October 2020

Project Report No. 626

Improving winter malting barley quality and developing and understanding of the interactions of introgressions with genetic background (IMPROMALT)

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This is the final report of a 60-month project (21130013) that started in April 2013. The work was funded through a BBSRC LINK award £1,724,221 and a contract for £106,040 from AHDB.

CONTENTS

1. Abstract

The IMPROMALT project aimed to improve the malting quality of winter barley in the UK. It utilised information from a genetic analysis of barley data from National List (NL) and AHDB Recommended List (RL) trials. The focus was the targeted backcrossing of three regions of the genome from elite spring barley into winter barley to improve malt extract level in the winter crop. The work built on a BBSRC SA LINK project, with AHDB funding, entitled 'Association Genetics of UK Elite Barley (AGOUEB)' that concluded in 2014.

The project involved a large consortium, including the James Hutton Institute (JHI) and NIAB, as academic partners, the AHDB, MAGB, SWRI and six UK breeding companies (KWS-UK, Limagrain, Syngenta, Secobra, RAGT and Ackermanns) as commercial partners. The project had six objectives, with the first being to extend the analysis of AGOUEB, which underpinned all the other objectives that were dependent upon AHDB support.

The project succeeded in its major aims. The AHDB-supported objective 1 exceeded the aims and increased the number of barley varieties studied by 20%. A change in the genotyping platform, designed by JHI, enabled the project to derive five times as much genotype data than anticipated. These data elucidated the genetic relationships between barley varieties that corresponded well to known pedigrees and varietal differences. These data were combined with the results of NL and RL trials (1988–2016) to carry out genome-wide association studies (GWAS) to correlate the detailed genetic fingerprint of the variety with its performance in the field. These studies highlighted several regions of the barley genome that are important in the genetic control of traits of agronomic and economic importance. For malting quality, associations were found. GWAS identified 24 independent regions in 13 malting traits in spring barley and 2 associations in 2 traits in winter barley. Importantly, the study confirmed the importance of the target regions in the malting quality backcrossing programme and helped to better delineate candidate genes in the target region on the top of chromosome 3H. Interestingly, the analyses suggested that most of the genomic regions associated with malting quality were moving to fixation or had been fixed in the most recent spring varieties, but more variation remained in the winter crop. The backcrossing programme succeeded in improving malting quality in the winter crop. This material has now been utilised within breeding programmes.

Ultimately, this project will help to improve the malting quality of UK winter barley varieties in the short-to-medium term. It will provide more choice to UK farmers and barley end-user. In particular, the earlier harvest of the winter crop may help bring resilience in the face of potential climate change scenarios.

2. Introduction

Analysis of National and Recommended Lists trial data for the AGOUEB project highlighted that the malt extract of the winter barley varieties under test was much reduced compared to that of the spring varieties. By contrast, winter barley varieties clearly had a superior crop yield. Closer analysis of the pedigrees of the winter and spring lines revealed that all the winter barleys that had been considered for recommendation by the Malting Barley Committee (MBC) featured Maris Otter in their pedigrees, which was derived from a Proctor cross. The Proctor lineage had largely been eliminated from the more current spring barley varieties as they all could be traced back to an old Czech malting line (Valticky) through the semi-dwarf variety Triumph, also known as Trumpf. The Triumph lineage appeared to be absent from winter barley varieties. Marker-trait Genome Wide Association Scans (GWAS) of the AGOUEB data set identified two distinct barley genomic regions, located on barley chromosomes 1H and 3H, that appeared to be associated with increased malt extract in the spring barley gene pool. (Thomas et al., 2014). Analysis of the genotypic data of all the lines highlighted the division of the lines into three genetically distinct sub-populations: spring 2-row, winter 2-row and winter 6-row. Targeted analysis of the genotypic data of the malting variety genotypes in the spring and winter gene pools identified the fact that these differed markedly between winter and spring barley, suggesting that they may represent regions where improved malting quality QTL alleles had been introduced through the breeding and selection of Triumph and its derivatives. Analysis of the genotypic data on the long arm of chromosome 4H also showed clear differences between winter and spring barley in the region where one of the vernalisation loci and a *beta*-amylase locus were located. No recombinants between the two loci could be found amongst winter barley lines that had been considered for promotion as malting varieties and the haplotype at the *beta*-amylase locus was the same as that found in Proctor, which had previously been found to be associated with low *beta*-amylase thermostability (Eglington et al., 1998).

The AGOUEB public dataset consisted of 547 spring and winter barley genotypes that had completed at least two years of official (National List) trials. All the lines had been genotyped with 3072 Single Nucleotide Polymorphism (SNP) markers using the Barley Oligo-nucleotide Pooled Assays one and two (BOPA1 and BOPA2). We combined these data with phenotypic data from 849 fungicide treated trials grown over harvest years 1988-2006 augmented with a set of common trials grown in the AGOUEB project over the years 2006-2008. By the end of the AGOUEB project, SNP genotyping had proceeded to the iSelect Illumina technology that provided a much more extensive set of 9000 individual SNP loci. Each year, an average of 22 additional barley genotypes were being added to the UK National List with phenotypic data being generated for each. The addition of an extra 20% genotypes in the period from 2007 (the end of the AGOUEB survey) and 2012 (submission of the IMPROMALT project), together with the more detailed genotyping technology, meant that we could refine the QTL intervals identified in the AGOUEB project and design crossing strategies that would efficiently introgress the spring QTL into a current winter

malting barley cultivar and potentially identify candidate genes underlying the QTL. Continually updating the genotypic and phenotypic datasets with the annual additions to the National List over the project's lifetime would also help further refinements of these intervals as well as providing up to date information upon barley breeding progress for a range of key characters.

The IMPROMALT project was, therefore, designed to test the hypothesis that introduction of spring QTL alleles at the regions on chromosomes 1H and 3H, together with a targeted recombination between the winter allele at the 4H vernalisation locus and a spring allele associated with a higher level of thermostability at the linked *beta*-amylase locus, would produce a significant improvement in the malting quality of winter barley. In order to conduct this task whilst maximising the winter barley genetic background, we needed to better delimit the spring regions to be introgressed and use molecular markers that flanked the region to select for the appropriate recombinations. The IMPROMALT project, therefore, had the following objectives:

1. Augment existing genotypic and phenotypic barley data sets by just under 20% to refine the QTL intervals for our target loci.
2. Design a rapid, cost-efficient and practical introgression strategy that will result in the development of several introgression lines for the same resource level as for one line.
3. Produce DH lines that combine the spring introgressions in different winter genetic backgrounds.
4. Introgress winter habit genes into spring barley with minimal winter genetic background.
5. Identify a candidate gene for at least one of the spring QTL.
6. Determine the effect of allelic substitutions at the introgressed loci upon the expression of other genes during the malting process.

A research programme designed to address all six objectives was submitted as a BBSRC-LINK project application that included an application for AHDB Cereals & Oilseeds funding to specifically address Objective 1 with the remaining five Objectives funded by BBSRC. We will, therefore, largely address work done under Objective 1 for this report but also summarise progress made for the other five objectives as all six are inter-related.

3. Materials and methods

3.1. Genotypic data

3.1.1. Germplasm

Genotypes in official trials (Objective 1)

At the time of writing, we had accumulated 809 lines ranging from those that had first entered National List trials in 1963 (Maris Otter) to those that had first entered National List Trial in 2017, the last year that could be included within the timescale of the project. Spring barleys comprised 53.4% of the total (432) with winter barley 2-rows being 40.3% (326) and the remainder being

winter barley 6 conventional 6-rows (not F1 hybrids). Thirty-five and 36 of the spring and winter (respectively) barley lines had entered National List Trials prior to 1992 (the start of the AGOUEB National List survey period) and there were one and four spring and winter (respectively) barley lines that either pre-dated National List Trials and/or were considered to be important parents in UK barley breeding. Over the 26 years of the combined AGOUEB and IMPROMALT survey, an average of 15 spring and 12 winter (respectively) barley had either completed two years of National List Trials and/or been added annually to the National List.

Introgression lines (Objectives 2, 3, 4, & 6)

Six breeding companies were part of the IMPROMALT Consortium and worked on developing Backcross Introgression Lines (BILs) to test the hypothesis that targeted selection of spring x winter crosses would produce lines with significantly improved malting quality (Table 1).

Company	Cross	Abbreviation
KWS (UK)	KWS Joy (Winter) x Shuffle (Spring	KWSJxS
Limagrain & Secobra	Etincel (Winter) x Overture (Spring)	EtxO
RAGT	Atlantick (Winter) x Overture (Spring)	AtxO
Saaten Union (Ackermann)	Acute (Winter) x Overture (Spring)	AcxO
Syngenta	SY Venture (Winter) x Overture (Spring)	SYVxO

Table 1 The distribution of the crosses amongst the breeder members for the construction of Backcross Introgression Lines (BILs).

All the winter parents were either accepted malting barley cultivars or at least entered into official trials with the view to becoming accepted malting barley cultivars. Atlantick and Etincel were 6-rowed French cultivars but all the rest were two-rowed. Overture and Shuffle were UK spring barleys that were placed on the MBC list of cultivars approved for use in distilling and prior genotyping identified both as carrying all three desired spring QTL alleles.

Each company used a backcrossing scheme to introgress the desired segments from the spring barley parent into the winter barley parent. The F1 of each cross was, therefore, re-crossed to the winter parent and the resulting BC1F1 seed screened for with markers that flanked the introgressed segments from chromosomes 1H and 3H to identify those lines that had retained the spring segment. The lines were also screened with markers that were linked to the flanking markers but on the other side of the introgressed segment to detect those lines that had retained more of the winter parental genome. Whilst both sides of the introgression were screened, our strategy was to identify recombinations close to any side of the introgression target as the likelihood of finding a double recombinant that just had the spring introgressed segment is very small and would require a much larger population than the ones that the companies had generated. The strategy for the recombination between the vernalisation and *beta*-amylase loci on

chromosome 4H was similar but we just needed to screen the interval between the two loci for the presence of spring alleles close to the *beta*-amylase locus and winter alleles close to the vernalisation loci. In all cases, the expectation is that the spring alleles would be present as heterozygotes because we were screening BC1F1 seed. Selected lines from the BCF1 of each cross were, therefore, crossed again back to the winter parent to produce BC2F1 seed, which were re-screened with the markers used in the BC1F1 screen but concentrating on selecting recombinants close to the introgression but on the other side to that identified in the BC1F1 screen. Again, lines carrying the spring alleles will be heterozygous and we needed to develop lines that carried the winter and spring alleles in homozygous form to test our hypothesis. The selected BC2F1 plants were then used to either derive Doubled Haploids from each or entered into a short selfing programme to generate homozygotes. These inbred (or near inbred) lines were then screened with the same set of markers to identify those that carried the three desired segments in various combinations as well as lines that lacked all three. Finally, the selected inbred and near inbred lines were genotyped with the 50K iSelect chip to provide an estimate of the overall background genotype of each.

QTL Segmental isolines (Objective 5)

In order to identify candidate genes for one of the QTL, a set of QTL segmental isolines was produced. We focused upon the 3H QTL as this was in a recombinogenic region of the barley genome and production of a set of a number of unique recombination events was, therefore, more probable. A line from the SYVxO cross that had the smallest introgression of the 3H QTL was re-crossed to SY Venture and the resulting BC3F1 was screened for with the two markers that flanked the introgression to identify individuals that were heterozygous for one but homozygous for the other as they would carry a recombination event within the target segment. These lines were then selfed and screened with a set of KASP markers that were designed to specific genes that were known from the first ordering of gene sequence data to be located in the region. This identified 80 lines that were homozygous and carried different segments of the spring introgression which were multiplied for trialling. These lines were then genotyped with a 50K iSelect chip (43,461 SNP markers) to validate the QTL introgressions and estimate the relative genetic contribution of the spring and winter parents.

3.1.2. Genotyping

Initially, all lines were genotyped with the barley 9K iSelect SNP genotyping platform (Comadran et al., 2012) but a much larger 50K iSelect SNP chip, representing polymorphic markers amongst worldwide elite barleys, was developed and validated (Bayer et al., 2017). As it included all the functional markers on the 9K chip, we therefore, used that for all subsequent genotyping to take advantage of the increased representation of markers in recombining regions of the barley genome. We also re-genotyped all the lines in the data set that had previously been genotyped

with the 9K chip to enable a complete analysis of all the data. Polymorphic SNPs for the crosses used in the production of the BILs were identified from the 9K iSelect genotyping at the start of the project and used to design KASP markers for use in the selection of BC1F1 individuals. After the first round of integrating the genotypic and phenotypic data for all individuals that had completed 2 years of official trials and/or been placed on the National List up to and including harvest year 2012, the intervals were refined and a new set of KASP markers delimiting smaller target segments designed and used in selection of BC2F1s and the inbred lines derived from the selections. The selected inbred lines were then genotyped with the 50K iSelect chip to provide a detailed estimate of the background genotype of each.

A set of 13 KASP markers that were polymorphic between SY Venture and Overture were designed from pre-existing exome capture and RNA-seq data. These were used to genotype the 3H QTL interval during the development of the 3H QTL segmental isolines. 10 of these generated reliable allele calls were used to separate the 88 uniquely recombinant isolines into 21 genotypically defined classes for advancing into phenotyping trials.

3.2. Phenotypic Data

3.2.1. Official trials (Objective 1)

The means from National and Recommended List trials data for each trial x year x site x treatment combination from harvest year 1988 to harvest year 2018 were combined with BSPB NL parallel trial data from harvest years 1988 to 2002 and AGOUEB trial data from harvest years 2006 to 2008 to form a data set with 1327 spring and 1185 winter barley fungicide treated trials and 1047 and 934 untreated trials, respectively. After merging synonymous data fields, there were 77 variates that had been recorded on at least one trial but, apart from yield, the data coverage was sparse. Choosing a threshold of at least 2000 data values resulted in 63 and 66 variates for the spring and winter treated trials, respectively and 27 and 29 variates for the spring and winter untreated trials, respectively. These variates were used for subsequent analyses.

3.2.2. Introgression Trials (Objectives 3 & 4)

Lines for KWSJxO, EtxO, and SYVxO were multiplied by the relevant breeding companies for harvest 2016 and used to sow trials for harvest 2017. As seed was limited, each cross was sown in a two-replicate trial at the originating company's site and in a combined trial of two replicates at the JHI site in Dundee. Where there was insufficient seed to sow all four plots, priority was given to sow at least one replicate at two sites. Seed for AtxO was multiplied for harvest 2017 and sufficient produced to sown in a combined trial series for harvest 2018, which were separated into a two- and a six-row set of trials to be grown at KWS and Syngenta's UK sites and Limagrain, RAGT and Secobra's French sites, although Syngenta did not grow the six-row trial. Both trials were also

grown at JHI's Dundee site in Scotland. Each trial was designed as a row and column design to fit in with each company's trial field under the local management regime to grow fungicide treated winter malting barley. There were problems in developing inbred lines from AcxO in time for the harvest 2018 trials, but seed was produced for a trial to be grown at Ackermann's site in Germany and JHI's site in Dundee for harvest 2019.

When the majority of plots were ripe in each trial, they were harvested with a small plot combine and plot weights and moisture recorded for each with some sites scoring supplemental developmental data during the growing season. Seed from each trial was sent to JHI where it was cleaned and graded over a 2.5mm sieve and analysed for grain size parameters using a MARVIN digital seed analyser (MARViTECH GmbH, Germany). Grain nitrogen content of each plot was then estimated using an Infratec 1241 grain analyser (FOSS, Denmark).

Plots were selected for micro-malting analyses by the Scotch Whisky Research Institute and seven member companies of the Maltsters Association of Great Britain (MAGB). Each MAGB member company had the capacity to micro-malt 16 samples and so we constructed batches of 14 test lines plus two controls, one of which was common to all batches. From harvest 2017, batches were formed to prioritise testing of the triple substitution of spring segments into a winter barley parent against control lines that lacked all three spring segments and, to prevent site differences being confounded with member company lab differences, samples from the breeding company site were matched with the same sample from the JHI site. In all cases, samples were grain for micro-malting which were formed by bulking over replicates when possible. From the 2018 harvest, the JHI and KWS sites were chosen to provide the majority of the samples for testing as they had low grain nitrogen contents for the two- and six-row trial series with any gaps being filled by samples from the Syngenta trial, which also had a low grain nitrogen content. A similar strategy was chosen to construct the batches of 16 samples for harvest 2018 but ensuring that the same company did not malt the same introgression x site combinations. This was ensured by the Scotch Whisky Research Institute, which undertook micro-malting three of the batches from harvest 2018.

3.2.3. Segmental Isoline Trials (Objective 5)

From the glasshouse multiplications, there was sufficient seed to sow out 182 individual lines in observation plots with controls for harvest 2017 at the JHI site only. Plots were managed in the same way as the JHI Introgression trial and plots were harvested when the majority were ripe. Plot yield and weight were recorded, and some developmental characters were recorded during the growing season. Plots were processed in the same manner as the Introgression trials from 2017 and 30 individual lines selected for micro-malting and analysis in two batches of 15 samples with a common control by the Scotch Whisky Research Institute. The 30 lines were distributed across recombination intervals identified by the genotyping (3.1.2). A subset of 92 lines from the original

182 was chosen for harvest 2018 trials, ensuring that all lines that were micro-malted from the harvest 2017 trials were represented. The two parents were added in as controls and two replicate trials were designed for growing at the Ackermann site in Germany and the JHI site in Dundee under the local standard winter malting barley management regime. The trials were harvested when ripe and all post-harvest processing was conducted at JHI's Dundee site as described for the Introgression trials from harvest 2018. Twenty-nine individuals were chosen for micro-malting analysis with 16 in common with the micro-malting samples analysed from the harvest 2017 trial. Samples from both sites were chosen for micro-malting with both reps of one sample being included to derive an estimate of biological error and were combined with a common control to form four batches of 16 samples. All the samples from the Ackermann site were represented in two batches and the JHI samples in the other two.

3.3. Statistical Analyses

3.3.1. Germplasm Relationships (Objective 1)

The genotypic data collected on all 809 lines was visualised in FlapJack (https://ics.hutton.ac.uk/flapjack/), which was also used to create a similarity matrix using Simple Matching. The similarity matrix was used to identify relationships between the different genotypes by Principal Components Analysis and also by Hierarchical Clustering Analysis. The subset of the 809 lines that had been placed on the AHDB Recommended List was supplemented with genotypic data from varieties that had been included on either the NIAB or SRUC barley recommended lists (pre-cursors of the AHDB Recommended List) since 1970. This set of 279 varieties was analysed in the same way as the Impromalt set above but the nexus file from the dendrogram was used to draw a 'Circle of Barley' using the Interactive Tree of Life (www.itol.embl.de) to illustrate the relationships between RL varieties over nearly 50 years. Finally, data was collected on pedigrees of lines that had been used in the project (where breeders were prepared to release the information) and merged with pedigree data collected from a range of sources for the ancestors of the IMPROMALT lines to produce a Helium (https://github.com/cardinalb/helium-docs/wiki) view of the pedigree relationships amongst the 809 lines.

3.3.2. Historical data analyses (Objective 1)

The individual trial means for all the variates where we had sufficient data were used to provide a Best Linear Unbiased Prediction (BLUP) for each genotype with harvest year and trial series as other main effects and trial sites nested within year. Other interactions fitted were those between genotype and year and genotype with site. Data was available from just the National List trials for many genotypes as they did not progress beyond National Listing and not one variety was present for the whole survey period. Each trial was grown with a set of five control varieties chosen from current Recommended Lists and these tended to persist in trials over a number of years. The

presence of these control varieties in the data set, together with our analysis model, provided a means of smoothing out differences between years and so reducing inflation and deflation of the means. The BLUPs were derived in several tranches. One was at the beginning of the project when we had supplemented the AGOUEB data with the phenotypic data from trials up to and including harvest year 2012. We then added in successive tranches of data to form sets that include up to harvest years 2014, 2016, and 2018 with the latter being the complete IMPROMALT phenotypic data set. For the subset of IMPROMALT lines that had been placed on the Recommended List, we regressed the BLUP for each variety against the year in which it was first recommended for a set of key performance characters. The significance of the regression slope indicates whether or not breeding progress has been made over time with the estimate of the slope providing an indication of the rate and direction of progress. When considering malting quality characters, we further restricted the set to just those lines that had either been placed on the Malting Barley Committee's approved list or had at least been tested for possible placement on the list.

The phenotypic data was combined with the relevant genotypic data for each tranche and used in Genome Wide Association Scans (GWAS) to identify specific chromosomal regions that were associated with the genetic control of 12 and 2 characters in spring and winter barley, respectively. For the 2012 and 2014 tranches, we used the 9K iSelect SNP data and used the Eigenanalysis GWAS method implemented in GenStat 14 (https://www.vsni.co.uk/software/genstat) to correct for underlying population sub-structure but analysed the spring and winter data sets separately as the phenotypes were collected from separate trials series. For the 2016 and 2018 analyses, we employed the 50K iSelect genotypic data and the R package GAPIT ((Wang and Zhang, 2018) to use a compressed Mixed Linear Model (cMLM) to detect significant associations after correcting for population sub-structure by fitting the first two principal components from a principal component analysis of the genotypic data. For the GenStat analyses, the most significant marker within a +/- 10cM window was chosen to represent a QTL with any other significant markers within that window being used to establish a putative confidence interval. For the GAPIT analyses, unique QTL were identified by establishing a window based on localised linkage disequilibrium ((Wang and Zhang, 2018). QTL and genotype information were combined for visualisation in FlapJack (https://ics.hutton.ac.uk/flapjack/). Finally, the selection history for QTL of interest was established by plotting the frequency of the desirable allele against time by grouping the first NL harvest year of each line into half-decadal groups.

4. Results

4.1. Genotyping of official trials (Objective 1)

The seed of varieties submitted to National and Recommended List trials were sourced from the breeders through the aegis of the British Society of Plant Breeders Ltd. (BSPB). DNA was extracted from germinated seedlings using standard protocols and the genotypic data determined with the 50K Illumina barley SNP Chip (Bayer et al., 2017). The resultant data was concordant with previous 9K SNP data on the AGOUEB varieties and also with the known relationships between the varieties.

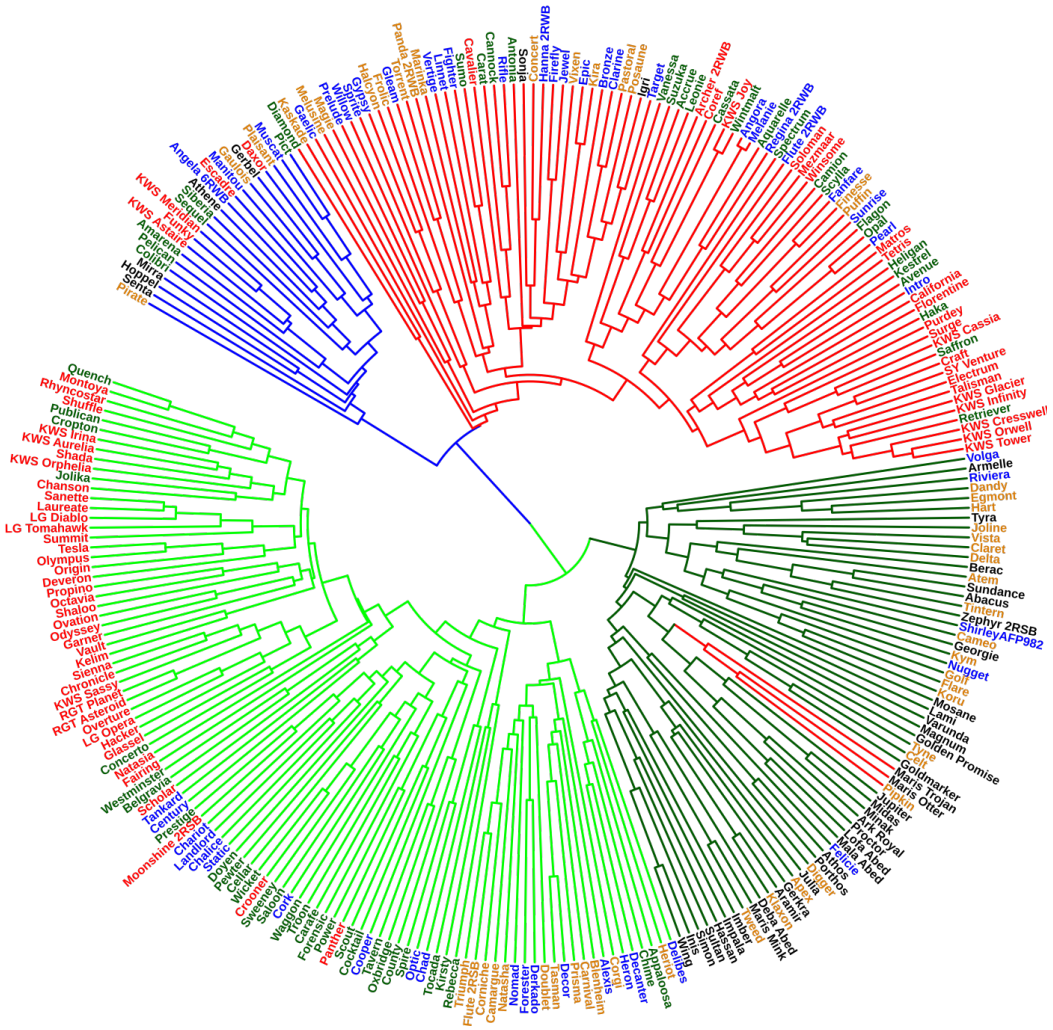

Figure 1. 'Circle of Barley' Dendrogram illustrating the genetic relationships between RL varieties over nearly 50 years based on 9K SNP data. The blue branches relate to 6-row winter barley, red branches to 2-row winter barley and green to 2-row spring barley with darker green representing older and light green the newer varieties. The colour of varietal names indicates the date of recommendation: pre-1980 in black, 1980-89 in amber, 1990-99 in blue, 2000-09 in green, 2010-18 in red.

The concordance of the genotypic data with expectations is shown by the relationship between the varieties recommended over the last 48 years illustrated by the ‘Circle of Barley’ dendrogram in Figure 1 constructed from the 9K SNP subset of the genotypic data. The dendrogram shows a clear division between spring and winter varieties and between six-row and two-row varieties within the winter varieties. Interestingly, there is a major division within the two-row spring barleys, denoted by the dark and light green branches in Figure 1 that relates to varieties pre- and post- the introduction of the variety Triumph in 1980 that was the source of introduction of the denso semi-dwarfing trait into UK varieties. There are three two-row winter varieties (Maris Otter and two closely related varieties Maris Trojan and Pipkin) that cluster with the older two-row springs which potentially relates to the pedigree of Maris Otter which was directly derived from a cross between the winter variety Pioneer and the spring variety Proctor. The patterns of relationship also work at the finer scale as shown by the very close relationship between the old winter varieties Angora and Melanie as shown by the short length of the branches in the dendrogram. These varieties were not able to be distinguished using standard DUS characters and necessitated the use of hordein patterns to separate them. The corresponding comparison of the 50K genotypes of these two varieties indicates one of the few regions of the genome that differ is at the top of the short arm of chromosome 1H which include the locus *Hor2* that encodes for B-Hordeins (Faulks et al., 1981). The dendrogram (Figure1) also demonstrates the changes in the varietal genetic composition over time with a clear decadal shift in both the two-rowed winter and spring varieties. Thus, for the two-row winters, many of the varieties recommended in the last decade group together on the right-hand side of the ‘circle of barley’. This grouping is centred around recent deficiens type barleys including KWS feed varieties that group with older varieties such as Saffron and Retriever which feature prominently in their pedigrees and introduced in 1993 which appears to be the source of the deficiens trait in modern UK winter varieties. The decadal shift is clearer in the spring varieties where almost all varieties recommended between 2010 and 2018 are on the left-hand quadrant bounded by the older varieties Quench and Concerto which feature heavily in the pedigrees of the later lines. It is noteworthy that many of the recent lines are also more similar to each other as indicated by their branch lengths in the dendrogram.

This close relationship between varieties is also evident in the pedigree data supplied by consortium members. Figure 2 shows the pedigree of the spring variety Laureate derived from a cross between the varieties Concerto and Sanette, together with their parents and its immediate daughter varieties.

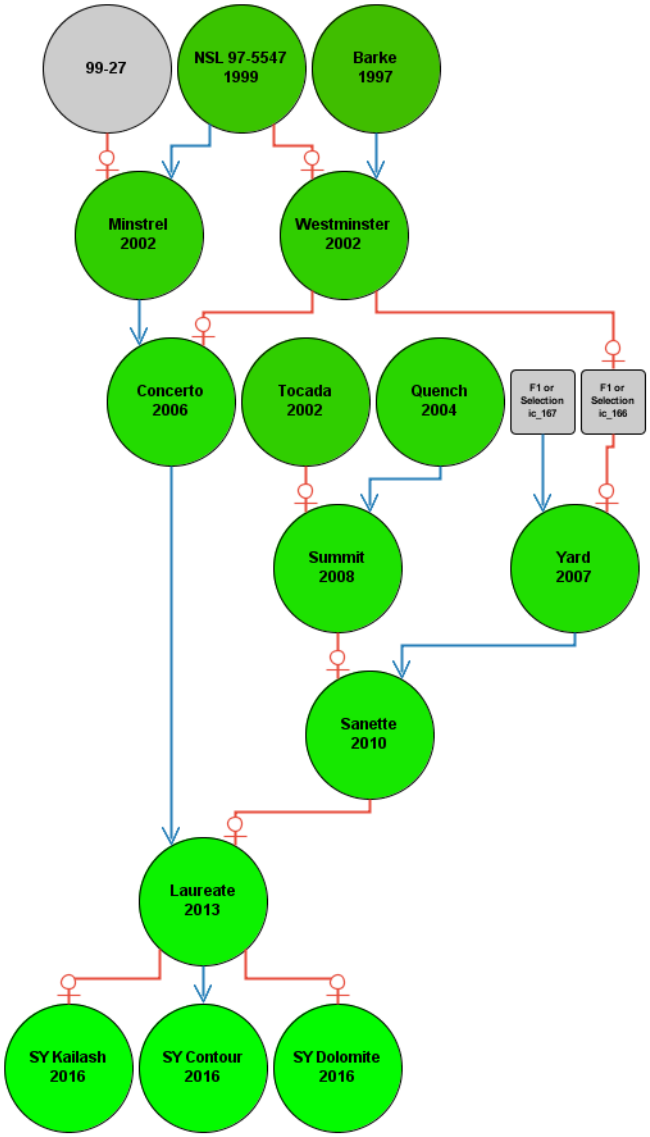

Figure 2 Cartoon showing the pedigree relationship of the variety Laureate drawn with Helium software. The date given is the year of entry to National List Trial

The interconnectedness of modern spring varieties such as Laurate is shown in Figure 2 as the variety Westminster features in the pedigree of Concerto and Sanette i.e. in both parents of Laureate and a Nickerson's breeder's line (NSL 97-5547) features as a grandparent twice in the pedigree of Westminster. Figure 2 also highlights the speed of modern breeding with daughter varieties being entered into National List trails only three years after the parent variety.

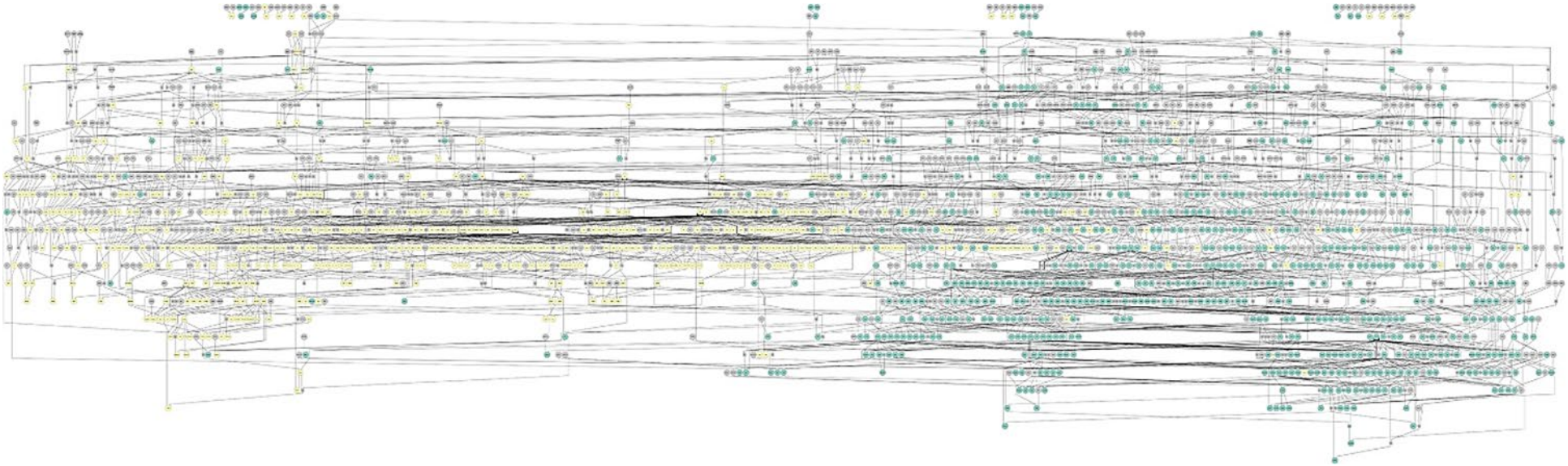

Figure 3. Helium diagram of all the varieties surveyed in the IMPROMALT project. Winter varieties are coloured amber and spring varieties cy

The complexity of the pedigree relationships is indicated by the small-scale view in Figure 3 of a large helium diagram of the varieties in the IMPROMALT project. This shows the complex pedigree relationships that exist within modern barley varieties thanks to the freedom of use of material supported by strong plant variety rights and also the general separation of winter and spring breeding programmes (although there are links as in this project). It also shows the greater number of generations within spring barley breeding reflecting the reduced generation time and potentially greater breeding progress in the period covered.

4.2. Historical malting data (Objective 1)

In total, data from 2862 individual trials were collated, representing 1520 spring, and 1342 winter barley trials (Table 2).

	NL	RL	AGOUEB
Spring	631 (1988-2016)	854 (1988-2016)	35 (2006-2008)
Winter	519 (1988-2016)	790 (1988-2016)	33 (2006-2008)

Table 2: Summary of the trials used to derive BLUPs for each variety. For each seasonal habit, the number of trials from which data was collected are shown for each trial series, along with the minimum and maxim year for each of these trials

From this data, BLUPs were calculated for 451 spring, and 407 winter varieties. These estimates were made for 26 spring malting quality traits and 25 winter traits, including grain quality, malt modification, wort attributes and process yield.
Considering just the cultivars that had been placed on the AHDB Recommended Lists and had also been considered for MBC approval, HWE BLUPs were regressed against the year in which they were first recommended to determine if genetic progress had been made.

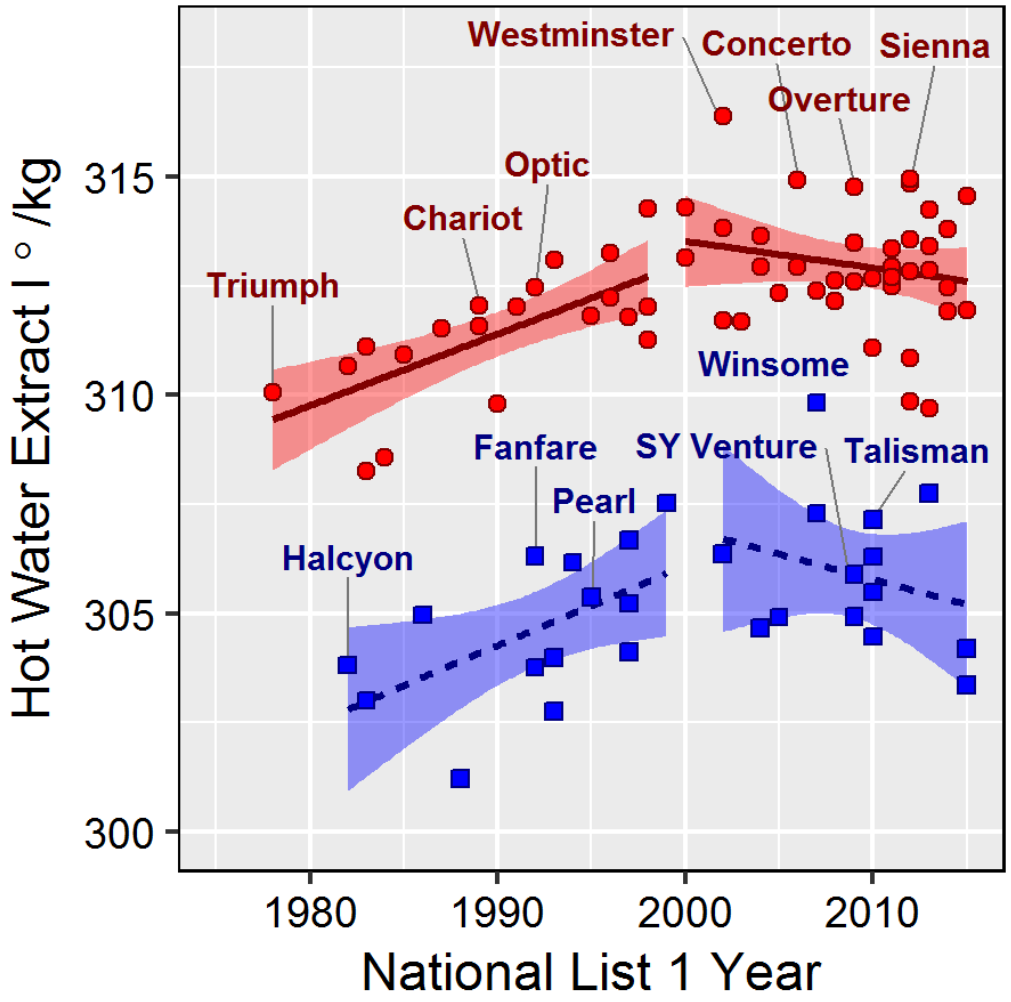

Figure 4 BLUPs for hot water extract plotted against year of introduction for varieties included on the Recommended List and considered for malting barley committee. Spring varieties are indicated by round symbols, and winter varieties by squares, with key varieties from each set labelled. Fits from a linear regression model (and 95% CI) are shown for winter and spring sets, with separate models for varieties pre-2000 and post-2000. Fitted values for the winter set are indicated by a dashed line, and solid lines for spring models.

Whilst there has been highly significant genetic progress in the spring two-row gene pool since the introduction of Triumph in 1980, and prior to 2000 (Figure 4) (β=0.16; p=0.001; R^2=0.48), the rate of progress since 2000 is non-significant (β=-0.06; p=0.245; R^2=0.04) with a large scattering of the datapoints, indicating that breeding progress for the character as stalled or that the maximum phenotype has been reached. The trend in the winter gene pool is less significant although the slope is similar to the springs both pre- and post-2000 (β=0.18; p=0.034; R^2=0.32) and (β=-0.12; p=0.368; R^2=0.07), respectively (Figure 4). There are, however, relatively few winter malting barley cultivars that have been released since 2000, so whilst there is no significant evidence of breeding progress, there are also too few numbers for an adequate test of genetic progress.

4.2.1 Phenotypic correlations (Objective 1)

BLUPs for the malting quality traits considered in the experiment were significantly correlated in many cases.

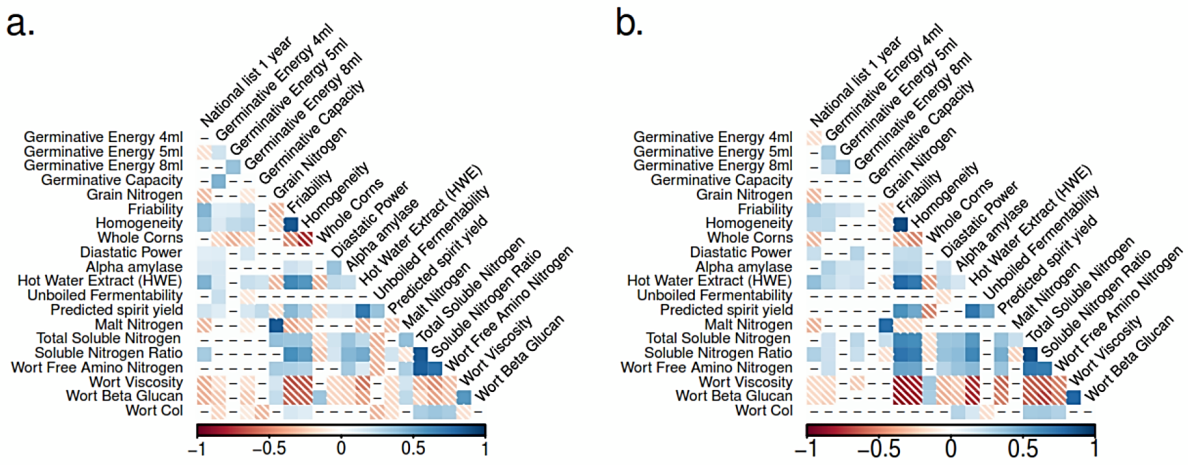

Figure 5. Significant correlations for BLUPs for malting quality traits examined in the current study in a: spring cultivars, and b: winter varieties. The shade corresponds to the magnitude of the correlation coefficient, with negative correlation coefficients being indicated by diagonal hatching. Where phenotypic data is not present for a pair of traits, this is indicated by a blank square.

A full correlation matrix is shown in Figure 5. In spring barley, measures of endosperm modification were highly correlated, with friability showing a strong positive correlation with homogeneity (r=0.85, p<0.001) and a negative correlation with whole corns (r=-0.46, p<0.001). Measures of endosperm modification, particularly friability, were also correlated with HWE (r=0.65, p<0.001) and, to a lesser extent, predicted spirit yield (PSY) (r=0.41, p<0.001). Germination traits generally showed low levels of correlation with other malting quality traits. Diastatic power showed moderate correlation with α-amylase activity, and both enzyme traits showed moderate levels of correlation with fermentable extract and one of its component traits (boiled fermentability). In the winter varieties, very strong positive correlations were observed between endosperm modification traits, protein modification, HWE and fermentable extract/ PSY (Figure 5). Similarly, strong negative correlations were seen between these traits and wort viscosity and *beta*-glucan (Figure 5). In both sets, there was a tendency for HWE, friability and homogeneity to be positively correlated with year of introduction, and grain/ malt nitrogen, wort *beta* glucan and wort viscosity to decrease with year of introduction (Figure 5). The magnitude of these correlations increased when they were restricted to varieties that were marketed as malting types. All the correlations of grain and malt nitrogen with other traits were very low which may reflect the fact that the sites chosen for micro-malting analyses are a subset of the whole trial set that have been selected to have a mean grain nitrogen content that would be acceptable for malting, i.e. the range of 1.4 to 1.7% grain nitrogen content. This would limit the influence of large variations in grain nitrogen content affecting malting quality parameters.

4.2.2 Genotyping (Objective 1)

From the phenotyped lines, genotypes were produced for 407 spring barley varieties and 352 winter varieties. In the overall set of spring genotypes, there were 22,748 markers that had a proportion of missing values less than 0.25 and a minor allele frequency greater than 0.1. In the winters, the corresponding figure was 25,575. Because there were different numbers of genotypes that had data for each malting quality trait, the filtration was applied again for each phenotype and seasonal growth habit combination. On average, 22,275 and 25,094 SNPs were used in GWAS for the spring and winter genotypes, respectively.

Set	Trait	Name	Chr	Genetic Interval cM	Position Mb (Interval)	Peak Marker (alleles)	Minor Allele (Freq)	-log10p	FDR adjusted p-value	Minor Allele Effect
Spring	Homogeneity	S-HM-1	1H	13.2-13.2	0.3	JHI-Hv50k-2016-284 (G/T)	T (0.19)	3.2	0.048	-0.45
	Diastatic Power	S-DP-1	1H	2.2-2.2	1.9	SCRI_RS_124234 (C/T)	C (0.18)	4.4	0.040	3.57
	Hot Water Extract	S-HW-1	1H	28.2-31.8	14.2	JHI-Hv50k-2016-13067 (A/G)	G (0.41)	3.8	0.041	-0.59
	Predicted Spirit Yield	S-PS-1	2H	78.7-78.7	647.3	JHI-Hv50k-2016-106390 (A/G)	G (0.23)	3.7	0.027	-0.73
	Wort Viscosity	S-WV-1	3H	1-6.9	1.5 (1.5-1.5)	JHI-Hv50k-2016-149225 (C/G)	C (0.15)	4.2	0.009	0.01
	Hot Water Extract	S-HW-2	3H	1-11.7	3.7 (1.7-8.9)	11_11453 (A/G)	A (0.23)	4.2	0.026	-0.9
	Whole Corns	S-WC-1	3H	27.2-27.2	22.5 (22.5-22.6)	JHI-Hv50k-2016-158667 (A/C)	C (0.1)	3.8	0.018	0.73
	Homogeneity	S-HM-1	3H	159.4-159.4	676.4	JHI-Hv50k-2016-218678 (A/G)	A (0.12)	3.6	0.023	0.62
	Friability	S-FR-1	4H	18.2-21.8	7.6 (7.6-8.7)	JHI-Hv50k-2016-228563 (A/G)	G (0.37)	3.8	0.037	-0.96
	Friability	S-FR-2	4H	61.2-62.4	569.2 (569.2-569.5)	JHI-Hv50k-2016-256147 (A/C)	C (0.12)	3.9	0.033	-1.42
	Homogeneity	S-HM-2	4H	62.4-62.5	569.2 (565.6-569.5)	JHI-Hv50k-2016-256147 (A/C)	C (0.12)	6.3	<0.001	-1.16
	Whole Corns	S-WC-2	4H	60-64.5	569.8 (559.6-569.8)	JHI-Hv50k-2016-256219 (A/G)	G (0.1)	5.7	<0.001	1.14
	Diastatic Power	S-DP-2	4H	118.7-123.3	642.3 (641-645.2)	JHI-Hv50k-2016-274747 (A/G)	A (0.37)	13.5	<0.001	-6.57
	Germinative Energy 8ml	S-G8-1	4H	118.7-123.3	643.5 (641.8-646.2)	JHI-Hv50k-2016-275320 (A/G)	G (0.31)	6	0.004	3.66
	soluble Nitrogen Ratio	S-SN-1	5H	31.8-43.1	19.4	JHI-Hv50k-2016-284122 (A/G)	A (0.45)	3.9	0.046	-0.46
	Wort Viscosity	S-WV-2	5H	51.2-52.5	445 (444.2-454.3)	JHI-Hv50k-2016-304397 (A/G)	A (0.22)	3.5	0.043	0.01
	Soluble Nitrogen Ratio	S-SN-2	5H	83.7-87.3	532.1 (531.9-534.3)	JHI-Hv50k-2016-312337 (G/T)	T (0.25)	5.5	0.002	0.74
	Free Amino Nitrogen	S-FA-1	5H	83.7-88.5	532.3 (532.1-534.7)	JHI-Hv50k-2016-312374 (A/G)	A (0.27)	5.6	0.033	3.16
	Predicted Spirit Yield	S-PS-3	6H	68.1-68.6	478.2 (478.2-479.1)	SCRI_RS_165986 (A/C)	C (0.12)	4.3	0.014	-0.93
	α Amylase	S-AA-1	6H	77.5-83.6	535.4 (532.8-535.8)	SCRI_RS_177093 (C/T)	C (0.13)	9.1	<0.001	2.88
	Diastatic Power	S-DP-3	6H	102.3-102.3	555.7 (554.9-555.7)	JHI-Hv50k-2016-421716 (C/G)	G (0.38)	5.3	0.006	3.6
	Whole Corns	S-WC-3	7H	41.1-45.9	42.7 (41.5-45.7)	JHI-Hv50k-2016-460614 (A/T)	T (0.1)	3.7	0.023	0.69
Winter	Hot Water Extract	W-HW-1	1H	55.9-58.1	117 (100.5-269.2)	11_10985 (A/C)	C (0.43)	5.8	0.035	-2.04
	Germinative Energy 4ml	W-G4-1	5H	65.6-65.6	494.3	JHI-Hv50k-2016-308754 (A/G)	G (0.49)	3.9	0.048	-0.26

Table 3: QTL identified for malting quality traits in spring and winter barley. Where multiple associated SNP markers were grouped into a single QTL, a QTL interval based on the positions of the significant markers is shown. FDR – false discovery rate.

4.2.3 Associations (Objective 1)

An association analysis was conducted using the BLUPs estimated from historical trial data and the derived and amalgamated 9k and 50k iSelect genotypes. This GWAS analysis identified 24 independent QTL in 13 traits in spring barley and 2 associations in 2 traits in winters (Table 3).

Endosperm Modification

Two QTL were identified for friability in spring varieties. These were both located on chromosome 4H, at 7.6 Mb and 569.2 Mb. Three QTL for homogeneity were detected: on chromosome 1H at 0.3 Mb; on chromosome 3H at 676.4 Mb and on chromosome 4H at 569.2 Mb. Three QTL for whole corns were identified on chromosomes 3H, 4H and 7H. Three of the QTL for modification traits on chromosome 4H (friability at 569.2 Mb; homogeneity at 569.2 Mb and whole corns at 569.8Mb) showed overlapping QTL intervals with minor allele effects consistent with the overall negative correlation between friability/ homogeneity and whole corns.

Protein Modification

QTL for measures of protein modification were restricted to two loci in spring barley on chromosome 5H at 19.4 Mb and 532.1Mb. The former was associated with soluble nitrogen ratio (SNR), whilst the latter showed a highly significant association with both free amino nitrogen (FAN) (-log10p = 5.5) and SNR (-log10p=5.6).

Diastase activity/ Germinative Energy

Three QTL for diastatic power were identified in spring barley on chromosomes 1H (at 1.9Mb); 4H (at 642.3Mb) and 6H (at 555.7Mb). The diastatic power QTL on chromosome 4H was collocated with a QTL for germinative energy (at 8ml). In neither case were these associated with QTL for α-amylase activity. A single, highly significant, QTL for α-amylase activity was detected on chromosome 6H at 535.4Mb (-log10p = 9.1). An additional QTL for germinative energy (at 4ml) was detected in winter barley on chromosome 5H at 494.3 Mb.

Wort Traits

Two QTL for wort viscosity were detected in spring barley at 1.5Mb on chromosome 3H and on chromosome 5H at 445Mb.

Process yield

Five QTL related to process yield were identified; four of which came from the spring barley cultivar collection. These comprised two QTL for HWE in the spring cultivar collection (on chromosome 1H at 14.2 Mb, and on 3H at 3.7 Mb) and a single QTL for HWE on chromosome 1H (at 117 Mb) in the winter cultivar collection. A single locus on chromosome 6H at 478.2Mb had a significant effect on predicted spirit yield in the spring cultivar collection.

4.2.4 Historical trends (Objective 1)

There was evidence for significant historical trends in allele frequencies for the majority of peak markers associated with QTL in the spring barley collection, although not for QTL identified in the winter collection (Table 4).

Marker	QTL	1963-1991	1991-1994	1994-1996	1996-1997	1997-1999	1999-2001	2001-2003	2003-2005	2005-2010	2010-2015	p	
JHI-Hv50k-2016-284	S-HM-1	0.37	0.43	0.33	0.38	0.19	0.28	0.18	0.31	0.04	0.02	<0.001	***
SCRI_RS_124234	S-DP-1	0.17	0.22	0.26	0.08	0.16	0.23	0.15	0.33	0.12	0.09	0.19	.
JHI-Hv50k-2016-13067	S-HW-1	0.47	0.30	0.29	0.08	0.26	0.23	0.40	0.29	0.81	0.66	<0.001	***
JHI-Hv50k-2016-106390	S-PS-1	0.23	0.25	0.22	0.23	0.24	0.15	0.10	0.11	0.22	0.60	<0.001	***
JHI-Hv50k-2016-149225	S-WV-1	0.63	0.45	0.30	0.23	0.21	0.10	0.15	0.03	0.03	0.02	<0.001	***
11_11453	S-HW-2	0.74	0.48	0.37	0.23	0.34	0.10	0.18	0.11	0.04	0.03	<0.001	***
JHI-Hv50k-2016-158667	S-WC-1	0.09	0.05	0.07	0.15	0.05	0.20	0.10	0.09	0.08	0.05	0.58	.
JHI-Hv50k-2016-218678	S-HM-1	0.11	0.21	0.12	0.23	0.11	0.21	0.13	0.06	0.13	0.09	0.35	.
JHI-Hv50k-2016-228563	S-FR-1	0.52	0.54	0.52	0.50	0.47	0.45	0.39	0.34	0.20	0.11	<0.001	***
JHI-Hv50k-2016-256147	S-FR-2; S-HM-2	0.34	0.23	0.15	0.38	0.24	0.08	0.08	0.06	0.07	0.05	<0.001	***
JHI-Hv50k-2016-256219	S-WC-2	0.28	0.14	0.12	0.33	0.24	0.05	0.08	0.06	0.07	0.05	<0.001	***
JHI-Hv50k-2016-274747	S-DP-2	0.54	0.43	0.52	0.46	0.50	0.53	0.50	0.33	0.22	0.11	<0.001	***
JHI-Hv50k-2016-275320	S-G8-1	0.24	0.38	0.29	0.31	0.32	0.38	0.28	0.26	0.23	0.19	0.09	*
JHI-Hv50k-2016-284122	S-SN-1	0.40	0.50	0.63	0.62	0.55	0.55	0.45	0.50	0.41	0.23	0.00	***
JHI-Hv50k-2016-304397	S-WV-2	0.20	0.28	0.26	0.38	0.32	0.28	0.33	0.25	0.14	0.09	0.01	**
JHI-Hv50k-2016-312337	S-SN-2	0.17	0.10	0.07	0.08	0.13	0.20	0.26	0.36	0.32	0.39	<0.001	***
JHI-Hv50k-2016-312374	S-FA-1	0.14	0.08	0.11	0.08	0.13	0.18	0.25	0.31	0.30	0.40	<0.001	***
SCRI_RS_165986	S-PS-3	0.20	0.34	0.26	0.15	0.13	0.13	0.08	0.09	0.05	0.00	<0.001	***
SCRI_RS_177093	S-AA-1	0.14	0.15	0.15	0.15	0.16	0.13	0.20	0.11	0.07	0.18	0.93	.
JHI-Hv50k-2016-421716	S-DP-3	0.37	0.30	0.26	0.23	0.32	0.25	0.15	0.33	0.41	0.71	<0.001	***
JHI-Hv50k-2016-460614	S-WC-3	0.09	0.13	0.04	0.08	0.08	0.03	0.08	0.08	0.10	0.22	0.07	*
11_10985	W-G4-1	0.63	0.52	0.58	0.50	0.64	0.56	0.40	0.27	0.50	0.71	0.97	.
JHI-Hv50k-2016-308754	W-HW-1	0.40	0.48	0.64	0.36	0.46	0.54	0.55	0.50	0.51	0.60	0.07	*

Table 4. Frequency of the minor allele of markers corresponding to peak QTL positions identified in the current study grouped by the year in which they were first entered into UK National list trials. In each case, the minor allele frequency is reported for the variety set from which the QTL was identified. Linear trends in allele frequency over time were tested using a logistic regression model. p-values from these tests are shown, with asterisks indicating statistical significance (***: <0.001; **: <0.01: *: <0.1)

In a number of cases, these represented large allele frequency changes over the period of time covered by the study. For example, the major allele of the marker 11_11453 associated with higher HWE in spring barley showed a steady increase in the frequency (with the minor allele decreasing from 0.74 to 0.03) throughout the period considered in this study, starting as the minor allele in cultivars released before 1991 and becoming nearly fixed in the most recently released cultivars. Similarly, alleles causing an increase in diastatic power (JHI-Hv50k-2016-274747& JHI-Hv50k-2016-421716) increased substantially, becoming the major allele in recent spring barley cultivars.

4.3. Investigation of QTL intervals (Objective 1)

Where QTL effects potentially coincided with previously reported malting quality genes or QTL, published markers/ sequences were related to the physical map. In total, 16 QTL/ sequences from 11 studies were identified. Figure 6 shows a diagram of a genetic map that includes these loci in relation to the QTL found in this study. Some clear co-location is evident with for example the position of the amylases and QTL for associated traits; *Bmy1* with the QTL S-DP-2 and *Amy1* with the QTL S-AA-1. However, for most of the QTL found in this study there were no clear candidates in previously reported malting quality genes or QTL.

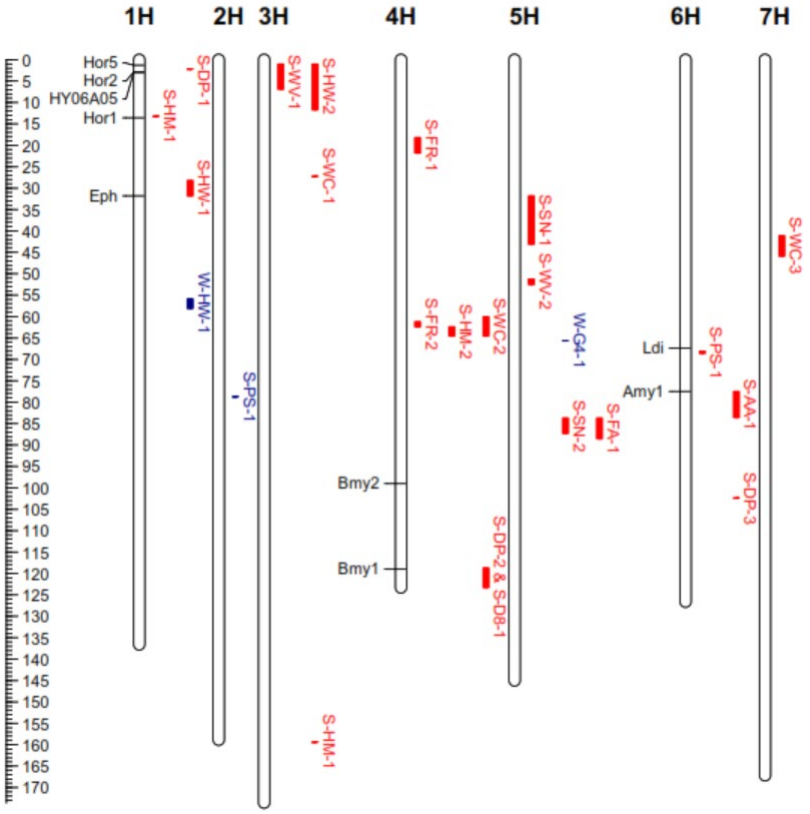

Figure 6: QTL positions identified in the current study relating to malting quality traits in winter and spring cultivar sets. QTL codes correspond to those described in Table 3. QTL codes with an 'S' prefix correspond to QTL identified in the spring set, and those with a 'W' prefix relate to those found in the winter set. Names and positions of genes or loci related to specific QTL or malting quality traits are indicated to the left of the bars. The scale on the far left of the figure indicates genetic positions (in cM units)

4.4 Development of Introgression lines via backcrossing programmes (Objective 2,3 & 4)

The six breeding companies that were part of the IMPROMALT Consortium worked on the development of the Backcross Introgression Lines (BILs) to test the hypothesis that targeted selection of spring x winter crosses would produce lines with significantly improved malting quality. This was carried out in five different background (Table 1), however due to delays in the development of the Acute (Winter) x Overture (Spring) cross, the results from the other four crosses are presented here to illustrate the results for Objective 3. BILs were also produced for Objective 4 to introgress winter habit genes into spring barley with minimal winter genetic background (i.e. the opposite of Objective 3) for the SY Venture (Winter) x Overture (Spring) cross. However, the results from these lines were agronomically less promising than the BILs from Objective 3 lines that brought the spring introgressions in a winter background and were not pursued as far as the other BILs.

The production of the BILs essentially followed a conventional backcrossing programme to Bc2 with genotypic marker selection of three regions that correspond to the QTL W_HW1 in the middle of chromosome 1H, S_HW2 on the distal end of the short arm of chromosome 3 and S_DP2 on the distal end of the long arm of 4H. The underlying genes were not known for the first two QTL so flanking genetic markers were used to introgress the genomic region whereas for S_DP2 on 4H the aim was to target a recombination between the likely causal gene *Bmy1* and the tightly linked vernalisation gene *VrnH2* (i.e. to introduce a spring allele at *Bmy1* while maintaining the winter allele at *VrnH2*).

Despite some problems at the subsequent selfing stage in the production of doubled haploid lines used in some crosses, BILs were produced from all crosses and these were grown over two years at multiple trial locations over two years. The field phenotypes confirmed that the BILs showed similar growth as the winter parents and genotyping with the 50K SNP platform also confirmed that the introgression lines were highly similar to the recurrent winter parent. Based on the field phenotyping and genotyping a subset of 60 BILs lines were selected for micro-malting and these samples were micro-malted by member companies of the Maltsters association of GB as part of the IMPROMALT consortium.

These data allowed multiple comparisons between crosses or different numbers of targets introduced to be calculated. However, the results presented here relate to the main contrast that was the focus of the project we were interested in: between Null lines (i.e. lines that had been through the backcrossing scheme, but without incorporating any of the target regions), and BILs with all three spring QTL.

Trait	**F**	**p**
HWE	4.7	0.004
Friability	6.5	0.01
Homogeneity	8.3	0.005
Grain Length	22.2	<0.001

Table 5. Significant Pairwise comparisons between Backcross Introgression Lines (BILs) with and without the three target spring QTL

The comparisons of the null and triple BILs showed a number of significant differences, in particular for Hot water extract, the target trait of the W_HW1 and S_HW2 QTL (Table 5). This improvement in HWE was associated with improvements in endosperm modification, in particular friability and homogeneity. As shown in Figure 7 this improvement was seen in three out of the four crossing programmes, Atlantick (Winter) x Overture (Spring) being the exception where no significant change was observed.

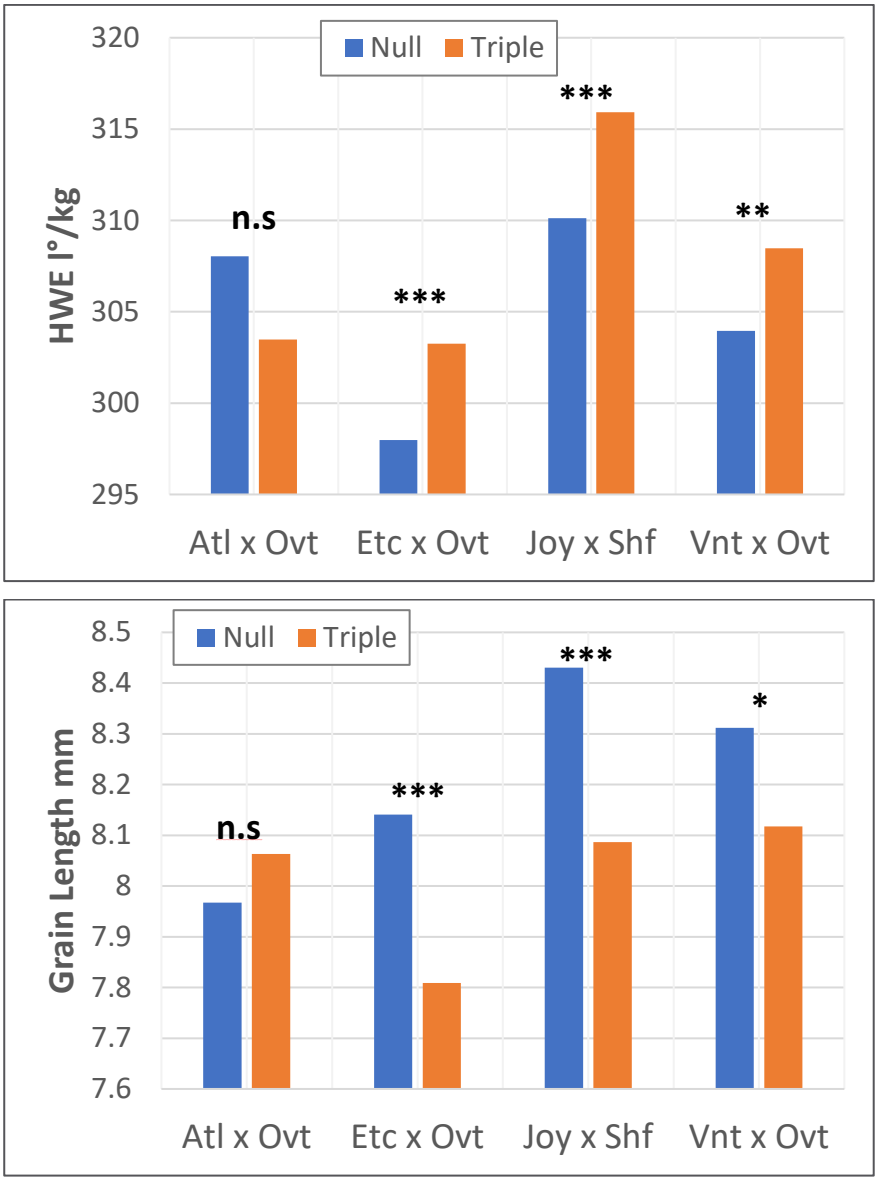

Figure 7, Histograms comparing the Null and Triple Backcross Introgression Lines for the four crosses (Table 1) for Hot Water Extract and grain length.

Interestingly, the comparisons of the null and triple BILs also showed a significant difference in grain length as well with the crosses that showed an improvement in hot water extract having shorter grain in the triple BILs compared to the null lines. Again, the Atlantick (Winter) x Overture (Spring) cross was an exception with no significant difference being shown (Figure 7).

4.5 Dissection of HWE QTL via segmental isoline development (Objective 5)

The hot water extract QTL on the distal region of the short arm of chromosome 3H (S_HW2) was the target for fine mapping. The strategy used was based on the use of a Bc2F1 individual from the SY Venture (Winter) x Overture (Spring) cross which was heterozygous across the QTL interval for a further round of backcrossing and the selection of recombinants in the interval through use of

flanking markers and additional KASP markers to delineate recombination breakpoints. These plants were then selfed, homozygotes selected and the resulting lines then multiplied for trialling.

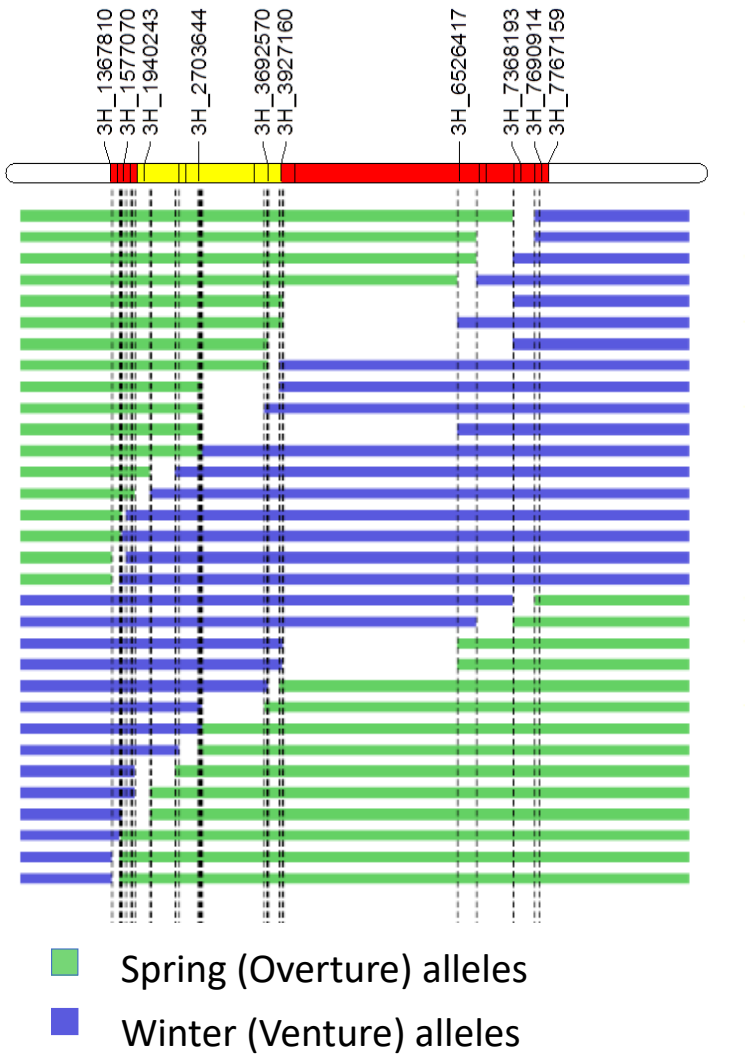

Figure 8. Cartoon showing the graphical genotype of the 3H segmental BILs derived from the Bc3F1 cross in the SY Venture (Winter) x Overture (Spring) cross.

A total of 800 Bc3F1 lines were screened, and 90 unique recombination events were identified across the QTL interval (Figure 8). The selfed homozygous progeny of the selected lines were genotyped with the 50K SNP platform and grown over two years of field trials (2016/17 &2017/2018) and 30 lines from the 2017 harvest were selected for micro-malting.
The field and malting trait data were combined with the genotypic information and a number of QTL effects were identified as shown in Figure 9. This study found significant effects on grain morphology and endosperm modification that were consistent with results from the BILs in the experiment. However, the segmental 3H BILs showed a separation of the QTL into two locations with grain size effects being distal within the introgressed region, whereas those relating to endosperm modification were more proximal and separated from the former.

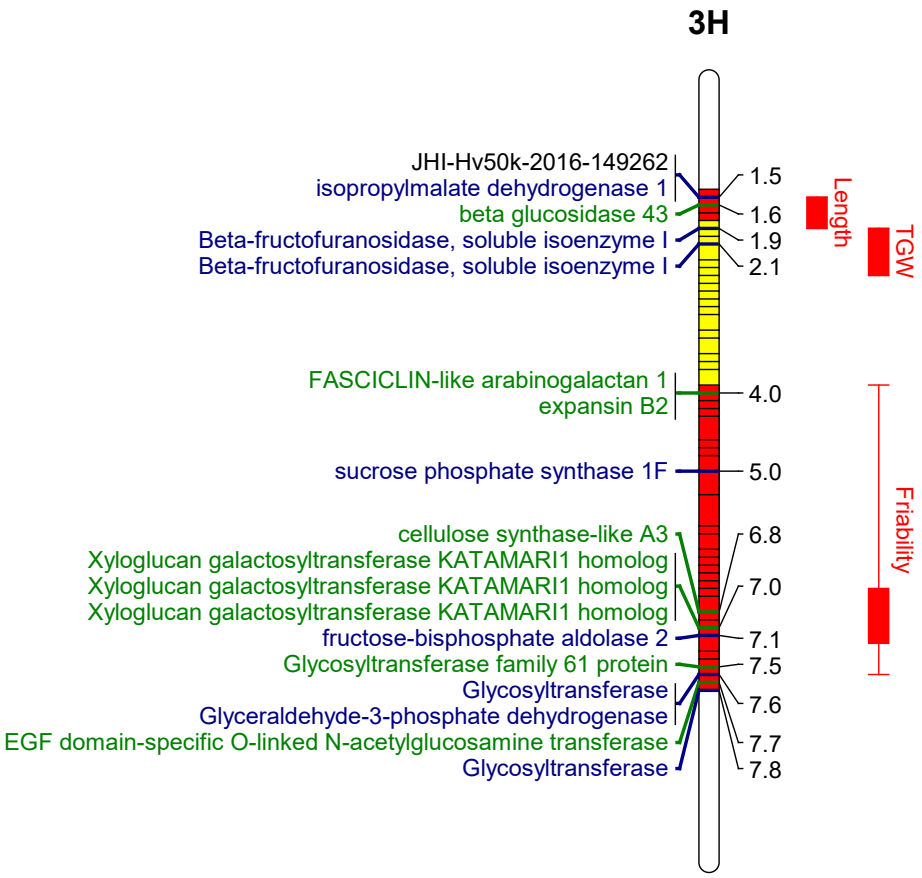

Figure 9. Diagram showing the S_HW2 QTL interval on 3H with the same potential candidate genes on the left and their position in cM on the right. The whisker plots of right indicate the delineated QTL positions found with the 3H segmental BILs.

The position of the peak of the friability QTL is in the region of a number of candidate genes that could potentially have a role in endosperm modification including a glycosyltransferase 61 protein as well a small family of potential xyloglucan galactosyltransferases.

5. Discussion

5.1 Objective 1

The results generated in this study provide a summary of the genetic variation at loci influencing major malting quality parameters in current and historical UK barley cultivars. The use of data from an extremely large number of historical trials allowed robust estimates of variety means for malting quality performance across a representative set of UK growing and testing environments. Such data would have been extremely expensive and time-consuming to generate for the large number of lines necessary to run association analyses. In addition, each GWAS (spring and winter) considered around 400 varieties, allowing the identification of high confidence QTL at high resolution. In addition, the composition of the association mapping panels, representing the complete range of genetic variation in UK barley germplasm over recent decades. Information on

QTL that have not become fixed in modern cultivars can be used to select for further improvement of malting quality parameters. Information on QTL that have become fixed in modern cultivars can be used in selecting appropriate parents in breeding for malting quality by ensuring that both parents contain the desirable QTL allele and relying on phenotypic selection to make further minor improvements in phenotypes.

The majority of malting quality QTL reported here were identified from spring barley. This may reflect stronger historical selection for malting quality traits in spring barley, and the incorporation (and selection of) variation that influences these traits. However, a previous study using subsets of this germplasm found substantial variation in winter varieties for a single malting quality trait (Diastatic Power) (Looseley et al., 2017), a finding not replicated here. This in turn may be due to a higher density of data for spring barley (reflecting the importance of this market in spring relative to winter barley breeding programs) in the current study, which may, provide more accurate BLUPs in this data compared to winter types, thus increasing the proportion of trait variation that can be attributed to genetic variation. Similarly, variety means were estimated for fewer winter barleys than for springs which will result in lower power to detect QTL. Alternatively, the relatively small number of winter malting varieties may lead to low minor allele frequencies at important malting quality loci when the entire collection is considered (unlike the study by Looseley et al., 2017), which considered equal numbers of high and low malting quality lines). In particular, current winter malting varieties are all descended from Maris Otter (Thomas et al., 2017), meaning that the genetic diversity represented in the winter malting data is much more restricted than that in the set of winter varieties overall, given that only varieties aimed at the malting market will have been extensively tested for malting quality traits. The crossing and selection strategies for winter malting barley are not so focused as for the spring crop, as winter malting barley is currently viewed as a declining market. The split of the winter barley market into feed and malting types is much more marked than the division in spring barley with AHDB UK Recommended List winter barley trials now largely being run under a feed management regime with a small number of selected sites run under a malting management regime (https://ahdb.org.uk/knowledge-library/recommended-lists-protocols). Nevertheless, the fact that significant breeding progress has been made since the release of Halcyon (a malting variety on the UK RL from 1985 until 2000) means that there is some genetic variation to be exploited and the inability of this study to detect more QTL may well reflect the lack of power due to the far fewer numbers of lines with phenotypic data. The lack of any significant genetic progress in the crop since 2000 together with the trend towards fixation of the beneficial alleles in the more recent spring genotypes suggests that progress may well have been achieved through the inter-crossing of good malting genotypes from different NW European gene-pools and that this process had largely been completed with the release of cultivars such as Westminster (introduced 2002) and Concerto (introduced 2006). Subsequent breeding progress has been more to improve other quality aspects and/or grain yield. In the winter crop, it appears that significant progress is still being made but there are too few malting varieties released since

2000 to test this. The fact that only one QTL for malt extract was detected suggests that the optimisation of alleles already present within winter germplasm across a large number of genetic loci might be leading to the progress. Nevertheless, a narrow crossing strategy that doesn't lead to any new beneficial alleles being introgressed into winter barley will not be capable of narrowing the gap between winter and spring quality. A new breeding strategy is required.

An analysis of correlations between BLUPs for each of the traits studied suggest that in many cases, the linear relationships between malting quality traits are strong. Thus, a number of the phenotypic characters examined in this study represent similar manifestations of the same underlying malting processes. For example, both homogeneity and friability measure similar aspects of endosperm modification, are highly correlated and share a QTL position on chromosome 4H. Correlations between the year in which a variety was introduced onto the National List (NL1 year) and a number of malting quality traits are likely to reflect overall levels of historical selection. This is particularly the case in spring varieties, where NL1 year shows a high level of correlation with both HWE and fermentability, and negative correlations with nitrogen content and wort viscosity. As expected under this interpretation, the correlation coefficients for these traits are greater in absolute magnitude when restricted to malting varieties only.

Central to the malting process is the synthesis or activation of enzymes that convert starch into sugar during the malting process. The major enzymes involved in starch degradation are α-amylase, β-amylase and limit dextrinase (Evans et al., 2010). Four QTL related to diastase function were identified from spring varieties. Three QTL for diastatic power were identified on chromosome 1H at 1.9Mb; on 4H at 642.3Mb and on chromosome 3H at 55.7Mb. The first of these co-locates with the position of the *Hor5* locus (encoding a γ-hordein endosperm storage polypeptide) (Shewry and Parmar, 1987; Cameron-Mills and Brandt, 1988). An EST (HY06A05) corresponding to a second hordein locus (*Hor2*) (Forde et al., 1985) previously associated with malting quality traits (but not diastatic power) (Potokina et al., 2004) also maps to this region (at 2.5Mb). This result supports previous observations that have suggested that hordein concentration is associated with diastatic power (Peltonen et al., 1994), and β-amylase specifically (Wei et al., 2009), although the mechanism behind this relationship is unclear. Despite the highly significant and strong minor allele effect at this locus, the beneficial (minor) allele has decreased in frequency over the period examined in this study, although this decrease is not statistically significant. This may reflect the fact that diastatic power has not been a major breeding target in UK spring barley, or linkage drag from selection against the producer allele at the *Eph* locus, that is located close by and has been subject to recent selection (Ehlert et al., 2019).

The second diastatic power QTL was located close to the known position of *Bmy1* (Yoshigi et al., 1995) and co-locates with a QTL reported by Looseley et al. (2017) for diastatic power in UK spring barley. β-Amylase has previously been reported to be the principle amylolytic enzyme, substantially correlating with DP (Delcour and Verschaeve, 1987; Gibson et al., 1995; Santos and Riis, 1996; Evans et al., 1997a, 1997b and 2008; Georg-Kraemer et al., 2001; Li et al., 2002; Duke

and Henson, 2008; Duke et al., 2013). It seems likely that this QTL represents an allelic effect of the *Bmy1* locus, confirming its importance to the genetic control of diastatic power.
A single QTL for α-amylase activity was identified on chromosome 6H at 535.4Mb, with an interval of 532.8-535.8Mb. This QTL was highly significant (-log10p =9.1), with the minor allele causing an increase of 2.77 DU. This QTL colocalises with the known position (533.9-542.9Mb) of a cluster of amylase genes at the *Amy1* locus (Zhang and Li 2017) and is likely to represent allelic effects or copy number variation at this locus (Mascher et al., 2017). The QTL is found in a near identical position to a QTL for α-amylase activity in US barley breeding programmes (Mohammadi et al., 2015), although it is not clear whether the alleles identified in the current study correspond to those previously reported. Despite a previous finding showing that α-amylase activity had a positive linear relationship with diastatic power (Gibson et al., 1995) (a finding supported by a moderate positive correlation between α-amylase activity and DP in the results reported here), QTL S-AA-1 (Table 3) was not associated with a corresponding QTL for DP. Whilst a QTL for DP was detected on the long arm of chromosome 6H (S-DP-3; Table 3), this was distinct and distal to the α-amylase activity QTL. Furthermore, despite the large effect associated with this locus, an analysis of allele frequency trends at the peak marker shows that the beneficial allele has remained at low frequency across the time period covered by the varieties in this study, suggesting that the allele has not been subject to positive selection in UK spring barley and may not influence primary malting quality characteristics that are under direct selection. Alternatively, there have been associations of increased α-amylase levels with sprouting in the ear (Pre-Harvest Sprouting) (Lin et al., 2008) and it may be that UK breeders have avoided excessively high AA levels due to the likelihood of wet harvests leading to excessive pre-germination and malting rejections. Additionally, the beneficial allele for this locus has remained at a consistently low frequency over time suggesting that it does not influence primary malting quality characteristics that are under direct selection. Other studies have similarly concluded that α-amylase activity is not the primary determinant of wort sugar in other germplasm collections (as described above) and that β-amylase is more strongly associated (Evans et al., 2008; Duke et al., 2013). Nevertheless, future genetic gains for β-amylase activity may require concurrent optimisation of other enzymes and this QTL effect represents an important determinant of α-amylase activity in current UK breeding germplasm.

Another key processes in the production of malt is the modification of starchy endosperm. In order to characterise this, physical properties of malted grain are assessed in malting quality analyses through friability (overall levels of modification), homogeneity (evenness of modification) and whole corns (the proportion of wholly unmodified grain). Despite strong correlations between BLUPs for these traits, they only co-located at one QTL (S-HM-2; S-FR-2 & S-WC-4), suggesting a degree of independence in the genetic control of each of these traits. This locus on chromosome 4H has not previously been implicated in malting quality variation and does not co-localise with known malting quality genes. Nevertheless, the QTL peak is located adjacent to a gene (HORVU4Hr1G069100.2)

that has high homology to a β-Xylosidase (HORVU6Hr1G075010.9) previously demonstrated to play a role in the hydrolysis of xylan oligosaccharides in barley (Lee et al., 2003) and which is expressed in both developing grain and embryos (https://ics.hutton.ac.uk/barleyGenes/).

In a number of cases, QTL for endosperm modification co-located with QTL for wort traits or process yield (discussed in detail below), emphasising the importance of modification to primary malt quality traits. The identification of 8 QTL related to endosperm modification in the spring cultivar set, a number of which are not fixed in current cultivars, offers significant opportunities for the optimisation of modification traits in UK cultivars.

The production of the potentially carcinogenic ethyl-carbamate during the distilling process is associated with barley varieties that produce a glycosidic nitrile known as epiheterodendrin (McGill and Morley. 1990) at the *Eph* locus. This locus has been mapped to the short arm of chromosome 1H (Swanston et al., 1999) and more recently, genes required for epihetrodendrin biosynthesis have been located at this locus (Knoch et al., 2016), representing a physical interval on the current genome assembly between 16.1 and 17.1Mb on 1H. Non-production of the compound is due to a deletion of this region (Ehlert et al., 2019) and null alleles at 9k iSelect SNPs located in the deletion have been shown to be perfectly correlated with non-production of epiheterodendrin. These SNPs behave like dominant markers and are of limited value in marker-assisted selection but the recent development of a SNP assay at 17.2 Mb on this chromosome (which is highly predictive of epihetrodendrin production (https://www.huttonltd.com/services/molecular-diagnostics) solves the problem. Two distinct sets of markers (S-GN-1 & S-GN-2) associated with opposing minor allele effects on glycosidic nitrile production were identified within this region, despite the fact that the physical position of these marker sets overlapped. Whilst it is highly likely that at least one of these QTL represent an effect of alleles at the *Eph* locus, the detection of two significant but opposing effects is more likely to reflect the fact that non-production is due to the deletion. Furthermore, there is some overlap in the BLUPs estimated for non-producers compared to producers, e.g. the producer Agenda has predicted mean of 216, whereas the non-producer Corsica has a predicted mean of 255. It is likely that seasonal variations lead to imprecision in the estimation of the phenotype and thus, over- or under-estimation of the marker effects at individual SNP loci and these are then compensated for by detection of a QTL of opposing effect, which is most likely to be a ‘ghost’ QTL. An analysis of allele frequencies shows that the allele associated with reduced glycosidic nitrile production has increased in frequency at both QTL in spring varieties, and that this increase is highly significant. This increase coincides with the release of a molecular marker for non-producers in the early 2000s (Bringhurst, 2015), illustrating the effectiveness of marker assisted breeding in the genetic improvement of malting barley. The marker is not, however, diagnostic for non-producers of GN as it is the major allele in the elite winter barleys, yet only five are non-producers. This is clearly a linked marker and its potential

effectiveness in deployment for Marker Assisted Selection will depend upon the genetic background of the gene pool that a breeding programme is using.

Two QTL for hot water extract (HWE) were identified from spring variety set and one from the winter set. In the spring varieties, these were located on chromosome 1H at 14.2Mb and on 3H at 3.7Mb. In the winters, the HWE QTL was located on chromosome 1H at 117Mb. This last QTL is in a (genetically) similar position to a malt extract QTL identified in a joint analysis of a European spring and winter barley cultivar collection (Matthies et al., 2014), although these QTL are located in a region of low-recombination. Whilst a number of genes with putative associations with cell wall or carbohydrate metabolism are located close to the HWE QTL peaks, little is known about the specific genetic control of malt extract traits, making the identification of candidate genes difficult. Despite the absence of shared QTL between HWE and measures of malt modification, there were strong correlations between BLUPs for friability, homogeneity and HWE illustrating the importance of endosperm modification to the output of the malting process.

A locus on chromosome 6H at 478.2Mb represented a QTL for PSY (predicted spirit yield). The peak marker for this QTL (SCRI_RS_165986) was located close (in genetic distance if not physical) to the position of a gene producing a known inhibitor of limit dextrinase (LD) (Stahl et al., 2007). Variation at this locus has previously been shown to influence the activity of limit dextrinase inhibitor (Huang et al., 2014), and whilst it is not clear if the QTL represents the effect of an allele at this locus, limit dextrinase is the only enzyme capable of cleaving α-1-6 linkages in branched dextrin molecules (Manners et al., 1970). As such, the activity of the inhibitor may have a substantial effect on the fermentability of the extract. The activity of limit dextrinase inhibitor has been shown to have considerable influence on starch biosynthesis and particularly the ratio of amylose to amylopectin during grain development (Stahl et al., 2004 and 2007), which may result in differential efficiency of the amylose enzymes and the capacity to reduce starch to fermentable sugars. Alternatively, the binding of limit dextrinase to its inhibitor can protect the enzyme during distillery mashing and its subsequent release during fermentation can, when coupled with α- and β-amylase activity, produce more fermentable sugars and hence, increase spirit yield (Stenholm and Home, 1999; Bringhurst et al., 2001; Walker et al., 2001). It is not known how allelic variation at the *Ldi* and/or *Ldx* loci may alter the degree to which limit dextrinase inhibitor exists in bound form, but it is a possible mechanism that may account for the observed QTL effect.

A single QTL position on chromosome 5H (532.1Mb) was detected for wort nitrogen traits, which influenced free-amino nitrogen (FAN) and soluble nitrogen ratio (SNR), suggesting that the locus is affecting protein modification and digestion rather than absolute levels of protein. This QTL corresponds to the physical position of a marker (12_31361) previously reported to be associated with Kolbach index in a mapping population derived from two elite German spring malting barleys (Kochevenko et al., 2018). A QTL for Soluble Nitrogen has previously been reported in a similar location on chromosome 5H in a European association mapping panel, but the position of this QTL

is somewhat distal to the effect reported here (Matthies et al., 2014). No gene candidate has previously been suggested for this effect, but the physical region contains a gene (HORVU5Hr1G071510) that is annotated as a Subtilisin-like protease and is expressed in early grain development and at a lower level in embryos (https://ics.hutton.ac.uk/barleyGenes/). This gene represents a strong candidate for a follow-up study. Historical allele frequency data suggest that the minor allele (increasing levels of FAN/soluble nitrogen) has increased in frequency over the period of the study but still represents the minor allele in recently released varieties. However, the requirement for protein modification will vary between brewing and distilling applications and as such strong directional selection for wort nitrogen traits is likely to be absent.

Together, these data represent an important summary of genetic variation for malting quality traits in elite UK breeding lines and, along with the associated markers, should be of considerable interest to breeders producing new varieties for the malting barley market; representing a comprehensive survey of genetic variation for malting quality in this material. In particular, the analysis of allele frequency trends will allow the selection of breeding targets that are currently segregating in elite varieties, considerably reducing the costs associated with the incorporation of novel genetic variation into existing breeding populations. In addition, by conducting a combined genomic analysis of a variety of malting quality traits, correlations between them (on an overall phenotypic level as well as at specific loci) have provided clues about their functional relationships. Candidate genes have been identified for a number of QTL, and further studies, including functional validation of these candidates, offers a route to a more complete understanding of the specific relationship between genetic variation at these loci and the physiological and biochemical processes that take place during crop development, malting, and fermentation. Such a detailed understanding of genetic relationships will provide the knowledge necessary for targeted genetic improvements in malting quality in new barley varieties.

5.2 Objectives 2-6

The main backcrossing programme succeeded in the aim of improving malting quality in the winter crop and this material has now been utilised within the companies breeding programmes. This built on and utilised the information gathered in the AGOUEB project and success of the IMPROMALT project is testament to quality of the data that underpinned the previous project. The significant improvements in hot water extract shown in three of the four crosses are substantial and commercially significant (Figure 7). The improvement achieved in the project by the utilisation of genetic information and traditional backcross breeding guided by molecular markers represents a potential step change in winter variety malting quality when compared to the progress shown over the last thirty years as indicated in Figure 4.

The fine mapping of the hot water extract on the distal end of chromosome 3HS (S_HW2) by the development of segmental backcross introgression lines was also a success having narrowed

down the interval and suggested a few candidate genes underlying the effect. The potential involvement of a glycosyltransferase 61 protein is of particular interest as this gene family plays a key role in the synthesis of arabinoxylans in grasses (Anders et al., 2012).
Interestingly, the analysis of both sets of introgression lines developed in the IMPROMALT indicated that the improvement of HWE was associated with changes in grain morphology. The segmental BILs indicated that this was due to close linkage and the effects could be separated. This does indicate the difficulty inherent in the improvement in multiple traits that barley breeders face as is also evident in the yield reduction shown relative to the recurrent parent in this first generation backcross material. However, this material has now entered into the commercial breeding programmes of the IMPROMALT partners and should result in RL entries in the near future. The results of this project will thus, ultimately help to improve the malting quality of UK winter varieties in the short to medium term and potentially help to spread the harvest load and provide more choice to UK farmers and barley end-users in increasingly uncertain future climate change scenarios.

5.3 Summary Points

(i) The use of genotypic data on NL and RL material has provided a unique insight into the genetic control of malting quality traits in elite barley cultivars adapted to the needs of UK industry and farming. The IMPROMALT project has built on the success of the previous AGOUEB (Association Genetics of UK Elite Barley) and shown the value of augmenting the existing dataset by ~20%.

(ii) The project has again shown the value of genotyping the RL entries and synthesising the genetic fingerprinting with the trialling results. The information gained on genetic control and shifts over time has real value and an important role in aligning academic research with UK-focussed applications. Given the relatively minor cost involved in genotyping, it would be advantageous to ensure that this continues into the future.

(iii) The genetic relationship between varieties recommended over the last 48 years illustrated by the ‘Circle of Barley’ dendrogram in Figure 1 shows a clear change overtime (as expected) but also shows the close similarity of more recent varieties to one another. This information will aid the maintenance of genetic progress through the careful selection of novel material in pre-breeding efforts of members of The IMPROMALT consortium.

(iv) The success of the IMPROMALT project was based on the meaningful data derived from the RL trials data that identified differences that are commercially significant amongst closely related material. This is illustrated by the significant difference between the mean hot water extract of varieties that were approved and that of those not approved by the UK Malting barley Committee on the 2018 RL and reiterates the robustness of the selection process.

(v) There is a clear improvement over time in malting quality traits, such as hot water extract of UK varieties, when plotted against year of introduction for varieties included on the RL and considered for the malting barley committee (Figure 4). However, there is evidence of a plateau in this improvement since 2000, especially in spring varieties, though malting quality has been maintained while yield has continued to improve.

(vi) The genome wide association study found a considerable number of QTL for malting quality traits in UK adapted material. These QTL provide a number of avenues for further research on, for example, the control and improvement of diastatic power, and levels of endosperm modification. It should be stressed that most of the QTL found do not relate to previously known major genes or published QTL in other studies, emphasising the specific nature of the differences found in this elite material that relates to UK industry needs.

(vii) Interestingly there is evidence that the larger effect malting QTL have predominantly been fixed in more modern spring varieties, reflecting the plateau seen in Figure 4. This is a testament to the strength and efficacy of selection in breeders' and official trials (see above) but potentially does represent a barrier to further improvement. Further research is needed to identify the genes underlying these QTL to allow the targeted introduction of novel allelic forms of these genes into elite material to obtain further improvements.

(viii) The IMPROMALT project has been successful in its primary aim of improving the malting quality in UK winter barley through the targeted introduction of spring material. This enabled beneficial spring alleles to be introduced in genomic regions known to be harbour malting quality QTL though a targeted backcrossing programmes by breeders in the IMPROMALT consortium. Further improvement could potentially be made by the introduction of more spring QTL found in the GWAS analysis in this project.

(ix) As part of the project, one of the targeted QTL, on the short arm of chromosome 3H, was subject to genetic dissection by fine mapping through the development of specific backcross lines. This indicated the potential involvement of gene family members involved in arabinoxylan synthesis that merits further research. In addition, this highlights the need for more research into the genes involved in the processes underlying germination and mobilisation of resources within the malting of barley using modern genomic technologies and material that is relevant to current industry needs.

(x) This significant improvement of winter barley malting quality up towards that of spring varieties could help reverse the long term decline in the winter malting barley crop in the UK and help the sustainability of end-user industries by taking advantage of its yield and agronomic benefits. In particular, the earlier harvest of the winter crop may help bring resilience in the face of potential climate change scenarios. The winter lines derived in the IMPROMALT are currently being used in the breeding programmes of the consortium members and will have an impact on UK farming through future NL/RL entries.

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