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Project Report No. 629

Integrated platforms for barley breeding and genetic research

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1. Abstract

This project aimed to discover new molecular diagnostics for use in barley breeding programmes. It exploited phenotypic data and high-density genetic marker data, generated by resequencing the germplasm assembled under the *Association genetics of UK elite barley – AGOUEB* project (AHDB Project Report 528). In AGOUEB, we assembled a vast collection of new and legacy phenotypic data and conducted preliminary genetic analyses. However, the initial AGOUEB work was of relatively low resolution (*c.* 1000 markers). As a result, the project lacked the highest level of informativeness. Additionally, most of the phenotypic data was not analysed optimally and remained unpublished.

This project had the following objectives:

1. To use a new high-density genetic marker dataset (*c.* 2,100,000 SNP markers) to analyse phenotypic data initially assembled and analysed in AGOUEB.
2. To discover and provide the information required to convert diagnostic markers into breeder-friendly marker systems.
3. To publicise findings to the scientific and end-user communities.

Reanalysis of the phenotypic dataset, with the new high density marker set, represented a greater than 130 fold increase in genetic marker density. This allowed us to define genomic regions that contain genes responsible for 31 traits. We were also able to identify SNPs that are significantly associated with each of these traits. For each of these SNPs, putative sequences for KASP markers have been identified. We are pursuing serveral of these traits, with various collaborators, with the view to identify and characterise the casual genes.

The progress made in AGOUEB and this project marks a significant step forward in the ability to better track key barley traits through breeding programmes.

2. Introduction

Advances in the assembly of DNA sequence data of barley means that it is now possible to compare variation amongst different barley lines across some 60,000 sequences representing barley genes. Previous work under the AGOUEB project had assessed AHDB Recommended List and National List data to identify markers associated with agronomic, yield, quality and DUS data. But, as only ~ 1000 SNPs were available at the time, the resolution was insufficient to resolve the differences down to potential candidate genes based purely on these markers (Cockram et al 2010). This last step is essential for effective and rapid mining of gene-bank collections for future improvements in breeding. Therefore, the main focus of the INTEGRA project has been the re-analysis of the AGOUEB data sets with the new SNP data revealed from the analysis of the data generated from exome capture sequences to test whether we can identify candidate genes for some of the un-resolved DUS traits characters.

The aim of this project is to rapidly exploit data from newly assembled genetic marker information to analyse phenotypic data collected – but, so far not optimally analysed – in a previous HGCA sponsored project. Analysis of the trait data with the new marker data will increase our ability to genetically resolve regions of the barley genome that control economically important traits, and then facilitate facile marker assisted selection in commercial plant breeding programmes. The project takes advantage of recent barley genomic research outcomes (IBSC 2012, Mascher et al 2013) and commercial technologies such as KASP and Affymetrix genotyping to generate a platform for genetic analysis in this crop.

The ability to monitor the inheritance of segments of the genome using ‘genetic markers’ in simple and complex populations is the core of modern genetics. Only 25 years ago, as few as tens or hundreds of markers were assayed in plant genetic studies, often through laborious techniques that involved the use of radioactivity (Beckmann and Soller 1983, Vos et al 1995). Today, as the result of a series of intricate and innovative technological developments, millions of markers can now be followed in a single experiment (Clark et al 2007) both quickly and at relatively low cost. For these 25 years, we have been at the forefront of genetic marker technology development and implementation in cultivated barley (Waugh et al 1997, Ramsay et al 2000, Close et al 2009). The most accessible and widely used resource currently available is an Illumina iSelect platform that assays genetic variation at 9000 sites across the genome that contain single nucleotide polymorphisms (SNPs) (Comadran et al 2012) While this platform has been an exceptionally powerful tool for genetic studies, it suffers from a combination of ascertainment bias (Moragues et al 2010), relatively high cost, and, due to marker numbers, and the inability to adequately exploit all of the recombination events (genetic power) present in experimental populations.

In INTEGRA-2.1 we plan to exploit a very high density 2.1M SNP dataset collected by resequencing the AGOUEB germplasm to analyse the phenotypic data that we collected in the AGOUEB and related programmes. This should provide unprecedented power to detect associations between molecular markers and phenotypic traits that can subsequently be deployed in accelerating genetic gain in plant breeding. Our analyses will provide many new leads (genes) and supporting information for several end-user relevant projects, facilitating the development of molecular diagnostics and enabling trait gene discovery.

The exome capture sequencing platform is relatively expensive and not routine in a breeding situation. To make the data more widely applicable, we are using 58,000 of the most robust and informative SNPs discovered using this approach to develop a generic genotyping platform for release to the broader community, through collaboration with the commercial organisation Affymetrix. This platform will provide a >97.5% reduction in cost per informative SNP datapoint over current technologies, making it financially accessible to entire communities.

The objectives of this project are below:

1. To use a new very high density genetic marker dataset (ca. 2,100,000 SNP markers) to analyse phenotypic data first assembled and analysed in a previous HGCA sponsored project - AGOUEB.
2. To discover and provide the information required to convert diagnostic markers into breeder-friendly marker systems
3. To publicise our findings to the relevant scientific and end-user communities

3. Materials and methods

3.1. Phenotypic Data

We have re-investigated a total of 32 DUS traits which were analysed previously by Cockram et al. (2010) having been scored in ~500 lines which were genotyped using the bOPA1 genotyping platform which consisted of 1536 SNPs. For two of the DUS traits, seasonal growth habit (spring or winter) and ear row number (2 or 6) we have 357 lines that have both phenotypic and genotypic data derived from exome capture. For the other 30 DUS traits, the number of lines with both phenotypic and genotypic data for the DUS traits varies from 159 to 179 depending on the trait.

3.2. Genotypic Data

A total of 138545 SNPs from exome capture were available for up to 357 lines from the BBSRC UK barley genome project (unpublished). These SNPs had passed the following filtering criteria:

1. >= 8x coverage for >= 50% of the samples (to ensure robust SNP and genotype calls)
2. >= 95% of samples represented at SNP locus (for maximum sample representation)

3. >= 5% minor allele frequency at the level of the sample, i.e., counting sample genotypes rather than individual reads (to exclude SNPs based on very rare alleles)
4. >= 30 SNP quality score, equating to a >= 1/1000 chance of the SNP having been called in error (for SNP robustness)
5. >= 98% of samples homozygous (to reduce false positives by removing variants that are the result of read mis-mapping or Illumina systematic sequencing error)
6. no indels

Missing SNP values were imputed for each chromosome separately using the R packages linkim (Xu and Wu, 2014) after recoding the SNP alleles 0 and 1.

3.3. Population Structure

The population structure of the initial 357 lines was investigated using Principal Component Analysis performed in R version 3.2.4 (R core Team, 2016).

3.4. Diversity for seasonal growth habit and ear row number

There were 357 lines characterised for seasonal growth habit (spring or winter) and ear row number (2 or 6) and for spring lines and whether it was exotic (exotic or non-exotic).

The divergency of the lines within different populations was investigated using the fixation indexes Φ_{ST} (Meirmans 2006), which is a locus-by-locus estimate of population diversity (defined as a function of the between population variance component and the within variance component) comparing the between population genetic variance to the total genetic variation over all populations and G_{ST} (Nei 1973) which is the locus-by-locus estimates of expected heterozygosity (gene diversity) between populations compared to the total genetic diversity. The fixation indexes range from zero which indicates no differentiation between the overall population and its subpopulations to a theoretical maximum of one. Negative values can be obtained (Meirmans 2006) and these can be interpreted as no population structure being present and are reset to a value of zero.

Where numbers allowed, further subpopulations were investigated (Table 1), Spring with ear row number two verses Spring with ear row number six, Winter with ear row number two verses Winter with ear row number six and Spring with ear row number two verse Winter with ear row number two. The number of SNP markers available for the comparison of these smaller populations reduces from the maximum of 138,545 as some of the SNPs become monomorphic with the smaller number of lines within the subpopulation comparisons.

Table 1: Number of lines in each subgroup

	Ear Row Number		**Total**
Seasonal growth habit	**2**	**6**	
Spring	230	29	259
Winter	70	28	98
	300	57	357

The library mmod (Winter 2012) within R version 3.2.4 (R core Team, 2016) was used to calculate the fixation indexes Φ_{ST} (Meirmans 2006) and G_{ST} (Nei 1973). Manhattan plots were used to show the minus inverse of the fixation indexes and created in using the R library qqman (Turner 2014).

3.5. Genome Wide Association Mapping

For each DUS trait with more than two responses (Supplementary S4), the association analysis fitted was a linear mixed model:

$$\boldsymbol{y} = \boldsymbol{X\tau} + \boldsymbol{Zu} + \boldsymbol{e}$$

where $\boldsymbol{y}$ is the vector of the mean response for each of n lines, $\boldsymbol{\tau}$ is a vector of fixed covariate terms consisting of an overall intercept term, the marker covariate and principal components, $\boldsymbol{X}$ is the associated design matrix, $\boldsymbol{u} \sim N(0, \sigma_g^2\boldsymbol{K})$ is the vector of random line effects with kinship matrix $\boldsymbol{K}$ representing relationships between lines which was generated by the method of VanRaden (2008) with design matrix $\boldsymbol{Z}$, $\boldsymbol{e} \sim N(0, \sigma^2\boldsymbol{I})$ is the residual effect, where $\boldsymbol{I}$ is the identity matrix. A separate model is fitted for each SNP marker. The marker effect represents the additive difference between the two homozygous individuals with opposing SNP alleles. The number of principal components included in the model was three and was chosen to reflect the structure in the population corresponding to season growth habit and row type. The kinship matrix (VanRaden 2008) and PCA components for analysis were created in GAPIT (Lipka et al 2012) for consistency and each model was run in GAPIT.

For the binomial DUS traits with two responses (Table 2), we fitted a generalised linear mixed model (GLMM).

$$\boldsymbol{\eta} = g(E(\boldsymbol{y}|\boldsymbol{\tau}, \boldsymbol{u})) = g(\pi) = \log\left(\frac{\pi}{1-\pi}\right) = \boldsymbol{X\tau} + \boldsymbol{Zu}$$

Where $\boldsymbol{\eta}$ is the linear predictor, g is the logit link function, $\boldsymbol{y}$ is the vector of responses with Bernoulli distribution, π is the unknown probability of response B (Table 2), $1 - \pi$ is the probability of response A; $\boldsymbol{\tau}$ is vector of fixed covariate terms, consisting of an intercept term, covariate terms for principal components and the SNP marker, $\boldsymbol{u} \sim N(0, \boldsymbol{K})$ is a random term for lines where $\boldsymbol{K}$ is the kinship matrix.

The significance of the fixed marker term is assessed using a Walds test, which is compared to an asymptotic chi-squared distribution.

The GLMM models were fitted using the library ASReml (Butler et al 2009) within R version 3.2.4 (R core Team, 2016). ASReml uses a penalised quasi-likelihood technique based on a first order Taylor series approximation to the likelihood. The kinship matrix (VanRaden 2008) and PCA components used in the GLMM were created in GAPIT (Lipka et al 2012).

Table 2: Binomial DUS Trait Summary

Binomial DUS Trait	Response A (Number of lines)	Response B (Number of lines)	Total Number of lines
Awn anthocyanin colouration of tips	Present (127)	Absent (32)	159
Awn spiculation of margins	Reduced (1)	Present (137)	138
Flag leaf anthocyanin colouration of auricle	Present (117)	Absent (30)	147
Grain disposition of lodicules	Frontal (3)	Clasping (158)	161
Grain husk	Present (160)	Absent (3)	163
Grain rachilla hair type	Short (36)	Long (143)	179
Grain ventral furrow presence of hairs	Present (31)	Absent (145)	176
Lower leaf hairiness of leaf sheaths	Present (66)	Absent (109)	175

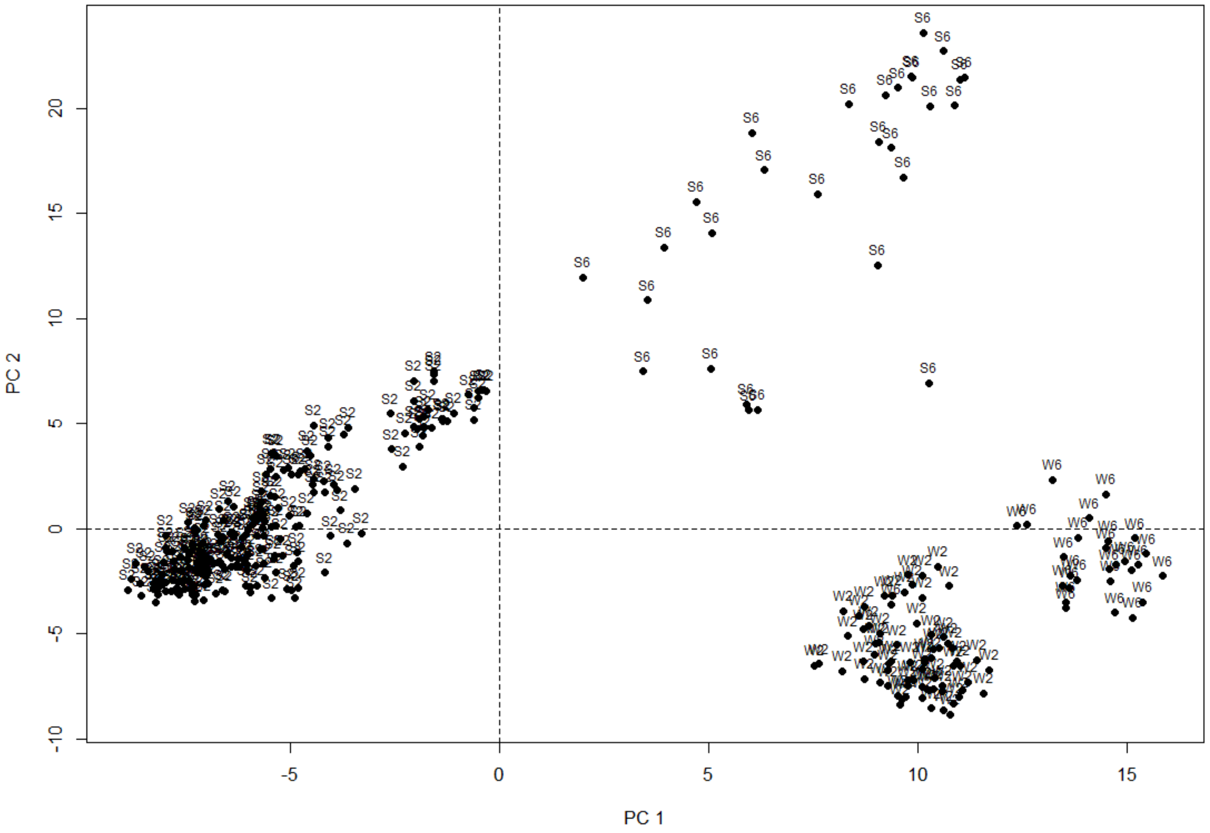
3.6. KASP marker sequence identification

After identifying the SNP with the most significant association for each trait included in the analysis, we referred back to the exome capture dataset to identify a sequence that would facilitate the conversion of this SNP into a KASP genotyping marker. In addition to identifying 100 nucleotides either side of the SNP (LGC Biosciences requirement is at least 50 nucleotides either side of the SNP) due to the available exome capture data, we were able to annotate SNPs that were variable between lines in this dataset with the appropriate IUPAC nucleotide code. Therefore, we have at least one sequence per trait available for KASP assay.

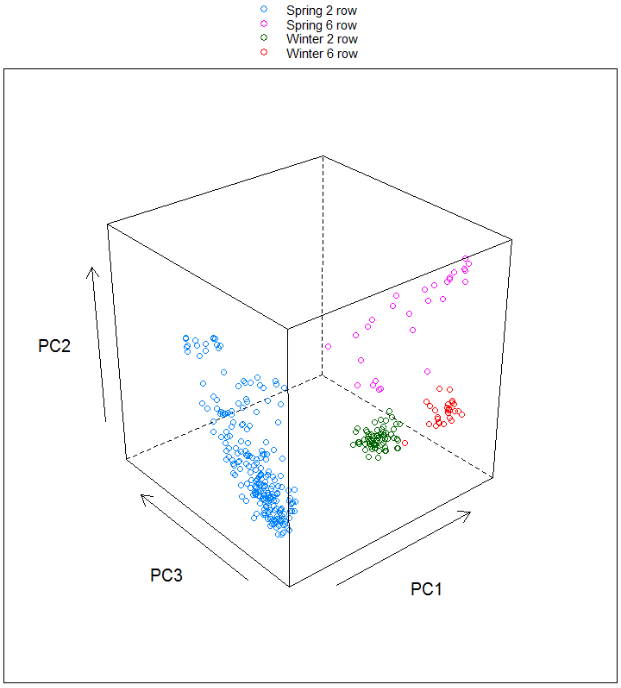
4. Results

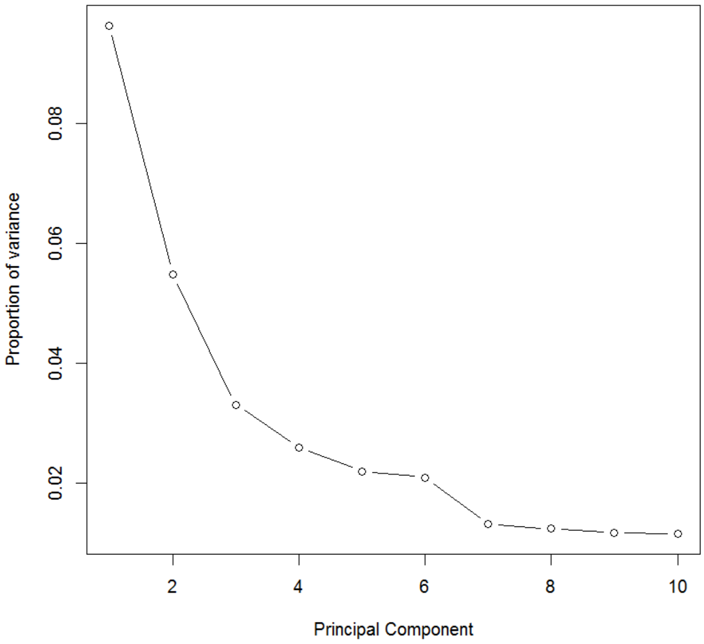
4.1. Population Structure

The initial population of 357 lines was investigated for genetic substructure using principal components analysis on the 138,545 SNPs. The first three principal components account for 18.4% of the variance in the population and distinguish between the phenotypic combinations of row number and seasonal growth habit.



S2=Spring 2 row, S6=spring 6 row, W2=Winter 2 row, W6=winter row





4.2. Diversity for seasonal growth habit and ear row number

Both seasonal growth habit and row type are also binomial variables, and as such, we attempted to fit a binomial GWAS model to the data. This brought problems related to the population structure of the data set. In GWAS, the principal components are included in the model to account for any population structure. However, as illustrated in the previous section, the population structure in this data set reflects both the seasonal growth habit and the row type - the two traits that we are interested in. Thus, in the initial binomial GWAS models fitted, the principal components accounted for all the genetic variation due to seasonal growth habit and row type. Therefore, we failed to identify any significant SNP markers which distinguished between the winter and spring or 2 row and 6 row. Conversely, failing to include the principal components in the model resulted in over 21,000 SNP markers with a –log10(pvalue) greater than 10. With so many significant SNP markers it is clearly impossible to distinguish 'real' significance from a false-positive. For this reason, we decided to investigate the seasonal growth habit and the row type using fixation indices.

There were 357 lines characterised for seasonal growth habit (spring or winter) and ear row number (2 or 6) for which there was a total of 138,545 SNPs from exome capture that were included in the fixation index analysis.

Plots of minus the inverse of the fixations index G_{ST} (Nei 1973) are shown in Figures 1 to 6, with plots of the fixation index Φ_{ST} (Meirmans 2006) shown in the supplementary material (S1). For the most part, the SNPs identified as defining the diversity between the studied populations were the same under both indexes. In cases where they differed, the SNPs were in the same genomic region. For Spring 2 vs 6 row, the Φ_{ST} fixation index identified a potential SNP at 7H which was not as obvious from the G_{ST} index, however, this SNP had a diversity value at least half as small as the most important SNPs on 2H.

The results for 6 row Spring vs Winter are not shown. There were a small number of lines in this group (57) with only 29 possible different allele frequencies within the 131,139 SNPs available and therefore, a limited number of possible fixation index values. For spring lines, we also had details of which were exotic lines and a comparison within spring lines was made, these are shown in Figure 6.

The regions identified using this analysis on the complete set of 357 lines for row type highlighted a region on 4HS that was strongly divergent for this trait, along with other regions on 1H, 2H and 4H being of potential interest (Figure 2). These overlapped with regions of the genome identified before for this agronomically important, and therefore, a well-studied trait, but both validate use of this approach for identifying regions important for traits in populations not suited to GWAS and provide opportunities to design markers specific to this germplasm. Previously, 5 *Vrs* loci have been indicated as regulating row type and located on 1HS (*Vrs3*), 2HL (*Vrs1*), 3HS (*Vrs4*), 4HS (*Vrs5*), and 5HL (*Vrs2*), and in the case of all loci except for *Vrs3* the causal gene has been published (Komatsdua et al 2007, Ramsay et al., 2011, Koppulu et al 2013, and Youssef et al., 2016). Several additional regions of the genome to those described above were highlighted in our analysis.

Similarly, for another important and well-studied trait, seasonal growth habit, several regions were identified as being divergent in our analysis (Figure 1) which had been previously identified contributing to this trait (summarised in Hill and Li 2016).

Figure 1

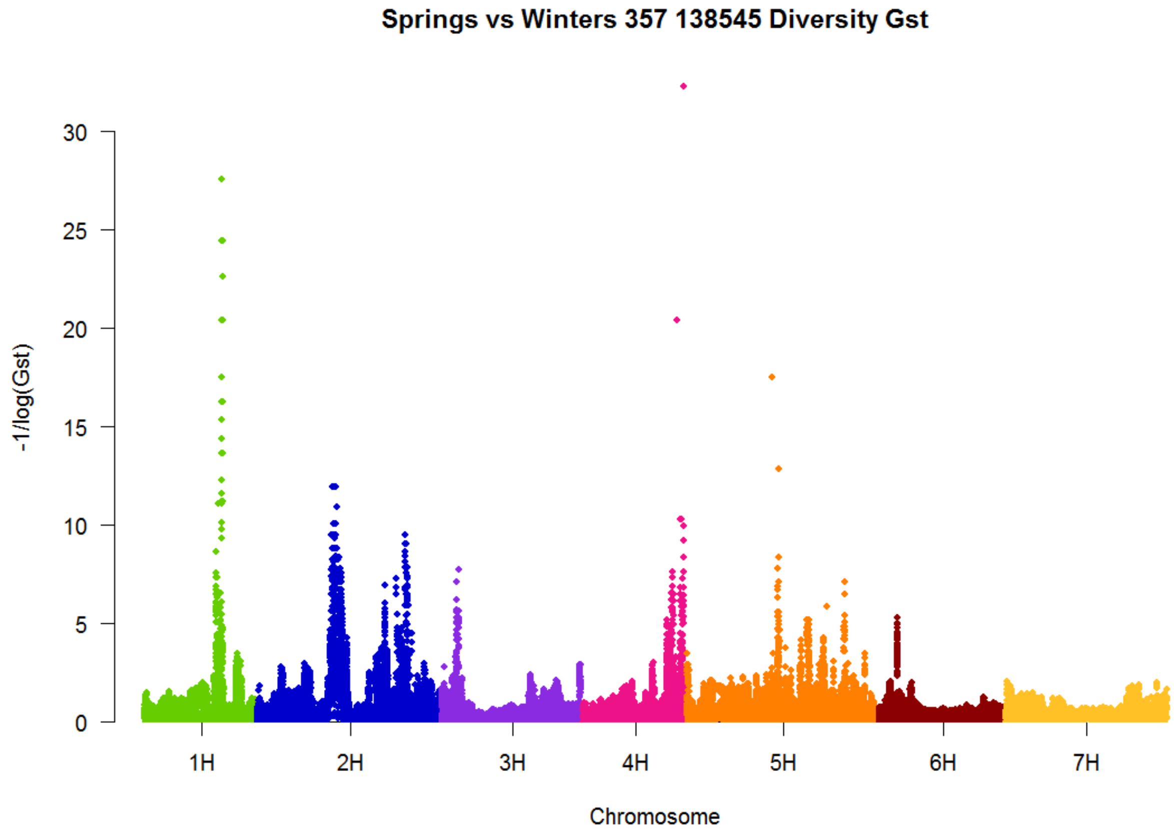


Figure 2

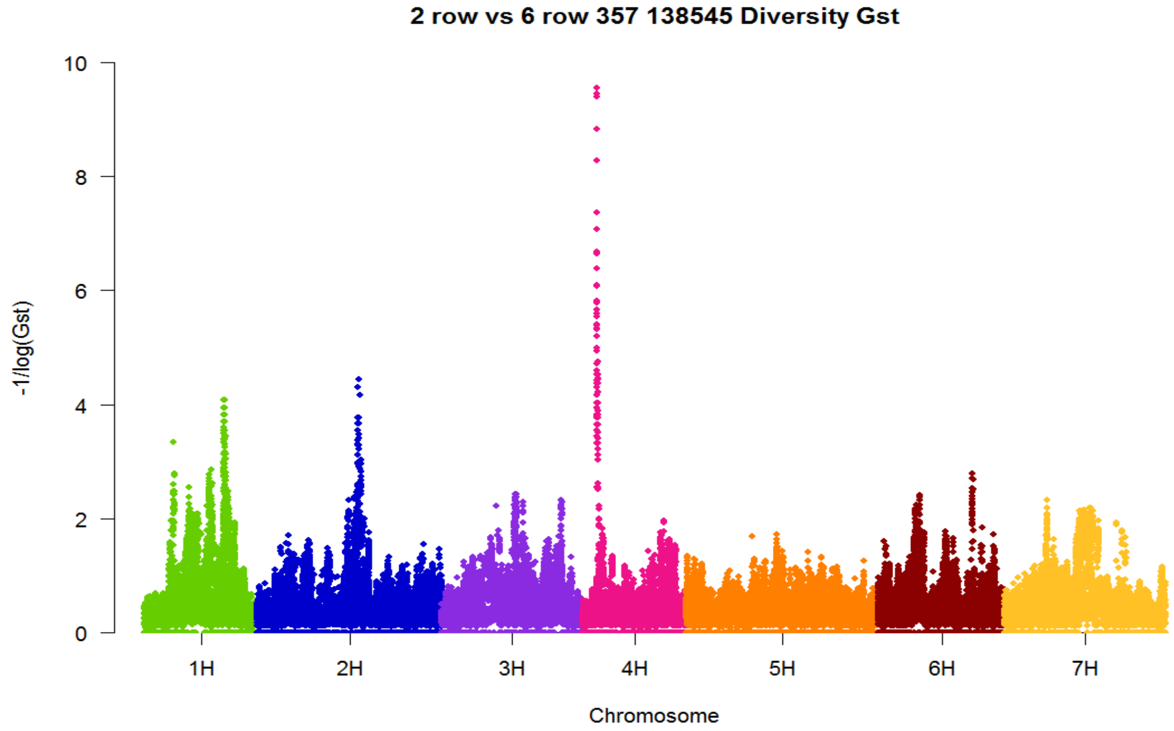


Figure 3

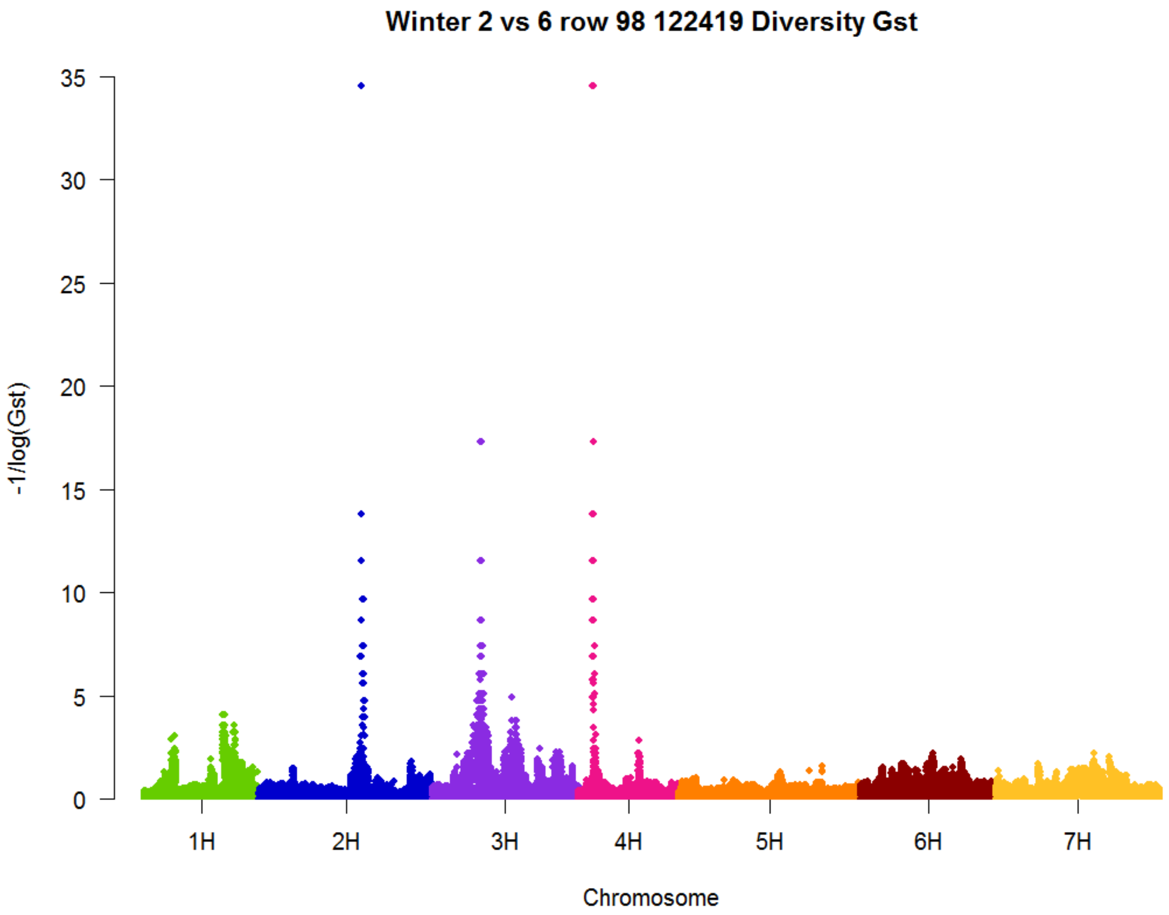

Winter 2 vs 6 row 98 122419 Diversity Gst
-1/log(Gst)
35
30
25
20
15
10
5
0
1H
2H
3H
4H
5H
6H
7H
Chromosome

Figure 4

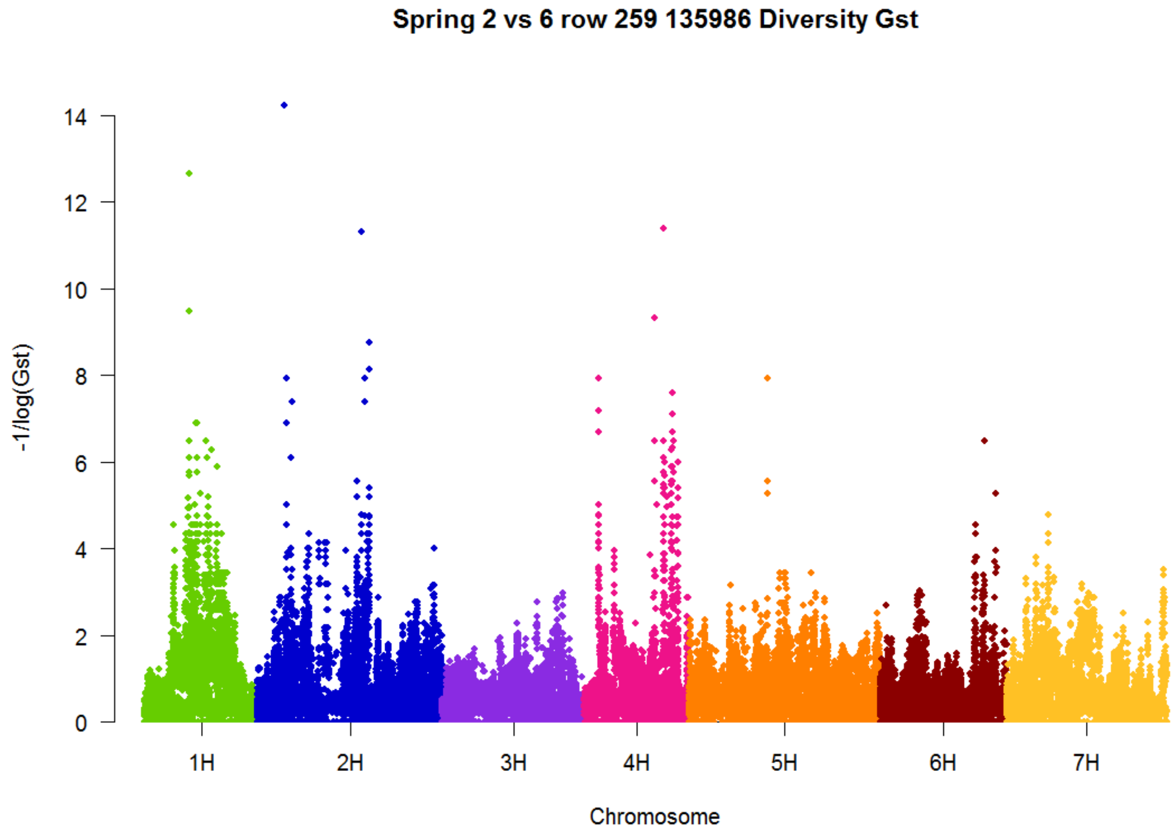


Figure 5

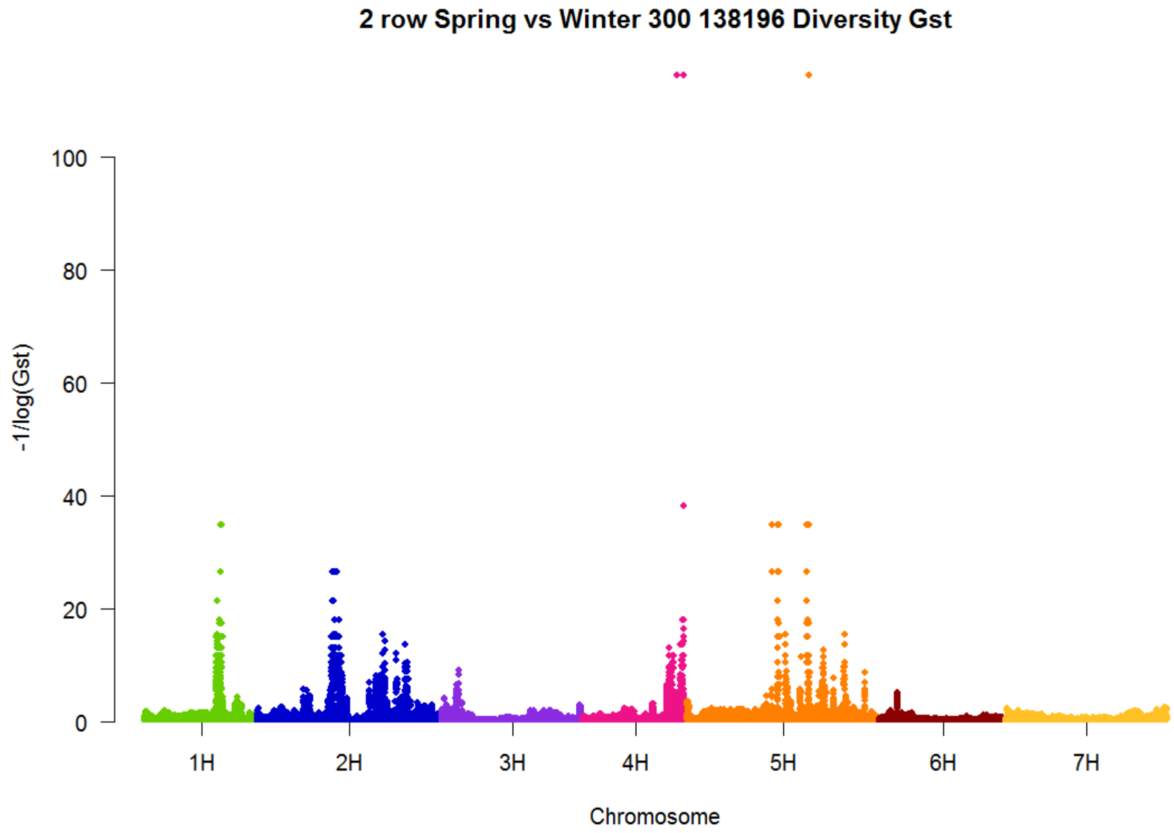
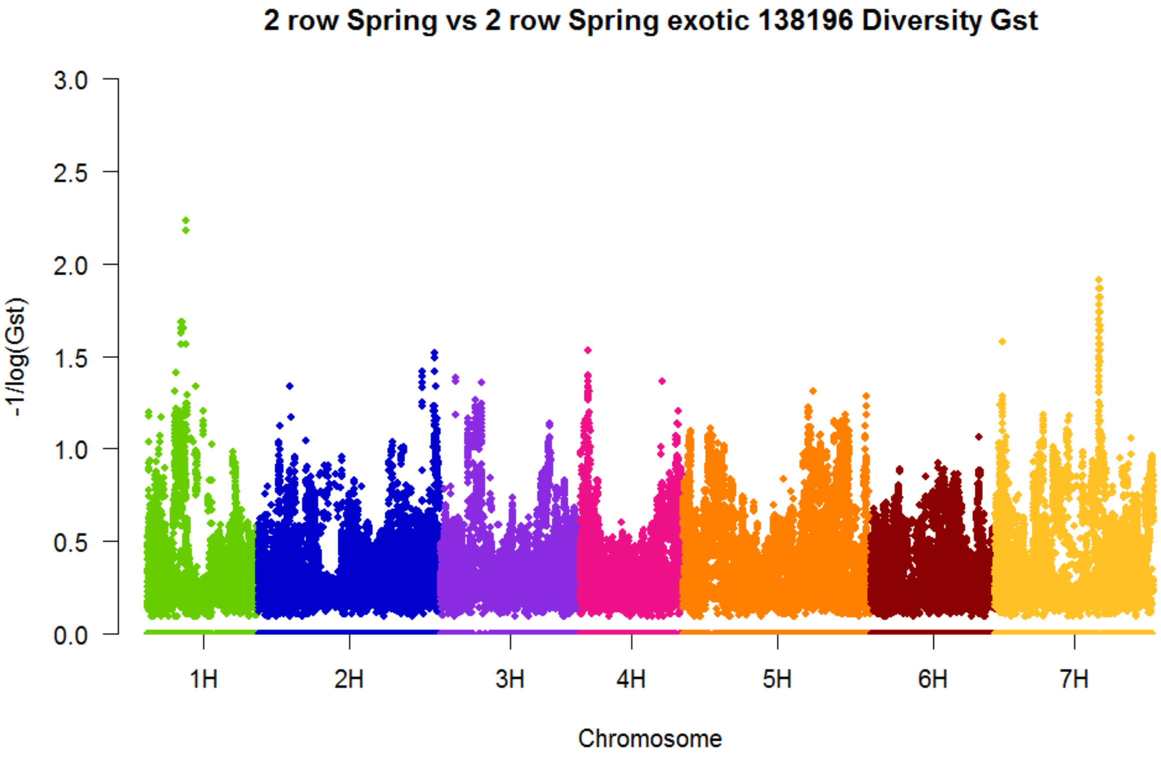


Figure 6



4.3. Genome Wide Association Mapping

4.3.1. Binomial DUS traits

In total, seven DUS traits were analysed using a binomial model. Initially, all the models for all traits were fitted including three principal components. However, we found that for the binomial DUS trait GWAS, model convergence was sensitive to the number of principal components included. Additionally, not all principal components were found to be significant. Therefore, to improve convergence, for five of the binomial DUS traits, we reduced the number of principal components included in the final GWAS model if they were not significant from three to either two or one (Table 3). It should be noted that the results were not materially changed by altering the number of principal components, but the convergence of the model improved.

Table 3: Final number of principal components included in the GWAS for each Binomial DUS trait

Binomial DUS Trait	Number of principal components included in the final model	Number of models not converged
Awn anthocyanin colouration of tips	2	1
Flag leaf anthocyanin colouration of auricle	2	1
Grain disposition of lodicules	3	1527
Grain husk	2	702
Grain rachilla hair type	3	0
Grain ventral furrow presence of hairs	1	0
Lower leaf hairiness of leaf sheaths	1	3014

Figures 7 - 10 show Manhattan plots of the $–\log_{10}$(pvalue) results (of converged models only) for responses where a clear peak is shown and include Awn anthocyanin colouration of tips, Flag leaf anthocyanin colouration of auricle, Grain ventral furrow presence of hairs and Grain rachilla hair, respectively. Significant SNPs have been identified and these are shown in Figures 7 – 10. Results for other binomial DUS traits, except Awn spiculation of margins, are shown in Supplementary material (S2). These latter DUS traits include Grain disposition of lodicules, Lower leaf hairiness of leaf sheaths and Grain husk where no peaks of any significance are shown for converged models. Grain disposition of lodicules has only 3 lines (Dew, Felicie and Prisma) representing the frontal group and Grain husk had only 3 lines (Cassata, Carola and Penthouse) representing the absent group so that the results from these variables should be treated with caution. The DUS traits Awn spiculation of margins had only one line representing the reduced category and no lines representing the absent category and therefore, this variable was not analysed.

We examined the SNP models which failed to converge to try and identify reasons for non-convergence. We found the reason for non-convergence could be explained by the distribution of counts across the trait and marker by cells with very low counts. For instance, the GWAS model for a SNP marker on chromosome 1 failed to converge for Lower leaf hairiness of leaf sheaths. When we examine the 2x2 table for trait and marker, we can see cells within the table with low counts.

Leaf sheaf Hair

Marker	Present	Absent
0	1	157
1	0	3

In a simple Chi-square calculation, cells with low counts cause issues with the calculation of pvalues. In the case of the binomial model, it makes estimation of components difficult and thus, convergence is compromised. In the case of lower leaf hairiness of leaf sheaths, none of the unconverged results would have resulted in significant SNP marker p-values.

However, for the Grain disposition of lodicule trait, the non-convergent may represent SNPs would have otherwise been significant had convergence been achieved. For example, a SNP on chromosome 2H has a 2x2 table as follows:

Grain lodicule

Marker	Frontal	Clasping
0	158	0
1	0	3

This SNP had a –log10(pvalue) of 4.45 estimated from the unconverged model. For this reason, figures for variables with unconverged models (Table 3), are provided (Supplementary 3) which also include the results of the unconverged models. However, these results should be treated with caution as in the case of the grain disposition of lodicules the significance of the non-converged model has to be weighed with the fact that there are only 3 varieties in the clasping response group.

Results from the INTEGRA analysis corroborated Cockram et al (2010). Like this previously published study we identified *HvbHLH1* as underlying anthocyanin pigmentation in several tissues (Figure 7 and 8). The increased marker density and therefore, improved resolution of the association mapping carried out as part of INTEGRA provides opportunities to identify markers linked to other DUS traits, for example, grain ventral furrow hair (Figure 9), and grain rachilla hair type (Figure 10). The results from grain rachilla hair which has a significant peak on 5H have been passed onto colleagues from the JHI who will pursue the identification of causal genes further.

Figure 7

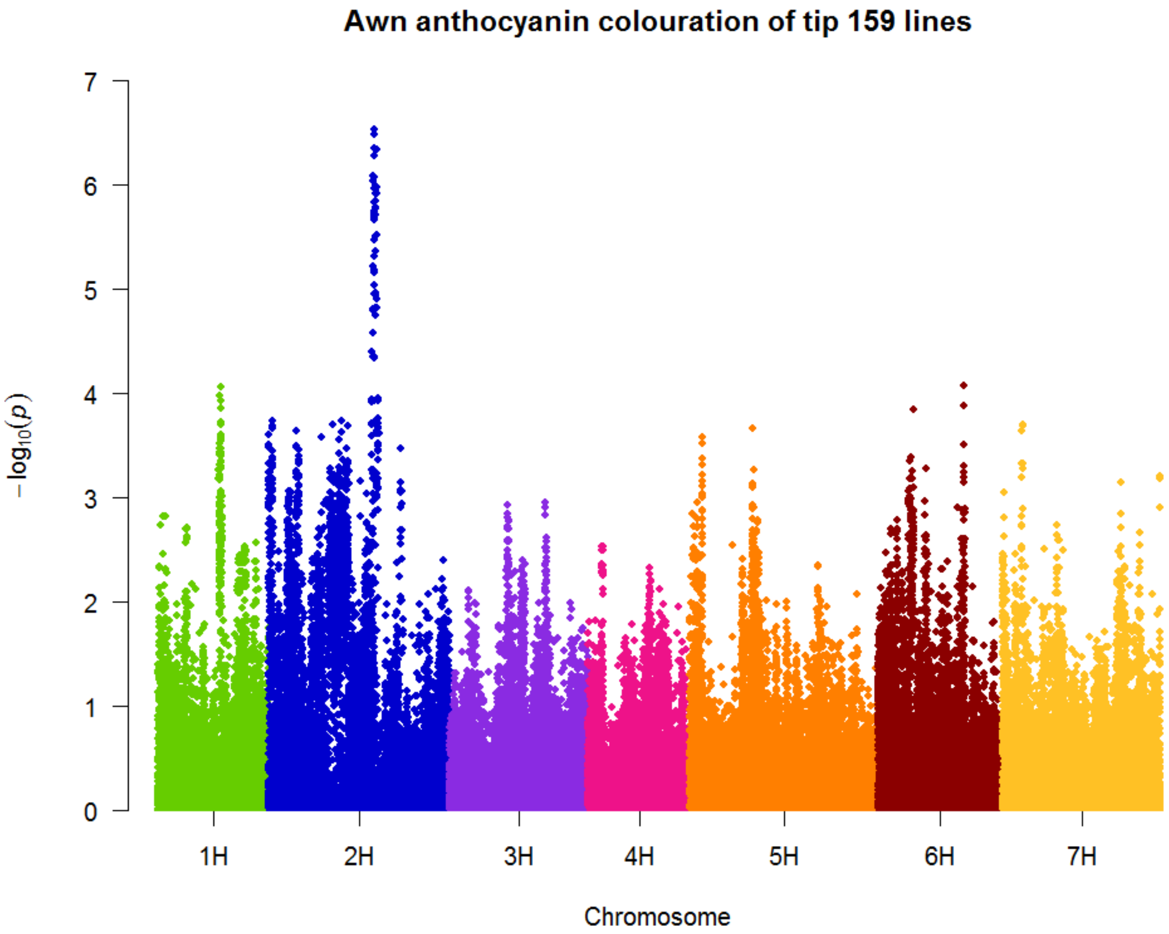


Figure 8

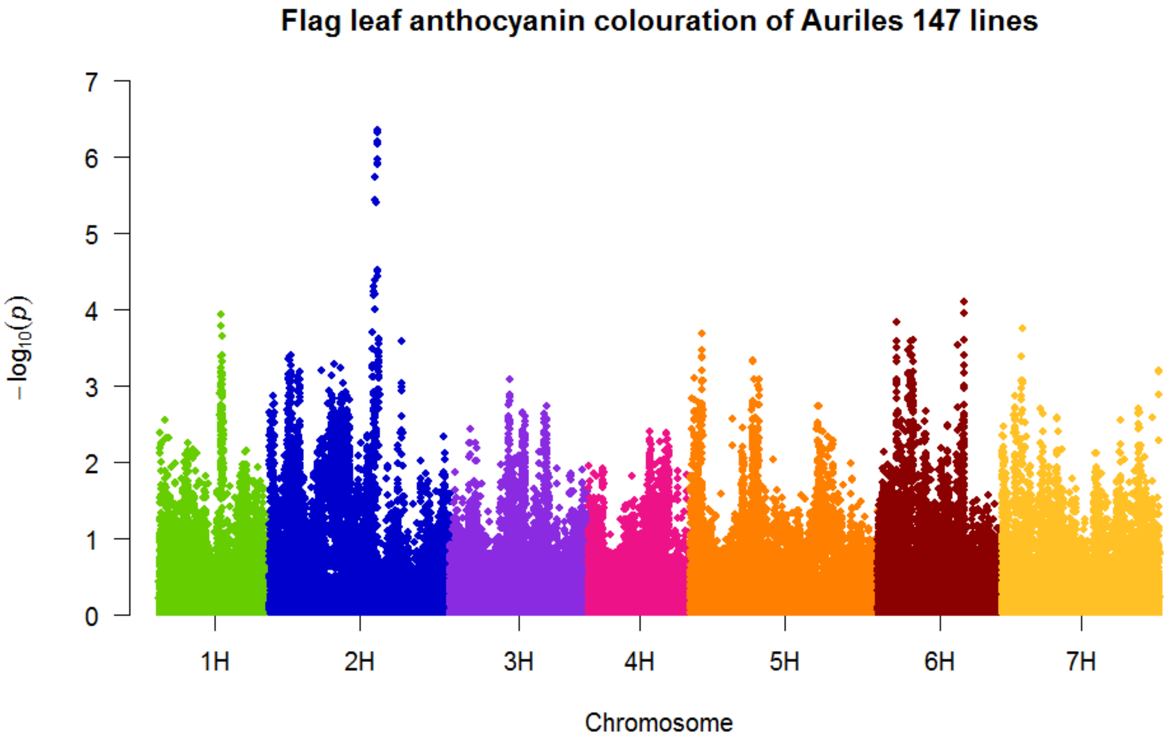


Figure 9

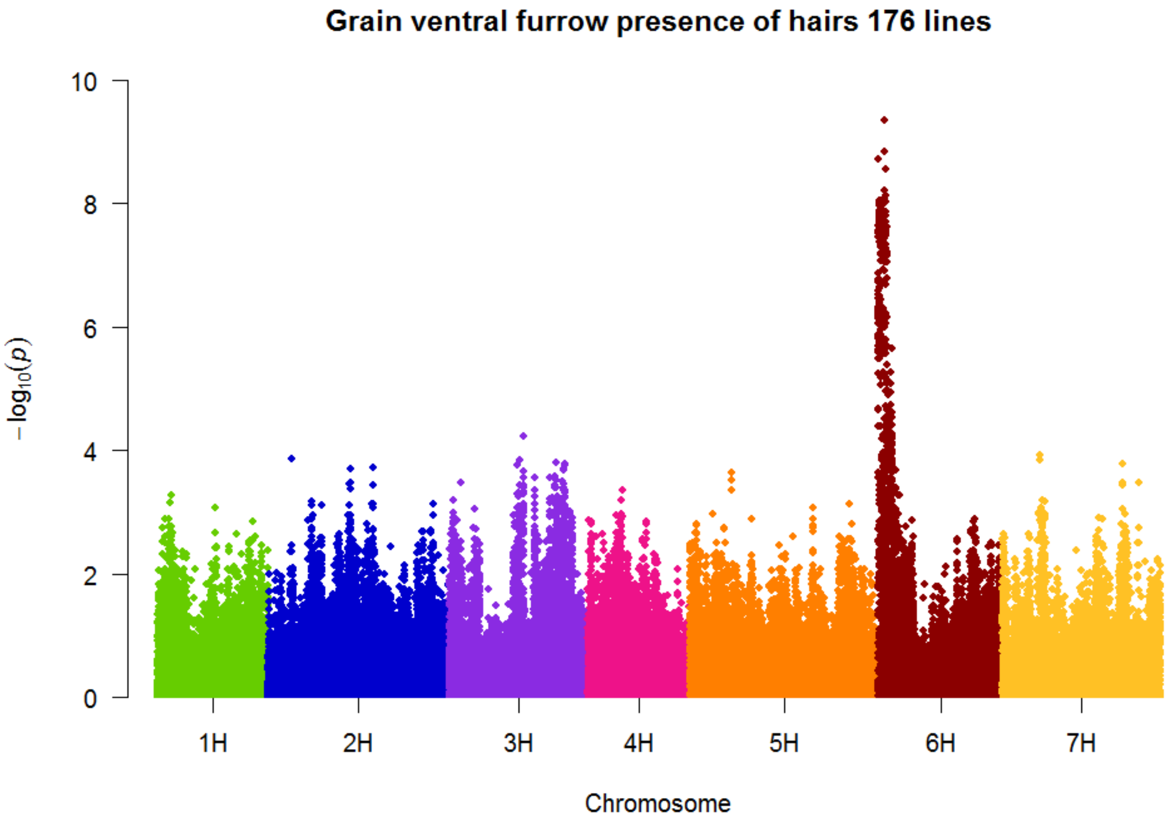
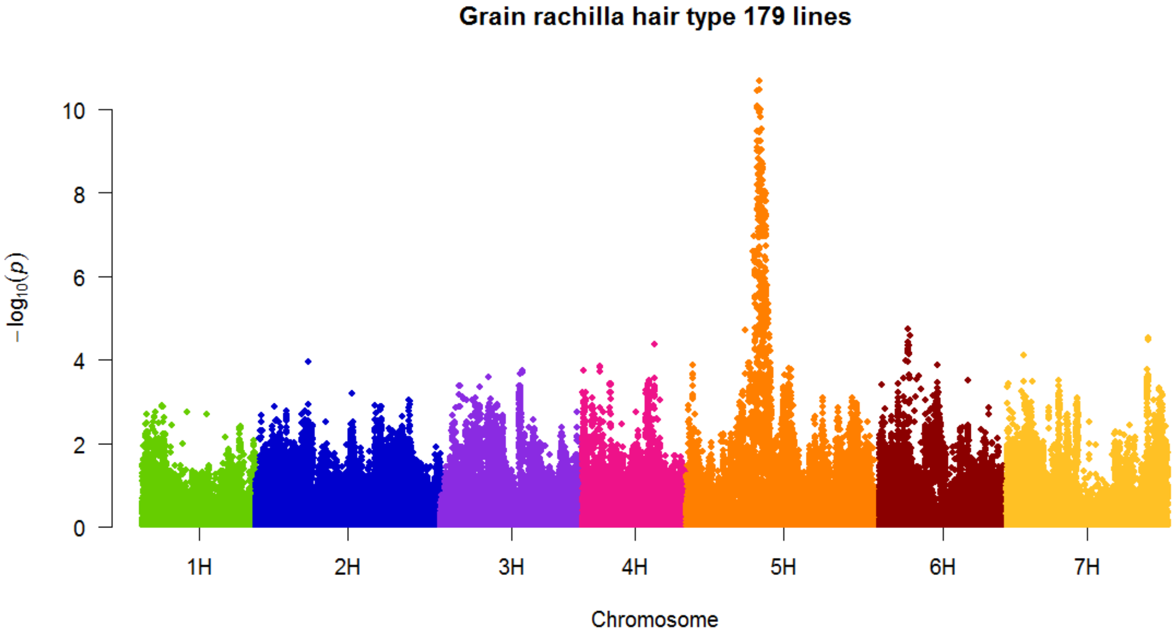


Figure 10



It is worth noting that the interpretation of marker effects in a binomial model is different from a standard association mapping model and is explained briefly as follows using an example. A marker on chromosome 5H has the marker effect of 6.49 for grain rachilla hair, thus, for this marker characterised by the C and T allele, a change in genotype from CC to TT INCREASES the odds of having long rachilla hair by 658.6 or 65,760%. (Note: genotypes CC and TT are represented as 0 and 1 in the SNP file and therefore, an increase in 1 unit represents the difference between the two homozygotes). Conversely, an alternative marker characterised by the A and T allele on chromosome 5H has a marker effect of -6.36 for grain rachilla hair, so a change in genotype from AA to TT DECREASES the odds of the long grain hair by 0.002 or 99.82%.

4.4. Other DUS traits

As expected, associations for all traits analysed were found in the same genomic regions when comparing to Cockram et al. (2010).

Figures 11 to 15 show –log10(p value) results for traits where a clear peak is shown and include Awn anthocyanin intensity of colouration of tips, Flag leaf anthocyanin intensity of colouration of auricle, Grain anthocyanin colouration of lemma nerves, grain speculation of inner later nerves and kernel colour of aleurone, respectively. The ~130 fold increase in marker density clearly provides an improvement in resolution, and more robust markers. Significant SNPs are clearly evident in Figures 11 – 15. We identified a strong association for the aleurone trait on 4H (Figure 15). It is the intention to further pursue the causal gene for aleurone layer colour, also known as blue aleurone, in collaboration with Ryan Whitford at the University of Adelaide. Aleurone colour can be considered a “quality trait with a preference for white aleurone. This preference can lead to the rejection of grain by barley buyers, for instance in Western Australia typically all blue aleurone grain is turned down (https://www.agric.wa.gov.au/barley/barley-blue-aleurone).

Results for other DUS traits are shown in Supplementary material (S5). These latter DUS traits include Awn length compared to ear, collar type, ear attitude, ear density, ear glaucosity, ear length, ear shape, flag leaf glaucosity, medium spikelet length of glume awn, plant frequency of recurved leaves, growth habit, plant length, rachis curvature, rachis length, sterile spikelet attitude, sterile spikelet shape and time of ear emergence.

The ear glaucosity has evidence of a peak on 1H which is of interest to colleagues who will pursue the identification of causal genes further.

Figure 11

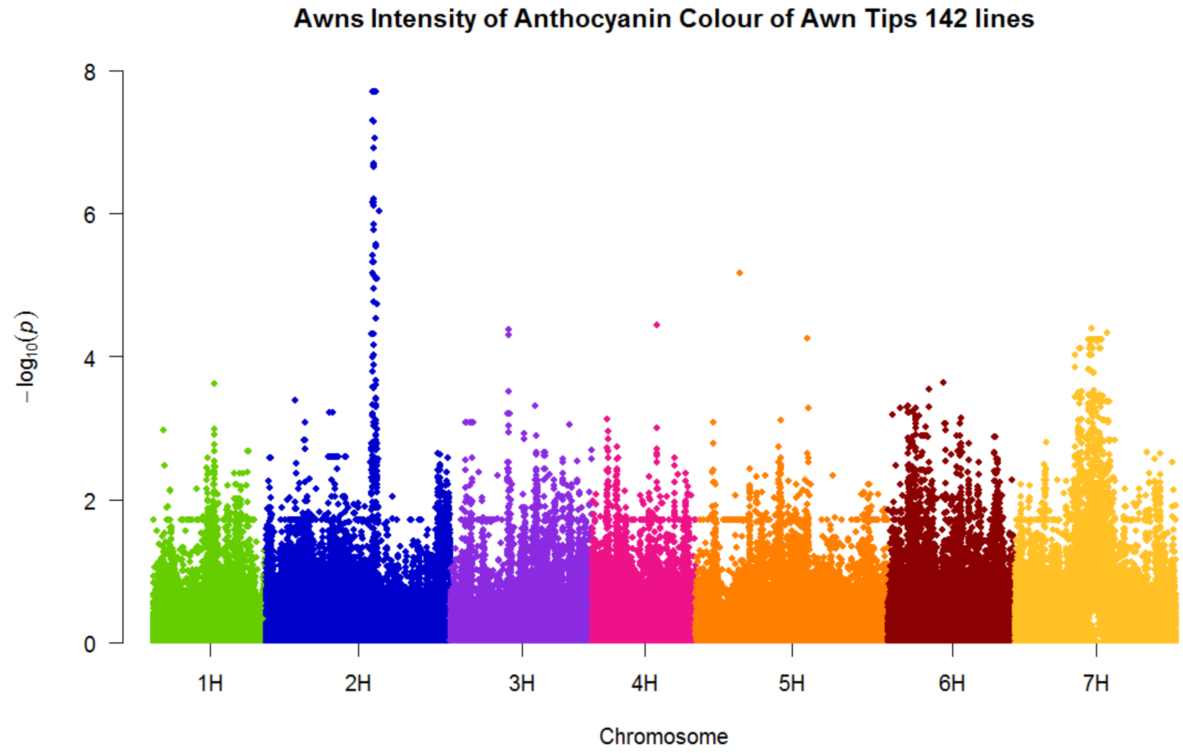


Figure 12

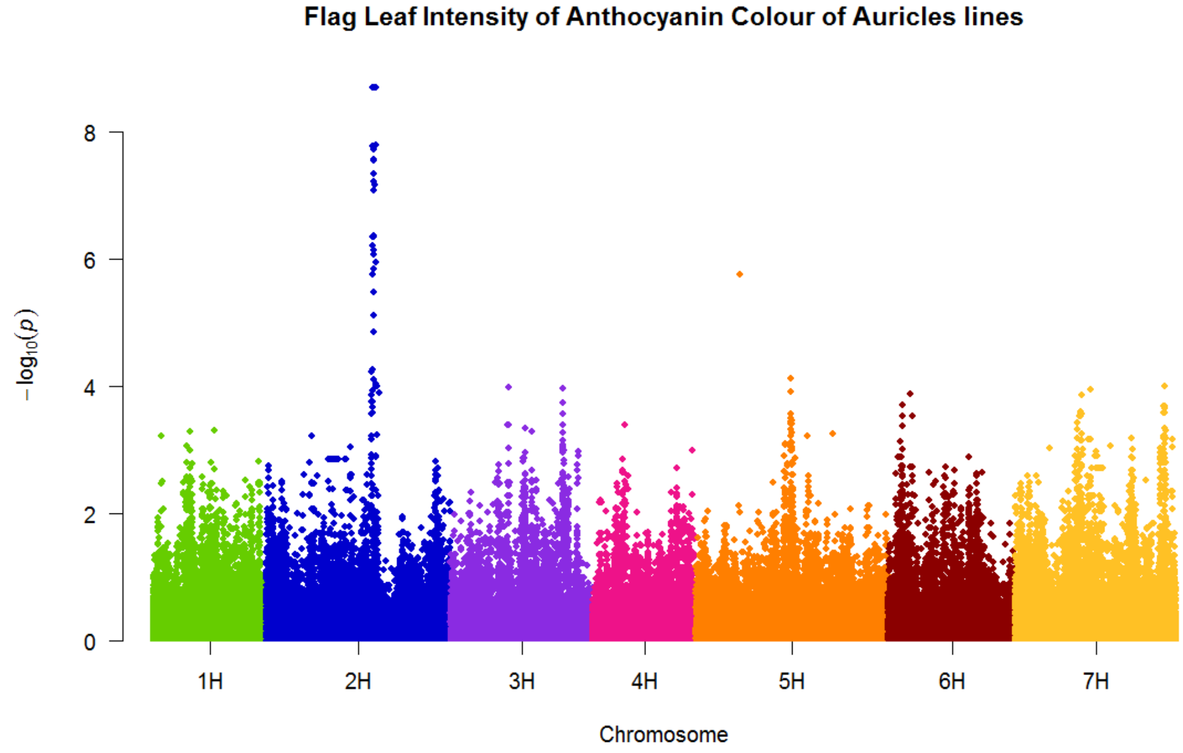


Figure 13

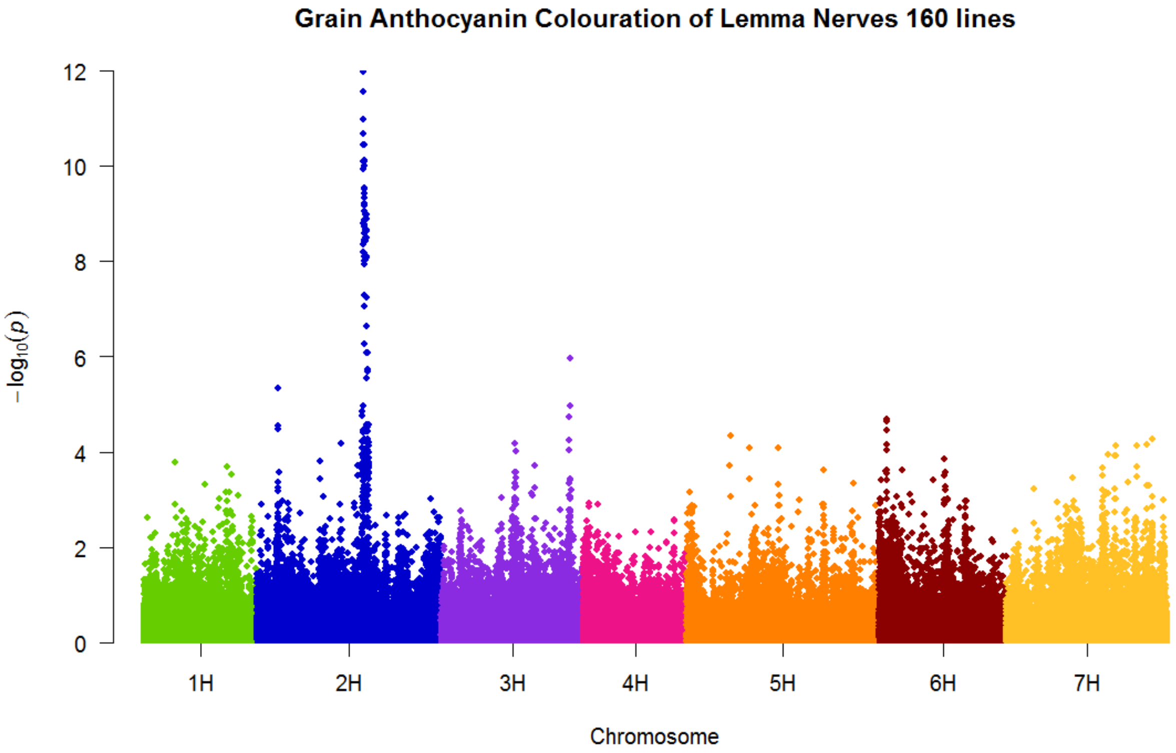


Figure 14

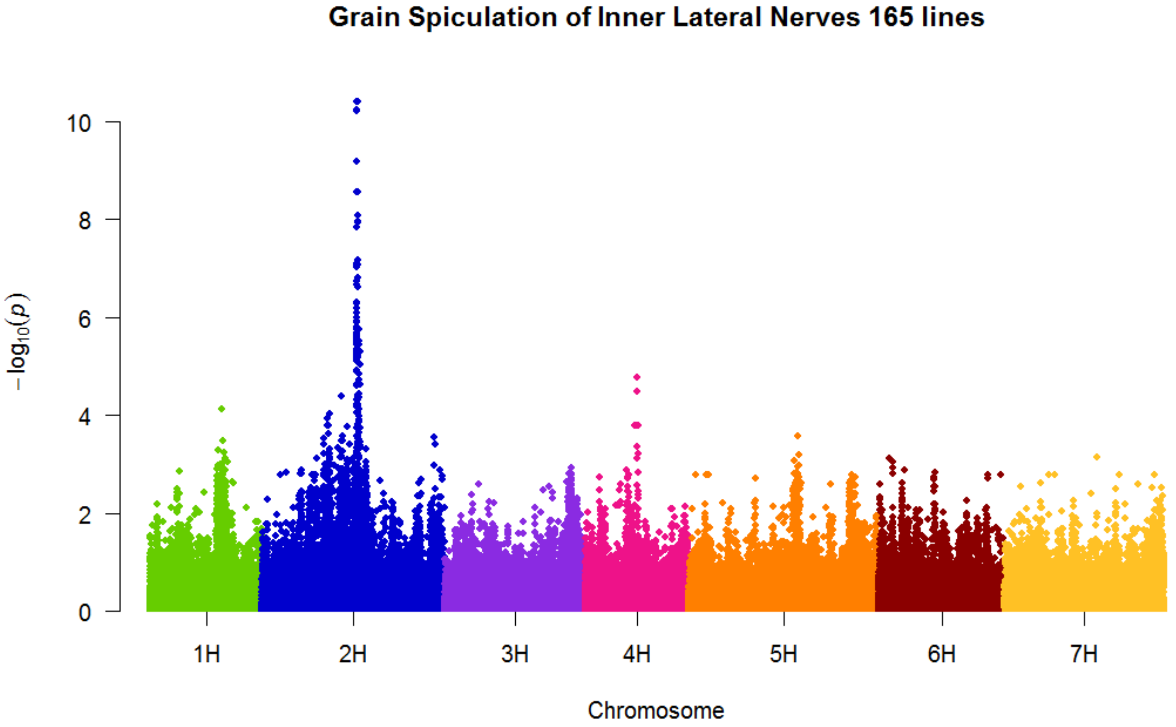
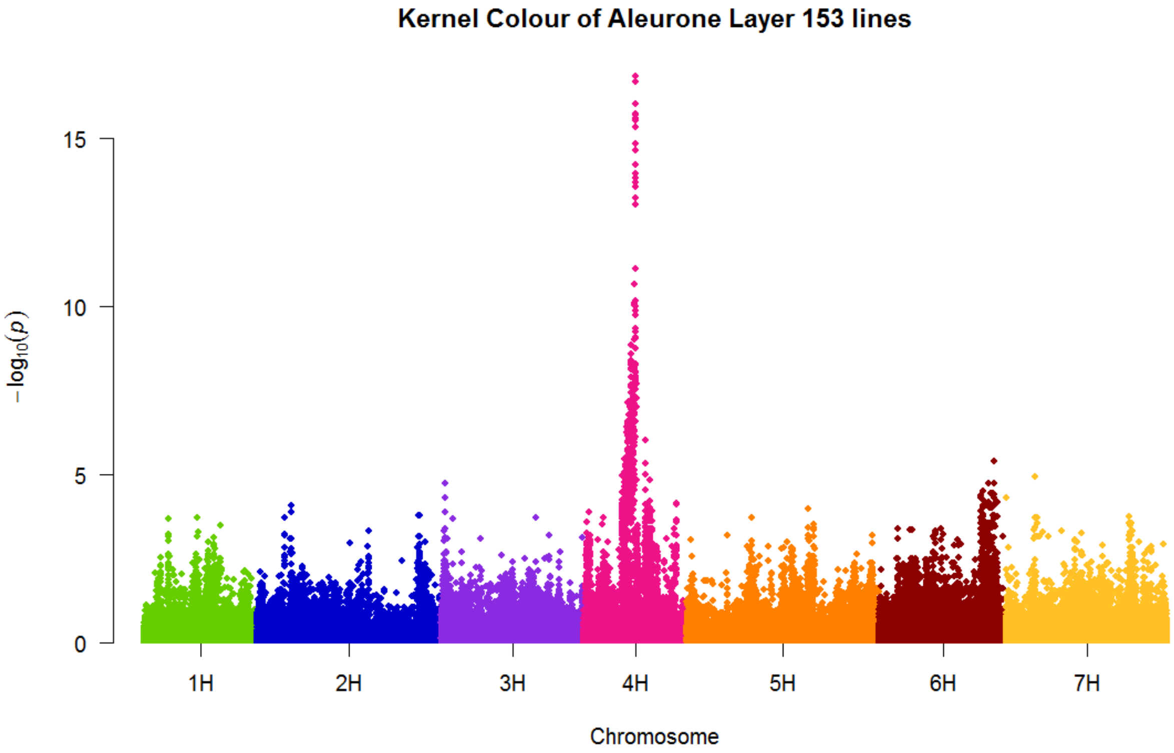


Figure 15



4.5. KASP marker sequence identification

One of the objectives of this project was to identify putative markers for the traits analysed and convert them into a format that can be used in plant breeding programs. From our analysis we identified associations for 25 traits with a LOD value of greater than the arbitrary threshold of LOD =3.0. However, we prefer to use the FDR (false discovery rate) threshold ($q<0.05$), and in this case, 12 of the characteristics analysed passed this threshold. Combining the outputs from the GWAS with the genotypic data from the most significant SNPs for these traits has allowed us to calculate the reliability of these markers based on the accessions used in the GWAS analysis, and the occurrence of mismatch between genotype and phenotype at each putative marker (data not shown).

As mentioned in section 4.3.1, for traits such as Awn spiculation of margins, Grain disposition of lodicules, Lower leaf hairiness of leaf sheaths and Grain husk there was issues with the analysis. For Awn spiculation of margins there was only one line representing the reduced category and no lines representing the absent category, and for the same reason the analysis of the other 3 traits did not identify QTLs that were considered appropriate for marker development.

Table 4: Traits which we have identified significant associations, and therefore, have sequences available for the development of KASP genotyping markers.

Trait - pass FDR (q<0.05)	Trait - LOD> 3.0
AWN SPICULATION OF MARGINS	AWN SPICULATION OF MARGINS
AWNS ANTHOCYANIN COLOURATION OF TIPS	AWNS ANTHOCYANIN COLOURATION OF TIPS
AWNS INTENSITY OF ANTHOCYANIN COLOUR OF AWN TIPS	AWNS INTENSITY OF ANTHOCYANIN COLOUR OF AWN TIPS
EAR NUMBER OF ROWS	COLLAR TYPE
FLAG LEAF ANTHOCYANIN COLOURATION OF AURICLES	EAR ATTITUDE at least 21 days after ear emerg
FLAG LEAF GLAUCOSITY OF SHEATH	EAR DENSITY
GRAIN ANTHOCYANIN COLOURATION OF LEMMA NERVES	EAR GLAUCOSITY
GRAIN DISPOSITION OF LODICULES	EAR LENGTH EXCLUDING AWNS
GRAIN HUSK	EAR NUMBER OF ROWS
GRAIN RACHILLA HAIR TYPE	EAR SHAPE
GRAIN SPICULATION OF INNER LATERAL NERVES	FLAG LEAF ANTHOCYANIN COLOURATION OF AURICLES
GRAIN VENTRAL FURROW PRESENCE OF HAIRS	FLAG LEAF GLAUCOSITY OF SHEATH
KERNEL COLOUR OF ALEURONE LAYER	GRAIN ANTHOCYANIN COLOURATION OF LEMMA NERVES
LOWER LEAVES HAIRINESS OF LEAF SHEATHS	GRAIN DISPOSITION OF LODICULES
SEASONAL TYPE	GRAIN HUSK
STERILE SPIKELET ATTITUDE MID 1 3 OF EAR	GRAIN RACHILLA HAIR TYPE
	GRAIN SPICULATION OF INNER LATERAL NERVES
	GRAIN VENTRAL FURROW PRESENCE OF HAIRS
	KERNEL COLOUR OF ALEURONE LAYER
	LOWER LEAVES HAIRINESS OF LEAF SHEATHS
	MEDIAN SPIKELET LENGTH OF GLUME AWN cf GRAIN
	PLANT FREQUENCY OF PLANTS WITH RECURVED LEAVES
	PLANT GROWTH HABIT
	PLANT LENGTH STEM EARS AND AWNS
	RACHIS CURVATURE OF FIRST SEGMENT
	RACHIS LENGTH OF FIRST SEGMENT
	SEASONAL TYPE
	STERILE SPIKELET ATTITUDE MID 1 3 OF EAR
	TIME OF EAR EMERGENCE 1st spk vis on 50 ears

5. Discussion

During this project we combined high density genetic data derived from exome capture with phenotypic data to identify and refine key regions of the barley genome for a collection of DUS traits. In the majority of cases, our analysis identified the same QTL's as Cockram et al (2010). However, in the current analysis we extended our analysis to account for the binomial distribution of several of these DUS traits, Awn anthocyanin colouration of tips, Flag leaf anthocyanin colouration of auricle, Grain disposition of lodicules, Grain husk, Grain rachilla hair type, Grain ventral furrow presence of hairs, and Lower leaf hairiness of leaf sheaths. As expected, applying a generalised linear mixed model (GLMM) to account for the binomial distribution of these traits the LOD values were lower compared to those when we used the standard model in GAPIT (Genome Association and Prediction Integrated Tool), but not necessarily lower than those identified in Cockram et al., (2010) due to different SNPs being present in the respective marker sets.

A second extension of the analysis in the current project was that to determine the efficiency of our analysis approach in removing loci contributing to variation in row type and Winter/ Spring growth habit. We exploited these differences using a diversity statistic analogous to F_{ST} known as G_{ST}. This revealed multiple loci already known to contribute to these traits, for example, in the case of the row type analysis we observed QTLs containing *Vrs1*, *Int- C* on 2H and 4H (Komatsuda et al 2007, Ramsay et al 2011). However, we also identified several novel QTLs including one in the region containing *Vrs3* on 1H.

The ~130 fold increase in marker density in the present study compared to the original analysis of Cockram et al (2010) has provided a greater number of potential markers which can be deployed in breeding programs than was previously available. This increase in marker density will have narrowed and refined the intervals containing putative candidate genes, and this combined with the availability of a barley genome sequence (Beier et al 2017, Mascher et al 2017) will no doubt facilitate the identification and characterisation of the gene responsible for variation in these traits. Nice et al (2016) reported using wild barley advanced backcross-nested association mapping (AB-NAM) population which consisted of 796 $BC_2F_{4:6}$ lines whose genotype data included 263,531 SNP imputed onto the population exome capture sequence of the parents to map a suite of traits. However, to our knowledge the current study is first to utilise exome capture data to genetically characterise a complete collection of barley accessions at such a large number of loci, subsequently using this data to map traits. In other cereal species using exome capture derived SNPs to construct a high marker density genotypic dataset suitable for GWAS has been used to important agronomic traits. Grabowski et al (2017) identified several loci, including a homolog of FLT, in switchgrass that contribute to variation in flowering time. Using a genetic dataset containing 1,377,841 SNPs helped the authors overcome some of the challenges normally faced when carrying out GWAS in switchgrass due to the nature of its genome; it is not only highly repetitive, large but also polyploid.

As indicated in the results sections we are actively pursuing the casual genes for several traits with local and international collaborators. Following the Grain Quality and Animal Feed Monitoring Meeting on the 11/01/2017 it was recommended that we needed to identify a clear route to dissemination to encourage industry uptake of the outputs generated by this project. To address this, we will take several approaches. We will hold a briefing for the relevant stakeholders, i.e., cereal breeders. We are presenting the results from this project at the 2017 Monogram Conference, held in Bristol on the 4-6th of April, with a poster (Integrated genetic analysis of barley phenotypic data using 2.1M SNPS (INTEGRA-2.1)) and flash presentation. This work could also be presented at the 4th Conference of Cereal Biotechnology and Breeding in Budapest, Hungary, in November 2017. A webpage describing INTEGRA is available http://www.barleyhub.org/integra/, and will be updated with publications when they are published.

6. References

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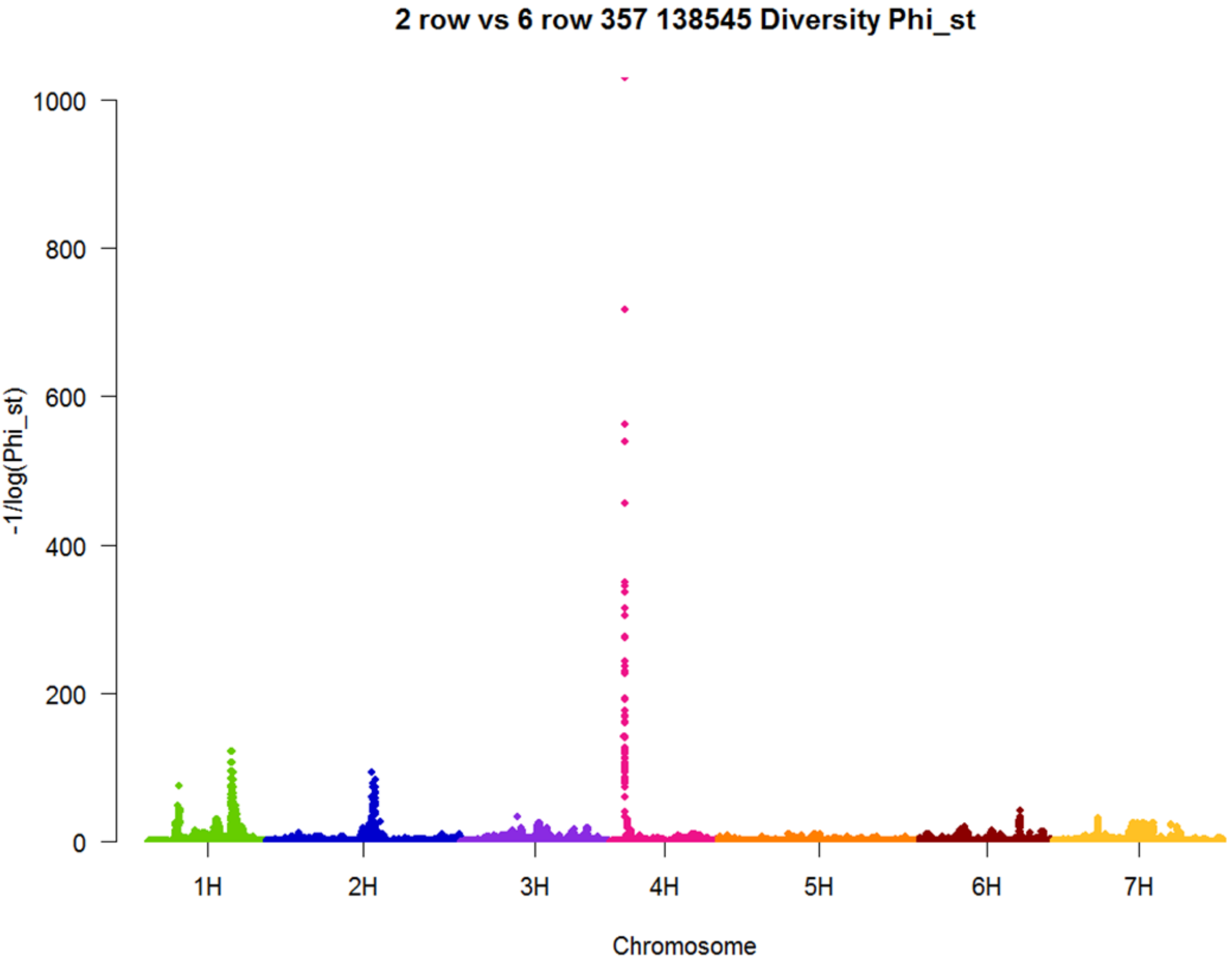
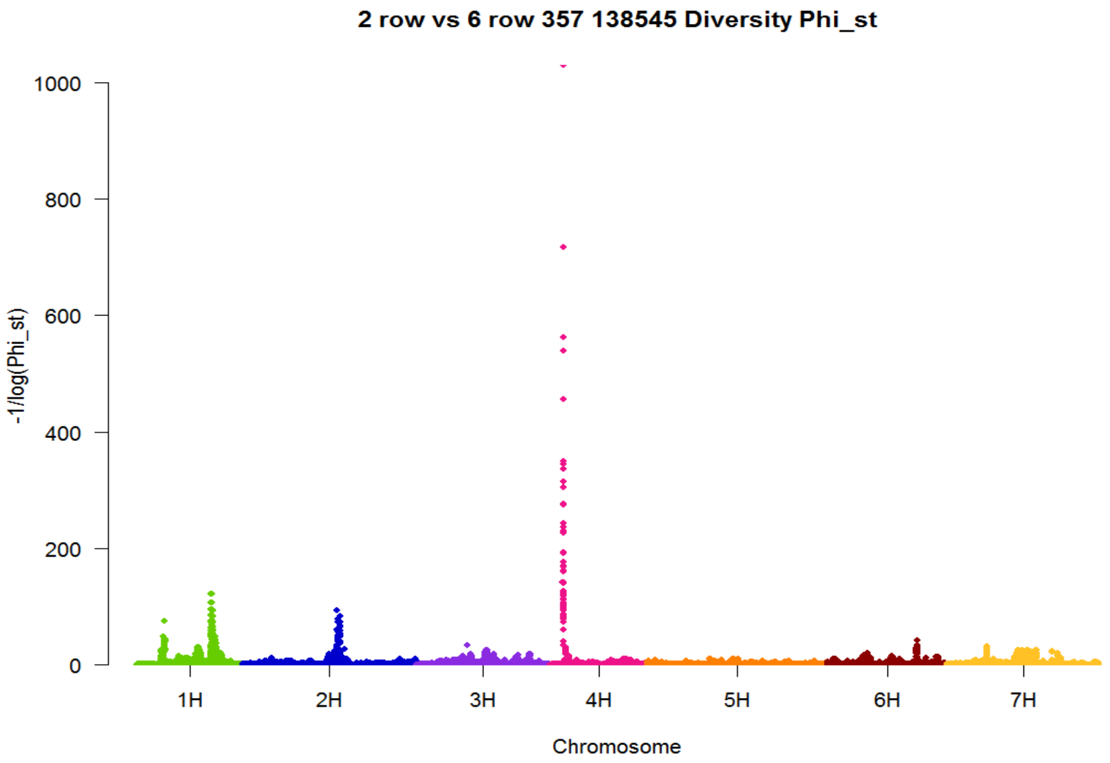
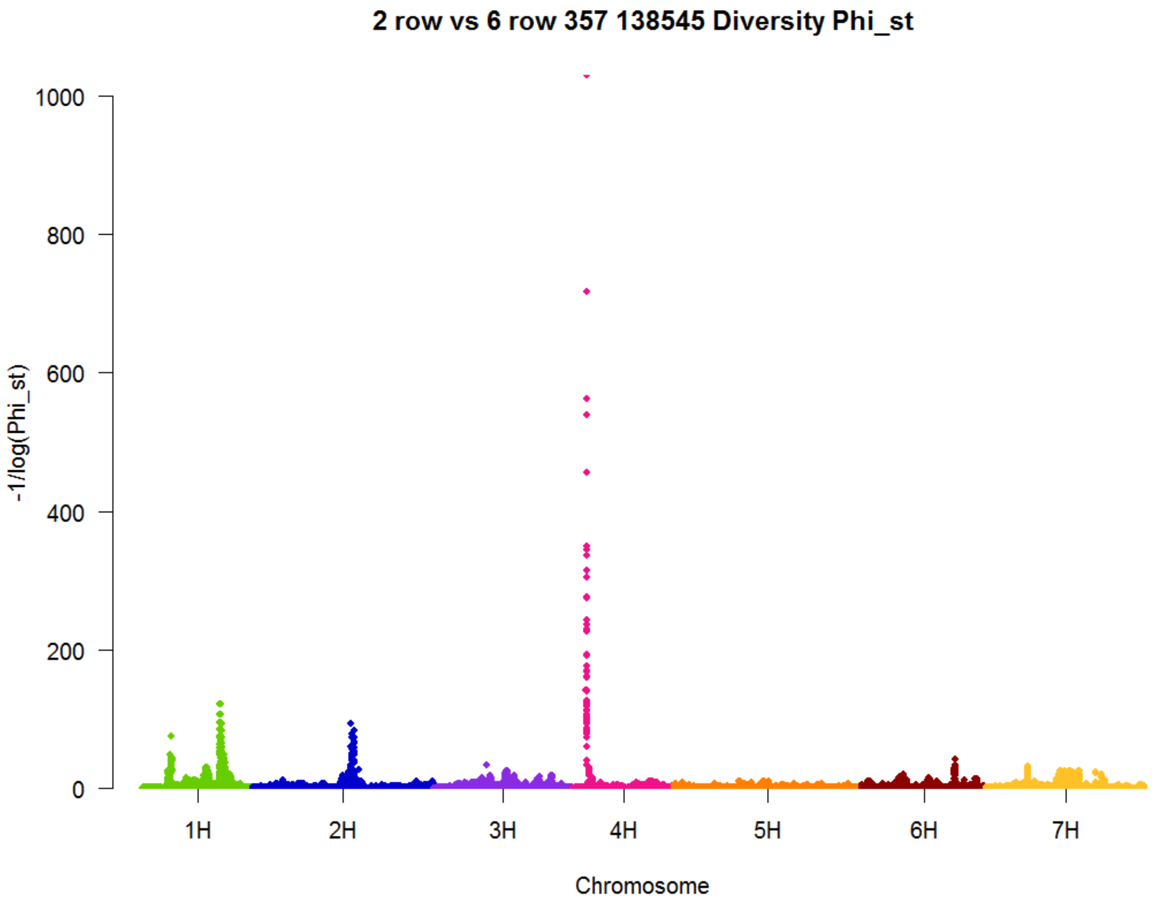
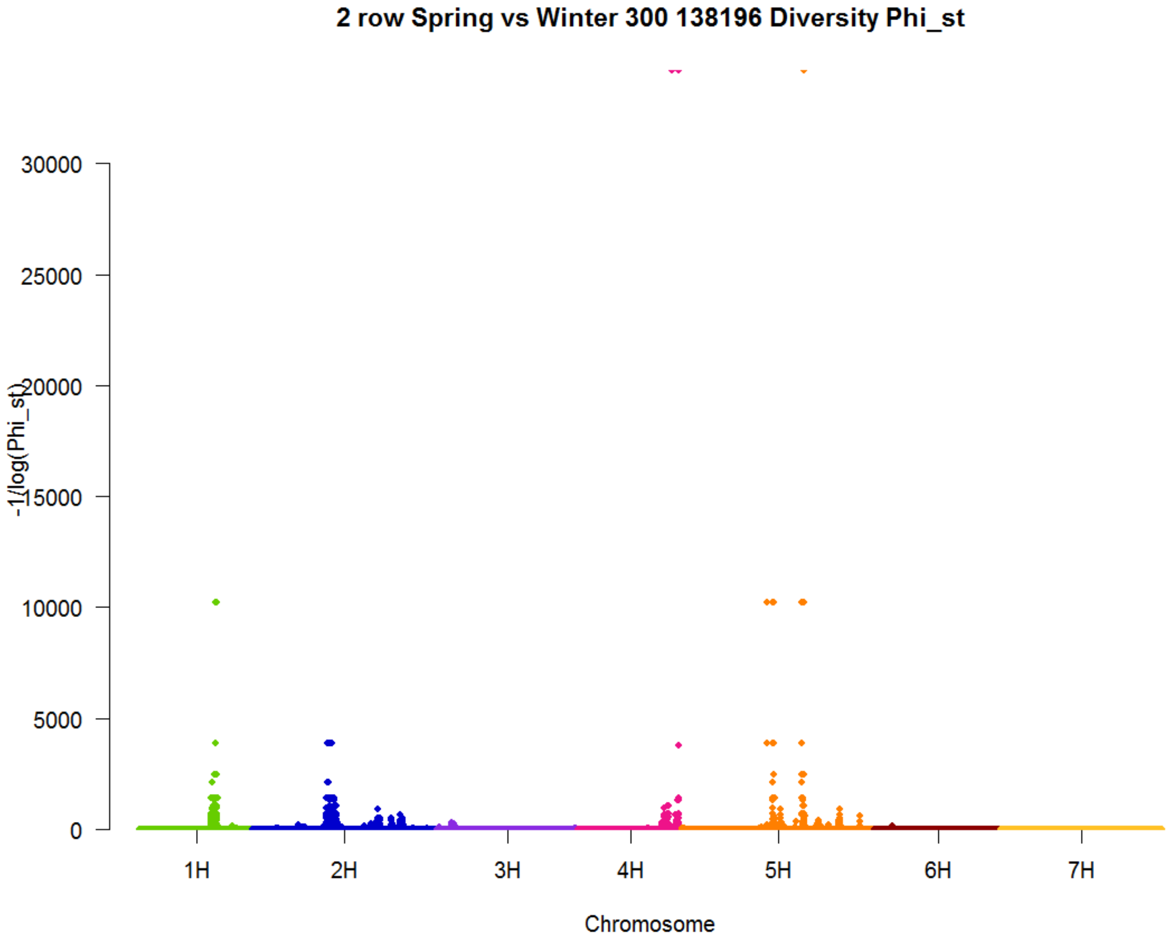
7. Supplementary 1

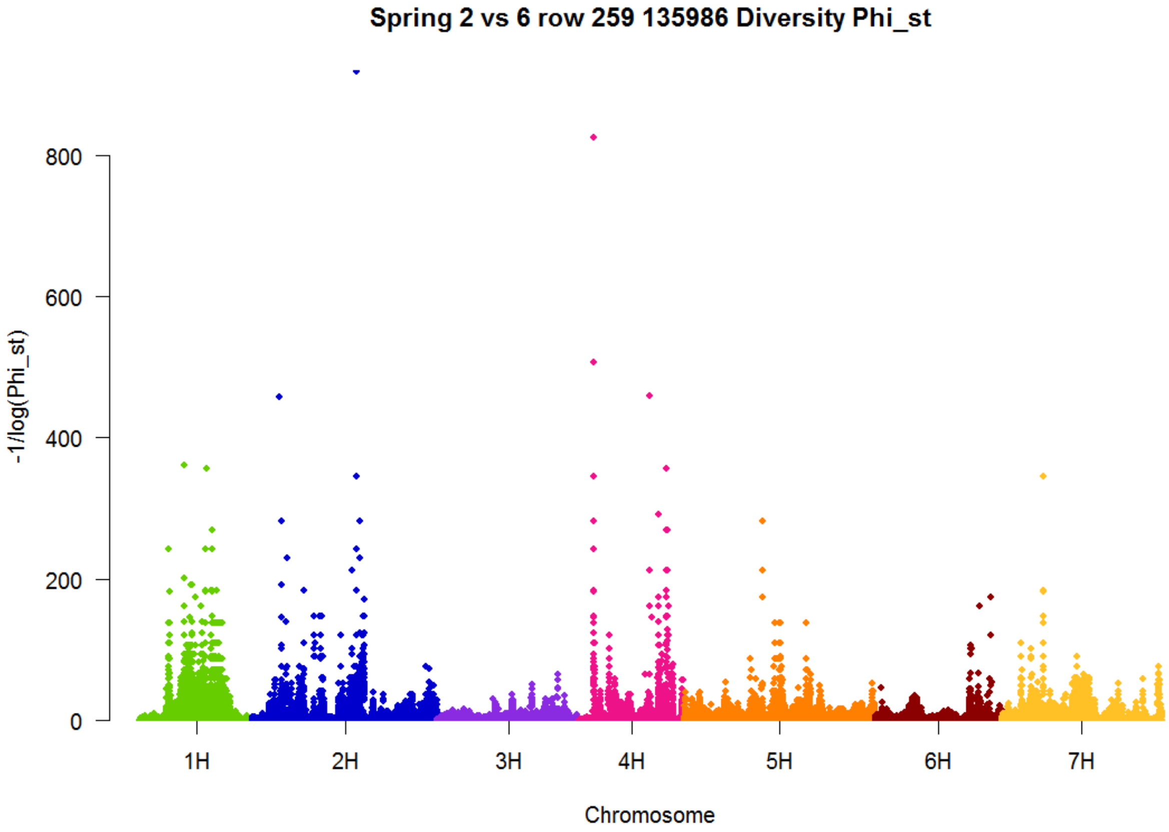
Figures of minus the inverse of the Diversity fixations index Φ_{ST} (Meirmans, 2006) are shown below. The annotation of the figures shows the comparison characterised by the fixation index, the number of lines and the number of SNPs.

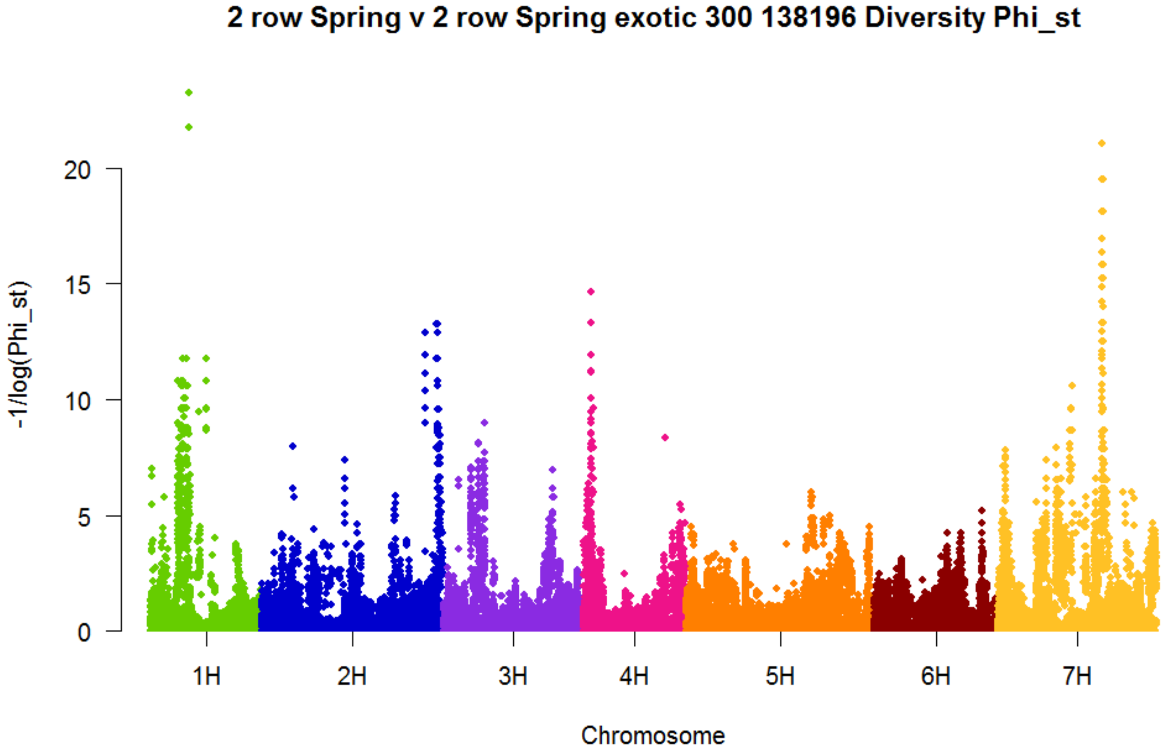



2 row vs 6 row 357 138545 Diversity Phi_st
-1/log(Phi_st)
1000
800
600
400
200
0
1H
2H
3H
4H
5H
6H
7H
Chromosome


2 row vs 6 row 357 138545 Diversity Phi_st
-1/log(Phi_st)
1000
800
600
400
200
0
1H
2H
3H
4H
5H
6H
7H
Chromosome

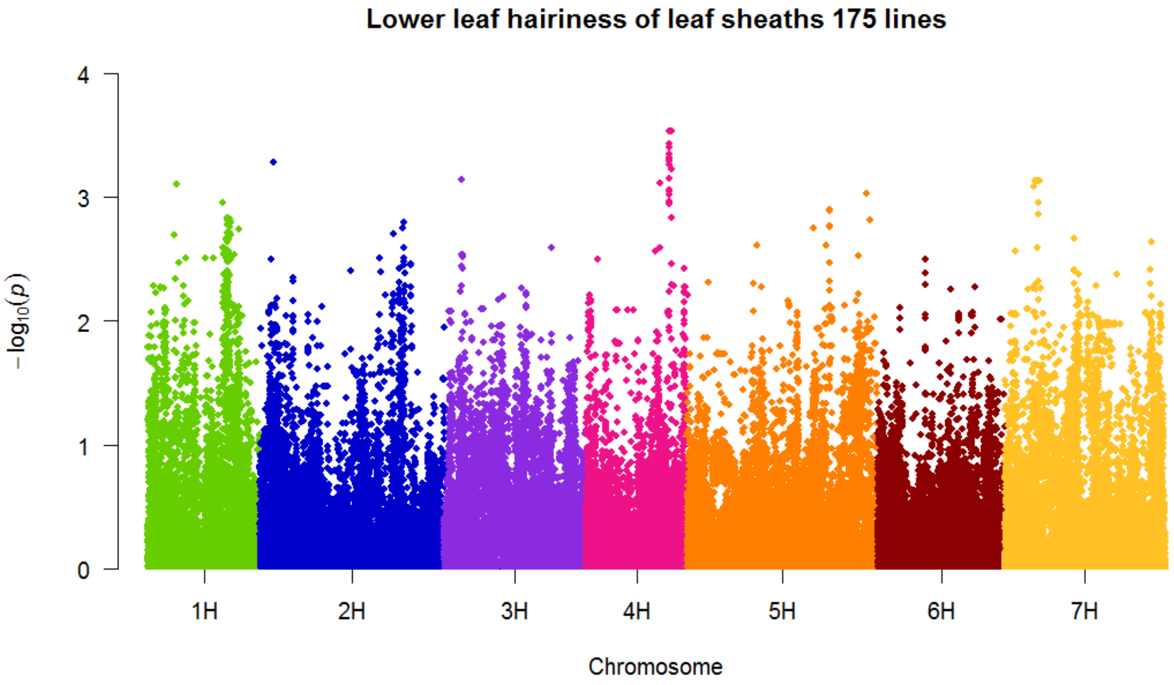

2 row Spring vs Winter 300 138196 Diversity Phi_st
-1/log(Phi_st)
30000
25000
20000
15000
10000
5000
0
1H
2H
3H
4H
5H
6H
7H
Chromosome

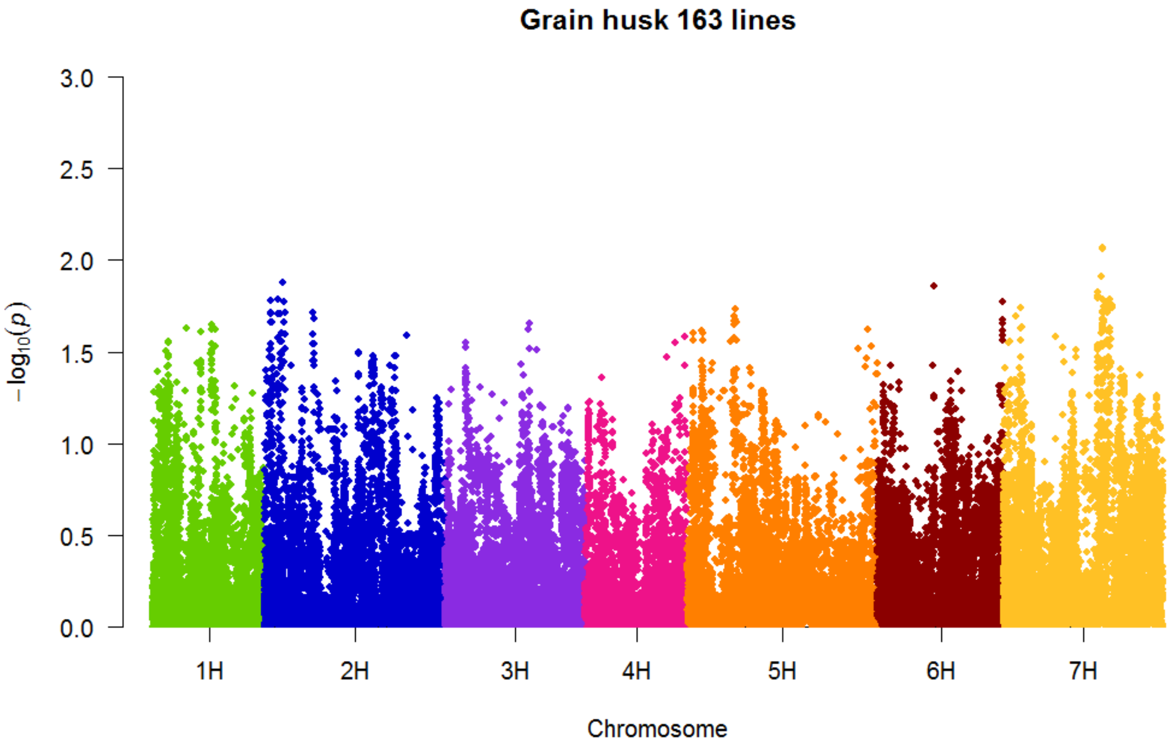

Spring 2 vs 6 row 259 135986 Diversity Phi_st
-1/log(Phi_st)
0
200
400
600
800
1H
2H
3H
4H
5H
6H
7H
Chromosome

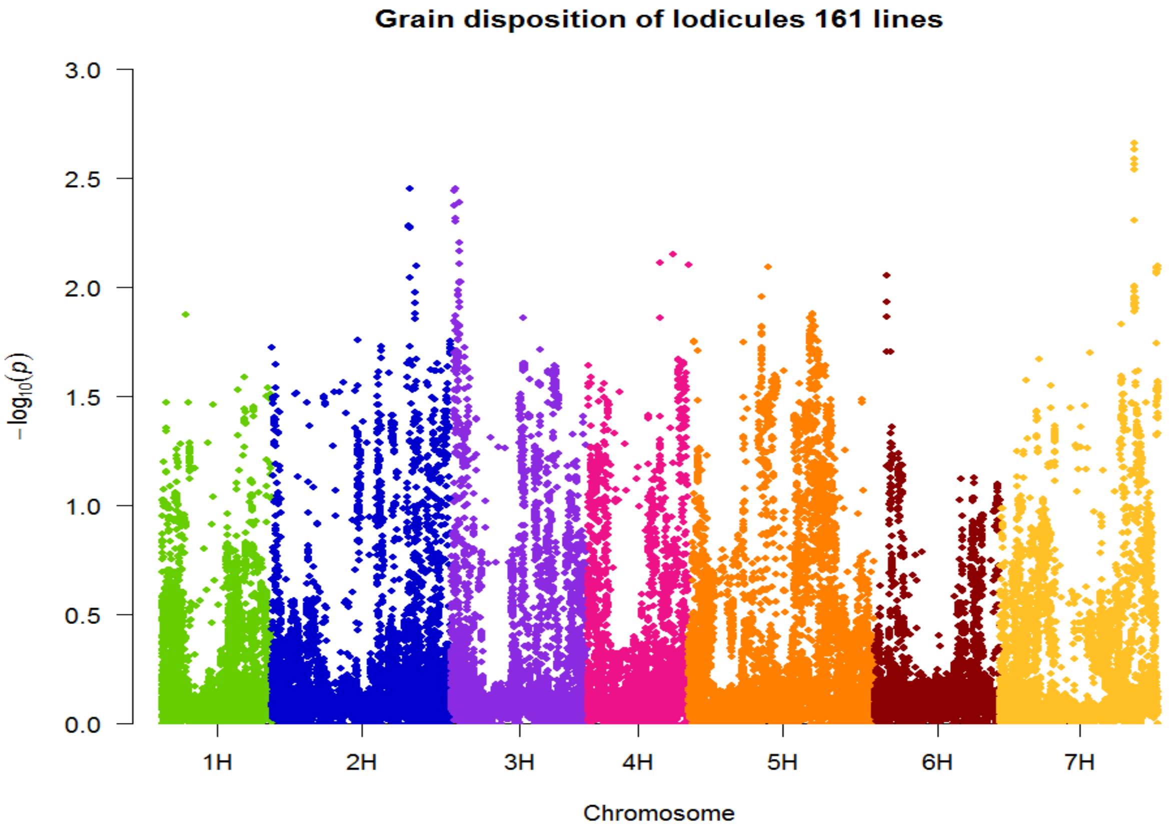

2 row Spring v 2 row Spring exotic 300 138196 Diversity Phi_st
-1/log(Phi_st)
0
5
10
15
20
1H
2H
3H
4H
5H
6H
7H
Chromosome

7.1. Supplementary 2

Binomial DUS traits

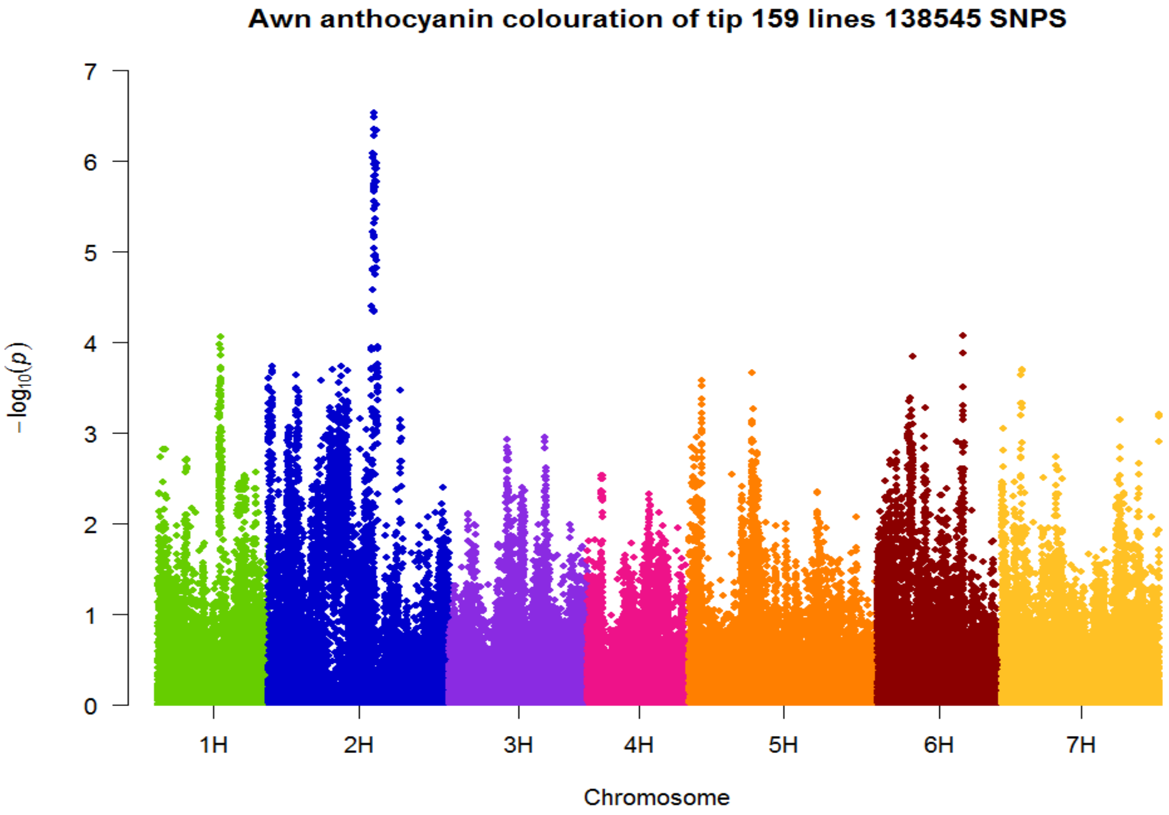


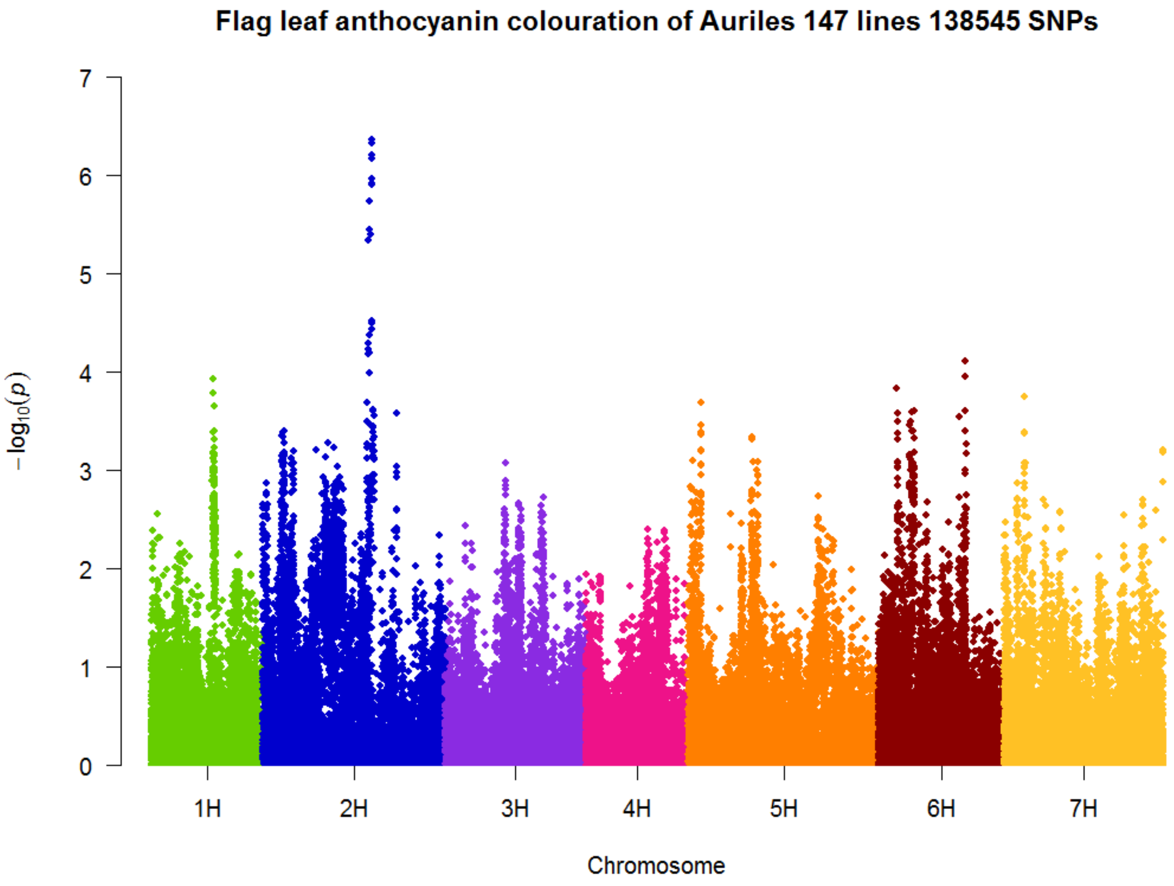


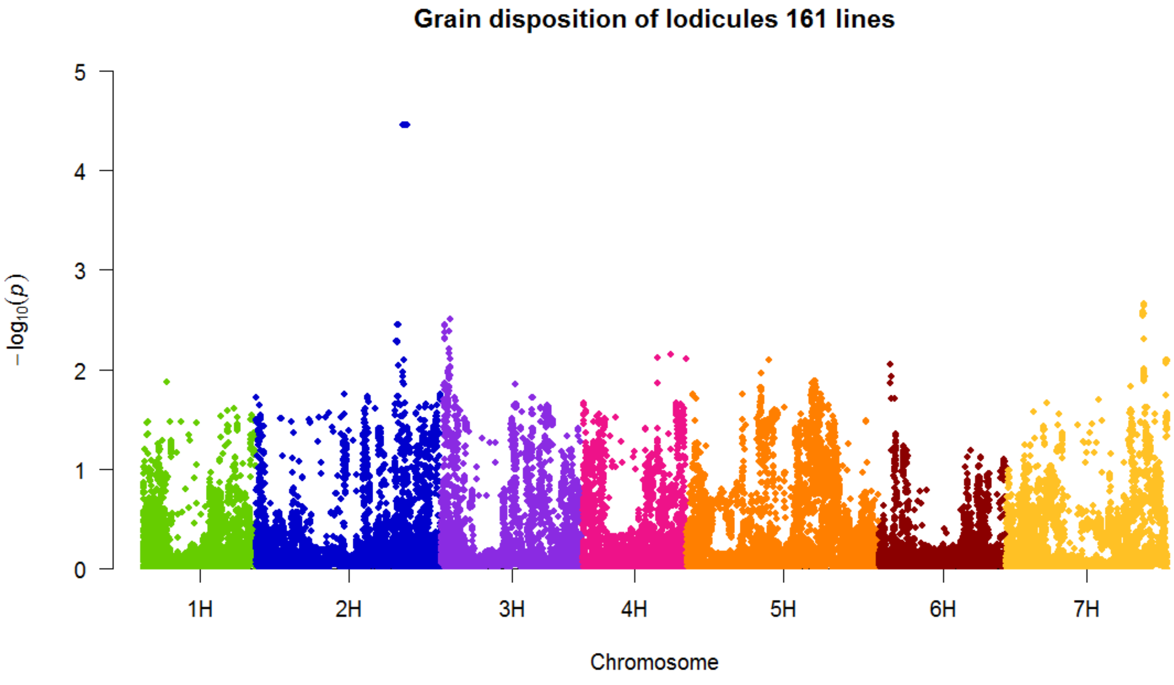

Grain disposition of lodicules 161 lines
3.0
2.5
2.0
1.5
1.0
0.5
0.0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome

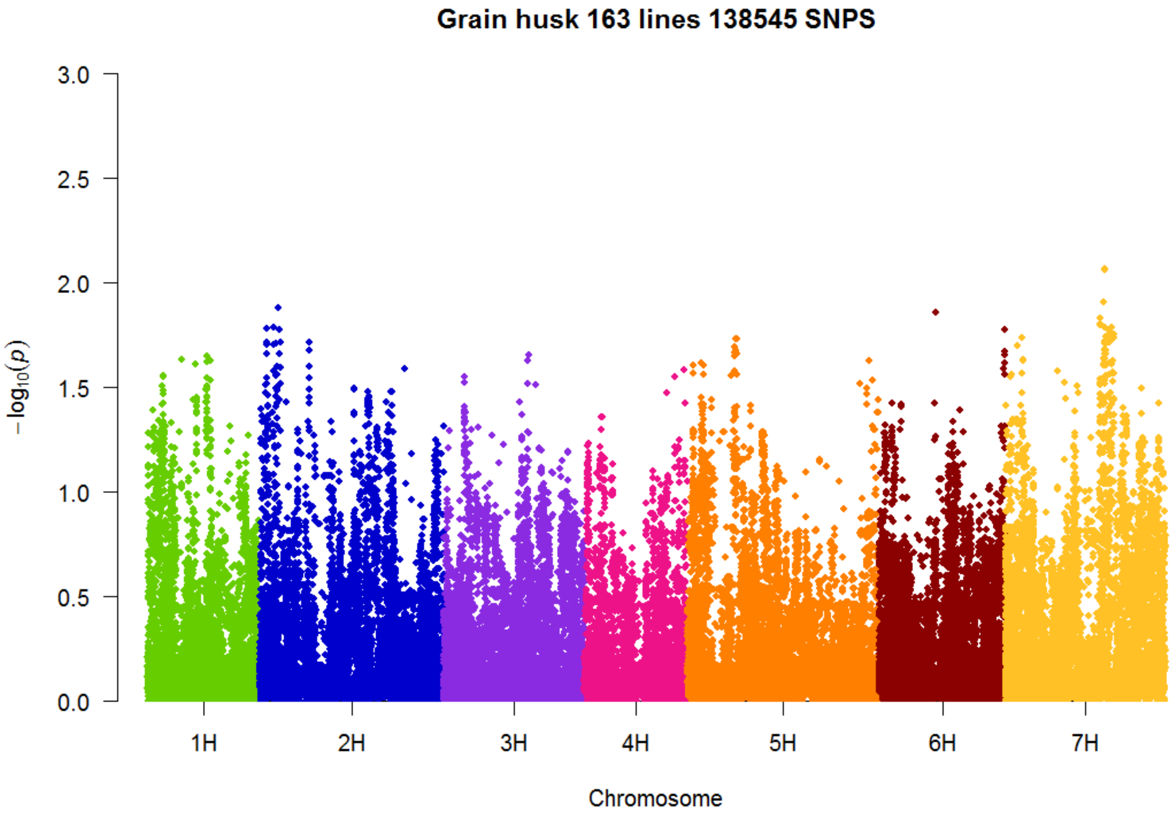
8. Supplementary 3

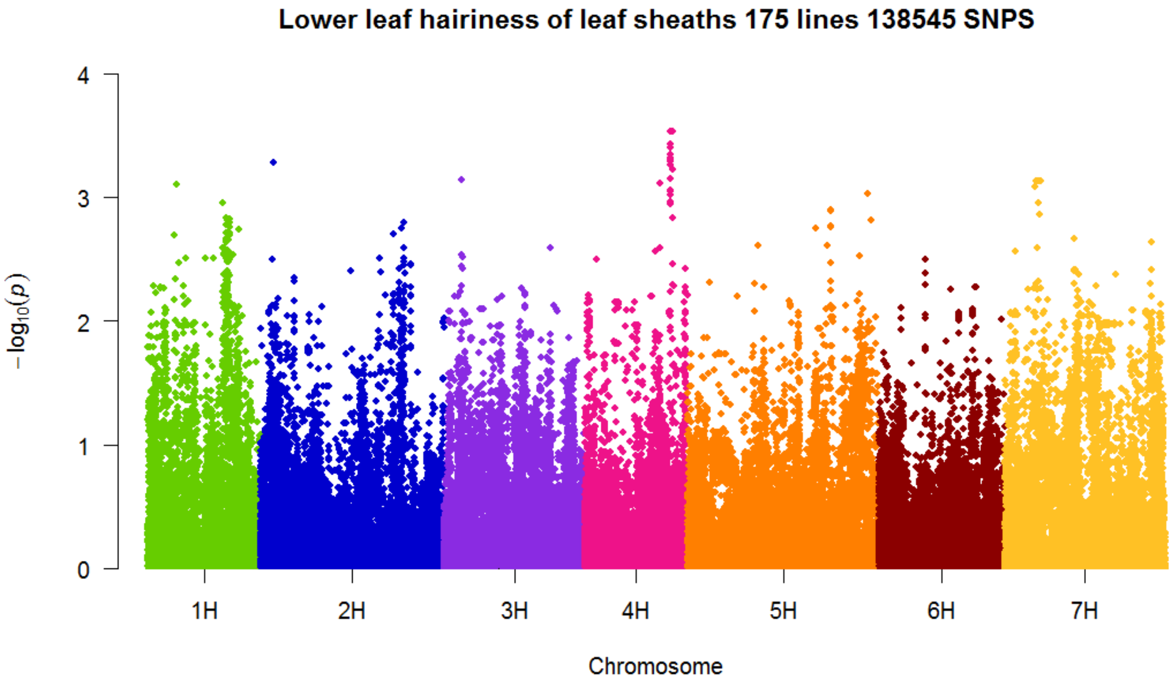
DUS TRAITS plots showing all SNPs including non-converged models



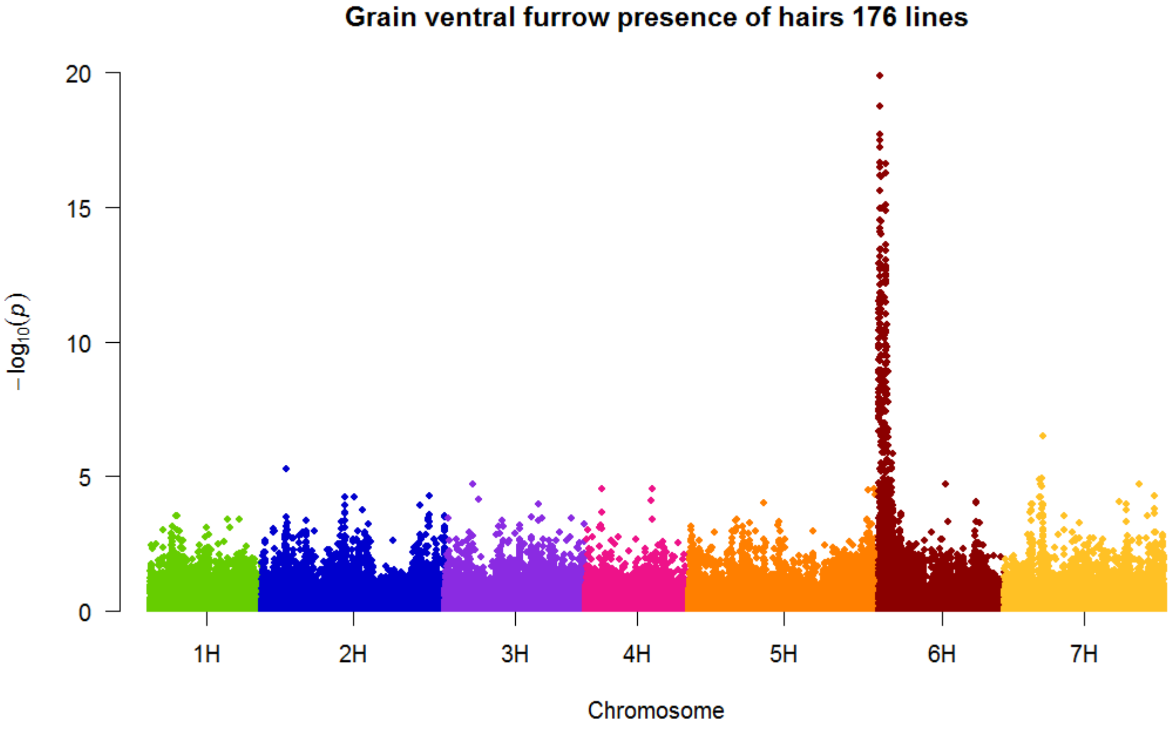

Flag leaf anthocyanin colouration of Auriles 147 lines 138545 SNPs
7
6
5
4
3
2
1
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome


Grain disposition of lodicules 161 lines
5
4
3
2
1
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome

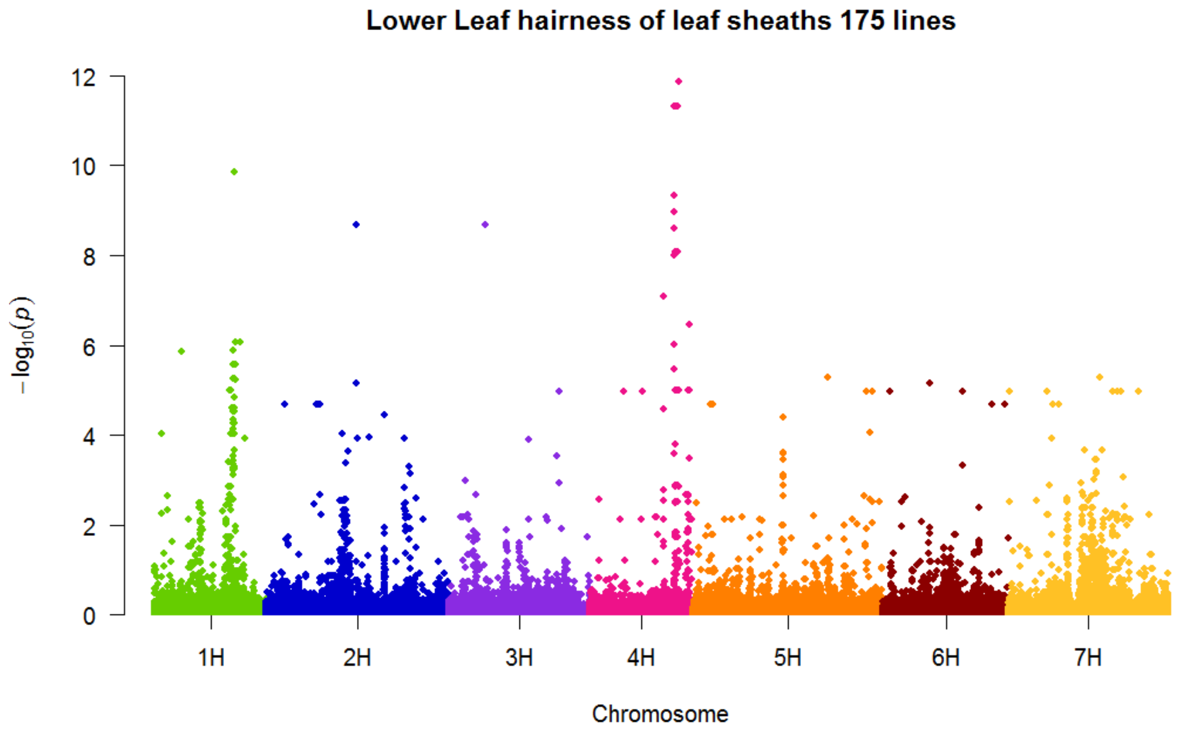

Grain husk 163 lines 138545 SNPS
3.0
2.5
2.0
1.5
1.0
0.5
0.0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome



Lower leaf hairiness of leaf sheaths 175 lines 138545 SNPS



Grain ventral furrow presence of hairs 176 lines


Lower Leaf hairness of leaf sheaths 175 lines
12
10
8
6
4
2
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome

9. Supplementary 4

DUS traits with more than 2 responses	**Response**									**Total Number of individuals**	**Response Description**
		2	**3**	**4**	**5**	**6**	**7**	**8**	**9**		
Awn Length Compared to Ear	-	-	3	1	33	26	99	0	0	162	Short to long
Awns Intensity of Anthocyanin Colour of Awn Tips	7	2	15	29	52	21	11	3	2	142	Absent to very strong
Collar Type	1		18	57	5	2	0	-	-	83	Recurrent-platfrom-cup
Ear Attitude at Least 21 Days After Ear Emergence	4	12	33	42	34	26	12	2	0	165	Erect to recurved
Ear Grain Density	0	5	33	42	60	18	5	1	0	164	Very lax to very dense
Ear Glaucosity	7	5	11	27	52	39	20	5	0	166	Absent to very strong
Ear Length excluding Awns	0	0	2	17	90	42	9	1	0	161	Very short to very long
Ear Shape	-	-	76	11	62	1	0	-	-	150	Tapering-parallel-fusiform
Flag Leaf Glaucosity of Sheath	0	0	1	2	27	39	51	43	2	165	Absent to very strong
Flag Leaf Intensity of Anthocyanin Colour of Auricles	5	1	4	9	29	29	38	21	4	140	Absent to very strong
Grain Anthocyanin	33	5	21	30	38	16	12	5		160	Absent to very strong

Colouration of Lemma Nerves											
Grain Spiculation of Inner Lateral Nerves	87	5	37	2	8	3	14	1	8	165	Absent to very strong
Kernel Colour of Aleurone Layer	126	12	15	-	-	-	-	-	-	153	None to strong
Median Spikelet Length of Glume Awn Cf Grain	11	133	15	-	-	-	-	-	-	159	Short to long
Frequency of Plants with Recurved Leaves	57	16	38	12	15	2	9	3	3	155	Absent to very high
Plant Growth Habit	1	3	20	25	47	28	31	6	3	164	Erect to prostrate
Plant Length Stem Ears and Awns		3	22	46	54	27	9	1	1	163	Very short to very tall
Rachis Curvature of First Segment	3	4	27	33	65	17	10	2	0	161	Absent to very strong
Rachis Length of First Segment	-	-	16	24	92	27	4	-	-	163	Short to long
Sterile Spikelet Attitude Mid 1/3 of Ear	12	25	100	-	-	-	-	-	-	137	Parallel to divergent
Sterile Spikelet Shape of Tip	13	99	15	-	-	-	-	-	-	127	Pointed, rounded, squared
Time of Ear Emergence 1st Spike Visible on 50 Ears	4	11	22	23	58	31	11	4	1	165	Very early to very late

PLANT - GROWTH HABIT	1	erect
	2	erect to semi-erect
	3	semi-erect
	4	semi-erect to intermediate
	5	intermediate
	6	intermediate to semi-prostrate
	7	semi-prostrate
	8	semi-prostrate to prostrate
	9	prostrate
PLANT - FREQUENCY OF PLANTS WITH RECURVED LEAVES	1	absent or very low
	2	very low to low
	3	low
	4	low to medium
	5	medium
	6	medium to high
	7	high
	8	high to very high
	9	very high
TIME OF EAR EMERGENCE (1st spk. vis. on 50% ears)	1	very early
	2	very early to early
	3	early
	4	early to medium
	5	medium
	6	medium to late
	7	late
	8	late to very late
	9	very late
FLAG LEAF - INTENSITY OF ANTH. COLOUR. OF AURICLES	1	absent to very weak
	2	very weak to weak
	3	weak
	4	weak to medium
	5	medium
	6	medium to strong
	7	strong

	8	strong to very strong
	9	very strong
EAR - GLAUCOSITY	1	absent or very weak
	2	very weak to weak
	3	weak
	4	weak to medium
	5	medium
	6	medium to strong
	7	strong
	8	strong to very strong
	9	very strong
FLAG LEAF - GLAUCOSITY OF SHEATH	1	absent or very weak
	2	very weak to weak
	3	weak
	4	weak to medium
	5	medium
	6	medium to strong
	7	strong
	8	strong to very strong
	9	very strong
EAR - ATTITUDE (at least 21 days after ear emerg.)	1	erect
	2	erect to semi-erect
	3	semi-erect
	4	semi-erect to horizontal
	5	horizontal
	6	horizontal to semi-recurved
	7	semi-recurved
	8	semi-recurved to recurved
	9	recurved
AWNS-INTENSITY OF ANTHOCYANIN COLOUR. OF AWN TIPS	1	absent to very weak
	2	very weak to weak
	3	weak
	4	weak to medium
	5	medium

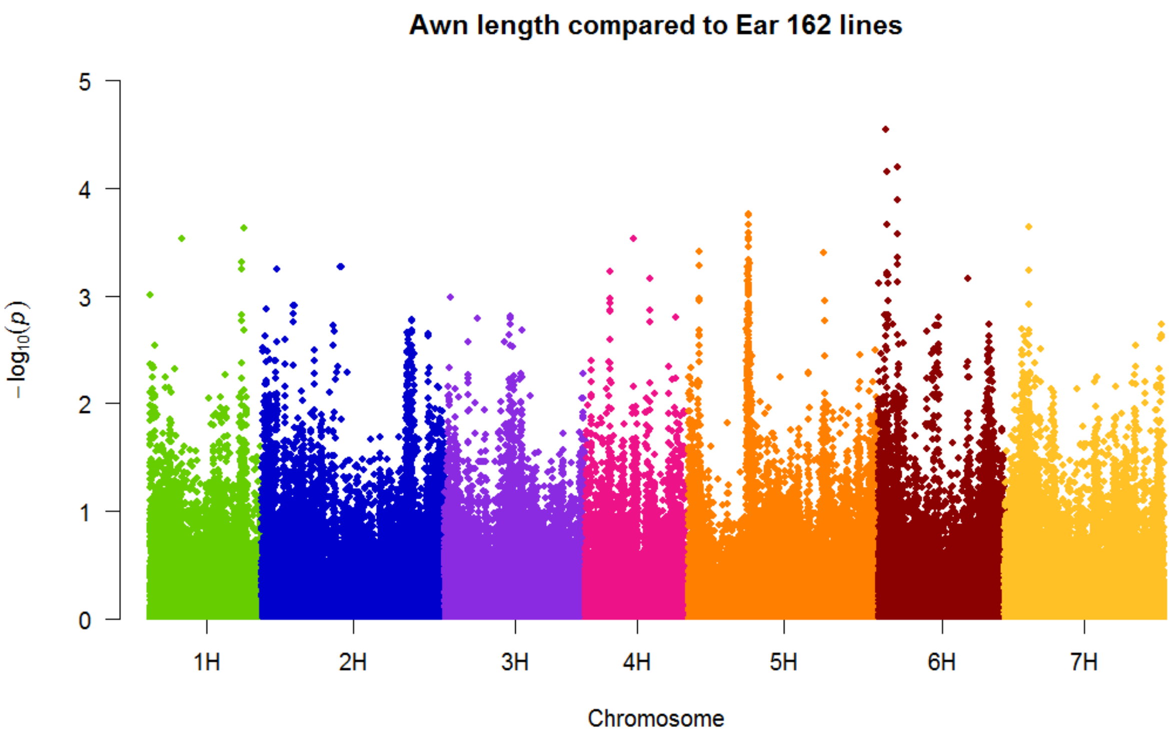
	6	medium to strong
	7	strong
	8	strong to very strong
	9	very strong
STERILE SPIKELET - ATTITUDE(MID 1/3 OF EAR)	1	parallel
	2	parallel to weakly divergent
	3	divergent
STERILE SPIKELET - SHAPE OF TIP	1	pointed
	2	rounded
	3	squared
MEDIAN SPIKELET - LENGTH OF GLUME+AWN cf GRAIN	1	shorter
	2	equal
	3	longer
EAR - LENGTH(EXCLUDING AWNS)	1	very short
	2	very short to short
	3	short
	4	short to medium
	5	medium
	6	medium to long
	7	long
	8	long to very long
	9	very long
AWN - LENGTH (compared to ear)	3	short (shorter than ear)
	4	shorter to +/- equal
	5	medium (+/- equal to ear)
	6	+/- equal to longer
	7	long (longer than ear)
PLANT - LENGTH(STEM,EARS AND AWNS)	1	very short
	2	very short to short
	3	short
	4	short to medium
	5	medium
	6	medium to long
	7	long

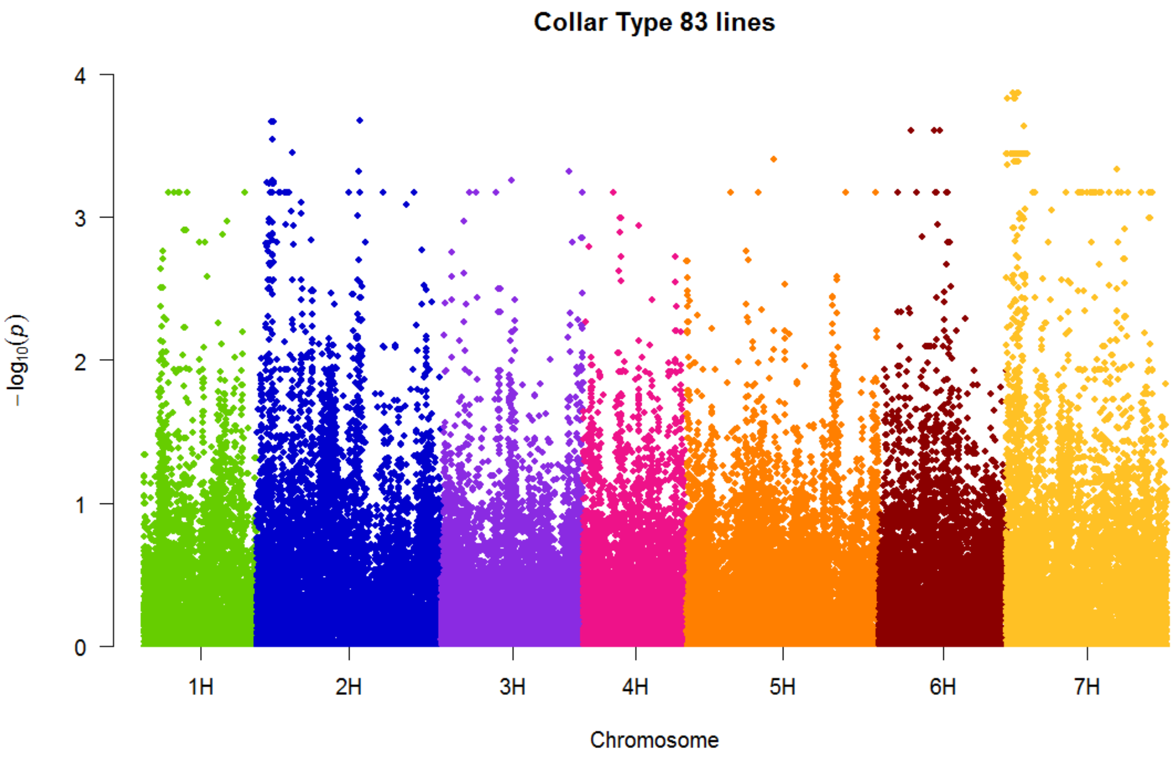
	8	long to very long
	9	very long
COLLAR TYPE	1	decurrent
	2	decurrent to platform
	3	platform
	4	platform to shallow cup
	5	shallow cup
	6	shallow cup to cup
	7	cup
EAR - DENSITY	1	very lax
	2	very lax to lax
	3	lax
	4	lax to medium
	5	medium
	6	medium to dense
	7	dense
	8	dense to very dense
	9	very dense
EAR - SHAPE	3	tapering
	4	tapering to parallel
	5	parallel
	7	fusiform
RACHIS - LENGTH OF FIRST SEGMENT	3	short
	4	short to medium
	5	medium
	6	medium to long
	7	long
RACHIS - CURVATURE OF FIRST SEGMENT	1	absent
	2	very weak
	3	weak
	4	weak to medium
	5	medium
	6	medium to strong
	7	strong
	8	strong to very strong

	9	very strong (angular)
KERNEL - COLOUR OF ALEURONE LAYER	1	whitish ("white")
	2	weakly coloured
	3	strongly coloured ("blue")
GRAIN - SPICULATION OF INNER LATERAL NERVES	1	absent/v. weak (0-2 per nerve)
	2	v. weak to weak
	3	weak (1-2 per nerve)
	4	weak to medium
	5	medium (3-5 per nerve)
	6	medium to strong
	7	strong (5-10 per nerve)
	8	strong to v. strong
	9	very strong (>10 per nerve)
GRAIN - ANTHOCYANIN COLOURATION OF LEMMA NERVES	1	absent or very weak
	2	very weak to weak
	3	weak
	4	weak to medium
	5	medium
	6	medium to strong
	7	strong
	8	strong to very strong
	9	very strong

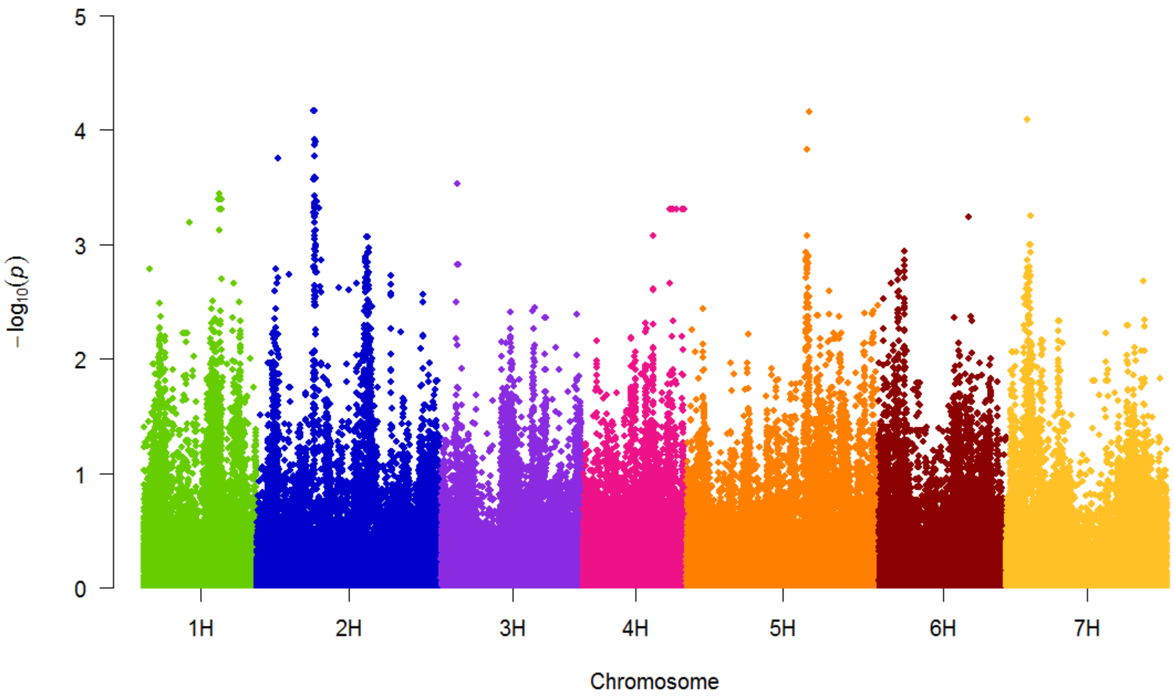
9.1. Supplementary 5

DUS Traits With More Than 2 Categories

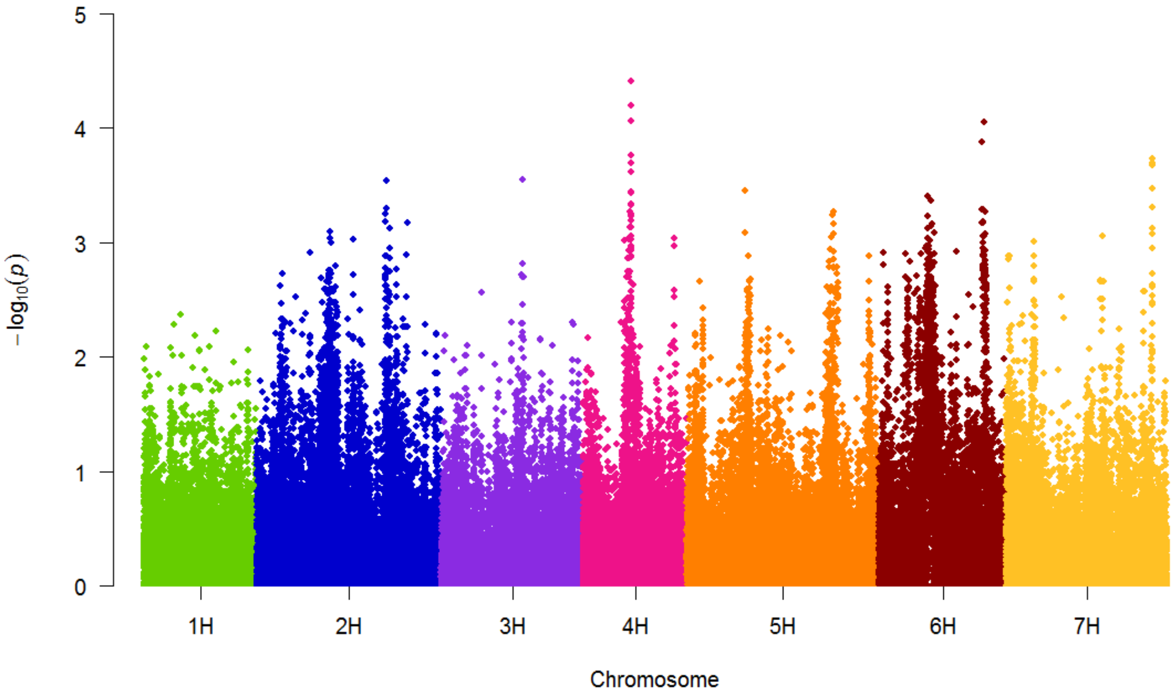

Awn length compared to Ear 162 lines

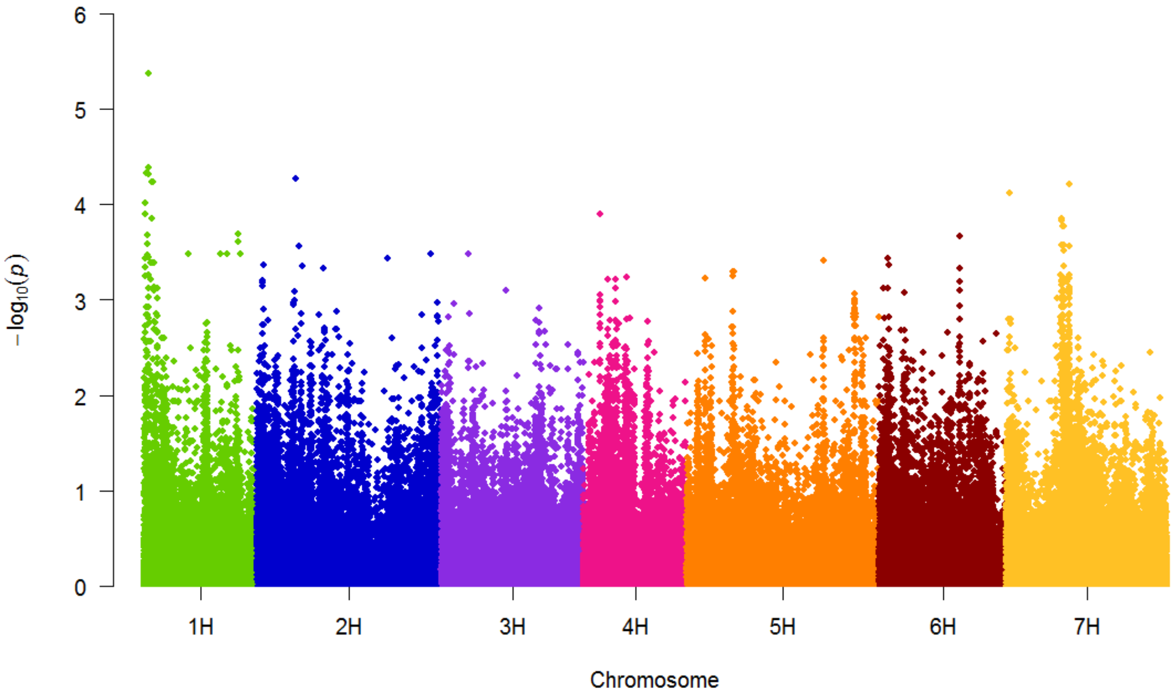

Collar Type 83 lines

Ear Attitude at Least 21 Days After Ear Emergence 165 lines

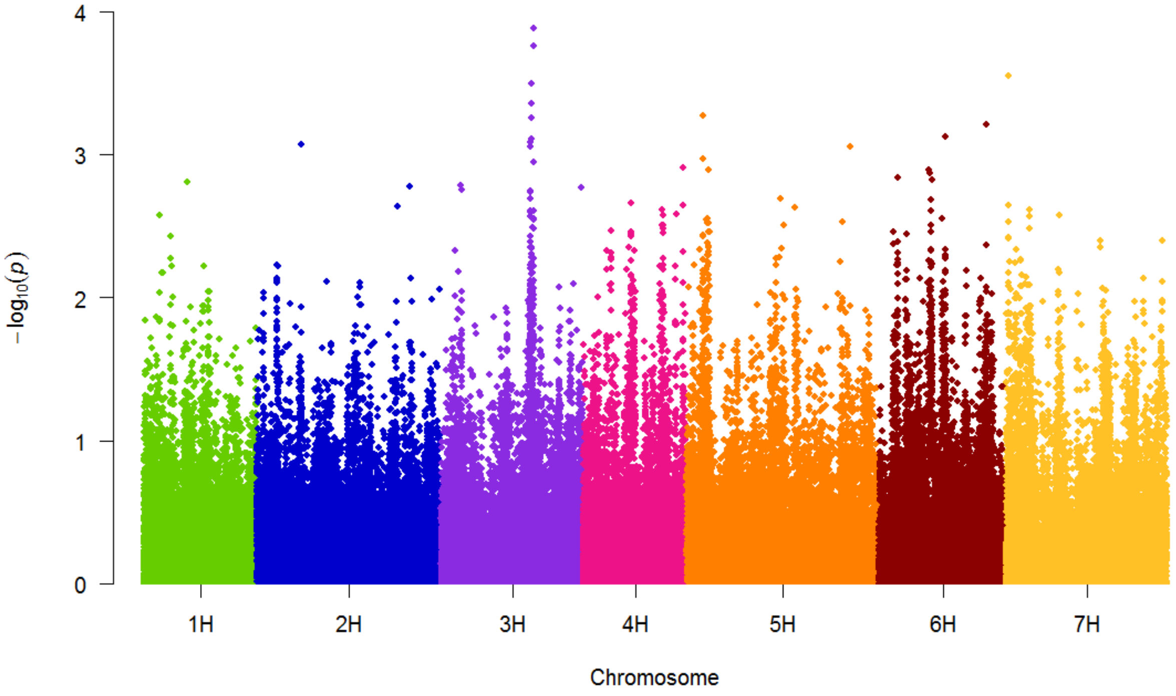


Ear Density 164 lines

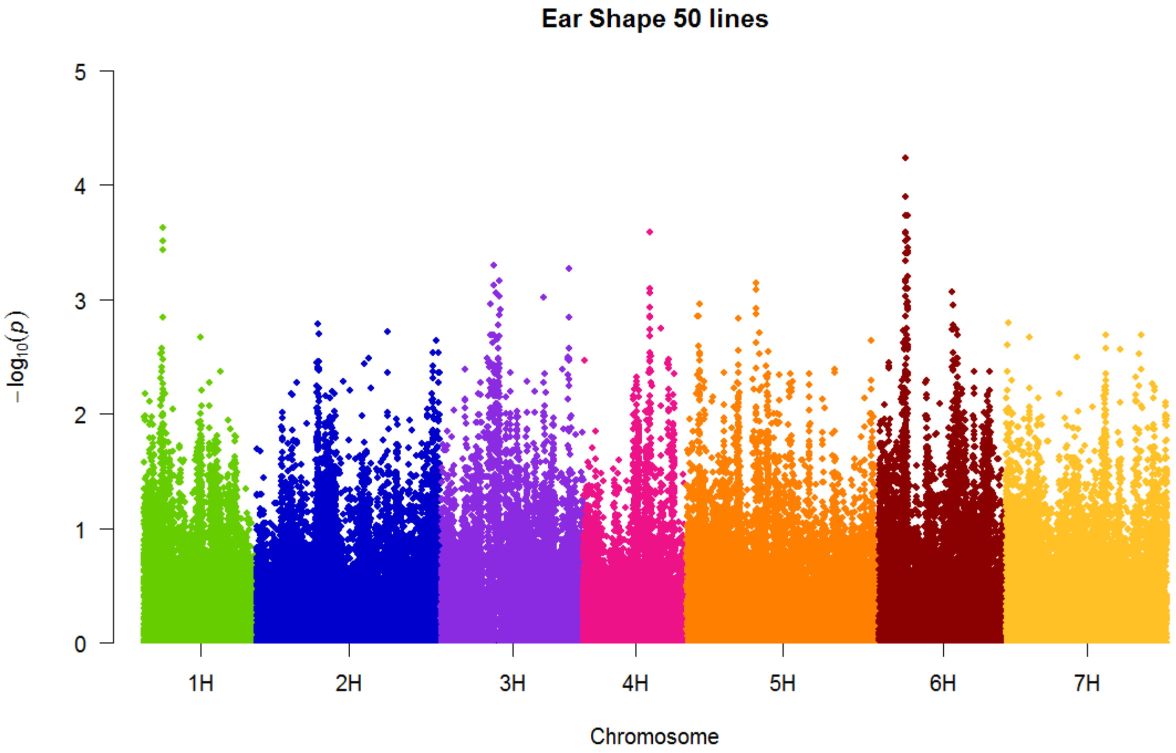


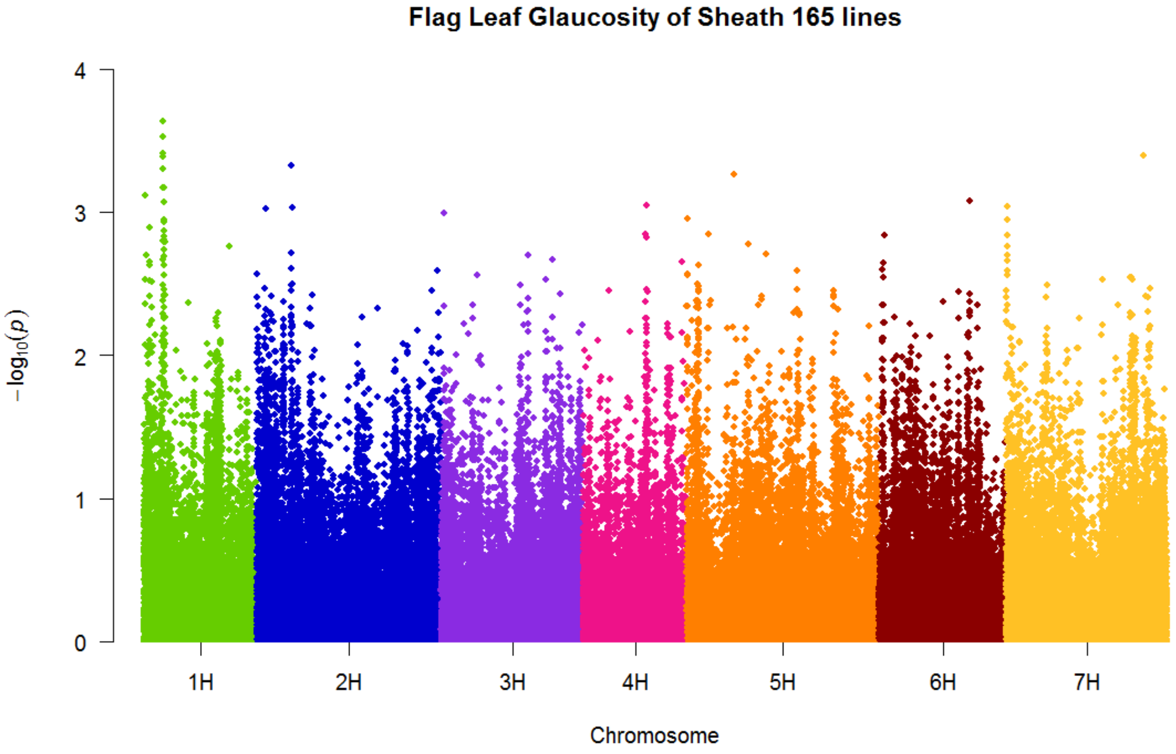


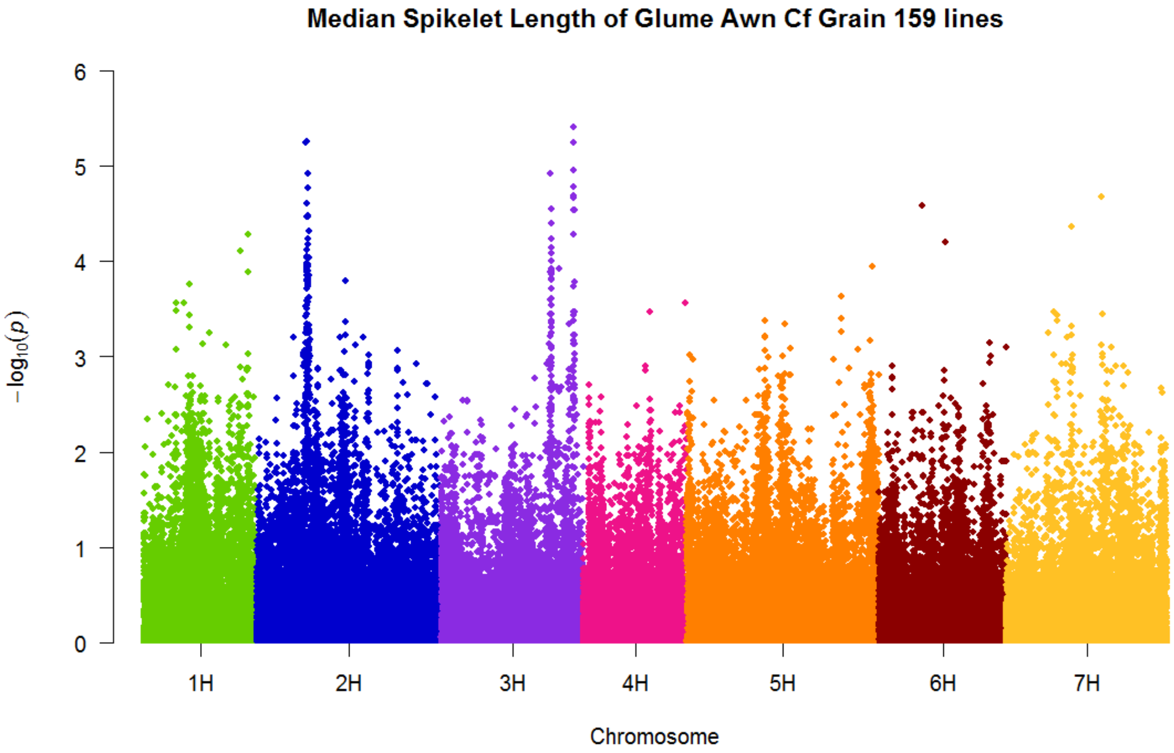
Ear Glaucosity 166 lines

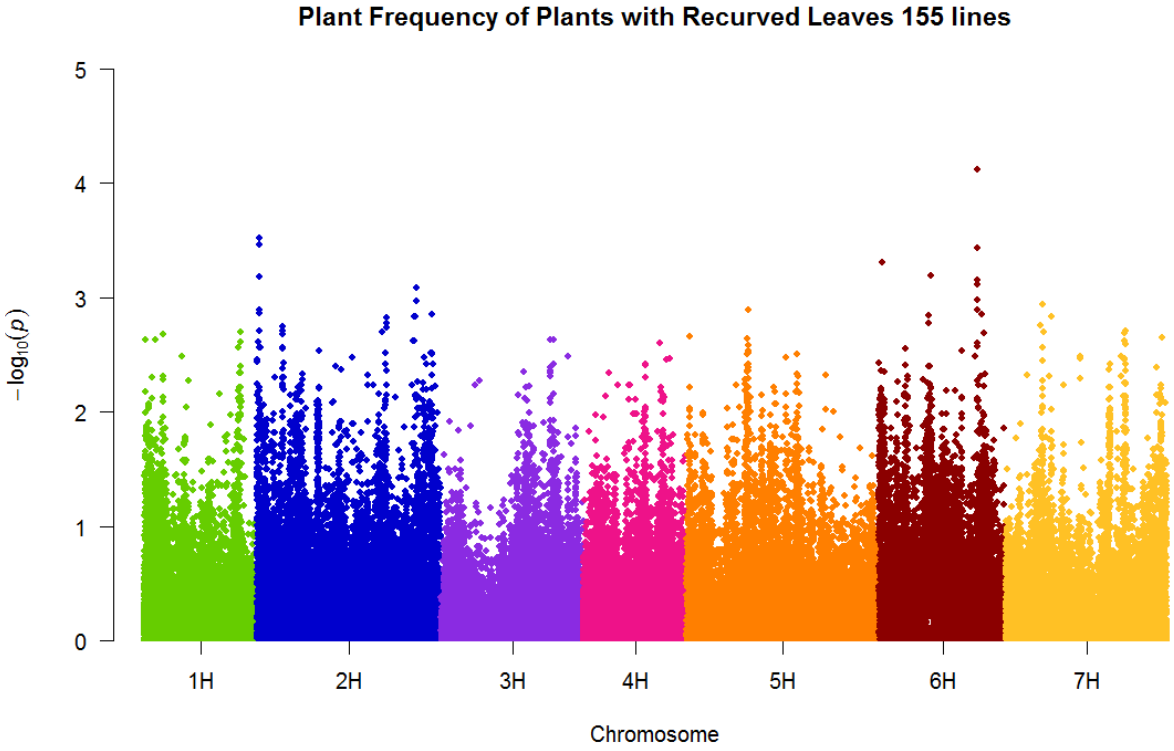


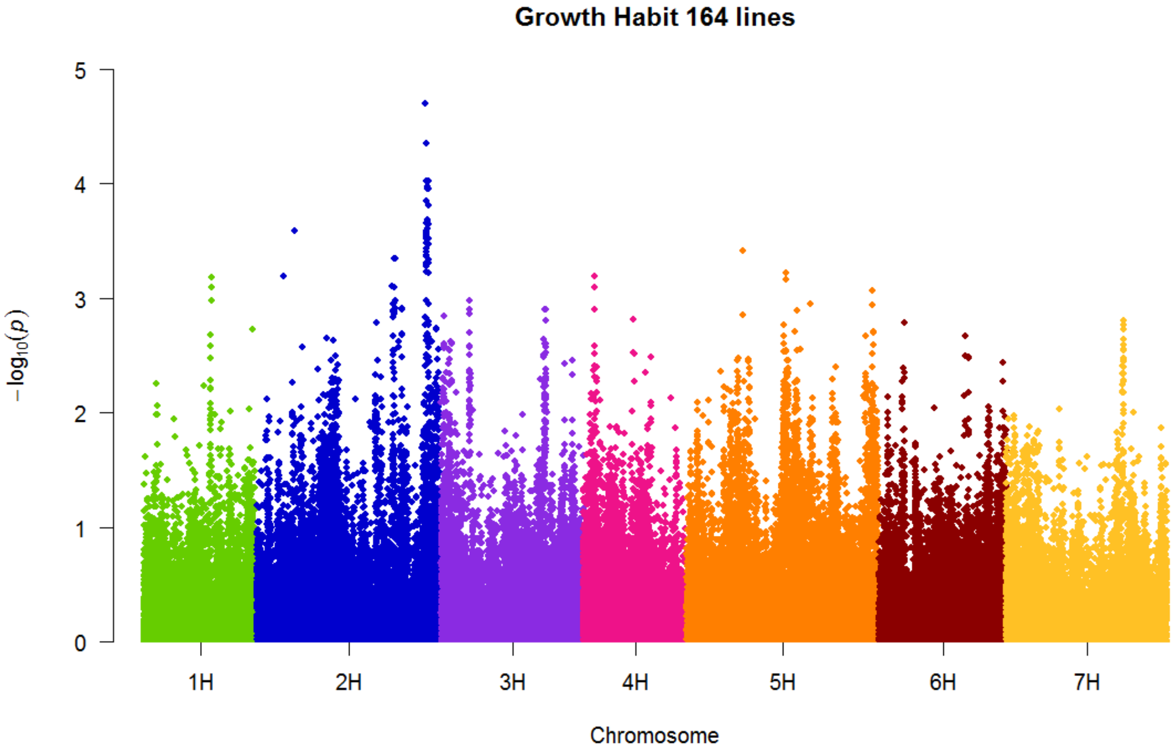
Ear Length excluding Awns 161 lines

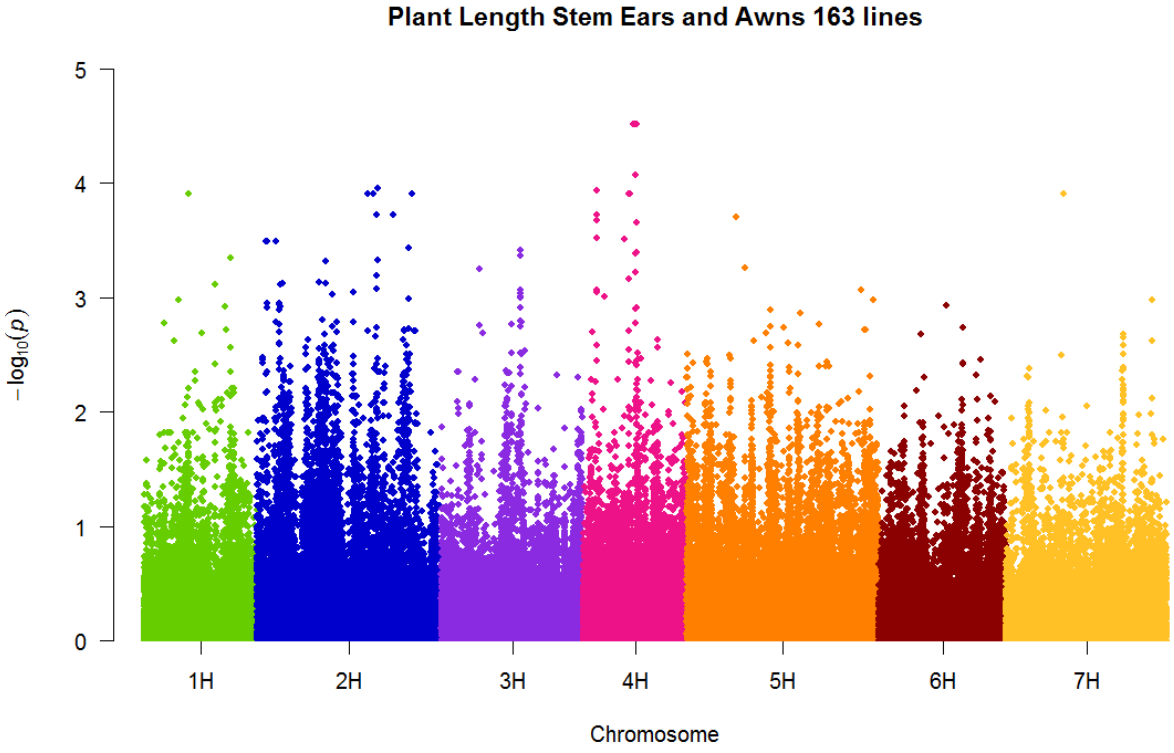

Ear Shape 50 lines
5
4
3
2
1
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome

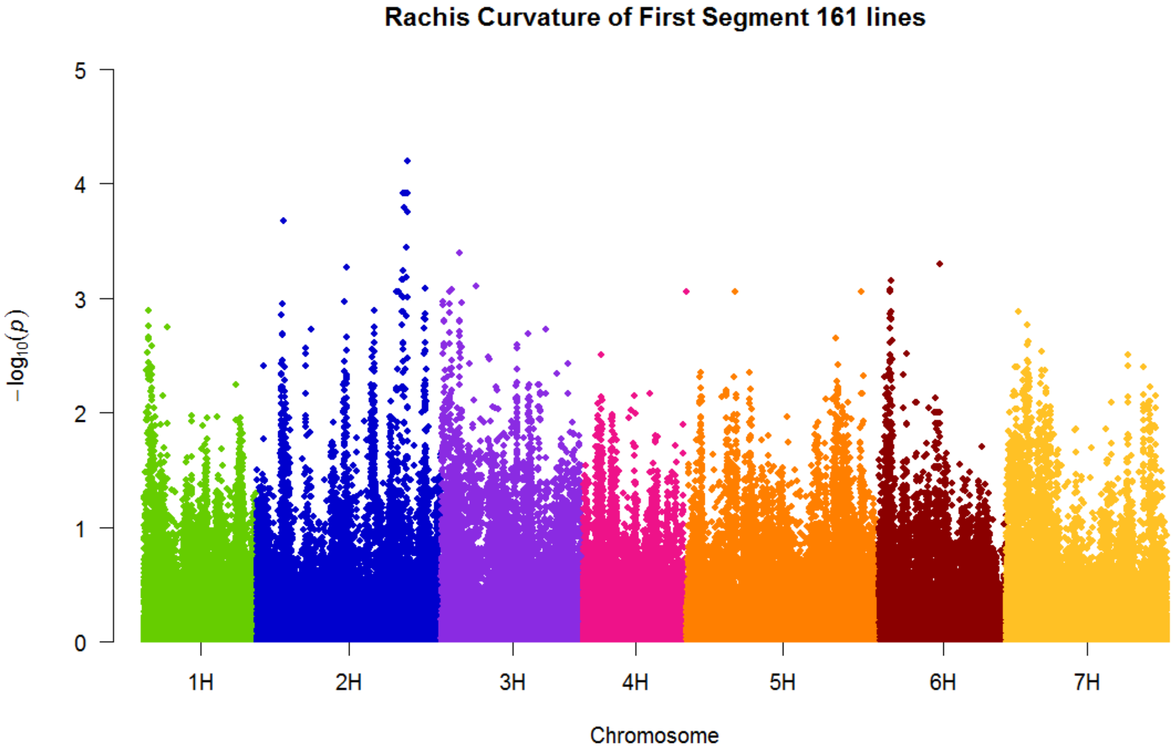

Flag Leaf Glaucosity of Sheath 165 lines
4
3
2
1
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome

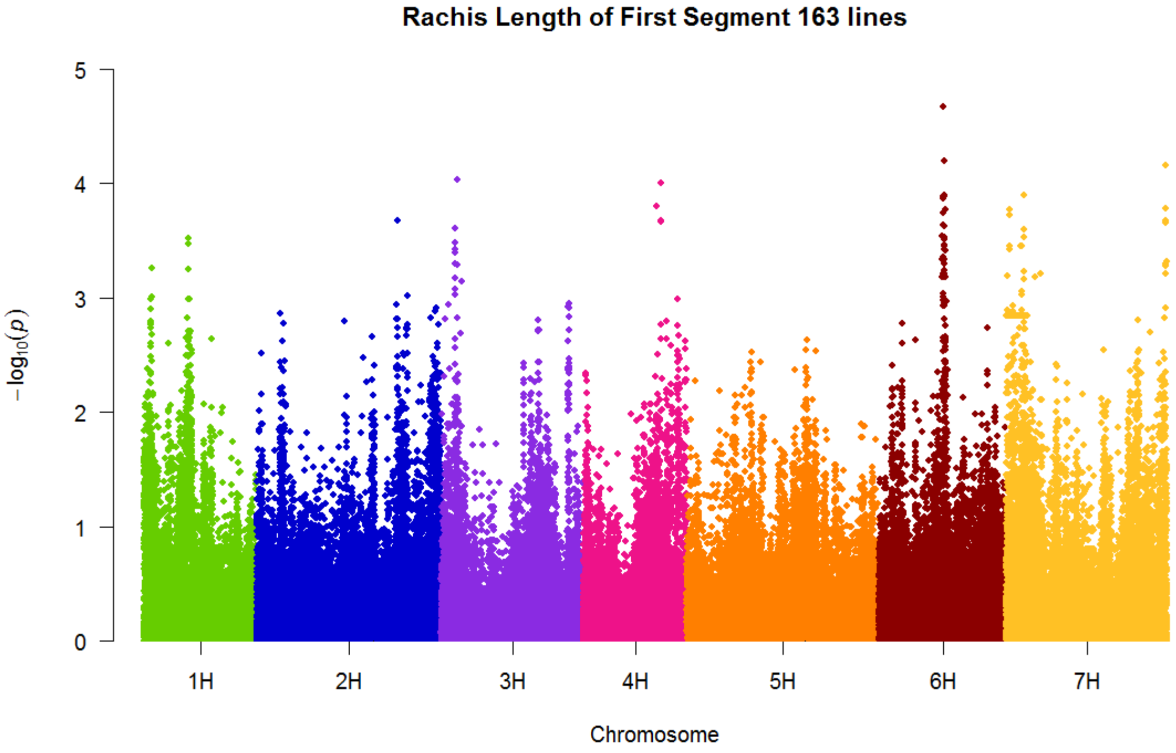

Median Spikelet Length of Glume Awn Cf Grain 159 lines
6
5
4
3
2
1
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome


Plant Frequency of Plants with Recurved Leaves 155 lines
5
4
3
2
1
0
$-\log_{10}(p)$
1H
2H
3H
4H
5H
6H
7H
Chromosome

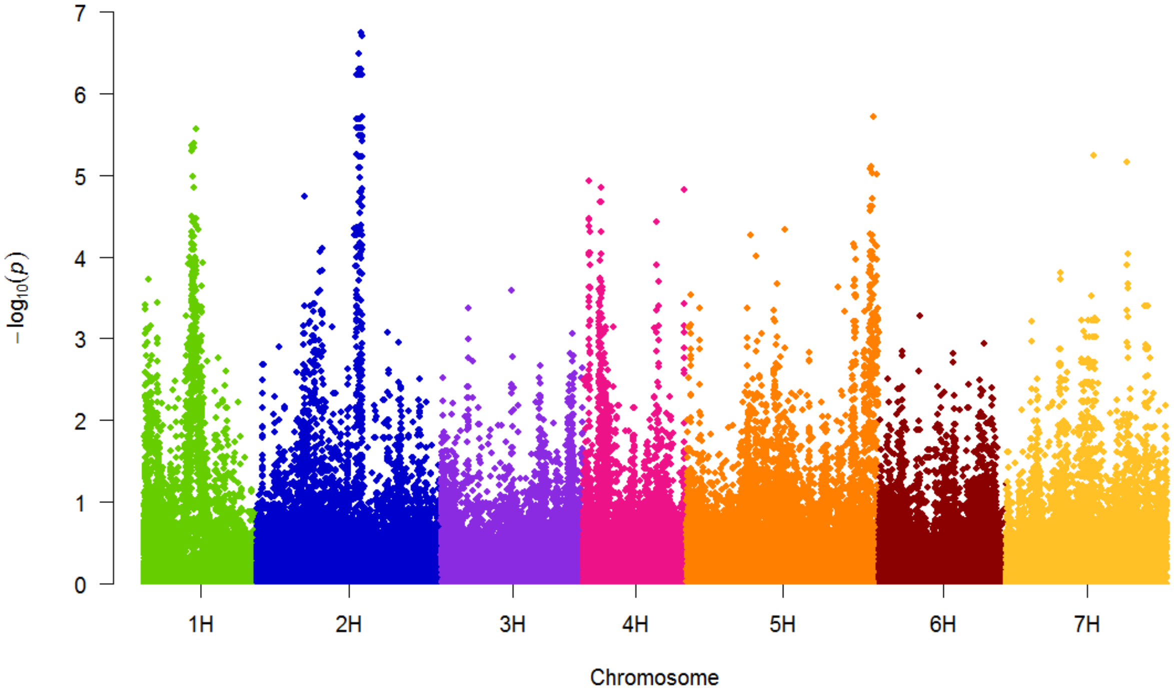

Growth Habit 164 lines
−log10(p)
0
1
2
3
4
5
1H
2H
3H
4H
5H
6H
7H
Chromosome


Plant Length Stem Ears and Awns 163 lines
−log10(p)
0
1
2
3
4
5
1H
2H
3H
4H
5H
6H
7H
Chromosome

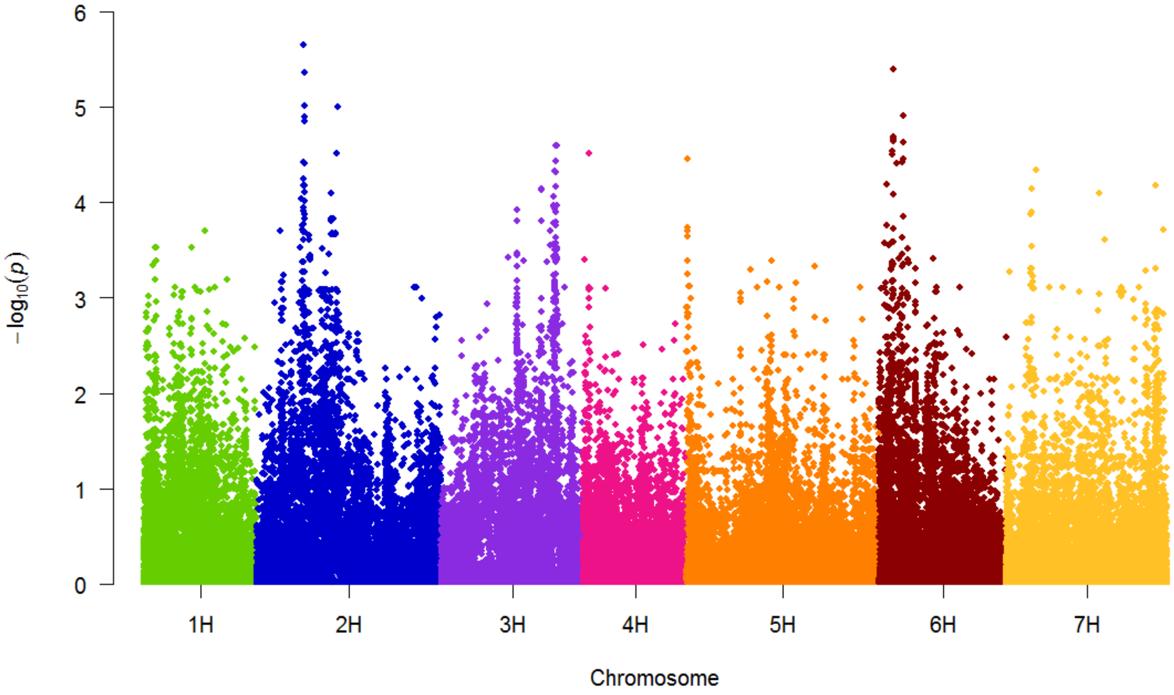

Rachis Curvature of First Segment 161 lines
$-\log_{10}(p)$
0
1
2
3
4
5
1H
2H
3H
4H
5H
6H
7H
Chromosome

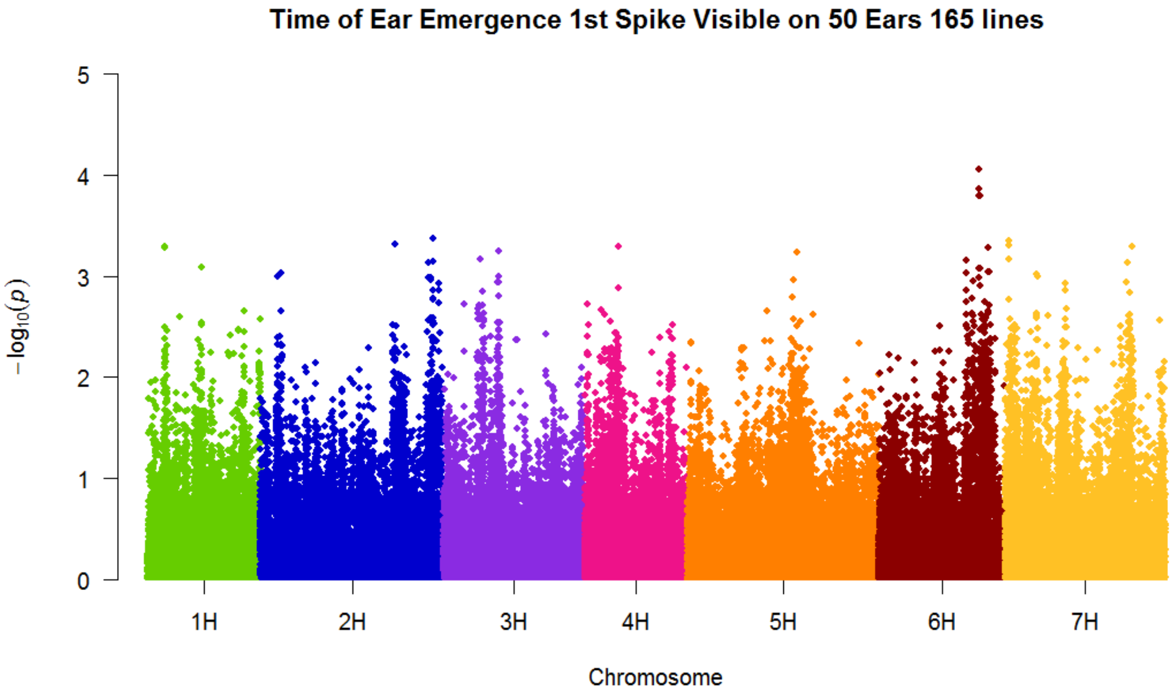

Rachis Length of First Segment 163 lines
$-\log_{10}(p)$
0
1
2
3
4
5
1H
2H
3H
4H
5H
6H
7H
Chromosome

Sterile Spikelet Attitude Mid 1/3 of Ear 137 lines



Sterile Spikelet Shape of Tip 127 lines




Time of Ear Emergence 1st Spike Visible on 50 Ears 165 lines
$-\log_{10}(p)$
5
4
3
2
1
0
1H
2H
3H
4H
5H
6H
7H
Chromosome