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Relationship Between Hardness Genes and Quality in Barley (*Hordeum vulgare*)

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ABSTRACT

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Barley (*Hordeum vulgare*) genotypes were sequenced for polymorphism in the hardness genes, these being the three hordoindoline (*hin a, hin b1* and *hin b2*) genes. The variation in haplotype was determined by sequencing for single nucleotide polymorphisms (SNPs). Polymorphism between each gene was then compared to grain hardness (three methods), malt quality characteristics (hot water extract and friability) and cattle feed quality. Two haplotypes were found in a set of forty barley genotypes. For *hin a,* two alleles were present, namely *hin a1* and *hin a2.* However, there was no specific *hin a* allele that was associated with grain hardness, malt and feed quality. Barley has two *hin b* genes, namely *hin b1* and *hin b2,* and the genotypes tested here had one of two alleles for each gene. However, there were no obvious effects on hardness or quality from either of these *hin b* alleles. Unlike wheat, where a clear relationship has been demonstrated between a number of SNPs in the wheat hardness genes and quality (soft or hard wheat), there was no such relationship for barley. Despite the wide range in hardness, malt and feed quality, there were only two haplotypes for each of the *hin a, hin b1* and *hin b2* genes and there was no clear relationship between grain hardness, malt or feed quality. The genotypes used in this study demonstrated that there was a low level of polymorphism in hardness genes in current commercial varieties as well as breeding lines and these polymorphisms had no impact on quality.

Key words: Barley, feed, genes, hardness, hordoindolines, malt quality.

INTRODUCTION

Genetic studies have identified a number of areas of the barley genome with QTLs associated with grain hardness. However, three specific genes (hordoindolines) for barley hardness have been identified on the short arm of chromosome 7 (5H), closely located to the Grain Softness Protein[6]. The three genes have been named *hin a, hin b1* and *hin b2.* The *hin a* sequence and *hin b* sequences are homologous to the puroindoline (*pin*) genes in wheat. Detailed studies have identified genetic and phenotypic variation in wheat hardness that can be associated with the *pin* genes[17].

Variation in trait expression can be due to allelic changes in the gene sequence. Any single base change in the gene sequence between two individuals is described as a single nucleotide polymorphism (SNP). SNPs have been identified in *pin* genes which have been associated with changes in wheat hardness. Similarly for barley, SNPs have been identified for each of the *hin a, hin b1* and *hin b2* genes[3,10]. The Genbank database (http://www.ncbi.nih.gov) holds hin sequence data for over 200 barley accessions. This data has been acquired by other researchers. Seventeen SNPs in fourteen alleles were catalogued for *hin a* (Caldwell et al. unpublished). These SNPs were at eight positions and resulted in 12 amino acid substitutions. Two additional alleles were also catalogued (*hin a13* and *hin a14*) but these had deletions at the start and end of the gene sequence.

Previous studies had identified the two *hin b* alleles[6,10]. Both of these studies sequenced numerous barley varieties and these are also catalogued in Genbank (http://www.ncbi.nih.gov). However, unlike the *hin a* alleles, there was no identification of the polymorphism between these two alleles for the varieties tested. Therefore, to provide a basis for identification of common or new SNPs and subsequent alleles, we have named the alleles for the *hin b1* and *hin b2.* For *hin b1,* there were 8 SNPs resulting in 8 amino acid substitutions. However, the common alleles were *hin b1-1* and *hin b1-2.* For *hin b2* 10 SNPs resulted in 10 amino acid substitutions with *hin b2-1* and *hin b2-2* being the common alleles (http://www.ncbi.nih.gov). While it is routine to align DNA sequences, to conserve space, we have used the transcribed amino acid sequence. Details of the amino acid sequence for *hin a, hin b1* and *hin b2* alleles are provided in Figs. 1 and 2 and are compared to the common wheat allele.

Darlington et al.[10] assessed a number of malt and feed varieties for *hin a* and *hin b* alleles and identified 3 haplotypes. There was no obvious effect of any particular allele or SNP on barley grain texture. Beecher et al.[2] identified an area on chromosome 7 (5H) where a QTL for hordoindoline (*hin*) was located. This region also coincided with

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a malt extract QTL suggesting possible interaction between malt extract and this particular region. However, of the eight varieties analysed in the Beecher et al.[2] study, again there was no relationship between variations in *hin* alleles and malt or feed varieties.

The early studies measuring barley grain hardness investigated the Milling Energy (ME) method where the resistance of the grain as it was being milled was calculated[1]. A number of studies have demonstrated the varying relationships between ME hardness and grain and malt quality parameters, including negative impacts of grain protein[10] and β-glucan[16], while there was a positive rela tionship between ME and hot water extract (HWE) and modification[19]. Further, a recent study showed the correlations between a Particle Size Index method and a number of malt traits[18]. These results showed positive relationships between softer grain and extract, Kolbach Index and friability but a negative relationship to β-glucan. Additional hardness measurements were carried out by calculating the grinding resistance. This was carried out in a

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Pina-D1a    MKALFLIGLLALVASTAFAQYSEVVGSYD VAGGGGAQQCPVETKLNSCRNYLLDRCSTMKDFPVTWRWWKWWKGG
Hina-1             ********A*****G******EGG**********LG***D**********T***********R*****
Hina-2             ********A*****G*******GG**********LE***D**********T***********R*****
Hina-3             ********A*****G*******GG**********LE***D**********T***********R*****
Hina-4             ********A*****G*******GG**********LE***D**********T***********T*****
Hina-5             ********A*****G******KGG**********LG***D**********T***********T*****
Hina-6             ********A*****G******KGG**********LG***D**********T***********T*****
Hina-7             ********A*****G*******GG**********LG***D**********T***********T*****
Hina-8             ********A*****G*******GG**********LG***D**********T***********T*****
Hina-9             ********A*****G*******GG**********LG***D**********T***********T*****
Hina-10            ********A*****G*******GG**********LE***D**********T***********T*****
Hina-11            ********A*****G*******GG**********LE***D**********T***********T*****
Hina-12            ********A*****G*******GG**********L****D**********T***********T*****
Hina-13                       G*******GG**********LG***D**********T***********T*****
Hina-14                       G*******GG**********L****D**********T***********R*****

Pina-D1a    CQELLGECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASKVIQEAKNLPPRCNQGPPCNIPGTIGYYW
Hina-1      *E***HD***Q*S*****************R***F*******TV****A***********A****S******
Hina-2      *L***HD***Q*G*****************R***V*******TV****A***********A****S******
Hina-3      *L***HD***Q*G*****************R***V*******TV****A***********A****S******
Hina-4      *L***HD***Q*G*****************R***V*******TV****A***********A****S******
Hina-5      *K***HD***Q*S************E****R***F*******TV****A***********A****S******
Hina-6      *E***HD***Q*S*****************R***F*******TV****A***********A****S******
Hina-7      *E***HD***Q*S*****************R***F*******TV****A***********A****S******
Hina-8      *E***HD***Q*S***************P*R***F*******TV****A***********A****S******
Hina-9      *L***HD***Q*G*****************R***V*******TV****A***********A****S******
Hina-10     *E***HD***Q*S*****************R***V*******TV****A***********A****S******
HinA-11     *L***HD***Q*G*****************R***V*******TV****A***********A****S******
Hina-12     *L***HD***Q*G*****************R***V*******TV****A***********A****S******
Hina-13     *E***HD***Q*G*****************R***V*******TV****A
Hina-14     *L***HD***Q*G*****************R***F*******TV****A
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Fig. 1. Amino acid sequences for alleles of *hin a* downloaded from Genbank (http://www.ncbi.nih.gov). These sequences were used to identify the alleles for the barley genotypes used in this current study.

Fig. 2. Amino acid sequences for alleles of *hin b1* and *hin b2* downloaded from Genbank (http://www.ncbi.nih.gov). These sequences were used to identify the alleles for the barley genotypes used in this current study.

Do-Corder instrument and showed harder grain with a higher value and as such had a positive correlation for β-glucan and negative correlations for extract, Kolbach Index and friability[18].

Most of the research into the association between hardness and feed quality has been carried out in North America. Bowman et al.[5] and Beecher et al.[3] reported a relationship between hardness and ruminant feed quality from the Steptoe × Morex mapping population. The region on 5H associated with hardness was also confirmed from this study[3]. Further, while there were genetic differences within both the malt and feed quality attributes assessed, there was a low level of variation in the texture of barley[3]. Results from an Australian study detailed the relationship between hardness methods and the impact of these methods in explaining feed and malt quality[13]. A review on the impact of the genetics associated with malt and feed quality is presented in Fox et al.[12]

This study investigated the diversity for the *hin a, hin b1* and *hin b2* genes in a range of commercial malt and feed barley varieties as well as barley breeding lines. In addition, these genotypes were tested for grain hardness, malt and feed quality to ascertain any relationship between haplotypes and end-product quality.

MATERIALS AND METHODS

Barley genotypes

The sample set comprised forty genotypes including breeding lines (25) and commercial varieties (15) that were selected from a combined intermediate/advanced stage breeding trial. The breeding lines represented a diverse range of genetic backgrounds including Australian and overseas parents of the breeding lines. These barleys were grown in a replicated trial at two sites (Kaimkillenbun and Breeza) over two years (2002 and 2003).

DNA extraction

DNA was extracted from barley following the method described by Maguire et al.[16] Barley grains were germinated under sterile conditions with MilliQ water in a 50 mL Falcon tube. When shoots were around 5 cm long, the shoot was cut from the grain and placed in 5 mL microtubes. Genomic DNA was extracted from shoots of young barley seedlings using DNeasy Plant Mini kits (Qiagen, Germany) according the manufacturer's protocol for fresh tissues. DNA yields were estimated by fluorescence intensity comparison on agarose gel and by spectrophotometric techniques.

PCR of *hin* genes

The Polymerase Chain Reaction (PCR) assays for the *hin* genes was carried as described in Beecher et al.[2] The *hin a* sequence was amplified from genomic DNA using the primers PHV5 (5′TAGGTCTGCTTGCTTTGG TAG′3) and PA3 (5′TCACCAGTAATAGCCAATA GTG′3). The *hin b* sequence was amplified from barley genomic DNA using the primers PB5 (5ATGAAGACCT TATTCCTCCTA3) and PB3 (5TCACCAGTAATAGC CACTAGGGAA3). The primers PA3, PB5, and PB3′ have been described previously[12]. Platinum *Taq* DNA polymerase was used in the reactions. The temperature regime used consisted of a 3 min initial denaturation step at 94°C, followed by 40 cycles of 94°C for 45 s, 54°C for 30 s, 72°C for 90 s, followed by a 5-min final extension at 72°C. The PCR product was subject to electrophoresis on a 1.5% agarose gel with a XIII ladder used for molecular weight comparison.

Sequencing analysis

PCR products were purified using the Qiagen Gel Purification Kit (Qiagen, Germany). Purified products were sequenced in both directions with BigDye Terminator v.3.0 (Applied Biosystems). Sequencing was carried out in an ABI3700 automated sequencer. Sequences were edited and aligned with *ChromasPro V1.32* using default settings.

Hardness, malt and feed quality analysis

All field grown barleys were tested for grain hardness, malt and feed quality. Three different methods were used to determine hardness, namely particle size index (PSI), Particle Size (PS) and Single Kernel Characterisation System (SKCS). Malt quality assessment was carried out using an in-house malting procedure. Friability and hot water extract (HWE) were carried out using industry methods. For feed quality assessment four traits were determined, namely starch, acid detergent fibre (ADF), hardness (PS) and *in sacco* Dry Matter Digestibility (ISDMD). These traits are used in a regression equation to derive Net Energy (NE) and Average Daily Gain (ADG). Details of all these methods are described in Fox et al.[13] For this study only ISDMD, NE and ADG are presented. The results for all four sites were averaged to ascertain a relationship between each genotype and the haplotypes identified.

RESULTS

Hordoindolines

Fifteen commercial varieties and twenty-five breeding lines were sequenced for SNPs in the hordoindoline genes. For *hin a,* only two alleles were detected, namely *hin a1* and *hin a2,* from the 14 alleles published (Fig. 1 http://www.ncbi.nih.gov). These sequences match those reported in Genbank. For *hin b1* and *hin b2* there were two alleles for each of these genes that were common for all of the sequenced genotypes. The genotypes either had *hin b1-1* and *hin b2-1* or *hin b1-2* and *hin b2-2.* These align with the data available on Genbank and shown in alleles provided with names by the authors (Fig. 2). The combined *hin a* and *hin b* haplotypes for each genotype are shown in Table I. For all the *hin* gene sequences identified in the barleys used in this study, it appeared that if a genotype had *hin a1* then it also had *hin b1-1* and *hin b2-1.* Conversely, where the *hin a2* allele was present then the *hin b1-2* and *hin b2-2* alleles were present.

Barley hardness

A summary of the hardness results is shown in Table II. Details of the relationship between these methods are described by Fox et al.[13] Two of the methods are similar,

these being PSI and PS, where the sample is ground and then sieved. The difference between the methods being that the PSI method uses only one sieve while the PS method uses five. These methods capture information related to the structure of the grain, in particular, the endosperm, in that harder grain has larger particles retained on the larger sieves. The SKCS method uses a crushing method and determines the resistance to the crushing. There was a good relationship between the PSI and SKCS methods[13]. However, the PS data did not correlate well with either of the PSI or SKCS methods. As all these methods have been used to measure barley hardness and relate that to malt quality we have included results from all three.

There was a range of greater than 10% for lowest to highest values for each method. For the PSI and PS methods, low values are indicative of harder grain, while for SKCS the higher values are for harder grain. A Box and Whisker plot (Fig. 3) suggests that there was no difference between either of the haplotype sets for each of the hardness methods, indicating that any change in the amino acid sequences for the various hardness genes did not influence hardness. While there were differences between hardness values for each of the genotypes tested in each of the methods used in this study[13], there would appear to be no influence on the hardness by the changes in hordoindoline gene sequences.

Malt quality

Detailed malt and feed analysis was carried out on forty genotypes grown in replicated trials at four sites grown over two years. Fig. 4 shows that there was also very little difference between the two haplotype groups for the malt quality traits of hot water extract and friability. There were differences in the ranges between the two haplotypes for all two traits but there was little difference between the averages. Again, this suggests that for the genotypes tested in this study, the two haplotypes had little effect on malt quality.

Table I. Haplotypes for the genotypes analysed in this study.

Haplotype	
hina1, hinb1-1, hinb2-1	***hina2, hinb1-2, hinb2-2***
Gairdner	Binalong
Grimmett	Grout
Fitzroy	Mackay
Kaputar	
Lindwall	
Schooner	
Scarlet	NRB01077
Tallon	NRB01126
Tantangara	NRB01133
Valier	NRB01134
	NRB01139
NRB01244	NRB01145
NRB01245	NRB01173
NRB01246	NRB01230
NRB01251	NRB01231
NRB01298	NRB01240
NRB01004	NRB01020
NRB01002	NRB01180
NRB01333	NRB01181
NRB01345	NRB01183
NRB01346	NRB01186

Grain hardness is known to impact on malt quality[19], and at the genetic level, markers have been identified for both hot water extract and friability on chromosome 5H[8,11]. However, these were not close to the region for the *hin* genes. Further, there are a number of grain components that can influence hardness, including protein and β-glucan[15,18].

The SNPs identified from the genotypes used here had no impact of hardness, and while there was quite a range in hardness, hot water extract and friability values, and the specific haplotypes have had no impact on malt quality. Hence the variation in hot water extract and friability may be due to other factors that impact on quality such as protein or modification.

Feed quality

Quality is generally not associated with grains being fed to animals. However, recent studies have indicated that there is variation in barley that does impact feed performance[4,5]. Further, a considerable portion of malting varieties in any season, and in any country do not meet

Table II. Averaged hardness data (PSI, PS and SKCS values[1]) from 40 genotypes grown at 4 sites × 2 replications.

Genotype	PSI	PS	SKCS
BINALONG	22.7	1250	76.1
FITZROY	25.9	1235	57.0
GAIRDNER	24.1	1258	62.0
GRIMMETT	21.3	1210	71.5
GROUT	24.0	1247	69.3
KAPUTAR	27.9	1290	53.3
LINDWALL	19.8	1205	73.3
MACKAY	20.0	1228	80.1
SCARLETT	21.8	1222	67.5
SCHOONER	24.7	1167	55.5
TALLON	23.3	1219	65.8
TANTANGARA	26.5	1211	60.0
VALIER	21.9	1196	71.3
NRB01002	25.6	1222	56.8
NRB01004	20.7	1205	75.5
NRB01020	23.7	1207	69.5
NRB01077	23.5	1293	67.7
NRB01126	27.2	1312	59.6
NRB01133	26.4	1275	67.3
NRB01134	25.7	1290	61.1
NRB01139	25.6	1275	55.0
NRB01145	24.0	1252	56.4
NRB01173	23.8	1267	55.7
NRB01180	22.7	1217	73.5
NRB01181[2]		1265	71.4
NRB01183	21.8	1288	64.4
NRB01186	22.3	1245	71.0
NRB01210	20.6	1221	73.6
NRB01230	22.5	1258	64.2
NRB01231	23.4	1252	67.3
NRB01240	20.8	1220	59.0
NRB01244	24.2	1191	56.5
NRB01245	25.3	1220	51.3
NRB01246	22.3	1243	55.0
NRB01251	20.6	1277	72.2
NRB01298	20.7	1218	70.8
NRB01333	19.5	1225	70.2
NRB01345	18.4	1211	79.2
NRB01346	21.1	1236	67.6
Average	21.7	1236	65.9
Standard Deviation	2.3	32.4	7.7

[1] PSI Particle Size Index; PS Particle Size; SKCS Single Kernel Characterisation System.
[2] Grown only in 2002.

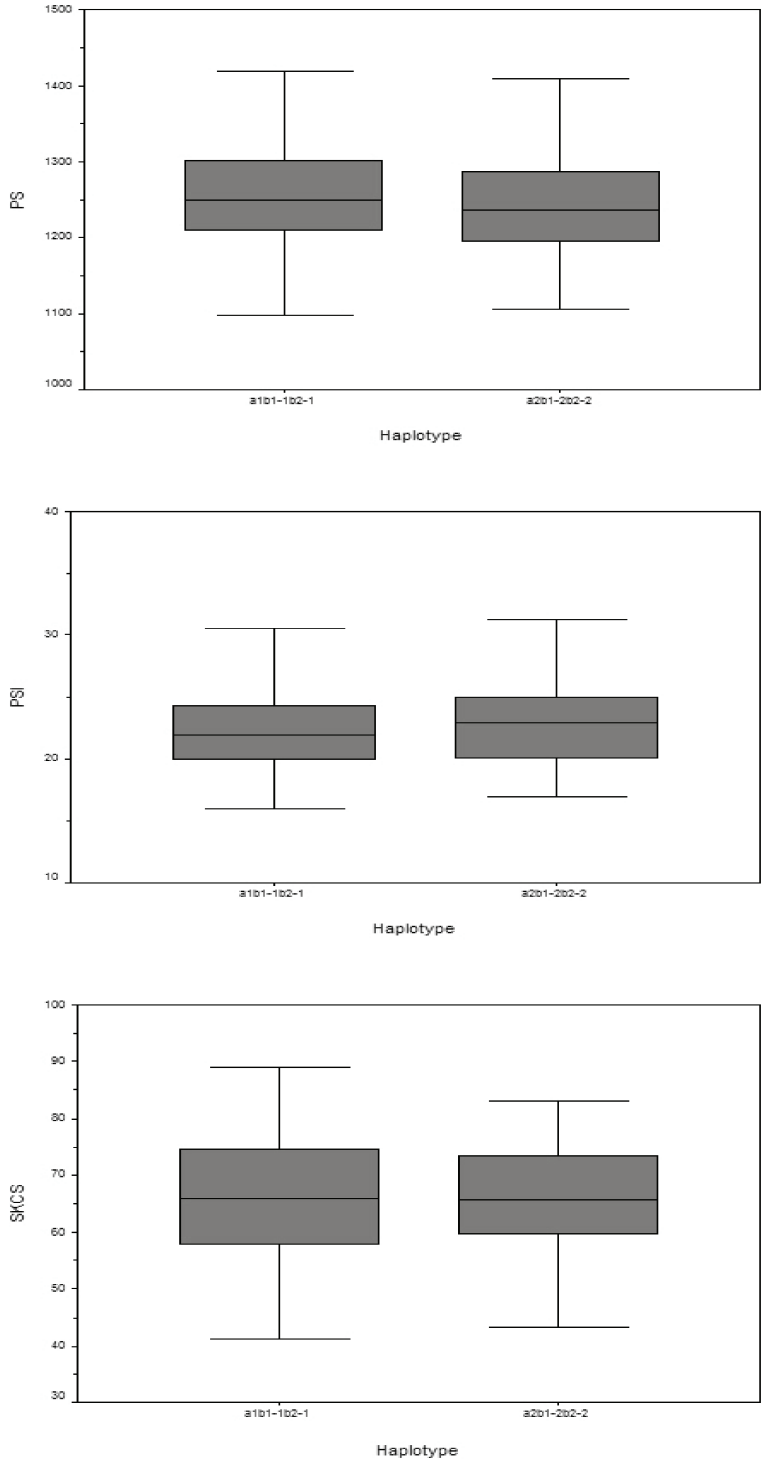

Fig. 3. Haplotypes for three barley hardness methods PS, PSI and SKCS for haplotypes a1b1-1b2-1 and a2b1-2b2-2.

malt industry delivery standards and go into a feed classification. The samples used in this study were assessed for malt and feed quality. Fig. 5 illustrates the difference between three feed traits, namely *in sacco* dry matter digestibility, net energy and average daily gain for the two haplotypes. The feed quality results were similar to the malt quality, in that there was little difference between these traits for either haplotype group.

DISCUSSION

The results from this study shows that in conserved breeding material and current Australian commercial malt and feed varieties, there is very little variation at the genetic level, ie. hardness genes. Based on the results from the three hardness methods, the barleys used in this study would generally be considered medium to soft, although

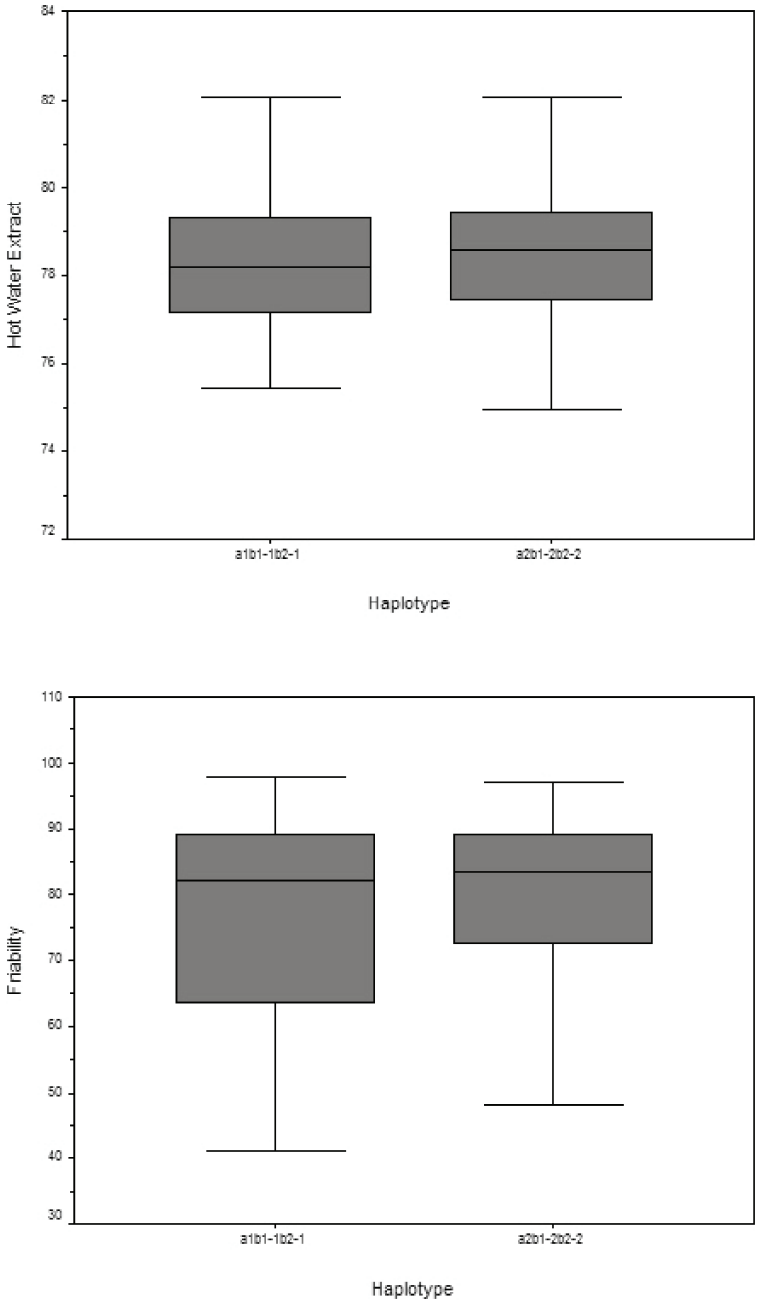

Fig. 4. Haplotypes for hot water extract and friability for a1b1-1b2-1 and a2b1-2b2-2.

some values for individuals would be classified hard. This may be for two reasons, firstly, barley is generally soft, when compared to wheat or durum and secondly, the commercial varieties (malt and feed) and breeding lines were initially selected as malt/potential malt quality. Hence, the selection was generally for softer textured endosperm.

The two haplotypes groups identified in this study are common as to those reported previously[3,10]. In addition, Caldwell et al.[7] described the three *hin* genes in DNA and amino sequences for over 70 commercial varieties in detail, but failed to identify any of the *hin b* haplotypes (unpublished http://www.ncbi.nih.gov). However, a number of SNPs on both *hin a* and *hin b* alleles have been described in wild accessions (Caldwell et al. unpublished). These wild accessions may provide more diverse protein structure and subsequently grain hardness. This notion is supported by the fact that most of the non-common hin alleles were from wild barley or landraces.

The results from this study as well as other studies highlight that while hardness does have an impact on quality, all the variation in hardness cannot be explained by the hardness genes. A number of gene regions, other than the region on chromosome 5H where the hardness genes are located, have been associated with indirect hardness measures such as friability[11] or malt tenderness[21] and β-glucan[18]. In studies where friability (malt tender-

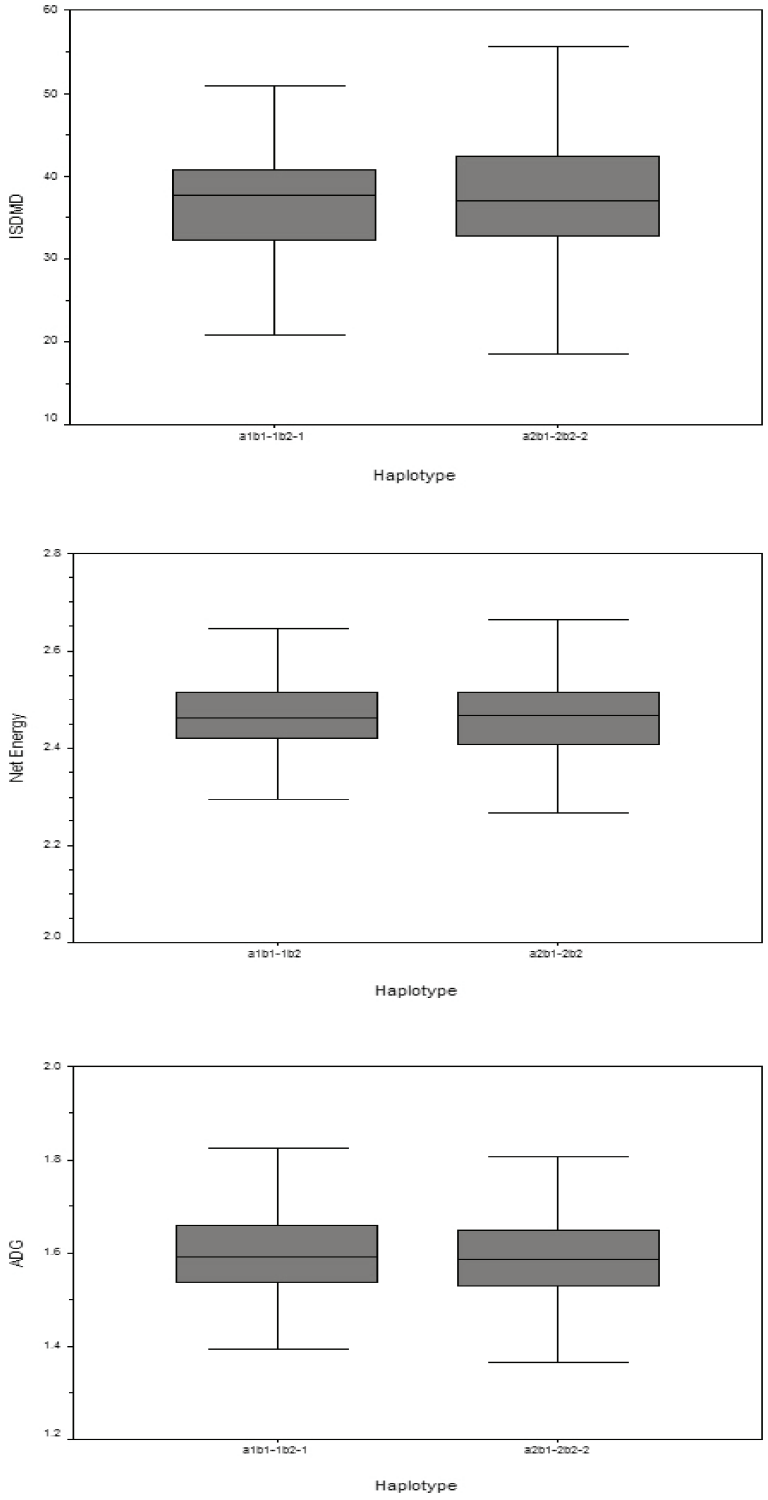

Fig. 5. Haplotypes for feed quality attributes ISDMD, Net Energy and ADG for a1b1-1b2-1 and a2b1-2b2-2.

ness) has been measured, the QTLs were not in the hardness region on chromosome 5H. In addition, other regions have been related to modification, which includes regions associated with malt β-glucan and hordein. This would suggest that while hardness has been associated with malt quality, there are a number of genetic regions that impact malt performance.

If it can be demonstrated that some SNPs in the *hin* gene sequences can result in changes in hardness then researchers may target those specific genes. The suggestion was put forward that if the wheat alleles were transformed into barley, then a greater variation in texture may be possible. This transformation may by achievable. However, it has been shown that barley transformed with a

maize storage protein resulted in little change to grain hardness and there was no malt or feed quality performed to ascertain any change in texture equated to a change in quality[22]. Alternatively, wild relatives of domesticated barley may provide new sources of hardness.

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