HGCA

PROJECT REPORT No. 183

USE OF DNA MARKER-BASED ASSAYS TO DEFINE AND SELECT MALTING CHARACTERISTICS IN BARLEY

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by

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SUMMARY

The main aim of the project was to show the value of using genetic markers to investigate characteristics of barley related to malting quality. Another objective was to gain some understanding of the contribution of starch granules, their morphology and their associated proteins on grain texture and malting behaviour. Malting quality is a complex character which depends on the barley cultivar, the weather and the expertise of the grower. By utilising the genotypes forming the pedigrees of three contemporary malting cultivars, the population investigated represented an evolution of malting barley. Concurrent with that has been a growing sophistication in selection technology and a vast increase in genetic knowledge. Since the development of Proctor and Maris Otter, in which relatively simple laboratory selection was used, tests have developed to mirror the changes in malting technology. Further, the breeding of Optic and Regina for example depended on a much greater understanding of how large numbers of genes act together to produce the better combinations of yield and grain quality.

The newest genetic techniques allow variation in the base sequences of the plant's DNA to be fingerprinted. This genetic information can then be used to find the location of particular genes on the seven chromosomes of barley, telling the breeder which characters can be easily modified without changing other important genes nearby. In addition, if end users define particular characters important to malting performance, the geneticist can use markers to identify the genes responsible for controlling these characters so allowing greater precision in breeding programmes. It also provides a simple means of demonstrating the genetic relationships between characters such as grain hardness (milling energy), hot water extract and starch properties. DNA marker assays only require small amounts of DNA so could be used to identify cultivars on the farm and in maltings. In addition the use of DNA markers has been shown to give greater discrimination of cultivars than plant morphology which is currently used in DUS tests.

The development and large scale application of DNA based markers in grain trading will aid profitability throughout the system from the farm to the consumer. More precise understanding of genetic relationships between characters will enable the consumer to identify genotypes for specific purposes and the breeder to produce suitable cultivars more rapidly and cost effectively. Growers will benefit from genotypes better targeted to give a high quality grain sample, in the environmental conditions under which they will be cultivated. This will be achieved by more precise location and manipulation of genetic factors already present in cultivated barley. Genetic markers are a short sequence of bases, constructed to match complimentary sequences in barley DNA. They are used as tags and are characteristic of cultivars but do not involve genetic modification.

BACKGROUND

When barley is traded there is a need to assay quality to ensure the fitness for use of particular lots. In the case of malting barley, it is possible for a local maltster to contract crops of a known variety and to monitor the crop throughout the growing season. At harvest the grain can be stored and dried precisely according to the maltster's requirements. In storage dry grain undergoes slow biochemical changes which can result in more rapid germination and more extensive modification during malting. As a result it may be necessary to change the malting conditions to ensure optimum malt production.

If a particular malting company follows a different trading policy, or if trading conditions result in a mismatch of supply and demand, barley or malt will be purchased from less controlled sources. While it is possible to purchase samples with passport data giving full traceability, the barley may be more variable than desired.

Maltsters use a range of tests to assess fitness for malting. Rapid assays of grain nitrogen, thousand corn weight, moisture and germination can be used to segregate loads as they arrive from the farm. In at least one case milling energy (Allison et al., 1979) has also been used at this stage (Maule 1990). Commercial malting conditions are usually adjusted for each batch of grain after micromalting small samples.

These initial tests of grain, while routine, only give a general indication of malting performance. Variety identification, the major determinant of malting quality, can be assayed by electrophoresis of grain proteins (Jarman et al., 1993). Grain size distribution, which depends on variety and growing conditions, is associated with grain composition and the rate of germination (Ellis & Marshall 1998; Marshall & Ellis 1998). Sieving fractions give a better indication of the distribution of grain size than thousand corn weight. Other assays of grain composition have been proposed e.g. β-glucan content, but their use in predicting malting performance is uncertain.

A basic reason for the uncertainty in predicting malting performance is genotype with environment interaction (GxE). It is possible to model GxE statistically (Findlay & Wilkinson, 1963) and to identify the extent of its genetic control. To date there has been no systematic application of such concepts to practical malting performance. The identification of key grain characteristics will allow such assessments to be made.

The development of new malting barley varieties shares many problems with the assay of intake samples. Malting quality is a complex character dependent on the development of genotypes with favourable alleles at many gene loci. Before the large scale application of micromalting techniques in barley breeding (Whitehouse & Whitmore, 1963), assessment of grain quality was based on visual selection for fine skinned types i.e. with low levels of lemma spiculation (Whitmore & Sparrow, 1957). The undesired effect of this selection was the poor yield performance of malting quality varieties relative to contemporary feed quality varieties (Riggs et al., 1981).

During the 1970s, a number of tests on barley grain were introduced, with a view to predicting malting performance of breeders' lines, and their suitability was subsequently reviewed (Ellis et al., 1979, Gothard, 1981). No test on unmalted grain

could infallibly predict hot water extract (HWE), but all modern malting cultivars were shown to have a friable endosperm structure with a low milling energy requirement (Allison, 1986). Milling energy is determined by use of a Comparamill (Allison et al., 1979), a modified hammer mill, in which the hammers are connected, via a drive shaft, to a rotating flywheel. Flywheel speed is monitored as the grain sample drops into the mill chamber and, subsequently, twice, at 2sec intervals. Deceleration during these two periods is used to calculate the milling energy 'A' and 'B' values respectively. The former, Milling Energy A (MEA) measures the resistance of the sample to mechanical disruption and is the figure quoted where a single milling energy is given. Milling Energy B (MEB) values are generally low, but it was noted that they were significantly increased in barley lines carrying starch mutations (Swanston, 1994). This was thought to result from a slower emptying of the mill chamber and may, therefore, reflect a different range of particle sizes within the flour sample.

Hard and soft wheats have been shown to produce different types of flour particle on milling, resulting from patterns of endosperm fracturing. This has been attributed to the presence or absence of a specific protein on the surface of the starch granules, which affects the adhesion between the granules and the surrounding protein matrix (Greenwell & Schofield, 1986). This protein, friabilin, has also been shown to occur in barley (Jagtap et al., 1993), but there are no data to show whether it differs significantly in content between high and low milling energy barleys.

There is also very little published data on associations between starch characteristics and malting quality. Starch mutations such as waxy and high amylose have been shown, in general, to have deleterious effects on HWE (Swanston et al., 1995), although some waxy lines with high extract levels have been observed (Swanston, 1996). Among cultivars with normal proportions of amylose and amylopectin, however, there have been few comparative studies, although Oliveira et al. (1994) suggested an association between granule size profiles and quality parameters.

The recent development of DNA marker based assays has permitted the simultaneous analysis of the genetic basis of malting quality (Thomas et al., 1996) and the deployment of new diagnostics. Quantitative trait loci for hot water extract have been detected in both 6-row and 2-row North American barleys (Han et al., 1997; Mather et al., 1997) and in European cultivars (Thomas et al., 1996). In addition, a number of loci affecting fermentability has also been discovered (Swanston et al., in press). This offers the potential to select for aspects of malting performance, by selecting specific DNA sequences in the unmalted grain. In this report we consider the role of simple grain assays such as milling energy, flour sieving characteristics and friabilin content and contrast them with the deployment of genetic markers such as simple sequence repeats. In addition, by deploying a range of barley cultivars, that have been developed over many years, we are able to monitor the inheritance of features common to contemporary malting barleys, through both changes in malting behaviour and in the underlying DNA sequences.

MATERIALS & METHODS

MATERIAL

A Genome Scanning Trial (GST) was assembled in 1995 by tracing the pedigrees of Prisma, Optic and Cooper (Figures 1-3). Seed of over 100 accessions (including those given in bold capitals in Figures 1-3) was obtained from the BBSRC Cereal Collection at the John Innes Centre (Dr M. Ambrose, Curator). Samples were grown in the glasshouse and DNA extracted. Grain was harvested and sown as rows in the field in 1996. After morphological verification of the plant phenotype, grain was harvested and the bulk used to sow replicated field trials in 1997 and 1998. Grain samples were dried and stored at ambient temperature before processing in laboratory tests.

MILLING ENERGY

The milling energies of 96 cultivars included in the GST were determined with the Comparamill as described by Allison et al. (1979) and further researched by Ellis et al. (1992). Triumph and Vada were used as controls in each run. Samples were precisely weighed to approximately 5g and the MEA values corrected to a sample weight of 5g.

A subset of 20 cultivars, comprising 15 with relatively low MEA and 5 with high MEA was chosen for detailed study. Samples from two field replicates of the 1997 and 1998 harvest of the GST were assessed for MEA. The milling energy data was subjected to analysis of variance (ANOVA) with and without sample weight as a covariate.

FLOUR SIEVING

Flour samples of Triumph and Vada obtained from MEA assays were separated using a range of sieves i.e. mesh sizes 75, 125, 250, 500 and 1000μ. A weighed flour sample was loaded onto the top sieve, the instrument sealed and shaken mechanically at 2mm amplitude for 10 min. Fractions retained by each sieve were collected and their weights recorded. As the best differentiation of these cultivars was achieved by measuring the proportion of the sample that passed through a 500μ sieve, the comparamill flour of 20 cultivars was separated into these two fractions.

MEASUREMENT OF FRIABILIN

Friabilin was assessed with a kit (Rhone Diagnostic Technologies Ltd) for a dipstick test and an ELISA assay both based on the reaction between grain proteins and a monoclonal antibody. The kits were designed to detect bread wheat in macaroni flour. Thus while it was possible to detect friabilin in barley flour with the commercial kits (Jagtap et al., 1993) it was still necessary to optimise grain milling, antigen extraction and ELISA plate coating and development.

FIGURE 1. Milling energy and hot water extract of cultivars in the pedigree of PRISMA

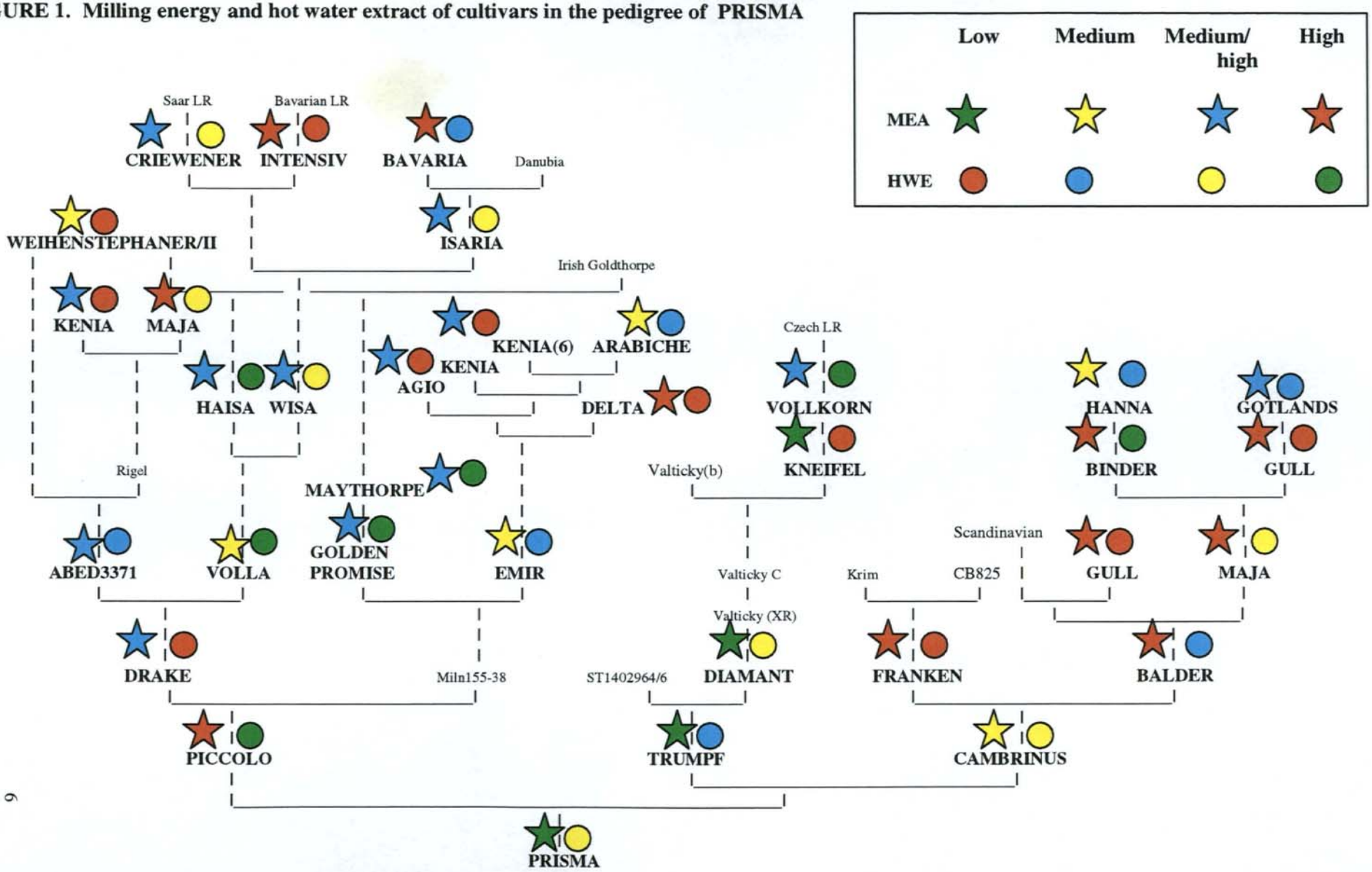


FIGURE 2. Milling energy and hot water extract of cultivars in the pedigree of COOPER.

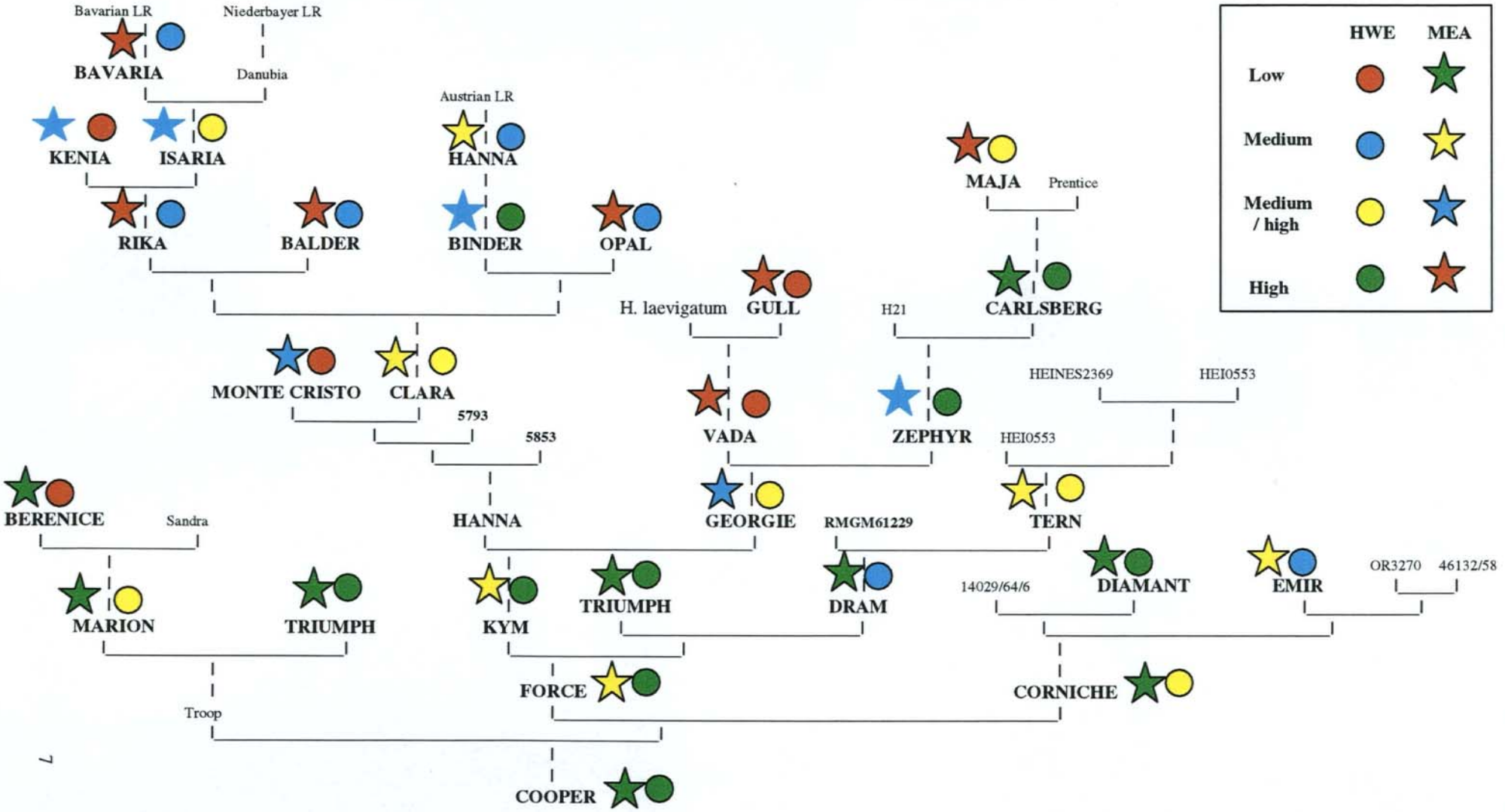
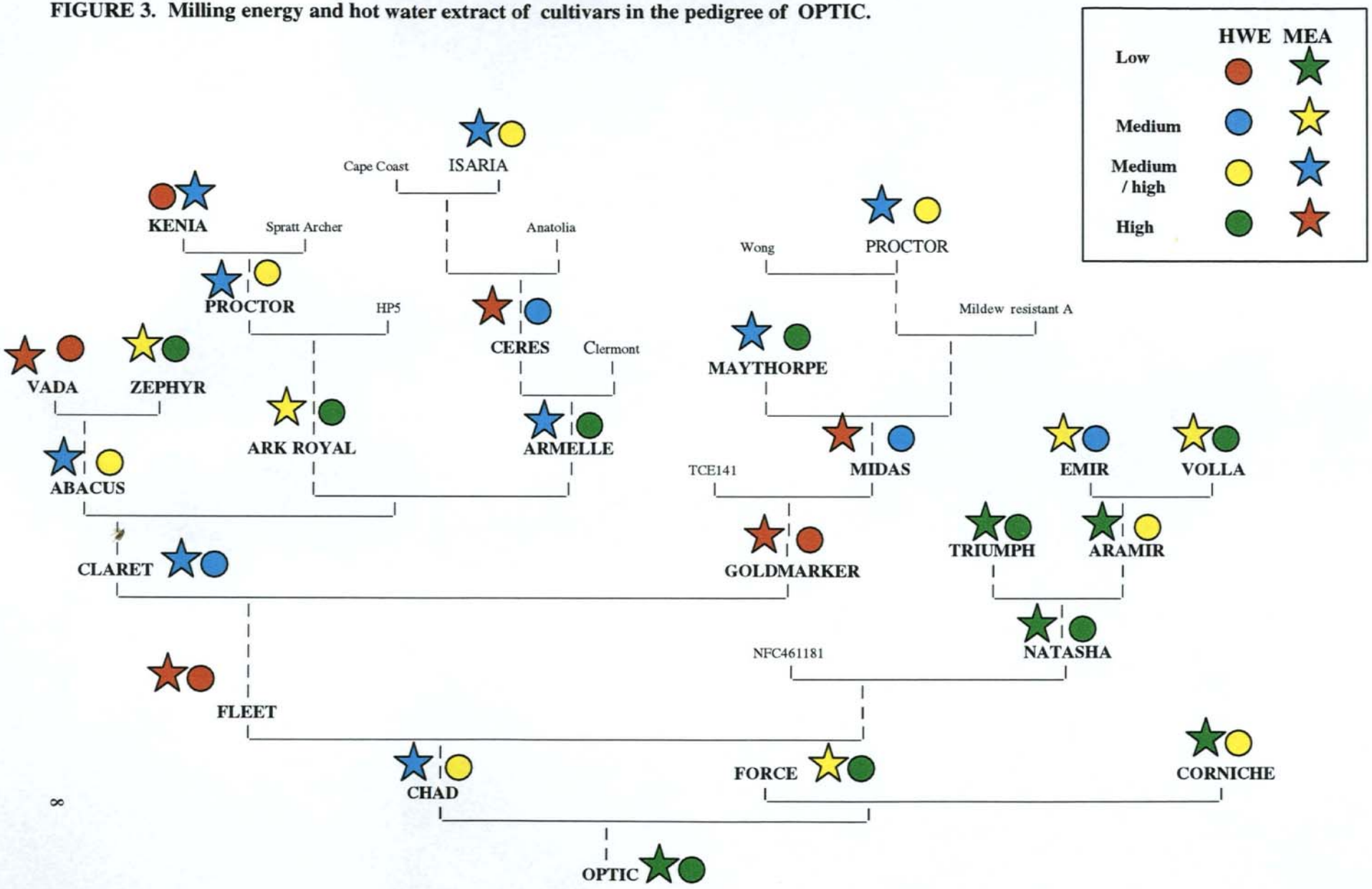


FIGURE 3. Milling energy and hot water extract of cultivars in the pedigree of OPTIC.



Identification of an optimum flour type

The Comparamill gives a heterogeneous, coarse flour, whilst the retsch mill produces a finer, more homogenous flour. An experiment was set up to compare the levels of friabilin detected by extracting flours from these mills. Triumph and Vada were extracted by adding 20mg of either flour to 1ml 100mM NaCl. Each extraction was carried out twice. The resultant extracts were serially diluted in plate coating buffer to concentrations of 0.5, 0.25, 0.125, 0.1 and 0.067% (vol/vol) and pipetted into the wells of an ELISA plate in a randomized layout, with 8 antigen negative wells containing only plate coating buffer included. The results (Figure 4.) indicated that there were significantly lower levels of friabilin extracted from the comparamill flour than the retsch mill ($P<0.01$). The 0.005% vol/vol dilution of the extracts gave absorbance values in the range 0.3 to 1.2 at 30 minutes after the addition of the substrate and was therefore considered the optimal antigen concentration for genotype screening.

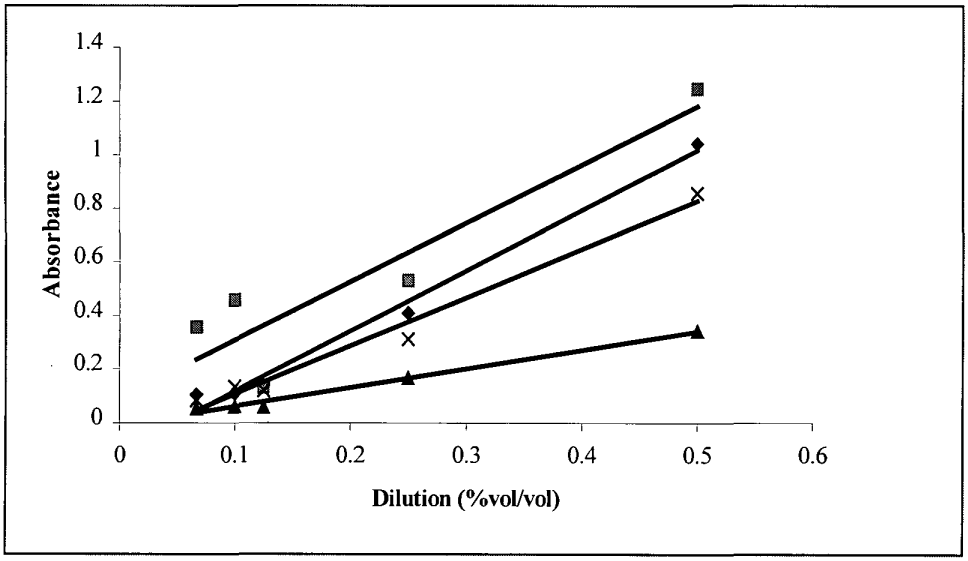


Figure 4. ELISA results for dilution series of friabilin antigen extracted with 100mM NaCl.

The treatments were:-

- ◆ Triumph - Comparamill flour
- ■ Triumph - Retch mill flour
- ▲ Vada - Comparamill flour
- x Vada - Retch mill flour

Selection of an antigen extraction medium

An extraction buffer can be purchased from Rhone Diagnostic Technologies Ltd as part of a dipstick or ELISA kit designed to assess the friabilin content of durum wheat. The composition of the "Durotest" extraction buffer is confidential and, as alternatives, 2% SDS in 50mM TRIS pH 7.2 was compared with 100mM NaCl solution. Samples of Triumph and Irish Archer were extracted and results from ELISA compared with results from extracts with the Durotest extraction buffer. The Durotest extraction buffer solubilised significantly greater friabilin than 2% SDS in 50mM TRIS pH 7.2.

The results for saline solution were not so clear. At an antigen concentration of 0.5% the absorbance readings with Durotest buffer extractions were significantly higher than those for NaCl extractions. At an antigen concentration of 5% the NaCl extractions gave similar absorbance values to the Durotest buffer extractions at 0.5%. These values were, however, considerably higher than the absorbance range recommended in the Durotest kit. While the 0.5% NaCl extraction might not extract as much protein as the Durotest buffer, the amount of friabilin present gave rise to absorbance values that differentiated the cultivars. Repeated extractions from flours derived from the same grain sample revealed that the results were consistent.

Antigen Extraction

Methods of extracting friabilin from wheat are well documented. Granule surface proteins are extracted from isolated starch by shaking the starch in 2% SDS for 30 min at 20°C (Sulaiman & Morrison, 1990), in 1M NaCl in a 3:1 v/w ratio for 30min at 5°C (Sulaiman et al., 1993) or with 1:1 propan-2-ol/100mM NaCl at room temperature for 30min (Bettge et al., 1995). For the development of a simple assay it is possible to use flour rather than extracted starch (Jagtap et al., 1993).

The first time ELISA was performed, 0.25ml Durotest Extraction Buffer (Rhone Diagnostic Technologies Ltd) was added to 5mg flour obtained from the Comparamill. The sample and buffer were mixed by drawing up and down a 1ml micropipette several times. The mixed sample was centrifuged for 3min and the supernatant serially diluted in plate coating buffer (50mM carbonate-bicarbonate pH 9.6, Sigma) to final concentrations of 5, 0.5 and 0.05% (v/v). Subsequently, so that the same treatment could be applied to each cultivar during cultivar screening, mixing, following the addition of the extraction solvent, was achieved by vortexing for 1-2min followed by incubation at room temperature with gentle mechanical rotation for 10-30min prior to centrifugation. These more vigorous extraction methods resulted in higher absorbance values being obtained for the same dilutions of antigen employed in the first ELISA, indicating a more efficient extraction.

ELISA plate coating and development

96 well microtitre plates (Greiner) were coated by pipetting 100µl antigen preparation into the wells and incubating at 37°C for 30min, then for 16h at 4°C. Plates were washed three times in TRIS buffered saline pH 8.0 with 0.05% Tween 20 (TBS-T, Sigma), dried and either developed immediately or covered with clingfilm and stored at -20°C for later use.

To develop the plates, 200µl of block solution (1% marvel/TBS-T) was added to each well and the plates were covered with clingfilm and incubated for 30min at 37°C.

Plates were washed three times with TBS-T and 100µl 1:500 dilution of anti-friabilin monoclonal antibody (stock supply at concentration of 3.1mg/ml (Rhone Diagnostic Technologies Ltd) in TBS-T was added to the wells. Plates were then wrapped in clingfilm and incubated at 37°C for 1h. Plates were washed three times with TBS-T and 100µl 1:2500 dilution of goat anti-mouse IgG/alkaline phosphatase conjugate (Sigma) was added to the wells. Plates were then wrapped in clingfilm and incubated at 37°C for 2h.

The plates were incubated with a secondary antibody, plates were washed three times with TBS-T and 100µl pNPP liquid substrate (Sigma) was added to 12 wells as a reagent blank for the absorbance measurement, and to all test/control wells. The plates were wrapped in clingfilm and incubated at room temperature for a minimum of 30min. Absorbance was read at 405nm (Titertek Multiskan Plus) directly following completion of the incubation period, or following the suspension of the reaction after 30min by the addition of 25µl 2M NaOH to all wells.

Screening of barley cultivars with methods established for wheat

Flour samples from the Comparamill of 96 cultivars were screened for friabilin using a Durotest P Kit (Rhone Diagnostic Technologies Ltd) developed for the detection of non-durum wheat adulterants in pasta. Following extraction of 75mg flour in 0.75ml Durotest extraction buffer, 300µl supernatant was added to 4.2ml Durotest plate coating buffer and the absorbance of the samples at 280nm was read using plate coating buffer as a blank. In order to normalise the protein concentration of all samples they were diluted in coating buffer according to the absorbance readings obtained. The following equations were employed to calculate the dilution required for each sample:-

0.25/Sample OD (to be diluted) x 2ml = Volume of Sample

Volume of plate coating buffer required = 2ml - Volume of Sample

As the levels of friabilin present in the samples to be tested are higher than those expected in adulterated pasta a 1:100 dilution was performed prior to ELISA. Thus the volume of sample required was mulitiplied by 0.1 and made up to 2ml in Durotest plate coating buffer and a further 10 fold dilution was prepared by adding 100ml of this solution to 900ml of plate coating buffer. 100µl of the diluted sample was delivered to each well of a 96 well microtitre ELISA plate (supplied as part of the kit). The coated plate was covered in clingfilm and incubated at room temperature overnight. The plate was then stored at 4°C for 3.5hr prior to development. The plate was washed x 4 with Durotest wash buffer and 100µl 1:3000 diluted Durotest antibody-HRP conjugate was added to the wells. The plate was covered in clingfilm and incubated at 37°C for 90min and then washed x 6 with Durotest wash buffer and 100µl Durotest TMB reagent was added to the wells. The plate was incubated for 20min at room temperature in the dark and 100µl Durotest stop solution was delivered to each well and the absorbance was measured at 450nm (Titertek Multiskan Plus blanked on air).

STARCH EXTRACTION

Starch was extracted from a subset of cultivars, selected on the basis of milling energy and friabilin assays. The methods employed to isolate starch from the grain were based on those developed by Sulaiman and Morrison (1990). 10g grain was ground in a malt mill and the coarse flour steeped at 4°C for 16h in 100ml 0.02M HCl. The solution was neutralised with 0.2M NaOH and ground using an automated mortar and pestle for 10min. The slurry was poured onto a 150μm sieve and the remaining material returned to the mortar for a further 5min grinding. The suspension was centrifuged at 3000g for 10min and the supernatant decanted. The pellet was layered on top of 25ml CsCl (80% w/v) and centrifuged at 18000g for 45min. The CsCl was decanted and the centrifugation of the pellet was repeated through 25ml fresh CsCl (80% w/v). The starchy pellet was washed three times with distilled water, centrifuging between washes at 3000g for 10min. Finally the pellet was washed in acetone and air dried in the centrifuge tube.

Following drying, the starch pellets required further purification steps to remove contaminating protein. The pellet was re-suspended in CsCl (80% w/v) and centrifuged at 18000g for 45min and the supernatant decanted. The pellet was then suspended in 30ml fresh CsCl and the centrifugation repeated. The supernatant was decanted and washed 4 times in distilled water, with centrifugation at 3000g for 10min between washes. The pellet was then washed twice in acetone. Following the second acetone wash the supernatant was decanted and the sediment was scooped out of the tube onto a glass surface and left to dry for 16h. A free-flowing white powder was obtained upon drying. Coulter counter analysis of starch granule size was carried out at Glasgow Caledonian University, according to the methods described by Morrison & Scott (1986).

MALTING

Grain samples (30g) were malted using the SCRI automated system (Swanston, 1997). Determinations of HWE and Fermentability were by the Institute of Brewing Recommended Methods (1982) as scaled down by Swanston & Thomas (1996), so that 20g of grist was extracted and the final volume made up to 206ml. Fermentabilities were assessed on 100ml samples after incubation with 0.5g yeast for 48hrs. Wort viscosity was measured using a Brookfield viscometer fitted with a low centipoise adaptor (Greenberg & Whitmore, 1974). Soluble nitrogen (mg/L) was determined by a spectrophotometric method (Haslemore & Gill, 1995) and the result was converted to a percentage by weight of the dry malt using an appropriate equation.

SIMPLE SEQUENCE REPEATS (SSR)

DNA was extracted from 3-week-old leaf material (Saghai Maroof et al., 1994), SSR-containing DNA sequences were obtained from enriched microsatellite libraries (Lui et al., 1996) or from Research Genomics, Inc. (Huntsville, USA) as described by Waugh et al. (1997). The association of SSR and traits in the germplasm was investigated by analysis of variance (Beer et al., 1997).

RESULTS

The results are presented in two main sections, the first involving 96 cultivars from the Genome Scanning Trial and in the second a subset of these cultivars assayed over two field replicates in two seasons. Malting assays differ from the grain tests in that all the cultivars were assessed in both seasons. The results derive from material grown in the East of Scotland where performance may not match that seen in environments where the genotypes are better adapted. For example Proctor was regarded as too late maturing and was not grown in Scotland while it was the leading spring malting barley in East Anglia. In comparison, the introduction of Golden Promise led to an expansion of the Scottish barley area but was too early, disease susceptible and low yielding to be profitable in East Anglia.

GENOTYPE SCREENING

Milling energy

The range of MEA values obtained for the cultivars tested was between 709J (for Tankard) and 998J (for Goldmarker) (Figure 5). The least significant difference (df = 95) was 22.2 J so that two "steps" in the histogram from 709 – 721 (Tankard, Natasha) to 751 – 795 (Prisma, Alexis) indicate significant changes in MEA.

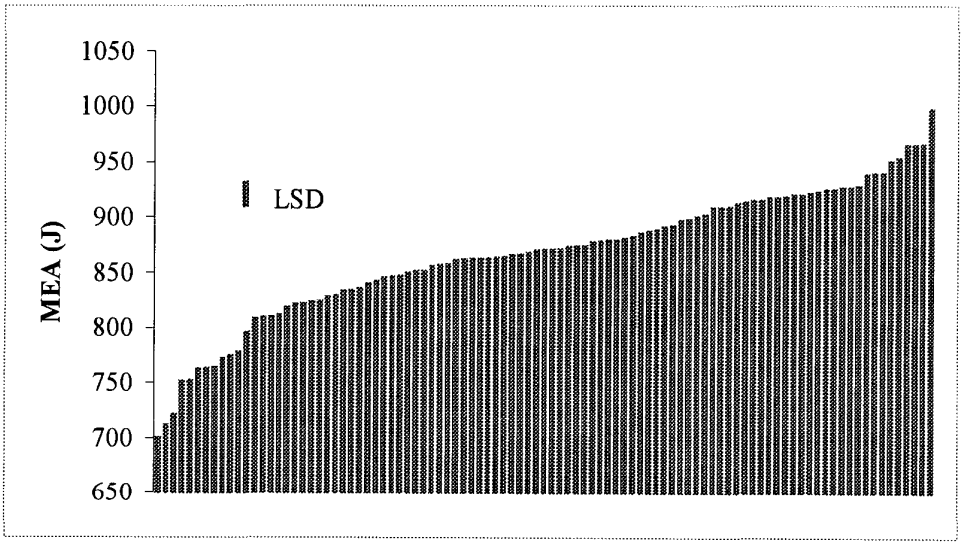


Figure 5. Milling energy (MEA) in 96 cultivars from the GST ranked in ascending order. LSD = least significant difference P = 0.05. Low MEA is indicative of high HWE.

Friabilin assay - Durotest P cultivar screen

The results of an assay of cultivars with the the Durotest P kit are presented in Figure 6. The lowest level of friabilin was in Emir (0.24) and the highest in Weihenstephaner II (1.14). The use of a protein assay to convert the results to a standard did not reduce of the population variances (CV 31% uncorrected and CV

27% corrected) nor did, within the limits of experimental error, the cultivar ranks change. The results of a similar cultivar screen for friabilin, but with the use of 100mM NaCl to extract samples are given in Figure 7. When these assays were plotted against each other it was obvious that the results were not closely related e.g. the lowest level occurred in Sherpa (0.35) and the highest in Haisa (1.98). Neither assay of friabilin indicated an association with HWE or MEA.

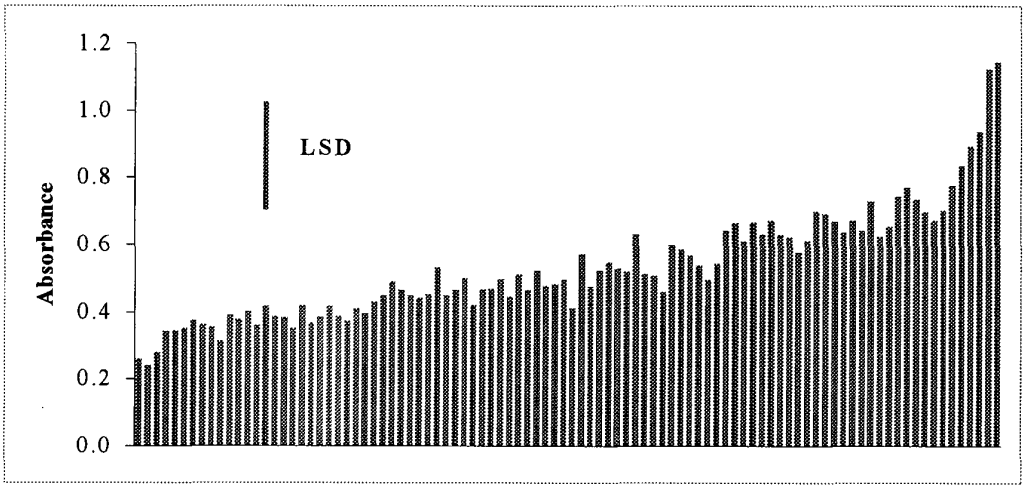


Figure 6. Friabilin assay results for flours from cultivars extracted with "Durotest" kit buffer, ranked according to absorbance in NaCl extract (Figure 7), and plated on an ELISA plate from the "Durotest" kit. Low friabilin is associated with soft texture in wheat. LSD = least significant difference at P = 0.05.

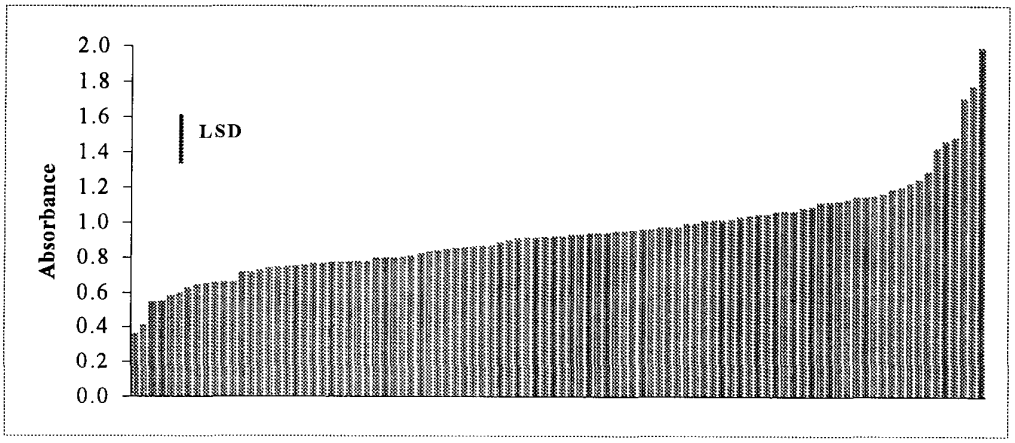


Figure 7. Friabilin assay results for flours from cultivars, ranked by absorbance, extracted with 100mM NaCl and plated onto an ELISA plate.

Malting Quality Results

Extract data covered a very wide range, lowest levels being associated with sources of mildew resistance, such as Monte Cristo (253 L°/Kg) and Weihenstaphener II or feed cultivars such as Hart and Vada. Highest extracts were found in contemporary malting cultivars Cooper (313 L°/Kg), Derkado and Optic. Plots of friabilin, extracted in either sodium chloride solution or Durotest buffer, against HWE (Figure 8a and b) showed no relationship, suggesting that proteins on the starch granule surfaces do not, over all cultivars, exert a major influence on endosperm modification.

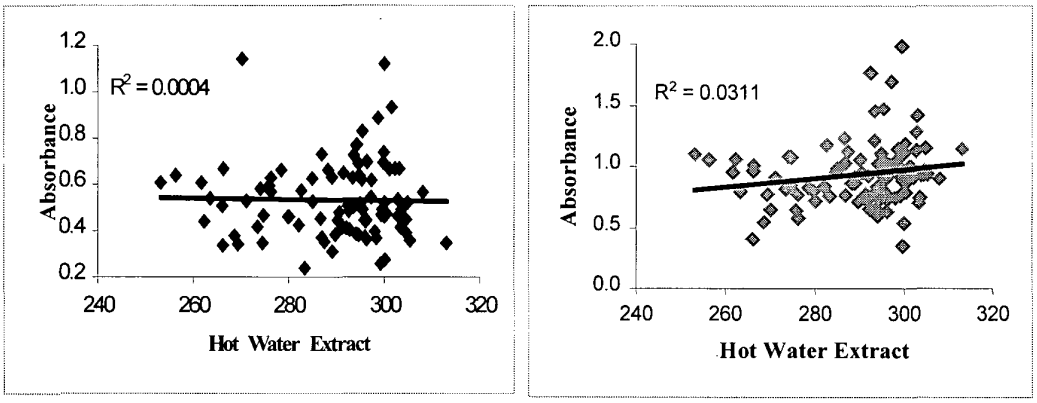


Figure 8. Friabilin content after extraction in either (a) Durotest buffer (left) or (b) sodium chloride solution (right) plotted against Hot Water Extract (L°/Kg) for 96 genotypes.

Milling energy plotted against HWE (Figure 9) did not demonstrate a significant correlation, but all genotypes with HWE above 300 L°/Kg were characterised by low or moderate milling energy. All genotypes with HWE below 280 L°/Kg had moderate or high milling energy, while genotypes between 280 and 300 L°/Kg demonstrated a very wide range of milling energies, in agreement with previously published results (Swanston et al., 1990).

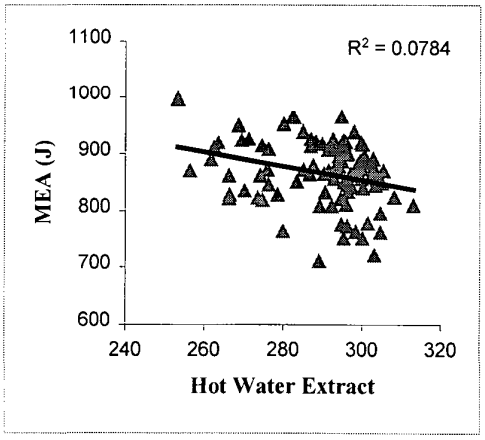


Figure 9. Milling energy plotted against Hot Water Extract (L°/Kg) for 96 barley genotypes.

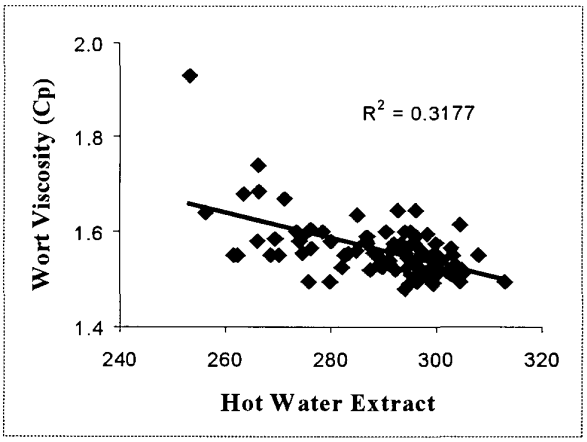


Figure 10. Wort viscosity and Hot Water Extract (L°/Kg) for 96 genotypes.

When factors such as wort viscosity and soluble nitrogen, which affect modification of the cell wall and proteinaceous material, respectively, were plotted against HWE, significant correlations were observed (Figures 10 and 11). Wort viscosity is affected by a number of factors such as grain β-glucan content and the rate at which it is solubilised and degraded during the malting and extraction processes. Soluble nitrogen reflects the integrity of the protein matrix surrounding the starch granules and the degree to which this is disrupted. Cell wall and protein modification are both potentially limiting factors in the shorter malting cycles used in contemporary malting plants, compared to traditional floor maltings, and inadequate modification will reduce the availability of starch for liquefaction and breakdown to fermentable sugars. Although HWE is predominantly starch derived, the actual profile of sugars and dextrins will determine fermentability. In addition, protein derived material, while contributing to specific gravity and, therefore, HWE, is not fermentable. Swanston and Thomas (1996) suggested that HWE and fermentability were under separate genetic control. The lack of any significant correlation between the two factors, here (Figure 12), supports that hypothesis.

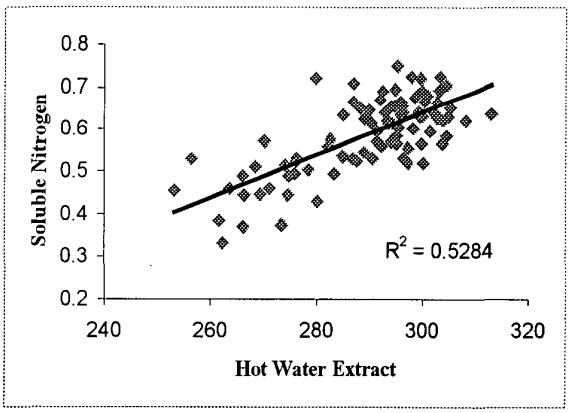


Figure 11. Soluble nitrogen plotted against Hot Water Extract for 96 genotypes.

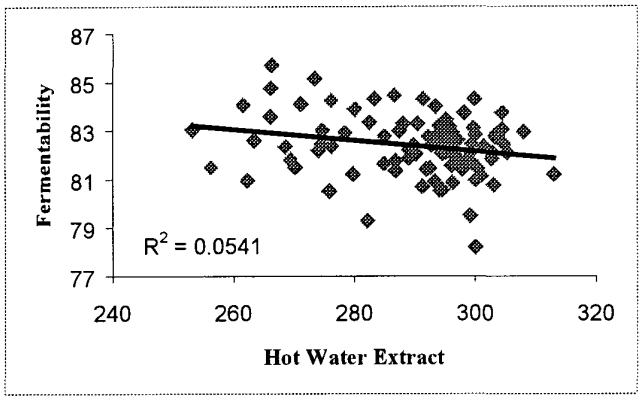


Figure 12. Fermentability plotted against Hot Water Extract L°/kg for 96 genotypes.

MEA has previously been considered to be a good predictor of malting potential because low values were obtained for cultivars with high levels of HWE. Genotypes with the lowest MEA may not have the highest hot water extract in a simple test as specific malting regimes may be necessary to produce the optimum result.

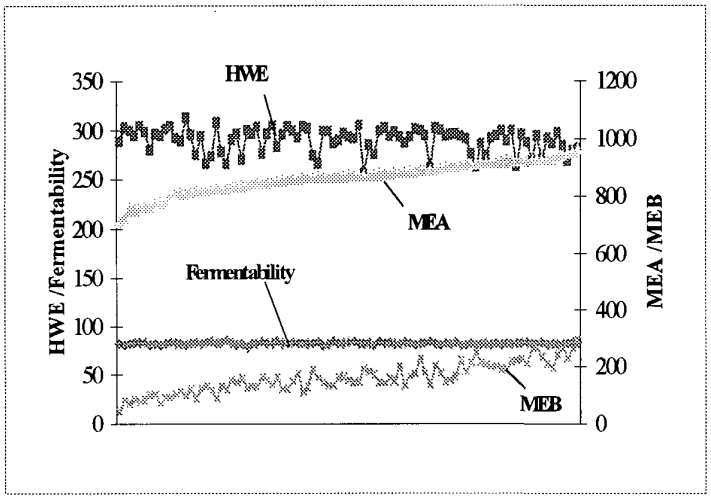


Figure 13. The relationship of MEA (J) and MEB (J) with Hot Water Extract L°/kg and Fermentability %. Cultivars are ranked by MEA (J).

To be viable, any screening programme, for both barley breeding and malting processes, must apply a single regime. Examples of the relationships between malting parameters and MEA/MEB are given in Figure 13 from which it can be seen that there is a consistent increase in HWE for a decrease in MEA or MEB. While a linear regression on cultivars ranked by MEA accounts for much of the variation (r^2 = 0.92 for MEA) (r^2 = 0.80 for MEB) only a small amount of the variation in HWE was accounted for by the regression (r^2 = 0.05). It would appear that this relationship would be improved by greater precision in the estimation of HWE. In contrast, fermentability showed no close relationship with milling energy or hot water extract.

The relationship between MEA and MEB is not linear (Figure 14) but it was possible to fit a curve that accounts for most of the variation. The implication of this relationship is that while MEA can be considered to be linear over a restricted range there is a tendancy for this to change at the extremes of the range.

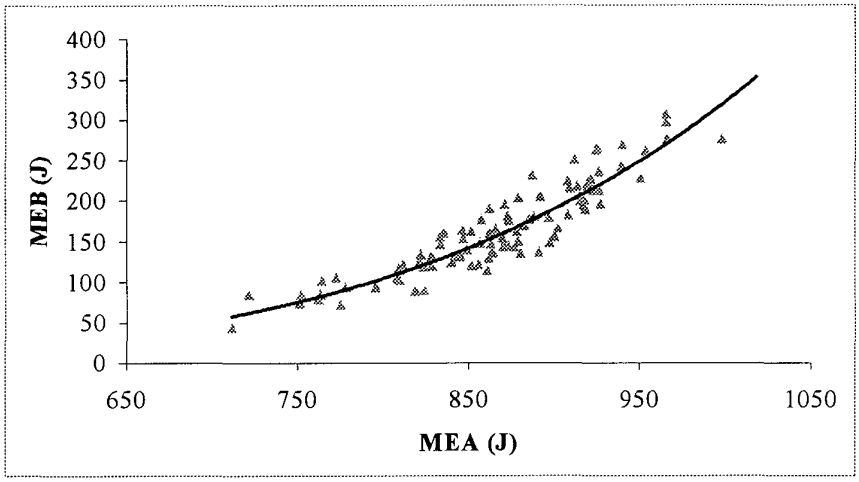


Figure 14. MEA plotted against MEB. The trend line fitted to the data points has the equation $y = 2E\text{-}13x^{5.0408}$ and $r^2 = 0.86$

ASSOCIATION WITH SSRs IN A SUBSET OF CULTIVARS

MILLING ENERGY

The milling energies of a subset of cultivars sampled from different field replicates and harvests are listed in Table 2. ANOVA indicated significant differences in the MEA values between cultivars ($P<0.001$), between seasons ($P<0.05$) and a significant interaction between these variables ($P<0.01$). The mean MEA values obtained for each year are plotted against each other in Figure 15 indicating a close relationship ($r^2 = 0.80$) between these assays.

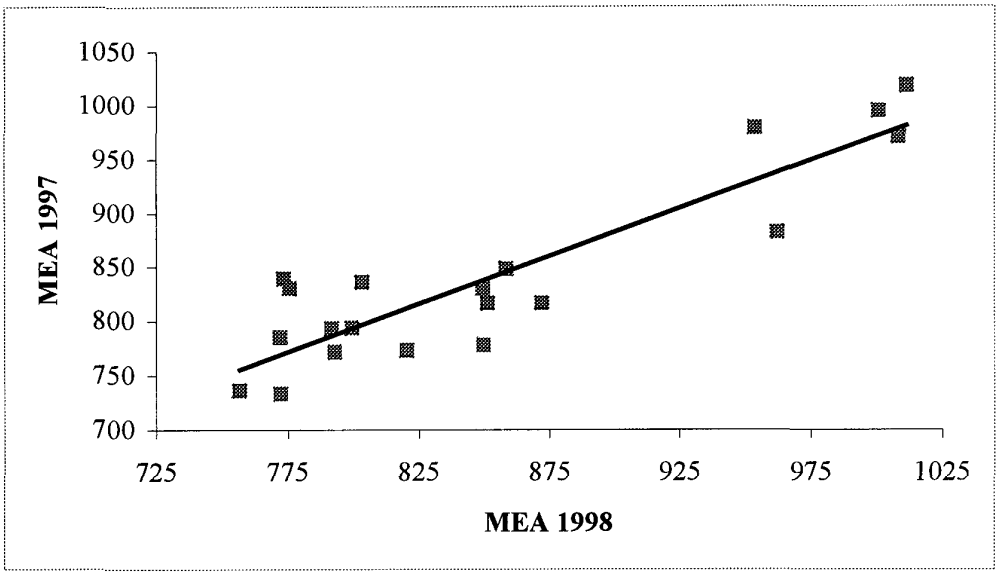


Figure 15. The relationship over two seasons for MEA in cultivars chosen for detailed studies of grain composition.

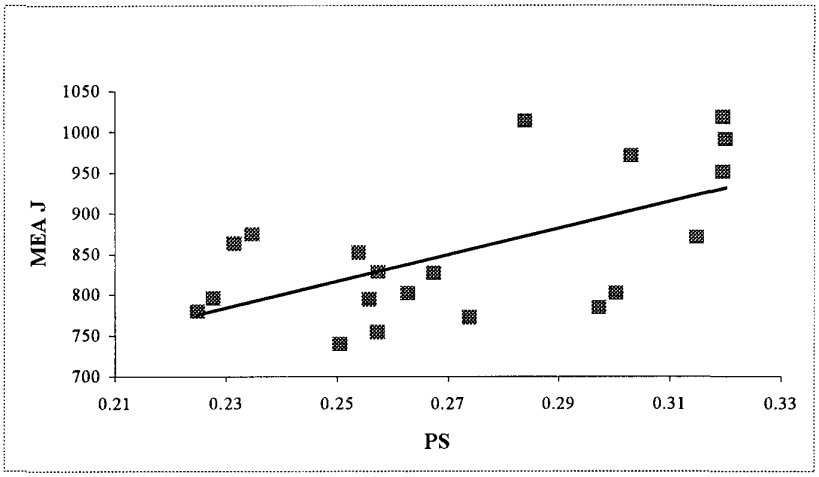


Figure 16. MEA and the proportion of flour samples retained by a 500μ sieve (PS).

SIEVING

The percentage of original weight represented by particles >500μ (PS) was plotted against milling energy (Figure 16). The relationship between MEA and PS resulted in an r^2 of 0.36, P = 0.05.

FRIABILIN

NaCl extraction

The mean absorbance of twenty cultivars from two field reps in two seasons was assessed for each cultivar and the results for duplicate extractions were plotted to assess the repeatability of the ELISA (Figure 17). ANOVA indicated that each time the experiment was performed there were significant differences between cultivars (first experiment, $P<0.05$, second experiment $P<0.001$) and between seasons (first experiment $P<0.05$, second experiment $P<0.001$), but no significant interaction between cultivar and year. ANOVA of the combined data indicated no significant interaction between cultivar and time of test. The mean absorbance values for 1997 were plotted against the mean absorbance values for 1998 (Figure 17).

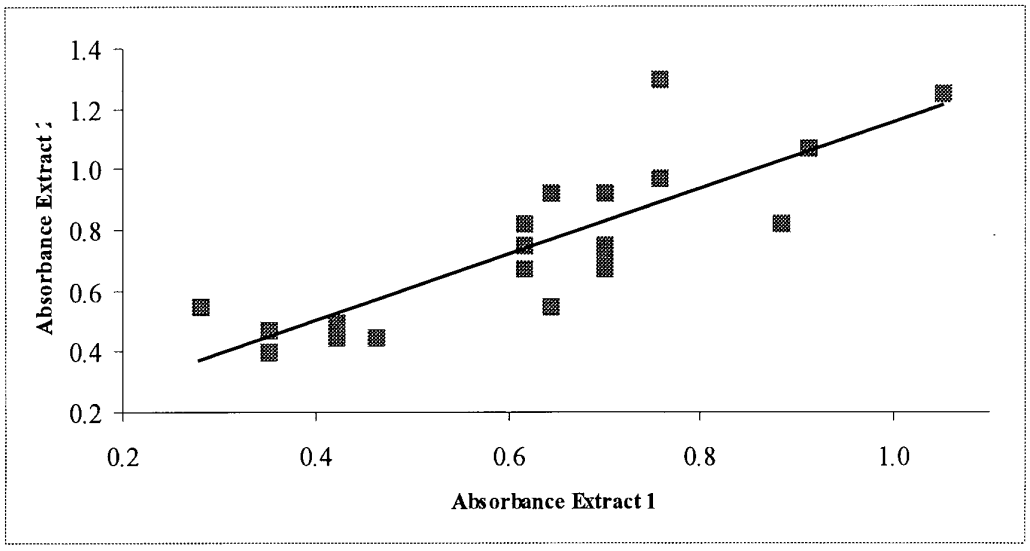


Figure 17. Repeatability of friabilin ELISA assays in flours of twenty cultivars, extracted in saline solution, assessed in two seasons.

STARCH EXTRACTION

The data obtained by Coulter counter analysis of starch isolated from cultivars are presented in Table 1. The mean diameter of A-granules was not closely correlated with HWE (Figure 18, $r^2 = 0.20$) but the mean HWE for cultivars with A-granules larger than 14.5μ was 14 L°/kg higher (at 295.7 L°/kg) than those with smaller granules. The percentage of B-granules by volume was also loosely associated with HWE (Figure 19, $r^2 = 0.26$) so that cultivars with less than 6% of the starch in B-granules by volume, had a mean HWE of 299.5 L°/kg in contrast to a mean of 287.2 L°/kg for those with a larger proportion of B-granules. The relationship of starch properties with HWE is summarised by the parameter specific surface area (SSA) which shows a correlation of $r = 0.55$ ($r^2 = 0.30$) with HWE (Figure 20, page 24).

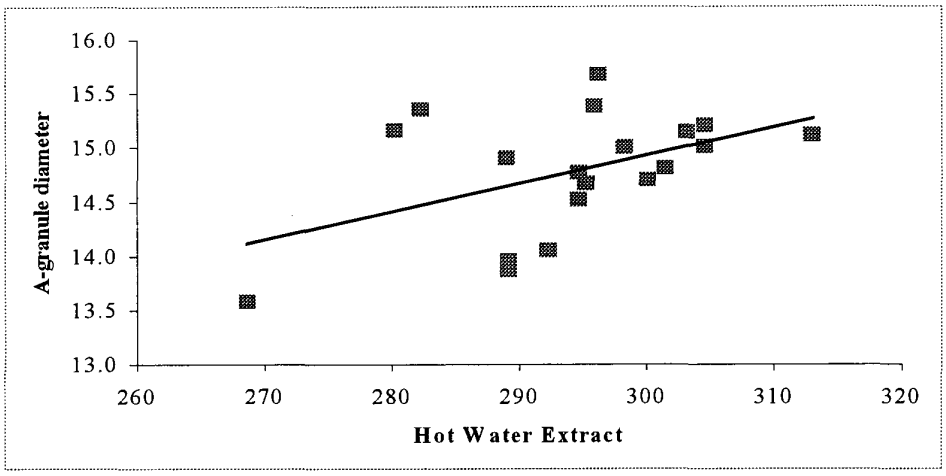


Figure 18. The relationship between HWE (L°/kg) and starch A-granule diameter in selected cultivars ($r^2 = 0.20$).

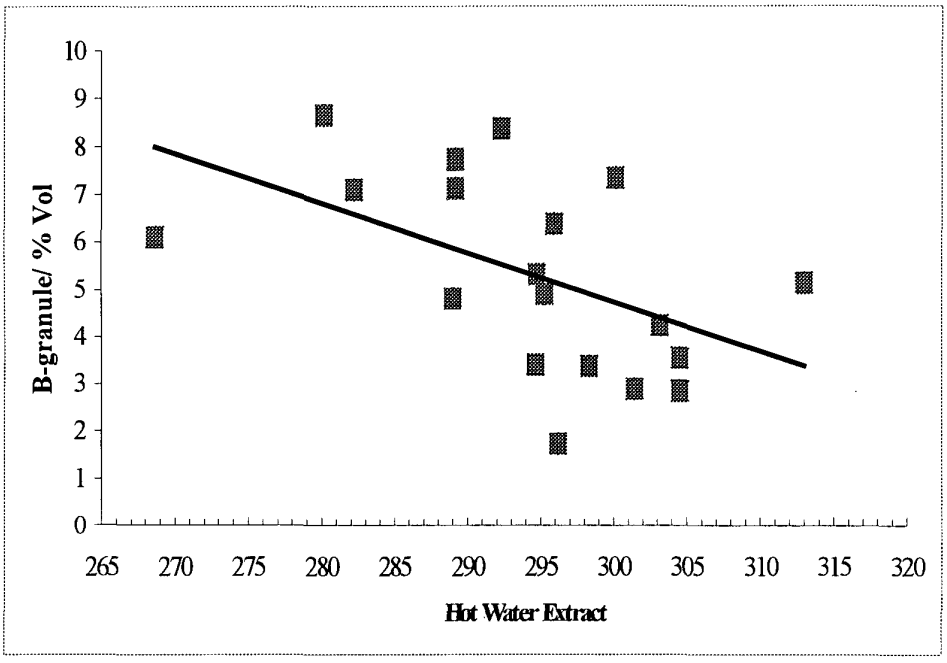


Figure 19. The relationship between the proportion of B-granules and HWE (L°/kg) ($r^2 = 0.26$).

Cultivar	HWE L°/kg	A- Granules				B- Granules						Specific Surface Area
		Mean diameter μ	Modal diameter μ	Surface Area μm^2	Volume μm^3	B- % by number	B-% by volume	Mean diameter μ	Modal diameter μ	Surface Area μm^2	Volume μm^3	
Vada	268.7	13.58	13.60	608.1	1509	81.67	6.12	3.314	3.014	40.25	29.88	0.336
Koru	280.2	15.16	14.74	754.8	2076	88.63	8.66	3.310	2.851	40.50	30.56	0.319
Balder	282.2	15.35	15.07	773.1	2154	86.68	7.10	3.405	2.851	43.47	35.08	0.308
Tankard	289.0	14.90	15.07	733.7	2008	76.07	4.81	3.696	3.340	49.29	39.52	0.294
Dram	289.2	13.87	13.28	635.1	1618	79.08	7.13	3.674	3.014	50.13	41.78	0.322
Kneifel	289.2	13.96	13.93	640.5	1626	81.32	7.73	3.563	3.340	45.69	35.07	0.331
Trumpf	292.3	14.05	13.77	649.3	1658	89.18	8.38	3.082	2.688	34.79	24.21	0.343
Chariot	294.7	14.77	14.58	720.5	1953	70.37	3.40	3.589	3.014	47.55	38.62	0.288
Tyne	294.7	14.52	15.23	701.7	1894	85.19	5.32	3.060	2.688	34.88	24.93	0.309
Prisma	295.3	14.67	15.23	707.8	1892	72.59	4.89	3.751	3.014	52.23	44.51	0.297
Diamant	295.9	15.38	15.23	782.6	2218	85.91	6.38	3.401	2.851	43.17	34.28	0.292
Aramir	296.2	15.67	15.88	812.3	2340	58.33	1.73	3.558	2.851	47.37	38.82	0.261
Corniche	298.3	15.00	15.40	743.3	2043	73.42	3.36	3.385	3.014	42.23	32.26	0.285
Carlsberg	300.1	14.70	14.25	711.6	1910	87.81	7.33	3.180	2.688	37.35	27.25	0.319
Triumph	301.5	14.81	14.58	726.7	1984	72.27	2.87	3.269	2.851	39.91	30.38	0.284
Natasha	303.2	15.14	14.74	754.6	2083	81.76	4.23	3.288	3.014	39.98	30.51	0.295
Alexis	304.6	15.01	15.40	743.8	2046	81.25	3.53	3.385	3.014	42.23	32.26	0.285
Derkado	304.6	15.20	14.91	762.3	2121	71.52	2.83	3.368	2.851	42.34	33.02	0.279
Cooper	313.0	15.11	14.09	751.2	2067	84.30	5.13	3.289	2.851	40.02	30.47	0.302
Mean	294.3	14.8	14.7	721.7	1957.9	79.3	5.3	3.4	2.9	42.8	33.3	0.3
SD	9.83	0.55	0.68	52.97	213.53	7.79	2.00	0.19	0.18	4.76	5.32	0.02

Table 1. Starch granule parameters measured in selected cultivars. Starch was extracted from flour and assessed with a Coulter counter. Cultivars are ranked by descending estimates for HWE in the micromalting of grain samples grown at SCRI near Dundee.

Cultivar	HWE	Ferment - ability	Fermen- table Extract	Wort Viscosity	Soluble Nitrogen%	ME A	ME B	Friabilin (NaCl)	Friabilin (Durotest)	Protein (Durotest)
Alexis	304.6	83.8	66.3	1.62	0.71	795.6	93.8	0.936	0.451	0.979
Aramir	296.2	81.6	62.7	1.53	0.66	772.2	105.2	0.637	0.365	1.097
Balder	282.2	79.3	58.2	1.53	0.56	965.0	306.9	0.840	0.427	0.968
Carlsberg	300.1	82.8	64.6	1.51	0.52	751.0	73.3	1.112	0.463	1.011
Chariot	294.7	80.6	61.7	1.52	0.57	775.0	72.5	0.736	0.382	1.031
Cooper	313.0	81.2	66.0	1.50	0.64	809.4	102.5	1.140	0.349	1.123
Corniche	298.3	83.8	64.9	1.60	0.60	762.8	85.1	1.042	0.370	1.080
Derkado	304.6	83.1	65.8	1.50	0.59	762.2	78.6	0.965	0.394	1.034
Diamant	295.9	82.6	63.5	1.58	0.67	811.4	122	1.039	0.375	0.991
Dram	289.2	81.9	61.5	1.53	0.63	808.6	115.9	0.864	0.312	1.165
Kneifel	279.9	81.2	59.1	1.50	0.72	763.9	101.7	0.803	0.462	0.979
Koru	280.2	83.9	61.1	1.58	0.43	953.2	261.6	0.723	0.463	0.939
Natasha	303.2	80.8	63.6	1.55	0.70	721.2	84.7	1.414	0.670	0.931
Prisma	295.3	83.5	64.0	1.51	0.57	751.7	84.9	1.007	0.622	1.038
Tankard	289.0	82.2	61.7	1.55	0.55	711.4	43.7	0.914	0.633	0.959
Triumph	301.5	82.4	64.5	1.53	0.60	777.5	93.5	1.002	0.934	0.937
Trumpf	292.3	82.8	62.9	1.52	0.56	808.0	104.6	0.654	0.511	1.006
Tyne	294.7	82.7	63.3	1.53	0.66	965.7	276.6	0.796	0.640	0.990
Vada	268.7	82.4	57.6	1.55	0.51	950.6	227.8	0.548	0.380	1.011
Mean	293.8	82.2	62.8	1.54	0.60	811.4	128.2	0.90	0.48	1.01
SD	10.30	1.19	2.45	0.03	0.07	80.65	75.41	0.20	0.15	0.06
Cv	3.5	1.4	3.9	2.2	12.0	9.9	58.8	22.3	30.9	6.2

Table 2. Malting quality, milling energy and friabilin scores for the cultivars studied.

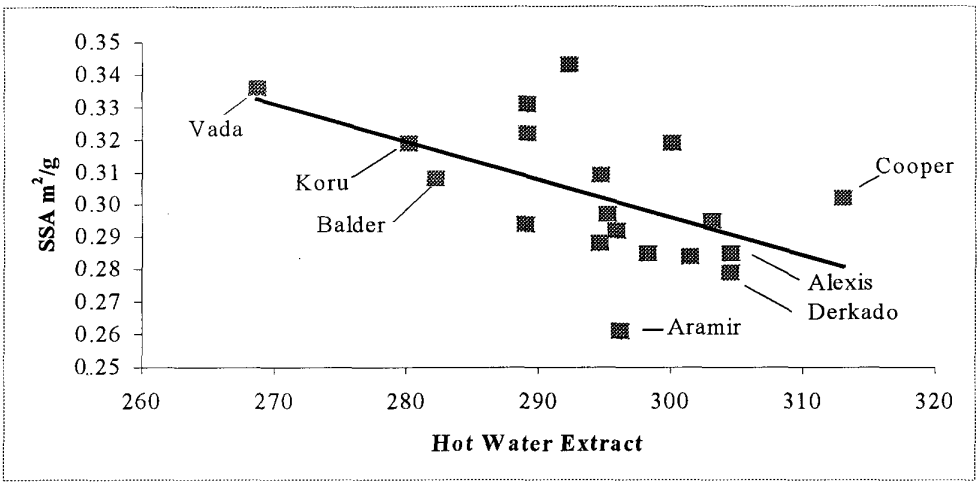


Figure 20. Starch specific surface area (m^2/g) in relation to HWE L°/kg of selected cultivars.

MALTING

The results of micromalting assays in the selected cultivars are given in Table 2. HWE varied from 268.7 L°/kg in Vada to 313.0 L°/kg in Cooper. The high HWE of Cooper resulted in Cooper having the highest Fermentable Extract although not having the most fermentable wort (Figure 21). A plot of Fermentable Extract against Soluble Nitrogen indicated the importance of endosperm protein degradation to the achievement of high HWE (Figure 22). While the highest level of Soluble Nitrogen was not always associated with the highest level of Fermentable Extract there appears to be a critical limit.

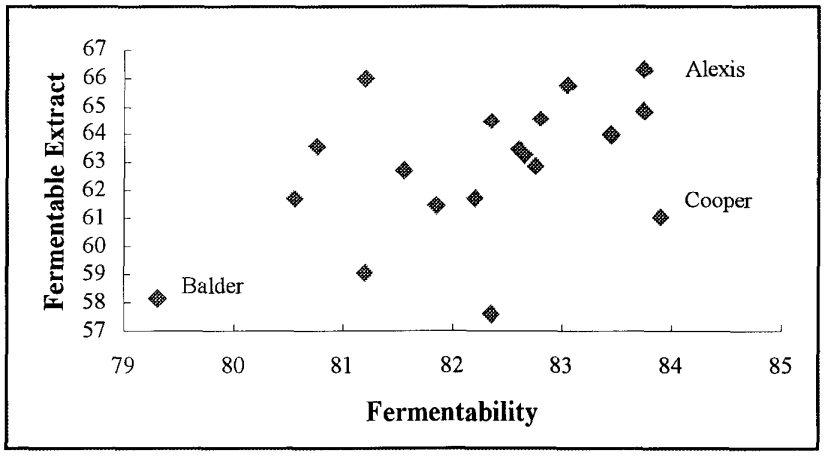


Figure 21. Wort fermentability and HWE L°/kg are not related in the cultivars examined so Fermentable extract shows a weak relationship to Fermentability.

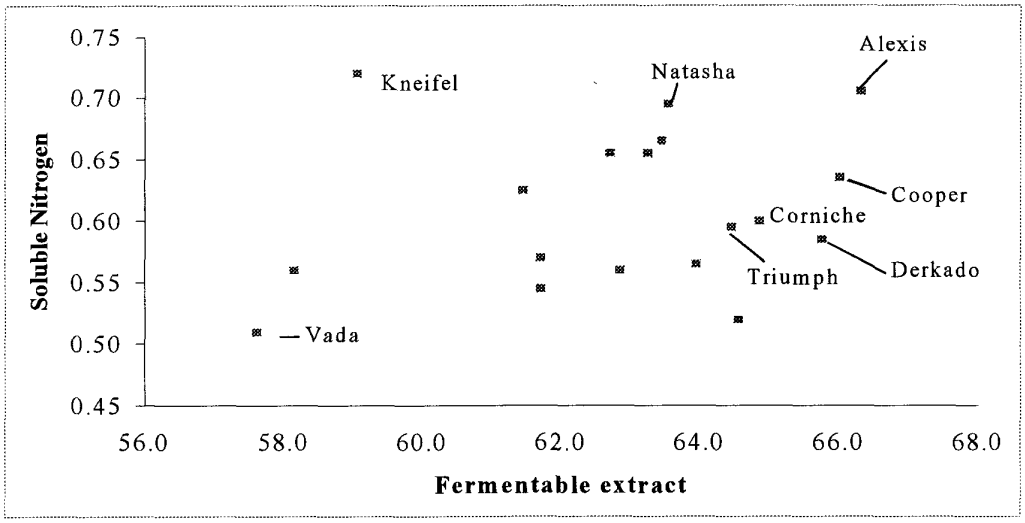


Figure 22. Fermentable Extract and Soluble Nitrogen.

Figure 24. Trait symbols - see page 27

Grain (blue)	MEA	milling energy A
	MEB	milling energy B
	PSI	particle size
	XPR	extractable protein
Malt (pink)	HWE	hot water extract
	FER	wort fermentability
	FERX	fermentable extract
	SOLN	soluble nitrogen
Starch (green)	B-dia	mean diameter of B-granules; B-mod B-granule modal daimeter
	B-SA	surface area of B-granules; B-vol B-granule volume
	B%	number of B-granules as % of total granule number
	DUR	friabilin – protein extracted with Durotest buffer
	NaCl	friabilin – protein extracted with NaCl
	SSA	specific surface area of the starch

Plant morphology – see characters given in black type.

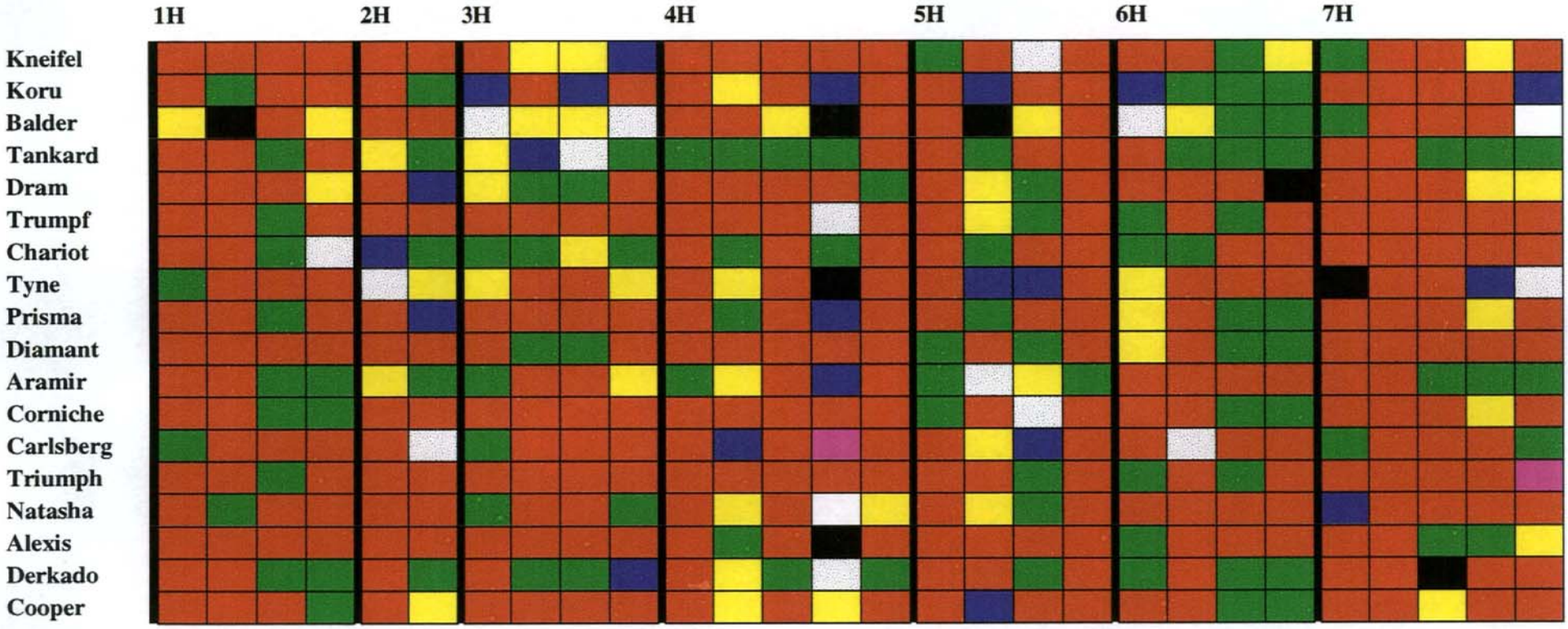


Figure 23. Genotypes of selected cultivars used to examine association between SSR and malting quality traits. The cultivars are ranked top to bottom in increasing levels of HWE and the alleles are colour coded in order of the frequency of their occurrence in these cultivars from red through green, yellow, blue, gray and white to pink (black = no data).

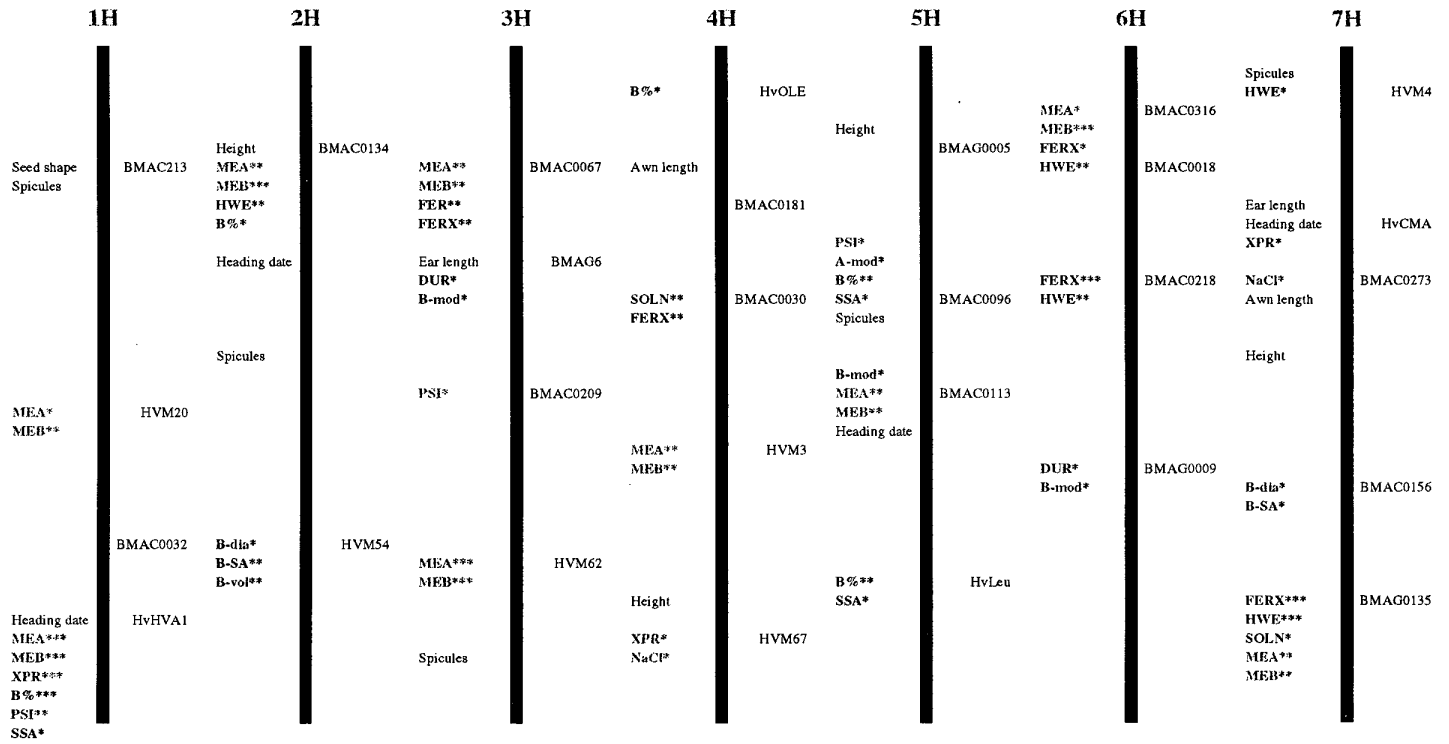


Figure 24. Associations detected by ANOVA between SSRs and traits related to grain, malt, starch and plant morphology. See text for trait symbols. Trait positions are given on the left of each chromosome while the marker positions are on the right.

*** P< 0.001

** P< 0.01

* P 0.05

SIMPLE SEQUENCE REPEATS (SSR)

SSR were assayed in the cultivars that make up the pedigree of Cooper, Optic and Prisma. It was not possible to obtain seed of all the lines recorded in these pedigrees and care had to be taken to ensure that all the material was authentic at the point of assay. As in the case of a similar, but smaller exercise, when AFLP were used to genotype spring barley cultivars (Ellis et al., 1997), SSR showed that considerable genetic change had occurred through the period of cultivar development. The pattern of variation for cultivars is shown graphically in Figure 23.

A comparison of cultivars with wide and close differences in HWE production showed nearly the same genetic diversity. For example Cooper (HWE 313 L°/kg) differs from Kneifel (HWE 279 L°/kg) at 14 loci while Cooper and Derkado (HWE 304.6 L°/kg) in turn differ at 11 loci (Figure 23).

The SSRs have been mapped to chromosome locations in a population of doubled haploids from the cross Lina x HS92 (Waugh et al., 1997). Associations between malting traits and starch characteristics were examined by analysis of variance. The results of significant associations are summarised in Figure 24, which indicates that HWE showed significant association with five loci (on chromosomes 2H, and two each on chromsomes 6H and 7H). The most significant association (***, $P < 0.001$) was at the position of BMAG0135 on chromosome 7H, which was also associated with Soluble Nitrogen, Fermentable Extract and Milling Energy. HWE and Milling Energy were also associated with the locus BMAC0134 on chromosome 2H, together with the proportion of B-granules by number. Milling Energy was associated with nine loci, most significantly with HvHVA1 on chromosome 1H and HVM62 on chromosome 3. HvHVA1 also showed association with Extractable Protein (Durotest), Particle Size after milling, the proportion of B-Granules by number and Specific Surface Area of the starch.

Fermentable extract was associated with five loci, BMAG0067 on chromosome 3H, as noted above, BMAC0030 on chromosome 4H, BMAC0018 and BMAC0218 on chromosome 6H and BMAG0135 on chromosome 7H. The combined associations at these loci seemed particularly interesting as they indicated possible interactions with traits influencing milling energy, endosperm protein and hot water extract.

Starch granule characters were associated with eleven loci and the majority of these associations were with B-granule characteristics. The most significant association was with the HvHVA1 locus on chromosome 1H but B-granule dimensions alone were associated with HVM54 on chromosome 2H, HvLeu on chromosome 5H and BMAC0156 on chromosome 7H. At other loci there were associations with other traits e.g. B-granule number as a percentage of the total number of starch granules together with milling energy and hot water extract on chromosome 2H. Surprisingly, starch A-granule characters only showed significant association with BMAC0096 in chromosome 5H. Starch specific surface area, a function of A- and B- granule size, showed the same associations as B-granule number (% total) i.e. with HvHVA1, BMAC0096 and HvLeu.

DISCUSSION

A unique aspect of the material considered in this project is that it represents a progression in the breeding of malting quality barleys. Allison (1986) demonstrated that the low milling energy character had been present in barley genotypes over a long period, possibly originating in genotypes such as Kneifel which were derived from central European land races. These genotypes were not necessarily of good malting quality (Swanston et al., 1990) and a number of more recent cultivars such as Aramir and Mazurka (Allison et al., 1979) have also been shown to have only moderate HWE despite low levels of milling energy.

Grain components form a complex matrix of different tissues and cell types that must be degraded during malting as efficiently as possible. The speed at which the protein matix is degraded will determine the extent to which embryo growth and respiration contribute to malting loss. The major components of barley grain, starch, protein and cell walls, are well known and their role in malting has been extensively studied. The role of the spatial distribution of hordein proteins has been described and the degradation of cell walls has been followed by fluorescence microscopy.

There are, however, extensive areas of ignorance that are amenable to a genetic marker assisted approach. This is particularly true in the case of HWE where many genes may be involved in a relatively lengthy process. Simple biometrical analysis of HWE may under-estimate the number of genes involved while the analysis of pedigrees without markers gives minimal information (Ellis et al., 1997).

Changes in malting technology have encouraged the development of malting barleys which can realise their malting potential in a shorter time scale. The associations observed here between parameters reflecting more rapid and extensive modification of endosperm components and HWE demonstrate the success of barley breeders in developing such genotypes. However, all modern malting cultivars continue to demonstrate low levels of milling energy, so it remains a valuable first screening technique.

Milling energy is influenced by the husk and aleurone tissues surrounding the endosperm. The husk is the most fibrous tissue, with the highest milling energy requirement (Camm et al., 1990), but represents a very small proportion of the grain. Consequently, most of the milling energy is determined by endosperm components and changes in milling energy associated with modification of cell walls and protein surrounding the starch granules have been studied (Swanston et al., 1992).

The major component of the barley grain, however, is starch which, in turn, is the major contributor to HWE. The synthesis and degradation of starch is, thus, central to good malting quality. While it has been clearly established that large changes from the "normal" ratio of amylose to amylopectin are detrimental to high HWE (Swanston, 1994), the positive effects of starch characters are not understood. A major target of this project is the identification of genetic control of starch related traits that might be of use in the improvement of malting quality.

The quantity of starch granule surface protein was shown to be strongly influenced by the methods used to mill the grain and to extract the protein, but a significant effect of genotype was also observed. However there was no association between friabilin content and either HWE or milling energy, suggesting that barley is different from wheat with regard to the constituents which determine grain hardness and quality. Milling energy was associated, to a limited degree, with particle size differences in the flour recovered, so there may be some differences between low and high milling energy barleys with respect to the pattern of fracture across the endosperm.

Correlations between starch granule parameters and HWE were not particularly high, but it was observed that small A-type granules or a high proportion by volume of B-type granules appeared to have deleterious effects on malting quality. Barley breeders have never actively bred for starch properties, however, due to the lengthy process involved in extracting and assessing the starch and the absence of any rapid screening tests which can be carried out without the need for starch extraction. A second aim of this project has been, therefore, to identify techniques that allow rapid identification of grain samples and an assessment of their quality. The use of DNA based markers allows easy cultivar identification from seed samples and molecular markers have been advocated to select for a range of malting characteristics, following the location of quantitative trait loci (QTLs) associated with both extract (Mather et al., 1997 Han et al., 1997; Thomas et al., 1996) and fermentability (Swanston et al, in press). SSR are an appropriate technology for this role as they are a codominant marker and can possess many alleles.

The empirical use of SSR to examine the "malting system" offers the great advantage that SSR occur in the intron of genes. It is possible to sequence the SSR and eventually it may be possible to find expressed sequences (EST) that result from gene expression. Work reported in this project has also confirmed the previous suggestion (Swanston and Thomas, 1996) that extract and fermentability are under separate genetic control. It is likely that genes controlling the synthesis of starch will also exert an influence on either or both of these parameters and may, therefore, map to the same location as other malting quality QTLs. Most quality characters appear to have a complex inheritance pattern but, as shown in this project, certain areas of the barley genome appear to be very important, either because of the major influence which they exert on a particular component or because of the number of parameters which they affect. The potential, therefore, exists to develop molecular diagnostics which will represent a major advance in the precision and effectiveness in breeding for starch characteristics or other malting quality parameters.

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