Screening for Drought Tolerance Using Polymorphic SNP Markers in Barley (*Hordeum vulgare* L.*)*

By Nawal Alsamdani

2160271

A thesis submitted for the Master's degree of biotechnology

Supervisors: Dr. Yuri Shavrukov and Assoc/Prof Peter Anderson

The School of Biological Sciences

Flinders University

South Australia

July 2018

Contents

Contents ..... **ii**

List of Figures ..... **iv**

List of Tables ..... **v**

Abstract ..... Error! Bookmark not defined.

Declaration ..... Error! Bookmark not defined.

Acknowledgments ..... **viii**

Abbreviations ..... Error! Bookmark not defined.

Chapter 1: Literature Review **1**

1.1 Introduction 1

1.2 The Production and Use of Barley 2

1.3 Drought 4

1.4 Drought-Tolerant Effects in Barley 5

1.5 Dehydration Genes 5

1.6 Molecular Markers 7

1.7 Single Nucleotide Polymorphism Markers 8

1.8 SNP Markers in Barley 9

1.9 Marker Assisted Selection 9

1.10 Amplifluor-Like SNP Markers ..... **Error! Bookmark not defined.**

1.11 Experimental Aims ..... **Error! Bookmark not defined.**

1.12 Biotechnology Significance ..... **Error! Bookmark not defined.**

Chapter 2: Materials and Methods ..... Error! Bookmark not defined.

2.1 DNA Extraction ..... **Error! Bookmark not defined.**

2.2 Primers Designs ..... **Error! Bookmark not defined.**

2.3 Polymerase Chain Reaction Amplification and Gel Electrophoresis 15

2.3.1 PCR Amplification with *HvDhn9* Using F-R and *HvDhn7* Using F6-R4 Primers 15

2.3.2 Agarose Gel Electrophoresis 16

2.3.3 Big-Scale PCR Protocol for Sequence Using *HvDhn9*-F1-R1, *HvDhn7* F2+R2 and F5+R4 Primers 16

2.3.4 Agarose Gel Electrophoresis 17

2.3.5 Purification of PCR Products 17

2.3.6 DNA Sequencing (Sanger) 18

2.3.7 Sequence Analysis Software 18

2.3.8 Design of Allele-Specific Primers and Universal Probes 18

2.3.9 Amplifluor-Like SNP Marker using (Protocol: PCR Cocktail Preparation for Genotyping) 19

2.3.10 Protocol: PCR Cocktail Preparation for Genotyping 20

Chapter 3: Results **21**

3.1 The PCR Amplification and Electrophoresis Gel Using *HvDhn9* F-R Primers 21

3.1.1 Checking Purified PCR Products Used for Sequencing with *HvDhn9* F1+R1 Primer (One Combination of Primers) 22

3.1.2 Sequencing Results of *HvDhn9*-F1 primer 23

3.2 PCR Amplification and Electrophoresis Gel Using *HvDhn7* F6-R4 24
3.2.1 Checking Purified PCR Products for Sequencing with *HvDhn7* F2+R2 Primers 25
3.2.2 Sequencing Results of *HvDhn7*-F2 primer 26
3.2.3 Checking of Purified PCR Products for Sequencing (One Combination of Primers) 27
3.2.4 Sequencing Results of *HvDhn7*-F5 primer 28
3.3 qPCR Fluorescence for Allele Discrimination 30
3.4 Comparison Between qPCR Fluorescence for Allele Discrimination and Quantification Curves 32

Chapter 4: Discussion 34

Chapter 5: Conclusion and Future Work 37

Reference List 39

Appendices ..... Error! Bookmark not defined.

List of Figures

Figure 1: Two universal probes (UPs) used for SNP analysis.....**Error! Bookmark not defined.**

Figure 2: Agarose gel showing PCR amplification products obtained with *HvDhn9* F+R primers. .....**Error! Bookmark not defined.**

Figure 3: Agarose gel showing PCR amplification products obtained with *HvDhn9* F1+R1 primers. .....**Error! Bookmark not defined.**

Figure 4: Comparison of sequences between samples AUK and NAT using *HvDhn9*-F1 primer.....23

Figure 5: Agarose gel showing PCR amplification products obtained with *HvDhn7* F6+R4 primers. 24

Figure 6: Agarose gel showing PCR amplification products obtained with *HvDhn7* F2+R2 primers.....25

Figure 7: Comparison of sequences between samples AUK and NAT using *HvDhn7*-F2 primer.....26

Figure 8: Agarose gel showing PCR amplification products obtained with *HvDhn7* F5+R4 primers.....27

Figure 9: Sequences of *HvDhn7* with the forward primer F5.....28

Figure 10: Amplifluor-like SNP genotyping of *HvDhn7*.....30

Figure 11: Comparison between allele discrimination and amplification curves for FAM and HEX using Amplifluor-like SNP genotyping using *HvDhn7*.**Error! Bookmark not defined.**

List of Tables

Table 1: Assessment of area harvested, production and yield of barley in 2016.....3
Table 2: PCR-based markers associated with drought tolerance in barley (de Mezer et al., 2014).....6
Table 3: Comparison of the most commonly used molecular makers (Kesawat and Kumar, 2009) 8
Table 4: List of primer sequences and characteristics used for PCR amplification..1314
Table 5: DNA concentration of purified PCR products.....17
Table 6: Sequence of Allele-Specific Primers (ASPs), Universal probes (Ups) and tails used for Amplifluor-like SNP markers 19

Abstract

This project deals with the screening of single nucleotide polymorphism (SNP) markers for drought tolerance in barley (*Hordeum vulgare* L.), the fourth largest cultivated crop worldwide. Barley seeds contain various valuable nutrients such as dietary fibers, starch, protein, free lipids, and trace amount of minerals. Barley is mainly used as animal feed and in the brewing industry. Barley plants have a good capacity of adaptation. Drought affects barley plants in different stages of its lifecycle, minimizing the overall yield of the crop. Dehydrin (*Dhn*) genes are abundant in barley, like other drought tolerant plants species. In this project, the variations in *Dhn* genes is analyzed using different molecular markers techniques. The aim is to find the suitable markers for selection of drought tolerant barley genotypes. In this study, two varieties of barley from Kazakhstan, known as Auksiniai (AUK) and Natali (NAT), as well as a collection barley cultivars were tested for genotyping of *Dhn* genes using SNP analysis. Twenty-six primers targeting two dehydrin genes, *Dhn9* and *Dhn7*, were designed using the National Centre for Biotechnology Information Genebank database and Oligocalculator. The amplification of the selected regions in two barley varieties was performed as per the standard protocol of PCR. The PCR product were visualised by 1% agarose gel electrophoresis and the purified for Sangers sequencing. The sequencing results were analysed using Chromas Lite software. Allele-specific primers (ASPs) were synthesised to identify the SNP in the studied genotypes for validation using Real-time quantitative polymerase chain reaction (qPCR). Using developed primers, clear single bands of DNA amplification were obtained. However, the Sangers sequencing showed an absence of SNPs in both of the barley varieties in three regions of *Dhn7* and *Dhn9* genes. Only one clear SNP was identified in the *Dhn7* gene among the collection of barley genotypes, while AUK and NAT remained monomorphic. All three possible variants of the SNP, both homozygous (‘TT’ and ‘AA’) and heterozygous (‘AT’) were detected. Amplifour-like SNP markers were developed based on the identified SNP. The distribution of FAM and HEX fluorescence in amplified PCR products of this allele (*Dhn7*-F7, F8 and R) using Real-time qPCR showed three distinct groups of barley collection with ‘TT’, ‘AA’ or ‘AT’ genotypes with the Amplifuor-like SNP marker application. This project has laid down the protocol to detect SNP markers in barley dehydrin genes. The method used could be applied to other plant species for screening different traits because it is suitable for high throughput analysis and is robust and

reporducible. The allele-specific PCR enables homozygous or heterozygous SNP genotypes involved in drought tolerance to be easily distinguished in varieties of barley. This is a modern approach for selecting suitable barley cultivars with tolerance to dehydration stress. However, the fruitfulness of this study can only be enhanced by testing more regions of dehydrin genes in other barley varieties.

Declaration

I certify that this thesis: 1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and 2. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Nawal Mohammed Alsamdani

Acknowledgments

In the Name of Allah, the most gracious and the most merciful.

Firstly and foremost, praise be to Allah, the almighty, the greatest of all, on whom ultimately we depend for sustenance and guidance. I would like to thank almighty Allah for giving me opportunity, determination and strength to do my research project. His continuous grace and mercy was with me throughout my life and ever more during the tenure of my research project. I would like to thank the Saudi Cultural Mission founded by the Saudi Arabia Government for their financial support. I am also very thankful to King Salman, the King of the Kingdom of Saudi Arabia, and the King Salman Center for Disability Research, for giving me the opportunity to continue my post graduate studies in Australia. I would like to thank my supervisor, Associate Professor Peter Anderson, for his support and supervision throughout this project. I would like to thank my supervisor Dr Yuri Shavrukov for her guidance and assistance through this project and my laboratory work. I would also like to thank Dr Paula Wiliams, disability advisor, for her support. Finally, I would like to thank my family, especially my mother, for their love and encouragement. I owe everything to my brother, Abdullah Alsamdani, who encouraged and helped me at this stage of my personal life.

Abbreviations

1. HV	*Hordeum vulgare* L. (barley)
2. NCBI	National Centre for Biotechnology Information
3. gDNA	Genomic DNA
4. DNA	Deoxyribonucleic Acid
5. Kb	Kilobase Pair
6. bp	Base Pair
7. dNTP	Deoxyribonucleotide
8. MW	Molecular Weight
9. PCR	Polymerase Chain Reaction
10. SNP	Single Nucleotide Polymorphism
11. TAE	Tris-Acetate-Ethylenediaminetetraacetic acid Buffer
12. EDTA	Ethylenediaminetetra Acetic Acid
13. UV	Ultra Violet
14. g/ng/ul	Grams/Nanograms/Volume
15. mL/µL	Millilitres/Microlitres
16. mM/µM	Millimolar/Micromolar
17. qPCR	Quantitative Polymerase Chain Reaction
18. Ta	PCR Cycle Annealing Temperature (°C)
19. Tm	Melting Temperature of Primers
20. v	Volume
21. v	Voltage
22. *Dhn*	Dehydrin
23. RFLP	Restriction Fragment Length Polymorphism
24. AFLP	Amplified Fragment Length Polymorphism
25. SSR	Simple Sequence Repeat

26. EST	Expressed Sequence Tag
27. CAPS	Cleaved Amplified Polymorphic Sequences
28. MAS	Marker Assisted Selection
29. FL	Fluorescein
30. UP	Universal Probe
31. T	Thymine
32. BHQ	Black Hole Quencher
33. FAM	6-Carboxy Fluorescein
34. HEX	6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein succinimidyl ester
35. AUK	Auksiniai
36. NAT	Natali
37. F-R	Forward and Reverse Primers
38. ASP	Allele-Specific Primer
39. T/T	Thymine/Thymine
40. A/A	Adenine/Adenine
41. A/T	Adenine/Thymine
42. RFU	Relative Fluorescence Unit
43. NRFU	Normatised Relative Fluorescence Unit
44. %	Percent
45. w/v	Weight/Volume
46. $MgCl_2$	Magnesium Chloride
47. Mins	Minutes
48. S	Second
49. FAO	Food and Agriculture Organization of the United Nations

Chapter 1: Literature review

1.1. Introduction

Barley

Barley is the world's most ancient cultivated crop. The scientific name of the crop is barley *Hordeum vulgare* L. which also contains the family of the plant. Barley crops were cultivated about 10,000 years ago along the Nile (Egypt) and Tigris Rivers (Iraq), and in Southern Turkey, Israel, Lebanon, Jordan, and Syria (Badr et al., 2000). The use and the production of this plant increased all over the world due to its diversity, multitude routine and archeological adaptations. Harlan (1995) proposed that the Fertile Crescent is the exclusive point of origin for *Hordeum vulgare* L. because the forefathers of these plants were present in two and six-rowed kinds. The Fertile Crescent was considered an ideal area to cultivate barley and it was assumed that the cultivation area stretched from Israel and Jordan to areas around Syria, Southern Anatolia, and the Zagros Mountain region of Iran (Von Bothmer and Jacobsen, 1985).

Barley belongs to the grass family (Poaceae) and the tribe Triticeae was the primary one that was having plants in the cultivated forms. There are a total of 45 taxa and 32 species of the *Hordeum* L. genus (Von Bothmer and Jacobsen, 1985, 1991). Cultivated barley has the progenitor as primary subspecies that is named as *H. vulgare* ssp. *spontaneum*. The general features of the progenitor include its being two-rowed, diploid, annually cultivated as well as self-pollinated. There is a common genome of barley in the cultivated and wild form that is 2n=14. The wild and cultivated barley are fertile and have compatibility with one another, as mentioned by Foster (2000). The chromosomes are normally paired as well as segregated in the hybrid form of barley (Zohary and Hopf, 1988).

The adaption of barley is wide due to dissimilar environments and it has the capability to grow in dry conditions even where wheat cultivation is not possible, that is also an adaptive feature of the barley crop (Gregory, 1994). Barley has the capacity to grow in various types of soils and it is cultivated as a food in many areas. Alkaline soils are the

best for cultivation of barley; acidic soils are not perfect for this plant species, especially in comparison to other cereal crops (Poehlmen, 1985). The soil pH ranges from 6.0-8.5 are perfect for barley plants. It was investigated that barley plants have the capacity to grow under diverse environment but there are specific conditions where it can grow fast, for example 15-30°C temperature and 500-1000 mm rainfall (Nilan and Ullrich, 1993)

1.2. The Production and Use of Barley

Barley is the plant species whereas the grains are used to make food for human and animal feed and malt in all over the world. It has been adapted to diverse ecological systems: feed grains as food utility versus the prominence of barley for malt preparation (Poehlman, 1985). There are countries that producing barley as a main crops are, such as Afghanistan, Algeria, Australia, Canada, Brazil, Denmark, Egypt, Ethiopia, France, Germany, India, Iran, Iraq, Italy, Kazakhstan, Morocco, Poland, Russian Federation, Spain, Turkey, Ukraine, United Kingdom and the USA.

Table1: Assessment of the area harvested, production and yield of barley in 2016.

Country	Area harvested (ha)	Production (tons)	Yield (Kg/ha)
Afghanistan	219208	301856	13770
Algeria	706429	919907	13022
Australia	4107648	8992274	21892
Canada	2336800	8704300	37249
Brazil	91055	379687	41699
Denmark	706900	3949600	55872
Egypt	77566	120100	15483
Ethiopia	959273	2024922	21109
France	1899615	10306008	54253
Germany	1605000	10730500	66857
India	590000	1505000	25508
Iran	1611423	2907572	18044
Iraq	259859	499222	19211
Italy	246370	988285	40114
Kazakhstan	1894068	3231268	17060
Morocco	1207615	619919	5133
Poland	926147	3441090	37155
Russian Federation	8133765	17992517	22121
Spain	2800628	7979590	28492
Turkey	2700023	6700000	24815
Ukraine	2859200	9435710	33001
United Kingdom	1122000	6655000	59314
USA	1035200	4338850	41913

Source: FAO (2017)

Barley is produced as a major crop in Australia. The annual production of barley in Australia is 8 million tons per year and barley occupies big area of cultivation. Barley is known to be the second largest crop in Australia due to its harvest, yield and the demand of area population geographically. Almost 40% of barley is used for malting, while rest of the crop is used as food. There is a high germination rate of barley plants in Australia. Therefore, harvesting is focused on increasing the annual yield rate. Brazil produces more barley compared with its area than Australia and there is a high yield of the crop. In Denmark, the area harvested is more but recent yields of barley crops are less than the production from 2015 to 2016 - the total yield reduced by 1 million. In 2016, Denmark was producing worth 145 million tonnes of barley seeds. Barley production in Egypt increased two-fold from 2015 to 2016; as indicated in FAOSTAT, 2017, with more production compared to the area harvested by the crop. (http://www.fao.org/faostat/ar/#data/QC).

The production of barley in Ethiopia, Algeria and Afghanistan was observed growing constantly as it was in 2015, but most of the area is harvested with the crop and there is less yield compared with other world countries. Germany and France have a wide area under production and these countries yield more production but the yield, on an annual basis, is less due to its constant usage. The area of barley harvested and the total productions are high in France, India and Germany due to safe farming. Iran, Kazakhstan and Morocco have wide areas under harvest as well as high production, but the overall output indicated in Table 1 is less than those in India, France and Germany. Morocco has a lower yield of barley than the production of this plant species. Canada, Russia, Spain, Turkey, Ukraine, the UK and the USA have more fertile areas for agriculture and barley is an essential food for the residents of these regions. The yield of this plant species is almost the same in all countries (http://www.fao.org/faostat/ar/#data/QC).

1.3. Drought

Drought is defined as a situation with low water availability for plants as compared with the required amount for its sustainable growth (Mueen et al., 2013). Drought hinders the growth, quality of seed and yield of crops. The effects of drought have been aggravated as a result of increased food demands for enormously growing population and limiting water resources. Occurrence and distribution of rainfall, moisture storing capacity of soil and evaporative demands are important factors that aggravate the severity of drought

(Riaz et al., 2013). Climate change has increased severity of drought and high temperature resulting in yield reduction of many cereal crops such as barley, wheat and maize (Lobell and Field, 2007). Water scarcity after seedings results in no plants production while after plant germinations results in low yield of crops. Water deficit results in loss of plant turgor which inhibits the cell elongation, reduced photoassimilation and metabolite required for cell division.

Obstructed cell elongation and impaired mitosis leads to reduced growth. Low water availability for plants results in reduced photosynthesis and cut down the overall plant growth including root growth. Water deficit results in stomatal closure to minimize the water loss, decline in Rubisco activity, which in turn leads to reduced photosynthesis (Farooq et al., 2009).

1.4. Drought tolerance effects in barley

The ability of plants to live, grow and reproduce despite a restricted supply of water or to face periodic changes in water shortage is drought tolerance (Turner, 1979). Tolerance can be achieved either by drought avoidance or dehydration tolerance (Kramer and Boyer, 1995). Different factors affect the plant response to drought stress: plant genotype, duration and severity of stress (Chaves et al., 2003), pattern of gene expression (Denby and Gehring, 2005), respiratory activities pattern (Ribas-Carbo et al., 2005) and photosynthetic activity pattern (Flaxes et al, 2004). Drought adversely affects the various stages in the barley plants life cycle, from seed germination to plants maturity (Aspinall et al., 1964). The effects of drought on barley crop yields depend on the duration and severity of stress (Anjum et al., 2011). Drought can affect the barley plants during very sensitive and important stages of development, such as spike formation, anthesis and the initial stages of grain development and grain filling (Aspinall, 1965). Pollination is affected by abortion of embryonic sac and dehydration of stigma and pollen under stress conditions. Drought can decrease the numbers of grains and spikes on a plants and ultimately decrease the yield (Mamnouie et al., 2010).

1.5. Dehydration genes

Dehydrins (Dhns) are belonging to Group II late embryogenesis abundant proteins. Dehydrins are quite thermos-stable and hydrophilic in nature, having many charged amino acids. These proteins are thought to protect plants from conditions of water stress.

Genes encoding Dhn proteins belong to a multigene family that are expressed in higher plants and mosses (Saavedra et al., 2006), algae (Close, 1996), during early embryogenesis and under drought stress conditions (Yang et al., 2012). Different *Dhn* genes are identified in various plant species; for example, there are 10 *Dhn* genes in Arabidopsis, 8 in rice, 6 in tomato, 4 in grapevine, 10 in poplar (Jing et al., 2016).

Using the IPK BLAST server, a homologous relation or correspondence study beside the existing barley genome structures properties is provided. The overall data sequences dataset consist of the whole genome shotgun assemblies of the cultivars Morex, Barke and Bowman and different gene sets with high and low confidence (Spannagl et al., 2016).

The *Dhn* genes are usually dispersed throughout the chromosomes in plant species. Barley presents the most comprehensive information on *Dhn* genes, in which eleven *Dhn* genes map to four chromosomes: 3H, 4H, 5H, and 6H. Several genetic studies have traced the locations of *Dhn* genes within chromosomes of barley.

These studies have shown that *Dhn1* is mapped on long arm of 5H; *Dhn3*, *Dhn4* and *Dhn7* are located on the long arm of chromosome 6H; *Dhn9* is present on the short arm of the chromosome 5H (Pan et al., 1994). In Table 2, five genetic regions of *Dhn* genes from two chromosomes (5H and 6H) are identified in the genome of barley, which are involved in the process of drought response. SNPs in these regions have previously been assessed for their role in tolerance against drought (de Mezer et al., 2014).

Table 2: PCR-based markers associated with drought tolerance in barley (de Mezer et al., 2014)

Dhn genes	Chromosome	Marker	Primers sequence (5'-3') F-R	Size bp
Dhn1	5H	*HvDhn1*	F: GATCAGCACACTTCCACAGC R: GCGCTGGTACACACGACATA	606
Dhn3	6H	*HvDhn3*	F: AGGCAACCAAGATCAACACC R: TTCTGCAAGGTAGCCAGACC	579
Dhn4	6H	*HvDhn4*	F: TTTTGGTGACAAGTTCAGCG R: ATTGATTCTGCAACGGTGGT	783
Dhn7	6H	*HvDhn7*	F: GCAAGATGGAGTACCAGGGA R: TACGTAGACCGGCACCTCTT	632
Dhn9	5H	*HvDhn9*	F: GCTGAAGGCACAACACTTCA R: CAACGTTTCGTTCAGCTTCA	624

Source: Pan et al., 1994. The locations of *Dhn* genes were obtained from (Choi et al., 2000)

1.6. Molecular Markers

The molecular markers are the latest technology that is helpful to investigate the localization of genes, and they can useful in plant breeding through desired gene selections. The history of molecular markers development indicates that genetic positions of markers has improved in genomes over the last two decades that is providing fast and, easy support for breeders and scientists. Molecular markers use the technology of genome analysis that helps to create information database for common use. There are a lot of molecular marker types readily available and used in the genetic analysis. The development of new markers is a continuous process, and most of the markers are classified on the basis of Polymerase chain reaction (PCR) or based on hybridization. The markers that used for the developmental studies are of great importance among all markers for identification of the genetic mapping, population studies and fingerprinting. The markers utilisation depends on the costs regarding the laboratory work and availability of the facilities (Kesawat and Kumar, 2009).

Molecular markers are classified as hybridization-based or PCR depending on their basic principles. Hybridization-based molecular makers use restriction enzyme digest of genome and hybridization with known DNA probe. Hybridization-based molecular markers include RFLP (Restriction fragment length polymorphism). In contrast, AFLP (Amplified fragment length polymorphism), SSR (Simple sequence repeat), SNP (Single nucleotide polymorphism), EST (Expressed sequence tag) and CAPS (Cleaved amplified polymorphic sequences) are PCR-based molecular markers and easily assessed. During PCR, a target DNA fragment is amplified using specific or arbitrarily designed primers and thermostable DNA polymerase (Nadeem et al., 2018).

Morphological and biochemical markers can vary in different environments and under control, with pleiotropic and epistatic effects, while molecular markers exhibit no phenotypic plasticity. DNA can be easily extracted from plants and its analysis is relatively cheap and labour-effective. The comparison of different molecular markers are present in Table 3, which shows that SNP is better type of molecular markers due to its ease of use, reproducibility, cost per analysis and amenability to automation.

Table 3: Comparison of the most commonly used molecular markers (Kesawat and Kumar, 2009)

N	Feature	RFLP	RAPD	AFLP	SSRs	SNPs
1	DNA requirement (μg)	10	.02	.5-1.0	.5-1.0	.05
2	DNA quality	High	High	Moderate	Moderate	High
3	PCR based	No	Yes	Yes	Yes	Yes
4	No. of polymorphic loci	1-3	1.5-50	20-100	1-3	1
5	Ease of use	Not Easy	Easy	Easy	Easy	Easy
6	Amenable to automation	Low	Moderate	Moderate	High	High
7	Reproducibility	High	Unreliable	High	High	High
8	Development cost	Low	Low	Moderate	High	High
9	Cost per analysis	High	Low	Moderate	Low	Low

1.7. Single nucleotide polymorphism (SNP) markers

The SNP markers have a single nucleotide base replacements that creates difference between individual DNA sequences. The nucleotide substitutions are helpful to categorize the SNP transversions (C/G, A/T, C/A or T/G) and transitions (C/T or G/A). In practice, single base variants in cDNA (mRNA) are considered to be SNPs as are single base insertions and deletions in the genome. The simplest type of molecular markers is SNP that has a single nucleotide base and is referred to as the smallest inherited unit. Both plants and animals possess the SNP and the range of SNP frequency is 100-300 bp in plants (Edwards et al., 2007; Xu, 2010). The coding regions of genes (exons) and introns non-coding gene regions with SNPs are present in various chromosome regions and dissimilar frequencies. The SNP genotyping techniques are developed on the basis of detection and allelic discrimination. The most reliable techniques for the identification of the SNP are the CAPS marker method and RFLP, where latter type is also known as SNP-RFLP.

Different length of chromosome segment can be produced if the restriction enzyme will bind to the recognition site in genome. There will produced fragments of various length through, depending presence of two SNP-RFLP alleles. The sequence can be analysed with the help of simple process through the major databases and these contribute to analysing the SNP. Four alleles can be separated when the DNA segment sequence is identified and shown as A, G, T and C in every SNP locus.

The SNP genotyping assays are diverse and these are based on the laboratory technologies like the primer extension, allele hybridization, invasive cleavage and oligonucleotide ligation (Sobrino et al., 2005). The differences in alleles can be observed according to the sensitivity of used method, such as mass spectroscopy, gel electrophoresis, fluorescence polarization, chromatography and arrays. Sequencing is the major tool to detect SNPs as the detailed techniques and elaborated by researchers (Xu, 2010).

SNPs are generally present in simple forms, such as the polymorphism, and these are co-dominant in nature. Therefore, they have become potential genetic markers, as they have in breeding and genetic studies. These SNPs can also be detected and automated through the highly effective polymorphism technique. Therefore, SNP can be used widely for multiple purposes, including the study of whole DNA sequence in maize, rice or soybean. The quality of DNA sequence is dependent on the high costs of marker development (Jiang, 2013).

1.8. SNP markers in barley

PCR-based markers are usually used to study *Dhn* genes in plants such as barley. SNPs in different chromosome regions can be identified by whole genome-wide SNP scanning and targeted SNP detection of performing PCR in these loci. If PCR is performed for SNP detection, the amplified product can be used for Sanger sequencing, or it can be treated with different restriction enzymes that digest the target DNA on SNP specific sites (Shavrukov, 2016). In this approach, the genomic regions containing high number of SNPs are analyzed. The CAPS technique is useful especially if the SNP is present in the recognition site of some restriction enzyme.

1.9. Marker assisted selection (MAS)

Marker assisted selection (MAS) can be helpful to check the genetic variability using studied plant germplasms (William et al., 2007). Using this method, a trait of interest is selected indirectly but with the help of linked markers. These markers may be morphological, biochemical or molecular. MAS is used in plant and animal breeding

procedures. The identified markers can differentiate between drought tolerant and sensitive varieties, selected as parental forms, P1 and P2, respectively. These were crossed to produce F_1 generation and F_2 generations, and plants from F_2 population can be analysed and selected using DNA markers.

1.10. Amplifluor-like SNP markers

Amplifluor-like SNP detection mechanism is a very sensitive, sophisticated, and robust method to detect Single Nucleotide Polymorphisms in a variety of DNA samples. The technique is based on special type of oligomers (probes), as shown in the Figure 1. The probes for Ampliflour-like SNP detection system comprise of 5 parts. First, a fluorescence dye is attached to 5'-end of the primer, second, a double stranded stem structure, third a loop structure, fourth, a quencher, and fifth a single stranded overhang on 3'-end of the primer. The allele specific primers (ASPs) are attached to 3'-end of the universal probes (Jatayev et al., 2017). Here, two forward primers and one reverse primer were used. The forward primers were GAAGGTGACCAAGTTCATGCTCGATTCCCCCGCAAAAA-AAAAAT, and GAAGGTCGGAGTCAACGGATTCGATTCCCCCGCAAAAAAA
AAAA, while the reverse primer was CATCTCATAACAGTCGCTCAGA. The amplification of the target relies on correct components of polymerase chain reaction (PCR). The Amplifluor-like SNP typing system can be used with general GoTaq-polymerase or Phusion Taq-polymerase. The GoTaq-polymerase is quite commonly used for DNA amplification, but for some difficult templates containing high GC contents, the Phusion Taq-polymerase can be used. For optimal working of polymerase enzyme, $MgCl_2$ in low concentration is also required. The Mg^{++} ions act as co-factor for the polymerase enzyme. Beside this, correct concentration of deoxy-nucleotide tri-phosphates (dNTPs) is also required in the reaction (Hadidi and Candresse, 2003).

The efficiency of this technique relies only on the hybridization/annealing of primers with the target. The allele specific primers anneal when the target is SNP is present in the sample DNA. The primers do not emit fluorescence as long as these are not annealed to the target DNA. This is because, in the free form, the primers maintain their loop

structure, whereby, the quencher lets not the fluorophore to emit fluorescence. As long as the primers anneal with the target, the stem-loop structure is opened up, as a result the fluorophore becomes far from the quencher. This causes to produce fluorescence which is detected in the Amplifluor-like SNP typing system (Hernández et al., 2004; Giancola et al., 2006).

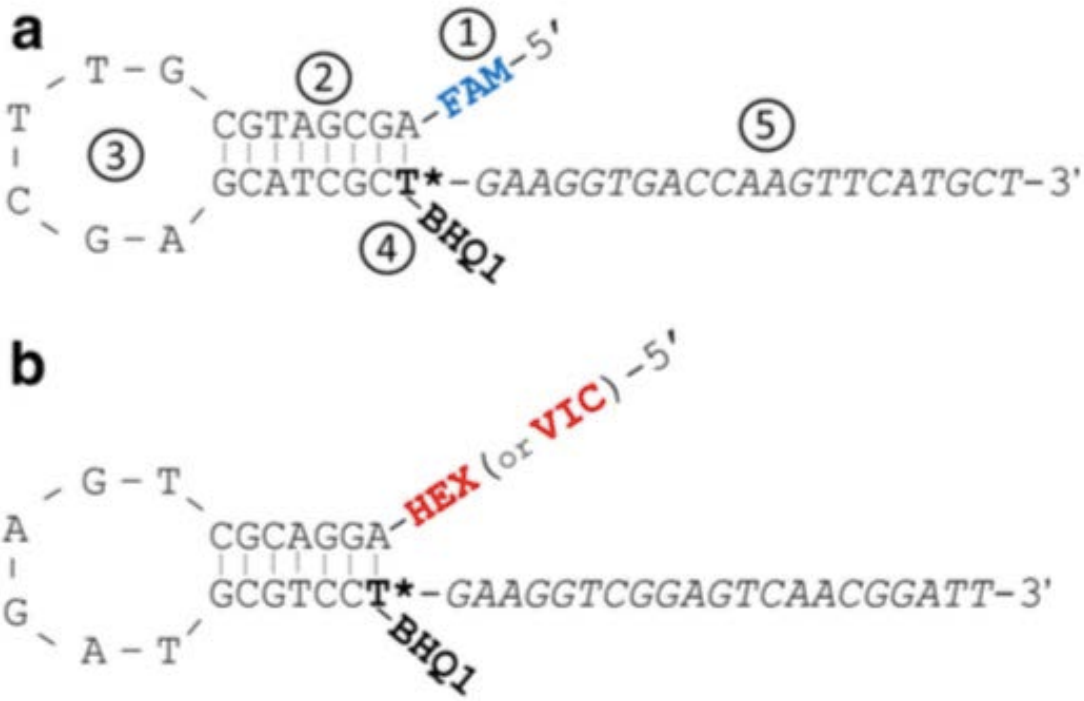


Figure 1: Two Universal Probes (UPs) are used in the Amplifluor SNP markers. The UPS are labelled with FAM (a) and HEX (b). These highlighted by colours. The numbers in cycles show elements where (1) is fluorophore present at the 5'-end, (2) and (3) are the hairpin stem and loop. (4) Modified oligonucleotide Thymine (T) indicated in Bold and asterisk with Black Hole Quencher 1 (BHQ1), (5) A 'tail' specific for each UP in the 3′-end, shown in Italics. Source**:** Jatayev et al., 2017

1.11. Experimental Aims

1- Identify PCR based markers linked to specific drought tolerance genes in barley varieties. The used DNA was extracted from barley collection in Kazakhstan, were selected germplasm have shown drought tolerance in the field.

2- The major aim of this experiment is to select a candidate gene for drought tolerance in Kazakhstan barley varieties, AUK and NAT, as well as in collection of barley showing drought tolerance in the field.

3- Screen possible drought tolerance genes in selected barley varieties using SNP markers.

4- Study of two cultivars of barley, AUK and NAT, and barley germplasm collection for genotyping of dehydrin genes, *Dhn* using SNP analysis.

1.12. Biotechnology Significance

The biotechnology significance of this project is to gain in-depth knowledge of the role of the SNPs analysis in *Dhn* genes that lead to the identification genetic background of barley cultivars tolerant to drought stress conditions.

Chapter 2: Materials and Methods

2.1. DNA extraction

DNA was extracted in Kazakhstan from barley parental varieties, Auksiniai (AUK) and Natali (NAT), and barley germplasm collection and from 200 lines produced from F_2 segregating population.

2.2. Primers designs

All primers were designed based on sequences retrieved from GenBank using web-site: https://www.ncbi.nlm.nih.gov. Primers were checked length, %GC, melting temperature (Tm) and presence of self-complementarity using OligoCalculator. All primers were synthesed by Sigma-Aldrich Pty Ltd (Australia). As a starting point for performing PCR, the annealing temperature (Ta) of each primer was estimated from the basic melting temperature (Tm) of primers technical datasheet. Initially, 100 µM stocks of the primers were prepared resuspending the lyophilised pellet for working concentration 5 µM of all primers. Twenty-six primers were designed for two *Dhn* genes, *Dhn9* and *Dhn7*. All primer sequences used with their corresponding Tm are shown in Table 4.

Table 4: List of primer sequences and characteristics used for PCR amplification.

Gene	Primer sequence (5' – 3')	Length (bp)	GC %	Tm (°C)	Expected PCR product size (bp)
Dhn9-F1	TCGCCACTAGTAAGCAAGGCAT	22	50	54.4	539
Dhn9-R1	TAGCTTTGGACTTCGGATGACAG	23	48	55.3	
Dhn9-R1 (RevCom)	CTGTCATCCGAAGTCCAAAGCTA				
Dhn9-F2	CACGTGATCTTCATTCAATAAT	22	32	47.4	576
Dhn9-R2	TATCGAGTATATGACCATTTATT	23	26	46.4	
Dhn9-R2 (RevCom)	AATAAATGGTCATATACTCGATA				
Dhn7-F1	AATGAGCAGGGTTATGATTTTTCA	24	33	50.6	738
Dhn7-R1	ATATACGCACCGAGCTGGAG	20	55	53.8	
Dhn7-R1 (RevCom)	CTCCAGCTCGGTGCGTATAT				
Dhn7-F2	ATCAAGGAGAAGCTGCCCGGAC	22	59	58.6	950
Dhn7-R2	CTTCACACCCTTACCCGGAGCA	22	59	58.6	
Dhn7-R2 (RevCom)	TGCTCCGGGTAAGGGTGTGAAG				
Dhn7-F4	TGAGATCCGCATCAACCTAAGG	22	50	54.8	1,007
Dhn7-R4	TGCGCAACTGGTGGCTGTGT	20	60	55.9	
Dhn7-R4 (RevCom)	ACACAGCCACCAGTTGCGCA				
Dhn7-F5	GCATCAACCTGACTCAACAACTAC	24	46	55.7	893
Dhn7-R4	TGCGCAACTGGTGGCTGTGT	20	60	55.9	
Dhn7-R4 (RevCom)	ACACAGCCACCAGTTGCGCA				
Dhn7-F6	CAGTATATCGTGATGATGTCTTCG	24	42	54	733
Dhn7-R4	TGCGCAACTGGTGGCTGTGT	20	60	55.9	
Dhn7-R4 (RevCom)	ACACAGCCACCAGTTGCGCA				
Dhn7-F7	CGATTCCCCCGCAAAAAAAAAAT	23	39	51.7	106
Dhn7-F7 (Rev Com)	ATTTTTTTTTTGCGGGGGAATCG				
Dhn7-F8	CGATTCCCCCGCAAAAAAAAAAA	23	39	51.7	
Dhn7-F7 (Rev Com)	TTTTTTTTTTGCGGGGGAATCG				
Dhn7-SNP-R	CATCTCATAACAGTCGCTCAGA	_	_	_	

2.3. Polymerase Chain Reaction (PCR), Amplification and Gel Electrophoresis

2.3.1. PCR Amplification with *HvDhn9* Using F-R primers and *HvDhn7* Using F6-R4 primers

PCR amplifications were performed in a MyCycler Thermal Cycler (Bio-Rad, USA). To study *HvDhn9* gene, PCR was carried out in a final volume of 20 µL with following composition of Master-mix: 150.3 µL of milli-Q H_2O, 72 µL of 5 x Green Go Taq® Flexi PCR buffer containing 36 µL of 2 mM dNTP, 28.8 µL of 25 mM $MgCl_2$, 18 µL of a 5 µM working concentration of both forward and reverse primers (*HvDhn9*), 0.9 µL of 5 units of Go Taq polymerase, where18 µL of the Master-Mix was loaded in each PCR microtube together with 2 µL of 10 ng/µL genomics DNA. The used regime of PCR cycles for amplification was as recommended by the manufacturer, (Promega, Australia) including: 94°C for 2 minutes for initial denaturation step, followed by 35 cycles of 94°C for 15 seconds for denaturation step, 55°C for 15 seconds annealing step and 72°C for 45 seconds extension step, with a final extension step at 72°C for 3 min and 15°C hold to hold, as shown in Table A1. For *HvDhn7* gene analysis, PCR was carried out in a final volume of 15 µL, where Master-mix proportionally contained: 42.7 µL of milli-Q H_2O, 21 µL of 5 x Green Go Taq® Flexi PCR buffer, 10.5 µL of 2 mM dNTP, 8.4 µL of 25 mM $MgCl_2$, 5.25 µL of 5 µM working concentration of both forward and reverse primers (*HvDhn7*), 1.4 µL of 5 units of Go Taq polymerase. The Master-Mix was loaded for 13.5 µL in each PCR tube together with 1.5 µL of 10 ng/µL genomic DNA of parental forms, NAT and AUK, in each reaction. The PCR cycling protocol was as follows: 95°C for 1min initial denaturation step, followed by 39 cycles at 95°C for 15 seconds denaturation step, at 55°C for 15 seconds annealing step, 72°C for 1 min extension step, with a final extension step at 72°C for 2 min and 15°C hold (Table A3).

2.3.2. Agarose Gel Electrophoresis

Regular 1% agarose gel, was prepared where 1.2 g agarose (LE, analytical grade, Bioline, Australia) was completely dissolved in 120 ml of 1x TAE Buffer. The solution was heated in a Sanyo microwave to dissolve the agarose powder. One µL of GelRed was added in agarose solution and mixed. The agarose solution was poured into the gel mould, fitted with a comb. The gel was cooled for 10-15 minutes and the comb was removed. Loading 20 µL of PCR product into the gel, and final lane with 3 µL of either 100 bp or 1 Kb markers (Hyper ladder II or I, BioLine, Australia) . Electrophoresis was carried out with 120 V voltage for 30 min using Bio-Rad power PAC 300. Agarose gel was visualised using Bio-Rad Gel Doc EZ imager and a tracked image capture system, the shown, for example, in Figure 2. For 15 µL loading PCR products into the gel, the procedure was the same as described above with minor adjustments including: 2.5 µL of either 100 bp or 1 Kb markers were used. The conditions for electrophoresis were exactly the same as mentioned above and example of tracked images are shown in Figure 5.

2.3.3. Big-Scale PCR Protocol for Preparation of Sequencing *HvDhn9*-F1-R1, *HvDhn7* F2+R2 and F5+R4 primers

PCR amplifications were performed using the same equipment as described in Sections 5.3.1 and 5.3.2. The PCR was carried out in a final volume of 60 µL where Master-mix contained following reagents: 18.7 µL of milli-Q H_2O, 12.0 µL of 5 x Colorless Go Taq® Flexi PCR buffer, 6.0 µL of 2 mM dNTP, 4.8 µL of 25 µM $MgCl_2$, 3.0 µL of 5 µM working concentration of both forward and reverse primers (*HvDhn7*), and 0.5 µL of 5 units of Go Taq polymerase. The Master-mix was loaded for 48.0 µL in each PCR tube together with of loading Master-Mix with 12.0 µL of 10 ng/µL DNA of two parents for AUK and NAT in each reaction. The PCR cycles when using PCR amplification protocol with Promega (Australia) as was mentioned above (See Appendix A2, A4, and A5).

2.3.4. Agarose Gel Electrophoresis

The same equipment as described above 2.3.2, shown in Figures 6 and 8.

2.3.5. Purification of PCR Products

The PCR products were purified using the FavorPrep™ Gel and PCR purification kit (Favorgen, Taiwan) following the manufacturer recommendations by(http://www.favorgen.com).

Table 5: DNA Concentration of purified PCR products.

Abbreviations: A, AUK and N, NAT.

Purified PCR product	Concentration, ng/ µL	Primers
Dhn9-A	6.8	F + R
Dhn9-N	7.0	
Dhn9-A	21.9	$F_1 + R_1$
Dhn9-N	21.3	
Dhn9-A	8.0	$F_2 + R_2$
Dhn9-N	4.7	
Dhn7-A	7.4	F + R
Dhn7-N	9.9	
Dhn7-A	1.7	$F_2 + R_2$
Dhn7-N	4.7	
Dhn7-N	8.4	F_1 + R
Dhn7-D1, Margret	4.9	F5 + R4
Dhn7-H3, Cmbh	25.5	
Dhn7-A4, Cmb89A	23.2	

2.3.6. DNA Sequencing (Sanger)

Purified PCR products with primers were submitted for sequencing to AGRF, Adelaide. Samples for sequencing were prepared according to the guidelines presented on AGRF web-site (http://www.agrf.org.au/).

2.3.7. Sequence Analysis Software

Chromas Lite software version 2.01(Technelysium Pty Ltd) was used for sequences analysis. Each sample had two complemented sequences with forward and reverse primers used in the sequencing reaction.

2.3.8. Design of Allele-Specific Primers (ASP) and Universal Probes (UPs)

In our experiments, three sets of allele-specific primers (ASPs). F7, F8 and R, were designed and used for each gene of interest with two corresponding Universal probes, UP1 and UP2 (Table 6C), respectively. The sequence of barley cultivar Morex, contig_135644 CAJW010135644, shown in Appendix B2.2, for ASPs F7, F8 and R, was used for design and checking based on web-site http://biotools.nubic.northwestern.edu/OligoCalc.html, and sequencing of Tails and UPs labelled with FAM and HEX were used from published earlier (Jatayev et al., 2017).

Table 6: Sequence of Allele-Specific Primers (ASPs), Universal Probes (UPs) and Tails used for Amplifiuor-like SNP markers.

Name	Sequence (5'-3')
A. Tails	
FAM tail 1	5'-GAAGGTGACCAAGTTCATGCT
HEX tail 2	5'-GAAGGTCGGAGTCAACGGATT
B. Universal probes (UPs)	
Universal-1	5'-FAM- AGCGATGCGTTCGAGCATCGCT*GAAGGTGACCAAGTTCATGCT
Universal-2	5'-HEX-AGGACGCTGAGATGCGTCCT*GAAGGTCGGAGTCAACGGATT
C. Allele specific primers (ASPs)	
Dhn7-SNP-F7	GAAGGTGACCAAGTTCATGCT-CGATTCCCCCGCAAAAAAAAAAT
Dhn7-SNP-F8	GAAGGTCGGAGTCAACGGATT-CGATTCCCCCGCAAAAAAAAAAA
Dhn7-SNP-R	CATCTCATAACAGTCGCTCAGA

In two Universal probes, fluorophores are located in the 5' end and the Thymidine (T) indicated by asterisks. Data were extracted from Jatayev et al. (2017) and allele-specific primers (ASPs) of barley with modified oligonucleotides. See in Appendix B2.2.

2.3.9. Amplifluor-like SNP marker using (Protocol: PCR Cocktail Preparation for Genotyping)

A Real-time qPCR system CFX96 was performed in a C1000™ Thermal Cycler (Bio-Rad, Australia). The PCR cocktail for genotyping contained 6.9 µL of milli-Q H_2O, 92.0 µL of 5xPCR Colorless buffer, 46.0 µL of 2 mM dNTP, 46.0 µL of allele-specific primer mix (two forward primers and one reverse primer for *Dhn7*, 46.0 µL of fluorescently labelled Universal probe mix (Uni1 and Uni2), 36.8 µL of 25 µM $MgCl_2$, 2.3 µL of 5 units of Go Taq polymerase. The Master-mix was loaded for 6.0 µL in each well of 96-well microplate using digital pipette with adding of 4.0 µL of template genomic DNA diluted to 10 ng/µL. Two samples contained 4.0 µL of water instead genomic DNA as No template control, NTC. The protocol for Amplifluor-like SNP genotyping with FAM and HEX using qPCR-instrument CFX96 was as follows: 94 °C for 2 min, and 16 cycles including 94 °C for 15 sec, 55 °C for 20 sec, 72 °C for 45 sec, 94 °C for 15 sec, 60 °C

for 30 sec, 72 °C for 45 sec, and final, 72 °C for one min followed by score genotyping (examples are shown in Figure 10 and Appendix A6).

2.3.10. Protocol: PCR Cocktail Preparation for Genotyping.

The same equipment as described in Sections 2.3.9, the showed in Figure 11 and see Appendix A7.

Chapter 3: Results

3.1. The PCR Amplification and Electrophoresis Gel using *HvDhn9* F-R primers

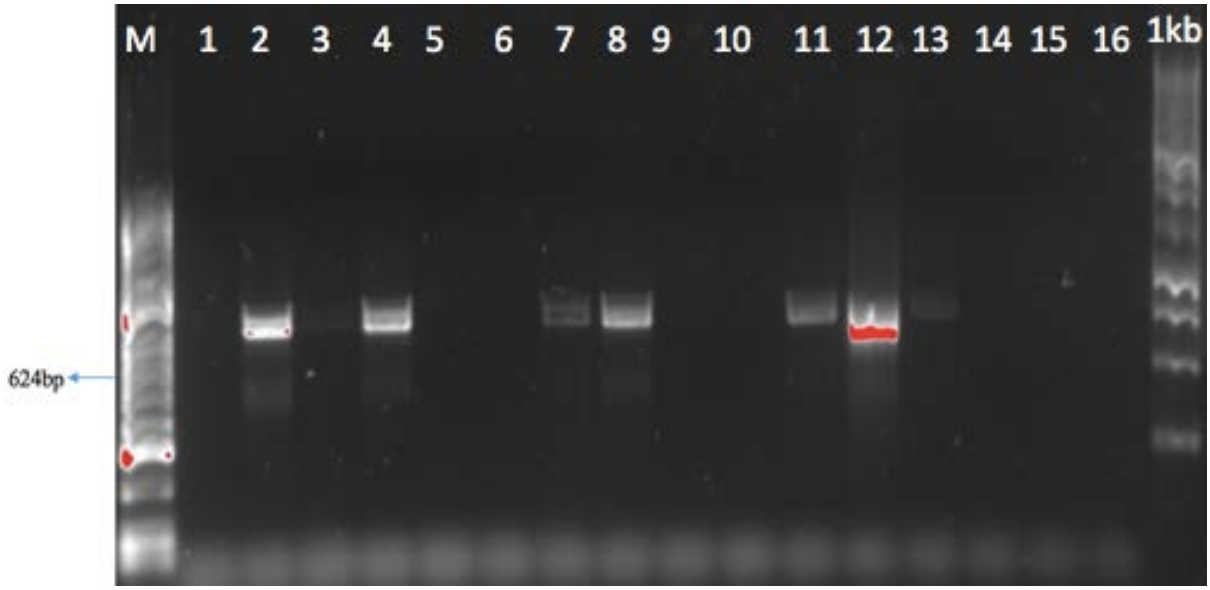


Figure 2: Agarose gel showing PCR amplification products obtained with *HvDhn9* F+R primers.

For M is the hyper ladder II marker 100 lane (Promega, Australia). Lanes1 to 16 are DNA is NAT and AUK progeny – plate 1, diluted 10 ng/ µL(Working concentration). 1 Kb is the DNA ladder marker (Promega, Australia). The expected size of PCR product is 624 bp, All samples were separated in 1% (w/v) agarose gel electrophoresis.

In Figure 2, PCR amplification failure may have resulted from a few different factors. For instance, concentration of DNA was low, or DNA synthesis itself failed. Therefore, it was necessary to confirm DNA synthesis using *HvDhn9* F+R primers. When the annealing temperature was 55 ^{0}C. PCR amplification failed when using diluted DNA, it was observed to enhance the quality of amplification for the detection of bands of 624 bp. When 2 µL of a diluted DNA (1:10) was used in the PCR, no bands were present for 1, 3, 5, 6, 9, 10, 14, 15 and 16 samples for AUK and NAT, plate 1.

3.1.1. Checking Purified PCR Products Used for Sequencing with *HvDhn9* F1+R1 primers (One Combination of Primers)

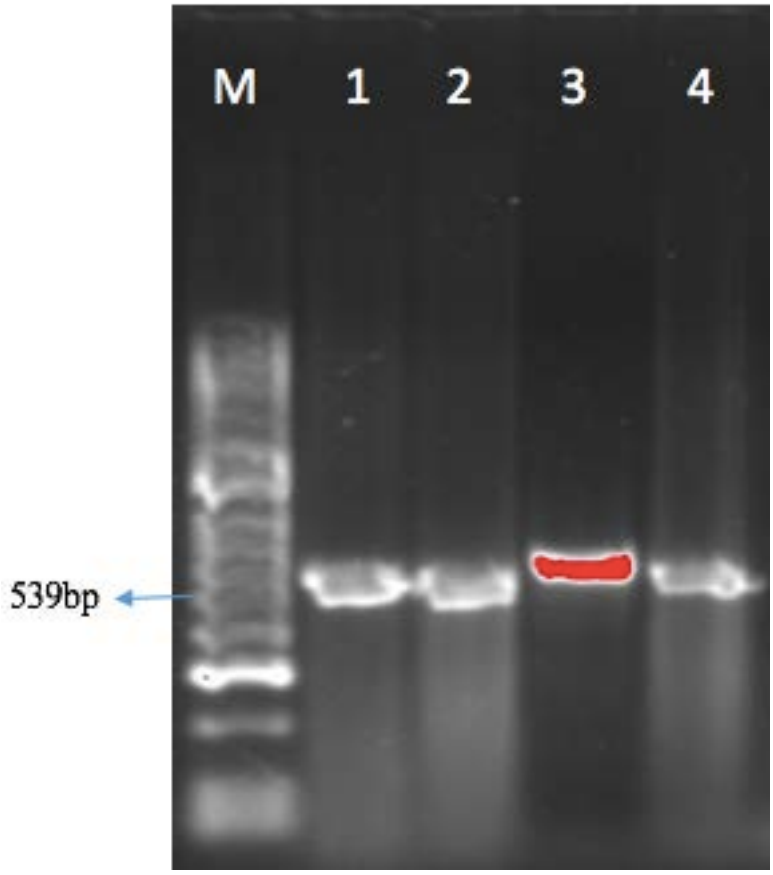


Figure 3: Agarose gel showing PCR amplification products obtained with *HvDhn9* F1+R1 primers.

For M is the hyper ladder II marker 100 lane (Promega, Australia). For genomic DNA, lane 1 and 2 for NAT progeny. For genomic DNA, lane 3 and 4 for AUK progeny. The expected sizes of PCR products are 539 bp. All samples were separated in 1% (w/v) agarose gel electrophoresis.

From the Figure 3, it was found that the PCR amplification of genomic DNA for NAT and AUK progeny using *HvDhn9* F1+R1 primers showed fragments bands (539 bp). This type of marker is not polymorphic. The amplified fragments in the same sizes. In PCR products amplified from genomic DNA may be fragments with the same or similar sizes. Since the cutting out the narrow bands with similar sizes on agarose gel for purification

of PCR products using FavorPrep™ Gel and PCR purification kit for Sanger sequencing see Figure 4.

3.1.2. Sequencing Results of *HvDhn9*-F1 primer

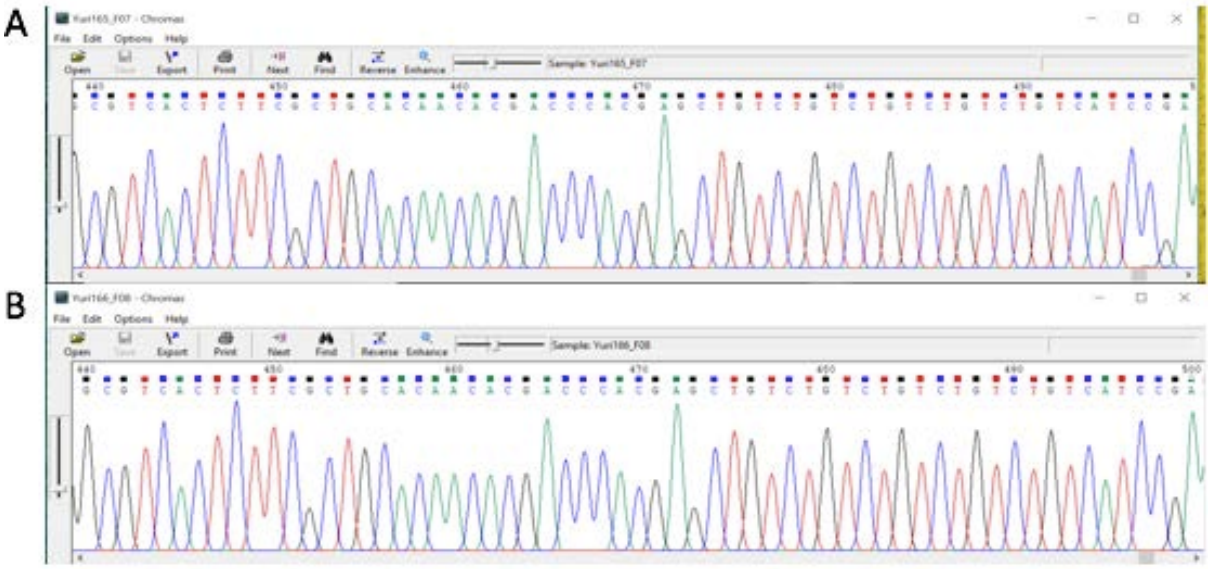


Figure 4: The figure is showing Sequences between samples AUK and NAT.

A and **B;** Samples AUK and NAT - sequence analysis. Primer used was *HvDhn9*-F1. There are no SNP. DNA concentrations were 21.9 and 21.3 ng/ µL for AUK and NAT, respectively. See Appendix C3.2.

3.2. PCR Amplification and Electrophoresis Gel using *HvDhn7* F6-R4

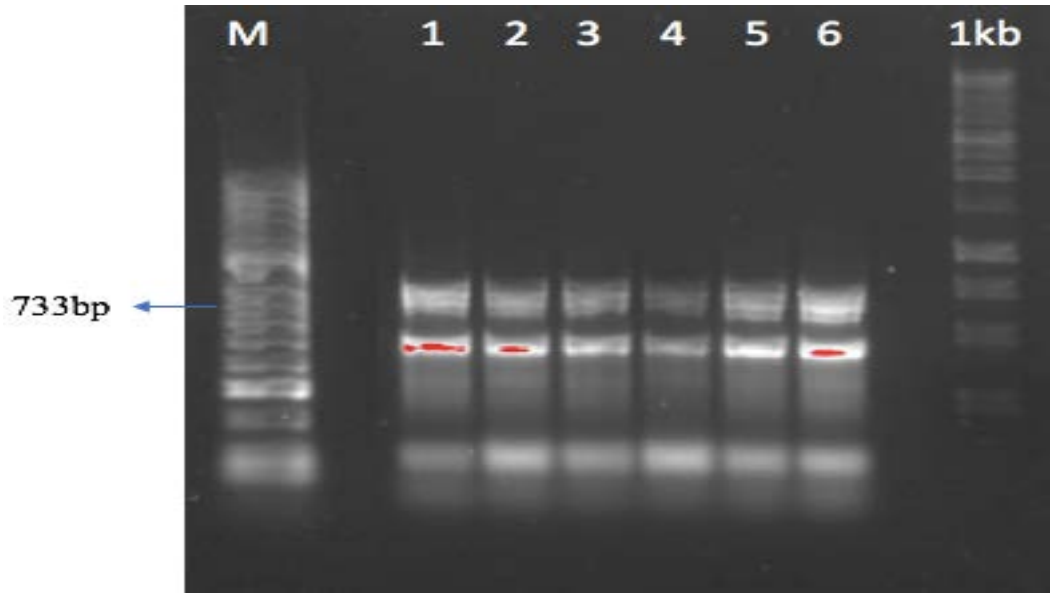


Figure 5: Agarose gel showing PCR amplification products obtained with *HvDhn7* F6+R4 primers.

For M is the hyper ladder II marker 100 lane (Promega, Australia). DNA, lane 1, 2 and 3 for AUK progeny it is diluted 10 ng/ µL. DNA, lane 4, 5 and 6 for NAT progeny it is diluted 10 ng/ µL. 1 Kb is DNA ladder marker (Promega, Australia). The expected size of PCR product is 733bp. All samples were separated in 1% (w/v) agarose gel electrophoresis

3.2.1. Checking Purified PCR Products for Sequencing with *HvDhn7* F2+R2 primers

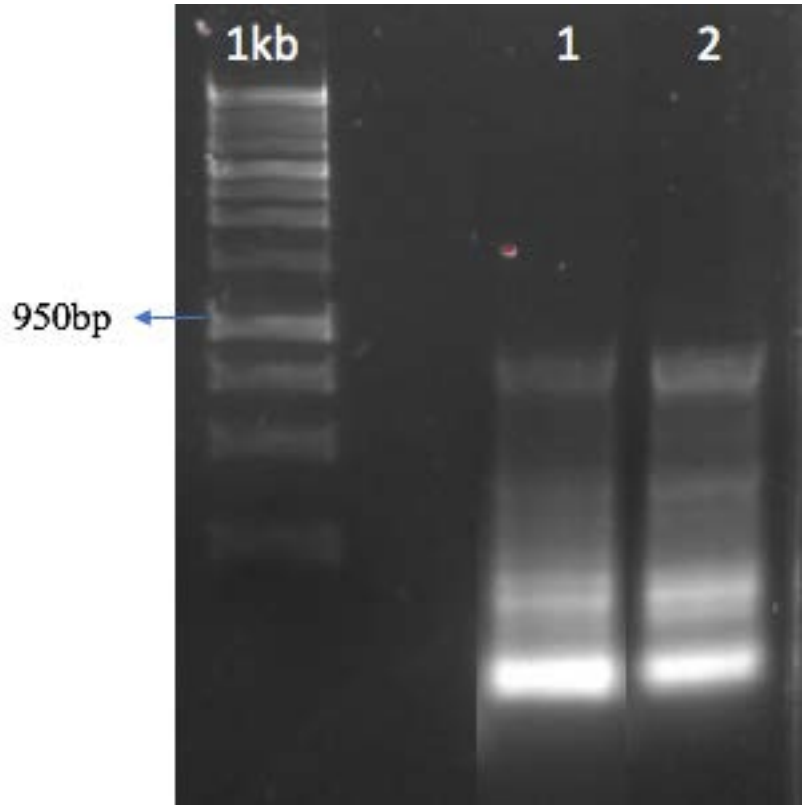


Figure 6: Agarose gel showing PCR amplification products obtained with *HvDhn7* F2+R2 primers.

For 1 Kb is DNA ladder marker (Promega, Australia). Two genotype for AUK and NAT progeny it is diluted 10 ng/ µL. Lane 1 it was AUK and lane 2 it was NAT gDNA. The expected size of PCR product is 950 bp, All samples were separated in 1% (w/v) agarose gel electrophoresis.

From the Figure 5. Initially, PCR amplification of the AUK and NAT with the *HvDhn7* F6 + R4 primers were performed. Using the F6 and R4 primers, when the annealing temperature was 55 ^{0}C lane 1, 2, and 3, AUK plants have specific band 733 bp. Lane 4, 5 and 6, NAT plants have specific bands between 733 – 600 bp. This type of marker is not polymorphic. The amplified fragments in the same sizes. In PCR products amplified

from genomic DNA, multiple fragments of similar size are observed. The narrow bands with similar sizes on agarose gel, shown on Figure 6, were cutting out for purification of PCR products using FavorPrep™ Gel and PCR purification kit for further Sanger sequencing (see Figure 7).

3.2.2. Sequencing Results of Sequenced with *HvDhn7*-F2 primer

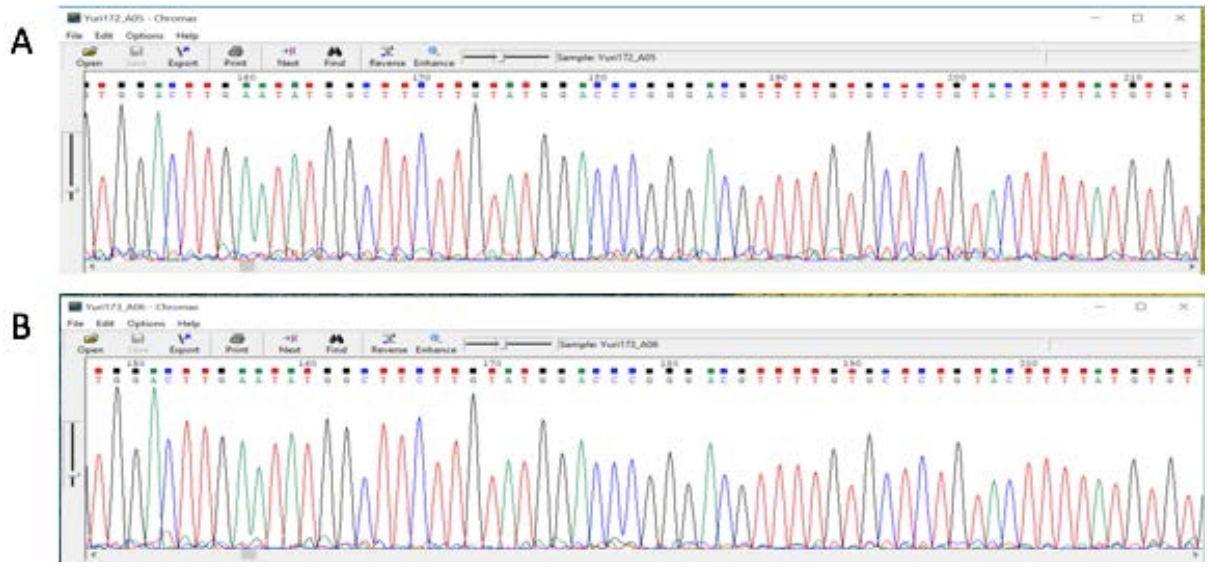


Figure 7: The figure is showing sequences of samples AUK and NAT.

A and **B**; Samples AUK and NAT- sequences analysis with background interference. Primer used was *HvDhn7*-F2. No difference between the parents sequences. No SNP marker detected for AUK and NAT. The concentrations of purified PCR products were low as 1.7 and 4.7 ng/ µL, respectively, for AUK and NAT. See Appendix C3.7.

3.2.3. Checking Purified PCR Products for Sequencing (One Combination of Primers)

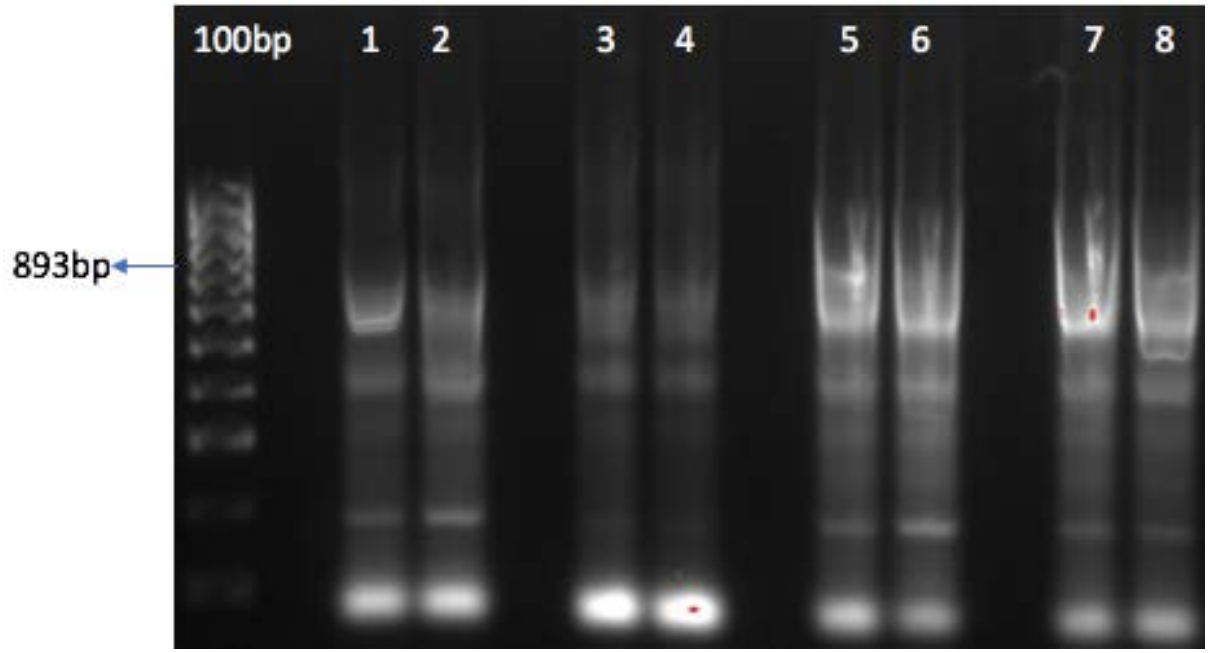


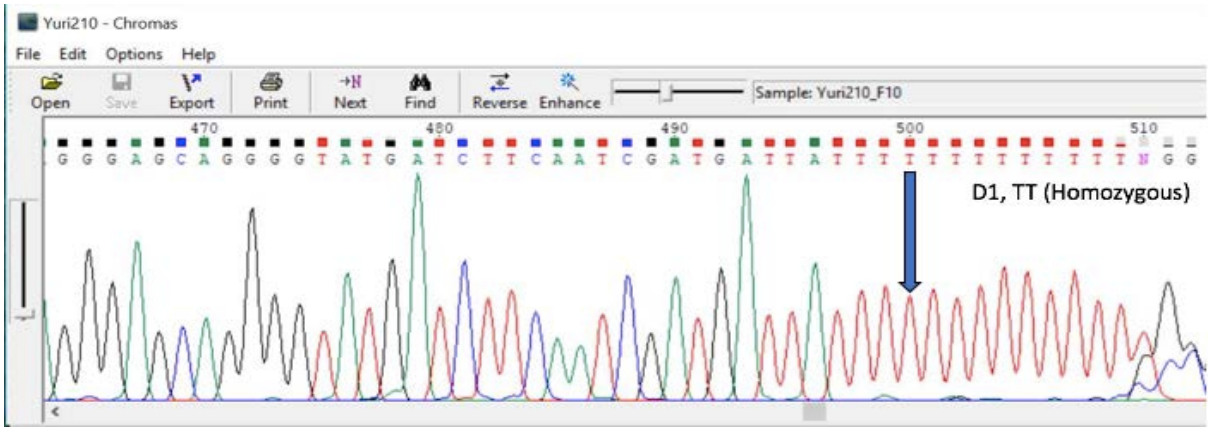
Figure 8: Agarose gel showing PCR amplification products obtained with *HvDhn7* F5+R4 primers.

For M is the ladder marker100bp (Promega, Australia). DNA for barley collection it is non diluted 100ng/ µL. Lane 1 and 2 (A1, Yukub). Lane 3 and 4 (D1, Margret). Lane 5 and 6 (H3, Cmbh). Lane 7 and 8 (A4, Cmb89A). All samples were separated in 1% (w/v) agarose gel electrophoresis

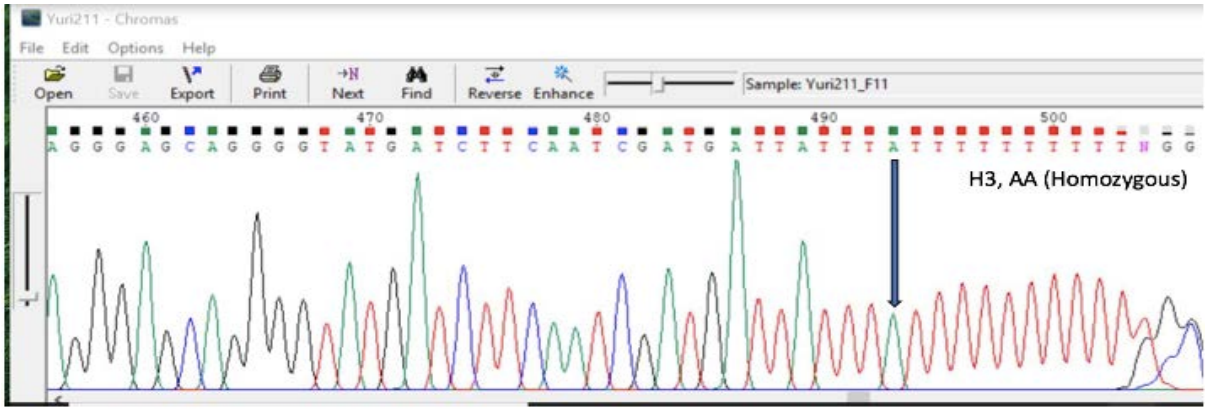
Figure 8 plant (A1, Yukub) has band 893 bp with no homozygote or heterozygous. Two plant (D1, Margret and H3, Cmbh) has band 893 bp with homozygote T/T. and A/A. Plant (A4, Cmb89A) has band 893 bp with heterozygote. Since the difficulty of either cutting out the narrow bands with similar sizes on agarose gel for purification of PCR products using FavorPrep™ Gel and PCR purification kit for Sanger sequencing (see Figure 9).

3.2.4. Sequencing results of *HvDhn7*-F5 primer

A



B



C

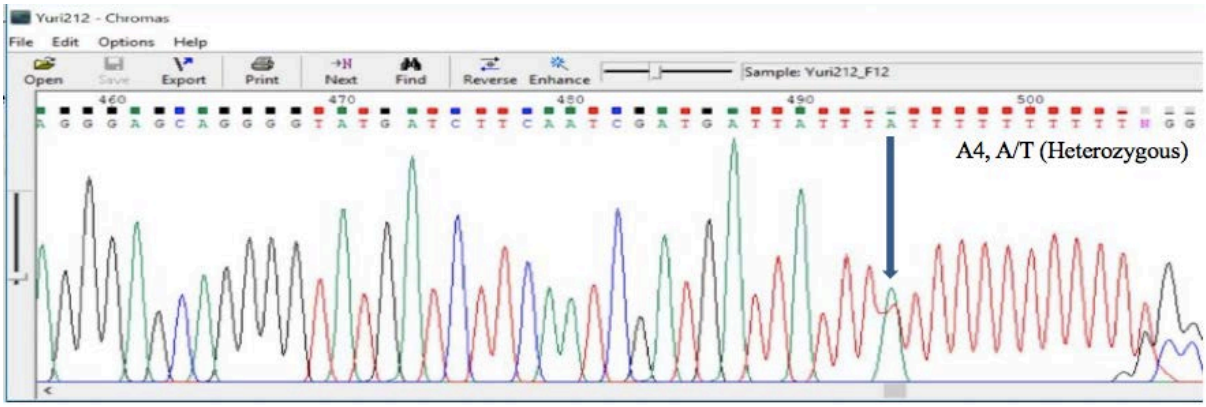


Figure 9: Sequences of *HvDhn7* with the forward primer F5.

A and **B**: homozygous T/T and A/A. **C**: heterozygous A/T. The arrows indicate the double diagonal that corresponds to heterozygous. DNA samples D1, H3 and A4 are corresponding to 500 bp, 493 bp and 494 bp. They were selected from barley germplasm collection. DNA concentrations were 4.9, 25.5 and 23.2 ng/uL for D1; the high is 25.5 ng/µL, respectively, for D1, H3 and A4. The details are present in Appendix C3.9.

3.3. qPCR Fluorescence for Allele Discrimination

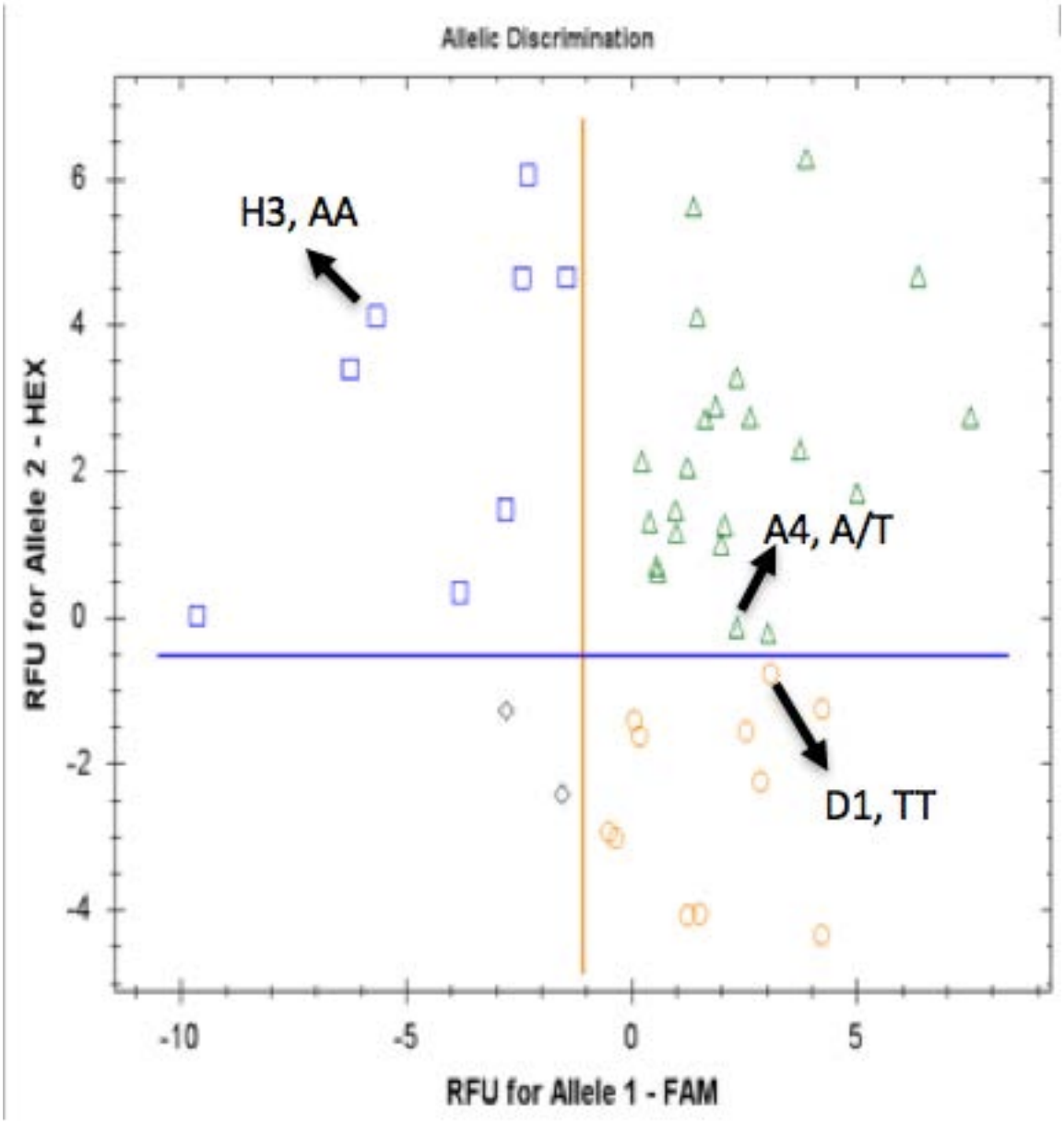


Figure 10: Image of Amplifuor-like SNP genotyping using *HvDhn7*.

Amplification with Real-time qPCR system, designed for FAM and HEX fluorescently labelled probes, using automatic calls for alleles, shown in different colours. DNA of barley germplams were diluted to 10 ng/µL. Homozygous for A/A and T/T were found in H3 and D1, corresponding to Allele 2 and Allele 1, barley genotypes, and heterozygous for A/T, was identified in A4, corresponding to heterozygous accession. Relative

fluorescence units (RFUs) for Allele 1 – FAM and Allele 2 – HEX were determined by the qPCR instrument. The details are present in Appendix A6 and Tables 7, 8 and 9.

3.4. Comparison between qPCR Fluorescence for Allele Discrimination and Quantification Curves

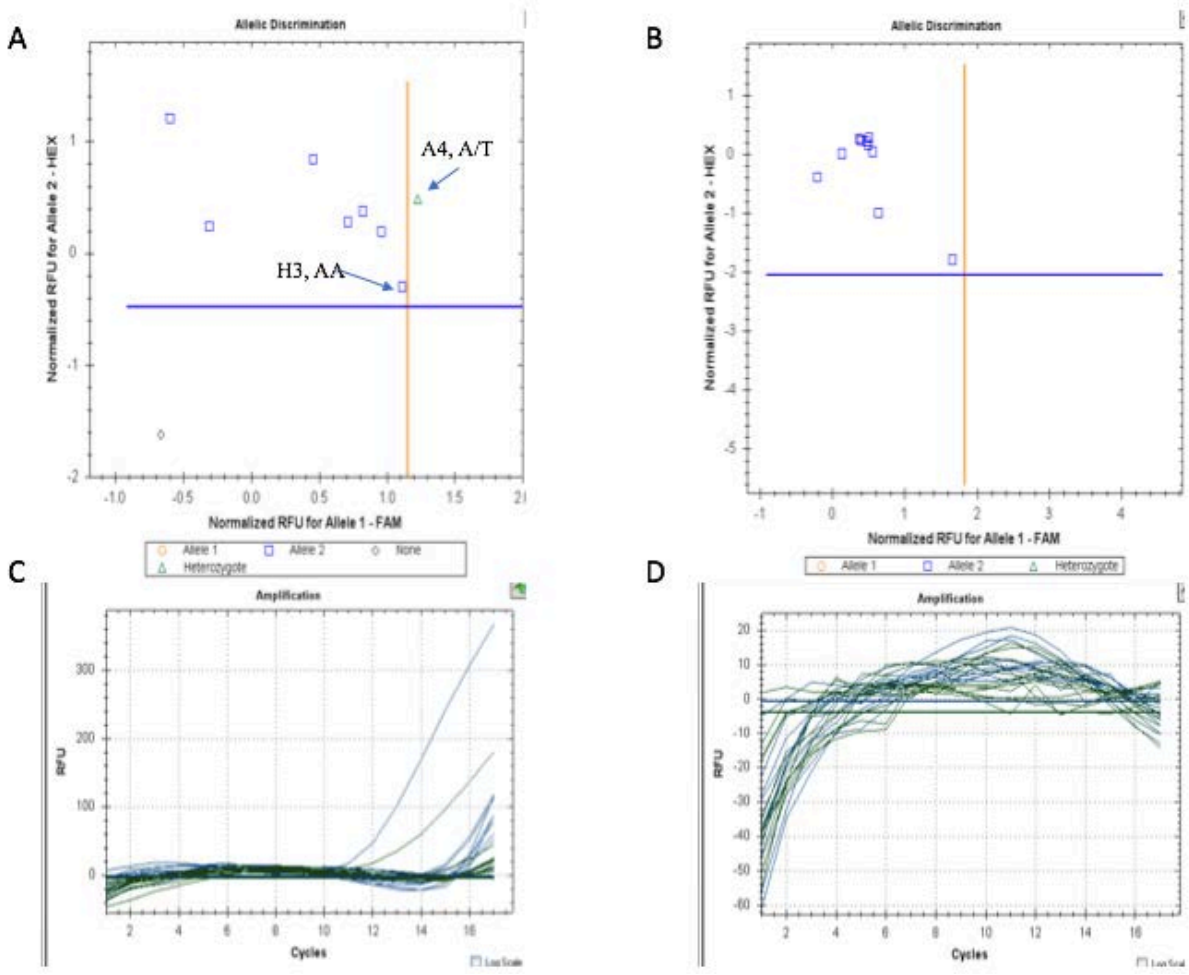


Figure 11: Shows image of comparison between allele discrimination and amplification curves for FAM and HEX of Amplifuor-like SNP genotyping using *HvDhn7*.

Amplification with Real-time qPCR system, designed for FAM and HEX fluorescently labelled probes, using automatic calls for alleles, shown in different colours. DNA of barley germplasm were diluted to 10 ng/ µL. **(a)** 4 µL of DNA, homozygous for A/A was found in H3, corresponding to Allele 2, barley genotypes, and heterozygous for A/T, was identified in A4, corresponding to heterozygous accession. **(b)** 2µL of DNA, homozygous for A/A, was found in H3, corresponding to Allele 2 and barley genotypes. Normalized Relative fluorescence units (NRFUs) for Allele 1 - FAM and Allele 2 - HEX. **(c)** start of

amplification between 10 and 12 cycles, and **(d)** low amplification, as determined by the qPCR instrument. The details are present in Appendix A7 and Table 10.

Chapter 4: Discussion

It is easy to find out similarities between genome sequences of different species, cultivars and subspecies due to public access to many genome sequences. SNPs are very abundant and widely distributed in the genome of organisms, including plants (Nasu et al., 2002). Dehydrins are a multigene family associated with the response to dehydration and low temperature in plants (Choi et al., 1999).

During this study, different members of the dehydrin family were studied in barley germplasm collections and in two different barley cultivars, AUK and NAT. The *HvDhn9* gene was amplified using DNA and forward and reverse primers; the expected size of the amplicon was 624 bp on agarose gel (Fig. 2). Suprunova et al. (2004) studied by PCR the role of the *HvDhn* family genes in different barley cultivars and suggested *HvDhn1* is involved in drought tolerance. After checking purified PCR products used for sequencing with *HvDhn9* for one combination of primers (Fig. 3), run on agarose gel and the expected sizes of NAT and AUK fragments in genomic DNA were estimated. The size of amplicon using *HvDhn9*- forward F1 and reverse R1 was 539 bp.

To fulfil the aims of this research and identify SNP in two barley cultivars, PCR amplifications were performed with *HvDhn9* from two combinations of primers. Bands were purified and sequenced (Fig. 4). With forward primer F1 for the AUK (Fig. 4a) and for NAT (Fig. 4b) samples, the same sequence analysis with overall no SNP were found. The genetic fragment of *HvDhn7* gene was amplified using primers forward 6 and reverse 4, with expected size of PCR product for 733 bp in both cultivars (Fig. 5).

To find SNPs in *HvDhn7*, PCR was performed in big scale with F2 and R2 primers. The length of the PCR band, was estimated using electrophoresis on agarose gel for 950 bp with F2+R2 primers (Fig. 6). The PCR bands were purified and sequencing was performed. The results of sequencing of AUK and NAT with F2 primers showed no SNP in either results (Fig. 7a and 7b).

The reason of SNPs absence in sequences may be related to an experimental error, handling of samples or a low amount of used template DNA. It is not always possible to find SNPs with sequence analysis of PCR products due to difficulties distinguishing between true polymorphism or sequencing artefacts (Segman et al., 2006). The main role of the *Dhn9* and *Dhn7* is to distinguish barley types such as Auksiniai and Natali from barley collections. It is basically a nutrient, which is developed by the National centre of biotechnology. It helps in specifying the plants and its species even in drought. It possess a higher tolerance level and so helps in maintaining the quality of the barley and other related species.

After checking of purified PCR products, they were used for sequencing with *HvDhn7* and primers F5 and R4 (Fig. 8). The purified PCR products of barley germplasm collection, were run in agarose gel and the expected size of PCR products was 893 bp. In the result of sequencing of the PCR fragments in studies barley genotypes with F5 primer, the SNPs were found in *HvDhn7*. Three variants, T/T and A/A (homozygous) and A/T (heterozygous), were identified in three accessions and it is shown in Fig. 9. Our results were similar to those published earlier for Tunisian olive germplasms using the SNP marker (Hakim et al., 2009). Although olive belongs to different family of plants, yet SNP markers have good potential to discriminate cultivars and assess diversity. This approach was found superior to the traditional biochemical approaches for studying plants diversity and certain traits (Muzzalupo and Perri, 2008).

Fig. 10 represents the application of Amplifluor SNP markers with FAM and HEX labelled Universal probes. It was observed in use of Real-time qPCR system, designed for FAM and HEX fluorescence scoring, and with automatic calls for alleles. The main point is for analysis of DNA in barley germplasm collection. The barley accessions were distinguished for SNP alleles using two types of fluorescent colours with the help of supplementary software in the instrument with manual checking. The homozygous for A/A and T/T SNP alleles between (Cmbh, H3 and Margret, D1) and heterozygous for A/T (Cmb89A, A4) along with Relative fluorescence units (RFUs) for Allele 1 - FAM and Allele 2 - HEX were found in this analysis (Fig. 10). Figure 11 shows such heterozygotes, A/T, and homozygotes, A/A, On this Fig. 11, both the X- and Y-axes represent a Relative amplification curves for HEX and FAM fluorescence signals. Note

was made for high concentrations of template DNA in Fig 11c and low concentration of DNA in Fig 11d. Additionally, different types of protocols were tested using various PCR cyclers.

Through previous studies, many genes have been associated with tolerance to stress conditions such as drought and frost in barley. The dehydrin proteins, encoded by *Dhn* genes, have been found to have profound effect on plant physiology. These proteins contain a K-loop structure which in enriched with lysing residues. This structural entity makes dehydrin proteins resistant to low water and low temperature conditions. The *Dhn* genes appear as cluster on chromosomes 5H and 6H. Studies have shown that all isoforms have synergistic role in providing tolerance to drought and low temperature (Holkova et al., 2010).

Chapter 5: Conclusion and Future Work

This study examined the role of Single nucleotide polymorphisms (SNPs) markers for the identification of drought tolerant barley (*Hordeum vulgare* L.). Previously, other molecular markers such as Cleaved amplified polymorphic sequences (CAPS), Restriction fragment length polymorphism (RFLP) and Amplified fragment length polymorphism (AFLP) have been utilised. Although these markers have provided useful information in the identification of important traits in many crops and plant species, the resolution power and/or accuracy was compromised in some instances. Further, these techniques are laborious and require more time for elucidating results. Hence, this study concluded that SNPs have more potential to determine trait specific varieties more precisely with less effort and time. The SNPs of *HvDhn* genes can be optimized for detection in Real-time qPCR assay to determine drought tolerant genes. For this, allele-specific primers (ASPs) can amplify the target region if specific SNP is present in *Dhn*. In Amplifluor-like SNP markers, UPs were labelled with fluorophores and the fluorescence produced by the associated complex ASP-UPs was detected with Real-time qPCR assay. This approach enables to determine genotypes based on different states of SNPs, the homozygous and heterozygous SNPs are used for the identification of different traitss. This was efficiently demonstrated in this study, where three different genotypes of one SNP with F7, F8 and R primers for *HvDhn7* gene was detected with Amplifluor-like SNP assay.

Although there are several *Dhn* genes in different plant species, the SNPs in all *Dhn* regions would not be detected by Amplifluor-like SNP assay, because each trait would carry certain types of SNPs. In this study, the SNPs in all genetic regions of *HvDhn7* and *HvDhn9* genes were not detected. Nevertheless, the SNP in F5 region of *HvDhn7* gene was detected successfully. The identification of SNPs in *HvDhn* genes leads to the identification of barley cultivars tolerant to drought stress conditions. The varieties here selected were drought tolerant. So, the presence of SNPs in *Dhn7* gene indicates the definitive role in resistance to stress of drought. However, further studies are required to identify in which region of *Dhn* gene the SNPs were found and what would be the effect of SNP on protein's structure and function. This task can be carried out through gene ontology approaches. This study is not limited to barley; and it can be applied for SNP

identification and study in other crops, such as wheat, cotton or maize. However, for these crops, different ASPs would be required for detection of drought specific SNPs. Barley is a good example for this. In fact, the impact of this study can be increased by using the combination of several SNPs in different *Dhn* genes. The larger number of SNPs means higher resolution power of SNPs, and it would be possible to distinguish various traits in different cultivars. This will help to manage better crops selection and increase their production in changing environmental conditions with respect to global warming, water shortages and increasing salinity in soil by cultivating varieties suitable to the atmosphere and soil conditions.

In future work, this project optimized the protocol for the fast detection of SNP markers in barley for dehydrin genes. Based on the knowledge of current study fast detection of different associated traits can be made possible in other crops. In case of abiotic stress the deposition of dehydrin serves as potential biochemical indicator. Although dehydrins exhibit protective function during cellular dehydration yet their precise role needs further elucidation. Besides, against various abiotic stress elements such as salinity, drought, heat or freezing, plants have evolved specific intracellular mechanisms for preventing dehydration, out of which dehydrin accumulation is the most eminent one. In future, the exploitation of structural, functional and physio-chemical attributes of these proteins could be helpful in improving the abiotic stress tolerance in crops. In contrast to abiotic stress, the function of these proteins during biotic stress is an open question, as wounding leads to biotic stress leads to water loss which creates dehydration stress. Therefore, in future wound triggered expression of dehydrin genes under hormonal signalling could be helpful in elucidation of stress signalling pathways. Moreover, future work should also broadly investigate other dehydrins to find whether dehydrin-dependent regulatory processes regulate pathogen responses in plants.

References list

1. Anjum, S.A., Xie, X.Y., Wang, L.C., Saleem, M.F., Man, C. and Lei, W., 2011. Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research*, *6*(9), pp.2026-2032.

2. Aspinall, D., 1965. The effects of soil moisture stress on the growth of barley. II. Grain growth. *Australian journal of agricultural research*, *16*(3), pp.265-275.

3. Aspinall, D., Nicholls, P.B. and May, L.H., 1964. The effects of soil moisture stress on the growth of barley. I. Vegetative development and grain yield. *Australian Journal of Agricultural Research*, *15*(5), pp.729-745.

4. Badr, A., Sch, R., Rabey, H.E., Effgen, S., Ibrahim, H.H., Pozzi, C., Rohde, W. and Salamini, F., 2000. On the origin and domestication history of barley (*Hordeum vulgare*). *Molecular Biology and Evolution*, *17*(4), pp.499-510.

5. Baik, B.K. and Ullrich, S.E., 2008. Barley for food: characteristics, improvement, and renewed interest. *Journal of Cereal Science*, *48*(2), pp.233-242.

6. Baum, M., Von Korff, M., Guo, P., Lakew, B., Hamwieh, A., Lababidi, S., Udupa, S.M., Sayed, H., Choumane, W., Grando, S. and Ceccarelli, S., 2007. Molecular approaches and breeding strategies for drought tolerance in barley. In *Genomics-assisted crop improvement*, Springer, Netherlands. pp. 51-79.

7. Chaves, M.M., Maroco, J.P. and Pereira, J.S., 2003. Understanding plant responses to drought—from genes to the whole plant. *Functional plant biology*, *30*(3), pp.239-264.

8. Choi, D. W., Koag, M. C., & Close, T. J. (2000). Map locations of barley Dhn genes determined by gene-specific PCR. *Theoretical and Applied Genetics*, 101(3), pp. 350-354.

9. Close, T.J., 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum*, *97*(4), pp.795-803.

10. de Mezer, M., Turska-Taraska, A., Kaczmarek, Z., Glowacka, K., Swarcewicz, B., & Rorat, T. (2014). Differential physiological and molecular response of barley genotypes to water deficit. *Plant physiology and biochemistry*, 80, pp. 234-248.

11. Denby, K. and Gehring, C., 2005. Engineering drought and salinity tolerance in plants: lessons from genome-wide expression profiling in Arabidopsis. *Trends in biotechnology*, *23*(11), pp.547-552.

12. Edwards, D.,J.W. Forster, D. Chagne, and J.Batley.2007.What is SNPs? In: N.C.Or aguzie, E.H.A. Rikkerink, S.E. Gardiner and H.N. de Silva (eds.), *Association Mapping in Plants*. Springer, Berlin, pp 41-52.

13. Flexas, J., Bota, J., Loreto, F., Cornic, G. and Sharkey, T.D., 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biology*, *6*(03), pp.269-279.

14. Foster, J.B., 2000. *Marx's ecology: Materialism and nature*. NYU Press.

15. FAOSTAT, 2017. Barley Crop Countries data 2016. [Online]
Available at: http://wwwfao.org/faostat/en/#data/QC

16. Farooq, M., A. Wahid, N. Kobayashi, D. Fujita and S.M.A. Basra, 2009. Plant drought stress: effects, mechanisms and management. *Agronomy for. Sustainable Development*, 29, pp. 185–212.

17. Giancola, S. McKhann, H.I., Berard, A., Camilleri, C., Durand, S., Libeau, P., Roux, F., Reboud, X., Gut, I. G. and Brunel, D., 2006. Utilization of the three high-throughput SNP genotyping methods, the GOOD assay, Amplifluor and TaqMan, in diploid and polyploid plants. *Theoretical and Applied Genetics*, *112*(6), pp.1115-1124.

18. Gregory, P.J., 1994. Root growth and activity. *Physiology and determination of crop yield*, (physiologyandde), pp.65-93.

19. Hadidi, A. and Candresse, T., 2003. Polymerase chain reaction. *Viroids*, pp.115-122.

20. Hakim, I.R., Kammoun, N.G., Makhloufi, E. and Rebaï, A., 2009. Discovery and potential of SNP markers in characterization of Tunisian olive germplasm. *Diversity*, *2*(1), pp.17-27.

21. Harlan, J.R., 1995. Barley. In: Smartt J, Simmonds NW (eds) *Evolution of Crop Plants*. Longman Scientific & Technical, Harlaw, pp 140-147.

22. Hernandez, M., Esteve, T., Prat, S. and Pla, M., 2004. Development of real-time PCR systems based on SYBR® Green I, Amplifluor™ and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21. *Journal of Cereal Science*, *39*(1), pp.99-107.

23. Holkova, L., Mikulkova, P., Hrstková, P., Prášil, I.T., Bradáčová, M., Prášilová, P. and Chloupek, O., 2010. Allelic variations at Dhn4 and Dhn7 are associated with frost tolerance in barley. *Czech Journal of Genetics and Plant Breeding*, *46*(4), pp.149-158.

24. Zohary, D. and Hopf, M., 1988. Domestication of plants in the Old World: the origin and spread of cultivated plants in West Asia. *Europe, and the Nile Valley Oxford University Press, New York.*

25. Jatayev, S., Kurishbayev, A., Zotova, L., Khasanova, G., Serikbay, D., Zhubatkanov, A., Botayeva, M., Zhumalin, A., Turbekova, A., Soole, K., Langridge, P. and Shavrukov, Y. , 2017. Advantages of Amplifluor-like SNP markers over KASP in plant genotyping. *BMC plant biology*, *17*(2), p.254.

26. Jiang, G.L., 2013. Molecular markers and marker-assisted breeding in plants.
In *Plant breeding from laboratories to fields*. InTech.

27. Jing, H., Li, C., Ma, F., Ma, J.H., Khan, A., Wang, X., Zhao, L.Y., Gong, Z.H. and Chen, R.G., 2016. Genome-wide identification, expression diversication of dehydrin gene family and characterization of CaDHN3 in pepper (Capsicum annuum L.). *PloS one*, *11*(8), p.e0161073.

28. Kesawat, M.S. and Kumar, B.D., 2009. Molecular markers: it's application in crop improvement. *Journal of Crop Science and Biotechnology*, *12*(4), pp.169-181.

29. Kramer, P.J. and Boyer, J.S., 1995. *Water relations of plants and soils*. Academic press.

30. Lobell, D.B. and Field, C.B., 2007. Global scale climate–crop yield relationships and the impacts of recent warming. *Environmental research letters*, *2*(1), p.014002.

31. Mamnouie, E., Fotouhi Ghazvini, R., Esfahani, M. and Nakhoda, B., 2010. The effects of water deficit on crop yield and the physiological characteristics of barley (*Hordeum vulgare* L.) varieties. *Journal of Agricultural Science and Technology*, *8*, pp.211-219.

32. Mueen A. K.H.A.N., Iqbal, M., Akram, M., Ahmad, M., Hassan, M.W. and Jamil, M., 2013. Recent advances in molecular tool development for drought tolerance breeding in cereal crops: a review. 100(3), pp. 325–334

33. Muzzalupo, I. and Perri, E., 2008. Genetic characterization of olive germplasms by molecular markers. *European Journal of Plant Science and Biotechnology*, *2*(1), pp.60-68.

34. Nadeem, M.A., Nawaz, M.A., Shahid, M.Q., Doğan, Y., Comertpay, G., Yıldız, M., Hatipoğlu, R., Ahmad, F., Alsaleh, A., Labhane, N. and Özkan, H., 2018. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. *Biotechnology & Biotechnological Equipment*, *32*(2), pp.261-285.

35. Nilan, R.A. and Ullrich, S.E., 1993. *Barley: Taxonomy, Origin, Distribution, Production,Ggenetics, and Breeding. Barley: Chemistry and Technology*, pp, 1-29

36. Pan, A., Hayes, P.M., Chen, F., Chen, T.H.H., Blake, T., Wright, S., Karsai, I., Bedo, Z. (1994). Genetic analysis of the components of winter hardiness in barley (*Hordeum vulgare* L.). *Theor Appl Genet*, 89: 900-910.

37. Poehlman, J.M., 1985. Adaptation and distribution. *Barley*, (barley), pp.1-17
35. Riaz, A.T.I.F., Younis, A., Taj, A.R., Karim, A., Tariq, U., Munir, S. and Riaz, S.I.T.W.A.T., 2013. Effect of drought stress on growth and flowering of marigold (*Tagetes erecta* L.). *Pak. J. Bot*, *45*(S1), pp.123-131.

38. Ribas-Carbo, M., Taylor, N.L., Giles, L., Busquets, S., Finnegan, P.M., Day, D.A., Lambers, H., Medrano, H., Berry, J.A. and Flexas, J., 2005. Effects of water stress on respiration in soybean leaves. *Plant Physiology*, *139*(1), pp.466-473.

39. Saavedra, L., Svensson, J., Carballo, V., Izmendi, D., Welin, B. and Vidal, S., 2006. A dehydrin gene in *Physcomitrella* patens is required for salt and osmotic stress tolerance. *The Plant Journal*, *45*(2), pp.237-249.

40. Shavrukov, Y., 2016. Comparison of SNP and CAPS markers application in genetic research in wheat and barley. *BMC plant biology*, *16*(1), p.11.

41. Singh, R.K., Singh, R., Ye, G., Selvi, A. and Rao, G.P., 2010. *Molecular plant breeding: principle, method and application*. Studium Press LLC.

42. Sobrino, B., M. Briona, and A. Carracedoa. 2005. SNPs in forensic genetics: a review on SNP typing methodologies. *Forensic Science International* 154: 181-194.

43. Spannagl, M., Alaux, M., Lange, M., Bolser, D.M., Bader, K.C., Letellier, T., Kimmel, E., Flores, R., Pommier, C., Kerhornou, A. and Walts, B., 2016. transPLANT resources for Triticeae genomic data. *The Plant Genome*, *9*(1).

44. Sreenivasulu, N., Graner, A. and Wobus, U., 2008. Barley genomics: an overview. *International Journal of Plant Genomics*, *2008*.

45. Turner, N.C., 1979. *Drought resistance and adaptation to water deficits in crop plants. Stress Physiology in Crop Plants* (No. 631.53 MUS. CIMMYT.).

46. von Bothmer, R. and Jacobsen, N., 1985. Origin, taxonomy, and related species. *Barley*, (barley), pp.19-56.

47. Von Bothmer, R. and Jacobsen, N., 1991. Interspecific hybrids within the genus *Hordeum*. In *Developments in Plant Genetics and Breeding* (Vol. 2, pp. 411-431). Elsevier.

48. William, H.M., Trethowan, R. and Crosby-Galvan, E.M., 2007. Wheat breeding assisted by markers: CIMMYT's experience. *Euphytica*, *157*(3), pp.307-319.

49. Xu, Y. 2010. Molecular plant breeding. CAB International.

50. Yang, Y., He, M., Zhu, Z., Li, S., Xu, Y., Zhang, C., Singer, S.D. and Wang, Y., 2012. Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. *BMC Plant Biology*, 12(1), p.140.

Appendixes

Appendix A: PCR protocol for Promega Taq-polymerase
A1: Order of Master-mix preparation and DNA adding

N	Component	x1(ul)	x18(ul)
1	Milli-Q H_2O	8.35	150.3
2	5 x PCR Green Buffer	4.0	72.0
3	dNTP, 2 µM	2.0	36.0
4	$MgCl_2$, 25 mM	1.6	28.8
5	Primer F, 5 µM (*Dhn9*)	1.0	18.0
6	Primer R, 5 µM (*Dhn9*)	1.0	18.0
7	Go-Taq Polymerase, 5 units/ µL	0.05	0.9
	Loading Master-Mix for 16 samples	18.0	
	Add DNA separately in each reaction		
8	DNA NAT and AUK, plate 1 (10 ng/µL)	2.0	2.0
	Total	20.0	

Yuri8: PCR amplification protocol

N	PCR steps	Temp (^{0}C)	Time	Cycles
1	Initial denaturation	94	2 min	
2	Denaturation	94	15 sec	35
3	Annealing	55	15 sec	
4	Extension	72	45 sec	
5	Final extension	72	3 min	
6	Hold	15	24 hrs	

A2. Big scale PCR protocol for sequencing
Order of Master-mix preparation and DNA adding

N	Component	x1(ul)	x8(ul)
1	Milli-Q H_2O	29.2	233.6
2	5 x PCR Colorless Buffer	12.0	96.0
3	dNTP, 2 mM	6.0	48.0
4	$MgCl_2$, 25 mM	4.8	38.4
5	Primer F1, 5 µM (*Dhn9*)	3.0	24.0
6	Primer R1, 5 µM (*Dhn9*)	3.0	24.0
7	Go-Taq Polymerase, 5 units/ µL	0.5	4.0
	Loading Master-Mix for 8 samples	58.5	
	Add DNA separately in each reaction		
8	DNA (10 ng/µL	1.5	1.5
	Total	60.0	480.0

Yuri8: PCR amplification protocol

N	PCR steps	Temp (^{0}C)	Time	Cycles
1	Initial denaturation	94	2 min	
2	Denaturation	94	15 sec	30
3	Annealing	55	15 sec	
4	Extension	72	45 sec	
5	Final extension	72	3 min	
6	Hold	15	24 hrs	

A3. PCR amplification
Order of Master-mix preparation and DNA adding

N	**Component**	**x1(ul)**	**x7(ul)**
1	Milli-Q H_2O	6.10	42.7
2	5 x PCR Green Buffer	3.0	21.0
3	dNTP, 2 mM	1.5	10.5
4	$MgCl_2$, 25 mM	1.2	8.4
5	Primer F6, 5 µM (*Dhn7*)	0.75	5.25
6	Primer R4, 5 µM (*Dhn7*)	0.75	5.25
7	Go-Taq Polymerase, 5 units/ µL	0.2	1.4
	Loading Master-Mix for 6 samples	13.5	
	Add DNA separately in each reaction		
8	DNA (AUK-NAT) (10 ng/µL	1.5	(10.5)
	Total	15.0	

Yuri7: PCR amplification protocol

N	PCR steps	Temp (^{0}C)	Time	Cycles
1	Initial denaturation	95	1 min	
2	Denaturation	95	15 sec	39
3	Annealing	55	15 sec	
4	Extension	72	1 min	
5	Final extension	72	2 min	
6	Hold	15	24 hrs	

A4. Big scale PCR protocol for sequencing
Order of Master-mix preparation and DNA adding

N	**Component**	**x1(ul)**
1	Milli-Q H_2O	18.7
2	5 x PCR Colorless Buffer	12.0
3	dNTP, 2 mM	6.0
4	$MgCl_2$, 25 mM	4.8
5	Primer F2, 5 µM (*Dhn7*)	3.0
6	Primer R2, 5 µM (*Dhn7*)	3.0
7	Go-Taq Polymerase, 5 units/ µL	0.5
	Loading Master-Mix for 2 samples	48.0
	Add DNA separately in each reaction	
8	DNA (AUK-NAT) (10 ng/µL	12.0
	Total	60

Yuri8: PCR amplification protocol

N	PCR steps	Temp (^{0}C)	Time	Cycles
1	Initial denaturation	94	2 min	
2	Denaturation	94	15 sec	30
3	Annealing	55	15 sec	
4	Extension	72	45 sec	
5	Final extension	72	3 min	
6	Hold	15	24 hrs	

A5. Big scale PCR protocol for sequencing
Order of Master-mix preparation and DNA adding

N	**Component**	**x1(ul)**	**x4 (ul)**	**x4 (ul)**
1	Milli-Q H_2O	5.55	28.0	22.0
2	5 x PCR Colorless Buffer	3.0	12.0	12.0
3	dNTP, 2 mM	1.5	6.0	6.0
4	$MgCl_2$, 25 mM	1.3	5.2	5.2
5	Primer F5, 5 µM (*Dhn7*)	0.75	3.0	3.0
6	Primer R4, 5 µM (*Dhn7*)	0.75	3.0	3.0
7	Go-Taq Polymerase, 5 units/ µL	0.15	0.8	0.8
	Loading Master-Mix for 8 samples	13.0		
	Add DNA separately in each reaction			
8.	DNA (barley collection, A1, D1, H3 and A4) (100 ng/µL	2.0	2.0 (A1)	8.0 (D1, H3, A4
	Total	15	60	60

Note: Barley collection for (A1, Yukub) (D1, Marget and H3, Cmbh) (A4, Cmb89A)

Yuri5: PCR amplification protocol

N	PCR steps	Temp (^{0}C)	Time	Cycles
1	Initial denaturation	94	2 min	
2	Denaturation	94	20 sec	39
3	Annealing	50	20 sec	
4	Extension	72	2 min	
5	Final extension	72	5 min	
6	Hold	4	24 hrs	

A6. PCR cocktail preparation for genotyping

N	Component	x1(ul)	x46(ul)
1	Milli-Q H_2O	0.15	6.9
2	5 x PCR Colorless Buffer	2.0	92.0
3	dNTP, 2 mM	1.0	46.0
4	Primers mix (F7, F8, R)	1.0	46.0
5	Probe mix (Uni1, Uni2)	1.0	46.0
6	$MgCl_2$, 25 mM	0.8	36.8
7	Go-Taq Polymerase, 5 units/ µL	0.05	2.3
	Loading Master-Mix for 43 samples	6.0	
	Add DNA separately in each reaction		
8.	DNA (barley collection) (10 ng/µL)	4.0	4.0
	Total	10	280

Using qPCR-CFX96 Thermal cycler (Genotyping)

1. 94C – 2 min
2. 94C – 15 sec
3. 55C – 20 sec
4. 72C – 45 sec
5. 94C – 15 sec
6. 60C – 30 sec
7. 72C – 45 sec
8. Go to step 2 to repeat 10
9. 72C 1 min
10. Score genotyping

Table 7: DNA samples in 96 well plate

Plate-Protocol												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	3	4	5	6	7	8	10	11	13	14	15	16
B	17	18	19	20	21	22	23	24	25	26	27	28
C	29	30	31	32	33	34	35	36	37	38	39	40
D	41	42	43	44	45	46	NTC					
E												
F												
G												
H												

Table 8: Barley collection, AUK and NAT for DNA samples in 96 well plate

Plate position	N	Name
A1	3	Odessky-100
A2	4	Don-9
A3	5	Don-8
A4	6	M30
A5	7	M32/95
A6	8	Tzelinniy-213
A7	10	Karabalyk-643
A8	11	Karabalyk-150
A9	13	Karagand-5
A10	14	Karagand-9
A11	15	Karagand-2
A12	16	Karagand-6
B1	17	Karagand-7
B2	18	Karagand-8
B3	19	Karagand-10
B4	20	Karagand-11
B5	21	Karagand-80
B6	22	Medicum-715
B7	23	Medicum-710
B8	24	Medicum-1040
B9	25	Prestizh
B10	26	Tzelin.goloz.
B11	27	Pamati Raisi
B12	28	Sabir
C1	29	Astana-2007
C2	30	Bozman
C3	31	Granal
C4	32	Boisheshek
C5	33	Karabalyk-110
C6	34	2775
C7	35	Don-9
C8	36	Karagand-6
C9	37	HXL-7
C10	38	A4-Cmb89A
C11	39	H3-Cmbh
C12	40	F3-Astana-2000 st
D1	41	B4-Icb97-1254
D2	42	D2-Astana-2000 st
D3	43	E1-Beatrie
D4	44	D1- Margret
D5	45	AUK
D6	46	NAT

Table 9: DNA samples for Amplifluor-like SNP genotyping

Well	RFU1	RFU2	Call
A01	1.23	2.06	Heterozygote
A02	1.86	2.89	Heterozygote
A03	-2.81	1.48	Allele 2
A04	-6.25	3.4	Allele 2
A05	0.971	1.47	Heterozygote
A06	0.57	0.632	Allele 1
A07	2.05	1.27	Heterozygote
A08	2.33	3.29	Heterozygote
A09	1.24	-4.07	Allele 1
A10	-3.82	0.345	None
A11	3.73	2.3	Heterozygote
A12	4.23	-1.25	Allele 1
B01	0.389	1.32	Heterozygote
B02	-1.44	4.65	Allele 2
B03	5	1.71	Heterozygote
B04	1.36	5.62	Heterozygote
B05	1.97	1.01	Heterozygote
B06	-9.65	0.0297	None
B07	0.0493	-1.4	Allele 1
B08	0.543	0.712	Allele 1
B09	1.44	4.12	Heterozygote
B10	4.21	-4.34	Allele 1
B11	-1.55	-2.41	None
B12	1.62	2.72	Heterozygote
C01	7.5	2.75	Heterozygote
C02	0.988	1.17	Heterozygote
C03	6.35	4.67	Heterozygote
C04	-2.31	6.05	Allele 2
C05	-0.517	-2.93	Allele 1
C06	2.86	-2.24	Allele 1
C07	1.5	-4.05	Allele 1
C08	0.173	-1.62	Allele 1
C09	-0.351	-3.02	Allele 1
C10	2.33	-0.13	Heterozygote
C11	-5.67	4.12	Allele 2
C12	3.02	-0.207	Allele 1
D01	0.216	2.15	Heterozygote
D02	2.62	2.75	Heterozygote
D03	-2.44	4.65	Allele 2
D04	3.08	-0.763	Allele 1
D05	3.86	6.29	Heterozygote
D06	2.53	-1.55	Allele 1
D07	-2.79	-1.27	None

A7. PCR cocktail preparation for genotyping

(G)

N	Component	x1(ul)	x15(ul)
1	Milli-Q H_2O	0.15	2.25
2	5 x PCR Colorless Buffer	2.0	30.0
3	dNTP, 2 mM	1.0	15.0
4	Primers mix (F7, F8, R)	1.0	15.0
5	Probe mix (Uni1, Uni2)	1.0	15.0
6	$MgCl_2$, 25 mM	0.8	12.8
7	Go-Taq Polymerase, 5 units/ µL	0.05	0.75
	Loading Master-Mix for 12 samples	6.0	
	Add DNA separately in each reaction		
8.	DNA (barley collection) (10 ng/µL)	4.0	4.0
	Total	10	90

(H)

N	Component	x1(ul)	x15(ul)
1	Milli-Q H_2O	2.1	31.5
2	5 x PCR Colorless Buffer	2.0	30.0
3	dNTP, 2 mM	1.0	15.0
4	Primers mix (F1, F2, R)	1.0	15.0
5	Probe mix (Uni1, Uni2)	1.0	15.0
6	$MgCl_2$, 25 mM	0.85	12.75
7	Go-Taq Polymerase, 5 units/ µL	0.05	0.75
	Loading Master-Mix for 12 samples	8.0	
	Add DNA separately in each reaction		
8.	DNA (barley collection) (10 ng/µL)	2.0	2.0
	Total	10	120

Using qPCR-CFX96 Thermal cycler (Genotyping)

1. 94C – 2 min
2. 94C – 15 sec
3. 55C – 20 sec
4. 72C – 45 sec
5. 94C – 15 sec
6. 60C – 30 sec
7. 72C – 45 sec
8. Go to step 2 to repeat 16
9. 72C 1 min
10. Score genotyping

Table 10: Barley collection, AUK and NAT for DNA samples

Plate position	N	Name
G1	20	Karagand-11
G2	15	Karagand-2
G3	38	A4-Cmb89A
G4	39	H3-Cmbh
G5	44	AUK
G6	45	NAT
G7	41	B4-Icb97-1254
G8	42	D2-Actaha-2000 st
G9	43	E1-Beatrie
G10	37	HXL-7
G11		NTC
G12		NTC

Plate position	N	Name
H1	20	Karagand-11
H2	15	Karagand-2
H3	38	A4-Cmb89A
H4	39	H3-Cmbh
H5	44	AUK
H6	45	NAT
H7	41	B4-Icb97-1254
H8	42	D2-Actaha-2000 st
H9	43	E1-Beatrie
H10	37	HXL-7
H11		NTC
H12		NTC

Plate-Protocol												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G	20	15	A4	H3	AUK	NAT	B4	D2	E1	37	NTC	NTC
H	20	15	A4	H3	AUK	NAT	B4	D2	E1	37	NTC	NTC

Well	NRFU1	NRFU2	Call
G01	-0.669	-1.62	None
G02	0.71	0.282	Allele 2
G03	1.22	0.489	Heterozygote
G04	1.11	-0.297	Allele 2
G05	-0.311	0.243	Allele 2
G06	0.822	0.379	Allele 2
G07	4.32	-5.28	Allele 1
G08	0.958	0.197	Allele 2
G09	-0.601	1.21	Allele 2
G10	0.453	0.84	Allele 2

Well	NRFU1	NRFU2	Call
H01	0.364	0.268	Allele 2
H02	1.65	-1.78	Allele 2
H03	0.499	0.291	Allele 2
H04	0.553	0.053	Allele 2
H05	0.631	-0.986	Allele 2
H06	0.126	0.0199	Allele 2
H07	-0.215	-0.387	Allele 2
H08	0.392	0.242	Allele 2
H09	0.468	0.218	Allele 2
H10	0.49	0.156	Allele 2

A8. Gel Electrophoresis Buffer

50 x TAE Buffer

242 g of Tris Base (MW = 121.1) or (MW = 121.14), 600 ml of ddH_2O, 100 ml of 0.5 M EDTA, 57.1 ml of Glacial Acetic Acid.

1xTAE Buffer

100 ml of 50 x TAE (stock), 4.900 ml of Milli-Q-H_2O 5.000 ml of 1 x TAE Buffer

Appendix B:

Allele-specific primers design and preparation for *HvDhn9*

B1. Location of primers

>AF181459.1 *Hordeum vulgare* dehydrin (*Dhn9*) gene, complete cds

TCGCCACTAGTAAGCAAGGCAT ACCGGGCTGGGGCCCCTCTCGTTGGGTGGCCCGGGGCGGCCGCCCCGC
TCGCCCTGCCCCATGACCGGGCCTGACTCTAACCCTAGGATCCAAACACGACAGATGAAGTCACGAAACA
AGGTGCACTGCCCTATAATTTGTTCATCTTACTCACCGTCACCGTGCTGTACTAAACTGTAGTACTACTG
TAACATCAAAAATAGAACGCCGAGCTAATCTGTCAAGTCACCCTTCCAGAACAGCCGAACGCGTACGTGT
CAGTGTCGCCACCGACACGTTCAGGACCGCGGCGCGTGCACGCGTCCCGGCTATCCGTGCGCCCACGCCG
ACCACTTGCGCCGCCGGACTCACCACGAAAGCACACGTACCCCCCCTGCAGTAGCCACCTGGCCTGCCAC
ATCCCGTTACAACCCGGCCTGTCGCCACGGTATAAATATCCACCCGGCGTCACTCTTCGCTGCACAACAC
GACCCACGAGCTGTCTGTCTGTCTGT CTGTCATCCGAAGTCCAAAGCTA GCTGAAGGCACAACACTTCAA
CTCGCAGTCCGGTTCAGGGAGAAGAGTCCATCATCCAAGGTAGTAGTAGCAGGCAAG ATGGAGTTCCAAG
GGCAGCACGACAACCCCGCCAACCGCGTCGACGAGTACGGCAACCCGTTCCCGCTGGCCGGCGGCATGGG
GGGAGCGCACGCCGCTCCCGGCACCGGCGGGCAGTTCCAGGCCCGCAGGGAGGAGCACAAGACCCGTGGG
ATACTGCATCGCTCCGGCAGCTCCAGCTCCAGCTCG GTATGATGCTCTGTCGACACACACATGTACGCAT
TCACGTGCTTCTGTATGTTTAGATGCTGATTTCCGTGCCTGTATGCGTGTGTGTGTGTGTGCAG TCCTCC
GAGGACGACGGCATGGGCGGGAGGAGGAAGAAGGGCATGAAGGAGAAGATCAAGGAGAAGCTCCCCGGCG
GCCACAAGGACAACCAGCAGCACATGGCGACGGGGACTGGAACCGGAGGAGCATACGGGCCGGGAACTGG
AACTGGTGGAGCCTACGGGCAGCAAGGGCACGCAGGAATGGCCGGCACCGGCGAGAAGAAGGGGATCATG
GACAAGATCAAGGAGAAGCTGCCGGGACAGCACT GAGCCGCCGGCTCCGGCTGGCTGCTTTCTTTGCATA
GCTACGCGTCGAAGCCTTCCAGTTC CACGTGATCTTCATTCAATAAT AAGATGAAGCTGAACGAAACGTT
GCCTCTGATTTCATCTGTGTCGGGGACACTTTTCTGTATACAGCGTGCGTTGTGTTTATGTTTGTGTGTT
CTTGTGTCTTCTTGCTGAAAAAAAATTCAGAATACAAGTGAACTTGTTTCGGTTCGTTCTTTCTTTCTTT
CTTTTTTTTTGCGAATCAACTGAACTTGTTTCTCTGACAAAGGAAATCAGGCCCTTATGTCTGTCCAACG
AAGGACCAATGTCATTTTTACCCTTGCACCCAGAACGTATGTTCTACTCCCTCCGTTACTAAATATAAGT
CTATATGTTCTAGTATGTTGTACACAAAGTAG AAAGCATGCTGGTGCAAAACGAAAAGAGCAACAGTTAC
ATCTTCTAAAAAA AAAGCATGCTGGTGCAAAACGAAAAGAGCAACAGTTACATCTTCTAAAAAA AATTGC
GGAAACATCGACTATAGCTAGCATTTGGTTTACCCCAATTATCATGACTATGTTAATATCTATGACGCTT
TTGGATGTCTCATGTCCA AATAAATGGTCATATACTCGATA

Dhn9-F GCTGAAGGCACAACACTTCA 20bp, 50% GC, 51.8C
Dhn9-R CAACGTTTCGTTCAGCTTCA 20bp, 45% GC, 49.7C
Dhn9-R (RevCem): TGAAGCTGAACGAAACGTTG
PCR Product size: 722 bp

Dhn9- 5'-UTR region:
Dhn9-F1 TCGCCACTAGTAAGCAAGGCAT 22 bp, 50% GC, 54.8C
Dhn9-R1 TAGCTTTGGACTTCGGATGACAG 23 bp, 48% GC, 55.3C
Dhn9-R1(RevCom): CTGTCATCCGAAGTCCAAAGCTA
PCR Product size: 539 bp

Dhn9- 3'-UTR region:
Dhn9-F2 CACGTGATCTTCATTCAATAAT 22 bp, 32% GC, 47.4C
Dhn9-R2 TATCGAGTATATGACCATTTATT 23 bp, 26% GC, 46.4C
Dhn9-R2 (RevCom): AATAAATGGTCATATACTCGATA
PCR Product size: 576 bp
PCR product size: 576 bp

F3
MEFQGQHDNPANRVDEYGNPFPLAGGMGGAHAAPGTGGQFQARR

EEHKTRGILHRSGSSSSSS SSEDDGMGGRRKKGMKEKIKEKLPGGHKDNQQHMATGTG
TGGAYGPGTGTGGAYGQQGHAGMAGTGEKKGIMDKIKEKLPGQH"F2

>>AF181459.1_*Hordeum_vulgare*_dehydrin_(*Dhn9*) Translated - Frame 3
ATSKQGIPGWGPSRWVARGGRPARPAP*PGLTLTLGSKHDR*SHETRCTAL*FVHLTHRHRAVLNCSTTV
TSKIERRANLSSHPSRTAERVRVSVATDTFRTAARARVPAIRAPTPTTCAAGLTTKAHVPPLQ*PPGLPH
PVTTRPVATV*ISTRRHSSLHNTTHELSVCLSVIRSPKLAEGTTLQLAVRFREKSPSSKVVVAGKMEFQG
QHDNPANRVDEYGNPFPLAGGMGGAHAAPGTGGQFQARREEHKTRGILHRSGSSSSSSV*CSVDTHMYAF
TCFCMFRC*FPCLYACVCVCSPPRTTAWAGGGRRA*RRRSRRSSPAATRTTSSTWRRGLEPEEHTGRELE
LVEPTGSKGTQEWPAPARRRGSWTRSRRSCRDSTEPPAPAGCFLCIATRRSLPVPRDLHSIIR*S*TKRC
L*FHLCRGHFSVYSVRCVYVCVFLCLLAEKKFRIQVNLFRFVLSFFLFFCESTELVSLTKEIRPLCLSNE
GPMSFLPLHPERMFYSLRY*I*VYMF*YVVHKVESMLVQNEKSNSYIF*KKSMLVQNEKSNSYIF*KKLR
KHRL*LAFGLPQLS*LC*YL*RFWMSHVQINGHILD

Allele-specific primers design and preparation for *HvDhn7*

B2. Location of primers

B2.1 >AF043092.1 *Hordeum vulgare* dehydrin 7 (*Dhn7*) gene, complete cds

TTTTTT**AATGAGCAGGGTTATGATTTTTCA**ATCCATTGTATATTTTCATGGGCACAGAAATGAATTTGAT
ATGCTCGACCATGTAACCCGAACGCGCCTACTTTTAATAATAGGATACCTTCGGTCTATATAGTTCCAAT
TTTGTTCCATGCCGACACTTTCTAGTTGTCATTTTCCTAAGCAAACAGGACGACTTTTGGAAGCGGAAAT
AAGTACCCCCTGTGCTGTGTGTCATCACCTCTCCGAGAACAAGCTGAGACGTGGCAACCCGAACGCGCCC
AGTAGCTGCACACGTICGGCCTCTCTTTATGGGCTAGTCATCACTCATCACTCATCAGTCACCGGCAACC
CACTCACGCGAGCACACGTAGCGCCTCCGACGACGATCCTATCCCGTCGATCGGCTATAAATTGTGCCTC
CTGCTGCACCTGCTTACACAACACAGCCACCAGTTGCGCAGCCAAGTGAGGAAGACAACCCACCAGATTT
CTGAGAGAACCAAGAGCAGCACCTGTGCAAGATGGAGTACCAGGGACAGCAGCAGCACGGCCAGGCGACC
AACCGCGTCGACGAGTACGGTAACCCGGTTGCCGGACACGGCGGTGGCACCGGCATGGGCGCGCACGGCG
GCGICGGCACCGGIGCGGCCGCTGGTGGTCATTTCCAGCCGACGAGGAAGGAACACAAGGCCGGCGGGAT
CCTGCAGCGCICCGGCAGCTCAAGCTCCAGCTCGGTGCGTATATAGGCTTTACTTACTTCTTACCAACAC
TTGCATAGTGGCATTTTCCCGTTTAGACTTTGAATGAGAAAGCATCAACGCCTGAATTTTCTCGTTGGTG
GTGATAGTCTGAGGACGATGGCATGGGCGGGAGGAGGAAGAAGGGTATCAAGGATAAGATCAAGGAGAAG
CTCCCTGGTGGCCACGGTGACCAGCAGCACGCCGCCGGCACCTACGGACAGCAGGGTATTGGCATGGCCG
GCACCGGCGCACCTATGGGCAGCAGGGTCACACTGGGATGACCGGCACGGGGCAACTGGCACCATGGC
CACAGGCGGTACCTACGGGCAGCCGGGACACACCGGTATGACTGGTACTGGGACGCATGGCACCGACGGC
ACCGGCGAAAAGAAGGGCATCATGGACAAA**ATCAAGGAGAAGCTGCCCGGAC**AGCACTGAGCCCGGCCCG
CGGCCCCTACTTGTGAAAGTTAAGAGGTGCCGGTCTACGTACCCACCTCTGCAGAAAAATAAGATGAAGA
TACAGTAAAACTTTCCGAAATGAAGTGAGCTTTAGTTCACTCGTGATATCTGAGTTCTCGGTTTTATGGA
CTTGAATATGGCTTCTTGTATGGACCCGGGACGTTTTGTGCTCTGTACTTTTATGTGTGAATTTTCTTTT
GGTTGAACTGTGTATCTGTTATGTATGTATATCATGATATAATTAATACTACAATATATTTTGCCCTTCT
ACTTGCGCTTTCTGTGCATTGCATGATTTTAAGTTGGGTAGTTTTGTCCACGTGGCATATTGTACATTGC
ATTTGAATTAGTTTTTGTGAGAGTTTAGAAGCATTCTATATAGTGTTTTGACGATTTTGAAGCATCCAAA
AGTAGGGTCCACCAAACATGGTTTAGTACAAAGCTACAACTTTGTATTAAACCAACATCAATTAATACAG
ATCAAAACAGCCGCAAAAAAGAAAAAAAATATAGATCAAAGGGACTTACAAAGCTACTTCTCATAAATTT
GGTTTTGATAGTAAGTTAGATGATGAGCACACCACATCCAAAAAATTACTTTCCATAAATTTATACTAAA
ATAATCATCATATAATACAACATATAAATGCATATAAAATATTCAAGATTGATAATATAATGGTATGAAA
CAATTTAAAATTATAGATATGTTGTATTCGAGATTGATAATATAATGGTATGAAACAATTTAAAATTATA
GATATGTTGAAGACGTATAACTTCCCTGCGTCGGTTTGTTCGAATGAGAGAGTTTATTACTCCTCGAGGC
ACAAGATCTAGGTGACCAAGGTTCCCACAGGAGCTTTCTTTTATTGTGTGCTCCGGGTAAGGGTGTGAAG
TGACCAAGGTGGTCAAGTTTCTCCGGA

MEYQGQQQHGQATNRVDEYGNPVAGHGGGTGMGAHGGVGTGAAAGGHFQPTRKEHKAGGILQRSGSSSSSSSE
DDGMGGRRKKGIKDKIKEKLPGGHGDQQHAAGTYGQQGIGMAGTGGTYGQQGHTGMTGTGATGTMATGGTYG
QPGHTGMTGTGTHGTDGTGEKKGIMDKIKEKLPGQH F2

Dhn7-F: GCAAGATGGAGTACCAGGGA 20 bp, 55%GC, 53.8C
Dhn7-R: TACGTAGACCGGCACCTCTT 20 bp, 55%GC, 53.8C
Dhn7-R (RevCom: AAGAGGTGCCGGTCTACGTA
PCR product size: 716 bp.

Dhn7-F1: AATGAGCAGGGTTATGATTTTTCA 24 bp, 33%GC, 50.6C
Dhn7-R1: ATATACGCACCGAGCTGGAG 20 bp, 55%GC, 53.8C
Dhn7-R1(RevCom: CTCCAGCTCGGTGCGTATAT
PCR product size: 738 bp.

Dhn7-F2: ATCAAGGAGAAGCTGCCCGGAC 22 bp, 59%GC, 58.6C
Dhn7-R2: CTTCACACCCTTACCCGGAGCA 22 bp, 59%GC, 58.6C
Dhn7-R2(RevCom: TGCTCCGGGTAAGGGTGTGAAG
PCR product size: 950 bp.

>EMBOSS_001

ATGGAGTACCAGGGCCAGCAGCAGCACGGCCAGGCCACCAACAGGGTGGACGAGTACGGC
AACCCCGTGGCCGGCCACGGCGGCGGCACCGGCATGGGCGCCCACGGCGGCGTGGGCACC
GGCGCCGCCGCCGGCGGCCACTTCCAGCCCACCAGGAAGGAGCACAAGGCCGGCGGCATC
CTGCAGAGGAGCGGCAGCAGCAGCAGCAGCAGC

>EMBOSS_001

ATGGGCGGCAGGAGGAAGAAGGGCATCAAGGACAAGATCAAGGAGAAGCTGCCCGGCGGC
CACGGCGACCAGCAGCACGCCGCCGGCACCTACGGCCAGCAGGGCATCGGCATGGCCGGC
ACCGGCGGCACCTACGGCCAGCAGGGCCACACCGGCATGACCGGCACCGGCGCCACCGGC
ACCATGGCCACCGGCGGCACCTACGGCCAGCCCGGCCACACCGGCATGACCGGCACCGGC
ACCCACGGCACCGACGGCACCGGCGAGAAGAAGGGCATCATGGACAAGATCAAGGAGAAG
CTGCCCGGCCAGCAC

Genomic sequence of conting in barley Morex

B2.2 >morex_contig_135644 CAJW010135644 carma=6HL POPSEQ: chr=6H cM=94.9008498583569

GCCTCTTGGGCCTTCAATACAGATAACTTGCTTTCCTAACATTAGCCACTCGAGCTAGCGAGCTTTCATG
CTTATAGAATAGCATAAAACCTTAAGACACATCAAGGCTGATTCGAATGTTTTCAAAAAATCCGACCATG
AAACTTATTTTTCAAAAACAAAAAGTGAATGTCTTATCAAACGGGAAACTCTAACATTTTTTAAACATGC
AAATGAATTTTGCAGAAGCGATTTTTTTTAAACTATCCAAAAACTGGGCAGGAACAATTTTTCAAAAACA
GAAACATTTTTAAAATTCCAGAACAAAATTTGGAAACACGAACAACTTTTGCATGCGAACATTTTTTAAA
CTCCAGAACGAAATTTTAAAACACGGGCATTTTTTAAAATTTGATAACTATTTTTGAAACAGAACATTTT
CTCAAAACTCCCGAACGAAACAAGAAAACATGAATTTTTTTGAAAACTTGTGAACAGATTTCAGAAATGG
GAACATTTTTAAACTACCCAAAAAACTGAACATGAACATTTTTTTTAAAACAGGAACATTTTTCAAATTC
CAAAACAAAATTTGGAAACACGAACAACTTTTGCATGCAAACAATTTTGAAACTCCAGAAACAAATTTGA
AAACACGAACAATTTTTAAAATTTGCAAACAATTTGTAAAACGGAACATTTTTTCAAAACTCCCGAACAA
AACATGAAGACATGATCTTTTTGAAAATTTGCGAACCAACTTTGAAATGCGACTATATTTTAAACTACCC
AAAAAAGTGAACAAGACCAATTTTTCAAAAACATGAACATTTTTGAAATTCCAAAAGAAAATTTGGAAAC
ACATACAACTTTTGCGTGGGAACATTTTTTAAACTCCAGAACGAAATATGAAAACAAGAACATTTTTCTA
AATTTGCAACCAGTTTTTGAAAGGGAATAATTTTTTAAATATCCCAAAAAAAAATTATAAAACCCAGCAC
ATTTTTTTTAAAAGACAAAAACATTTTTCTAATTTGAAGAACATTTTTTGAAAATGCTGAACATTATTTG
AAAATACAGATTTTTTTTGGAAAATCCTAAACAGTTTTTGAAAATCCAGAACAGTTTTCGAAAAAGTCAA
AACATTTTTTGAATTGGAGAAACATTTTTAAAACAGAAACATAAAAGAAGAAAAAATAAAAAAGAAACAA
AAAATGAAATAAAAACCAGTTTAGGAGACCTTCTATAAGTTTCCCAAAACCGGAAAAAAACGGTTGGGAC
CTCTAGAAGGTTTTCAAAACGAGGGCGCTGGAGCAGTTAAACAGGCCGGCCCGATGGGTCGCTCCTCGGT
CGCTCGCCTGTGTGAAGCGTCGACAGCTTGACGCAACGAGGCGCAAATAGTATATTCTCAACAACTAACA
CGGTGACGGGAGCTCCCAATTGATGTTGCAGGCGCCAATTTCCCTAGCTGGCGCCCACCCACCGTCGTCT
CGTGAGCCGGCCCATATAGAAGCGCTTTGCTCGGTCGCTGGTGGGTTCGCTCCATCGATGGCTAGTGGGT
GCCCTCCGGTTCGATCGCTGCTGGGTTCGATCGAGTCAAACGATGATTAAATGTGATTTTTTTAAATGCA
AAAAACAGAAATGTTTGTGAAATAATTTATTTTACGAATTTTGAAGAAAAGTTCATGAATTTATAAAAAA
GTGCACCAAAATTTTAAAAAAGTTCATGCAATTTTGAAAAAAGTTCACCGAATTTCATTGAATTTGAAAT
AATTTCATCAGATCTGAAAAAAGTTCATCGAATATGAAAAAAAGTTCAGCAAACTTGAAAAAAGTTCATT
GGATTTGAAAAAGCTTCATCGATTTT TGAGATCCGCATCAACCTAAGG CGAGAAGATATTTATTATATTT
TCACTAGGGCGAAAAGATATTTATCATATTTTCACGTAGGGCAAGAAGATACTTATTATATTTGGCCTCT
GCATCAACCTGACTCAACAACTAC TCAAGATATCAAAAATACACGCAACCACATTGTTAGAAAAGAAAGA
AAGAAAGGTGAACAACATAGTAGCCGCAGCAAACAATAACATCTACAACAACAACAACAACAAAGCAATA
ATGGTAGCAACCATATGTAA CAGTATATCGTGATGATGTCTTCG AAAAGGGAAATACAAATGAGAGTCGC
TGTCATTGGAATCAACCATCAAGGCTAGATCATGAATTTTCACTCCGAAGAATAGTTGAGTAAGCTCTAT
GCAATGTCTTCAACTAGGGAATGTGACATGAACGACCTCATTACCATGTTGAACCAATAAAGATCAGATC
CTTTAATTTTCATCCCAAGAACTCGAGCCCTTGCACTGAAAAGCACCACCTAGCCGACTTCGAACTTGTG
CTGCCACCACTTCTCGATC CATCTCATAACAGTCGCTCAGA CCGAATCGACCCATTTCCCATTTTTAGGG
AGCAGGG GTATGATCTTCAATCGATGATTATTT ATTTTATTTTTGCGGGGGAATCG ATGCATATTTTCGT
AGACGTGCAATGGCATCCTGGGCATAAAGATGAATTCCATGCCGATACTTTCTAGTTGCGATTTTCCTAA
GCAAACAGGACGACTTTTGGAAGCGGAAATAAGTACCCCCTGTGCTGTGTGTCATCACCTCTTCGAGAAC
AAGCTGAGACGTGGCAACCCGAACGCGCCCAGTAGCTGCACACGTCCGGCCTCTCTCTATGGGCTGGTCA
CCAGTCACCGGCAACCCACTCACGCGAGCACAC (mix GTAGCGCCTCCGACGATCGTATCCCGTCGATCGCCTA
TAAATTGTGCCTCCTGCTGCACCTGCTCACACA ACACAGCCACCAGTTGCGCA GCCAAGTGAGGAAGACA
ACCCACCAGATTTCTGAGAGAACCAAGAGCAGCACCTGTGCAAGATGGAGTACCAGGGACAGCAGCAGCA
CGGCCAGGCGACCAACCGCGTCGATGAGTACGGTAACCCGGTTGCTGGACACGGCGTAGGCACCGGCATG
GGCGAGCACGGCGGCGTCGGCACCGGGATGGGCGCGCACGGCGGCGTCGGCACCGGTGCGGCCGCTGGTG
GGCATTTCCAGCCGACGAGGAAGGAGCACAAGGCCGGCGGGATCCTGCAGCGCTCCGGCAGCTCAAGCTC
CAGCTCGGTGCGTGTATATATACTTTACTTGCTTCTTGCCAGCATTTGCAGAGTGGCATTTTCCCGCTTA
GACTTTGAATGAAAAAGCATCAACGCCTTGAATTTTTCTCGTTGGTGGTGATAGTCTGAGGACGATGGCA
TGGGCGGGAGGAGGAAGAAGGGTATCAAGGATAAGATCAAGGAGAAGCTCCCTGGTGGCCACGGTGACCA
GCAGCACGCCGCCGGCACCTACGGACAGCAGGGTATTGGCATGGCCGGCACCGGCGGCACCTATGGGCAG
CAGGGTCACACTGGGATGACCGGCACGGGGGCAACTGGCACCATGGCCACAGGCGGTACCTACGGTCAGC
CGGGACACACCGGTATGACTGGTACTGGGACGCATGGCACCGACGGCACCGGCGAAAAGAAGGGCATCAT
GGACAAAATCAAGGAGAAGCTGCCCGGACAGCACTGAGCCCGGCCCGCGGCCCCTACTTGTGAAAGTTAA
GAGGTGCCGGTCTACGTACCCACCTCCGCAGAAAAATAAGATGAAGATACAGTAAAACTTCCCGAAATGA
AGTGAGCTTTAGTTCACATGTGATATCTGAGTTCTCGGTTTTGTGGACTTGAATTTTCTTTTGGTTGAAC
TTCTTGTATGGACCCGGGACGTTTTGTGCTCTGAACTTTTATGTGTGAATTTTCTTTTGGTTGAACTGTG

TATCTGTTATGTATGTATATCATGATATAATTAATACTACAATATATTTTGCCCTTCTACTTGCGCTTTC
TGTGCATTGCATGATTTTAAGTTGGGTAGTTTTGTCCACGTGGCATATTGTACATTGCATTTGAATTAGT
CTTTGTGAGAGTTTAGAAGCATTCTATATAGTGTTTTGACGATTTTGAAGCATCCAAAAGTAGGGTCCAC
CAAACATGGTTTAGTACAAAGCTACAACTTTGTATTAAACCAACATCAATTAATACAGATCAAAACAGCC
GCAAAAAAGAAAAAAAATATATAGATCAAAGGGACTTACAAAGCTACTTCTCATAAATTTGGTTTTGATA
GTAAGTTAGATGATGAGCACACCACATCCAAAAAATTACTTTCCATAGATTTATACTAAAATAATCATCA
TATAATACAACATATAAATGCATATAAAATATTCAAGATTGATAATATAATGGTATGAAACAATTTAAAA
TTATAGATATGTTGTATTCAAGATTGATAATATAATGGTATGAAACAATTTAAAATTATAGATATGTTGA
AGACGTATAACTTCCCCGCGTCGGTTTGTTCGAATGAGAGAGTTTATTACTCCTCGAGTCACAAGATCTA
GGTGACCAAGGTTCCCACAGAAGCTTTCTTTATTGTGTGCTCCGGGTAAGGGTGTGAAGTGATCAAGGTG
GTCAAGTTCCTCCGGAAGCTTCGGTGTTTGTGGAGTGCTCCGGAGAAGTTCGTAAAGGTGTGCTGGCGCC
TTCAAGGCCAACCCCAACCCAAATGAACTAAGGCTCATCCGTTGGGGTGAATCGAAGGTGAGAATACGGT
GAGCCACTTGGTAGCGCTCCGGAGCTTTGGCTTCGGCACCGTTCCAACGGAGATTAACACTCTCACAAGT
GTGAACTTCGATATAACTCTGCGTCCCCGACTCACTTGTGGTTATGTCATTCCCACCCCTTTACTTCCCA
CAATTCATACTTGCTTATATTGATATATCTTATGCTAGTTAGAATTGCTTTGTTGCTCCTACATTTTCAT
ATCTTGTGATATAGTTGTGTGCTTGCTAGTTTCTTTAAGTGCTTATCTTATTTAACATAGGTTGTTGGTG
CATTTAGTTGAGCCTAGCATATTTAAAATTTATGTTTGAAAAGTTTATGTTTAGTTTAATTTCACATTAG
TATAAACCAAATCCGTAAAATTTTCAAAACGCATATTCACTCTCCTATCATTTCACATGTCATCACCAGC
ATTTCGTACGCTCGGTGTTGCTGGTATCTTGTTATCAGCCGCATCACCATACCGCCTTCCCGCCTGGACA
TTGCTAGAAGGTGATTTAGAATGCTGCAATTAGGGGTAGTTCCTAATGCCTCCACAGTGTAGGAGGAGGC
TGGATTGACGGGAGCACAGATGTAATTAATAAAATTCATACTGATAATGTCACTCTAAGGTTGCGCTTAC
GACGGATTCGAATCTCCTCCTTGCATGATTGTTTTCCAGCCGTAATATTTACCCTCGTATGCCCGCTACA
CCCGTATTTAGCTTATAGAAAATACATCCCATCTGCGGCCAGTTTTCCCCACCTTCGTATCCTTTCTTGA
CCCATCCACTTGCCCGCACAGCTGCTTCCTTCCTCTTGGAGCACGGCCTGCAGGAGCAGTGCTAGCCGCG
GGTGCACGTGGTGTGCGCCACCGCCGCAGGGGTTCACTGGCACCGCCCTGCTGGGGAACGTAGTAATTTA
AAATTTTCCTACGCCATACAAGGACCTATCTATGGAGAGACCAGCAACGAGCATGGAAAAGAGCATCTTC
ATATCTTTGAAGATCGCTGAGCGGAAGCGTTACTATGAACGCGGTTGAGGGAGTTGTACTCGTAGCGATT
CAGATCGCCGTGGA

Dhn7-F4: TGAGATCCGCATCAACCTAAGG 22 bp, 50%GC, 54.8C
Dhn7-R4: TGCGCAACTGGTGGCTGTGT 20 bp, 60%GC, 55.9C
Dhn7-R4 (RevCom): ACACAGCCACCAGTTGCGCA
PCR product size: 1,007 bp

Dhn7-F5: GCATCAACCTGACTCAACAACTAC 24 bp, 46%GC, 55.7C
Dhn7-R4: TGCGCAACTGGTGGCTGTGT 20 bp, 60%GC, 55.9C
Dhn7-R4 (RevCom): ACACAGCCACCAGTTGCGCA
PCR product size: 893 bp

Dhn7-F6: CAGTATATCGTGATGATGTCTTCG 24 bp, 42%GC, 54C
Dhn7-R4: TGCGCAACTGGTGGCTGTGT 20 bp, 60%GC, 55.9C
Dhn7-R4 (RevCom): ACACAGCCACCAGTTGCGCA
PCR product size: 733 bp

***Dhn7*-F7: CGATTCCCCCGCAAAAAAAAAAT** 23 bp, 39%, 51.7C
Dhn7-F7 (Rev Com): ATTTTTTTTTTGCGGGGGAATCG

***Dhn7*-F8: CGATTCCCCCGCAAAAAAAAAAA** 23 bp, 39%, 51.7C
Dhn7-F7 (Rev Com): TTTTTTTTTTGCGGGGGAATCG

***Dhn7*-SNP-R: CATCTCATAACAGTCGCTCAGA**

Size of PCR product: 106 bp

Ordered primers:

***Dhn7*-SNP-F7:** GAAGGTGACCAAGTTCATGCT**CGATTCCCCCGCAAAAAAAAAAT**

***Dhn7*-SNP-F8:** GAAGGTCGGAGTCAACGGATT**CGATTCCCCCGCAAAAAAAAAAA**
***Dhn7*-SNP-R: CATCTCATAACAGTCGCTCAGA**

>morex_contig_135644 CAJW010135644 carma=6HL POPSEQ: chr=6H cM=94.9008498583569

Fragment F6-R4

CAGTATATCGTGATGATGTCTTCGAAAAGGGAAATACAAATGAGAGTCGCTGTCATTG
GAATCAACCATCAAGGCTAGATCATGAATTTTCACTCCGAAGAATAGTTGAGTAAGCTCT
ATGCAATGTCTTCAACTAGGGAATGTGACATGAACGACCTCATTACCATGTTGAACCAAT
AAAGATCAGATCCTTTAATTTTCATCCCAAGAACTCGAGCCCTTGCACTGAAAAGCACCA
CCTAGCCGACTTCGAACTTGTGCTGCCACCACTTCTCGATCCATCTCATAACAGTCGCTC
AGACCGAATCGACCCATTTCCCATTTTTAGGGAGCAGGGGTATGATCTTCAATCGATGAT
TATTTATTTTATTTTTGCGGGGGAATCGATGCATATTTTCGTAGACGTGCAATGGCATCCT
GGGCATAAAGATGAATTCCATGCCGATACTTTCTAGTTGCGATTTTCCTAAGCAAACAGG
ACGACTTTTGGAAGCGGAAATAAGTACCCCCTGTGCTGTGTGTCATCACCTCTTCGAGAA
CAAGCTGAGACGTGGCAACCCGAACGCGCCCAGTAGCTGCACACGTCCGGCCTCTCTCT
ATGGGCTGGTCACCAGTCACCGGCAACCCACTCACGCGAGCACACGTAGCGCCTCCGAC
GATCGTATCCCGTCGATCGCCTATAAATTGTGCCTCCTGCTGCACCTGCTCACACA**ACAC
AGCCACCAGTTGCGCA**

C3. Nano-Drop measurement of purified PCR products

C3.1. Nano-Drop measurement of purified PCR products *Dhn9*-F-R primers:

1- Sample (purification *Dhn9*-A) = 6.8 ng/ul
2- Sample (purification *Dhn9*-N) = 7.0 ng/ul

Dhn9-F-R primers for sequencing of AUK and NAT

Code	Name	Purification PCR protocol	Add primers *Dhn9*-F-R= 100 mM stock	Add water	Total volume
Yuri 148	*Dhn9*-AUK-F	12.0	0.15	0	12.15
Yuri 149	*Dhn9*-AUK-R	12.0	0.15	0	12.15
Yuri 150	*Dhn9*-NAT-F	12.0	0.15	0	12.15
Yuri 151	*Dhn9*-NAT-R	12.0	0.15	0	12.15

C3.2. Nano-Drop measurement of purified PCR products *Dhn9*-F1-R1primers:

1- Sample (purification *Dhn9*-A) = 21.9 ng/ul
2- Sample (purification *Dhn9*-N) = 21.3 ng/ul

Dhn9-F1 primer for sequence of AUK and NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn9*-F1= 5 mM (ul)	Add water	Total volume
Yuri 165	*Dhn9*-AUK-F1	4.0	3.0	5.0	12.0
Yuri 166	*Dhn9*-NAT-F1	4.0	3.0	5.0	12.0

C3.3. Nano-Drop measurement of purified PCR products *Dhn9*- F2-R2primers:

1- Sample (purification *Dhn9*-A) = 8.0 ng/ul
2- Sample (purification *Dhn9*-N) = 4.7 ng/ul

Dhn9-F2 primer for sequence of AUK and NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn9*-F2= 100 mM stock(ul)	Add water	Total volume
Yuri 167	*Dhn9*-AUK-F2	10.0	0.15	2.0	12.15
Yuri 168	*Dhn9*-NAT-F2	12.0	0.15	0	12.15

C3.4. Nano-Drop measurement of purified PCR products *Dhn9*- F2-R2primers:

1- Sample (purification *Dhn9*-N) = 4.7 ng/ul

Dhn9-R2 primer for sequence of NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn9*-R2= 100 mM stock(ul)	Add water	Total volume
Yuri 169	*Dhn9*-NAT-R2	12.0	0.15	0	12.15

C3.5. Nano-Drop measurement of purified PCR products *Dhn7*-F-Rprimers

1- Sample (purification *Dhn7*-A) = 7.4 ng/ul
2- Sample (purification *Dhn7*-N) = 9.9 ng/ul

Dhn7-F primer for sequence of AUK and NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn7*-F= 100 mM stock(ul)	Add water	Total volume
Yuri 170	*Dhn7*-NAT-F	8.0	0.15	4.0	12.15
Yuri 171	*Dhn7*-AUK-F	11.0	0.15	1	12.15

C3.6. Nano-Drop measurement of purified PCR products *Dhn7*-F2-R2 primers

1- Sample (purification *Dhn7*-A) = 1.7 ng/ul
2- Sample (purification *Dhn7*-N) = 4.7 ng/ul

Dhn7-F2 primer for sequence of AUK and NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn7*-F2= 100 mM stock(ul)	Add water	Total volume
Yuri 172	*Dhn7*-AUK-F2	12.0	0.15	0	12.15
Yuri 173	*Dhn7*-NAT-F2	12.0	0.15	0	12.15

C3.7. Nano-Drop measurement of purified PCR products *Dhn7*-F2-R2 primers

1- Sample (purification *Dhn7*-A) = 1.7 ng/ul
2- Sample (purification *Dhn7*-N) = 4.7 ng/ul

Dhn7-R2 primer for sequence of AUK and NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn7*-R2= 100 mM stock(ul)	Add water	Total volume
Yuri 174	*Dhn7*-AUK-R2	12.0	0.15	0	12.15
Yuri 175	*Dhn7*-NAT-R2	12.0	0.15	0	12.15

C3.8. Nano-Drop measurement of purified PCR products *Dhn7*-F1-R primers

1- Sample (purification *Dhn7*-N) = 8.4 ng/ul

Dhn7-F1 primer for sequence of NAT

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn7*-F1= 100 mM stock(ul)	Add water	Total volume
Yuri 176	*Dhn7*-NAT-F1	12.0	0.15	0	12.15

C3.9. Nano-Drop measurement of purified PCR products *Dhn7*-F5-R4 primers

1- Sample (purification *Dhn7*-A1, Yukub) = 4.1 ng/ul
2- Sample (purification *Dhn7*-D1, Margret) = 4.9 ng/ul
3- Sample (purification *Dhn7*-H3, cmbh) = 25.5 ng/ul
4- Sample (purification *Dhn7*-A4, cmb89A) = 23.2 ng/ul

Dhn7-F5 primer for sequence of DNA

Code	Name	Purification PCR protocol (ul)	Add primers *Dhn7*-F5= 100 mM stock(ul)	Add water	Total volume
Yuri 209	*Dhn7*-A1, yukub –F5	12.0	0.15	0	12.15
Yuri 210	*Dhn7*-D1, margret –F5	12.0	0.15	0	12.15
Yuri 211	*Dhn7*-H3, cmbh –F5	3.0	0.15	8.85	12.00
Yuri 212	*Dhn7*-A4, cmb89A –F5	3.0	0.15	8.85	12.00