Genetic diversity of the DREB1 gene in *Hordeum vulgare* and *Hordeum spontaneum*

By

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Abstract

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Environmental stresses often disrupt and alter the growth of plants and their life cycles. This causes molecular responses to upregulate gene expression, inducing important stress responsive genes. Dispersal and continuous domestication of crop plants may cause a loss of genetic diversity that may be detrimental to these stress responsive genes. A population-based resequencing and phylogenetic analysis of the dehydration responsive element binding 1 (DREB1) stress responsive gene was used to determine its genetic diversity in three populations of wild and cultivated barley. Variation between China wild, China cultivated, and Middle Eastern wild populations were examined to understand gene-pool exchanges with the spread and development of barley cultivation. My results showed genetic differentiation among and within the three populations of barley by examination of nucleotide diversity (π), theta (per site) (θ), and number of haplotypes. Middle East wild-type and China wild-type were statistically significant with Fu and Li's D and F tests however, China cultivated-type was not. It was found that the highest diversity occurred in Middle Eastern wild-type, with the second highest diversity value as China wild-type, and lowest diversity in China cultivated type. These results provide important observations about the domestication processes of crop plants like barley where selection processes may be detrimental to the survival of stress responsive genes.

After this thesis was completed, some questions arose about sequence clarity and reliability, and how they affected alignment and phylogenetic analysis. These questions were analysed at the end of this thesis in an addendum (page 40).

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1. Introduction

1.1 Environmental Stress in Plants

Plants are often subjected to biotic and abiotic stresses that can alter their growth and development throughout their life cycle (Sachs and Ho 1986). This may be especially concerning for farmers who grow and sell crop plants for food as it may lower the quality of their yield (Sachs and Ho 1986). In recent years, climate trends in many agricultural areas around the world show an increase in atmospheric carbon dioxide (CO_2) and ozone levels (O_3) (Flamant et al. 2005; Lobell and Gourdji 2012). Due to the growth of the world's human population, urban development, agricultural, economic, and industrial activities have caused this increase in these gas emissions and result in atmospheric warming, global temperature increases, changes in wind events and altered precipitation patterns (Manning and Tiedemann 1995). Increased temperatures may cause crops to develop at a faster rate, thus leading to a shorter crop duration which has been known to correlate with lower yields. (Stone 2001). However, increased global temperatures are not always associated with warm temperatures in a particular area. North America, Europe and east Asia have experienced extremely cold conditions as well as heavy snowfall during their winter months (Liu et al. 2012). One particularly interesting explanation for this phenomenon is that diminishing Artic sea ice is responsible for these colder conditions and increased snowfall due to disruption on midlatitude atmospheric circulation (Liu et al 2012).

Domesticated and native plants may experience a direct biological effect from climate change (Krupa and Manning 1988). Direct effects of climate change may occur through the normal process of gas exchange between a leaf and its natural environment (Rich et al. 1970). Increased amounts of O_3 may enter plant leaves through open stomata changing the composition of cells due to alterations in membrane permeability (Lee 1985). The result of these changes may lead to cells collapsing and eventual death (Krupa and Manning 1988).

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Alternatively, increased CO_2 atmospheric levels influences stomatal function and closes the stomatal aperture causing the cells to collapse (Rogers et al. 1994). Several studies also suggest that CO_2 and O_3 can indirectly affect the severity of plant diseases induced by biotic agents (Dowding 1988; Manning and Keane 1988; Colls and Unsworth 1992; Rogers et al. 1994; Runeckles and Krupa 1994). In their review, Manning and Tiedemann (1985) found that increasing CO_2 may result in canopy structure becoming denser due to more biomass accumulation which combined with changing precipitation, causes the microclimate to become moister and provides a breeding ground for bacteria and pathogens.

The combination of direct factors (salinity, drought and temperature) and indirect factors (plant diseases) from climate change can be detrimental to the growth and survival of both domesticated and native plants (Rich et al. 1970; Dowding 1988; Manning and Keane 1988; Colls and Unsworth 1992; Rogers et al 1994; Runeckles and Krupa 1994). It is often assumed that direct or indirect climate change factors affecting plants is the predominant determinant of ranges and may limit species (Louthan et al. 2015).

Gene flow from pollen and seed dispersal are important sources of genetic variation throughout species range and selection against poorly adapted genotypes and genetic recombination are expected throughout species range as the plant species attempt to migrate and adapt to new locations (Davis and Shaw, 2001). To counter the challenges climate change imposes on plants, they will require proper responses and defence mechanisms.

1.2 Plant Responses to Stress

Plants respond to stresses such as cold, heat, drought, and high salinity at the molecular and cellular levels, as well as the physiological and biochemical level (Kidokoro et al. 2015), where responses are often interconnected (Wang et al. 2003). Physiological changes experienced by plants under stress include leaf wilting, reduction in leaf area, leaf abscission,

and stimulation of root growth (Lata and Prasad 2011). At the cellular level, osmotic stress induced by drought and salinity may result in the disruption of ion distribution and homeostasis by causing a decrease in potassium uptake and an increase in sodium influx (Serrano et al. 1999; Zhu 2001). Some metabolic reactions may be compromised by an influx of sodium such as the Hal2p phosphate in sulphate metabolism, forcing the salt stressed plant to maintain homeostasis (Serrano et al 1999). As a response to these changes, activation of cell signalling pathways (Figure 1) and cell responses occur (Shinozaki and Yamaguchi-Shinozaki 2000) where defence mechanisms may then activate functional proteins due to changes in protein and nucleic acid conformation, membrane fluidity and nutrient uptake (Chinnusamy et al. 2007). Functional proteins that aid in cellular stress response include chaperones, Late Embryogenesis Abundant (LEA) proteins, detoxification enzymes, transporters, and enzymes for metabolite biosynthesis (Kidokoro et al. 2015). For example, salinity stress may induce severe oxidative stress in the leaves of plants causing protective antioxidant enzymes to act against the salt stress. This happens by producing high amounts of hydrogen peroxide to eliminate toxicity of superoxide radicals (Lee 2001).

Other signalling mechanisms used by plants when subjected to abiotic stress include regulatory proteins and abscisic acid (ABA) (Lata and Prasad 2011). ABA is a plant growth regulator and stress hormone responsible for regulating leaf stomata closure to reduce water loss which decreases the photosynthetic rate to improve water-use efficiency (Lata and Prasad 2011). ABA also plays a role in seed development, seed and bud dormancy, seed germination, root growth, fruit ripening and the activation of stress-responsive genes (Agarwal and Jha, 2010). Alongside the ABA hormone, regulatory proteins also play a role in response to stress and include various transcription factors and cis-acting elements that function as molecular switches (Kidokoro et al. 2015), protein kinases, and other signalling molecules (Akhtar et al. 2012). Some transcription factors important in stress response include myelocytomatosis

oncogene (MYC), myeloblastosis oncogene (MYB), basic leucine zipper (bZIP) and dehydration responsive element binding protein (DREB) (Lata and Prasad 2011).

Although ABA treatment induces regulatory genes in response to dehydration and cold (Zhu 2002; Shinozaki et al. 2003) genes also exist that do not respond to ABA treatments (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2005) suggesting there is an ABA-dependent and ABA-independent signalling pathway. These may be pathways that control acclimation to stress through the activation of regulons which are a group of genes controlled by the same regulatory gene. These included the myelocytomatosis oncogene/Myeloblastosis oncogene (MYC/MYB) regulon in the ABA-dependent pathway (Abe et al. 1997; Busk and Pagés 1998) and the cold-binding factor/dehydration responsive element binding (CBF/DREB) regulon in the ABA-independent pathway (Saibo et al. 2009). Although there are many known regulons and transcription factors responsive to stress, the best studied group of transcription factors are the dehydration responsive element binding (DREB) genes due to their ability to activate the expression of various target genes during times of abiotic stress (Hussain et al. 2011).

1.3 DREB 1 Gene Expression

Some of the most important transcription factors for regulating plant responses to stress are contained within a family known as the APETLA2 (AP2)/ethylene responsive element binding protein (EREBP) which consists of a total of 145 genes (Yamaguchi-Shinozaki and Shinozaki 2005). The AP2 transcription factors are known to consist of an AP2 binding domain which is a three-dimensional structure consisting of approximately 60 amino acid residues in the form of three β-sheets and one α-helix (Allen et al. 1998). Within the AP2/EREBP family there are separate sub families known as AP2 (14 genes), DREB (56 genes), ERF (65 genes), RAV (6 genes) and others (Sakuma et al. 2002). The DREB subfamily was first discovered in the genus *Arabidopsis*, and homologs have since been found in several other plants (Khan

2011). The DREB subfamily was discovered in a response element in the promoter region of the rd29A gene which is responsible for drought resistance (Yamaguchi-Shinozaki and Shinozaki 1993) and can be further divided into six small groups termed A-1 to A-6. Of these small groups, A-1 and A-2 make up the two largest groups that are involved in two separate signal transduction pathways (Sakuma et al. 2002). The DREB1 gene, which is the focus of this study, belongs to the A-1 subgroup which is most commonly expressed during periods of cold and drought stress, whereas the DREB2 gene belongs to the A-2 subgroup and is most commonly expressed during periods of salt stress and drought (Khan 2011).

When under stress, functioning DREB transcription factors activate target genes that have cis-acting dehydration-response elements/C-repeats (DREs/CRTs; A/GCCGAC) in their promoters, which results in an improved tolerance to drought and cold through regulated gene expression (Agarwal et al. 2006) (Figure 1). These genes are activated in an ABA-independent pathway during drought and cold stress, however it has been suggested that some DRE/CRT motifs can respond to ABA-dependent pathways resulting in a crosstalk between these regulatory systems (Xu et al. 2009). The induction of DREB1 transcripts are organ specific and correlated to the length of stress treatment. For example, DREB1 in rice is expressed in almost all tissues and organs (Wang et al. 2008), whereas in wheat, DREB1 is highly expressed in roots but less in leaves and stems (Shen et al. 2003).
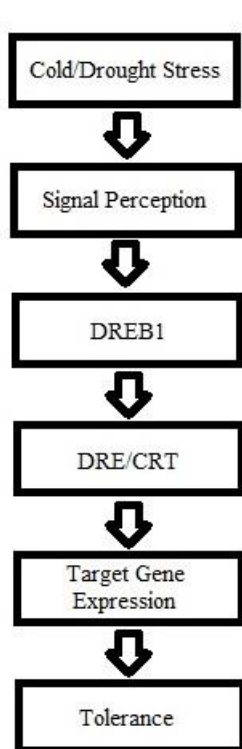
Figure 1: Visual representation of the DREB1 stress signal perception and gene expression in response to stress.

1.4 Structural Analysis of DREB1

All transcription factors have a DNA-binding domain comprised of a conserved amino acid sequence containing a small region called the DNA-binding motif that is responsible for recognizing single or double-stranded DNA (Alberts et al. 2002; Akhtar et al. 2012). The DREB proteins ERF/AP2 DNA-binding domain contain amino acids that show high sequence similarity in the nuclear localization signal at the N-terminal region and some similarity in the C-terminal acidic domain. Xu et al. (2009) demonstrated that the DREB1 gene in barley (HvDREB1) could specifically bind DRE/CRT elements, and that the acidic N-terminus is critical for the ability of DREB1 to act as a potential transcriptional activator (Khan 2011) and the entry of the DREB proteins occurs by one or two nuclear localization signals (NLS) (Akhtar et al. 2012). Within the ERF/AP2 domain, two amino acids, the 14th valine (V14) and the 19th glutamic acid (G19) play a significant role in the determination of DNA-binding specificity to the DRE core sequences and are a characteristic of the DREB proteins (Liu et al. 1998; Cao et al. 2001; Sakuma et al. 2002). The DREB1-type proteins also have two highly conserved motifs known as the DSAW motif and the LWSY motif and are located at the one end of the ERF/AP2 domain and at the opposite end of the C-terminal (Rasool and Ahmed 2014) (Figure 2).
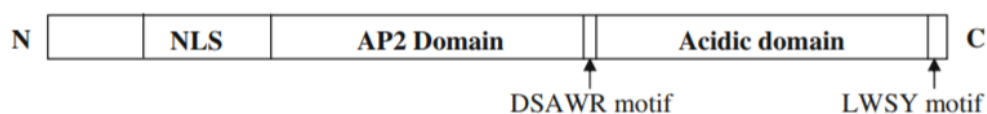
Figure 2: Illustration of DREB1 transcription factor structure and domains from Akhtar et al. (2012).

There are also other amino acids that are conserved in the DREB1-type transcription factors that facilitate direct contact with DNA for DNA binding activity which include: arginine (6), arginine (8), tryptophan (10), glutamic acid (16), arginine (25) and tryptophan (27) (Allen et al. 1998). Conservation of alanine at the position 37 in the AP2/ERF domain

indicates that it plays an important role in binding to the DRE element (Liu et al 2006). Furthermore, adjacent to the ERF/AP2 domain is a conserved Ser/Thr-rich region that is responsible for phosphorylation of the DREB proteins (Liu et al. 1998) which is important for regulation of protein function in response to stress (Nestler and Greengard 1999).

1.5 The Domestication of Wild barley (Hordeum spontaneum) to Cultivated barley (Hordeum vulgare)

The genus *Hordeum,* is in the tribe Triticeae of the grass family, Poaceae and contains 32 species (total 45 taxa), and 51 cytotypes exist at three ploidy levels (diploid, tetraploid or hexaploidy) with a basic chromosome number of x = 7 (Taketa et al. 1999). At least 4 different genomes were identified by meiotic analysis of interspecific hybrids, cpDNA, karyotypes, isoenzymes, and sequence analysis and were given the names: H, I, X, and Y in the *Hordeum* species (Bothmer et al. 1986). Interestingly, the genus *Hordeum* contains both annual species (e.g.: *Hordeum vulgare*), as well as perennial species (e.g.: *Hordeum bulbosum*) (Bothmer 1986). Barley (*Hordeum vulgare*), often referred to as cultivated barley, is known to be a founder crop of Old-World agriculture and is a model experimental system due to its short life cycle (Bothmer 1986). *Hordeum spontaneum* is a wild barley, from which cultivated barley originated, and can be found in various areas around the globe including south western Asia and the Mediterranean (Bothmer 1986). It has been speculated that barley was domesticated around 8,000 BCE. in the East Fertile Crescent. Today, the wild form of barley still exists in the East Fertile Crescent including but not limited to the locations: Israel, Jordan, south Turkey, Iraq, and Iran (Harlan et al. 1992). Cultivated barley and wild barley are morphologically similar including three, one-flowered spikelets at each rachis node known as a triplet (Von Bothmer et al. 2003) however some differences are detectable. The domestication (genetic modification of a wild species to create a new altered form) and genetic isolation led to differences in the wild and cultivated form of barley. As cultivated barley was domesticated,

larger leaves, smaller stems and awns, tough ear rachis, smaller and thicker spike, and larger grains were eventually selected (Zohary, 1969). The genetic distribution of diversity of cultivated barley corelates with genes for adaptation to various areas and ecological needs (Von Bothmer et al. 2003).

The geographical distribution of wild barley in the near East Fertile Crescent was considered the only location where barley was domesticated, however other domestication centres such as Tibet, central Asia, Morocco, Libya, Egypt, Crete, and Ethiopia have also been suggested (Molina-Cano et al. 2002; Azhaguvel and Komatsuda. 2005; Molina-Cano et al. 2005; Morrell et al. 2007; Orabi J et al. 2009; Bjornstad and Abay et al. 2010; Von Bothmer et al. 2011; Dai F et al. 2012; Ren et al. 2013). The spread of these crops from their domestication areas involved the dispersal of crop plants far beyond their native range and may need to adapt to new environments (Jones et al. 2008). As the plants disperse, their response to stress may vary given the location. Zhen and Ungerer (2008) suggested that as range expansion of thale cress (*Arabidopsis thaliana)* into warmer climates occurred, relaxed selection on the DREB1 resulted in multiple mutations that arose independently in both regulatory and coding regions and that these mutations persisted in local populations. The mutations then resulted in diminished freeze tolerance among populations in southern regions of the species range (Zhen and Ungerer, 2008). It may be possible that wild barley plants that originated in warmer climates such as the middle eastern countries (Zhang et al. 2005) and expanded their range into colder climates may have experienced changes in gene expression patterns to accommodate colder climates. Range expansion combined with domestication of plants may reduce the plants ability to adapt due to mutations in important stress responsive genes.

Domestication of plants by humans often reduces genetic diversity and can lead to a bottleneck effect (an event that drastically reduces the size of a population) where one population's gene pool is slowly reduced through continuous selection (Doebley et al. 2006).

Often, each generation during the domestication process, just one seed from the desirable plants forms the next generation (Doebley et al. 2006). Because of domestication, bottlenecked crops contain around 70% of the neutral genetic diversity seen in their wild ancestors (Bucker et al. 200+1). Analysis of the DREB1 gene in *Hordeum vulgare* and *Hordeum spontaneum* from different environmental conditions could reveal natural selection and adaptive genetic diversity of the DREB1 gene in barley populations. Analysis may also reveal the loss of genetic diversity due to selection pressures created by domestication bottlenecks. The importance of examining and researching these variations in gene expression may be useful as crop domestication and modern breeding strategies continue to result in serious reductions of genetic diversity in many different species (Gross and Olsen 2010). This may cause a loss of alleles that may contribute to stress tolerance in crops (Dwivedi et al. 2017) and as climate change becomes more prominent, domesticated plants may be less responsive to stressful conditions through mutations and the loss of genetic diversity (Zhen and Ungerer, 2008).

1.6 Objective of Study

The objective of this study was to compare the genetic variation of the DREB1 gene in *Hordeum vulgare* and *Hordeum spontaneum* amongst and within three different populations: The Middle East wild-type, China wild-type and China cultivated-type. This was completed to determine whether domestication processes of wild barley into cultivated barley resulted in the reduction of genetic variation of the stress responsive gene DREB1. This is important because without the stress responsive genes that allow pants to acclimate and adapt, survival against the increasing rate of climate change may become impossible. These results may provide further insight into the challenges barley and other crop plants may face as the rate of climate change increases. Whether or not the adaptation of barley to different regions resulted in DREB1 changes has never been assessed, I expected to see higher levels of genetic diversity in wild barley compared to cultivated barley.

2. Materials and Methods

2.1 Plant Materials: Hordeum vulgare and Hordeum spontaneum

Seeds of each species were obtained from the United States Department of Agriculture (Table 1) and were randomly selected to be planted in three-by-three-inch pots. Twenty seed packets from different locations across the globe were chosen for each of the three species, resulting in a total of 119 samples. Each pot was filled with value-tier Signal Potting Mix leaving approximately five cm of space at the top. A glass stir rod was then used to create seven holes in each pot roughly one inch deep. The seven smallest seeds from each packet were then placed in each hole and covered over with the potting mix. The plants were watered with 50 mL of tap water every two days for four weeks and left by a window to grow at room temperature (approximately 25°C -28°C).

2.2 DNA Isolation

Samples were collected by cutting leaves from the plants and placed into labelled centrifuge tubes. The leaf samples were snap frozen and crushed into a fine powder by adding liquid nitrogen and using an autoclaved metal rod. The metal rod was sterilized between each sample by putting it over a flame after being dipped in 70% ethanol. After all samples were ground, DNA was isolated using an EZ-10 Column Plant Genomic DNA Purification Kit according to the manufacturer's instructions (Bio Basic Inc.).

2.3 DNA Amplification of DREB1 *with Polymerase Chain Reaction*

I designed primers for amplifying the DREB1 gene based on *Triticum aestivum* AP2-containing protein (DREB1) mRNA (GeneBank: AF303376.1) using Primer 3 software. The forward primer sequence was 5'-GAAGAAAGTGCGCAGGAGAAG-3' (DREB1F) starting at bp 305, and the reversed primer was 5'- TCCCTATTGCTCCGCATGAC-3'(DREB1R) starting at bp 1130 (Figure 4). The resulting product size of the amplified gene was roughly 825 bp. The sequence was amplified in a 50 µL reaction containing: 30 ng template DNA, 0.25

mM dNTPs, 2.0 mM MgCl$_2$, 0.25 µM of each primer and 2.0 U Taq polymerase which were all acquired from New England BioLabs Ltd. Taq polymerase was kept on ice and added last. Each reaction was vortexed for approximately 30 s and placed into a BioRad T100^{TM} Thermal Cycler PCR machine. PCR settings were set-up such that initial denaturation occurred at 95°C for 5 min and 36 cycles of 95°C for 45 s, 58°C for 50 s, and 72°C for 150 s. The cycling ended with 72°C for 10 min.

2.4 Preparation of Gel Electrophoresis

After amplifying the PCR product, a gel was prepared using 150 mL of 1x Tris/Borate/ 0.5 M EDTA pH 8.0 (TBE) solution and 1.8 g of Agarose A (BioBasic Inc. Canada) and mixed together in an Erlenmeyer flask. The gel solution was microwaved for two min then left to cool for 15 min. Next, the solution was poured into a gel mould with a 15 well comb piece. After solidifying, the comb was taken out of the gel and the gel was placed into the electrophoresis chamber.

2.5 Gel Electrophoresis

To begin, 5 µL of DNA tracker 100 – 1500 bp size standard (BioBasic Inc. Canada) was added into the first well in the agarose gel. Then, 2 µL of gel dye solution consisting of 0.05% bromophenol blue and 30% glycerol in water (Sambrook et al, 2001) were added onto a sheet of Parafilm along with 5 µL of PCR product and mixed together. The product was transferred into the second well in the prepared agarose gel and then repeated for each sample. The gel was completely covered with 1x TBE solution. The power supply was set to 130V and left for 45 minutes. The remaining 45 µL of amplified product was stored at -20°C until commercial sequencing was performed.

After gel electrophoresis, the gel containing the PCR product was carefully transferred to a container consisting of 200 mL of distilled H$_2$O with 30 µL ethidium bromide (10 mg/mL)

for 30 minutes. The gel was then placed in a BioRad Molecular Imager Transilluminator System to view single bands at approximately 825 bp.

2.6 Sequence and DNA Analysis

After detection of visible bands on the electrophorized gel, amplified PCR products were sent for commercial sequencing by the company Eurofins Genomics (Toronto, Ontario, Canada). After receiving the sequenced product, chromatographs received from the company were used to determine the quality of sequences. An NCBI BLAST search was conducted on each clean sequence to ensure that the amplified product was DREB1.

The DREB1 sequences were then aligned using ClustalX software (Larkin et al. 2007) and imported to GeneDoc software (Nicholas and Nicholas, 1997) where genetic variation between each sequence could be examined. Number of haplotypes, haplotype diversity, theta (per site) (θ), and nucleotide diversity (π) were calculated using DNAsp5 software (Librado and Rozas 2009) to determine the genetic diversity between and within each species of cultivated and wild barley. DNAsp5 was also used to perform statistical tests: Fu and Li's D and F test and Tajima's D test. Fu and Li's D tests statistic is based on the differences between the number of singletons (mutations appearing only once among the sequences) and the total number of mutations and Fu and Li's F statistic is based on the haplotype frequency distribution conditional the value of theta. Tajima D statistic is calculated by determining the differences between two measures of genetic diversity; the mean number of pairwise differences and the number of segregating sites. Statistical significance was determined using * for $P < 0.05$ and ** for $P < 0.01$.

A phylogenetic analysis was conducted using the Neighbour Joining (NJ) and maximum parsimony methods from PAUP software (Swofford, 2002) to determine bootstrap percentages and relatedness among the samples.
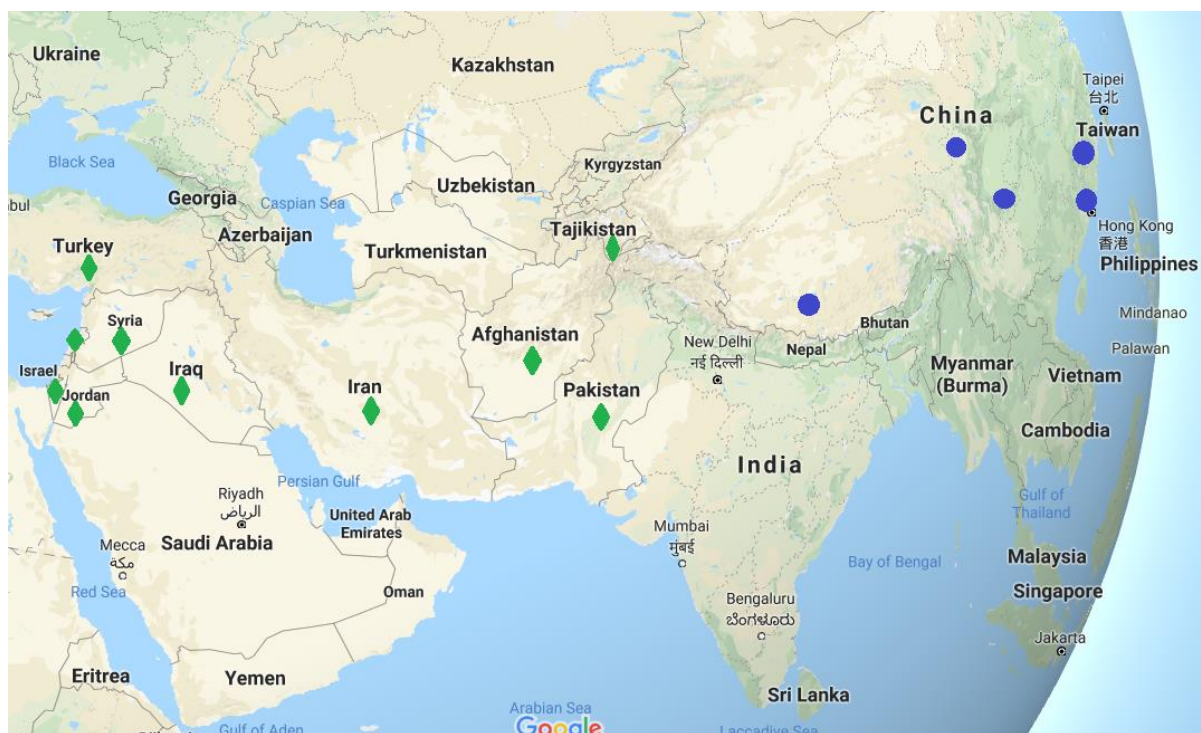
Figure 3: Global locations of wild barley (*Hordeum spontaneum*) ◆ and cultivated barley (*Hordeum vulgare*) ● used in this study.

Table 1: The code, accession number, origin and characteristic of 119 barley used in this study.

Code	Accession Number	Origon	Characteristic	Code	Accession Number	Origon	Characteristic
HS1	PI212305	Afghanistan	wild, two-row	HS69	PI662080	Tajikistan	wild, two-row
HS3	PI219796	Iraq	wild, two-row	HS70	PI662109	Tajikistan	wild, two-row
HS4	PI220664	Afghanistan	wild, two-row	HS71	PI662118	Tajikistan	wild, two-row
HS5	PI227019	Iran	wild, two-row	HS72	PI662138	Turkey	wild, two-row
HS7	PI236386	Syria	wild, two-row	HS73	PI662158	Turkey	wild, two-row
HS8	PI244772	Pakistan	wild, two-row	HS74	PI662170	Turkey	wild, two-row

HS9	PI244774	Afghanistan	wild, two-row	HS75	PI662178	Turkey	wild, two-row
HS10	PI244776	Afghanistan	wild, two-row	HS76	PI662188	Turkey	wild, two-row
HS11	PI244777	Afghanistan	wild, two-row	HS77	PI662204	Turkey	wild, two-row
HS12	PI245739	Turkey	wild, two-row	HS78	PI662214	Turkey	wild, two-row
HS13	PI253933	Iraq	wild, two-row	HS79	PI662218	Turkey	wild, two-row
HS14	PI254894	Iraq	wild, two-row	HS80	042	Sichuan, China	cultivated, six-row
HS15	PI268243	Iran	wild, two-row	HS81	043	Sichuan, China	cultivated, six-row
HS18	PI284752	Israel	wild, two-row	HS82	045	Sichuan, China	cultivated, six-row
HS19	PI293411	Tajikistan	wild, two-row	HS83	046	Sichuan, China	cultivated, six-row
HS20	PI293412	Tajikistan	wild, two-row	HS84	0177	Hunan, China	cultivated, six-row
HS24	PI296849	Israel	wild, two-row	HS85	087	Henan, China	cultivated, six-row
HS25	PI296862	Israel	wild, two-row	HS86	091	Sichuan, China	cultivated, six-row
HS27	PI296908	Israel	wild, two-row	HS87	0274	Sichuan, China	cultivated, six-row
HS28	PI354948	Israel	wild, two-row	HS88	0276	Shandong, China	cultivated, six-row
HS31	PI391100	Israel	wild, two-row	HS89	0384	Zhejiang, China	cultivated, six-row
HS32	PI401368	Iran	wild, two-row	HS90	056	Sichuan, China	cultivated, two-row
HS34	PI420911	Jordan	wild, two-row	HS91	0248	Sichuan, China	cultivated, two-row
HS35	PI420912	Jordan	wild, two-row	HS92	0252	Sichuan, China	cultivated, two-row

HS37	PI420915	Jordan	wild, two-row	HS93	0279	Sichuan, China	cultivated, two-row
HS38	PI420916	Jordan	wild, two-row	HS94	0353	Sichuan, China	cultivated, two-row
HS39	PI420917	Jordan	wild, two-row	HS95	065	Sichuan, China	cultivated, two-row
HS40	PI466040	Syria	wild, two-row	HS96	0381	Sichuan, China	cultivated, two-row
HS41	PI466048	Syria	wild, two-row	HS97	0382	Zhejiang, China	cultivated, two-row
HS42	PI466060	Syria	wild, two-row	HS98	0383	Zhejiang, China	cultivated, two-row
HS43	PI466086	Syria	wild, two-row	HS99	0385	Zhejiang, China	cultivated, two-row
HS44	PI466118	Syria	wild, two-row	HS100	01	Tibet, China	wild, two-row
HS45	PI466130	Syria	wild, two-row	HS101	03	Tibet, China	wild, two-row
HS47	PI466206	Syria	wild, two-row	HS102	04	Tibet, China	wild, two-row
HS48	PI466238	Syria	wild, two-row	HS103	013	Tibet, China	wild, two-row
HS49	PI466249	Lebanon	wild, two-row	HS104	018	Tibet, China	wild, two-row
HS50	PI466256	Lebanon	wild, two-row	HS105	022	Tibet, China	wild, two-row
HS51	PI466264	Lebanon	wild, two-row	HS106	023	Tibet, China	wild, two-row
HS52	PI466296	Israel	wild, two-row	HS107	026	Tibet, China	wild, two-row
HS53	PI466328	Israel	wild, two-row	HS108	027	Tibet, China	wild, two-row
HS54	PI466388	Israel	wild, two-row	HS109	028	Tibet, China	wild, two-row
HS55	PI466498	Israel	wild, two-row	HS110	02	Tibet, China	wild, six-row

HS56	PI466524	Israel	wild, two-row	HS111	05	Tibet, China	wild, six-row
HS57	PI466554	Israel	wild, two-row	HS112	06	Tibet, China	wild, six-row
HS58	PI466586	Israel	wild, two-row	HS113	07	Tibet, China	wild, six-row
HS59	PI466605	Iran	wild, two-row	HS114	09	Tibet, China	wild, six-row
HS63	PI554426	Turkey	wild, two-row	HS115	010	Tibet, China	wild, six-row
HS64	PI466632	Iran	wild, two-row	HS116	011	Tibet, China	wild, six-row
HS65	PI466699	Iran	wild, two-row	HS117	014	Tibet, China	wild, six-row
HS66	PI554428	Turkey	wild, two-row	HS118	015	Tibet, China	wild, six-row
HS67	PI559556	Turkey	wild, two-row	HS119	019	Tibet, China	wild, six-row
HS68	PI662052	Tajikistan	wild, two row				
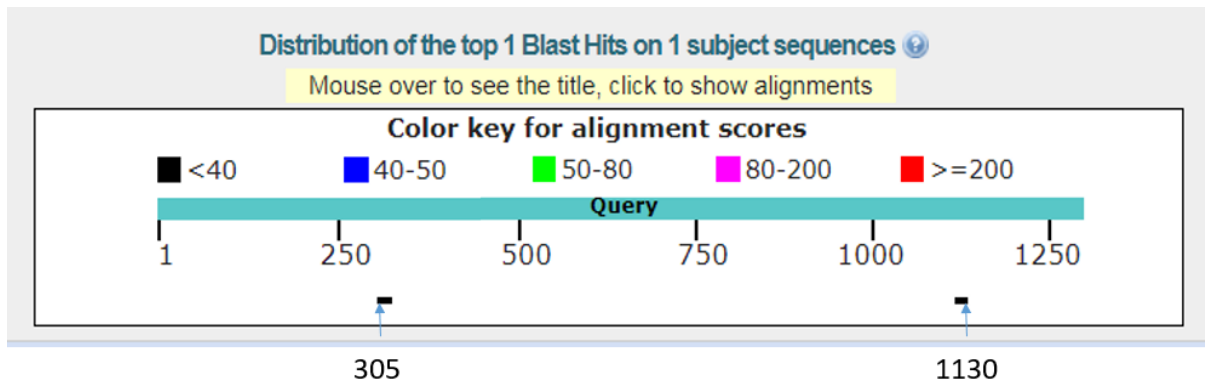
Figure 4: *Triticum aestivum* AP2-containing protein (DREB1) Gene, complete cds GenBank: AF303376.1 containing 1250 base pairs. Forward primer started at 305 bp and reverse primer started at 1130 bp resulting in an 825 bp strand.

3. Results

3.1 Amplified DNA Samples

Thirty of the 119 DNA samples were successfully amplified with the DREB1 primers (Figure 5). An example sequence is shown in Figure 6. The 30 sequenced samples were from three populations of barley: The Middle East wild-type, China wild-type and China cultivated-type. Their origin, code, accession number and characteristics are listed in Table 2. The Middle East population consisted of accessions from Syria, Israel, Lebanon, Iran, and Turkey and were all wild-type barley. China cultivated-type populations consisted of accessions from Sichuan and Zhejiang and China wild-type populations consisted of accessions from Tibet. Each accession included their own code to make labelling easier and their own accession number for identification from the manufacturer.

Table 2: The region of seed origin, code, accession number, origin and characteristics of the 30 successfully sequenced accessions out of 119 barley accessions amplified in this study.

Region	Code	Accession Number	Origin	Characteristics
Middle East wild-type	HS7	PI236386	Syria	wild, two-row
	HS18	PI284752	Israel	wild, two-row
	HS24	PI296849	Israel	wild, two-row
	HS51	PI466264	Lebanon	wild, two-row
	HS53	PI466328	Israel	wild, two-row
	HS55	PI466498	Israel	wild, two-row
	HS57	PI466554	Israel	wild, two-row
	HS64	PI466632	Iran	wild, two-row
	HS72	PI662138	Turkey	wild, two-row
	HS73	PI662158	Turkey	wild, two-row
	HS74	PI662170	Turkey	wild, two-row
	HS75	PI662178	Turkey	wild, two-row
	HS76	PI662188	Turkey	wild, two-row
	HS77	PI662204	Turkey	wild, two-row
	HS78	PI662214	Turkey	wild, two-row
	HS79	PI662218	Turkey	wild, two-row
	HS 54	PI466388	Israel	wild, two-row
China cultivated-type	HS80	42	Sichuan, China	cultivated, six-row
	HS81	43	Sichuan, China	cultivated, six-row
	HS83	46	Sichuan, China	cultivated, six-row
	HS89	384	Zhejiang, China	cultivated, six-row
	HS97	382	Zhejiang, China	cultivated, two-row
	HS98	383	Zhejiang, China	cultivated, two-row
	HS99	385	Zhejiang, China	cultivated, two-row
China wild-type	HS102	4	Tibet, China	wild, two-row
	HS103	13	Tibet, China	wild, two-row
	HS104	18	Tibet, China	wild, two-row
	HS105	22	Tibet, China	wild, two-row
	HS106	23	Tibet, China	wild, two-row
	HS118	015	Tibet, China	wild, two-row

Figure 5: Image of an electrophorized gel showing amplified DNA from PCR at 800 to 1000 bp indicated by a red arrow. Samples included in the gel from left to right were HS 89, 97, 98, 99, 80, 81 and 83 China cultivated-type.

DNA from 17 accessions of Middle East wild-type, seven accessions of China cultivated-type and six accessions of China wild-type were PCR amplified using the DREB1 primer pair. By visually analyzing the electrophorized gel in Figure 5, clear bands were present indicating the amplified product was around 800 to 1000 bp. An NCBI BLAST search found that the 30 sequences (Table 2) shared 90 to 100% identity with *Hordeum vulgare* subsp. *vulgare* DREB1 complete coding sequences (DQ012941.1).
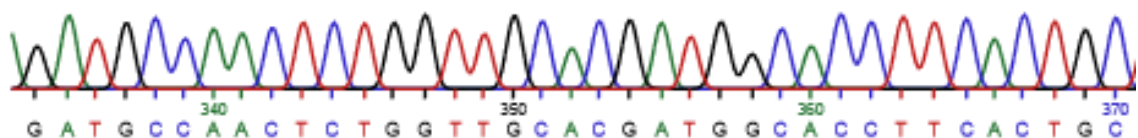
Figure 6: Colour coordinated chromatograph of a partial sequence of the *DREB1* gene amplified in HS 7 wild-type barley produced by sequence manufacturer.

The 30 amplified DNA sequences were aligned with the program ClustalX. This illustration of a partial sequence can be seen of sample HS 7 (wild-type barley) at around 300 to 400 base pairs. Each strand of DNA had its own chromatograph with clear distinguishable, high peaks that indicated each nucleotide was reliable (see addendum, page 40). Unclear chromatographs were discarded, and DNA

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amplification will need to be repeated. Green, blue, black, and red peaks represented

nucleotides A, C, G, and T respectively.

Figure 7: Example of comparison of partial sequences within China cultivated barley formulated by GeneDoc software. Samples include HS 89, 97, 98, 99, 80, 81, 83 and show base pairs 20 to 80.

 After determining which sequences were reliable from the chromatographs in Figure 6,

sequences from the amplified products were all aligned and compared using GeneDoc software
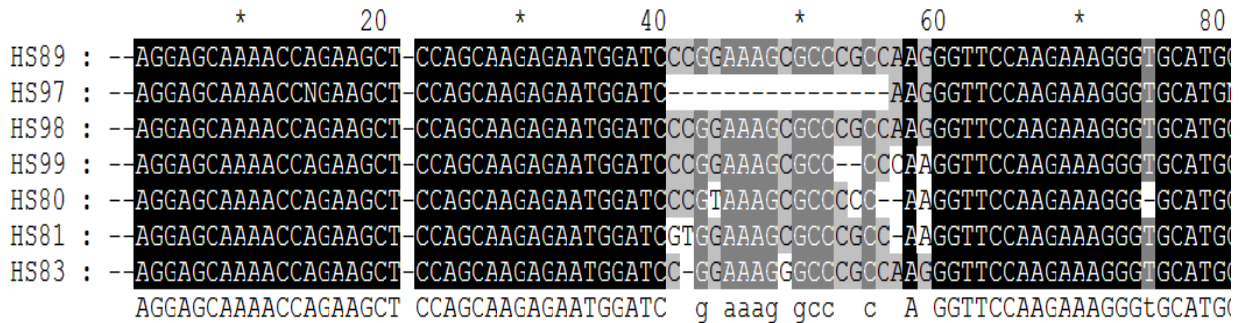
(Figure 7). This example of a comparison of partial sequences was comprised of China

cultivated barley. Code numbers of the samples include HS 89, HS 97, HS 98, HS 99, HS 80,

HS 81, and HS 83 and show variation between sequences from base pair numbers 40 to 60 and

grey highlighted base pairs 40 to 60 indicate positions with differences. The dashes occurring

between base pairs 40 to 60 in sequence HS 97, HS 99, HS 80, HS 81 and HS 83 and between

base pairs 60 to 80 in sequence HS 80 indicate that a deletion of a nucleotide occurred.

 The number of haplotypes of DREB1 sequences from wild-type and cultivated-type

barley were calculated (Table 3). A total of 20 haplotypes were identified in the 30 accessions

which included: 9 haplotypes from 17 sequences of Middle East wild-type, 5 haplotypes from

6 sequences of China wild-type and 6 haplotypes from 7 sequences of China cultivated-type.

The highest haplotype diversity was seen in China cultivated-type as 0.952, with the second

highest seen in China wild-type as 0.933 and the smallest haplotype diversity seen in Middle

East wild-type as 0.787.

3.3 Nucleotide Diversity

Nucleotide diversity π and θ were calculated for each of the wild and cultivated species (Table 3). The highest nucleotide diversity value for π was found in China wild-type barley as 0.01002, followed by Middle East wild-type as 0.00619, and the smallest value found in China cultivated-type as 0.00618. The highest nucleotide diversity value for θ was found in Middle East wild-type as 0.01466, followed by China wild-type as 0.01139 and the smallest value found in China cultivated-type barley as 0.00602. Tajima (1989) and Fu and Li's (1993) D and F statistics were also calculated for each of the wild and cultivated barley species. Tajima's D values for Middle East wild-type, China wild-type and China cultivated-type were calculated to be -2.14252, -0.71767 and 0.1321 respectively. Tajima's D values for Middle East wild-type and China wild-type were found to be statistically significant, however China cultivated type was not statistically significant. Fu and Li's D values for Middle East wild-type, China wild-type and China cultivated-type were calculated to be -2.72204, -0.7751, and 0.22792 respectively and Fu and Li's F values for Middle East wild-type, China wild-type and China cultivated-type were calculated to be -2.95194, -0.82236 and 0.22446 respectively. Fu and Li's D and F values were both statistically significant for Middle East wild-type and China wild-type but not statistically significant for China cultivated-type.

Table 3: Estimates of nucleotide diversity within each barley population. Number of haplotypes, haplotype diversity, theta (θ) and nucleotide diversity (π) were calculated with DNAsp5 software. Tests used to determine statistical significance include Fu and Li's D and F test and Tajima's D test where * indicates $P < 0.05$ and ** indicates $P < 0.01$ which were also created using DNAsp5 software.

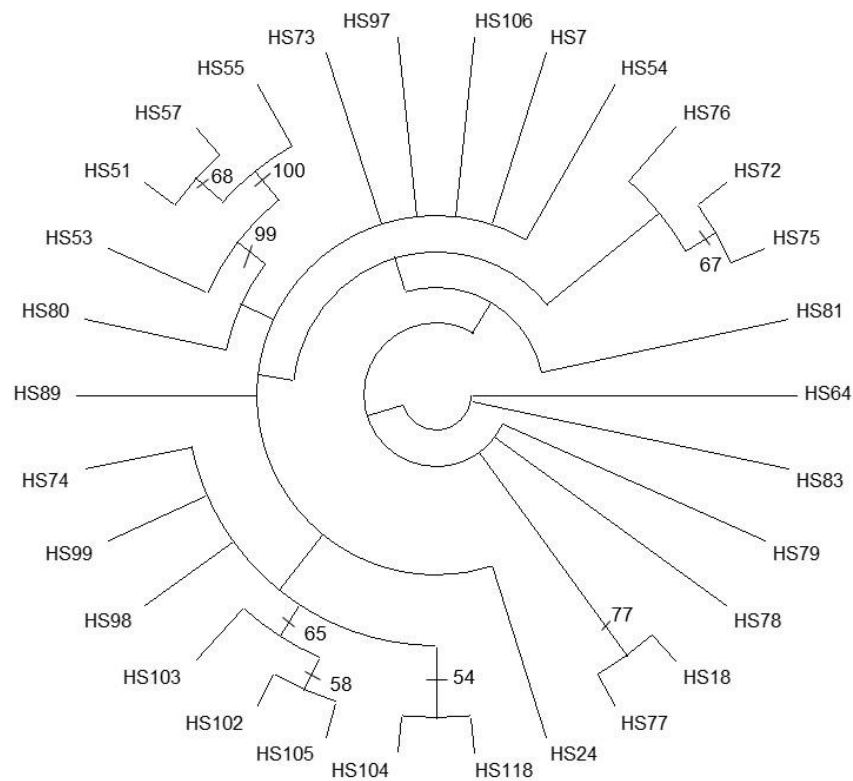| Populations	No. of Haplotypes (H)	Haplotype Diversity (Hd)	Theta (per site) (θ)	Nucleotide Diversity (π)	Fu and Li's D Test	Fu and Li's F Test	Tajima's D Test
Middle East, Wild-type	9	0.787	0.01466	0.00619	-2.72204*	-2.95194**	-2.14252**
China, Wild-type	5	0.933	0.01139	0.01002	-0.7751*	-0.82236*	-0.71767*
China, Cultivated-type	6	0.952	0.00602	0.00618	0.22792	0.22446	0.1321

3.4 Phylogenetic Analysis

Figure 8: Phylogenetic tree and bootstrap values indicating percent support of 30 sequences in the DREB1 gene created with PAUP software.

Table 4: Organization of 5 groups of related DREB1 DNA samples from phylogenetic tree (Figure 8). Regions include China wild-type, China cultivated-type and Middle East wild-type.

Location	Group 1	Group 2	Group 3	Group 4	Group 5
China wild-type				HS 106	HS 103 HS 102 HS 105 HS 104 HS 118
China cultivated-type	HS 83	HS 80	HS 81	HS 97	HS 89 HS 99 HS 98
Middle East Wild-type	HS 77 HS 78 HS 64 HS 18 HS 24 HS 79	HS 51 HS 57 HS 55 HS 53	HS 75 HS 72 HS 76	HS 54 HS 7 HS 73	HS 24

A phylogenetic tree based on Neighbour Joining (NJ) method was modelled and illustrates the relatedness of the 30 sequenced DREB1 genes from Middle East wild-type populations, China wild-type populations and China cultivated-type populations of barley (Figure 8). Based on relatedness, groups were formed and were recorded in Table 4. The composition of the 1st group was found to be primarily wild-type, however one cultivated-type (HS 83) was present. The 2nd group was found to be mostly wild-type with one cultivated-type (HS 80). The composition of the 3rd group was comprised of primarily wild-type with one cultivated-type (HS 81) present. The composition of the 4th group was wild-type with one cultivated-type (HS 97) present. The 5th group was primarily wild-type with three cultivated-type including (HS 89, 99 and 98). The Middle East wild populations and China cultivated populations were represented in all five groups and the China wild-type population was only represented in group 4 and 5.

The phylogenetic relationship of the 30 DREB1 sequences from cultivated and wild-type barley was also analyzed using the maximum parsimony method. The maximum parsimony analysis resulted in 183 most parsimonious trees (263 constant characters, 83

parsimony-informative characters, 42 parsimony uninformative characters, CI = 0.885; RI = 0.886).

The strict consensus phylogenetic tree (Figure 8) yielded bootstrap values for group 1 (Table 4) Middle East wild-type samples HS 77 and HS 18 which had 77% support. Group 2 yielded highly supported bootstrap values between Middle East wild-type samples HS 53 and HS 51, HS 57, and HS 55 which was determined to be 99% support. Even higher bootstrap values of 100% were among Middle East wild-type samples HS 51, HS 55 and HS 57; however, the bootstrap value between HS 57 and HS 51 was only 68%. Group 3 yielded one bootstrap value of 67% between HS 72 and HS 75 samples in Middle East wild-type. Group 5 yielded bootstrap values between China wild-type samples HS 102, HS 105 and HS 103 which was determined to be 65%. Also belonging to group 5, bootstrap values were given for samples HS 104 and HS 118 which showed 54% support.

4. Discussion

Cultivable lands that are important factors for contributing to food security in the developing world have been greatly suffering due to the exploitation of the population and urbanization (Govindaraj et al. 2014). Agricultural practices have been domesticating and exploiting crop species from the beginning of industrialization to meet preference, food requirements and surplus for the growing population. These activities over time have led to an extinction of primitive and adaptive genes and a loss of genetic diversity (Govindaraj et al. 2014). Genetic diversity is important in almost all aspects of biology including molecular genetics, evolutionary adaptation, gene expression, conservation and many others. It allows natural selection to decrease or increase the frequency of alleles already in the population through genetic and drift or selection by humans. This loss of genetic diversity may cause deleterious mutations (Wang, 2016). The objective of this study was to compare the genetic diversity among the Middle East wild-type, China wild-type and China cultivated-type where

we expected to see more genetic diversity contained within the wild species compared to the cultivated species.

4.1 Sequence Analysis

Plant molecular, cellular, physiological, and biochemical level responses to stress (including cold, heat, drought and high salinity) may fail to activate defence mechanisms like important cell signalling pathways and cell responses if functional and regulatory proteins are compromised from deletions and mutations in their genes. This could lead to the plants inability to acclimate to new areas and eventually become unadaptable. Within a partial sequence of China cultivated barley (Figure 7), deletions were indicated by dashes and nucleotides mutations were indicated by grey highlight. These deletions and mutations within the DREB1 gene in cultivated barley show they are no longer similar to their wild form. This may be a result of the barley attempting to acclimate to a new area, or the barley has mutated through domestication processes.

In this study, the combined wild population of barley (23 accessions) showed 14 haplotypes of the DREB1 gene in the 30 sequenced accessions (Table 3, also see addendum, page 40). This was compared to the 6 haplotypes found in the cultivated barley (7 accessions). This agrees with previous statements made that these domesticated lines have lost most alleles compared to the wild species. Also included in Table 3 is per site nucleotide diversity which indicated there was around a 57.8% reduction in the China cultivated barley compared to the Middle East wild barley, and a 22.3 % reduction between the Middle East wild species and the China wild species. These results agree with Fu (2012) and Morrell et al. (2003), that suggested barley landraces may have suffered a population bottleneck causing a reduction of genetic diversity due to domestication. This loss is apparent in the Tajima's D values for the China cultivate-type population because the values shift towards positive values compared to both

wild species. These results also match other studies (Wright et al. 2005) and supports that the bottleneck effect may result in a loss of genetic diversity (Govindaraj et al. 2014). The results from Table 3 reveal that wild germplasms originating in the Middle East (East Fertile Crescent) are of great importance when it comes to contributing to cultivated barley gene pool (Wang et al. 2016). The DREB1 gene showed statistically significant negative values of Fu and Li's F and D test which may indicate that there was a deviation from neutrality that could be due to positive selection in the wild species (Kilian et al. 2006). Because the negative values for the Middle East wild-type are more negative than China wild-type, they may have more positive selection through genetic diversity. Positive values in the cultivated-type may indicate the opposite is happening. Tajima's D and both of Li and Fu's tests are all statistically insignificant and positive in the cultivated-type which may have resulted from the bottleneck effect (Wang et al. 2016).

4.2 Phylogenetic Analysis

A phylogenetic analysis of the 30 accessions of barley used in this study was conducted and resulted in the construction of a phylogenetic tree (Figure 8, also see addendum, page 40). Relatedness of the 30 accessions were illustrated and show the closest related sequences with the bootstrap values. This phylogenetic tree was then divided into groups based on their relatedness (Table 4). Accessions HS 80, HS 53, HS 51, HS 57 and HS 55 show a close relationship with a bootstrap value of 99%. These accessions were collected from the same region (The Middle East, wild) except for accession HS 80 which is China cultivated-type (Table 4). This may indicate that not much genetic change on the DREB1 gene sequence occurred among these accessions. Accessions HS 51, HS 57 and HS 55 from group 2 come from the same region (The Middle East, wild) and share a 100% bootstrap value indicating they have a high certainty of being related. HS 51 (Lebanon) and HS 57 (Israel) was grouped together with 68% support (Table 2). Cultivated accessions were grouped together with wild

accessions from different regions and may indicate multiple contributions from gene pools from wild-type to domesticated barley.

4.3 Conclusion

In summary, this study shows a clear pattern of genetic diversity difference in the DREB1 gene between wild and cultivated barley populations, supporting the bottleneck effect of genetic diversity during domestication. As previously mentioned, loss of diversity in the DREB1 gene may cause reduction in acclimation to stress especially as the rate of climate change increases from urbanization and human population growth. The pattern of domestication in barley is still controversial and information on geographically based genetic differentiation of barley populations is not well documented (Wang et al. 2016). The purpose of this study was to help understand the complications with domestication of barley and to provide further understanding to successful crop production in the future. To further this research and to provide more insight to the issues surrounding the loss of genetic diversity in the DREB1 gene, both wild and cultivated species may be subjected to stress treatments (i.e. cold) and sequenced once again to compare between each population. In a study such as this, the genetic diversity from the Middle East wild, China cultivated and China wild barley should be compared from the DREB1 gene after subjection to cold stress as well as comparing any mutations that occurred.

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5. Addendum

Found in the promoter region of target stress responsive genes such as rd29a, there is a cis-acting element responsible for cold and dehydration induced expression. The 9 bp conserved sequence TACCGACAT, termed the dehydration responsive element (DRE) is responsible for the regulation of this gene expression. cDNAs encoding DRE binding proteins, known as DREB1 specifically interact with the DRE sequence in the promoter region of the rd29a gene from dehydration and cold stress where DREB1 acts as a transcriptional activator for DRE-dependent transcription. Should this conserved region become altered, it is possible the function of the gene may be altered or lost.

At the beginning of this study, the goal was to examine adaptive mutations in the DREB1 gene in all 119 barley accessions after subjecting the plants to cold stress. To achieve this goal, I amplified and sequenced the coding region of DREB1 in cultivated and wild barley. Within this coding region, it may have been possible that we would see a silent mutation, where a base substitution occurs in the third position of the codon producing greater chances of generating a synonymous codon. This would mean the amino acid sequence encoded by the gene is not changed and the mutation would be deemed "silent." These mutations may be seen in conserved regions of genes such as in the AP2 DNA-binding domain in DREB1 genes as it is an important attachment site to the promoter of stress responsive target genes. Another mutation we may have seen is a deletion or insertion where a base pair is taken out or added to the sequence, respectively. This could result in a frameshift if the number of nucleotides added or removed is not a multiple of 3. In this case the resulting translation frame leaves a non-functional product. We expect to observe deletions and insertions more commonly in non-coding regions of the gene (i.e. promoter region, introns, untranslated regions), however, should it occur in conserved regions, the gene may no longer function properly as it should.

In eukaryotic DNA, coding regions (exons) are interrupted by non-coding regions (introns). During transcription, the gene is copied into pre- mRNA which excludes the exons and introns and eventually during RNA-splicing, introns are removed, and exons joined to form a mature coding sequence. Once this occurs, the mRNA is ready for translation. In Figure A1 of this addendum, the DREB1 intron is indicated in the Genebank sequence (DQ012941.1) with green highlight and based on the PCR primers also indicated in Figure 1, the intron is not located in the DNA amplified by PCR in this study. The PCR product in this study is in exon 2. Mutations in introns are less likely to affect gene function because the intron sequence is discarded before the mRNA is translated. Although this may be the case for introns, if the mutation causes the intron to be incorrectly spliced out of the mRNA, there may still be a negative effect on the gene expression, as sequences from the intron could be translated into the protein. Mutations in exons may be especially detrimental to the function of a gene as the segment of DNA or RNA molecule contains information coding for a protein or peptide sequence thus directly affecting the function.

CAAAACCAAGGCGGCGGCAGCGGGGTGGGAGAGCCGGGAGCACCGACCGACACCGGGGGCTGCATGCGGAGCTGAGGCGAGGCGAGGAG
GATCCGGCGCGGGTGCCACCGCCGCCCGCCCGCGGGAGATCTGGTTGGCGGCGCCGCCGCCCGGACAAGGAAGCGGCCGCGGAGGCGGC
GTGGGGCGAGCTGCCGGGGAGGCCGACGAAGCTAGAGGAGATCTCTCTCTCCCTTCCTCCCTCCTCTTCCGCCTCGATGGAGACCGGGGGTAG
CAAGCGGGAAGGAGACTGCCCCGGGCAGGAAAG|GAC<u>GAAGAA</u><mark>AGT</mark><u>GCGCAGGAGAAC</u>CACTGGTCCAGATTCGGTTGCTGAAACTATCAAGA
AGTGGAAGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCGGAAAGCGCCCGCCAAGGGTTCCAAGAAAGGGTGCATGGCAGGGAAA
GGAGGTCCAGAGAATTCAAACTGCGCTTACCGCGGTGTGAGGCAGCGCACGTGGGGCAAATGGGTGGCTGAGATCCGTGAGCCCAACCGTGGC
AACCGGCTGTGGCTTGGTTCATTCCCTACCGCAGTCGAAGCTGCACGTGCATATGATGATGCCGCAAGGGCAATGTATGGCGCCACAGCGCGTG
TCAACTTCCCAGAGCATTCCCCAGATGCCAACTCTGGTTGCACGATGGCACCTTCACTGCTGACGTCTAATGGGGCAACCGCTGTGTCACATCCGT
CTGATGGGAAGGATGAATCAGAATCTCCTCCTTCTCTTGTCTCAAATGCGCCGACAGCTGCGCTGCATCGGTCTGATGCCAAGGATGAGTTTGAGT
CTGCAGGGACTGTGGCGCATAAGGTGAAAACAGAAGTGAGCAATGATTTGGGAAGTACCCATGAGGAGCACAAGGCCCTGGAAGTATTCCAACC
AAAAGGGAAGGCTTTACATAAAGAAGCGAACGTAAGTTATGATTACTTCAACGTTGAAGAAGTTGTCGACATGATAATTGTGGAATTGAGTGCTGAT
GTAAAAATGGAAGCACATGAAGAGGTCATGTACCAAGAGGGCGATGACGGGTTTAGTCTTTTCTCATATTAGGGTTC<mark>TAG</mark>CTATGAGGGTTGTA<u>TG
GCGGAGCAATAGGGA</u>TAACTTCATTCTAGCTGCTAGGAAATACTTCAAATTATCTGCAACCTGAAGCTTTGTAGTCACTTATGGTTTTAATCTTACTG
GAGAGAATAGCTTTATACCATAAGTCAACGGGTACAAGAAGTTGTCCTGTGTGTCGAGTTCATGTACTGTGGTAAAAATTGAGTCCATATTTAATGAG
CTTACTCTGTTGA

Figure A1: *Hordeum vulgare* DREB1 mRNA complete cds (DQ12941.1). Indicated in bold and underlined are the PCR primers used in the methods of this study. The green highlight and vertical line indicate the position of the intron. The amplified region does not span the intron and it lies between the start and stop codon (indicated by yellow highlight).

After receiving only 30 clear samples that were relatively short, we chose to study the nucleotide diversity in the DNA sequences to examine the genetic variation between wild barley and cultivated barley, instead of translating the sequences to examine the effects of adaptive mutations. Although we changed the focus of our study, it is important to note that these variations in nucleotide sequences may have a resulting effect on the sequences of the amino acids. In Figure A2 and A3 of this addendum, I have aligned amino acid sequences of cultivated barley and wheat as well as mRNA sequences. This is to show their similarities and differences especially in important regions such as the AP2 DNA-binding domain. Highlighted in turquoise in each of these sequences (Fig. A3) is the AP2 DNA-binding domain that shows very similar sequences between the comparisons. Cultivated barley and wheat only have one mutation in the sequences comparison as they are closely related and belong to the same family and tribe. This provides evidence that a significant amount of variation in these regions should not occur as it may alter the stress responsive effect of the gene.

In Figure 7 of the thesis, partial sequences of cultivated barley were aligned that show possible deleterious mutations that could have resulted in a loss of function for the DREB1 gene. In Figure 4 of the addendum, I have aligned all sequences from Figure 7 in the thesis including their amino acids to show variation between the reference gene found on GeneBank (DQ012941.1) and the accessions I tested of cultivated barley. Indicated by yellow highlight are changed in sequence or deletions which have resulted in frameshift mutations, thus changing the amino acid sequence. As previously mentioned, the sequences in this study did not include introns, and are therefore coding regions where any change to the coding region may cause a loss of function for the protein. The barley sequences in Figure A4 are expected to contain less variation than between barley and wheat because cultivated barley originated from wild barley and is a subspecies, however, this is not what we observed. While the wheat species only had one difference in sequence, the barley sequenced in our study had multiple.

This analysis is important because it may indicate the sequences in this study may not be accurate and should be retested. Low sequence quality such as this will also create changes to the phylogenetic tree (Fig. 8) in the results of the thesis. In the future, to check the reliability of the phylogenetic tree a reference sequence such as those in GeneBank should be added.

Barley vs. Wheat: DREB1 mRNA

Cultivated Barley (Top)

Wheat (Bottom)

```
DQ012941.1 CAAAACCAAGGCGGCGGCAGCGGGGTGGGAGAGCCGGGAGCACCGACCGACA------- 52
AF303376.1 CAAAACCAAGGCGGCGGCAGCGGGGCGGGAGAGCGGGGAGCACCGACCGACACCGGCCGA 60
           ************************* ******* ****************

DQ012941.1 --CCGGGGGCTGCATGCGGAGCTG----------AGGCGAGGCGAGGAGAGATCCGGCGC 100
AF303376.1 CAGGGTGGGCTGCATGCGGAGCTGAGGCGAGGCGAGGCGAGGCGGGGAGAGATCCGGCGC 120
             * ***************** ********** ***************

DQ012941.1 GGGTGCCACCGCCGCCCGCCCGCGGGAGATCTGGTTGGCGGCGCCGCCGCCCGGACAAGG 160
AF303376.1 GGGTGCCACCGCCGGCCGGCCGCGGGAGATCTGGTTGGTGGCGCCGCCCGGATAAGGGAG 180
           ************* *** ****************** ********* * *

DQ012941.1 AAGCGGCCGCGGAGGCGGCGTGGGGCGAGCTGCCGGGGAGGCCGACGAAGCTAGAGGAGA 220
AF303376.1 AGGCGGCGA------------GGGGAGAGCAGCCGGGGG--AGACCGAGGCGAGAGGAG- 225
           * ***** **** **** ******* *** ** *******

DQ012941.1 TCTCTCTCTCCCTTCCTCCCTCCTCTTCCGCCTCG[ATG]GAGACCGGGGGTAGCAAGCGGG 280
AF303376.1 ---------ATCTCTCTCGTCCCTCTTCTCGCTCC[ATG]GAGACCGGGGGTAGCAAGCGGG 276
                    ** *** ******* *** ***********************

DQ012941.1 AAGGAGACTGCCCCGGGCAGGAAAGGA[C]GAAGAAAGTGCGCAGGAGAA[C]CACTGGTCCAG 340
AF303376.1 AAGGAGACTGCCCCGGGCAGGAAAGGA[A]GAAGAAAGTGCGCAGGAGAA[G]CACTGGTCCTG 336
           *************************** ******************** ********* *

DQ012941.1 ATTCGGTTGCTGAAAC[T]ATCAAGAAGTGGAAGGAGCAAAACCAGAAGCTCCAGCAAGAGA 400
AF303376.1 ATTCGGTTGCTGAAAC[C]ATCAAGAAGTGGAAGGAGGAAAACCAGAAGCTCCAGCAAGAGA 396
           *************** ***************** **********************

DQ012941.1 ATGGATCCCGGAAAGCGCCCGCCAAGGGTTCCAAGAAAGGGTGCATGGCAGGGAAAGGAG 460
AF303376.1 ATGGATCCCGGAAAGCACCGGCCAAGGGTTCCAAGAAAGGGTGCATGGCAGGGAAAGGAG 456
           *************** ** **********************************
```

```
DQ012941.1  GTCCAGAGAATTCAAACTGCGCTTACCGCGGTGTGAGGCAGCGCACGTGGGGCAAATGGG 520
AF303376.1  GTCCAGAGAATTCAAACTGCGCTTACCGCGGTGTGAGGCAGAGGACGTGGGGGAAATGGG 516
            ***************************************** * ******** *******

DQ012941.1  TGGCTGAGATCCGTGAGCCCAACCGTGGCAACCGGCTGTGGCTTGGTTCATTCCCTACCG 580
AF303376.1  TTGCTGAGATCCGTGAGCCCAACCGTGGCAATCGGCTGTGGCTTGGTTCATTCCCTACCG 576
            * *************************** *** **************************

DQ012941.1  CAGTCGAAGCTGCACGTGCATATGATGATGCCGCAAGGGCAATGTATGGCGCCACAGCGC 640
AF303376.1  CAGTCGAAGCTGCACGTGCATATGATGATGCGGCAAGGGCAATGTATGGCGCCAAAGCAC 636
            ****************************** ********************** *** *

DQ012941.1  GTGTCAACTTCCCAGAGCATTCCCCAGATGCCAACTCTGGTTGCACGATGGCACCTTCAC 700
AF303376.1  GTGTCAACTTCTCAGAGCAGTCCCCGGATGCCAACTCTGGTTGCACGCTGGCACCTCCAT 696
            *********** ******* ***** ******************** ******* **

DQ012941.1  TGCTGACGTCTAATGGGGCAACCGCTGTGTCACATCCGTCTGATGGGAAGGATGAATCAG 760
AF303376.1  TGCCGATGTCTAATGGGGCAACCGCTGCGTCACATCCTTCTGATGGGAAGGATGAATCGG 756
            *** ** ****************** ********* ***************** *

DQ012941.1  AATCTCCTCCTTCTCTTGTCTCAAATGCGCCGACAGCTGCGCTGCATCGGTCTGATGCCA 820
AF303376.1  AGTCTCCTCCTTCTCTTATCTCAAATGCGCCGACAGCTGCGCTGCATCGGTCTGATGCTA 816
            * ************** ****************************************** *

DQ012941.1  AGGATGAGTTTGAGTCTGCAGGGACTGTGGCGCATAAGGTGAAAACAGAAGTGAGCAATG 880
AF303376.1  AGGATGAGTCTGAGTCTGCAGGGACCGTGGCACGTAAGGTGAAAAAAGAAGTGAGCAATG 876
            ********* ************** ***** * ********** ************

DQ012941.1  ATTTGGGAAGTACCCATGAGGAGCACAAGGCCCTGGAAGTATTCCAACCAAAAGGGAAGG 940
AF303376.1  ATTTGAGAAGTACCCATGAGGAGCACAAGACCCTGGAAGTATCCCAACCAAAAGGGAAGG 936
            ***** ********************** ********** ****************

DQ012941.1  CTTTACATAAAGAAGCGAACGTAAGTTATGATTACTTCAACGTTGAAGAAGTTGTCGACA 1000
AF303376.1  CTTTACATAAAGCAGCGAACGTAAGTTATGATTACTTCAACGTCGAGGAAGTTCTTGACA 996
            ************ ******************************* ** ****** * ****

DQ012941.1  TGATAATTGTGGAATTGAGTGCTGATGTAAAAATGGAAGCACATGAAGAGTACCAAGAGG 1060
AF303376.1  TGATAATTGTGGAATTGAGTGCTGATGTAAAAATGGAAGCACATGAAGAGTACCAAGATG 1056
            ********************************************************* *

DQ012941.1  GCGATGACGGGTTTAGTCTTTTCTCATAT`TAG`GGTTCTAGCTATGAGGGTTGTAGTCATG 1120
AF303376.1  GTGATGATGGGTTTAGTCTTTTCTCATAT`TAG`GGTTTTAGCTATGAGGGTTGCAGTCATG 1116
            * ***** *************************** ************** *******

DQ012941.1  CGGAGCAATAGGGATAACTTCA-TTCTAGCTGCTAGGAAATACTTCAAATTATCTGCAAC 1179
AF303376.1  CGGAGCAATAGGGATAACTTTCATTCTAGCTGCTAGGAAATACT---TCAAATCTGCAAC 1173
            ******************** ******************** *********
```

44

```
DQ012941.1 CTGAAGCTTTGTAGTCACTTATGGTTTTAATCTTACTGGAGAGAATAGCTTTATACCATA 1239

AF303376.1 CCGAAGCTTTGTAGTCACTTATGGTTTTCATCTTACTGGAGAGAATAGCTTTATACCATA 1233

           *  ************************** ******************************


DQ012941.1 AGTCAACGGGTACAAGAAGTTGTCCTGTGTGTCGAGTTCATGTACTGTGGTAAAAATTGA 1299

AF303376.1 AGTCAACGGGTACAAGAAGTTGTCCTGTGCGTTGAGTTCATGTACTATGGTAAAAGTTG- 1292

           ***************************** ** ************* ******* ***
```

Figure A2: DREB1 mRNA sequence in cultivated barley DQ012941.1 aligned with DREB1 mRNA in wheat AF303376.1. Between the start and stop codons (green highlights) single nucleotide variation is evident (e.g. yellow highlights, no star in the third row). Some deletions and insertions are present in the 5' and 3' untranslated regions.

Translations

DREB1 Cultivated Barley (Top)

DREB1 Wheat (Bottom)

```
METGGSKREGDCPGQERTKKVRRRTTGPDSVAETIKKWKEQNQKLQQENGSRKAPAKGSK      60
METGGSKREGDCPGQERKKKVRRRSTGPDSVAETIKKWKEENQKLQQENGSRKAPAKGSK      60
*****************.*****:****************:********************


KGCMAGKGGPENSNCAYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAA     120
KGCMAGKGGPENSNCAYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAA     120
***********************************************************


RAMYGATARVNFPEHSPDANSGCTMAPSLLTSNGATAVSHPSDGKDESESPPSLVSNAPT     180
RAMYGAKARVNFSEQSPDANSGCTLAPPLPMSNGATAASHPSDGKDESESPPSLISNAPT     180
******.***** *:**********:** *    ******.*****************:*****


AALHRSDAKDEFESAGTVAHKVKTEVSNDLGSTHEEHKALEVFQPKGKALHKEANVSYDY     240
AALHRSDAKDESESAGTVARKVKKEVSNDLRSTHEEHKTLEVSQPKGKALHKAANVSYDY     240
**********  ******* :***.****** *******:*** :*** ********* *******
```

Figure A3: Translated protein sequences of DREB1 *Hourdeum vulgare* (cultivated barley) ABA08424.1 and *Triticum aestivum* (wheat) ABA08424.1 aligned. Turquoise highlighting indicates the AP2 DNA-binding domain amino acid sequence and yellow highlights indicates amino acids that are altered by mutations. The underlined region corresponds to the aligned sequences in Figures 7 and A4.

GeneBank Barley (DQ012941)

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCGGAAAGCGCCCGCCAAGGGTTCCAAGAAAGGGTGCATG

  E Q N Q K L Q Q E N G S R K A P A K G S K K G C `Met`

GeneBank Wheat (AF303376.1)

AGGAGGAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCGGAAAGC`A`CCGGCCAAGGGTTCCAAGAAAGGGTGCATG

HS 83 (FS)

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCC-GGAAAG`GG`CC`C`GCCAAGGGTTCCAAGAAAGGGTGCATG

 E Q N Q K L Q Q E N G S G K G P P R V P R K G A

HS 80 (FS)

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCG`T`AAAGC`G`CC`C`-CCAAGG`-`TTCCAAGAAA`-`GG`G`GCATG

  E Q N Q K L Q Q E N G S R K A P P R F Q E R G `Met`

HS 99 (FS)

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCGGAAAGC`GCCC`-CCAAGG`-`TTCCAAGAAAGGGTGCATG

 E Q N Q K L Q Q E N G S R K A P P R F Q E R V H

HS 97 (FS)

AGGAGCAAAACC`N`GAAGCTCCAGCAAGAGAATGGATC`NNNNNNA NNNNNNNN`CAAGGGTTCCAAGAAAGGGTGCATG

 E Q N X K L Q Q E N G X   XXXXX   K G S K K G C `M`

HS 98

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCGGAAAGC`GCCC`GCCAAGGGTTCCAAGAAAGGGTGCATG

 E Q N Q K L Q Q E N G S R K A P A K G S K K G C `M`

HS 89

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATCCCGGAAAGC`GCCC`GCCAAGGGTTCCAAGAAAGGGTGCATG

 E Q N Q K L Q Q E N G S R K A P A K G S K K G C `M`

HS 81 (FS)

AGGAGCAAAACCAGAAGCTCCAGCAAGAGAATGGATC`GT`GGAAAGC`G`CC`C`GCCAAGG`-`TTCCAAGAAAGGGTGCATG

 E Q N Q K L Q Q E N G S W K A P A K V P R K G A

Figure A4: Partial sequences of cultivated barley from Figure 7 in results of thesis aligned with GeneBank reference sequence DQ012941 and AF303376.1. Yellow highlight indicates changes in barley reference sequence. (FS) indicated in each title represents a sequence containing a frameshift mutation.

PCR is an important tool in molecular biology; however, it is a process that can fail in a number of ways if not done properly. Weak bands, such as those seen in figure 5 of the thesis may be a result of various issues including degraded PCR primers, inaccurate concentrations, or inaccurate PCR settings (i.e. number of cycles or temperature). For example, if the denaturing time is too short, the DNA will not completely denature or if the annealing time is too short, the primers will not have enough time to bind to the template. Only bands

that are completely clear should be sent away for commercial sequencing to eliminate the chance of receiving inaccurate samples.

To conclude this addendum, it is important to acknowledge the importance of translating DNA sequences as it provides a quality check and is useful when finding amino acid changes that may be adaptive or detrimental to the function of a gene. Although variation was seen in the results of this thesis, a clearer result could be obtained by sampling all 119 accessions rather than the 30 used in this study. Sequences should then be translated to determine any major changes to the amino acids and function of the gene.