GENETIC EXPLORATION OF SPIKE AND SEED MORPHOLOGY IN A TWO-ROWED BARLEY NESTED ASSOCIATION MAPPING PANEL

by

Megan Marie Getz

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ABSTRACT

Barley (*Hordeum vulgare*) is unique as it has six-rowed cultivars and two-rowed cultivars. The six-rowed has three fertile florets on each spikelet, while two-rowed has only a single fertile center floret per spikelet. This by itself affects seed uniformity as seed produced in the lateral florets tend to be less symmetrical and smaller in size than the center floret. Yield and plumpness are also affected by spike morphology. While the genes and alleles affecting six-rowed vs. two-rowed spikes are known, there are a number of other genes that affect additional spike morphological traits such as number of seeds per head, length of head, and compaction of spikelets. Here, a two-rowed Nested Association Mapping (NAM) population was used to genetically dissect barley spike and seed morphology. To create the population, 13 diverse founder lines of barley were crossed with Conlon, a high yielding, plump, spring variety. Approximately 80 progenies were selected from each of the Conlon by founder crosses, and then backcrossed to Conlon in hopes of exposing the advantageous traits while adapting lines to the Great Plains of the Northwest. The population varying for spike and seed morphology was grown out in a field trial in Bozeman, MT and Logan, UT where phenotypic data was collected throughout the stages of growth and harvest. At maturity (Zadok's 50) stage five heads from each of the lines were collected. Digital Image Analysis was used to measure spike length, kernel count, density, and seed size. Genetic maps were created for each family as well as a consensus map for the whole population. Associations between the phenotypic data and the genotypic data observed in the individual families and consensus map allowed us to identify QTLs conserved across multiple families as well as unique to individual families. Evidence of pleiotropic effects between traits was observed. Some of the QTLs previously were identified, and some are novel. One of the families was of particular interest due to unique QTLs impacting seeds per spike and seed weight. Additionally, several novel QTLs were identified on chromosome 7H that highly impact seed traits.

INTRODUCTION

Barley (*Hordeum vulgare* L.) has a variety of end uses, including animal feed in the form of grain or forage, biofuels, distilling, and malting, or as a whole grain food for human consumption. Specific end uses require released varieties containing specific qualities. Some desired traits are consistent across end uses, for example, yield; while other traits are specific to end use. Seed size and uniformity is essential for most uses. Kernel size is negatively correlated with grain hardness and particle size and positively correlated with dry matter digestibility, one of the significant parameters for cattle feed quality according to Turuspekov et al. (2008). Kernel size and uniformity are also important determinants of malting quality (SýkoroVá et al., 2009). A general rule of thumb is that larger seeds are better for malt, and smaller seeds are better for feed (Bowman et al., 2001). Furthermore, Spilde (1989) reported the importance of seed size uniformity for planting, finding that grain yield produced from small seed was significantly less than that from large seed. Grain yields from crops planted with small seed averaged 4 to 6 % less than those from medium to large seed size.

Breeders require genetic diversity to make improvements and sometimes use founder lines in crosses to provide new alleles, particularly for disease resistance. A more difficult prospect, because of linkage drag of poor alleles, is using un-adapted lines to improve quality or yield. The first step in this process requires identification of favorable alleles for quality attributes existing in otherwise poorly adapted germplasm. In an attempt to access new alleles from founder lines, a two-row Nested

Association Mapping (NAM) population was created from the Barley World Core germplasm collection.

The goal of this study is to exploit the two-rowed NAM population and identify QTLs associated with spike and seed morphology and uniformity, which are essential traits for both yield and quality. Major genes that control the six-row versus two-row spike type have been found to affect yield, plumpness, and seed uniformity. Here, the NAM population is being used to genetically dissect genes specific to two-rowed lines that impact spike and seed morphology. To create the population, lines from the world core were genotyped, and 100 that were genetically diverse were selected to be crossed with Conlon, a high yielding, plump, spring variety, creating approximately 100 families each with about 90 lines. Each line was then backcrossed to Conlon in hopes of exposing new alleles while eliminating some un-adapted traits.

The population was then grown in Bozeman, MT where phenotypic and phenological data were collected. Initially, five heads from each of the lines were collected for 30 families that were observed as segregating for spike morphology. Digital Image Analysis was used to measure the length and width of heads as well as seeds per head, seed density, and seed size to determine a smaller, more manageable, population for the 2016 field season. In 2016, 13 families including 1,133 lines were grown in Utah and Montana. Agronomic data was collected. The phenotypic data along with the genotypic maps were used to discover QTLs that affect spike morphology, yield, and kernel uniformity of size and shape.

LITERATURE REVIEW

History and Economics of Barley

Roughly 10,000 years before present (BP), ancient families pioneered a new way of life by initiating the domestication of barley (Hordeum *vulgar* L.). Early farmers recognized that barley could handle harsh abiotic factors such as saline soils, drought, low nutrients, late frosts, and harsh direct UV levels and still produce seed (Kurowska et al. 2012). The origin of domesticated barley is still not clear. However, Badr et al. (2000) suggest that Israel-Jordan in the southern part of the Fertile Crescent is the most likely geographical area for barley domestication, which then spread towards the Himalayas. They noted that many landraces from the Himalayas indicate that allelic substitution took place during barley migration across Asia. Thus, calling the Himalayas a region of domesticated barley diversification, not domestication. In contrast, Zohary et al. (2012) support the theory that domestication of barley has two independent origins, one within the Fertile Crescent and a second event further east at the edge of the Iranian Plateau.

Since barley was a primary food source, farmers saved seed from their favorite plants, resulting in the selection of germplasm. Throughout history, barley was found in most civilizations. It has served as the staple crop for many locations and even as the grain of choice for the well-known Roman Gladiators, the “Hordearii” or “barley men” due to its ability to provide a consumer with strength and stamina (Percival, 1921).

Barley has many dietary benefits such as low fat, complex carbohydrates, well-balanced protein to meet amino acid requirements, minerals, vitamins, especially Vitamin E and other antioxidants. High in fiber, barley impacts cardiovascular health, diabetes control and lowers blood cholesterol. (Baik & Ullrich, 2008).

In 1324, English King Edward standardized the "inch" based on three kernels of sun-dried barley placed end-to-end lengthwise (Watson, 1915). During both World Wars, roasted malt was used as a coffee substitute, especially in Italy. Even today we see barley in many diverse locations fulfilling multiple end uses. Barley is cultivated in the highest plots in the world near Lake Titicaca at 15,420 feet above sea level, providing the people a carbohydrate to eat. On the other side of the world, approximately 6,145,130 bushels of barley was malted in the United States of America to provide, on average 323.5 million gallons of beer for the last five Super Bowl football games (Council, 2017).

Looking economically, barley is a significant U.S. crop with an estimated value of $1.2 billion as a raw agricultural commodity in the 2012 Census (Committee, 2018). Barley exports also benefit the US economy with around $60 million for raw barley and milled products, $225 million for malt and extracts, $600 million and $1.06 billion for beer and whiskey respectively (Committee, 2018). Domestically, approximately 70% of all barley is malted for beer production, 20% for feed, 3% human food, and 2.5% for each whiskey and seed. Unpredictable yield is impacting barley production. Minnesota, North Dakota, and South Dakota have historically been the highest barley producing

states, but since 2013, when Montana planted 400,639 hectares of barley, Montana had the most planted hectares. In 2017, Montana planted 283,280 hectares of barley, accounting for approximately 30% of all United States grown barley ((USDA), 2017). The movement of production out of the Midwest has been due to two important factors. First, production in the Midwest has been threatened by Fusarium head blight. Second, movement of corn and soybeans into the northern Midwest has been possible due to the creation of cold tolerant varieties. Corn and soybeans are lower risk crops, as barley growers can often have their crop rejected for malt quality reducing its value by half. Therefore, as more northern varieties of corn and soybeans become available, the more barley production is pushed north and west directly to Montana where barley can thrive in environmental conditions unsuitable for many other crops.

Genes Impacting Spike and Seed Morphology

Barley, a diploid cereal grain, consists of 2x= 14 chromosomes with a haploid genome of 5.1 gigabases. During the process of cereal domestication, humans selected for traits that increase yield, one of which was the appearance of six-rowed barley about 8,000 years ago (M. Pourkheirandish & Komatsuda, 2007). All barley heads consist of a series of nodes alternating side to side along the center rachis. Each node consists of three spikelets (Baik & Ullrich, 2008). In two-rowed barley, the earlier row type, only the center spikelet is fertile, and the outside two spikelets are sterile so that a cross-section of the spike shows two kernels.

Figure 1 illustrates row type. Top picture is a two-rowed barley spike. Bottom picture is a six-rowed barley spike.

Figure 1 illustrates the difference between a two-rowed barley spike and a six-rowed barley spike. Due to the limitation of size and nutrient uptake six-rowed barley kernels tend to be smaller and less uniform than two-rowed kernels, this is especially true for the two outer seeds called the side laterals. S. D. Tanksley and McCouch (1997) stated that often a substantial portion of genetic variation in a population could be explained by a few genes of moderately large effects. This is true for spike type in

barley. There are five well understood, independent loci controlling the six-rowed spike phenotype (Pourkheirandish & Komatsuda, 2007). The first is *vrs1,* this recessive gene, located on chromosome 2HL, determines row type by effecting sterility of the side lateral florets. Observed in all cultivated six-rowed barley, the mutation causing recessive vrs1 during barley domestication is the cornerstone for the origin of six-rowed barley (Pourkheirandish & Komatsuda, 2007). Cloning of vrs1 gene revealed that Vrs1 encodes a member of the homeodomain-leucine zipper (HD-ZIP) class of transcription factors. The dominant nature suggests Vrs1 codes for a repressor protein that binds to the DNA of genes and regulates the development of lateral spikelets (Doebley et al., 2006; Komatsuda et al., 2007). Next, int-c is a recessive gene on 4HS and is detected in many two-rowed barley cultivars. This gene alters the degree of side lateral floret sterility by impacting anther development, and occasionally causes random seed formation in the lateral florets. Lastly, vrs2, vrs3, and vrs4 are all independent recessive genes on 5HL, 1HL, 3HL respectively, but these three are not naturally occurring mutations and not observed in barley cultivars. Like int-c, vrs2, vrs3, and vrs4 enhance lateral spike development. The impact of the six-rowed versus two-rowed row genes on seed and head morphology is well understood. Less well understood are other genes impacting these traits. Hence the focus of this study on two-rowed barley types to isolate variation for seed and spike morphology.

While row type does impact seed size and uniformity, it is not the only factor. Independent of spike type, the position of seed on the spike also affects seed size.

Spikelets in central positions have earlier floral development, e.g., pollen and stigma development, then spikelets at the apical, or basal part of the spike (Arisnabarreta & Miralles, 2006). The second kernels from the basal end of the spike tend to be largest, with variation in seed size as the seeds travel up the spike. Not only does seed size change within a two-rowed spike, but also the number of seeds, spike length, and the shape varies. Many previous studies have reported genes identified in barley. Review "Description of Barley Genetic Stocks" for 2016 (Franckowaik et al., 2016). However, below are genes known to impact traits of interest in this study. Many exhibit pleiotropic effects.

Eam-1/ Ea / Ppd-H1, reported by Nilan (1964), D. Laurie, Pratchett, Snape, and Bezant (1995), controls a strong photoperiod response to short nights in some cultivars, causing earlier plant maturity. Located on 2H, the *Eam1* locus is weakly linked to *vrs1* (Luna Villafaña, 1995; Wexelsen, 1934) and is most likely homoeologous to the *Ppd1*, *Ppd2*, and *Ppd3* loci in wheat (Laurie et al., 1995). Other members of this gene family alter flowering time and include *Eam10* on 3H, *Eam7* on 6H, *Eam8* on 1H, and *Eam9* on 4H (Franckowiak & Lundqvist, 2012).

The semi-dwarfing and dwarfing genes were an important contributor to the major increase in yield during the Green Revolution (Sakamoto & Matsuoka, 2004). In barley, *Denso* on chromosome 3H, also called *sdw1*, is found in many European cultivars, while in Asia, the *uzu1* (3H) and *dsp1* (7H) genes generally impact plant stature (Bezant et al., 1996; Franckowiak & Lundqvist, 2012; Kicherer et al., 2000).

Sameri et al., (2006) reported pleiotropic effects from *uzu1* on spike length. *Denso* and *dsp1* share similar dense spike characteristics by exhibiting shorter spikes with more seeds (Sameri et al., 2009). Furthermore, the semi-dwarf (*sdw*) and slender dwarf (*sld*) suites of genes include *sdw3* on 2HS, *sdw2* on 3HL, *sdw7* on 4HL, *sdw4* on 7H and, *sld2* on 2HS. All reduce plant height affecting culm and/or spike length. Thinner seeds and altered heading dates are generally associated with the *sdw* genes (Franckowiak & Lundqvist, 2012). *Sld2* is linked to an *Eam1* gene, explaining the earlier heading observed in plants carrying *sld2* (Franckowiak, 1994). The dense spike genes (*dsp*) alter spike length as result of reduced rachis internode lengths. The *dsp* genes include *dsp11* on 1H, *dsp10* on 3H, *dsp9* on 6H, and *dsp1* on 7H. Like *sdw4*, the *dsp1* and *uzu1* alleles are additive and can have pleiotropic effects on grain size (Sameri et al., 2009; Takahashi & Yamamoto, 1951).

Zeocritons (*Zeo*) 1, 2, and 3 are also known as erectoides “c” and “r.” They regulate spike density and seed width. *Zeo1*, *Zeo2,* and *Zeo3* loci are very close to each other on chromosome 2H (Druka et al., 2011). Plants carrying *Zeo1* have very compact spikes and reduced fertility, while *Zeo2* have two to four extra fertile spikelets per spike, but have lower test weights. *Zeo3* had shorter rachis internodes. Height may also be affected in some environments (Druka et al., 2011; Franckowiak & Lundqvist, 2012). In a study by Druka et al. (2011), some barley plants had reduced heights, but all seed shape and weights were similar. An erectoides gene has been identified on every chromosome in barley. All alleles impact spike density by controlling rachis internode

length and can shorten plant height in some environments. Some, such as *ert-u*, elongates rachis internode length, *ert-zd* shorten rachis internode length and widens seed and *ert-q* results in slightly irregular spikelet positions. For a complete list of *ert* genes see Tsuchiya (1976).

The *glo-c* and *glo-a* genes, on chromosome 2H and 4H respectively, promote more round, or globe-shaped seeds when compared to the parents' oblong seed shape. As a result, the spikes are often more laxed and less dense (von Wettstein-Knowles, 1992).

In summary, a number of genes have been identified that could impact spike and seed morphology on every chromosome. However, most of the previous studies were bi-parental and so the number of alleles observable at one time is reduced. Here the NAM (nested association mapping) allows us to observe individual family alleles, while also allowing comparison across the families.

Requirements for Various End Uses

Though many barley traits are desired across all uses, like hardiness of plant, high yielding, and seed size uniformity, many barley traits are specific to each end use, and many are based on seed morphology. A general rule of thumb is larger seeds are better for malt, and smaller seeds are better for feed (Bowman et al., 2001). In a study done by Y Turuspekov et al. (2008), six-rowed barleys were more variable in grain hardness when compared to two-rowed. The authors further found that seed size is

negatively correlated with grain hardness and particle size and positively correlated with dry matter digestibility, one of the major parameters for cattle feed quality. Additionally, Spilde (1989) showed that grain yield produced from drilled small seed was significantly less than that from the drilled large seed, averaging a four to six percent difference in yield. Also, the small grains, generally found in six-rowed types, tend to have lower starch and higher protein levels. Alternately, large grains have increased levels of starch, and therefore they have more extract potential, which can impact the malting process. Kumar et al. (2013) and SýkoroVá et al. (2009) agree that for consistent processing and high malt extract, barley grain used for malting needs to be uniform and plump. Hence, two-rowed barley grain is preferred for malting as the central florets produce the largest and most uniform seed. The malting industry also requests hulled barley while the food industry prefers hull-less. According to Pomeranz and Shands (1974), barley has many characteristics that make it a good food candidate. Ideal food barley is clean, bright yellow-white, plump, thin-hulled, medium-hard and uniform in size.

Ultimately, the ideal seed morphology varies per end use, but within each use, seed size uniformity is desired to produce the best products. This is a hard goal to achieve while simultaneously targeting a high yield under our changing environments.

Increasing Genetic Diversity

Badr et al. (2000) claim barley is one of the most genetically diverse cereal grains however due to the many bottlenecking events such as domestication, selection of six-row type, and hundreds of years of selection of elite cultivars for each specific end use, has resulted in a narrowing gene pool for grain improvement. There is a tendency to cross elite cultivar by elite cultivar, which exacerbates the issue. Feuillet et al. (2008) state that relatively little germplasm from outside the primary gene pool has been introduced into elite cultivated materials. New germplasm is needed. Tanksley and McCouch (1997) plea that the world cannot expect to maintain, let alone increase the current level of agricultural inputs. Resources are running low; farmland is being lost. In an attempt to keep crop production at levels needed to keep pace with the predicted increase in the human population, genetic improvement of crops is the most viable approach to do so. Many beneficial alleles have undeniably been left behind.

Looking to landraces and wild relatives is a solid starting point. However, this can be difficult for a few reasons in barley. First, the brittle rachis. Evolutionarily, a brittle rachis allowed wild barley to plant itself through seed shattering, but it made harvesting seed intense, and almost impossible. Domestication events selected against a brittle rachis. In many landraces and almost all wild relatives (e.g., *Hordeum bulbosum, Hordeum spontaneum*) a brittle rachis is still seen, making it hard to evaluate potential agronomic traits (Pourkheirandish et al., 2015; Pourkheirandish &

Komatsuda, 2007). Secondly, wild barley is not adapted to agronomic growing conditions (Nice et al., 2016). Evaluating accessions that were collected from environments around the world that vary in basic climate conditions is challenging. Dormancy, vernalization requirements, photoperiod sensitivity, germination temperature and moisture are a few examples of hurdles to be overcome before evaluation even occurs.

Many backcrossing schemes were developed to address these challenges efficiently. Backcrossing, simply put, is crossing a specific offspring (noted as F_1 with allelic parental proportions of 50:50) of two parents back to one of the parents. The backcross results in allelic proportions to skew towards that parent (BC_1 with allelic parental proportions of 75:25). An additional cross to that same parent may be done again resulting in an advanced backcross (BC_2 with allelic parental proportions of 87.5:12.5) and so on. The number of backcrossing events is dependent on the goal. If the goal is to scout for new alleles from un-adapted barley under adapted conditions, then the fewest backcrossing events that allow the plant to perform in adapted conditions while maintaining the highest allelic portions of the wild barley is preferred. Discovering and mobilizing these desired alleles from wild relatives into cultivated barley was termed “Advanced backcross quantitative trait loci” by Tanksley and Nelson (1996).

Use of backcrossing methods has been very successful in many crops to identify new germplasm that can increase yield and quality, as well as provide biotic and abiotic

stress resistance. An example of success using wild germplasm in barley is a broad-spectrum resistance against *Bulmeria graminis* f.sp. *hordeii,* known commonly as mildew, found in Ethiopian landraces (Feuillet et al., 2008; Pickering et al., 2006). Another example is the introgression of resistance alleles against scald (*Rhynchosporium secalis)* from H. *bulbosum* (Pickering et al., 2006).

While there is success in identifying and using beneficial alleles found in un-adapted germplasm, one common issue is linkage drag of poor alleles. Simply put, linkage drag of poor alleles means that non-beneficial, or even deleterious alleles link to the desired alleles. Breaking that linkage is sometimes extremely hard. One way to combat this is to combine backcrossing schemes, which can help break the linkage. This allows for more thorough investigation of alleles and their benefits and relations (Liu et al., 2016).

QTL Mapping

Simply put, quantitative trait loci (QTL) mapping is like a road trip. The roads traveled are chromosomes on the linkage genetic map, and the molecular markers are like signs letting the driver know where they are and what is at that location. The QTLs, are points of interest found along the way that give us the information we are curious about. They are chromosomal regions identified by using markers that associate with quantitative trait variation.

Researchers utilize QTL mapping projects for many reasons. One reason is that the population structure can help dissect complex traits within a population, we can increase our knowledge of the biology and genetics through inheritance and architecture (Mackay, 2001). Another reason is QTL mapping in plants can help identify molecular markers for potential selection purposes. The focus can be major effect QTLs to be introgressed into the breeding material or minor effect QTLs, which can be stacked and serve as a base germplasm selection (Bernardo, 2008). The goal of the QTL mapping project will determine the level of resolution for identifying the QTL, as well as, the level of power, robustness, or stringency of calling the presence of a QTL is needed. A genetic mapping study consists of a population that has been phenotyped for specific traits and genotyped. A linkage map is developed from the population's genotypes, then a statistical analysis of phenotypic variations associated with genotypes results in QTLs.

The linkage map is constructed by determining the amount of recombination between morphological markers and/or molecular markers. The lower the recombination frequency, the closer the markers are. The overall goal of each step is to create the shortest map possible with the given marker set. To achieve this, first, markers are grouped as linkage groups where recombination frequencies between markers are lower than 0.30. Only polymorphic markers can be positioned in the groups. Generally, each linkage group represents a single chromosome. The likelihood ratio test statistic, also called logarithm of odds (LOD), between marker pairs, or pair-

wise recombination frequencies determine initial groups. Recombination frequencies are calculated by taking the number of recombined gametes between two loci, divided by the total number of gametes.

$$\text{Recombination frequencies (or fractions)} = \frac{Number\ of\ recombined\ gametes}{Total\ number\ of\ gametes}$$

A recombinant gamete has a different allele at one of the two marker loci when compared to one of the two parental genotypes, while a non-recombinant gamete would have the same alleles at two markers as one of the two parental genotypes. While LOD scores are also based on this recombination fraction, they are more complicated. LOD scores are equal to the $\log_{10}$ of the probability that two loci are linked divided by the probability that two loci are unlinked (Xu, 2013).

$$\text{LOD Score} = \log_{10}\frac{Probablility\ that\ two\ loci\ are\ linked}{Probablility\ that\ two\ loci\ are\ UNlinked}$$

According to (Xu, 2013) a general guideline for evidence of linkage between two markers is a LOD score of three or more. A LOD of three means the likelihood of having linkage between two specific markers is 1,000 times greater than the likelihood of not having linkage between the markers.

Once linkage groups are formed, the markers within each group need to be ordered. The ordering process is like the Traveling Salesman problem, the shortest distance is desired between markers. There are many methods for this; we will only provide a brief introduction here of three methods. In 1987, the Seriation method was published as an ordering algorithm, which considers a distance matrix of pairwise recombination frequencies for n loci where r_{ij} is the estimated recombination

frequencies between the i^{th} and the j^{th} locus in the matrix (Buetow & Chakravarti, 1987). In 2005, recombination counting and ordering method, known as RECORD, was published. It calculated the pairwise expected number of recombination events in a specific mapping population from genotype data (Van Os et al., 2005). Then, in 2015, nnTwoOpt became available. It proposed to use the nearest neighbor algorithm for a tour construction which results in an arbitrary vertex where the closest vertex to the last vertex is considered to include it in the tour, repeating until all vertex has been considered. Then it links the last vertex to the first. The tour is then broken at the longest interval (Meng et al., 2015).

Following marker orderings, rippling was used to fine-tune marker placement. Rippling used permutation tests to compare all possible map orders within a marker window of a select size. The shortest distance between markers indicates the highest probability of correct order. Many rippling methods are available, but two popular methods include Sum of Adjacent Recombination Frequencies, or SARF, which uses an estimation of recombination frequencies noted within the window, and Sum of Adjacent Distances instead of recombination frequencies (Meng et al., 2015). Ultimately, any map is the best statistical representation of the data possible.

After the linkage map is constructed, the segregation of genotypes and the segregation of phenotypes are associated statistically. Many methods are available for use (see Broman et al., (2003)). However, an innovative method was used by the authors of the ICIM-mapping software which we have selected to use (Meng, 2015).

They named the method, the inclusive composite interval mapping (ICIM). The approach uses a stepwise regression to identify the most significant regression variables. Then, a one-dimensional scan is used to map additive effects, or a two-dimensional scan is used to map digenetic epistasis. A QTL is detected when a significant LOD score is observed for a given trait/genotype association. The LOD threshold of significance is set by the intent of the mapping purpose. A much more stringent LOD score is needed for gene identification and cloning than exploratory mapping. For each QTL identified for an individual trait chromosomal position, the LOD score, the additive effect of a parental allele, the direction of the effect (positive or negative), and the amount of phenotypic variance explained (PVE) by the QTL is determined. The significance of epistatic interactions between QTLs could also be determined.

Nested Association Mapping

Linkage mapping and association mapping are two of the most common genetic mapping strategies to help dissect the genetics that underlies phenotypes measured in the field. While both techniques utilize genetic markers, a primary difference between the two is that linkage mapping provides higher power, with lower resolution, while association mapping has high resolution with the lower power. In an attempt to combine the benefits from both strategies, Nested Association Mapping (NAM) was

created. To understand the structure and strengths of a NAM panel, a basic understanding of linkage mapping and association mapping is needed.

A linkage mapping approach uses a structured population created by a known cross. This structure provides high power enabling rare alleles if present to be detected as there are only two possible allelic variations per loci. However, since only the alleles present in the two parents are being tested the genetic diversity across barely is not represented. Also, a bi-parental population is created through limited recombination events, resulting in low resolution for mapping (McMullen et al., 2009). First reported by Sax in 1923, linkage mapping was used to look at an F_2 population of common-bean to link Mendelian loci for seed color to a novel QTL for seed size. However, until molecular markers were made readily available in the 1980's, linkage mapping was not commonly used (Lander & Botstein, 1989). Linkage mapping is a robust approach if interested in rare alleles. The population size is generally small and includes the two parents, but it is time-consuming and laborious to develop the population.

On the other hand, association mapping also referred to as linkage disequilibrium mapping uses an unstructured (unrelated plants and/or parents are unknown) and very diverse population. The unstructured population results in low power so that QTLs associated with rare alleles are not observable due to low statistical power. (Nice et al., 2016). This powerful tool was initially developed in human and animal genetics as there are population structure limitations that prevent the formation of linkage mapping populations. For example, inbreeding, or selfing, is a

common practice in plants but can cause significant side effects elsewhere, including fatality in animals. Instead, association mapping takes advantage of ancestral recombination events. A very high marker coverage is needed, but coupled with a large, diverse population association mapping offers high resolution (Rafalski, 2002; Yu et al., 2008). Association mapping in plants has gained popularity for several reasons. First, time, resources, and labor are not spent in developing a structured population. Secondly, larger association populations can be grown as prices for high density genotyping, even full genome scans, decrease. Thirdly, as high throughput phenotyping practices are developed, the ability to accurately and quickly collect data from larger association mapping populations increase. (See Zhu et al., (2008) for review).

In an attempt to combine the power of linkage mapping and the resolution of association mapping, nested association mapping (NAM) was designed (Bajgain et al., 2016; McMullen et al., 2009; Yu et al., 2008). McMullen et al. (2009) developed the first NAM population in corn (*Zea mays*); it used multiple structured subpopulations nested within an unstructured population. To do this one common parent, B73 was crossed to 25 diverse founders, creating a "family" of 200 for each of the initial crosses. Within each family, the F_1 seed underwent single seed descent for three generations creating recombinant inbred lines (RILs). These 25 families, each having 200 lines if looked at individually are bi-parental, linkage mapping populations. The author's high density genotyped the entire population. Power and resolution of the first maize NAM was compared to multiple line crosses with multiple different numbers of significant QTLs

and heritability levels using simulated QTLs (Yu et al., 2008). In each circumstance, the NAM led with significantly higher power and higher resolution based on the rapid linkage disequilibrium decay within 2000 base pairs (bp) in maize.

In a NAM design, the structured family populations create high statistical power allowing QTLs with broad effect ranges to be detected, including rare alleles. The common parent allows comparison between the unstructured founders and families by normalizing the genetic background (Bajgain et al., 2016; Blanc et al, 2006; Yu et al., 2008). Furthermore, the broad diversity allows more QTLs to be observed. Analysis of many alleles is performed, but the ability to look at the two alleles within a given family also exists. NAM has been very successful in many crops, such as maize, wheat (*Triticum aestivum)*, rice (Oryza sativa), and sorghum (Sorghum bicolor) (Mace, et al., 2013; McMullen et al., 2009; Nice et al., 2016; Saade et al., 2016; Schnaithmann, Kopahnke, & Pillen, 2014), for a variety of traits with a variety of goals, including interrogation founder parental lines for new alleles or clues about origin, domestication, and introgression as seen in maize (Liu et al., 2016). It is important to keep in mind that to have both high resolution and high power the population needs to be very large. The large population creates an issue when trying to phenotype in multiple environments and for multiple traits. A lot of labor, funding, and time is needed to exploit a Nested Association Mapping population successfully. We utilized a two-rowed barley NAM population to identify regions of genetic control (QTL) for improvement of uniformity, size and shape of spike and seed. We have three

objectives. The first objective is to determine the relationships between spike and seed morphology and other agronomic traits. Secondly, genetically explore spike and seed morphology of two-rowed barley. Lastly, identify unique alleles (genes) from diverse lines for barley improvement. We hypothesize that the two-rowed barley NAM will present regions of genetic control (QTL) of spike and seed morphology that are novel and independent from known genes.

MATERIALS AND METHODS

Field Population and Design

The two-row NAM population was created under the TCAP grant 2011-68002-30029. The population was created by crossing 100 founder parents to the adapted two-rowed parent Conlon to create F_1's. The F_1's were then backcrossed to Conlon to create the BC_1F_1. A BC_1F_1 plant from each founder by Conlon cross, was selected and underwent single seed descent for four generations, which created RILs (recombinant inbred lines). The NAM population consisted of over 8,000 lines from 100 founder parents backcrossed to Conlon. Dr. Richard Horsley's group at NDSU based the population structure on the six-rowed barley NAM (Nice et al., 2016). However, only one backcross to the common parent was utilized by Dr. Horsley. The entire two-rowed barley NAM population, ~8,000 lines, was grown in single row plots in the field season of 2015 at the Montana State University (MSU) Post farm in Bozeman, MT (45.403981°, -111.9630W°).

We evaluated the entire population in the field for spike morphology variation. Of that 30 families, which consisted of 2,179 lines, were visually assessed to vary for spike morphology and selected for further study. We collected five intact spikes from each of those 2,179 lines. Fifteen lines from each family of the 30 families were randomly selected, and spike length was measured. After the spikes were threshed, we used the WinSeedle software (Regent Instruments: http://www.regentinstruments.co

m/assets /winseedle_about.html) and Epson scanner V700 (Epson America: https://epson.com/Support/Scanners/Perfection-Series/Epson-Perfection-V700-Photo/s/SPT_B11B178011) to count the number of seeds per spike, measure seed length and seed width, and calculate seed volume and surface area.

Based on the preliminary evaluation described above 13 families with 1,133 lines that were most variable for seed and spike morphology were included in the further study. Table 1 lists the family number, the founder's plant identification ID, the family size, the improvement status and the origin of the parent. Of these 13 families, nine were landraces, and four had a malting background, each with a unique origin. Family 123 had the smallest family size with 56 RILs, and Family 64 with the largest family size of 88 RILs. The families had an average family size of 75 RILs per family, but a median of 80 RILs per family.

In the 2016 field season, we planted the 13 families at the Montana State University Post farm in Bozeman, MT as well as in the Utah State University (USU) Small Grains field plots in Logan, Utah (41.763303° -111.816162°). The Logan plots were irrigated and 1.4 m x 1.4 m with four rows, while the Bozeman plots were rain-fed and 0.9 m X 2.7 m with three rows. Due to limited seed amount and large size of the population, both locations utilized an augmented randomized complete block design. Each variety check was randomized within a block, and the experimental lines, including the founder parents, were randomized across the whole experiment. Logan consisted of 25 blocks, with 48 plots per block. Each block consisted of 46 lines plus two

replicated checks, Conlon (as it is the common parent for the NAM), and Hays (to give us a low yield comparison). Bozeman consisted of 40 blocks, with 32 plots per block that contained 29 un-replicated lines and three replicated checks. As in Logan, both Conlon and Hays were checks, but Champion (which is a large seed variety) was added as the third check.

Table 1 identification and origin of founder parents used in constructing the nested association mapping population for a two-rowed barley spike and morphology study.

Family Number	Parent ID	Family Size	Improvement Status	Origin
Conlon		Common Parent	Malt variety	United States
2	CIho11588	85	Landrace	Israel
5	CIho14250	87	Landrace	Afghanistan
6	CIho14394	74	Landrace	Armenia
18	PI227449	87	Landrace	Iran
32	PI286388	80	Landrace	Eritrea
39	PI296472	86	Landrace	Ethiopia
51	PI330397	59	Malt variety	Germany
52	PI342139	60	Landrace	Turkey
61	PI357314	82	Malt variety	Denmark
64	PI362203	88	Malt variety	Belgium
67	PI371056	57	Landrace	Switzerland
79	PI392451	77	Malt variety	South Africa
123	PI270692	56	Landrace	Peru

Phenotype Measurements

Agronomic Data Collection

We collected the following data in Logan and Bozeman. Heading date, was determined when 50 % of the plot had the first spikelet of inflorescence visible, based on cereal grain development stage 50 on the Zadoks scale (Commission, 2003; Zadoks et al., 1974). Maturity dates were determined when 50% of the plot had fully senesced glumes, which corresponds with the cereal grain development stage 92 on the Zadoks scale (Commission, 2003; Zadoks et al., 1974). We measured plant height right before harvest in centimeters from the base of the plant at soil surface up to spike top, excluding awns. Two height measurements were taken and averaged per plot. Plot length was measured from first to the last plant of plot rows. Spike type was identified as two-rowed or six-rowed inflorescences, to ensure all test lines were two-rowed. We calculated kilograms per hectare yield by using plot yield and length. Five intact heads from main tillers for each plot were randomly collected five days after the maturity date to ensure intact heads with full seed development. The difference between maturity date and heading date determined the grain-fill period. Culm length was calculated by subtracting the spike length from plant height. To ensure accuracy and consistency between locations we used the Fieldbook app for Android for field data collection (Trevor Rife, 2015).

Digital Image Analysis

We selected WinSeedle, from Regent Instruments, Québec Canada, (Inc., 2016) for the digital image analysis. The five intact heads collected for each line were scanned using an Epson scanner at 360 dpi. Following procedures in the WinSeedle manual, the Scanner and WinSeedle program were calibrated (Inc., 2016). However, to ensure consistency of calibration, and that no unseen program errors occurred, a permanent three-centimeter ruler was placed on the backdrop of the scanner and randomly measured throughout image processing. The rachis was then traced for spike length starting at the base of the rachis and extending to the top of the rachis in millimeters (Jantasuriyarat et al., 2004). The individual heads within each line were assigned a number, 1-5 (Figure 2). Using a single spike Winterstiger research thresher, we threshed the spikes one at a time to ensure no seed loss or mixing. Density, also known as rachis internode length, was calculated by the following formula to allow across family comparison, where the seeds per spike were divided by two for each spike row, divided by spike length:

$$Density\ (rachis\ internode\ length) = \frac{\left(\frac{Seeds\ per\ Spike}{2}\right)}{Spike\ Length}$$

We scanned the threshed seed from the individual heads on an Epson scanner with a tray system at 700 dpi (Figure 3). Scanned images were directly uploaded into WinSeedle for seed data collection. In addition to seeds per spike, each seed was measured for length, width, surface area, volume, and width-to-length ratio. The

length measured as a straight line from tip to tip in millimeters. Seed width was measured at the widest point perpendicular to the seed length line in millimeters. Surface area and volume were estimated by using the following formulas under the assumption of the ellipsoid model:

$$SA = LW\pi\sqrt{\frac{(1+\frac{H}{W})}{2}} \qquad Vol = L\pi * \frac{W}{2} * \frac{H}{2}$$

where L is seed length, W is seed width, H is estimated height. The width-to-length ratio, called seed roundness, was calculated by straight seed width divided by seed straight length. For quality control, size parameters were set to alert if an object was too small or large to be considered one seed. The filter prevented any two touching kernels to be combined as one large measurement or a piece of larger debris to be considered a seed. Seed weight, measured in milligrams, was a combined weight of threshed seeds from the five plot heads divided by the total seeds per spike of the five spikes.

Figure 2 illustrates the orientation of spikes for the scanned image.

Figure 3 illustrates the orientation of seed for the scanned image.

Statistical Analysis

Plots means were computed for each response variable. Where data from individual seeds were obtained, this was done by computing a mean for each individual spike and then computing the mean of all heads, and where data were collected from individual heads, the mean of all heads from a plot was computed. For each of the phenotypic traits describing spike and seed morphology, a Levene's statistic was calculated and transformed using the following procedure. For a given spike trait the median measurement from each plot was determined, and then the difference between each spike measurement and its respective plot median was calculated (Sangster et al., 2008; Schultz, 1985). For a given seed trait the median measurement from each spike was determined, and the difference between each seed and its respective spike median was calculated. Those deviations from the median constitute the data for the transformed spike and seed traits. Below is the formula used:

$$LS = | X_{ij} - \tilde{X}_{i.} |$$

Data for all response variables were checked for departures from normality by examining normality probability plots (QQ plots) and histograms. All response variables were then analyzed via analysis of variance using a model described in Wolfinger et al., (1997) for a randomized block augmented design treating the blocks as random and all entries as fixed effects using the lme function from nlme package in R (Pinheiro et al., 2014). The least squares means adjusted for field variation, were obtained from this

model. The least squares means from each location were then used in a model that included location, family, and line within family where location and family were treated as fixed and line within family as random effects using the lmer function in the lme4 package (Bates et al., 2014) in R. Empirical Best Linear Unbiased Predictors (EBLUP) were obtained for each line within each family. The EBLUPS were then used for subsequent QTL analysis.

Correlations among traits were computed within each family and across all families using the pair.panels function in the psych package in R (Revelle, 2011).

QTL Mapping

Linkage Map Construction

Eastern Regional Small Grains Genotyping Laboratory, located in Raleigh, NC used Genotyping by sequencing, GBS, to genotype each line in the population. They used tissue from the F_4 plants and parents for genotype sequencing. The lab sent a file for each family which contained the identified markers and the corresponding allelic calls. GBS includes a random sequence reduction step. Therefore all markers were not observed in all families. The genotype files were uploaded into TASSEL 5.0 for quality control (Bradbury et al., 2007). Working one family at a time, each family file was trimmed to remove any markers that were monogenetic for alleles between Conlon and the founder parent, as detection of QTLs in any mapping procedure is limited to

only those that are polymorphic in the population. Markers that were missing 25% or more calls were removed and not included in the linkage map construction.

Next, a genetic map for each family was made by using the software QTL IciMapping (Meng et al., 2015). Working family by family, the data was uploaded and underwent binning of redundant markers using the BIN function to identify non-informative markers. The BIN function identifies co-segregating markers as well as markers with segregation ratios significantly different from the expected Mendelian segregation ratios. Initial map construction was done using the "MAP: linkage map construction in Biparental population" function. We determined linkage groups by the anchors associated with identified markers in the raw GBS chromosome data. The nnTwoOpt algorithm determined initial linkage group order. Visual inspection of the linkage groups was done to ensure coverage. Rippling by Sum of Adjacent Recombination Frequencies (SARF) using a window size of 5 was used to investigate alternate orders. We repeated ordering and rippling until the best order, which resulted in the shortest linkage map, was discovered. We repeated this method for each of the 13 individual families.

Focus then turned to the consensus map. A marker was included for construction in the consensus map if it was present in two or more of the family populations. The BIN function was again used before the consensus map was created by using the "CMP: Construction of consensus mapping" function in the IciMapping software and implemented the same parameters as used in the family populations.

QTL Analysis

QTL mapping was conducted for each family using its specific family map and the inclusive composite interval mapping (ICIM) approach in IciMapping 4.1. A manual LOD threshold of 2.5 was chosen because the focus of this study is on discovery and any QTLs will be confirmed by further study, and for each QTL an estimation of allelic effects and phenotypic variance explained (R^2) was collected. The confidence interval was set by a one-LOD drop from the estimated QTL position. Using the Joint Inclusive Composite Interval Mapping (JICIM) approach in IciMapping 4.1, joint QTL mapping overall 13 family populations was carried out using the consensus map. For the JICIM approach, the LOD threshold was set by running the 1,000 permutations of trait data resampling method, with a type I error at $\alpha = 0.05$. An interval window of 5 cM with a walking speed of 1 cM was used. For a QTL to be declared significant, it must be greater than the highest LOD threshold found by permutation test with 1,000 permutations. In the consensus analysis, significant LOD determination by permutation was chosen as the NAM design offers higher mapping power (Buckler et al., 2009; C. Li et al., 2016).

RESULTS

Phenotypic Summary

Non-recurrent parents for this experiment were originally selected from the barley world core based on genetic variation (Table 1). The lines originated from around the world with seven from the Middle East, two from Africa, four from Europe and one from South America. Most of the lines are landraces; however, four of the lines are varieties used for malting. The parents and families included were selected because they varied in spike morphology, seed size and seed shape (Figures 4 and 5). Parent 123 has six-rowed heads while Conlon is two-rowed. Several studies have indicated row type has a significant impact on seed morphology. Therefore, lines showing uniform two-rowed head type were selected from the segregating 123 family. Several of the non-recurrent parents have longer spikes than Conlon, e.g., 61 and 6; while several of the parents have shorter spikes than Conlon, e.g., 18 and 123. The density of the seed along the spike varies, e.g., parent 79 more dense than Conlon and parent 18 less dense. The number of seeds per spike also varied between Conlon and non-recurrent parents, with family 123 having the most and family 18 having the least. Seed size also varied between Conlon and non-recurrent parents with parents 32 and 67 appearing bigger; while parents 51 and 61 were smaller. Also, note that most of the parents had more variable seed size than Conlon. Conlon seed appears rounder than

all the other parents. Note parent 52 had black seed; while the remainder had seed similar in color to Conlon.

Measurements of material from field study confirmed previous observations as well as confirming variation within families for traits. Conlon differed from some of the non-recurrent parents for agronomic traits (Table 2).

Agronomic Traits

Most of the non-recurrent parents had later heading, and maturity dates but a few were earlier than Conlon. The family with the greatest range in heading dates was 64. Maturity was more compressed than heading, and the family with the greatest range was 2. Some of the non-recurrent parents had significantly shorter lengths of grain-fill (52, 61, 64, 67, and 79). In all cases, these parents had latter heading dates shortening grain-fill. In all cases, transgressive segregation was observed for all the families with family 51 having the greatest range for grain-fill.

Although none of the parents were significantly different in plant height and yield many of the families showed transgressive segregation with Family 123 having the greatest range in plant height and Family 6 having the greatest range in yield.

Spike Morphology

Seven of the 13 non-recurrent parents had spike length longer than Conlon, although not always significant. Three of the parents had significantly shorter spikes than Conlon. Family 79 had the greatest range in spike length. Only one of the non-

recurrent parents had significantly fewer seeds per spike than Conlon (18); while four had significantly more seeds per spike (51, 64, 79, and 123). Family 123 had the greatest range of seeds per spike. None of the non-recurrent parents had significantly different seeds per millimeter on the heads, although all families have transgressive segregation for density, with family 123 having the greatest range.

Seed Size and Shape

Most of the non-recurrent parents were not significantly different from Conlon in seed weight, although one was significantly smaller (51) and two significantly larger (52, and 123) than Conlon. Family 32 had the largest range in seed weight, but all the families have transgressive segregation for seed weight. Conlon has wider seed than all the non-recurrent parents, although the difference between Conlon and parent 52 is not significant. The family with the greatest range of seed widths was 123. Most of the non-recurrent parents have longer seed than Conlon. The family with the greatest variation in seed length was family 123. The non-recurrent parents with the longest seed also have the greatest difference in surface area when compared to Conlon, although parent 51 has a significant difference in surface area without a significant difference in length and so in that case differences in width is impacting differences in surface area. Family 123 had the greatest variation in surface area. Seed volume was significantly larger in three of the non-recurrent parents (6, 39, and 52) when compared to Conlon, while seed volume was significantly smaller in four of the

recurrent parents (18, 51, 61, and 64) as compared to Conlon. Family 123 had the greatest range in volume. All the non-recurrent parents were significantly less round than Conlon, with Family 123 having the greatest range in seed roundness.

Figure 4 compares the parental spikes with a 1:1 ratio.

Figure 5 compares the parental seed from one spike with a 1:1 ratio.

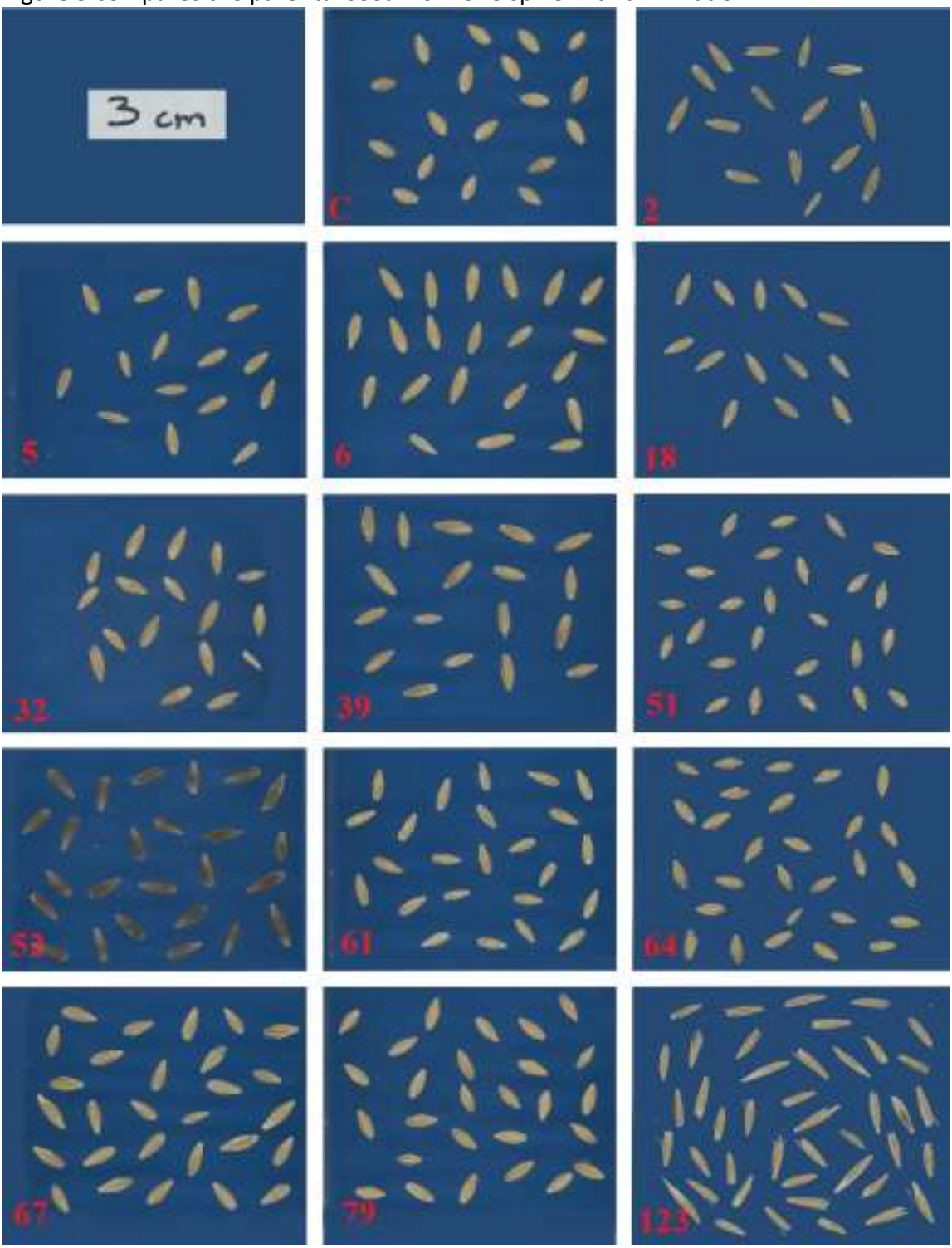


Table 2 Summary statistics for agronomic, spike, and seed traits for parents and the 13 families comprising the NAM spike and morphology study.

	Heading Date (julian days)						
Family	Conlon	Founder Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	171.35	172.46	170.32	165.82	174.62	8.80	2.64
5		167.28**	170.44	164.29	174.32	10.03	5.61
6		173.52	171.76	169.69	174.55	4.86	1.22
18		168.59*	169.62	163.62	174.71	11.08	6.88
32		167.74**	170.13	167.55	175.41	7.86	2.81
39		169.3	170.75	165.17	175.10	9.93	4.51
51		179.21***	175.98	171.23	184.18	12.95	8.25
52		175.84***	172.61	167.45	180.71	13.26	9.95
61		180.48***	173.13	170.04	180.48	10.45	2.07
64		184.95***	175.02	170.45	184.95	14.50	7.16
67		179.97***	174.37	169.41	182.35	12.94	11.16
79		181.93***	174.34	170.33	181.93	11.60	4.75
123		174.93**	175.19	169.23	180.32	11.09	8.21
	Maturity Date (julian days)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	201.89	202.35	200.55	197.06	205.27	8.21	2.05
5		196.72**	199.97	196.67	202.74	6.08	2.29
6		203.52	202.99	199.99	205.76	5.77	1.29
18		196.90**	200.15	196.90	204.68	7.78	2.99
32		197.19*	200.46	197.19	203.22	6.03	1.81
39		197.68*	200.74	196.63	203.95	7.32	2.67
51		207.48**	205.43	202.35	207.48	5.13	1.30
52		202.37	203.02	199.57	207.12	7.56	2.78
61		207.15**	203.63	200.44	207.15	6.70	1.79
64		205.80*	204.52	200.86	207.00	6.14	1.86
67		206.21*	204.11	200.76	207.34	6.57	2.15
79		205.41	204.79	201.81	207.19	5.38	1.66
123		205.82*	205.94	200.15	208.78	8.63	3.27

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level

Table 2 Continued

	Grain-fill (days)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	30.54	29.88	30.23	26.2	33.36	7.17	2.02
5		29.43	29.53	22.71	34.72	12.01	6.38
6		30	31.23	26.66	34.96	8.30	2.32
18		28.3	30.53	24.94	36.4	11.45	4.23
32		29.45	30.32	27.33	33.48	6.15	1.92
39		28.38	30	26.49	35.03	8.54	3.24
51		28.27	29.45	24.56	36.73	12.17	4.93
52		26.53*	30.42	24.91	32.68	7.77	2.54
61		26.66*	30.51	20.85	34.68	13.83	3.93
64		20.85***	29.5	24.83	35.14	10.31	4.84
67		26.24**	29.74	24.42	33.68	9.25	5.53
79		23.47***	30.45	26.24	35.69	9.45	2.99
123		30.88	30.75	25.78	36.44	10.65	4.46
	Plant Height (cm)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	58.43	55.88	57.88	53.39	61.93	8.54	3.69
5		56.76	59.67	54.07	64.70	10.63	4.67
6		59.14	62.8	58.43	67.02	8.59	4.01
18		56.45	58.2	54.26	63.76	9.50	5.33
32		60.93	60.92	55.86	66.19	10.33	4.38
39		66.21	63.32	56.78	69.53	12.75	5.72
51		56.94	60.61	54.04	64.32	10.28	5.43
52		62.52	61.51	56.31	67.92	11.61	6.30
61		60.83	59.49	55.74	67.19	11.45	3.13
64		61.66	61.84	57.75	65.58	7.83	3.16
67		65.18	64	60.68	69.41	8.73	4.71
79		63.23	62.54	58.83	67.92	9.10	3.44
123		70.89	70.65	61.55	79.15	17.60	9.85

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level

Table 2 Continued

	Yield (kg ha^{-1})						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	3589.58	3345.33	3490.05	3243.65	3721.93	477.75	3.77
5		2837.99	3028.44	2568.45	3335.65	767.2	6.97
6		3713.33	3726.78	3070.95	3951.13	880.18	5.29
18		3636.39	3825.77	3576.13	4108.76	532.63	4.94
32		3277.54	3287.76	2977.33	3516.42	539.08	4.12
39		3240.42	3370.62	3060.19	3632.63	571.9	4.30
51		3690.73	3595.5	3300.67	3791.88	490.66	4.34
52		3737.54	3717.63	3480.37	3990.4	510.03	4.83
61		3330.8	3525.02	3222.13	3755.29	533.17	4.48
64		3826.31	3841.37	3544.93	4079.71	534.78	3.28
67		3865.58	3803.71	3609.49	4021.6	412.11	3.45
79		3657.91	3888.18	3657.91	4132.43	474.52	4.05
123		3548.7	3599.81	3272.7	3975.34	702.64	6.27
	Spike Length (mm)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	84.86	75.28*	87.77	75.28	97.07	21.79	16.89
5		78.75	86	75.70	96.74	21.04	14.72
6		90.85	88.31	79.97	94.06	14.09	8.60
18		73.71**	83.11	73.40	93.80	20.39	17.43
32		86.91	88.23	77.46	96.59	19.12	18.71
39		83.09	83.82	73.38	96.47	23.09	24.17
51		91.11	88.34	75.01	101.06	26.05	38.37
52		89.67	88.03	79.02	99.17	20.15	19.55
61		101.89***	88.6	80.62	101.89	21.27	13.76
64		90.33	93.31	85.18	102.68	17.51	17.19
67		93.60 *	88.23	76.20	97.66	21.46	23.36
79		79.46	88.45	74.35	102.59	28.24	32.59
123		73.48**	87.18	71.33	96.52	25.19	27.84

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Table 2 Continued

	Seeds per spike (seed/spike)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	21.03	18.49	21.32	18.49	23.54	5.05	0.76
5		18.71	20.74	18.05	22.55	4.50	1.21
6		21.47	21.49	17.82	23.02	5.21	0.71
18		17.00*	20.14	16.88	23.52	6.64	1.71
32		19.24	20.31	18.20	28.34	10.14	1.58
39		21.15	21.39	19.29	23.36	4.07	0.82
51		23.76*	21.56	19.46	23.76	4.30	1.11
52		21.53	21.43	19.28	23.98	4.70	1.13
61		23.1	21.68	20.38	23.35	2.97	0.40
64		25.12**	23.96	21.77	30.33	8.56	1.81
67		23.17	22.44	19.50	25.61	6.12	1.65
79		25.13**	22.76	20.25	25.13	4.88	1.12
123		30.03***	23.13	19.37	33.51	14.14	4.42
	Spike Density (seed/mm)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	1.24	1.19	1.22	1.13	1.37	0.24	0.002
5		1.16	1.21	1.02	1.35	0.32	0.003
6		1.18	1.22	0.97	1.34	0.37	0.002
18		1.11	1.21	1.02	1.36	0.34	0.006
32		1.10	1.15	0.97	1.80	0.84	0.008
39		1.27	1.28	1.16	1.49	0.33	0.004
51		1.32	1.22	1.13	1.32	0.19	0.002
52		1.20	1.22	1.10	1.31	0.21	0.002
61		1.17	1.23	1.09	1.32	0.22	0.001
64		1.39	1.29	1.22	1.74	0.52	0.004
67		1.25	1.27	1.19	1.38	0.20	0.002
79		1.60	1.29	1.18	1.60	0.42	0.004
123		2.23	1.35	1.16	2.59	1.43	0.053

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level

Table 2 Continued

	Seed Length (mm)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	8.29	10.32***	8.9	8.35	10.32	1.97	0.089
5		9.07***	8.52	7.53	9.22	1.69	0.071
6		10.11***	8.62	8.13	10.11	1.98	0.092
18		9.00***	8.42	7.98	9.00	1.02	0.052
32		9.77***	9.08	8.51	9.94	1.43	0.087
39		10.04***	8.87	8.24	10.04	1.81	0.084
51		8.28	8.62	8.15	9.09	0.95	0.039
52		10.27***	9.04	8.36	10.27	1.91	0.169
61		8.98***	8.38	7.95	9.16	1.21	0.036
64		8.54	8.51	8.24	8.98	0.74	0.025
67		8.96***	8.51	8.20	8.96	0.76	0.034
79		8.98***	8.39	8.00	8.98	0.97	0.030
123		13.33***	9.22	8.40	13.33	4.94	0.547
	Seed Width (mm)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	3.92	3.65***	3.9	3.65	4.09	0.44	0.006
5		3.58***	3.87	3.58	3.99	0.40	0.005
6		3.72**	3.95	3.72	4.06	0.34	0.003
18		3.54***	3.83	3.54	4.02	0.48	0.005
32		3.64***	3.87	3.64	4.04	0.41	0.005
39		3.71***	3.89	3.71	4.13	0.42	0.006
51		3.66***	3.85	3.66	3.98	0.31	0.006
52		3.82	3.9	3.69	4.04	0.34	0.006
61		3.58***	3.91	3.58	4.05	0.47	0.004
64		3.70***	3.86	3.65	4.02	0.37	0.005
67		3.70***	3.86	3.70	4.00	0.31	0.005
79		3.67***	3.82	3.65	4.03	0.38	0.006
123		3.38***	3.93	3.38	4.06	0.68	0.014

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level

Table 2 Continued

	Seed Surface Area (mm2)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	54.01	60.92***	57.97	53.29	62.8	9.51	3.5861
5		54.78	55.13	50.59	60.14	9.55	2.014
6		62.81***	56.57	52.48	61.58	9.10	4.0499
18		53.39	54.14	50.82	56.99	6.17	1.8056
32		59.56***	58.51	53.16	63.18	10.02	2.8827
39		62.63***	57.95	54.06	64.8	10.74	4.7167
51		49.21***	55.17	51.02	58.96	7.94	2.2799
52		65.99***	59.15	53.75	66.36	12.61	4.7923
61		53.13	54.71	51.21	59.83	8.62	1.9246
64		52.2	54.68	51.42	57.34	5.92	1.2106
67		55.17	55.04	52.44	57.99	5.55	1.5013
79		54.26	53.68	51.09	58.31	7.22	1.8019
123		68.75***	59.93	49.38	66.76	17.37	7.8332
	Seed Volume (mm^3)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	14.66	15.43	15.69	14.05	17.77	3.72	0.474
5		13.79	14.78	13.43	16.39	2.95	0.219
6		16.29**	15.49	14.07	16.91	2.84	0.430
18		13.27**	14.41	13.27	15.46	2.19	0.234
32		15.13	15.67	13.80	16.94	3.14	0.311
39		16.25**	15.7	14.15	18.45	4.31	0.607
51		12.45***	14.62	12.45	16.14	3.69	0.415
52		17.55***	16.03	14.15	17.81	3.66	0.441
61		13.31*	14.78	13.31	16.36	3.05	0.267
64		13.36*	14.59	13.25	15.74	2.50	0.229
67		14.24	14.73	13.83	16.11	2.29	0.229
79		13.75	14.22	13.14	16.16	3.01	0.281
123		15.63	16.35	12.31	18.12	5.81	0.726

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level

Table 2 Continued

	Seed Weight (mg)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	49.71	53.47	51.7	46.28	54.90	8.62	3.27
5		49.67	50.22	47.57	54.75	7.18	1.65
6		53.28	52.7	48.77	56.03	7.26	2.84
18		50.02	50.28	47.10	53.91	6.80	2.18
32		51.69	52.47	35.09	56.55	21.46	6.26
39		51.97	52.78	47.78	57.26	9.49	3.39
51		43.51*	49.37	44.77	52.74	7.98	2.86
52		58.33***	54.7	50.61	60.59	9.97	2.92
61		44.33*	50.11	47.47	53.23	5.76	1.31
64		45.67	49.04	38.42	52.53	14.11	4.21
67		48.04	49.11	44.75	54.46	9.72	3.91
79		45.61	48.93	45.00	52.32	7.32	2.14
123		58.53***	54.5	39.12	57.58	18.45	7.92
	Seed Roundness (width/length)						
Family	Conlon	Parent†	Family Mean	Minimum	Maximum	Range	Family Variance
2	0.47	0.36***	0.44	0.36	0.47	0.11	0.0003
5		0.39***	0.46	0.39	0.53	0.14	0.0004
6		0.37***	0.46	0.37	0.48	0.11	0.0003
18		0.39***	0.46	0.39	0.50	0.11	0.0003
32		0.37***	0.43	0.37	0.46	0.09	0.0003
39		0.37***	0.44	0.37	0.47	0.10	0.0002
51		0.44***	0.45	0.42	0.49	0.07	0.0002
52		0.38***	0.43	0.38	0.48	0.10	0.0006
61		0.40***	0.47	0.40	0.49	0.09	0.0002
64		0.43***	0.46	0.41	0.48	0.07	0.0002
67		0.41***	0.46	0.41	0.48	0.07	0.0003
79		0.41***	0.46	0.41	0.49	0.08	0.0002
123		0.27***	0.43	0.27	0.47	0.20	0.0011

† Significance of variation between parental lines.

* Significant at the 0.05 probability level.

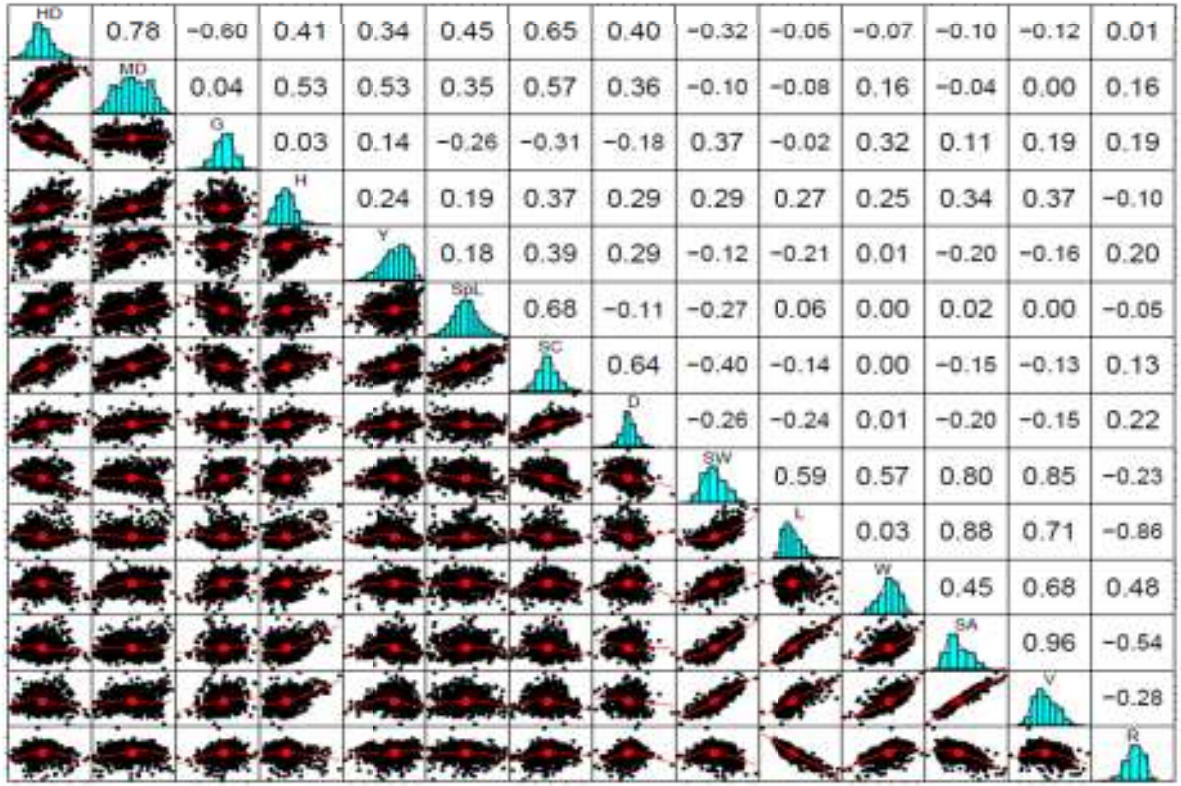
** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level

Correlation Summary

All the traits approached normal distribution (Figure 6). Several of the traits were correlated across the whole population. Heading date and maturity were correlated but not perfectly. Therefore differences in grain-fill were observed. Grain-fill was more highly negatively correlated with heading date indicating differences in grain-fill were more often related to differences in heading date and not differences in maturity. Late maturity correlated more highly with height and yield than heading date does. Both late heading and late maturity correlated with more seeds per spike. In some families early heading correlated with seed weight, as did longer grain-fill. The length of the heads correlated with the number of seeds per spike, but not with seed density per spike across populations. However, seeds per spike did positively correlate with seed density and negatively correlated with seed weight. Longer seed correlated with heavier seeds, greater surface area, and volume, but inversely correlated with seed roundness. Wider seed is rounder, with increased volume, but tend to have less surface area. Even though volume and surface area are correlated, we included both traits in QTL map due to their different interactions with seed width and length.

Figure 6 shows correlations between traits across the 13 NAM families used in the study. HD-heading date, MD-maturity date, G-grain-fill, H-plant height, Y-yield, SpL-spike length, SC-seeds per spike, D-density, SW-seed weight, L-seed length, W-seed width, SA-surface area, V-seed volume, R-seed roundness.



Linkage Map Summary

GBS markers used for genetic mapping had fewer than 25% missing data and major and minor allelic frequencies of no greater than 0.90 and no less than 0.10. Linkage maps consisted of 239 to 4,565 markers with an average of 2,429 markers per family. See Table 3 for the distribution of markers across families and linkage groups. Each family had seven linkage groups with linkage groups varying from 39 to 204 cM with an average size of 114.47 cM. The average distance between markers was 3.1 cM. It is important to note that the size of linkage groups and number of markers in a linkage group varies from family to family.

The consensus map resulted in a total number of 7,900 markers to be considered for construction by the allowance of any marker present in two or more families. However, due to many markers co-segregating only 204 “skeletal” marker positions were identified. This resulted in an average length of 99 centimorgans per chromosome with an average marker coverage of 3.2 resulting in a total length of 691 centimorgans. Table 3 summarizes all linkage maps. The lack of overlapping markers made the consensus linkage groups smaller and with fewer markers than linkage groups from individual families. We then interrogated the markers used for map construction for allelic distribution as shown in Figure 7. The percent alleles from the non-recurrent parent varied from 10 to 37%. With backcrossing we expected non-recurrent alleles to represent about 25%. Most families vary from 18% to 24%, which is within our expected range considering the backcross of the population structure. Some

of the families may have had different allelic frequencies due to sampling error inherent to GBS. Another possibility is fewer polymorphisms due to human selection of the parents. For example, Families 51, and 61 had the smallest percentage of non-recurrent parent alleles with only 10%. These two varieties are malt lines and could share more alleles with Conlon. However, parent 64 is also a malt line, and Family 64 has the highest non-recurrent parent allelic distribution. A final possibility skewing population toward the adapted parent is unintended selection during NAM population development. This could occur if certain un-adapted or deleterious traits in the non-recurrent parent did not allow the line to produce seed to be included in the final population, e.g., dormancy, unfavorable photoperiod reactions, plant height, seed set, or plant survivability.

Table 3 Summary of markers used for linkage map construction for individual families and consensus map for the two-rowed NAM spike and seed morphology study.

Chromosome	Markers for map construction	Unique markers in map	Length of chromosome (cM)	Average marker coverage (cM)
		Family 2		
1	505	67	129.56	1.93
2	754	40	39.75	0.99
3	789	71	50.52	0.71
4	562	32	39.86	1.25
5	865	42	119.49	2.85
6	582	31	42.48	1.37
7	507	31	98.97	3.19
Total	4564	314	520.63	1.66
		Family 5		
1	453	28	85.24	3.04
2	814	41	86.42	2.11
3	737	71	197.12	2.78
4	455	105	117.57	1.12
5	845	42	139.2	3.31
6	670	31	85.28	2.75
7	827	31	75.45	2.43
Total	4801	349	786.28	2.25
		Family 6		
1	80	19	42.03	2.21
2	269	19	59.27	3.12
3	158	34	76.56	2.25
4	152	16	35.76	2.24
5	176	24	60.33	2.51
6	335	30	72.81	2.43
7	105	16	47.21	2.95
Total	1275	158	393.97	2.49
		Family 18		
1	303	39	136.47	3.5
2	460	61	172.9	2.83
3	575	71	204.88	2.89
4	391	43	133.76	3.11
5	678	49	126.89	2.59
6	350	40	93.2	2.33
7	314	59	195.42	3.31
Total	3071	362	1063.52	2.94

Table 3 Continued

	Family 32			
1	236	50	98.43	1.97
2	49	49	56.98	1.16
3	115	47	107.5	2.29
4	39	39	170.93	4.38
5	40	40	45.46	1.14
6	81	51	94.3	1.85
7	132	47	117.7	2.5
Total	692	323	691.3	2.14
	Family 39			
1	257	59	136.43	2.31
2	341	80	185.69	2.32
3	683	46	201.79	4.39
4	206	47	190.43	4.05
5	674	74	189.59	2.56
6	216	26	206	7.92
7	640	39	172.74	4.43
Total	3017	371	1282.67	3.46
	Family 51			
1	46	46	50.16	1.09
2	17	17	72.25	4.25
3	20	20	80.52	4.03
4	20	20	53.63	2.68
5	79	79	98.4	1.25
6	23	23	54.91	2.39
7	34	34	119.72	3.52
Total	239	239	529.59	2.22
	Family 52			
1	394	21	79.77	3.8
2	420	86	208.72	2.43
3	401	33	155.14	4.7
4	136	24	172.69	7.2
5	471	44	153.23	3.48
6	410	24	89.95	3.75
7	282	52	184.86	3.56
Total	2514	284	1044.36	3.68

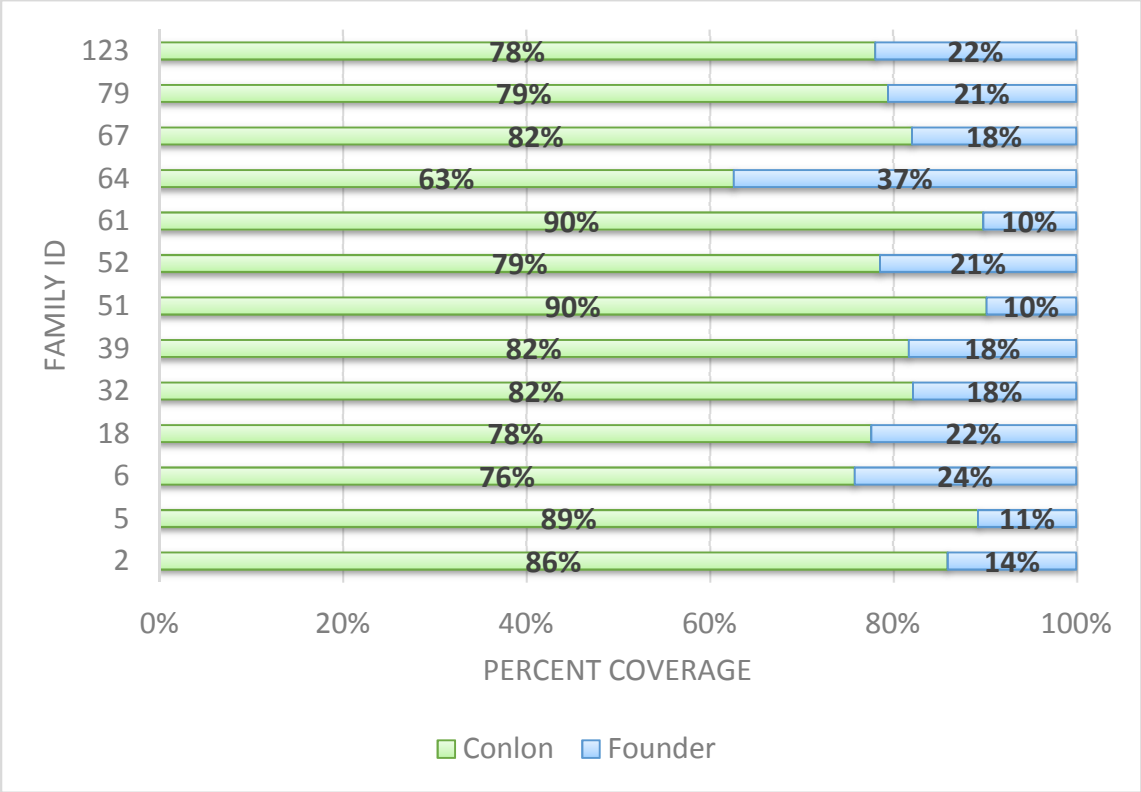
Table 3 Continued

	Family 61			
1	288	26	78.57	3.02
2	154	35	59.14	1.69
3	278	20	62.75	3.14
4	132	32	101.47	3.17
5	529	39	51.54	1.32
6	464	22	53.68	2.44
7	443	22	58.9	2.68
Total	2288	196	466.05	2.38
	Family 64			
1	148	35	80.08	2.29
2	85	42	86.38	2.06
3	16	19	87.55	4.61
4	19	19	158.98	12.23
5	45	17	42.42	2.5
6	163	39	73.09	1.87
7	282	66	183.8	2.78
Total	758	237	712.3	3.08
	Family 67			
1	403	25	103.26	4.13
2	530	38	208.73	5.49
3	479	36	180.34	5.01
4	380	40	194.29	4.86
5	456	70	196.21	2.8
6	625	36	94.54	2.63
7	654	29	185.07	6.38
Total	3527	274	1162.44	4.24
	Family 79			
1	126	35	194.22	5.55
2	223	25	117.17	4.69
3	232	30	158.12	5.27
4	118	32	127.39	3.98
5	133	17	90.74	5.34
6	108	19	71.21	3.75
7	541	52	121.13	2.33
Total	1481	210	879.98	4.19

Table 3 Continued

	Family 123			
1	429	34	109.5	3.22
2	511	24	46.24	1.93
3	236	46	188.05	4.09
4	429	40	115.88	2.9
5	728	14	57.67	4.12
6	426	40	151.43	3.79
7	642	45	187.46	4.17
Total	3401	243	856.23	3.52
	Consensus			
1	830	18	50.25	2.79
2	1289	28	84.58	3.02
3	1155	44	191.13	4.34
4	740	35	132.04	3.77
5	1480	31	84.04	2.71
6	1036	25	74.65	2.99
7	1370	23	74.59	3.24
Total	7900	204	691.28	3.38

Figure 7 Proportion of markers contributed from parent genomes in the 13 families in the two-rowed barley NAM spike and seed morphology study.



QTL Analysis

The consensus analysis identified fourteen QTLs. Due to low marker count chromosome 1H only had one QTL identified. We detected two separate QTLs on chromosomes 2H,4H,5H, 6H, and 7H. Chromosome 3H had three distinct QTLs. Table 4 summarizes all consensus QTLs by chromosome, position, and trait. For each QTL the likelihood of odds score (LOD), percent of phenotypic variation explained (PVE) or R^2, and the additive effect of Conlon allele at that position for each family is given. QTLs for heading date, maturity date, grain-fill, height, and yield were observed in similar chromosome regions. Note for any trait at any locus the allelic effects could be opposite depending on the family. Also, note that the relationship between traits at a given locus also varied across families. For example, when seeds per spike increased seed size often decreased (chromosome 1H family 32), but exceptions to this were observed (Chromosome 1H family 51).

Table 5 records QTLs observed in individual families. To be able to compare maps between families and between individual maps and consensus maps, marker positions needed to be standardized since there was variation in maps in sizes of linkage groups and in markers involved. This was accomplished by matching the left and right flanking markers of an individual family QTL with the marker's position within the consensus map, see "Consensus Position" column in Table 5. We determined family QTL "Consensus Position" by comparing relative marker position along chromosomes by dividing QTL left marker position by length of chromosome. (e.g., family 6 QTL at

position 24 cM on chromosome 4H, which had a total length of 35.76 cM. So, it had a relative position of 67.11%. Family 52 had a QTL at position 119 cM on chromosome 4H, which had a length of 172.69 cM. So it had a relative position of 68.91%.) Also reported in Table 5 are the significance of QTL (LOD), percent variation explained (PVE), and the additive effect of Conlon allele. Each family varied in the number of mapped QTLs. Family 18 had one significant QTL while Family 64 had 21. In the following comparison between consensus and individual family analyses, all significant individual family QTLs are included.

Agronomic Traits

For the consensus analysis at least one QTL impacting maturity and yield was identified on every chromosome. Heading date was impacted by at least one QTL on every chromosome except for chromosome 5H. A height QTL was detected on every chromosome except for 4H and 6H. No QTLs significantly impacted grain-fill in the consensus map.

In family analyses, heading date QTLs appeared on 2H, 3H, 5H, and 7H. Maturity appeared on all but 5H and 6H. Only Family 123 had a significant QTL impacting grain-fill on 6H. A QTL impacting plant height was identified on all chromosomes except for 1H. A QTL impacting yield was identified on all chromosomes, except for 3H.

Spike Morphology

For the consensus analysis, a QTL impacting spike length was identified on all chromosomes except for 3H. QTLs impacting seeds per spike were identified on chromosomes 1H and 2H. No density QTLs were identified in the consensus analysis. The largest PVE in the consensus analysis was identified on 2H impacting seeds per spike.

Family analyses exhibit QTLs altering spike length on chromosome 2H, 3H,6H, and 7H. Seeds per spike QTLs were identified on all chromosomes except 1H, while density QTLs were on all chromosomes except 6H.

Seed Size and Shape

In the consensus analysis, we observed QTLs for seed width and seed roundness on all seven chromosomes. Surface area was altered by QTLs on every chromosome except 1H. No significant QTLs impacting seed length, volume, or seed weight were identified in the consensus.

In the family analyses, QTLs for seed length and width were observed on all chromosomes except 5H and 3H respectively. QTLs for surface area were noted on 1H, 2H, 4H, 6H, and 7H. While QTLS impacting volume were identified on all chromosomes except for 6H. Excluding chromosome 7H, at least one QTL on each chromosome altered seed weight. Seed roundness exhibited QTLs on 1H, 2H, 3H, 6H, and 7H. Family 39 and 52 were the only families to have a QTL identified for a Levene statistic. In

Family 39 the Conlon allele offered uniformity to seed width and in Family 52 surface area.

Table 4 Quantitative trait loci (QTL) identified from the consensus map from the two-rowed NAM spike and morphology population.

Chrom	Consensus Position± (cM)	Trait	LOD$^{\Psi}$	PVE%$^{\tau}$	Family Additive Effect$^{\delta}$												
					2	5	6	18	32	39	51	52	61	64	67	79	123
1	17-34	Heading Date	20	2.8	-0.18	2.14	-1.83	0.55	0.17	-0.09	2.48	0.29	-1.2	-0.48	-0.06	-0.93	-2.33
1	17-34	Height	21.81	1.79	-0.14	1.22	0.96	2.76	0.87	0.2	-1.01	0.89	0.61	0.85	-0.01	-0.51	2.07
1	17-34	Spike Length[1]	18.95	1.61	-1.65	0.89	2.53	-0.11	-0.31	0.02	7.41	-0.33	-6.15	-2.95	-2.06	1.75	-2.23
1	17-34	Maturity	24.58	1.2	-0.54	0.78	-0.5	-0.15	0.55	0.05	-1.51	0.14	-2.13	-1.34	-0.54	-0.64	-1.93
1	17-34	Seeds per spike[1]	19.41	4.59	0.04	0.56	-0.13	0.59	0.92	0.05	-0.43	-0.61	-0.11	-1.22	0.68	-0.25	0.46
1	17-34	Width[2]	18.99	1.07	-0.04	0.03	-0.07	0.02	-0.01	-0.01	-0.06	-0.05	-0.06	0	-0.06	-0.01	-0.07
1	17-34	Surface Area[2]	30.61	1.99	-0.79	1.07	-2.43	0.65	0.48	-0.98	0.69	-2.1	-2.04	0.75	-0.64	0.72	-2.79
1	17-34	Roundness[2]	22.76	1.93	-0.01	0.01	0.01	0	0.01	0	-0.02	0	0	-0.01	-0.02	-0.01	0.01
1	17-34	Yield	33.8	1.3	0.52	4.51	2.01	-0.16	2.58	0.58	1.69	-0.44	1.35	0.12	-0.4	-0.05	0.09
2	9-30	Heading Date	20.97	2.4	-0.5	1.22	-1.83	0.81	0.11	-0.44	-2.09	-0.61	-0.51	-2.19	-1.28	-0.97	1.29
2	9-30	Height	10.5	1	-1.78	-1.4	0.96	0.85	0.39	-0.16	-0.28	0.32	0.67	-0.31	-0.36	0.18	-2.4
2	9-30	Spike Length[1]	26.84	0.9	0.58	0.33	2.53	1.34	-0.51	1.24	-4.88	0.25	1.1	-3.64	-1.54	-3.47	2.37
2	9-30	Maturity	12.84	0.98	-0.52	0.95	-0.52	0.23	-0.11	-0.19	-0.34	-0.24	-0.39	-0.97	-0.21	-0.03	2.97
2	9-30	Seeds per Spike[1]	18.31	4.02	-0.01	0.8	-0.13	0.34	-0.64	0.23	-0.07	0.11	0.02	-1.01	-0.52	-0.29	-1.26
2	9-30	Width[2]	18.77	1.41	0	0.04	-0.07	0.01	0.02	0.03	0.01	-0.01	0.01	0.02	0.04	0.02	0.13
2	9-30	Roundness[2]	17.67	0.72	0	0.01	0	0	0.01	0.01	0	0	0	0.01	0.01	0.01	0.01
2	44-66	Heading Date	25.11	3.14	0.22	2.14	-1.83	0.38	-0.11	-0.02	-1.46	-0.13	-0.71	-1.78	-1.82	-0.04	2.84
2	44-66	Height	10.27	1.82	-2.36	1.27	-0.12	1.1	0.58	-0.41	-0.37	-0.53	1.42	-0.63	-0.79	-0.52	3.5
2	44-66	Spike Length[1]	29	1.52	-0.08	1.7	1.14	1.13	0.01	0.82	-2.77	0.1	-6.03	-3.04	-2.78	-2.99	6.52
2	44-66	Maturity	15.64	1.83	-0.21	1.25	0.04	0.17	-0.14	0.04	-0.09	-0.28	-2.46	-0.92	-0.46	0.26	3.35
2	44-66	Seeds per Spike[1]	18.84	14.36	-0.04	0.96	0.26	0.4	-0.62	0.05	-0.29	0.08	-0.37	-0.88	-0.52	-0.24	-3.91
2	44-66	Width[2]	21.46	1.47	-0.04	0.05	-0.02	0.02	0.03	0.03	0.04	0	-0.08	0.03	0.02	0.02	0.15
2	44-66	Surface Area[2]	11	0.4	0.33	0.32	-0.88	-0.01	-0.02	-0.4	0.05	-0.45	-1.85	0.43	-0.16	-0.16	-1.11
2	44-66	Roundness[2]	16.92	0.94	0	0.01	0.01	0	0.01	0	0.01	0	0	0.01	0	0.01	0.02
2	44-66	Yield	11.49	0.35	-0.78	1.77	0.17	-0.09	-1.07	-0.49	-0.2	0.08	1.56	-0.15	-0.19	0.09	0.36

Table 4 Continued

3	11-37	Heading Date	10.32	3.15	-0.55	2.58	0.28	0.12	-0.82	-0.65	0.37	-0.9	-0.1	-2.02	-2.11	-0.1	2.61
3	11-37	Height	11.56	0.87	1.18	-0.11	-0.71	-1.85	0.81	0.27	-0.49	1.11	-0.34	0.52	-0.83	-0.23	1.62
3	11-37	Maturity	10.12	0.67	0.34	0.92	-0.16	-0.09	-0.76	-0.22	0.22	-1.61	-0.61	-0.97	-0.06	-0.53	1.26
3	11-37	Surface Area^2	14.04	1.22	-1.39	-1.32	-0.85	-0.21	1.79	-1.33	-0.67	-1.1	-0.41	0.16	0.06	0.29	-3.28
3	11-37	Yield	28.29	1.03	1.16	4.51	-0.17	-0.2	1.4	0.88	-0.29	0.62	0.08	-0.09	-0.08	0.08	0.61
3	104-136	Height	10.91	0.95	1.02	-0.29	-0.61	-1.47	-0.66	-0.17	1.07	0.01	1.16	0.79	-1.35	-0.34	1.92
3	104-136	Maturity	12.17	1.21	0.11	1.02	-0.27	0.01	-0.38	0.21	1.02	0.29	-2.9	-0.53	0.2	-0.52	-0.41
3	104-136	Surface Area^2	15.52	0.77	0.01	-0.43	-0.82	-0.1	-0.03	-0.63	0.44	-1.55	-2.19	0.73	-0.09	1.05	-1.53
3	104-136	Roundness^2	9.62	0.37	0	0.01	0.01	0	0	0	0	0.01	0	-0.01	0	0	-0.01
3	104-136	Yield	33.86	1.18	1.12	4.51	-0.04	0.04	-1.18	0.3	-0.69	0.1	1.87	-0.19	-0.03	0.15	-0.45
3	144-173	Maturity	13	1.59	0.06	1.06	-0.26	0.11	-0.01	-0.45	-1.43	0.13	-0.52	-0.5	-0.52	-0.48	-0.65
3	144-173	Width^2	9.65	0.44	0.02	0.05	-0.01	-0.01	0.01	0.01	-0.01	-0.01	0.01	0	-0.02	-0.01	-0.09
3	144-173	Surface Area^2	13.91	1.16	-0.11	-0.75	-0.74	-0.71	0.21	-0.63	0.02	-1.1	-1.78	0.34	-0.34	0.47	-4.07
3	144-173	Roundness^2	9.94	1.04	0.02	0.01	0.01	0	0	0	-0.01	0.01	0	0	0	0	0.01
3	144-173	Yield	24.37	0.95	-0.54	4.43	-0.21	-0.36	0.06	0.61	0.63	-0.32	-0.01	-0.14	-0.17	-0.19	-0.44
4	9-31	Maturity	12.36	0.69	0.43	1.02	-0.53	-0.29	0.14	0.2	0.14	0.09	0.29	-0.72	-1.21	-1.06	-1.76
4	9-31	Width^2	9.74	0.69	0.03	0	-0.07	-0.02	0.03	0	0.02	0.04	0.01	0	-0.04	-0.07	-0.02
4	9-31	Surface Area^2	15.97	1.06	1.09	-0.44	-0.74	0.07	2.2	0.59	-0.17	0.07	-2.27	0.44	0.58	1.08	-0.9
4	9-31	Roundness^2	14.32	1.27	0	0	0.01	0	-0.01	0.01	0	0.01	0	0	-0.02	0	0.02
4	98-130	Heading Date	11.65	1.94	0.33	4.51	-0.13	-0.17	1.09	-0.3	-0.17	-0.6	-0.17	-0.02	0.24	0.09	0.48
4	98-130	Spike Length^1	21.14	1.38	0.32	0.46	0.08	0.14	0.42	0.26	1.04	-1.07	0.44	-2.24	-2.51	-0.06	1.21
4	98-130	Maturity	16.84	0.98	0.58	0.68	0.33	0.03	0.45	2.51	1.1	-2.22	-6.9	-3.23	-2.78	1.32	4.33
4	98-130	Width^2	12.25	0.59	0.35	0.67	-0.19	-0.1	1	0.11	0.23	-1.29	-1.76	-1.15	-0.55	-0.04	0.33
4	98-130	Surface Area^2	13.33	0.87	0.03	0.01	-0.01	0	0.04	0	0	-0.01	-0.05	0.03	0.04	0.02	0.09
4	98-130	Roundness^2	15.43	1.18	1.54	-0.36	-0.78	-0.1	1.63	-0.13	-0.2	-1.01	-1.5	0.07	-0.4	0.38	-2
4	98-130	Yield	29.39	1.03	0	0	0	0	-0.01	0	0	0	-0.01	0.01	0.01	0	0.02
5	26-50	Height	11.11	1.71	-0.91	1.08	-0.92	-2.43	-0.05	-2.31	-1.94	-0.57	-0.42	-0.09	0.13	0.15	2.25
5	26-50	Width^2	14	0.94	0.58	0.31	-0.04	-0.09	0.43	0.32	1.35	-1.01	-0.23	-1.15	0.18	-0.29	2.75

Table 4 Continued

5	26-50	Maturity	15.22	1.03	-0.04	0.14	0.06	-0.14	-0.09	-0.04	-0.06	-0.05	-0.03	0.04	0.09	-0.22	-0.15
5	26-50	Surface Area[2]	18.8	0.99	0.72	-0.17	-1.22	-0.05	-0.2	0.74	0.52	-1.66	-0.48	0.15	-0.32	-0.05	3.45
5	26-50	Roundness[2]	20.61	1.13	0	0.01	0.01	0	0	0.01	0	0	0	0	0.01	-0.02	0.01
5	55-83	Spike Length[1]	17.43	1.01	-0.34	0.12	-0.03	-0.93	-1.35	-2.26	1.1	-1.07	-6.9	-2.53	-0.66	0.79	3.08
5	55-83	Maturity	13.19	1.16	0.17	0.12	0.01	0.13	0.63	-0.06	-1.1	-1.86	0.12	-0.74	0.12	-0.89	2.8
5	55-83	Width[2]	15.19	1.29	-0.04	-0.02	-0.01	0	0.07	0.01	-0.01	-0.04	-0.02	-0.01	0.03	-0.09	0.11
5	55-83	Surface Area[2]	17.06	1.01	0.42	-0.09	-1.14	0.04	-0.18	0.75	-1.2	-1.69	-0.38	0.3	-0.34	0.03	3.45
5	55-83	Roundness[2]	20.5	1.61	0	0.01	0.01	0	0.01	0.01	-0.01	0	0	0	0.01	-0.02	0.01
5	55-83	Yield	12.63	0.45	0.15	2.51	-0.05	-0.27	0.03	-0.51	-1.19	-0.04	1.55	-0.02	-0.18	-0.01	0.24
6	14-46	Heading Date	10.25	1.44	-0.36	1.84	-0.15	0.14	-0.24	-0.5	-0.03	1.27	0.2	-1.4	-1.61	-0.2	0.89
6	14-46	Maturity	11.51	1.01	0.31	1.29	-0.16	-0.94	0.13	-0.47	-0.13	-0.7	-2.32	-0.76	-0.55	0.26	-0.49
6	14-46	Width[2]	13.3	1.06	0.03	0.05	-0.01	0	-0.03	-0.02	0.01	-0.01	-0.08	0	-0.03	-0.09	0.06
6	14-46	Surface Area[2]	15.67	0.94	0.38	1.59	-0.67	-0.6	-0.67	-1.12	0.06	-1.29	-2.25	0.49	0.08	0.03	-1.46
6	14-46	Roundness[2]	11.57	1.19	0	0.01	0	-0.01	-0.01	0	0	0	0	0	-0.01	0	0.02
6	14-46	Yield	11.09	0.43	0.06	2.34	-0.11	0.34	0.62	-0.43	-0.73	0.06	1.87	-0.18	-0.04	-0.18	0.01
6	55-74	Spike Length[1]	23.53	1.3	-1.06	1.82	0.58	-0.93	1.41	2.71	2.5	-3.75	-6.79	-0.97	2.59	0.93	1.58
6	55-74	Maturity	12.11	0.85	0.48	0.27	-0.21	0.19	-0.07	0.13	-0.37	0.2	-2.46	-1.1	-0.61	0.2	-0.09
6	55-74	Width[2]	15.87	0.88	0.02	0.05	-0.01	0	-0.02	-0.01	-0.01	-0.05	-0.06	-0.02	-0.03	-0.09	0.02
6	55-74	Surface Area[2]	17.16	1.02	0.05	1.5	-0.66	-0.19	-0.48	-1.27	0.37	-1.69	-2.24	0.62	0.02	-0.29	-1.84
6	55-74	Roundness[2]	12.04	1.38	0	0.01	0	0	0	0.01	0	-0.01	0	-0.01	-0.01	-0.02	0
6	55-74	Yield	15.49	0.32	-0.2	1.95	-0.01	-0.18	1.36	0.38	0.4	-0.04	1.24	-0.14	-0.07	-0.4	0.55
7	10-30	Heading Date	13.88	2.34	0.37	1.13	-0.53	0.22	-0.16	0.32	-0.26	0.48	0.59	-2.33	-1.46	-0.27	2.95
7	10-30	Height	9.32	1.79	-0.75	0.06	0.59	0.92	-0.53	-0.31	0.34	0.63	-0.97	0.04	-0.91	0.36	4.96
7	10-30	Spike Length[1]	22.88	1.24	0.69	0.79	1.81	-0.2	-1.63	-0.29	1.33	-0.45	-6.9	-3.68	-2.15	0.82	3.58
7	10-30	Maturity	19.82	1.83	0.15	0.53	-0.5	0.23	0.37	-0.1	-0.03	-0.01	-2.46	-1.47	-0.38	0.07	3.33
7	10-30	Width[2]	19.46	0.94	-0.01	0.03	-0.01	0.01	0.03	-0.05	0.02	-0.01	-0.09	0.03	0.03	0.01	0.08
7	10-30	Surface Area[2]	10.04	1.24	0.25	-0.4	0.15	0.01	-0.21	-0.55	0.3	0.22	-2.3	-0.03	-0.31	0.15	3.92
7	10-30	Roundness[2]	21.15	1.44	0	0.01	0	0	0.01	-0.01	0.01	-0.01	0.01	0.01	0.01	0	0.01

Table 4 Continued

7	10-30	Yield	13.74	0.52	-0.69	3	-0.36	0.08	0.14	-0.56	0.56	-0.21	1.1	-0.27	-0.28	0.15	-0.05
7	57-66	Heading Date	21.77	2.4	-0.11	2.58	-1.03	0.74	0.13	0.69	0.43	-0.5	-0.1	-1.74	-1.91	-0.1	-1.2
7	57-66	Height	14.52	1.42	-1.44	1.29	1.05	1.19	-0.36	0.07	-0.34	-0.79	1.09	0.28	-0.92	0.55	-3.24
7	57-66	Spike Length[1]	16.16	1.36	0.22	1.37	2.92	0.34	-1.56	-0.51	-1.84	0.02	-8.02	-3.12	-2.42	0.38	0.21
7	57-66	Maturity	18.68	0.84	0.07	1.21	-0.93	0.47	0.03	-0.38	1.02	-0.28	0.54	-1.01	-0.56	-0.29	-1.91
7	57-66	Width[2]	11.72	0.73	0	0.07	-0.02	0.01	0	-0.01	-0.01	0	-0.09	0.03	0.01	0.02	-0.01
7	57-66	Surface Area[2]	9.49	0.57	-0.19	-0.56	0.25	-0.02	-0.7	-0.82	-0.78	-1.14	-2.08	0.25	-0.31	0.25	1.19
7	57-66	Roundness[2]	11.06	0.87	0	0.01	-0.01	0	0.01	-0.01	0.01	0	0	0	0.01	0	-0.01

[1] Referring to spike trait.

[2] Referring to seed trait.

± Position in centimorgans on the noted chromosome.

Ψ Likelihood of odds score.

† Amount of phenotypic variation explained by QTL for given trait (R^2).

δ Additive effect estimation of Conlon allele.

Table 5 Quantitative trait loci (QTL) identified for 13 individual families for the two-rowed NAM spike and morphology population.

Chromosome	Family Position[±]	Consensus Position[±]	Trait	LOD[ψ]	PVE[†]	ADD[δ]
Family 2						
6	11	14-46	Plant Height	2.58	14.25	0.91
6	23	14-46	Roundness[2]	2.67	14.01	0.03
Family 5						
1	59	17-34	Density[1]	5.43	5.75	-0.02
2	31	9-30	Surface Area[2]	5.86	28.57	-0.96
2	31	9-30	Volume[2]	7.38	18.17	-0.34
2	48	44-66	Heading Date	7.71	37.77	1.44
2	48	44-66	Maturity Date	11.42	47.14	1.41
2	48	44-66	Seeds per Spike[1]	12.03	48.17	1.12
3	35	11-37	Plant Height	5.39	16.34	1.35
3	77	11-37	Roundness[2]	4.01	26.12	0.01
3	133	104-136	Seed Weight[1]	5.01	25.05	-1.53
3	179	144-173	Heading Date	6.1	15.15	0.96
4	51	9-31	Density[1]	14.32	18.85	-0.04
5	14	26-50	Heading Date	5.55	14.9	-0.85
5	66	26-50	Seeds per Spike[1]	6.52	11.69	0.66
5	77	26-50	Plant Height	7.21	21.13	-1.41
6	24	14-46	Seeds per Spike[1]	4.52	7.59	-0.78
Family 6						
1	17	17-34	Yield	3.34	4.25	5.69
4	9	9-31	Seed Weight[1]	3.89	15.29	-0.58
4	9	9-31	Maturity Date	3.8	21.38	-0.61
4	11	9-31	Surface Area[2]	3.48	21.24	-0.80
4	11	9-31	Width[2]	3.49	20.20	-0.03
4	11	9-31	Volume[2]	4.64	25.53	-0.3
4	24	98-130	Yield	3.35	4.25	5.7
5	16	26-50	Seeds per Spike[1]	4.37	23.99	0.41
5	19	26-50	Yield	3.60	4.25	5.7
5	44	55-83	Seed Weight[1]	7.28	32.55	-0.73
6	39	14-46	Seed Weight[1]	4.91	20.25	-0.58
7	19	10-30	Density[1]	2.77	5.36	0.02
7	19	10-30	Plant Height	7.68	38.85	-1.24
Family 18						
7	147	57-66	Density[1]	3.74	4.37	0.07

Table 5 Continued

Family 32						
2	14	9-30	Surface Area[2]	3.734	14.17	2.04
2	101	44-66	Yield	2.98	15.91	0.94
5	82	26-50	Density[1]	2.58	5.09	-0.02
5	101	55-83	Width[2]	6.03	29.3	0.06
5	102	55-83	Volume[2]	3.69	21.35	0.46
7	70	10-30	Surface Area[2]	5.48	24.31	-0.98
7	70	10-30	Seed Length[2]	3.69	24.75	-0.15
7	73	10-30	Roundness[2]	4.48	25.57	0.01
Family 39						
1	17-34	39	Width[2]*	2.7	12.76	0.01
3	82	11-37	Density[1]	7.3	21.64	0.04
4	163	98-130	Seed Length[2]	2.58	5.78	-0.4
6	176	55-74	Spike Length[1]	2.81	14.86	2.12
Family 51						
1	24	17-34	Width[2]	2.58	15.74	0.07
2	18	9-30	Spike Length[1]	8	47.29	-4.95
2	18	9-30	Seeds per Spike[1]	3.6	24.81	-0.59
2	20	9-30	Density[1]	3.5	24.28	0.02
6	21	14-46	Surface Area[2]	4.28	18.67	-0.67
6	22	14-46	Seed Length[2]	3.34	17.08	-0.08
7	7	10-30	Width[2]	3.57	15.74	0.03
7	7	10-30	Volume[2]	3.35	23.04	0.29
7	27	10-30	Surface Area[2]	4.46	19.77	-0.81
7	27	10-30	Seed Length[2]	7.28	40.96	-0.14
7	30	10-30	Roundness[2]	3.3	21.82	0.01
7	41	10-30	Heading Date	2.98	20.49	-1.47
Family 52						
2	126	44-66	Volume[2]	3.34	23.09	-0.32
2	191	44-66	Surface Area[2]	2.9	19.61	-1.03
4	119	98-130	Yield	2.86	21.31	-1.17
4	120	98-130	Plant Height	2.57	18	-1.29
7	10-30	52	Surface Area[2]*	3.32	19.8	0.27
Family 61						
3	32	104-136	Maturity Date	3.97	18.87	-0.55
4	51	98-130	Seeds per Spike[1]	4.41	22.11	0.29
Family 64						
1	76	17-34	Maturity Date	4.55	14.88	-0.52
2	2	9-30	Spike Length[1]	3.17	11.92	1.14
2	12	9-30	Density[1]	23.18	15.98	-0.08

Table 5 Continued

2	45	44-66	Plant Height	3.08	16.54	-0.74
2	54	44-66	Density[1]	5.35	3.08	-0.13
2	56	44-66	Seed Weight[1]	5.47	8.57	1.12
2	61	44-66	Seeds per Spike[1]	12.12	5.99	-0.64
2	67	44-66	Heading Date	11.99	33.79	-0.93
2	69	44-66	Maturity Date	9.32	34.94	-0.78
2	75	44-66	Seed Length[2]	7.88	17.65	-0.07
2	77	44-66	Width[2]	3.88	32.69	0.03
2	77	44-66	Roundness[2]	9.02	37.06	0.01
3	81	144-173	Seed Weight[1]	4.37	10.21	3.25
4	8	9-31	Density[1]	5.53	3.08	-0.13
5	35	55-83	Seeds per Spike[1]	2.56	1.35	-0.56
5	36	55-83	Seed Weight[1]	4.43	8.74	1.83
7	34	10-30	Spike Length[1]	4.54	18.13	-1.2
7	74	10-30	Roundness[2]	5.41	20.35	0.01
7	121	57-66	Heading Date	14.02	43.92	-1.09
7	131	57-66	Seeds per Spike[1]	45.51	59.02	-2.17
7	132	57-66	Spike Length[1]	2.63	9.09	-0.9
			Family 67			
1	37	17-34	Volume[2]	4.34	17.4	-0.22
1	50	17-34	Seed Weight[1]	6.8	17.18	-1.5
1	52	17-34	Surface Area[2]	6.1	30.95	-1.65
2	50	9-30	Roundness[2]	5.72	43.26	0.01
2	52	9-30	Width[2]	5.14	34.41	0.05
3	118	104-136	Volume[2]	2.72	10.16	-0.24
3	155	144-173	Seeds per Spike[1]	5.1	6.9	0.83
7	34	10-30	Plant Height	6.96	29.57	-1.46
7	34	10-30	Seed Length[2]	3.68	29.3	-0.07
7	38	10-30	Maturity Date	2.59	21.96	-0.63
			Family 79			
4	64	98-130	Seeds per Spike[1]	4.34	3.18	-0.6
5	22	26-50	Plant Height	2.73	15.88	1.07
7	116	57-66	Seed Length[2]	5.02	4.45	0.07
			Family 123			
1	54	17-34	Surface Area[2]	8.25	25.46	-1.65
1	54	17-34	Seed Length[2]	11.17	43.76	-0.41
1	54	17-34	Volume[2]	7.63	12.32	-0.46
1	54	17-34	Roundness[2]	10.47	17.57	0.02
3	34	11-37	Density[1]	34.32	15.61	0.63
3	34	11-37	Seed Weight[1]	7.33	10.97	-5.66
3	34	11-37	Seeds per Spike[1]	17.31	11.47	4.77

Table 5 Continued

3	34	11-37	Volume[2]	6.87	10.45	-1.4
3	130	104-136	Spike Length	4.32	31	-2.15
4	46	9-31	Seed Length[2]	8.11	25.37	-0.52
6	44	14-46	Grain-fill	3.05	16.14	-0.9
6	44	14-46	Yield	2.79	7.51	-0.88
6	94	55-74	Plant Height	2.57	15.83	-1.17
7	82	10-30	Width[2]	3.36	11.45	0.02
7	96	10-30	Heading Date	4.14	22.11	-1.33
7	131	55-66	Heading Date	5.13	28.49	2.6
7	174	55-66	Yield	4.49	13.88	4.34

[1] Referring to spike trait.

[2] Referring to seed trait.

* Levene's Statistic for a given trait.

± Position in centimorgans on the noted chromosome.

Ψ Likelihood of odds score.

† Amount of phenotypic variation explained by QTL for given trait.

δ Additive effect estimation of Conlon allele.

DISCUSSION

Comparison to other NAM Studies

Nested Association Mapping (NAM) populations combine the strengths of both linkage mapping and association mapping by taking advantage of historical recombination events giving the analysis high power and high resolution (Yu et al., 2008). NAM is quite flexible for field, population structure, and analysis. The structure of the two-rowed barley NAM originally constructed by the TCAP had 100 diverse founder parents crossed to the spring malting cultivar Conlon; the F_1 progeny were backcrossed to Conlon. The final population resulted in approximately 100 families containing on average 75-85 lines per family. However, due to limited resources and intensity of phenotyping, we utilized 13 of the most variable families for our traits of interest to study. To provide accurate spike and seed morphology measurements we utilized digital analysis. The size of the population utilized here compares with many other NAM structures with 1,133 lines (Maurer et al., 2015; Nice et al., 2016; Rostoks et al., 2005). The population was genotyped using genotype by sequencing (GBS) technology.

The choices made to reduce the family number, number of RILs per family, the backcross, and use of GBS data limited the power and resolution of the QTL analysis. To better understand these limitations, we compared the structure and size of our NAM to other successful NAM panels. The very first NAM was constructed in maize. The

reference design was 25 diverse families of 200 recombinant inbred lines (RILs) (McMullen et al., 2009). Since then, the maize NAM has been a backbone to many genomic studies. In Buckler et al. (2009), they phenotyped the whole 5,000 lines within the maize NAM for days to silking and days to anthesis. Kump et al. (2011) phenotyped all 5,000 NAM maize lines for southern leaf blight resistance. In sorghum, a NAM population of, 56 backcross-derived families ranging from 30 to 90 lines per family, was created using a standard elite parent. The approximately 1,000 lines were phenotyped for a range of traits, for example in Jordan et al. (2011), plant height, days to flower, and grain yield were analyzed. Germplasm was screened and mapped using 197 Diversity Array Technology (DArT) markers. Mace et al. (2013) used the same sorghum NAM to interrogate flowering by screening 1,005 DArT markers. Like ours, the population was backcrossed derived.

In barley, the first NAM was created by using the six-rowed spring barley elite cultivar Barke, to cross with 25 exotic barley accessions which they backcrossed back to Barke. The population was named 'Halle Exotic Barely 25' or HEB-25, and it resulted in 25 families with up to 75 lines per family with a total of 1,420 BC_1S_3 lines (Nice et al., 2016). Authors used an iSelect 9k chip for genotyping and assigned marker map position based on (Comadran et al., 2012) with remaining SNPs placed by chi-square tests of independence (Maurer et al., 2015). Additionally, in another six-rowed barley NAM, 25 wild barley parents were crossed to the six-rowed malting cultivar Rasmusson, and backcrossed two generations to Rasmusson. Only 31 lines on average

were within each family. The 796 $BC_2F_{4:6}$ lines were genotyped using the barley 9K SNP Illumina, iSelect platform, and exome capture sequenced (Nice et al., 2016). Four phenotypic traits were measured, glossy spike, glossy sheath, black hull, and grain protein content.

The resolution and power of the current NAM study were reduced due to several choices. The greatest limitation of this study was the use of genotyping by sequencing, GBS, to generate marker data. During the GBS process, there is an intentional reduction of the genome that is sequenced. This reduction is somewhat random; therefore, the sequenced portions of the genome are not the same between the families, resulting in few overlapping markers. Most NAM populations, as illustrated above use genotyping methods that result in the same markers in each family. A few have used GBS, but have run into similar issues of few consensus markers, such as in the maize NAM (Liu et al., 2016). Therefore, a consensus map was unable to be made; the authors used the initial maize consensus map for analysis instead of creating a new one. In a wheat NAM, Nice et al., (2016) created a consensus map with GBS marker data by allowing markers that were present in two or more families but had a few single sequence repeats (SSR) markers to help anchor the maps. We too created a consensus map by allowing markers which overlapped in two or more families, but this resulted in noise in the consensus map. A marker present in a handful of families that significantly associated with a trait did not exist in other families, but a different marker in close proximity did, resulting in two peaks during QTL mapping,

with a smaller LOD score and lower PEV instead of one peak with a more significant LOD score and higher PVE. Therefore, noise resulted in QTLs explaining a smaller portion of phenotypic variance than observed in individual family maps in part due to the phenotypic variance explained only included the portion of the population that shared the marker being tested. Pillen et al., (2003) reported the average number of markers in bi-parental barley maps ranged from 50-129. While we had more markers than this, they were not all present in each family or in the consensus map. Furthermore, lack of overlapping markers especially limited the number of QTL on chromosome 1H, especially in the consensus map. 1H was the smallest linkage group in the consensus map. All the overlapping family markers which associated with traits in individual family QTL analyses mapped within positions 17-34 cM on the consensus. This is most likely because we had such few overlapping markers for the consensus map on this chromosome. Due to lack of overlapping markers, the resolution of the consensus map is less than expected, providing less precision of QTL positions. However, we still captured associations between genotypes and phenotypes.

The impact of the backcross of the F_1 generation to Conlon resulted in more Conlon alleles and fewer founder alleles as well as a large number of monomorphic genotypic calls. Originally the choice was made to backcross as these landraces, and malting parents were not adapted to the northern plains. The backcross was made to ensure lines were adequately adapted. The backcross can explain why chromosomes vary so much in length. Founder alleles could have been lost due to sampling error in

the creation of the population. The backcross is also the reason we had to use the reference chromosomes to anchor initial linkage groups. During the creation of the barley NAM HEB-25, this backcross design was successful as they used a chip array for genotyping, so the identified SNPs existed in every family (Maurer et al., 2015) increasing the number of useful consensus markers once monomorphic markers were removed. Again, a maize NAM, (Liu et al., 2016) was backcrossed four times and genotyped using GBS data, but a consensus map was unable to be constructed. However, in both the maize NAM as well as the barley two-rowed even though little of the founder genome was represented; the phenotypic variation could be measured and mapped especially using individual families.

Another future consideration is genotyping of parents. Multiple individuals of each parent were used in the crosses, but those same individuals were not genotyped. Studies have shown that even elite varieties are not genetically uniform enough for this type of study. For example, Cooke et al. (2003) found only 24 of the tested 45 wheat varieties were uniform and stable enough to meet requirements for the European Union by comparison of 7-9 different SSR loci. In tomato varieties, using six SSR loci, nine out of ten were stable enough to meet requirements (Cooke et al., 2003; Li-Xin et al., 2009; Li Wang et al., 2014; Wang et al., 2015). Therefore, allelic difference or lack thereof could be due to variation within a parental line instead of differences between the parents. This problem has impacted other studies but here was magnified due to the lack of overlapping markers. While we do not feel this is the case with the QTLs

reported here as many of the QTLs align with each other within a family, and the Conlon background seems uniform, we feel it should be noted for future awareness.

Due to limited resources, investigators often weigh the pros and cons of utilizing a larger number of families versus bigger families (McMullen et al., 2009). In our case, the small size of families in conjunction with the lack of overlapping markers resulted in maps with lower resolution. Due to the use of GBS marker data and the lack of overlapping makers adding more families to the study would not likely have been helpful. Additionally, we selected the families based on their variation for the traits of interest. To include families that did not vary phenotypically for the traits would not improve our ability to map QTLs. Other families created for the barley two-rowed NAM would prove useful if other traits were mapped. While the limitations discussed here are real, we were able to overcome them to still make use of the population. Overall the resolution of our QTL analysis was reduced due to our mapping resolution. However, this does not mean that real and robust associations within the consensus were not observable, but it does mean that the QTL locations have larger confidence intervals. Additionally, the left and right flanking markers of the individual family QTLs aligned well with the consensus QTLs assuring us the QTL regions identified were real. We identified multiple QTLs that align with the literature and a few unique family QTLs.

Success of Digital Image Analysis

This project would not have been possible without digital image analysis. Using it, we created an efficient pipeline to collect spike specific seed measurements which allowed us to detect even small differences between genotypes for the traits measured. Currently, two-dimensional image analyses are commonly used to predict three-dimensional traits. While model interrogation for best fit is still under investigation, many agree that image analysis using key concept models, including ellipsoid, is a quick, yet useful alternative to physical measurements (Ayoub et al., 2002; SýkoroVá et al., 2009; Walker & Panozzo, 2012). Due to the assumption of seed shape symmetry, we made these 3D calculations.

Image analysis is just another method of collecting the phenotypic data, Gebhardt et al. (1993) employed digital image analysis for quantifying seed morphology variation in six-rowed barley in relation to malting quality where they confirmed the central seed is more uniform and desirous for malting than the two asymmetrical lateral kernels. Nielsen (2003) and del Moral et al. (1998) attempted to evaluate malting barley quality by using digital image analysis. Ninomiya et al. (1992) used images for husk wrinkle evaluation on malting barley as a predictor of malt quality (del Moral et al., 1998). Additionally, many studies in wheat and rice implemented image analysis as indicators of grain quality for milling processes and food grades (Neuman et al., 1987; Sakai et al., 1996; Symons & Fulcher, 1988; Van Dalen, 2004). More recently in barley, Amaral et al. (2009) successfully applied multivariate partial least squares

(PLS) analysis to predict barley weight distribution from images of seed with high accuracy. Walker and Panozzo (2012) applied PLS and linear regression to determine seed measurements, width (in terms of % plumpness) and weight. We utilized similar methods to ensure no debris or touching seeds were considered as measurements.

QTLs in the Consensus Map

We observed QTLs in the consensus map that were not observed in family maps, QTLs in family maps not observed in the consensus, and QTLs in both. When a trait is mapped to a QTL in the consensus and does not appear within a family analysis, it means no family had enough variation alone to create a significant phenotypic-to-genotypic association, but when the small insignificant associations within multiple families combined for consensus analysis, they were significant. A rare association existed if a trait mapped to an individual family or a few individual families, and not in the consensus.

The trend within the consensus map involved traits that are seen as highly heritable and conserved across most of the families such as heading date, maturity date, and plant height. For the most part, these traits have a one to one correlation between QTLs in families and the QTLs in the consensus map. For example, we only observed two family unique QTLs for heading date out of a total of eight heading date QTLs in the consensus. While different QTLs impact different families, the same theme is noted for plant height, and no unique family QTLs were detected for maturity. The

detection of strong QTLs impacting these traits aligns well with the literature. QTLs have been found on every chromosome for each of the agronomic traits above, and involve a number of genes such as for heading date (EA, eam, Ppd-H1) for height (sdw, Denso , uzu1, ert). An expectation in our map is the lack of association of heading date to chromosome 5H (De la Pena et al., 1999; Pillen et al., 2003). Although our QTLs overlap with those reported, due to our limited resolution, we cannot know if our QTLs encompass these same genes above. Another issue could be that because of population size or distribution of an allele amongst the populations we may not have the power to detect a QTL. For example, we saw a peak for heading date on the second QTL on chromosome 5H, but the LOD score was just over two, and therefore, not deemed significant. The high correlation between heading and maturity resulted in the appearance of these QTLs together on the consensus, each time we saw a QTL for heading a QTL for maturity was in the same location, on every chromosome but 5H. The correlation between heading date and maturity was strong in all the families. This resulted in limited variation in grain-fill duration. However, we were successful in mapping variation in grain-fill in family 123.

The overlap between consensus and family maps is not as strong for the spike and seed traits. We identified QTLs impacting spike length, seeds per spike, seed width and surface area in both the consensus analysis and individual family analysis. Furthermore, spike density, seed weight, length, and volume did not have significant associations in the consensus QTL analysis. This is most likely because the genes that

control these traits are different within each family. This is supported by the lack of overlap between most QTLs for these traits across families. A few exceptions include chromosome 2H QTL for spike density in Families 51 and 64, and a QTL on chromosome 7H for seed length in Families 32, 51, and 67. In other studies that reported these traits, they had the resolultion to link their QTLs to known genes such as spike density genes (dsp10, sld, sdw1, denso, zeo3, int-c, vrs1, dsp9) and roundness genes (glo-a, glo-a), (Baum et al., 2003; Chen et al., 2009; Franckowaik et al., 2016; J. Franckowiak, 1994; Laurie et al., 1993; Li et al., 2006; Marquez-Cedillo et al., 2001; Pillen et al., 2003; Tinker et al., 1996; von Wettstein-Knowles, 1992). Our QTLs align well with the previous studies results, except seed weight on 5H and 6H, reported by (Baum et al., 2003) and on 7H, reported by (Tinker et al., 1996). However, in addition to what has been reported by others, we report additional associations impacting spike and seed traits in specific families on every QTL. We observed maturity (1H, 2H, 3H. 4H), grain-fill (6H), novel QTLs for density (1H, 3H, 4H, 5H), seeds per spike (3H, 4H, 6H), seed length (4H), volume (1H, 2H, 3H, 4H, 5H, 7H), and surface area (1H, 6H, 7H) were observed.

Additionally, the Levene Statistic we computed and mapped did not provide insight other than the Conlon allele is providing stability at all significant QTLs. One within Family 39 and one within Family 52, informed us about the uniformity of the populations' seed width and surface area respectively. It is not surprising that Conlon

provided stability as it is a variety of malting barley commonly cultivated in the state of Montana while the founder parents were not.

Interpretation of the Results

Agronomic Traits

In this study, we observed that on average Families 6, 51, 52, 61, 64, 67, 79, and 123 have later heading and maturity dates, higher yield, and taller plants than Conlon. We noticed that Families 51, 61, 64, and 79, which all had malting founder parents, were included in this group. The only exceptions to this trend being Family 61 and 67 which on average had lower yield and an earlier heading date, respectively. Most of the QTLs for the agronomic traits in the families listed above mapped to the same loci, and all had a negative additive effect from Conlon. However, in Family 123 the Conlon allele had a positive effect on heading and yield on chromosome 7H. Making the second group on average Families 5, 18, and 32 had earlier heading and maturity dates, shorter plants, and ultimately, lower yields than Conlon. The two exceptions being Family 18, having a higher yield, and Family 32 being shorter than Conlon. For most of the QTLs for these traits for this group, the Conlon allele had positive additive effects. Two exceptions came from Family 5 and received a negative effect for heading and height from the Conlon allele on chromosome 5H.

We observed a positive correlation between heading, height, and yield across the population. Borem et al. (1999) suggested that an extended period before heading

permits more accumulation of source carbohydrates, and taller plants may have higher biomass and photosynthetic capacity both favoring higher yield. Qi, et atl., (1998) and Hayes et al., (1993) reported positive correlations between heading date and height. These results agree with our study. This suggests the same and/or linked genes may affect these traits. We repeatedly saw QTLs for heading date and plant height in the same positions. Similarly, a correlation between maturity and height and subsequent co-segregation of QTLs were exhibited in the consensus on Chromosome 1H, 2H, 3H, and 7H. For all families, except Family 5, the QTLs for these traits have the same direction of effect. Both late heading and late maturity correlated with more seeds per head, which could in part explain heading date and maturity date's relationship to yield. (Baum et al., 2003; Pillen et al., 2003) each reported seeing QTLs for heading date, height, yield, and seeds per spike present on the same chromosome. Perhaps later heading allows the initiation of more seed primordia (Arisnabarreta & Miralles, 2006). In some families early heading correlated with kernel weight, indicating extending grain-fill can increase seed size. We saw similar results for extended grain-fill to those described in (Backes et al., 1995). In fact, the one extended grain-fill QTL observed in family 123 on chromosome 6H co-segregated with yield, with the increased grain-fill/yield QTL from the non-recurrent parent.

Spike Morphology

When compared to Conlon the spike traits measured, on average, from Families 6, 51, 52, 61, 64, 67, 79, and 123, had longer, denser spikes with more seed. In most cases, the Conlon allele reduced spike length, the density of seeds on a spike, and number of seeds per spike. However, in four of the families (6, 61, 67, 123), the Conlon allele increased the number of seeds per spike. Families 5, 18, and 32, on the other hand, displayed a lower density of seeds resulting in a smaller amount of seed per spike than Conlon, with only Family 18 and 39 exhibiting shorter spikes than Conlon.

The co-segregation of spike length, seeds per spike, and density, in this and other studies (Wang et al., (2010)) could be explained by all three traits impacted by single QTLs with pleiotropic effects or by several linked genes and either option could be true depending on the QTL. For example, spike length and seeds per spike co-segregated on chromosome 1H and 2H in the consensus, and in Families 51 and 64 on chromosome 2H. Also, spike length and density only appeared together in Family 51 and Family 64 on chromosome 2H. Importantly, the QTL explaining the largest PVE (14.36%) in the consensus was for seeds per spike on chromosome 2H. The family additive effect identified Family 123 as the highest contributor to seeds per spike. Parent 123 was six-rowed, but all family members in this study were two-rowed. Powell et al. (1990) reported QTLs for several traits co-segregating with *vrs1* (six-rowed gene), including spike length, height, and seeds per spike. They had evidence to suggest that the *vrs1* locus was linked to genes for spike length and height, but pleiotropic to

the number of seeds per head. Our evidence suggests that seed number is not wholly pleiotropic to vrs1 as family 123 does not vary for row type and yet we see variation in seed number on 2H supporting the existence of linked genes as described by J. Wang et al. (2010) and Franckowiak (1994).

Seed Size and Shape

The seeds in the NAM population were all longer than Conlon, on average, and the only families to have wider seeds than Conlon were Family 6 and Family 123. The narrower widths resulted in oblong seeds compared to the circular seed of Conlon. This result is not unexpected as Conlon is a North American, two-rowed, malting barley, requiring more round, uniform size seed (Kumar et al., 2013). Families 18, 51,61, 64, and 79, displayed the shortest, narrowest, roundest seed with the smallest surface area, volume, and weight. Not surprisingly, all the families mentioned above, but Family 18, have malting backgrounds. We would expect these malting lines to have more uniform seed, than the landraces. Also, it was possible that having undergone selection as malt varieties that these lines would have been less polymorphic when compared to Conlon. The diversity among the founder parent malt varieties is most likely because these malting varieties were each from different countries. Looking closer, correlation analysis showed strong correlations between most seed traits, which is in agreeance with a wheat kernel size study by Kumar et al. (2016). Similarly, QTLs impacting seed length, width, surface area, volume, and roundness appear together in the same genomic region for a specific family. For example, volume and surface area

co-segregate to the same region within Families 5, 6, 51, 52, 67, and 123 individually. In past studies as in our study, seed traits for size and shape have been detected on every chromosome. Although our QTLs align nicely with QTLs recorded by Ayoub et al. (2002), there are a few exceptions. They reported QTLs on all the chromosomes impacting seed length and width and roundness. On 3H they detected seed length where we did not, on 4H, we report seed length where they did not. On 6H, they detected seed length and roundness; we report roundness and seed width. However, Rajasekaran et al. (2004) reported seed width and length on 6H. On 7H, Ayoub et al. (2002) only observed QTL for seed roundness while we in addition to roundness detected seed length QTL on 7H.

Furthermore, we discovered that while the calculations for volume and surface area of each seed were dependent on both seed length and seed width, seed width had more impact on volume than seed length, conversely surface area relied more on length than width. This could be of importance to selection if end-users desire seed with larger volume then seed width should be increased, or if they desire more surface area, then seed length is key. Moreover, as shown above, all the families have different mechanisms of controlling seed width and length creating great potential for future breeder stock. We expected volume and seed weight to co-segregate due to very strong correlation. However, we only saw these traits appear together on chromosome 1H in Family 67 and chromosome 4H in Family 6; the positive effect was from the founder parent alleles. Interestingly, the QTL with the greatest additive effect on kernel

weight was on chromosome 3H and was contributed by the six-rowed parent, 123, and co-segregated for increased volume and reduced seed number. Although overall, six-rowed barley (referring to parent 123) on average had smaller seed than two-rowed barley, positive alleles for seed size could be contributed by six-rowed parents. This result indicates that lower average seed size in six-rowed is due to more seeds per spike, rather than specific genetics for smaller seed.

Special interest in Family 64

One particular family of interest is Family 64 for two reasons. First, all families exhibited a negative correlation between seeds per spike and seed weight. Family 64, was no exception. But two QTLs unique to the family may be of interest to breeders. When we considered the effect of the Conlon allele on chromosome 2H and 5H, they negatively impacted seeds per spike with PVEs of 6% and 1.3% respectively, and seed weight was positively impacted with PVEs of approximately, 8.6% each. However, on chromosome 3H Conlon's allele positively impacted seed weight with a PVE of over 10% with no significant impact on any other trait. Furthermore, parent 64 contributes an allele for a QTL on chromosome 7H that increased seeds per spike with a PVE of almost 60%with no impact on seed weight, although there was a negative impact on spike length. Importantly, these QTLs from 3H and 7H could break the commonly seen negative correlations between seeds per spike and seed weight.

Secondly, Family 64 is of interest because we detected several QTLs affecting multiple traits on chromosome 2H. We saw many of the expected trait relationships such as a less dense spike due to few seeds per spike, and the seeds weighed more. The high number of observed QTLs could be due to a higher resolution map or a stronger association between genotypes and phenotypes. Family 64 exhibited a positive correlation between seeds per spike and seed length, which was unique to this family. The founder allele contributed the positive effect for both traits on chromosome 2H. Parent 64 is a malt line, although there is an assumption that rounder seeded lines are preferred for malt. Malting of this family segregating for seed shape could give important information about the relationship between seed shape and malt quality.

Chromosome with Highest
Proportion of Seed Morphological Traits

Another observation we made is that chromosome 7H had a higher proportion of seed trait QTLs than any other chromosome for both consensus analysis and individual family analyses. Two QTLs were identified on chromosome 7H in the consensus analysis. Both QTLs impacted heading date, maturity, height as well as spike length, seed width, surface area, and roundness. Yield was only detected on the first QTL on chromosome 7H, and surprisingly, seeds per spike was not identified in either QTL. Family 6, 51, 64, and 123 had QTLs impacting agronomic traits on chromosome 7H. The same loci in Families 6 and 64 also impacted spike morphology. Additionally, the only

QTL identified in Family 18 was on chromosome 7H and it impacted spike density. However, seed size and shape was altered in Families 32, 51, 64, 67, 79, and 123. Within a given family, all seed traits co-segregated to the same locus. The seed traits co-segregated with the same locus with either agronomic traits, and/ or spike traits, except for families 32, 64, and 79. Further investigation of this chromosome with high-resolution mapping is needed as there are unique relationships between the agronomic, spike and seed morphology traits within each family on this chromosome. Some of the highest PVEs for seed traits were also seen on chromosome 7H, e.g., 59% PVE for seeds per spike occurred in family 64; while 41% and 30% PVE for seed length occurred in Families 51 and 67, respectively. Dsp1 and sdw4 are located on chromosome 7H and have been reported to impact spike density and grain size (Franckowiak & Lundqvist, 2012), our maps lack the resolution to determine if these are the same genes. Also, although height associated with both 7H QTLs in the consensus, it does not associate with all families that have spike density or grain size QTLs in the two-rowed NAM families.

CONCLUSION

While we lost resolution and power due to the limited number of members in each family, the number of families our resources could support, the backcross to Conlon, and the use of GBS marker data we successfully created linkage maps and ran QTL analyses. The high-density genotyping approach of GBS marker data in a NAM could be beneficial for QTL mapping within individual families, but known markers are needed in each family for construction of a stable consensus map. Additionally, since our resolution was limited, we could only identify QTLs with wide confidence intervals on the consensus map leaving us unsure of comparative QTL and gene identification, but we did identify several seed size traits in specific families which overlapped with previously reported QTLs.

An innovation of this project that made gathering a large amount of phenotypic data feasible was the creation a high-throughput digital imaging pipeline for the collection of spike and individual seed morphological traits.

While the founder parents included a mix of landraces and malting barleys from around the world we saw more phenotypic similarities between the malting families vs. the landraces, with each of the malting lines having rounder and more uniform seed size and shape. While we expected to see this, the level of genotypic differences between the malt lines was unexpected and provides more opportunities for improvement.

This two-rowed spring barley NAM shed insight into the conserved genomic regions that impact agronomic traits observed in the consensus map and the diverse mechanisms of increasing yield through traits associated with spike and seed morphology of each of the families.

We were surprised that some of our expected correlations were not as conserved in the QTLs. For example, a QTL for longer spike did not always co-segregate with a QTL for increased in seeds per spike. Only Family 51 and Family 64 (7H) had a single QTL for both traits, indicating number of seeds per head can be modulated without impacting head length. Other unexpected results were that volume was impacted more by seed width and surface area by seed length as observed in Families 6, 32, 51, and 123. The outcome of this project leaves us with a desire to investigate further Family 64 with use of NILs and its many associated traits on chromosome 2H as well as potential breeder selection markers to help break the strong negative correlation of seeds per spike and weight. Also on chromosome 2H, Family 123 exhibited a QTL of interest impacting seeds per spike. Additionally, while the families all have different mechanisms for influencing spike density, seeds per spike, and seed size and shape, chromosome 7H is a very important chromosome for these traits. Without the combined analysis of these families, we would not have seen this trend. High density and high-resolution mapping of this chromosome should be done to dissect further traits associated with the chromosome.

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