GENETIC ROUTES TO MODULATE RATE OF DRY-MATTER DISAPPEARANCE OF BARLEY GRAIN IN THE RUMEN OF CATTLE

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Plant Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2007

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of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to to the Division of Graduate Education.

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Jeremy Burke Jewell
April 2006

ACKNOWLEDGEMENTS

Thanks and respect go to the members of my graduate committee: Dr. Tom Blake, Dr. Jack Martin, Dr. Jan Bowman, and Dr. Mike Giroux.

Gratitude to the members of the Oscar Thomas Nutrition Center: Liz, Brenda, Nicole, and Allison. Gratitude to Pat Hensleigh, Stan Bates, and MacKenzie Ellison.

Much respect for Dr. Tom Blake and Hope Talbert. They have been the two greatest influences on my intellectual development during this time.

Much love and respect for my wife Angie Matule Jewell. She has been the greatest influence on my emotional development during this time.

Much love and respect for my parents Wallace Jewell and Pearl Jewell and my brother Greg Jewell for their continued love and support.

Love and respect to Dr. Vladimir Kanazin.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF TABLES CONTINUED

LIST OF FIGURES

ABSTRACT

Recent research has identified important characteristics of barley grain as feed for cattle. Of these, low ruminal dry-matter digestibility (DMD) is of particular importance as it is highly correlated with animal performance and with animal health. This research attempts to identify genetic loci that contribute to the ruminal DMD of barley grain.

The utility of the barley *ant*18 mutation for decreasing ruminal DMD was investigated. The DMD of several barley cultivars and their *ant*18 mutations was investigated in a randomized complete block design in two environments. Genotype by environment interaction was present: in the greenhouse the DMD of *ant*18 mutants was less than that of the wildtype, and in a dryland field the reverse was true. Because of this interaction, *ant*18 is not likely to be a reliable method of modulating DMD.

With the aim of identifying markers for marker-assisted selection (MAS), a 123-member inbred population was developed from a cross of Haxby and PI 28624. PI 28624 is a low DMD accession from the USDA barley collection. The grain of this population was evaluated for DMD at the F6 generation and F5 DNA genotyped using SSR and AFLP markers, allowing genetic map construction and quantitative trait locus (QTL) analysis. Two QTL were detected on chromosome 6H and 7H explaining 19 and 17% of phenotypic variation, respectively. Due to the low estimated genome coverage of this map (50 to 65%), 86 F7 lines were genotyped using the GoldenGate SNP genotyping technology. Use of this technology allowed accurate assessment of genome coverage, which is quite complete with the exception of the extreme short arms of 2H, 5H, and 6H. 3H and 6H, though apparently quite complete, are of much shorter centiMorgan length than the consensus maps of these chromosomes. Possible causes of this phenomenon are discussed. In addition to the previously detected QTL, two new QTL for DMD were detected in this F7 sub-population, on chromosomes 1H and 7H. These QTL may be useful for MAS if they can be validated in other populations. This population will be useful for other genetic studies in barley.

CHAPTER 1

LITERATURE REVIEW

Introduction

The modern feedlot is a value-added system for converting inexpensive commodities (corn, barley, etc.) into a more expensive commodity, beef. The digestive system of the cow (*Bos taurus*) is a system capable of fermenting cellulose due to its complex organization and microbial ecology, and has evolved for this purpose. In a feedlot environment, the microbial fauna of this system are presented with a novel substrate, starch. This perturbation can lead to digestive upset and reduced animal performance. Rapidly digestible starch sources such as wheat and barley have been particularly implicated as causative factors in these disorders. This review will detail these disorders and common methods of prevention. Further, it will highlight aspects in which barley may conceivably be improved to reduce these effects and to improve animal performance.

Cattle and Feed Barley for Montana Agriculture

Agriculture continues to be the dominant industry in Montana, accounting for an average of 34% of the economic production by Montana's five major industries from 2002-2005 (USDA National Agricultural Statistics Service, 2007). Agricultural output in 2005 was 3.1 billion dollars (ibid.). Cattle operations and barley production are important components of Montana's agricultural sector, accounting for 50.3% and 4.9% of agricultural receipts from 2001-2005(ibid.). From 2003 to 2005, 47% of harvested barley was sold as malting barley (ibid.); the remainder was sold as feed barley or used on the farm where it was produced. Of beef cattle produced in Montana from 2001-2005, only 0.8% were slaughtered commercially in-state (this figure does not include beef cattle slaughtered non-commercially on farms) (ibid.). The remaining cattle were "backgrounded," i.e. weaned and shipped out of state to be fattened and slaughtered elsewhere. This beef production system where calves are weaned and sent directly to a feedlot is termed an intensive system, and is favored by many large producers due to its greater feed efficiency and corresponding economic advantage (Lewis et al., 1990a, b). The export of these cattle represents a sizable loss of revenue in the state

of Montana due to the high cost of transport, and mortality and morbidity of transported cattle (Dr. Tom Blake, pers. comm.).

Starch Digestion by Ruminants

In cattle, the rumen is the primary site of starch digestion; 50 to 90 percent of fed dietary starch is digested ruminally, depending on grain source and grain processing (Huntington, 1997). For barley, this value is approximately 80 % based on a review of 5 publications (Huntington, 1997). Starch digested ruminally is fermented by microbes to volatile fatty acids (VFAs), methane, and carbon dioxide (Russell and Rychlik, 2001). The VFAs thus produced are absorbed ruminally and used as an energy source for the animal (Van Soest, 1994).

Starch not digested in the rumen may be digested post-ruminally in the small intestine or the large intestine, or escape digestion by elimination. The fraction of fed starch digested post-ruminally is between 5 and 20%, depending on source and processing of grain; for barley this value is approximately 13% (Huntington, 1997). The extent of digestion of starch digested post-ruminally varies between 46 and 93%, again depending on source and

processing of grain; for barley this value is approximately 75% (ibid.). Starch digestion in the small intestine is accomplished by endogenous α-amylase, and the glucose produced by this digestion is absorbed into the epithelium of the small intestine primarily by Na^+ co-transport (Wright, 1993). Ruminal fermentative starch digestion is only 70 to 75 percent as energetically efficient as enzymatic starch digestion (Owens *et al*, 1986; Harmon and McLeod, 2001). Energy lost by fermentative digestion is due to methane and heat production (Black, 1971). Despite the increased energetic efficiency of small intestine enzymatic digestion, there may be limits to the amount of starch that can be digested in this manner (Kreikemeier K.K. *et al*, 1991; Owens *et al*, 1986).

Fermentative starch digestion in the large intestine is accompanied by the same energetic losses as ruminal digestion. Also, though VFAs may be absorbed in the large intestine, excessive passage and large-intestinal fermentation of starch produces high levels of organic acids (VFAs) that the large intestine is not able to completely absorb. This hindgut acidosis has been shown to be positively correlated with residual feed intake. Channon *et al* (2004) reported that low fecal pH was associated with lower animal efficiency.

Diets high in irregularly fed rapidly fermentable carbohydrates i.e. starch or sugars may result in a situation where the rate of accumulation of VFAs is greater than the rate of absorption of VFAs. As a result, the pH of the rumen will decrease, a condition called acidosis. Ruminal acidosis results in inflammation of the rumen lining, accumulation of lactate producing bacteria which further reduce ruminal pH, reduction in numbers of protozoa, and reduced efficiency of cellulose fermentation (Owens *et al*, 1998; Goad *et al*, 1998). Further, lactate accumulation promotes the accumulation of *Fusobacterium necrophorum*, a toxin producing bacterium that, if it escapes the rumen, may colonize the liver and form abscesses (Nagaraja and Chengappa, 1998). Prolonged periods of ruminal acidosis may lead to metabolic acidosis; a condition in which the pH of the blood itself is reduced below normal, reducing the blood's ability to carry oxygen and even resulting in death (Owens *et al*, 1998). Also, metabolic and ruminal acidosis are theorized to result in or exacerbate laminitis, a painful and economically important condition of the bovine foot (Nocek, 1997).

A further complication of too-rapid ruminal grain digestion is a condition called grain bloat. In grain bloat, there is excessive production of bacterial polysaccharides which trap fermentation gases

(Cheng *et al*, 1976). These trapped fermentation gases increase the intra-ruminal pressure, even to the point that the expanding rumen will compress the lungs, killing the animal.

Methods to Modulate Rates of Digestion

Because of the general acceptance that too rapid ruminal digestion of starch adversely affects animal health and performance (Huntington, 1997; Owens *et al*, 1998; Nocek, 1997; Channon and Rowe, 2004; Cheng *et al*, 1998), numerous methods have been evaluated to modulate starch rate of digestion and/or alleviate its adverse effects. These methods include varying the extent of grain processing, the addition of ionophores and/or antibiotics, or buffers to the diet, and treatment of the grain with formaldehyde, sodium hydroxide, or polyphenolics. A final method relies on differences between grain varieties.

Lykos and Varga (1995) observed that rumen starch digestibility decreased with increasing grain particle size. Similarly, in an experiment using corn processed by three different methods, Rémond *et al* (2004) observed, both in situ and in vivo, that increased particle size decreased ruminal starch digestibility. Reduced ruminal

digestibility led to reduced small intestinal digestibility, although the amount of starch digested in the small intestine increased. As might be expected, they also noted a decrease in total tract digestibility. Zinn (1993) observed an increase in the ruminal digestibility of barley when it was steam-flaked rather than dry-rolled. In an experiment attempting to determine the optimal extent of processing of barley grain, Beauchemin *et al* (2001) observed that processing barley to a smaller particle size resulted in a small increase in total tract starch digestibility, which they warned could lead to problems associated with acidosis. Although less processed grain had a slower rate of digestion, total ruminal digestibility was not significantly affected. They noted that ruminal pH tended to decrease with increased grain processing; however, they also noted that fecal pH *increased* with increased grain processing, probably as a result of decreased starch flow to the large intestine.

Ionophores are molecules that disrupt the membranes of certain microbes and have sometimes been used as antibiotics. Ionophores are generally agreed to have positive effects on the performance of animals in the feedlot and are widely used as feed additives (McGuffey *et al*, 2001). In a diet of 75% corn supplemented with the ionophore monensin, Zinn and Borques (1993) observed reduced ruminal

digestion of organic matter and an increased fraction of organic matter digested post-ruminally. Also, they noted decreased energetic loss due to decreased methane production, and increased metabolizable energy of the diet. Nagaraja *et al* (1981) noted that the ionophores monensin and lasalocid were effective in reducing lactic acid accumulation in steers on a high starch diet. Later (Nagaraja *et al*, 1987), they showed that a range of other ionophores had the same effect and that ionophores reduced the population of lactic acid producing bacteria such as *Streptococcus bovis* and *Lactobacillus* species.

The antibiotic virginiamycin is a fermentation product of *Streptomyces virginiae* and is widely used as a poultry, swine, and cattle feed additive. In 7 experiments over 4 years, Rogers *et al* (1995) showed that virginiamycin decreased incidence of liver abscess in feedlot steers on high energy diets from 30% to less than 20%. They also observed increased feed efficiency and average daily gain. Godfrey *et al* (1992) noted that virginiamycin was effective in stabilizing the large intestinal pH and lactate levels of sheep fed high barley diets. Later, in an experiment with sheep fed wheat without an adjustment period, they observed increased ruminal pH and lower ruminal L-lactate when virginiamycin was fed (Godfrey *et al*, 1995).

Although it makes intuitive sense that the feeding of buffers to acidotic animals would stabilize the ruminal pH, in many cases it has had no effect on ruminal pH. Zinn and Borques (1993) were unable to detect any effect of buffers on rumen pH, site or extent of starch digestion, or performance of animals on high grain diets. Similarly, Xu *et al* (1994) found no effect of buffers on ruminal pH. In seeming contrast, Phy and Provenza (1998) found that sheep fed high-wheat diets drank more water buffered with sodium bicarbonate than un-buffered water. Also, they noted decreased acidosis in animals that drank buffered water rather than salt water.

Formaldehyde treatment has been shown to decrease ruminal starch digestion. Michalet-Doreau *et al* (1997) showed that cereal grains treated with formaldehyde were less ruminally digestible than untreated cereal grains and that the effect was dose-dependent, i.e. treatment with 5% formaldehyde reduced digestibility more than treatment with 1% formaldehyde. Van Ramshorst and Thomas (1988) found that formaldehyde treatment increased nitrogen and starch absorption in the intestines relative to ruminal absorption. After formaldehyde treatment, starch passed from the rumen to the small intestine increased from 3.8 to 7.4 % of starch ingested. They found no effects, however, on rumen pH. Ortega-Cerilla *et al* (1999), also,

found that formaldehyde treatment reduced rumen digestibility of starch. Schmidt *et al* (2006) found that either sodium hydroxide treatment or formaldehyde treatment significantly increased the amount of starch reaching the small intestine.

Mahmoudzadeh *et al* (1989) infused starch post-ruminally into lambs and administered gel capsules containing the phenolic monomers *p*-coumaric or ferulic acids. No effect was observed on starch digestibility. Martínez *et al* (2005) treated barley grain with 4 concentrations of the polyphenolic compound tannic acid and found that at the highest level of treatment, the ruminal digestibility of dry-matter was reduced. Measurements of starch digestibility were not reported.

Manipulation of Grain for Improved Animal Performance

By feeding 200 steer calves 10 different barley cultivars, Ramsey *et al* (2002) tested the hypothesis that barley ruminal digestibility affects the health and performance of feedlot steers. The percentage of animals that bloated at least once was positively correlated with rate of dry-matter and starch digestion. Incidence of liver abscess, too, was positively correlated with dry-matter digestibility. In other words, increased rate of digestion was associated with increased bloat

and abscess. Surber *et al* (2000) reported that ruminal dry-matter digestibility was negatively correlated with average daily gain and feed efficiency. In addition, they reported that barley with a slower rate of digestion had a higher energy value. Particle size was negatively correlated with both in situ and in vivo digestibility. In contrast to these results, Boss and Bowman (1996a, b) found that the barley cultivar showing the greatest weight gain, Harrington, was also the cultivar of most rapid ruminal digestion. However, the grain producing greatest average daily gain, corn, was the grain with the slowest rate of digestion. Also, Harrington did reduce ruminal pH more significantly than corn (Boss and Bowman, 1996b).

Because proanthocyanidins (or "tannins") have been shown to reduce the digestibility of forages, Wang *et al* (1999) explored whether naturally occurring barley proanthocyanidins could also affect digestibility. Their approach was to evaluate the digestibility of Harrington and three proanthocyanidin-free mutant lines. They found no significant differences in digestibility between the four lines.

Puroindolines are the primary proteins in determining wheat grain hardness (Giroux and Morris, 1998). Hordoindolines are the orthologous genes in barley (Darlington *et al*, 2001). Beecher *et al* (2002) showed that in the cross of the barley cultivars Steptoe and

Morex, lines differing in the chromosomal region containing the hordoindolines also differed in extent of ruminal dry-matter digestion after 3 hours. It has also been shown that wheat puroindolines can effect ruminal digestion of wheat dry-matter and starch (Swan *et al*, 2006).

Based on the results of 18 feedlot trials over 7 years, Surber *et al* (2000) used results of laboratory analyses of barley varieties in an attempt to predict animal performance. They found that barley grain particle size was negatively correlated with average daily gain ($r=-0.36$, $P=0.007$), feed efficiency ($r=-0.37$, $P=0.007$), energy for maintenance ($r=-0.59$, $P<0.001$), and energy for gain ($r=-0.60$, $P<0.001$). These results indicate that not only is barley ruminal digestion rate an important factor for ruminal and over-all animal health, but that it is also an important factor for animal productivity.

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CHAPTER 2
EVALUATION OF THE *ANT18* MUTATION AS A POTENTIAL TOOL TO MODULATE RATE OF BARLEY RUMINAL DRY-MATTER DISAPPEARANCE

Abstract

Rate of ruminal dry-matter digestion (DMD) is an important parameter of cereal grain as feed for ruminants. Ruminal digestion is primarily the result of microbial attack. The flavonoid dihydroquercetin, and flavonoids in general, are antimicrobial agents. Dihydroquercetin accumulates in the testa layer of mutant *ant*18 barley grain. Therefore, experiments were conducted to test whether the *ant*18 mutation could be used to modulate rate of ruminal digestion. The barley cultivar Klages and its *ant*18 mutant were grown in a dryland field. The barley cultivars Advance and Cougbar and their *ant*18 mutants were grown in a greenhouse and a dryland field. The *in situ* DMD of Klages and its mutant were evaluated in a time course experiment, and Klages was found to have a higher rate of digestion than its *ant*18 mutant. This result was confounded by the smaller average particle size and higher starch content of Klages relative to the mutant. To negate the effects of differing particle size, Advance, Cougbar, their mutants were cracked to a finer particle size and

evaluated for *in situ* DMD after 3 hours in rumen. Genotype by environment interaction was important in this second experiment, with the *ant*18 mutation reducing DMD in greenhouse grown grain but increasing DMD in dryland field grown grain. Because of the inconsistent effects of the *ant*18 mutation on barley grain DMD across environments, and because of it may reduce grain starch content, *ant*18 mutants are unlikely to be useful as feed for ruminants.

Introduction

Barley and cattle are important segments of Montana's agriculture, accounting for an average of 4.9 and 50.3 percent, respectively, of agricultural receipts between 2001 and 2005 (USDA National Agricultural Statistics Service). Approximately 850,000 metric tons, or 13.6%, of Montana barley grain sold in market year 2005 was sold as feed barley (USDA National Agricultural Statistics Service), with the remainder sold as malting barley.

Rate of ruminal digestion is an important parameter of cereal grain as feed for ruminants. It has been reported that the extent of barley dry-matter digestibility (DMD) after 3 hours in the rumen was negatively correlated with the metabolizable energy content of barley

grain as a feed for feedlot steers, and that DMD was negatively correlated with animal performance as measured by average daily gain and feed efficiency (Surber *et al*, 2000). Similarly, it was later reported that average daily gain was positively correlated with the proportion of rolled barley dry-matter that slowly disappeared in the rumen (Ramsey *et al*, 2002). Further, the proportion of slowly disappearing dry-matter was negatively correlated with incidence of bloat and liver abscess (ibid.) and it is generally accepted that high rate of ruminal digestion of cereal starch is a contributing factor to bloat, acidosis, and liver abscess (for reviews see: Cheng *et al*, 1998; Owens *et al*, 1998; Nagaraja and Chengappa, 1998).

Along with widely-used feed additives such as ionophores that ameliorate the negative effects of rapid ruminal digestion (Nagaraja *et al*, 1987; Rogers *et al*, 1995), several feed treatments have been reported to decrease the rate of ruminal digestion of cereal grain dry-matter. These treatments include less intensive processing to increase grain particle size (Beauchemin *et al*, 2001), treatment of grain with formaldehyde or sodium hydroxide (Michalet-Doreau *et al*, 1997; Schmidt *et al*, 2006), and treatment with tannic acid (Martínez *et al*, 2005).

Genetic effects have also been explored as a route to altering the rate of digestion of cereals. In general, six-row barleys digest less rapidly in the rumen than two-row barleys (Bowman *et al*, 2001). In addition to controlling wheat grain hardness, functional puroindolines are able to decrease rate of wheat dry-matter and starch digestion (Swan *et al*, 2006). Further, the genetic locus encoding barley's orthologous proteins has been associated with changes in ruminal dry-matter digestibility (Beecher *et al*, 2002). In a digestibility comparison of Harrington and three proanthocyanidin-free mutants, Wang *et al* (1999) reported that the endogenous proanthocyanidins of the barley testa layer do not affect DMD.

Certain flavonoids possess anti-microbial activity (reviewed by Cushnie and Lamb, 2005). The flavonoid dihydroquercetin accumulates in small amounts in the grain testa layer of mutant anthocyanin-free *ant*18 barley plants, and dihydroquercetin has been shown to be an inhibitor of the fungi *Fusarium poae, F. culmorum, and F. graminearum* (Skadhauge *et al*, 1997). Because ruminal digestion is primarily microbial (Russell and Rychlik, 2001), and because of the anti-microbial effects of flavonoids and dihydroquercetin in particular, it is reasonable to hypothesize that accumulation of dihydroquercetin in *ant*18 barley grain will result in a slower rate of ruminal digestion.

This paper reports the results of experiments intended to test this hypothesis.

Materials and Methods

Plant Material

Seed of the cultivars Advance (CIho 15804) and Cougbar (PI 496400) were obtained from the USDA National Small Grains Collection (http://www.ars-grin.gov/npgs/). Seed of Klages was available here in the stores of the Montana State barley breeding program. Seed of anthocyaninless-18 mutants was kindly provided by Dr. Diter von Wettstein. These anthocyaninless-18 (*ant*18) mutants were *ant*18.623 in the Klages background, *ant*18.660 in the Cougbar background, and *ant*18.592 and *ant*18.621 in the Advance background.

Cougbar, Advance, and their mutants were planted both in the greenhouse and in the field. In the greenhouse, they were planted in a randomized complete block design with two replications. They were planted in ten-inch pots with five plants per pot. In the field they were hand-planted in three meter dry-land rows in a randomized complete block design, twenty seeds of each line in each of three replications.

Klages and its mutant were planted in the field in a randomized complete block design. After harvest, we realized that enough seed for a time-course experiment could be obtained only by bulking the seed. Therefore, the time-course experiment was performed using these bulked grains.

Particle size, starch content, and DMD Analysis

After seed harvest and cleaning, seed of each line was cracked using a Buehler disc mill (Buehler-Miag, Braunschweig, Germany) with disc spacing set to 1.5 mm or 1.25 mm, depending on the experiment. Fifty grams or ten grams, depending on the amount available, of each cracked sample was placed on the top sieve of a stack of 5 International Standards Organization sieves. Sieves used were 2,360, 1,700, 850, 425, and 90 µm in screen opening diameter. The sieve stack was shaken for five min using a RoTap shaker (Tyler Co., Mentor, OH). Geometric mean particle size (d_{gw}) of each line was calculated on a weight basis of the geometric mean of the diameter openings in 2 adjacent sieves in a stack using the equation (Pfost and Headley, 1976) (d_{gw}) = $\log^{-1}$ [$\sum$ (W_i log d_i)/$\sum$ W_i] in which W_i = weight of material in sieve i and d_i = diameter of the sieve i. Dry-matter content of each line was determined using AOAC method 930.15 (2000) for

oven drying and was replicated twice. Before measuring starch content, grain samples were ground through a 0.5 mm screen using an UDI Cyclone Sample Mill (UDI Corporation, Boulder, Colorado, USA). Starch content was determined using the Megazyme starch assay kit (Megazyme International, Brey, Ireland) and was replicated twice.

The cows used for the DMD analysis were maintained on a diet of low quality grass hay consumed *ad libitum* and 3.6 kg per animal per day dry-rolled barley. The cows had access to fresh water at all times and were maintained on the diet for 14 days before conducting the DMD analysis. DMD was determined as in Vanzant *et al* (1998). Five g of each cracked grain sample was weighed into each of four 10- by 20-cm, 50 µm pore size polyester bags (Ankom Technology, Fairport, NY) and sealed with a Clamco impulse sealer (Clamco Corp., Cleveland, OH). For the time-course experiment, for each time point two replications of each sample were placed into the rumen of each of two ruminally cannulated cows. Bags were placed in the rumen at 12.0, 9.0, 6.0, 3.0, 2.0, 1.5, 1.0, and 0.5 hours before the time of removal and removed at the same time as recommended in Vanzant *et* al (1998). For the similar particle size experiment, two replications of each sample were placed in the rumen of each of two ruminally cannulated cows at the same time, and removed at the same time

after three hours. Also included in each incubation were two empty bags to correct for DM content from microbial contamination and two bags of the variety 'Harrington.' After removal from the rumen, the bags were rinsed under cold water until the rinse water ran clear. The bags were dried at 60° for 48 h and then weighed. Percent DMD was calculated as (sample weight in * mean DM content) – (dry weight out – bag weight)/(sample weight in * mean DM content) * 100.

Statistical Analysis

The time-course experiment was analyzed using PROC MIXED of SAS (SAS Institute, Incorporated, Cary, North Carolina, USA) as outlined in Littell *et al*, (1998). Values of DMD of replications of a sample within a cow at a time were averaged, and cows were treated as random blocks. Covariance was modeled with an autoregressive structure.

Data from the similar particle size experiment were analyzed as a split-plot design using PROC GLM of SAS (SAS Institute, Incorporated, Cary, North Carolina, USA). Cows and blocks within environment were treated as random. *Ant*18 status (mutant versus wildtype), cow, environment, sample, block within environment, and genotype by environment interaction were included in the model. One

row of Advance in the field produced no seed because of water stress, and one row of *ant*18.592 was misplaced.

Results and Discussion

Time course experiment

As an initial test of the effects of the *ant*18 mutation on DMD, grain from Klages and grain from *ant*18.623(Klages) were evaluated in a DMD time-course experiment. Grain from each line was cracked in a Buehler disc mill with the disc spacing set to 1.5 mm. After particle-sizing, the samples were evaluated for DMD at various times of incubation in the rumen. Klages had a mean particle size of 1355 μm, while *ant*18.623(Klages) had a mean particle size of 1603 μm.

Dry-matter disappearance of Klages and *ant*18.623(Klages) increased as time increased (Figure 2-1). Differences between the two lines were significant at each time point, i.e. line x time interactions were not significant (P>0.65). Averaged over time points, DMD of *ant*18.623(Klages) was 7.9 percentage units less than DMD of Klages (P=0.03).

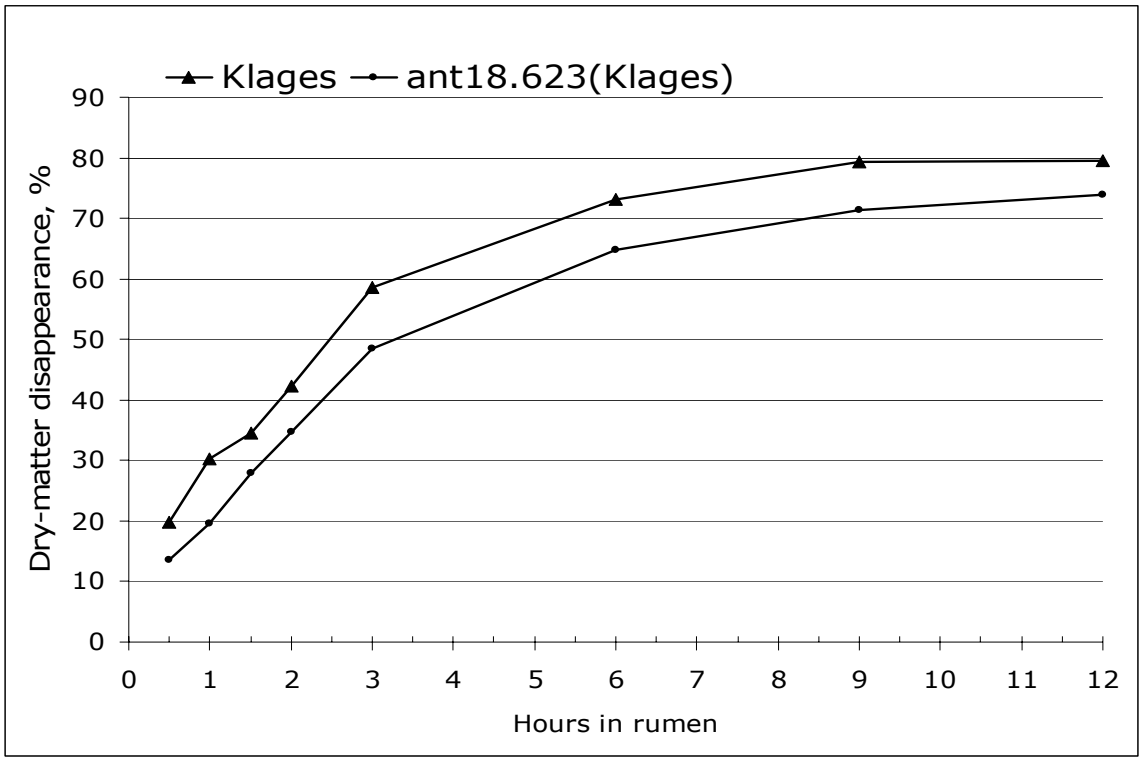

Figure 2-1. In situ dry-matter disappearance of Klages and *ant*18.623(Klages) at 0.5, 1.0, 1.5, 2.0, 3.0, 6.0, 9.0, and 12.0 hours.

The starch content of Klages was greater than the starch content of its *ant*18 mutant (47% and 41%, respectively). It has repeatedly been emphasized that grain digestibility cannot be evaluated without taking into account the effects of grain-processing on digestibility, and the mean particle size of *ant*18.623(Klages) was greater than the mean particle size of Klages. Because of the differences in starch content and particle size, further experimentation was conducted to reduce any possible effects of particle size on DMD. We also wanted to determine if the *ant*18 mutation reduced starch content in other *ant*18 mutants.

Similar particle size experiment

All ground grain of Klages and its *ant*18 mutant had been expended in the time course experiment, so grain of two other cultivars and their *ant*18 mutants was used instead: Cougbar, *ant*18.660(Cougbar), Advance, *ant*18.592(Advance), and *ant*18.621(Advance). All five of these lines were grown in two replications in the greenhouse, and three replications in the field. Individual samples were cracked with a disc mill spacing of 1.25 millimeters and particle sized. The decreased disc spacing was an attempt to minimize any differences in particle size between cultivars and their *ant*18 mutants. This attempt was successful. After this grain processing, *ant*18 effects were insignificant (Table 2-1).

Table 2-1. Estimated differences and standard error of difference in particle size of wildtype and *ant*18 lines* after cracking with disc mill spacing of 1.25 mm

Difference	Environment	Estimate (μm)	Standard Error	P Value
wt-*ant*18	Field	62	47	0.205
wt-*ant*18	Greenhouse	66	52	0.222
wt-*ant*18	Both	64	35	0.089

*wt-wildtype lines, *ant*18-*ant*18 lines

Factorial analysis of variance of the DMD data revealed significant interaction between *ant*18 effects and environmental effects

(Table 2-2). This result is due to differing significance of *ant*18 between environments (Figure 2-2). In the greenhouse, *ant*18 had significantly reduced DMD (P=0.02), while in the field *ant*18 lines had significantly higher DMD (P=0.02).

Table 2-2. Sources of variation in DMD between Klages and ant18 lines after cracking with disc mill spacing of 1.25 mm

Source	Sum of Squares	F Value	P Value
Cow	3595.8	149.07	<0.001
*ant*18[a]	0.3	0.01	0.915
Background	306.2	12.70	0.001
Block(Env.)	137.3	1.90	0.147
Environment	729.4	30.24	<0.001
*ant*18 X Env.	294.5	12.21	0.001

[a]wildtype versus *ant*18 averaged over genetic background

A possible reason for the varying digestibility between environments is revealed by Figures 2-3. Starch is a highly digestible component of cereal grains (Huntington, 1997). In the greenhouse, wildtype lines tended toward more starch than their mutants. In the field the reverse was true. Starch was measured on bulk ground grain of lines (for example, bulked Advance of blocks 1,2, and 3 in the field), rather than on individual plantings. It could be that measuring starch on each individual planting could give enough statistical power to declare significant differences in starch content

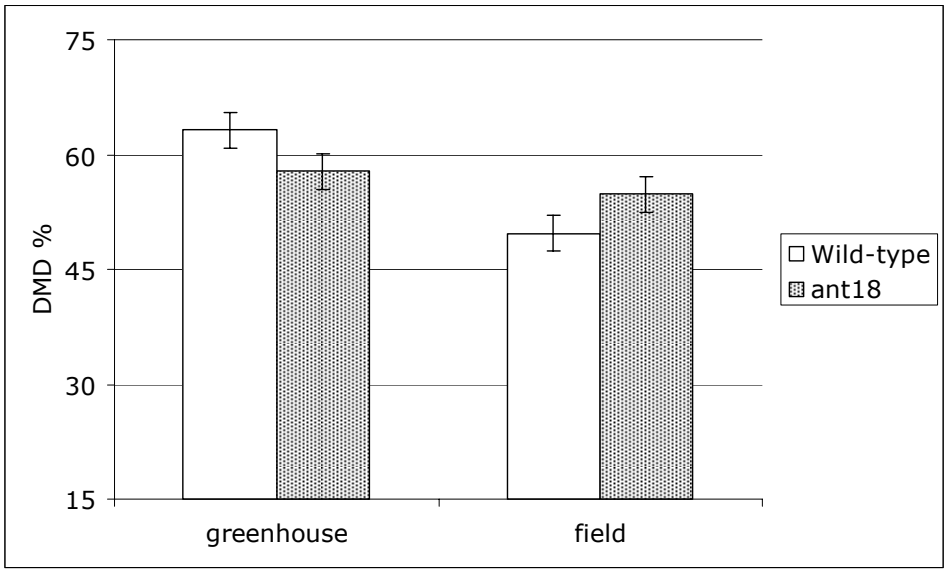

Figure 2-2. DMD of wildtype lines and their ant18 mutants. Error bars indicate standard error of 2.3.

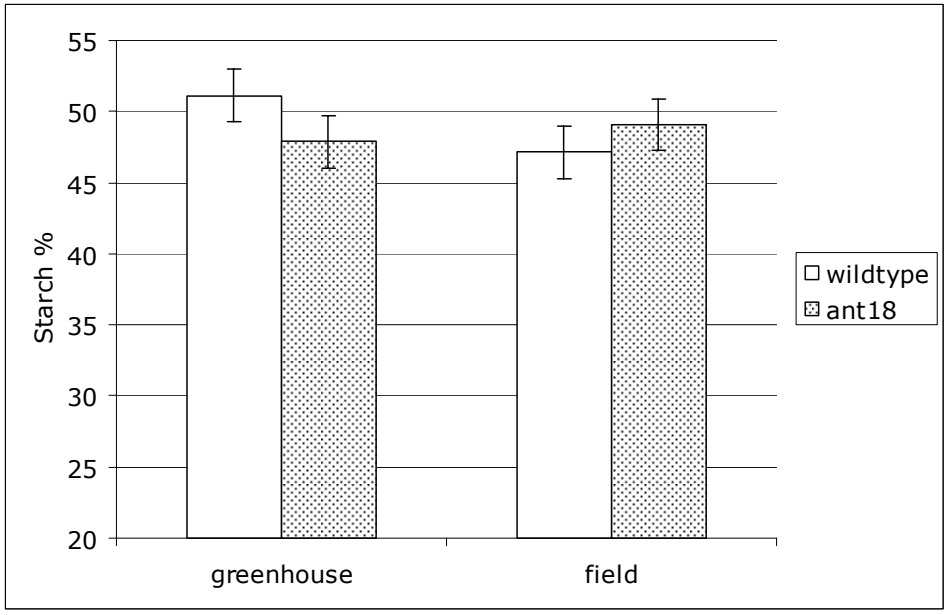

Figure 2-3. Starch content of wildtype lines and their *ant*18 mutants. Error bars indicate standard error of 1.8.

Starch content of the field-grown *ant*18 line was decreased in the time course experiment, while in this experiment the starch

content tends toward an increase in the field-grown *ant*18 lines. If repeated starch measurements do indicate that the starch content of the *ant*18 mutants are increased relative to the wildtype lines, the question is "Why?" Advance, Cougbar, and their mutants were planted one-week later than Klages and its mutant. Also, they were planted by hand at a much shallower depth than Klages and its mutant. It seems clear that environment does impact the effects of *ant*18 on starch content.

Conclusions

The time-course experiment shows that the *ant*18 mutation can reduce dry-matter digestibility, though this experiment can not differentiate whether the mode of action is via reduced particle size, reduced starch content, or by the hypothesized accumulation of dihydroquercetin. Further, as the starch content of the *ant*18 line was reduced relative to Klages, the *ant*18 mutation in the Klages background is likely to be detrimental to animal performance, rather than beneficial.

The experiment using Advance, Cougbar, and three *ant*18 mutations of these lines showed a significant genotype by environment

interaction, suggesting that this gene may be an unreliable way to modulate barley grain DMD.

Acknowledgments

I thank Jack Martin for his advice on statistical analyses. I am thankful to Dr. Diter von Wettstein for providing the seed of the *ant*18 mutants. Thanks go to MacKenzie Ellison for starch assays.

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CHAPTER 3

MAPPING OF QUANTITATIVE TRAIT LOCI FOR FEED-QUALITY RELATED TRAITS IN A TWO-ROWED INBRED BARLEY POPULATION

Abstract

Barley and cattle are important components of Montana's economy. Recent research has identified important characteristics of barley grain as feed for cattle; these characteristics include high starch content, large particle size after dry-rolling, and low extent of dry-matter digestibility (DMD) after 3 hours incubation in the rumen. Evaluation of particle size and DMD is time-consuming and relatively expensive, therefore it would be desirable to identify molecular markers useful for marker-assisted selection. With this aim, grain from 123 recombinant inbred lines (RILs) derived from a cross of the cultivars Haxby and Baku were evaluated for DMD, starch, and particle size and genotyped with 218 AFLP, SSR, and STS markers. By composite interval quantitative trait locus (QTL) analysis, two loci were detected on barley chromosomes 6H and 7H that together explain 43% of phenotypic variation for DMD. An 86-member F7 subset of the original population was also genotyped using the Illumina GoldenGate

SNP genotyping assay. The loci on 6H and 7H were again detected. The effects of the *Nud* locus were also detected as was a new, but minor QTL for DMD on 1H. One marker with significant effects on DMD and particle size was genotyped in a RIL population derived from a cross of the cultivars Drummond and Baku. The locus was not found to be significant in this population, though this result may be due to masking by the *Vrs1* locus. It was concluded that the very low DMD of Baku is due to many genes of minor effect.

Introduction

Barley and cattle are important segments of Montana's agriculture, accounting for an average of 4.9 and 50.3 percent, respectively, of agricultural receipts between 2001 and 2005 (USDA National Agricultural Statistics Service). Approximately 850,000 metric tons, or 13.6%, of Montana barley grain sold in market year 2005 was sold as feed barley (USDA National Agricultural Statistics Service), with the remainder being sold as malting barley.

Despite the importance of barley grain as feed for ruminants, relatively little work has been done to determine characteristics of a quality feed barley. However, in an experiment utilizing the results of

18 feedlot experiments over the course of 7 years, Surber *et al* (2000) reported that high starch content and low acid-detergent fiber (ADF) content are correlated with feedlot steer performance. Also, it was noted that the extent of barley dry-matter digestibility (DMD) after 3 hours in the rumen was negatively correlated with the metabolizable energy content of barley grain as a feed for feedlot steers and that DMD was negatively correlated with animal performance as measured by average daily gain and feed efficiency (Surber *et al*, 2000). Similarly, it was later reported that average daily gain was positively correlated with the proportion of rolled barley dry-matter that slowly disappeared in the rumen (Ramsey *et al*, 2002). Further, the proportion of rolled barley dry-matter that slowly disappeared in the rumen was negatively correlated with incidence of bloat and liver abscess (ibid.). Grain particle size after cracking or dry-rolling is negatively correlated with ruminal DMD (Lykos and Varga, 1995; Surber *et al*, 2000; Rémond *et al*, 2004), therefore particle size may be a useful indicator of the DMD of a potential barley line.

As the assays (in situ or in vitro) for DMD are relatively time consuming and expensive, it would be preferable to use molecular markers for early selection of lines in barley breeding programs. Previously, markers have been identified associated with variation in

DMD on barley chromosomes 1H, 3H, and 4H in the Steptoe/Morex population (Bowman *et al*, 1996), 1H and 3H in the Lewis/Baronesse population (Abdel-Haleem, 2004), and 2H in the Valier/PI 370970 population (*ibid*.).

The objective of this research is to identify molecular markers linked to loci that decrease extent of barley DMD after three hours in the rumen or that increase cracked grain mean particle size, with the ultimate aim of deploying these markers for marker-assisted selection.

Materials and Methods

Plant Material

A 123-member F_5 derived recombinant inbred line (RIL) population was developed by single seed descent from a cross between the 2-rowed feed cultivar 'Haxby' (PI 646160) and the 2-rowed USDA barley collection accession 'Baku' (PI 28624). Seed from greenhouse grown F_5 plants was bulked and planted at the Arthur H. Post Research Farm near Bozeman, MT in the spring of 2006. These F_5 derived F6 seed were planted in rain-fed un-replicated 3 meter rows, with a check variety planted as every twelfth row. Another 96-member F_5 derived F6 RIL population was developed by the same

means from the same parents to be used as a validation population. An additional 94-member F_5 derived validation population was derived by the same means from a simple cross of 'Baku' and the 6-rowed variety 'Drummond'. The two validation populations were also planted in the spring of 2006 at the Arthur Post Research Farm in rainfed un-replicated 3 meter rows with a check variety every twelfth row. Harvested seed was cleaned and de-awned.

Phenotypic Data

Flowering date for each line was defined as the Julian day when 50% of heads had emerged from the sheath. Plant height was measured on three individuals of each line at physiological maturity and averaged. The weight of 500 kernels of each RIL were counted in triplicate using an electronic seed counter (The Old Mill Company, Savage, MD) and weighed. 500 kernel weight rather than the more frequently reported 1000 kernel weight was reported because several entries had fewer than 1000 kernels, but all entries had at least 500 kernels. Seed of each line was cracked using a Buehler disc mill (Buehler-Miag, Braunschweig, Germany) with disc spacing set to 0.05 inches. The geometric mean particle size of the cracked seed was determined in duplicate as in Swan *et al* (2006). Fifty grams of each

cracked sample was placed on the top sieve of a stack of 5 International Standards Organization sieves. Sieves used were 2,360, 1,700, 850, 425, and 90 µm in screen opening diameter. The sieve stack was shaken for 5 min using a RoTap shaker (Tyler Co., Mentor, OH). Geometric mean particle size (d_{gw}) of each line was calculated on a weight basis of the geometric mean of the diameter openings in 2 adjacent sieves in a stack using the equation (Pfost and Headley, 1976) (d_{gw}) = $\log^{-1}$ [$\sum$ (W_i log d_i)/$\sum$ W_i] in which W_i = weight of material in sieve i and d_i = diameter of the sieve i. Dry matter (DM) content of thirty random lines was determined using AOAC method 930.15 (2000) for oven drying and replicated twice. The mean DM content of the thirty lines was considered to be representative of the DM content for all lines.

The cows used for the DMD analysis were maintained on a diet of low quality grass hay consumed *ad libitum* and 3.6 kg per animal per day dry-rolled barley. The cows had access to fresh water at all times and were maintained on the diet for 14 days before conducting the DMD analysis. DMD was determined as in Vanzant *et al* (1998). Five g of each cracked grain sample was weighed into each of four 10- by 20-cm, 50 µm pore size polyester bags (Ankom Technology, Fairport, NY) and sealed with a Clamco impulse sealer (Clamco Corp.,

Cleveland, OH). Twenty eight polyester bags (representing 14 RILs) were placed in the rumen of each of two ruminally cannulated cows at the same time. Also included in each incubation were two empty bags to correct for DM content from microbial contamination and two bags of the variety 'Harrington.' After removal from the rumen, the bags were rinsed under cold water until the rinse water ran clear. The bags were dried at 60° for 48 h and then weighed. Percent DMD was calculated as (sample weight in * mean DM content) – (dry weight out – bag weight)/(sample weight in * mean DM content) * 100.

Anchor Markers

DNA was extracted from individual two-week-old F_5 plants using the DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). PCR was performed in a 15 µL reaction mix consisting of 0.3 µ*M* of forward and reverse primers, 0.5 units of Taq DNA polymerase (Promega Corporation, Madison, WI), 0.2 m*M* of each dNTPs, 1 X PCR buffer (50 m*M* KCL, 10 m*M* Tris-HCl, 1 g L^1 Triton X-1000), 2.5 m*M* MgCl2, 25 ng of template DNA, and distilled H2O to a volume of 15 µL. The PCR amplification consisted of an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of three steps: denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and elongation at 72°C for

40 seconds. The final step was an elongation step at 72°C for 5 minutes.

Simple sequence repeat (SSR) (Li *et* al, 2003; Ramsay *et al*, 1997; Struss *et al*, 1998) and sequence-tagged site (STS) molecular markers (Blake *et al*, 1996) were screened against DNA from 'Baku' and 'Haxby', those markers showing polymorphism between the parents were then used to genotype the entire population. Markers amplifying fragments that differed by more than 10 nucleotides between the parents were amplified in the population and separated by ethidium bromide stained 6% polyacrylamide gels in 0.5 X TBE buffer. Markers that produced bands differing by less than 10 nucleotides were amplified in the population using a three-primer amplification method (Schuelke, 2000). The three primers are: 1) the standard reverse primer, 2) the M13 primer (of sequence 5' CAC GAC GTT GTA AAA CGA C) labeled with one of the WellRED fluorescent dyes (Sigma-Aldrich, St. Louis, MO) and 3) the forward primer to which the M13 primer sequence has been added at the 5' end. Fifty ng of total DNA was amplified in a volume of 15 µL of 1 X *Taq* buffer, 0.1 m*M* each DNTP, 2.5 m*M* $MgCl_2$, 0.5 units *Taq* DNA polymerase (Promega), distilled water to volume, 0.3 µ*M* of the M13 oligo and the reverse oligo, and 0.02 µ*M* of the M13/forward concatenation oligo. In the

case of two primer amplification, both forward and reverse primers were at 0.3 µ*M*. The thermocycler program was 94°C for 3 min, 40 cycles of three steps: 94°C for 30 sec, 52°C for 30 sec, 72°C for 40 sec, and a final extension at 72°C for 5 min. The amplified products were separated using a CEQ 8800 capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA) and scored using Genographer (Benham, 1999).

In addition to DNA markers, the state of the *Nud* locus was scored by visually assessing the adherence of the glumes to the caryopsis.

Amplified Fragment Length Polymorphism Markers

Amplified fragment length polymorphism (AFLP®, Vos *et al.*, 1995) markers were employed essentially as in See *et al* (2002). One hundred nanograms of total DNA from the parents, the RILs, and a simulated heterozygote (i.e. a sample containing equal amounts of both parental DNA) were digested in a 15 µL reaction volume for 3 h at 37°C with three units of *Eco*RI and three units of *Hpa*II. After digestion, the reaction was terminated by incubation at 65° for 20 min. A 15 µL mix of ligating adapters was added to the digested DNA, containing

*Hpa*II adapters: 5’ GAC GAT GAG TCC TGA G, 150 ng

5’ CGC TCA GGA CTC AT, 132 ng

*Eco*RI adapters: 5’ CTC GTA GAC TGC GTA CC, 16.8 ng

5’ AAT TGG TAC GCA GTC TAC, 17.4 ng

in 1 X ligation buffer and 1 unit of T4 DNA ligase (New England Biolabs, Inc., Ipswich, MA). This ligation mix was then incubated at 4°C for 24 h.

1 µL of ligation reaction product was preamplified in 30 µL of 0.1 m*M* each DNTP, 1 X PCR buffer (Promega), 2.5 m*M* $MgCl_2$, 0.5 units *Taq* DNA polymerase (Promega), and 30 ng of both *Eco*RI (5’ GAC TGC GTA CCA ATT CA) and *Hpa*II (5’ GAT GAG TCC TGA GCG GC) preamplification primers. Pre-amplification conditions were as follows: 94°C for 2 min, 30 cycles of three steps: 94°C for 30 sec, 56°C for 1 min, 72°C for 1 min, and a final extension incubation at 72°C for 5 min. These amplification products were diluted 1:2 and frozen to be used later as templates for selective amplification.

Selective amplification included 1 µL of the diluted preamplification products in 15 µL of 0.1 m*M* each DNTP, 1 X PCR buffer (Promega Corporation, Madison, WI), 2.5 m*M* $MgCl_2$, 0.5 units *Taq* DNA polymerase (Promega Corporation, Madison, WI), distilled water, and 5 ng of fluorescently labelled *Eco*RI primer (5’ ACT GCG

TAC CAA TTC + 3 selective bases) and 30 ng of *Hpa*II primer (5' GAT GAG TCC TGA GCG GC + 2 selective bases). Five *Eco*RI and six *Hpa*II primers were used in combination, for a total of thirty primer pairs used (Table 3-1). The *Eco*RI primers were labeled with WellRED fluorescent dyes (Sigma-Aldrich, St. Louis, MO) to enable AFLP® product visualization on a CEQ 8800 automated capillary DNA sequencer. AFLP® segregation was scored using Genographer version 1.6.0 (Benham, 2001).

Table 3-1. Selective primers used for AFLP® analysis[a]

Name	Sequence	Name	Sequence
e	5'GACTGCGTACCAATTC**A**	H	5'GATGAGTCCTGAGCGG**C**
e33	5'e + **AG**	h49	5'h + **AG**
e40	5'e + **GC**	h50	5'h + **AT**
e41	5'e + **GG**	h55	5'h + **GA**
e42	5'e + **GT**	h56	5'h + **GC**
e43	5'e + **TA**	h58	5'h + **GT**
		h60	5'h + **TC**

a. Bold letters indicate selective nucleotides

Illumina GoldenGate Assay

Further genotyping of the population was performed with the Illumina GoldenGate™ SNP genotyping platform. The GoldenGate™ assay is a single-nucleotide extension assay making use of universal primers and fiber-optic arrays (Shen *et al*, 2005). An oligonucleotide pool assay (OPA) denotes the collection of all SNPs to be genotyped in the assay. The OPA used for GoldenGate™ genotyping of the Haxby/Baku population was PilotOPA1 (Close, 2006). As SNP allelic state is detected using a 96-array configuration (Shen *et al*, 2005), a subset of the original Haxby/Baku mapping population was chosen for further characterization. Ninty-four RILs were chosen at random to be genotyped along with Haxby and Baku. DNA was extracted from individual two-week-old F_7 plants using the DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). DNA quantity and quality was evaluated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). After quantitation, DNA was diluted to 50 ng/uL with TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA). The SNP genotyping was performed by Dr. Tom Blake in the laboratory of Dr. Shiaoman Chao in Fargo, North Dakota. The SNP assay was performed as recommended by the manufacturer.

Map Construction and QTL Analysis – F5

Heterozygous data points were scored as missing data. JoinMap 3.0 was used for linkage grouping and map construction (Van Ooijen and Voorrips, 2001). The complete data set was first analyzed to separate linkage groups. After this initial analysis, anchor markers were used to assign linkage groups to chromosomes.

After the initial mapping, data quality and consistency was evaluated in the following manner. Within linkage groups, loci with mean chi-square contributions greater than 3.0 were removed. Also, genotype probabilities at loci were examined. For a specified map order, JoinMap calculates the probability of each genotypic data point for an individual, conditional on the genotypes of neighboring loci. Loci in which the average of these probabilities was less than 0.05 were excluded. After removing suspect loci based on the above criteria, map orders and distances were recalculated. This process was repeated until no further markers could be removed. Each marker included in the map was tested for segregation distortion using the χ^2 test implemented in JoinMap. Map distances were calculated as Kosambi centiMorgans (Kosambi 1944).

QTL analysis was performed with the composite interval mapping module of Windows QTL Cartographer Version 2.5 (Wang *et al*, 2006).

Cofactors to be used in the analysis were selected using forward and backward regression. The walk speed was 2 cM and the window size was 10 cM. The mean value of DMD from the four replications was used for the analysis. 1000 permutations were used to estimate a LOD score for which genome-wide significance was P=0.05 (Churchill and Doerge 1994).

Results of map construction and QTL analysis were displayed graphically using MapChart 2.1 (Voorrips 2002).

Map Construction and QTL Analysis– F7

During the SNP assay, 8 DNA samples were lost because of a malfunction in the centrifuge. Therefore, data were available for 88 lines; 86 RILs, Haxby, and Baku. Before mapping, data were manually checked for quality. The complete dataset contains the allelic state of 1,481 loci for all genotyped lines, regardless of polymorphism in the population. First, loci missing data for more than eight individuals were removed, leaving 1,205 high quality marker loci. Next, 825 obviously monomorphic markers were removed, leaving the final dataset of 380 polymorphic loci and 86 RILs.

JoinMap 3.0 was used for linkage grouping and map construction (Van Ooijen and Voorrips, 2001). Linkage groups were formed using a

maximum recombination percentage of 35 and a minimum LOD of 4. The 380 markers were separated into linkage groups at a logarithm of odds (LOD) score of 8.0. HarvEST:Barley, Version 1.55 (available at http://harvest.ucr.edu/) contains consensus map positions for SNP loci from PilotOPA1 that have been genetically mapped. This resource was used to assign linkage groups to chromosomes. Based on chromosome assignment, linkage groups from the same chromosome were combined and marker order was recalculated. The stringent LOD score of 8.0 used as the criteria for initial linkage grouping means that the marker order within groups is highly self-consistent, therefore these linkage group orders were used as fixed orders in the final map calculation.

Data quality and consistency were evaluated as described for the F5 population.

QTL analysis was performed as described for the F5 population with the exception that window size for QTL analysis was 2 cM.

Results and Discussion

Phenotypic Data

The mean values and standard deviations for all measured traits are summarized in Table 3-2. Haxby had higher DMD and smaller mean particle size than Baku (46.4 versus 36.6 % and 1101 versus 1284 μm, respectively). Haxby had lower average 500-kernel weight than Baku (18.77 g versus 20.09 g). All parental differences except starch content and height were significant ($P<0.03$) over six random independent replications. There was considerable variation for many traits measured in the RILs; DMD, starch, 500-kernel weight, and plant height all had minimum and maximum values greater than two phenotypic standard deviations from the population mean. All traits were normally distributed except particle size, which was skewed strongly to the right. An inverse square transformation of the particle size data resulted in a normal distribution and this transformed data was used for QTL analysis.

Table 3-2. Average values (ave)of dry-matter digestibility (DMD), particle size (PS), starch content (SC), 500-kernel weight (KW), height at maturity (Ht), heading date (HD) and standard deviations (s) for 'Haxby' and 'Baku' and the RILs

Trait	'Haxby'	'Baku'	RILs		
	ave (s)[a]	ave (s)[a]	ave (s)	Min	Max
DMD (%)	46.4 (1.9)	36.6 (3.8)	41.2 (6.6)	18.9	55.2
PS (μm)	1101 (40)	1284 (53)	1183 (150)	945	1884
SC (%)	62.7 (6.2)	57.5 (2.8)	60.4 (5.2)	46.0	76.0
KW (g)	18.77 (0.97)	20.09 (0.78)	19.80 (2.42)	14.19	25.64
Ht (cm)	81 (4)	86 (5)	80 (8)	60	107
HD (day)	182 (1)	181 (1)	184 (3)	179	194

[a]N=6

Significant phenotypic correlation existed between many of the traits (Table 3-3). DMD was positively correlated with starch content and average plant height, and negatively correlated with heading date. As in previous studies of barley DMD (Lykos and Varga, 1995; Surber *et al*, 2000; Rémond *et al*, 2004), particle size was negatively correlated with DMD. Particle size was negatively correlated with kernel weight and starch content, and positively correlated with heading date.

Table 3-3. Pearson correlation coefficients among field measurements and feed-quality characteristics of the Haxby/Baku RIL population

	HD	Ht	KW	SC	DMD
HD	1	—	—	—	—
Ht	0.04	1	—	—	—
KW	-0.20*	-0.10	1	—	—
SC	-0.01	0.34***	-0.05	1	—
DMD	-0.41****	0.30***	0.17	0.32***	1
PS	0.33***	-0.15	-0.43****	-0.23*	-0.63****

* indicates significance at P<0.05, *** indicates significance at P<0.001, **** indicates significance at P<0.0001

Map Construction

Of 240 STS and SSR primer pairs tested, 97 revealed polymorphism between 'Haxby' and 'Baku.' Thirty five of these, chosen at random, were used to genotype the population and act as anchor markers. An additional 183 markers were generated using 30 AFLP primer pairs. Two AFLP markers, e33h55(514/517) and e42h60(475/484), were scored as co-dominant markers. In the initial analysis of the complete segregation data, 11 linkage groups were formed. Forty markers remained un-linked to linkage groups containing anchor markers. The presence of previously mapped markers in the linkage groups allowed the groups to be assigned to a chromosome. In this way, a linkage map was constructed covering

680 Kosambi centiMorgans (Figure 3-1). This map length indicates approximate genome coverage of between 49 and 64% (Costa *et al*, 2001; Qi *et al*, 1998). Chromosomes 6H and 7H each have regions containing no markers: the two linkage groups comprising 6H are separated by a recombination fraction of approximately 0.4, while the two linkage groups comprising 7H are separated by a recombination fraction of approximately 0.5. The estimate of genome coverage is likely an underestimate as there are several regions of segregation distortion in this genetic map, and segregation distortion at multiple loci usually results in reduced apparent genetic distance (Zhu *et al*, 2007). These markers that show distortion occur in blocks in this map. Sixty-three markers showed segregation distortion at the level of $P \leq 0.05$. Thirty-two markers showed segregation distortion at the level of $P \leq 0.01$ (Figure 3-1). If it is assumed that intervals between distorted markers are also distorted, 5% (35.3 cM) of this map is distorted at the level of $P \leq 0.01$, and 14% (96.0 cM) is distorted at the level of $P \leq 0.05$. Distorted markers were included in the QTL analysis.

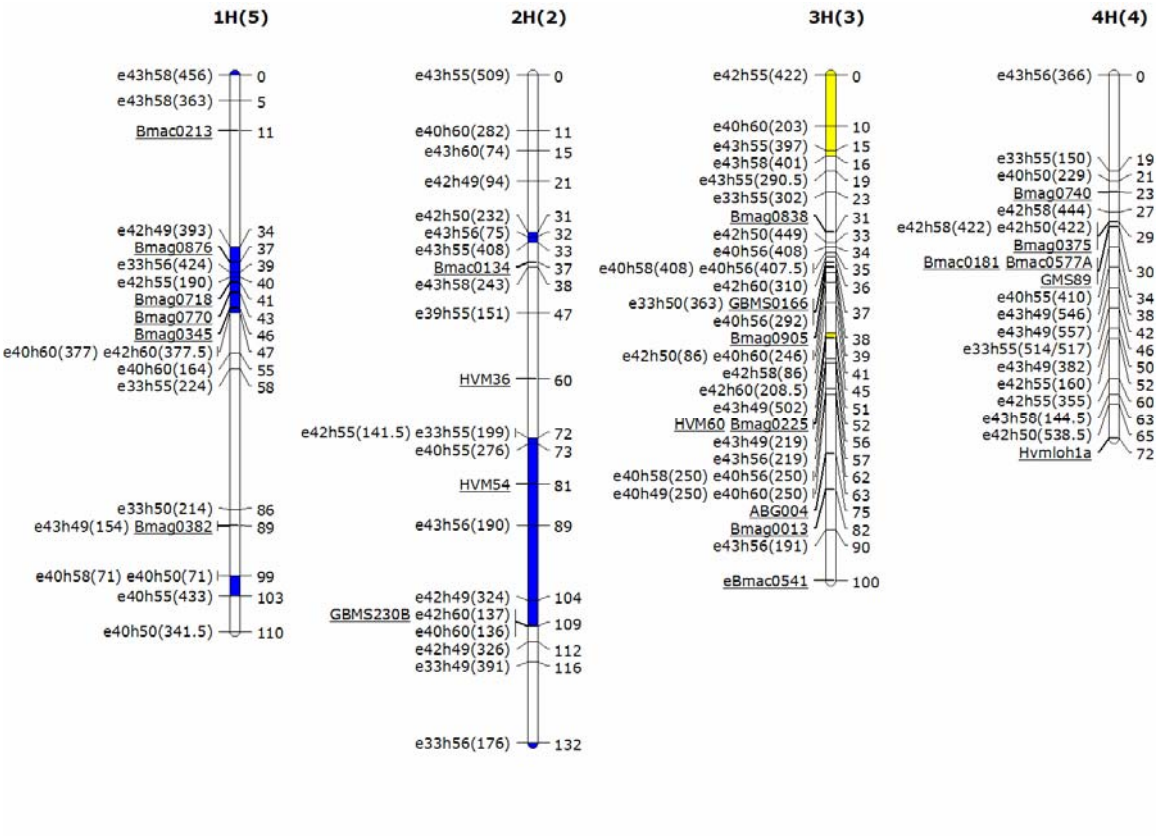
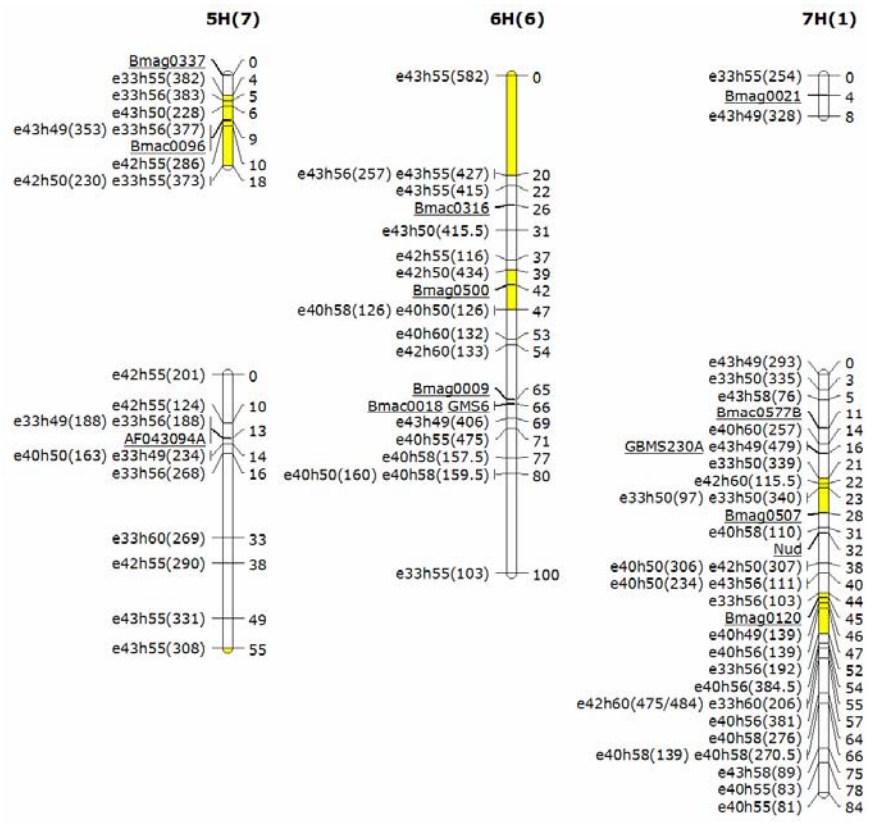


Figure 3-1. Linkage map of Haxby/Baku RIL population. Distances are in Kosambi centimorgans. Anchor markers are underlined. Blue intervals are distorted (P≤0.05) in favor of Baku alleles, yellow toward Haxby.

Figure 3-1. continued.



QTL Analysis

Using simple Composite Interval Mapping, 2 putative QTL of significance greater than LOD 2.8 were detected for ruminal dry-matter digestibility (Table 3-4). These loci also reached the critical value by single-marker analysis and simple interval mapping. Two-way analysis of variance detects no epistasis between these loci. These two QTL were on chromosomes 6H and 7H and explained 19% and 17% of phenotypic variation, respectively. Total phenotypic variation explained by the QTL model was 43%. The additive effect of

a Haxby allele at the 6H locus is 3.3 percentage units, while the additive effect of a Haxby allele at the 7H locus is -2.8 percentage units. The LOD peak of the 6H locus is at 65 cM in the present map, nearest to the SSR marker Bmag0009, and the 2-LOD support interval is 5 cM in length. The LOD peak of the 7H locus is at 16.5 cM, nearest the AFLP marker e43h49(479). The 2-LOD support interval is 10 cM in length, although the portion of the LOD profile above the critical value of 2.8 is very broad, extending from 5 to 28 cM on the present map.

Table 3-4. Locations, LOD scores, and effects of QTL detected by simple Composite Interval Mapping in the Haxby/Baku RIL population

Trait	Position[a]	Interval[b]	LOD	Effect[c]	R^{2} [d]	TR^{2} [e]
DMD (%)	6H(64.7)	64.2-69.4	7.39	3.33	19	
	7H(16.5)	14.8-25.4	6.63	-2.83	17	43
PS[f] (µm)	5H(4.2)	0-15.4	6.01	96	15	
	6H(33.1)	25-41.4	2.80	80	7	
	6H(64.7)	58.2-70.4	2.83	-64	7	
	7H(66.3)	55.2-71.8	4.34	-80	11	45

[a]Chromosome and cM position of maximum LOD score, [b]2-LOD support interval, [c]average effect of one Haxby allele, [d]Percent phenotypic variation explained by the indicated QTL, [e]Percent phenotypic variation explained by QTL model, [f]Particle Size

Although only two regions of the genome exceeded the critical value of 2.8 for DMD, a region extending from 42 to 54 on 6H is near the critical value, reaching a maximum LOD of 2.5 and a maximum additive effect of -2.0 (Figure 3-2). This locus is suggestive of a QTL for DMD.

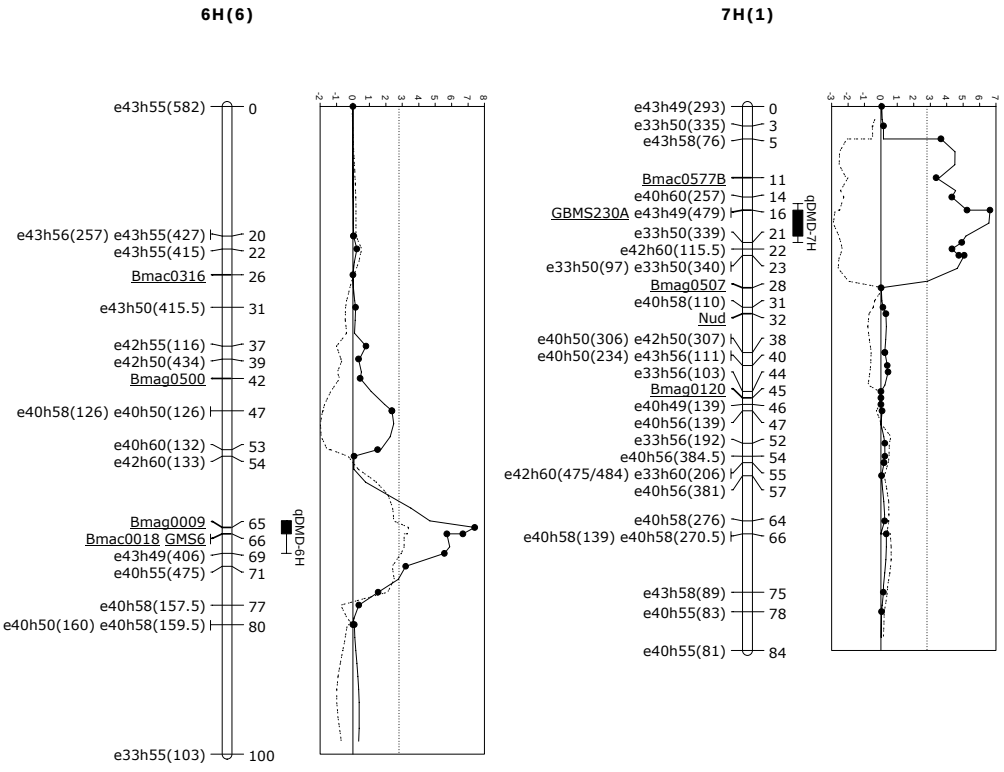


Figure 3-2. Scans of barley chromosomes 6H and 7H for trait DMD. Dashed line indicates allelic effect at loci. Solid line indicates LOD scores at loci. Dotted line is LOD of 2.8. Bars and lines to right of chromosomes are 1- and 2-LOD support intervals, respectively.

Previously, QTL for DMD have been identified on barley chromosomes 1H, 3H, and 4H in the Steptoe/Morex population (Bowman *et al*, 1996), 1H in the Lewis/Baronesse population (Abdel-Haleem, 2004), and 2H in the Valier/PI 370970 population (*ibid*.). Therefore, the putative QTL detected in the Haxby/Baku population seem to be previously unidentified.

Four QTL were detected for mean particle-size (Table 3-4). In two QTL, the Haxby allele increases mean particle-size. One QTL in which the Haxby allele decreases particle size is coincident with the 6H QTL for DMD in which the Haxby allele increases DMD, thus it is likely that this locus impacts DMD indirectly by its effects on particle size. Although the DMD 7H locus does not reach the critical value of 2.8 by composite interval mapping, by F-test the interval e43h58(76)-Bmag0507 is significant at $P<0.02$, and the Haxby allele that decreases DMD is also associated with increased particle size. Forty-five percent of total phenotypic variation was explained by the four detected QTL. No epistasis was detected between these loci.

QTL for particle size have previously been identified on 2H, 3H, 4H, and 7H in the Steptoe/Morex map, 5H and 7H in the Lewis/Baronesse map, and 2H and 7H in the Valier/PI 370970 map.

The QTL on 7H in the Valier/PI 370970 is towards the short arm relative to the *nud* locus, while in the Haxby/Baku map it is towards the long arm. In the Steptoe/Morex map the 7H QTL is again on the short-arm. In the Lewis/Baronesse map there are limited points of reference so it is difficult to make any comparisons.

Further Analysis of the Haxby/Baku Population

Two QTL were identified in the initial mapping of this population. They are on chromosomes 6H and 7H and are estimated to explain 19 and 17 percent of phenotypic variation, respectively. Because these loci are approximately equal in additive effect but opposite in sign, we presume that there must be more loci conferring the extremely low DMD to Baku. Further, as the map used to detect these QTL is estimated to cover 50-60% of the barley genome, it is quite possible that important DMD loci remain undetected in this population. With the recent development of an extremely rapid and reliable single-nucleotide polymorphism (SNP) genotyping method in barley (Rostoks *et al*, 2006), it has become feasible to genotype up to 1,536 SNPs in a single experiment. Due to the apparently limited coverage of the initial mapping, a subset of the Haxby/Baku mapping population was genotyped using this new system, Illumina

GoldenGate, in an attempt to extend genome coverage and identify more loci with important effects on DMD.

Map Construction:SNPs

At a LOD threshold of 8.0, the SNP markers were grouped into 26 linkage groups. The 26 linkage groups were assigned to chromosomes based on chromosomal assignment of markers in HarvEST:Barley, Version 1.55 (available at http://harvest.ucr.edu/). On further inspection, one linkage group was comprised of SNP markers previously mapped to chromosomes 2H and 5H. This group was split at a LOD of 9.0. The 27 linkage groups were combined into their respective chromosomes at a LOD of from 2.0 to 7.0. Several properties of the resulting genetic maps are interesting to note and are summarized in Figure 3-3.

Gross marker order is in most cases consistent with the HarvEST consensus map (Figure 3-3). The most obvious areas of marker order discrepancy are located in regions of high marker density in either of the maps (for example: 1H:130 cM, 2H:147 cM). Differences in marker order between the two maps are probably the result of sampling error or lack of recombination information.

Comparison to the HarvEST consensus map allows direct estimation of genome coverage. Genomic coverage is apparently quite complete with several exceptions (Figure 3-3). Approximately eighteen cM of the extreme short arm of chromosome 2H contains no markers. Also, twenty cM of the extreme short arm of chromosome 5H is not covered by markers. For the purposes of this mapping project, it is unfortunate that the 5HS region contains no markers. It has been reported that the genes encoding the grain softness proteins that reside in this area, the barley hordoindolines and the wheat puroindolines, are important contributors to DMD (Beecher *et al*, 2002; Swan *et al*, 2006). This problem could easily be rectified by genetic mapping of either of the hordoindolines or of GSP Seventeen centiMorgans of the short arm of 6H contains no markers. A final area of incomplete genome coverage is the short arm of 7H, where 29 centiMorgans in the Haxby/Baku map contains no markers, though this region in the consensus map is only 6.7 centiMorgans in length.

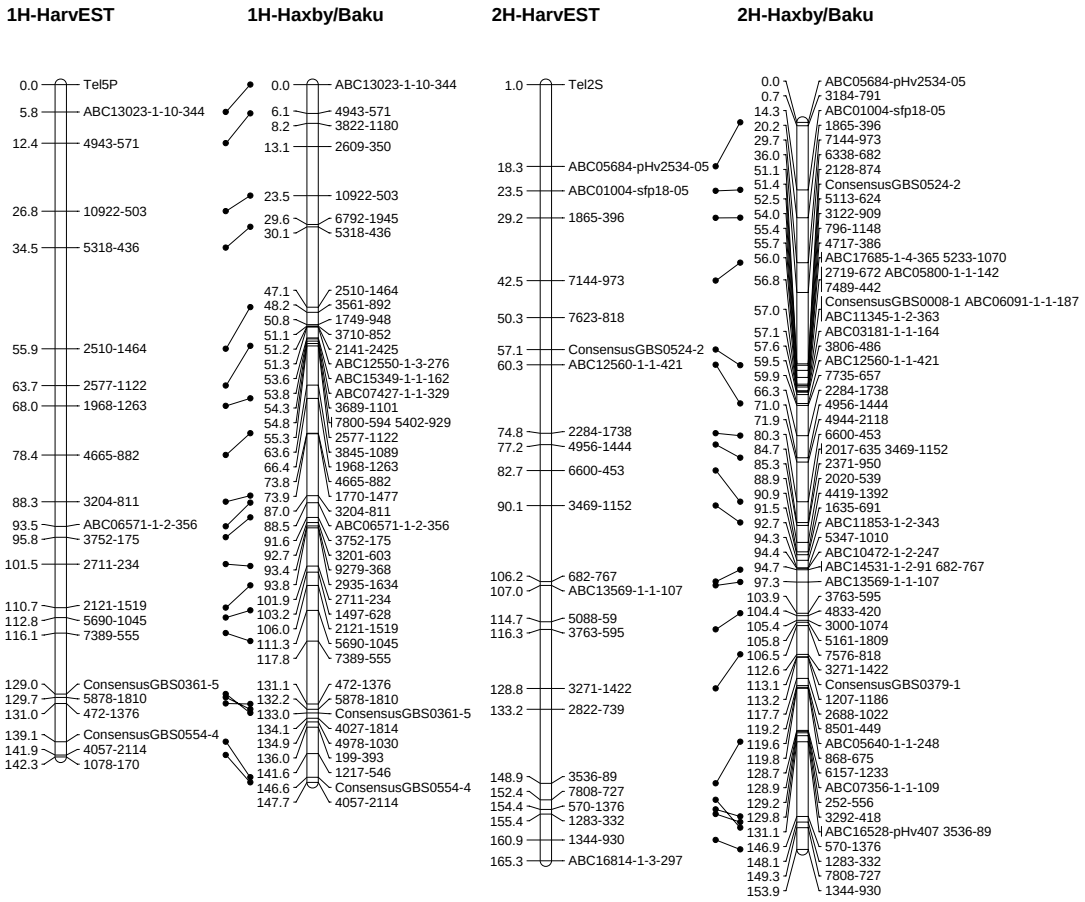


Figure 3-3. Comparison of Haxby/Baku RIL population and HarvEST:Barley consensus linkage maps

Figure 3-3. Continued. All SNPs mapped in the Haxby/Baku population are shown. For clarity, only a subset of consensus SNPs are shown.

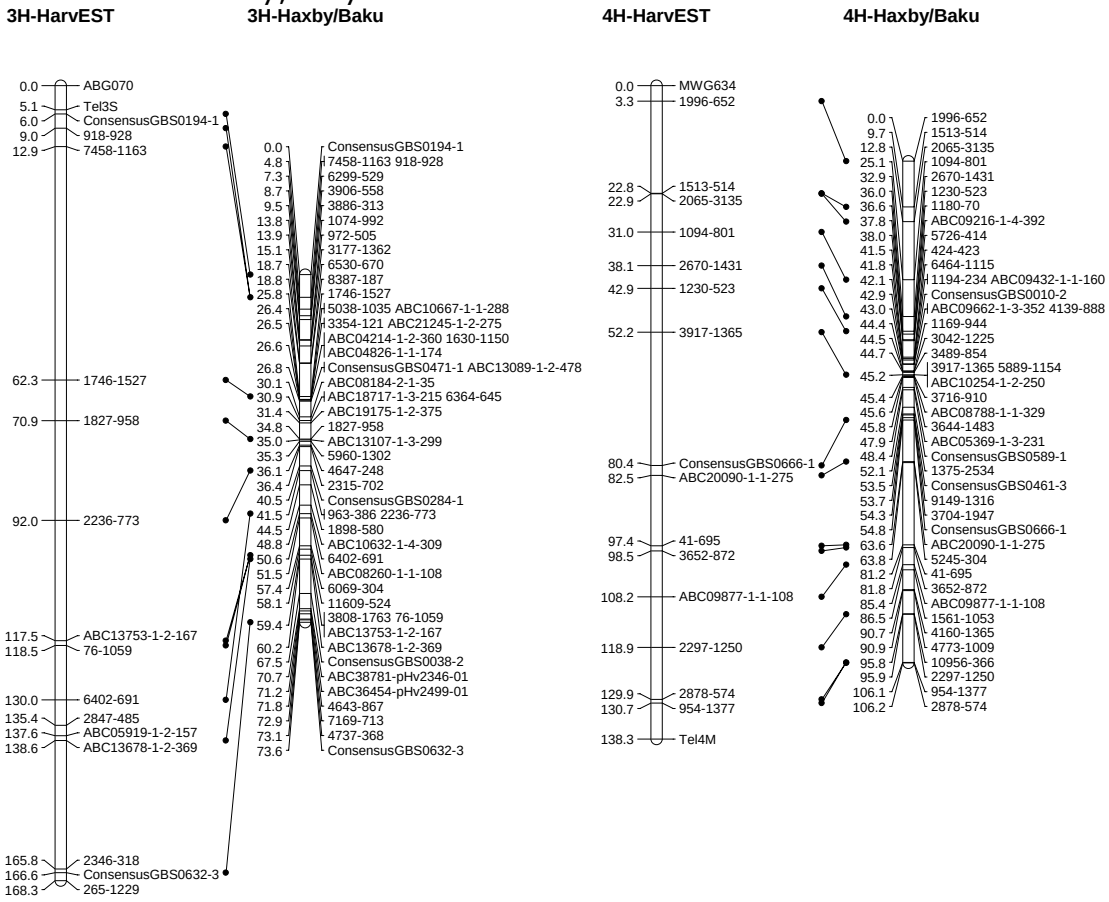


Figure 3-3. Continued.

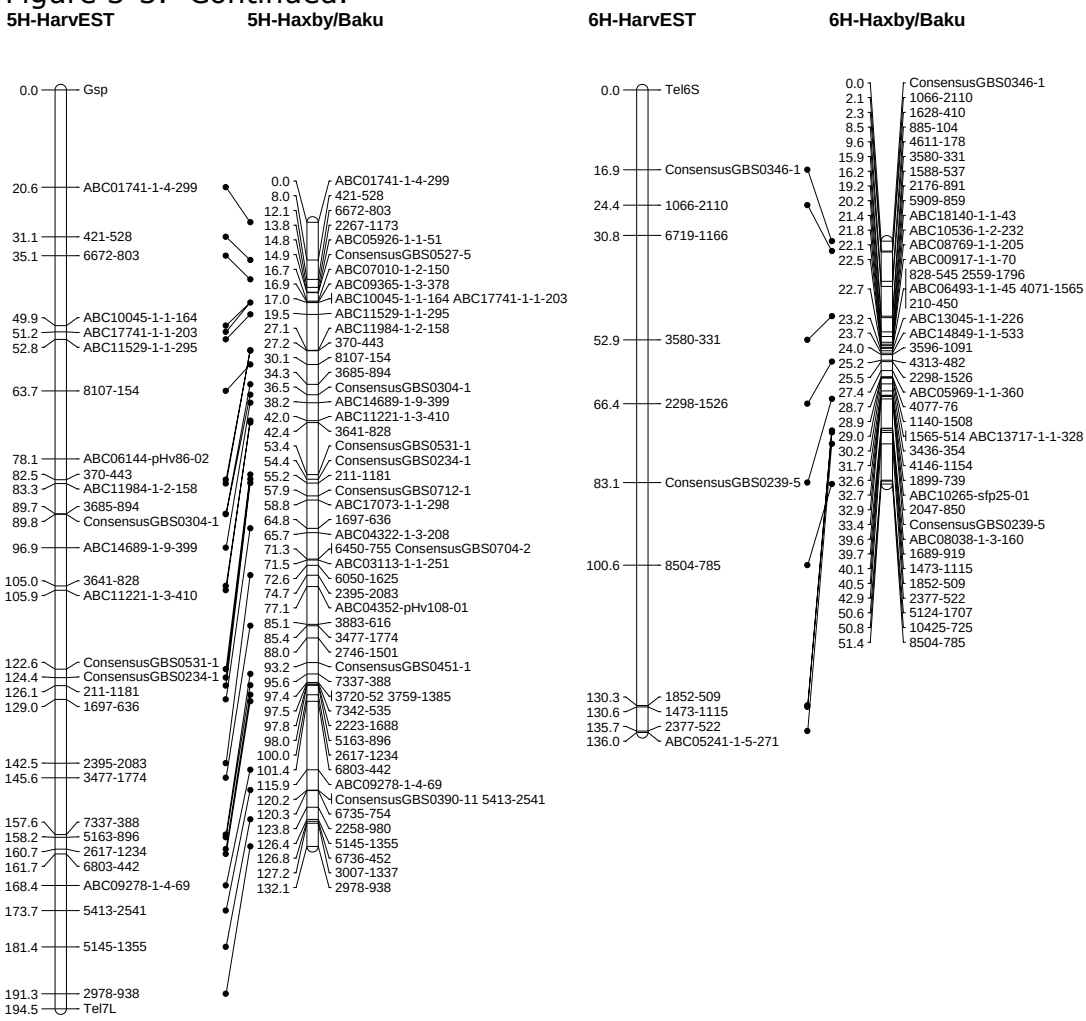
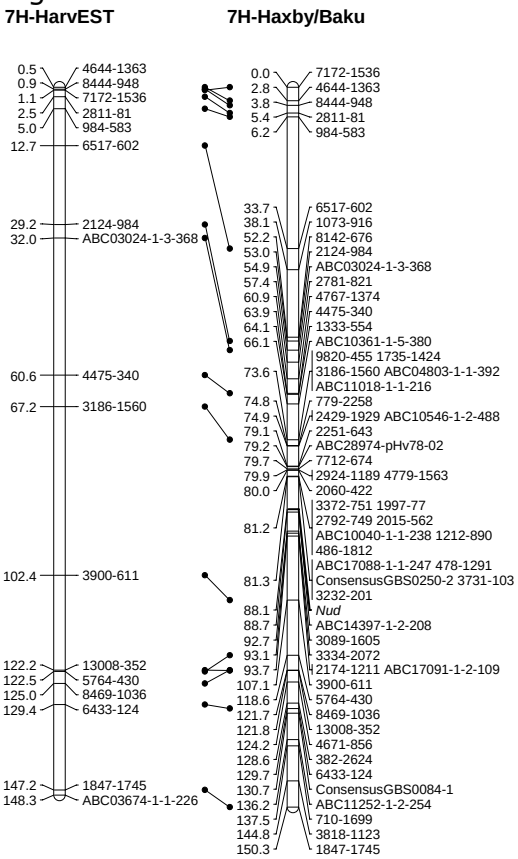


Figure 3-3. Continued.



Despite apparently complete coverage of the genome (based on comparison with the consensus maps obtained in HarvEST), two of the chromosomal maps are of dramatically shorter centiMorgan length (Figure 3-3). 3H and 6H are only 46% and 43% of the centiMorgan length of the consensus maps, respectively (Table 3-5). From the standpoint of QTL mapping, this is unfortunate because any QTL mapped on these short chromosomes will likely be mapped to larger

intervals due to less recombination information, and the effects of linked genes may not be separated (Noor *et al*, 2001).

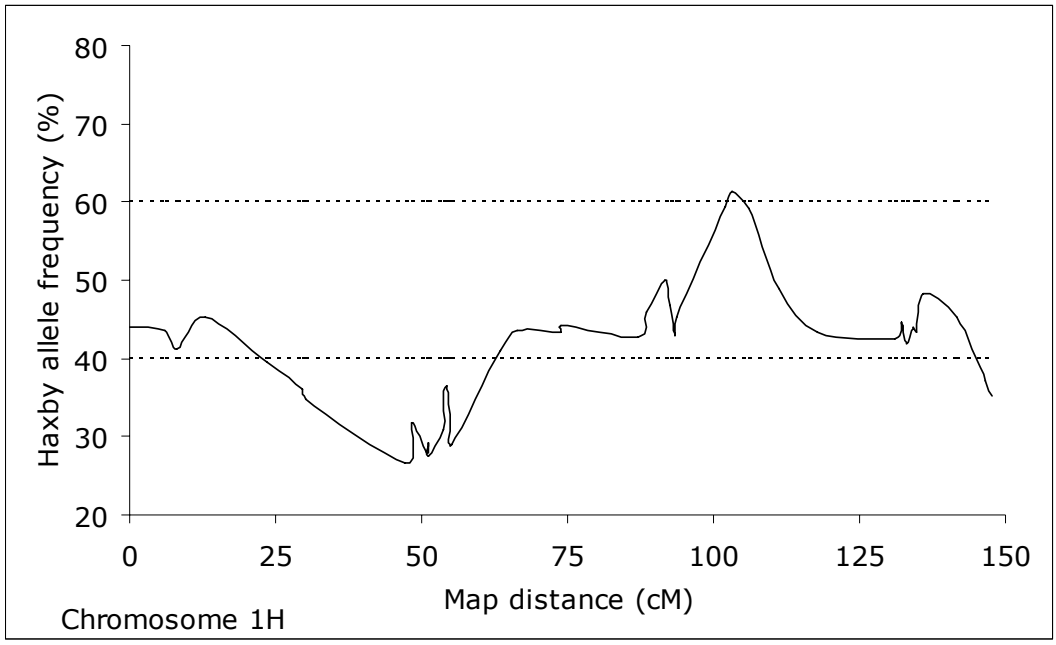
Table 3-5. Comparison of HarvEST consensus and Haxby/Baku map lengths.

Chromosome	Map length (cM)		%[a]
	HarvEST	Haxby/Baku	
1H	136.1	147.7	109
2H	142.6	153.9	108
3H	160.6	73.6	46
4H	127.4	106.2	83
5H	170.7	132.1	77
6H	119.1	51.4	43
7H	147.2	150.2	102
all	1003.7	815.1	81

[a] Haxby/Baku chromosomal map lengths expressed as % of HarvEST chromosomal map lengths

An appealing hypothesis to explain the reduction in map length is selection against heterozygotes (or selection for a homozygous class) in early generations. This population is an F7 recombinant inbred population. Calculation of map length in F7 recombinant inbreds assumes that residual heterozygosity beyond the first generation allows increased opportunity for informative recombination. JoinMap corrects for this expected increase in observed pair-wise

recombination by reducing recombination frequencies used in the calculation of map length (Stam, 1993). Therefore, selection resulting in less than expected heterozygosity would reduce calculated map length. However, because informative recombination frequency in an RIL is expected to be twice that of a single-meiosis population, reduction in map length of greater than 50% cannot be explained even by complete lack of heterozygotes in early generations. Also, selection for a homozygous class (such as could be used to help explain map compression) would be expected to result in non-random segregation of alleles, i.e. segregation distortion. Though chromosome 3H does show extensive and significant segregation distortion, the chromosome that shows the greatest map compression, 6H, shows very little segregation distortion (Figure 3-4). For completeness, all chromosomes are depicted in Figure 3-4.



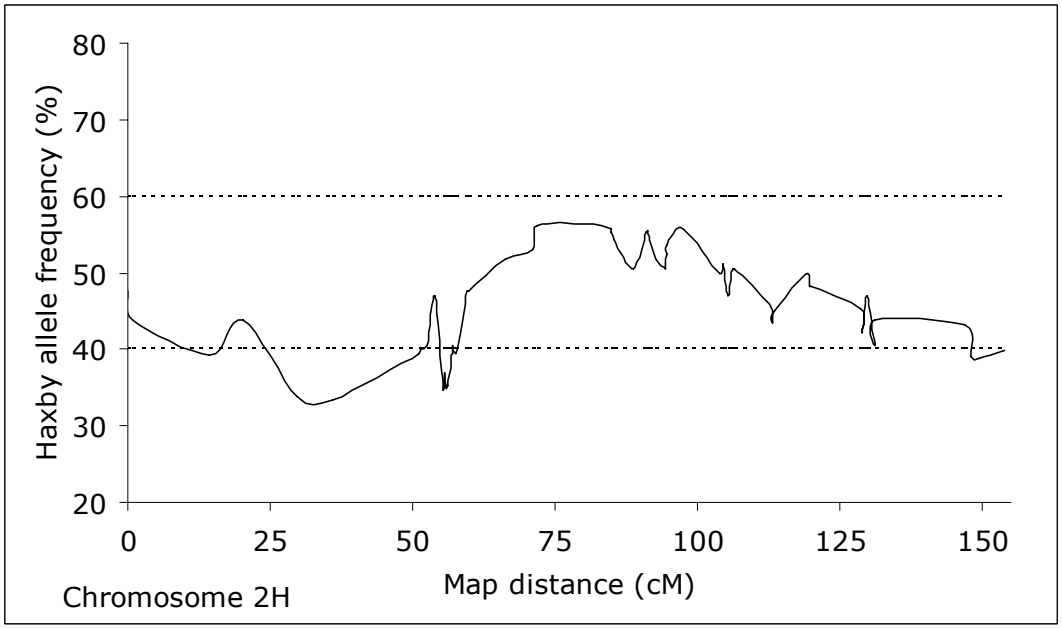
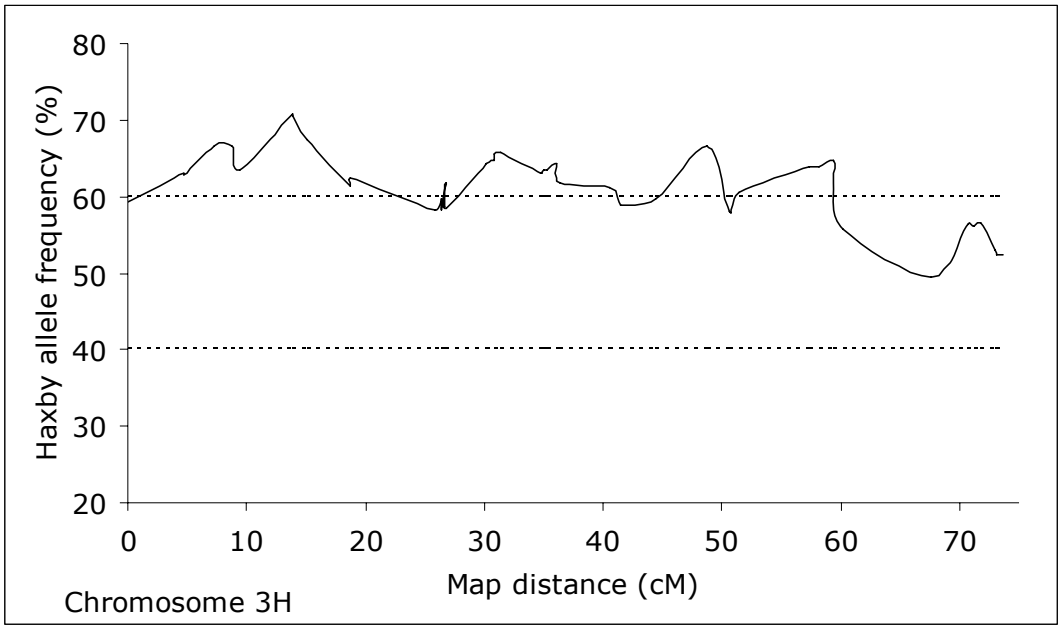


Figure 3-4. Allele frequency in the Haxby/Baku mapping population. Dashed line indicates different from expectation at P=0.05.

Figure 3-4. Continued.



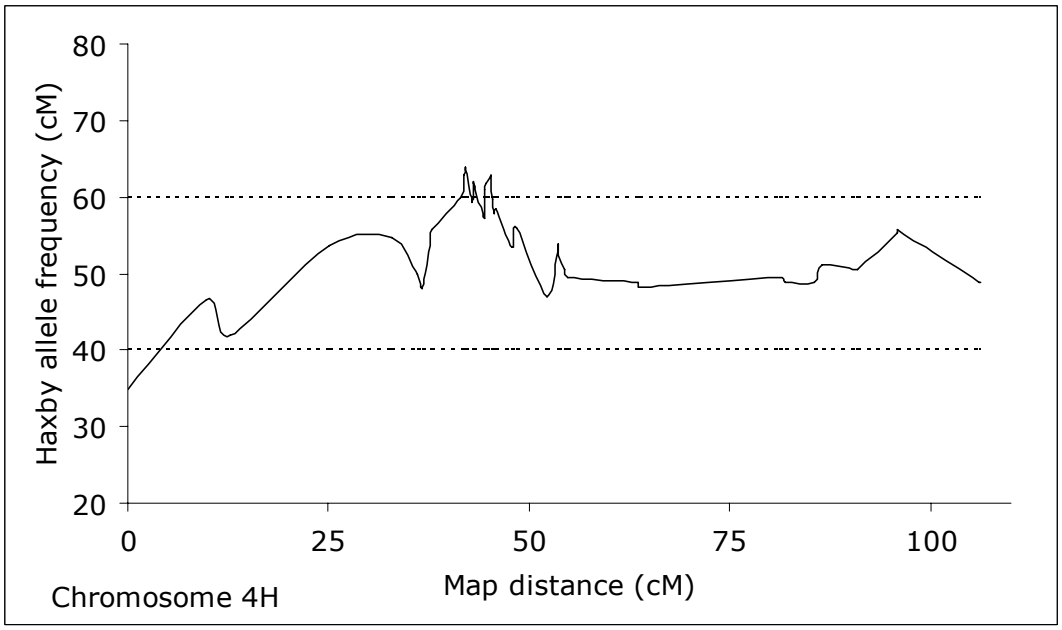


Figure 3-4. Continued.

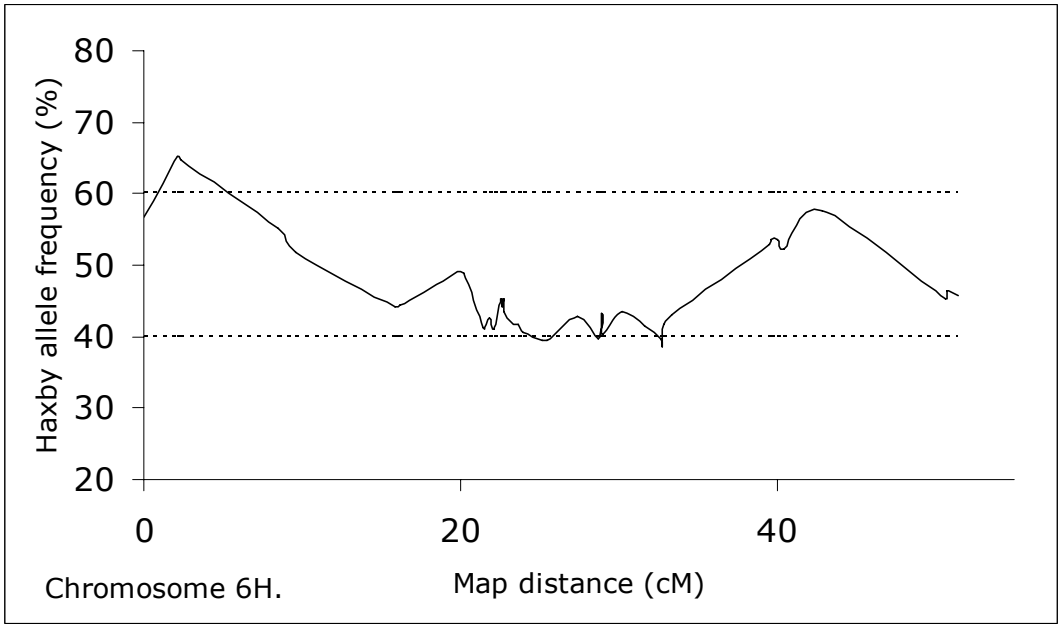


Figure 3-4. Continued.

Although the cause of the reduced Haxby/Baku map length relative to the consensus map is unclear, differences in meiotic recombination frequency and therefore map length have been observed by other investigators, and some of this variability in recombination frequency has been attributed to genetic effects rather than sampling or environmental effects (Cornu et al, 1989; Hadad *et al*, 2006). Further, Barth *et al* (2001) used fourteen mapped antibiotic resistance gene insertions to estimate recombination frequencies in *Arabidopsis* and found that recombination frequencies differed depending both on the parental ecotypes used in the cross and on the chromosome or chromosomal region being evaluated.

Comparison of the F5 and F7 maps

Comparisons of F5 and F7 maps length are summarized in Table 3-6. Based on map length, the AFLP mapping of the full F5 population covered approximately 83% of the F7 SNP genetic map length. The discrepancy between the map lengths of 3H and 6H between generations is likely due to AFLP marker error. The main effect of genotyping errors on genetic mapping is to inflate the map length (Hackett and Broadfoot, 2003). Based on duplicate genotyping of samples, the error rate of AFLP genotype calls is estimated to be between 2 and 5% (Bonin *et al*, 2004). The estimated error rate of GoldenGate genotype calls is estimated to be as low as 0.01% (Fradin and Bougneres, 2007).

Table 3-6. Comparison of F5 and F7 Haxby/Baku map lengths.

Chromosome	Map length (cM)		%[a]
	F7	F5	
1H	147.7	110.4	75
2H	153.9	132.4	86
3H	73.6	99.6	135
4H	106.2	72.2	68
5H	132.1	73.1	55
6H	51.4	99.5	194
7H	150.2	91.2	61
all	815.1	678.4	83

[a] F5 chromosomal map lengths expressed as % of F7 chromosomal map lengths

QTL Analysis: SNPs

A number of phenotypic traits were measured in the population. All QTL detected will be presented (Table 3-7), but only those QTL for DMD and PS will be discussed in detail.

A QTL for particle size was detected on the short arm of chromosome 5H (Table 3-7). This QTL explained fourteen percent of phenotypic variation and, on average, increased particle size by seventy micrometers. This QTL for particle size has been detected previously. In the F5-based mapping population, four QTL for particle size were detected, and the QTL with the greatest R^2 was located on the short arm of 5H centered approximately at the AFLP marker

e33h55(382). By integrating markers from the AFLP map into this map, it can be shown that the two QTL are coincident (Figure 3-6). In Figure 4-3, the integrated map is used as a bridge between the F5 and F7 maps rather than simply using an integrated map for QTL analysis because broad-scale integration of the two maps resulted in unacceptable changes in SNP marker order, changes in distance between markers, and exclusion of SNP markers (results not shown).

By composite-interval mapping, four QTL explaining between 11 and 14 % of DMD phenotypic variation were detected on chromosomes 1H, 6H, and 7H (Table 3-7). The 1H QTL was not previously detected, though this is not due to incomplete coverage in this region (Figure 3-5), it may be that the different QTL detection algorithm is the cause. The 6H QTL and the 7H QTL at 66 cM were detected in the F5 mapping population (Figure 3-5). Because three of the four QTL detected are QTL in which Haxby decreases DMD, it is concluded that the very low DMD of Baku is due to the combined effects of several minor genes rather than the effects of a small number of genes with major effects.

Table 3-7. Locations, LOD scores, and effects of QTL detected by simple Composite Interval Mapping in the Haxby/Baku RIL population

Trait	Position[a]	Interval[b]	LOD	Effect[c]	R^{2d}	TR^{2e}
Starch (%)	6H(37.5)	33.3-41.7	5.2	2.6	22	22
Kernel Wt. (g)[f]	2H(97.4)	94.7-101.5	3.1	0.7	11	
	7H(72.2)	67.3-74.3	8.9	-1.5	27	
	7H(88.2)	85.1-88.5	16.4	2.3	57	65
Height (cm)	2H(22.6)	14.1-29.2	3.1	2.4	7	
	2H(56.9)	55.4-58.1	11.4	4.9	29	
	7H(73.8)	67.4-79.0	4.5	-3.0	10	62
Head Date (day)	1H(88.5)	79.0-91.3	5.7	-1.4	11	
	1H(145.6)	142.9-end	7.4	-2.0	24	
	2H(63.9)	59.7-70.3	3.8	1.2	12	
	7H(52.2)	43.4-53.4	7.4	1.7	26	54
Particle Size (μm)	5H(17.1)	15.0-19.3	4.3	65.0	14	14
DMD (%)	1H(47.1)	39.1-48.1	3.4	-3.0	13	
	6H(23.0)	20.2-25.3	3.6	2.7	11	
	7H(66.4)	64.1-72.5	3.3	-3.0	11	
	7H(85.5)	81.5-88.6	3.5	-3.4	14	48

[a]Chromosome and cM position of maximum LOD score, [b]2-LOD support interval, [c]average effect of one Haxby allele, [d]Percent phenotypic variation explained by the indicated QTL, [e]Percent phenotypic variation explained by QTL model, [f]weight of 500 kernels

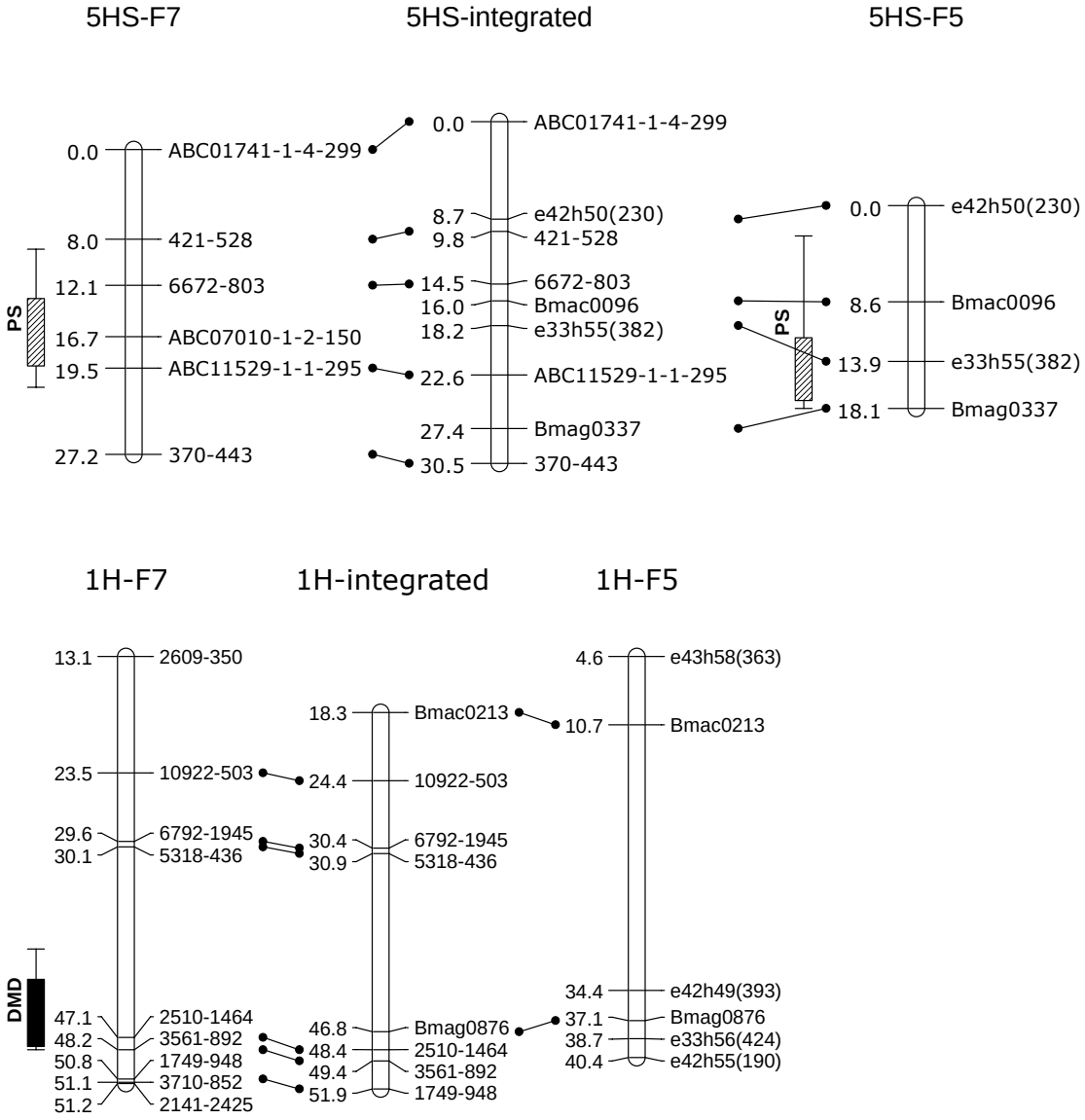
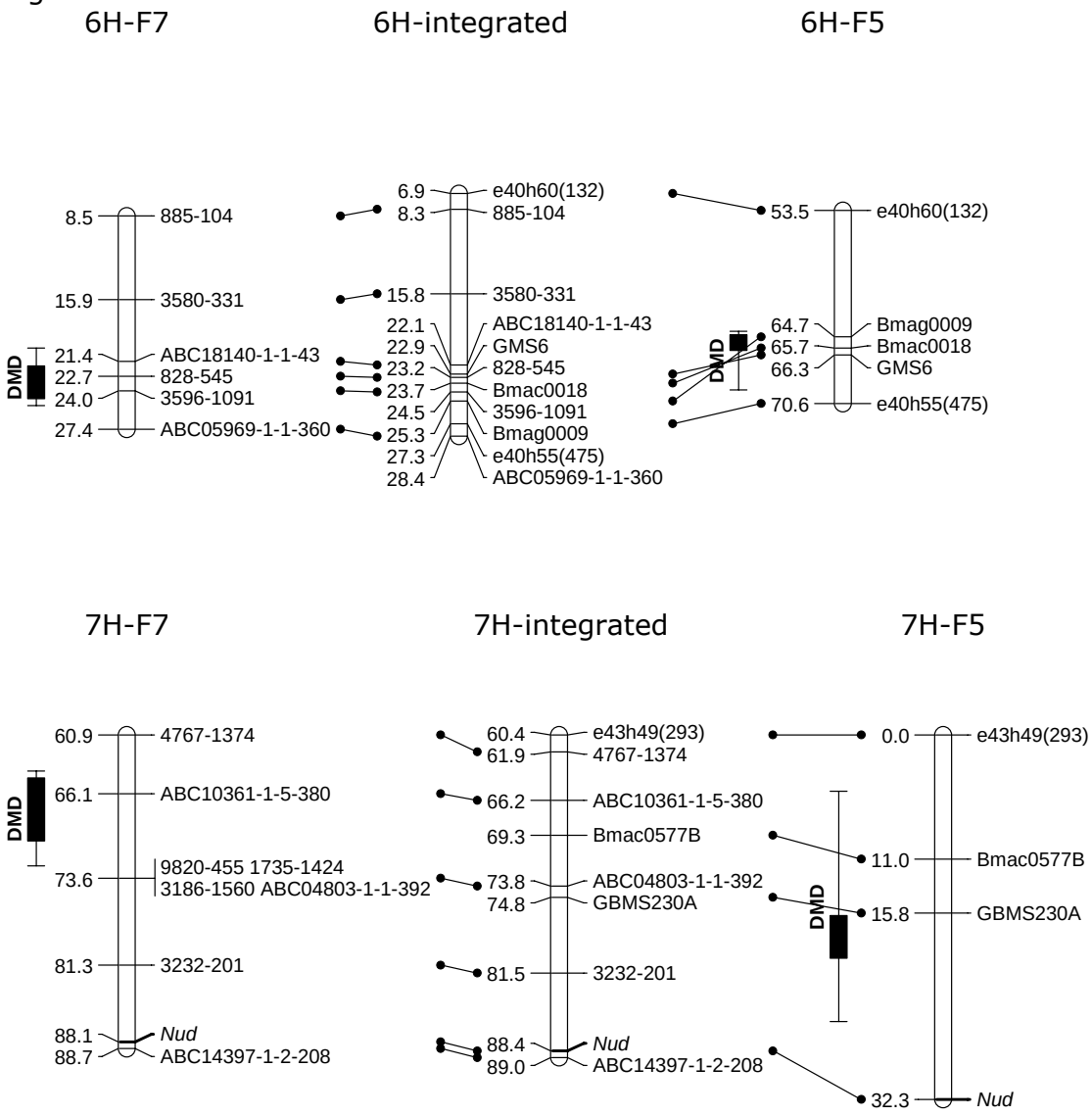


Figure 3-5. Comparisons of QTL for mean particle size (PS) and DMD detected in the 123-member F5 population and in the 86-member F7 population. Marker positions (cM) are indicated to the left of the maps. QTL regions are to the left of the F5 and F7 genetic maps and the 1-LOD QTL support interval is indicated by shaded bars, the 2-LOD interval is indicated by lines.

Figure 3-5. Continued.



One possible candidate gene of the 7H QTL for DMD (*qDMD*-7H) at 66 cM is that it is simply the *Nud* locus. Covered barley grains may be expected to disappear more slowly in the rumen due to a protective effect of the hull. While the Haxby allele of the QTL reduces DMD and

Haxby grain is covered, which is consistent with the hypothesis that *qDMD*-7H is *Nud*, I find it unlikely for several reasons. By single-marker QTL analysis, the most significant marker identified as impacting DMD is ABC10361-1-5-380. This marker is more than twenty centiMorgans away from the *Nud* locus (Figure 3-5). Further, by composite-interval mapping, the *Nud* locus rises above the significance threshold, but the LOD profile between the two peaks decreases to zero. In other words, both QTL (*qDMD*-7H and *Nud*) seem to have been detected as individual loci.

Further evidence exists to counter the claim that *qDMD*-7H is *Nud* in the form of consecutive QTL mapped for kernel weight. Two QTL were detected for kernel weight on 7H at 72 and 88 cM (Figure 3-6). The peak of the 88 cM kernel weight QTL LOD profile is at the precise position of *Nud* and the Haxby allele increases kernel weight, which is to be expected. The Haxby allele at 72 cM decreases average kernel weight and is coincident with the QTL in which the Haxby allele decreases average DMD. It seems that two loci impacting both DMD and kernel weight exist within twenty centiMorgans of each other. Both Haxby alleles at both QTL on 7H reduced DMD, but Haxby *Nud* allele increased kernel weight, while the Haxby allele at ~ 70 cM decreased kernel weight. The reduction in kernel weight by *qDMD*-7H

may not deleterious to animal performance as long as it does not reduce test-weight. Barley test-weight has been shown to be an important indicator of animal performance (Mathison *et al*, 1991; Grimson *et al*, 1987). These plant materials will be grown again this year at the Post Farm in Bozeman, and test-weight will be measured.

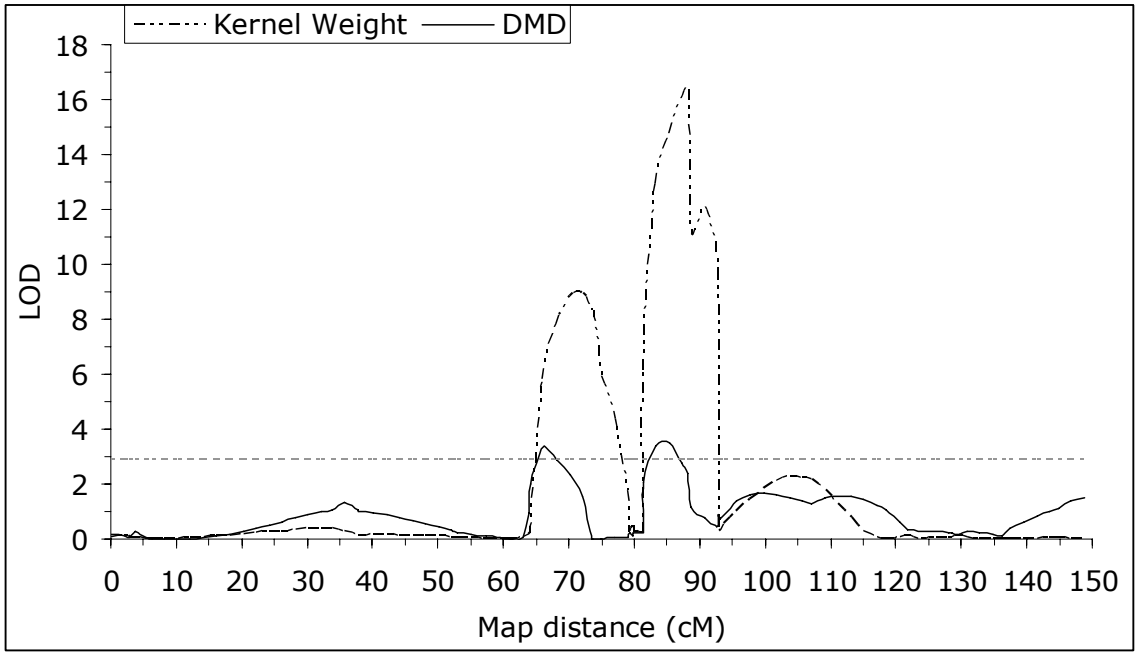


Figure 3-6. 7H LOD scores for kernel weight and for DMD. LOD threshold for significance is shown as a dotted line at LOD 2.9. *Nud* is at 88 cM.

It is interesting to note that a significant QTL explaining 10 % of height variation was also detected at approximately 70 cM on chromosome 7H (Table 3-7, Figure 3-7). It may be that a developmental gene or gene complex with pleiotropic effects on DMD,

kernel weight, and height exists in this region of 7H. The homologous region in rice consists of nearly four Megabases of DNA and nearly 600 predicted genes, therefore identification of candidate genes by comparison to the rice genome is daunting if not impossible, especially given that the physiological basis of this locus (or loci) is unknown.

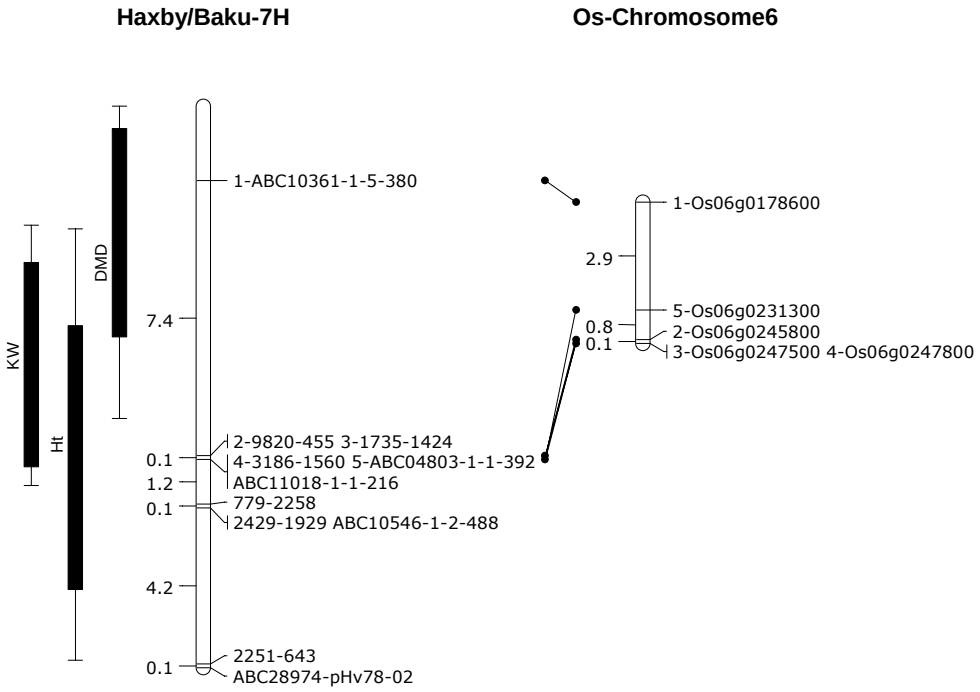


Figure 3-7. Comparison of the barley 7H QTL region and the orthologous rice chromosome 6. 1- and 2-LOD QTL support intervals are indicated by bars and lines, respectively, to the left of the barley 7H QTL region. Distance between loci on 7H are in cM, distances between loci on rice 6 are in Megabases. Orthologous rice and barley loci are preceded by the same number.

It is conceivable that a locus impacting DMD may do so indirectly by its effect on starch content, as starch is highly digestible in the rumen. Further, starch is the principle source of energy in barley grain (Huntington, 1997). Therefore, a QTL that reduces DMD by reducing starch content may be of limited use: grain energy content would likely be reduced. In order to assess pleiotropic or linked effects of DMD QTL loci on starch content, interval analysis was conducted using ANOVA. The starch content of RILs was compared at contrasting alleles in the detected QTL intervals GMS6-Bmag0009 and e43h58(76)-Bmag0507 (Table 3-8). The DMD QTL on 6H is nearly significant (P=0.08) for starch content and, as may be expected, the Haxby allele that increases DMD is also associated with increased starch content. This QTL requires further research to ensure that it does not have deleterious effects on barley feed quality. The DMD QTL on 7H is not associated with changes in starch content (P=0.97).

Table 3-8. Type III tests of qDMD-6H and qDMD-7H effects on starch content

	Mean SC[a]	F Value	P Value
qDMD-6H, Haxby allele	61.4	4.08	0.08
qDMD-6H, Baku allele	58.0		
qDMD-7H, Haxby allele	59.6	0.01	0.97
qDMD-7H, Baku allele	59.8		

[a] Least squares means starch content (%)

QTL Validation

Ninety-four RILs of a cross between Drummond and Baku were evaluated for digestibility and particle size and genotyped with the marker Bmag0009 in an attempt to validate the putative QTL on 6H. Genotyping this population for markers at the 7H locus would not make sense, as low DMD at that locus is associated with the Haxby allele. By F-test, the 6H locus did not significantly affect DMD or mean particle size in the validation population (Table 3-9). In fact, contrary to expectation, the mean DMD of lines with the Baku allele is greater than the mean DMD of those lines with the Drummond allele.

Table 3-9. Type III tests of Bmag0009 effects on DMD and mean particle size in an F_5 validation population

Trait	Allele	Mean[a]	F Value	P Value
DMD (%)	Drummond	40.4	2.55	0.114
	Baku	43.5		
Particle Size (μm)	Drummond	1249	1.56	0.216
	Baku	1197		

[a]Least squares means

The inability to validate this locus in a second population is disappointing. There are several possible causes for this lack of validation. DMD variation in this population may be a result of multiple loci with minor effects. It has repeatedly been shown by computer simulations and by QTL validation experiments that QTL effects are often overestimated in QTL detection populations (Melchinger *et al*, 1998; Utz *et al*, 2000). It may be that the QTL detected on 6H is a true QTL, but its effect is smaller than estimated, small enough even that its effect is undetectable in another population. A second possibility is that the detected QTL is actually false and has no effect on DMD whatsoever. This possibility seems unlikely given that the LOD score of 7.4 obtained by composite interval analysis equates to a P value of approximately 1e-8. With single marker regression, the LOD of 3.37 equates to a P value of less than

0.001. It must be admitted that this value is greater than the value of 0.0001 that was suggested by Bernardo (2004) for use in marker-trait regression analysis.

The final possibility is that the 2-row/6-row gene, *Vrs1*, is masking the effects of the 6H DMD QTL. Drummond is a 6-row cultivar and Baku is a 2-row cultivar and it has been shown that 6-row cultivars generally have lower DMD than 2-rows (Bowman *et al*, 2001). In populations segregating for *Vrs1*, head type accounts for 30-60% of variation in DMD. It has been suggested that the reduced DMD is the result of lateral kernels escaping extensive cracking due to their reduced size and that less damaged kernels allow less microbial colonization. The Drummond/Baku population again shows that the 6-row head-type is associated with reduced DMD (Table 3-10).

Table 3-10. Type III tests of head type effects on DMD and mean particle size (PS) in an F_5 validation population

Trait	Head Type	Mean[a]	F value	P Value	R^2 (%)
DMD (%)	2-row	46.8	41.89	<0.0001	35
	6-row	35.5			
PS (µm)	2-row	1125	63.26	<0.0001	47
	6-row	1372			

[a]Least squares means

Contrasts between Bmag0009 alleles within head type were not significant (Table 3-11). Because of the small numbers involved in the contrasts, however, it is difficult to say whether the lack of significance is meaningful.

Table 3-11. Type III tests of Bmag0009 within head type

Head Type	Bmag0009	Mean DMD (%)[a]	F Value	P Value
6-row[n=52]	Drummond	35.6	0.01	0.93
	Baku	35.4		
2-row[n=42]	Drummond	47.0	0.02	0.88
	Baku	46.7		

[a]Least squares means

We have evaluated the DMD of a 96 member population derived from the same cross of Haxby and Baku. This population may be more useful for validation of the DMD loci.

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