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DISCOVERY OF NOVEL GENES FOR IMPROVING PROTEIN QUANTITY AND GRAIN TEXTURE BY MARKER-MEDIATED GENETIC ANALYSIS

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DISCOVERY OF NOVEL GENES FOR IMPROVING PROTEIN QUANTITY AND GRAIN TEXTURE BY MARKER-MEDIATED GENETIC ANALYSIS

by

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ABSTRACT

A series of precise genetic stocks of wheat were investigated to discover new, novel loci controlling grain texture and protein quantity, and grain size and shape, which would be of use for directed plant breeding by marker-assisted selection.

Analysis of a population of Hobbit Sib (Avalon 5A) recombinant substitution lines revealed two year-on-year consistent loci associated with both protein content and texture, although these were not statistically significant.

Analysis of Hobbit Sib (Avalon 5D) recombinant substitution lines revealed strong consistent associations, as expected, between the hardness locus, *Ha,* and grain texture, and between a quantitative trait locus (QTL) on the long arm and grain protein content. Both associations were statistically significant.

Analysis of a population of Hobbit Sib x Avalon recombinant inbred lines (RILs) revealed year-on-year consistent statistically significant QTL for protein content on chromosomes 1A and 6B, and for texture on 5D (*Ha*). Single-year significant QTL were found for protein content on chromosomes 6A and 7A.

Analysis of neo-RILs from the cross Hope x Pastiche (both hard-grained) revealed year-on-year consistent associations between markers on chromosome 1B and protein content and markers on 6B and grain texture.

Analysis of the International Triticeae Mapping Initiative (ITMI) population for grain size and shape revealed a year-on-year consistent statistically significant QTL related to the ratio seed length / seed width on chromosome 2D.

Finally, a survey of hardness-conferring polymorphisms in puroindoline loci *pina* and *pinb* was conducted on a suite of commonly grown UK varieties. The allele *pinb-D1b* (Gly_{46} to Ser_{46}) was by far the most common hardness-conferring allele detected in the hard wheats.

SUMMARY

Background

To remain competitive, UK agriculture requires a constant supply of new, genetically improved, varieties. Breeders produce new varieties by artificially generating crosses between established varieties to release new genetical variation in the generations derived from the F_1. However, in the main, they have had little genetical information to guide their choice of crosses or subsequent breeding strategy. To date, most of the selection for improved genotypes has been based on empirical selection of observed phenotypes, rather than for the specific, desirable genes known to improve those traits. However, genetical studies of crop plants have now reached a very exciting phase of development. Advances in molecular biology are now providing the tools to study the genetical makeup of plants with a precision never previously possible. This is coming about because of the development of genetic maps and discoveries in the new science of genomics, which allow us to unravel the inheritance of all traits, whether they are controlled by single (major) genes, or-by many genes (of smaller effect) acting together, known as quantitative trait loci (QTL). Thus, the genetical control of end-use quality and its components is becoming amenable to discovery in terms of the effects of known, identified and mapped genes, which can be utilized by breeders for producing improved strains.

Objectives

The objectives of this study were to investigate, with the aid of precise genetic stocks, the genetic basis of grain protein content and texture in wheat and to attempt to characterise loci within the genome influencing these characteristics.

Experimental Approaches

In this study, four populations were used. Firstly, series of recombinant substitution lines containing the Avalon 5A and 5D chromosomes in a Hobbit Sib background, and a series of Avalon x Hobbit Sib recombinant inbred lines (recombinant for all chromosomes). Hobbit Sib is a soft, low protein, high yielding feed wheat which has in its pedigree Capelle-Desprez, Viking and Prof Marchal, and has been used extensively in UK high-yielding and biscuit wheat breeding programmes. Avalon (derived from Bilbo (the progeny of Hobbit Sib) and Maris Ploughman (which contains Viking germplasm) is the ancestor of several of the modern UK bread-making wheat varieties and is a hard, high quality, high protein variety. In the relationship between protein levels and yield, Avalon consistently outperformed most other bread wheat varieties having an intrinsically higher protein content than expected on its yield level. The small differences in yield and yet large differences in quality made these two

parents suitable for producing the populations in this study. Additionally, in order to investigate more exotic sources of genes for high protein, a series of neo-RILs were produced by single-seed descent from the cross Hope x Pastiche. Both these parents are hard, high protein varieties which are suitable for bread-making.

Results

Genetics of endosperm texture

Previous studies by a number of workers worldwide, has shown the importance of chromosome 5D in the genetic control of grain hardness, and the major effect of a single gene, designated *Ha*, on the short arm of this chromosome. This study is the first demonstration that this gene (although perhaps not surprisingly) is also the major determinant of grain texture in UK bread-making quality wheats. This gene has obviously been maintained in the UK gene pool since the beginning of the 20th century for its contribution to hard grain texture. Selection for this gene via NIR is straight forward, and thus although we could generate diagnostic molecular markers, they are unlikely to be necessary for plant breeders seeking to separate hard segregants from soft.

However, these results also indicate that the 5D locus is not the whole story of the genetic control of grain texture in UK wheats. There is clearly other genetic variation present, and some of these genes have been identified here as

QTL of minor effect relative to the over-riding effect of the *Ha* locus. It is interesting that two presumptive QTL have been found on chromosome 5A, one in position on the short arm likely homoeologous to the *Ha* locus, and the other in a position on the long arm in the region of the vernalization gene, *Vrn-A1*. These effects are minor, and, in these experiments, not statistically significant, but can be seen visually in ground samples. The latter locus may be homoeologous to one found on 5D in a study of the variety Hope.

In addition to the group 5 chromosomes, using the RIL lines, QTL consistent over years were found on chromosomes 1B, 4B, 4D, 5BS/7BS. Avalon had the allele for increasing hardness on 1B and 4D whilst Hobbit Sib consistently contributed the effect for increasing hardness on chromosomes 4B and 5BS/7BS. In simple terms, this means that hard grain texture varieties such as Avalon carry minor alleles for soft texture and soft-varieties like Hobbit carry minor alleles for hard texture. This was also confirmed in the Hope x Pastiche cross where although both varieties were hard, significant genetical variation for grain texture was found. This is important, since it means that by manipulation of these minor effects it should be possible to breed different grain textures into new varieties leading to a series of varieties that could be described as soft-softs, softs, hard-softs, soft-hards, hards, hard-hards etc. There is a capacity to breed for a spectrum of textures rather than simply hard or soft. This opens up interesting

possibilities for fine-tuning the texture of varieties for different end uses as desired using the available genetic variation in UK or exotic wheats.

Genetics of grain protein content

This study has provided the first complete analysis of grain protein content in UK wheat varieties, and it has shown that it is possible to dissect the genetics of this difficult-to-measure and highly-environmentally sensitive character using modern methods of genetic analysis. It has shown that the genetic control is complex and that there are no underlying major genes as in the case of grain texture. However, it again highlights the effects of the group 5 chromosomes, shown in studies of wheat varieties from other countries. Of particular note is the fairly large effect of a QTL on chromosome 5D that completely co-segregates with the *Ha* locus on the short arm. It is open to speculation and further study as to whether this is a pleiotropic effect of the *Ha* locus or an effect of a closely linked independent locus. Linkage seems most likely, as it is difficult to easily think of a mechanistic link of how variation in the grain texture locus could affect protein amounts. Nevertheless, in plant breeding terms this means that selection for hard grain texture by NIR will carry the allele for higher grain protein content along with it – a fortuitous but useful association. Similarly, selection for soft texture, will result in lower protein levels. Conversely, if soft high protein or hard low protein varieties are needed for specific end-use products, these results will suggest that this will require selection for alternative genes than that on 5D. Luckily, the analysis of the RILs indicates that additional genetic variation is present and QTL on several chromosomes make a contribution.

Significant QTL were identified on chromosomes 2B and 6B (Avalon contributing increasing effect) and chromosomes 6A and 7A (Hobbit Sib contributing increasing effect) in data from both years of this study. These effects were also relatively large, and, interestingly were dispersed between the parents. So, analogous to the situation for grain texture, although Avalon is generally regarded as a high protein wheat, it carries alleles at certain loci for reduced grain protein relative to their homologues from Hobbit sib, and vice versa. Thus, in this cross, genes for higher levels of grain protein are dispersed between the parents and transgressive segregation for higher protein containing lines than Avalon is possible from this cross. Diagnostic markers for particular alleles could be sought/designed to enable this to be a tool by plant breeders for protein content selection.

In these studies, detailed examination of the relationship between grain protein content and yield was only carried out for the recombinant substitution lines. This showed that there was no yield penalty associated with carrying the Avalon 5D gene for higher levels. This gene is thus very useful for maintaining high levels as it hitch-hikes in breeding programmes with the hardness locus. It may be that some of the other alleles discovered for higher grain protein

could be associated with a yield penalty. Further studies of the presumptive loci discovered are needed before their deployment can be recommended to breeders.

Genetics of agronomic traits

Although the prime purpose of this study was to discover new genes for grain protein content and grain texture, the opportunity was also taken to evaluate the genetics of traits of agronomic importance. Several new QTL were discovered in the Avalon x Hobbit Sib cross and, overall, this indicates that there is quite a lot of genetic variation for traits even in the relatively narrow genetic pool of UK winter wheats.

Several QTL mediating variation for plant height were discovered. These can be regarded as minor effects relative to the large effect of the major dwarfing gene *Rht-D1* that both parents are known to carry. However, the QTL on chromosomes 2D and 6A, in particular, may be useful to plant breeders as they each mediated differences between 10-15 cm in height, which were consistent over years. As with the grain quality traits, 'useful' alleles were dispersed between the parents, with Avalon having the shortness allele at the 2D locus and Hobbit Sib the shortness allele at the 6A locus.

Significant QTL were also found for flowering time, but only mediated differences by 1-2 days. However, such small difference may be useful for 'fine-tuning' flowering to environment, although, probably of low agronomic significance in the UK.

Both height and flowering time are traits with high heritabilities, and thus QTL are relatively easy to detect consistently over experiments. Yield and yield components have much lower heritabilities and are more environmentally sensitive. Hence it would be expected in these studies that such QTL would be more difficult to detect consistently over years and with high statistical significance, and this appeared to be the case. Although QTL for biomass, spikelet number, grain size and grain number were detected by single marker ANOVA, few of the effects could be confirmed by marker regression and interval mapping that were consistent over years. Nevertheless, these results do highlight putative QTL for yield components worthy of future study.

Seed morphology studies

The second part of this study encompassed an investigation into the genetics of seed morphology using the International Triticeae Mapping Initiative (ITMI) population. This was derived from crossing a hexaploid wheat, "Synthetic M6", re-synthesised by crossing a durum wheat and an *Aegilops tauschii* accession (a "D" genome donor), with a CIMMYT variety, Opata. A recombinant inbred line population was developed by single seed descent. A comprehensive genetic map of this population has been built up using Restriction Fragment Length Polymorphism (RFLP) markers and Simple Sequence Repeat (SSR) markers. The

mapping data on these lines is publicly available on the GrainGenes website (http://wheat.pw.usda.gov/) and they have been and are being grown at several locations around the world for phenotypic analysis. They are known to differ significantly in grain size and shape and therefore present a good starting point for genetic analysis of these traits. Hopefully, new loci found in this population will have relevance to UK wheats.

The shape and size of wheat grains is a primary determinant in the value of the grain. It has an effect on many factors of the agronomy on end use of the crop and therefore is an important trait for genetic study. Millers obtain higher flour yields from larger grains and therefore place pressure on breeders to ensure new varieties exhibit large grains. This has an effect on the specific weight of the grain sample, with large, full grains producing higher specific weights than small, shrivelled grains. As grain value in the UK is partly determined by specific weight values, again seed size and shape may be very important in increasing the premium on grain samples. It has been suggested in a number of studies that increasing kernel size is an important factor in increasing yield from a crop. However, this may have some negative implications on the end use of the crop, with larger kernelled lines often showing a decrease in protein content due to the extra grain size being composed mainly of starch rather than protein. It has also been shown that large seeds may give a better start for plants grown in the field, with greater seedling size and speed of growth coming from larger seeds.

Using image analysis techniques developed in these studies, it was easy to measure a large number of grain, and to process the results on a computer, avoiding laborious hand measurements. It was thus been possible to correlate variation in grain dimensions with hectolitre weight for the large number of samples needed for QTL mapping studies of such traits. This study has shown that grain dimensions and hence hectolitre weight are under the genetical control of many genes. Interestingly, there are different 'types' of QTL. First, there are those that influence the components of grain shape, grain height, width and length, independently. For example, there is a QTL on 2B that influences grain width, and one on 4B that influences grain height. The importance of such QTL is in the fact that they indicate that it should be possible to breed for different grain shapes, such as long thin grain, short thin grain etc, through utilising such variation. Secondly, there are QTL which influence more than one trait, for example, the ratios of length:width for a the QTL on chromosome 2D. These imply that there are 'general' grain size factors which influence all dimensions. Such variation means that it should be possible to increase grain size through simultaneously increasing all dimensions. By judicious selection of both types of QTL it should be possible to breed for larger grains, and hence increase yield, with different grain shapes, as desired. Several QTL control hectolitre weight, and these seemed to reflect the probable pleiotropic effect of QTL for grain volume and grain weight. Thus, the variation in hectolitre weight is

due to grain shape and size differences rather than differences in grain density. For certain co-incident QTL, eg on chromosome 1A, QTL for greater volume meant lower hectolitre weight. This indicates, not surprisingly, that to maximise hectolitre weight means breeding for many small grains, clearly not desirable from a quality point of view. Thus, selection has to be a compromise between 'good' grain shape, size and hectolitre weight. This could be achieved by directed plant breeding for the types of QTL identified here.

These experiments have utilized the ITMI population rather than an adapted UK cross, because no suitable UK population with a good genetic map was available at the beginning of the project. However, this has changed with the recent development of several maps in this and DEFRA funded project work. So, it would now be useful to use other crosses and to revisit the Avalon x Hobbit Sib cross to measure grain size difference to see how far these results can be extrapolated to UK germplasm.

Hardness-conferring alleles at the puroidoline loci

The final part of this study investigated the nature of the alleles at the hardness-conferring puroindoline loci on the group one chromosomes of wheat. A protein known as friablin (Latin *friablis*-friable), which is associated with the *Ha* locus described above, has been located on the surface of water-washed starch granules from soft textured wheats, but is absent in those of hard wheat cultivars and durum wheats. The mature friablin protein consists of two proteins, puroindoline a and b (pin-a and pin-b) which act together as a heterodimer to bind starch granules to membrane lipids within the endosperm. The interaction between pin-a and pin-b is altered by the effect of mutations that may occur in either of the genes. Wild-type bread wheats have soft endosperm texture, and express the functional, wild-type puroindoline alleles: pina-D1a and pinb-D1a, while in hard wheats, one or other of the puroindoline genes or its product has been rendered non-functional by mutation.

A suite of commonly grown UK varieties was analysed for hardness, protein content and alleles at the *pin* loci. The distribution of puroindoline hardness-conferring alleles was comparable to that found in studies or other cultivar suites from the USA and Europe, with the allele *pinb-D1b* (Gly_{46} to Ser_{46}) being by far the most common. At least one other mutation was found to be present. Serine was present at amino acid 46 in most of the soft wheats and the one *T. spelta* variety assayed in this study.

General conclusions

Implications for wheat breeding

For wheat, as most arable crops, conventional plant breeding is still, and will be for the foreseeable future, the mainstay for the production of new arable crop varieties for the UK market. This, of course, involves creating variation through making crosses between

established varieties with complementary characteristics. New genetical variation is then released in the progenies, and improved variants selected and multiplied. This has been very successful in the UK over the latter half of the last century. For example, wheat yields have continued to rise at about, on average, 1%/year since the 1970's. On the other hand, though, only limited increases in quality have been obtained in recent times. One of the reasons for this situation is that most of the characters being manipulated have a very complex inheritance and are highly affected by environment, such that breeders have had little information to guide their choice of parents or crosses, and subsequent breeding strategy. Most of the selection for improved genetic type has been based on empirical selection of observed phenotypes, rather than for the specific, desirable genes known to improve those traits. However, because of the development of molecular marker systems, good genetic maps can finally be developed in wheat, and as here, applied to discover new genes which can be used by plant breeders for marker-assisted selection.

This study has shown that QTL controlling different aspects of grain quality variation can be discovered in UK wheats, which can subsequently be targets for plant breeders for directed plant breeding. This study has established that the well-known major gene, *Ha* on chromosome 5D, controls the major distinction between hard and soft wheats in the UK gene pool. However, modifier genes have been detected and mapped, which give plant breeders other options to modulate grain texture for different purposes. Similarly, *Ha*, or a closely linked gene, has a major effect on increasing grain protein content, as have several other genes that have been discovered in these studies. These can give plant breeders options for breeding higher and lower grain proteins levels than currently usually obtained. In particular, they give the option to breed consistently higher levels, such as that found in the variety Hereward, by directed plant breeding, rather than by chance alone.

Although this study has discovered new QTL, this is only the start of the process of their application for directed plant breeding. It will require further mapping work to validate the QTL discovered, using other crosses, and more molecular work to convert nearest markers into diagnostic tools. Then, the genes controlling such traits can be associated with gene 'tags', and then selection using linked DNA markers ('marker-assisted selection') can be used the laboratory, rather than the field. This could bring huge increases in breeding efficiency. Eventually, also, studies such as this will allow the ability to search for new alleles in germplasm collections, which are, at the present time, under-used in their contribution to UK plant breeding, particularly for quality traits.

Implications for Levy Payers

The results of these studies will contribute indirectly to levy payers through their future contribution to plant breeding. Such studies provide plant breeders with the genetic variation to enable them to produce new series of varieties adapted to traditional markets, but also allow the flexibility to breed for new markets. However, genetic studies can contribute directly to levy payers through attaching specific genetic information to named varieties, thereby informing them of which varieties are suited to their particular farm, process or markets. A good example of this is knowing which varieties have the presence of the 1B/1R translocation. Studies have shown that this chromosome can increase the yield of a variety, but that also, on average, varieties possessing it are poorer for animal feed (Wiseman et al, HGCA report). In the context of the present results, it is shown that varieties that are hard, are expected to have intrinsically higher protein, and vice versa for soft varieties, a fortuitous, but important association. In general, however, before levy payers can make such informed choices, they will need easy access to information on the genetical characteristics of varieties and the consequences of possessing particular genes. This could be regarded as a form of 'genetic screening' where varieties are characterised not only by their agronomic performance, but also on their genetic make-up. However, at the present time, this begs the question on the best way of informing levy payers concerning the genetic characteristics of particular varieties. Perhaps, in future, such information could be included in the Recommended Lists, for example. It is to be hoped that when a large body of information on the genetical control of important agronomic and quality characteristics in UK varieties is accumulated, it will be presented in a easily digestible form to enable levy payers to practise 'genetic screening' for varieties with potential for their economic situation.

TECHNICAL REPORT

General Introduction

In terms of human and animal nutrition, hexaploid wheat (*Triticum aestivum*) is perhaps the most economically important of all crop plants. Tens of thousands of landraces and cultivars have evolved locally, or have been produced by plant breeders to suit a wide range of climatic and agronomic conditions. In addition, human cultural preference and technological advance have dictated the end use to which wheat grain is put and have led to further proliferation of wheat diversity. In China and Japan, for example, varieties suitable for the manufacture of noodles are required (Nakamura, 2000), while in the West, bread making and livestock feeding are the predominant uses of wheat.

Endosperm texture

The endosperm, which forms the major part of the wheat grain, is the storage organ of the seed and it is from here that the embryo derives its nutrition. It is formed from a fusion of two female polar cells with a single sperm cell. Seed storage proteins within the endosperm form a matrix in which starch granules are dispersed (MacRitchie, 1980). Based upon the texture of the endosperm, wheats are categorised either as hard or soft and the suitability of wheat for a particular end use depends, to a large extent, upon this factor. Hard wheats are usually employed in bread-making. When milled these give free-flowing flour that has a high water holding capacity. The ability to hold water is important in the bread-making process as endosperm proteins known as glutens become visco-elastic when wetted and trap gases in the dough that cause bread to rise. Soft wheats are often more easily digested by livestock, especially when fed as whole grain to poultry and when milled produce a less free-flowing flour with poorer water-absorbing qualities, which is used in cake and biscuit manufacture. The main genetic effect on grain texture, determining classification into hard and soft wheats, is due to a single locus on the short arm of 5D (Law *et al*. 1978) and this has been postulated to be a locus controlling the production of puroindoline proteins. In hard wheats, mutations at this locus reduce the amount or efficacy of these proteins in separating the starch granules, resulting in flour particles consisting of the fragments of several cells bound together, rather than individual starch granules as found in soft wheats. However, there is still variation in texture levels which cannot be accounted for by this locus alone, and therefore other loci must be contributing to the overall phenotype. However, these other loci have not yet been discovered and characterized.

Protein Quantity

The storage proteins in wheat grains are responsible for the visco-elastic properties of bread dough. They are also important in determining the nutritional value of the wheat, both for human and animal consumption. Therefore, the composition and overall amounts of these proteins will ultimately determine the end use to which the grain can be put. The levels of protein found in commercial UK winter wheat varieties vary between 9 and 14%. For the grain to be used in bread making, the levels need to be above 12.5% and contain "high quality" subunits of the High Molecular Weight glutenins (HMWg). To achieve this, millers and bakers frequently have to blend flours to obtain the correct protein composition.
Much is known about the genetics of the glutenin and gliadin fractions of the gluten and their functionality has been investigated. However, much less is known about the genetics of protein quantity. Previous studies have shown this to be highly influenced by environmental effects, making genetic analysis more difficult to reproduce. Increased protein has also traditionally been linked to a reduction of yield potential of the variety, although several studies have suggested that it should be possible to split the two phenotypes and breed for high yielding, high protein varieties.

The importance of seed size to grain end use.

The shape and size of wheat grains is a primary determinant in the value of the grain (Campbell *et al.* 1999). It has an effect on many factors of the agronomy on end use of the crop and therefore is an important trait for genetic study. Millers obtain higher flour yields from larger grains (Wiersma *et al*. 2001, Giura and Saulescu 1996) and therefore place pressure on breeders to ensure new varieties exhibit large grains. This has an effect on the specific weight of the grain sample, with large, full grains producing higher specific weights than small, shrivelled grains. As grain value in the UK is partly determined by specific weight values, again seed size and shape may be very important in increasing the premium on grain samples.
It has been suggested in a number of studies that increasing kernel size is an important factor in increasing yield from a crop. However, this may have some negative implications on the end use of the crop, with larger kernelled lines often showing a decrease in protein content due to the extra grain size being composed mainly of starch rather than protein. It has also been shown that large seeds may give a better start for plants grown in the field, with greater seedling size and speed of growth coming from larger seeds (Bredemeier *et al*. 2001).

The complexity of seed size genetics.

Several studies have been published which show linkage of seed size and shape traits to a number of different loci in wheat (Snape *et al*. 1985, Campbell *et al*. 1999, Ammiraju *et al*. 2001). There is no doubt, therefore, that this character is a polygenic trait. It also seems, from the differences in numbers and positions of the QTL found in these studies, that different combinations of these genes may be responsible for producing the different phenotype of size and shape in different wheat varieties, although, of course, environment and genotype x environment interactions play a part.

Difficulties of Quantitative Trait Locus (QTL) analysis in wheat.

Much of the variation for important quality traits in wheat is quantitative in nature and controlled by many genes of small effect acting together, so called QTL. QTL analysis in crop species with complex genomes is an important tool which allows the location of multiple loci important in controlling complex characters such as yield and protein. This analysis is complicated in wheat by the complexity of its polyploid genome, with the three genomes interacting in the regulation of one trait. In addition, low levels of polymorphism in molecular markers (Chao et al. 1989), especially in the "D" genome of wheat make it very difficult to construct complete genetic maps. To produce complete maps, researchers have in the past resorted to studying very wide crosses such as that of the ITMI population to increase the chances of finding polymorphic markers. However, to study quality traits, crosses need to be made between much more closely related varieties to ensure the results are relevant to the modern market.

The difficulties in finding polymorphisms have been alleviated to some degree by the introduction of alternative markers. Microsatellite or Simple Sequence Repeat (SSR) markers and Amplified Fragment Length Polymorphism (AFLP) markers tend to be more polymorphic than the traditionally used Restriction Fragment Length Polymorphism (RFLP) markers and the numbers of new markers becoming available is increasing rapidly. It is still the case, however, that many QTL studies are hampered by incomplete genetic maps, or large genetic distance between markers because of the large amount of work required to find enough polymorphic markers to construct a complete map.

Creating lines which only vary in part of the genome has two major advantages over a standard Recombinant Inbred (RI) population when used in a QTL study. Firstly, the amount of work to produce a complete genetic map is greatly reduced. Secondly, there is less possibility of epistatic or opposing genetic effects within the population, and background genetic variation is greatly reduced, so that QTL with smaller effects can be more finely

mapped. Therefore, although these lines can take several years to produce they are a very valuable resource in genetic studies, and have been one of the major approaches in this study.

Objectives of the present study

In this study, populations of recombinant substitution lines segregating for specific chromosomes, and recombinant inbred lines segregating for all the genome, have been used to dissect the genetic variation for grain texture, grain protein content and grain size. The identification of new loci for these traits will provided plant breeders with opportunities for directed plant breeding by marker assisted selection, there-by enabling the more consistent production of varieties with good quality attributes. Additionally, genetic information on particular varieties gives more information to farmers to decide whether to grow a particular variety suited to their farming system and markets.

Section 1. Investigations of QTL for grain texture and protein concentration in Avalon x Hobbit Sib and Hope x Pastiche genetic stocks

1.1 Introduction

The objectives of this study were to investigate, with the aid of the precise genetic stocks, the genetic basis of grain protein content and texture and to attempt to characterise loci within the genome influencing these characteristics. In this study, four populations were used. Firstly, a series of recombinant substitution lines containing the Avalon 5A chromosome in a Hobbit Sib background. Secondly, a second series of recombinant substitution lines containing the Avalon 5D chromosome in a Hobbit Sib background. Thirdly, a series of Avalon x Hobbit Sib recombinant inbred lines (recombinant for all chromosomes). Fourthly, a series of Hope x Pastiche single-seed descent (neo-RIL) lines.

1.2. Materials & Methods

1.2.1 Choice of parental lines.

The parental varieties used to generate the recombinant substitution lines in this study were Hobbit Sib and Avalon. Hobbit Sib is a soft, low protein, high yielding feed wheat which has in its pedigree Capelle Desprez, Viking, and Prof Marchal and has been used extensively in UK high-yielding and biscuit wheat breeding programmes. Avalon (derived from Bilbo (the progeny of Hobbit Sib) and Maris Ploughman (which contains Viking germplasm) is the ancestor of several of the modern UK bread-making wheat varieties and is a hard, high quality, high protein variety. Also, in the relationship between protein levels and yield, Avalon consistently outperformed most other bread wheat varieties having an intrinsically higher protein content than expected on its yield level. The small differences in yield and yet large differences in quality made these lines suitable for producing the populations in this study. In order to examine QTL influencing grain protein concentration and texture on chromosomes other than 5A and 5D, a series of all-chromosome recombinant inbred lines (RILs) was produced by single seed descent. Finally, in order to investigate more exotic sources of genes for high protein, a series of neo-RILs were produced by single-seed descent from the cross Hope x Pastiche. Both these parents are hard, high protein varieties which are suitable for bread-making.

1.2.2 Production of Recombinant Inbred Lines

Single seeds from each plant of a population of the cross in question were selected at random, grown out, and a single seed from the next generation selected at random. This process was

repeated until a high degree of homozygosity at all loci was achieved (lines at F_7 for this study).

1.2.3 Production of single chromosome substitution lines.

Single chromosome substitution lines, substituting an Avalon chromosome into a Hobbit Sib background, were produced according to Law and Worland (1973). A cross between a recipient (Hobbit Sib) monosomic parent and donor variety (Avalon) was backcrossed to the recipient monosomic variety, selecting the monosomic lines at each generation for 6 generations until a homozygous recipient background was obtained.

1.2.4 Recombinant substitution line production.

F_{1s} between the single chromosome substitution lines and the recipient variety (Hobbit Sib) were crossed to the monosomic of the recipient variety. The recombinant monosomic offspring are selected and selfed. The disomics from these were selected, resulting in homozygous recombinant substitution lines as described in Law (1966).

1.2.5 Genetic mapping.

DNA was extracted from the leaves of seedlings of individual lines by either the method of Devos *et al*. (1992) for RFLP or as described in Magrath *et al*. (1994) for microsatellite markers.

Restriction Fragment Length Polymorphism Markers were analysed as described in (Devos *et al.* 1992), using markers from The John Innes Centre and PBI,C. Due to the low number of markers on chromosome 5D, an enriched library was made from which further markers were produced (Aitken 1993).

SSRs were analysed as described in Roder *et al*. (1998), being run on 5% polyacrylamide gels and visualised by silver staining (Sourdille *et al*. 1998). Five different groups of microsatellites were used “gwm” (Roder *et al*. 1998), “gdm” (Pestsova *et al*. 2000), specifically designed to map to the D genome, “wmc” (Wheat Microsatellite Consortium), psr (M.D. Gale, John Innes Centre, UK) and barc (http://www.scabusa.org).

The mapping population sizes were 71 for the 5A population, 83 for the 5D population, 97 for the Avalon x Hobbit Sib RILs and 138 for the Hope x Pastiche neo-RIL's. Mapping data were analysed using the doubled haploid or SSD algorithms and Kosambi mapping function in Mapmaker v. 3.0 (Lander et al. 1987) and JoinMap (Stam & Van Ooijen 1995) to produce the genetic maps.

1.2.6 QTL Analysis

An eclectic approach was used for QTL analysis, where different statistical methods were applied and cross-checked. The software packages QTL Café (http://web.bham.ac.uk/g.g.seaton/) and MapQTL (Van Ooijen & Maliepaard, 1996) were used independently to locate QTL using single marker ANOVA, interval mapping (Haley and Knott, 1992), and marker regression (Kearsey and Hyne, 1994) techniques.

1.2. 7 Field trials.

The results from several years of field trials have been gathered, analysed, and collated in this study. In 2000, all 4 populations were planted out in spaced plant field trials consisting of one-metre row plots of 11 plants of each line, hand-dibbed, in a triplicated block experiment. In 2001, 1m x 6m drilled plots of each line were sown out in a triplicated experiment, alongside dibbed rows of all lines. Yield components were measured for all lines (spikelet number, seed weight, grain number per ear, tiller number) in the dibbed rows, and in addition, a yield assessment was made from the drilled plots in 2001.

1.2.8 Protein and Texture measurements.

Protein and texture were measured using Near Infra Red Reflectance spectroscopy (NIR) on a Bran and Luebbe Infra-alyser 2000. The protein measurements were calibrated according to calibration samples sent from CCFRA analysed by the Dumas method.

For some experiments in 2000, texture was also measured using the Single Kernel Characterisation System of CCFRA. A subset of samples was put through the SKCS system in 2001 to check the validity of the NIR data.

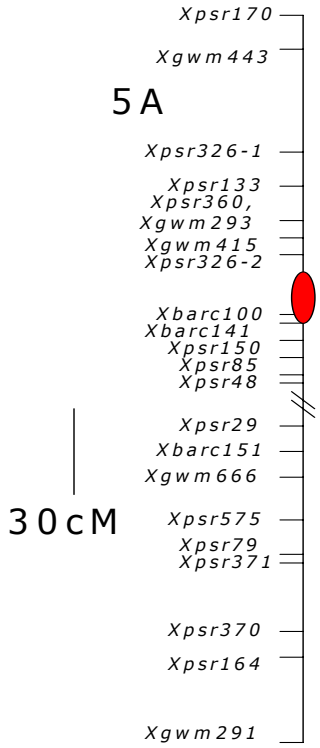
1.3. Results

1.3.1 Analysis of the Hobbit Sib (Avalon 5A)) Recombinant Substitution Lines

1.3.1.1. Mapping

The RFLP and SSR map produced of the population consisted of 22 markers. It had one gap of around 50cM which could not be filled with available markers (Figure 1.3.1.1). The map agrees well in order and overall length with published maps (Roder et al. 1998).

Figure 1.3.1.1. Map of 5A based on recombinant substitution population.



1.3.1.2. QTL Analysis: Marker Means Analysis

Of the 22 polymorphic markers mapped in the 5A recombinant substitution population, 18 showed significant ($P<0.05$) differences between allele groups for one or more of the 8 traits quantified in either of the two years of the study (Table 1.3.1.2). In 2002 only, grain protein content was significantly associated with marker loci *Xbarc100* and *Xbarc141* with the Avalon allele increasing protein. Grain texture was significantly associated with locus *Xpsr3261* in 2001 only, with the Avalon allele increasing texture. Height was significantly associated with a number of marker loci from *Xpsr133* to *Xpsr150* (51 to 107 cM) in 2002 only, with the Hobbit Sib allele increasing height. Flowering date was the only trait with which markers were significantly associated in both years of the study, where the Avalon alleles at marker loci *Xpsr575*, *Xpsr79* and *Xpsr371* (206-220 cM) were significantly associated with later flowering.

Table 1.3.1.2. Marker means analysis for the Hobbit sib (Avalon 5A/Hobbit Sib 5A) RSL population over two years. Only significant (P<0.05) differences are presented. Where the additive effect (in appropriate units) is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Marker	Position (cM)	Trait	Additive effect	P value
2001	*Xm3261*	38	texture	0.21	0.0264
2002	*Xpsr133*	51	height	-0.97	0.0127
2002	*Xpsr360*	62	height	-0.95	0.0162
2002	*Xgwm293*	64	height	-1.01	0.0133
2002	*Xgwm415*	70	height	-0.93	0.0303
2002	*Xpsr3262*	76	height	-0.78	0.0436
2002	*Xbarc100*	96	grain protein content	0.2	0.0161
2002	*Xbarc100*	96	yield	-250.56	0.0477
2002	*Xbarc100*	96	height	-0.81	0.0391
2002	*Xbarc141*	101	grain protein content	0.17	0.0363
2002	*Xbarc141*	101	height	-0.82	0.0358
2002	*Xpsr150*	107	height	-0.81	0.039
2002	*Xpsr150*	107	flowering date	0.37	0.014
2001	*Xpsr85*	112	biomass	1.06	0.048
2001	*Xbarc151*	183	50 grain weight	0.09	0.045
2001	*Xbarc151*	183	biomass	1.46	0.0111
2001	*Xgwm666*	191	flowering date	0.32	0.0413
2001	*Xpsr575*	206	flowering date	0.54	1.00E-04
2002	*Xpsr575*	206	flowering date	0.34	0.0191
2001	*Xpsr79*	218	flowering date	0.38	0.0083
2002	*Xpsr79*	218	flowering date	0.35	0.0162
2001	*Xpsr371*	220	flowering date	0.38	0.0084
2002	*Xpsr371*	220	flowering date	0.33	0.0243
2001	*Xpsr370*	243	spikelet number	-2.52	0.0491
2001	*Xpsr370*	243	tiller no.	4.98	0.0238
2001	*Xpsr370*	243	flowering date	0.32	0.0315
2001	*Xpsr164*	252	tiller no.	4.46	0.0373
2001	*Xgwm291*	283	flowering date	0.35	0.0116

Increased plant biomass was significantly associated with two loci (112 and 183 cM) in 2001 only (Avalon increasing allele). Also in 2001 only, there were two further significant associations between markers and later flowering date at the distal end of the chromosome (Avalon increasing allele), and two significant associations with tiller number at markers *Xpsr370* and *Xpsr164* (243 and 262 cM, Avalon increasing allele). Single markers showed significant associations for 50 grain weight (*Xbarc151*, 183 cM, Avalon increasing allele), and spikelet number (*Xpsr370*, 243 cM, Hobbit Sib increasing allele) in 2001 only.

1.3.1.3 QTL analysis by Multiple Marker Regression

Significant ($P<0.05$) QTL as defined by multiple marker regression (1000 simulations) were present for biomass (143 cM; Avalon increasing allele) in 2001 and height (91 cM; Hobbit Sib increasing allele) in 2002 (Table 1.3.1.3). No QTL consistent across years were discovered.

1.3.1.4. QTL analysis by Interval Mapping

Location of QTL for grain protein content by interval mapping showed a consistent effect at 100 cM (*Xbarc100 – Xbarc141*) in both years (Fig. 1.3.1.4.1). However, this QTL was not significant in either year as assessed by permutation test (1000 permutations). Location of QTL for grain texture showed a consistent effect at 45 cM (*Xpsr133*) with a peak Lod score of 0.7 in both years (Fig. 1.3.1.4.2). However, again this QTL was not significant in either year as assessed by permutation test (1000 permutations). The location of a QTL for height by interval mapping showed inconsistent effects between years. Lod scores greater than 1.0 were only observed in 2002, and the peak Lod score of 1.4 at 112 cM was not significant as assessed by the permutation test (1000 permutations) (Figure 1.3.1.4.3). The location of a QTL for yield also showed inconsistent effects between years. Lod scores greater than 0.5 were only observed in 2002, and the peak Lod score of 0.9 at 101 cM was not significant as assessed by permutation test. The location of a QTL for flowering date showed a consistent peak Lod score at 206 cM in both years (Fig. 1.3.1.4.4). However, this peak was only significant in 2001 as assessed by the permutation test. The peak of the QTL in 2001 had a Lod score of 3.55 ($P<0.01$) and explained 29.9% of the variation across the chromosome, with Avalon contributing the increasing effect. Interestingly, this QTL appears near/at at the location of the major vernalization locus *Vrn-A1,* so although both Avalon and Hobbit Sib are winter wheats, they may differ in their vernalization response and hence, flowering date.

Table 1.3.1.3. Marker regression analysis (1000 simulations) of the Hobbit Sib (Avalon 5A) RSL population over two years. Only significant (P<0.05) differences are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Trait	Position (cM)	Additive effect	Units	P value
2001	Biomass	143.5 +/- 57.3	1.83 +/- 1.59	g	0.022
2002	Height	90.76 +/- 41.3	-1.30 +/- 0.77	cm	0.006

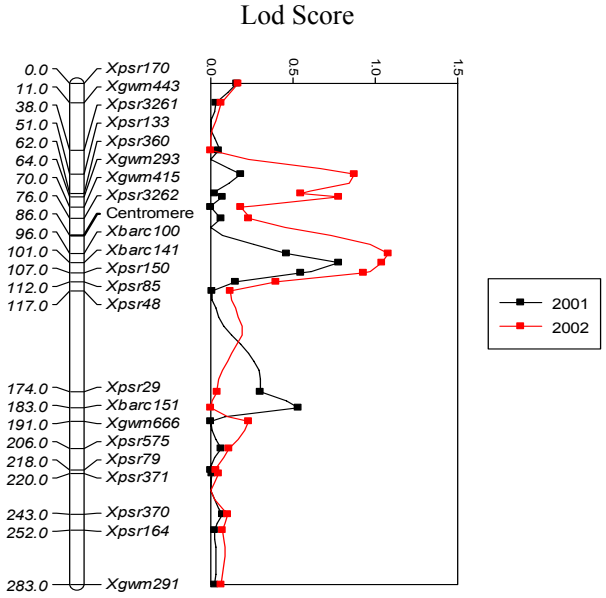


Figure 1.3.1.4.1. Interval mapping of chromosome 5A for protein content. No significant QTL detected.

However, a presumptive QTL is consistent between marker loci *Xbarc100* - *Xbarc141*

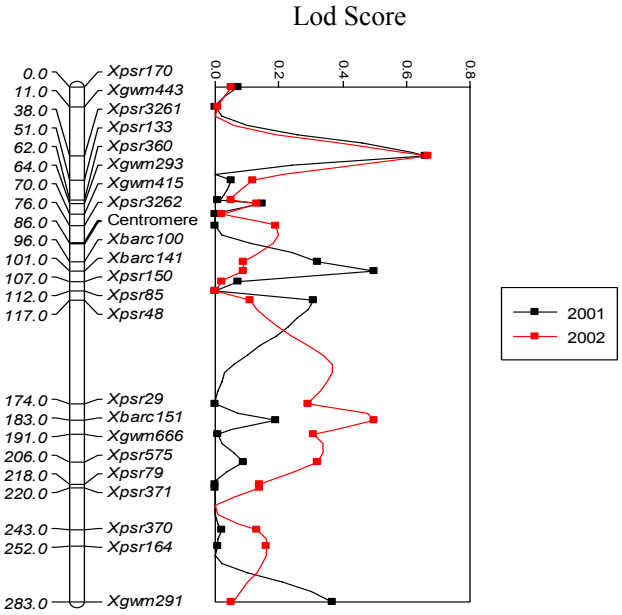


Figure 1.3.1.4.2. Interval mapping of chromosome 5A for grain texture. No significant QTL detected.

However, a consistent effect is observed at marker *Xpsr133*

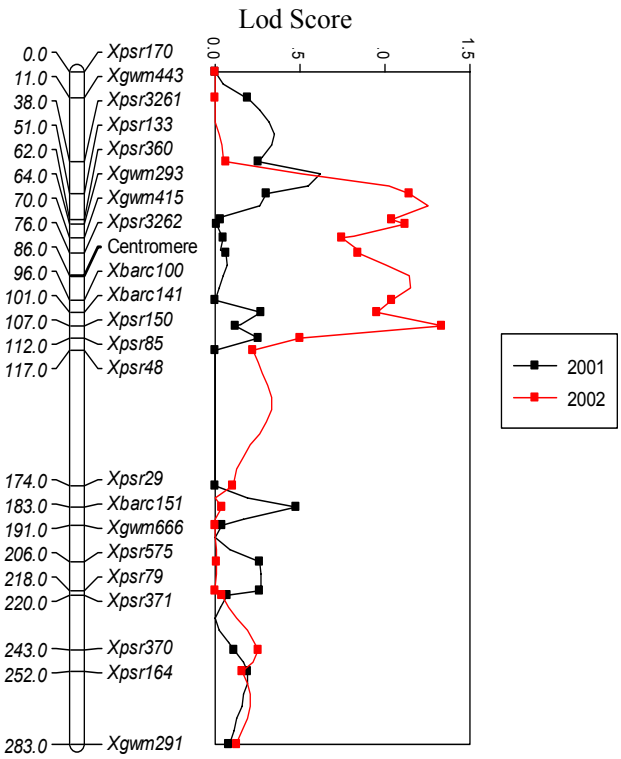


Figure 1.3.1.4.3. Interval mapping of chromosome 5A for height. No significant QTL detected.

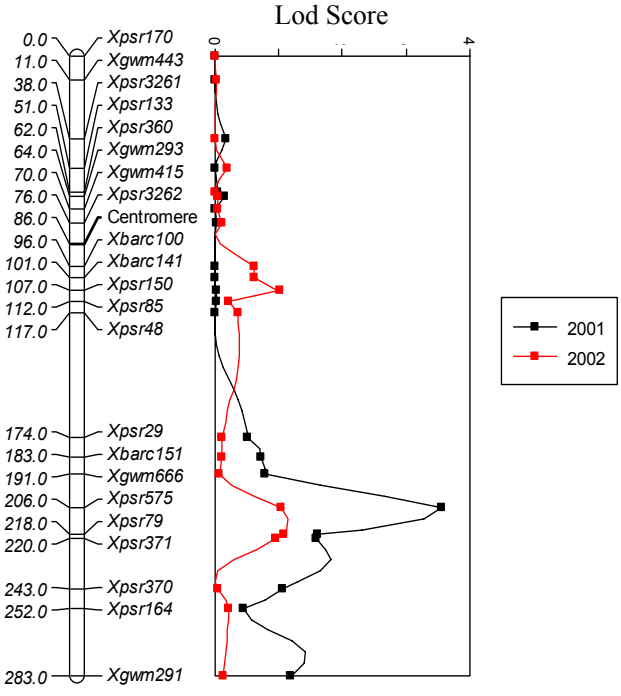


Figure 1.3.1.4.4. Interval mapping of chromosome 5A for flowering date. Significant QTL detected in 2001 only.

Lod Score : 3.55
Significance*: 0.006
Peak of QTL: 206 cM
Additive effect: + 0.5362
% variation explained: 29.9

* permutation test, 1000 permutations.

1.3.1.5. Summary of QTL on 5A significant by interval mapping

A Summary of QTL on 5A significant by interval mapping is presented in Figure 1.3.1.5.

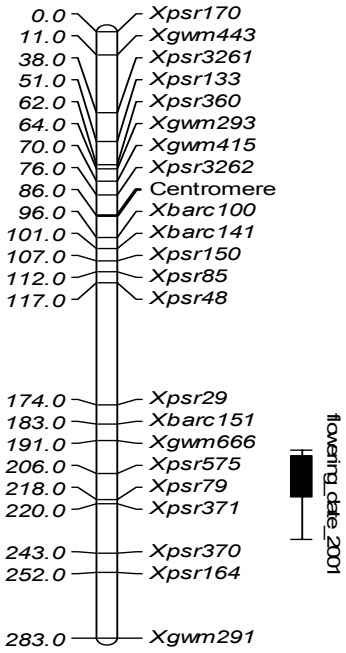


Figure 1.3.1.5. Summary of positions of significant QTL in the 5A substitution line population.

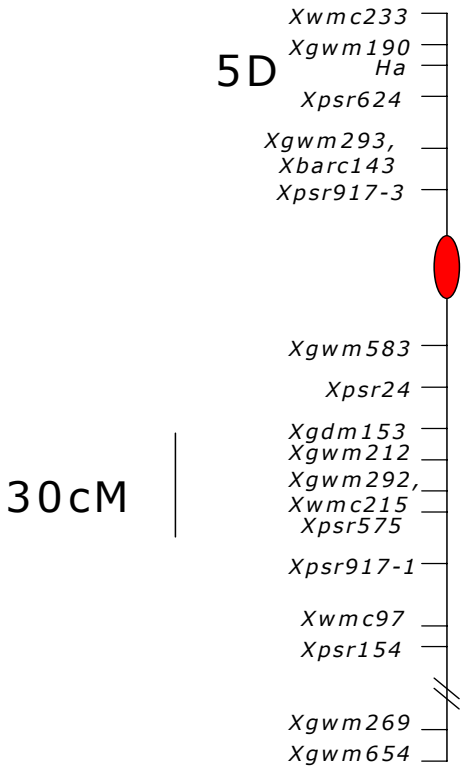
The outer (narrow) line indicates the 1-Lod interval, the inner (broad) line indicates the 2-Lod interval.

1.3.2. Analysis of the Hobbit sib (Avalon 5D) Recombinant Substitution Lines

1.3.2.1 Mapping

The RFLP and SSR map produced of the population consisted of 18 markers. It had one gap of around 50cM which could not be filled with available markers (Figure 1.3.2.1). The map agrees well in order and overall length with published maps (Roder et al. 1998).

Figure 1.3.2.1. Map of 5D based on recombinant substitution population.



1.3.2.2 QTL analysis : Marker Means Analysis

Of the 19 marker loci mapped in the 5D recombinant substitution line population, eleven were associated with significant ($P<0.05$) differences of one or more of the five traits assayed (Table 1.3.2.2). The majority of these differences were for grain texture, with six markers surrounding the *Ha* locus showing a significant association with increased grain texture, as expected, due to the Avalon allele. Marker loci were significantly associated with increased grain protein content at 15, 162 and 179 cM. Late flowering was associated with the Hobbit Sib alleles at 125 and 135 cM, and increased height was associated with the Avalon allele at a single locus at 162 cM.

Table 1.3.2.2. Marker means analysis for the Hobbit sib (Avalon 5D) RSL population. Only significant (P<0.05) differences are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Marker	Position (cM)	Trait	Additive Effect	P value
Xwmc233	0	grain texture	1.23	0
Xgwm190	15	grain protein content	0.07	0.0075
Xgwm190	15	grain texture	1.54	0
Ha	20	grain texture	1.87	0
Xpsr624	29	grain texture	1.45	0
Xgwm293	42	grain texture	1.28	1.00E-04
Xbarc143	43	grain texture	1.04	0
Xpsr917-3	55	grain texture	0.57	0.0226
Xgdm153	125	flowering date	-0.1	0.0365
Xgwm212	135	flowering date	-0.11	0.0197
Xpsr917-1	162	grain protein content	0.06	0.0238
Xpsr917-1	162	height	0.62	0.0439
Xwmc97	179	grain protein content	0.06	0.0136

1.3.2.3 QTL analysis by Multiple Marker Regression

QTL analysis by multiple marker regression revealed three significant ($P<0.05$) QTL (Table 1.3.2.3). A QTL for grain texture was located at 22 cM with Avalon contributing the increasing effect; a QTL for grain protein content was located at 156 cM with Avalon contributing the increasing effect; and a QTL for flowering date was located at 122 cM with Hobbit Sib contributing the increasing effect.

1.3.2.4. QTL analysis by Interval Mapping

QTL analysis by interval mapping revealed a significant QTL for grain protein quantity with a peak Lod score of 1.82 at 219 cM. This QTL explained 20% of the variation across the chromosome, with Avalon contributing the increasing effect (Figure 1.3.2.4.1). The majority (75%) of grain texture variation across the chromosome was explained by a QTL at the *Ha* locus (20 cM, Lod 24.75, $P<0.001$) with Avalon contributing the increasing effect (Figure 1.3.2.4.2). The QTL for flowering date detected using multiple marker regression (above) was not significant when analysed by interval mapping (Figure 1.3.2.4.3).

1.3.2.5. Summary of QTL on 5D significant by interval mapping

A summary of QTL on 5D significant by interval mapping is presented in Figure 1.3.2.5.

Table 1.3.2.3. Marker regression analysis (1000 simulations) of the Hobbit Sib (Avalon 5D) RSL population. Only significant (P<0.05) differences are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Trait	Position (cM)	Additive effect	Units	P value
grain texture	21.8 +/- 3.6	1.986 +/- 0.314	arbitrary	0.002
flowering date	122.4 +/- 35.1	-0.126 +/- 0.07	days	0.03
grain protein content	155.8 +/- 49.1	0.075 +/- 0.048	percent	0.049

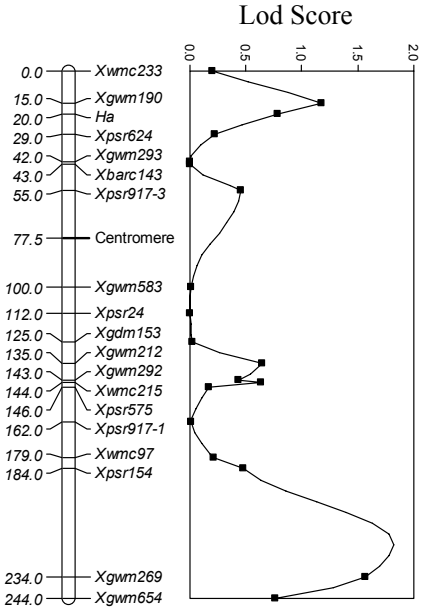


Figure 1.3.2.4.1. Interval mapping of chromosome 5D for protein quantity. One significant QTL detected.

Lod Score : 1.82
Significance*: 0.049
Peak of QTL: 219 cM
Additive effect: + 0.096
% variation explained: 19.4

* permutation test, 500 permutations.

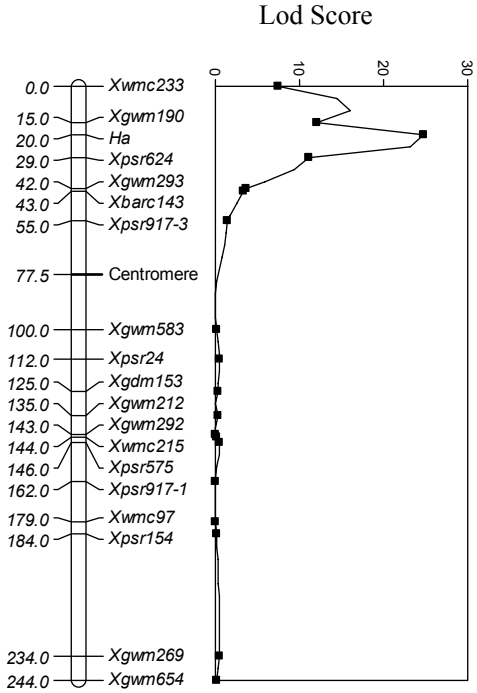


Figure 1.3.2.4.2. Interval mapping of chromosome 5D for grain texture. One significant QTL detected.

Lod Score : 24.75
Significance*: <0.001
Peak of QTL: 20 cM
Additive effect: + 1.869
% variation explained: 75.5

* permutation test, 500 permutations.

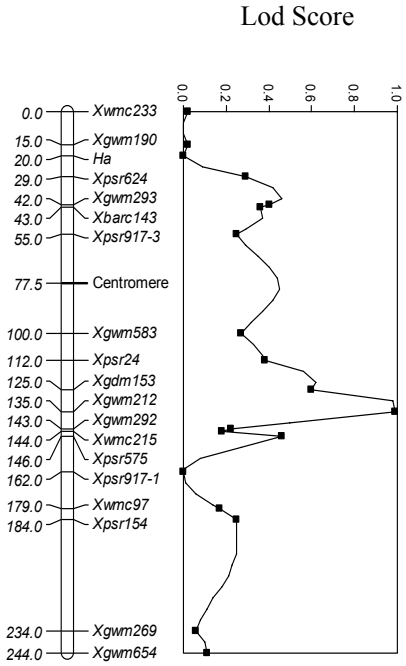


Figure 1.3.2.4.3. Interval mapping of chromosome 5D for flowering date. No significant QTL detected.

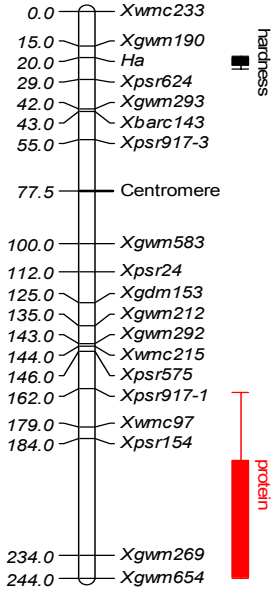


Figure 1.3.2.5. Summary of positions of significant QTL in the 5D substitution line population.

The outer (narrow) line indicates the 1-Lod interval, the inner (broad) line indicates the 2-Lod interval. Where the peak of the QTL is significant at a Lod score of less than 2.0, the inner interval is set to the peak Lod score of the QTL, here 1.8 for grain protein content.

1.3.3 Analysis of the Hobbit sib x Avalon Recombinant Inbred Lines

1.3.3.1 Mapping

A total of 260 RFLP and SSR markers were mapped in this population. The maps giving the best fit with the recombination data observed are presented below (Figure 1.3.3.1).

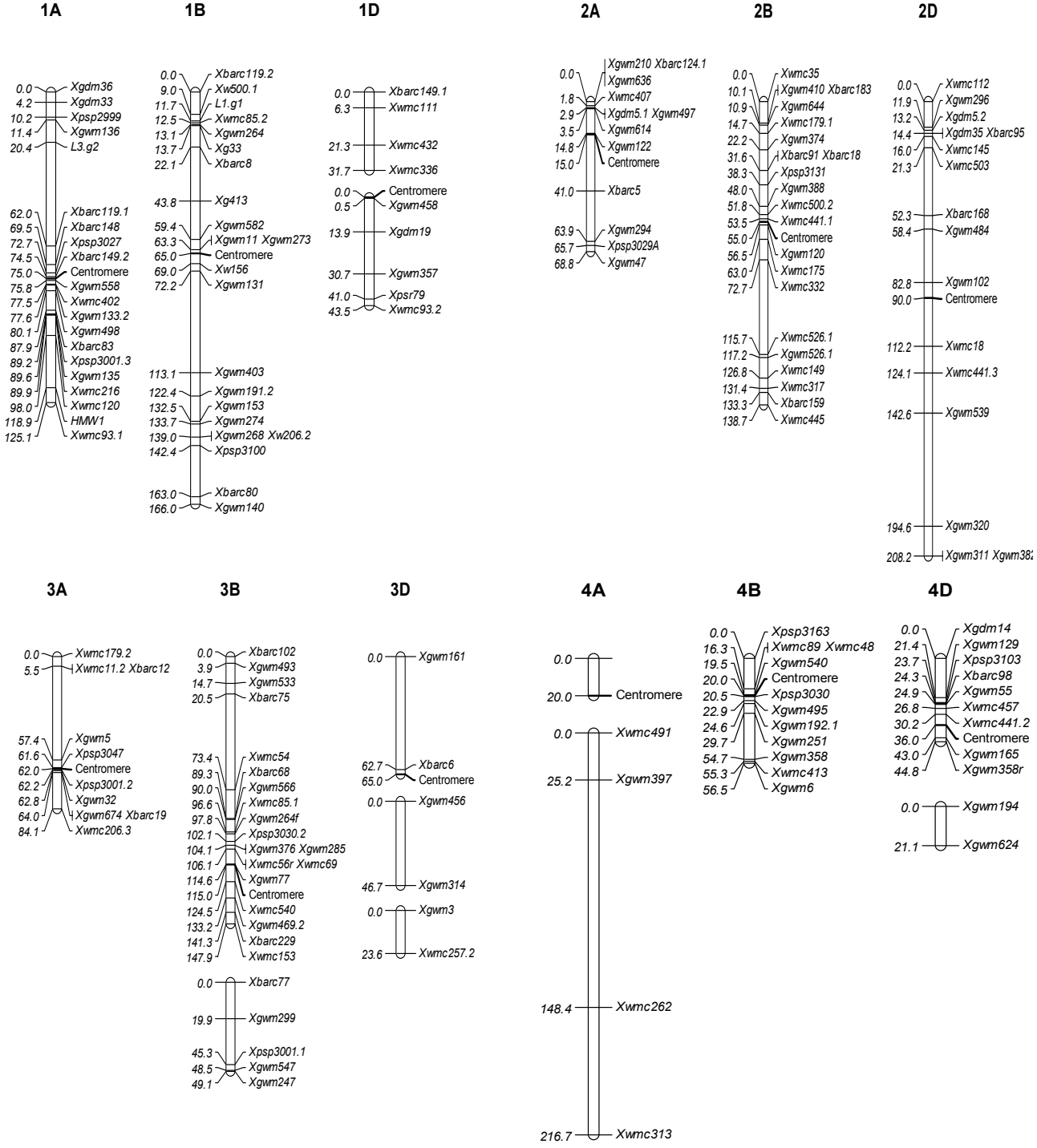
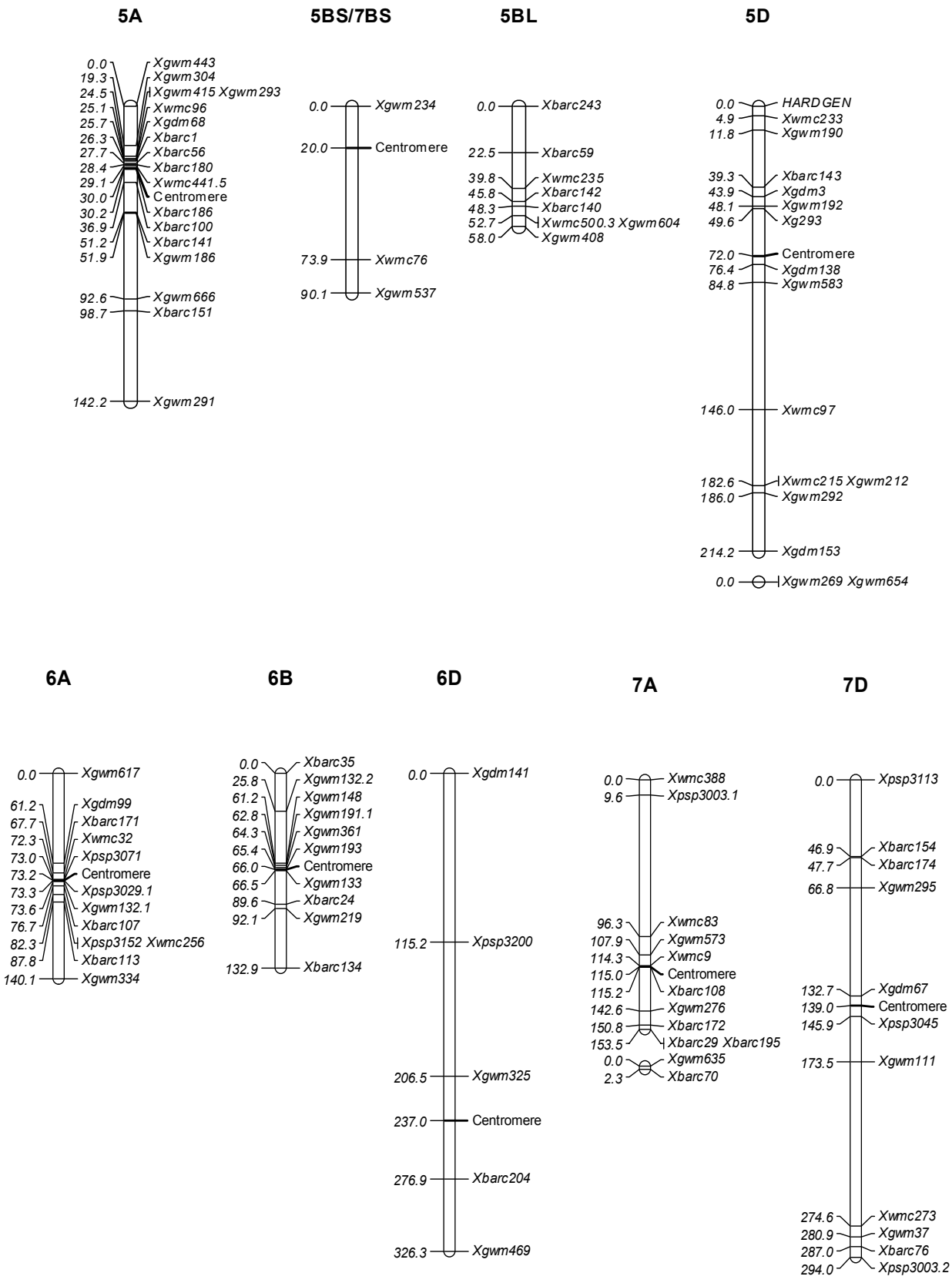


Figure 1.3.3.1. continued.



1.3.3.2 Grain Protein Content

1.3.3.2.1 Marker Means Analysis of Grain Protein Content

A total of 35 markers across 13 chromosomes were associated with significant ($P<0.05$) differences in grain protein content. Of these, 12 markers showed consistent differences in both years of the study (Table 1.3.3.2.1). Increased grain protein content was consistently associated with markers on chromosomes 1A, 2B (3 markers), 2D, 3B, 5BS/7BS and 6B with Avalon contributing the increasing allele, and markers on chromosomes 3A, 6A and 7A (2 markers) with Hobbit Sib contributing the increasing allele.

1.3.3.2.2 QTL analysis by Multiple Marker Regression of Grain Protein Content

Nine significant ($P<0.05$) QTL were identified by multiple marker regression over the two years of the study, located on six chromosomes (Table 4.3.2.2). Effects consistent over both years were located on chromosomes 2B and 5BS (Avalon allele increasing) and 7A (Hobbit Sib allele increasing). Single year significant QTL were found on chromosomes 6B and 6D (Avalon allele increasing) and 1B (Hobbit Sib allele increasing).

1.3.3.2.3. QTL analysis by Interval Mapping of Grain Protein Content

Significant QTL were identified by interval mapping on chromosomes 2B and 6B (Avalon contributing increasing effect) and chromosomes 6A and 7A (Hobbit Sib contributing increasing effect). The QTL on chromosome 2B (Figure 1.3.3.2.3.1) was located between 15 and 20 cM, explained between 11 and 15.5 % of variation across the chromosome, and had significant Lod scores in both years. The QTL on chromosome 6A (Figure 1.3.3.2.3.2) was located at 140 cM, explained between 11.1 and 12.9 % of variation across the chromosome, and had significant Lod scores in 2002 only. The QTL on chromosome 6B (Figure 1.3.3.2.3.3) was located between 76 and 87 cM, explained between 15.9 and 18.2 % of variation across the chromosome, and had significant Lod scores on both years. The QTL on chromosome 7A (Figure 1.3.3.2.3.4) was located at 114 cM, explained between 8.8 and 10.3 % of variation across the chromosome, and had significant Lod scores in both years.

Table 1.3.3.2.1. Marker means analysis of grain protein concentration on the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) consistent year-on year differences are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Chromosome	Marker	Position (cM)	Additive effect (%)	P value
2001	1A	*Xbarc83*	87.9	0.36	0.014
2002	1A	*Xbarc83*	87.9	0.3	0.0086
2001	2B	*Xgwm410*	10.1	0.28	0.0066
2002	2B	*Xgwm410*	10.1	0.21	0.0062
2001	2B	*Xgwm644*	10.9	0.28	0.006
2002	2B	*Xgwm644*	10.9	0.21	0.0069
2001	2B	*Xwmc179.1*	14.7	0.29	0.006
2002	2B	*Xwmc179.1*	14.7	0.21	0.0063
2001	2D	*Xwmc441.3*	124.1	0.29	0.0045
2002	2D	*Xwmc441.3*	124.1	0.17	0.0251
2001	3A	*Xgwm32*	62.8	-0.22	0.0463
2002	3A	*Xgwm32*	62.8	-0.18	0.0216
2001	3B	*Xbarc229*	141.3	0.25	0.0141
2002	3B	*Xbarc229*	141.3	0.17	0.0288
2001	5BS/7BS	*Xgwm537*	90.1	0.27	0.014
2002	5BS/7BS	*Xgwm537*	90.1	0.22	0.0062
2001	6A	*Xgwm334*	140.1	-0.29	0.0056
2002	6A	*Xgwm334*	140.1	-0.25	0.0022
2001	6B	*Xbarc24*	89.6	0.23	0.0452
2002	6B	*Xbarc24*	89.6	0.23	0.0076
2001	7A	*Xwmc9*	114.3	-0.31	0.0029
2002	7A	*Xwmc9*	114.3	-0.2	0.012
2001	7A	*Xbarc108*	115.2	-0.44	0.00007
2002	7A	*Xbarc108*	115.2	-0.29	0.0037

Table 1.3.3.2.2 Marker regression analysis of grain protein content on the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (%)	P value
2002	1B	56.084 +/- 20.767	-0.302 +/- 0.121	0.012
2001	2A	10.404 +/- 6.29	-0.562 +/- 0.146	0.004
2002	2A	10.543 +/- 6.603	-0.39 +/- 0.102	0.011
2001	2B	18.34 +/- 20.419	0.316 +/- 0.132	0.01
2002	2B	17.085 +/- 27.867	0.227 +/- 0.114	0.001
2001	5BS/7BS	32.03 +/- 23.691	0.525 +/- 0.291	0.026
2002	5BS/7BS	79.582 +/- 20.753	0.240 +/- 0.127	0.026
2001	6B	104.15 +/- 22.691	0.444 +/- 0.205	0.028
2002	6D	256.384 +/- 59.077	0.358 +/- 0.22	0.016
2001	7A	117.854 +/- 18.446	-0.443 +/- 0.182	0.003
2002	7A	118.797 +/- 20.958	-0.318 +/- 0.149	0.006

2B

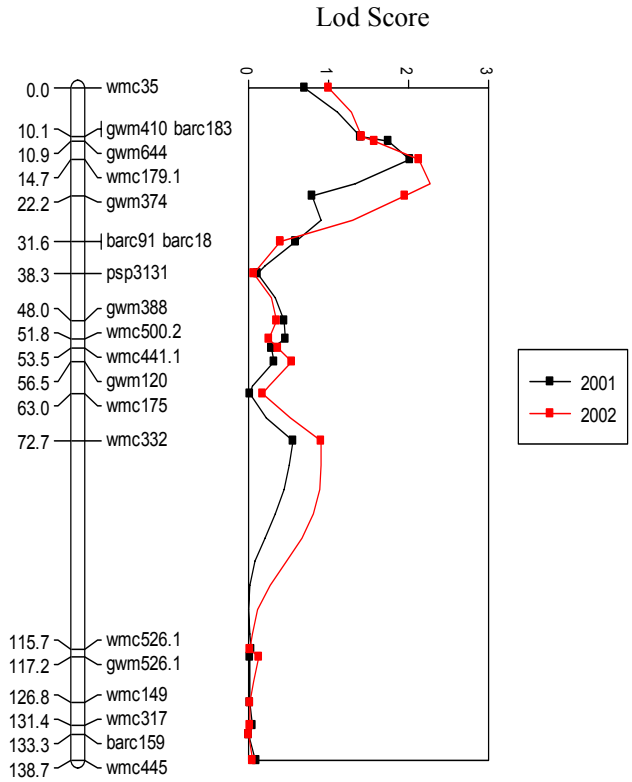


Figure 1.3.3.2.3.1. Interval mapping of chromosome 2B for grain protein content. One significant QTL detected (both years).

<u>2001</u>
Lod Score : 2.27
Significance*: 0.03
Peak of QTL: 19.7 cM
Additive effect: +0.2559 %
% variation explained: 15.5

<u>2002</u>
Lod Score : 2.01
Significance*: 0.042
Peak of QTL: 14.7 cM
Additive effect: +0.3024 %
% variation explained: 10.9

* permutation test, 500 permutations.

6A

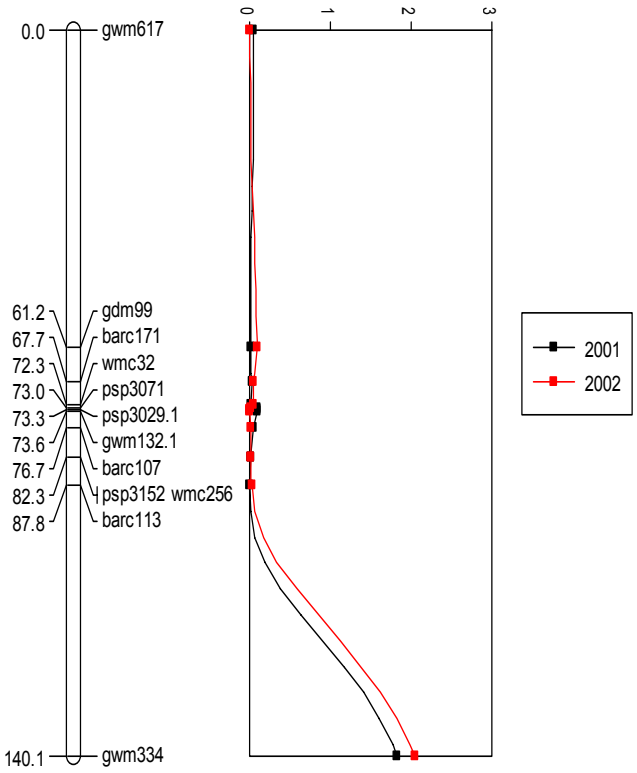


Figure 1.3.3.2.3.2 Interval mapping of chromosome 6A for grain protein content. One significant QTL detected (2002 only).

<u>2001</u>
Lod Score : 1.83
Significance*: 0.10
Peak of QTL: 140.1 cM
Additive effect: -0.3009 %
% variation explained: 11.1

<u>2002</u>
Lod Score : 2.05
Significance*: 0.01
Peak of QTL: 140.1 cM
Additive effect: -0.2342 %
% variation explained: 12.9

* permutation test, 500 permutations.

6B

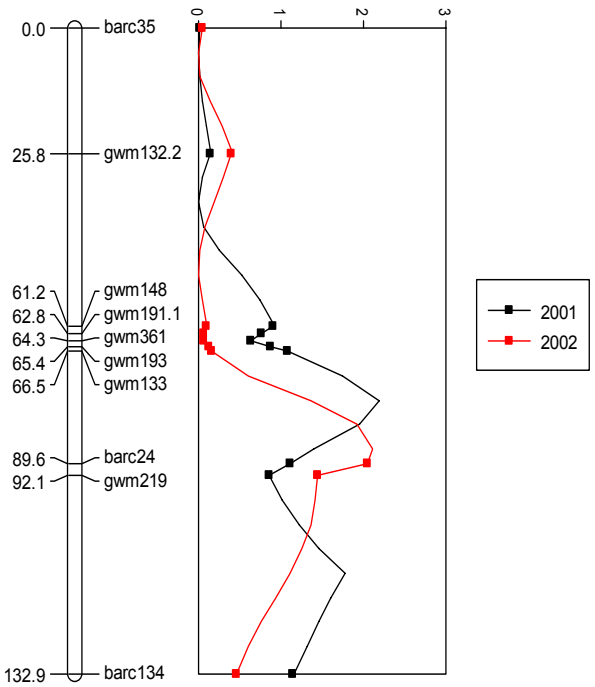


Figure 1.3.3.2.3.3. Interval mapping of chromosome 6B for grain protein content. One significant QTL detected.

2001
Lod Score : 2.19
Significance*: 0.03
Peak of QTL: 76.5 cM
Additive effect: +0.6635 %
% variation explained: 18.2

2002
Lod Score : 2.11
Significance*: 0.01
Peak of QTL: 86.5 cM
Additive effect: +0.2590 %
% variation explained: 15.9

* permutation test, 500 permutations.

7A

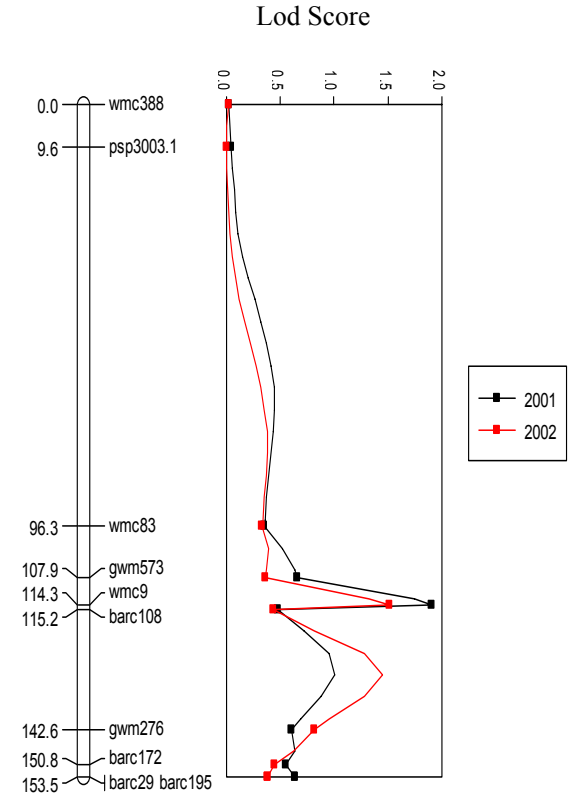


Figure 1.3.3.2.3.4. Interval mapping of chromosome 7A for grain protein content. One significant QTL detected (2001 only)

2001
Lod Score : 1.9
Significance*: 0.032
Peak of QTL: 114.3 cM
Additive effect: -0.2941 %
% variation explained: 10.3

2002
Lod Score : 1.51
Significance*: 0.068
Peak of QTL: 114.3 cM
Additive effect: -0.1945 %
% variation explained: 8.8

* permutation test, 500 permutations.

1.3.3.3 Grain Texture

1.3.3.3.1. Marker Means Analysis of Grain Texture

A total of 18 markers across five chromosomes were associated with significant ($P<0.05$) differences in grain texture. Of these, 11 markers on six chromosomes showed consistent differences in both years of the study (Table 1.3.3.3.1). Avalon consistently contributed the increasing effect towards hardness to nine of these markers, on chromosomes 1B (5 markers), 4D and 5D (3 markers) whilst Hobbit Sib consistently contributed the increasing effect to two markers, on chromosomes 4B and 5BS/7BS.

1.3.3.3.2. QTL analysis by Multiple Marker Regression of Grain Texture

Six significant ($P<0.05$) QTL were located by multiple marker regression over the two years, corresponding to a single QTL, consistent over both years, on each of chromosomes 1B, 5D and 5BS/7BS (Table 1.3.3.3.2). Avalon contributed the increasing effect towards hardness on chromosomes 1B and 5D whilst Hobbit Sib contributed the increasing effect towards hardness on chromosome 5BS/7BS.

1.3.3.3.3 QTL analysis by Interval Mapping of Grain Texture

Not surprisingly, one significant QTL was identified by interval mapping on chromosome 5D, at the *Ha* locus, with Avalon as the increasing allele (Figure 1.3.3.3.3.1). The peak Lod score was 27.5 at 0 cM in 2001, and this QTL explained 79% of the variation in grain texture across this chromosome. Interval mapping of chromosome 1B showed Lod scores approaching 2 in 2001 (Figure 1.3.3.3.3.2). However, this QTL was not significant by permutation test in either year.

Table 1.3.3.3.1. Marker means analysis of grain texture (Arbitrary Units) for the Avalon x Hobbit Sib RIL population over two years. Only differences significant (P<0.05) in both years are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Chromosome	Marker	Position (cM)	Additive effect (AU)	P value
2001	1B	*Xbarc119.2*	0	0.7	0.017
2002	1B	*Xbarc119.2*	0	0.62	0.023
2001	1B	*Xw500.1*	9	0.84	0.004
2002	1B	*Xw500.1*	9	0.86	0.002
2001	1B	*L1.g1*	11.7	0.74	0.012
2002	1B	*L1.g1*	11.7	0.72	0.01
2001	1B	*Xwmc85.2*	12.5	0.7	0.021
2002	1B	*Xwmc85.2*	12.5	0.6	0.035
2001	1B	*Xg413*	43.8	0.74	0.015
2002	1B	*Xg413*	43.8	0.61	0.034
2001	4B	*Xpsp3030*	20.5	-0.97	0.001
2002	4B	*Xpsp3030*	20.5	-0.81	0.005
2001	4D	*Xgwm194*	0	0.76	0.008
2002	4D	*Xgwm194*	0	0.73	0.007
2001	5BS/7BS	*Xwmc76*	73.9	-0.71	0.014
2002	5BS/7BS	*Xwmc76*	73.9	-0.65	0.017
2001	5D	*Ha*	0	2.46	0
2002	5D	*Ha*	0	1.95	0
2001	5D	*Xwmc233*	4.9	1.79	0
2002	5D	*Xwmc233*	4.9	1.47	0
2001	5D	*gwm190*	11.8	1.66	0
2002	5D	*gwm190*	11.8	1.29	0

Table 1.3.3.3.2 Marker regression analysis of grain texture (arbitrary units) on the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (AU)	P value
2001	1B	36.28 +/- 28.088	1.071 +/- 0.498	0.005
2002	1B	20.601 +/- 34.358	0.841 +/- 0.436	0.018
2001	5BS/7BS	58.456 +/- 20.938	-1.26 +/- 0.689	0.019
2002	5BS/7BS	64.134+/-20.326	-0.912 +/- 0.557	0.042
2001	5D	1.025 +/- 3.223	2.27 +/- 0.379	0
2002	5D	1.585 +/- 6.306	1.777 +/- 0.361	0.001

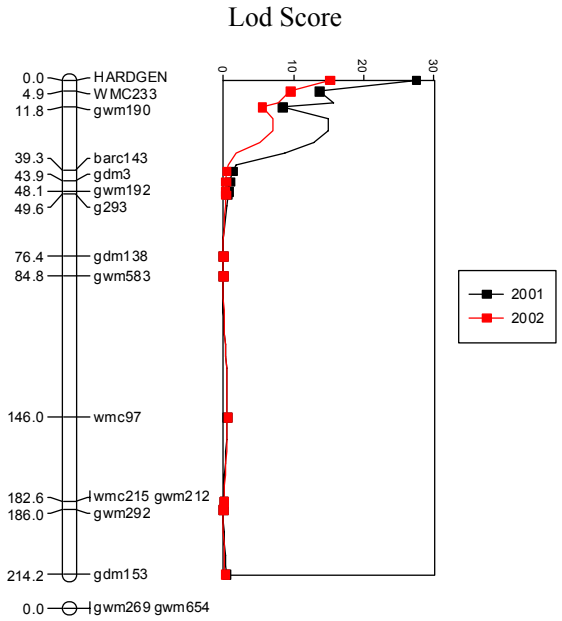


Figure 1.3.3.3.3.1. Interval mapping of chromosome 5D for grain texture. One significant QTL detected.

<u>2001</u>
Lod Score : 27.48
Significance*: <0.001
Peak of QTL: 0.0 cM
Additive effect: +2.33 arbitrary units
% variation explained: 79.0

<u>2002</u>
Lod Score : 15.21
Significance*: <0.001
Peak of QTL: 0.0 cM
Additive effect: +1.894 arbitrary units
% variation explained: 63.7

* permutation test, 500 permutations.

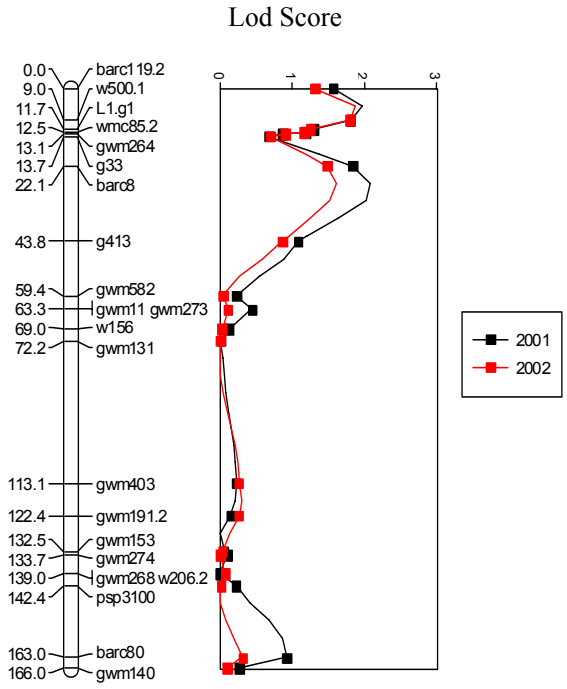


Figure 1.3.3.3.3.2. Interval mapping of chromosome 1B for grain texture. No significant QTL detected.

<u>2001</u>
Lod Score : 2.08
Significance*: 0.098
Peak of QTL: 27.1 cM
Additive effect: + 1.1396 arbitrary units
% variation explained: 19.0

<u>2002</u>
Lod Score : 1.61
Significance*: 0.10
Peak of QTL: 27.1 cM
Additive effect: + 0.9159 arbitrary units
% variation explained: 14.9

* permutation test, 500 permutations.

1.3.3.4. Agronomic Traits

1.3.3.4.1. Flowering Time

1.3.3.4.1.1. Marker Means Analysis of Flowering Time

Over the entire dataset, analysing each year's data separately, 43 markers were significantly ($P<0.05$) associated with differences in flowering date. Of these, only six markers on three chromosomes showed differences consistent over both years of the study (Table 1.3.3.4.1.1). Three markers on chromosome 4B showed consistent effects with Avalon as the late allele, whilst two markers on chromosome 6A and one on chromosome 7D showed consistent effects with Hobbit Sib as the late allele.

1.3.3.4.1.2. QTL analysis by Multiple Marker Regression of Flowering Time

Seven QTL were significant by multiple marker regression over the two years' data, located on five chromosomes (Table 1.3.3.4.1.2). Of these, QTL which were consistent over two years were located on chromosome 4B (38-39 cM, Avalon late effect) and chromosome 6A (47-66 cM, Hobbit Sib late effect). Single year significant QTL were located on chromosomes 5A, 7A and 7D.

1.3.3.4.1.3. Interval Mapping of Flowering Time

The only significant QTL as defined by interval mapping was located on chromosome 4B, with Avalon contributing the late allele (Figure 1.3.3.4.1.3). This QTL was significant in 2001 only, with a peak Lod score of 2.14 at 25 cM and accounting for 11% of variation across the chromosome. The Lod score varied across the chromosome in a similar manner in both years, but was not significant in 2002.

Table 1.3.3.4.1.1 Marker means analysis of flowering date (days) on the Avalon x Hobbit Sib RIL population over two years. Only differences significant (P<0.05) in both years are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Chromosome	Marker	Position (cM)	Additive effect (days)	P value
2001	4B	*Xwmc89*	16.3	0.72	0.0049
2002	4B	*Xwmc89*	16.3	0.33	0.0267
2001	4B	*Xgwm192.1*	24.6	0.75	0.003
2002	4B	*Xgwm192.1*	24.6	0.34	0.0289
2001	4B	*Xgwm358*	54.7	0.73	0.0051
2002	4B	*Xgwm358*	54.7	0.35	0.0248
2001	6A	*Xgdm99*	61.2	-0.7	0.0181
2002	6A	*Xgdm99*	61.2	-0.4	0.0467
2001	6A	*Xbarc171*	67.7	-1.6	2.00E-04
2002	6A	*Xbarc171*	67.7	-0.6	0.0222
2001	7D	*Xbarc76*	287	-0.6	0.0202
2002	7D	*Xbarc76*	287	-0.5	0.0012

Table 1.3.3.4.1.2 Marker regression analysis for flowering date (days) on the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (days)	P value
2001	4B	39.535 +/- 9.822	1.115 +/- 0.337	0
2002	4B	38.873 +/- 12.591	0.449 +/- 0.209	0.034
2001	5A	37.522 +/- 14.247	-0.848 +/- 0.309	0.045
2001	6A	65.514 +/- 11.524	-1.105 +/- 0.396	0.016
2002	6A	47.021 +/- 29.101	-0.643 +/- 0.387	0.025
2001	7A	121.487 +/- 20.98	-1.009 +/- 0.479	0.001
2002	7D	264.603 +/- 59.363	-0.567 +/- 0.423	0.002

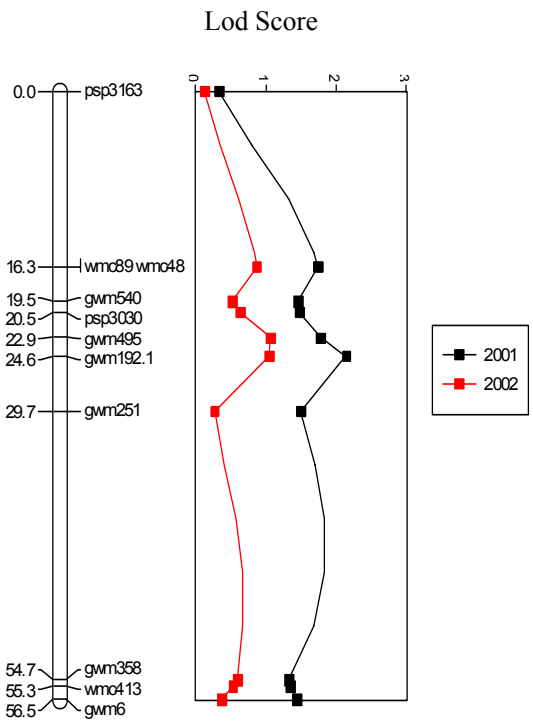


Figure 1.3.3.4.1.3. Interval mapping of chromosome 4B for flowering date. One significant QTL detected (2001 only).

<u>2001</u>
Lod Score : 2.14
Significance*: 0.014
Peak of QTL: 24.6 cM
Additive effect: +0.7329
% variation explained: 11.1

<u>2002</u>
Lod Score : 1.06
Significance*: 0.18
Peak of QTL: 22.9 cM
Additive effect: +0.3240
% variation explained: 6.4

* permutation test, 500 permutations.

1.3.3.4.2 Height

1.3.3.4.2.1. Marker Means Analysis of Height

Over the entire dataset, analysing each year's data separately, 39 markers on 11 chromosomes were significantly ($P<0.05$) associated with differences in height. Of these, 15 markers on four chromosomes showed differences consistent over both years of the study (Table 4.3.4.2.1). Avalon contributed the tall allele to two markers on chromosome 3B, eight markers on chromosome 6A and four markers on chromosome 7A, whilst Hobbit Sib contributed the increasing effect to two markers on chromosome 2D.

1.3.3.4.2.2. QTL analysis by Multiple Marker Regression of Height

Nine QTL were significant by multiple marker regression over the two years' data, located on six chromosomes (Table 1.3.3.4.2.2). Of these, QTL which were consistent over both years were located on chromosomes 6A (70-112 cM, Avalon tall allele), 7A (120-122 cM, Avalon tall allele) and 2D (5-40 cM, Hobbit Sib tall allele). Single year significant QTL were located on chromosomes 1D, 6B and 6D.

1.3.3.4.2.3 QTL analysis by Interval Mapping of Height

Three significant QTL were identified by interval mapping, of which one was significant in both years of the study. A QTL on chromosome 2D was significant in 2001 only with a peak Lod score of 4.2 at 0 cM, explaining 22% of the variation across the chromosome and with Hobbit Sib contributing the tall allele (Fig. 1.3.3.4.2.3.1). A QTL on chromosome 6A was significant in both years with a peak Lod score of 3.3-4.0 at 73 cM, explaining 17-22% of the variation across the chromosome and with Avalon contributing the tall allele (Fig. 1.3.3.4.2.3.2). A QTL on chromosome 6B was significant in 2002 only with a peak Lod score of 1.9 at 80 cM, explaining 19% of the variation across the chromosome and with Hobbit Sib contributing the increasing effect (Fig. 1.3.3.4.2.3.3).

Table 1.3.3.4.2.1 Marker means analysis of height on the Avalon x Hobbit Sib RIL population over two years. Only differences significant (P<0.05) in both years are presented. Where the additive effect (cm) is positive, Avalon contributes the tall allele; where the additive effect is negative, Hobbit Sib contributes the tall allele.

Year	Chromosome	Marker	Position (cM)	Additive effect (cm)	P value
2001	2D	*Xwmc112*	0	-4.49	0
2002	2D	*Xwmc112*	0	-2.92	0.0038
2001	2D	*Xwmc145*	16	-4.41	3.00E-04
2002	2D	*Xwmc145*	16	-2.66	0.029
2001	3B	*Xgwm533*	14.7	2.55	0.0152
2002	3B	*Xgwm533*	14.7	2.35	0.0182
2001	6A	*Xwmc32*	72.3	4	5.00E-04
2002	6A	*Xwmc32*	72.3	3.97	2.00E-04
2001	6A	*Xpsp3071*	73	3.78	2.00E-04
2002	6A	*Xpsp3071*	73	4.03	0
2001	6A	*Xpsp3029.1*	73.3	4.58	0
2002	6A	*Xpsp3029.1*	73.3	4.34	0
2001	6A	*Xgwm132.1*	73.6	3.92	2.00E-04
2002	6A	*Xgwm132.1*	73.6	3.65	1.00E-04
2001	6A	*Xbarc107*	76.7	4.13	1.00E-04
2002	6A	*Xbarc107*	76.7	3.77	3.00E-04
2001	6A	*Xpsp3152*	82.3	2.77	0.0077
2002	6A	*Xpsp3152*	82.3	2.96	0.0026
2001	6A	*Xwmc256*	82.3	2.52	0.0223
2002	6A	*Xwmc256*	82.3	2.6	0.0144
2001	6A	*Xgwm334*	140.1	2.9	0.0065
2002	6A	*Xgwm334*	140.1	3.65	3.00E-04
2001	7A	*Xwmc9*	114.3	2.64	0.0154
2002	7A	*Xwmc9*	114.3	2.37	0.0221
2001	7A	*Xbarc108*	115.2	2.95	0.0301
2002	7A	*Xbarc108*	115.2	3.1	0.0202
2001	7A	*Xgwm276*	142.6	2.78	0.0105
2002	7A	*Xgwm276*	142.6	2.07	0.0445
2001	7A	*Xbarc29*	153.5	3.08	0.0073
2002	7A	*Xbarc29*	153.5	2.91	0.0072

Table 1.3.3.4.2.2 Marker regression analysis on the Avalon x Hobbit Sib population for height over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (cm)	P value
2001	1D	36.841 +/- 9.148	2.688 +/- 1.222	0.031
2001	2D	5.638 +/- 10.402	-5.285 +/- 1.289	0.01
2002	2D	39.55 +/- 35.31	-3.652 +/- 1.744	0.008
2001	6A	70.783 +/- 9.66	4.688 +/- 1.514	0.012
2002	6A	78.0 +/- 16.194	8.672 +/- 3.082	0.004
2002	6B	77.103 +/- 19.214	-3.385 +/- 1.416	0.029
2002	6D	99.069 +/- 102.271	-7.683 +/- 7.073	0.034
2001	7A	122.176 +/- 23.092	4.417 +/- 2.105	0.006
2002	7A	120.234 +/- 22.918	3.967 +/- 1.912	0.007

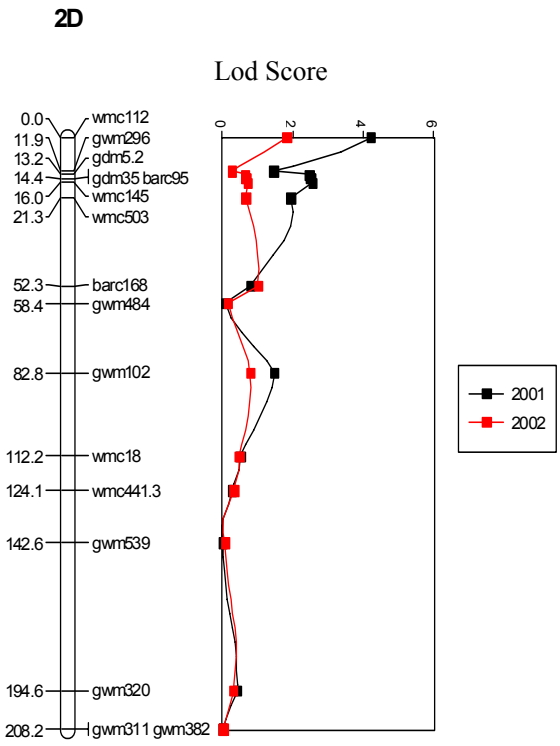


Figure 1.3.3.4.2.3.1 Interval mapping of chromosome 2D for height. One significant QTL detected (2001 only).

<u>2001</u>
Lod Score : 4.22
Significance*: 0.03
Peak of QTL: 0.0 cM
Additive effect: -4.6078 cm
% variation explained: 22.3

<u>2002</u>
Lod Score : 1.84
Significance*: 0.09
Peak of QTL: 0.0 cM
Additive effect: -2.87 cm
% variation explained: 10.8

* permutation test, 500 permutations.

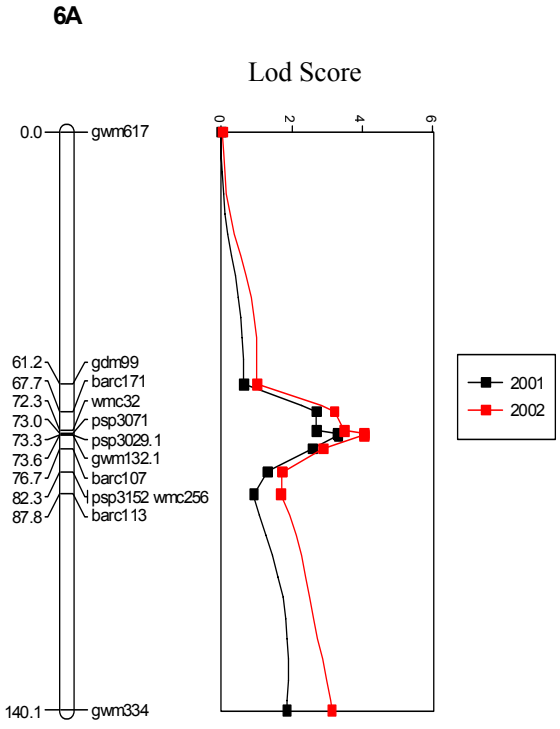


Figure 1.3.3.4.2.3.2. Interval mapping of chromosome 6A for height. One significant QTL detected.

<u>2001</u>
Lod Score : 3.29
Significance*: 0.002
Peak of QTL: 73.6 cM
Additive effect: +4.0316 cm
% variation explained: 17.6

<u>2002</u>
Lod Score : 4.04
Significance*: <0.001
Peak of QTL: 73.3 cM
Additive effect: +4.0256 cm
% variation explained: 22.1

* permutation test, 500 permutations.

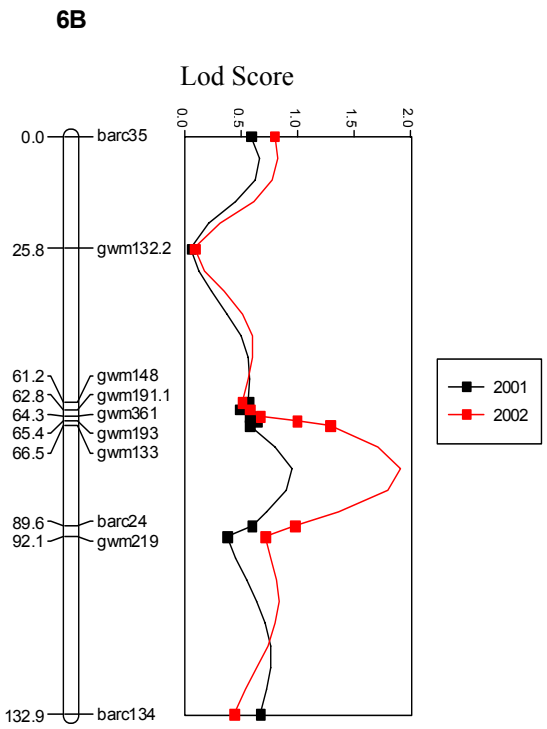


Figure 1.3.3.4.2.3.3. Interval mapping of chromosome 6B for height. One significant QTL detected (2002 only)

2001
Lod Score : 0.91
Significance*: 0.22
Peak of QTL: 81.5 cM
Additive effect: -3.03541 cm
% variation explained: 9.4

2002
Lod Score : 1.91
Significance*: 0.01
Peak of QTL: 76.5 cM
Additive effect: -3.853 cm
% variation explained: 19.1

* permutation test, 500 permutations.

1.3.3.4.3. Yield

1.3.3.4.3.1. Marker Means Analysis of Yield

Over the entire dataset, analysing each year's data separately, 36 markers on 14 chromosomes were significantly ($P<0.05$) associated with differences in yield. Of these, only one marker on chromosome 1B showed differences consistent over both years of the study. Marker *Xpsr413*, at 43.8 cM, had an additive effect of +16.9 g in 2001 (Avalon increasing allele; $P = 0.0047$) and an additive effect of +203.1 g in 2002 (Avalon increasing allele; $P = 0.0335$)

1.3.3.4.3.2. QTL analysis by Multiple Marker Regression of Yield

Six QTL were significant by multiple marker regression over the two years' data, located on six chromosomes (Table 1.3.3.4.3.2). None were consistently significant in both years of the study.

1.3.3.4.3.3 QTL analysis by Interval Mapping of Yield

No significant QTL for yield were detected by interval mapping.

1.3.3.4.4. Biomass

1.3.3.4.4.1. Marker Means Analysis of Biomass

Over the entire dataset, analysing each year's data separately, 38 markers on 14 chromosomes were significantly ($P<0.05$) associated with differences in plant

Biomass. Of these, three markers showed differences consistent over both years of the study (Table 1.3.3.4.4.1). Single markers on each of chromosomes 1B, 3B and 6A showed significant association with biomass, with Avalon contributing the increasing allele in all cases.

1.3.3.4.4.2. QTL analysis by Multiple Marker Regression of Biomass

Eight QTL were significant by multiple marker regression over the two years' data, located on seven chromosomes (Table 1.3.3.4.4.2). QTL on chromosome 2A were significant in both years, but at different locations (4.7 cM in 2001 and 33.4 cM in 2002, Avalon contributing increasing effect). Single year effects were found on chromosomes 1D, 3B, 5BL and 7A (Avalon contributing increasing effect) and 2B and 3A (Hobbit Sib contributing increasing effect).

1.3.3.4.4.3. QTL analysis by Interval Mapping of Biomass

No significant QTL for plant biomass were detected by interval mapping.

Table 1.3.3.4.3.2 Marker regression analysis of yield in the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (g)	P value
2001	1A	81.964 +/- 16.157	-19.729 +/- 8.229	0
2002	2B	13.475 +/- 23.868	-316.997 +/- 140.242	0.002
2002	2D	68.903 +/- 29.979	-406.227 +/- 181.471	0.02
2002	6B	107.479 +/- 26.889	-385.402 +/- 201.194	0.046
2001	6D	237.75 +/- 62.231	-34.595 +/- 21.347	0.023
2002	7A	112.675 +/- 20.781	363.128 +/- 183.762	0.004

Table 1.3.3.4.4.1. Marker means analysis of biomass on the Avalon x Hobbit Sib RIL population over two years. Only differences significant (P<0.05) in both years are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Chromosome	Marker	Position (cM)	Additive effect (g)	P value
2001	1B	*Xgwm582*	59.4	1.15	0.0328
2002	1B	*Xgwm582*	59.4	1.06	0.0304
2001	3B	*Xwmc540*	125	1.82	0.0231
2002	3B	*Xwmc540*	125	1.41	0.0257
2001	6A	*Xgwm334*	140	1.42	0.0114
2002	6A	*Xgwm334*	140	1.41	0.0034

Table 1.3.3.4.4.2 Marker regression analysis of biomass in the Avalon x Hobbit Sib population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (g)	P value
2001	1D	37.794 +/- 8.169	1.607 +/- 0.624	0.012
2001	2A	4.755 +/- 4.603	2.706 +/- 0.625	0.006
2002	2A	33.361 +/- 9.217	3.139 +/- 0.869	0.014
2002	2B	30.801 +/- 25.719	-1.343 +/- 0.652	0.015
2002	3A	36.883 +/- 19.949	-1.746 +/- 0.913	0.024
2001	3B	60.603 +/- 28.494	2.487 +/- 1.421	0.021
2001	5BL	49.504 +/- 9.902	1.508 +/- 0.607	0.013
2002	7A	106.999 +/- 24.336	1.592 +/- 0.951	0.028

1.3.3.4.5. Ear Weight

1.3.3.4.5.1. Marker Means Analysis of Ear Weight

Over the entire dataset, analysing each year's data separately, 34 markers on 13 chromosomes were significantly ($P<0.05$) associated with differences in ear weight. Of these, none showed effects consistent over both years of the study.

1.3.3.4.5.2. QTL analysis by Multiple Marker Regression of Ear Weight

Eight QTL were significant by multiple marker regression over the two years' data, located on eight chromosomes (Table 1.3.3.4.5.2). None of these QTL were consistent over both years of the study. Single year effects were found on chromosomes 1D, 2A, 2B, 3B and 5BL (Avalon contributing increasing effect) and 2D, 4D and 6B (Hobbit Sib contributing increasing effect).

1.3.3.4.5.3 QTL analysis by Interval Mapping of Ear Weight

No significant QTL for ear weight were detected by interval mapping.

1.3.3.4.6 50/100 grain weight

1.3.3.4.6.1 Marker Means Analysis of Grain Weight

Over the entire dataset, analysing each year's data separately, 29 markers on 9 chromosomes were significantly ($P<0.05$) associated with differences in 50/100 grain weight. Of these, four showed effects consistent over both years of the study (Table 1.3.3.4.6.1). A single marker on chromosome 1D was consistently significant with Avalon as the increasing allele. Two markers on chromosome 3A and a single marker on chromosome 6B were consistently significant with Hobbit Sib as the increasing allele.

1.3.3.4.6.2. QTL analysis of Grain weight by Multiple Marker Regression

Three QTL were significant by multiple marker regression over the two years' data, located on four chromosomes (Table 1.3.3.4.6.2). None of these QTL were consistent over both years of the study. Single year effects were found on chromosomes 2A and 6A (Avalon contributing increasing effect) and 3A (Hobbit Sib contributing increasing effect).

1.3.3.4.6.3 QTL analysis of Grain Weight by Interval Mapping

No significant QTL for 50/100 grain weight were detected by interval mapping.

Table 1.3.3.4.5.2 Marker regression analysis of ear weight in the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect	P value
2002	1D	37.001 +/- 0.9015	0.942 +/- 0.384	0.019
2001	2A	4.731 +/- 4.933	1.713 +/- 0.398	0.009
2002	2B	39.081 +/- 25.99	3.804 +/- 2.02	0
2001	2D	96.951 +/- 31.202	-1.532 +/- 0.748	0.034
2001	3B	107.582 +/- 15.618	1.14 +/- 0.408	0
2002	4D	23.405 +/- 10.027	-3.389 +/- 1.577	0.045
2001	5BL	51.101 +/- 9.004	0.966 +/- 0.376	0.017
2001	6B	106.637 +/- 22.831	-1.486 +/- 0.695	0.036

Table 1.3.3.4.6.1. Marker means analysis of 50 grain weight (2001) and 100 grain weight (2002) on the Avalon x Hobbit Sib RIL population over two years. Only differences significant (P<0.05) in both years are presented. Where the additive effect is positive, Avalon contributes the increasing allele; where the additive effect is negative, Hobbit Sib contributes the increasing allele.

Year	Chromosome	Marker	Position (cM)	Additive effect (g)	P value
2001	1D	*Xpsr79*	41.0	0.06	0.0257
2002	1D	*Xpsr79*	41.0	0.15	0.0019
2001	3A	*Xwmc179.2*	0	-0.05	0.0309
2002	3A	*Xwmc179.2*	0	-0.11	0.0191
2001	3A	*Xbarc12*	5.5	-0.05	0.0417
2002	3A	*Xbarc12*	5.5	-0.11	0.0392
2001	6B	*Xbarc134*	132.9	-0.05	0.0422
2002	6B	*Xbarc134*	132.9	-0.11	0.0324

Table 1.3.3.4.6.2 Marker regression analysis of 50/100 grain weight in the Avalon x Hobbit Sib RIL population over two years. Only significant (P<0.05) regressions are presented.

Year	Chromosome	Position (cM)	Additive Effect (g)	P Value
2002	1D	38.288 +/- 7.465	0.147 +/- 0.058	0.009
2001	2A	5.393 +/- 6.586	0.097 +/- 0.029	0.015
2002	3A	20.464 +/- 18.336	-0.171 +/- 0.096	0.045
2001	6A	66.472 +/- 14.224	0.089 +/- 0.039	0

1.3.3.4.7. Spikelet number

1.3.3.4.7.1. Marker Means Analysis of Spikelet Number

Over the entire dataset, analysing each year's data separately, 29 markers on 11 chromosomes were significantly ($P<0.05$) associated with differences in spikelet number. Of these, only locus *Xgwm374* on chromosome 2B showed effects consistent over both years of the study. In 2001, it was significant at $P=0.0183$ with an additive effect (Hobbit Sib allele) of 1.32; in 2002, it was significant at $P=0.0296$ with an additive effect (Hobbit Sib allele) of 4.77.

1.3.3.4.7.2. QTL analysis by Multiple Marker Regression of Spikelet Number

No significant QTL for spikelet number were detected by multiple marker regression.

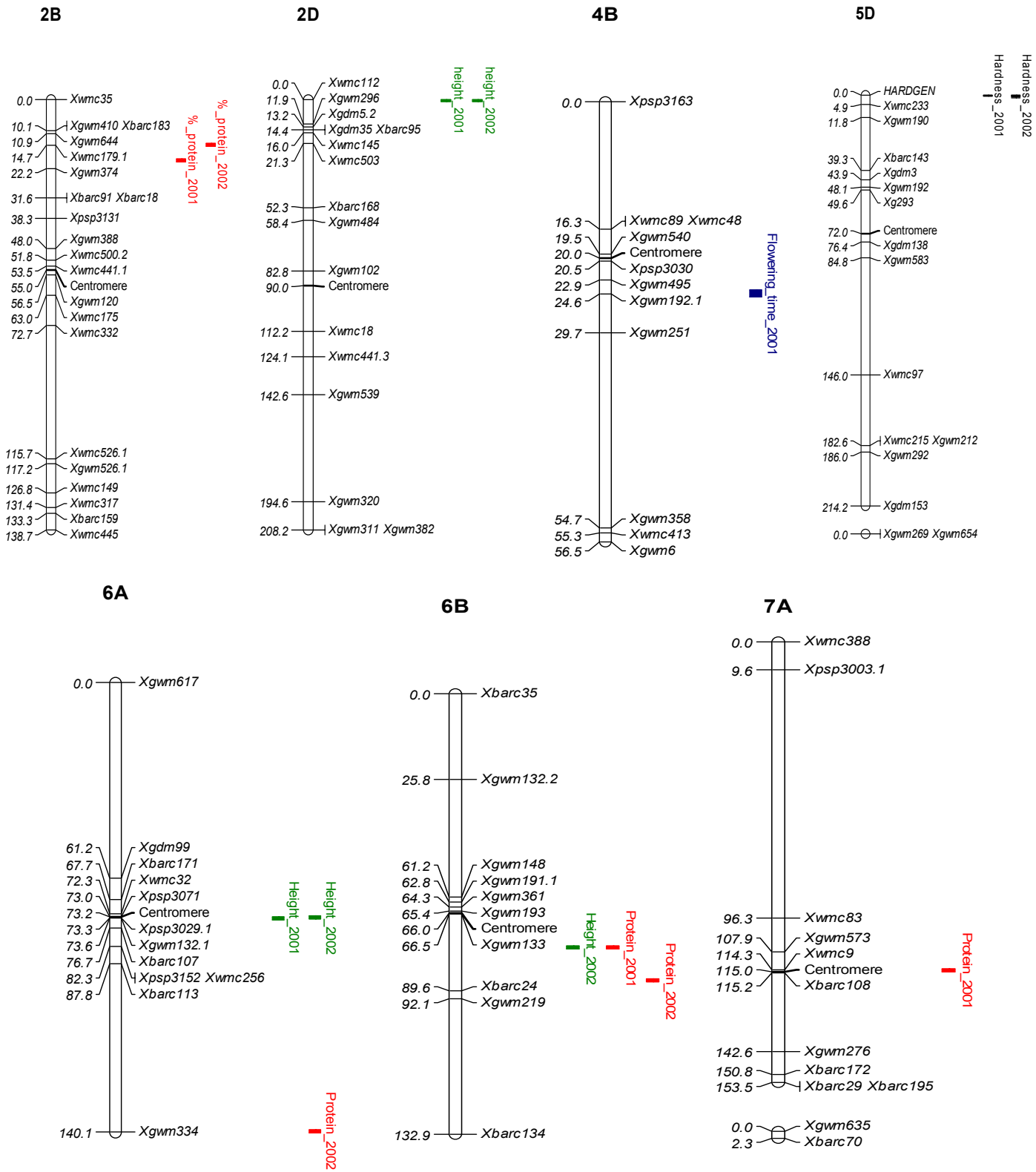
1.3.3.4.7.3. QTL analysis by Interval Mapping of Spikelet Number

No significant QTL for spikelet number were detected by interval mapping.

1.3.3.5. Summary of QTL Significant by Interval Mapping in the Avalon x Hobbit Sib Recombinant Inbred Population

A summary of QTL significant by interval mapping in the Avalon x Hobbit Sib recombinant inbred population is presented in Figure 1.3.3.5.

Figure 1.3.3.5. Summary of QTL significant by interval mapping in the Avalon x Hobbit Sib recombinant inbred population. Positions indicated are the peaks of the QTL.



1.3.4. Analysis of the Hope x Pastiche Single Seed Descent Lines

1.3.4.1 Mapping

A skeletal map of 93 of the 138 lines comprising the population was constructed as follows: A suite of SSR markers was selected on the basis of equal coverage of the genome (2 to 4 per chromosome) and polymorphism between the parental varieties.

These SSR's were then scored in the 93 lines. Altogether, 39 markers were scorable in these lines. The distribution of the markers across chromosomes is summarised in Table 1.3.4.1. The coverage of the genome was not sufficiently dense to permit mapping of the markers. Rather, investigations of allelic association with trait data were conducted using a map-independent non-parametric test, the Kruskal-Wallace rank test.

1.3.4.2 Trait Association analysis by Kruskal-Wallace Rank Test

Trait association analysis by Kruskal-Wallace rank test indicated that alleles at 30 of the 39 markers used to map the population differed significantly ($P<0.05$) for at least one of the nine traits assayed in at least one of the two years of the study. Of these, nine markers on nine chromosomes were consistently associated with the same effects in both years (Table 1.3.4.2). The Hope allele of marker *Xgwm274.1* on chromosome 2B was associated with increased grain protein concentration, whilst the Pastiche allele of *Xbarc178* on chromosome 6B was associated with increased grain texture. Three markers were associated with flowering date: *Xbarc12* on chromosome 3A and *Xbarc196* on chromosome 6D, both with Hope having the late allele; and *Xbarc151* on chromosome 5A with Pastiche having the late allele. Height was associated with marker *Xgwm349* on chromosome 2D, yield with *Xbarc69* on chromosome 5B, and increased ear weight with *Xbarc97* on chromosome 7D, all with Pastiche carrying the increasing allele. A second marker associated with ear weight, *Xbarc15* on chromosome 2A, was also associated with increased 50 grain weight, with Pastiche as the increasing allele in both cases.

Table 1.3.4.1 Skeletal mapping of the Hope x Pastiche population: Distribution of markers across chromosomes

Chromosome	No. of markers scored
1A	2
1B	3
1D	1
2A	2
2B	2
2D	1
3A	2
3B	0
3D	1
4A	2
4B	2
4D	0
5A	3
5B	4
5D	2
6A	2
6B	2
6D	2
7A	2
7B	2
7D	2

Table 1.3.4.2. Markers significantly ($P<0.05$) associated with traits in the Hope x Pastiche population over both years of the study.

Chrom-osome	Marker	Trait	Increasing allele	Significance 2001	2002
1B	*Xgwm274.1*	Grain Protein Content	Hope	<0.01	<0.05
2A	*Xbarc15*	Ear Weight	Pastiche	<0.001	<0.05
2A	*Xbarc15*	50 grain weight	Pastiche	<0.001	<0.01
2D	*Xgwm349*	Height	Pastiche	<0.001	<0.05
3A	*Xbarc12*	Flowering Date	Hope	<0.01	<0.01
5A	*Xbarc151*	Flowering Date	Pastiche	<0.05	<0.01
5B	*Xbarc69*	Yield	Pastiche	<0.01	<0.01
6B	*Xbarc178*	Grain Texture	Pastiche	<0.01	<0.05
6D	*Xbarc196*	Flowering Date	Hope	<0.01	<0.05
7D	*Xbarc97*	Ear Weight	Pastiche	<0.05	<0.05

1.4. Discussion

Genetics of grain texture

Previous studies by a number of workers worldwide has shown the importance of chromosome 5D in the genetic control of grain hardness, and the major effect of a single gene, designated *Ha*, on the short arm of this chromosome. However, this study is the first demonstration that this gene (perhaps not surprisingly) is also the major determinant of grain texture in UK bread-making quality wheats. This gene has obviously been maintained in the UK gene pool since the beginning of the 20th century for its contribution to hard grain texture. Selection for this gene via NIR is straight-forward, and thus although we could generate diagnostic molecular markers, they are unlikely to be necessary for plant breeders seeking to separate hard segregants from soft.

However, these results also indicate that the 5D locus is not the whole story of the genetic control of grain texture in UK wheats. There is clearly other genetic variation present, and some of these genes have been identified here as QTL of minor effect relative to the over-riding effect of the *Ha* locus. It is interesting that two presumptive QTL have been found on chromosome 5A, one in position on the short arm likely homoeologous to the *Ha* locus, and the other in a position on the long arm in the region of the vernalization gene, *Vrn-A1*. These effects are minor, and, in these experiments, not statistically significant, but can be seen visually in ground samples. The latter locus may be homoeologous to one found on 5D in a study of the variety Hope.

In addition to the group 5 chromosomes, using the RIL lines, QTL consistent over years were found on chromosomes 1B, 4B, 4D, 5BS/7BS. Avalon had the allele for increasing hardness on 1B and 4D whilst Hobbit Sib consistently contributed the effect for increasing hardness on chromosomes 4B and 5BS/7BS. In simple terms, this means that hard grain texture varieties such as Avalon carry minor alleles for soft texture and soft-varieties like Hobbit carry minor alleles for hard texture. This was also confirmed in the Hope x Pastiche cross where although both varieties were hard, significant genetical variation for grain texture was found. This is important, since it means that by manipulation of these minor effects it should be possible to breed different grain textures into new varieties leading to a series of varieties that could be described as soft-softs, softs, hard-softs, sort-hards, hards, hard-hards etc. There is a capacity to breed for a spectrum of textures rather than simply hard or soft. This opens up interesting possibilities for fine-tuning the texture of varieties for different end uses as desired using the available genetic variation in UK or exotic wheats.

Genetics of Protein content

This study is the first to provide a complete analysis of grain protein content in UK wheat varieties, and it has shown that it is possible to dissect the genetics of a difficult-to-measure and highly-environmentally sensitive character such as grain protein content using modern methods of genetic analysis. It has shown that the genetic control is complex and there are no underlying major genes as in the case of grain texture. However, it again highlights the effects of the group 5 chromosomes, shown in studies of wheat from other countries. Of particular note is the fairly large effect of a QTL on chromosome 5D that completely co-segregates with the *Ha* locus on the short arm. It is open to speculation and further study as to whether this is a pleiotropic effect of the *Ha* locus or an effect of a closely linked independent locus. Linkage seems most likely, as it is difficult to easily think of a mechanistic link of how variation in the grain texture locus could affect protein amounts. Nevertheless, in plant breeding terms this means that selection for hard grain texture by NIR will carry the allele for higher grain protein content along with it – a fortuitous but useful association. Similarly, selection for soft texture, will result in lower protein levels. Conversely, if soft high protein or hard low protein varieties are needed for specific end-use products, these results will suggest that this will require selection for alternative genes than that on 5D. Luckily, the analysis of the RILs indicates that additional genetic variation is present and QTL on several chromosomes make a contribution.

Significant QTL were identified on chromosomes 2B and 6B (Avalon contributing increasing effect) and chromosomes 6A and 7A (Hobbit Sib contributing increasing effect) in data from both years. These effects were also relatively large, and, interestingly were dispersed between the parents. So, analogous to the situation for grain texture, although Avalon is generally regarded as a high protein wheat, it carries alleles at certain loci for reduced grain protein relative to their homologues from Hobbit sib, and vice versa. Thus, in this cross, genes for higher levels of grain protein are dispersed between the parents and transgressive segregation for higher protein containing lines than Avalon is possible from this cross. Diagnostic markers for particular alleles could be sought/designed to enable this to be a tool by plant breeders for protein content selection.

In these studies detailed examination of the relationship between grain protein content and yield was only carried out for the recombinant substitution lines. This showed that there was no yield penalty associated with carrying the Avalon 5D gene for higher levels. This gene is thus very useful for maintaining high levels as it hitch-hikes in breeding programmes with the hardness locus. It may be that some of the other alleles discovered for higher grain protein could be associated with a yield penalty. Further studies of the presumptive loci discovered are needed before their deployment can be recommended to breeders.

Genetics of Agronomic traits

Although the prime purpose of this study was to discover new genes for grain protein content and grain texture, the opportunity was also taken to evaluate the genetics of traits of agronomic importance. Several new QTL were discovered in the Avalon x Hobbit Sib cross and, overall, this indicates that there is quite a lot of genetic variation for traits even in the relatively narrow genetic pool of UK winter wheats.

Several QTL mediating variation for plant height were discovered. These can be regarded as minor effects relative to the large effect of the major dwarfing gene *Rht-D1* that both parents are known to carry. However, the QTL on chromosomes 2D, 6A in particular may be useful to plant breeders as they each mediated differences between 10-15 cm in height, which were consistent over years. As with the grain quality traits, 'useful' alleles were dispersed between the parents, with Avalon having the shortness allele at the 2D locus and Hobbit Sib the shortness allele at the 6A locus.

Significant QTL were also found for flowering time, but only mediated differences by 1-2 days. However, such small difference may be useful for 'fine-tuning' flowering to environment, although, probably of low agronomic significance in the UK.

Both height and flowering time are traits with high heritabilities, and thus QTL are relatively easy to detect consistently over experiments. Yield and yield components have much lower heritabilities and are more environmentally sensitive. Hence it would be expected in these studies that such QTL would be more difficult to detect consistently over years and with high statistical significance, and this appeared to be the case. Although QTL for biomass, spikelet number, grain size and grain number were detected by single marker ANOVA, few of the effects could be confirmed by marker regression and interval mapping that were consistent over years. Nevertheless, these results do highlight putative QTL for yield components worthy of future study.

Section 2. Seed Morphology Studies
(Based on the HGCA student bursary awarded to Elizabeth O'Connor)

2.1 Introduction

The ITMI mapping population.

Due to the difficulty of finding enough polymorphic genetic markers to produce a saturated genetic map of wheat from intervarietal crosses, an international collaborative project (the International Triticeae Mapping Initiative) was set up in the early 1990s. A hexaploid wheat "Synthetic M6" was re-synthesised by crossing a durum wheat and an *Aegilops tauschii* accession (a "D" genome donor). This 'Synthetic' was then crossed with a CYMMIT variety, Opata and a recombinant inbred line population developed by single seed descent. A comprehensive genetic map of this population has been built up using Restriction Fragment Length Polymorphism (RFLP) markers and Simple Sequence Repeat (SSR) markers. The mapping data on these lines is publicly available on the GrainGenes website (http://wheat.pw.usda.gov/) and they have been and are being grown at several locations around the world for phenotypic analysis. They are known to differ significantly in grain size and shape and therefore present a good starting point for genetic analysis of these traits. Hopefully, new loci found in this population will have relevance to UK wheats.

Objectives of study

The objectives of this study were to examine the characteristics of seed morphology in the ITMI population and attempt to relate these characteristics to QTL in specific areas of the genome by association with molecular markers.

2.2 Materials & Methods

Field Trials.

These were conducted at Morley Research Centre, Norfolk. Dibbed trails were sown in both years. Dibbed trails consisted of five replicates of ten plants hand sown in single lines in beds of sixty to seventy lines. Both parents and controls were included in the trial design.

ITMI mapping data.

Mapping data were obtained from the GrainGenes website. Data were converted to JoinMap format and markers were chosen to give coverage of the genome with, where possible, gaps

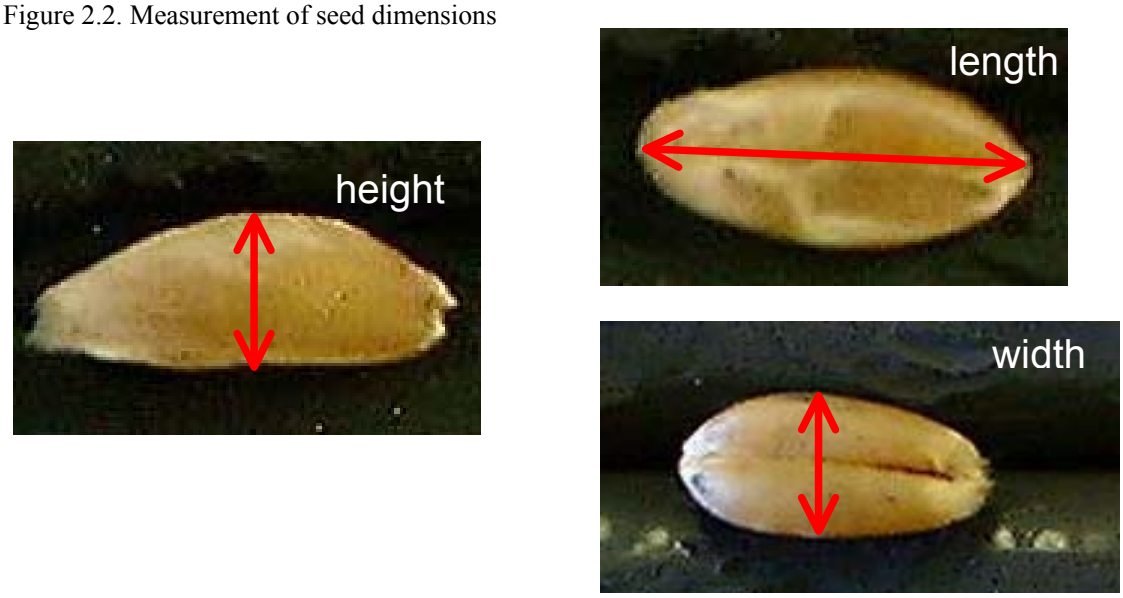
between the markers of around 10 cM, as this is optimum for QTL mapping. Markers significantly associated with the traits of interest were determined by marker means analysis using QTL Café. QTL analysis by multiple marker regression (1000 simulations) was also performed using QTL Café. Interval Mapping was conducted using the program MapQTL (Van Ooijen & Maliepaard, 1996) and permutation tests (1000 permutations) conducted to determine significance of the QTL.

Phenotypic assessment

Seed weight was measured as 50 grain weight (50GW) on cleaned seed samples. Hectolitre weight was measured using a hectolitre weight measuring cylinder (using the standard protocol as suggested by the manufacturers) on the 2000 samples when there was enough grain from a single plot. Samples were cleaned of any excess chaff and weed seeds and left to equilibrate to even moisture (approximately 12%) in a drying barn before measurement of hectolitre weights.

Seed shape was determined using digital imaging. Two different photographs of the seeds were taken. Seeds were laid on their crease on a light box and photographed from a set distance from above. They were then lined up and photographed from the side from a set distance using a digital camera (Figure 2.2). These images were then analysed using Scion Image software. Measurements of seed length, seed width (from the overhead image) and seed "height" (from the side image) were taken from these images. From these, the ratio of the variables were calculated to give an indication of the shape of the seed. The equation for the volume of an elipsoid (4/3*(pi*l/2*w/2*h/2)) was used to estimate seed volume.

Figure 2.2. Measurement of seed dimensions



2.3 Results

2.3.1 Seed Dimensions

2.3.1.1 Marker Means Analysis of Seed Dimensions

Of the 43 markers on 19 chromosomes significantly associated with either seed height, width or length, 14 markers on nine chromosomes were consistently associated with the same effects in both years (Table 2.3.1.1). A single marker at each of chromosomes 1A and 3B was associated with seed length with Opata contributing the increasing allele, whilst three markers on chromosome 5B, two on 6A and one on 6D were associated with seed length with Synthetic M6 contributing the increasing allele. Two markers, both on chromosome 2B were associated with seed width (Synthetic M6 contributing the increasing allele). Two markers on chromosome 2A were both linked with seed height, with Opata contributing the increasing allele, and a single marker on each of 3A and 4B were associated with height with Synthetic M6 contributing the increasing allele.

Table 2.3.1.1. Marker Means analysis of seed dimensions (cm) on the ITMI population over two years. Only markers significant (P<0.05) in both years are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Trait	Year	Chrom-osome	Marker	Position (cM)	Additive effect	F	P
Length	1999	1A	*Xmwg912-1A*	155.6	-0.01168	6.475	0.0126
Length	2000	1A	*Xmwg912-1A*	155.6	-0.01033	6.231	0.0144
Length	1999	3B	*Xmwg69-3B*	146.3	-0.01055	5.591	0.0199
Length	2000	3B	*Xmwg69-3B*	146.3	-0.00895	5.476	0.0213
Length	1999	5B	*Xfba367-5B*	12.1	0.015235	6.327	0.015
Length	2000	5B	*Xfba367-5B*	12.1	0.013743	6.036	0.0174
Length	1999	5B	*Xfba393-5B*	17.9	0.020278	12.591	8.00E-04
Length	2000	5B	*Xfba393-5B*	17.9	0.017723	11.152	0.0016
Length	1999	5B	*Xcdo412-5B*	36.8	0.009416	4.669	0.033
Length	2000	5B	*Xcdo412-5B*	36.8	0.007541	4.289	0.0408
Length	1999	6A	*Xbcd758-6A*	79	0.010617	5.767	0.0181
Length	2000	6A	*Xbcd758-6A*	79	0.008485	5.157	0.0253
Length	1999	6A	*Xcdo388-6A*	98	0.010327	5.891	0.0169
Length	2000	6A	*Xcdo388-6A*	98	0.008736	5.573	0.0201
Length	1999	6D	*Xpsr889-6D*	0	0.012413	6.804	0.0106
Length	2000	6D	*Xpsr889-6D*	0	0.00869	4.28	0.0414
Width	1999	2B	*Xfba272-2B*	20	0.00374	5.658	0.021
Width	2000	2B	*Xfba272-2B*	20	0.004452	4.679	0.0352
Width	1999	2B	*Xbcd18-2B*	30.7	0.004174	12.498	6.00E-04
Width	2000	2B	*Xbcd18-2B*	30.7	0.003067	4.908	0.0289
Height	1999	2A	*Xbcd152-2A*	112.8	-0.00352	7.895	0.0059
Height	2000	2A	*Xbcd152-2A*	112.8	-0.00331	4.944	0.0283
Height	1999	2A	*Xbcd161-2A*	119.1	-0.00324	6.846	0.0102
Height	2000	2A	*Xbcd161-2A*	119.1	-0.00321	5.081	0.0263
Height	1999	3A	*Xabc172.2-3A*	195.1	0.002931	5.15	0.0253
Height	2000	3A	*Xabc172.2-3A*	195.1	0.002996	4	0.0481
Height	1999	4B	*Xbcd1262-4B*	19.8	0.003008	8.905	0.0035
Height	2000	4B	*Xbcd1262-4B*	19.8	0.000866	4.023	0.0475

Table 2.3.1.2. QTL analysis by multiple marker regression of seed dimensions (cm) on the ITMI population over two years. Only significant (P<0.05) effects are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Trait	Year	Chromosome	Position	Additive Effect	P Value
Length	1999	2D	61.958 +/- 31.743	0.015 +/- 0.0070	0.034
Length	1999	5B	20.643 +/- 22.994	0.014 +/- 0.0060	0.031
Length	2000	5B	19.267 +/- 13.211	0.014 +/- 0.0050	0.016

2.3.1.2. QTL analysis by Multiple Marker Regression of Seed Dimensions

Two QTL for seed dimensions were located by multiple marker regression (Table 2.3.1.2). A QTL for seed length was present in both years on chromosome 5B at 20 cM with Synthetic M6 contributing the increasing effect. A second seed length QTL on chromosome 2D (62cM, Synthetic M6 contributing the increasing effect) was present in 1999 only.

2.3.1.3. QTL analysis by Interval Mapping of Seed Dimensions

Seven QTL were identified by interval mapping in the two years' data, located on six chromosomes (Table 2.3.1.3). None were consistent between years. Two closely positioned QTL on chromosome 1A, for seed length at 71cM and seed height at 57 cM, were detected in 1999 only (Synthetic M6 contributing the increasing effect). QTL for seed width were detected on chromosome 2B in 1999 at 25 cM with Synthetic M6 contributing the increasing effect, and on chromosomes 2D at 45 cM and 4A at 0 cM in 2000 with Opata contributing the increasing effect. A further QTL for seed height was located on chromosome 4B at 20 cM in 2000 with Synthetic M6 contributing the increasing effect; and a further QTL for seed length was located on chromosome 5B at 138 cM in 2000 with Synthetic M6 contributing the increasing effect. The latter was not coincident with the QTL detected for seed length on chromosome 5B detected by multiple marker regression (section 2.3.1.2, above) which were based around 20 cM.

2.3.2. Ratios Between Seed Dimensions

It is possible that the ratios between the seed dimensions measured could provide a more consistent marker for seed morphology than the absolute dimensions themselves. Therefore, these ratios were calculated and QTL analysis performed.

2.3.2.1 Marker Means Analysis of Seed Dimension Ratios

Of the 100 markers on 18 chromosomes significantly ($P<0.05$) associated with any of the ratios length:height, length:width or width:height in either year, 16 markers on six chromosomes were consistently associated with the same effects in both years (Table 2.3.2.1). The ratio length:width had by far the greatest number of consistent associations with markers. Markers on chromosomes 2D (4), 4A (2), 5A (1) and 5B (8) were associated with Synthetic M6 contributing the increasing allele. A single marker on chromosome 3B was associated with length:width with Opata as the increasing allele. The ratio length:height was associated

Table 2.3.1.3. QTL analysis by interval mapping of seed dimensions (cm) on the ITMI population over two years. Only significant (P<0.05) effects are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Year	Chromosome	Trait	Location of peak (cM)	Lod score	P value	% of variation explained	Additive Effect
1999	1A	Length	71.5	1.95	0.045	7.8	0.012736
1999	1A	Height	57.2	2.62	0.011	10.1	0.004257
1999	2B	Width	25	2.95	0.008	15	0.004894
2000	2D	Width	45.5	2.4	0.012	11.8	-0.00491
2000	4A	Width	0	3.23	0.003	16.7	-0.0058
1999	4B	Height	19.8	1.88	0.029	7.5	0.003645
2000	5B	Length	138.4	2.81	0.006	12.1	0.013837

Table 2.3.2.1. Marker Means analysis of seed dimension ratios on the ITMI population over two years. Only markers significant (P<0.05) in both years are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Trait	Year	Chromosome	Marker	Position (cM)	Additive effect	F	P
Length:Height	1999	2B	*Xbcd152-2B*	64	-0.03	4.026	0.0473
Length:Height	2000	2B	*Xbcd152-2B*	64	-0.04	7.636	0.0068
Length:Width	1999	2D	*W2I-2D*	0	0.03	5.406	0.0219
Length:Width	2000	2D	*W2I-2D*	0	0.02	4.009	0.0478
Length:Width	1999	2D	*Xbcd102-2D*	40.5	0.03	4.183	0.0437
Length:Width	2000	2D	*Xbcd102-2D*	40.5	0.03	7.006	0.0096
Length:Width	1999	2D	*Xfba400-2D*	48.9	0.06	10.295	0.0023
Length:Width	2000	2D	*Xfba400-2D*	48.9	0.05	9.099	0.004
Length:Width	1999	2D	*Xcdo1379-2D*	65	0.03	6.282	0.0137
Length:Width	2000	2D	*Xcdo1379-2D*	65	0.03	7.465	0.0074
Length:Width	1999	3B	*Xmwg69-3B*	146.3	-0.03	4.335	0.0398
Length:Width	2000	3B	*Xmwg69-3B*	146.3	-0.02	4.157	0.0441
Height:Width	1999	4A	*Xfba40-4A*	8.6	0.01	5.568	0.0221
Height:Width	2000	4A	*Xfba40-4A*	8.6	0.02	8.325	0.0057
Length:Width	1999	4A	*Xbcd8-4A*	30.1	0.03	5.087	0.0262
Length:Width	2000	4A	*Xbcd8-4A*	30.1	0.03	5.972	0.0163
Length:Width	1999	5A	*Xfbb2-5A*	51.5	0.04	4.982	0.0299
Length:Width	2000	5A	*Xfbb2-5A*	51.5	0.04	5.74	0.0202
Length:Height	1999	5A	*Xfbb2-5A*	51.5	0.05	5.157	0.0272
Length:Height	2000	5A	*Xfbb2-5A*	51.5	0.07	11.72	0.0012
Length:Width	1999	5B	*Xfba367-5B*	12.1	0.04	4.225	0.0448
Length:Width	2000	5B	*Xfba367-5B*	12.1	0.04	4.339	0.0422
Length:Width	1999	5B	*Xfba393-5B*	17.9	0.06	12.206	0.001
Length:Width	2000	5B	*Xfba393-5B*	17.9	0.06	10.723	0.0019
Length:Height	1999	5B	*Xfba393-5B*	17.9	0.06	7.59	0.008
Length:Height	2000	5B	*Xfba393-5B*	17.9	0.05	6.851	0.0116
Length:Width	1999	5B	*Xmwg561-5B*	31.9	0.03	4.107	0.0455
Length:Width	2000	5B	*Xmwg561-5B*	31.9	0.04	10.452	0.0017
Length:Width	1999	5B	*Xcdo412-5B*	36.8	0.03	4.06	0.0464
Length:Width	2000	5B	*Xcdo412-5B*	36.8	0.03	7.794	0.0062
Length:Width	1999	5B	*Xbcd1140-5B*	47.8	0.03	4.455	0.0372
Length:Width	2000	5B	*Xbcd1140-5B*	47.8	0.03	6.843	0.0103
Length:Width	1999	5B	*Xabg473-5B*	55.4	0.03	4.447	0.0376
Length:Width	2000	5B	*Xabg473-5B*	55.4	0.04	9.258	0.003
Height:Width	1999	5B	*Xfba166-5B*	81.8	0.01	5.122	0.0277
Height:Width	2000	5B	*Xfba166-5B*	81.8	0.02	7.105	0.0102
Length:Width	1999	5B	*Xcdo1326-5B*	113.1	0.03	6.142	0.0148
Length:Width	2000	5B	*Xcdo1326-5B*	113.1	0.02	4.124	0.0448

with single markers on chromosomes 2B (Opata increasing allele), 5A and 5B (Synthetic M6 increasing alleles). The ratio height:width was associated with a single marker on chromosome 5A with Synthetic M6 contributing the increasing allele.

2.3.2.2. QTL analysis by Multiple Marker Regression of Seed Dimension Ratios

Over both years of the study, 18 QTL for seed dimensions were located on nine chromosomes by multiple marker regression (Table 2.3.2.2). Three QTL were consistent over both years of the study. A QTL influencing the length:width ratio was identified on chromosome 2D at 60 cM with Synthetic M6 contributing the increasing effect; a second QTL influencing the length:width ratio was identified on chromosome 5B with Synthetic M6 contributing the increasing effect at between 18 and 40 cM (although the location was different in each year so it is possible that these are separate QTL); and a QTL influencing the length:height ratio was identified also on chromosome 5B at 25 cM with Synthetic M6 contributing the increasing effect; This last QTL could also be coincident with the 5B length:width QTL described above. Several QTL significant in one year only were also identified by multiple marker regression. On chromosome 2B in 2000, either a single or two closely linked QTL were identified influencing the ratios length:width and length:height (17 and 25 cM, Opata contributing the increasing effect). In 1999, two QTL influencing height;width and length:height were identified on chromosome 2D (160 and 52 cM, both with Synthetic M6 contributing the increasing effect). Single QTL influencing length:height were located on chromosomes 3B in 1999 (150 cM, Opata increasing) and 3D and 5A in 2000 (77cM, Opata increasing, and 55cM, Synthetic M6 increasing, respectively). On chromosome 4A in 2000, either a single or two closely linked QTL were identified influencing the ratios length:width and height:width (6 and 8 cM, Synthetic M6 contributing the increasing effect). A QTL influencing height:width was identified in 2000 only at 63 cM with Synthetic M6 contributing the increasing effect, and two single QTL influencing length;width were identified in 1999 only on chromosomes 6B and 6D (114 and 16 cM, Synthetic contributing increasing effect).

Table 2.3.2.2. QTL analysis by multiple marker regression of seed dimension ratios on the ITMI population over two years. Only significant (P<0.05) effects are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Trait	Year	Chromosome	Position	Additive Effect	P Value
Length:Width	2000	2B	17.485 +/- 39.683	-0.035 +/- 0.021	0.028
Length:Height	2000	2B	25.75 +/- 47.609	-0.035 +/- 0.024	0.036
Length:Width	1999	2D	61.391 +/- 26.192	0.051 +/- 0.021	0.001
Length:Width	2000	2D	57.752 +/- 24.811	0.045 +/- 0.018	0.004
Height:Width	1999	2D	159.55 +/- 32.51	0.013 +/- 0.0060	0.047
Length:Height	1999	2D	52.274 +/- 18.201	0.058 +/- 0.02	0.001
Length:Height	1999	3B	150.018 +/- 45.339	-0.044 +/- 0.029	0.026
Length:Height	2000	3D	77.11 +/- 13.202	-0.049 +/- 0.015	0.002
Length:Width	2000	4A	6.352 +/- 18.042	0.046 +/- 0.017	0.034
Height:Width	2000	4A	7.66 +/- 14.943	0.015 +/- 0.0050	0.005
Length:Height	2000	5A	54.887 +/- 20.216	0.037 +/- 0.017	0.015
Length:Width	1999	5B	18.398 +/- 15.374	0.047 +/- 0.017	0.001
Length:Width	2000	5B	40.17 +/- 9.052	0.054 +/- 0.015	0.011
Height:Width	2000	5B	63.443 +/- 14.557	0.012 +/- 0.0040	0.028
Length:Height	1999	5B	25.722 +/- 28.966	0.036 +/- 0.023	0.042
Length:Height	2000	5B	23.423 +/- 21.331	0.04 +/- 0.019	0.018
Length:Width	1999	6B	113.634 +/- 26.433	0.043 +/- 0.023	0.042
Length:Width	1999	6D	16.294 +/- 38.523	0.036 +/- 0.021	0.04

2.3.2.3. QTL analysis by Interval Mapping of Seed Dimension Ratios

Seven QTL were identified by interval mapping in the two years' data, located on six chromosomes (Table 2.3.2.3). A QTL influencing the ratio length:width was located on chromosome 2D in both years of the study at 54 cM with Synthetic M6 contributing the increasing effect (Figure 2.3.2.3). This location also coincided with a QTL influencing length:height in 1999 only. A single QTL influencing both length;width and length:height was identified on chromosome 3D in 2000 only (77 cM, Opata contributing increasing effect). Single-year QTL for length:width were identified on chromosomes 3B (24 cM, Synthetic M6 increasing), 5B (32 cM, Synthetic M6 increasing), and 6D (9 cM, Synthetic M6 increasing). Single-year QTL affecting the ratio length:height were identified on chromosomes 5A (58 cM, Synthetic M6 increasing) and 5B (133 cM, Synthetic M6 increasing).

2.3.3. Seed Volume, Hectolitre Weight and 100 Grain Weight

2.3.3.1 Marker Means Analysis of Seed Volume and Weight

Of the 75 markers on 20 chromosomes significantly ($P<0.05$) associated with any of the measurements seed volume, hectolitre weight and 100 grain weight in either year, 9 markers on 5 chromosomes were consistently associated with the same effects on seed volume and 100 grain weight in both years (Table 2.3.3.1.1). The majority of consistent effects were on seed volume. Three markers on chromosome 1A were linked to seed volume, one with Synthetic M6 contributing the increasing allele and two with Opata contributing the increasing allele. Single markers were significantly associated with seed volume on chromosomes 2A and 6D (Opata increasing alleles) and 4B (Synthetic M6 increasing allele). Two markers on chromosome 6A were linked to seed volume, both with Synthetic M6 contributing the increasing allele and one of which was also associated with 100 grain weight, again with Synthetic M6 contributing the increasing allele.

Hectolitre weight was only measured in 2000; the 21 markers significantly associated with hectolitre weight on 7 chromosomes in this year are presented in Table 2.3.3.1.2.

Of these, five markers were located on chromosome 1A (Opata increasing), five on 2D (Synthetic M6 increasing), two on 3A (Opata increasing), two on 3D (one with Opata increasing and one with Synthetic M6 increasing), three on 5B (all Opata increasing), three on 6B (one with Synthetic M6 increasing and two with Opata increasing) and one on 7D (Opata increasing).

Table 2.3.2.3. QTL analysis by interval mapping of seed dimension ratios on the ITMI population over two years. Only significant (P<0.05) effects are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Year	Chromosome	Trait	Location of peak (cM)	Lod score	P value	% variation explained	Additive Effect
1999	2D	Length:Width	53.9	2.24	0.034	17.3	0.055852
2000	2D	Length:Width	53.9	2.61	0.008	16.7	0.049446
1999	2D	Length:Height	53.9	3.11	0.005	19.9	0.066589
2000	3B	Length:Width	23.7	2	0.049	8.2	0.03427
2000	3D	Length:Width	77.5	2.35	0.021	9.5	-0.03687
2000	3D	Length:Height	77.5	2.69	0.006	10.7	-0.0441
2000	5A	Length:Height	58.3	2.27	0.027	9.1	0.040682
2000	5B	Length:Width	31.9	2.92	0.002	11.7	0.041028
2000	5B	Length:Height	133.4	2.27	0.027	19.5	0.060171
1999	6D	Length:Width	9	2.6	0.012	10.3	0.042956

Figure 2.3.2.3. Lod score chart of QTL for length:width ratio on chromosome 2D of the ITMI population in both years of the study.

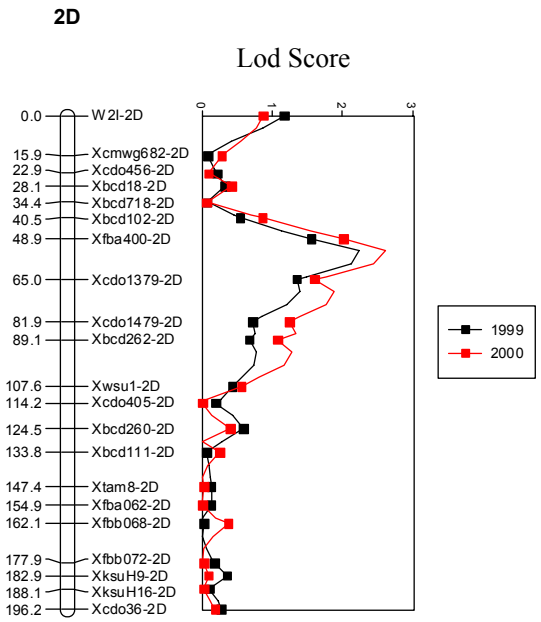


Table 2.3.3.1.1. Marker Means analysis of seed volume (cm^3) and 100 grain weight (g) on the ITMI population over two years. Only markers significant (P<0.05) in both years are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Trait	Year	Chromo-some	Marker	Position (cM)	Additive effect	F	P value
Volume	1999	1A	*Xcdo473-1A*	62.4	1.29	9.345	0.0028
Volume	2000	1A	*Xcdo473-1A*	62.4	1.03	5.077	0.0263
Volume	1999	1A	*Xmwg632-1A*	144.8	-1.03	5.279	0.0237
Volume	2000	1A	*Xmwg632-1A*	144.8	-1.17	6.177	0.0146
Volume	1999	1A	*Xmwg912-1A*	155.6	-1.52	11.681	9.00E-04
Volume	2000	1A	*Xmwg912-1A*	155.6	-1.22	5.591	0.0202
Volume	1999	2A	*Xbcd152-2A*	112.8	-0.89	3.989	0.0484
Volume	2000	2A	*Xbcd152-2A*	112.8	-1.09	5.384	0.0223
Volume	1999	4B	*Xbcd1265-4B*	26.3	1	5.198	0.0247
Volume	2000	4B	*Xbcd1265-4B*	26.3	0.95	4.411	0.0382
Volume	1999	6A	*Xbcd758-6A*	79	1.17	7.219	0.0084
Volume	2000	6A	*Xbcd758-6A*	79	1.05	5.312	0.0232
Grain Weight	1999	6A	*Xbcd758-6A*	79	0.13	6.261	0.0139
Grain Weight	2000	6A	*Xbcd758-6A*	79	0.08	5.015	0.0281
Volume	1999	6A	*Xtam36-6A*	91	1.26	7.323	0.0081
Volume	2000	6A	*Xtam36-6A*	91	1.2	6.429	0.0129
Volume	1999	6D	*XksuE14-6D*	142	-1.17	7.026	0.0093
Volume	2000	6D	*XksuE14-6D*	142	-0.93	4.086	0.0459

Table 2.3.3.1.2. Marker Means analysis of hectolitre weight (g) on the ITMI population in 2000 only. Only significant (P<0.05) markers are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Year	Chromo-some	Marker	Position (cM)	Additive effect	F	P value
2000	1A	*XGli1-1A*	5.1	-5.26	4.744	0.0327
2000	1A	*Xcdo473-1A*	62.4	-5.06	4.555	0.0362
2000	1A	*Glu-1-1A*	80.7	-6.73	7.325	0.0086
2000	1A	*Xcmwg733-1A*	93.8	-5.01	4.177	0.0447
2000	1A	*Xbcd265-1A*	96	-5.27	4.698	0.0337
2000	2D	*Xcmwg682-2D*	15.9	5.71	5.307	0.0245
2000	2D	*Xcdo456-2D*	22.9	6.79	7.86	0.0065
2000	2D	*Xbcd18-2D*	28.1	6.92	9.147	0.0034
2000	2D	*Xbcd718-2D*	34.4	7.58	9.986	0.0023
2000	2D	*Xbcd102-2D*	40.5	5.79	4.966	0.0294
2000	3A	*Xglk683-3A*	0	-5.93	5.986	0.017
2000	3A	*Xtam61-3A*	10.1	-5.25	4.652	0.0344
2000	3D	*Xfbb370-3D*	11.6	7.54	5.993	0.0192
2000	3D	*Xfbb237-3D*	102.2	-8.38	8.064	0.0075
2000	5B	*Xfba393-5B*	17.9	-6.55	4.599	0.0386
2000	5B	*Xmwg914-5B*	67.3	-7.96	6.494	0.0144
2000	5B	*Xfba166-5B*	81.8	-7.32	5.967	0.0195
2000	6B	*Xbcd342-6B*	0	4.83	4.49	0.0376
2000	6B	*Xfba251-6B*	114.5	-6.37	4.273	0.0458
2000	6B	*Xfbb59.2-6B*	131.5	-7.92	7.138	0.0112
2000	7D	*Xmwg710-7D*	24.5	-5.2	4.099	0.047

2.3.3.2. QTL analysis by Multiple Marker Regression of Seed Volume and Weight

Over both years of the study, 11 QTL for seed volume, 100 grain weight and hectolitre weight were located on nine chromosomes by multiple marker regression (Table 2.3.3.2). No QTL were consistent over both years of the study. The QTL on chromosome 1A linked to both 100 grain weight and seed volume in 1999 (65 and 62 cM, both Synthetic M6 increasing) co-locates with a QTL for hectolitre weight in 2000 (75cM, Opata increasing). Other QTL influencing hectolitre weight were located on chromosomes 2D (35 cM, Synthetic M6 increasing), 5B and 6B (67 and 113 cM, both Opata increasing). Seed volume was associated in 1999 with a QTL on chromosomes 4B (22 cM, Synthetic M6 increasing) and in 2000 with QTL on 6A and 7A (73 and 196 cM, both Synthetic M6 increasing). A QTL influencing 100 grain weight in 1999 was located on chromosome 4A (32 cM, Synthetic M6 increasing) and in 2000 QTL influencing 100 grain weight were located on chromosomes 3B (124 cM, Opata increasing) and 6A (35 cM, Synthetic M6 increasing).

2.3.3.3. QTL analysis by Interval Mapping of Seed Volume and Weight

Over both years of the study, six QTL for seed volume, 100 grain weight and hectolitre weight were located on four chromosomes by interval mapping (Table 2.3.3.3). No QTL were consistent over both years of the study. A single QTL was linked to both 100 grain weight and seed volume on chromosome 1A in 1999 only (67 cM, Synthetic M6 contributing increasing effect). Hectolitre weight was associated with a QTL on chromosome 2D in 2000 at 28 cM with Synthetic M6 contributing the increasing effect. Two closely located QTL on chromosome 7A in 2000 influenced 100 grain weight and seed volume (213 and 233 cM respectively, Synthetic M6 contributing the increasing effect). Similarly, either a single or two closely located QTL on chromosome 7D in 1999 influenced 100 grain weight and seed volume (141 and 139 cM respectively, Opata contributing the increasing effect).

2.4 Summary of Seed Morphology QTL Significant by Interval Mapping in the ITMI Population

A summary of seed morphology QTL significant by interval mapping in the ITMI population is presented in Figure 2.4.

Table 2.3.3.2. QTL analysis by multiple marker regression of seed volume, 100 grain weight and hectolitre weight on the ITMI population over two years (hectolitre weight 2000 only). Only significant (P<0.05) effects are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Trait	Year	Chromosome	Position	Additive Effect	P Value
100 grain weight	1999	1A	65.351 +/- 15.287	0.174 +/- 0.063	0
Volume	1999	1A	62.4 +/- 18.334	1.343 +/- 0.537	0.004
Hectolitre weight	2000	1A	74.583 +/- 23.859	-7.532 +/- 3.624	0.015
Hectolitre weight	2000	2D	34.603 +/- 34.489	7.701 +/- 3.718	0.016
100 grain weight	2000	3B	124.465 +/- 47.797	-0.084 +/- 0.064	0.049
100 grain weight	1999	4A	32.252 +/- 21.665	0.14 +/- 0.066	0.025
Volume	1999	4B	21.992 +/- 12.254	0.949 +/- 0.517	0.049
Hectolitre weight	2000	5B	67.251 +/- 20.877	-6.417 +/- 3.278	0.031
Volume	2000	6A	73.357 +/- 29.415	1.193 +/- 0.661	0.026
100 grain weight	2000	6A	35.245 +/- 29.606	0.108 +/- 0.053	0.013
Hectolitre weight	2000	6B	112.511 +/- 27.802	-8.548 +/- 4.737	0.032
Volume	2000	7A	196.128 +/- 47.237	0.143 +/- 0.079	0.012

Table 2.3.3.3. QTL analysis by interval mapping of seed volume (cm^3), 100 grain weight (g) and hectolitre weight (g) on the ITMI population over two years (Hectolitre weight 2000 only). Only significant (P<0.05) effects are presented. Where the additive effect is positive, Synthetic M6 contributes the increasing allele; where the additive effect is negative, Opata contributes the increasing allele.

Year	Chromosome	Trait	Location of peak (cM)	Lod score	P value	% variation explained	Additive Effect
1999	1A	100 Grain Weight	67.4	2.55	0.012	12	0.181959
1999	1A	Volume	67.4	2.23	0.036	10.5	1.47789
2000	2D	Hectolitre Weight	27.9	2.07	0.049	12.4	7.3769
2000	7A	100 Grain Weight	213.4	3.13	0.005	35.2	0.184761
2000	7A	Volume	233.4	1.98	0.048	10.1	1.48107
1999	7D	Volume	138.6	2.17	0.034	10.9	-1.51062
1999	7D	100 Grain Weight	141.3	2.15	0.031	8.6	-0.15314

Figure 2.4. Summary of Seed Morphology QTL Significant by Interval Mapping in the ITMI Population. Positions indicated are the peaks of the QTL.

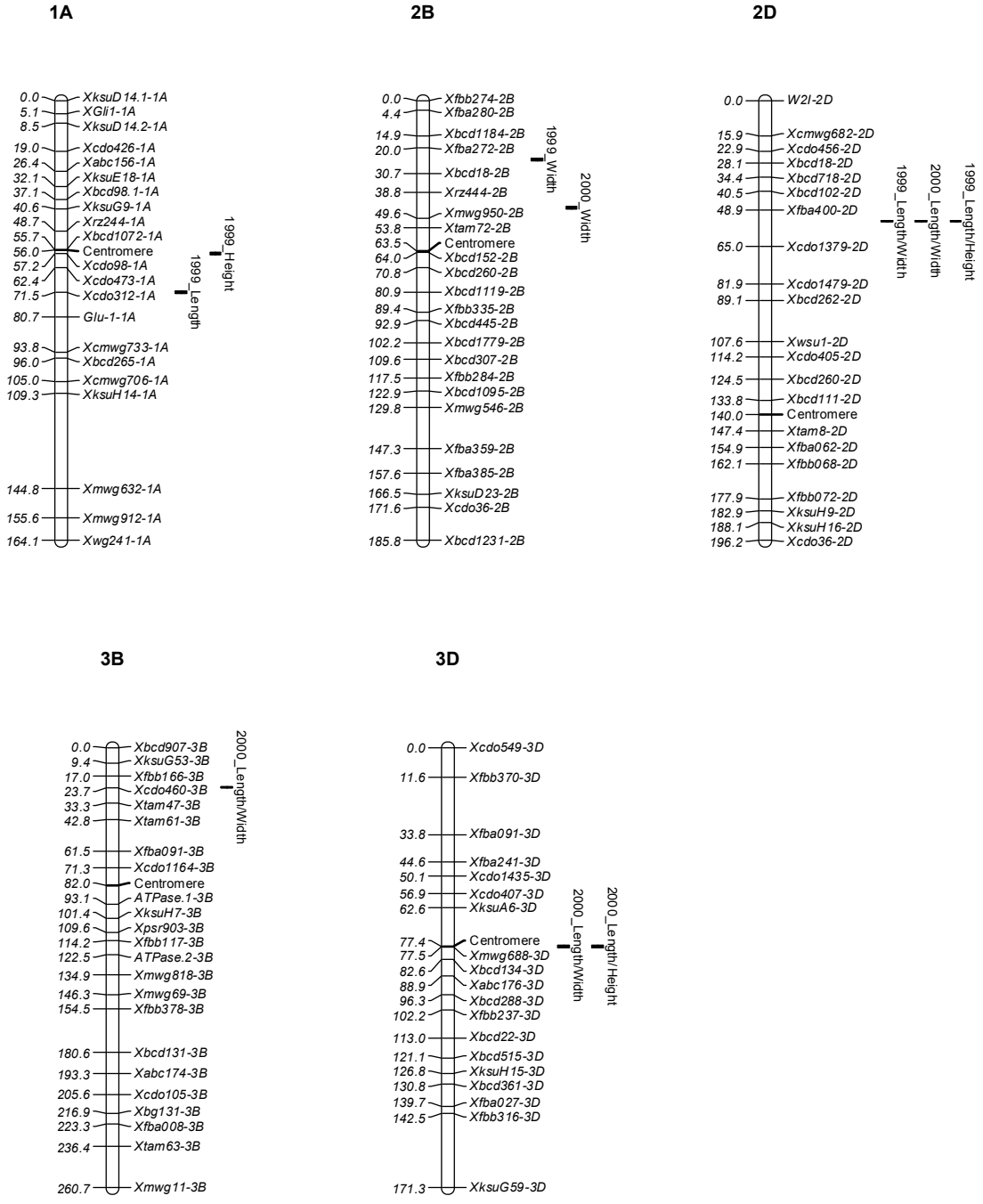
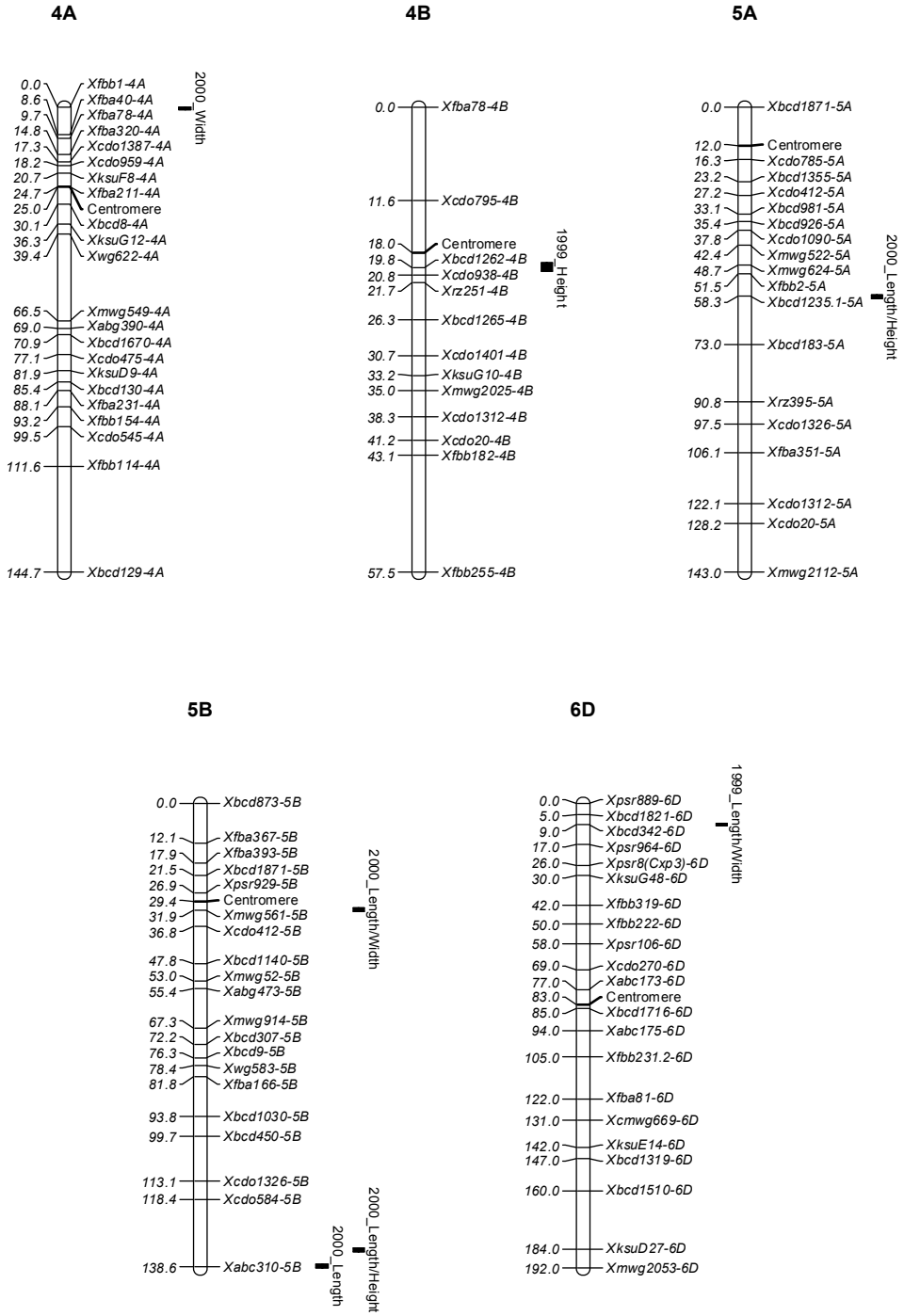


Figure 2.4 continued



2.5 Discussion

Using the image analysis techniques developed in these studies it has been easy to measure a large number of grain, and to process the results on a computer, avoiding laborious hand measurements. It has thus been possible to correlate variation in grain dimensions with hectolitre weight for the large number of samples needed for QTL mapping studies of such traits.

This study has shown that grain dimensions and hence hectolitre weight are under the genetical control of many genes. Interestingly, there are different 'types' of QTL. First, there are those that influence the components of grain shape, grain height, width and length, independently. For example, there is a QTL on 2B that influences grain width, and one on 4B that influences grain height. The importance of such QTL is in the fact that they indicate that it should be possible to breed for different grain shapes, such as long thin grain, short thin grain etc, through utilising such variation. Secondly, there are QTL which influence more than one trait, for example, the ratios of length:width for a the QTL on chromosome 2D. These imply that there are 'general' grain size factors which influence all dimensions. Such variation means that it should be possible to increase grain size through simultaneously increasing all dimensions. By judicious selection of both types of QTL it should be possible to breed for larger grains, and hence increase yield, with different grain shapes, as desired. Several QTL control hectolitre weight, and these seemed to reflect the probable pleiotropic effect of QTL for grain volume and grain weight. Thus, the variation in hectolitre weight is due to grain shape and size differences rather than differences in grain density. For certain co-incident QTL, eg on chromosome 1A, QTL for greater volume meant lower hectolitre weight. This indicates, not surprisingly, that to maximise hectolitre weight means breeding for many small grains, clearly not desirable from a quality point of view. Thus, selection has to be a compromise between 'good' grain shape, size and hectolitre weight. This could be achieved by directed plant breeding for the types of QTL identified here.

These experiments have utilized the ITMI population rather than an adapted UK cross, because no suitable UK population with a good genetic map was available at the beginning of the project. However, this as changed with the recent development of several maps in this and DEFRA funded project work. So, it would now be useful to use other crosses and to revisit the Avalon x Hobbit Sib cross to measure grain size difference to see how far these results can be extrapolated to UK germplasm.

Section 3. Texture-conferring mutations in puroindoline genes and their relation to texture and protein levels in a suite of UK varieties
(Based on the HGCA student bursary project conducted by Douglas Arkell)

3.1 Introduction

Quantifying endosperm texture

As discussed earlier, based upon the texture of the endosperm, wheats may be categorised either as or soft. The larger particles produced from some wheat genotypes will feel coarse or "hard", while flour from "soft" varieties will feel smooth to the touch. Traditionally, this difference has allowed the experienced miller or baker to distinguish between hard and soft flours at the point of use. In present day milling and baking industries however, accurate and repeatable methods of endosperm texture evaluation are essential. The prediction of milling times, energy expenditure and, in some cases, end product quality, can be important economic considerations and several ways have been devised to measure grain texture. These have included both biochemical and physical methods. Detection of the grain-texture-associated protein friabilin (Bettge *et al.*1995) and the exploration of the relationship between gliadin composition and grain texture (Huebner and Gaines, 1992) have been among the former. Physical methods include the single kernel wheat characterisation system (SKWCS) developed in the USA. This automated method, which measures the amount of energy required to crush a single grain, has a throughput of 180 seeds per minute. As well as measuring texture, it can determine grain moisture and size characteristics (Martin *et al.* 1993). Other methods are based on the determination of starch granule size after milling.

Although microscopic studies such as that by Mattern (1988), show distinctive fraction patterns for crushed hard and soft grains, such methods are not easily quantifiable, so are not practical for large-scale studies or for the milling and baking industries. Near infrared reflectance (NIR) spectroscopy (Rubenthaler and Pomeranz, 1987) is the most widely used large-scale evaluation method and is the method of choice for the food industry. Here light at a range of infrared wavelengths is shone onto a flour sample and reflectance from the sample is measured. Total protein, moisture, as well as texture are derived from the spectrum via precise calibrations. In modern systems, such as that used in this study, the spectroscope is linked to a computer and data is analysed, recorded and displayed automatically.

Friabilin proteins

The genetic basis for the difference in texture phenotypes is mainly controlled by a single major locus, (of more than one closely linked genes) located on the short arm of chromosome 5D (Law *et al.,* 1978). This locus, named *Ha*, is also closely associated with or pleiotropic to

a gene designated *Fpl-1*, which controls levels of extractable polar lipids (Morrison *et al.* 1989). A 15 kD protein known as friablin (Latin *friablis*-friable), which again is associated with the *Ha* locus, has been located on the surface of water-washed starch granules from soft textured wheats, but is absent in those of hard wheat cultivars and durum wheats (Greenwell and Schofield 1986). It is now accepted that the mature friablin protein consists of two proteins, puroindoline a and b (pin-a and pin-b) (Gautier *et al.,* 1994), which may act together as a heterodimer (Giroux and Morris, 1998) to bind starch granules to membrane lipids within the endosperm. These components are members of a group of a number of low-molecular-weight cystine-rich proteins found in cereal seeds. Those characterised to date include thionins, ∝-amylase inhibitors and lipid transfer proteins (Gautier *et al*, 1989).

Figure 3.1.2. Three-dimensional representation of puroindoiline-b : A bundle of four alpha-helices connected with flexible loops. In this model the tryptophan-rich domain is in the loop between helix one and two (arrowed), and is probably in a beta-sheet conformation (Bihan *et al.* 1996)

Members of the puroindoline (Greek puro-wheat and indoline-the ring formed by the tryptophan-rich domain) protein family are basic and characterised by a unique tryptophan-rich domain, which forms a ring (Blochet *et al.*,1993)(Figure 3.1.2). This structure has also been found in barley and oat endosperm proteins (Fabijanski *et al.,* 1988). Some mammalian polypeptides responsible for antimicrobial activity have been found to contain a similarly high proportion of tryptophan indicating a seed defence function for these proteins (Selsted *et al*, 1992). The fact that puroindoline-a is also known to strongly bind polar lipids, reinforces

the possibility that these proteins might perform this role as membranotoxins with the ability to attack fungi and bacteria cell membranes.

As mentioned above, the friablin protein is probably the product of the mature pin-a and pin-b precursor proteins, acting as a dimer, or at least associating closely. The pin-a and pin-b cDNA sequences show around 55% homology and share similar propepides at both their N- and C- terminals, so it is likely that they are targeted towards the same part of the endosperm – the vacuole within the aleurone cells (Matsuoka and, Nakamura, 1991).

The presence of closely related genes in oats and barley and in one of the diploid ancestors (*Aegilops tauschii*) of bread wheat points to a long evolutionary history for puroindolines. The genes are not present in tetraploid wheats such as *T. durum,* which always has a very hard endosperm structure. The hybridisation of the tetraploid *T. diccoccum* with *Ae. tauchii,* the D genome donor, was probably responsible for introducing the softness trait to *T. aestivum* (Allaby, 1999).

Hardness-conferring mutations in puroindoline genes

The likelihood of interaction between pin-a and pin-b is further reinforced by the effect of mutations that may occur in either of the genes. Wild-type bread wheats have soft endosperm texture, and express the functional, wild-type puroindoline alleles: pina-D1a and pinb-D1a, while in hard wheats, one or other of the puroindoline genes or it product has been rendered non-functional by mutation. The first hardness conferring mutation to be described was a glycine to serine change at position 46 in the puroindoline b protein (pinb-D1b, Giroux and Morris 1998). Since that date a number of hardness conferring of alleles have been discovered (Table 3.1, Puroindoline alleles).

Table 3.1. Characterised *pin* alleles. To date, six hardness mutations have been found in *pin-b,* including three to stop codons. The nature of the mutation that causes *pin-a* not to function has not, so far, been characterised

pin-a	*pin-b*	molecular change	phenotypye	reference
pina-D1a	*pin-bD1a*	-	Soft, wild-type	Giroux and Morris, 1997
pina-D1b	*pin-bD1a*	unknown	hard, *pin-a* null	Giroux and Morris, 1998
pina-D1a	*pin-bD1b*	Gly-46 to Ser-46	Hard	Giroux and Morris, 1997
pina-D1a	*pin-bD1c*	Leu-60 to Pro-60	Hard	Lillemo and Morris, 2000
pina-D1a	*pin-bD1d*	Trp-44 to Arg-44	Hard	Lillemo and Morris, 2000
pina-D1a	*pin-bD1e*	Trp-39 to stop codon	Hard, *pin-b* null	Morris *et al.,* 2001
pina-D1a	*pin-bD1f*	Trp-44 to stop codon	Hard, *pin-b* null	Morris *et al.,* 2001
pina-D1a	*pin-bD1g*	Cys-56 to stop codon	Hard, *pin-b* null	Morris *et al.,* 2001

Grain softness protein

In addition to the puroindolines, a protein known as grain softness protein (GSP-1) is thought by some researchers to have associations with grain endosperm texture. (GSP-1 is not to be confused with the mature friabilin protein, which is sometimes also referred to as GSP (reviewed by Morris, 2002)). Rahman and his colleagues (1994) reported the isolation of three closely related GSP-1 sequences from cDNA libraries. They located these to the distal ends of each of the group 5 chromosomes. The GSP-1 locus on 5DS is closely linked to the *Ha* locus and shows a 42% homology to pin-a. It is possible that GSP-1 is also a component of friabilin, but its role is debatable. Giroux and Morris (1998) have, for example, isolated the GSP-1 transcript from durum varieties. There is, therefore, no evidence that this protein is concerned with grain hardness and current understanding of grain hardness is that the two puroindolines alone combine to maintain grain softness.

Relationships between texture and protein content

The interdependence of endosperm protein content and grain texture has long been a subject of discussion (P.I.Payne personal communication and Bushuk, 1998). In general hard wheats have a higher protein level than do soft wheats, though the traits do not correlate in all mapping populations and may have a high dependence on environmental factors (Campbell et al., 1999). Mapping studies shown in Chapter 2, have however, shown that the *Ha* locus has a major effect on both, alleles showing hard/high protein or soft/low protein.

Objectives of the present study

Here, techniques were employed to explore several aspects of grain texture and its link with grain protein levels.

1. Flour samples from a suite of 58 hard and soft cultivars were analysed by NIR for grain texture, protein content and moisture content to examine relationships between these traits.
2. The glutenin HMW profile of those varieties suspected of being genetically mixed was examined using SDS-PAGE.
3. The same group of cultivars was PCR-screened for the presence of puroindoline genes, hardness-conferring mutations within those genes, and for the presence of GSP-1 and wild-type pin-b sequences.

3.2 Materials & Methods

Plant Material

Seed of cultivars used for mutant screening and grain texture measurements were obtained from the Germplasm Resources Unit and other sources within the John Innes Centre. The varieties selected included hard and soft, spring and winter, modern and historically important cultivars classified by reference to Farmers Recommended lists (NIAB, 1935 – 2002). In total, 58 seed samples were used, 36 of which had been harvested in 2001 after being grown as part of a germplasm maintenance program. For a further 19 varieties, the year of harvest was unknown. Seven varieties were represented in both the above groups. Up to 30g of seed was set aside for NIR analysis (Chapter 2). After overnight cold treatment at 4^{o}C, six seeds from each sample were sown in 1.0 litre pots and set out on the glasshouse bench during mid-January. Growing conditions were augmented with artificial light, thus giving a day length of 16 hours. Plant leaves were harvested 22 days after sowing, when a sufficient amount of green material had been produced and before the leaves became fibrous.

NIR Analysis

Measurements of grain texture (texture score), percentage moisture content and percentage protein content were obtained by near infrared reflectance (NIR) analysis. Seed samples were visually inspected and weed seeds, straw and chaff were removed. Samples were then milled using a Trecato Cylotec mill. Approximately 6 g of the resultant wholemeal flour was packed into the Bran-Luebbe "doughnut" following manufacturer's instructions. The wholemeal flour samples were then placed in a BRAN+LUEBBE®, Infra Alyzer 2000 machine, which had been previously calibrated with samples milled by the Trecato Cyclotec mill. Where enough flour was available - in most cases - three replications were carried out for each sample.

Moisture content

In order to increase wholemeal flour moisture levels, samples were placed overnight in a humid growth room prior to NIR analysis. They were then left on the lab bench for 24 hrs before re-analysis. Texture scores from high moisture and low moisture samples were compared statistically.

Verification by SDS – PAGE

Cultivar genotype was verified with the use of SDS-PAGE, using the method of Payne (1983). A single seed from each of 22 genotypes and the variety "Sicco" as a control, was crushed with a pair of pliers and the flour extracted in sample buffer. The gels were stained using the method devised by Caldwell and Kasarda (1978). The gels were destained in water for 24 hours.

Using a digital camera, gels were photographed. The banding pattern of each lane was compared to that previously recorded for the cultivar (Payne *et al*, 1987 and personal communication from J. Simmonds, JIC). Those cultivars with bands that did not match those previously recorded were removed from the study.

DNA extraction

Plant material was freeze-dried. It was then placed in a 30ml soft plastic tube with two or three ball bearings. The tubes were shaken mechanically until a fine talcum powder-like consistency was achieved. DNA was then extracted using the procedure described by Devos *et al.,* (1992) and diluted to 20ng per µl with sterile water.

PCR- Screening

Information on appropriate sequence specific primers was obtained from several sources within the literature (Table 3.2.1). PCR conditions were amended to suit laboratory equipment and available consumables (Table 3.2.2). Oligonucleotides were obtained from Sigma-Genosys Ltd., Cambridge UK. DNA samples were screened for the presence of *pin-a* and *pin-b* alleles, hard texture-causing mutations, and sequences expected to be present in genomic DNA from soft wheats, by PCR amplification, as described by Mullis and Faloona (1987) in a Peltier PTC – 225 tetrad thermal cycler under conditions as in Table 3.2.3.

Table 3.2.1. Primer sequences for PCR amplification of pin-a and pin-b wild–type genes (*pin-aD1a* and *pinbD1a)*, *pin-a* and *pin-b* mutations (*pin-aD1b*- null*, pin-b*D1b- Gly-46 to Ser-46, *pin-bD1c* –Leu-60 to Pro 60) *GSP-1* and *CS* (wild-type). Wild-type *pin-b*, *CS* and *pin-bD1b* share their left primer.

Giroux and Morris, 1997	*CS pin-b* right	CTCATGCTCACAGCCGCC
Giroux and Morris, 1997	*pin-b* left	ATGAAGACCTTATTCCTCCTA
Giroux and Morris, 1997	*pin-bD1b* mutation right	CTCATGCTCACAGCCGCT
Gautier *et al.* 1994	*pin-a* left	ATGAAGGCCCTCTTCCTCA
Gautier *et al.* 1994	*pin-a* right	TCACCAGTAATAGCCAATAGTG
Gautier *et al.* 1994	*pin-b* right	TCACCAGTAATAGCCACTAGGGAA
Giroux and Morris, 1998	*GSP-1* left	GTAGTGAGCACTACTATTGC
Giroux and Morris, 1998	*GSP1* right	GCACTTGGAGGGAAGGCTC
Lillemo and Morris, 2000	*pinb D1c* mutation left	ATGAAGACCTTATTCCTCCTA

Table 3.2.2. PCR conditions for PCR amplification of puroindoline wild-type, puroindoline mutation and grain softness protein sequences. All volumes are in µl.

name	*pin-a*	*pin-aD1b*	*pin-b*	*pin-bD1b*	*pin-bD1c*	*GSP*	*CS*
DNA (100ng)	2.5	2.5	2.5	5	2.5	2.5	5
10 x buffer	3.1	3.1	3.1	2.5	3.1	3.1	2.5
dNTP (200mM)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
primer l (2mM)	5	5	5	2.5	5	5	2.5
primer r (2mM)	5	5	5	2.5	5	5	2.5
taq (0.125U)	0.125	0.125	0.125	0.125	0.125	0.125	0.125
Sterile H_2O	6.775	6.775	6.775	9.875	6.875	6.775	9.875
total	25	25	25	25	25	25	25

Table 3.2.3 Thermal cycling programs for PCR amplification of target sequences. In the first step the samples were denatured. They then went through 35 cycles of denaturing, annealing and elongation. A final cycle with an extention step completed the reaction. All temperatures are in degrees C.

pin-a		*pin-aD1b*		*pin-b*		*pin-bD1b*		*pin-bD1c*		*GSP*		*CS*	
temp	time (mins)	temp C	time (mins)	temp	time (mins)	temp	time (mins)	temp	time (mins)	temp	time (mins)	temp	time (mins)
93	4	93	4	94	4	94	4	94	4	93	4	94	4
94	1	94	1	94	0.5	94	0.5	94	0.5	94	1	94	0.5
50	1.5	50	1.5	60	0.5	60	0.5	60	0.5	58	1.5	60	0.5
72	2	72	2	72	0.75	72	0.75	72	0.75	72	2	72	0.75
72	10	72	10	72	7	72	7	72	7	72	10	72	7

PvuII Digestion

As a method of detection of the Lue-60 to Pro-60 mutation, 10µl of the unpurified PCR product of pin-bD1c was digested with 2U of endonuclease PvuII (Cambio, Cambridge UK) in 1.5µl x 10 no. 5 buffer and sterile water to make up to 15µl total volume. The mixture was incubated at 37^{o}C for 2 hours (Lillemo and Morris, 2000).

Agarose gel Analysis

10µl of PCR amplified product (or PvuII digestion product for pin-bD1c) together with 5µl of orange G loading buffer were loaded onto 1.2% agarose/1 x TBE gels, stained with ethidium bromide. Gels were visualised and photographed under UV light and scored for the presence/absence of the target sequences or for detection of the Lue-60 to Pro-60 mutation, the presence of two bands.

3.3 Results

NIR measurement variation between replicate samples

In all varieties, percentage moisture and protein scores varied little between replicates (mean of sd 0.0765 and 0.0825, respectively). However, texture scores varied considerably (mean sd 0.2908) between varieties. During NIR screening of wholemeal flour samples, it was noticed that percentage moisture levels varied little between samples and between replicates assayed on the same day (sd = 0.243). There were, however, larger differences between percentage moisture content in the same sample when assays were performed days or weeks apart. These fluctuations seemed to be associated with changes in atmospheric moisture. As flour samples absorbed more water from the atmosphere, moisture levels increased and a decreased NIR texture score was recorded. Hard and soft samples appeared to be affected in a similar way and a similar trend was seen in the relationship between texture within a subset of cultivars assayed after exposure to different atmospheric moisture levels (Figure 3.3.1). Protein measured by NIR did not fluctuate significantly with wholemeal flour moisture content (one-way ANOVA: F = 0.06, P = 0.801).

Figure 3.3.1. Fluctuation of texture score with changes in atmospheric moisture. Texture score values were consistently lower where samples had a greater moisture content and a consistent change across texture scores was observed

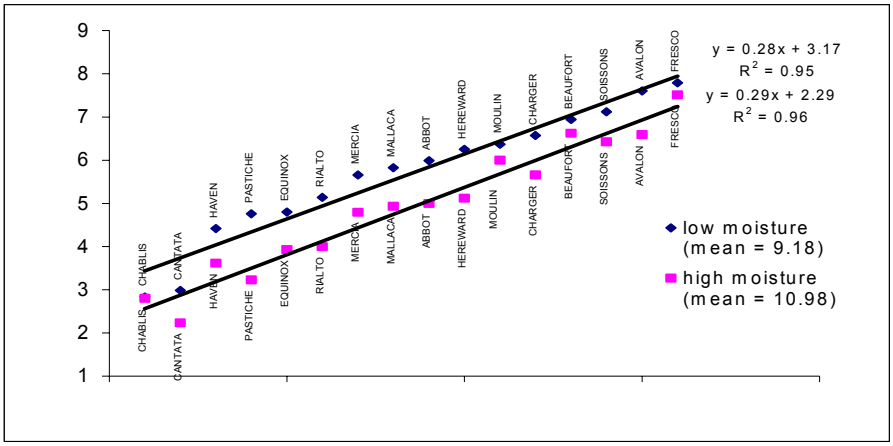


Table 3.3.1. NIR percentage moisture, endosperm texture and percentage protein mean data of 59 flour wholemeal flour samples from a series of cultivars. Those varieties harvested in years other than 2001 are marked with an asterix. HW = hard winter, HSp = hard spring, and SW = soft winter, PB = poor breadmaking. Those varieties hi-lighted in grey are suspected of being contaminated and were subsequently found to possess HMW subunits differing from those previously reported for the variety These were removed from NIR data analysis, as was cultivar Carmen, which exhibited an unusually high protein content score.

	moisture	NIR hardness	protein	Class
FIORELLA	10.04	2.42	15.18	SW
CANTATA *	10.55	2.77	14.67	SW
TILL221	10.38	3.34	12.66	SW
CHAMPLEIN	10.23	3.36	12.14	SW
VIMORIN	10.34	3.81	12.73	SW
CLAIRE	10.29	3.92	10.33	SW
HAVEN *	10.44	4.30	14.97	SW
DESPREZ 80	10.82	4.39	11,53	SW
BEAVER	10.34	4.41	10.55	SW
PASTICHE *	10.38	4.45	16.12	HW
EQUINOX *	10.48	4.80	13.23	HW
SPELTA	9.96	4.99	14.59	H -SPELT
SAVA	10.17	5.01	12.18	-
DWA	10.40	5.06	10.34	SW
2552	10.33	5.19	11.80	SW
WEK0609	10.25	5.27	10.29	SW
MALLACCA *	10.52	5.37	13.06	HW
RIALTO *	10.37	5.28	11.72	HW
RENDEZVOUS *	10.38	5.30	11.69	HW
BRIMSTONE *	10.36	5.71	15.68	HW
POROS	10.16	5.71	11.03	-
BERSEE	**10.15**	**5.88**	**11.39**	**PB**
HEREWARD *	10.54	6.07	14.49	HW
TRINTELLA	10.29	6.08	9.83	-
CARMEN	9.43	6.37	21.90	HW
MOULIN *	10.44	6.39	14.16	HW
AVALON *	10.42	6.85	15.59	HW
SOISSONS *	10.46	7.10	13.76	HW
ABBOT *	10.44	6.97	14.68	HW
GRANA	10.10	7.18	11.03	HW
STARKE	9.88	7.25	14.25	HW
CHABLIS *	10.65	7.45	13.70	HSp
SAVANNAH	10.06	7.52	10.58	HW
BEAUFORT *	10.25	7.62	14.98	HW
HYBNOS	10.22	7.78	9.70	HW
FRESCO *	10.53	8.06	13.71	HW
CHEYENNE	10.20	8.07	11.69	HW
BADGER	9.86	8.11	11.47	HW
FAVORITS	9.87	8.14	15.43	HW
MOULIN	10.50	8.17	10.15	HW
CHARGER *	11.39	8.29	12.97	HW
SOISSONS	10.16	8.47	10.11	HW
PEKO	10.04	8.72	10.06	HW
MARIS HUNTSMAN	9.91	8.99	10.94	SW
LITTLE JOSS	9.95	9.02	14.33	PB
CHARGER	10.05	9.08	10.58	HW
BEZOSTOYA	9.99	9.25	13.65	HW
RIALTO	10.22	9.47	11.40	HW
MYRANOVSKA	9.90	9.72	11.86	HW
HOPE	9.62	9.90	11.24	HSp
WEMBLEY *	10.19	9.93	14.18	HSp
MERCIA	10.72	10.29	10.78	HW
CADENZA	9.78	10.56	10.81	HSp
CAROLA	9.74	10.56	14.15	HSp
SPARK	10.16	10.65	12.27	HW
PASTICHE	9.29	11.11	16.29	HW
AVALON	10.18	12.58	11.24	HW

Analysis of NIR Data

Distribution Frequency

A histogram plot of NIR texture scores (Table 3.3.1) showed an approximately bimodal distribution with two peaks, indicating overlapping normal distributions. These two peaks represent the two endosperm texture classes (Figure 3.3.2a). A histogram plot of percentage protein scores also showed a tendency to form two distinct groups (Figure 3.3.2b). A normal distribution was observed for moisture percentage scores (Figure 3.3.2c).

Relationships between NIR moisture, texture and protein scores

The relationship between protein and texture scores was not significant ($R^2 = 0.0632$, $p = 0.152$) for all hard and soft varieties together, but was nearly significant ($p= 0.092$) for hard varieties alone (Figure 3.3.3a). There was a significant negative relationship between texture and moisture scores over all samples ($p= 0.009$, Figure 3.3.3b). There was no significant relationship between moisture and protein scores for all samples (Figure 3.3.3c).

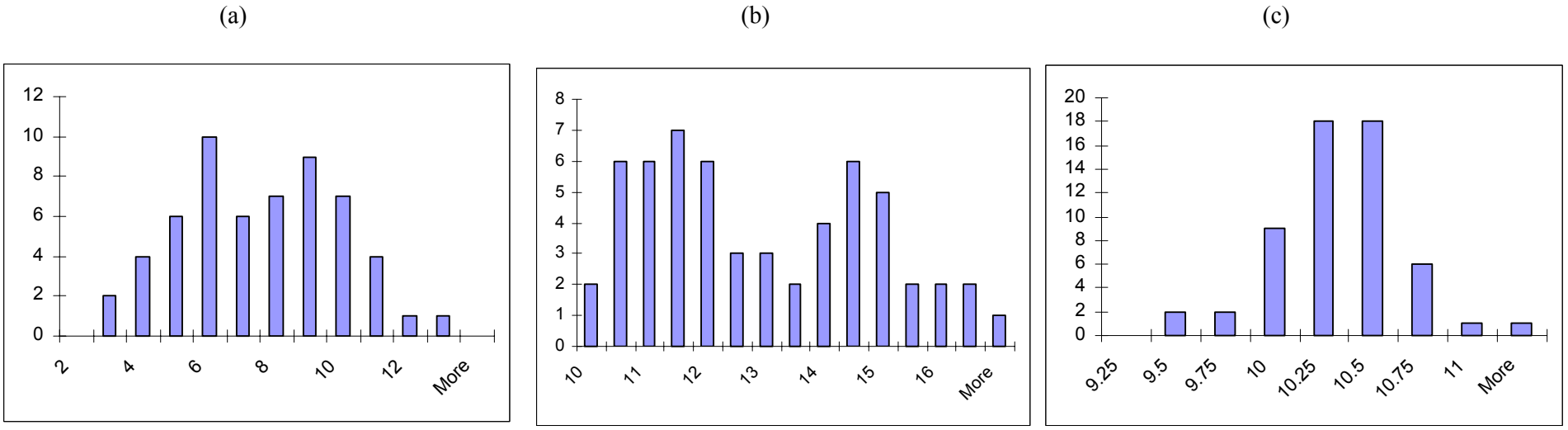


Figure 3.3.2. Distribution frequencies of NIR score for (a) endosperm texture (b) percentage endosperm protein and (c) percentage moisture content from wholemeal flour samples from a range of cultivars (Table 3.3.1)

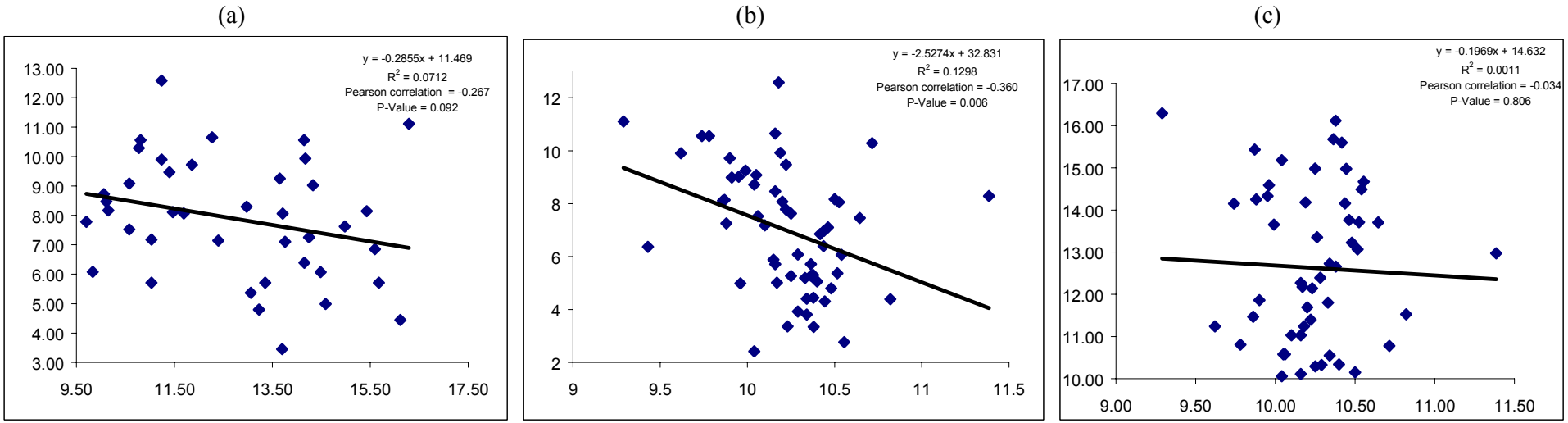


Figure 3.3.3. Relationships between NIR texture, moisture and protein scores. (a) protein x texture for hard endosperm cultivars; (b) moisture x texture for all cultivars; (c) moisture x protein for all cultivars.

SDS-PAGE analysis

It was noticed that some NIR texture and/or protein scores were not as expected. For example, some varieties listed as hard lines produced a texture score of less than 6.0. (Table 3.3.1). Using SDS-PAGE analysis, verification of genotype was carried out on those seed samples suspected of having been contaminated (Figure 3.3.4, Table 3.3.2). Of the 22 cultivars analysed three (Bersee, Badger and Hereward) were found not to possess the expected glutenin HMW subunits and were eliminated from the study. For a further three varieties (Starke, Mallacca and Carmen) no record of previous SDS-PAGE profile could be found in the literature. Although this was the case for Spelta Grey this variety did show a distinctive spelt pattern of glutenin sub units; Figure 3.3.4 gel 1 lanes 6 and 7 (Radic et al., 1997).

Figure 3.3.4. SDS Page gels. For layout and glutenin HMW subunit patterns see Table 3.3.2

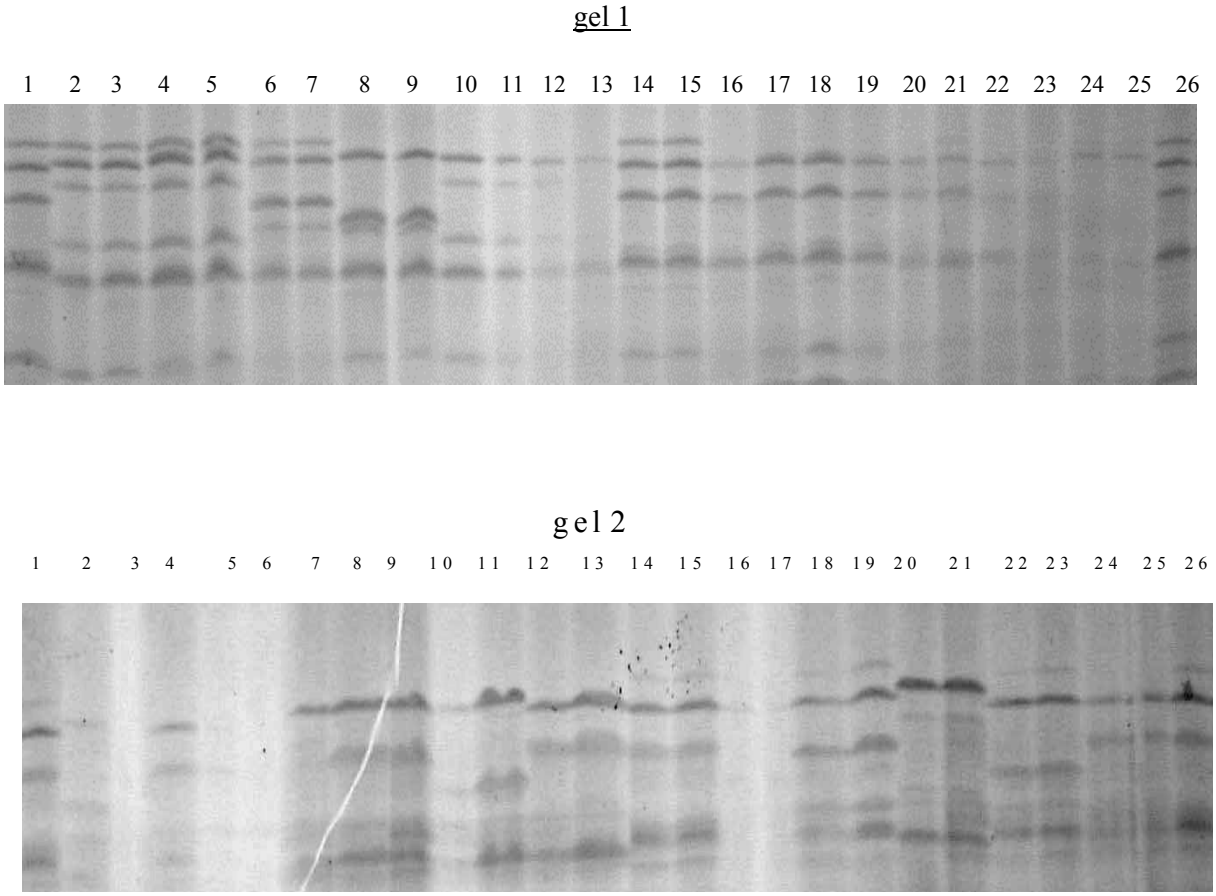


Table 3.3.2. Cultivar verification. Comparison between glutenin HMW subunits from the cultivars in the present study, and those recorded in the literature. N = null for subunit.

Gel and lane	variety	expected subunits	observed subunits	verification
1.1	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
1.2	Avalon	1, 3+12, 6+8	1, 3+12, 6+8	yes
1.3	Avalon	1, 3+12, 6+8	1, 3+12, 6+8	yes
1.4	Abbot	1, 6+8, 2+12	1, 6+8, 2+12	yes
1.5	Abbot	1, 6+8, 2+12	1, 6+8, 2+12	yes
1.6	Spelta Grey	spelt type	1, 2+12, 13+16	yes
1.7	Spelta Grey	spelt type	1, 2+12, 13+16	yes
1.8	Moulin	N, 17+18, 2+12	N, 17+18, 2+12	yes
1.9	Moulin	N, 17+18, 2+12	N, 17+18, 2+12	yes
1.1	Maris Huntsman	N, 2+12, 6+8	N, 2+12, 6+8	yes
1.11	Maris Huntsman	N, 2+12, 6+8	N, 2+12, 6+8	yes
1.12	DwarfA	N, 3+12, 6+8	N, 3+12, 6+8	yes
1.13	DwarfA	N, 3+12, 6+8	N, 3+12, 6+8	yes
1.14	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
1.15	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
1.16	Cheyenne	2, 7+9, 5+10	2, 7+9, 5+10	yes
1.17	Cheyenne	2, 7+9, 5+10	2, 7+9, 5+10	yes
1.18	Carmen	?	N, 7+9, 5+10	?
1.19	Carmen	?	N, 7+9, 5+10	?
1.20	Bersee	1, 7+8, 4+12	1, 7+?, 3 +12	no
1.21	Bersee	1, 7+8, 4+12	1, 7+?, 3 +12	no
1.22	Badger	N, 14+15, 5 +10	7+?, 5+10	no
1.23	Badger	N, 14+15, 5 +10	7+?, 5+10	no
1.24	Avalon	1, 6+8, 2+12	1, 6+8, 2+12	yes
1.25	Avalon	1, 6+8, 2+12	1, 6+8, 2+12	yes
1.26	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
2.1	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
2.2	Charger	N, 17+18, 2+12	N, ?+?, 2+12	yes
2.3	Charger	N, 17+18, 2+12	-	yes
2.4	Fresco	N, 7+9, 5+10	N, 7+ 9, 5 +10	yes
2.5	Fresco	N, 7+9, 5+10	-	yes
2.6	Haven	N, 6+8, 2+12	-	-
2.7	Haven	N, 6+8, 2+12	-	-
2.8	Hereward	N, 7+9, 3+12	-	no
2.9	Hereward	N, 7+9, 3+12	?+?, 5+10	no
2.10	Mallacca	?	N, 6+8, 17,18	-
2.11	Mallacca	?	N, 6+8, 17,18	-
2.12	Pastiche	N, 7+ 8, 4 + 12	N, 7+ 8, 4 + 12	yes
2.13	Pastiche	N, 7+ 8, 4 + 12	N, 7+ 8, 4 + 12	yes
2.14	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
2.15	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control
2.16	Rialto	1, 5+10, 17,18	-	-
2.17	Rialto	1, 5+10, 17,18	-	-
2.18	Savannah	N, 6+8, 3+12	-	-
2.19	Savannah	N, 6+8, 3+12	-	-
2.20	Brimstone	N, 6+8, 2+12	N, 6+8, 2+12	yes
2.21	Brimstone	N, 6+8, 2+12	N, 6+8, 2+12	yes
2.22	Rialto (2001)	1, 5+10, 17,18	1, 5+10, 17,18	yes
2.23	Rialto	1, 5+10, 17,18	1, 5+10, 17,18	yes
2.24	Starke	?	N, 5+10, 17+18	-
2.25	Starke	?	N, 5+10, 17+18	-
2.26	Sicco	1, 7+9, 5+10	1, 7+9, 5+10	control

Screening for known puroindoline mutations

A total of 48 DNA samples were isolated from plant material and PCR-screened for the presence of sequences that would confirm the presence or absence of friabilin component genes pina and pinb. Two further PCR screenings were carried out on the same samples for mutations in pin-b (pin-bD1b and pin-bD1c) that lead to differences in endosperm texture. Additional PCR screening was carried out for the presence of GSP (putative grain softness protein) and CS (characteristic of soft endosperm wheats) sequences (Figure 3.3.5). Data are presented in Table 3.3.3.

Figure 3.3.5. Agarose gels of *puroindoline* and *GSP* sequences. (a) The presence of *pin-a* amplifies *a* 450 bp fragment in all varieties (b) the presence of *pin-b* wild-type allele amplifies a 450 bp fragment in all varieties (c) *pin-bD1c* hardness allele (Leu-60 to Pro-60) mutation. amplifies a 448 fragment (not shown), which remains uncut when digested with PvuII (d) *pin-bD1b* hardness allele (Gly-46 to Ser-46) produces a 250 bp fragment (e) in the presence of *GSP-1* amplifies a 430 bp fragment in all varieties. (f) A 250bp fragment is amplified in wild-type (*CS*) soft wheats where glycine residues persist at position 46.

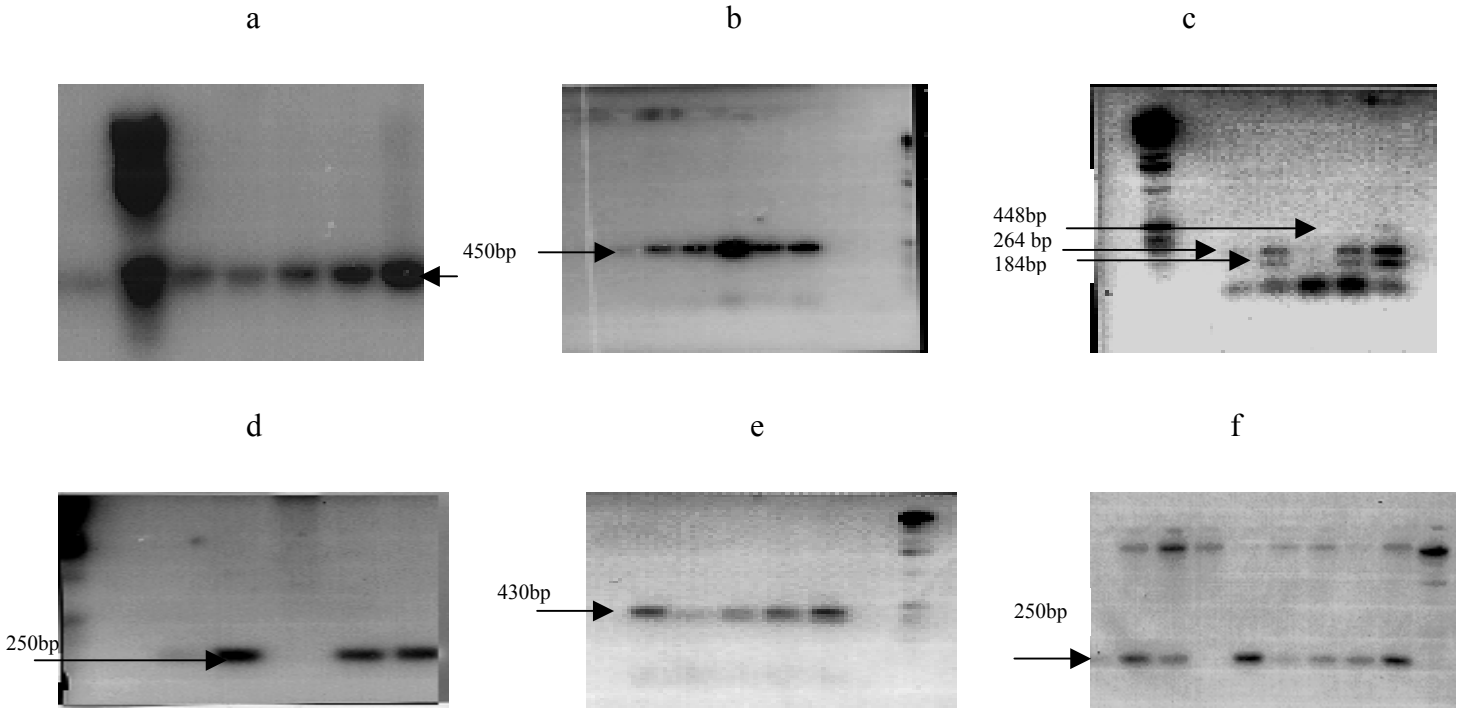


Table 3.3.3. Wheat varieties arranged into puroindoline gene mutation class. The presence of the expected agarose gel band or bands is denoted by the letter p.

VARIETY	hardness class	pina	pinb	GSP	CS	pinbD1b	pinbD1c
PASTICHE	HW	p	p	p	-	p	-
AVALON 1	HW	p	p	p	-	p	-
BEZOSTOYA	HW	p	p	p	-	p	-
CHARGER	HW	p	p	p	-	p	-
CHEYENNE	HW	p	p	p	-	p	-
LITTLE JOSS	Sp - PB	p	p	p	-	p	-
MYRANOVSKYA	HW	p	p	p	-	p	-
POVOS	HW	p	p	p	-	p	-
SAVANNAH	HW	p	p	p	-	p	-
STARKE	-	p	p	p	-	p	-
ABBOT	HW	p	p	p	-	p	-
BEAUFORT	HW	p	p	p	-	p	-
BRIMSTONE	HW	p	p	p	-	p	-
CHARGER	HW	p	p	p	-	p	-
FRESCO	HW	p	p	p	-	p	-
MOULIN	HW	p	p	p	-	p	-
HOPE	HSp	p	p	p	-	p	-
RIALTO	HW	p	p	p	-	p	-
SPARK	HW	p	p	p		p	
CHABLIS	HSp	p	p	p		p	-
	hard varieties bearing pinbD1c						
CADENZA	HW	p	p	p	-	-	p
CAROLA	HSp	p	p	p	-	-	p
PEKO	HSp	p	p	p	-	-	p
WEMBLEY	HSp	p	p	p	-	-	p
	hard varieties where no mutation found						
GRANTA	HW	p	p	p	-	-	-
RENDEZVOUS	HW	p	p	p	-	-	-
FAVORITS	HW	p	p	p	-	-	-
MERCIA	HW	p	p	p	-	-	-
SOISSONS	HW	p	p	p	-	-	-
HYBNOS	HW	p	p	p	p	-	-
MALLACCA	HW	p	p	p	-	-	-
CARMEN	-	p	p	p	p	-	-
EQUINOX	HW	p	p	p	-	-	-
	spelt						
SPELTA	H-spelt	p	p	p	p	-	-

Contd.

Table 3.3.3. Continued

CANTATA	SW	p	p	p	p	-	-
BEAVER	SW	p	p	p	-	-	-
TILLH221	SW	p	p	p	p	-	-
DESPREZ	SW	p	p	p	p	-	-
VILMORIN	SW	p	p	p	P	-	-
2552	SW	p	p	p	p	-	-
WEK	SW	p	p	p	-	-	-
DWARFA	SW	p	p	p	p	-	-
TRINTILLA	SW	p	p	p	-	-	-
CLAIRE	SW	p	p	p	p	-	-
FIORELLO	SW	p	p	p	p	-	-
CHAMPLEIN	SW	p	p	p	P	-	-
SAVA	SW	p	p	p	p	-	-
HAVEN	SW	p	p	p	-	-	-

The presence of 450 bp single bands indicated the presence of a *pin-a* and *pin-b* gene in all varieties. Most DNA samples from hard wheats were shown to have mutations in *pin-b.* The *pin-bD1b* mutation was the most common, present in 20 varieties. Four varieties carried the mutation *Pin-bD1c.* No puroindoline mutations could be found in nine other varieties. Chinese Spring (*CS*) wild type *pin-b* sequences were found in most soft wheats, in the one spelt variety and in two hard wheat varieties where no hardness conferring allele was detected. *GSP* sequences were present in all samples. No varieties were found to be null for *pin-a.*

3.4 Discussion

This study has investigated some of the factors that contribute to grain texture and has attempted to provide a more meaningful definition of the term "hardness". Results have, on the whole, reinforced conclusions arrived at by other researchers, but they have also posed some additional questions.

NIR analysis

An NIR texture assay is, in effect, a measurement of apparent particle size after grain has been milled, as particle size increases the logarithm of the reciprocal of reflectance increases at every wavelength in the NIR spectrum (Norris *et al,* 1989). Wholemeal flour samples derived from the same genotype should, therefore, produce similar reflectance profiles. In this study, however, considerable differences between replicates of the same sample were observed even when the flour had been well mixed and care had been taken to ensure that lumps had been broken up. In some cases the differences were over 0.5 of a unit. Texture scores were also seen to change as atmospheric moisture fluctuated though differences here were predictable and were of similar magnitude in all those varieties tested. Differences in texture score were also noted between samples from the same variety, which had been grown in different years. This indicates an environmental dimension to grain texture. In addition, some samples shown to belong to one or other of the genetic texture classes produced texture scores lower than would have been expected.

The accuracy of NIR spectroscopy as a measurement of grain texture is dependent upon several factors. In this study an increase in wholemeal flour moisture levels of less than 2% caused grain texture scores to fall by up to one unit. There was no corresponding change in protein percentage score, which had been corrected to 14% moisture. Wholemeal flour brought from conditions of high humidity to a drier atmosphere will quickly desiccate, so fluctuation in humidity caused by,

for example by space heating systems, will quickly change flour humidity levels. Ideally, a laboratory where NIR analysis is to be carried out would be kept at a constant humidity level. The consistent nature of changes in texture score due to fluctuations in atmospheric moisture suggests, however, that an algorithm could be devised to correct such fluctuations by a similar method to that used to correct protein percentage scores.

Variation in pre-milling grain moisture content, which will differ with storage conditions, can also lead to variation in texture score (Windham *et al.*, 1993). As stated above, NIR measures apparent particle size. Hard wheats produce larger particles when milled than do soft wheats. However, wheats milled when high in moisture, also tend to produce higher texture scores. Microscopic studies (Gaines and Windham, 1998) have shown that after milling, the pericarp will break into larger pieces in wheat stored at high humidity, leading to higher apparent particle size at NIR analysis. It is suggested that uneven distribution of pericarp fragments may also be responsible for variation in texture scores between replicates. In the present study, no account was taken of pre-grinding moisture levels in whole grains. It is suggested, therefore, that grain moisture levels be ascertained before milling. Windham *et al.* (1993) have developed an algorithm to correct NIR texture scores to 11% whole grain moisture. The use of such methods should be investigated for use in future projects.

Amongst those samples assayed in this study was a single variety of spelt wheat (*T. spelta*). Although the present Spelta Grey is known to be hard, and generally of good breadmaking quality (S.Reader, JIC, personal communication), NIR texture scores were, nevertheless, low at below 5.0, and the glycine allele was present at amino acid 46. Spelt has A,B and D genomes but is probably of more recent origin than is common wheat. Its evolutionary history is distinct, as are patterns of HMW glutenin subunits when visualised by SDS Page (Blatter *et al.,* 2002). It is probable that spelt varieties also fall into hard and soft catagories.

NIR is undoubtedly a fast and easy method of differentiating between different classes of wholemeal flour, hard from soft. For comparing texture within classes however, other methods such as SKCS may be superior. There are, nevertheless, several ways of improving its accuracy. Mills should thoroughly cleaned between samples and well maintained, moisture levels both pre and post grinding should be corrected mathematically, and environmental factors should be eliminated as far as is possible.

SDS analysis

Contamination of grain samples of one genotype with grain from another or erroneous labelling can be a major problem and failsafe handling systems should be employed where ever possible. In this study, cultivar purity was examined using the SDS-PAGE protocol, which was developed over 30 years ago and has proved efficient in predicting bread making quality. Because the genes governing the inheritance of HMW glutenin subunits are found at three separate loci on the long arms of each of the group 1 chromosomes (Payne *et al,* 1982), HMW glutenin inheritance is complex and distinct patterns of separation are apparent on acrylamide gels. Many varieties will, of course, possess a uniform subunit array, but variation is sufficient to ensure polymorphism between cultivars in most cases. Although this method could be termed "old technology", it is still effective in that it is cheap and provides a wide range of polymorphism in a single and relatively simple process.

Puroindoline alleles

It seems likely from the evidence of this and other studies which have explored the distribution of mutations across wheat varieties, that the Gly-46 to Ser-46 (*pin-bD1b*) mutation is the most common of the texture conferring mutations in bread wheats. In a large a study on the North American wheats, Morris *et al.* (2001) found this mutation to be present in all but a very few hard winter varieties. It was also present in the majority of spring hard varieties, though they carried a greater proportion of other alleles. Similarly, Lillemo and Morris (1999) reported results from hard wheats, mostly spring types, from Northern and Eastern Europe. Of 166 hard genotypes 100 bore the Gly-46 to Ser-46 point mutation. In this study of mostly UK varieties, 20 of the 24 where a mutation was found, carried this mutation. In most of the soft lines tested, the glycine residue was found to be present at amino acid 46. The rate of occurrence of other rarer mutations was comparable to that of the Northern European study. None of the varieties analysed here were null for *pin-a* (*pin-aD1b*). Lillemo and Morris (2000) found only one European variety with this mutation, but found it to be the most common texture allele among 23 South American wheats they investigated. The possibility that this mutation may be of New World origin is strengthened by results reported in the above-mentioned North American study where it is fairly common in spring hard wheat cultivars originating in North America. The Leu-60 to Pro-60 (*pinb-D1c*) point mutation was found in four varieties in this study, three of which are spring varieties. This is a smaller proportion than reported by Lillemo and Morris (2000) who found a concentration of this allele amongst Scandinavian genotypes in both spring and winter varieties. Among those North American wheats screened by Morris and his colleagues (2001), this was a very rare allele found

only in spring wheat genotypes introduced into, or bred in that region, during the latter19th and the early 20th centuries. Other recently discovered single-point *pin-b* alleles (*D1d, D1e, D1f* and *D1g*) were not screened for in the present study. It is possible that these may have been responsible for conferring hardness in some unresolved hard wheat cultivars here, though their extreme rarity (Morris, 2002) would seem to make this unlikely and difficulties with experimental procedure were probably responsible for negative results here.

As discussed above, mutations expressed in either of the puroindoline proteins have the effect of rendering the mature friabilin protein dysfunctional. Point Mutations in the *pin-b* gene have been well characterised and their putative location on the expressed protein give indications to the consequences of the changes in amino acid sequence. As seen above, the tertiary structure of Pin-b is thought to consists of a bundle of four helices linked together by flexible loops, with a tryptophan- rich domain included in the loop between helices one and two (Figure 3.1.2). A change from Gly to Ser at nucleotide 46 may introduce rigidity to the loop between the tryptophan-rich domain and the second helix. Similarly change at position 44 introduces a positively charged argenine residue into the tryptophan-rich domain. The Leu to Pro change at position 60 is probably responsible for altering the helix configuration. All these changes have the potential to reduce the affinity between polar lipids and pin-b, and therefore, to cause loss of protein function (Lillemo and Morris, 2000). The molecular nature of the mutation that causes a genotype to be null for *pin-a* is not known. That the absence of either functional protein has a similar effect on hardness indicates that each puroindoline plays a separate role here. Differences in tryptophan-rich domains (Dubriel *et al.*, 1997) probably mean that each binds lipids differently.

As has been demonstrated in this and other studies, the presence or absence of functional puroindoline genes is easily detected by simple PCR amplification. It is, therefore, a straightforward matter for the researcher or the plant breeder to classify the individual lines of a segregating population not only into hard or soft lines but also by hardness – bestowing allele. Because of the vast differences in the backgrounds of randomly selected cultivars, such as those examined here, it is difficult to see whether differences the nature of any hardness mutation has any appreciable effect on end use quality. Additionally, the relatively few samples screened and the uneven distribution of the hardness alleles make such comparisons beyond the scope of this project. Data from the study of a much larger suite of cultivars by Lillemo and Morris (2000) indicates that cultivars bearing the *pin-a* null genotype may produce harder grain, though here also, some hardness-mutation classes contained comparatively few

varieties. Martin and his colleagues (2001) have used a recombinant inbred line (RIL) population derived from crosses between varieties with different hardness alleles (*pin-aD1b* and *pin-bD1b*), which are simply inherited. A strong association was found between trait and allele, with the *pin-a* mutation null giving a consistently higher texture score. When considering texture along with other breadmaking quality traits they suggest that those lines where the *pin-aD1b* mutation occurs were inferior, as very hard grain is not desirable. Their conclusions seem to suggest either that the pin-a protein has some softening effect without pin-b or that the mutated pin-b retains some residual activity. Further comparisons between the breadmaking qualities of RILs derived from crosses between other combinations of alleles may be useful.

All the cultivars, both hard and soft, screened here for *GSP-1* were positive for this protein indicating that it does not have any bearing on grain texture. These results support the findings of Giroux and Morris (1998), who found evidence for the presence of GSP-1 transcripts after Northern blot analysis in both soft and hard cultivars as well as in the durum variety Langdon and 5D substitution lines of Langdon. Although it has been shown that *GSP-1* is located on the short arm of 5D (Jolly *et al.,* 1996), it is also present at similar locations on the other group 5 chromosomes Giroux and Morris, 1998). The evidence for a direct link between this protein and grain softness is therefore, not strong.

Conclusions

It has been shown here that although NIR is a very useful tool in distinguishing between grain texture, it is limited in its precision. NIR texture score is a measure of apparent particle size and is subject to distortion from several exogenous factors, the most important being atmospheric moisture, both pre and post-milling. Control of laboratory environment and/or mathematical correction should be utilised to minimise such fluctuation in texture score.

Although SDS-Page detection of HMW glutenin separation patterns was introduced around 30 years ago, it remains a quick and effective way of confirming genotype, where contamination may have occurred.

The distribution of puroindoline hardness-conferring alleles amongst those varieties assayed here is comparable to that found in studies or other cultivar suites, with the Gly-46 to Ser-46, being by far the most common. At least one other mutation was found to be present. Serine is present at amino acid 46 in most of the soft wheats and the one T. spelta variety assayed in this study.

General Conclusions

Implications for wheat breeding

For wheat, as most arable crops, conventional plant breeding is still, and will be for the foreseeable future, the mainstay for the production of new arable crop varieties for the UK market. This, of course, involves creating variation through making crosses between established varieties with complementary characteristics. New genetical variation is then released in the progenies, and improved variants selected and multiplied. This has been very successful in the UK over the latter half of the last century. For example, wheat yields have continued to rise at about, on average, 1%/year since the 1970's. On the other hand, though, only limited increases in quality have been obtained in recent times. One of the reasons for this situation is that most of the characters being manipulated have a very complex inheritance and are highly affected by environment, such that breeders have had little information to guide their choice of parents or crosses, and subsequent breeding strategy. Most of the selection for improved genetic type has been based on empirical selection of observed phenotypes, rather than for the specific, desirable genes known to improve those traits. However, because of the development of molecular marker systems, good genetic maps can finally be developed in wheat, and as here, applied to discover new genes which can be used by plant breeders for marker-assisted selection.

This study has shown that QTL controlling different aspects of grain quality variation can be discovered in UK wheats, which can subsequently be targets for plant breeders for directed plant breeding. This study has established that the well known major gene, *Ha* on chromosome 5D, controls the major distinction between hard and soft wheats in the UK gene pool. However, modifier genes have been detected and mapped, which give plant breeders other options to modulate grain texture for different purposes. Similarly, *Ha*, or a closely linked gene, has a major effect on increasing grain protein content, as have several other genes that have been discovered in these studies. These can give plant breeders options for breeding higher and lower grain proteins levels than currently usually obtained. In particular, they give the option to breed consistently higher levels, such as that found in the variety Hereward, by directed plant breeding, rather than by chance alone.

Although this study has discovered new QTL, this is only the start of the process of their application for directed plant breeding. It will require further mapping work to validate the QTL discovered, using other crosses, and more molecular work to convert nearest markers into

diagnostic tools. Then, the genes controlling such traits can be associated with gene 'tags', and then selection using linked DNA markers ('marker-assisted selection') can be used the laboratory, rather than the field. This could bring huge increases in breeding efficiency. Eventually, also, studies such as this will allow the ability to search for new alleles in germplasm collections, which are, at the present time, under-used in their contribution to UK plant breeding, particularly for quality traits.

Implications for Levy Payers

The results of these studies will contribute indirectly to levy payers through their future contribution to plant breeding. Such studies provide plant breeders with the genetic variation to enable them to produce new series of varieties adapted to traditional markets, but also allow the flexibility to breed for new markets. However, genetic studies can contribute directly to levy payers through attaching specific genetic information to named varieties, thereby informing them of which varieties are suited to their particular farm, process or markets. A good example of this is knowing which varieties have the presence of the 1B/1R translocation. Studies have shown that this chromosome can increase the yield of a variety, but that also, on average, varieties possessing it are poorer for animal feed (Wiseman et al, HGCA report). In the context of the present results, it is shown that varieties that are hard, are expected to have intrinsically higher protein, and vice versa for soft varieties, a fortuitous, but important association. In general, however, before levy payers can make such informed choices, they will need easy access to information on the genetical characteristics of varieties and the consequences of possessing particular genes. This could be regarded as a form of 'genetic screening' where varieties are characterised not only by their agronomic performance, but also on their genetic make-up. However, at the present time, this begs the question on the best way of informing levy payers concerning the genetic characteristics of particular varieties. Perhaps, in future, such information could be included in the Recommended Lists, for example. It is to be hoped that when a large body of information on the genetical control of important agronomic and quality characteristics in UK varieties is accumulated, it will be presented in a easily digestible form to enable levy payers to practise 'genetic screening' for varieties with potential for their economic situation.

References:

Aitken KS (1993). Genetic Analysis of Grain Protein Content in Wheat. Ph. D. Thesis, University of East Anglia.

Allaby RC, Banerjee M, Brown TA (1999) Evolution of the high molecular weight glutenin loci of the A, B, D, and G genomes of wheat *Genome* 42 (2): 296-307

Ammiraju JSS, Dholakia BB, Santra DK, Singh H, Lagu MD, Tamhankar SA, Dhaliwal HS, Rao VS, Gupta VS, Ranjekar PK (2001). Identification of inter simple sequence repeat (ISSR) markers associated with seed size in wheat. *Theoretical and Applied Genetics* **102** (5): 726-732

Beltsville Agricultural Research Centre, Maryland USA website. www.barc.ac.usa

Bettge AD, Morris CF, Greenblatt GA (1995) Assessing genotypic softness in single wheat kernels using starch granule-associated friabilin as a biochemical marker *Euphytica* **86 (1):** 65-72

Bihan TL, Blochet JE, Desormeaux A, Marion D, Pezolet M (1996) Determination of the secondary structure and conformation of puroindolines by infrared and Raman spectroscopy *Biochemistry* **35 (39):** 12712-12722

Blatter RHE, Jacomet S, Schlumbaum A (2002) Spelt-specific alleles in HMW glutenin genes from modern and historical European spelt (*Triticum spelta* L.) *Theoretical and Applied Genetics* **104 (2-3):** 329-337

Blochet JE, Chevalier C, Forest E, Pebaypeyroula E, Gautier MF, Joudrier P, Pezolet M, Marion D (1993) Complete amino-acid-sequence of puroindoline, a new basic and cystine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by triton X-114 phase partitioning
Febs Letters **329 (3**): 336-340

Bredemeier C, Mundstock CM, Buttenbender D (2001). Effect of seed size on initial plant growth and grain yield of wheat. *Pesquisa Agropecuaria Brasileira* **36** (8): 1061-1068

Bushuk W (1998) Wheat Breeding for End-Product Use (Reprinted From Wheat: Prospects For Global Improvement, *Euphytica* **100 (1-3):** 137-145

Caldwell KA, Kasarda DD (1978) Assessment of genomic and species relationships in Triticum and Aegilops by polyacrylamide gel electrophoresis and by differential staining of seed alumins and globulins *Theorhetic and Applied Genetics* **52:** 273-280

Campbell KG, Bergman CJ, Gualberto DG, Anderson JA, Giroux MJ, Hareland G, Fulcher RG, Sorrells ME, Finney PL (1999) Quantitative trait loci associated with kernel traits in a soft x hard wheat cross. *Crop Science* **39 (4):** 1184-1195

Chao S, Sharp PJ, Worland AJ, Warham EJ, Koebner RMD, Gale MD (1989). RFLP-based genetic maps of wheat homologous group-7 chromosomes. *Theoretical And Applied Genetics* **78** (4): 495-504

Devos KM, Atkinson MD, Chinoy CN, Lloyd JC, Raines CA, Dyer TA, Gale MD (1992). The coding sequence for sedoheptulose-1,7-bisphosphatase detects multiple homologs in wheat genomic DNA. *Theoretical And Applied Genetics* **85** (2-3): 133-135

Dubreil L, Compoint JP, Marion D (1997) Interaction of puroindolines with wheat flour polar lipids determines their foaming properties *Journal of Agricultural and Food Chemistry* **45 (1)**: 108-116

Fabijanski S, Chang Sc, Dukiandjiev S, Bahramian MB, Ferrara P, Altosaar (1988) The Nucleotide-Sequence of a cDNA for a major prolamin (avenin) in oat (*Avena-sativa* l cultivar Hinoat) which reveals homology with oat globulin. *Biochemie Und Physiologie Der Pflanzen* **183 (2-3):** 143-152

Gaines CS, Windham WR (1998) Effect of wheat moisture content on meal apparent particle size and hardness scores determined by near-infrared reflectance spectroscopy *Cereal Chemistry* **75 (3):** 386-391

Gautier MF, Alary R, Kobrehel K, Joudrier P (1989) Chloroform Methanol-Soluble Proteins Are The Main Components Of *Triticum durum* Sulfur-Rich Glutenin Fractions *Cereal Chemistry* **66 (6):** 535-535

Gautier MF, Aleman ME, Guirao A, Marion D, Joudrier P (1994) *Triticum aestivum* puroindolines, 2 basic cystine-rich seed proteins - cDNA sequence-analysis and developmental gene-expression. *Plant Molecular Biology* **25 (1):** 43-57

Giroux MJ and Morris CF (1998) A glycine to serine change in puroindoline b is associated with wheat grain hardness and low level of starch-surface friablin *Proceeding of the National Academy of Science of the United States of America* **95:** 6262-6266

Giura A, Saulescu NN (1996). Chromosomal location of genes controlling grain size in a large grained selection of wheat (*Triticum aestivum L*). *Euphytica* **89** (1): 77-80 1996

Greenwell P, Schofield JD (1986) A starch granule protein associated with endosperm softness in wheat *Cereal Chemistry* **63 (4):** 379-380

Haley CS, Knott SA (1992). A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. *Heredity* **69**: 315-324

Huebner FR, Gaines CS (1992) Relation between wheat kernel hardness, environment, and gliadin composition *Cereal Chemistry* **69 (2):** 148-151

Jolly CJ, Glenn GM, Rahman S (1996) *GSP-1* Genes are linked to the grain hardness locus (H alpha) on wheat chromosome 5D *Proceedings of the National Academy of Sciences of the United States of America*

Kearsey MJ, Hyne V (1994). QTL analysis - a simple marker-regression approach *Theoretical and Applied Genetics* **89** (6): 698-702

Lander E, Abrahamson J, Barlow A, Daly M, Lincoln S, Newburg L, Green P (1987). Mapmaker: A computer package for constructing genetic-linkage maps. *Cytogenetics And Cell Genetics* **46** (1-4): 642

Law CN (1966). *Genetics* **53** 487-98

Law CN, Worland AJ (1973). *Plant Breeding Institute Annual Report for 1972* pp 25-65

Law CN, Young CF, Brown JWS, Snape JW & Worland AJ (1978) The study of grain protein control in wheat using whole chromosome substitution lines. *In,* Seed Protein Improvement by Nuclear Techniques I.A.E.A., Vienna, Austria 483-502

Lillemo M, Morris CF (2000) A leucine to proline mutation in puroindoline b is frequently present in hard wheats from Northern Europe *Theoretical and Applied Genetics* **100 (7):** 1100-1107

MacRitchie F (1980) in *Advances in Cereal Science and Technology,* ed. Pomeranz Y. (American Association of Cereal Chemists, St. Paul) **3**: 271- 313

Magrath R, Bano F, Morgner M, Parkin I, Sharpe A, Lister C, Dean C, Turner J, Lydiate D, Mithen R (1994). Genetics of aliphatic glucosinolates .1. Side-chain elongation in *Brassica napus* and *Arabidopsis thaliana*. *Heredity* **72**: 290-299

Martin CR, Rousser R, Brabec DL (1993) Development of a single-kernel wheat characterization system
Transactions of the ASAE **36 (5):** 1399-1404.

Martin JM, Frohberg RC, Morris CF, Talbert LE, Giroux MJ (2001). Milling and bread baking traits associated with puroindoline sequence type in hard red spring wheat *Crop Science* **41 (1):** 228-234

Matsuoka K, Nakamura K (1991) Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting *Proceedings of The National Academy of Sciences of The United States of America* **88 (3):** 834-838

Mattern PJ (1988) Wheat hardness - a microscopic classification of individual grains
Cereal Chemistry **65 (4):** 312-315

Morris CF (2002) Puroindolines: the molecular genetic basis of wheat grain hardness *Plant Molecular Biology* **48 (5):** 633-647

Morris CF, Lillemo M, Simeone MC, Giroux MJ, Babb SL, Kidwell KK (2001) Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats
Crop Science **41 (1):** 218-228

Morrison WR, Law CN, Wylie LJ, Coventry AM, Seekings J (1989)The effect of group-5 chromosomes on the free polar lipids and breadmaking quality of wheat *Journal Of Cereal Science* **9 (1):** 41-51

Mullis KB, Faloona FA (1987) Specific synthesis of dna invitro via a polymerase-catalyzed chain-reaction
Methods In Enzymology **155:** 335-350

Nakamura H (2000). Allelic variation at high-molecular-weight glutenin subunit loci, *Glu-A1, Glu-B1* and *Glu-D1*, in Japanese and Chinese hexaploid wheats. *Euphytica* **112** (2): 187-193

Norris KH Hruschka WR, Bean MM, Slaughter DC (1989) A definition of wheat hardness using near-infrared reflectance spectroscopy *Cereal Foods World* **34 (9)**: 696-702

Payne PI (1983) Breeding for protein quantity and protein quality in seed crops. In: Daussant, Mosse, Vaughan (eds) Seed Proteins (London/New York, Academic) pp. 223-253

Payne PI, Holt LM and Macartney DP (1987) Glutenin subunit composition of wheat varieties and the presence/absence of 1BL/1RS Tempory Catalogue. Unpublished

Payne PI, Holt LM, Worland AI, Law CN (1982) Structural and genetic-studies on the high-molecular-weight subunits of wheat glutenin .3. telocentric mapping of the subunit genes on the long arms of the homoeologous group-1 chromosomes *Theoretical And Applied Genetics* **63 (2):** 129-138

Pestsova E, Ganal MW, Röder M (2000). Isolation and mapping of microsatellite markers specific for the D genome of bread wheat. *Genome* 43: 689-697

Radic H, Gunther T, Kling CI, Hesemann CU (1997) Characterization of spelt (Triticum spelta L) forms by gel-electrophoretic analyses of seed storage proteins .2. The glutenins *Theoretical and Applied Genetics* **94 (6-7):** 882-886

Rahman S, Jolly CJ, Skerritt JH, Wallosheck A. (1994). Cloning of a wheat 15-KDa grain softness protein (GSP) - GSP is a mixture of puroindoline-like polypeptides.
European Journal Of Biochemistry **223** (3): 917-925

Roder MS, Korzun V, Wendehake K, Plaschke J, Tixier MH, Leroy P, Ganal MW (1998) A microsatellite map of wheat *Genetics* **149 (4):** 2007-2023

Rubenthaler GL, Pomeranz Y (1987) Near-infrared reflectance spectra of hard red winter wheats varying widely in protein-content and breadmaking potential *Cereal Chemistry* **64 (6):** 407-411

Selsted ME, Novotny MJ, Morris WL, Tang YQ, Smith W, Cullor JS (1992) Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils *Journal of Biological Chemistry* **267 (7):** 4292-4295

Snape JW, Law CN, Parker BB, Worland AJ (1985). Genetic analysis of chromosome-5A of wheat and its influence on important agronomic characters. *Theoretical And Applied Genetics* **71** (3): 518-526

Sourdille P, Perretant MR, Charmet G, Leroy P, Gautier MF, Joudrier P, Nelson JC, Sorrells ME,

Bernard M (1996)Linkage between RFLP markers and genes affecting kernel hardness in wheat *Theoretical and Applied Genetics* **93 (4):** 580-586

Stam P & Van Ooijen JW 1995. JoinMap ™ version 2.0: Software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen, Netherlands.

Wiersma JJ, Busch RH, Fulcher GG, Hareland GA (2001). Recurrent selection for kernel weight in spring wheat. *Crop Science* **41** (4): 999-1005

Windham WR, Gaines CS, Leffler RG (1993) Effect of wheat moisture-content on hardness scores determined by near-infrared reflectance and on hardness score standardization *Cereal Chemistry* **70 (6):** 662-666

Van Ooijen, JW & Maliepaard C, 1996. MapQTL ™ Version 3.0: software for the calculation of QTL positions on genetic maps. CPRO-DLO, Wageningen, Netherlands

Appendix 1 : List of Figures

Appendix 2: List of Tables