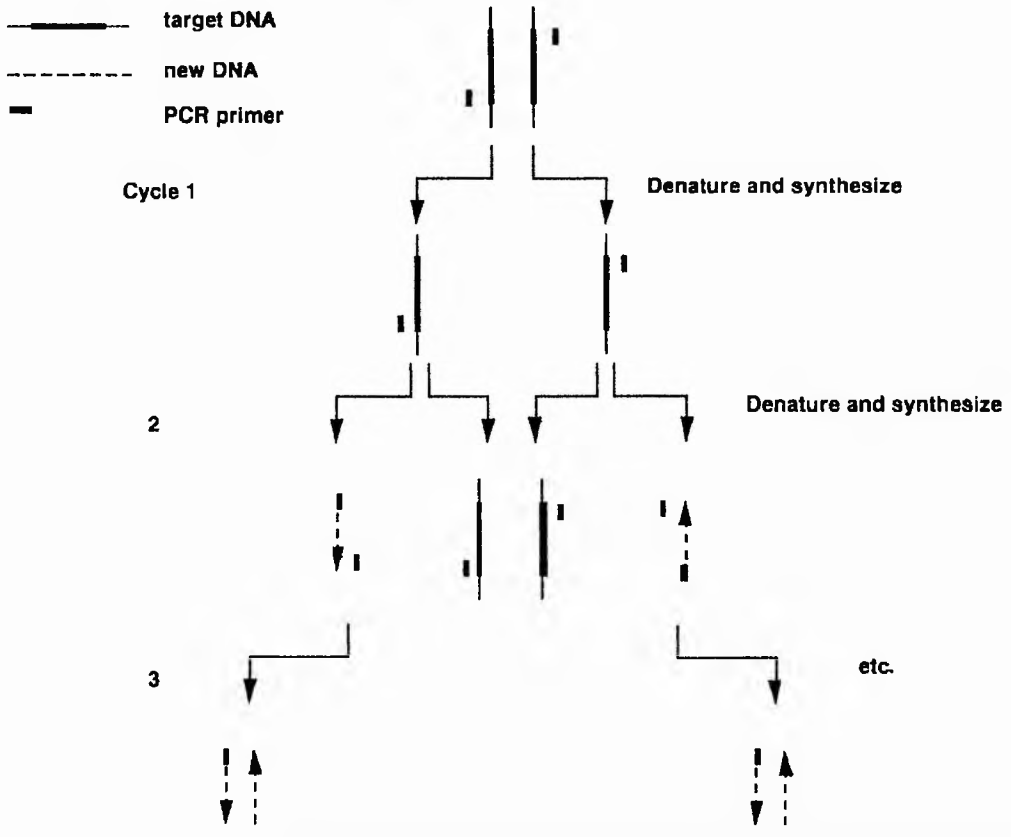
PCR is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers hybridizing to opposite strands of the target sequence with their 3' ends pointing towards each other (Saiki *et al.*, 1985;

Mullis and Faloona, 1987). The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotide primers and the four deoxyribonucleotide triphoshates (dNTPs). The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. Repeated cycles of heat denaturation of the template, annealing of the primers to their complimentary sequences and extension of the annealed primers with a thermostable DNA polymerase (*taq*) isolated from the thermophilic bacterium *Thermus aquaticus*, result in the amplification of the segment defined by its 5' ends. Since the extension product of each primer can serve as a template for the other primer, each successive cycle essentially doubles the amount of DNA fragment produced in the previous cycle (Figure 1.2). This results in the exponential accumulation of the specific target fragment, up to several million fold in a few hours. When genomic DNA is used as the template, it is possible to amplify a specific single gene by the selection of the appropriate primers.

1.5.3. The use of known DNA sequences in PCR amplification of Plant gene sequences

Recently, polymerase chain reaction based markers have been exploited in plant genetic studies for the amplification of a wheat genomic sequence (D'Ovidio *et al.*, 1990). The two

Figure 1.2. PCR based amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target DNA sequence. The primers are oriented with their 3' ends pointing at each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers.



oligonucleotides used as primers for the PCR reaction were synthesized on the basis of the published sequence of a gene coding for a wheat storage protein belonging to the gamma-gliadin fraction. Two oligonucleotide primers representing the first twenty nucleotides of the 5' transcribed region and nineteen nucleotides of the complementary strand were used as PCR primers. The electrophoretic analysis of the PCR products showed specific bands which revealed both inter- and intra-cultivar genetic polymorphism among the genotypes. examined. The authors proposed this form of PCR analysis as a simple and efficient alternative to the use of RFLP markers.

1.5.4. Randomly Amplified Polymorphic DNA (RAPD) markers

A modification of the PCR technique involves the use of random oligonucleotide primers, rather than known defined primers. This modified PCR technique is known as Randomly Amplified Polymorphic DNA (RAPD) markers (Williams *et al.*, 1990). Under conditions of reduced stringency, primers will anneal to many sequences throughout the genome with a variety of miss-matches. Where these primers anneal to opposite strands of the DNA within approximately 2.5 Kb of each other, sequences between these primers will be amenable to amplification by PCR (Williams *et al.*, 1990).

The length and the sequence of the primers were found to exert a major influence on the resulting amplification products. For plants the nucleotide sequences of each primer must be at least 9-10 base pairs long, between 50 and 80% G+C

in composition and contain no palindromic sequences. The rational behind this is that sequences shorter than 9-10 bases and with a low G+C content have a low Tm and thus fail to remain hybridized to the DNA template as the temperature is increased to allow the *Taq* polymerase to function. Additionally the primers were non-palindromic in order to limit the orientation in which the primer could bind to the template DNA sequence.

Results from many workers have now confirmed the high specificity and reproducibility of PCR based marker techniques. These techniques have several advantages over RFLPs. These include the high levels of specificity, speed and particularly the avoidance of the use of hybridization with radioactive probes. The numerous published nucleotide sequences provide a substantial source of oligonucleotides for use as primers. This coupled with the ability to use random oligonucleotide sequences suggest a great potential for the use of PCR markers in plants.

1.6.1. Applications of Polymorphic Markers to Varietal identification

As the number of polymorphic markers identified in a particular plant species increases so does the potential for their use in discriminating between strains and varieties of agricultural importance. The most immediate application of RFLPs in plant breeding may be in the area of seed quality control and strain and varietal identification (Soller and

Beckmann, 1983). RFLPs can provide DNA fingerprints for the identification of lines or individuals. Probes homologous to loci possessing multiple alleles may be particularly suitable for this purpose (Helenjartis and Gestland, 1983; Jeffreys *et al.*, 1985) as they can generate highly specific fingerprints for strain identification. Beckmann and Soller (1983) have calculated that 10-20 RFLP markers would be sufficient to differentiate tomato and maize inbred strains, with even fewer required if multiple allelic forms were identified for each marker.

1.6.2. The use of hypervariable minisatellite DNA sequences for varietal identification

The discovery of minisatellite DNA sequences which are hypervariable in many species has potentially provided one of the most suitable classes of probes for varietal identification purposes in plants. These sequences consist of a series of tandem repeats of a core consensus sequence. These sequences are often dispersed throughout the genome and thus represent several loci. At a given locus, numerous alleles differing in the number of core repeats may occur. In humans these sequences are highly variable and when hybridized with a radioactively labelled probe, yield a genetic fingerprint unique to each individual (Jeffreys *et al.*, 1985a,b). Some of these same probes have detected intraspecific variation in bacteria, protozoa, fungi, several animal species, gymnosperms and angiosperms (Burke and Bruford, 1987; Jeffreys and Morton, 1987; Vassart

et al., 1987; Dallas, 1988; Rogstad *et al.*, 1988; Ryskov *et al.*, 1988).

1.6.3. The use of the M13 probe for varietal identification

A sequence present within the protein III gene of the M13 bacteriophage vector (Messing *et al.*, 1977) has also been shown to detect a distinct set of hypervariable minisatellites in human and animal DNA and also in a range of gymnosperms and angiosperms (Rogstad *et al.*, 1988). Nybom *et al.* (1989) have also used this probe to "fingerprint" genera and species in the angiosperm family *Rosaceae*. They found that four apple cultivars could be differentiated with any one of a combination of five restriction enzymes and the M13 probe. Similarly 4 individual trees of *Prunus serotina* (black cherry) exhibited different "fingerprints" with each of four restriction enzymes and extensive inter- and intraspecific variation was found when a total of 14 *Rubus* (blackberries and raspberries) genotypes representing 4 species were investigated with 2 enzymes. These studies demonstrate that the M13 probe may be used in a manner similar to that of the human minisatellite used to 'fingerprint' cultivated rice (Dallas, 1988).

1.6.4. Varietal identification based on the use of PCR markers

Identification procedures based on PCR techniques are also currently being evaluated in a number of species (Welsh and McClelland, 1990; Wilde *et al.*, 1991). The use of a range of randomly chosen primers to generate a genetic fingerprint is

essentially equivalent to the use of a selection of RFLPs as described above. The main advantage this procedure has over the RFLP technique is that the time taken to generate the fingerprint is reduced and only small quantities of DNA are required. This coupled with the technical simplicity of RAPD markers may indicate a potentially ideal role for this technique in cultivar identification.

1.7.1. Mapping of polymorphic genetic markers

Perhaps the primary application of genetic marker techniques is in the construction of genetic linkage maps. As the first step in the generation of a genetic linkage map the widest sexual cross possible should be chosen as this increases the probability of detecting polymorphism. Beckmann and Soller (1986) proposed the use of crosses between two very divergent but compatible landraces. Such a mapping population would be expected to demonstrate maximum levels of variation for genetic markers. In particular, the level of variation would be expected to be greatly in excess of that of crosses between closely related cultivars. This has been demonstrated in a range of plant species. It is interesting to note, however, in maize where high levels of DNA polymorphism exist it is sufficient to make crosses between unrelated inbreds. In the case of rice a cross between *indica* and *javanica* rice cultivars was used to generate the linkage map (McCouch *et al.*, 1988). However, for tomato both the creation and the exploitation of linkage maps have involved inter-specific hybridization between

Lycopersicon esulentum and *L. chmilelwskii* (Tanksley *et al.*, 1989).

For inbreeders the creation of a linkage map can be based on either the backcross or F_2 generations. In theory the F_2 population provides the maximum resolution for a given number of individuals (Allard, 1956; Tanksley *et al.*, 1988), but as the number of loci being evaluated increases and as destructive sampling of plant tissue is often required this may limit the number of characters that it is possible to evaluate on a single plant basis. Recombinant inbred lines offer an attractive alternative for perpetuating a mapping population where one cannot vegetatively propagate individuals (Burr *et al.*, 1988). Recombinant inbreds are usually generated either by selfing from the F_2 or by producing doubled haploids. These also allow accurate mapping with a much smaller number of individuals than an F_2 (Taylor, 1978). However, one major benefit of using recombinant inbreds is that within individual families, plants are genetically uniform and therefore different plants within a family may be used for destructive sampling. Allelic classification may therefore be based on families rather than individual genotypes.

1.7.2. The generation of RFLPs by insertional mutagenesis

In cases where polymorphic markers cannot be found distinguishing particular individuals there may possibly be a role for the use of mutagenized plants. DNA insertion techniques (Beckmann and Soller, 1985) may allow the

generation of novel RFLP polymorphisms. The insertion of a foreign DNA sequence into an individual chromosome will result in the interruption of the indigenous sequence, resulting in the generation of an RFLP at the site of insertion. A possible problem with this approach is that using current transformation procedures the inserted DNA sequences are not specifically targeted and will tend to be randomly inserted into the host genome. As a consequence there is no control over whether or not the inserted sequence disrupts gene functions in its vicinity such as by interrupting coding or control sequences. This form of mutational event is classed as insertional mutagenesis (Martin *et al.*, 1985). This technique does however offer the possibility of generating RFLPs in areas of the genome where it may prove otherwise difficult to obtain suitable markers. However, this approach is dependant on gene insertion techniques which are relatively well advanced in dicotyledonous species but restricted in monocotyledonous crops such as barley (reviewed by Potrykus, 1991).

1.7.3. Comparison of RFLP Maps in Related Species

The construction of genetic maps may enable a comparison of genomic structure and organisation in related species. Studies of the genetic maps of humans and mouse have indicated that in several instances closely associated loci tend to maintain linkage relationships despite the species divergence that has taken place (Nadeau and Taylor, 1984). Similarly, RFLP maps based on a common set of tomato clones

have been used as the basis for the unification of the previously separate disciplines of tomato and potato genetics (Bonierbale *et al.*, 1988). They reported that since the divergence of potato and tomato from their last common ancestor, the only detectable chromosomal changes were found to be paracentric inversions. That is, three of the 12 homologous chromosomes were found to differ by an inversion with one breakpoint at or near the centromere. This comparison of the gene order in tomato and potato determined that there was a lack of both translocations and pericentric inversions and is consistent with the cytogenetical theory relating to the relative fitness of paracentric and pericentric inversions. As a result of these findings it is possible to predict the chromosomal location of additional mapped tomato markers in the potato genome. However, care must be exercised in assuming that map positions will be conserved between other related species. Comparison of the RFLP linkage-maps of the two solanaceous species tomato and pepper based on a common set of tomato clones indicated that although the genetic make up was conserved between the two species, the gene order had been significantly altered by extensive chromosomal rearrangement (Tanksley *et al.*, 1988). The lack of linkage conservation between these closely related species suggest that caution must be exercised when extrapolating linkage maps from one species to another. This is especially important when considering the construction of RFLP maps in related plant species where heterologous probes may be used.

If however, linkage groups are highly conserved, the map from one species could be valid for related species or genera, but without directly testing, it may be unwise to assume that related species will have the same gene order.

1.8. Marker-assisted introgression of major loci

It is clear from the work with isozymes that molecular markers can also be of use in improving the speed and precision of gene introgression from related species into an adapted cultivar (Tanksley and Rick, 1980; Tanksley *et al.*, 1981; Tanksley *et al.*, 1983). Marker based selection is the area where molecular markers are most likely to have the most immediate impact in plant breeding. The fact that DNA may be isolated from plants at almost any stage of growth should enable an increase in the efficiency of breeding programmes. This is because molecular markers should allow the plant breeder to make earlier selections while examining fewer plants. This may be of most use in situations where the donor (e.g., a wild race) has poor agronomic characters apart from the gene of interest, while the recipient is of superior merit. The objective of the breeder is to eliminate as much of the donor genome in as few crosses as possible, while retaining the gene of interest from the donor. This is normally accomplished by successive rounds of backcrossing. It is possible that if the chromosomes of the donor strain were to carry isozyme or molecular markers differentiating it from the recipient strain then the number of backcrosses required may be reduced by

selecting against the donor markers. Due to the general lack of between cultivar variation at the isozyme level, Tanksley and Rick (1980) and Tanksley (1983) have proposed that this method is best suited for the introgression of genes from wild species. The use of molecular markers and their potential to detect greater levels of polymorphism may, broaden their application to intervarietal gene transfer.

1.9.1. Quantitative Trait Loci

With the exception of specific disease resistances and morphological and colour traits, most characters of economic importance such as yield, quality and abiotic stress resistance are determined by allelic differences at several, but generally an unknown number of loci having relatively small individual effects (Mather and Jinks, 1971). These traits are characterised by phenotypic variation of a continuous nature and loci associated with these traits are termed quantitative trait loci, abbreviated to QTL (Geldermann, 1975).

1.9.2. Marker based analysis of QTLs

Because the genes controlling quantitative traits are difficult to resolve individually, quantitative geneticists have dealt with the effects of these genes *en masse*, using biometrical procedures which allow an approximate estimation of the number of QTLs (Mather and Jinks, 1971; as outlined by Powell *et al.*, 1985). The biometrical approach is, however, unable to identify, characterize and directly manipulate the

individual loci involved. Using marker genes to identify specific regions of the genome which are involved in the expression of quantitatively controlled characters leads to the possibility of dissecting and manipulating these traits. The basic approach of using marker genes in segregation was proposed as a viable technique for the systematic analysis of quantitative variation by Thoday (1961) and more recently by Geldermann (1975), Mather and Jinks (1982), Tanksley *et al.* (1982), and Beckmann and Soller (1983). Morphological markers have been used for limited mapping of QTLs from as early as 1923 (Sax, 1923). Isozyme polymorphisms have also been used to map some QTLs controlling characteristics such as leaf ratio, fruit weight and seed weight in tomato (Tanksley *et al.*, 1982) and ear number and grain yield in maize (Stuber *et al.*, 1982). The low number of morphological and isozyme markers segregating in a particular cross is a major limitation to the identification of loci contributing to the quantitative trait. Clearly the more genetic markers available, the greater the proportion of the genome that can be compared and the greater the likelihood of identifying an important chromosomal region and locating the quantitative trait within it. High-density RFLP and isozyme maps make it technically feasible to effectively map quantitative trait loci (Lander and Botstein, 1989). These maps provide reasonably complete genome coverage and increase the probability of finding a QTL. Segregation of a large number of dispersed RFLP markers in a single cross would enable the entire genome to be assayed for loci contributing to the

quantitative traits (Tanksley, 1983). Analysis of QTLs based on linkage to RFLP markers have been widely used in crop species including tomato (Osborne *et al.*, 1987; Tanksley and Hewitt, 1988; Young *et al.*, 1988) and maize (Edwards *et al.*, 1987).

1.9.3. The use of High-density genetic maps in QTL analysis

High-density genetic maps also enable the use of flanking marker genetic models to identify QTLs and are based on the hypothesis that a particular quantitative trait lies between codominant marker loci (Weller, 1987; Lander and Botstein, 1989). These models are more efficient than individual marker models for estimating the effects of quantitative trait loci (Lander and Botstein, 1989). The theoretical application of methods based on flanking marker genetic models using high-density maps has been reported in doubled haploids, recombinant inbred lines and F_2 and F_3 progeny (Knapp *et al.*, 1990).

Hence genetic markers provide a means for the resolution of QTLs into Mendelian entities that can be manipulated more readily and enable breeding programmes to proceed with greater efficiency.

1.10. Genome Organisation

Molecular marker based analysis has provided an opportunity for a more extensive investigation of genome organisation. Studies of DNA reassociation kinetics (Marmur and Doty, 1961; Evans *et al.*, 1983) have previously provided

the only method of assessing genome organisation at the DNA sequence level. This form of analysis provided only gross estimates of genome organisation. RFLP markers enable the analysis of the organisation and variation of specific components of the genome (Engels, 1981; Ewens *et al.*, 1981; Hudson, 1982). Estimations of neutral mutation rate, genetic diversity and phylogenetic relationships are also possible from RFLP analysis.

1.11.1. Organelle Genomes

Although the vast majority of genetic information is contained in the nucleus, there are important functions encoded by the DNA of chloroplasts and mitochondria. These organelles are central to the production and utilization of energy, and accordingly, affect the performance of the plant (Lever *et al.*, 1986). Since organelle genomes are much less complex than nuclear genomes, restriction endonuclease digestion generally yields fragments that are easily resolved and thus directly observable.

1.11.2. RFLP Analysis of the Mitochondrial Genome

RFLP analysis of the mitochondrial genome has provided evidence for the co-evolution between specific nuclear and mitochondrial genes (Cann *et al.*, 1984). RFLP analysis of the maize genome has provided evidence that large rearrangements have occurred during its evolution. The duplications, deletions and recombinations that have occurred

in the maize mitochondrial genome contrast with the observation that base substitutions account for most of the polymorphisms in animal mitochondrial genomes (Sederoff *et al.*, 1981).

1.11.3. RFLP Analysis of the Chloroplast Genome

In angiosperms the chloroplast genome has been particularly useful in clarifying phylogenetic relationships. The low frequency of structural changes in the chloroplast genome is complemented by a conservative rate of sequence evolution. The structural and sequence stability of the angiosperm chloroplast genome suggests that it is well suited to tracing the evolutionary history of plant species.

Although the use of chloroplast DNA analysis in plant systematics is relatively recent there is evidence of its potential value in the *Triticeae* (Vedel and Quetiner, 1978), *Zea* (Timothy *et al.*, 1979), *Nicotiana* (Kung *et al.*, 1981), *Lycopersicon* (Palmer and Zamir, 1982), *Coffea* (Berthou *et al.*, 1983), *Brassica* (Palmer *et al.*, 1983) and *Daucus* (de Bonte *et al.*, 1984). In addition to taxonomic variation, chloroplast DNA restriction patterns provide a unique marker to follow the inheritance of organelles. In most angiosperms chloroplast DNA is inherited maternally, but biparental inheritance has also been reported (Smith *et al.*, 1986). Wagner *et al.* (1987) have also reported that chloroplasts are inherited paternally in lodgepole pine (*Pinus controta*) and jack pine (*P. banksiana*).

1.12. Dynamics of Plant Populations

The potential of wild plant species as genetic resources for enhancing the germplasm of cultivated crops is well established (Frankel and Bennett, 1970; Nevo, 1986). Wild species are important sources of genetic adaptations to extreme environments and of disease resistance genes not possessed by their cultivated relatives. Isozymes and molecular markers may provide a convenient means of assessing population structure and diversity. If one assumes that polymorphism for the marker system is indicative of variability for other characters, then this form of screening may ensure that germplasm resources represent a wide spectrum of genetic variability. Biochemical and molecular markers have an important role in the characterisation and eventual exploitation of these resources efficiently in breeding programmes.

Isozymes have already been used extensively in many areas of population biology. These include: the analysis of the genetic structure of populations and measurements of genetic variability (Brown *et al.*, 1978), detection and measurement of natural selection (Clegg, 1983), studying phylogenetic relationships and investigating the role of autopolyploidy and alloploidy in the origin of polyploid species (Crawford, 1983).

The availability of molecular techniques has improved the precision with which genetic variation in cultivated and natural plant populations can be examined. RFLPs have been used in the same way as isozymes for population analyses. The

RFLP analysis of ribosomal DNA (rDNA) variation has been particularly informative and has been used extensively to monitor levels of diversity. rDNA is organised into tandem repeat units, with each repeat containing a highly conserved transcription unit and a more variable intergenic spacer (IGS) region. Spacer length variation (slv) has been found to be common in many plant species (Rogers and Bendich, 1987). A map of a typical barley rDNA repeat unit is shown in Figure 1.3.

rDNA variation has been used to describe both the genetic structure within plant populations and the phylogenetic relationships among species of plants. Variability in the IGS region has provided informative genetic markers for population studies, and variation both within and among individual populations has been detected (Saghai-Maroof *et al.*, 1984; Learn and Schaal, 1987; Schaal *et al.*, 1987). Changes in rDNA IGS composition have also been observed in *H. vulgare* (Saghai-Maroof *et al.*, 1984), *Triticum dicoccoides* (Flavell *et al.*, 1986), *Zea mays* (Rocheforde *et al.*, 1990) and *Drosophila melanogaster* (Cluster *et al.*, 1987) that have undergone natural or artificial selection.

1.13.2. Genetic Linkage maps in barley

The first systematic isozyme studies of barley were carried out by Frydenberg and Neilsen, (1965) and Nilson and Hermelin (1966). More extensive mapping of barley isozymes is limited primarily by their general lack of polymorphism.

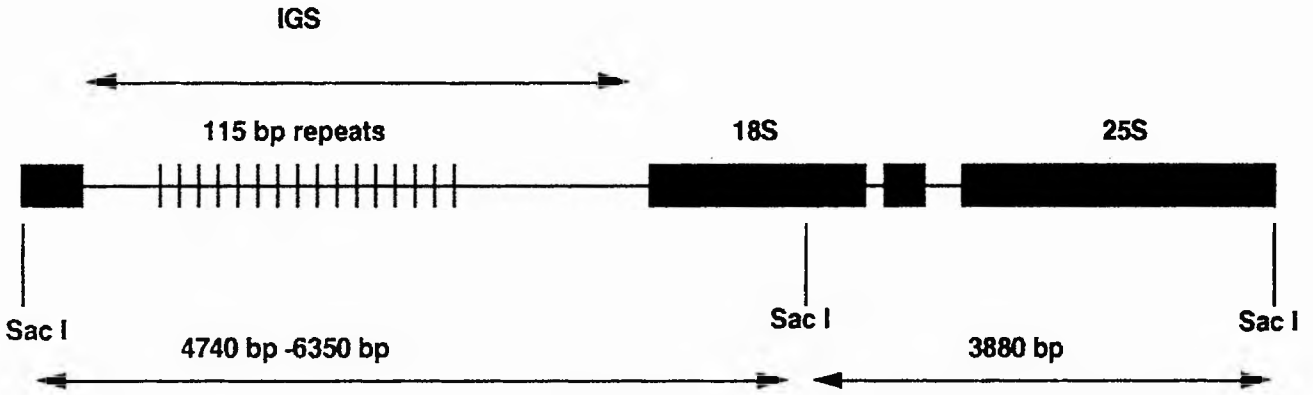


Figure 1.3 Diagramatic representation of a barley rDNA repeat unit. Regions coding for the 18S and 25S rRNA genes are labeled. The intergenic spacer (IGS) region between the 18S and 25S genes contain the 115 bp subrepeats. Sac I restriction sites are as indicated. Variation in the number of 115 bp subrepeats results in variation in the length of the Sac I fragment carrying the IGS. (Addapted fromSaghai-Maroof *et al.*, 1984)

Many barley isozyme genes have been mapped to chromosomes by aneuploid analysis using wheat/barley addition lines. The polyploid nature of wheat has allowed this organism to tolerate the loss and gain of chromosomes. Hybrids between wheat and barley were produced by Islam *et al.* (1978), Fedak (1980) and Sethi *et al.* (1986). By crossing the hybrid with wheat and subsequent backcrossing, Islam *et al.* (1981) produced wheat/barley disomic addition lines which have been invaluable in the location of genes to specific barley chromosomes. However, a lack of polymorphism has inhibited the intra-chromosomal mapping of isozyme loci (Brown, 1983). During the past few years the development of RFLPs has provided a new impetus for the mapping of barley chromosomes (reviewed by Wettstein-Knowles, 1989).

The restricted number of isozyme loci and relatively low levels of polymorphism has limited the application of biochemical markers in barley breeding programs. Associations between isozyme and protein loci with genes controlling spring/winter growth habit in barley cultivars (Forster *et al.*, 1991; Ellis *et al.*, 1991) and with a range of quantitatively controlled traits in barley doubled haploids (Powell *et al.*, 1990) have been reported. In general, isozyme techniques have been more frequently applied to population genetic studies and to aid varietal identification.

1.13.3. Barley varietal identification

Barley identification on the basis of seed characters is presently the most important method for the regulation of trade in barley grain. Hordein protein analysis after separation in polyacrylamide gels has been adopted for the commercial classification of barley varieties (Cooke and Morgan, 1986). The hordein storage proteins of barley serve as an important electrophoretic marker system, about 40% of the protein in the barley grain is hordein. Hordein contains two major groups of polypeptides both of which are highly polymorphic in cultivated barley (Doll and Anderson, 1981). These polypeptides have been shown to be encoded by two linked complex loci *Hor1* and *Hor2*, located on the short arm of chromosome 1H (Shewry *et al.*, 1980). The levels of diversity at these two loci have been shown to greatly exceed that of any isozyme locus. Storage protein loci of cereals rank among the most variable loci in plants. Several alternative marker systems have been proposed for use in barley varietal identification (Thompson *et al.* 1990). These include various isozyme and protein systems, the most common systems are starch gel and isoelectric focusing gel electrophoresis techniques (Almgard and Landegren, 1974; Bassirri, 1976; Anderson, 1982; Thompson *et al.*, 1990). Recently the use of RFLPs at the hordein loci has been evaluated for 'fingerprinting' barley and has demonstrated the usefulness of RFLPs for varietal identification (Bunce *et al.*, 1986).

1.13.4. The application of RFLPs to barley genetics

RFLPs have proved to be useful genetic markers in barley and have been used for a range of purposes in addition to varietal identification. As with other plant species the availability of molecular techniques has improved the precision with which genetic variation in cultivated and natural plant populations can be examined. RFLP analyses of variation in chloroplast and mitochondrial DNA in populations of cultivated and wild barley were amongst the first applications of RFLPs in barley (Holweda *et al.*, 1986; Neal *et al.*, 1988).

Analysis of ribosomal DNA (rDNA) variation has also proved particularly informative. Associations between alleles at the rDNA loci and a significant portion of the genetic variation for a range of agronomic characters have been detected (Powell *et al.*, 1991). However, it is in the field of barley population genetics that rDNA has proved most interesting. Changes in rDNA IGS composition have been observed in *H. vulgare* (Saghai-Maroof *et al.*, 1984), that have undergone natural or artificial selection. A number of studies have described the structure and diversity of *H. spontaneum* in Israel (reviewed by Nevo, 1991) and have emphasized the considerable isozyme variability present in these populations. Importantly the patterns of variation appear, at least in part, to be related to both ecological and climatic variables. Similarly, studies of rDNA IGS variability in these populations have

sought to relate rDNA variability to a range of ecogeographical factors (Saghai Maroof *et al.*, 1990).

The association of the barley *Rrn2* locus with traits that have a strong reproductive advantage has been demonstrated in *H. vulgare* (Powell *et al.*, 1991). It is thus possible that loci associated with the various slvs may influence reproductive advantage in natural populations, and hence influence the observed frequencies of the slvs. Saghai-Maroof *et al.* (1990) and Allard *et al.* (1990) have also quantified the extent of rDNA variability in *H. spontaneum* and and found that genetic diversity and genotypic frequencies were significantly correlated with ecogeographical factors, indicating that the alleles and genotypes marked by the slvs differ in their adaptive properties.

To date no direct evidence has been produced to substantiate the claims of Allard *et al.* (1990) nor Saghai-Maroof *et al.* (1990) that selection, acting on specific alleles of the *Rrn1* and *Rrn2* genes played a major role in the development and maintenance of the observed patterns of molecular and genetic organisation of rDNA variability in wild and cultivated barley.

1.14. Project Aims

The broad aims of this project are to consider the role of molecular and conventional genetic markers in characterising

barley germplasm and to identify opportunities for the exploitation of genetic markers in barley improvement.

The specific aims are:-

1) To develop the methodologies to detect isozyme, protein, RFLP and RAPD polymorphisms in barley.

2) To determine the level of variation for isozyme, protein, RFLP and RAPD markers in a selection of European barley cultivars (*H. vulgare*) and *H. spontaneum* genotypes.

3) To monitor the transmission of polymorphic isozyme, protein, RFLP and RAPD markers, in a barley doubled haploid population, and to investigate the relationships between these markers and a range of quantitative traits.

4) To examine the spectrum of genetic variability present in natural populations of *H. spontaneum* sampled from its entire ecological range in Israel and to compare and relate this variability to ecogeographical factors.

Chapter Two

MATERIALS AND METHODS

2.1. Plant Material

A range of spring and winter barley cultivars were used in this study. F_1 derived doubled haploid populations were used to monitor the segregation of alleles at isozyme and RFLP loci. The specific material used is described in the appropriate chapter. Plants were maintained in the greenhouse under a 14-16 hour day length regime and a minimum temperature of 18°C.

2.2. Isozymes

2.2.1. Starch gel electrophoresis.

Electrophoresis was carried out on 12% (w/v) starch gels using the discontinuous buffer system described by Ashton and Braden (1961). Leaf samples were cut at 10-14 days following germination. Extracts were prepared by grinding 4-6 cm of first leaf in 50 µl of the appropriate extraction buffer. Alternatively, alcohol dehydrogenase (ADH) extracts were prepared from root material of 5 day old seedlings. ADH activity was induced by a period of flooding (normally 2 days). The extraction buffer and the choice of gel buffer system are dictated by the particular enzyme being assayed and are noted below.

2.2.2. Electrophoresis buffers

2.2.2.1 Borate gel: Electrode buffer

Per litre: Lithium hydroxide 1.2 g, Boric acid 11.9 g. pH was adjusted to 8.3 with anhydrous boric acid.

2.2.2.2 Borate gel: Gel buffer

Per litre: Tris base 5.45 g, Anhydrous citric acid 1.28 g, 100 ml electrode buffer. pH was adjusted to 8.3 with citric acid or 1 M sodium hydroxide.

2.2.2.3. Histidine gel: Gel buffer 50 mM histidine.

2.2.2.4. Histidine gel: Electrode buffer 0.41 M sodium citrate.

2.2.3. Extraction buffers

Standard extraction buffer was used for most stains:

50 ml borate gel buffer, 37 mg potassium chloride, 10 mg magnesium chloride, 18 mg EDTA (tetrasodium salt), 25 g PVPP, 0.5 mg Triton-X 100, and 2 ml 2-mercaptoethanol.

Peroxidase and Esterase extraction buffer:

50 ml gel buffer, 37 mg potassium chloride, 10 mg magnesium chloride, 18 mg EDTA (tetrasodium salt).

2.2.4. Enzyme stains used in staining starch gels

The following are the enzyme assays used in this study. All are modifications of established procedures (Brewer 1970; Brown *et al.*, 1978). All quantities are listed, as the amount required to stain one gel slice.

2.2.4.1. Stains used with the Borate gel system

Acid phosphatase (ACP) EC 3.1.3.2

Presoak gel in 50 ml 0.4 M acetate for 10 minutes.

Staining solution: 50 ml 0.2 M acetate pH 5.0, 50 mg sodium alpha-napthyl acid phosphate, 40 mg Fast Garnet GBC, 0.5 ml 10% magnesium chloride

Alcohol dehydrogenase (ADH) EC1.1.1.1

Staining solution: 50 ml 0.1 M Tris-HCL pH 8.0, 5 ml ethanol, 30 mg NAD, 20 mg MTT, 5 mg PMS

Esterase (EST) EC 3.1.1.2

Staining solution: 40 ml distilled water, 2 ml 1% alpha-napthyl acetate, 1 ml 1% beta-napthyl acetate, 125 mg Fast Blue RR salt dissolved in 1 ml acetone, 50 ml 0.2 M dihydrogen sodium orthophosphate, 10 ml 0.2 M disodium orthophosphate

Glucose-6-phosphate dehydrogenase (G-6-PDH) EC 1.1.1.49

Staining solution: 50 ml 0.1 M Tris-HCl pH 7.5, 50 mg glucose-6-phosphate (monosodium salt), 10 mg NADP, 15 mg MTT, 3 mg PMS, 0.5 ml magnesium chloride

Glutamate dehydrogenase (GDH) EC1.4.1.2

Staining solution: 50 ml 0.1M Tris-HCl pH 7.5,210 mg L-glutamic acid, 10 mg NADP, 15 mg MTT, 1 mg PMS.

Glutamate oxaloacetate (GOT) EC 2.6.1.1

Staining solution: 50 ml 0.1 M Tris-HCl ,18 mg alpha-ketoglutaric acid, 65 mg aspartic acid, 250 mg PVP , 25 mg EDTA (disodium salt), 710 mg disodium orthophosphate, 1 mg pyridoxal-5-phosphate, 200 mg Fast Blue BB.

Hexokinase (HEX) EC 2.7.1.1

Staining solution: 50 ml 0.1 M Tris-HCl pH 8.0, 90 mg glucose, 20 mg EDTA (tetrasodium salt), 10 mg NADP, 15 mg MTT, 1 mg PMS, 0.5 ml 10% magnesium chloride, 65 mg ATP.

Malate dehydrogenase (MDH) EC 1.1.1.37

Staining solution: 50 ml 0.1M Tris-HCl pH 7.5, 1 g malic acid, 12 mg NAD, 10 mg MTT, 3 mg PMS.

Phosphoglucomutase (PGM) EC 2.7.5.1

Staining solution: 50 ml 0.1M Tris-HCl pH 7.5, 100 mg glucose phosphate, 20 units glucose-6-phosphate dehydrogenase, 15 mg ATP, 10 mg NADP, 15 mg MTT, 1 mg PMS, 0.5 ml 10% magnesium chloride.

Phosphoglucose isomerase (PGI) EC 5.3.1.9

Staining solution: 50 ml 0.1 M Tris-HCl pH 8.5, 40 mg fructose-6-phosphate, 20 units glucose-6-phosphate dehydrogenase, 10 mg NADP, 15 mg MTT, 1 mg PMS, 0.5 ml 10% magnesium chloride.

2.2.4.2. Stains used with the Histidine gel system

Isocitrate deydrogenase (ICDH) EC 1.1.1.42

Staining solution: 50 ml Tris-HCl pH 8.0, 50 mg DL-isocitric acid (trisodium salt), 10 mg NADP, 10 mg MTT, 3 mg PMS, 0.5 ml 10% magnesium chloride.

Peroxidase (PER) EC 1.11.1.7

Staining solution: 40 mg 3-amino-9-ethylcarbazole dissolved in 5 ml Dimethyl formanide, 50 ml 0.2 M acetate pH 5.0, 1 ml calcium chloride 0.1 M, 1 ml hydrogen peroxide.

6-phoshogluconate dehydrogenase (6-PGD) EC1.1.1.44

Staining solution: 50 ml 0.1 M Tris-HCl pH 8.0, 50 mg 6-phosphogluconic acid (trisodium salt), 10 mg NADP, 15 mg MTT, 3 mg PMS, 0.5 ml 10% magnesium chloride.

Shikimate dehydrogenase (SkDH) EC 1.1.1.25

Staining solution: 50 ml Tris-HCl pH 8.0, 50 mg shikimate,10 mg NAD, 10 mg MTT, 3 mg PMS.

Succinate dehydrogenase (SuDH) EC1.3.99.1

Staining solution: 50 ml 20 mM sodium phosphate, 210 g succinic acid, 185 mg EDTA (disodium salt), 25 mg NAD, 20 mg MTT, 3 mg PMS, 25 mg ATP.

Xanthine dehydrogenase (XDH) EC1.2.1.3

Staining solution: 50 ml 0.1 M Tris-HCl pH 8.0, 50 mg hypoxanthine, 10 mg NAD, 10 mg MTT, 3 mg PMS.

2.3.1. Polyacrylamide gel Isoelectric Focusing

Analytical isoelectric focusing (IEF) was performed in a horizontal Pharmacia-LKB multiphor apparatus essentially as described by the manufacturer. Ultra-thin polyacrylamide gels 0.1 mm thick containing 4.85% acrylamide and 0.15% of *N,N'*-methylenebisacrylamide were prepared containing 1/16 th final volume ampholines. Ampholines (Pharmalytes, Pharmacia) of differing pH gradients were mixed according to

the manufacturers instructions to generate the required pH gradient The ampholyte mixtures used for each IEF marker system are given in Table 2.1 together with the protein symbols and gene loci.. The anodal electrode buffer was dependent on the pH of the gel used. 0.01 M phosphate was used at pH 3.0 and 0.04 M glutamic acid pH 4.0. The cathodal electrode buffer was 0.02 M sodium hydroxide at pH 10 and 0.2 M histidine at pH 6.0. IEF was performed at a constant power of 1.0 W/cm of gel length and maximum voltage (3000 V). Gels were prerun for 500 V/hours . Following the prerun, samples were applied to the gel on filter-paper wicks and the gels run for a further period of 500 V/hours after which the wicks were removed. Isoelectric focusing was continued for 2500 V/hours after which bands were visualized using histochemical stains. Extracts of barley grains were prepared from crushed whole grains extracted in water at room temperature. Samples were applied to the gel on filter paper wicks wetted in the extraction liquid.

2.3.2. Sample preparation

β-amylase and grain esterase sample extractions.

Portions of endosperm (20 mg) from mature dry grains were hand milled and mixed with 50 µl of distilled water. Samples were left to extract for 1 hour and then centrifuged briefly prior to loading onto the gel.

Table 2.1 Proteins, loci and ampholyte mixtures used for isoelectric focusing.

Protein symbol	EC number	Gene symbol	Chromosome location*	Ampholyte 5% mixture v/v
EST10	EC 3.1.1	*Est10*	3H	pH (4-6.5) (4.2-4.9) (4.5-5.4) 1:1:1
EST3	EC 3.1.1	*Est3*	7H	pH (4-6.5) (4.2-4.9) (4.5-5.4) 1:1:1
β-AMY1	EC 3.2.1.2	β-amy1	4H	pH 4-6.5
ADH1	EC 1.1.1.1	*Adh1*	4H	pH (4-6.5) (4.2-4.9) (4.5-5.4) 1:1:1
WSP2,3		*Wsp2, Wsp3*	5H, 4H	pH (4-6.5) (4.2-4.9) (4.5-5.4) 1:1:1

* Chromosome nomenclature is base on homology in the *Triticeace*.

Water soluble protein and alcohol dehydrogenase.

Portions of endosperm (40 mg) from mature dry grains were hand milled and mixed with 70 µl of 10 mM dithiothreitol (DTT) with sucrose added at a concentration of 200 mg/ml. Samples were left to extract for 1 hour then centrifuged briefly prior to loading onto the gel.

2.3.3. The following staining procedures were used in conjunction with IEF:

Grain esterases (EST3 and EST10).

Gels were incubated in a solution of 50 mg α-napthyl acetate and 100 mg Fast Blue RR salt dissolved in 2 ml dimethyl sulphoxide and made up to 100 ml with 1 M $NaHPO_4$ (pH 7.6) for 30 minutes at 37°C and then destained in 7% acetic acid.

β-amylase (β-AMY-1).

Gels were immersed in 30 g / litre soluble starch for 10 minutes, drained and washed to remove superficial starch then stained with 3% stock iodine (65 g/litre I_2, 1.95 g/litre KI) and 1% acetic acid. Gels were then developed in 3% stock iodine for a further 10 minutes.

Water-soluble protein.

Gels were placed in a solution of of 34.6 g sulphosalicylic acid and 115 g trichloroacetic acid made up to 1 litre, for 15 minutes. Gels were then immersed in a Coomassie Blue R

solution (0.46 g Coomassie Blue R dissolved in 400 ml of 25% absolute ethanol with 7% acetic acid and heated to 70°C) for 10 minutes and then destained in 25% absolute ethanol with 7% acetic acid).

Alcohol dehydrogenase.

Gels were placed in a solution containing 30 mg NAD, 1 ml absolute alcohol, 20 mg MTT, 5 mg PMS and 100 ml 0.05 M Tris (pH 8.6) until dark blue bands developed (30 minutes).

2.4.1. Barley endosperm proteins analysis

Barley endosperm proteins were extracted as described by Smith and Payne (1984). Single grains were crushed between a folded filter paper with a hammer and transfered to an eppendorf tube where they were ground to a fine powder with a micro-pestle. 20 mg of flour was mixed with 0.2 ml extraction buffer (12% glycerol, 2.4% SDS, 75 mM Tris-HCl, 1.7 M 2-mercaptoethanol, 0.12 mg/ml Pyronin-G and 20% dimethylformamide) and incubated for 1 hour at room temperature, followed by boiling for 2 minutes. Following brief centrifugation, the proteins present in the supernatant were separated by SDS-PAGE.

2.4.2. Polyacrylamide Gel Electrophoresis in the presence of Sodium Dodecyl Sulphate (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed using the system of Laemmli (1970). 3% (w/v) polyacrylamide

stacking gels pH 6.8, were cast above 17.5% (w/v) separating gels pH 8.6. The protein extracts (40 µl) were loaded onto the gel and run at a constant current of 10 mA until the pyronin G dye front had run off the gel. Following electrophoresis the proteins were visualized employing a stain consisting of 0.02% (w/v) Coomassie blue R 250 in 25% (v/v) methanol and 10% (v/v) acaetic acid followed by destaining in 25% (v/v) methanol and 10% (v/v) acetic acid.

2.5.1. Bacterial Growth Conditions

All media used in this study have been described previously. Solid media contained 15 g of agar per litre.

2.5.2. LB Medium (Maniatis *et al.* 1982)

Per litre: Bacto-tryptone 10 g, Yeast extract 5 g, Sodium chloride 10 g.

2.5.3. LM Medium

2.5.4. LM medium was LB medium containing 10mM magnesium sulphate

2.5.5. SOB Medium (Hanahan 1983)

Per litre: Bacto-tryptone 20 g, Yeast extract 5 g, Sodium chloride 0.5 g, Potassium chloride 0.1 g. After autoclaving, 10 ml of filter sterilized 1 M magnesium sulphate, 1 M magnesium chloride was added.

2.5.6. TFB Medium (Standard Transformation Buffer) (Hanahan 1983)

10 mM-K-MES (ph 6.2), 100 mM rubidium chloride, 45 mM magnesium chloride, 10 mM calcium chloride, 3 mM cobalt chloride. All salts were added as solids. TFB was sterilized by filtration through a 0.22 µm filter and stored at 4°C.

2.5.7. Antibiotics and other additives to growth media

Antibiotic selective liquid media and plates were prepared by the addition of the appropriate antibiotic stock. In the case of solid media the antibiotic was added to the molten (45-50°C) agar.

2.5.7.1. Ampicillin

A stock solution of 25 mg/ml of ampicillin in water, was sterilized by filtration and stored in aliquots at -20°C. Ampicillin was used at a final concentration of 50 µg/ml.

2.5.7.2. Tetracycline

A stock solution of 12.5 mg/ml tetracycline hydrochloride in ethanol/water (50%v/v) was sterilized by filtration and stored in a light-tight container at 4°C. Tetracycline was used at a final concentration of 12.5 µg/ml.

2.5.7.3. Chloramphenicol

A stock solution of 34 mg/ml chloramphenicol in 100% ethanol was stored as alliquots at -20°C. A final concentration of 170 µg/ml was used for the amplification of plasmids.

2.5.8. Assay for β-galactosidase activity in lac- bacteria

The chromogenic substrate for β-galactosidase, 5-Bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal) was prepared as a 2% (w/v) solution in dimethyl formamide and stored at 4°C. The gratuitous inducer of β-galactosidase activity, isopropylthiogalactoside (IPTG) was prepared as a 2.4% (w/v) solution in water and stored at -20°C. 35 µl of X-gal and 10µl of IPTG were spread across an ampicillin selective plate.

2.5.9. Storage of bacterial strains and plasmids

Bacterial strains were stored as either -20°C working stocks or at -80°C for long term storage. Permanents were prepared from an overnight liquid culture by the addition of either glycerol to 20% for the -20°C permanent or by the addition of dimethylsulphoxide to 7% for the -80°C permanent. Permanents were rapidly frozen in liquid nitrogen prior to storage at -80°C.

Plasmids were stored as either ethanol precipitates of purified DNA or within plasmid bearing strains which were stored as above.

2.5.10. Bacterial transformations

E.coli strain DH5α was transformed with purified plasmid DNA essentially as described by Hanahan (1983). Single colonies were picked from fresh streaks of frozen stock cells grown overnight on LM plates and dispersed in 1 ml of SOB medium. This was used to inoculate 50 ml of SOB medium in a 500 ml flask. The culture was incubated at 37°C, 300 rpm until the cell density was approximately measured to be OD_{550} 0.45-0.55. The cells were collected in 80 ml centrifuge tubes and placed on ice for 10 minutes, and pelleted at 2500 rpm for 5 minutes at 4°C. The cells were resuspended in 1/3 the original volume of cold TFB medium and kept on ice for 10 minutes. The cells were pelleted as before and resuspended in 1/12.5 of the original volume of cold TFB medium. Fresh DMSO was added to 3.5%, swirled and placed on ice for 5 minutes. 2-mercaptoethanol solution containing 750 mM 2-mercaptoethanol, 10 mM-K-MES (pH 6.2) was added (7 µl per 200 µl), swirled and placed on ice for 10 minutes. Another equal portion of DMSO was added, and the cells incubated on ice for 5 minutes. These cells remained 'competent' for up to two days if stored on ice. For transformation approximately 100 ng of DNA was added to 0.2 ml of competent cells. The mixture was heat pulsed without agitation at 42°C for 90 seconds, and placed on ice for 2 minutes after which 800 µl of LB broth was added and the cells incubated at 37°C for 1 hour to allow expression of the plasmids antibiotic resistance determinant. The cells were pelleted and resuspended in LB

broth prior to spread plating onto the relevant antibiotic selective plate.

2.6.1. DNA Manipulations

In general the methods used were those described by Maniatis *et al.* (1982).

2.6.2. General methods and reagents

TE buffer was 10 mM Tris-HCl pH 7.5, 1 mM EDTA. DNA in TE buffer was stored at 4°C. RNase A was prepared by boiling a solution of 2 mg/ml RNase A (Sigma) in 25 mM Tris-HCl, pH 7.5 for 15 minutes to remove any contaminating DNase activity. The enzyme was aliquoted and stored at -20°C. Bovine serum albumin was prepared as a solution of 2mg/ml and sterilized by filtration through 0.22 μm filters.

2.6.3. DNA Precipitations

DNA was precipitated by the addition of 0.6 volumes of isopropanol or by thee addition of 2 volumes of 95% ethanol, 150 mM sodium acetate and incubation at -20°C, followed by centrifugation in either an Eppendorf or Wifuge 500E benchtop centrifuge.

2.6.4. Phenol and Chloroform extractions

Phenol solution was prepared by dissolving 'Analar' grade phenol crystals (BDH) in 1 M Tris-HCl, pH 8.0 with the addition of 8-hydroxy-quinoline to a final concentration of

0.1%. Phenol solution was stored in the dark at 4°C. Phenol extractions were carried out using a mixture of phenol and chloroform (1:1 v/v). Chloroform extractions were carried out using a mixture of chloroform and isoamyl alcohol (24:1 v/v).

2.6.5. Determination of DNA concentration

Determination of DNA concentration was performed by the spot method described by Silhavy *et al.* (1984). 2 µl drops of a dilution series of the unknown DNA were spotted onto agarose containing 0.5 µg/ml ethidium bromide. A series of salmon sperm DNA concentration standards ranging from 1.0 µg/ml to 30 µg/ml in 5 µg increments were also spotted onto the agar. After drying at 37°C for 1 hour, the plates were placed on the transilluminator and the intensity of the unknown DNA solution compared with that of the known, allowing an approximation of the concentration of the unknown DNA sample to be made. This method was suitable for the estimation of relatively crude extracts and was not sensitive to contaminating phenol or proteins.

2.6.6. Spectrophotometric Determination of DNA and RNA concentration

Spectrophotometric determination of DNA and RNA concentration was performed as described by Maniatis *et al.* (1982). Spectrophotometric readings were taken at 260 nm and 280 nm. Concentration was calculated from the reading at 260 nm. An OD of 1 corresponds to approximately 50 µg/ml double

stranded DNA and 40 µg/ml RNA. The ratio of the readings at 260 nm and 280 nm provided an estimation of the purity of the nucleic acids. Pure samples of DNA and RNA have ratios of 1.8 and 2.0 respectively. Samples contaminated with phenol or proteins show significantly lower ratios and concentration cannot be measured using this method.

2.6.7. DNA Isolations

DNA was isolated essentially as described by Murray and Thompson (1980). Total DNA was prepared from 5 g fresh or 500 mg freeze-dried material. Fresh material was frozen in liquid nitrogen and ground to a fine powder in a pestle and mortar followed by the addition of 4.5 ml of 2 times CTAB extraction buffer and 4.5 ml of 1 times CTAB extraction buffer. This was incubated at 65°C for 60 minutes with occasional mixing. The freeze-dried material was ground in a coffee-grinder prior to the addition of 9 mls of 1 times CTAB extraction buffer then treated exactly the same way as the fresh material. Following extraction the crude DNA solution was purified by sequentially extracting with chloroform: isoamylalcohol (24:1 v/v), phenol: chloroform (1:1 v/v) and chloroform: isoamylalcohol (24:1 v/v) twice. The aqueous supernatant was recovered and the DNA precipitated by the addition of 0.6 volumes of isopropanol. The DNA was recovered by centrifugation at 2000 rpm for 2 minutes, vacuum dried and resuspended in 4 ml of TE. RNase A was then added to 10µg\ml and the solution incubated at 65°C for 15 minutes.

The DNA was then precipitated by the addition of 2 volumes 95% ethanol, 150 mM sodium acetate. Following recovery by centrifugation, the DNA pellet was washed with 70% ethanol, briefly vacuum dried and resuspended in 2 mls of TE.

2.6.8. Plasmid DNA isolations

A modified procedure of that described by Maniatis *et al.* (1982) was used for both large scale and small scale 'minipreps' of plasmids. 5ml of LB broth containing the appropriate antibiotic was inoculated with a single bacterial colony and grown at 37°C overnight at 300 rpm in an orbital shaker. Cells were harvested and resuspended in 0.2ml of lysozyme solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 and 5 mg/ml lysozyme), and incubated on ice for 5 minutes. 0.4 ml of 0.2 M sodium hydroxide, 1% (w/v) SDS was added with vortexing and incubated on ice for a further 5 minutes, prior to the addition of 0.3 ml of potassium acetate and incubation at -70°C for 15 minutes. The resulting precipitate was removed by centrifugation in an Eppendorf bench top centrifuge for 15 minutes at top speed. 0.75 ml of the supernatant was removed and 0.45 ml of propan-2-ol added, mixed by inversion and incubated at -70°C for 15 minutes to precipitate the plasmid DNA which was collected by centrifugation in an Eppendorf bench top centrifuge at top speed for 15 minutes. The resulting pellet was washed with 70% ethanol, dried under vacuum and resuspended in 0.1 ml

TE. RNase A was then added to 10 µg/ml and the sample incubated at 65°C to remove contaminating RNA.

For large scale plasmid isolations 200 ml of overnight culture was used. The same procedure, scaled up 40 times was followed.

2.6.9. Restriction endonuclease digestions

Total genomic DNA was digested with the following enzymes *EcoRI*, *EcoRV*, *Bam HI*, *Hind III*, *Bgl II* and *Hae III* in Spermidine Digestion Buffer (SDB) (33 mM Tris-HCl, pH 7.8, 64 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine and 5 mM ditiothreitol). 10 µg aliquots of genomic DNA were digested overnight at 37°C in a volume of 300 µl with 5 times excess of enzyme to ensure complete digestion. Purified plasmid DNA was digested according to the manufacturers instructions using the supplied restriction endonuclease buffers or alternatively, SDB. 1µg of plasmid DNA was usually digested with 1 unit of enzyme at the recommended temperature.

2.6.10. Dephosphorylation of plasmid vector

Calf intestinal phosphatase (CIP) was purchased from Pharmacia. DNA was dephosphorylated according to the manufacturers instructions except SDB was used in place of the supplied reaction buffer. Two units of CIP diluted to 0.1 unit/µl in 1 X SDB were added to 10 µg of plasmid DNA, previously digested to completion with the appropriate restriction

endonuclease. The DNA was incubated in the presence of the CIP for 30 minutes at 37°C, followed by heat inactivation at 85°C for 15 minutes. The DNA was purified by phenol extraction and ethanol precipitation.

2.6.11. Ligations

EcoRI terminated cDNAs were ligated into *EcoRI* dephosphorylated pUC18. A series of dilutions of the cDNA containing column effluent were mixed with 20 ng of vector. The combined DNA samples were precipitated by addition of 2 volumes 95% ethanol, 45 mM sodium acetate and incubation at -20°C for 2 hours. Following recovery by centrifugation, the DNA pellet was washed with 70% ethanol, briefly vacuum dried and resuspended in 1 X SDB with 1 µg/µl BSA 5 mM ATP and 10 units T4 DNA ligase. Ligation was carried out overnight at 12°C.

2.6.12. Neutral agarose gel electrophoresis

10 µg aliquots of digested genomic DNA were electrophoresed on 1% agarose gels for 16-18 hours at 75 V. Lambda DNA digested with *Hind III* was used to provide size markers. Following electrophoresis the gels were stained with 0.5 µg/ml ethidium bromide, briefly destained, examined on the transilluminator and the position of the lambda fragments marked with india ink.

2.6.13. Southern Transfer

The gel was then either blotted onto nylon membrane (Hybond N+, Amersham) using alkaline transfer (Reed and Mann, 1985) or denatured with 0.5 M NaOH/1.5 M NaCl for 15 minutes followed by 30 minutes neutralization in 0.5 M Tris-HCl, pH 7.5/1.5M NaCl and transfered using 20X SSC as the transfer buffer (Southern,1975). After blotting the membranes were rinsed in 6 X SSC exposed on the transilluminator for 2 minutes, dried and baked at 80°C for two hours to fix the DNA to the membrane.

2.7. RNA extractions

2.7.1. Guanidine Hydrochloride extraction

Total RNA was extracted using a modification of the protocol described by Logemann *et al.* (1987). 20 g of young etiolated shoots were frozen in liquid nitrogen and homogenized to a fine powder in a mortar and pestle followed by the addition of 2 volumes of guanidine buffer. Guanidine buffer is 8 M guanidine hydrochloride, 20 mM MES (4-morpholineethan-sulphonic acid), 20 mM EDTA, and 50 mM 2-mercaptoethanol at pH 7.0. The extract was centrifuged at 4000 rpm for 10 minutes to remove plant debris. The supernatant was extracted sequentially with phenol: chloroform (1:1) and three times with chloroform: isoamylalcohol (24:1). The RNA was recovered by centrifugation at 4000 rpm for 10 minutes, washed twice with 3 M sodium acetate, once with 70% ethanol

and briefly vacuum dried before being resuspended in 5 ml sterile distiled water. CsCl was added to a final concentration of 2 g/ml and the RNA pelleted by isopicnic ultracentrifugation at 35000 rpm for 18 hours. The resulting RNA pellet was stored as an ethanol precipitate at -70°C until ready for use when it was washed with 70% ethanol, vacuum dried and resuspended in 1ml TE buffer.

2.7.2. Lithium Chloride extraction

Total RNA was isolated using the Lithium Chloride protocol (Guerineau, personal communication). Young shoots were frozen in liquid nitrogen and homogenized to a fine powder in a mortar and pestle followed by the addition of 5 volumes of TLES extraction buffer, (50 mM Tris-HCl pH 9.0, 5 mM EDTA, 150 mM lithium chloride, 5% (w/v) SDS). Following two phenol:chloform extractions, nucleic acids were precipitated by the addition of 2.5 volumes of absolute ethanol and incubation at -80°C for 45 minutes. The precipitate was recovered by centrifugation, washed with 70% ethanol (v/v), dried briefly under vacuum and resuspended in sterile distiled water. RNA was selectively precipitated by the addition of lithium chloride to 2 M and incubation of the sample in iced water for 30 minutes. Following centrifugation, washing with 70% ethanol (v/v) and drying, the RNA was resuspended in sterile distilled water.

2.7.3. Formaldehyde Gel Electrophoresis

The quality of the isolated total RNA was analysed by electrophoresis in 1.5% formaldehyde gels by a modified procedure of that described by Maniatis *et al.* (1982). The electrophoresis buffer was 1 X MOPS gel buffer (10 X MOPS gel buffer was, 0.4 M morpholinopropanesulpfonic acid (MOPS), 100 mM sodium acetate and 10 mM EDTA), with 80 ml/litre of formaldehyde added. The gel buffer contained 2.2 M formaldehyde and 1 X MOPS gel buffer. Samples to be run on the gel were prepared by the addition of 3 volumes of sample buffer (see below), incubated at 65°C for 5 minutes then immediately transfered on to ice. 0.1 volume of loading buffer was added and the sample loaded onto the gel. Electrophoresis was carried out at 100 V until the bromophenol blue dye front reached the end of the gel. Following electrophoresis the gel was stained for 1 hour with 0.01% Toluidine Blue and destained by diffusion in water overnight.

Sample Buffer: 0.2 ml MOPS gel buffer, 0.35 ml formaldehyde, 1.0 ml formamide.

2.7.4. Isolation of mRNA

Poly-adenylated RNA was selected using two cycles of selection on oligo (dT)-cellulose columns. Oligo (dT)-cellulose columns with a bed volume of 1ml were poured in sterile 1ml disposable syringes (Maniatis *et al.*, 1982). The columns were washed with 2.5 ml 0.1 M sodium hydroxide followed by

equilibration with 5 ml TE, 0.5 M sodium chloride. Total RNA was resuspended in 1 ml TE, denatured at 65°C for 5 minutes, followed by rapid cooling on ice. 1 ml of 1M sodium chloride was added and the mixture applied to the column. The effluent was collected and reapplied to the column followed by washing with 2.5 ml TE, 0.5 M sodium chloride. The RNA was eluted by the application of 1.5 ml TE buffer collecting 0.2 ml fractions. RNA-containing fractions were identified by mixing 2 µl of each fraction with 20 µl ethidium bromide solution (1 µg/ml). Samples were spotted onto 'clingwrap,' placed on the transilluminator and the RNA-containing fractions, identified by their fluorescence, pooled. The pooled fractions were heated to 65°C for 5 minutes, chilled on ice and 1 volumes of 1 M sodium chloride added. Following the same procedure the sample was applied to the second column, the effluent reapplied, RNA eluted with TE buffer and the RNA-containing fractions pooled. RNA was precipitated by the addition of 0.2 volumes 2 M NaCl and 3 volumes of cold ethanol and stored at -70°C until required.

2.8. cDNA Synthesis

EcoRI tailed cDNAs were synthesized from purified mRNA using a cDNA synthesis kit (Pharmacia).

2.9. High Resolution Denaturing Polyacrylamide Gel Electrophoresis.

Denaturing polyacrylamide gel electrophoresis was performed as described by Maniatis *et al.* (1982). RNA samples and DNA digests with 4 base-pair cutting enzymes were run on 5% (w/v) polyacrylamide gels containing 1 X TBE and 8 M urea. Gels were cast on custom made gel apparatus: dimensions 1.0 mm X 20 cm X 40 cm. DNA and RNA samples were ethanol precipitated and resuspended in 10 µl sample buffer heated to 65°C and rapidly cooled on ice prior to loading. The gel was run at a constant voltage of 700 V until the xylene cyanol dye front had run off the gel.

Sample Buffer: Formamide 100 µl, Xylene cyanol 23 µl, 1M sodium hydroxide 12 µl, 0.4 M EDTA 0.6 µl.

2.10. Electroblotting

DNA and RNA run on denaturing high resolution polyacrylamide gels was transfered to nylon membrane by electroblotting.

2.11. Isolation of DNA probe sequences

Plasmid inserts of 2 kb or less were first separated from the plasmid vector by restriction enzyme digest and by gel electrophoresis. Probe sequences were recovered from the gel by electroelution either as described by Maniatis *et al.* (1982) or using an IBI unidirectional electroluter (IBI USA). Probe

inserts present within pUC plasmids were isolated by use of the M13 sequencing primers as primers for polymerase chain reaction (PCR) multiplication.

2.12. Polymerase Chain Reaction (PCR)

Polymerase chain reaction amplification of cloned cDNA sequences was carried out as described by Saiki *et al.* (1988) using a Programable Dry Heating Block (Hybaid, Middlesex, UK). PCR amplification of pUC plasmid insert was accomplished by the use of the M13 plus and minus strand sequencing primers flanking the multiple cloning site. Taq polymerase was obtained from either IBI or Boeringer Mannheim. The PCR reactions were carried out in the buffer supplied by IBI (10 X Taq reaction buffer:100 mM Tris-HCl, pH 8.3, 500 mM potassium chloride,15 mM magnesium chloride, 0.1% (w/v) gelatin, 0.1% (v/v) Tween20 and 0.1% (v/v) NP40). The reaction was carried out in 1 X Taq reaction buffer with the addition of 1 µg/ml M13 of each sequencing primer, each of the four deoxynucleotide-triphosphates to 2 mM and 2 units of Taq polymerase in a final volume of 100 µl and were overlaid with 50 µl of mineral oil. DNA template for the reaction was supplied either as 20 ng of purified plasmid or as 1 µl of cell suspension from a bacterial glycerol permanent. Thirty cycles of the PCR reaction were routinely used in the amplification of cloned cDNA inserts. Each cycle consisted of denaturation of the DNA template at 96°C for 1.5 minutes, annealing of the primers at 56°C for 1 minute followed by primer extension at 72°C for

1.5 minutes. Amplification was completed by a final incubation at 72°C for 5 minutes. PCR products were purified by column chromatography as described previously. Following ethanol precipitation, two washes in 70% ethanol and vacuum drying, each sample was resuspended in 0.1 ml TE, 10 µl analysed on 1% agarose gel and DNA concentration estimated by comparison with the lambda marker.

2.13. Randomly Amplified Polymorphic DNA markers (RAPDs).

PCR amplification of genomic DNA with single 10-mer oligonucleotides to generate Randomly Amplified Polymorphic DNA markers was carried out essentially as described by Williams *et al* 1990. PCR reactions contained approximately 2.5-25 ng DNA and were, 100 µM dNTPs, 200 nM primer, 1 X Taq reaction buffer and 1.5 units taq polymerase. Reactions were run for 45 cycles of 1 minute at 92°C, 1 minute at 36°C, and 2 minutes at 72°C. Amplification was completed by a final incubation at 72°C for 5 minutes. Products were analysed on 1.5% or 2% agarose gels.

2.14. Random priming radiolabelling

DNA probes were labelled with 30 µCi 32P-dCTP (3000 Ci/mM, ICN) as described by Feinberg and Vogelstein (1982, 1984), except that random octamers were used in place of random hexamer primers. Approximately 50 ng of DNA was labelled per reaction. Incubation was carried out for 12-18 hours at 15°C, after which labelled probe was separated from

unincorporated nucleotides by chromatography on Sepharose CL 6B (Maniatis *et al.*, 1982).

2.15. Polynucleotide Kinase (PNK) labelling of oligonucleotides

PNK end-labelling of dephosphorylated oligonucleotides was performed essentially as described by Maniatis *et al.* (1982). The reaction was carried out in 1 X PNK reaction buffer(100 mM Tris-HCl, pH 7.6, 20 mM magnesium chloride, 10 mM dithiothreitol, 2 mM spermidine and 1 mM EGTA), with the addition of 70 µCi gamma-^{32}P ATP (4500 Ci/mM) and 5 units of PNK. Approximately 150 ng of DNA was labelled per reaction. Incubation was carried out for 30 minutes at 37°C, after which labelled probe was phenol extracted and ethanol precipitated.

2.16. Prehybridization and hybridization conditions

Prehybridization and hybridization reactions were carried out in a hybridization oven (Hybaid, Middlesex, UK) at 65°C. Blots were prehybridized for several hours at 65°C in 50 ml of 1.5 X SSPE, 0.5% dried milk powder, 1% SDS, 6% PEG and 30 µg/ml denatured salmon sperm DNA. The prehybridization solution was then removed and replaced with 25 ml of hybridization solution, which is the same as prehybridization solution but with the addition of denatured radioactive probe. Hybridization was carried out overnight at 65°C.

2.17. Post Hybridization Conditions

Following hybridization the membranes were washed twice for 15 minutes in 2 X SSC, 0.5% SDS and twice for 15 minutes in 2 X SSC, 0.1% SDS at room temperature. For probes detecting high copy number genes or repeat sequences a further stringent wash of 0.1 X SSC, 0.1% SDS at 65°C for 15 minutes was required. Blots were prepared for reuse by washing in 0.4 M NaOH at 45°C for 15 minutes followed by neutralization in 0.2 M Tris-HCl, pH 7.5, 0.1 X SSC at 45°C for 30 minutes.

2.18. Autoradiography

Autoradiography was carried out at -80°C using Fuji RX X-ray film and Kodak X-omatic intensifying screens.

2.19. Materials

All chemicals used were of the highest grade commercially available and were obtained from BDH Chemicals LTD. (Poole, Dorset, U.K.). unless otherwise stated. Yeast-extract, Tryptone and Bi-Tek agar were obtained from Difco Laboratories (Detriot, Michigan,USA). Antibiotics, RNase A, DNase, T4 DNA Ligase, Calf Intestinal Phosphatase, Lysozme, X-gal, IPTG, Sepharose CL 6B, Hepes, Urea, Hexadecyltrimethyl-ammonium bromide(CTAB), Dimethylformamide, Hydrolysed potato starch and Bovine Serum Albumin were obtained from Sigma (London) Chemical Co. (Kingston upon Thames, Surry,

U.K.). Restriction endonucleases and DNA Polymerase klenow fragment were obtained from either Pharmacia Fine Chemicals AB., (Sweden), BRL (Gaithersburg, MD,USA) or Boheringer Mannheim. Hybond N+ nylon charged nylon membrane was obtained from Amersham International (Amersham, U.K.). Radioisotope was obtained from ICN., Taq polymerase and Taq polymerase reaction buffer were Taq polymerase was also obtained from BRL (Gaithersburg, MD, USA). cDNA synthesis kit was obtained from Pharmacia Fine Chemicals AB., (Sweden).

Chapter Three

The Identification and Use of Polymorphic Marker Systems for Varietal Identification in Barley

3.1. Introduction

The large numbers of barley varieties grown and traded in the UK require systems for testing and registering varieties. Each variety must conform to a set of requirements of distinctness, uniformity and stability (DUS). Varietal identification requires detailed morphological descriptions and field comparisons of seed stocks (Eade and Law, 1983). Identification on the basis of seed characters alone is presently the most important method in the regulation of trade in barley grain and malts. Hordein protein separation using polyacrylamide gel electrophoresis is currently the only electrophoretic technique which has been adopted for the commercial classification of barley varieties (Cooke and Morgan, 1986). The advantages of this over more conventional methods are that it is carried out on the endosperm and hence the embryo can be retained, only small samples are required and importantly the storage protein patterns produced are largely unaffected by the growing conditions of the plants. Although analysis of the hordein protein fraction has proved useful, additional criteria are generally required for the unequivocal identification of barley varieties.

Several alternative marker systems have been proposed for use in barley varietal identification. These include various isozyme and protein systems based on both starch and isoelectric focusing gel electrophoresis techniques (Almgard and Landegren, 1974; Bassirri, 1976; Anderson, 1982;

Thompson *et al.*, 1990). The use of RFLPs at the hordein loci have also been evaluated for 'fingerprinting' barley (Bunce *et al.*,1986).

In addition to the techniques reported in barley, DNA 'minisatellite' probes, have been demonstrated to detect a distinct set of hypervariable minisatellites in a range of organisms and have been used to 'fingerprint' genera and species in the angiosperm family *Rosaceae* (Vassart *et al.*, 1987; Rogstad *et al.*,1988; Nybom *et al.*, 1989). Identification based on PCR techniques are is currently being evaluated in a range of crop species.

The objective of this study was to evaluate the marker systems available for their ability to detect useful polymorphism in a range of barley accessions and to determine their potential value for use in varietal identification and in subsequent genetical studies.

3.2. Survey of isozyme polymorphism using Starch Gel Electrophoresis

The isozyme systems investigated are presented in Table 3.1 and these were used to investigate the level of polymorphism in thirty accessions of barley (Table 3.2), including 19 cultivars, two breeding lines of *H. vulgare*, and 9 accessions of *H. spontaneum*. Five enzyme systems detected polymorphism in the genotypes studied. The phenotypes obtained for each of the polymorphic isozyme systems are

Table 3.1. Isozyme systems investigated using starch gel electrophoresis.

Marker symbol	Marker
AAT	Aspartate aminotransferase
ACP	Acid Phoshatase
ADH	Alcohol dehydrogenase
ALD	Aldolase
CAT	Catalase
Leaf-est	Leaf-esterase
G6PD	Glucose-6-phosphate dehydrogenase
GADH	Galactose dehydrogenase
GDH	Glutamate dehydrogenase
GO	Glucose oxidase
HEX	Hexokinase
IDH	Isocitrate deydrogenase
LAP	Leucine aminotransferase
MDH	Malate dehydrogenase
ME	Malic enzyme
PER	Peroxidase
PGI	Phosphglucose isomerase
PGM	Phosphogluco mutase
6PGD	6-Phoshogluconated dehydrogenase
SKDH	Shikimate dehydrogenase
SUDH	Succinate dehydrogenase
XDH	Xanthine dehydrogenase

Table 3.2. Starch gel electrophoresis isozyme phenotypes of 30 barley accessions.

H. vulgare	Leaf-est	6PGD	AAT	PGI	ADH
Blenheim	1	1	1	1	1
Corniche	1	1	1	1	1
Digger	1	1	1	1	1
Doublet	1	1	1	1	1
Ermo	2	1	1	1	1
Gimple	2	1	1	1	1
Golden Promise	2	2	1	1	1
Halcyon	1	2	1	1	1
Heriot	2	1	1	1	1
Joline	1	1	1	1	1
Kaskade	1	1	1	1	1
Magie	2	1	1	1	1
Marinka	1	1	1	1	1
Natasha	2	1	1	1	1
Panda	2	1	1	1	1
Pipkin	1	2	1	1	1
Plaisant	1	2	1	1	1
Triumph	1	1	1	1	1
Tyne	1	2	1	1	1
E224/3	1	2	1	1	1
TS264/22	1	1	1	1	1
H. spontaneum					
H.S.2	1	2	2	1	1
H.S.4	3	2	1	1	1
H.S.6	1	2	1	2	1
H.S.11	4	2	1	1	2
H.S.16	4	1	1	1	1
H.S.19	5	2	1	1	1
H.S.24	1	2	1	1	1
H.S.36	1	2	1	1	1
H.S.38	1	2	1	1	1
Total Number of phenotypes	5	2	2	2	2

summarised in Table 3.2. Of these systems only leaf-esterase and 6-phosphogluconate dehydrogenase detected polymorphism in both *H. vulgare* and *H. spontaneum.* Aspartate amino transferase, phosphoglucose-isomerase and alcohol dehydrogenase only detected polymorphism within *H. spontaneum.* The polymorphism detected was, however, limited to an individual accession. The esterase system was markedly more variable than the other systems. In addition to the two esterase phenotypes detected in *H. vulgare*, three additional phenotypes were found in the *H. spontaneum* lines. Examples of the phenotypes obtained for the polymorphic isozyme systems are shown in Figure 3.1.

The remarkably low level of polymorphism detected in barley cultivars using starch gel electrophoresis is the major limitation to the application of this technique to both breeding and genetic research. The selection of barley cultivars investigated in this study represent a reasonably broad cross-section of commercial cultivars, comprising both winter and spring types. Yet on the whole their isozyme phenotypes were remarkably similar. Of the 22 enzyme systems investigated, only two showed the presence of allelic differences among the 19 barley cultivars. Leaf-esterase and 6-phoshogluconate dehydrogenase, while both detecting differences, were each able to distinguish only two allele types amongst the barley cultivars, and as such are limited in their usefulness. Several other isozyme systems have been investigated using starch gel electrophoresis. The most notable of these is based on seed

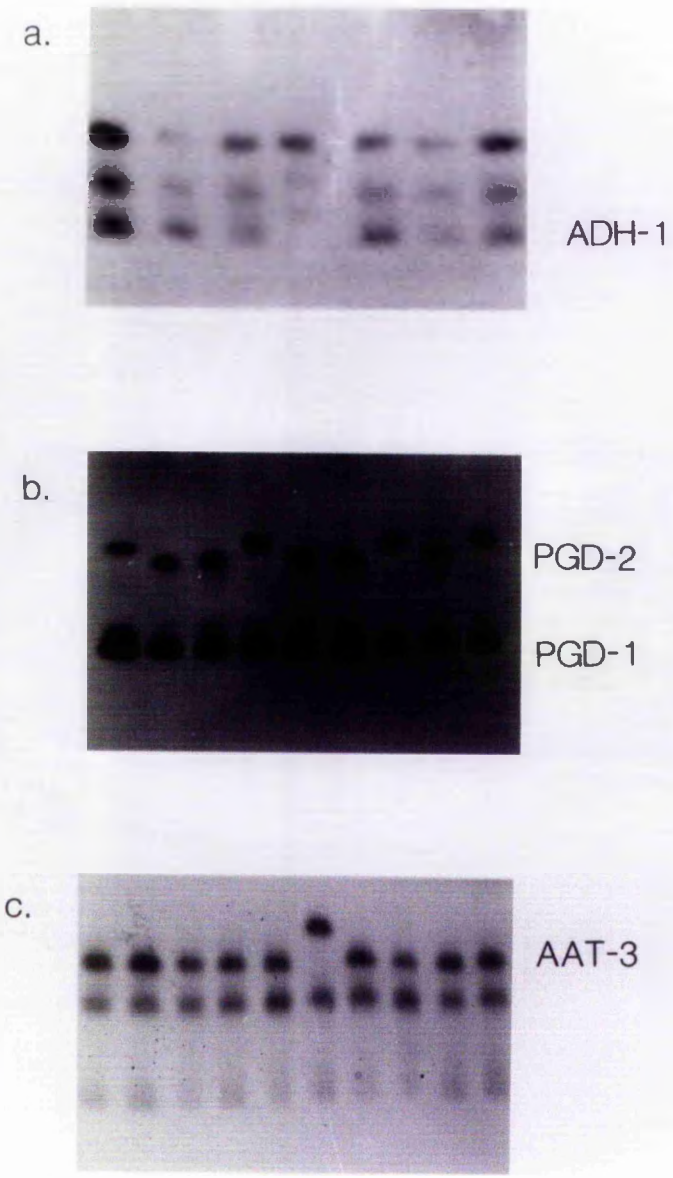


Figure 3.1. Examples of polymorphic starch isozyme systems, a) alcohol dehydrogenase (ADH), b) phosphoglucose dehydrogenase (PGD), c) aspartate amino transferase (AAT).

esterase, which has been found to be polyallelic and may comprise upwards of 10 loci (Hivid and Neilsen, 1977). Although the grain based isozyme system was not considered in this study the range of isozymes and cultivars studied give a broadly representative indication of the low level of isozyme polymorphism detectable using starch gel electrophoresis.

3.3. Comparison of Starch Gel Electrophoresis and Isoelectric Focusing

Isoelectric focusing has a number of advantages over starch gel electrophoresis. A considerable improvement in the resolution of protein bands is possible as the flat-bed cooling apparatus used allows a much higher voltage to be applied across the gel. In addition, the sample is separated according to its isoelectric point rather than its overall electrical charge. This results in an increase in the definition of the protein bands obtained, and any further migration in the gel beyond the point of focusing is minimised. As a direct consequence of this, it is generally possible to resolve a greater number of component bands which would co-migrate to the same point using conventional electrophoresis.

Following the detection of only low levels of isozyme polymorphism in the European cultivars of barley using starch gel electrophoresis, a comparison of the resolving capabilities of isoelectric focusing and starch gel electrophoresis was made. Leaf-esterase and 6-phosphogluconate dehydrogenase were initially investigated as they were polymorphic using starch gel

electrophoresis. In addition, three further systems were compared. Two of these, phosphoglucose-isomerase (PGI), and alcohol dehydrogenase (ADH), detected allelic variants in *H. spontaneum,* while the third, shikimate dehydrogenase (SKDH), was monomorphic in all the accessions studied. All the IEF samples were prepared from extracts of individual crushed grain, with the exception of, leaf-esterase samples which were prepared from the first leaf of newly germinated barley grains (see Materials and Methods chapter). Thus, although this comparison is confounded with tissue source, tissue specific expression of these enzymes have only been reported for alcohol dehydrogenase. Thus the same isozyme loci were evaluated despite the use of different tissue (Brown, 1983).

Of the five enzyme systems used to compare starch and isoelectric focusing gel electrophoresis only leaf-esterase and 6PGD showed polymorphism in the cultivars examined (Table 3.2). The range of phenotypes detected using both starch and IEF gel electrophoresis for leaf esterase are shown in Figure 3.2. Five phenotypes were detected using IEF compared to two using starch. The various phenotypes identified with each system are given in Table 3.3. The variation detected for leaf esterase probably represents three loci located on the short arm of chromosome 3H. Although further segregation analysis is required to resolve individual loci, the IEF approach is capable of resolving a greater number of phenotypes for this particular isozyme than starch gel electrophoresis. In this case IEF is capable of resolving a greater number of putative loci.

Table 3.3. A comparison of leaf-esterase phenotypes detected by IEF and Starch gel electrophoresis.

H. vulgare	Leaf-est	
	IEF	Starch
Blenheim	A	1
Corniche	D	1
Digger	C	1
Doublet	C	1
Golden Promise	B	2
Halcyon	E	1
Magie	D	2
Marenka	D	1
Natasha	B	2
Panda	B	2
Pipkin	C	1
Plaisant	D	1
Triumph	D	1
Tyne	E	1

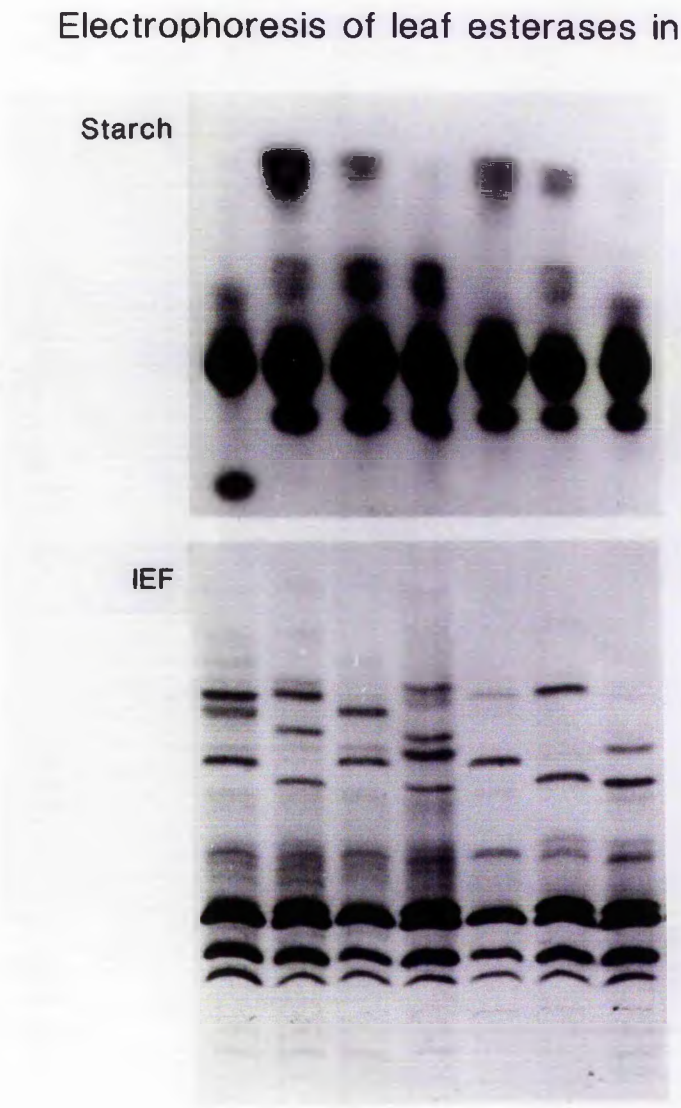


Figure 3.2. A comparison of the resolutions achieved using starch and isoelectric focusing gel electrophoresis to assay the leaf esterases in barley.

Further indication of the increased resolution of the IEF system is clear from the shikimate dehydrogenase gels (Figure 3.3.). Using starch gel electrophoresis, shikimate dehydrogenase produces a single monomorphic band. Using IEF it was resolved into six discrete bands. It was however still monomorphic, even though a much greater number of bands were resolved. Similarly, although a greater level of resolution for both alcohol dehydrogenase and phosphoglucose-isomerase was demonstrated using IEF, both systems remained monomorphic for all the cultivars studied. Thus, in this study the greater resolution with IEF did not result in a greater level of detectable polymorphism. However, when polymorphic systems have been identified IEF would appear to offer advantages over starch gel electrophoresis. In comparing the two, further evidence of the increased resolution of IEF comes from studies with α-amylase, β-amylase and grain esterases. Thompson *et al.* (1990) reported an increased number of phenotypes using IEF compared to those reported by Neilson and Johansen (1986) using starch gel electrophoresis. Two β-amylase and grain-esterase phenotypes were identified using starch compared to four using IEF and two α-amylase phenotypes were identified using starch compared to three using IEF.

The separation of the protein and isozyme bands according to their isoelectric point rather than their electrical charge, and the large number of bands obtained, enables phenotypes to be described as distinctive banding patterns

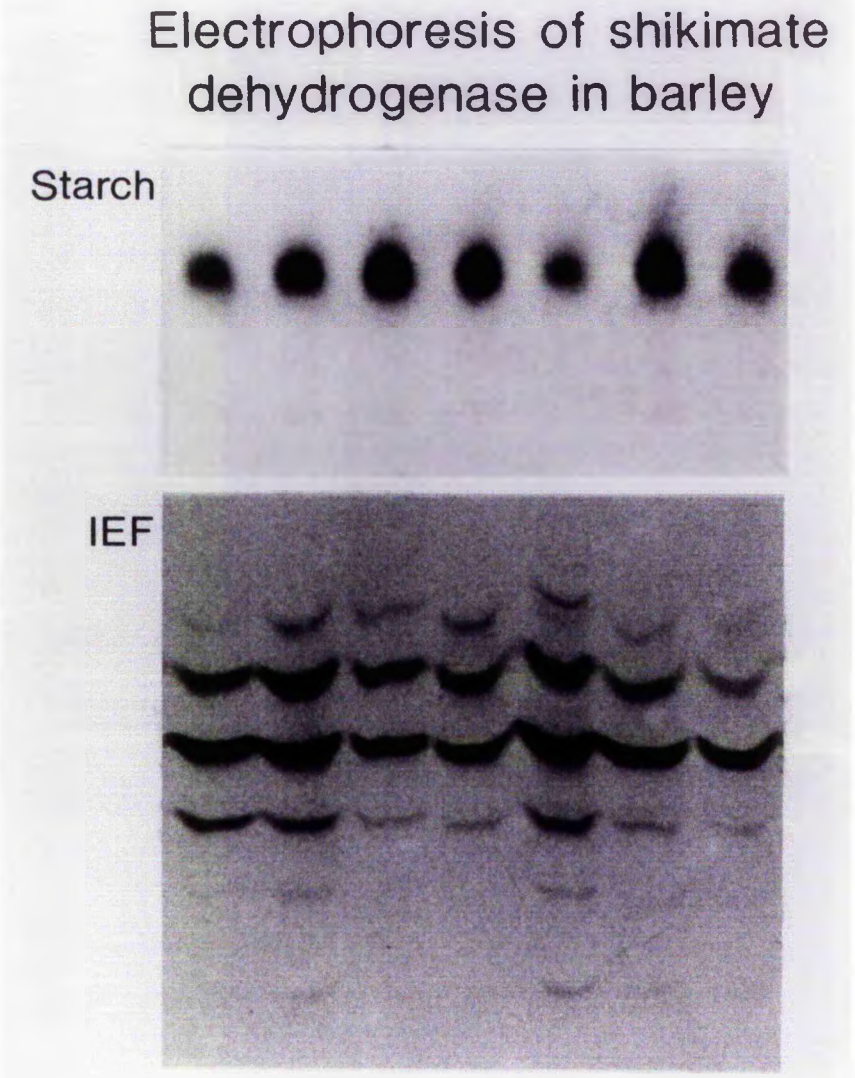


Figure 3.3. A comparison of the resolutions achieved using starch and isoelectric focusing gel electrophoresis to assay the shikimate dehydrogenase in barley.

rather than as relative mobilities. As a consequence, IEF systems are reproducible and standardisation between gels is readily obtainable. This offers the most important advantage of IEF over starch gel electrophoresis particularly when comparing the derived pattern with known phenotypes in varietal identification. In addition the greater resolution of IEF may prove to be useful for mapping isozyme loci to specific chromosomes using wheat barley addition lines.

3.4. Electrophoretic characterisation of the storage proteins of barley grains

Electrophoretic characterisation of the storage proteins of barley grains have proved to be the single most powerful tool in varietal identification (Shewry *et al.*, 1981). The test is not only attractive to seed merchants and commercial users, but also to seed registration and testing authorities in defining the distinctive features of the variety. The test is readily carried out on the endosperm providing a suitable method for assessing the homogeneity of seed stocks. Numerous electrophoretic methods are available but of these acidic polyacrylamide gel electrophoresis (Marchylo and Laberge, 1980; Gebre *et al.*, 1986) and SDS-PAGE (Shewry *et al.*, 1978; Smith and Simpson, 1983; Smith and Payne, 1984) provide the best resolution. The advantages of these methods are that they are used on the seed, the commercially important part of the plant; only small samples are required and the electrophoretic patterns are largely unaffected by growing conditions. Cooke *et*

al. (1983), classified 68 barley varieties into 19 groups using acid polyacrylamide gel electrophoresis of hordeins, while Marchylo (1987) classified 100 barley varieties into 65 groups based upon silver stained SDS-PAGE of their B, C and D hordeins.

The twenty accessions of barley listed in Table 3.4 were characterised according to their storage protein phenotypes. Total storage protein was prepared from individual grains according to the method of Smith and Payne (1984). This method solubilizes most of the barley storage proteins present but is not specific for the hordein proteins. The phenotypes obtained following SDS-PAGE are shown in Figure 3.4.a. Six phenotypes were detected in the twenty accessions, although only four were present within the *H. vulgare* cultivars. The ability of this system to distinguish between cultivars is an improvement over many of the isozyme systems evaluated but is still limited. However the method of storage protein analysis used in this study may be less discriminating than other systems employing hordein specific extraction techniques (Shewry *et al.*, 1980).

The exploitation of hordein polymorphism for varietal identification may be restricted due to the linkage of a mildew resistance gene complex (Mla) to the hordein loci (Shewry *et al.*, 1981). Selection for mildew resistance by barley breeders may therefore result in an artificially low level of hordein variability. Problems may also arise due to the number of

Table 3.4. 20 accessions of barley selected for use in molecular studies.

Accession	Growth habit
Doublet	Spring
Tyne	Spring
Prisma	Spring
Golden Promise	Spring
Regatta	Spring
Triumph	Spring
Klaxon	Spring
Blenheim	Spring
TS264/3	Spring
E224/22	Spring
Maris Otter	Winter
Halycon	Winter
Igri	Winter
Plaisant	Winter
Panda	Winter
Gerbil	Winter
HS.33	-
HS.28	-
HS.24	-
HS.26	-

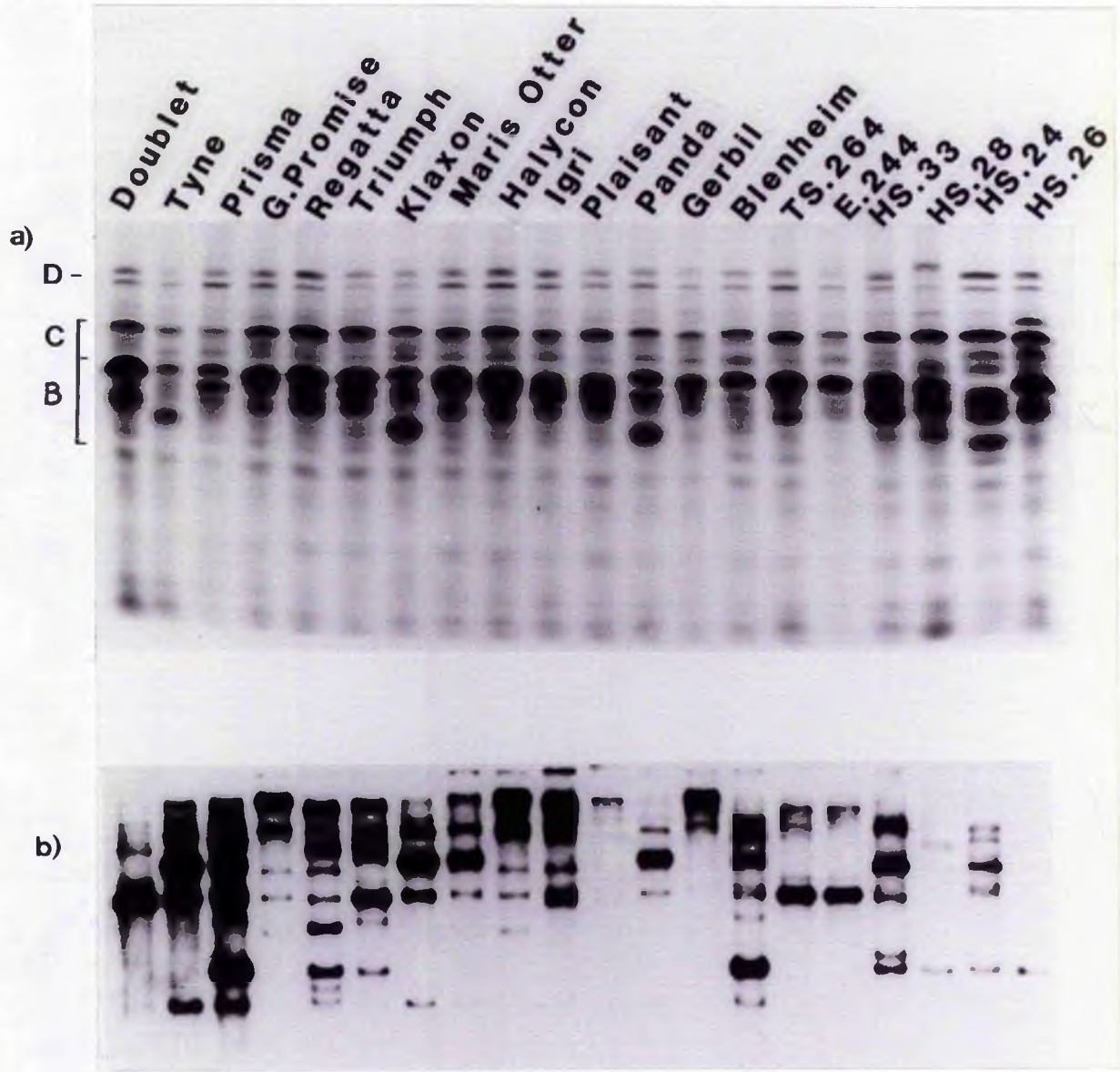


Figure 3.4. a) SDS-PAGE of hordein fractions extracted from single seeds from 20 accessions of *H. vulgare* and *H. spontaneum*. b) Hind III digests of total genomic DNA from the same accessions hybridized with the B-hordein probe pB11.

biotypes present within barley cultivars. Thus although polymorphism at the hordein loci provides a useful tool for cultivar discrimination, further assay methods are required to complement this approach.

3.5. Restriction Fragment Length Polymorphism Analysis of Barley

RFLPs at the *Hor2* locus were also studied by 'Southern blot' analysis using a barley B-hordein cDNA clone pB11 (see Table 3.5.). Hind III and EcoRV digests of DNAs from 20 different accessions (Table 3.4) were examined. The pB11/Hind III probe enzyme combination identified nine phenotypes within the 16 *H. vulgare* accessions while the four *H. spontaneum* accessions each had an unique phenotype (Figure 3.4.b.). Similarly, with the EcoRV/pB11 probe enzyme combination ten phenotypes were identified within the 16 *H. vulgare* accessions while the four *H. spontaneum* accessions again had unique phenotypes. Comparison of the SDS-PAGE and the RFLP phenotypes of the hordeins shown in Figures 3.4.a. and 3.4.b. demonstrates the advantage of the RFLPs over conventional protein markers in their ability to detect a greater level of polymorphism at the same locus.

It is clear from the results presented that the barley B-hordein probe (pB11) detects a greater degree of polymorphism than 1 dimensional SDS-PAGE. The use of the hordein DNA probe with a range of enzyme digests of the DNA further demonstrates the potential variation detectable using

Table 3.5. Probe information.

Clone	Source Plant Material	Probe	Reference
pB11	barley	B-hordein	Forde *et al.* 1985.
pcP387	barley	C-hordein	Forde *et al.* 1985.
pBG35	flax	ribosomal repeat DNA unit	Gouldsbrough and Cullis, 1981
pHvc 1-10	barley	Chloroplast	Day and Ellis, 1985
pbNIRp10	barley	Nitrate reductase	Cheng *et al.* 1986

RFLPs and the ability to use hordein probes to discriminate between cultivars which had shown identical hordein patterns using SDS-PAGE. Bunce *et al.* (1986), reported similar findings and showed that the hordein probes could even discriminate between cultivars with the same hordein phenotype using two dimensional SDS-PAGE. The most important limitation to this technique is that the DNA used in 'Southern blot' analysis is generally prepared from fresh tissue, unlike hordein protein analysis where the sample is prepared from an individual grain. The DNA extraction procedures that are available for use with dried endosperm require bulked material from several grains to produce enough DNA for analysis.

In addition to the use of the medium copy number hordein probe, a range of high and low copy number probes were used (see Table 3.5.). EcoRI, BamHI, Hind III and EcoRV digests of the twenty accessions were probed with the barley nitrate reductase clone pbNIRp10, the flax ribosomal probe pBG35 and a selection of anonymous barley cDNA probes generated for this study. The phenotypes obtained for the barley nitrate reductase probe with BamHI digested DNA are shown in Figure 3.5. Three phenotypes were present within the 16 barley cultivars. pBG35 was used to detect variation in the intergenic spacer of the rRNA genes following SacI digestion of the barley DNA (Saghai-Maroof *et al.*, 1984). Three phenotypes were detected in the barley cultivars (Figure 3.6.). In addition to the clones of known function, a selection of 20 anonymous cDNA clones were screened with the twenty accessions using a

Figure 3.5. RFLP variation in 20 accessions of barley for the barley nitrate reductase clone pbNIR10.

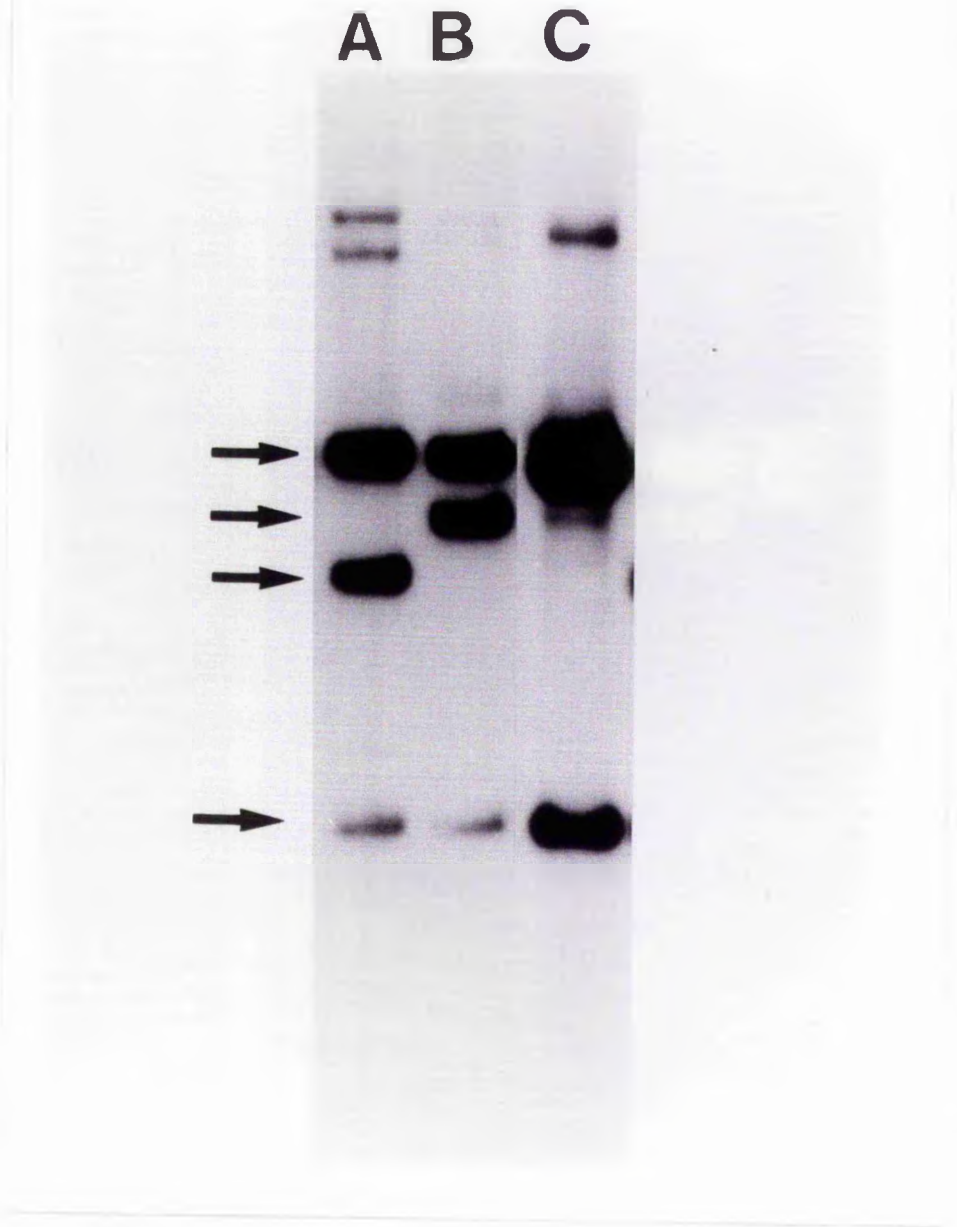


Figure 3.6. RFLP ribosomal DNA phenotypes observed in the 16 *H. vulgare* accessions examined.

range of enzymes. None of the cDNA clones detected polymorphism within the *H. vulgare* lines. The most likely explanation for the inability of the cDNA library to detect polymorphism is that the quality of the library was poor. The only cDNA probe from the library to detect an RFLP was pBC247, which was found to be polymorphic in the *H. spontaneum* line HS.26.

RFLPs offer the most promising method for characterising and identifying cultivars unequivocally. The advantage RFLPs have over conventional methods is that they are generally capable of detecting a much greater level of polymorphism within the genome. The large number of probe enzyme combinations means that almost the whole genome can be covered. The drawback, however, is that in comparison to conventional techniques, RFLP systems are technically more difficult to implement. This coupled with the time and expense involved may limit their application in certain fields such as routine identification of germplasm.

3.6. Chloroplast Variation in European Cultivars of Barley

Although the vast majority of genetic information is contained in the nucleus, there are important functions encoded by the chloroplast DNA. This organelle is central to the energy production and is accordingly important to the performance of the plant. The chloroplast genome is much less complex than the nuclear genome. Restriction digests yield

fragments that are easily resolved and are thus amenable to study.

Previous studies of chloroplast variation in *Hordeum spp.* based on restriction enzyme patterns have investigated the level of variation present within a selection of both *H. spontaneum* and *H. vulgare* accessions. In general it was found that chloroplast DNA was highly conserved in both sub-species, although *H. spontaneum* was shown to be more variable than *H. vulgare.* It was suggested that cytoplasmic diversity may have been restricted during the domestication of cultivated barley.

The present study differs from previously published results in that it constitutes an analysis of chloroplast DNA within a selection of European cultivars based on variability as revealed by two restriction endonucleases previously shown to uncover variability (Neale *et al.* 1988). The barley accessions in Table 3.4 were chosen to represent a range of barley cultivars exhibiting both spring and winter growth habit. A sample of *H. spontaneum* lines and two *H. vulgare* breeding lines were included to increase the range of diversity examined. Blots were prepared from total extracted DNA digested to completion with BamHI or Hind III. Chloroplast specific fragments were identified by hybridization to a series of Pst I barley chloroplast clones pHvcP1 to pHvcP10. These clones are comprised of sequences covering most of the chloroplast genome (Day and Ellis, 1985). Map locations of the barley

chloroplast pHvc clone series are shown (Figure 3.7. reproduced from Day and Ellis, 1985). pHvcP1-pHvcP10 were hybridized sequentially to each of the BamHI and Hind III blots. The number of major hybridizing fragments detected following autoradiography is shown in Figure 3.7. The ten probes detected a range of hybridizing fragment patterns from single bands, to highly complex patterns comprising up to 11 hybridizing fragments. A selection of the hybridization patterns are presented in Figures 3.8. and 3.9. The patterns produced were, with the exception of one probe, identical for all accessions investigated. The clone pHvcP3 identified a single accession displaying an alternative phenotype with both BamHI and Hind III blots (Figures 3.10.a and 3.10.b.). The two phenotypes identified by the pHvc P3/ BamHI probe enzyme combination have previously been reported (Neale *et al.*, 1988). The *H. spontaneum* accession HS.28 shows the presence of a 5.6 kb fragment which is not found in the remaining accessions. The additional fragment appears to be restricted to *H. spontaneum* and is not present within ***H. vulgare*** accessions. This supports the findings of Neale *et al.* (1988), who attributed the additional 5.6 kb fragment to a single point mutation present within a Hind III restriction enzyme recognition sequence in the chloroplast genome.

The *H. vulgare* accession TS264/3 shows the presence of an additional 2.0 kb fragment. The additional fragment appears only in this specific *H. vulgare* genotype and is not present within any of the other *H. vulgare* or *H. spontaneum* accessions

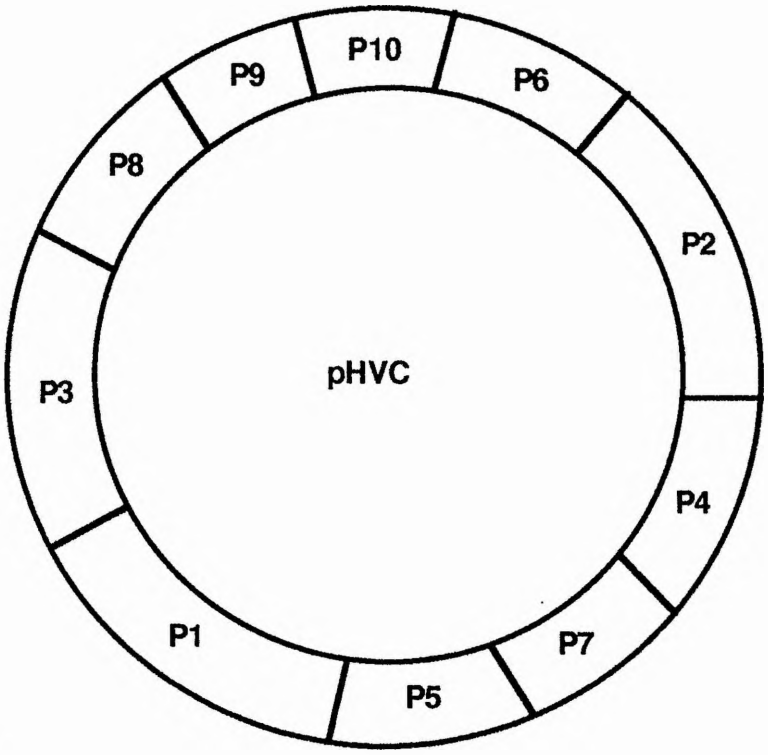


Figure 3.9. Restriction Map of the Barley Chloroplast genome showing the pHvc clone series P1-P10. Clone sizes and the number of major hybridizing fragments detected for Bam HI and Hind III digests.

Clone	Insert size (kb)	Number of major hybridising fragments	
		Bam HI	Hind III
pHcvP1	20.7	8	6
pHcvP2	20.1	6	7
pHcvP3	18.9	7/8	4/5
pHcvP4	13.4	3	5
pHcvP5	11.9	5	4
pHcvP6	10.7	2	7
pHcvP7	9.90	1	1
pHcvP8	8.11	8	11
pHcvP9	5.51	1	11
pHcvP10	5.19	2	7

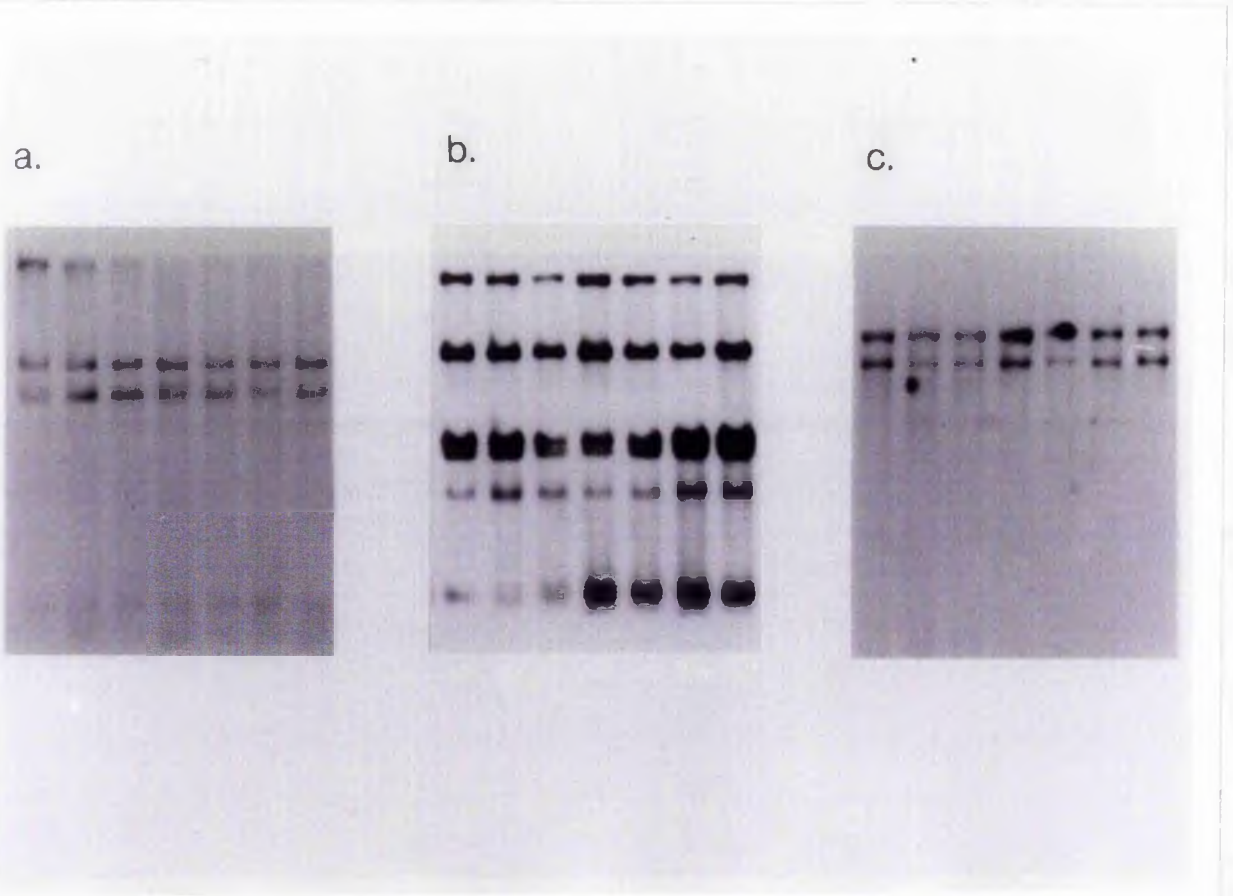


Figure 3.8. Chloroplast RFLP phenotypes observed in *H. vulgare* when Southern blots of BamHI digested barley total genomic DNA were hybridized with the chloroplast clones a) pHvc P4 b) pHvc P5 c) pHvc P6.

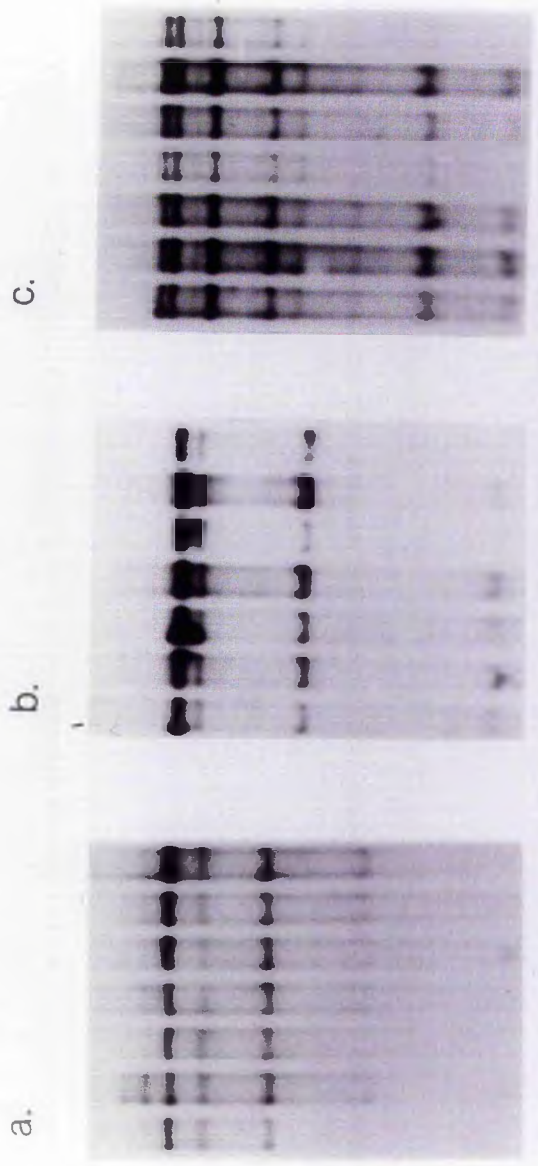


Figure 3.9. Chloroplast RFLP phenotypes observed in *H. vulgare* when Southern blots of HindIII digested barley total genomic DNA were hybridized with the chloroplast clones a) pHvc P4 b) pHvc P5 c) pHvc P6.

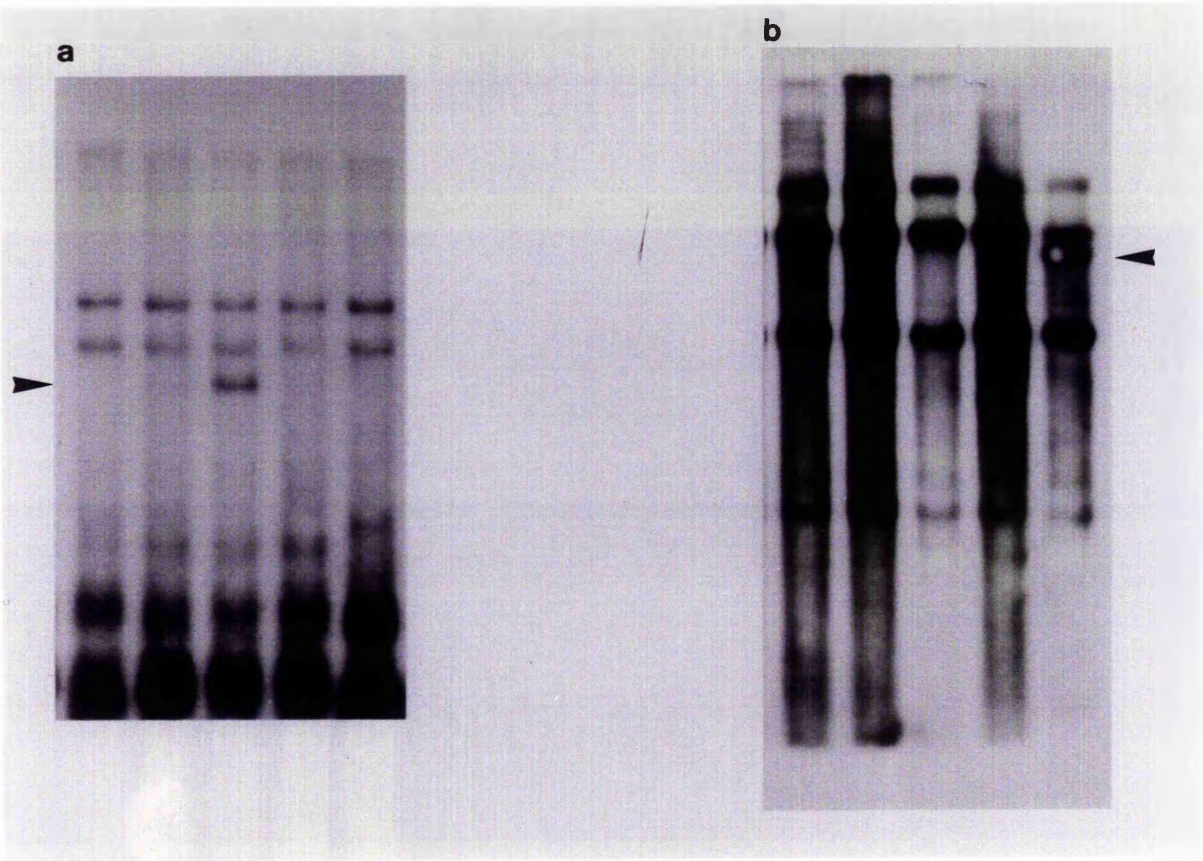
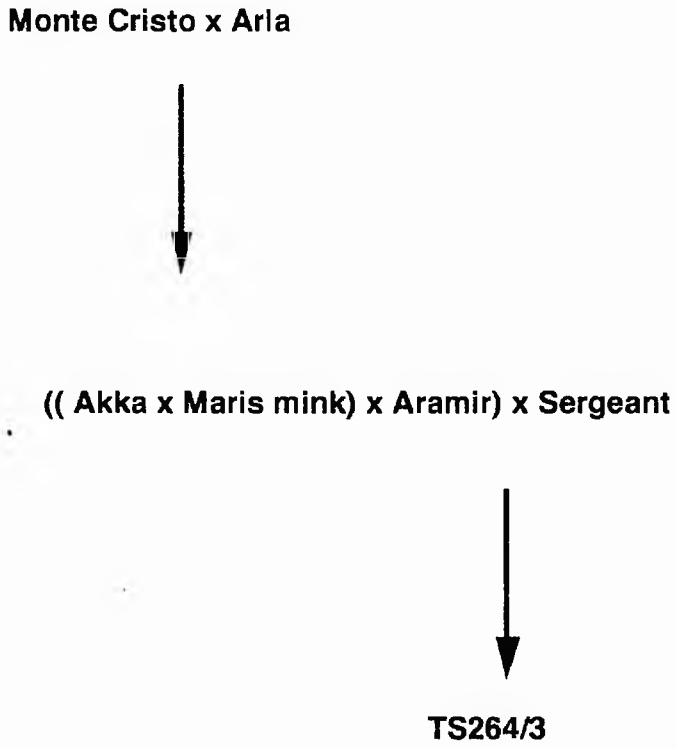


Figure 3.10. Chloroplast probe pHvc P3 phenotypes observed in *H. vulgare* and *H. spontaneum*. The arrows indicate the presence of additional hybridizing fragments in: Figure 3.10a. Genomic DNA from the *H. vulgaure* accession TS264/3 digested with BamHI; Figure 3.10b. Genomic DNA from the *H. spontaneum* accession HS28 digested with Hind III.

studied. The pedigree of TS264/3 is given in Figure 3.11. Its female ancestory traces to the cultivar Akka which has Monte Cristo as its maternal parent. This particular genotype was used as a donor of mildew resistance genes and the chloroplast DNA phenotype detected in TS264/3 may have originated from Monte Cristo.

The aim of this study was to assess DNA variation in *H. vulgare* in a representative range of cultivars. The high level of conservation of the chloroplast genome is almost certainly due to the narrow genetic base of cultivated barley which has been restricted during the process of domestication coupled with the maternal mode of inheritance of this organelle. Nuclear genomes are afforded greater opportunity to recombine through syngamy and meiosis with other variants during crossing. The restricted range of chloroplast variability observed in the barley cultivars examined is also a reflection on the tendency of breeders not to use exotic barley germplasm as the female parent in a crossing programme. *H. spontaneum* and other wild barleys are characterised by possessing the brittle rachis trait which ensures seed dispersal in their natural habitat. However, this adaptively important feature of wild barley would result in the dispersal of hybrid seed if such plants were used as female parents. Thus in most cases such crosses would be constructed with the *H. vulgare* genotype as the female parent.

Figure 3.11. Female Pedigree of TS264/3



3.8. Randomly Amplified Polymorphic DNA Markers (RAPDs)

Under conditions of reduced stringency, oligonucleotide primers will anneal to many sequences throughout the genome. Where these primers anneal to opposite strands of the DNA within approximately 2.5 kb of each other, sequences between these primers will be amenable to amplification by the polymerase chain reaction (PCR). PCR amplification was carried out on the Dissa and Sabarlis cultivars using single 10-base pair oligonucleotide primers. Primer sequences were derived from a computer generated random arrangement of the four possible nucleotides. The primer sequences used are shown in Table 3.6. The 12 primers each produced a unique and characteristic banding pattern of amplified DNA sequences in the size range from 100 base pairs to 2.5 kb. The numbers of products generated was characteristic of the primer used and ranged from 3 products for primer 'Oligo 9' to 8 bands for primer 'Oligo 3'. Examples of the molecular profiles produced for eight of the primers are presented in Figure 3.12. An example of RAPD polymorphism detected with R004 is shown in Figure 3.13. The two genotypes Dissa and Sabarlis exhibit polymorphism with Dissa possessing an additional amplification product. This is most likely the result of a point mutation creating or destroying a primer recognition site. The ability of the PCR technique to produce accurate and reproducible results is demonstrated in Figure 3.15 which shows the amplification products of 19 barley accesions with the primer 'Oligo 10'. As can be clearly seen from this figure, each of the 19

Table 3.6. Polymerase Chain Reaction primers evaluated in *H. vulgare* cultivars.

Primer	Sequence
Oligo 1	5' GGTAGCAGTC
Oligo 2	5' GGTCCTCAGG
Oligo 3	5' CAGTTCGAGG
Oligo 4	5' TACCGACACC
Oligo 5	5' TCGGAGTGGC
Oligo 6	5' ACTCAGGAGC
Oligo 7	5' CCACCGCCAG
Oligo 8	5' AGAGATGCCC
Oligo 9	5' CAGTTCTGGC
Oligo 10	5' CGTGCTAGCA
R003*	5' TCACTGGCGA
R004*	5' CTCGCGGCTA

* Received from Peter Jack, PBI, Cambridge.

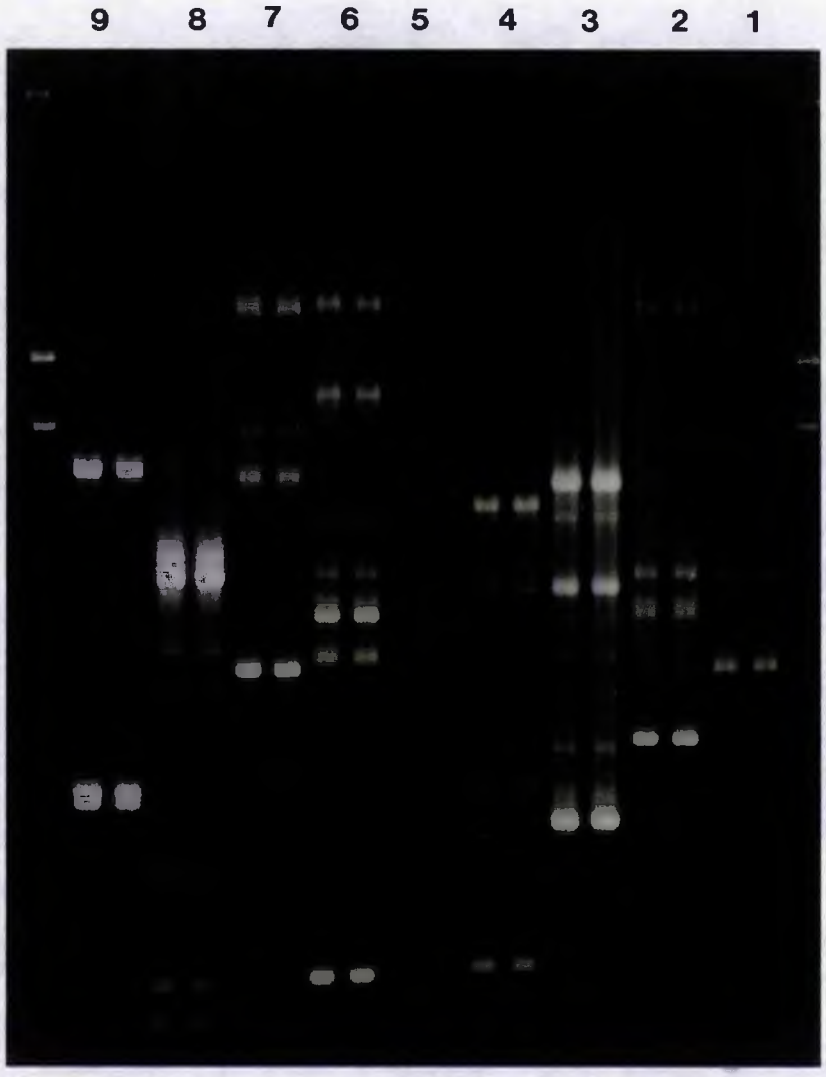


Figure 3.12. PCR amplification products of Oligos 1-9. In each case the phenotype observed was the same for Dissa and Sabarlis.

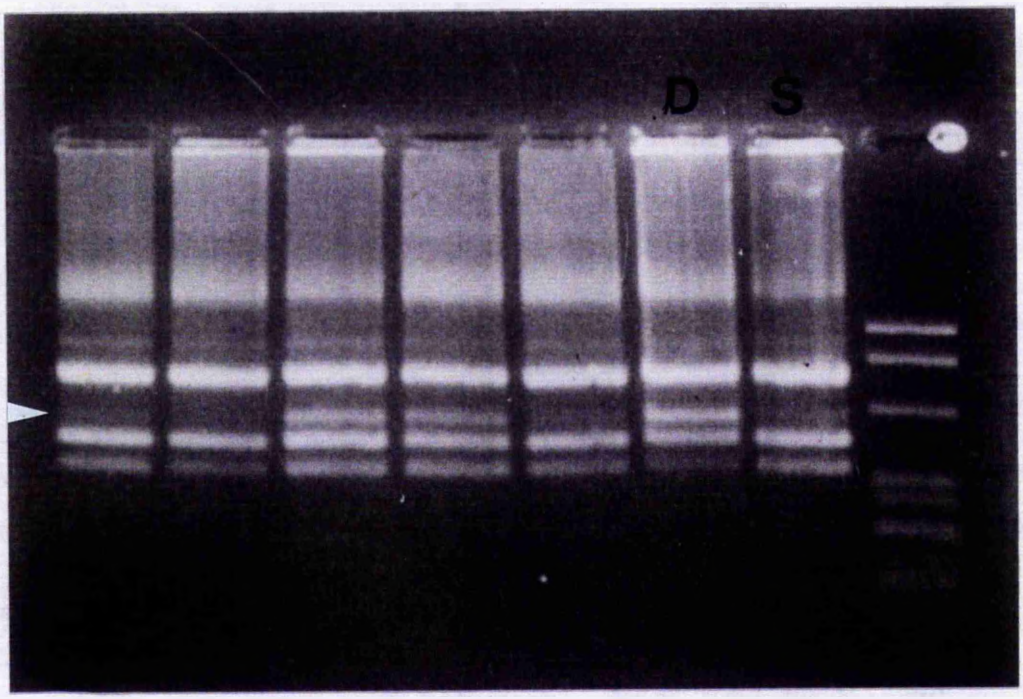


Figure 3.13. Segregation of the RAPD marker R004 in Dissa x Sabarlis DHs. The presence of an additional amplification product in the Dissa phenotype is indicated.

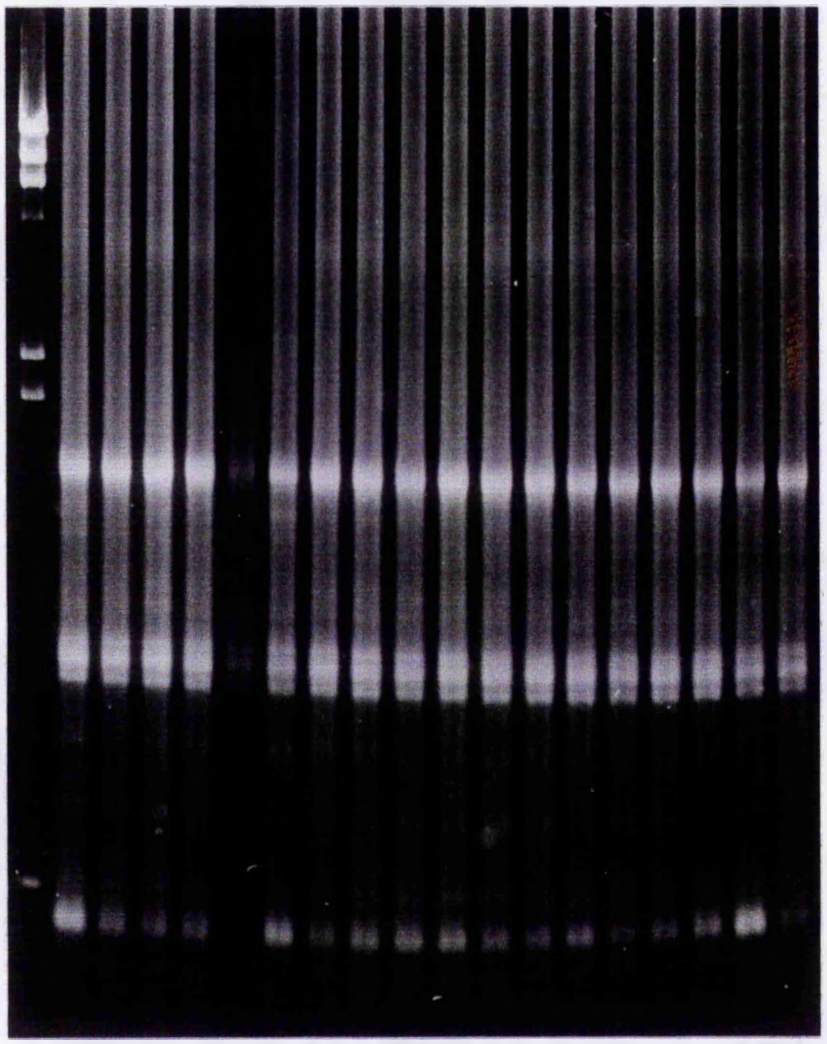


Figure 3.14. RAPD marker 'Oligo 10' amplification products from 19 barley accessions demonstrating the reproducibility of the RAPD markers. Each accession demonstrates the same phenotype.

accessions produced exactly the same amplification products when genomic DNAs were amplified using primer 'oligo 10'.

RAPD markers offer one of the potentially most useful of the molecular marker systems investigated. The initial ten primers used however, demonstrate the low levels of polymorphism detected within the barley genotypes assayed. Using a range of random 10 base-pair oligonucleotide primers barley has been shown to exhibit only 1-2% percent polymorphism in the Dissa x Sabarlis cross; only wheat has been shown to exhibit less (Peter Jack personal communication). This contrasts with the levels of polymorphism detected by these same primers in a range of other crops. For example, *Vicia faba, Solanum tuberosum* and *Theobroma* genotypes exhibit on average 15, 85 and 100 percent polymorphism respectively (Robbie Waugh personal communication). However, once polymorphic primers have been identified, they offer a technically simple and efficient means of characterising a range of barley cultivars. The two polymorphic primers R003 and R004 demonstrate the simple polymorphic patterns detected by these primers. In all the crop species examined to date, individual alleles at a particular locus are revealed by the presence or absence of a particular amplification product enabling each locus to be easily and unambiguously scored. In some species, individual primers may be able to identify several loci. This is possible as each band on the gel effectively represents a specifically amplified DNA sequence, each of which can be considered as an

individual marker locus. Hence if two or more of the priming sites are polymorphic they can each be identified from the same PCR reaction.

The usefulness of this technique in barley however depends on whether or not sufficient numbers of polymorphic primers can be identified. Furthermore, the inability to identify heterozygous genotypes with RAPD markers is also a limitation. However, the use of doubled haploids or recombinant inbred lines would circumvent this problem.

3.9. Conclusions

Currently varietal identification is based on the use of morphological traits and field testing of grain to determine their phenotype. The only alternative method presently being employed commercially to complement these techniques is that of storage protein analysis. Relatively high levels of polymorphism exist in the barley storage proteins, however, this is insufficient to discriminate between all the currently available barley cultivars. The presence of biotypes within cultivars further complicates identification and additional methods are required for the complete characterization of barley genotypes.

The use of starch gel electrophoresis has been proposed as a method of classifying cultivars but this technique is not capable of unequivocal identification. Alternatively Thompson *et al.* (1990) have proposed that a range of isozyme and protein

markers be employed for the routine testing of seed. IEF has some advantages over conventional starch gel electrophoresis and has the advantage of producing results in a fraction of the time required by more conventional electrophoretic methods. SDS-PAGE of hordein proteins however has the advantage that only one system need be assayed to provide a good indication of the cultivars identity rather than a range of isozymes.

It is to molecular markers that we must look in order to obtain a more comprehensive classification of cultivars. The potentially unlimited numbers of probes makes the completely unambiguous classification of any cultivar possible. This power is unquestionable. However the practical application of the relevant techniques is the main limitation. RFLP analysis for cultivar identification is very time consuming and requires a relatively high degree of technical sophistication. However it is likely that these techniques will be simplified in the future. There are other drawbacks. RFLP analysis requires a relatively large amount of DNA, which at present relies upon extracting sufficient amounts from leaf material. Although it is possible to extract DNA from dry embryos, bulked grain is required to provide sufficient amounts of DNA for RFLP analysis, thus preventing RFLP phenotypes to be accurately determined from individual grains as may be required for routine purity testing of stocks.

RAPD markers potentially offer a viable alternative to the application of RFLPs for cultivar identification. RAPDs offer the

advantage of only requiring small quantities of crude DNA to act as the initial template for amplification. It is feasible that DNA can be extracted from endosperm, thus allowing the embryo to be retained. In addition PCR based assays do not require the use of radio-isotopes. However, the eventual exploitation of RAPDs in barley cultivar identification will depend on the level of polymorphism detected.

Chapter Four

Monitoring the segregation and mapping of both mono and polygenic traits in Doubled Haploid (DH) populations of barley

4.1. Introduction

Although there are many examples of characters in agricultural species inherited as discrete monogenic traits, most economically-important traits, such as yield, quality and stress resistance are inherited quantitatively. That is, phenotypic variation of a given trait is characterised by continuous variation between individuals. Genetic differences affecting such traits are thought to arise from the collective effects of numerous loci, each having a small positive or negative contribution to the final expression of the trait. Such loci are termed quantitative trait loci (QTLs) Gelderman (1975).

In addition to genetic factors, quantitative traits are also affected by environmental factors, with the effect that particular environmental regimes can result in a quantitative increase or decrease in the final expression of a trait. As a result, it is generally not possible to determine how many loci are involved in producing the observed variation, or to determine their individual effects.

Because the genes controlling quantitative traits have not generally been resolvable individually, quantitative geneticists have dealt with their effects *en masse*, using biometrical procedures which allow an estimation of the number of QTLs (Mather and Jinks 1971; as outlined by Powell *et al.* 1985). However, these procedures give no indication as to the chromosomal location of these factors. Using marker genes to

identify specific regions of the genome which enhance the expression of quantitatively controlled characters leads to the possibility of the dissection and manipulation of these traits.

Reports of linkage between major genes and QTLs were first reported by Sax (1923) who found an association in *Phaseolus* of seed size (a quantitatively inherited character) with seed coat pigmentation (a discrete monogenic character). He attributed this correlation to linkage of seed size genetic factors to the alleles for seed colour. The basic approach of using marker genes in segregation was proposed as a viable technique for the systematic analysis of quantitative variation by Thoday (1961) and more recently by Gelderman (1975), Mather and Jinks (1982), Tanksley *et al.* (1982), and Beckman and Soller (1983).

One of the first steps towards the identification and manipulation of genes involved in the control of agronomically important characters is the development of a genetic linkage map in the species of interest. Genetic studies in barley have led to the establishment of a well developed classical genomic map based on morphological and more recently protein and isozyme markers. However, linkage relationships have been established based on information from many crosses (Sogaard and von-Wettstein-Knowles 1987). While these mapping techniques have provided important insights into the genetics and cytology of barley, they embody the inherent limitations common to all classical maps. The majority of the markers

consist of morphological mutant loci which are undesirable in breeding populations. In addition, alleles of these loci are normally recessive, seriously limiting the types of crosses in which they can be utilized.

The use of isozymes avoids many of the problems associated with morphological markers, and isozymes have been used in a range of crop plants (Tanksley and Orton 1983). These biochemical markers are generally selectively neutral, well distributed over the genome and interact in a codominant manner. Useful as isozymes have been for genetic research, the limited number of these loci has restricted their use, particularly in genetic mapping and linkage analysis.

Currently, mapping programmes now include the use of molecular markers particularly RFLPs, and more recently RAPD markers (Williams *et al.* 1990, Welsh and McCleland 1990), to overcome the limitations imposed by the lack of polymorphic isozymes. Both of these molecular techniques share a number of advantages with isozymes and have the potential to generate virtually unlimited numbers of markers for use in genetic analysis. This enables the construction of a linkage map based on information from a single cross thus enabling all the markers to be mapped relative to each other, rather than inferring relationships as in previous mapping studies. This leads to a greater degree of accuracy in determining the genetic distances between linked loci. The use of RFLPs in the generation of linkage maps has been reported in several

important crop plants, including tomato (Bernatzky and Tanksley 1986; Tanksley *et al.* 1987), maize (Helenjaris 1987; Burr 1988), lettuce (Landry *et al.* 1987) and potato (Bonierbale *et al.* 1988; Gebhardt *et al.* 1987) and the use of PCR markers based on primers identifying known gene sequences, has recently been reported in barley (Shin *et al.* 1990).

The choice of the population in which the mapping of these genes is undertaken is also of particular importance. In order to maximise the chances of detecting polymorphism, a number of studies have used inter-specific crosses. For example in both tomato (Bernatzky and Tanksley, 1986) and potato (Bonierbale *et al.* 1988) interspecific crosses were employed for linkage map creation. In other crops relatively divergent parents within a species were analysed, eg. lettuce and maize. Linkage mapping in self-pollinating plants has traditionally used generations derived from the F_1 between homozygous parents differing at the appropriate loci - either the F_2 or first backcross. In these cases the individual plant is considered the unit of segregation but this limits the number of markers that can be assayed on a single plant basis. The selfed seed of these generations may also be required to be progeny tested to obtain a fuller characterisation of genotypes. An alternative is to use homozygous lines generated using doubled haploid (DH) techniques. The major benefit of DH is that within individual families, plants are genetically uniform and do not segregate. As a consequence, each family can be replicated, enabling large amounts of tissue to be analysed. Furthermore

the inbred lines can be evaluated in a range of environments for agronomic traits. Another advantage is that dominance relationships at any locus are irrelevant since only homozygotes are present and thus, for a diploid such as barley only two allelic combinations are present at each locus.

Using an appropriate technique DHs can be derived from any filial generation, however, the most common approach is to extract from the F_1 between two inbred lines. Doubled haploids extracted from F_1 hybrids are likely to exhibit a higher linkage disequilibrium relative to other generations due to the reduced opportunities for recombination. Furthermore, the greater additive genetic variation associated with doubled haploids together with the absence of within-family segregation indicates that this approach is well suited to relating markers detected at the biochemical and nucleic acid level to whole plant phenotypic variation. This approach has been used previously to analyse the effects of morphological (Powell *et al.* 1985; Powell *et al.* 1990; Thomas *et al.* 1990), isozyme loci (Powell *et al.* 1990) and RFLP loci (Powell *et al.* 1991) on quantitative traits in random inbred lines in barley.

Doubled haploid populations, therefore, provide the ideal genetical resource with which to investigate a range of marker systems. Both the initial mapping and the evaluation of associations between marker loci and quantitative traits can be considered. A DH population consisting of sixty-six families derived by the *H. bulbosum* technique from the F_1 hybrid of

the *H. vulgare* cross, Dissa x Sabarlis was chosen as the mapping population for this study. This population had previously been scored for the range of quantitative traits listed in Table 4.1 (Wayne Powell personal communication). Thus, this population was suitable as a mapping population and for assessing the correlations of morphological, isozyme, protein and molecular markers with a range of quantitative traits.

4.2 Materials and methods

Sixty-six doubled haploids (DH) were produced by the *H. bulbosum* technique (Kasha and Kao, 1970) from the F_1 hybrid of the *H. vulgare* cross, Dissa x Sabarlis. The parents, DH lines and F_1 hybrids were grown in a randomised complete block field experiment with two replicates in 1986. Within a block each family was represented by a row of up to ten seeds, sown at 5 cm spacing, with a wheat guard at each end of the row. Rows were 22.5 cm apart. After harvest, sheaves were returned to the laboratory and five randomly chosen plants from each row were scored for each of the traits listed in Table 4.1. Milling energy (joules) was assessed by the modified comparamill method (Cowe *et al.*, 1989).

All isozyme and RFLP analysis were carried out essentially as described in the Materials and Methods section. All isozyme data were derived from isoelectric focusing gels. Isozyme extractions were carried out on individual grains, or on the first leaf for the Leaf esterase assay. DNA extractions were conducted on leaf tissue pooled from 5 individuals, 6

Table 4.1 Quantitative traits scored in the Dissa x Sabarlis doubled haploid population.

EL	Ear length measured from the base to the tip of the the ear
Ht	Final height measured from the base of the stem to the collar
GN	Number of grains on the main-stem
MSW	Weight of grain on the main stem
STG	Weight of main stem
TN	Number of fertile tillers per plant
RS	Weight of straw
WAG	Weight of grain from the whole plant
TGW	Thousand grain weight, calculated from GN and MSW
SW	Weight of straw from the whole plant, straw weight
SPY	Grain yield from the whole plant, single plant yield
Bio	Biomass
Ind A	Harvest Index A
Ind B	Harvest Index B
ME	Milling energy (joules)

weeks after germination. The DNA manipulations were as previously described (Materials and Methods).

Segregation analysis, tests of significance and estimation of linkage between linked loci were as described by Mather (1938). The Mapmaker computer program of Lander *et al.* (1987) was used to further confirm linkage relationships for the hordein loci.

4.3. Results and Discussion

4.3.1. Screening of Dissa x Sabarlis for polymorphic marker loci

The cultivars Dissa and Sabarlis, the parents of the doubled haploid population chosen for the mapping study, were screened for a range of morphological, isozyme, protein, RFLP and RAPD markers. Those morphological, protein and biochemical markers found to exhibit polymorphism are listed in Table 4.2. The probe enzyme combinations detecting polymorphic RFLP patterns and the polymorphic RAPD marker are listed in Table 4.3. Segregation data is presented in Appendix 4.1. Markers found to be non-polymorphic in this cross are presented in Appendix 4.3

4.3.2. Assigning chromosomal locations using wheat-barley addition lines

Isozyme, and protein markers were assigned to specific barley chromosomes using wheat-barley disomic addition lines (Islam *et al.* 1981). The presence of marker isozyme and

Table 4.2. Morphological and biochemical characters evaluated.

Locus designation	Phenotype	Chromosomal location
2/6	2 row / 6 row	2L
Wsp-2	Water soluble protein	5
Wsp-3	Water soluble protein	4L
Wsp-4	Water soluble protein	2
Est-3	Esterase	7S
Est-10	Esterase	3L
Leaf Est	Esterase	3L

Table 4.3. Informative RFLP and PCR markers.

Clone name	Phenotype	Locus designation	Chromosome location	Enzymes†	Source	Reference
pBII	B-hordein	*Hor-2*	1S	B1,H3,R1,R5	Barley	Ford B.G., 1974
pCP387	C-hordein	*Hor-1*	1S	R5	Barley	Ford B.G., 1974
pBG35	rDNA	*Rrn-2*	5S	S1	Flax	Goldsborough and Cullis, 1982.
PCR Primer	**Sequence**					
R004	5'CTCGGGGCTA		unassigned	-	-	Jack, P. (pers. comm)

† B1 = Bam HI, H3 = Hind III, R1 = EcoRI, R5 = EcoRV, S1 = Sac I.

protein bands specific to Betzes barley in an addition line and their absence in Chinese Spring wheat or other addition lines indicates the barley chromosome on which the marker is located.

Wheat-barley addition lines were used to assign the chromosomal location of previously unmapped isozyme loci and in order to further characterise isozyme systems possibly detecting several loci within a complex banding pattern. In the case of grain esterase two loci were detected on the same gel. Figures 4.1a and 4.1b show the use of the wheat-barley addition lines to map two isozyme loci using isoelectric focusing gels. Figure 4.1a shows the identification of the β-amylase locus using wheat-barley addition lines. The presence of the protein bands specific to Betzes barley in the addition line 4H and their absence in Chinese Spring wheat and the other addition lines indicates that this set of protein bands map to chromosome 4, confirming that this is the β-Amy-1 locus. Using the same method, it is possible to confirm that the isozyme banding patterns of esterase in Figure 4.1b relate to the Est-3 locus.

4.3.3. Identification of polymorphic marker loci in Dissa x Sabarlis

Examples of the protein, isozyme and molecular marker polymorphisms detected are shown in Figures 4.2a and 4.2b The polymorphic marker loci identified are distributed over six of the seven pairs of barley chromosomes. The chromosomal location of the isozyme and RFLP markers studied are given in

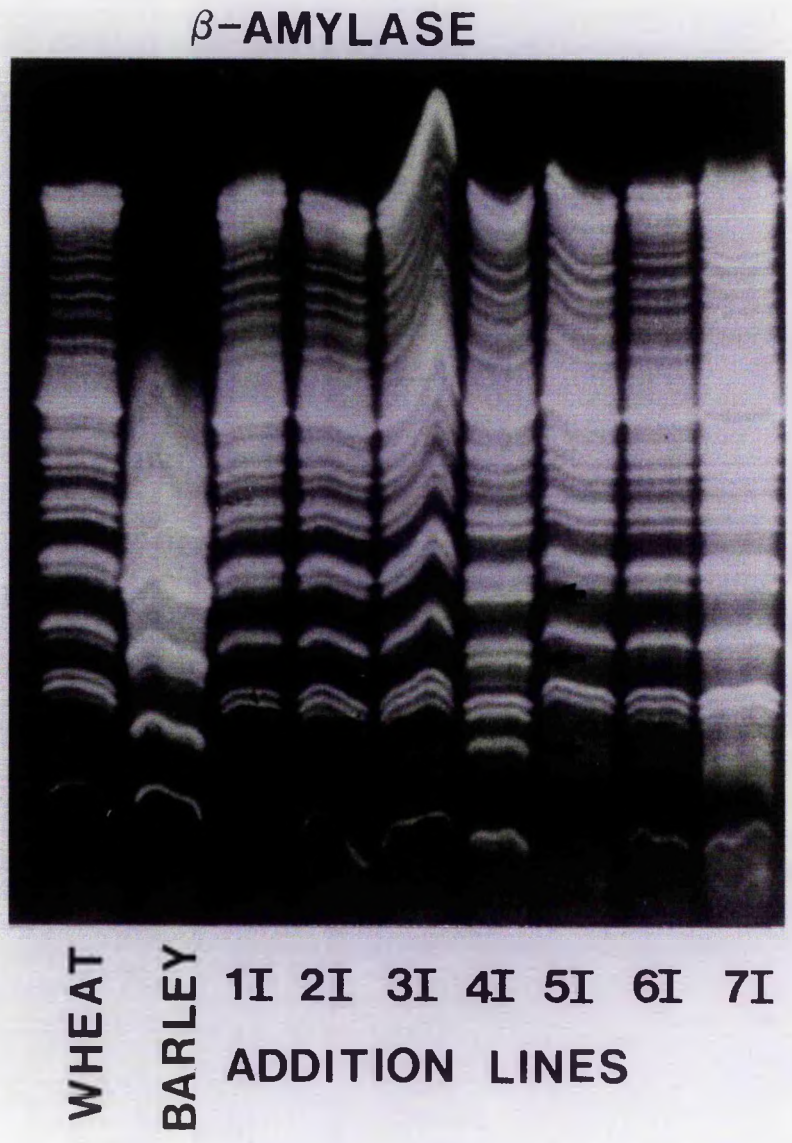


Figure 4.1a. β-AMY1 phenotypes of 'Betzes' barley and 'Chinese Spring' wheat-barley addition lines, showing the location of the β-AMY1 locus to chromosome 4I.
(Chromosome 4I is equivalent to chromosome 4H).

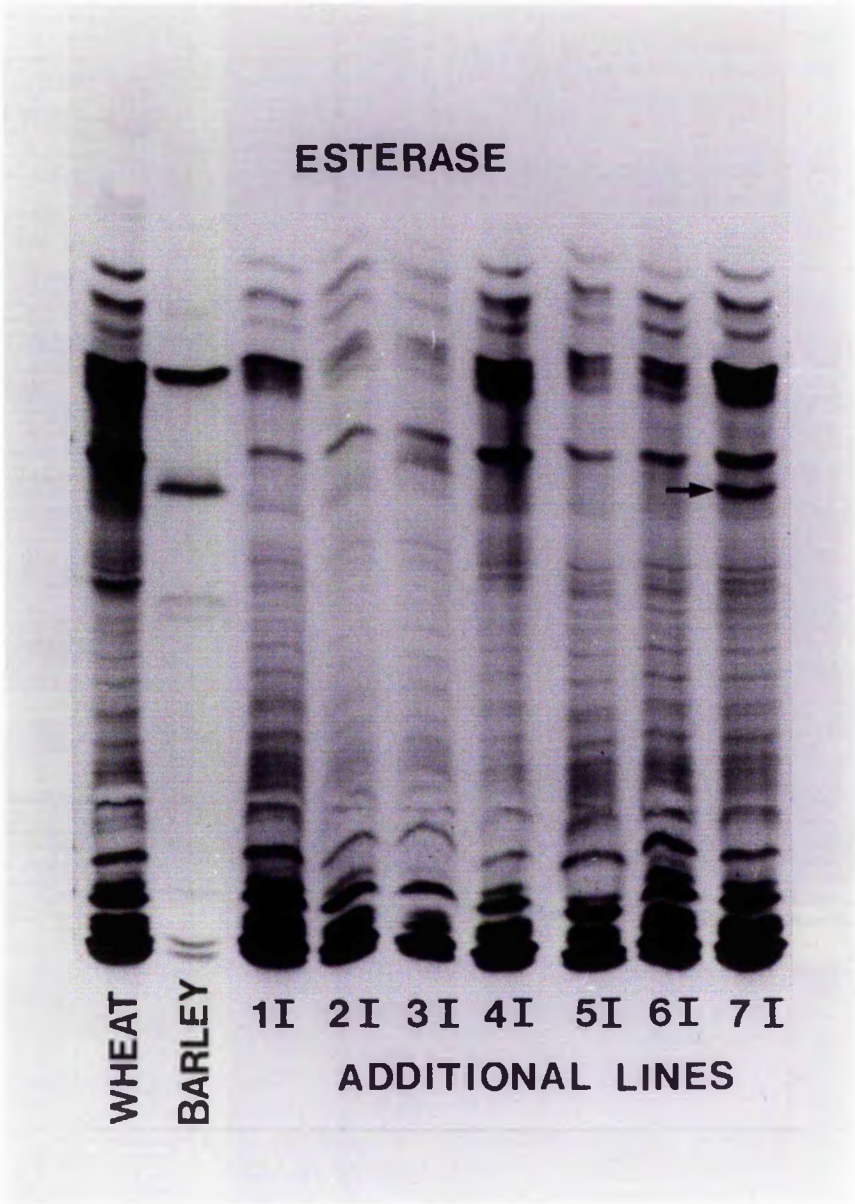


Figure 4.1b. Est3 phenotypes of 'Betzes' barley and 'Chinese Spring' wheat-barley addition lines, showing the location of the Est3 locus to chromosome 7I.
(Chromosome 7I is equivalent to chromosome 7H).

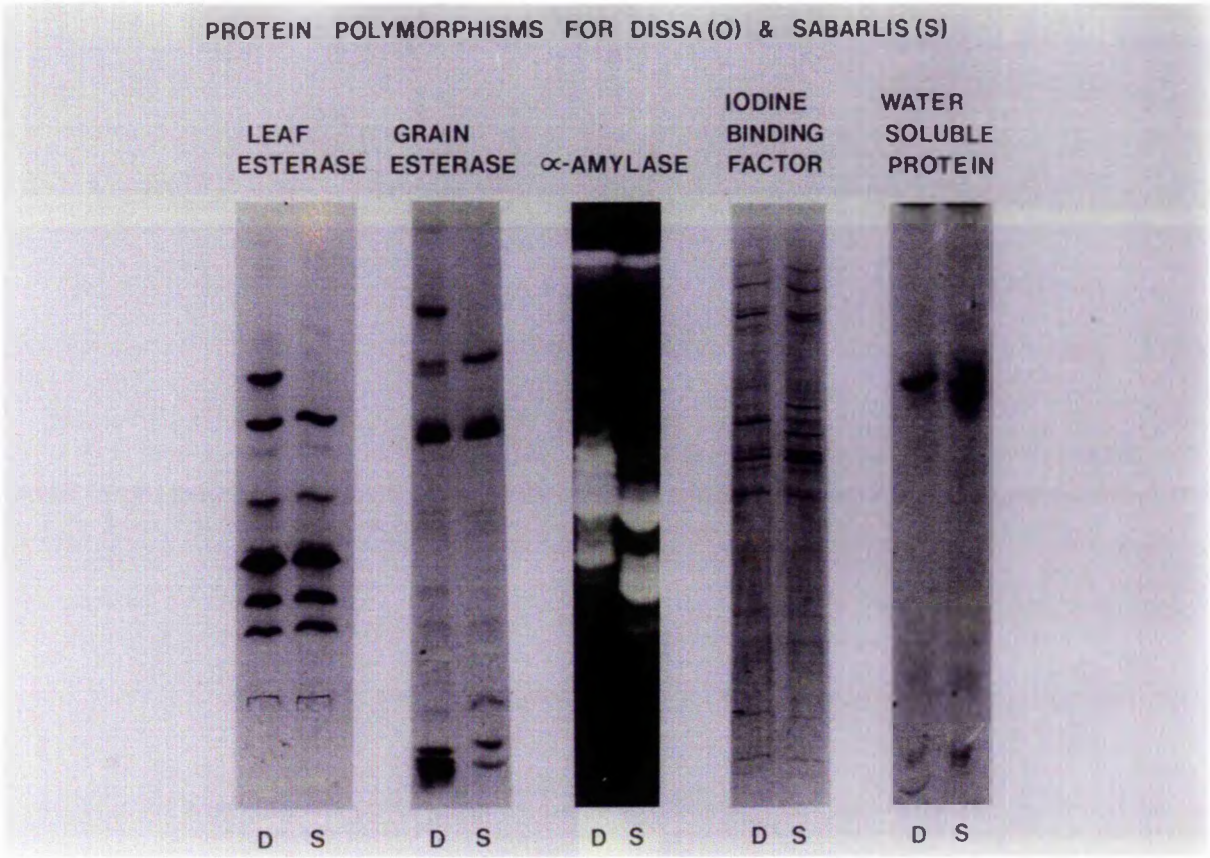


Figure 4.2a. Protein polymorphism detected in the parental genotypes (Dissa x Sabarlis) for Leaf-esterase, grain esterase, iodine-binding factor and water soluble protein.

Iodine binding factor (IBF) is equivalent to WSP2,3 and water soluble protein has been provisionally classed as WSP4.

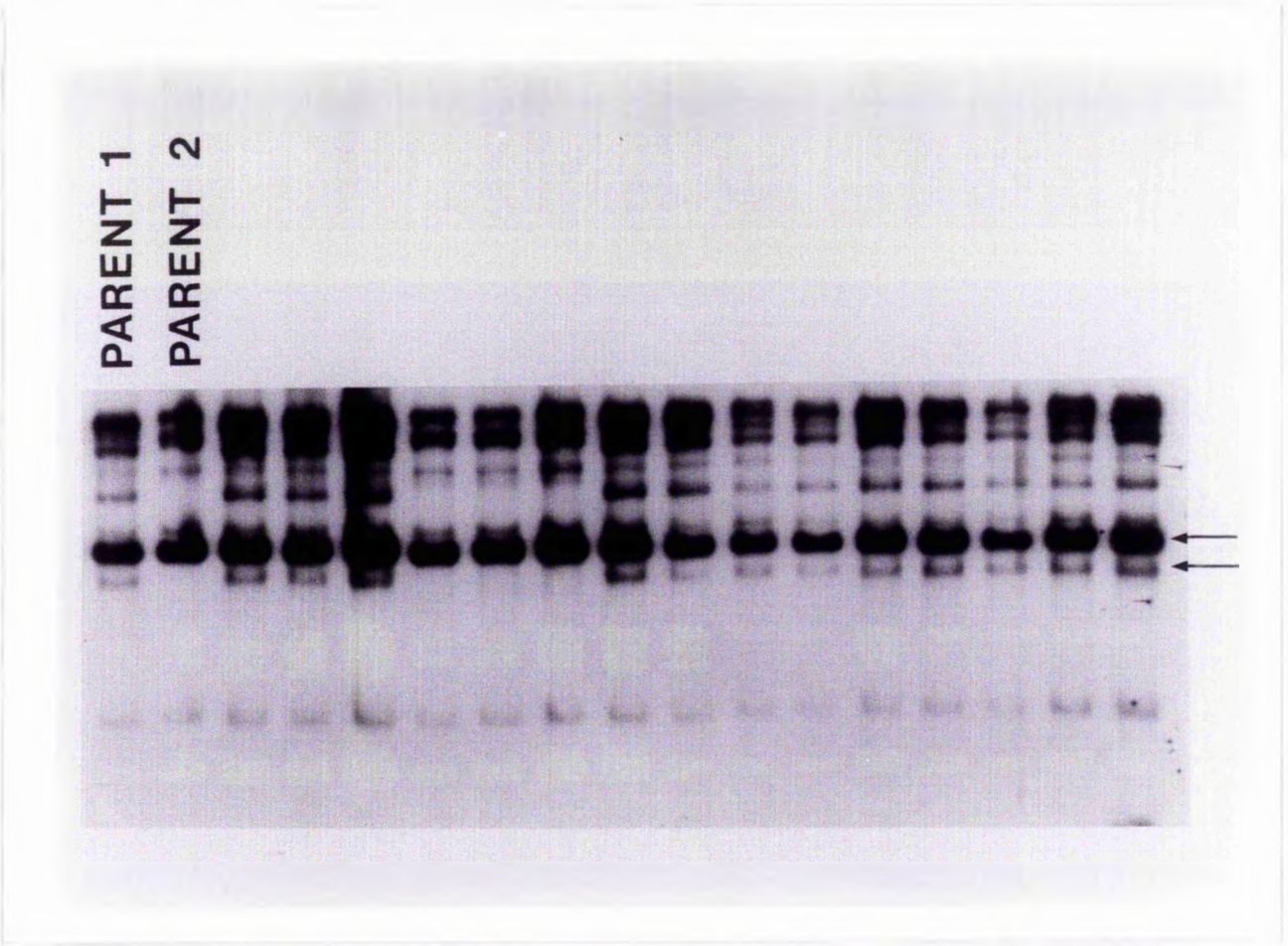


Figure 4.2b. RFLP polymorphism at the Rrn2 locus as detected by the pBG35 ribosomal DNA probe in BamHI digests of barley genomic DNA. An additional restriction fragment in Parent 1 (Dissa) and its absence in Parent 2 (Sabarlis) can be seen to segregat in their DH progeny.

Tables 4.2 and 4.3. Chromosome nomenclature is based on homeology in the *Triticeae* (Dewey, 1984). The RAPD marker R004 had not previously been assigned to an individual chromosome or linkage group.

4.3.4. Segregation analysis

The segregation of alleles at the *Hor2* locus as detected by the B-hordein probe pB11 was unusual in that while the expected segregation of two allelic forms was observed in blots prepared from DNA digested with each of BamHI, EcoRI and Hind III, two additional sets of alleles were observed when autoradiographs of blots prepared from EcoRV digested DNA were examined. These bands were much fainter than those observed to be segregating in the other blots. The three segregating loci were classified as follows: the major segregating band detected with DNA digested with BamHI, EcoRI and Hind III were designated *Hor2*. The additionally segregating loci were designated *Hor2a* and *Hor2b*. The segregation of alleles at each locus was tested for deviation from the expected 1:1 ratio (Table 4.4). The morphological marker locus for ear type (2/6) and the protein marker *Wsp-3* were found to deviate significantly from the expected 1:1 segregation ratio in the Dissa x Sabarlis population ($P < 0.05$). Wsp3 showed an excess of Dissa phenotypes while 2/6 showed an excess of Sabarlis phenotypes. The remaining isozyme, protein and molecular markers did not deviate significantly from the expected 1:1 ratio.

Table 4.4. Segregation and χ^2 goodness-of-fit analysis for marker loci in the Dissa x Sabarlis doubled haploid population.

Marker loci	Marker Penotype		$\chi^2_{(1)}$
	Dissa	Sabarlis	1:1
Hor2	29	36	0.75
Hor1	33	30	0.14
Hor2a	28	24	0.31
Hor2b	25	26	0.02
Wsp-4	32	34	0.06
2/6	24	41	4.45*
Est-10	34	32	0.06
Leaf-est	36	30	0.55
Wsp-3	42	24	4.91*
Wsp-2	35	31	0.24
Rrn2	31	34	0.14
α-Amy-1	34	32	0.06
R004	30	30	0.00
Est-3	32	34	0.06

* $P < 0.05$

4.3.5. Joint segregation analysis

Joint segregation analysis was carried out for each of the previously unmapped marker and mapped loci in order to determine if any of these loci were linked. The joint segregation analysis for those pairs shown to differ significantly from the expected 1:1:1:1 ratio are shown (Tables 4.5 to 4.12).

The joint segregation analysis of EST10 and Leaf-Est is given in Table 4.5. The alleles at each individual locus segregate in the expected 1:1 ratio. However, the joint segregation item is significant ($P < 0.001$) indicating that the two loci are linked with a recombination value of 0.212 ± 0.050 (Table 4.13). The chromosomal location of these markers was known previously, however this set of results confirms that these loci are linked on the long arm of chromosome 3H.

The joint segregation analysis of EST3 and R004 is given in Table 4.6. The alleles at each individual locus segregate in the expected 1:1 ratio. However, the joint segregation item is significant ($P < 0.05$) indicating that the two loci are linked with a recombination value of 0.350 ± 0.062 (Table 4.13). This would therefore place the marker locus R004 on the short arm of chromosome 7H.

The alleles at each of the 3 loci homologous to the *Hor2* locus, detected by pB11, segregate in the expected 1:1 ratio, as do the alleles at the *Hor1* locus. The joint segregation item was

Table 4.5. Joint segregation analysis for *Est-10* and *Leaf-esterase*

	Est-10	
Leaf esterase	Dissa	Sabarlis
Dissa	28	8
Sabarlis	6	24

	d.f	χ^2
Segregation for *Est-10*	1	0.06
Segregation for *Leaf esterase*	1	0.55
Joint segregation	1	21.88***
Total	3	22.49

*** P < 0.001.

Table 4.6. Joint segregation analysis for *R004* and *Est-3.*

	R004	
Est-3	Dissa	Sabarlis
Dissa	19	10
Sabarlis	11	20

	d.f	χ^2
Segregation for *R004*	1	0.00
Segregation for *Est-3*	1	0.07
Joint segregation	1	5.40*
Total	3	5.47

* P < 0.05.

Table 4.7. Joint segregation analysis for *Hor-1* and *Hor-2* loci.

	Hor-2	
Hor-1	Dissa	Sabarlis
Dissa	26	7
Sabarlis	3	27

	d.f	χ^2
Segregation for *Hor-2* alleles	1	0.75
Segregation for *Hor-1* alleles	1	0.14
Joint segregation	1	29.35***
Total	3	30.24

*** P < 0.001.

Table 4.8. Joint segregation analysis for *Hor-2* and *Hor-2a* loci.

	Hor-2	
Hor-2a	Dissa	Sabarlis
Dissa	20	8
Sabarlis	4	20

	d.f	χ^2
Segregation for *Hor-2* alleles	1	0.31
Segregation for *Hor-2a* alleles	1	0.31
Joint segregation	1	15.08***
Total	3	15.70

*** P < 0.001.

Table 4.9. Joint segregation analysis for *Hor-2* and *Hor-2b* loci.

	Hor-2	
Hor-2b	Dissa	Sabarlis
Dissa	18	7
Sabarlis	5	21

	d.f	χ^2
Segregation for *Hor-2* alleles	1	0.49
Segregation for *Hor-2b* alleles	1	0.02
Joint segregation	1	14.29***
Total	3	14.80

*** P < 0.001.

Table 4.10. Joint segregation analysis for *Hor-1* and *Hor-2a* loci

	Hor-1	
Hor-2a	Dissa	Sabarlis
Dissa	21	7
Sabarlis	6	16

	d.f	χ^2
Segregation for *Hor-1* alleles	1	0.32
Segregation for *Hor-2a* alleles	1	0.72
Joint segregation	1	11.52***
Total	3	12.56

*** P < 0.001.

Table 4.11. Joint segregation analysis for *Hor-1* and *Hor-2b* loci.

	Hor-1		
Hor-2b	Dissa	Sabarlis	
Dissa	21	4	
Sabarlis	5	19	
		d.f	χ^2
Segregation for *Hor-1* alleles		1	0.18
Segregation for *Hor-2b* alleles		1	0.02
Joint segregation		1	19.61***
Total		3	19.81

*** P < 0.001.

Table 4.12. Joint segregation analysis for *Hor-2a* and *Hor-2b*

	Hor-2a		
Hor-2b	Dissa	Sabarlis	
Dissa	22	3	
Sabarlis	5	21	
		d.f	χ^2
Segregation for *Hor-2a* alleles		1	0.18
Segregation for *Hor-2b* alleles		1	0.02
Joint segregation		1	24.02***
Total		3	24.22

*** P < 0.001.

found to be significant ($P < 0.001$) for each pairwise combination of the 4 hordein loci, indicating these loci are linked and map to the short arm of chromosome 1H. Table 4.13 presents a pairwise recombination analysis of the *Hor1* and *Hor2* loci along with the two additional hordein loci *Hor2a* and *Hor2b*. From the recombination values obtained, it is not possible to unambiguously assign the order of these markers relative to each other.

4.3.6 Analysis of the hordein couples

As it had not been possible to order the hordein loci, the segregation data for the Dissa x Sabarlis doubled haploid population was analysed using the computer program Mapmaker (Landers *et al.* 1987). Table 4.14. shows the LOD scores and recombination fractions derived from the computer analysis of the data. Taking the criterion of linkage as a LOD score greater than 2.5 and a recombination fraction less than 0.4, four groups of possibly linked loci were identified (Table 4.15).

Using the maximum likelihood method (Weller 1986, 1987), it was possible to derive 12 possible orders of the 4 hordein loci that did not conflict with the three point linkage data. The predicted order and log-likelihood values are presented (Table 4.15). The order with the lowest log-likelihood value is predicted to represent the most likely order of the 4 hordein loci relative to each other. The recombination percentage, r, was converted to map distance, D, using the

Table 4.13. Recombination frequencies of linked loci in the Dissa x Sabarlis population.

Linked loci	Phenotypes		P	S.E.
	Parentals	Recombinants		
Hor-2 - Hor-1	53	10	0.159	0.046
Hor-2 - Hor-2a	40	12	0.231	0.058
Hor-2 - Hor-2b	39	12	0.235	0.059
Hor-1 - Hor-2a	37	13	0.260	0.062
Hor-1 - Hor-2b	40	9	0.184	0.055
Hor-2a - Hor-2b	41	8	0.163	0.053
Est-10 - Leaf est	52	14	0.212	0.050
Est-3 - R004	39	21	0.350	0.062

P = (recombinants/parentals+recombinants)

S.E. = $(P(1-P)/n)^{1/2}$; n = parentals+recombinants

Table 4.14 LOD scores (lower value) and recombination fractions (upper value) calculated for the segregating loci in the Dissa x Sabarlis population

	1	2	3	4	5	6	7	8	9	10	11	12	13
2	0.38 1.70												
3	0.50 0.00	0.50 0.00											
4	0.23 9.01	0.45 0.24	0.50 0.00										
5	0.41 0.95	0.45 0.24	0.48 0.03	0.42 0.66									
6	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00								
7	0.50 0.00	0.50 0.00	0.50 0.00	0.44 0.42	0.41 0.95	0.42 0.66							
8	0.50 0.00	0.45 0.33	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00						
9	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.42 0.81	0.50 0.00	0.41 0.98					
10	0.50 0.00	0.50 0.00	0.48 0.06	0.50 0.00	0.50 0.00	0.50 0.00	0.40 1.14	0.45 0.24	0.38 1.76				
11	0.45 0.21	0.47 0.08	0.50 0.00	0.49 0.01	0.37 1.46	0.50 0.00	0.50 0.00	0.41 0.69	0.45 0.21	0.27 4.67			
12	0.38 1.26	0.44 0.31	0.50 0.00	0.46 0.14	0.46 0.14	0.50 0.00	0.50 0.00	0.44 0.31	0.34 2.26	0.26 5.22	0.14 12.52		
13	0.47 0.11	0.50 0.00	0.50 0.00	0.47 0.11	0.50 0.00	0.50 0.00	0.44 0.45	0.48 0.06	0.36 2.09	0.16 13.54	0.29 4.04	0.23 6.46	
14	0.50 0.00	0.33 2.95	0.47 0.12	0.47 0.12	0.50 0.00	0.47 0.12	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.50 0.00	0.42 0.60

Key
1. *Est10*
2. *Est3*
3. *amy1*
4. Leaf est
5. *Wsp4*
6. *Wsp3*
7. *Wsp2*
8. 2/6
9. *Rrn2*
10. *Hor2*
11. *Hor2a*
12. *Hor2b*
13. *Hor1*
14. R004

Table 4.15. Possible orders of the 4 hordein loci in the cross Dissa x Sabarlis based on log-likelihood analysis. Orders derived from Mapmaker analysis of the segregation data.

Sequence	Predicted order				log-likelihood
Order 1	*Hor-2*	*Hor-1*	*Hor-2b*	*Hor-2a*	-64.781
Order 2	*Hor-1*	*Hor-2*	*Hor-2b*	*Hor-2a*	-66.609
Order 3	*Hor-1*	*Hor-2*	*Hor-2a*	*Hor-2b*	-67.413
Order 4	*Hor-2*	*Hor-1*	*Hor-2a*	*Hor-2b*	-67.594
Order 5	*Hor-2b*	*Hor-1*	*Hor-2*	*Hor-2a*	-72.920
Order 6	*Hor-2*	*Hor-2a*	*Hor-2b*	*Hor-1*	-73.586
Order 7	*Hor-2b*	*Hor-2*	*Hor-1*	*Hor-2a*	-74.894
Order 8	*Hor-2*	*Hor-2b*	*Hor-2a*	*Hor-1*	-75.559
Order 9	*Hor-1*	*Hor-2b*	*Hor-2*	*Hor-2a*	-80.811
Order 10	*Hor-2*	*Hor-2b*	*Hor-1*	*Hor-2a*	-81.062
Order 11	*Hor-2*	*Hor-2a*	*Hor-1*	*Hor-2b*	-81.932
Order 12	*Hor-2b*	*Hor-2*	*Hor-2a*	*Hor-1*	-83.558

Table 4.16. Map distances calcuated by the Mapmaker-1 program for the hordein loci 'Order 1' (Table 15).

Linked loci	Recombination fraction r.	Map distances D.
Hor-2 - Hor-1	15.8%	19.0 cM
Hor-1 - Hor-2a	28.7%	28.7 cM
Hor-2a - Hor-2b	13.8%	16.2 cM

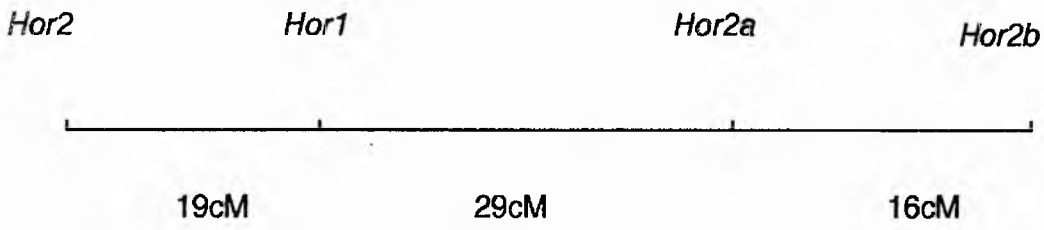
Kosambi function (Kosambi 1944) to correct for undetected double crossovers (Table 4.16).

The *Hor1* and *Hor2* loci have been previously located on the short arm of chromosome 1H, with the *Hor2* locus being variously estimated to lie 7 to 17 centi-Morgans distal with respect to the *Hor1* locus (Doll and Brown 1979; Jensen 1981). The 19 centi-Morgan distance separating these loci, calculated from the Dissa x Sabarlis cross is in general agreement with such an estimate. From the known position of the *Hor1* and *Hor2* loci it is possible to determine the relative orientation of the two loci *Hor2a* and *Hor2b*. These must lie between the *Hor1* locus and the centromere on chromosome 1H, as shown in Figure 4.3.

The results of the computer generated map of the segregating marker alleles indicate that two additional hordein loci are present within this cross. The fact that they were detected using the pB11 clone indicates that they are structurally related to the B hordeins. Two major hordein loci have previously been identified. These are the *Hor1* and *Hor2* loci encoding the C and B hordeins respectively (Oram *et al.* 1975; Doll and Brown 1979; Shewry *et al.* 1980).

Two dimensional electrophoresis of the C hordein fraction has demonstrated the presence of a large number of component polypeptides showing extensive variation in the numbers, molecular weight and isoelectric point. There is however, a high degree of structural homology between

Figure 4.3 Linkage groups showing the determined order and genetic distance of the hordein loci on chromosome 5.



individual polypeptides (Shewry *et al.* 1981; Brandt *et al.* 1981), which are all encoded by a multigene family comprising of 20-30 copies at the *Hor1* locus. Similarly the B hordein fraction consists of 8-16 major components which vary in their molecular weight and isoelectric point (Faulks *et al.* 1981).

Analysis of cDNA clones derived from endosperm specific mRNAs have shown the presence of two major B hordein related cDNAs and several minor groups indicating that the B hordein fraction is a heterogeneous mixture of polypeptides (Forde *et al.* 1981). In addition to the *Hor2* locus, two B hordein like proteins have been located proximally to *Hor1* on the short arm of chromosome 1H (Netsvetaev and Sozinov 1984). One of these loci, designated *Hor4*, has been demonstrated to be structurally related to the major B hordein protein encoded by the *Hor2* locus and is thought to have been derived from *Hor2* by translocation of a small number of genes (Shewry *et al.* 1988). Molecular studies of the B hordein loci support these findings indicating that the two major subfamilies of B hordeins are encoded by spatially separated groups of genes within the locus (Shewry *et al.* 1990). These findings suggest that a similar duplication has taken place in the cultivar Dissa leading to the presence of the two additional hordein loci. The identification and mapping of these loci demonstrates the power of RFLP techniques for genetical analysis of related loci and the construction of linkage maps. More importantly this study has demonstrated the use of

RFLPs to identify putative duplicated regions of the barley genome.

4.3.7. Detection of associations between marker loci and quantitative traits

4.3.7.1. Student t-test

Although the number of polymorphic genetic markers identified in the Dissa x Sabarlis cross is relatively small, it is still of interest to establish whether any of these markers are associated with QTLs. Furthermore, since the genetic markers have been mapped to specific regions of the barley genome, any significant associations would allow loci affecting quantitative traits to be mapped to particular barley chromosomes. An objective of this study was to determine if specific marker loci affect any of the quantitative traits studied. Classification of the doubled haploid lines into two groups carrying the alternative alleles for each marker system, allows the assessment of their effects on the mean performance of the quantitative traits in a random genetic background. For each trait measured, the mean scores and standard errors for each allelic class at isozyme, protein, RFLP and RAPD loci are given in Appendix 4.2. Using a Students t-test, significant differences were detected between the mean scores for a range of traits classed according to allelic types. Each of the 14 marker systems investigated showed significant associations with several of the quantitative traits. In total 63 significant associations based on the t-test were detected between the

marker systems and the quantitative traits (Table 4.17). Only IND A and IND B were found not to be significantly associated with any marker system analysed. Clearly, selection of alleles at each of the marker loci can influence the expression of a quantitative loci. Although this test of significance has been used previously, it is not a particularly rigourous method of analysis and can inflate the number of associations between markers and QTLs.

4.3.7.2 Mean squares analysis

A more stringent test of significance and hence a more useful one, can be obtained from a mean squares analysis. The mean squares for the between groups item in the analysis of variance were tested against the between lines within groups item. A summary of these results are presented in Table 4.18. The significant difference between the two groups indicates that a substantial portion of the additive genetic variation is associated with allelic differences at the marker locus. This form of analysis reduces the number of significant associations observed to a subset of 21 of the 63 derived from the t-test. Three additional significant associations were detected between 2/6 and Harvest index A and B, and between *WSP-4* and Harvest index B (Table 4.17). Of the 21 significant associations, 12 were found to be due to the 2 row / 6 row locus. These results confirm previous observations that this morphological locus effects many of the quantitative characters studied in

Table 4.17. Significant t-tests " * " and mean square analysis, () .

Locus	EL	Ht	GN	MSW	SWT	TN	RS	WAG	TWG	MS	SPY	Bio	IND A	IND B	ME
Est-10		*	*												*
Est-3	*	*	*						*						
α-Amy-1			*			*	*	*			*	*			*
Leaf-est		(*)	*												*
WSP-2		*							*			*			*
WSP-3			*						*						
WSP-4	*	*	(*)						*					()	*
'2/6'	(*)	(*)	(*)		(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	()	()	
Rrn2									*						(*)
Hor2	*	*							*						*
Hor2a			(*)	(*)											*
Hor2b			(*)			*	*		*	*					(*)
Hor1		*				*	*	*		*	*	*			*
R004									*						(*)

* t-test; () analysis of variance.

Table 4.18 Mean square analysis

Locus	Item	d.f.	EL	Ht	GN	MSW	STG	TN	RS	WAG	TCW	SW	SPY	Bio	IND A	IND B	ME
Est-10	Between groups	1	7.07	281.46	1590.95	2.43	1.10	0.23	13.59	13.61	74.56	21.83	26.35	96.00	0.95	3.10	259.48
	Within groups	64	21.20	494.05	1736.00	1.12	0.77	18.48	18.89	19.74	1235.92	24.60	26.10	95.10	20.60	19.10	156.48
	Variance Ratio		0.33	0.57	0.92	2.17	1.43	0.01	0.72	0.69	0.06	0.89	1.01	1.01	0.05	0.16	1.66
Est-3	Between groups	1	30.56	313.64	880.57	0.20	0.35	12.10	0.00	0.64	471.23	0.62	0.02	0.41	0.02	0.33	57.36
	Within groups	64	20.83	493.55	1747.10	1.15	0.78	18.30	19.10	19.94	1229.72	24.93	26.51	96.58	20.60	19.13	159.95
	Variance Ratio		1.47	0.64	0.50	0.17	0.45	0.66	0.00	0.03	0.38	0.02	0.00	0.00	0.00	0.02	0.36
a-Amy-1	Between groups	1	4.95	339.81	3660.60	3.05	0.39	19.02	41.59	68.38	2.38	53.12	106.16	309.16	14.58	28.62	0.37
	Within groups	64	21.23	493.14	1703.66	1.11	0.78	18.20	18.45	18.88	1237.05	24.11	24.85	91.75	20.42	18.68	160.86
	Variance Ratio		0.23	0.69	2.15	2.75	0.50	1.05	2.25	3.62	0.00	2.20	4.27	3.37	0.71	1.53	0.00
Leaf-est	Between groups	1	19.67	2266.56	512.68	2.03	1.56	2.03	14.99	16.13	862.49	25.08	27.36	164.63	4.93	0.47	57.49
	Within groups	64	21.10	463.04	1752.84	1.12	0.77	18.45	18.87	19.70	1223.61	24.55	26.08	94.95	20.57	19.12	159.95
	Variance Ratio		0.93	4.89†	0.29	1.81	2.03	0.11	0.79	0.82	0.70	1.02	1.05	1.73	0.24	0.02	0.36
WSP-2	Between groups	1	1.61	202.96	2654.02	0.61	0.04	4.08	1.65	0.14	821.02	0.94	1.79	0.13	5.93	4.61	49.83
	Within groups	64	21.28	495.28	1719.39	1.14	0.79	18.42	19.08	19.95	1224.26	24.92	26.48	96.58	20.55	19.06	160.81
	Variance Ratio		0.08	0.41	1.54	0.54	0.05	0.22	0.09	0.01	0.67	0.04	0.07	0.00	0.29	0.24	0.31
WSP-3	Between groups	1	8.77	4.70	1154.59	0.21	0.04	0.29	0.18	4.43	220.52	0.047	6.81	8.02	6.03	5.93	3.51
	Within groups	64	21.17	498.38	1742.81	1.15	0.79	18.48	19.10	19.88	1233.64	24.94	26.40	96.46	20.55	19.04	160.81
	Variance Ratio		0.41	0.01	0.66	0.18	0.05	0.02	0.01	0.22	0.18	0.00	0.26	0.08	0.29	0.31	0.02
WSP-4	Between groups	1	35.44	615.18	7718.43	2.76	0.01	0.71	0.16	9.53	1799.14	0.04	21.78	23.81	46.27	71.01	5.61
	Within groups	64	20.75	488.84	1640.25	1.11	0.79	18.48	19.10	19.80	1208.98	24.98	26.17	96.21	19.92	18.02	160.77
	Variance Ratio		1.71	1.26	4.71†	2.49	0.01	0.04	0.01	0.48	1.49	0.00	0.83	0.25	2.32	3.94†	0.03
'2/6'	Between groups	1	640.79	7151.46	46278.51	0.01	11.11	140.61	240.61	110.43	52057.96	353.18	100.27	829.17	397.02	552.52	2.25
	Within groups	64	11.41	389.63	1054.64	1.16	0.62	16.02	15.57	18.50	426.91	19.72	25.29	84.93	14.14	10.60	161.8
	Variance Ratio		56.16†	18.35†	43.88†	0.01	17.92†	8.78†	15.45†	5.97†	121.94†	17.91†	3.96†	9.76†	28.08†	52.12†	0.01
Rrn2	Between groups	1	19.10	1018.38	912.38	0.02	0.27	5.64	3.88	6.81	353.17	7.11	0.68	12.13	44.97	43.39	644.24
	Within groups	64	21.00	482.54	1746.60	1.15	0.79	18.40	19.04	19.94	1231.57	24.83	26.50	96.39	19.94	18.45	150.64
	Variance Ratio		0.91	2.11	0.52	0.02	0.34	0.31	0.20	0.34	0.29	0.29	0.03	0.13	2.26	2.35	4.28†

† Significant mean square value

Table 4.18 Mean square analysis

Hor2	Between groups	1	1.42	92.40	500.94	0.09	0.02	0.28	0.32	4.04	684.92	0.22	5.49	7.85	3.92	9.00	141.84
	Within groups	64	21.62	503.40	1760.00	1.14	0.80	18.73	19.29	19.83	1244.69	25.19	26.25	96.66	20.49	18.96	157.6
	Variance Ratio		0.07	0.18	0.28	0.08	0.03	0.01	0.02	0.20	0.55	0.01	0.21	0.08	0.19	0.47	0.90
Locus	Item	d.f.	EL	Ht	GN	MSW	STG	TN	RS	WAG	TCW	SW	SPY	Bio	IND A	IND B	ME
Hor2a	Between groups	1	7.57	18.25	13280.24	5.75	0.51	1.95	5.15	10.27	2105.24	9.88	32.43	78.11	0.37	48.43	253.27
	Within groups	49	21.49	489.76	1752.17	1.09	0.79	17.47	19.91	19.67	1189.06	26.01	25.56	96.31	21.85	17.90	152.54
	Variance Ratio		0.35	0.04	7.58†	5.28†	0.65	0.11	0.26	0.52	1.77	0.38	1.27	0.81	0.02	2.71	1.66
Hor2b	Between groups	1	13.10	113.19	8100.81	3.99	0.18	21.00	3.54	0.19	1461.50	2.15	5.24	0.68	19.99	47.23	608.41
	Within groups	48	21.14	491.32	1880.80	1.15	0.80	17.35	20.02	20.18	1182.04	26.24	26.50	98.76	21.66	18.29	144.36
	Variance Ratio		0.62	0.23	4.31†	3.47	0.23	1.21	0.18	0.01	1.24	0.08	0.20	0.01	0.92	2.58	4.21†
Hor1	Between groups	1	0.18	272.57	1727.57	0.83	0.05	5.66	1.78	1.51	1100.64	2.78	5.29	15.81	3.24	13.82	180.44
	Within groups	60	22.65	490.61	1779.01	1.15	0.81	19.53	20.00	20.74	1290.98	26.02	27.31	100.14	21.36	19.41	162.86
	Variance Ratio		0.01	0.56	0.97	0.72	0.06	0.29	0.09	0.07	0.85	0.11	0.19	0.16	0.15	0.71	1.11
R004	Between groups	1	13.35	53.63	890.37	3.65	1.00	4.32	8.29	10.31	177.14	14.77	27.48	82.40	16.15	13.12	637.99
	Within groups	58	21.71	482.98	1829.66	1.10	0.78	18.89	19.10	19.48	1205.31	24.72	25.62	94.29	21.17	19.47	153.61
	Variance Ratio		0.61	0.11	0.49	3.32	1.28	0.23	0.43	0.53	0.15	0.60	1.07	0.87	0.76	0.67	4.15†
† Significant mean square value																	

doubled haploid and single seed descent populations between two row and six row barley cultivars (Powell *et al.* 1990).

Only two of the protein and isozyme markers demonstrate associations with quantitative traits based on mean squares analysis. Leaf esterase appears to be significantly associated with genes controlling the expression of height (Ht). *WSP-4* appears to be significantly associated with genes controlling the expression of the number of grains on the main-stem (GN), Harvest index B and milling energy (ME). Three of the RFLP markers and the RAPD marker demonstrate significant associations with the several of the quantitative traits. The ribosomal DNA locus, *Rrn2* on the short arm of chromosome 5H, (as detected by the flax rDNA clone pBG35) showed a significant association with a quantitative locus controlling milling energy. This is in agreement with the findings of Powell *et al.* (1991), who also report significant associations between the *Rrn2* locus and milling energy. Additionally, the hordein locus *Hor2b* on the short arm of chromosome 1H and the RAPD marker locus *R004* on the short arm of chromosome 7H both show significant associations with milling energy. This indicates that variation in the milling energy requirement is influenced by at least three separate loci on different chromosomes.

Since the chromosomal location of the markers used in this study are known it is possible not only to show association of quantitative trait loci to these markers but also to locate

these loci to specific chromosomes. Of particular interest is the fact that two closely linked hordein loci, *Hor2a* and *Hor2b* show significant association with the quantitative trait grain number (GN). The fact that both loci are associated with this trait may indicate that the observed genetic variation associated with allelic differences at each marker locus may be due to the same quantitative trait locus. This may allow the mapping of the QTL in relation to the two linked marker loci on the short arm of chromosome 5H.

The expected mean squares may be used to obtain estimates of $\underline{D}$, the additive genetic variance for the doubled haploid families. This is the heritable portion of the phenotypic differences between homozygotes and is the fixable portion of the variability and reflects the genetic variation available for manipulation (Mather and Jinks 1971). Values for the additive genetic variance were calculated for each of the significant associations obtained from the mean squares analysis. The extent of the genetic variation associated with allelic variation at the individual loci is given in Table 4.19. In the case of the 2 row / 6 row locus, a large portion of the additive genetic variation available for manipulation for each of these traits can be accounted for by allelic variation at this locus. A graphical representation of the distribution of this variation is presented in Figure 4.4. Likewise, graphical representations of the distribution of this variation for the isozymes and molecular markers with significant associations obtained from the mean squares analysis are presented in Figures 4.5 to 4.10.

Table 4.19. Estimates of the additive genetic variation associated with allelic differences at morphological, isozyme and molecular marker loci in the Dissa x Sabarlis doubled haploid population.

Locus / Trait	2/6 EL	Ht.	GN	STG	TN	RS	WAG	TCW	SW	SPY	Bio	IND A	IND B
σ^2_D	1.92	30.70	149.40	0.064	1.048	0.956	0.923	109.40	0.999	1.406	5.282	1.55×10^{-3}	1.42×10^{-3}
σ^2_{D1}	0.93	20.13	78.73	0.048	0.853	0.605	0.779	28.73	0.921	1.289	4.119	9.48×10^{-4}	5.74×10^{-4}
*	0.98	10.57	70.67	0.016	0.195	0.352	0.144	80.67	0.078	0.117	1.163	5.98×10^{-4}	8.47×10^{-4}
%	51.32	34.43	47.30	24.65	18.61	36.76	15.57	73.74	7.79	8.32	22.02	38.69	59.61

Locus / Trait	L-est Ht.	Hor2a MSW	Hor2a GN	Hor2b GN
σ^2_D	30.42	0.09719	175.982	178.169
σ^2_{D1}	27.65	0.08787	152.926	165.475
*	2.77	0.00932	23.056	12.694
%	9.11	9.5	13.10	7.12

Locus / Trait	pBG35 ME	Hor2b ME	R004 ME
σ^2_D	7423.50	7206.7	7590.0
σ^2_{D1}	7038.05	6869.7	7172.5
*	385.45	337	417.5
	5.19	4.68	5.50

σ^2_D = Total genetic variance
σ^2_{D1} = Within family genetic variance
* = Genetic variance associated with allelic differences at the locus of interest
% = * above expressed as a percentage of the total genetic variance

Figure 4.4. TCW distribution classified according to 2 row / 6 row phenotypes.

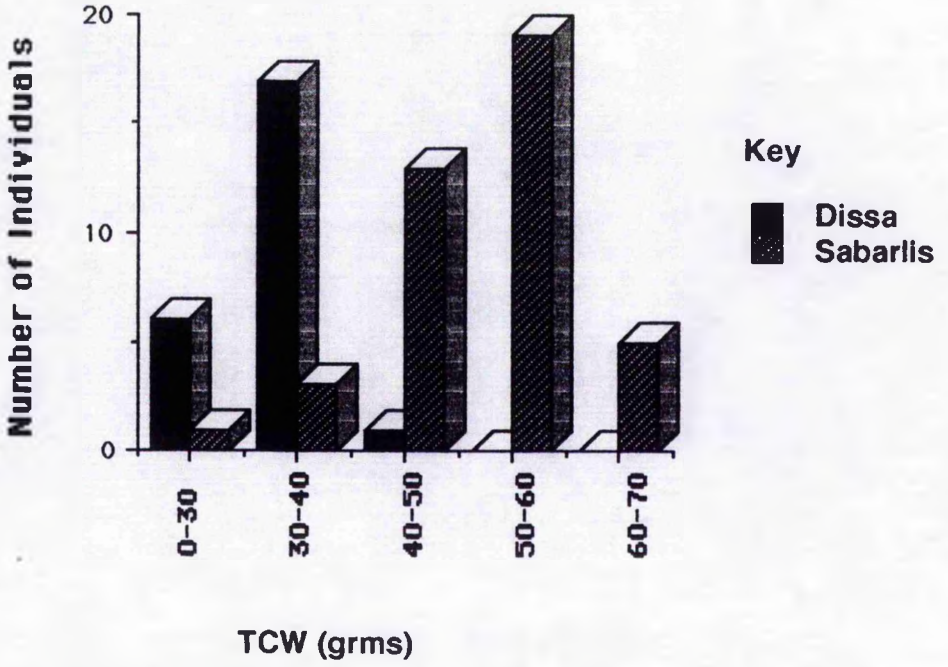


Figure 4.5. Height Distribution classified according to Leaf esterase phenotype.

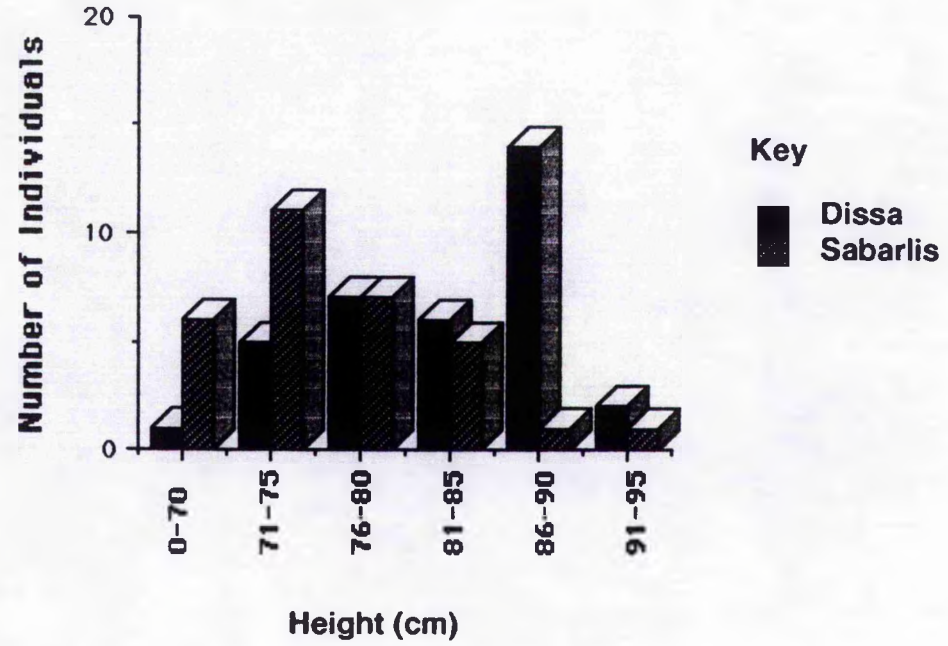


Figure 4.6. Grain Number distribution classified according to Hor2a phenotypes.

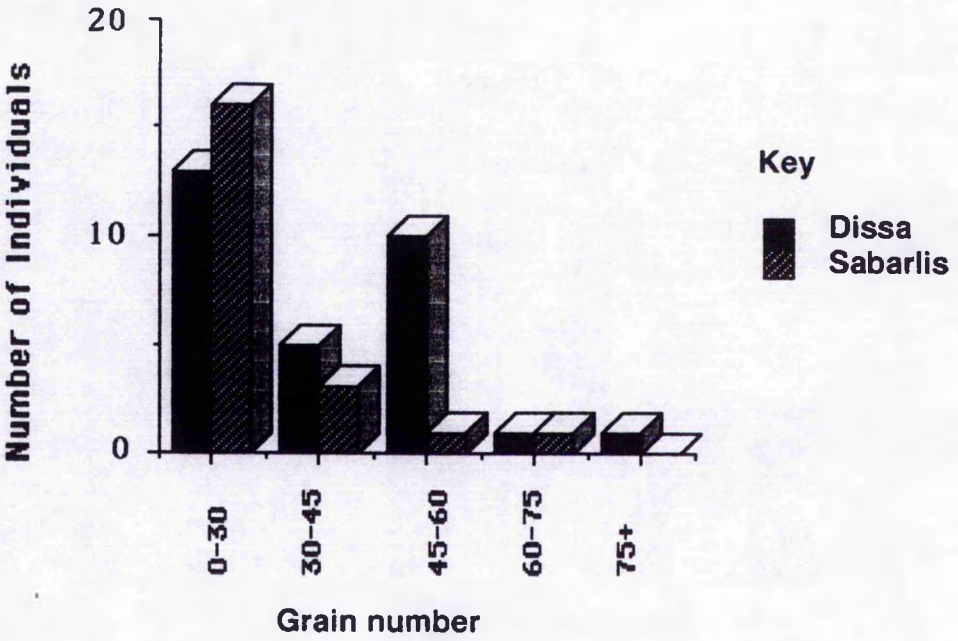


Figure 4.7. Grain Number classified according to Hor2b phenotypes.

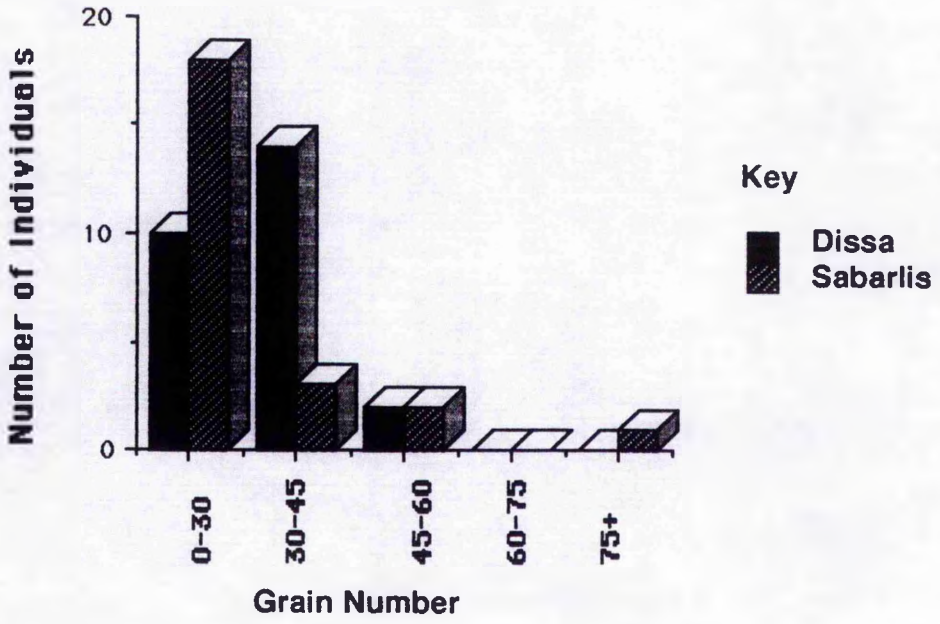


Figure 4.8. Milling Energy distribution classified according to Rrn2 phenotypes.

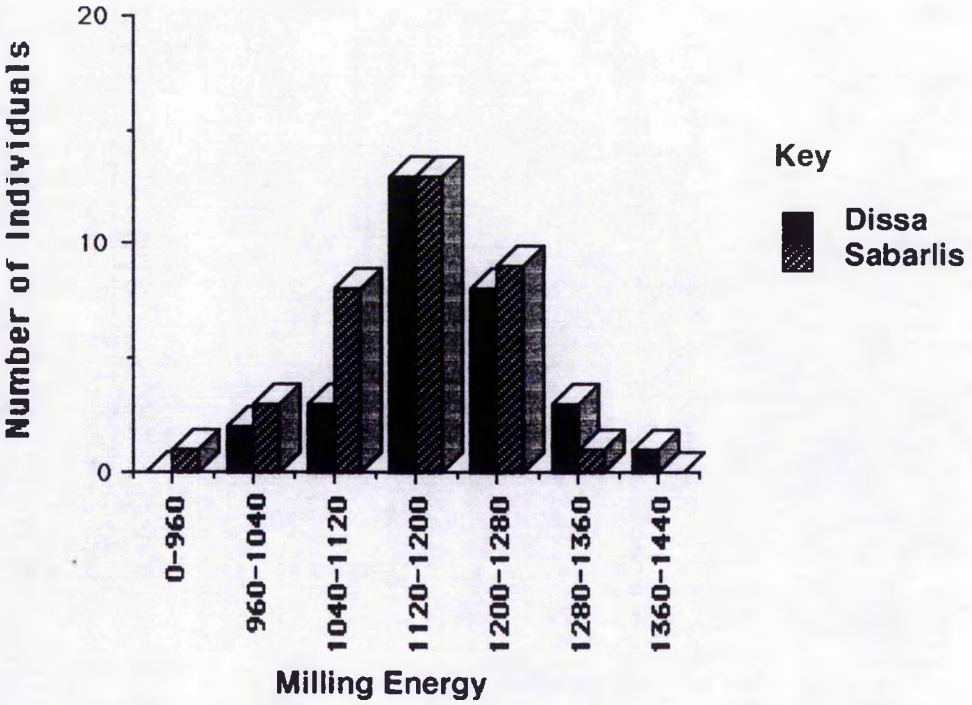


Figure 4.9. Milling Energy distribution classified according to R004 phenotypes.

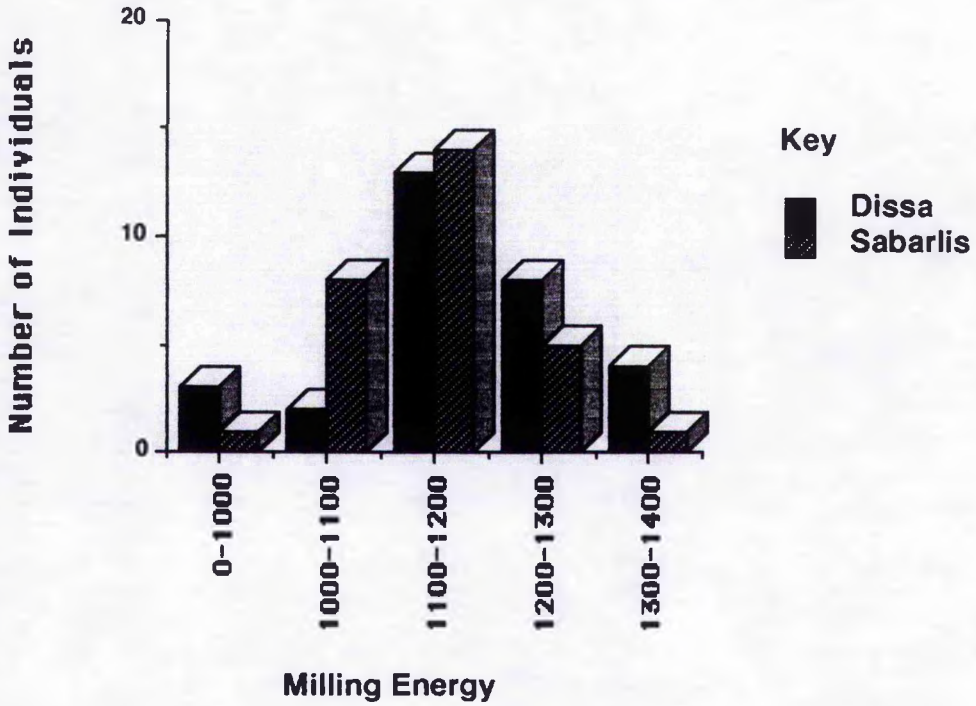
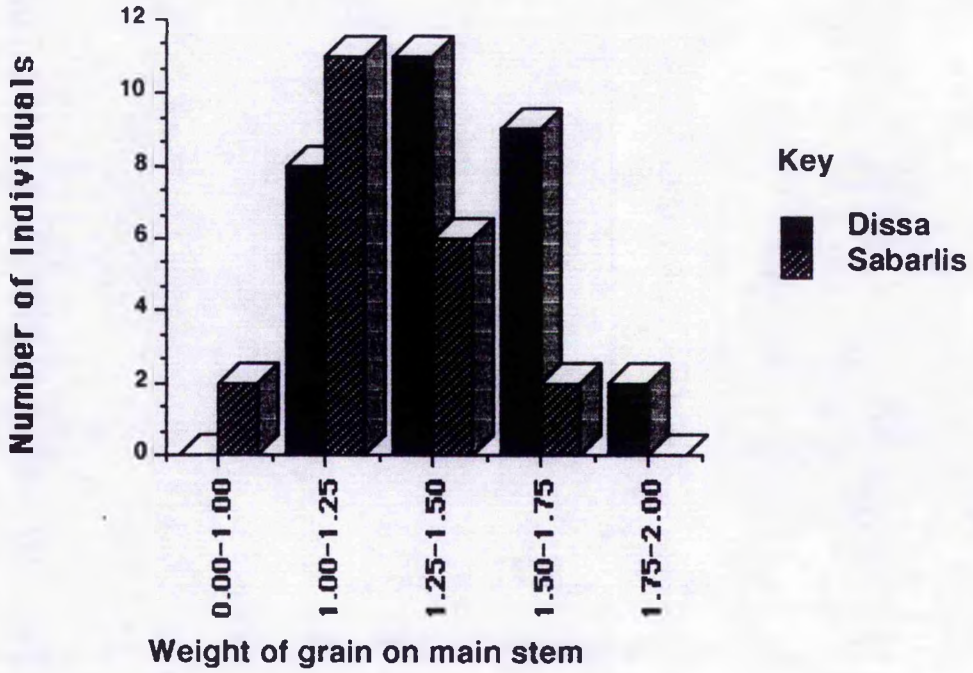


Figure 4.10. Weight of grain on main stem distribution classified according to Hor2a phenotypes.



4.3.7.3 Assessment of the analysis of quantitative trait loci in Dissa x Sabarlis

The mapping of components of polygenic systems depends on the ability to detect the effects of allelic differences which are independent of the variation associated with genetic segregation and environmental variation (Thompson and Thoday 1979). It has been previously shown that the expression of quantitative traits in barley can be significantly altered by the selection of particular isozyme phenotypes (Powell *et al.* 1990). In a similar manner, both RFLP and RAPD markers may be utilised to the same effect.

This study has demonstrated the association of alleles at morphological, biochemical and molecular marker loci with a number of quantitatively inherited characters in barley. In order to predict the level of expression of a given quantitative trait a high level of linkage disequilibrium is required (Brown *et al.* 1988). Doubled haploid lines extracted from F_1 hybrids are likely to exhibit a higher linkage disequilibrium relative to other generations and are therefore well suited to identify such associations (Powell *et al.* 1990).

The associations detected between the markers studied and the quantitative traits indicates that a significant portion of the genetic variation for these traits is determined by regions of the genome linked to these marker loci. This demonstrates that such markers may be used to detect, map and monitor genes controlling quantitative variation. Of particular value are

the molecular markers, both RFLP and RAPDs as they currently represent the largest source of available markers in many crop species. RFLP markers have been used to construct high density linkage maps in tomato (Tanksley *et al.* 1987), in maize (Helenjaris 1987) and most recently in barley (Heun *et al.* 1991).

Quantitatively-inherited traits have been studied with molecular markers in tomato (Tanksley *et al.* 1982; Osborn *et al.* 1987; Zamir *et al.* 1984), pepper (Tanksley and Ingesias-Olivas 1984), maize (Stuber *et al.* 1987) and barley (Powell *et al.* 1991). In all of these studies it was possible to detect genetic loci controlling characters of interest. There is, however, evidence that the genes underlying quantitative variation may not function in a predictable manner over a range of genetic backgrounds. Tanksley and Hewitt (1988) found that although chromosomal segments containing a QTL influencing the soluble solids content of tomato could be efficiently transferred into different breeding lines, they could not accurately predict whether the genes transferred would have the desired effect in a new genetic background. Similar studies have indicated that associations between the *Rrn2* locus in barley and QTLs may be inconsistent over crosses, even when these crosses shared the same nuclear DNA. This may indicate that cytoplasmic and or maternal effects may be involved in determining the effects of QTLs (Powell *et al.* 1991).

In order to be able to predict the effect of a QTL it is necessary to first test it in different genetic backgrounds. Additionally, the transferred QTL must be tested for effects on other agronomically important traits. Only when the effects of the QTL are determined over a range of genetic backgrounds, will the modification of quantitative traits based on the selection of linked loci be predictable, reproducible and hence of value to breeders. The large number of associations detected between the 2/6 row locus and QTL is also worthy of discussion. This morphologically important locus may be linked to agronomically important traits or the association may be due to pleiotropy. In both tomato and maize, RFLP markers associated with QTL are often chromosomally linked to extreme phenotypic mutants. For example the three traits analysed by Patterson *et al.* (1988) have large effects which map to chromosome 6 and are linked to sp (spinelessness) a morphological marker which alters plant development. Helentjaris and Shattuck-Fidens (1987) have identified RFLP markers on chromosome 9 of maize which have a significant impact on plant development. These molecular markers are located near the centromere adjacent to a known gibberelic acid biosynthesis dwarf mutant (d_3). Robertson *et al.* (1989) have postulated that extreme mutant phenotypes which map to specific chromosomal regions may also be expected to be implicated in the expression of quantitatively controlled characters. These results emphasise the importance of chromosomally mapping morphological markers and examining

their effects on the expression of QTL. The identification of polymorphic molecular markers adjacent to the 2/6 row locus on chromosome 2 of barley would allow further detailed mapping of this region of the barley genome.

Given more time the analysis presented in this chapter could be extended in two possible ways. First, the effect of recombination between the linked loci identified could be examined in relation to the expression of the QTLs. Secondly, maximum likelihood methods (Weller *et al.* 1983; Weller 1986) could be used to estimate the intensity of the linkage between a given marker and a QTL. Both approaches would bring greater refinement and precision to the localisation of QTLs to specific regions of the barley genome.

Chapter Five

Genetic resources and Macrogeoraphical differentiation in *Hordeum spontaneum* from Israel

5.1 Introduction

The potential of wild plant species as genetic resources for enhancing the germplasm of crops is well established (Frankel and Bennett, 1970; Nevo, 1986). Wild species are important sources of genetic adaptations to extreme environments and of disease resistances not possessed by their cultivated relatives. Genetic analysis of population structure and genetic diversity within these wild relatives is an important step towards exploiting these resources efficiently in breeding programs.

Hordeum spontaneum, the wild ancestor of cultivated barley (Harlan, 1979) exhibits considerable variability particularly in the Near East Fertile Crescent. A number of studies have described the structure and genetical basis of diversity in *H. spontaneum* in Israel (Nevo *et al.*, 1979, 1986; Nevo, 1991) and have emphasised the extensive isozyme variability present in these populations. Importantly the patterns of variation appear, at least in part, to be predictable both ecologically and climatically. These studies have also indicated that the geographical distribution of isozyme variation within *H. spontaneum* may be of adaptive significance.

Modern plant breeding practices have reduced the range of genetic variability amongst cultivars of many crops including barley, and recent studies of isozyme variability in *H. vulgare*

cultivars have confirmed this trend (Thompson *et al.*, 1990; Foster *et al.*, 1991). Future improvement of barley lies in exploiting its abundant wild genepool (Nevo *et al.* 1979a; Nevo *et al.* 1979b). Genetic diversity is the raw material essential to meet the diverse goals of modern plant breeding, namely producing cultivars with increased yield, wider adaptation, desirable quality and pest and disease resistance. The naturally occurring wild progenitors of crop plants are one potential source of such diversity.

The availability of molecular techniques has improved the precision with which genetic variation in cultivated and natural plant populations can be examined and analysis of ribosomal DNA (rDNA) variation has been particularly informative. rDNA is organised into tandem repeat units, with each repeat containing a highly conserved transcription unit and a more variable intergenic spacer (IGS) region.

In barley, the restriction enzyme Sac I cleaves each of the several thousand rDNA repeats twice, once on each side of the IGS region, resulting in two fragments of DNA from each repeat unit. One of the fragments is invariant in length (≈ 3880 bp) while the other fragment varies in length as a result of differences in the number of subrepeats in the IGS. A schematic map of a typical barley rDNA repeat unit is shown in Figure 5.1. Twenty rDNA spacer length variants (slvs) have been identified in barley (Allard *et al.* 1990). Each variant differs from the the next by 115 bp so as to form a complete

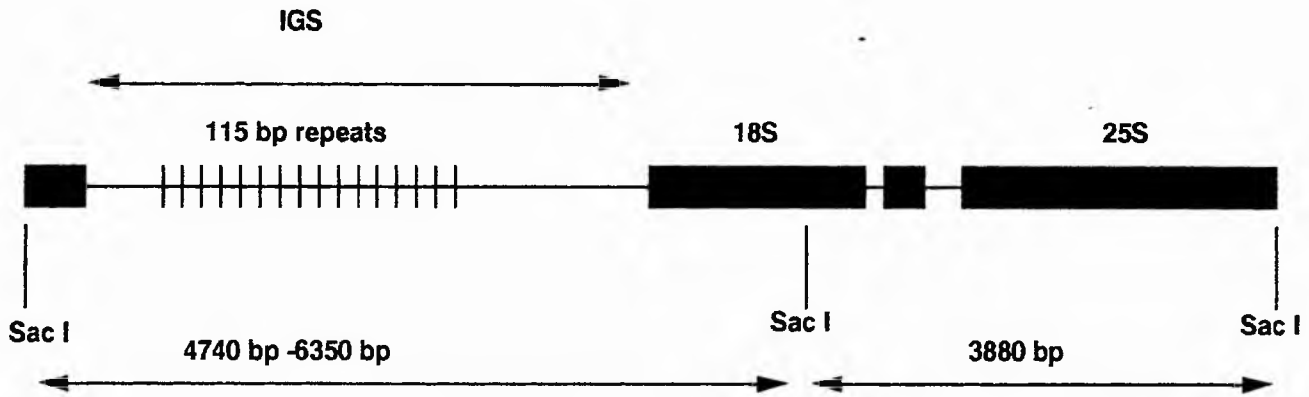


Figure 5.1 Diagramatic representation of a barley rDNA repeat unit. Regions coding for the 18S and 25S rRNA genes are labeled. The intergenic spacer (IGS) region between the 18S and 25S genes contain the 115 bp subrepeats. Sac I restriction sites are as indicated. Variation in the number of 115 bp subrepeats results in variation in the length of the Sac I fragment carrying the IGS.

ladder. These variants have been classed as slv 100 to slv 118. Only one exception to this has been found in barley, slv 108a, which is approximately 42 bp shorter than slv 108. Analysis of Mendelian segregation ratios of these slvs have established that slvs 100-107 segregate as codominant alleles of the locus *Rrn2*, associated with the nucleolar organizer region of chromosome 5, and slvs 108a-118 segregate as codominant alleles of locus *Rrn1*, associated with the nucleolar organizer region of chromosome 6. Changes in rDNA composition have been observed in *H. vulgare* (Saghai-Maroof *et al.*, 1984), *Triticum dicoccoides* (Flavell *et al.*, 1986), *Zea mays* (Rocheforde et al., 1990) and *Drosophila melanogaster* (Cluster *et al.*, 1987) following natural or artificial selection.

Isozyme and protein loci have been shown to be linked to genes controlling spring/winter growth habit in barley cultivars (Foster and Ellis, 1991) and with a range of quantitatively controlled traits in barley doubled haploids (Powell *et al.*, 1990). Associations between alleles at the rDNA loci and a range of agronomically important characters have also been detected (Powell *et al.*, 1991). These studies have emphasised the need to monitor the spectrum of molecular variability available in natural populations of *H. spontaneum*.

Saghai-Maroof *et al.* (1990) have also examined ribosomal variability in wild and cultivated barley but the present study has included an analysis of five mature endosperm proteins together with rDNA IGS variability in

H. spontaneum sampled from its entire ecological range in Israel. Thus allowing me to compare and relate protein and rDNA variability in *H. spontaneum* to a range of ecogeographical factors.

5.2. Materials and Methods

A total of 135 accessions of *H. spontaneum* were collected from 27 sites in Israel (Nevo *et al.*, 1979, Nevo *et al.*, 1984) with five plants being sampled per site. The 29 cultivars of *H. vulgare* were a selection of spring and winter varieties supplied from standard seed stocks of the National Institute of Agricultural Botany, UK. used in previous studies (Thompson *et al.*, 1990). The sites shown in Figure 5.2. were chosen to cover the ecological and geographical range in which *H. spontaneum* grows under natural conditions in Israel. The specific geographical locations and climatic background for each of the sampling sites are presented in Table 5.1. (reproduced from Nevo *et al.* 1984). The symbols used to identify the ecogeographical variates are listed as follows:

(a) Geographical variables: Lon = Longtitude, in decimals; Lat = Latitude, in decimals; Alt = altitude, in meters.

(b) Temperature: Tm = mean annual temperature, (°C); Ta = mean August temperature, (°C); Tj = mean January temperature, (°C); Tdd = day-night temperature difference, (°C); Trd = mean number of tropical days; Sh = mean number of Sharav days, i.e., hot and dry days.

(c) Water availability: Rn = mean annual rainfall, in mm, Rd = mean number of rainy days; Hu an = mean annual humidity; Hu 14 = mean humidity at 14:00 (%); Dw = mean number of dew nights in summer; Th = Thornthwaite's moisture index (Thornthwaite 1948); Ev = mean annual evaporation (cm); Rv = mean interannual variability of rainfall (%); Rr = mean relative variability of rainfall (%).

(d) Edaphitic variables: So = Soil type; 1 = terra rossa; 2 = rendzina; 3 = alluvium; 4 = sandy loam; 5 = loess.

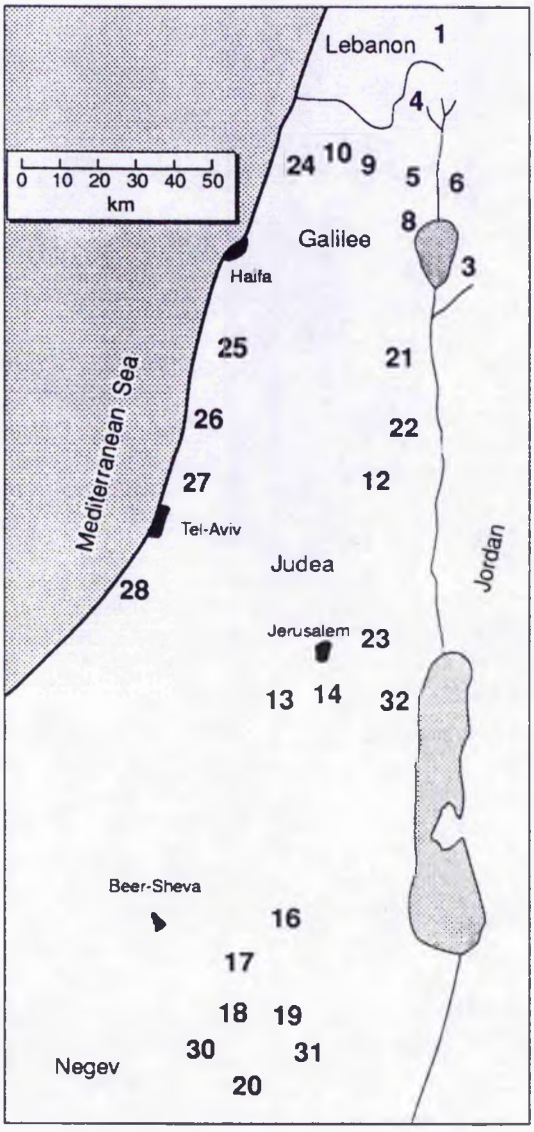
(e) Biotic variables: Pl = Plant community; 1 = Marginal Mediterranean desert batha; 2 = Tragacantic batha (phyrgana); 3 = Marginal Mediterranean batha with *Zizyphus loti*; 4 = Marginal Mediterranean batha with perennial weeds; 5 = Primary climax of *Quercus ithaburensis* (sagital); 6 = Primary climax of *Quercus ithaburensis* (batha).

5.3. Ecological and climatic background

The relatively small area of Israel is exceptionally variable and represents the main ecosystems characteristic of the Near East. In Israel, *H. spontaneum* is abundant, covering a large diversity of habitats ranging from the northern mesic Mediterranean area to the southern xeric steppes and deserts. It is commonly found in the north and central regions where it often builds up large continuous stands, while in the arid Negev

Table 5.1 Geographical locations and climatic background for each of the 27 *H. spontaneum* populations from Israel. For explaination of the titles see text.

Pop.	Locality	Lon.	Lat.	Alt.	Tm	Ta	Tj	Td	Tdd	Rn	Rd	Hu 14	Hu an	Dw	Th	Trd	Ev	So	Pl
1	Mt. Hermon	35.75	32.28	1530	11	20	1	19	6	1600	70	52	58	60	-	0	160	1	2
3	Afiq	35.7	32.78	325	21	27	11	16	11	450	50	41	57	50	-30	130	170	5	2
4	Tel Hay	35.55	33.23	400	19	25	8	17	10	769	64	50	61	40	10	75	150	1	1
5	Rosh Pinna	35.52	32.95	700	18	25	9	16	10	697	50	48	58	50	-10	35	150	1	1
6	Gadot	35.62	33.02	100	20	28	11	17	12	500	42	45	58	45	-30	150	150	3	2
8	Zefat	35.5	32.93	800	17	24	8	16	10	718	60	48	59	50	10	25	150	2	1
9	Mt. Meron	35.05	33.05	1150	14	22	6	16	8	1010	65	49	61	50	50	0	155	1	3
10	Maalot	35.27	33	500	17	23	8	15	10	785	55	50	64	55	10	0	150	2	3
12	Shechem	35.23	32.23	400	18	24	9	15	10	618	45	46	60	42	10	0	160	2	1
13	Bar Giyyora	35.08	31.72	760	17	26	10	15	9	537	43	49	61	58	-10	75	155	1	3
14	Talpiyyot	35.23	31.75	800	18	24	9	15	9	486	42	50	61	40	-10	0	160	2	2
16	Tel Shoket	34.92	31.32	375	19	26	11	15	12	280	32	45	58	55	-50	100	163	6	5
17	Bor Mashash	34.83	31.07	350	20	27	10	15	13	150	18	38	55	65	-50	125	167	6	5
18	Revivim	34.75	31.02	320	20	27	10	15	14	130	18	38	55	65	-50	130	170	3	5
19	Yeroham	34.9	30.98	490	19	26	10	16	13	130	18	35	53	65	-50	100	168	6	5
20	Sede Boqer	34.78	30.78	450	19	26	9	15	13	91	15	36	53	70	-50	100	168	6	5
21	Bet Shean	35.5	32.5	-120	22	30	13	17	14	300	42	40	55	30	-50	180	170	2	5
22	Mehola	35.48	32.13	-150	22	30	13	17	13	270	39	34	53	22	-50	183	180	3	5
23	Wadi Qilt	35.38	31.83	50	23	30	14	16	13	170	32	40	55	25	-50	125	180	3	5
24	Akhziv	35.1	33.05	10	20	26	12	13	10	620	56	60	67	59	-10	15	130	3	4
25	Atlit	34.95	32.7	50	20	26	13	13	9	500	48	65	72	75	-10	0	133	4	4
26	Caesarea	34.9	32.5	10	20	26	13	13	9	539	48	65	72	75	-10	10	130	4	4
27	Herzliyya	34.8	32.17	25	20	26	13	13	10	530	50	65	72	75	-30	0	130	4	4
28	Ashquelon	34.6	31.63	50	20	27	14	13	10	424	38	64	72	55	-30	80	120	4	4
30	Avedat	34.77	30.82	525	19	25	9	16	13	100	15	36	53	70	-50	95	168	5	6
31	Ha-Machtesh	34.97	30.97	600	19	26	9	16	13	120	19	35	53	-	-50	100	170	5	6
32	Ein Zukim	35.44	31.74	-200	24	32	15	16	12	200	20	35	52	-	-50	200	210	5	6



1	Mt. Hermon
3	Afiq
4	Tel Hay
5	Rosh Pinna
6	Gadot
8	Zefat
9	Mt. Meron
10	Maalot
12	Shechem
13	Bar Giyyora
14	Talpiyyot
16	Tel Shoket
17	Bor Mashash
18	Revivim
19	Yeroham
20	Sede Boqer
21	Bet Shean
22	Mehola
23	Wadi Qilt
24	Akhziv
25	Atlit
26	Caesarea
27	Herzliyya
28	Ashquelon
30	Avedat
31	Ha-Machtesh
32	Ein Zukim

Figure 5.2 Geographical distribution of sampling localities for *H.spontaneum* in Israel.

and Dead Sea regions it is more locally distributed. Its morphology is notably variable. Robust plants with large spikes and relatively large seeds occur in the the mesic and warm environments whereas slender, smaller forms grow in the arid steppes.

5.4. Isozyme and RFLP techniques

All isozyme and RFLP analysis were carried out essentially as described in the Materials and Methods section. Isozyme data were derived from isoelectric focusing gels and extractions were carried out on individual grains. Extractions for the esterase loci were carried out as described, however, due to the small size of grain from several populations, a complete characterisation of the *Est3* locus was not always possible. As a result the phenotype scores derived are based only on the major staining bands and may be an underestimate of the number of phenotypes present. DNA extractions were carried out on leaf tissue pooled from 5 individuals per accession 6 weeks after germination. The DNA manipulations were carried out exactly as previously described. The genomic clone pBG35 which contains the entire flax rDNA repeat unit was used as the hybridization probe.

5.5. Statistical Analysis of Genetic diversity

Shannon's index of phenotypic diversity (King and Schaal, 1989) was used to quantify the level of polymorphism detected and to partition this variability into between and within

population components. Estimates of diversity (H_o) were calculated as follows: $Ho = -\Sigma$ pi $\log_2$ pi, where pi is the phenotypic frequency.

Results

The phenotypes observed in the *H. spontaneum* populations for the five endosperm protein systems are given in Figure 5.3a together with a selection of the rDNA RFLP profiles (Figure 5.3b). Phenotypic scores for each of the five enzyme systems and rDNA variation in the populations of *H. spontaneum*, are presented in Appendix 5.1. Phenotypic data for 'protein variation in the European cultivars (*H. vulgare*) for EST3, EST10, WSP1 and β-AMY1 have been published previously (Thompson *et al.*, 1990). Several new isozyme and protein phenotypes were detected in *H. spontaneum* that had not been previously observed in the cultivated barley genepool. For example, three phenotypes were observed at the ADH1 locus which is monomorphic in cultivated barley. Similarly seven WSP1 phenotypes, five EST3, four EST10, and three β-AMY1 phenotypes were detected in *H. spontaneum* that were not present in the the *H. vulgare* cultivars examined by Thompson *et al.*, (1990). A total of eleven rDNA phenotypes were detected in the *H. spontaneum* populations whereas only three rDNA phenotypes have been detected in the *H. vulgare* genepool (Chapter 3).

Phenotypic frequencies for the six marker systems sampled from 27 geographical locations in Israel are given in

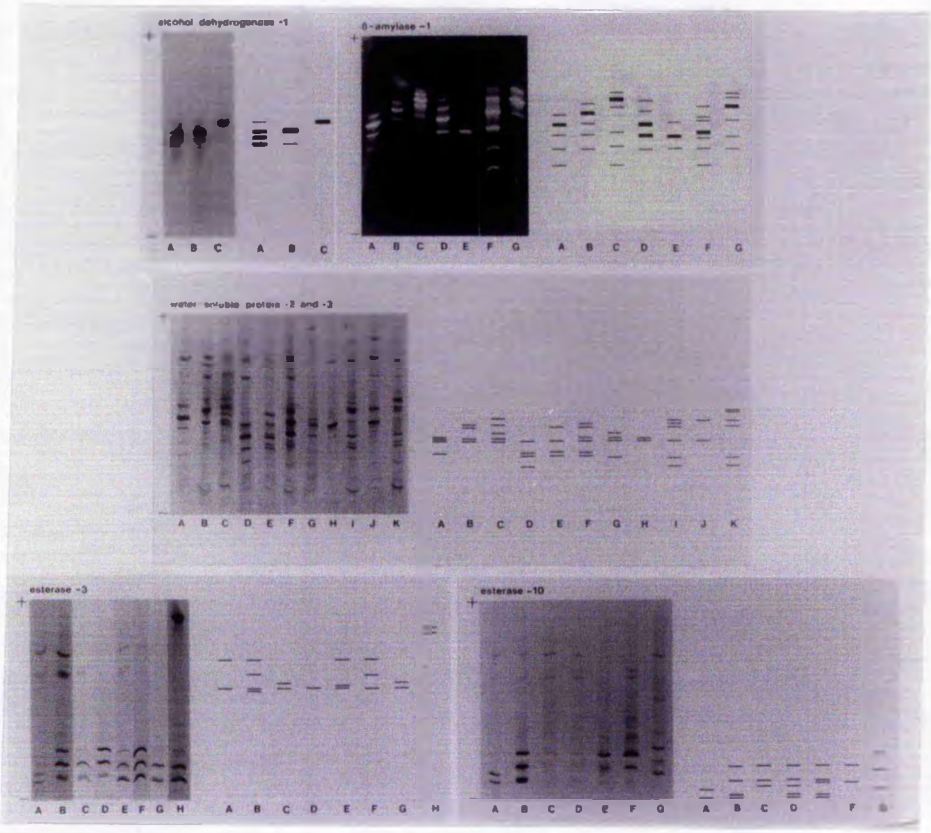


Figure 5.3a. Grain isozyme phenotypes identified in the *H. spontaneum* populations sampled from Israel.

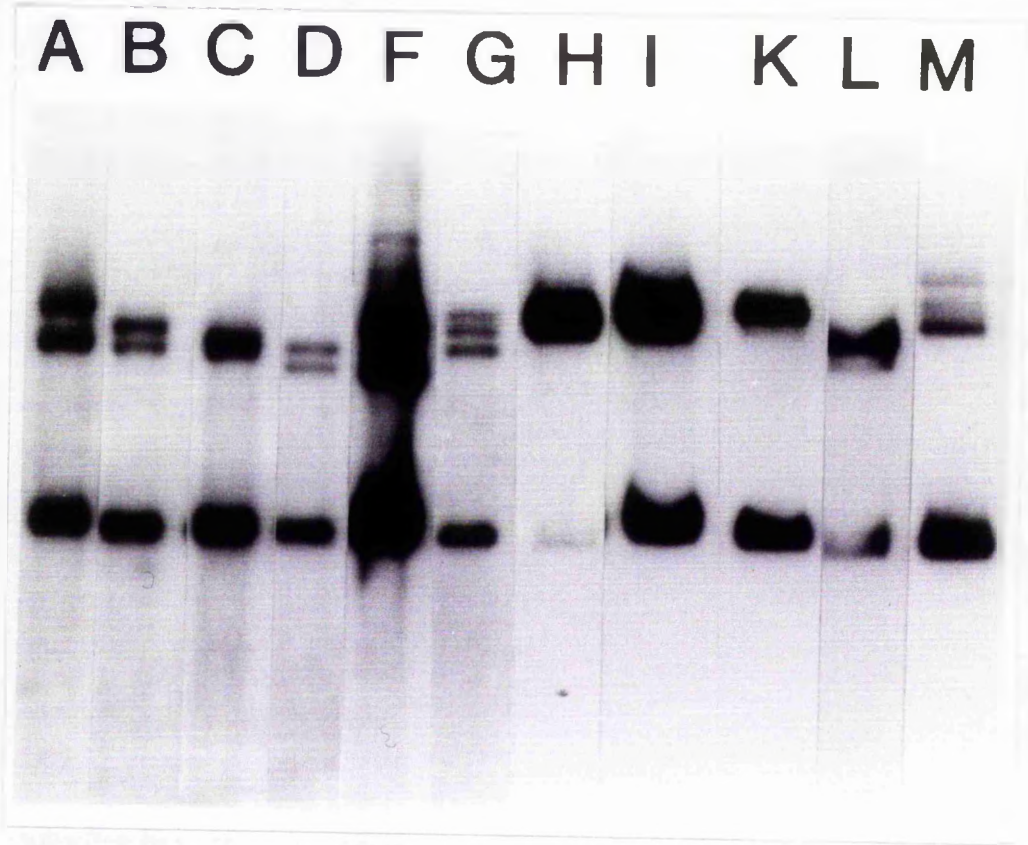


Figure 5.3b. RFLP ribosomal DNA profiles indicating the 10 phenotypes identified in *H. spontaneum* populations sampled from Israel.

Appendix 5.2. Estimates of diversity (H_o) within populations were calculated using Shannon's information measure. The results show that for each system examined there are marked differences between populations in phenotypic frequencies. In each case a proportion of populations are monomorphic while the remainder vary in degree of polymorphisms. For example, at the ADH1 locus, two phenotypes which are unique to wild barley were restricted to three sites: Tel Hay (phenotype A), Mt. Meron (C), and Bar Giyyora (C), while populations at other sites were monomorphic for the phenotype found in *H. vulgare*. Considerable variability both within and between populations was observed for the rDNA phenotypes, with six populations exhibiting three or more distinct rDNA profiles.

Shannon's index of phenotypic diversity (King and Schaal, 1989) was used to quantify the levels of polymorphism detected and to partition this variability into between and within population components (Table 5.2). H_{pop} provides a measure of the average diversity within populations. ADH1 and EST10 show relatively low levels of polymorphism, while in contrast, rDNA and EST3 exhibit relatively high levels of diversity within populations. The greatest levels of diversity within *H. spontaneum* (H_{ssp}) are found for rDNA and WSP2,3. An examination of the proportion of diversity present within populations (H_{pop}/H_{ssp}) and between populations (H_{ssp}-H_{pop}/H_{ssp}) indicates that on average most of the phenotypic variation occurs between *H. spontaneum* populations.

Table 5.2. Partitioning of the genetic diversity between and within populations of the six marker systems using Shannon's Diversity index.

	Hpop *H.spontaneum*	Hssp *H.spontaneum*	Hpop/Hssp *H.spontaneum*	(Hssp-Hpop)/Hssp *H.spontaneum*
WSP2,3	0.469	2.42	0.194	0.806
ADH1	0.102	0.31	0.329	0.671
EST3	0.691	1.692	0.408	0.592
EST10	0.143	0.695	0.208	0.794
β-AMY1	0.418	1.842	0.227	0.773
rDNA	0.800	2.689	0.298	0.702
Mean	0.437	1.608	0.277	0.723

Correlation coefficients were calculated between estimates of genetic diversity (H_o) for the six marker systems and a range of ecogeographical variables given in Table 5.1. Thirteen of the 114 correlations coefficients computed were significant (Table 5.3). In particular, ADH1 diversity was negatively correlated with soil type (So; r = -0.498; P<0.010) while genetic diversity for β-AMY1 was negatively correlated with mean annual evaporation (Ev; r = -0.386) and positively correlated with plant community (Pl; r = 0.405; P<0.01). Only for EST10 was there no evidence of a correlation between the level of genetic diversity within a population and one or more ecogeographical variables.

To extend the analysis further, a stepwise multiple regression analysis was performed using Shannon's information measure (H_o) as the dependent variable and the ecogeographical factors as the independent variables. The results (Table 5.4) indicate that a combination of ecogeographical factors can account for 29.9%, 30.7%, 22.3%, 38.4% and 33.1% of the variation observed for ADH1, β-AMY1, EST3, WSP and rDNA. The relationship between specific phenotypic frequencies and environmental factors was also examined (Table 5.5) for marker phenotypes with a frequency greater than 10%, i.e. the A, B and G phenotypes for β-AMY1 and the A and B phenotypes for rDNA. It was found that the mean number of rainy days (Rd) and the mean temperature in January (Tj) significantly influence the occurrence of the A and G phenotypes of β-AMY1. Thus 78.4% of the variation in the

Table 5.3. Correlation coefficients (r) between genetic diversity (H_o) and ecogeographical variables in *H. spontaneum* for six marker systems in the 27 sites sampled.

Variable	ADH	Beta-amylase	Est-3	Est-10	WSP 2,3	rDNA
Lon	0.43	0.065	0.079	0.009	0.039	0.009
Lat	0.255	0.034	0.034	0.015	0.306	0.269
Alt	0.395	-0.109	-0.423 *	-0.172	-0.269	-0.378
Tm	-0.384	0.011	0.395 *	0.118	0.135	0.194
Ta	-0.269 *	-0.065	0.477 *	0.080	0.051	0.102
Tj	-0.296	0.093	0.519 **	0.084	0.228	0.334
Td	0.127	-0.242	-0.189	0.092	-0.326	-0.268
Tdd	-0.371	-0.168	0.049	0.082	-0.104	-0.160
Rn	0.335	0.124	-0.118	-0.065	0.012	0.173
Rd	0.377	0.298	0.035	0.035	0.117	0.349
Hu14	0.096	0.329	0.202	-0.027	0.298	0.465 *
Huan	0.076	0.324	0.186	-0.004	0.330	0.449 *
Dw	-0.095	0.036	-0.240	-0.107	0.166	-0.203
Sw	0.283	-0.364	-0.200	-0.107	-0.455 *	-0.213
Th	0.597	0.229	-0.316	-0.050	0.162	0.079
Trd	-0.165 **	-0.133	0.293	0.127	-0.110	-0.121
Ev	-0.089	-0.386 *	-0.040	-0.009	-0.297	-0.433 *
So	-0.498 **	-0.041	0.164	0.131	0.222	-0.103
PI	-0.297	0.405 * *	0.206	0.040	0.153	-0.176

TABLE 5.4. Coefficients of multiple regression (R^2) of dependent variable Ho and independent ecogeographical variables in 27 populations of *H. spontaneum* in Israel.

	Stepwise model				
	X_1 X_2 X_3 X_4	$R^2 1$	$R^2 2$	$R^2 3$	$R^2 4$
Constant					
(H_o) ADH	So Lon Alt Trd	20.1*	23.8*	26.1*	29.9*
(H_o) β-amylase	Ev Pl So Trd	5.9^{ns}	8.2^{ns}	25.6*	30.7*
(H_o) WSP	Ev So Lat Pl	15.0*	19.2*	36.5**	38.4**
(H_o) Est-3	Tj	22.3*			
(H_o) rDNA	Alt Rd Lat Rn	23.5*	25.9*	28.8*	33.1*

Level of significance:** = $P < 0.01$; * = $P < 0.05$; ns= $P > 0.05$

Table 5.5. Coefficients of multiple regression (R^2) of dependent variable phenotypic frequencies and independent ecogeographical variables in 27 populations of *H. spontaneum* in Israel.

	Stepwise model					
	X_1 X_2 X_3 X_4 X_5	$R^2 1$	$R^2 2$	$R^2 3$	$R^2 4$	$R^2 5$
Constant						
β-amylase-A	Rd Tj Alt Dw Pl	10.8*	21.8*	33.4*	39.8**	46.1**
β-amylase-B	Lat So Ev Rn Alt	25.0**	32.6**	35.8**	39.2**	48.8**
β-amylase-G	Rd Tj Dw Tdd Th	63.6***	78.4***	83.2***	88.8***	89.5***
rDNA-A	Tj Pl Ev Huan Rn	19.6*	40.2**	44.9**	59.9**	63.3**
rDNA-B	Huan Ev Trd Lon	31.6**	51.6**	65.1***	77.3***	82.9***

Level of significance: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$.

frequency of the β-AMY1 G phenotype can be explained by Rd and Tj. These two factors are also responsible for a significant portion of the variation in the frequency of phenotype A. In contrast, over 25.0% of the variation in the frequency of phenotype B could be explained by latitude. These results indicate that the individual phenotypes of β-AMY1 are associated with certain geographical regions where specific environmental regimes predominate. The geographical distribution of the β-AMY1 phenotypes is presented in Figure 5.4a the distribution of the A and G phenotypes are quite distinctive with phenotype G being restricted to the Negev desert and the Dead Sea regions.

Multiple regression analysis of the A and B rDNA phenotypic frequencies (Table 5.5) indicate that 63.3% of the distribution of phenotype A and 82.9% of the distribution of phenotype B could be accounted for by five environmental factors. The distribution of rDNA phenotypes is given in Figure 5.4b and highlights the predominance of B phenotypes in the Negev desert region of Israel. These data demonstrate that the occurrence of certain rDNA and β-AMY1 phenotypes in Israel can be predicted from a limited number of ecological and environmental factors.

5.6. Discussion and Conclusions

Several previous studies have examined isozyme variation in *H. spontaneum* from Israel (Nevo *et al.* 1979, Nevo *et al.* 1981) but this is the first examination of the level of

Figure 5.4a

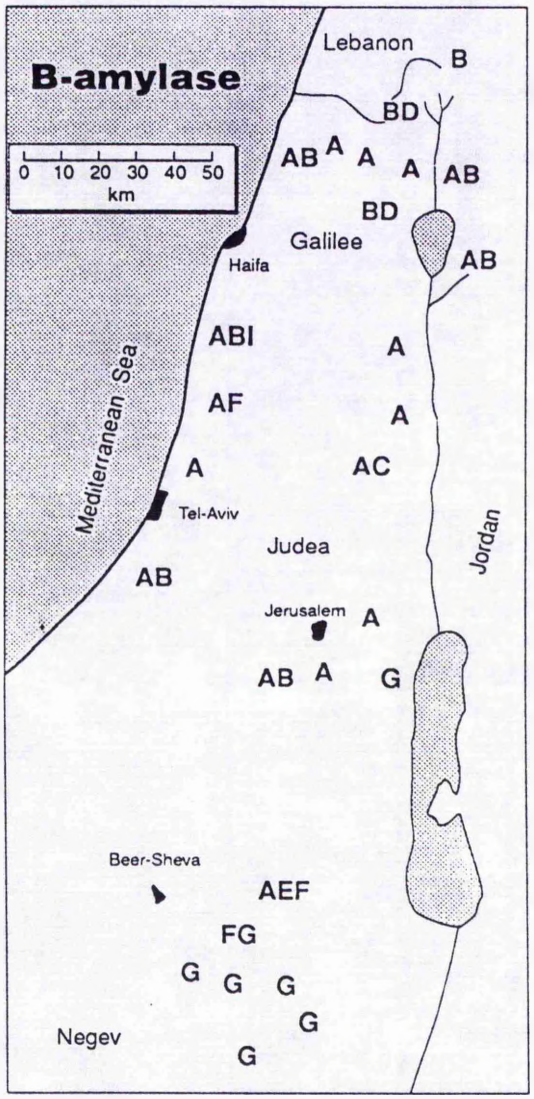


Figure 5.4b

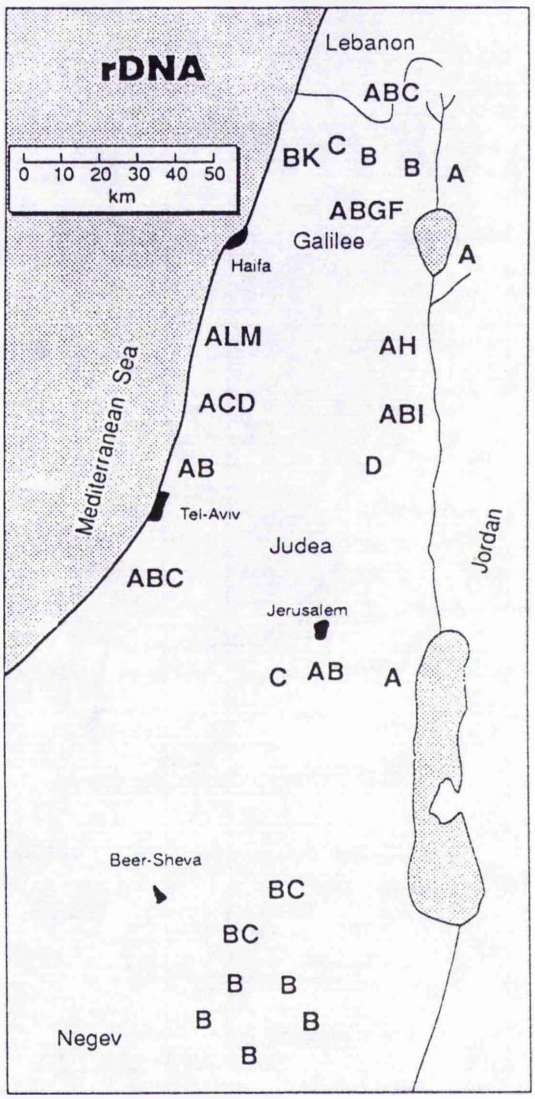


Figure 5.4a The distribution of β-amylase phenotypes in Israel.

Figure 5.4b The distribution of rDNA phenotypes in Israel.

polymorphism observed in *H. spontaneum* for endosperm derived isozymes. Several new phenotypes were observed which have have not been detected in the *H. vulgare* genepool (Thompson *et al.* 1990). For *EST-3, EST-10* and β-*AMY-1* these novel phenotypes correspond to new alleles. For ADH1, three phenotypes were found with a distribution in good agreement to that found for ADH1 phenotypes based on leaf extracts (Nevo *et al.*, 1979). The common phenotype 'B' is equivalent to 'b' in Nevo *et al.* (1979), the rare phenotype 'A' (equivalent to Nevo *et al.*'s 'a') was found in population 13 (Bar Giyyora), and by Nevo *et al.* (1979) in the neighbouring population, 14, (Talpiyyot), similarly the 'C' phenotype has a similar distribution to Nevo *et al.*'s 'c' phenotype. For WSP it is not possible to identify specific alleles since more than one locus is involved in the control of this marker system (Foster *et al.*, 1991). Judged over all marker systems, a greater proportion of phenotypic diversity was found between rather than within populations of *H. spontaneum.*

Both the overall levels of genetic diversity and the frequency of certain phenotypes were significantly correlated with a number of ecological or environmental factors. Similar observations have been reported for isozymes (Nevo *et al.* 1979) and hordein polymorphisms (Nevo *et al.*, 1983) in *H spontaneum.* These examples serve to illustrate the importance of wild relatives of cultivated barley as a source of useful genetic variability. Modern plant breeding practices have reduced the range of genetic variability available in the

cultivated *H. vulgare* genepool and *H. spontaneum* germplasm represents an important source of genetic variability that can be introduced into barley cultivars through conventional hybridization. An improved understanding of the genetic structure of a population can facilitate the exploitation of wild relatives of crop plants such as *H. spontaneum*. In the present study an examination of the geographical distribution of the β-AMY1 alleles revealed a replacement of the A phenotype by the G phenotype in the more arid regions of Israel. Furthermore, regression analysis showed that over 63% of the variation in the frequency of the G phenotype can be accounted for by the mean number of rainy days (Rd) per year. Recent studies have demonstrated that biochemical markers, including β-AMY1 on chromosome 4H are linked to genetical factors controlling the time to ear emergence in barley (Ellis *et al.*, 1991). Further genetical studies are now required to elucidate the underlying mechanism for the restricted distribution of the G phenotype in the *H. spontaneum* populations studied.

Considerable rDNA IGS variability was observed both within and between *H. spontaneum* populations, and much of the variation was correlated with a combination of ecological and climatic variables (Table 5.4). A previous study by Flavell *et al.* (1986) of variation in the IGS of rDNA of tetraploid wheat (*T. dicoccoides*) originating from 12 sites in Israel has also shown significant correlation between spacer length variation and a range of environmental variables. A general question arising from these studies is the role of selection in generating

and maintaining the extensive heterogeneity between and within populations. There are at least two possibilities: selection is operating directly upon the rDNA or alternatively the rDNA loci are linked to adaptively important traits.

When considering the possibility that selection may act on the intergenic spacer region of the rRNA gene, it is important to consider the possible functions of this DNA region. The method of supplying the enormous demand for rRNA in most eucaryotes, is to maintain a large number of rRNA genes tandemly arranged. Plants usually carry from 500 to 40,000 copies per diploid cell (Ingle *et al.*, 1975). All available evidence suggests that superfluous rRNA copies exist in plants and that these extra copies are in considerable excess to that required to sustain ribosome synthesis (Ingle *et al.* 1975). It is not known whether the extra rDNA is important or non-functional and simply tolerated. It is possible that environmental conditions play some role in selecting for rDNA copy number variants, but the most likely explanation is that the changes and variability are both caused by recombination events that lead to an adequately large rDNA pool, but most of the rDNA is in excess of that needed to produce rRNA and is merely tolerated.

The heterogeneity in rDNA repeat length seen among members of the same genus or species is attributable to variability in the IGS. In the cases investigated, the variability is in the number of a subrepetitive element that is generally

between 100 and 400 bp long and is species-specific in sequence; in barley the repeat is 115 base pairs (Saghai-Maroof *et al.*, 1984), in wheat 135 base pairs (Appels and Dvorak 1982), in peas about 180 base pairs (Jorgensen *et al.*, 1982) and in *Vicia faba* about 325 base pairs (Yakura and Tanifuji, 1983). The variability in the number of these units from one IGS to another has been attributed to unequal crossing over between subrepeats either on the same chromosome or its homologue. Crossing over between distant genetic loci does not appear to be common (Dvorak, 1986; Polans *et al.*, 1986) although in onion the rDNA loci have been inferred to jump between loci (Schubert *et al.*, 1978). The exchanges probably occur during both mitosis and meiosis since different numbers of rRNA genes can be found among tissues of the same plant (Rodgers and Bendich, 1987). So far every IGS examined has contained subrepetitive elements at a similar location.

It appears that the subrepeats within the IGS are "hotspots" for recombination and that by increasing exchanges in the rDNA regions they promote copy number change (Rodgers and Bendich, 1987). Part of their function may be maintaining the multiplicity of rDNA but, it is probably not their only function. The IGS itself contains several different regions that control rRNA transcription and or processing in animals. Plants also contain different IGS regions similar in form at least to animal IGS regions. One of the types of subrepetitive units in the IGS of *Xenopus* appears to be involved in nucleolar dominance, a phenomenon in which one

class of rRNA is transcribed while the other is not (Reeder, 1985). Repetitive sequences near the 5' end of the 18S gene may bind a protein factor involved in RNA polymerase I attachment to the spacer region (Reeder, 1985). These sequences have been termed "enhancers" since they can affect transcription in either a forward or reverse orientation and may sequester limiting transcription factors in nucleolar dominance. The subrepeats in wheat have been considered as enhancers similarly involved in nucleolar dominance partly because of sequence homology with a *Xenopus* promoter region (Flavell, *et al.*, 1986).

Another possibility is that the subrepeats in plants are either RNA processing or transcription termination sites as indicated in several animal studies (Labhert and Reeder, 1986; DeWinter and Moss 1986). Their effect is apparently to allow the polymerase to be retained within the IGS instead of falling off the DNA at the end of the 28S gene. Within the authentic promoter region in *Xenopus* the terminator sequence is repeated again within close proximity to the transcription start site. It has been found that this terminator is essential for transcription of the adjacent gene (Baker and Platt, 1986). In plants a similar arrangement has been reported, but only sequence and not functional data are available.

These subrepeat sequences may be enhancers or promoters but it is also possible that they are terminators and still occur within the promoter vicinity because they are

required for promoter activity. The repeat units are located a few hundred bp from the 3' end of the 25S gene, similar to the location in *Xenopus* (Baker and Platt 1986; Henderson and Sollner-Webb, 1986). Also, stem-loop structures bordered on one end by pyrimidine rich regions have been inferred to be important in transcription termination (Henderson and Sollner-Webb, 1986). Thus positional and structural characteristics suggest that the subrepetitive units are transcription termination regions important for control of polymerase retention and movement, although any functional assignment will require experimental verification. It has been suggested that terminators in the IGS of animals may modulate rRNA production in response to metabolic demand by providing stop/restart control of transcription at each ribosomal gene (Baker and Platt, 1986). If the subrepeats in the upstream region of the IGS do contain terminators, their high copy number in plants relative to animals may be useful in preventing unnecessary transcription of so many superfluous copies of rDNA (Baker and Platt, 1986).

Several studies of the effect of selection acting on alleles marked by spacer length variation have been reported in barley. In a study of 54 generations of a *H. vulgare* composite cross population, Saghai-Maroof *et al.* (1984) observed that specific classes of spacer length variants (slv) that were originally present at low frequency at the *Rrn2* locus became predominant in later generations, whereas the originally predominant class decreased markedly and others disappeared

entirely. In addition the *Rrn1* locus became fixed for a specific slv. They concluded that the enduring phenotypes were strongly favoured over all other rDNA alleles by natural selection under the environmental conditions in which the populations were grown. However, as the barley composite cross population studied was generated from a wide genetic base with diverse parents from contrasting geographical regions, changes in the observed slv frequencies may therefore have been due to the loss of unadapted genotypes. The association of the barley *Rrn2* locus with traits that have a strong reproductive advantage has been demonstrated in *H. vulgare* genotypes fully adapted to the conditions of cultivation (Powell *et al.*, 1990). Doubled haploids generated from reciprocal F_1 hybrids which were heterozygous for alleles at the *Rrn2* locus were used to assess the the effect of this locus on several quantitative trait loci. Allelic frequencies of the slvs in the progeny did not deviate significantly from the expected 1:1 ratio. Variation at the *Rrn2* locus was shown to be responsible for a significant proportion of the genetic variation for yield, thousand corn weight and water sensitivity. This would indicate that the *Rrn2* locus is associated with traits that have a strong reproductive advantage. The fact that the two adapted allele types assessed were able to account for a significant amount of variation in *H. vulgare* may suggest that the greater levels of diversity present in *H. spontaneum* would likely lead to even more pronounced effects on these traits. It is thus possible that loci associated with the various slvs may

influence reproductive advantage in natural populations, and hence influence the observed frequencies of the slvs.

In a study of rDNA variability in *H. spontaneum* similar in kind to that of the present investigation Saghai-Maroof *et al.* (1990) demonstrated that genetic diversity and genotypic frequencies among populations sampled from Israel and Iran were also significantly correlated with ecogeographical factors suggesting that alleles and genotypes marked by the slvs differ in adaptive properties. They concluded that the most common slv behaved as a well adapted wild type allele, while other locally frequent slvs acted to enhance adaptedness to locally specialised habitats. Slvs detected at low frequencies, and in the presence of the adapted phenotypes, were considered to be subvital or semi-lethal, and maintained as part of a compound allele along with a favoured slv. A genetic analysis of the inheritance of slvs in barley (Allard *et al.*, 1990) has shown that homozygotes for rare slvs frequently occur in the F_2 at a frequency significantly lower than expected. From this it was concluded that rare slvs have adverse effects on reproductive capacity and/or viability under glasshouse conditions and possibly in natural populations also.

Allard *et al.* (1990) have favoured the possibility that selection acts directly on the rDNA loci in barley stemming from the sequence variability in the transcription units associated with the slvs. They have concluded that natural selection acting directly on the rDNA alleles of *Rrn1* and *Rrn2*

plays a major role in the development and maintenance of the observed patterns of molecular and genetic organisation of rDNA variability in both wild and cultivated barley. It cannot be ruled out, however, that traits governed by genes associated or linked to the rRNA loci may be responsible for the observed differences in reproductive advantages as suggested by Powell *et al.* (1991). Allard *et al.* (1990) have suggested that one possible way to resolve the dilemma is to sequence the slvs and transcription units of a selected set of alleles and then attempt to relate sequence data to differences in selective advantage.

In conclusion, this study has demonstrated that both grain protein and rDNA phenotypes are distributed in a non-random manner in *H. spontaneum* populations sampled in Israel. The restricted distribution of the β-AMY1 G phenotype which is largely confined to the Negev desert region of Israel suggests that variation at this locus may be of adaptive significance for abiotic stresses in barley. Consequently, allelic variation at the β-AMY1 locus is likely to be an useful indirect marker for abiotic stresses in barley. Since *H. spontaneum* is easily crossed with *H. vulgare* the phenotypic variation detected in *H. spontaneum* can be readily transferred into the cultivated genepool for evaluation by barley breeders.

Chapter Six

Discussion and Conclusions

6.1. Introduction

The detection and exploitation of polymorphism in plants represents one of the most important developments in plant biology. Although recombinant DNA technology is used in generating molecular markers the strategy does not include the genetic engineering of plants. Gene mapping research is therefore not constrained by regulations relating to the release of genetically engineered organisms. The role of molecular markers in barley cultivar identification is discussed together with strategies for the location and manipulation of both mono and polygenic traits. The potential of molecular markers in gene introgression from exotic barley germplasm into adapted cultivars is also considered.

6.2 Barley Varietal Identification

Conventional barley varietal identification procedures essentially involve detailed morphological descriptions and field comparisons of seed stocks (Eade and Law 1983). The use of SDS-PAGE analysis of hordein storage proteins has gained some acceptance, although this technique is unable to characterise varieties unequivocally. Several isozyme systems have been proposed as a means of varietal identification, although the only system to offer a significant improvement over conventional procedures is based on the analysis of grain proteins using IEF (Thompson *et al.* 1990). It is the use of molecular markers that offer the most promising method for

varietal identification in barley. Bunce *et al.* (1986) have demonstrated the use of RFLPs to fingerprint barley cultivars.

The major advantage of varietal identification based on both grain isozymes and the hordeins is that they require only a small portion of the endosperm for extraction while the embryo may be retained for germination. This is particularly important in breeding programmes where individual plants need to be selected. The ability to carry out analysis on a single grain may also be important for determining the purity of genetic stocks. Although it is possible to extract DNA from dry embryos, bulked grain is required to provide sufficient amounts of DNA for RFLP analysis, thus preventing RFLP phenotypes from being accurately determined for individual grains as would be required for routine purity testing of stocks.

The fact that PCR based techniques require only nanogram quantities of DNA to act as a template offers an alternative approach to the use of RFLPs. Most plant DNA extraction techniques are capable of isolating sufficient quantities of DNA from a single barley endosperm thus enabling RAPD markers to be used. The small amount of DNA required for these reactions should enable each endosperm to be assayed simultaneously for several RAPD markers offering a more accurate classification than that based on conventional protein and isozyme markers. Hence RAPD markers may in future be adopted as a suitable method for routine varietal identification either in combination with existing techniques or

if suitable polymorphic primers are identified, potentially replacing these techniques.

6.3 Genetic maps and gene introgresion

The first step towards the identification and manipulation of genes involved in the control of agronomically important characters is the development of genetic linkage maps. To determine linkage relationships amongst genetic loci one must generate crosses which segregate for the genes of interest. Using conventional markers it has been difficult to score more than a few segregating markers simultaneously in a single cross. The generation of classical genetic maps has thus required hundreds of crosses and the analysis of thousands of segregation progeny.

More recently isozyme markers have assumed greater importance, however the limited number of polymorphic loci in barley has restricted their use in genetic mapping and linkage analysis. Current mapping programmes now include the use of molecular markers, particularly RFLPs (see Wettstein-Knowles 1989), and more recently PCR markers (Shin *et al.* 1990), to overcome the restrictions imposed by the limited numbers of isozymes. Both of these molecular techniques share a number of advantages with isozymes and have the potential to generate large numbers of markers for use in genetic analysis. The present study has involved the mapping of morphological, biochemical, RFLP and RAPD markers in a doubled haploid population of barley. Although the number of polymorphic

markers identified was limited, the methodology for further development in this area has been established. Recently, Heun *et al.*, (1991) have reported the production of a linkage map in barley with more than 100 markers. These workers exploited a doubled haploid population produced by anther culture from a cross between Proctor and Nudinka. The parents were chosen to maximise diversity and hence increase the probability of detecting polymorphisms. However, no isozyme or RAPD markers have been reported in this population.

The availability of high density genetic linkage maps offer an important tool for the plant breeder particularly for marker assisted introgression of new traits into breeding populations. Plant breeders have recognised the potential of exotic germplasm for varietal improvement and significant successes have been achieved as reviewed by Stalker (1980). The most common method of transferring genes from exotic germplasm into adapted varieties is through backcross breeding. The objective is to eliminate the exotic donor germplasm as rapidly as possible, replacing it with the recipient cultivar genome whilst retaining the gene of interest from the donor. RFLP markers which are tightly linked to traits of interest can be used to select backcross derivatives with the least amount of undesirable donor DNA. In this way RFLP markers can be used to expedite the transfer of small amounts of genetic information into commercial varieties. The introgression of important genes from wild species into cultivated plants is regarded as the most significant

contribution of RFLP technology to plant breeding (Tanksley *et al.*, 1989).

The analysis of biochemical and molecular variability in *H. spontaneum* from Israel (Chapter 5) revealed considerable genetic variability in the *H. spontaneum* genepool. Furthermore, this variability was not distributed in a random manner. Specific phenotypes for β-amylase and rDNA were restricted to certain geographical areas. This suggests that variation at these loci or alleles linked to them may be of adaptive significance. These particular alleles may therefore be of value for directed gene introgression from *H. spontaneum* into the *H. vulgare* genepool. The present study has identified *H. spontaneum* genotypes possessing relatively rare alleles for both biochemical and molecular markers. The availability of these and other genetic markers is expected to have a major impact on gene introgression studies in barley. In other words, these genetic markers will provide the means to improve the precision and speed with which alien genetic information is introduced into adapted barley cultivars.

The exploitation of genetic markers in barley will place greater emphasis on the maintenance and conservation of genetic resources. It is therefore imperative that maximum levels of diversity are retained in germplasm collections to ensure that future barley improvement programmes have access to the appropriate sources of genetic variability. In this context, it is also important to have information on the

distribution of diversity in relation to ecological, climatic and geographical factors. This spatial distribution of diversity within the *H. spontaneum* genepool of Israel was shown to be non-random and information of this nature may help formulate and optimise strategies for the collection and maintenance of variability in the *H. spontaneum* genepool.

Information on the molecular diversity available in crop genepools is limited and this in part reflects the labour and resources require to process the large number of samples involved in such studies. Methods for processing large numbers of samples quickly and cheaply are required. PCR based assay methods are obvious candidates for such studies. Indeed Anderson and Fairbanks (1990) have proposed that RAPDs should be use to estimate diversity levels in germplasm collections.

6.4 Quantitative Trait Analysis

One of the major challenges in plant breeding is the reliable manipulation of quantitative traits. An important future development will be the integration of genetic markers with DHs and recombinant inbred lines. This will allow genetic markers including RAPDs to be efficiently targeted towards regions of the barley genome which are important in the control and expression of quantitative traits. This study has initiated the process of identifying markers linked to important agronomic traits. For example, the hordein loci were shown to be associated with genetic factors controlling grain number.

The availability of more genetic markers would allow this approach to be extended. Although the present study focused on agronomic traits, the same procedures may also be used to analyse disease resistance genes. Of particular relevance to barley breeders will be the manipulation of polygenic sources of resistance to mildew (*Erysiphe graminis*) and other diseases. The use of genetic markers may then allow the assembly of major and polygenic sources of disease resistance genes in a single genotype.

Although these approaches offer considerable promise for the precise manipulation of QTLs, further advances in our understanding of quantitative traits may emerge from the development and evaluation of recombinant backcross inbred lines (Beckmann and Soller, 1989). In this procedure genetic markers are used in conjunction with backcrossing to selectively introduce specific regions of a chromosome from a donor into a recipient genome. Molecular markers are used to select for specific segments of the donor and recurrent genotypes. The end result of such an approach is the creation of chromosome substitution lines which allow a detailed dissection of characters associated with this region of the genome. The value of similar substitution lines has been documented in wheat (Law, 1987). The obvious candidate for such an approach in barley would be the selective transfer of chromosome regions from *H. spontaneum* into adapted *H. vulgare* backgrounds.

6.5 Future Prospects

As the genetic maps of many species have becomes relatively saturated the continuing mapping of random markers becomes progressively less efficient. In order to concentrate the mapping of markers to specific areas of interest or areas that are sparsely covered isogenic lines have been employed. Hinze *et al.* (1991) have recently used this approach to identify RFLP markers linked to the barley *Ml-o* mildew resistance locus on the long arm of chromosome 4. Several sets of Near Isogenic Lines (NIL) are available in barley and these include NILs for cereal cyst nematode (*Heterodera avenae*) and scald (*Rhynchosporium secalis*). RFLPs in conjunction with NILs therefore offer an opportunity to identify markers linked to disease resistance genes in barley. However, it should be noted that the identification of linkage between five RFLP markers and the *Ml-o* locus required the screening of approximately 1100 anonymous genomic PstI barley clones (Hinze *et al.*, 1991). In order to improve the efficiency of identifying markers, Martin *et al.* (1991) have proposed using a combination of RAPD markers and near isogenic lines to identify DNA sequences that are closely linked to important plant genes. Martin *et al.* (1991) working with tomato NILs identified three markers linked to the gene conferring resistance to the pathogen *Pseudomonas syringae* from a total of 600 primers screened over the period of one month. This approach contrasts markedly with the use of RFLP markers which required the screening of 800 probes to

identify 5 markers linked to the same resistance gene over the course of 3 years.

6.6. Bulked segregational analysis

RAPD markers in conjunction with NILs offers an efficient means of identifying linkage between a marker and the gene of interest. However, this approach is dependent on the availability of appropriate genetic stocks. Michelmore *et al.* (1991) have proposed an alternative method for the rapid identification of markers linked to important traits which has general applicability. The procedure involves the creation of two bulked DNA sample from a segregating population. Each bulk contains individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The two bulks can be made from any genomic region and from any segregating population. However, if the F_2 is to be used then heterozygotes are excluded usually by progeny testing (F_3 analysis). Michelmore *et al.* (1991) have used this method to identify RAPD markers linked to downy mildew resistance genes in lettuce and have stressed that such an approach can also be used to identify markers in regions of the genome which are devoid of markers. In addition bulked segregation analysis can be used to identify markers linked to QTLs. Bulked analysis of barley DHs would provide an interesting opportunity to identify molecular markers linked to QTL. Further advances in gene mapping will demand an integrated multi-disciplinary approach in which the skill of the barley

breeder to identify well characterised germplasm will be of paramount importance.

6.7. Map based gene cloning

As tight linkage between molecular marker loci and characters of interest is achieved, it may then become possible to use map based cloning strategies in order to clone the genes controlling these characters. Several approaches are available based on first identifying markers linked to the target gene as a starting point. One method of molecular cloning that has proved effective is chromosome walking and jumping (Rommens *et al.*, 1989); that is the successive isolation of overlapping clones, beginning at the marker locus and ending at the gene of interest. *Arabidopsis* has been proposed as the plant of choice in which to carry out such mapping (Meyerowitz *et al.*, 1989). There is little dispersed repetitive DNA present within the *Arabidopsis* genome and what there is found mainly in large tandem arrays (Pruitt and Meyerowitz, 1986). Therefore there are few obstacles to the isolation of overlapping clones by repetitive screening of genomic libraries. However, the extension of this technology to barley will require a highly saturated linkage map and eventually a reliable transformation system. Barley may therefore not be a good candidate and rice (*Oryza sativa*) would be a more appropriate cereal for map based gene cloning (Prince and Tanksley, 1992).

6.8. Conclusions.

1. This study has established the methodologies to detect and analyse polymorphism at isozyme and RFLP loci. Both nuclear and chloroplast RFLP variability was detected.

2. The segregation of alleles at isozyme and RFLP loci has been monitored in a DH population of barley. Several loci were shown to be linked and were mapped intra-chromosomally.

3. Associations between genetic markers and a range of agronomically important quantitative traits have been studied. Several genetic markers were identified which influenced the expression of QTL.

4. A rapid marker R004 was shown to be linked to the *Est3* locus on the short arm of chromosome 7.

5. The spectrum of genetic variability for five grain isozymes and rDNA variability has been studied in *H. spontaneum* sampled in Israel. The distribution of both isozyme and rDNA phenotypes was non-random and correlated with a range of ecogeographical factors.

6. Opportunities for the exploitation of genetic markers in barley improvement have been identified.

6.9. Significance and Future Research.

The significance of this research is that the methodology to detect polymorphism at the molecular level has been

established in a Research Centre which has a strong commitment to barley improvement. In particular, RAPD markers in conjunction with DH and bulk segregation analysis will allow areas of the barley genome to become saturated with genetic markers. However, the success of this approach will depend on identifying appropriate populations exhibiting sufficient polymorphism but also segregating for traits of importance to barley breeders.

Barley breeders have recognised the importance of *H. spontaneum* as a source of genetic variability but its utilization in breeding programmes has been limited. The availability of "user friendly" genetic markers will act as a catalyst for an expansion in the use of *H. spontaneum* in barley improvement programmes.

Appendix 4.1. Dissa (D) x Sabarlis (S) phenotypic scores *** representing missing data

	Hor 2	Hor 1	Hor 2a	Hor 2b	pBG35	R004	Est-3	Est-10	L-Est	A-amy	Wsp4	Wsp3	Wsp2	'2/6'
91	D	D	D	D	D	D	D	D	D	S	S	S	S	S
92	D	D	D	D	D	S	S	D	D	S	D	D	D	S
93	D	D	D	D	D	D	S	S	S	D	D	D	D	S
94	S	S	D	S	S	S	S	D	D	S	D	D	S	D
95	S	S	D	S	S	S	D	S	S	S	D	D	S	S
96	S	S	S	S	D	D	S	S	S	D	S	D	S	D
97	S	S	S	D	S	D	D	D	D	S	S	D	S	D
98	S	D	S	S	S	D	S	D	D	D	D	D	S	S
99	S	***	S	S	S	D	D	D	D	S	D	D	D	S
100	S	S	S	S	D	S	S	D	D	S	D	D	D	S
101	D	D	D	D	D	S	D	D	D	D	D	D	D	S
102	D	D	D	D	D	D	D	S	D	S	D	D	D	S
103	S	S	S	S	S	D	D	S	S	D	S	S	S	S
104	D	D	D	D	S	S	S	S	S	D	S	S	D	S
105	D	D	D	D	S	D	D	D	D	D	D	S	S	D
106	D	D	D	D	S	D	S	D	D	S	D	D	S	S
107	S	S	S	S	S	D	D	S	S	D	D	D	D	S
108	D	D	D	D	D	S	D	S	S	S	S	D	D	D
109	D	D	D	D	D	D	D	D	D	D	D	D	S	D
110	D	D	D	D	D	S	S	D	D	D	D	S	D	S
111	D	S	D	S	S	S	S	S	S	D	D	D	D	S
112	D	D	D	D	S	D	S	S	D	S	S	D	S	D
113	S	D	D	D	D	D	S	D	S	S	S	D	S	S
114	S	S	S	S	S	D	S	D	D	D	S	S	S	S
115	S	***	S	S	S	***	S	S	D	S	S	D	D	S
116	S	S	S	S	S	D	D	D	D	D	S	D	D	S
117	D	D	D	D	D	S	D	D	D	S	D	S	D	D
118	S	D	S	D	***	S	D	D	D	S	D	S	D	S
119	S	S	S	S	D	D	D	S	D	S	D	D	D	D
120	D	D	D	D	D	S	S	D	D	D	S	S	S	D
121	D	D	D	D	D	D	S	D	D	D	S	S	S	D
122	D	S	D	S	S	S	S	S	D	S	S	D	D	D
123	S	S	S	S	D	D	S	S	S	D	S	D	S	S
124	S	S	S	S	S	S	S	S	S	S	S	D	S	S
125	S	S	S	S	D	S	D	S	S	S	D	D	S	S
126	S	S	S	S	S	D	D	D	S	S	S	D	D	S
127	S	S	S	S	S	S	S	S	D	D	D	S	S	S
128	D	D	S	S	D	S	S	D	D	D	D	D	D	D
129	D	D	***	***	S	***	S	S	D	S	S	D	D	S
130	D	D	***	***	D	***	S	D	S	S	D	S	D	S
131	S	D	***	***	D	D	S	S	D	S	S	D	D	***
132	S	S	***	***	D	S	D	D	S	D	S	D	S	D
133	D	S	***	***	D	S	S	S	S	D	S	D	S	S
134	S	D	***	***	D	D	D	S	S	D	S	D	S	D
135	***	***	***	***	S	***	D	D	D	D	D	D	D	S
136	S	S	***	***	D	S	S	S	S	D	S	D	S	D
137	D	D	***	***	S	S	S	S	S	D	D	S	D	S
138	S	S	***	***	D	***	D	D	D	D	S	D	D	S
139	S	S	***	***	S	S	S	S	S	D	D	S	D	S
140	S	S	***	***	D	S	S	D	S	S	D	D	S	D
141	S	S	***	***	S	D	D	S	S	D	D	S	D	D

Appendix 4.1. (cont.) Dissa (D) x Sabarlis (S) phenotypic scores *** representing missing data

	Hor 2	Hor 1	Hor 2a	Hor 2b	pBG35	R004	Est-3	Est-10	L-Est	A-amy	Wsp4	Wsp3	Wsp2	'2/6'
142	D	D	***	***	S	***	D	D	D	D	S	S	S	S
143	S	S	S	S	S	D	D	D	D	D	S	S	D	S
144	D	D	S	S	D	D	D	S	S	S	S	D	S	S
145	S	D	D	S	S	D	D	S	S	S	D	D	D	D
146	D	D	D	***	S	S	S	S	S	D	S	D	S	D
147	D	D	S	S	D	D	S	S	S	D	S	S	D	S
148	D	D	D	D	D	D	D	S	S	D	S	D	D	D
149	S	S	D	D	S	S	S	D	D	D	D	S	D	S
150	S	D	D	D	S	D	D	D	D	S	D	S	S	S
151	S	S	D	D	S	S	D	D	D	S	D	S	D	D
152	D	D	D	D	S	S	D	D	S	S	S	S	S	D
153	S	S	S	S	S	S	D	D	D	S	S	D	D	S
154	D	D	S	D	D	S	S	S	S	S	S	D	D	S
155	S	S	S	S	D	S	S	S	S	S	S	S	S	D
156	S	S	D	D	S	D	D	D	D	D	D	S	S	S

Appendix 4.2. Mean Squares analysis.

EL		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	7.46	1.51	34	0.0444	0.7073
	Sabarlis	7.25	1.40	32	0.0438	
Est-3	Dissa	6.83	1.56	32	0.0488	2.2872
	Sabarlis	7.50	1.26	34	0.0371	
A-amy	Dissa	7.53	1.44	34	0.0424	0.3342
	Sabarlis	7.63	1.51	32	0.0472	
Leaf-est	Dissa	7.56	1.07	36	0.0297	0.9014
	Sabarlis	7.29	1.80	30	0.0600	
WSP-2	Dissa	7.37	1.52	35	0.0434	1.0597
	Sabarlis	7.04	1.66	31	0.0535	
WSP-3	Dissa	7.40	1.47	42	0.0350	0.9307
	Sabarlis	7.11	1.49	24	0.0621	
WSP-4	Dissa	7.69	1.48	32	0.0463	2.9801
	Sabarlis	6.80	1.46	34	0.0429	
2row6row	Dissa	6.03	1.24	24	0.0517	7.5697
	Sabarlis	8.19	1.22	41	0.0298	
Rrn2	Dissa	8.19	1.32	31	0.0426	0.4297
	Sabarlis	8.08	0.78	34	0.0229	
Hor2	Dissa	7.12	1.63	29	0.0562	1.9656
	Sabarlis	7.71	1.22	36	0.0339	
Hor2a	Dissa	7.80	1.63	28	0.0582	0.5944
	Sabarlis	7.60	1.32	24	0.0550	
hor2b	Dissa	7.16	0.37	25	0.0148	1.6366
	Sabarlis	7.59	1.41	26	0.0542	
Hor1	Dissa	7.58	1.90	33	0.0576	0.1556
	Sabarlis	7.63	1.37	30	0.0457	
R004	Dissa	7.20	1.41	30	0.0470	0.3880
	Sabarlis	7.32	1.46	30	0.0487	

Appendix 4.2. Mean Squares analysis (cont).

Ht		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	80.89	6.74	34	0.1982	1.9596
	Sabarlis	79.61	7.31	32	0.2284	
Est-3	Dissa	80.43	8.95	32	0.2797	1.9046
	Sabarlis	79.13	6.33	34	0.1862	
A-amy	Dissa	79.27	5.95	34	0.1750	1.1515
	Sabarlis	79.99	6.91	32	0.2159	
Leaf-est	Dissa	82.94	6.16	36	0.1711	6.9135
	Sabarlis	78.30	8.38	30	0.2793	
WSP-2	Dissa	80.05	8.44	35	0.2411	4.2288
	Sabarlis	77.23	6.31	31	0.2035	
WSP-3	Dissa	79.50	6.93	42	0.1650	1.2247
	Sabarlis	80.34	7.33	24	0.3054	
WSP-4	Dissa	81.38	4.84	32	0.1513	3.4810
	Sabarlis	79.21	8.07	34	0.2374	
2row6row	Dissa	75.48	6.88	24	0.2867	11.0930
	Sabarlis	82.91	6.64	41	0.1620	
Rrn2	Dissa	82.70	5.80	31	0.1871	0.4889
	Sabarlis	83.00	6.44	34	0.1894	
Hor2	Dissa	80.38	6.70	29	0.2310	3.6217
	Sabarlis	82.73	6.84	36	0.1900	
Hor2a	Dissa	82.29	7.99	28	0.2854	1.3883
	Sabarlis	81.25	6.62	24	0.2758	
Hor2b	Dissa	80.76	0.05	25	0.0020	0.2146
	Sabarlis	80.87	6.78	26	0.2608	
Hor1	Dissa	76.60	6.51	33	0.1973	9.4951
	Sabarlis	82.85	7.08	30	0.2360	
R004	Dissa	80.35	4.81	30	0.1603	0.2912
	Sabarlis	80.54	7.96	30	0.2653	

Appendix 4.2. Mean Squares analysis (cont).

GN		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	35.85	14.73	34	0.4332	3.5205
	Sabarlis	32.73	11.27	32	0.3522	
Est-3	Dissa	33.80	11.99	32	0.3747	2.0430
	Sabarlis	32.09	11.08	34	0.3259	
A-amy	Dissa	31.02	9.40	34	0.2765	5.4179
	Sabarlis	35.86	16.69	32	0.5216	
Leaf-est	Dissa	38.77	17.55	36	0.4875	5.3304
	Sabarlis	33.79	11.56	30	0.3853	
WSP-2	Dissa	33.05	12.56	35	0.3589	0.6854
	Sabarlis	33.61	9.57	31	0.3087	
WSP-3	Dissa	34.39	12.23	42	0.2912	8.1464
	Sabarlis	28.31	6.38	24	0.2658	
WSP-4	Dissa	29.48	9.59	32	0.2997	7.0261
	Sabarlis	35.27	12.90	34	0.3794	
2row6row	Dissa	47.21	13.80	24	0.5750	19.7697
	Sabarlis	29.05	11.02	41	0.2688	
Rrn2	Dissa	28.14	9.36	31	0.3019	0.6068
	Sabarlis	27.69	8.43	34	0.2479	
Hor2	Dissa	33.78	10.95	29	0.3776	0.0548
	Sabarlis	33.83	16.41	36	0.4558	
Hor2a	Dissa	40.67	19.89	28	0.7104	9.9381
	Sabarlis	29.89	11.19	24	0.4663	
Hor2b	Dissa	42.95	27.93	25	1.1172	12.6375
	Sabarlis	28.01	7.29	26	0.2804	
Hor1	Dissa	32.92	11.10	33	0.3364	1.5099
	Sabarlis	31.54	14.97	30	0.4990	
R004	Dissa	34.48	14.73	30	0.4910	1.6725
	Sabarlis	32.80	15.54	30	0.5180	

Appendix 4.2. Mean Squares analysis (cont).

MSW		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	1.38	0.36	34	0.0106	0.8427
	Sabarlis	1.26	0.31	32	0.0097	
Est-3	Dissa	1.25	0.29	32	0.0091	0.1471
	Sabarlis	1.27	0.32	34	0.0094	
A-amy	Dissa	1.26	0.26	34	0.0076	0.7520
	Sabarlis	1.37	0.44	32	0.0138	
Leaf-est	Dissa	1.45	0.41	36	0.0114	0.9573
	Sabarlis	1.31	0.30	30	0.0100	
WSP-2	Dissa	1.27	0.34	35	0.0097	0.0757
	Sabarlis	1.26	0.24	31	0.0077	
WSP-3	Dissa	1.33	0.34	42	0.0081	0.9184
	Sabarlis	1.22	0.15	24	0.0063	
WSP-4	Dissa	1.27	0.30	32	0.0094	0.4311
	Sabarlis	1.33	0.34	34	0.0100	
2row6row	Dissa	1.39	0.46	24	0.0192	0.1207
	Sabarlis	1.41	0.34	41	0.0083	
Rrn2	Dissa	1.40	0.31	31	0.0100	0.8886
	Sabarlis	1.28	0.28	34	0.0082	
Hor2	Dissa	1.31	0.32	29	0.0110	0.3381
	Sabarlis	1.36	0.39	36	0.0108	
Hor2a	Dissa	1.59	0.46	28	0.0164	2.1529
	Sabarlis	1.24	0.24	24	0.0100	
Hor2b	Dissa	1.46	0.58	25	0.0232	1.4313
	Sabarlis	1.21	0.19	26	0.0073	
Hor1	Dissa	1.32	0.20	33	0.0061	0.2297
	Sabarlis	1.29	0.33	30	0.0110	
R004	Dissa	1.32	0.37	30	0.0123	0.3846
	Sabarlis	1.26	0.36	30	0.0120	

Appendix 4.2. Mean Squares analysis (cont).

STG		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	1.06	0.30	34	0.0088	0.6145
	Sabarlis	0.98	0.26	32	0.0081	
Est-3	Dissa	0.99	0.32	32	0.0100	0.0779
	Sabarlis	0.98	0.22	34	0.0065	
A-amy	Dissa	1.00	0.22	34	0.0065	0.0000
	Sabarlis	1.00	0.30	32	0.0094	
Leaf-est	Dissa	1.08	0.29	36	0.0081	0.5899
	Sabarlis	1.00	0.31	30	0.0103	
WSP-2	Dissa	1.01	0.26	35	0.0074	0.4820
	Sabarlis	0.95	0.25	31	0.0081	
WSP-3	Dissa	1.02	0.28	42	0.0067	0.0730
	Sabarlis	1.01	0.29	24	0.0121	
WSP-4	Dissa	1.08	0.28	32	0.0088	0.6789
	Sabarlis	0.99	0.30	34	0.0088	
2row6row	Dissa	0.86	0.26	24	0.0108	2.5730
	Sabarlis	1.18	0.19	41	0.0046	
Rrn2	Dissa	1.17	0.30	31	0.0097	0.5559
	Sabarlis	1.10	0.21	34	0.0062	
Hor2	Dissa	0.98	0.25	29	0.0086	0.8015
	Sabarlis	1.08	0.25	36	0.0069	
Hor2a	Dissa	1.20	0.29	28	0.0104	1.6120
	Sabarlis	0.97	0.24	24	0.0100	
Hor2b	Dissa	0.99	0.07	25	0.0028	0.0000
	Sabarlis	0.99	0.26	26	0.0100	
Hor1	Dissa	1.05	0.30	33	0.0091	0.2190
	Sabarlis	1.02	0.29	30	0.0097	
R004	Dissa	0.99	0.25	30	0.0083	0.0000
	Sabarlis	0.99	0.28	30	0.0093	

Appendix 4.2. Mean Squares analysis (cont).

TN		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	3.63	1.01	34	0.0297	0.0000
	Sabarlis	3.63	1.63	32	0.0509	
Est-3	Dissa	3.37	1.76	32	0.0550	1.2890
	Sabarlis	3.78	1.57	34	0.0462	
A-amy	Dissa	3.39	1.25	34	0.0368	3.0018
	Sabarlis	4.25	1.45	32	0.0453	
Leaf-est	Dissa	3.87	1.12	36	0.0311	0.7889
	Sabarlis	3.65	1.40	30	0.0467	
WSP-2	Dissa	3.61	1.48	35	0.0423	0.1612
	Sabarlis	3.56	1.67	31	0.0539	
WSP-3	Dissa	3.55	1.50	42	0.0357	1.6413
	Sabarlis	4.06	1.46	24	0.0608	
WSP-4	Dissa	3.51	1.11	32	0.0347	0.5404
	Sabarlis	3.66	1.44	34	0.0424	
2row6row	Dissa	3.14	1.04	24	0.0433	3.8616
	Sabarlis	4.23	1.49	41	0.0363	
Rrn2	Dissa	3.95	0.86	31	0.0277	0.0374
	Sabarlis	3.96	1.49	34	0.0438	
Hor2	Dissa	3.89	1.47	29	0.0507	0.5941
	Sabarlis	3.71	1.48	36	0.0411	
Hor2a	Dissa	3.74	0.66	28	0.0236	0.3306
	Sabarlis	3.84	1.63	24	0.0679	
Hor2b	Dissa	2.70	0.42	25	0.0168	3.6177
	Sabarlis	3.72	1.63	26	0.0627	
Hor1	Dissa	2.86	0.85	33	0.0258	4.8968
	Sabarlis	4.28	1.75	30	0.0583	
R004	Dissa	3.63	1.68	30	0.0560	0.8354
	Sabarlis	3.91	1.69	30	0.0563	

Appendix 4.2. Mean Squares analysis (cont).

RS		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	3.07	1.30	34	0.0382	0.8334
	Sabarlis	2.83	1.43	32	0.0447	
Est-3	Dissa	2.76	1.74	32	0.0544	0.3975
	Sabarlis	2.88	1.25	34	0.0368	
A-amy	Dissa	2.59	1.02	34	0.0300	3.1189
	Sabarlis	3.46	1.53	32	0.0478	
Leaf-est	Dissa	3.33	1.08	36	0.0300	1.2005
	Sabarlis	2.98	1.65	30	0.0550	
WSP-2	Dissa	2.84	1.42	35	0.0406	0.1976
	Sabarlis	2.78	1.60	31	0.0516	
WSP-3	Dissa	2.88	1.30	42	0.0310	1.4023
	Sabarlis	3.32	1.62	24	0.0675	
WSP-4	Dissa	2.98	1.39	32	0.0434	0.2319
	Sabarlis	2.91	1.62	34	0.0476	
2row6row	Dissa	2.24	1.17	24	0.0488	5.2058
	Sabarlis	3.73	1.36	41	0.0332	
Rrn2	Dissa	3.53	1.08	31	0.0348	0.6973
	Sabarlis	3.34	1.34	34	0.0394	
Hor2	Dissa	2.99	1.34	29	0.0462	0.5084
	Sabarlis	3.14	1.47	36	0.0408	
Hor2a	Dissa	3.30	1.26	28	0.0450	0.8110
	Sabarlis	3.03	1.58	24	0.0658	
Hor2b	Dissa	2.38	0.46	25	0.0184	2.1481
	Sabarlis	2.98	1.55	26	0.0596	
Hor1	Dissa	2.40	0.99	33	0.0300	3.5398
	Sabarlis	3.43	1.64	30	0.0547	
R004	Dissa	2.82	1.64	30	0.0547	0.5051
	Sabarlis	2.98	1.37	30	0.0457	

Appendix 4.2. Mean Squares analysis (cont).

WAG		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	3.37	1.40	34	0.0412	0.7577
	Sabarlis	3.15	1.38	32	0.0431	
Est-3	Dissa	3.04	1.77	32	0.0553	0.5713
	Sabarlis	3.21	1.13	34	0.0332	
A-amy	Dissa	2.92	1.07	34	0.0315	3.2356
	Sabarlis	3.84	1.58	32	0.0494	
Leaf-est	Dissa	3.77	1.19	36	0.0331	1.7726
	Sabarlis	3.25	1.59	30	0.0530	
WSP-2	Dissa	3.13	1.49	35	0.0426	0.1341
	Sabarlis	3.09	1.44	31	0.0465	
WSP-3	Dissa	3.22	1.21	42	0.0288	0.5433
	Sabarlis	3.38	1.39	24	0.0579	
WSP-4	Dissa	3.09	1.41	32	0.0441	0.4021
	Sabarlis	3.21	1.53	34	0.0450	
2row6row	Dissa	3.04	1.64	24	0.0683	2.4236
	Sabarlis	3.80	1.23	41	0.0300	
Rrn2	Dissa	3.77	1.14	31	0.0368	1.1501
	Sabarlis	3.46	1.22	34	0.0359	
Hor2	Dissa	3.33	1.20	29	0.0414	0.2804
	Sabarlis	3.41	1.44	36	0.0400	
Hor2a	Dissa	3.50	1.22	28	0.0436	0.2175
	Sabarlis	3.43	1.44	24	0.0600	
Hor2b	Dissa	3.26	1.46	25	0.0584	0.0296
	Sabarlis	3.25	1.44	26	0.0554	
Hor1	Dissa	2.44	0.99	33	0.0300	4.4441
	Sabarlis	3.71	1.55	30	0.0517	
R004	Dissa	3.21	1.44	30	0.0480	0.1926
	Sabarlis	3.27	1.47	30	0.0490	

Appendix 4.2. Mean Squares analysis (cont).

TGW		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	42.66	11.74	34	0.3453	0.7810
	Sabarlis	42.02	10.44	32	0.3263	
Est-3	Dissa	40.68	12.63	32	0.3947	2.6260
	Sabarlis	42.82	9.16	34	0.2694	
A-amy	Dissa	43.81	10.57	34	0.3109	1.7005
	Sabarlis	42.45	10.52	32	0.3288	
Leaf-est	Dissa	42.71	11.07	36	0.3075	0.8522
	Sabarlis	42.00	11.60	30	0.3867	
WSP-2	Dissa	42.28	9.63	35	0.2751	2.3136
	Sabarlis	40.40	11.94	31	0.3852	
WSP-3	Dissa	42.48	11.92	42	0.2838	3.2682
	Sabarlis	45.33	11.44	24	0.4767	
WSP-4	Dissa	45.61	8.63	32	0.2697	5.8812
	Sabarlis	41.02	11.54	34	0.3394	
2row6row	Dissa	29.80	2.46	24	0.1025	42.4414
	Sabarlis	51.26	6.28	41	0.1532	
Rrn2	Dissa	51.99	9.55	31	0.3081	6.4841
	Sabarlis	47.54	5.54	34	0.1629	
Hor2	Dissa	40.65	9.15	29	0.3155	4.4615
	Sabarlis	44.01	9.06	36	0.2517	
Hor2a	Dissa	44.96	12.35	28	0.4411	0.8349
	Sabarlis	44.18	10.36	24	0.4317	
Hor2b	Dissa	37.39	11.03	25	0.4412	8.6064
	Sabarlis	45.26	10.27	26	0.3950	
Hor1	Dissa	43.95	13.79	33	0.4179	0.3655
	Sabarlis	44.27	10.46	30	0.3487	
R004	Dissa	41.54	11.80	30	0.3933	2.5007
	Sabarlis	43.71	10.79	30	0.3597	

Appendix 4.2. Mean Squares analysis (cont).

SW		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	4.14	1.53	34	0.0450	1.0091
	Sabarlis	3.83	1.58	32	0.0494	
Est-3	Dissa	3.76	1.95	32	0.0609	0.3462
	Sabarlis	3.87	1.36	34	0.0400	
A-amy	Dissa	3.60	1.16	34	0.0341	2.9092
	Sabarlis	4.47	1.77	32	0.0553	
Leaf-est	Dissa	4.41	1.29	36	0.0358	1.2724
	Sabarlis	4.01	1.89	30	0.0630	
WSP-2	Dissa	3.87	1.59	35	0.0454	0.4066
	Sabarlis	3.74	1.76	31	0.0568	
WSP-3	Dissa	3.91	1.46	42	0.0348	1.2906
	Sabarlis	4.34	1.83	24	0.0763	
WSP-4	Dissa	4.07	1.63	32	0.0509	0.4943
	Sabarlis	3.91	1.83	34	0.0538	
2row6row	Dissa	3.11	1.38	24	0.0575	5.9869
	Sabarlis	4.92	1.39	41	0.0339	
Rrn2	Dissa	4.70	1.29	31	0.0416	0.8643
	Sabarlis	4.45	1.43	34	0.0421	
Hor2	Dissa	3.97	1.49	29	0.0514	0.8710
	Sabarlis	4.24	1.61	36	0.0447	
Hor2a	Dissa	4.52	1.45	28	0.0518	1.4466
	Sabarlis	4.01	1.74	24	0.0725	
Hor2b	Dissa	3.38	0.52	25	0.0208	2.0302
	Sabarlis	3.98	1.73	26	0.0665	
Hor1	Dissa	3.47	1.26	33	0.0382	3.1488
	Sabarlis	4.46	1.82	30	0.0607	
R004	Dissa	3.82	1.78	30	0.0593	0.5118
	Sabarlis	3.99	1.53	30	0.0510	

Appendix 4.2. Mean Squares analysis (cont).

SPY		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	4.77	1.66	34	0.0488	1.0616
	Sabarlis	4.44	1.53	32	0.0478	
Est-3	Dissa	4.32	1.90	32	0.0594	0.5753
	Sabarlis	4.50	1.31	34	0.0385	
A-amy	Dissa	4.20	1.21	34	0.0356	3.2668
	Sabarlis	5.21	1.92	32	0.0600	
Leaf-est	Dissa	5.23	1.50	36	0.0417	1.9541
	Sabarlis	4.61	1.77	30	0.0590	
WSP-2	Dissa	4.44	1.69	35	0.0483	0.1920
	Sabarlis	4.38	1.53	31	0.0494	
WSP-3	Dissa	4.58	1.43	42	0.0340	0.0974
	Sabarlis	4.61	1.46	24	0.0608	
WSP-4	Dissa	4.37	1.64	32	0.0513	0.5673
	Sabarlis	4.55	1.68	34	0.0494	
2row6row	Dissa	4.46	2.03	24	0.0846	2.2310
	Sabarlis	5.22	1.29	41	0.0315	
Rrn2	Dissa	5.18	1.34	31	0.0432	1.4985
	Sabarlis	4.75	1.33	34	0.0391	
Hor2	Dissa	4.65	1.40	29	0.0483	0.4564
	Sabarlis	4.79	1.65	36	0.0458	
Hor2a	Dissa	5.12	1.53	28	0.0546	1.2185
	Sabarlis	4.70	1.54	24	0.0642	
Hor2b	Dissa	4.72	2.04	25	0.0816	0.6413
	Sabarlis	4.48	1.52	26	0.0585	
Hor1	Dissa	3.83	1.08	33	0.0327	3.9580
	Sabarlis	5.02	1.73	30	0.0577	
R004	Dissa	4.57	1.57	30	0.0523	0.0906
	Sabarlis	4.54	1.72	30	0.0573	

Appendix 4.2. Mean Squares analysis (cont).

Bio		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	8.91	3.08	34	0.0906	1.5101
	Sabarlis	8.26	3.03	32	0.0947	
Est-3	Dissa	8.09	3.81	32	0.1191	0.6351
	Sabarlis	8.37	2.56	34	0.0753	
A-amy	Dissa	7.80	2.31	34	0.0679	4.4292
	Sabarlis	9.67	3.53	32	0.1103	
Leaf-est	Dissa	9.65	2.64	36	0.0733	2.3547
	Sabarlis	8.62	3.54	30	0.1180	
WSP-2	Dissa	8.32	3.22	35	0.0920	0.4526
	Sabarlis	8.12	3.20	31	0.1032	
WSP-3	Dissa	8.49	2.74	42	0.0652	1.0077
	Sabarlis	8.94	3.22	24	0.1342	
WSP-4	Dissa	8.44	3.19	32	0.0997	0.0668
	Sabarlis	8.47	3.46	34	0.1018	
2row6row	Dissa	7.57	3.38	24	0.1408	5.6866
	Sabarlis	10.14	2.60	41	0.0634	
Rrn2	Dissa	9.88	2.50	31	0.0806	1.6716
	Sabarlis	9.21	2.72	34	0.0800	
Hor2	Dissa	8.62	2.76	29	0.0952	0.9578
	Sabarlis	9.03	3.17	36	0.0881	
Hor2a	Dissa	9.64	2.81	28	0.1004	1.9290
	Sabarlis	8.71	3.17	24	0.1321	
Hor2b	Dissa	8.10	2.56	25	0.1024	0.7601
	Sabarlis	8.46	3.17	26	0.1219	
Hor1	Dissa	7.30	2.31	33	0.0700	5.0776
	Sabarlis	9.48	3.43	30	0.1143	
R004	Dissa	8.39	3.19	30	0.1063	0.3038
	Sabarlis	8.53	3.18	30	0.1060	

Appendix 4.2. Mean Squares analysis (cont).

IND A		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	0.54	0.04	34	0.0012	0.0000
	Sabarlis	0.54	0.05	32	0.0016	
Est-3	Dissa	0.55	0.05	32	0.0016	0.1816
	Sabarlis	0.54	0.05	34	0.0015	
A-amy	Dissa	0.54	0.03	34	0.0009	0.0000
	Sabarlis	0.54	0.05	32	0.0016	
Leaf-est	Dissa	0.54	0.05	36	0.0014	0.1809
	Sabarlis	0.55	0.05	30	0.0017	
WSP-2	Dissa	0.54	0.03	35	0.0009	0.2012
	Sabarlis	0.55	0.05	31	0.0016	
WSP-3	Dissa	0.54	0.05	42	0.0012	0.1748
	Sabarlis	0.53	0.05	24	0.0021	
WSP-4	Dissa	0.52	0.03	32	0.0009	0.6525
	Sabarlis	0.55	0.04	34	0.0012	
2row6row	Dissa	0.58	0.04	24	0.0017	1.2252
	Sabarlis	0.52	0.03	41	0.0007	
Rrn2	Dissa	0.53	0.04	31	0.0013	0.2145
	Sabarlis	0.52	0.03	34	0.0009	
Hor2	Dissa	0.54	0.05	29	0.0017	0.1878
	Sabarlis	0.53	0.04	36	0.0011	
Hor2a	Dissa	0.53	0.04	28	0.0014	0.3375
	Sabarlis	0.55	0.05	24	0.0021	
Hor2b	Dissa	0.57	0.06	25	0.0024	0.4563
	Sabarlis	0.54	0.05	26	0.0019	
Hor1	Dissa	0.54	0.03	33	0.0009	0.0000
	Sabarlis	0.54	0.05	30	0.0017	
R004	Dissa	0.55	0.06	30	0.0020	0.3464
	Sabarlis	0.53	0.04	30	0.0013	

Appendix 4.2. Mean Squares analysis (cont).

IND B		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	0.57	0.04	34	0.0012	0.2030
	Sabarlis	0.56	0.04	32	0.0013	
Est-3	Dissa	0.56	0.05	32	0.0016	0.0000
	Sabarlis	0.56	0.04	34	0.0012	
A-amy	Dissa	0.56	0.04	34	0.0012	0.2030
	Sabarlis	0.57	0.04	32	0.0013	
Leaf-est	Dissa	0.57	0.05	36	0.0014	0.0000
	Sabarlis	0.57	0.05	30	0.0017	
WSP-2	Dissa	0.56	0.04	35	0.0011	0.1905
	Sabarlis	0.57	0.05	31	0.0016	
WSP-3	Dissa	0.56	0.04	42	0.0010	0.1815
	Sabarlis	0.55	0.05	24	0.0021	
WSP-4	Dissa	0.54	0.03	32	0.0009	0.8151
	Sabarlis	0.58	0.05	34	0.0015	
2row6row	Dissa	0.61	0.03	24	0.0013	1.5725
	Sabarlis	0.54	0.03	41	0.0007	
Rrn2	Dissa	0.55	0.04	31	0.0013	0.2145
	Sabarlis	0.54	0.03	34	0.0009	
Hor2	Dissa	0.57	0.04	29	0.0014	0.4008
	Sabarlis	0.55	0.04	36	0.0011	
Hor2a	Dissa	0.56	0.04	28	0.0014	0.0000
	Sabarlis	0.56	0.05	24	0.0021	
Ho2b	Dissa	0.58	0.08	25	0.0032	0.2905
	Sabarlis	0.56	0.04	26	0.0015	
Hor1	Dissa	0.56	0.04	33	0.0012	0.0000
	Sabarlis	0.56	0.05	30	0.0017	
R004	Dissa	0.57	0.05	30	0.0017	0.1732
	Sabarlis	0.56	0.05	30	0.0017	

Appendix 4.2. Mean Squares analysis (cont).

ME		mean	standard deviation	Sample size	sample variance	t-test
Est-10	Dissa	117.91	7.87	34	0.2315	3.8900
	Sabarlis	115.06	9.77	32	0.3053	208.2342
						220.4740
Est-3	Dissa	115.88	8.84	32	0.2763	1.7008
	Sabarlis	114.58	10.47	34	0.3079	206.4785
						210.1470
A-amy	Dissa	116.56	10.46	34	0.3076	2.1781
	Sabarlis	115.00	6.57	32	0.2053	253.7991
						242.5274
Leaf-est	Dissa	117.50	8.45	36	0.2347	3.3195
	Sabarlis	115.11	8.51	30	0.2837	216.1269
						198.1985
WSP-2	Dissa	114.25	11.63	35	0.3323	3.3259
	Sabarlis	116.79	7.78	31	0.2510	233.1292
						269.6798
WSP-3	Dissa	116.96	7.90	42	0.1881	0.6975
	Sabarlis	117.45	7.33	24	0.3054	212.5234
						190.5138
WSP-4	Dissa	117.73	12.22	32	0.3819	3.5687
	Sabarlis	114.96	7.50	34	0.2206	
2row6row	Dissa	116.50	7.19	24	0.2996	0.7016
	Sabarlis	117.00	8.54	41	0.2083	
Rrn2	Dissa	120.93	8.84	31	0.2852	8.9749
	Sabarlis	114.25	9.14	34	0.2688	
Hor2	Dissa	112.46	9.73	29	0.3355	3.9759
	Sabarlis	115.41	7.74	36	0.2150	
Hor2a	Dissa	117.70	9.35	28	0.3339	4.3445
	Sabarlis	114.15	8.01	24	0.3338	
Hor2b	Dissa	113.25	10.25	25	0.4100	2.0021
	Sabarlis	114.97	8.53	26	0.3281	
Hor1	Dissa	120.60	8.04	33	0.2436	11.4570
	Sabarlis	112.32	8.36	30	0.2787	
R004	Dissa	116.75	9.48	30	0.3160	4.1073
	Sabarlis	113.65	7.61	30	0.2537	

Isozyme markers screened in the Dissa x Sabarlis DH population and found to be non-polymorphic.

AAT	Aspartate aminotransferase
ACP	Acid Phoshatase
ADH	Alcohol dehydrogenase
ALD	Aldolase
CAT	Catalase
G6PD	Glucose-6-phosphate dehydrogenase
GADH	Galactose dehydrogenase
GDH	Glutamate dehydrogenase
GO	Glucose oxidase
HEX	Hexokinase
IDH	Isocitrate deydrogenase
LAP	Leucine aminotransferase
MDH	Malate dehydrogenase
ME	Malic enzyme
PER	Peroxidase
PGI	Phosphglucose isomerase
PGM	Phosphogluco mutase
6PGD	6-Phoshogluconated dehydrogenase
SKDH	Shikimate dehydrogenase
SUDH	Succinate dehydrogenase
XDH	Xanthine dehydrogenase

Appendix 5.1. Phenotypic scores for the *H.spontaneum* populations.

Population	WSP	b-amy	Est-10	Est-3	ADH	pBG35
1	?	B	B	A		
	?	B	B	A		
	G	B	B	A		
	G	B	B	A	A	
	G	B	B	E		
3	A	A	B	?	A	A
	A	B	B	?	A	
	-	-	-	-	-	A
	E	A	B	?	A	
	A	B	B	C	A	A
	A	A	B	A	A	
4	F	B	D	A	A	C
	F	B	D	A	A	C
	F	D	D?	A	A	A
	F	D	B	A	B	
	F	D	B	A	A	B
5	A	A	B	?	A	B
	A	A	B	A	A	
	A	A	B	A	A	
	A	A	B	A	A	
	A	A	B	A	A	B
6	A	A	B	E	A	
	E	B	B	F	A	
	A	A	B	F	A	
	E	B	B	C	A	
	B	B	B	F	A	
8	B	B	B	A		
	B	G	B	A		B
	J	D	B	E		G
	B	B	B	A		A
	A	B	B	A		F
9	J	A	B	A		B
	-	-	-	-	B	
	J	A	B	A	B	
	J	A	B	A	B	
	J	A	B	A	A	B
	J	A	B	A	A	B
10	A	A	B	A	A	C
	A	A	B	A	A	C
	H	A	B	A	A	C
	A	A	B	A	A	C
	A	A	B	A	A	C

Appendix 5.1. (continued).

Population	WSP	b-amy	Est-10	Est-3	ADH	pBG35
12	A	A	B	A	A	D
	A	A	B	A	A	D
	K	C	B	A	A	
	A	A	B	A	A	
	K	C	B	A	A	D
13	F	A	B	C	A	
	F	A	B	D	A	C
	F	B	B	D	C	
	F	A	B	D	C	C
	F	A	B	C	A	C
14	A	A	B	A	A	A
	A	A	B	D	A	
	A	A	B	D	A	
	A	A	B	D	A	A
	A	A	B	D	A	B
16	D	E	B	C	A	
	A	A	C	A	A	?
	D	F	B	E	A	B
	A	A	B	A	A	B
	A	A	B	E	A	C?
17	A	F	B	D	A	
	H	G	B	A	A	B
	H	G	B	A	A	
	-	-	-	-	-	G
	H	G	B	A	A	B
	H	G	B	E	A	
18	A	G	B	A	A	
	A	G	B	A	A	
	A	G	B	A	A	
	A	G	B	A	A	
	A	G	B	A	A	
19	G	G	B	A	A	
	G	G	B	A	A	
	G	?	B	A	A	
	G	G	B	A	A	
	G	G	F	A	A	B
20	H	G	B		A	B
	H	G	B		A	
	H	G	B		A	
	H	G	B		A	B
	H	G	B		A	

Appendix 5.1. (continued).

Population	WSP	b-amy	Est-10	Est-3	ADH	pBG35
21	A	A	B	A	A	A
	A	A	B	D	A	
	A	A	B	D	A	
	A	A	B	D	A	
	A	A	B	E	A	H
22	A	A	B	G	A	B
	A	A	B	A	A	
	A	A	B	A	A	I
	A	A	F	G	A	
	A	A	B	G	A	A
23	G	A	B	C	A	
	G	A	B	A	A	
	G	A	B	?	A	
	G	A	B	A	A	
	G	A	B	E	A	
24	B	B	B	A	A	
	A	A	B	C	A	K
	A	A	B	A	A	B
	B	B	B	A	A	
	B	B	B	E	A	
25	I	B	B	E	A	A
	I	B	B	A	A	A
	A	A	B	A	A	L
	I	B	B	A	A	A
	B	B	B	A	A	M
26	F	A	E	A	A	A
	F	A	E	A	A	
	A	A	E	E	A	D
	A	A	B	A	A	C
	F	A	E	A	A	A
27	A	A	B	D	A	A
	A	B	B	A	A	A
	A	A	B	A	A	E
	A	A	B	A	A	E
	A	A	B	A	A	E
28	A	A	B	A	A	C
	A	A	B	C	A	A
	A	A	B	?	A	
	B	B	B	A	A	A
	A	A	B	D	A	E

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Appendix 5.2. Phenotypic frequencies and diversity estimates (Ho) for the six marker systems analysed from 27 geographical locations in Israel

Marker		Population																									
		1	3	4	5	6	8	9	10	12	13	14	16	17	18	19	20	21	22	23	24	25	26	27	28	30	31	32
ADH1	A			0.2							0.4																	
	B	1.0	1.0	0.8	1.0	1.0	1.0	0.4	1.0	1.0	0.6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	C							0.6																				
	Ho			0.722				0.971			0.971																	
WSP2,3	A		0.8		1.0	0.4	0.2		0.8	0.6		1.0	0.6	0.2	1.0			1.0	1.0		0.4	0.2	0.4	1.0	0.8	1.0		0.8
	B					0.2	0.6				0.4										0.6	0.2			0.2			
	D																											
	E		0.2			0.4																						
	F			1.0							0.6												0.6					
	G	1.0														1.0											0.2	0.2
	H													0.8			1.0			1.0							0.8	
	I								0.2													0.6						
	J						0.2	1.0																				
	K									0.4																		
	Ho		0.722			1.520	1.370		0.722	0.971	0.971		0.971	0.722							0.971	1.370	0.971		0.722		0.722	0.722
EST3	A	0.8	0.5	1.0	1.0		0.8	1.0	1.0	1.0		0.2	0.4	0.6	1.0	1.0		0.2	0.4	0.5	0.6	0.8	0.8	0.8	0.5	1.0	1.0	0.4
	B										0.4																	0.6
	C		0.5			0.2					0.6		0.2							0.25	0.2				0.25			
	D											0.8		0.2				0.6						0.2	0.25			
	E	0.2					0.2						0.4	0.2				0.2		0.25	0.2	0.2	0.2					
	F					0.8																						
	G																		0.6									
	Ho	0.722	1.000			0.722	0.72				0.971	0.722	1.520	1.370				1.370	0.971	1.500	1.370	0.722	0.722	0.722	1.500			0.971
EST10	B	1.0	1.0	0.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.8	1.0	1.0	1.0	0.2	1.0	1.0		1.0	
	C			0.6																								
	E																						0.8					
	F												0.2						0.2									
	G																									1.0		1.0
	Ho			0.971									0.722						0.722				0.722					
BMY1	A				1.0	0.4	1.0	0.6		0.8																		
	B	1.0	1.0	0.4		0.6				0.2	0.6	1.0			0.6	1.0	1.0	0.4	0.2	1.0	0.8	0.8	0.4					
	C														0.4			0.6	0.8		0.2	0.2	0.6					
	D			0.6							0.2																	
	E							0.2																				
	F							0.2	0.2																			
	G								0.8		0.2		1.0	1.0												1.0	1.0	1.0
	Ho			0.971		0.971		1.370	0.722	0.722	1.370				0.971			0.971	0.722		0.722	0.722	0.971					
rDNA	A		1.0	0.25			0.2					0.67						0.5	0.33			0.6	0.5	0.4	0.5			1.0
	B			0.25	1.0		0.4	1.0				0.33	0.5	0.67		1.0	1.0		0.33		0.5			0.6	0.25	1.0	1.0	
	C			0.5					1.0		1.0		0.3										0.25		0.3			
	D									1.0													0.25					
	F						0.2																					
	G						0.2							0.33				0.5										
	I																		0.33									
	K																				0.5							
	L																					0.2						
	M																					0.2						
	Ho			1.500			1.920					0.918	1.500	0.918				1.000	1.370		1.000	0.971	1.500	0.971	1.500			