Evaluation of Drought Resistance Accession of Wheat (*Triticum aestivum*) Using ISSR Markers

By

Wondi Haso Kabato

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfilment of the Requirement for the Degree of Master's of Science in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

December 2018

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APPROVAL BOARDS OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defence by Wondi Haso Kabato have read and evaluated his thesis entitled "Evaluation of drought resistance accession of wheat (*Triticum aestivum*) Using ISSR Markers" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfilment of the requirement of the Degree of MSc in Biotechnology.

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DECLARATION

I hereby declare that the work which is being presented in this research entitled as **Evaluation of drought resistance accession of wheat (*Triticum aestivum*) Using ISSR Markers** in partial fulfillment of the requirement for the award of the degree of MSc in Biotechnology. It was authentic record of my original work carried out from October 2017 to November 2018 under the advisors of Dr. Mulugeta Kebede, Department of Applied Biology, School of Applied Natural Science, Adama Science Technology University and Mrs. Belainesh Hailu, Dr. Dejene Girma Department of Plant Biotechnology, Wheat Researcher Group, National Agricultural Biotechnology Research Center, Holeta. The matter embodied in this has not been submitted by other person or me for the award of any other degree. All relevant resources of information used in this body have been duly acknowledged.

Wondi Haso Kabato _____ _____

Signature Date

This MSc thesis has been submitted for examination with my approval as thesis advisor

Mulugeta Kebede (PhD) _____ _____

Signature Date

Date of submission; December 2018

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To: Applied Biology Department

Subject: Thesis Submission

This is to certify that the thesis entitled "**Evaluation of drought resistance accession of wheat (*Triticum aestivum*) Using ISSR Markers**" submitted in partial fulfillment of the requirements for the degree of Master's in Applied Biology (Biotechnology), the Graduate program of the department of Applied Biology, and has been carried out by **Wondi Haso Kabato**, with ID. No. GSR/0198/09, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

_____	_____	_____
Name of Major Advisor	Signature	Date

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LIST OF ACRONYMS AND ABBREVIATIONS

ABA	Abscisic Acid
ABREs	Abscisic Acid Responsive Elements
Bp	Base Pair
DArT	Diversity Arrays Technology
DNA	Deoxy Ribose Nucleic Acid
DREs	Drought Responsive Element
EDTA	Ethylene Diamine Tetra Acetic acid
ISSR	Inter Simple Sequence Repeat
Kb	Kilobases
kDa	Kilo Dalton
LEA	Late Embryogenesis Abundant
MW	Molecular weight
NCBI	National Center of Biotechnology Information
NVT	National Varieties
OA	Osmotic Adjustment
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait loci
RAPD	Randomly Amplified Polymorphic DNA
ROS	Reactive Oxygen Species
TBE	Tris Boric acid Ethylene diamine tetra acetic acid

ABSTRACT

Triticum aestivum is the world's leading cereal grain where more than one-third of the population of the world uses as a staple food and it is the third most widely produced cereal crop in the world after rice and maize. However, It is very difficult to find areas "without stress," where Triticum aestivum can reach their maximum yield potential due to abiotic stresses such as drought. Drought is one of the most common external stresses affecting plant growth and development through changes in metabolism and gene expression. Thus it is important to use genetic markers to evaluate drought resistance accessions of wheat. Thus the main objective of this study was to evaluate drought resistance accession of hexaploid wheat (Triticum aestivum) using ISSR markers. Genomic DNA was extracted from 55 selected T. aestivum varieties using DArT plant DNA extraction protocol. The quality and quantity extracted genomic DNAs were measured using spectrophotometry and approximately 1.8 genomic DNA ratio of 260nm/280 absorbance was taken and diluted to 50ng/µl for PCR. PCR reactions for fifty five samples were performed using ten ISSR primers. A total of 73 amplified DNA fragments (locus) ranging from 200-2961bp were produced from 10 ISSR markers. 39 fragments were polymorphic (53.4%) and the rest 34 fragments were monomorphic (46.6%) with the average of 3.9 to 3.4 polymorphisms and monomorphism per primer, respectively. Among ten ISSR primers used for this study, primer 844A, 844B, 17898A, 17898B, 17899A and HB11 have produced specific fragments on expected ranges of primer molecular weight that used to distinguish drought resistance LEA gene in each samples except, primer HB12, HB13 and HB15 those were produced undifferentiated faint fragments. Most of accessions produced specific fragment in each primer, where as some accessions was produced faint bands or no any band formation. This indicated that, most of selected Triticum aestivum accessions were drought resistance. Since, the evaluation of drought resistance accessions has great values for wheat crop improvement through genetic engineering and modern breeding programs, further investigation should be give attention to fragment sequencing, LEA gene isolation and grouping based on amino acid sequences.

Keywords; Drought Resistance, Fragments, ISSR marker, *Triticum aestivum*, PCR

1. INTRODUCTION

1.1 Background and Justifications

World food production is primarily restricted by the impact of the environment. It is very difficult to find areas "without stress," where crops can reach their maximum yield potential (Jalal, 2014). Abiotic stresses such as drought, high salinity and freezing temperatures affect most areas of the world and they impact in plants by directly reducing its survival in the natural environment and its productivity in agriculture. About half of the annual world crop production is lost due to abiotic stresses (Mahpara *et al.*, 2014). Abiotic environmental factors are considered the main source (71 %) of yield reduction. Different in quantity and intensity, abiotic factors affect the plants in various ways, but certain quantity of each abiotic environmental factor is necessary for the optimal plant development. Higher plants dominate terrestrial ecosystems and play most important economic and social roles in human life, which contribute to their strong adaptive ability owing to a long period of evolution under the pressure of natural and human selection. However, any excess or deficit of the abiotic stress factors such as drought, adversely affects the plant growth and productivity (Koyro *et al.*, 2012).

Agricultural sector uses 75% of global water to solve food security problem. Amazing, in a world which access to abundant, clean, freshwater is becoming more difficult, therefore the amount of agricultural water use threatens future global water security. So our ability to produce staple crops such as wheat, which comprise the majority of the agricultural sector and constitute a large parts of people's diets, will become a growing concern as water supplies dwindle (FAO, 2017). Drought is one of the most common external stresses affecting plant growth and development through changes in metabolism and gene expression. Global climate change leads to a higher probability of occurrence of dry years in the regions where drought was a rare event, and also to appearance of new areas with arid conditions that require urgent development of new crop varieties resistant and/or tolerant to drought conditions (Jalal *et al.*, 2014). Drought effects on crop production have been investigated for decades. However, studying the drought response is still a challenge because of the complex and quantitative nature of the trait (Budak *et al*., 2013).

Drought tolerance depends on the environmental interactions. Duration and severity of the dehydration stress and its dependence on other abiotic and biotic stress factors are also significant. One of the most effective methods for increasing the crop productivity is to increase the adaptation to growth conditions (Rashed *et al*., 2010). It's continues to be an important challenge/ threat to global agricultural researchers and plant breeders. It is assumed that by the year 2025, around 1.8 billion people will face absolute water shortage and 65% of the world's population will live under water-stressed environments (Gorji *et al*., 2010). Tolerance to water stress is a complicated parameter in which crops' performance can be influenced by several characteristics (Farooq *et al.,* 2011). Tolerance can be divided into two parts including drought avoidance and dehydration tolerance. Drought avoidance includes root depth, reasonable use of available water by plants, and changes in plants' lifestyle to use rainfall. Dehydration tolerance consists of plants' capability to partially dehydrate and grow again when rainfall continues. Therefore, the adaption of plants to drought stress is a vital issue to develop new improved methods for increasing stress tolerant plants (Arash *et al.,* 2013).

Drought stress induces a range of physiological, cellular and molecular responses in plants towards stress tolerance. Among the diversity of responses, plants can adapt to water deficit by the induction of specific gene including the changing of gene expression related to drought tolerance (Savitr *et al.,* 2013). One of the genes related to drought tolerance is late embryogenesis abundant (*LEA*) gene encoding family dehydrin protein. LEA proteins are a family of hydrophilic proteins that are presumed to play a protective role during exposure to different abiotic stresses. As the name suggests, Late Embryogenesis Abundant proteins were originally discovered in the late stages of embryo development in crop seeds like Cottons (Tang *et al.,* 2016). LEA proteins those encodes for LEA genes are mainly low molecular weight (10–30 kDa) proteins in many crops, which are involved in protecting higher plants from damage caused by environmental stresses, especially drought (Marina, 2013).

Dehydrin are part of these LEA proteins (group II) and are built up by many charged and polar amino acids without cysteine and tryptophan ever occurring. LEA is a functional protein which plays a role in stabilization of membrane structures and protected macromolecules. It is accumulated in the cytoplasm, nucleus, plasma membrane and mitochondria, and no tissue-specific LEA gene expression has been considered as one main regulatory mechanism on the basis of extensive studies with the different model plant (Cesar, 2017).

The evolution of LEA gene is one of these changes, which plays an important role in resistance to drought. This implies that studies on tolerant proteins, and the isolation, identification and functional analysis of their genes will be of great benefit to genetic engineering and breeding of drought-resistant crops. Drought stress not only affects plant growth and development but ultimately productivity in almost all the cereals, thus it is one of the most serious threats to world agriculture. The situation demands crop breeding for drought stressed areas utilizing using traditional along with modern molecular techniques. The genes for drought tolerance are regulated at once under drought conditions and produced the respected products that response to signal transduction, stress response and help the plant to withstand under drought stress (Mahpara *et al.*, 2014).

Plants are exposed to multiple environmental stresses along their life cycle, during such time span, higher plants have developed multi-pathway, multi-level and multiscale survival strategies for continual changes in the environment, which include anatomical, physiological, biophysical, biochemical, genetic, developmental and reproductive biological changes in response to adverse conditions (Shao *et al;* 2005). Most plants encounter transient decreases in relative water content at some stages of their life, and many produce a highly desiccation tolerant structure, such as seeds, spores or pollen. Although in a few vascular plants i.e. resurrection plants, desiccation tolerance also occurs in the vegetative tissues, in general plant vegetative tissues, leaves and roots, are very sensitive to water deficit and they can only withstand reduced and transient water losses (Imen *et al.*, 2014).

At the global level, agricultural production and consumption will need to be raised by 70 %, and food production in the developing world will need to double, which also makes it necessary to increase the wheat production all over the world. Wheat is a very valuable food crop, which occupies a leading place in grain balance for most of the world population. It is strategically important foodstuff for about 2 billion people (about 36 % of the world population). However, many hectares of wheat grain was lost due to abiotic stress especially drought that lends to hunger and food security in many parts of the world including our countries. Food security is invariably interconnected with water security because water is needed to produce the food that feeds the billions of the people on our planet (FAO, 2013)

Triticum aestivum (hexaploid bread wheat) is one of the most important and significant staple cereal food crops in the world, both in terms of food production and for providing the total amount of food calories and protein in the mankind diet. It contributes 30% of the world's edible dry matter and 60% of the daily calorie intake in several developing countries. Wheat is produced for a wide range of end-users and a critical staple food for a large proportion of the world's consumers. It's the world's leading cereal grain where more than one-third of the population of the world uses as a staple food and it is the third most widely produced cereal crop in the world after rice and maize.Currently wheat is supplying more than 20% of the world's caloric needs to 4.3 billion people annually while being produced on more than 220.4 million hectares worldwide (FAO, 2017

Wheat production is adversely affected by drought in 50 % of the area under cultivation in the developing countries. Genetic gain for yield under these environments is estimated to be half of the yield compared to well-watered environments. It is difficult to make progress for grain yield and yield components under drought as they are complex characters influenced by many environmental factors and are characterized by low heritability and large genotype environment interactions under drought conditions. Breeding for drought tolerance of wheat cultivars is a major objective of wheat breeding program in arid and semi-arid regions of the world due to inadequate precipitation, shortage of water for irrigation and high water demand due to crop Evapotranspiration in such climates. Development of drought resistant cultivars will considerably improve the rain failed wheat production (Trethowan and Pfeiffer 2000).

Systematic evaluation of molecular diversity with respect to drought tolerance swathed in wheat genetic resource is essential in optimizing both conservation and utilization strategies. Morphological characteristics are used to ascertain proper selection of stress tolerant crops. While the sensitivity of morphological observations to environmental and developmental variations limits their applicability, molecular markers can be efficiently utilized to screen and identify proper tolerant plants even at an early stage. Since, drought tolerance is contributed by several regions of the genome; it requires identification which can be accomplished by the use of DNA markers as they offer a faster way to identify drought tolerance related regions (Deshmukh *et al.,* 2012). One of the latest genetics and biotechnologies achievements are ISSR molecular markers. Inter-simple sequence repeats (ISSRs) are a new type of DNA markers which involve the use of microsatellite sequences directly in the polymerase chain reaction (PCR) for DNA amplification. ISSRs have been used successfully in genome mapping of a variety of crop species including maize, rice, barley and wheat. ISSR markers are useful in gene tagging and can be used for finding markers linked to the gene of interest. The linked ISSR molecular markers can now be used in marker-assisted selection (MAS) programs. Moreover these markers have potential importance in facilitating selection procedure, particularly for pyramiding two or more different genes aiming at a more durable and broad-spectrum resistance (Ben *et al.,* 2004).

Research on the biology and genetics of drought resistance and breeding of drought-resistant crops have, thus, become important field of contemporary research in plant molecular biology and molecular breeding. It is common knowledge that drought related food shortages is a worldwide problem, especially in sub-Saharan Africa including Ethiopia (Jalal, 2014). However, transgenic plant carrying genes for drought tolerance has been developed by the introduction of LEA gene, proline synthesis and betaine (Savitr *et al.,* 2013). In this regard, studies on the development of adaptive breeding techniques and screening of drought resistance *T. aestivum* cultivars based on the latest achievements of genetics and biotechnology are of particular relevance and allow expanding the range of variability of the existing genotypes and creating forms with new features and properties in a short period of time.

Wheat production zones in Ethiopia lie between 6°and 16°N, and 35° and 42°E, at altitudes ranging from 1500 to 3000 m.a.s.l. The national average yield of wheat is 2.11t/ha in Ethiopia, which is very low as compared to Africa and world yield productivity. The low productivity is because of both biotic (diseases, insect pest and weeds) and abiotic (drought, soil fertility, etc.) factors and due to lagging behind in adoption of new agricultural technologies (Endale and Getaneh, 2015). Ethiopia facing declining of soil fertility, frequent drought and unreliable rain fall, and increased pest infestations even though the population number will be rapidly increasing (Jalal, 2014). Therefore, development of the drought-resistant high-yielding varieties of wheat and other staple crop is very important to overcome the above problem.

1.3 Objective of the Study

1.3.1 General Objective

The main objective of this study was:-

Evaluation of drought resistance selected modern and advanced accession of wheat (*Triticum aestivum)* using ISSR markers

1.3.2 Specific Objectives

The specific objectives of this study were:-

- To select best primers used for evaluation of drought resistance accession of wheat (Triticum aestivum)
- To amplify the fragment possessing drought responsive ability in all selected modern and advanced accession of *Triticum aestivum using ten primers*
- To determine fragment length generated by each primer used for this study in all samples
- To determine the percentage of polymorphism and genetic diversity among selected modern and advanced lines of *Triticum aestivum*

2. LITERATURE REVIEW

2.1 Origin and Geographic Distribution of Wheat

The precise origin of the wheat plant as we know it today is not known. Wheat evolved from wild grasses, probably somewhere in the Near East. The debate over wheat's origin has never been fully resolved, and although the region of greatest development seems to lie in the eastern region (Mediterranean to Asia), it cannot be determined whether wheat stemmed from the Far or Near East. Two scientists in particular have aided to this debate, and through research, have helped to discover the origin of this grain. Nikolai Vavilov, a Russian botanist, studied the diversity of wild wheats in South East Asia. From his research on gene pool of wild wheats and barley, he concluded that this region is the ancestral homeland of wheat, for where diversity is greatest so must be the point of crop origin, fixing mutations and inbreeding. In contradiction, Robert Braidwood, an archaeologist from the University of Chicago, claimed the Near East of the Mediterranean must have been the birthplace of wheat. His studies focused mostly on the modern ecological range of wild wheats, reasoning that the first wheat farmers chose to "settle in" to these regions due to the great variety of wild wheat that allowed them to successfully adapt both culturally and ecologically (Valerie, 2008).

Wheat is one of the earliest domesticated crop species. It was domesticated at least as early as 7500 B.C. in the Near East, somewhere in the Fertile Crescent (which comprises the mountain chains flanking the plains of Mesopotamia and Syrian Desert including Iran, Jordan, Syria, Turkey, Israel and Palestine) and also in Anatolia and the Balkans. Similarly, Wheat spread from western Asia, the primary center of development, to Europe through the Caucasus and the Balkan Mountains and then to other parts of the world. Today, it is the most widespread crop in the world and the staple food of over a third of the world's population (Yifru, 2012).

2.2 Phylogeny and Taxonomy of Modern Wheat

Modern varieties are selections caused by natural mutation starting with emmer wheat up to husk less modern wheat. Cytological and cytogenetic evidences showed that wheat consists of diploid, tetraploid and hexaploid. All cultivated wheats belong to the genus *Triticum*, to the tribe *Triticeae* in the family *Poaceae (Gramineae*) and sub-family *Pooideae*. Chromosome numbers in the genus *Triticum* is a function of the basic number (x = 7) and the ploidy level.

The chromosomal basis of the three natural groups or species of wheat (*Triticum* spp.), which is an allopolyploid plant with chromosome numbers 2n=2x=14 diploid, 2n=4x=28 tetraploid and 2n=6x=42 hexaploid (Tariku, 2012).

Triticum aestivum which is the most common wheat was finally evolved through years of cultivation in the southern Caspian plains. This evolution was accelerated by an expanding geographical range of cultivation and by human selection, and had produced bread wheat as early as the sixth millennium BC. These varieties are the most drought tolerant species due to the presence of late embryogenesis abundant gene with glycine max (Ewan, 2007).

2.3 Effects of Drought Stress on Plants

The ultimate detrimental effect of drought stress is reduction in yield, as reported in crops such as rice (Brevedan and Egli, 2003), wheat (*Triticum aestivum*) (Cabuslay *et al.*, 2002), soybean (*Glycine max*) (Kirigwi *et al.*, 2004), and chickpea (*Cicer aerietum*) (Khanna-Chopra and Khanna-Chopra, 2004). Various United States Department of Agriculture (USDA) reports have identified drought as the most frequent yield-reducing factor in arid and semiarid regions, although water deficit may occur even in high rainfall areas (Vamerali *et al.*, 2003). Indian production of cereals and pulses dropped by about 30% in 1971 due to drought (Swindale and Bidinger, 1981)

In the Sahel in Mauritania and Ethiopia, cereal production decreased by the same magnitude during the same period. The USDA reported that droughts in 1980, 1983, and 1988 significantly reduced US maize and soybean yields (Taiz and Zieger, 1998). A heat and drought wave of 2003 caused significant reductions in gross primary productivity and reduction in maize yield in both Eastern and Western Europe (Ciais *et al.*, 2005). This information presents drought as a potential cause of disaster, especially since it affects almost all areas of the world; and crops, such as rice, that feed much of the world's population are easily affected by drought.

Water stress due to drought can lead to major physiological and biochemical changes, such as reduced photosynthesis (Lawlor and Cornic, 2002; Tezara *et al.*, 1999) and reprogramming of gene expression (Romo *et al.*, 2001). Physiological changes under drought stress are often reflected at the transcription level, where the levels of mRNA related to key processes such as photosynthesis are down-regulated (Bartels and Salamani, 2003). When plants experience

water deficits, stomatal pores progressively close (Lawlor and Cornic, 2002).This process is regulated largely by leaf water potential but can be mediated by abscisic acid (ABA).

Stomatal closure leads to decreases in photosynthetic CO2 assimilation due to restricted diffusion of CO2 into the leaf and altered CO2 metabolism. Pelleschi *et al.*, (1997) found that reduced CO2 diffusion during stomatal closure is mainly responsible for the decline in photosynthesis in C3 plants subjected to dehydration. However, Tezara *et al.*, (1999) reported that, in sunflower (*Helianthus annuus*) (a C3 plant) under water stress, the photosynthetic rate is limited more by altered CO2 metabolism than by reduced diffusion. The lower CO2 availability inhibits carbon assimilation, and ultimately photosynthetic capacity is lost as a consequence of the reduced stomatal conductance and/or direct damage to carbon metabolism (Colom and Vazzana, 2003).

Closure of stomata, as a result of water deficit and consequent decrease in CO2 concentration in the leaf mesophyll, results in the accumulation of reducing power (NADPH) in the chloroplasts. This is because electrons produced by photolysis of water are in excess compared to NADP. Under such conditions, where NADP is limiting, O2 acts as an alternative electron acceptor, resulting in the formation of superoxide radical (O2-). The superoxide radical, through a series of univalent reduction reactions, produces hydrogen peroxide (H2O2) and hydroxyl radical (OH.). In addition to these species, superoxide in a reaction with 2H+ can also generate single oxygen (1O2). These molecules (O2-, H2O2, OH, and 1O2), are called reactive oxygen species (ROS). They are highly toxic and can damage important cellular biomolecules such as lipids, proteins, nucleic acids, and chlorophyll (Cruz de Carvalho, 2008). According to Zhang and Kirkham (1996), the harmful effects of the ROS are due primarily to their ability to initiate auto-oxidative chain reactions on unsaturated fatty acids leading to lipid peroxidation and membrane destruction. Tissue damage due to ROS under drought has been reported in pea (*Pisum sativum*) (Moran *et al.*, 1994) and *Phillyrea angustifolia* (Munne-Bosch and Penuelas, 2003).

On the other hand, as ROS levels increase under drought stress, they might function as an "alarm" signal that triggers defense responses. A specific transduction pathway that involves H2O2 as a secondary messenger has been reported by Cruz de Carvalho (2008). As stated by Dat *et al.*, (2000), ROS seem to have a dual effect under abiotic stress conditions that depend on their overall cellular amount. If present at relatively low levels, they are likely to function as components of a stress signaling pathway, triggering stress defense/acclimation responses. However, when they reach a certain level, ROS can become phytotoxic, damaging cellular components and eventually causing tissue death. Thus, ROS can be viewed both as toxins indicating cellular stress and as secondary messengers involved in stress-signal transduction.

2.4 Mechanisms to Cope With Drought in Plants

Mechanisms that plants use to cope with drought can, more or less intuitively, be grouped into drought avoidance and drought tolerance. A combination of these defines drought resistance in its physiological context according to Levitt (1972). Each of these general strategies is described below.

2.4.1 Drought Avoidance Mechanisms

Drought-avoiding plants have the ability to complete their life cycle without severe water deficits developing. Some ephemerals have a very short life cycle that can be completed during a brief rainy season. Other plants exhibit adaptations to increase water uptake and reduce water loss and thereby avoid the debilitating consequences of drought that other plants might feel (Bartels and Salamini, 2003).

Accordingly, developing a more extensive root system is a drought-avoiding strategy. It is an almost universal observation that the root: shoot ratio increases with water stress. Increases in root weight may be due to a greater density or depth of roots. Under water stress, new root growth extends into moist soil zones. As water deficits progress, the upper soil layers usually dry first. Thus shallower roots are common in wetter soils as opposed to deeper roots systems in dryer soils (Taiz and Zeiger, 1998). The possession of a deep and thick root system, which allows access to water deeper in the soil is considered important in determining drought resistance in rice (Price *et al.*, 2002), *Arabidopsis thaliana* (Xiong *et al*., 2006), pea (Chiantante *et al*., 1999), tall fescue (*Festuca arundinacea*) (Ervin and Koski, 1998), and

Wilwitchia mirabilis (Fitter and Hay, 2002). Therefore, greater root growth and improved morphological development can result in drought avoidance.

Development in shoots also plays an important role in water stress responses. At the onset of the dry season, a desert plant *Zygophyllum qatarense* responds to water stress with a leaf polymorphism in which it replaces the wet-season foliage with unifoliate, xeromorphic leaves. As the dry season progresses, the plant reduces its leaf area substantially (Sayed, 1996). Leaves of many plants frequently wilt and droop (or roll, in the case of many grasses) under water stress, and this response reduces the interception of radiation, thereby counteracting the increase in leaf temperature arising from stomatal closure and preventing further development of leaf water deficit (Turner, 1979). Reduced leaf area can be a mechanism for moderating water use and injury under drought stress. Severe drought stress may result in increased levels of ABA and subsequent leaf abscission, thereby reducing transpiration demand. Such developmental changes within a plant during water stress are important morphological drought-avoiding adaptations that help maintain the plant's water potential at some functional level in the midst of potential water limitation ((Blum, 2005).

2.4.2 Drought Tolerance Mechanisms

As drought stress becomes more severe, it becomes increasingly more difficult to avoid dehydration; and mechanisms that allow plants to withstand reduced water content (more negative water potentials) become increasingly important. Drought tolerance is defined as the ability of plants to continue to function at lowered tissue water potentials. Drought-tolerating mechanisms often involve the maintenance of turgor (by accumulation of solutes) and/or desiccation tolerance (by protoplasmic resistance) (Jones *et al.,* 1981). "Desiccation tolerant" plants can recover from a fully air-dried state (Vicre *et al.,* 2004). When dehydrated, these plants are in a metabolically dormant, or crypto biotic, state. Mesophytes plants, including all crop plants, lack the ability to enter the crypto biotic state. In fact, mesophytes typically cannot recover from severe (approximately 50% or greater) decreases in water content (Verslues *et al*., 2006). However, many plants have some ability to tolerate significant water loss, while maintaining metabolic activity.

Biochemical and physiological studies have shown that sugars (e/g., raffinose family oligosaccharides (RFO), sucrose, sorbitol, and mannitol), amino acids (e.g., proline), and amines (e.g., glycine betaine) accumulate under drought stress in different plant species (reviewed by Seki *et al*., 2007). As soil dries, its water potential becomes more negative. Accumulation of solutes (osmolytes) by plant tissues makes their water potentials more negative, allowing them to take up water and avoid reductions in turgor. This drought-tolerating mechanism is called osmotic adjustment (OA). By contributing towards OA, osmolytes act as protectants for plants subjected to low water potential (Pandey *et al.,* 2004). OA helps maintain cell turgor, which can allow cell enlargement and plant growth during water stress; and it can allow stomata to remain at least partially open and CO2 assimilation to continue at water potentials that would be otherwise inhibitory (Alves and Setter, 2004). OA by osmolytes has been reported to be a cause of improved productivity in wheat (Flower and Ludlow, 1987), sorghum (*Sorghum bicolor*), barley (*Hordeum vulgare*), chickpea (*Cicer arietnum*), pigeon pea (*Cajanus cajan*) (Khanna-Chopra and Khanna-Chopra, 2004), and rice (Lanceras *et al.,* 2004). In addition to acting as osmolytes to maintain turgor for a longer period of time, some of the accumulated solutes are thought to participate in stress-protective functions as free radical scavengers and stabilization of macromolecules during drought (Abebe *et al*., 2003).

2.4.3 Biochemical/ Metabolic/Genomic Responses Associated with Drought Resistance

In many plants that adapt to water stress, a set of genes are transcriptionally activated, leading to accumulation of new proteins in seeds and vegetative organs and greater tolerance to drought. Proteins termed LEA (Late Embryonic Abundant), which were first characterized in cotton (*Gossypium hirsutum*), are a set of proteins that accumulate in embryos late in seed development (Xu *et al.,* 1996) where they are associated with acquisition of desiccation tolerance in maturing seeds. These proteins are also found in vegetative tissues in response to exogenous ABA, as well as osmotic and dehydration stress (Liang *et al.,* 2006), at any stage of plant development (Baker *et al.,* 1988). At least six groups of LEA proteins have been categorized by virtue of the similarity in their deduced amino acid sequences; and group 2, also referred to as the dehydrin, contain proteins that are induced by dehydration-related stresses such as osmotic stress and drought (Wang, *et al*., 2006).

Their hydrophilic nature and high solubility indicate that the proteins are maintained in the cytosol, where they are assumed to function as chaperone-like protective molecules to combat cellular damage and to act as hydrophilins, retaining water (Reys *et al.*, 2008) during dehydration. An association between tolerance to drought stress and these groups of proteins has been observed in some crop plants. In blueberry (*Vacinium* spp.), the dehydrin were found to accumulate in response to changes in ABA levels during drought stress (Panta *et al.*, 2001). *LEA* genes, when over-expressed in rice (Xiao *et al*., 2007), tobacco (*Nicotiana tabacum*) (Wang *et al.*, 2006), and *Arabidopsis thaliana* (Figueras *et al*., 2004) led to drought tolerance in transgenic plants. Although the specific roles of the LEA proteins are not known, it is clear that they are regulated by ABA and cellular water loss.

Plant cells, including photosynthetic cells, are protected against the detrimental effects of reactive oxygen species (ROS) by an antioxidant system that has been associated with stress tolerance in plants. The system is composed of enzymatic and non-enzymatic detoxification mechanisms, which mitigate and repair damage initiated by the ROS (Fu and Huang, 2001). In wheat, proteomic studies identified differential expression of a *thioredoxins* (TRX) between drought-tolerant and -susceptible genotypes expression of the protein was higher in drought-tolerant than in -susceptible genotypes. Therefore, TRX can be seen to play a role in drought tolerance by taking part in antioxidant defense systems in plants (Hajheidari *et al*., 2007).

In summary, the ROS generated under water deficit can be counteracted in drought-tolerating plants by the activation of antioxidant systems to remove the ROS from the affected cells. Comparative and transgenic studies have identified association between drought tolerance and activities of the antioxidant systems in different plants. However, efficiency of the system might depend on the degree of water deficit, as high levels of stress could also cause damage to the antioxidant system itself. Another group of proteins that are induced by abiotic stress is the heat shock proteins (HSP). Five major families of HSP are recognized: The HSP70 (DnaK) family, the chaperonins (HSP60), the HSP90 family, the HSP100, and the small heat shock protein (sHSP) family (Wang *et al*., 2004). Of these families, the sHSP have been most associated with abiotic stress.

The involvement of HSP in drought has been studied through comparative and transgenic approaches**.** Qualitative differences in the pattern of synthesis of HSP were observed in maize lines differing in drought and heat tolerance. A unique HSP was observed in a drought- and heat-resistant line compared to a sensitive line. Differential HSP expression was also observed in potato genotypes differing in drought tolerance. While HSP70, HSP40, and sHSP whose products localize to the chloroplast were induced in a drought-tolerant genotype, HSP genes targeted to the cytosol were induced in the sensitive genotype. This differential expression in different genotypes and organelles implies roles for HSP in drought tolerance. Overexpression of sHSP enhanced drought tolerance in rice (Sato and Yokoya, 2008) and Arabidopsis (Sun *et al*., 2001).

2.4.4 Drought Avoidance and Tolerance Strategies May Be Integrated

Since drought-avoidance and -tolerance mechanisms were first proposed by Levitt (1972) and others, our understanding of molecular and cellular events during drought stress has increased. It has become clear that many of the molecular events initiated by drought do not fit exclusively into avoidance or tolerance categories. For example, accumulated solutes may play a role as osmolytes, allowing plants to take up more water and as such constitute drought avoidance. At the same time, solutes such as amino acids and sugars may in addition play a protective role against protein and membrane damage. Likewise, dehydrin can function as chaperone-like protective molecules (tolerance mechanism), while their hydrophilic activity helps retain water (an avoidance mechanism). The involvement of ABA in ABA-regulated stomatal conductance and root growth is important in avoidance; but, at the same time, ABA accumulation has been reported to cause synthesis of dehydrin, which are important for drought tolerance (Schroeder *et al*., 2001).

2.5 Recent Advances in Identifying Drought-Resistance Genes and Pathways

Many of the genes that are known to respond to drought stress have been identified, and the products of these genes can be classified into two groups. The first group includes proteins that probably directly protect against stress such as enzymes for osmolytes biosynthesis, LEA proteins, and detoxification enzymes. The second group consists of proteins involved in the regulation of gene expression and signal transduction of stress responses, such as transcription factors (TF), protein kinases, protein phosphatases, and enzymes involved in biosynthesis of signaling molecules.

The phytohormone ABA accumulates under water deficit. Increasing ABA concentrations lead to many changes in plant development, physiology, and growth. Some of these changes bring about avoidance and tolerance mechanisms (discussed above). At the molecular level, ABA induces expression of several genes mediated through ABA-responsive *cis*- and *Trans*-acting factors and protein kinases or phosphatases in a Ca2+-dependent or -independent signaling cascade (Bray, 2002).

A number of genes are known to be induced by drought, salinity, and cold in *aba* (ABA-deficient) and (ABA-insensitive) Arabidopsis mutants. This suggests the existence of alternative, ABA-independent regulatory systems of gene expression during stress responses. Indeed there is an ABA-independent pathway whose genes have one or several copies of the CRT/DRE *cis* elements in their promoters. A family of TF known as CBF or DREB1 binds to this element and activates transcription of the downstream dehydration-responsive genes. DREB1 TF is believed to interact with CRT/DRE and induce expression of stress-tolerance genes in response to cold, whereas DREB2 TF is involved in drought responses (Liu *et al.*, 2001).

Overexpression of the Arabidopsis DREB1A TF under the control of a stress-inducible promoter (RD29A) increased drought tolerance in wheat (Pellegrineschi *et al.*, 2004). In a study with DREB1-expressing tobacco, transgenic plants had improved drought- and low-temperature-stress tolerance (Kasuga *et al*., 2004). This information indicates that, even in ABA-independent pathways, plants have common mechanisms in their physiological responses and tolerance to drought.

2.5.1 Late Embryogenesis Abundant (LEA) Proteins

Plant species diversity is important for preserving the natural relations in ecosystems. Currently in seed gene banks various seeds can be stored for decades or even centuries, but tree propagation from seeds is not easy, especially for species that produce seeds irregularly or rarely. It is even more difficult if the seeds cannot be stored for a long time, or at least no effective storage conditions have been identified yet. There is usually a reserve of dormant seeds in the soil in habitats where the plants grow. However, seed production can be seriously limited by various environmental stresses. Some environmental stresses, such as drought stress, cold stress and salinity stress, result in cellular dehydration. Water is one of the factors that limit seed development, storage, and germination, so seeds must have some mechanisms that allow them to withstand the loss of water (Ewa, 2007).

Water-protein interactions are one of the main forces in protein folding and stabilizing the conformation, but also in determining the dynamics and properties of bonds in proteins and their catalytic activity. LEA proteins were first identified in seeds, when mRNA and proteins that occur in large quantities were investigated. LEA proteins are located in the cytoplasm, nucleus, mitochondria, vacuoles, near the cellular membrane, as well as in amyloplasts but primarily they accumulate in the cytoplasm and nucleus. After synthesis they are transported to cell organelles and membranes, where they stabilize cell structures and molecules. The partition of LEA proteins into various cell compartments determines their possible function in the protection or regulation of essential biochemical processes, like replication or respiration (Muhammad, 2012).

2.5.1.1 LEA Proteins Distribution and Types in Higher Plants

LEA proteins are formed during the late period of seed development accompanied by dehydration. They are proteins with small molecular weight ranging mainly from 10 to 30kDa and above 30kDa in some crops like wheats (200kDa). LEA proteins first studied in developing cotton seeds. Subsequently, researchers detected their existence in wheat, barley, maize, rice, sunflower, potato, grape, apple, bean, Arabidopsis, tomato, rye, soybean, carrot and so on (Shao *et al;* 2005). LEA proteins exist mainly in higher plant seeds, but have also been found in seedlings, roots and other organs and they are mainly localized in cytoplasm and nuclear regions.

LEA proteins can be classified into 3 or 6 groups. The classification is based on sequence similarity and properties. Proteins of group 1 contain a 20-amino-acid region, which was first identified in the wheat Emprotein (Osmoprotective molecule). LEA proteins of group 2 are widespread and widely analyzed in respect of water stress. Group 3 of LEA proteins is characterized by the 11-aa motive. It has been predicted that this region forms an amphipathic a-helix, which probably participates in structural interactions. And they are involved in response to cold stress. Usually they are located in the cytoplasm, nucleus and mitochondria, where they can be associated with membranes. Groups 4 and 5 have a less conserved structure and are possibly involved in the protection of membrane stability (Shao *et al;* 2005). Group 2 of LEA proteins is also called the D-11 family or dehydrin. They are glycine- and lysine-rich proteins with molecular weight of 9-200 kDa. Glycine, histidine, lysine and threonine comprise 56% of all amino acid in LTI30 water stress-responsive protein (Cesar, 2017).

2.5.1.2 LEA Gene Expression and Protein Structure

LEA genes are sensitive to ABA because of the presence of ABREs (ABA responsive elements), i.e. regulatory elements in promoter regions, which contain the ACGT sequence called cassette G. Sensitivity to ABA also depends on the presence of MYC elements that include the sequence CACCTG, and MYB elements that include the TAACTG motive. ABA-independent expression of dehydrin is based on activation of DREs, also known as CRT (C-repeat) elements. The ABA responsive elements can interact with the bZIP transcription factors. ABA responsive elements motifs are also present in the promoter regions in drought-stress. Inducible regulatory genes that are involved in modulation of the expression of multiple genes related to plant response to stress (Yiling *et al.,* 2015).

LEA proteins in higher plants are mainly composed of hydrophilic amino acids ordered in repeated sequence (e.g. Glycine and Lysine), forming hyper-hydrophilicness and thermal stability. Advanced structure of such protein contains non-periodic linear and a-helixes structure without thermal dominative state and corresponding dehydrated proteins exist in a natural form of dimers. Group 1 proteins (such as D19) have a conservative sequence made up of 20 amino acid residues in repeated copies, which can absorb a large amount of bound water. Group 1 protein genes play a role in endosperm development and osmotic protection of vegetative organs in higher plants. Group 2 proteins (e.g. D11) include a 15-amino acid

conservative structure (EKKGIMDKIKEKLPG) at the C-terminus, and play an important role in metabolism as molecular chaperones and defending protein structure with a close connection to higher plant resistance to drought (Ewa, 2007).

2.5.1.3 Apparent functions of LEA Proteins

LEA proteins have been widely analyzed in plants. They play an important role in seed maturation and osmotic stress. LEA proteins in seeds are related to desiccation tolerance. In seeds the germination rate, electrolyte leakage, presence of heat-stable proteins and LEA accumulation can be measured to detect the moment of desiccation tolerance acquisition. The mRNA of LEA genes appears in maturing seeds and becomes the most abundant mRNA species in the dry seed, but disappears shortly after imbibition. LEA proteins are hydrophilic. In drought-tolerant seeds, numerous polar groups situated on the surface of LEA proteins participate in preferential hydration. During water deficit in desiccation-tolerant seeds, they can interact with other groups and replace water in the cell (Yiling *et al.*, 2015).

LEA proteins mainly play functions in dehydration tolerance and storage of seeds and in whole-plant stress resistance to drought, salt, and cold. Further study showed that LEA proteins are expressed through all the developmental stages with different expression levels and no tissue specificity. For instance, RAB21 and dehydrin in seeds can be found in the root, stem, leaf, callus and suspension cultures of higher plants under ABA or/and NaCl induction. In cotton seedlings, in vitro treatment could lead to the accumulation of LEA protein mRNAs (Shao *et al.*, 2005).

2.5.1.4. LEA Proteins in Relation to Environmental Stresses

Various LEA proteins have been found to accumulate in plants during cold acclimation. Therefore, LEA proteins might participate in cryoprotection mechanisms, by accumulation in the tissues where primary ice nucleation occurs. Enzyme protection is important during long-term seed storage in seedbanks (usually low temperature) and in the soil during winter. In various plant tissues LEA proteins are able to prevent the inactivation of cyclic frozen and unfrozen lactate dehydrogenase, so they probably display the same protective role in seeds. Moreover, during germination, LEA proteins may regulate a-amylase activity and starch grain degradation throughout the time of water deficit. The regulatory role in relation to enzymatic

activity also can be detected at the reaction level, because in dry seeds and whole plants LEA proteins can store the water molecules needed to catalyze biochemical reactions, especially in winter time.

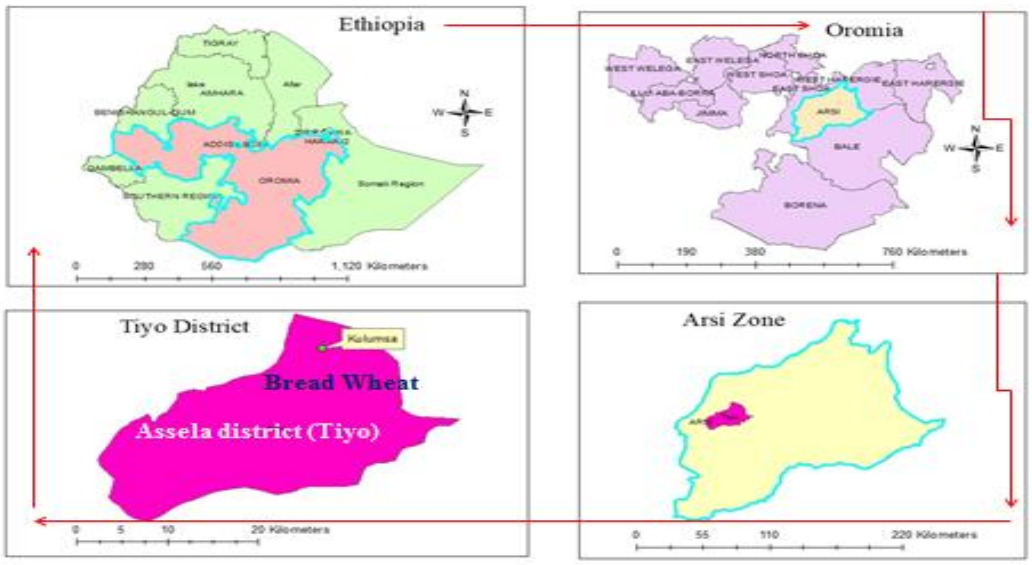
2.5.1.5 Biotechnological Applications of LEA Genes

Numerous transgenic studies revealed a positive effect of LEA gene expression on plant stress tolerance. The phenotypes of the transgenic plants show enhanced stress tolerance, often related to drought or salt stress. Most studies report enhanced growth rates and reduced wilting of the aerial parts under stress under laboratory conditions and in some field trials, demonstrating a real potential of LEA proteins in engineering crops more tolerant to stress. Apart from agronomical purposes, LEA proteins could be useful for other biotechnological applications in relation to their capacity to prevent aggregation of proteins. The use of a group 3 LEA protein as a fusion partner facilitates recombinant expression of recalcitrant proteins in *E. coli* in a soluble form. Moreover, the anti-aggregation properties of other group 3 LEA proteins have been applied to reduce the formation of *in vivo* aggregates; thus, co-expression of aggregation-prone proteins containing long poly-glutamine (polyQ) or poly-alanine (polyA) sequences, with group 3 AavLEA1, LEA protein in mammalian cells reduces substantially the expansion of protein aggregates associated to neurodegenerative diseases (Imen *et al.,* 2014).

3. MATERIAL AND METHODOLOGY

3.1 Description of sample collection Area

The study was conducted during the period from October 2017- November 2018 in Holeta National Agricultural Biotechnology Research Center. About fifty five wheat (*Triticum aestivum*) accessions were collected from Kulumsa Agricultural Research Center, Oromia Regional State, and East Arsi Zone around Assela town that far away 167 km from Addis Ababa, the capital city of Ethiopia (Figure 1). The center was established in 1966 by government of Ethiopia and the Swedish International Development Agency (SIDA) on the total area of 442.7 ha with the mandate to wheat, malt barely, and highland pulse crops research nationally and serve as bread wheat (Triticum aestivum) center of excellence for East Africa (Ethiopia, Kenya, Uganda, Tanzania), regionally. The center is located on 8°2'N and 39°10'E latitude and longitude, respectively with cool highland to semi-arid agrological zone at 2200 m.a.s.l altitude. It was also known with 10-22 minimum and maximum temperature and 840 mm annual rainfall.



Source: www.eiar.gov.et/Index.php/kulumsa-agricultural-research-center

Figure 1: Samples collection site

3.2 Plant material

A total of fifty five *Triticum aestivum accessions* were selected for this study. Out of this, thirty three accessions were released (from 1974–2016) whereas twenty four advanced accession (Table 1)

Table 1: Lists of *Triticum aestivum* genotypes collected from Kulumsa Research Center

1S. No	Name of accessions	Characteristics of selected accessions
1	Kakaba	Released, local checked
2	Ogolcho	Released, local checked
3	Kingbird	Released , local checked
4	Danda'a	Released, local checked
5	Hidase	Released , local checked
6	Hogana	Released, local checked
7	Gambo	Released, local checked
8	Dure	Released, local checked
9	Dashen	Released, local checked
10	Mararo	Released, local checked
11	Digalu	Released, local checked
12	Hawi	Released, local checked
13	Kubsa	Released, local checked
14	Sofumar	Released, local checked
15	Mada wolabu	Released, local checked
16	Wane	Released, local checked
17	Sirbo	Released, local checked
18	Galama	Released, local checked
19	Tuse	Released, local checked
20	TOA19	Released, local checked
21	Bika	Released, local checked
22	Kulkulu	Released, local checked
23	Pavon	Released, local checked
24	TOA17	Released, local checked
25	Millennium	Released, local checked
26	Honkolo	Released, local checked

27	Bobitcho	Released, local checked
28	Enkoy	Released, local checked
29	Huluka	Released, local checked
30	Shorima	Released, local checked
31	Alidaro	Released, local checked
32	NVT-msa2	Advanced , international checked
33	NVT-msa3	Advanced , international checked
34	NVT-msa4	Advanced, international checked
35	NVT-msa5	Advanced, international checked
36	NVT-msa6	Advanced, international checked
37	NVT-msa7	Advanced, international checked
38	NVT-msa8	Advanced, international checked
39	NVT-msa9	Advanced, international checked
40	NVT-msa10	Advanced, international checked
41	NVT-msa11	Advanced, international checked
42	NVT-msa12	Advanced, international checked
43	NVT-msa13	Advanced, international checked
44	NVT-msa14	Advanced, international checked
45	NVT-msa15	Advanced, international checked
46	NVT-msa16	Advanced, international checked
47	NVT-msa17	Advanced, international checked
48	NVT-msa18	Advanced, international checked
49	NVT-msa19	Advanced, international checked
50	NVT-msa20	Advanced, international checked
51	NVT-msa22	Advanced, international checked
52	NVT-msa29	Advanced, international checked
53	BWNVTOA2	Advanced, international checked
54	BWNVTOA17	Advanced, international checked
55	BWNVTOA19	Advanced, international checked

3.3 DNA Extraction and Purification

Total genomic DNA was extracted from green-house grown rust symptom free young leaf of selected *T. aestivum*. About 200-300 mg leaf from two week-old seedlings were independently harvested in sterilized 2 ml Eppendorf tube and DNA was extracted using Diversity Arrays Technology (DArT) protocol (Keb-lianes, *et al*., 2002). Initially, stock buffer solutions like, extraction buffer, lysis buffer and sodium disulfide (SDS) stock 5% (w/v) were prepared before extraction (appendix 1). Briefly, fresh buffer working solution was prepared from the stock solutions as standard procedure for DArT. Polyvinyl pyrroliodine (PVP) was added again to fresh working buffer solution and incubated in water bath at 65 °C for 1 hr. Then, each sample was added into liquid nitrogen with Eppendorf tube, immediately to dry and stop oxidation. All samples were crushed using sterilized pistils with care of any cross contamination until the leaves were powdered.

About 800 µl cooled fresh working buffer solution was added to each powdered leave samples and incubated immediately at 65 °C for 1 hrs. an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm, for 20 min. Then the supernatant of centrifuged samples were transferred to fresh 1.5 ml sterilized Eppendorf tubes and the same volume of ice cold isopropanol was immediately added for each samples separately and shaked 10 times invertly. All mixtures were centrifuged again at 10,000 rpm for 30 min and the supernatants were immediately discarded from each sample.

The DNA pellets were washed twice with 800µl of 70% (v\v) ethanol and dissolved in 100 µl of nucleus free water after being ethanol was discarded and DNA pellets were air dried in laminar air hood flow machine. Obtained DNAs were quantified and qualified by using 1% agarose gel electrophoresis in 1× TBE buffer at 120 V for 40 min. The DNA samples were stained with 6- x loading dye containing gel red and visualized under UV light. On the other hand, the purity and concentration of extracted DNA was confirmed and estimated spectrophotometrically using Ultra spec. 800, pharmaceutical Biotech, Cambridge, UK Nano drop at 260 nm. Finally, genomic DNAs with an approximately $A_{260\ nm}/A_{280\ nm}$ ratio of 1.8-2.1 were diluted to 50ng/µl for PCR amplification (appendix 2).

$$\text{Thus; DNA purity} = \frac{\text{Absorbance at 260 nm}}{\text{Absorbance at 280 nm}} \quad \text{ratio} \ldots\ldots\ldots\ldots \text{appendix 3}$$

Electrophoretic profiles of genomic DNA by using 1% agarose in 1xTBE buffer for 55 selected *T. aestivum* after run under electrophoresis at 120 V for 40 minutes. Letter 'M' represents DNA marker/ladder. The lanes from serial no. 1-55 were represented the order of samples given in Table 1.

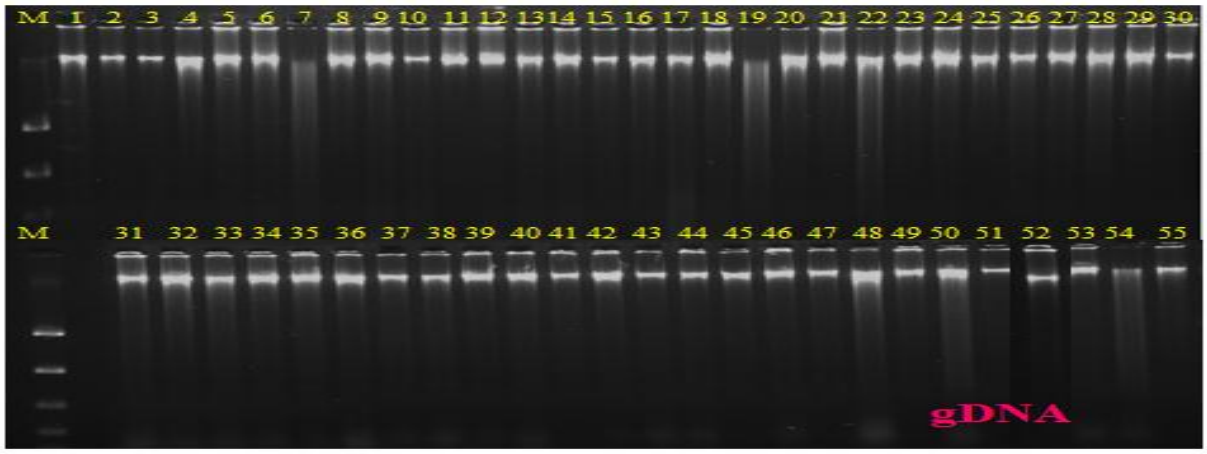


Figure 2: Electrophoretic pattern of 55 selected varieties of *T. aestivum* genomic DNA

3.4 PCR amplification

The PCR reaction of ten ISSR primers were conducted with 25 µl total reaction volume containing; 12.5 µl of master mix with standard buffer, 9.5 µl of nucleus free water, 2 µl of DNA template and 1 µl of primer in Altay Scientific S.P.A via Tuscolana,242 Thermocycler 00046 Grottaferata, Rome (Italy) machine programmed to full fill 40 cycles after an initial step of denaturation for 3 min at 94 °C followed by final denaturation step of 1min at 94 °C, an annealing step at temperature of 48 °C for 1 min and an extension step of 1min at 72 °C. Seven minutes were given after the last cycle to the extension step at 72 °C to ensure the completion of the primer extension reaction and the products were infinitely stored at +4 °C (appendix 4).

Eight µl of PCR products were taken from the total products and mixed with 2 µl 6×loading dye containing nonhazardous gel red that serve for clearly separation and visualization of each fragments. Lastly, the gel electrophoresis was performed on 2.5 % high-resolution agarose gel in 1× TBE buffer at 120 V for 2 hr. PCR products were visualized and all necessary images were scored under Altay Scientific S.p.A via Tuscolana, 242, 00046 Grottaferata, Rome, (Italy) gel documentation system for gene analysis.

3.5 Screening of fragment produced by ten ISSR primers

The fragments that were generated among each primers during PCR amplification was used to LEA gene screening as well as drought resistance genotype identification. It was also used to identify and validate molecular markers linked to drought resistance genes. To identify DNA band polymorphism between selected *T. aestivum* genotypes, sufficient number of drought resistance genes ISSR markers were selected from designed ISSR-based linkage maps of wheat. The primer sequences and annealing temperatures for all selected ISSR markers Afiah, *et al*., 2016) (Table 2)

Table 2: Lists of ten ISSR primer and their nucleotide sequences selected for this study

No.	Primer name	Barcode	Sequence	GC% min\max	Base length	nmol	Anne. Tm
1	844A	S3B19	(CT)8AC	50\50	18	67.2	48
2	844B	S3B1A	(CT)8GC	55.56\55.56	18	79.66	49
3	17898A	S3B1B	(CA)6AC	50\50	14	61.79	54
4	17898B	S3B1C	(CA)6GT	50\50	14	60.64	54
5	17899A	S3B1D	(CA)6AG	50\50	14	61.03	48
6	HB11	S3B1E	(GT)6CC	57.14\57.14	14	68.39	58
7	HB12	S3B1F	(CAC)3GC	72.73\72.73	11	61.39	44
8	HB13	S3B20	(GAG)3GC	72.73\72.73	11	68.49	48
9	HB14	S3B21	(CTC)3GC	72.73\72.73	11	67.61	50
10	HB15	S3B22	(GTG)3GC	72.73\72.73	11	58.92	58

Source; Inqaba biotechnology industries, Ltd, South Africa and Aliah, *et al.,* (2016)

Selected primers were used for the detection of the absence or presence of certain drought resistance genes based on fragment analysis in selected wheat (*Triticum aestivum*) released and advanced accessions. For all markers detected alleles, polymorphism was identified by presence or absence of band or fragments of different size (bp) detected between all genotypes. Present band was scored as 1 whereas absent band was scored as 0 (appendix 5). Scoring started from lower band (small fragment size) and then to upper band (large fragment size). The significant size of each fragment was determined using 100 bp and 1kb DNA ladder (Bioneer, USA) as reference.

3.6 Molecular Marker polymorphism

For all markers detected alleles, number and percentages of polymorphic and dominance Polymorphism information content (PIC) average was presented using Scores system 1 for present and 0 for absent (appendix 5).

$$\%\ of\ polymorphic\ bands = \frac{no.of\ polymorphic\ bands}{Total\ no.of\ bands} \times 100\%$$

3.7. Data analysis

Genomic DNA from all selected wheat (*Triticum aestivum*) samples was extracted using DArT plant DNA extraction protocol. Genomic DNAs were quantified and qualified under gel electrophoresis and spectrophotometry (Nano drop). DNA dilution/ normalization was conducted using Microsoft excel. The PCR product fragments produced from all samples using ten ISSR markers were analyzed using tabular, figure, Microsoft excel.

4. RESULT AND DISCUSSION

4.1 DNA amplification and ISSR Analysis using PCR

Ten ISSR primers were used to evaluate/ identify and characterize the 55 Wheat (*Triticum aestivum*) accessions. The following figures (3-12) shows the banding patterns produced from each primer for the 55 accessions. The highest number of PCR amplified fragments by using all primers was presented in a hexaploid wheat accession TOA 19 (64 fragments), Dashen (61 fragments) and Kakaba (59 fragments) respectively. While the hexaploid accession NTV-msa 6 gave the lowest number of 36 fragments. On the other hand, the primer HB12 gave the highest number of amplified fragments (426 fragments) for all studied accessions. While the primer HB14 showed the lowest number of fragment (114 fragments) as shown in table 5

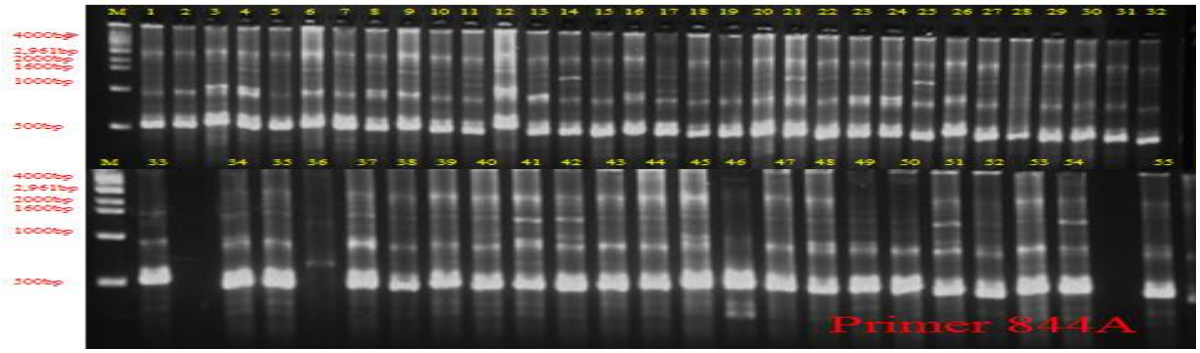


Figure 3: Electrophoretic pattern of 55 selected varieties of *T. aestivum* - PCR product for primer 844A

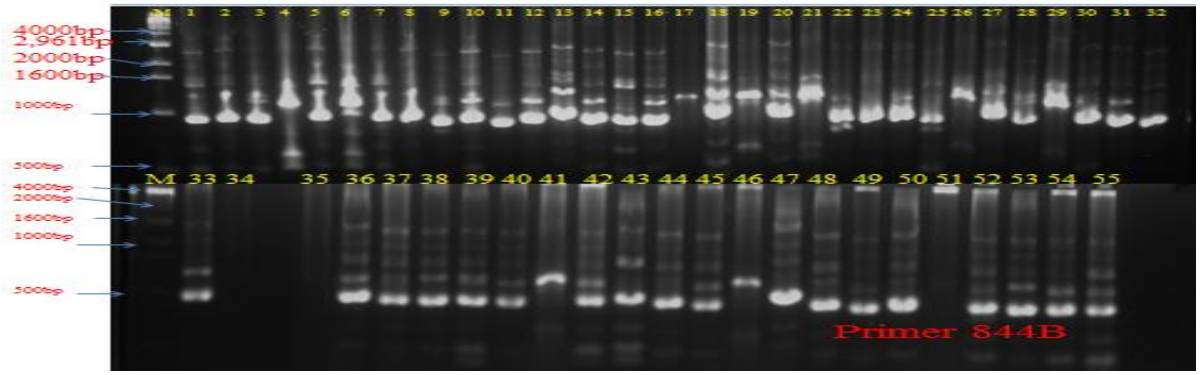


Figure 4: Electrophoretic pattern of 55 selected varieties of *T. aestivum* - PCR product for primer 844B

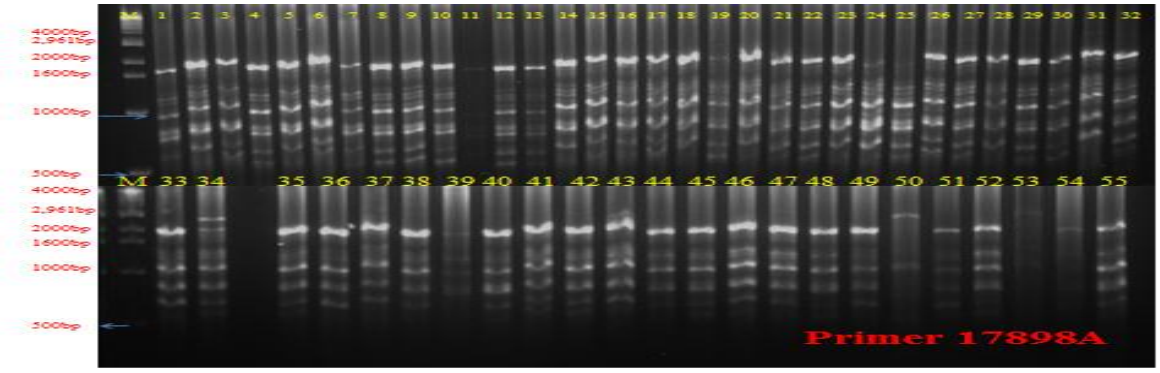


Figure 5: Electrophoretic pattern of 55 selected varieties of *T. aestivum*-PCR product for primer 17898A

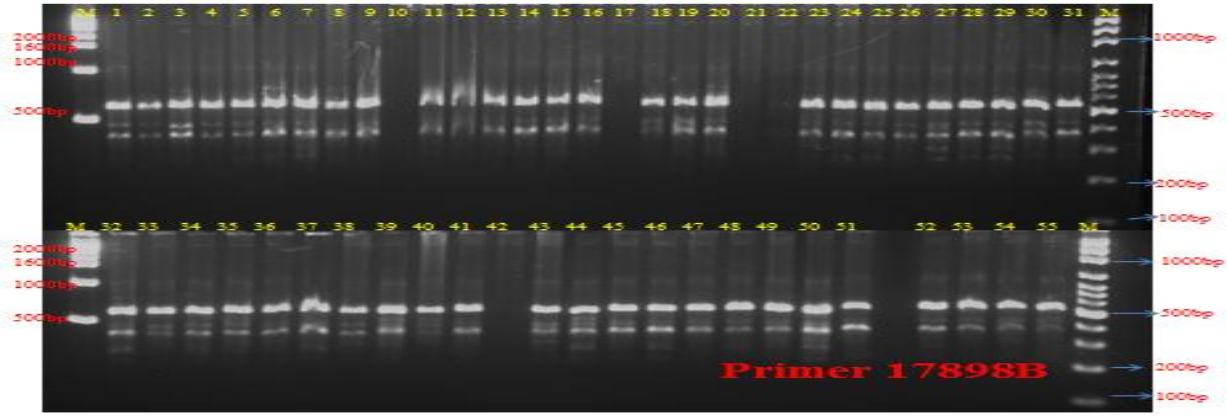


Figure 6: Electrophoretic pattern of 55 selected varieties of T. aestivum - PCR product for primer 17898B

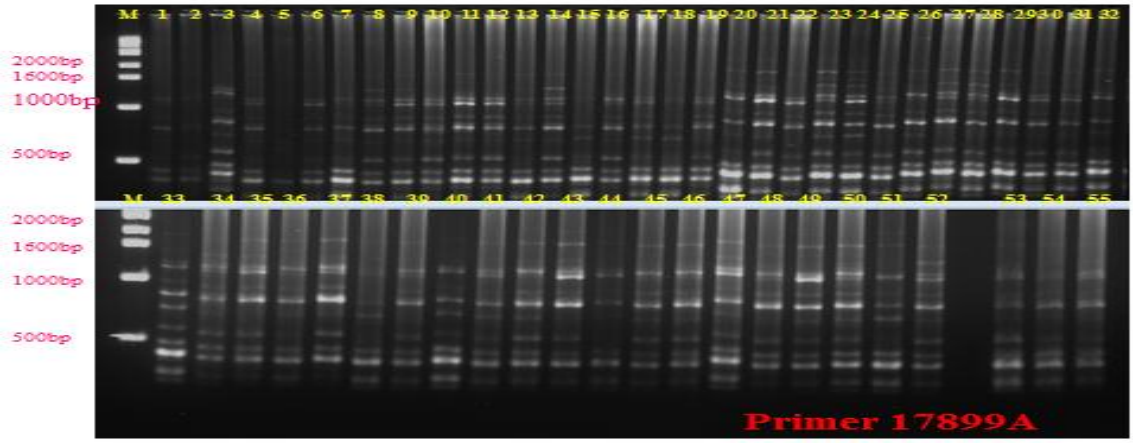


Figure 7: Electrophoretic pattern of 55 selected varieties of T. aestivum-PCR product for primer 17899A

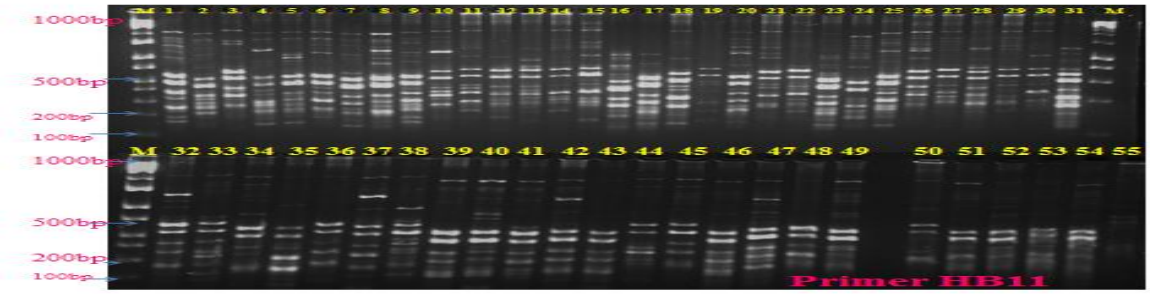


Figure 8: Electrophoretic pattern of 55 selected varieties of T. aestivum - PCR product for primer HB11

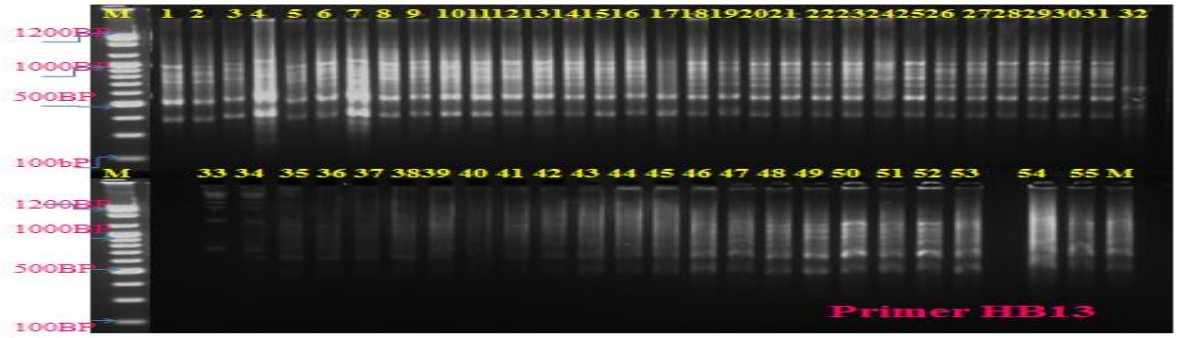


Figure 9: Electrophoretic pattern of 55 selected varieties of T. aestivum - PCR product for primer HB13

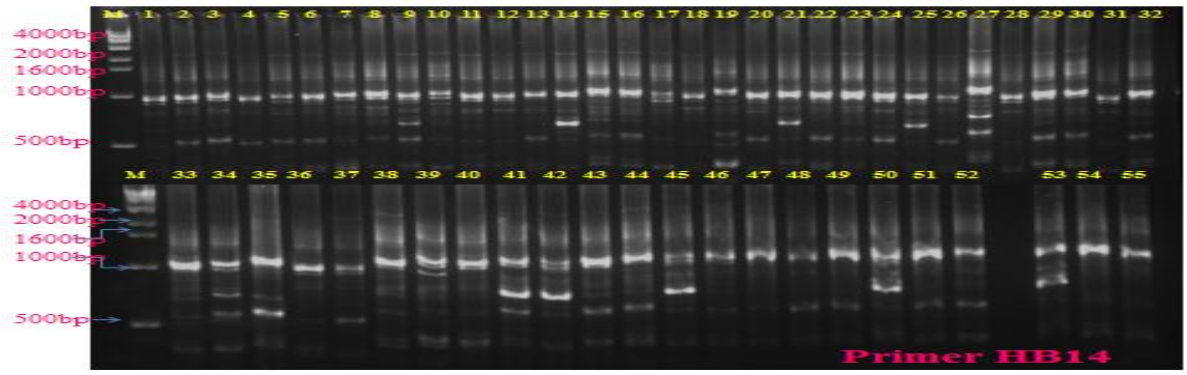


Figure 10: Electrophoretic pattern of 55 selected varieties of T. aestivum- PCR product for primer HB14

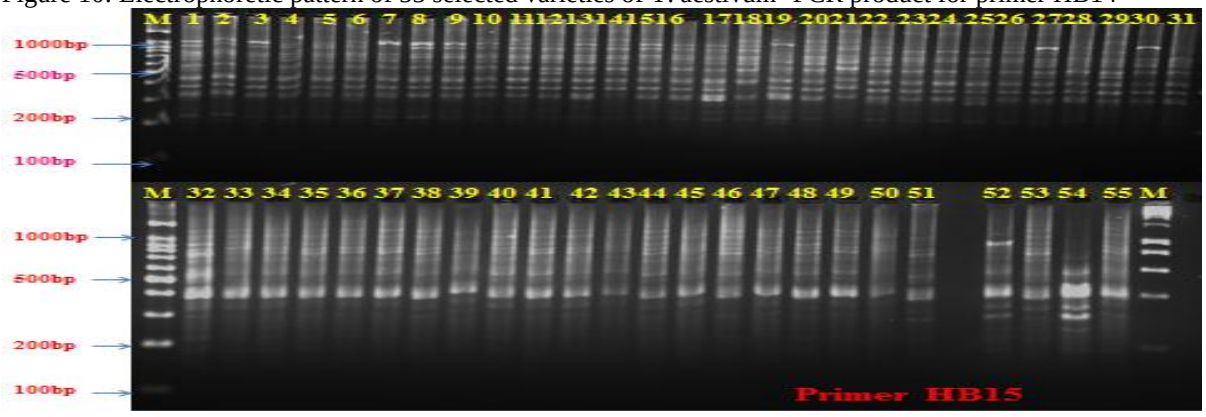


Figure 11: Electrophoretic pattern of 55 selected varieties of T. aestivum- PCR product for primer HB15

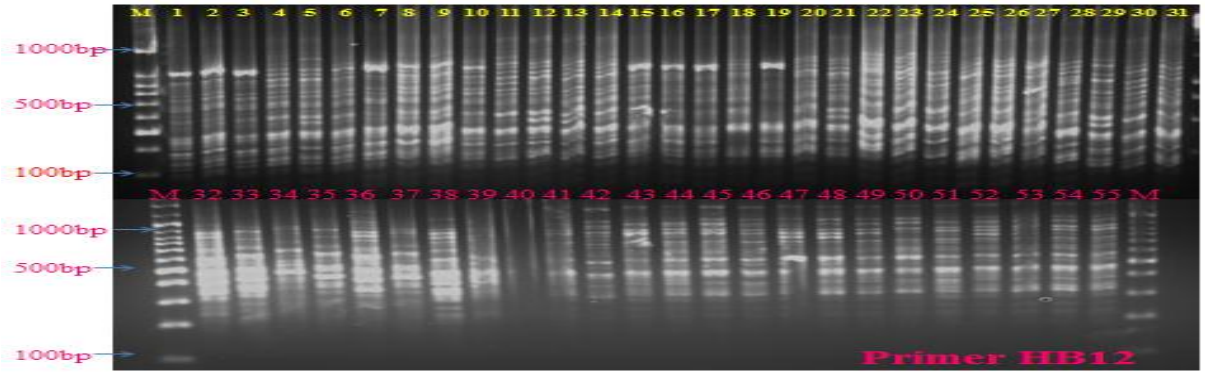


Figure 12: Electrophoretic pattern of 55 selected accessions of T. aestivum- PCR product for primer HB12

Thus, Electrophoretic profiles of PCR products by using 844A (fig. 3), 844B (fig. 4), 17898A (fig. 5), 17898B (fig. 6), 17899A (fig.7), HB11 (fig. 8), HB13 (fig. 9), HB14 (fig.10), HB15 (fig.11) and HB12 (fig. 12) ISSR primers for 55 bread wheat accession were given below. Letter 'M' represents DNA marker. The arrangement of accessions is the same in Table 1.

The above Figures were showed that, electrophoretic patterns of 55 selected genotypes of *T. aestivum* obtained from polymerase chain reaction using ten ISSR primers. Most of primers generate bright and specific fragment in samples containing drought resistance gene that help to distinguish drought tolerant genotypes among the selected one. However some primers produced faint bands on the same samples used for this study with the same; PCR reaction volume, voltage as well as the same time used for gel electrophoresis. The reason behind this variation may be due to; the characteristics of primers used, electric fluctuation on PCR processing and gel electrophoresis or the primers generate faint bands were not work for gene screening for selected genotypes. Specific bands that were briefly used to identify the expression of drought resistance gene in each selected genotypes were amplified by primer 844A, 844B, 17898A, 17898B, 17899A, HB11 and HB14. Unspecific and faint fragments were produced by primers; BH12, HB13 and HB15 respectively. Therefore, primer 844A, 844B, 17898A, 17898B, 17899A, HB11 and HB14 were selected as best primers used for drought resistance gene screening for this study.

4.2 Screening of ISSR polymorphism

The maximum numbers of 12 bands were amplified by primers HB11 while the minimum numbers of bands were obtained by primer HB14. Out of 73 fragment produced by ten ISSR markers ,39 fragments were polymorphic (53.4 %) across all bread wheat (Triticum aestivum) accession selected for this study. Polymorphic percentage ranged from 33- 75% (table 3).

Table 3: Primer sequence, Total amplified bands (loci), number of polymorphic bands and % of polymorphism in 10 ISSR primers

No	Primer	Primer Sequence 5' to 3'	Total number of bands generated	No. Polymorphic bands	% Polymorphism
1	844A	(CT)8AC	7	4	57.1
2	844B	(CT)8GC	6	2	33.3
3	17898A	(AC)6AC	8	3	37.5
4	17898B	(CA)6GT	5	3	60.0
5	17899A	(CA)6AG	9	5	55.5
6	HB11	(GT)6CC	12	6	50.0
7	HB12	(CAC)3GC	9	6	66.7
8	HB13	(GAG)3GC	6	3	50.0
9	HB14	(CTC)3GC	4	3	75.0
10	HB15	(GTG)3GC	7	4	57.1
Total			73	39	53.4
Mean			7.3	3.9	

4.3 Fragment Analysis for ISSR primers

This experiment was conducted on fifty five *T. aestivum* genotypes for evaluation of drought resistance accession of wheat (*Triticum aestivum*) using ISSR Markers at genetic level. Out of 10 primers used for the study, seven (7) primers were produced very bright and specific reproducible bands in most accessions containing drought resistance ability; three primers (HB12, HB13, and HB15) were generated faint fragments. The size of amplified DNA fragments ranged between 500-2961bp for primer 844A and 844B, 500-2000bp for primer 17898A, 300-550bp for primer 17898B, 300-1600bp for primer HB11, 200-800bp for primer HB12, 200-1000bpfor primer HB13,300-1000bp for primer HB14, while 400-1000bp DNA fragments were produced for primer HB15 respectively (Table 4).

Table 4: Summary of gene amplification using ten ISSR marker in all selected *T. aestivum*

No.	Primer	Location of amplified gene fragments	Fragment size (bp)	Genotype present
1	844A	L5	500	All except, 36
2	844B	L5	490	All except, 34,35& 51
3	17898A	L2	1900	All except, 39, 50, 51,54 with faint band and 11 &53 no band at all
4	17898B	L1	600	All except, 19, 17,21,42 no band and 22 with faint
5	17899A	L2, L4 and L8	900, 800 &300	All except, 1, 2 and 5
6	HB11	L4, L5 and L6	450, 500 &550	All except, 19, 50 &53
7	HB12	L1 and L5	800 and 500	Faint fragment except, 4
8	HB13	L4	400	All, but faint from 33-55
9	HB14	L2	1000	All except, 17, 37 and 48
10	HB15	L5	400	All but, faint in 1-31

Note: L- represent **locus**

Primer 844A produced specific and bright fragment/band on L5 in all accessions containing drought resistance ability except, variety number 36 which produced very faint band. Primer 844B produced specific and bright band on L5 in all accessions containing drought resistance ability except variety number 34, 35 and 51 which was not produced any specific band. Primer17898A was produced specific and bright band on L2 in all accessions containing drought resistance ability except variety number 39,50,51 and 54 which produced very faint band whereas, no specific band was produced for accession number 11 and 53 as shown above. Primer17898B produced specific and bright band on L1 in all accessions containing drought resistance ability except accession number 10, 17, 21 and 42 which shows any band formation at all and faint band on number 22. Primer 17899A produced specific and bright band on L2, L4 and L8 respectively in most of accessions containing drought resistance ability except accession number 1, 2 and 5 which produced very faint bands. Primer HB11 was produced specific and bright band on L4 and L5 in most of accessions containing drought resistance ability as shown in figure 3-12 respectively.

In general, Primer HB12, HB13 and HB15 didn't produce clear specific fragments on well-known molecular weight, most generated fragments were faint. However the genomic amplification shown on L4 for primer HB12 in most accessions except accession number four.

Primer HB13 generated fragment on L4 in all accessions containing drought resistance ability except advanced lines of selected accessions from number 33-55, that were produced faint fragments whereas, PrimerHB14 generated band on L2 in most accessions containing drought resistance ability except accession number 17, 37 and 48. This may be due to; the nature of marker, fluctuation of electric power during electrophoresis. A total of 2685 fragments were produced from ten ISSR markers, primer HB12, 17899A, HB11, 17898A, 844A, HB15, HB13, 17898B, 844B and HB14 were produced 426, 384, 316, 299, 271, 238, 236, 212, 189 and 114 number of fragments respectively. Among the selected released accessions, from number one-thirty produced high numbers of fragments that possess drought resistance ability than advanced genotypes from serial number thirty –fifty five as shown in below Table 5

Table 5: Total fragments generated from 10 ISSR primers for selected Triticum aestivum

	Primer										
S.No	844A	844B	17898A	17898B	17899A	HB11	HB12	HB13	HB14	HB15	*
1	5	4	7	5	5	12	9	4	1	7	59
2	5	3	6	4	6	6	9	4	2	7	52
3	6	2	6	4	9	7	9	5	3	7	58
4	6	2	7	4	5	7	9	5	2	6	53
5	6	5	7	4	1	9	9	4	2	6	53
6	7	3	7	3	6	7	9	4	2	6	54
7	6	2	7	5	6	8	9	5	1	6	55
8	6	2	6	3	7	9	8	5	2	7	55
9	6	2	7	3	8	11	9	5	4	6	61
10	6	5	7	0	9	8	9	5	2	6	57
11	6	3	2	3	8	8	9	5	2	4	50
12	5	5	6	2	8	8	9	5	2	6	56
13	6	5	6	3	6	5	9	5	2	6	53
14	5	5	7	4	8	9	9	5	3	5	60
15	5	3	7	5	7	3	9	5	3	7	54
16	6	4	7	4	6	6	9	5	3	6	56
17	5	1	7	0	8	3	9	2	2	3	40
18	6	4	4	4	6	4	9	6	1	6	50
19	4	1	6	4	6	2	6	6	2	7	44
20	7	6	5	4	9	9	9	6	3	6	64
21	5	2	5	0	9	9	9	6	2	5	52
22	6	1	6	3	6	4	9	6	2	5	48
23	4	1	6	5	9	6	9	6	3	4	53
24	6	2	5	5	9	8	9	6	3	6	59
25	4	1	6	5	8	9	9	6	2	4	54
26	3	1	7	5	9	6	9	6	2	4	52
27	5	2	8	5	9	5	9	6	4	6	59
28	1	3	5	5	9	8	9	6	1	4	51
29	4	2	8	5	9	1	9	6	4	4	52
30	4	5	6	5	9	3	8	6	3	5	54
31	5	6	4	4	9	5	7	6	1	3	50
32	3	5	6	4	5	6	9	2	2	3	45
33	4	4	4	4	7	4	9	4	2	3	45
34	5	0	6	4	5	2	6	6	3	3	40
35	6	0	5	5	5	5	9	2	3	3	43
36	1	6	4	3	6	4	6	2	1	3	36
37	6	6	5	4	6	9	7	2	2	3	50
38	5	6	4	5	5	7	8	2	2	3	47
39	5	6	4	3	7	5	2	2	2	1	37
40	7	6	5	4	9	7	0	0	1	2	41
41	6	1	5	2	7	6	6	1	3	3	40
42	5	5	5	4	9	8	5	3	2	3	49
43	4	3	5	5	9	6	6	2	2	1	43
44	5	4	4	5	6	3	7	2	2	3	41
45	5	4	6	4	8	3	7	2	2	3	44
46	3	1	6	4	6	5	7	2	1	3	38
47	4	4	5	4	7	6	5	3	1	3	42
48	5	5	5	4	7	5	6	6	2	3	48
49	4	5	5	4	7	4	8	5	1	3	46
50	3	5	2	5	7	6	8	5	2	1	44
51	4	0	5	3	7	2	8	6	2	3	40
52	4	5	4	5	7	2	6	6	1	5	45
53	5	5	2	4	5	4	5	3	2	3	38
54	5	5	1	4	5	2	7	4	1	4	38
55	6	5	6	4	3	0	7	2	1	3	37
Total	271	189	299	212	384	316	426	236	114	238	2685

During the study high polymorphic percentages (53.4%) were produced from 10 primers, out of 73 total locus generated by all samples. This showed the selected genotypes were polymorphic with high genetic variation among them. (Rana *et al.,* 2017) reported that, the reason behind the high polymorphism is perhaps due to multi-directional selection of genotypes during breeding for many traits. This could be due to multi- national and international phenotypic selection for different types of genotypes in the past. Genomic DNA was amplified in all bread wheat (*Triticum aestivum*) genotypes selected for this study except in some varieties by some primers as it were described in (Table 4). Thus LEA genes were found in all bread wheat genotypes selected for this study, even though they varies in numbers and size of amplified fragment.

The current finding in agree with the work of Hong *et al*., (2005) that reports, LEA proteins gene are formed during the late period of seed development accompanied by dehydration and the existence of LEA gene was first studied in cotton seeds. Subsequently, Scientists detected their existence in some varieties of wheat, barley, maize, rice, sunflower, potato, grape, apple, bean, Arabidopsis, tomato, rye, soybean, carrot and so on. LEA protein genes were exist mainly in higher plant seeds, but have also been found in seedlings, roots and other organs.

This finding shows that LEA genes were found in leaf parts of all selected *Triticum aestivum* since reproducible fragments were generated in all samples as shown in (Figure 3-12) above, therefore the gene play a great role in genetic engineering and modern breeding. This in agreement with the work of Hong, (2017) that reported ISSR markers were used to detect major genes that contribute to agronomical value for efficiency of agricultural staple crop production. Taking advantage of a superior genome, high yielding varieties exhibit outstanding performance compared to traditional varieties. However, selecting a few desirable traits for the long term is one of the main factors causing genetic erosion. Once genetic diversity narrows, plants could be susceptible to drought.

The current finding will help in the cloning and breeding program of multi drought resistance wheat varieties in genetic resource of wheat genotype. The result showed drought resistance LEA genes were amplified in both genomic DNA of released and advanced *T. aestivum* genotypes in most of used primers. This indicated that both released and advanced line of control and treatment were possessing LEA gene, which shows most of drought moderate and

drought resistant genotypes has LEA gene even though the levels of expression in different parts of crops may be varies this shows, drought may not be only altered by LEA gene.

As the study indicated that, most of released accessions of the selected Wheat (Triticum aestivum) were drought resistance as compared to advanced line. For instance accessions like; Kakaba, Hogana, Digalu, Wane, Ogolcho, Gambo, Hawi, Sirbo. Kingbrid, Dure, Kubsa, Galema, Danda'a, Dashen, Sofomer, Tuse, Hidase, Mararo, M/wolabu, TOA19, Bika ,Kulkulu, Pavon, TOA17, Millenium, Honkolo, Bobicho, Enkoy, Huluka, Shorima, Alidaro, were generate large number of fragments which possess drought responsive elements like LEA gene Table 5.

Ming *et al.,* (2008) reported that, Detection of genomic responses of plants to water stress is so important to know; Transcriptional reactions of plants to drought stress, Functions of genes in stress environments, Distinguish promoters which are crucial for crop engineering. Arash *et al.,* (2013) also reported that, Wheat (*Triticum aestivum*) genotypes with good water management are able to: - bear high, yields in drought conditions, create new breeding lines and cultivars with developed drought resistance

5. CONCLUSION AND RECOMMENDATIONS

In this study drought resistance accessions were screened from 55 selected *Triticum aestivum* genome using ISSR markers and best ISSR markers used for drought resistance accession identification and their expression pattern was detected. The present study not only revealed the means for identification of ISSR markers associated with drought tolerant loci but also defined polymorphism between susceptible and drought tolerant wheat cultivars. Though some more sophisticated methodologies are required to generate greater specificity and tight associations with drought tolerant loci, the ISSR markers have nevertheless proved to be an easy, fast, simple and efficient means to identify such regions. Present effort have provided a base to critically judge these characters further by utilizing more advanced techniques wherein the marker termini will be sequenced to design longer primers such as SCAR for specific amplification of a particular locus. Therefore, primer 844A, 844B, 17898A, 17898B, 17899A, HB11, and HB14 were selected as the best marker for screening drought resistance accessions and as well as to screening resistance gene in wheat (*Triticum aestivum)* based on PCR-ISSR markers techniques. The identification of such loci would consequently help breeders to introgress such traits in wheat.

Considering the above conclusion and based on the results of the study, the following recommendations were suggested:

- Further study should focus on the sequence of the gene and chromosomal location as well as the numbers of proteins it encoded to compare with the LEA gene found in other staple crops like *Arabidopsis thaliana.*
- Since the breeding and genetic engineering of drought tolerant varieties would be of great values in agriculture, from the basis of searching for anti-drought inducible genes and their characterization, clearly identifying and characterization of drought resistance Wheat (Triticum aestivum) accessions will be needs further work.

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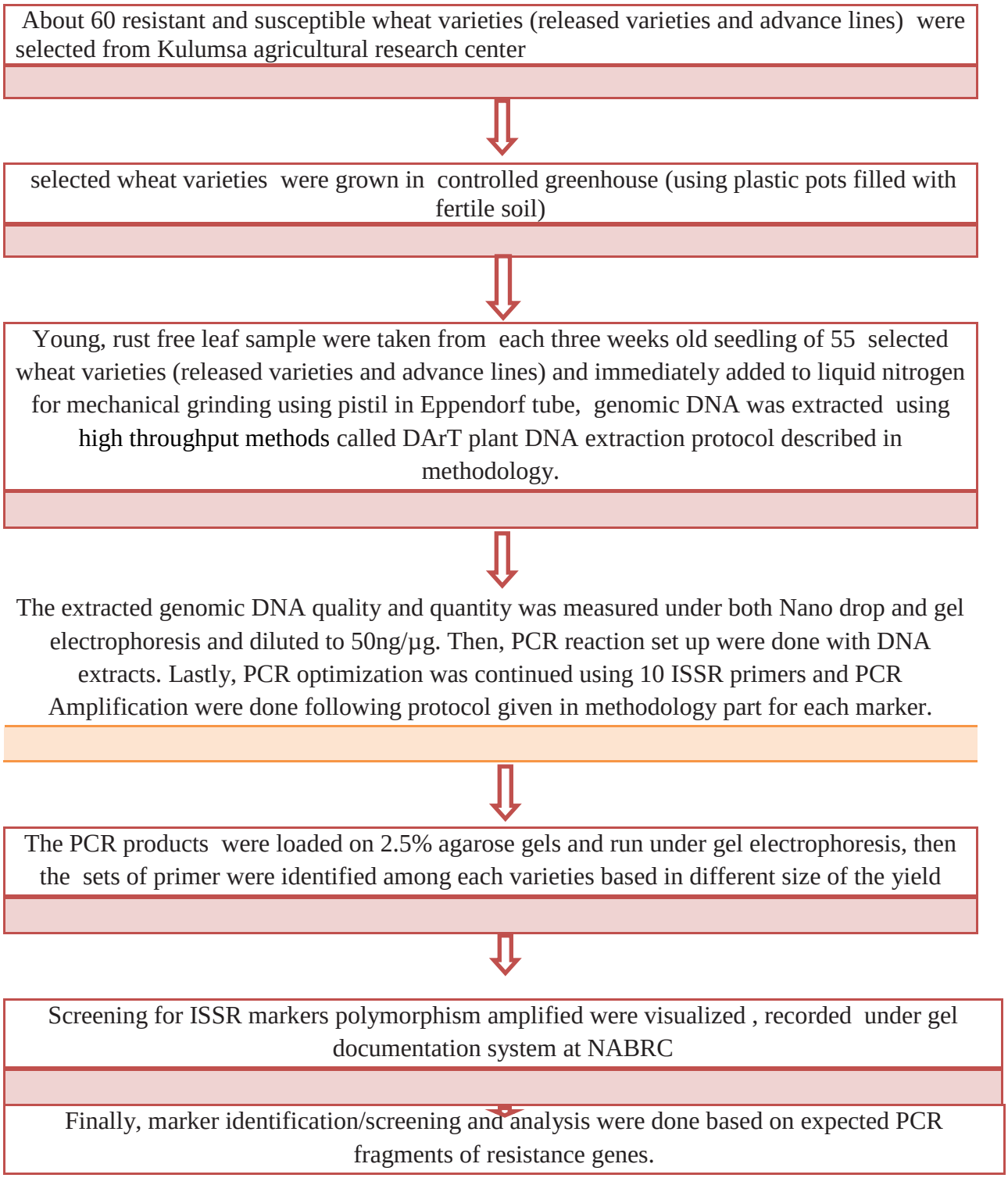
APPENDIXES

Appendix 1: Components of 20xTBE buffer, agarose gel preparation for genomic and PCR product analysis under electrophoresis and 25 total volume of PCR reaction ingredients

A). 20x TBE buffer components		
No.	Chemicals	Amount
1	Tris base	216g
2	Boric acid	110g
3	EDTA	14.88g
4	H_2O (dd) up to	1L
B). Components of gel electrophoresis for genomic DNA analysis		
No.	Chemicals	Amount
1	Agarose	1gram (1% agarose),but 2.5% for PCR
2	1x TBE buffer	100ml (based on sample size)
C). Components of sample preparation for 25 total reaction volume of PCR		
No.	Components	Amount in µL
1	Master mix with standard buffer	12.5
2	Nucleus free water	9.5
3	Primer	1
4	DNA template	2

Appendix 2: Workflow for Evaluation of drought resistance accession of wheat (*Triticum aestivum*) Using ISSR Markers



Appendix 3: DNA quality and quantity measured by Nano drop

Sample ID	Initial Conc.	gDNA	water	A260 nm	A280 nm	260/280nm ratio	Fin.conc. ng/µl
1	1521 ng/µl	3	97	30.422	14.563	2.09	50
2	1437 ng/µl	3	97	28.737	13.611	2.11	50
3	544.7 ng/µl	9	91	10.893	5.327	2.04	50
4	587.7 ng/µl	9	91	11.754	5.867	2	50
5	807.5 ng/µl	6	94	16.149	7.823	2.06	50
6	899.1 ng/µl	6	94	17.982	8.654	2.08	50
7	1146 ng/µl	4	96	22.916	11.052	2.07	50
8	1373 ng/µl	4	96	27.467	13.36	2.06	50
9	898.6 ng/µl	6	94	17.972	8.447	2.13	50
10	1039 ng/µl	5	95	20.775	9.86	2.11	50
11	976.5 ng/µl	5	95	19.53	9.47	2.06	50
12	1710 ng/µl	3	97	34.194	16.482	2.07	50
13	1233 ng/µl	4	96	24.661	12.053	2.05	50
14	550.7 ng/µl	9	91	11.014	5.513	2	50
15	1556 ng/µl	3	97	31.129	14.963	2.08	50
16	944.7 ng/µl	5	95	18.894	9.074	2.08	50
17	2131 ng/µl	2	98	42.618	20.113	2.12	50
18	801 ng/µl	6	94	16.02	7.768	2.06	50
19	2426 ng/µl	2	98	48.528	23.429	2.07	50
20	729.5 ng/µl	7	93	14.591	7.42	1.97	50
21	1074 ng/µl	5	95	21.488	10.507	2.05	50
22	2791 ng/µl	2	98	55.811	27.5	2.03	50
23	646 ng/µl	8	92	12.919	6.554	1.97	50
24	378.8 ng/µl	13	87	7.576	3.817	1.98	50
25	496.1ng/µl	10	90	9.921	5.059	1.96	50
26	1693 ng/µl	3	97	33.864	15.966	2.12	50
27	1156 ng/µl	4	96	23.11	11.014	2.1	50
28	1663 ng/µl	3	97	33.258	15.811	2.1	50
29	368.3 ng/µl	14	86	7.367	3.631	2.03	50
30	1930 ng/µl	3	97	38.596	18.282	2.11	50
31	2451 ng/µl	2	98	49.028	23.929	2.05	50
32	2738 ng/µl	2	98	54.765	27.034	2.03	50
33	1607 ng/µl	3	97	32.136	15.414	2.08	50
34	3067 ng/µl	2	98	61.34	29.952	2.05	50
35	1270 ng/µl	4	96	25.395	12.601	2.02	50
36	2316 ng/µl	2	98	46.329	22.562	2.05	50
37	3075 ng/µl	2	98	61.51	30.381	2.02	50
38	799.3 ng/µl	6	94	15.986	7.751	2.06	50
39	2117 ng/µl	2	98	42.348	20.496	2.07	50
40	457.9 ng/µl	11	89	9.159	4.57	2	50
41	908.5 ng/µl	6	94	18.17	8.631	2.11	50
42	2025 ng/µl	2	98	40.5	19.596	2.07	50
43	650.4 ng/µl	8	92	13.008	6.718	1.94	50
44	785.2 ng/µl	6	94	15.704	7.704	2.04	50
45	2723 ng/µl	2	98	54.458	26.814	2.03	50
46	1170 ng/µl	4	96	23.397	11.277	2.07	50
47	922.3 ng/µl	5	95	18.445	9.252	1.99	50
48	1118 ng/µl	4	96	22.368	10.694	2.09	50
49	4118 ng/µl	1	99	82.36	44.002	1.87	50
50	1560 ng/µl	3	97	31.2	14.956	2.09	50
51	3456 ng/µl	1	99	69.118	35.585	1.94	50
52	864.5 ng/µl	6	94	17.291	8.472	2.04	50
53	966.3 ng/µl	5	95	19.326	9.477	2.04	50
54	4554 ng/µl	1	99	91.079	49.127	1.85	50
55	2127 ng/µl	2	98	42.544	20.61	2.06	50

Appendix 4: List of ISSR Primers and their Thermocyclers

No	ISSR Primer	No of cycle	Inti.den. in (°C)	Final.den in (°C)	Annealing in (°C)	Intial ext. in (°C)	Final ext. in (°C)	Final store in (°C)
1	844A	40	94 for 3'	94 for 1'	48 for 1'	72 for 1'	72 for 7'	at +4
2	844B	40	94 for 3'	94 for 1'	49 for 1'	72 for 1'	72 for 7'	at +4
3	17898A	40	94 for 3'	94 for 1'	54 for 1'	72 for 1'	72 for 7'	at +4
4	17898B	40	94 for 3'	94 for 1'	54 for 1'	72 for 1'	72 for 7'	at +4
5	17899A	40	94 for 3'	94 for 1'	48 for 1'	72 for 1'	72 for 7'	at +4
6	HB11	40	94 for 3'	94 for 1'	58 for 1'	72 for 1'	72 for 7'	at +4
7	HB12	40	94 for 3'	94 for 1'	44 for 1'	72 for 1'	72 for 7'	at +4
8	HB13	40	94 for 3'	94 for 1'	48 for 1'	72 for 1'	72 for 7'	at +4
9	HB14	40	94 for 3'	94 for 1'	50 for 1'	72 for 1'	72 for 7'	at +4
10	HB15	40	94 for 3'	94 for 1'	58 for 1'	72 for 1'	72 for 7'	at +4

Note: - Initial denaturation and final extension were not included in the cycle

Appendix 5: Scoring bands for each primer for all selected varieties

A. Scoring band for primers 844A

S.no	L1	L2	L3	L4	L5	L6	L7
1	1	0	0	1	1	1	1
2	1	0	0	1	1	1	1
3	1	1	0	1	1	1	1
4	1	1	1	0	1	1	1
5	1	1	0	1	1	1	1
6	1	1	1	1	1	1	1
7	1	1	1	0	1	1	1
8	1	1	0	1	1	1	1
9	1	1	0	1	1	1	1
10	1	1	1	1	1	1	0
11	1	1	1	1	1	1	0
12	1	1	1	1	1	0	0
13	1	1	1	1	1	1	0
14	1	0	1	1	1	1	0
15	1	0	0	1	1	1	1
16	1	1	1	1	1	1	0
17	0	1	1	1	1	1	0
18	1	1	1	1	1	1	0
19	1	0	0	1	1	1	0
20	1	1	1	1	1	1	1
21	1	0	1	1	1	1	0
22	1	0	1	1	1	1	1
23	1	0	0	1	1	1	0
24	1	1	1	1	1	1	0
25	1	0	1	1	0	1	0
26	1	0	0	1	1	0	0
27	1	1	0	1	1	1	0
28	0	0	0	0	0	1	0
29	1	0	0	1	1	1	0
30	1	0	0	1	1	1	0
31	1	0	1	1	1	1	0
32	1	0	0	1	0	1	0
33	0	0	1	1	1	1	0
34	0	0	1	1	1	1	1
35	1	0	1	1	1	1	1
36	0	0	0	0	1	0	0
37	1	1	1	1	1	1	0
38	1	0	1	1	1	1	0
39	1	0	1	1	1	1	0
40	1	1	1	1	1	1	1
41	1	0	1	1	1	1	1
42	1	0	1	1	1	1	0
43	1	0	0	1	1	1	0
44	1	0	1	1	1	1	0
45	1	0	1	1	1	1	0
46	0	0	0	0	1	1	1
47	1	0	0	1	1	1	0
48	1	0	1	1	1	1	0
49	0	0	1	1	1	1	0
50	0	0	0	1	1	1	0
51	1	0	1	1	1	0	0
52	0	0	1	1	1	1	0
53	1	0	1	1	1	1	0
54	1	0	1	1	1	1	0
55	1	1	0	1	1	1	1

B. Scoring band for primer 844B

S.no.	L1	L2	L3	L4	L5	L6
1	1	0	0	1	1	1
2	1	0	0	1	1	0
3	0	0	0	0	1	1
4	0	0	0	1	1	0
5	1	1	0	1	1	1
6	0	0	1	1	1	0
7	0	0	0	1	1	0
8	0	0	0	1	1	0
9	0	0	0	1	1	0
10	1	1	1	1	1	0
11	1	0	0	1	1	0
12	1	1	1	1	1	0
13	1	1	1	1	1	0
14	1	1	1	1	1	0
15	1	0	1	0	1	0
16	1	0	0	1	1	1
17	0	0	0	1	0	0
18	1	0	1	1	1	0
19	0	0	0	1	0	0
20	1	1	1	1	1	1
21	0	0	0	1	1	0
22	0	0	0	0	0	1
23	0	0	0	0	0	1
24	0	0	0	1	1	0
25	0	0	0	0	0	1
26	0	0	0	1	0	0
27	0	0	0	0	1	1
28	0	0	0	1	1	1
29	0	0	1	1	0	0
30	1	1	1	1	1	0
31	1	1	1	1	1	1
32	1	1	1	1	1	0
33	1	1	0	0	1	1
34	0	0	0	0	0	0
35	0	0	0	0	0	0
36	1	1	1	1	1	1
37	1	1	1	1	1	1
38	1	1	1	1	1	1
39	1	1	1	1	1	1
40	1	1	1	1	1	1
41	0	0	0	1	0	0
42	1	1	1	1	1	0
43	1	0	1	0	1	0
44	1	1	1	0	1	0
45	1	0	1	1	1	0
46	0	0	0	1	0	0
47	1	1	1	0	1	0
48	1	1	1	1	1	0
49	1	1	1	1	1	0
50	1	1	1	1	1	0
51	0	0	0	0	0	0
52	1	1	1	1	1	0
53	1	1	1	1	1	0
54	1	1	1	1	1	0
55	1	1	1	1	1	0

C. Scoring band for primer 17898A

S.no	L1	L2	L3	L4	L5	L6	L7	L8
1	1	0	1	1	1	1	1	1
2	1	1	1	1	1	1	0	0
3	1	0	1	1	1	1	1	0
4	1	0	1	1	1	1	1	1
5	1	0	1	1	1	1	1	1
6	1	1	1	1	1	1	1	0
7	1	0	1	1	1	1	1	1
8	1	0	0	1	1	1	1	1
9	1	0	1	1	1	1	1	1
10	1	0	1	1	1	1	1	1
11	1	0	0	1	0	0	0	0
12	1	0	0	1	1	1	1	1
13	1	0	0	1	1	1	1	1
14	1	1	1	1	1	1	1	0
15	1	1	1	1	1	1	1	0
16	1	1	1	1	1	1	1	0
17	1	1	1	1	1	1	1	0
18	1	0	0	0	1	1	1	0
19	1	0	0	1	1	1	1	1
20	1	0	0	1	1	1	1	0
21	1	0	0	1	1	1	1	0
22	1	0	1	1	1	1	1	0
23	1	1	1	1	1	1	0	0
24	1	1	1	1	1	0	0	0
25	0	0	1	1	1	1	1	1
26	1	1	1	1	1	1	1	0
27	1	1	1	1	1	1	1	1
28	1	0	0	1	1	1	1	0
29	1	1	1	1	1	1	1	1
30	1	1	0	1	1	1	1	0
31	1	0	0	1	1	1	0	0
32	1	1	1	1	1	1	0	0
33	1	0	0	1	1	1	0	0
34	1	1	0	1	1	1	1	0
35	1	0	0	1	1	1	1	0
36	1	0	0	1	1	1	0	0
37	1	1	0	1	1	1	0	0
38	1	0	0	1	1	1	0	0
39	1	0	1	1	1	0	0	0
40	1	0	0	1	1	1	1	0
41	1	0	0	1	1	1	1	0
42	1	0	0	1	1	1	1	0
43	1	0	0	1	1	1	1	0
44	1	0	0	1	1	1	0	0
45	1	0	1	1	1	1	1	0
46	1	0	1	1	1	1	1	0
47	1	1	1	1	1	0	0	0
48	1	1	1	1	1	0	0	0
49	1	0	1	1	1	1	0	0
50	1	0	0	1	0	0	0	0
51	1	0	1	1	1	1	0	0
52	1	0	1	1	1	0	0	0
53	1	0	1	0	0	0	0	0
54	1	0	0	0	0	0	0	0
55	1	1	1	1	1	1	0	0

D1. Scoring band for primer 17898B

S.no	L1	L2	L3	L4	L5
1	1	1	1	1	1
2	1	1	1	0	1
3	1	1	1	0	1
4	1	1	1	0	1
5	1	1	1	0	1
6	1	0	1	0	1
7	1	1	1	1	1
8	1	0	1	0	1
9	1	0	1	0	1
10	0	0	0	0	0
11	1	0	1	0	1
12	1	0	0	0	1
13	1	0	1	0	1
14	1	1	1	0	1
15	1	1	1	1	1
16	1	1	1	0	1
17	0	0	0	0	0
18	1	1	1	0	1
19	1	1	1	0	1
20	1	1	1	0	1
21	0	0	0	0	0
22	1	0	0	1	1
23	1	1	1	1	1
24	1	1	1	1	1
25	1	1	1	1	1
26	1	1	1	1	1
27	1	1	1	1	1
28	1	1	1	1	1
29	1	1	1	1	1
30	1	1	1	1	1
31	1	1	1	0	1
32	1	1	1	0	1
33	1	1	1	0	1
34	1	1	1	0	1
35	1	1	1	1	1
36	1	0	1	0	1
37	1	1	1	0	1
38	1	1	1	1	1
39	1	0	1	0	1
40	1	1	1	0	1
41	1	0	0	1	0
42	1	1	1	1	0
43	1	1	1	1	1
44	1	1	1	1	1
45	1	1	1	0	1
46	1	0	1	1	1
47	1	1	1	0	1
48	1	1	1	0	1
49	1	1	1	0	1
50	1	1	1	1	1
51	1	0	0	1	1
52	1	1	1	1	1
53	1	1	1	0	1
54	1	0	1	1	1
55	1	1	1	0	1

E. Scoring band for primer 17899A

S.no	L1	L2	L3	L4	L5	L6	L7	L8	L9
1	1	0	1	0	0	0	1	1	1
2	1	0	1	0	1	1	1	0	1
3	1	1	1	1	1	1	1	1	1
4	1	0	1	0	0	1	1	0	1
5	0	0	0	0	0	0	0	1	0
6	1	0	1	1	1	1	0	0	1
7	1	1	1	0	0	1	1	0	1
8	1	1	1	1	0	1	1	1	0
9	1	1	1	1	1	1	1	1	0
10	1	1	1	1	1	1	1	1	1
11	1	1	1	1	0	1	1	1	1
12	1	1	1	1	1	1	1	0	1
13	1	0	1	1	0	1	1	0	1
14	1	1	1	1	1	1	1	1	0
15	0	0	1	1	1	1	1	1	1
16	1	1	1	0	1	1	1	0	0
17	1	0	1	1	1	1	1	1	1
18	1	0	1	1	0	1	1	0	1
19	1	1	1	0	1	1	1	0	0
20	1	1	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	1	0	0	0
23	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1
25	1	1	1	0	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1
27	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1
31	1	1	1	1	1	1	1	1	1
32	1	1	0	0	1	1	1	0	0
33	1	1	1	1	1	1	1	0	0
34	1	0	1	0	1	1	1	0	0
35	1	0	1	0	1	1	1	0	0
36	1	0	1	0	0	1	1	1	1
37	1	1	1	0	1	1	1	0	0
38	0	0	1	1	0	0	1	1	1
39	1	0	1	0	1	1	1	1	1
40	1	1	1	1	1	1	1	1	1
41	1	1	1	1	1	1	1	0	0
42	1	1	1	1	1	1	1	1	1
43	1	1	1	1	1	1	1	1	1
44	1	0	1	0	0	1	1	1	1
45	1	0	1	1	1	1	1	1	1
46	1	0	1	1	1	1	1	0	0
47	1	1	1	1	1	1	1	0	0
48	1	1	1	1	1	1	1	0	0
49	1	1	1	1	1	1	1	0	0
50	1	1	1	1	1	1	1	0	0
51	1	1	1	1	1	1	1	0	0
52	1	1	1	1	1	1	1	0	0
53	0	0	1	1	1	1	1	0	0
54	1	0	1	1	0	1	1	0	0
55	1	0	1	0	0	1	0	0	0

F. Scoring band for primer HB11

S.no	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	0	1	0	0	1	1	1	1	0	0	0
3	1	1	0	1	1	1	1	1	0	0	0	0
4	0	1	1	0	1	1	0	1	1	0	1	0
5	0	1	1	1	1	1	1	1	1	0	1	0
6	1	1	1	1	1	0	1	1	0	0	0	0
7	1	0	0	1	0	1	1	1	1	1	0	1
8	1	0	1	1	1	1	1	1	1	1	0	0
9	1	1	1	1	1	1	1	1	1	1	1	0
10	1	0	1	1	1	1	1	1	1	0	0	0
11	1	0	0	1	1	1	1	1	1	1	0	0
12	0	1	0	1	1	1	1	1	1	1	0	0
13	0	0	0	1	1	1	1	1	0	0	0	0
14	1	0	1	1	1	1	0	1	1	1	1	0
15	0	0	0	0	0	1	0	1	1	0	0	0
16	0	0	0	0	1	1	1	1	1	1	0	0
17	0	0	0	0	0	0	1	1	1	0	0	0
18	0	0	0	0	0	0	1	1	1	1	0	0
19	0	0	0	0	1	1	0	0	0	0	0	0
20	0	0	1	1	1	1	1	1	1	1	1	0
21	1	0	0	1	1	1	1	1	1	1	1	0
22	0	0	0	0	1	1	0	1	1	0	0	0
23	0	0	0	0	0	0	1	1	1	1	1	1
24	0	0	0	1	1	1	1	1	1	1	1	0
25	1	0	1	0	1	1	1	1	1	1	0	1
26	1	0	1	0	1	1	1	1	0	0	0	0
27	0	0	0	0	0	1	1	1	1	1	0	0
28	0	1	1	0	1	1	1	1	1	1	0	0
29	0	0	0	0	0	1	0	0	0	0	0	0
30	0	0	0	0	0	1	1	1	1	0	0	0
31	0	0	0	0	0	1	1	1	1	1	0	0
32	0	0	1	0	1	1	1	1	1	0	0	0
33	0	0	0	0	0	1	1	1	1	0	0	0
34	0	0	0	0	0	0	1	1	0	0	0	0
35	0	0	0	0	0	1	1	0	1	1	1	0
36	0	0	0	0	1	1	0	1	1	0	0	0
37	1	0	1	1	1	1	1	1	1	1	0	0
38	0	0	1	1	1	1	1	1	1	0	0	0
39	1	0	0	0	0	1	1	1	1	0	0	0
40	1	0	0	1	1	1	1	1	1	0	0	0
41	1	0	0	0	0	1	1	1	1	1	0	0
42	1	0	1	0	1	1	1	1	1	1	0	0
43	0	0	0	0	1	1	1	1	1	1	0	0
44	0	0	0	1	0	1	1	0	0	0	0	0
45	0	0	0	1	0	1	1	0	0	0	0	0
46	0	0	0	0	1	1	1	1	1	0	0	0
47	0	0	1	0	1	1	1	1	1	0	0	0
48	0	0	0	0	1	1	1	1	1	0	0	0
49	1	0	0	0	1	1	1	0	0	0	0	0
50	0	0	0	1	1	1	1	1	1	0	0	0
51	0	0	0	0	1	1	0	0	0	0	0	0
52	0	0	0	0	1	1	0	0	0	0	0	0
53	0	0	0	0	1	1	0	1	1	0	0	0
54	0	0	0	0	1	1	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0

G. Scoring band for primer HB12

S.no	L1	L2	L3	L4	L5	L6	L7	L8	L9
1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1	0
9	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1
19	1	1	0	0	0	1	1	1	1
20	1	1	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	1	1	1	1
23	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1
27	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1
30	0	1	1	1	1	1	1	1	1
31	0	0	1	1	1	1	1	1	1
32	1	1	1	1	1	1	1	1	1
33	1	1	1	1	1	1	1	1	1
34	1	0	1	1	1	1	0	1	0
35	1	1	1	1	1	1	1	1	1
36	0	1	1	1	1	1	1	0	0
37	0	1	1	1	1	1	1	1	0
38	1	1	1	1	1	1	0	1	1
39	0	0	0	0	1	1	0	0	0
40	0	0	0	0	0	0	0	0	0
41	1	1	1	1	1	1	0	0	0
42	1	1	1	0	1	1	0	0	0
43	1	1	1	1	1	1	0	0	0
44	1	1	1	1	1	1	1	0	0
45	1	1	1	1	1	1	1	0	0
46	1	1	1	1	1	1	1	0	0
47	1	1	1	1	1	0	0	0	0
48	1	1	1	0	1	1	1	0	0
49	1	1	1	1	1	1	1	1	0
50	1	1	1	1	1	1	1	1	0
51	1	1	1	1	1	1	1	1	0
52	1	1	1	1	1	0	1	0	0
53	0	0	0	0	1	1	1	1	1
54	1	1	1	1	1	1	1	0	0
55	1	1	1	1	1	1	1	0	0

H. Scoring band for primer HB13

S.no	L1	L2	L3	L4	L5	L6
1	1	1	1	1	0	0
2	0	1	1	1	1	0
3	1	1	1	1	1	0
4	1	1	1	1	1	0
5	1	1	0	1	1	0
6	1	1	1	1	0	0
7	1	1	1	1	1	0
8	1	1	1	1	1	0
9	1	1	1	1	1	0
10	1	1	1	1	1	0
11	1	1	1	1	1	0
12	1	1	1	1	1	0
13	1	1	1	1	1	0
14	1	1	1	1	1	0
15	1	1	1	1	1	0
16	1	1	1	1	1	0
17	0	0	0	1	1	0
18	1	1	1	1	1	1
19	1	1	1	1	1	1
20	1	1	1	1	1	1
21	1	1	1	1	1	1
22	1	1	1	1	1	1
23	1	1	1	1	1	1
24	1	1	1	1	1	1
25	1	1	1	1	1	1
26	1	1	1	1	1	1
27	1	1	1	1	1	1
28	1	1	1	1	1	1
29	1	1	1	1	1	1
30	1	1	1	1	1	1
31	1	1	1	1	1	1
32	0	0	0	1	1	0
33	1	1	1	1	0	0
34	1	1	1	1	1	1
35	1	0	0	0	0	1
36	1	0	0	0	0	1
37	1	0	0	0	0	1
38	1	0	0	0	0	1
39	1	0	0	0	0	1
40	0	0	0	0	0	0
41	0	1	0	0	0	0
42	1	0	0	1	1	0
43	0	0	0	0	1	1
44	0	0	0	0	1	1
45	0	0	0	0	1	1
46	0	0	0	0	1	1
47	0	0	1	1	1	0
48	1	1	1	1	1	1
49	1	1	1	0	1	1
50	1	1	1	0	1	1
51	1	1	1	1	1	1
52	1	1	1	1	1	1
53	1	0	0	0	1	1
54	1	0	0	1	1	1
55	1	0	0	0	1	0

I. Scoring band for primer HB14

S.no	Locus1	L2	L3	L4
1	0	1	0	0
2	0	1	0	1
3	0	1	1	1
4	0	1	0	1
5	0	1	0	1
6	0	1	0	1
7	0	1	0	0
8	0	1	0	1
9	1	1	1	1
10	1	1	0	0
11	1	1	0	0
12	1	1	0	0
13	0	1	0	1
14	1	1	1	0
15	1	1	0	1
16	1	1	0	1
17	1	1	0	0
18	0	1	0	0
19	0	1	0	1
20	1	1	0	1
21	0	1	1	0
22	0	1	0	1
23	1	1	0	1
24	0	1	1	1
25	0	1	1	0
26	0	1	0	1
27	1	1	1	1
28	0	1	0	0
29	1	1	1	1
30	1	1	0	1
31	0	1	0	0
32	0	1	1	0
33	0	1	0	1
34	0	1	1	1
35	0	1	1	1
36	0	1	0	0
37	0	1	0	1
38	1	1	0	0
39	0	1	1	0
40	0	1	0	0
41	0	1	1	1
42	0	1	1	0
43	0	1	0	1
44	0	1	0	1
45	0	1	1	0
46	0	1	0	0
47	0	1	0	0
48	0	1	0	1
49	0	1	0	0
50	0	1	1	0
51	0	1	0	1
52	0	0	0	1
53	0	1	1	0
54	0	1	0	0
55	0	1	0	0

J. Scoring band for primer HB15

S.no	Locus 1	L2	L3	L4	L5	L6	L7
1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1
4	0	1	1	1	1	1	1
5	0	1	1	1	1	1	1
6	0	1	1	1	1	1	1
7	1	1	1	1	1	1	0
8	1	1	1	1	1	1	1
9	1	1	1	1	1	1	0
10	0	1	1	1	1	1	1
11	0	0	1	1	1	1	0
12	0	1	1	1	1	1	1
13	0	1	1	1	1	1	1
14	0	1	1	1	1	1	0
15	1	1	1	1	1	1	1
16	1	1	1	1	1	1	0
17	0	0	1	1	1	0	0
18	0	1	1	1	1	1	1
19	1	1	1	1	1	1	1
20	0	1	1	1	1	1	1
21	0	1	1	1	1	1	0
22	0	1	1	1	1	1	0
23	0	0	0	1	1	1	1
24	0	1	1	1	1	1	1
25	0	0	0	1	1	1	1
26	0	0	0	1	1	1	1
27	1	1	1	1	1	1	0
28	0	0	0	1	1	1	1
29	0	0	0	1	1	1	1
30	0	0	1	1	1	1	1
31	1	1	1	0	0	0	0
32	1	1	1	0	0	0	0
33	1	1	1	0	0	0	0
34	1	1	1	0	0	0	0
35	1	1	1	0	0	0	0
36	1	1	1	0	0	0	0
37	1	1	1	0	0	0	0
38	1	1	1	0	0	0	0
39	0	0	0	1	0	0	0
40	0	0	1	1	0	0	0
41	1	1	1	0	0	0	0
42	1	1	1	0	0	0	0
43	0	0	0	1	0	0	0
44	1	1	1	0	0	0	0
45	1	1	1	0	0	0	0
46	1	1	1	0	0	0	0
47	1	1	1	0	0	0	0
48	1	1	1	0	0	0	0
49	1	1	1	0	0	0	0
50	0	0	0	1	0	0	0
51	1	1	1	0	0	0	0
52	1	1	1	1	1	0	0
53	1	1	1	0	0	0	0
54	0	1	1	1	1	0	0
55	1	1	1	0	0	0	0