GENETIC DIVERSITY OF ETHIOPIAN BREAD AND DURUM WHEAT VARIETIES AND ADVANCED LINES AS REVEALED BY SSR MARKERS

MSc THESIS

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June, 2015

Jimma, Ethiopia

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DEDICATION

This thesis is dedicated to my late mother Nigatwa Metaferiya.

STATEMENT OF THE AUTHOR

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List of Abbreviations and Acronyms

ADARC	Adet Agricultural Research Center
AFLP	Amplified Fragment Length Polymorphism
APS	Ammonium per Sulphate
CTAB	Cetyl-trimethylammonium bromide
CIMMYT	International Maize and Wheat Improvement Center
DBARC	Debre Birhan Agricultural Research Center
DH	Doubled Haploids
dNTP	Deoxy-ribose Nucleoside Tri-phosphate
DZARC	Debrezeit Agricultural Research Center
EDTA	Ethylene Diamine Tetra Acetic acid
EIAR	Ethiopian Institute of Agricultural Research
EST	Expressed Sequence Tag
GD	Genetic Distance
HARC	Holeta Agricultural Research Center
HPLC	High Performance Liquid Chromatography
ISSR	Inter Simple Sequence Repeats
KARC	Kulumsa Agricultural Research Center
MAB	Marker Assisted Breeding
MoARD	Ministry of Agriculture and Rural Development
NIL	Near Isogenic Lines
OARI	Oromia Agricultural Research Institute
PAGE	Poly-Acryl amide Gel Electrophoresis

PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant Inbred Lines
SARC	Sinana Agricultural Research Center
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Micro-satellite Sites
TBE	Tris-Borate EDTA
TEMED	Tetra-Methyl Ethylene Di-amine
UPGMA	Un-weighted Pair Group Method using Arithmetic mean
VNTR	Variable Number Tandem Repeats

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Abstract

About fifty eight bread and 34 durum wheat varieties have been released in Ethiopia until 2012. However, enough work has not been done on the genetic relationships among these varieties. Hence, the present study was initiated with the aims of determining the genetic variation among wheat varieties released in the country and to assess the achievement of wheat variety development program of Ethiopia in terms of developing genetically diverse varieties. Forty one bread and twenty four durum wheat varieties obtained from different breeding centers were used for this study. DNA was extracted using the CTAB method (Doyle & Doyle, 1990). PCR was conducted using 15 SSR markers, and allele separation was performed using polyacrylamide gel on vertical electrophoresis apparatus. The result showed that the number of alleles per locus ranged from 4-16 and 2-15 for bread and durum wheat varieties, respectively. PIC ranged from 0.16 (Xgwm257) to 0.83 (Xgwm136) with an average of 0.56 among bread wheat and from 0.36 (Xgwm257) to 0.89 (Xgwm292) with an average of 0.63 among durum wheat varieties. The genetic distance between Galema and K-6290 bulk was the lowest (0.07) whereas that of Shina and Bobicho was the highest (0.12) among bread wheat varieties. On the other hand, the genetic distance between Werer and Gerardo was the lowest (0.07) whereas that of Selam and Candidate#4 was the highest (0.14) among durum wheat varieties. Both bread wheat varieties with the lowest genetic distance were obtained from KARC and that of durum wheat were obtained from DZARC. The most distant bread wheat variety (Shina) and durum wheat variety (Selam) were obtained from ADARC. There were three main clusters for both bread and durum wheat varieties. Based on the pair wise comparison in the distance matrix all of bread as well as durum wheat varieties shared more than 88% of their marker alleles, which indicates low genetic diversity among the varieties. It could be concluded that the wheat breeding program in Ethiopia was unable to produce more diverse varieties with a wide genetic base. Therefore, it is recommended that future breeding programs should focus on widening the genetic base of the country's commercial varieties to minimize risks emanating from biotic and abiotic production constraints.

Key words: *T. aestivum*, *T. durum*, PCR, PAGE, Genetic Distance

1. INTRODUCTION

Wheat (*Triticum spp*.) belongs to the family Poaceae, and is a result of crossing between three wild species. The wild diploid wheat (*T. urartu*, AA) crossed with goat grass (*Aegilops speltoid*, BB) 300, 000 years ago produced wild Emmer (*T. dicoccoides*, AABB) after spontaneous chromosome doubling. The domestication of wild emmer 10, 000 years ago produced the cultivated Emmer (*T. dicoccum*). Later cultivated emmer again crossed with goat grass (*Aegilops tauschii*, DD) in the wild to produce the hexaploid spelt wheat (*T. spelta*, AABBDD) after spontaneous chromosome doubling. Finally, *T. spelta* was domesticated to produce the present day allohexaploid, free threshing bread wheat (*T. aestivum* L.). On the other hand, cultivated emmer gave rise to the present day durum wheat (*T. durum* L.), which is free threshing allotetraploid wheat- through domestication (Peng *et al*., 2011).

Wheat has a determinate, composite spike inflorescence. A spike may be awnless, awnleted, or awned. A spikelet consists of 1–5 flowers (or florets) attached alternatively to opposite sides of the rachilla (central axis). A floret consists of a lemma and palea, which enclose these stamens and a pistil, plus two lodicules that regulate the opening of the flowers and anthers. Wheat flowers bloom under temperatures of 13–25°C. The flowering is usually diurnal, the highest peak occurring in the morning, and a lower peak in the afternoon. Blooming begins in the spikelets located above the middle of the spike and proceeds both upward and downward. It takes about 2–3 days for a wheat spike to complete blooming, after the appearance of the first anthers. The flowering period may last from 14 to 21 days. Wheat is predominantly self-pollinated with about1– 4% natural cross-pollination. Pollen shed usually starts inside the floret, but about 80% of anther dehiscence occurs outside the floret. The primary and secondary florets produce larger and more viable pollen grains than other florets. Wheat pollen remains viable for up to about 30 minutes after shedding. Once pollinated, the pollen tube growth starts within 15– 60 minutes. Even though the stigma remains receptive for up to 13 days, it is most receptive within 3 days of anthesis (Acquaah, 2007).

Wheat has been one of the world's major food sources since its time of domestication (Peleg *et al*., 2011). In Sub-Saharan Africa Ethiopia is the second largest producer of wheat next to South Africa (Demeke and Di Marcantonio, 2013). According to CSA (2014) wheat is the third largest cereal produced in Ethiopia next to maize and tef. Unlike other cereal crops

wheat in Ethiopia is grown in altitude range of 1600 to 3200 m.a.s.l, average annual rainfall of 400-1200mm and average annual temperature of 25^{o}C (NRC, 1996).

Wheat is used for the manufacture of flour for different purposes such as bread, biscuits and pasta products such as macaroni, spaghetti and noodles. Traditionally, wheat is used for making bread ("*dabo*"), *dabokolo*, *ganfo*, *kinche*, local beer ("*tella*"), *chachabsa* (Namara and Manig, 2000) and other types of food. The straw is good source for animal feed and is also used for thatching roofs (Demeke and Di Marcantonio, 2013).

Wheat breeding in Ethiopia dates back to 1949 with the start of durum wheat breeding at the Paradiso Experiment Station near Asmara, Eritrea Administrative Region. The main objective of wheat breeding in Ethiopia has been to search for widely adapted high yielding and disease resistant durum wheat varieties through selections from indigenous germplasm, introductions and hybridization. Initially, the program partly consisted of mass selection from local cultivars and introductions from abroad (van Ginkel and Tanner, 1987).

Production of wheat is usually impaired by biotic and abiotic factors. Among the biotic factors rust is one of the most important diseases causing a greater loss of wheat from field. Stem rust (caused by *Puccinia graminis* Pers.) is the most important among the rust diseases (Acquaah, 2007).

Up to the year 2012, 58 bread wheat and 34 durum wheat varieties have been released across the country (MoARD, 2012).

Plant breeding is highly dependent on identification and utilization of genetic variation. Thus, breeders have to make decisions on the selection of the most appropriate parents for initial crosses and then on the selection strategies of the most desirable individuals from their progenies. Large breeding programs for annual crops may need hundreds to thousands of lines to produce a new variety once in a few years. These make field trials and evaluation of quality and yield stability expensive to assess. Molecular markers have proved to be a powerful tool in replacing bioassays and there are now many examples available to show the efficacy of such markers (Langridge and Chalmers, 2004; Sönmezoğlu *et al*., 2012).

Even though, the development of molecular markers has enabled breeders to assess the genetic distance among plant varieties with in a population and/or between different plant populations, enough work has not been done on assessment of genetic diversity of wheat varieties released in Ethiopia. Hence, determining the genetic relatedness is important to determine the level of diversity among the released wheat varieties, and to develop an appropriate breeding strategy in the country. Therefore, the present study was initiated with the aim of determining the genetic variation among wheat varieties released in the country using SSR markers.

Objectives:

- To determine the genetic distance among released bread and durum wheat varieties and advanced lines.
- To assess the achievement of the wheat variety development of Ethiopia in terms of developing genetically diverse varieties.

2. LITERATURE REVIEW

2.1. The Biology, Ecology, Taxonomy and Genomics of wheat

Wheat (*Tricticum* spp.) is an annual plant having a spikelet inflorescence. A floret is composed of a lemma, palea, and a caryopsis or grain that has a deep furrow and a hairy tip or brush. The floret may be awned or awn less. Awned varieties are common in regions of low rainfall and warm temperatures. The presence of awns also tends to influence transpiration rate, accelerating the drying of ripe grain. Consequently, the tips of awn less spikes tend to be blasted in hot dry weather. The grain may also be amber, red, purple, or creamy white in color (Acquaah, 2007).

Under normal high density production conditions, a wheat plant may produce 2-3 tillers. However, when amply spaced on fertile soils, a plant may produce 30-100 tillers. The spike (head) of a plant may contain 14-17 spikelets, each spike containing about 25-30 grains. Large spikes may contain between 50 and 75 grains. The grain size varies within the spikelet, the largest being the second grain from the bottom and decreasing in size progressively towards the tip of the spike. Wheat is predominantly self-pollinated plant with about 1– 4% natural cross pollination. Pollen shed usually starts inside the floret, but about 80% of anther dehiscence occurs outside the floret. Anthers assume a pendant position soon after the flower opens. Blooming occurs at temperatures between 13 and 25°C starting with the spikelet around the middle of the spike and proceeding upwards and downwards. The wheat kernel or berry is a caryopsis about 3-10mm long and 3-5 mm wide. It has a multilayered pericarp that is removed along with the testa, nucellus, and aleurone layers during milling. The endosperm makes up about 85% of a well-developed kernel. Below the aleurone layer occurs a complex protein called gluten that has cohesive properties. It is responsible for the ability of wheat flour to hold together, stretch, and retain gas as fermented dough rises. This property is available to the flour of only one other species, rye flour (Acquaah, 2007).

Wheat (*Triticum* ssp.) is a monocotyledonous plant of the Gramineae family and of the Triticeae tribe and belongs to the genus Triticum. The species of *Triticum* are grouped into three ploidy classes: diploid (2n= 2X =14), tetraploid (2n=4X =28), and hexaploid (2n=6X =42). Three genomes (A, B, and D) comprise the polyploid series of wheat. The A genome

comes from *T. monococcum*, while the D comes from *T. tauschii*. The origin of the B genome is debatable. The genomic formula of the ploidy classes are AA for diploids and AABB for tetraploids or emmer wheat. Common wheat (*T. aestivum* L.) is an allohexaploid of genomic formula AABBDD. In hexaploid wheat, the 21 chromosomes are divided into seven homeologous groups (partially homologous chromosomes) identified with numbers from 1 to 7. The three chromosomes within the ABD homeologous group usually share some loci in common for a specific trait. An example of this is that there are two genes for rust resistance on chromosome 2A, three genes on 2B, and three genes on 2D.Tetraploid and hexaploid wheat reproduce naturally as diploids (2n=28 or 2n =42). This reproductive mechanism is made possible by the presence of a gene on chromosome 5B, Ph1, which enables diploid pairing to occur. The Ph1gene causes truly homologous paring within the same genome. When absent, paring between one chromosome and a homeologous chromosome from another genome is possible. The homeology that exists in its three component genomes allows the species to tolerate a range of aneuploidy (Acquaah, 2007).

Durum wheat is a separate species from the other commercially grown wheat classes grown in Canada (which are almost entirely *T. aestivum* L.), and possesses unique quality characteristics that differentiates durum wheat from other classes of wheat. The principal use of durum wheat grain is the production of semolina for use in pasta products. However, in North Africa, durum is preferred for the production of couscous and bulgur. Traditional breads are also made with durum flour, particularly in Morocco. Durum (derived from the Latin word for hard) has the hardest kernel of all wheat types. Durum wheat with high protein content and gluten strength is the preferred choice of processors for producing pasta products. Durum kernels are amber-colored and larger than those of other wheat classes. Also unique to durum is its yellow endosperm, which gives pasta its golden color. Durum wheat with strong gluten characteristics forms strong, non-sticky dough ideal for pasta production. Semolina with strong gluten properties also results in pasta products with superior cooking characteristics. In Canada, two sub-classes of durum wheat are recognized: conventional varieties with moderate gluten strength, and extra strong varieties with extra-strong gluten properties similar to the USA desert durum wheat varieties (Clarke *et al*., 2005).

2.2. Origin and Distribution of Wheat

Evidences from archaeological findings reveal that the domestication of wheat took place over a very long period. Wheat cultivation was reported about 6000 years ago in the middle Mesopotamian Fertile Crescent, and from there it apparently spread to the Middle East, North Africa, Asia and Europe. Wheat spreads to the Americas and South Africa around 1500 AD, and it was introduced into Australia in 1790. In the present day wheat is the most widely and diversely grown food crop in the world. It is grown under varied agro-ecologic conditions and at different altitudes, from sea level to 4500m, which reflects its cultivability and adaptability (Tiwari and Shoran, 2000).

According to Vavilov, the diversity in Ethiopian wheat varieties comprises six wheat species: *Triticum durum* subsp. Abyssinicum; *T. turgidum* subsp. abyssinicum; *T. dicoccum*; *T. aestivum*; *T. polonicum* and *T. compactum*. Currently, the five tetraploid species listed above are classified under *T. turgidum*. All these species of wheat observed by Vavilov in the mid-1920s are still grown by farmers as landraces. Although Vavilov regarded the Ethiopian region as a centre of origin and diversity for tetraploid wheat, the absence of wild relatives and lack of archaeological evidences suggest that Ethiopia could be a secondary centre of origin. The diploid einkorn and the hexaploid wheat do not seem to be native to the Ethiopian gene centre (IBC, 2007).

2.3. Molecular Characterization

Molecular characterization of plant germplasm has gained great importance for both the quantity and quality of results obtained. Previously, these results could not be obtained, as they were based on characterizing the phenotype especially morphologically, and, to a lesser extent, biochemically. Now, a large variety of molecular methodologies, based on DNA, are available, making the direct characterization of the genotype possible. Hence, these modern methodologies provide the means of knowing the genetic diversity of a germplasm collection. The measurement of genetic distance (GD) can be used as an estimation of expected genetic variances in different sets of progenies derived from different crosses, which is vital for plant breeding programs to allow the production of new varieties that are aimed towards the improvement of crop productivity and able to withstand damage from biotic and abiotic factors (Korzun, 2003).

Currently, markers are now being used. These markers have a DNA molecular nature and are highly sensitive to changes in the genotype of individuals. This situation permitted major advances in studies on the genetic characterization of plant germplasm. The selection of the marker to use depends on the study objectives, the availability of germplasm to characterize, cost, and, the markers inherent characteristics. Research on DNA- based technologies has been favored with the availability of numerous markers such as those based on restriction fragment length polymorphisms (RFLP) and the polymerase chain reaction (PCR). From these two techniques multiple techniques have derived, for example, random amplified polymorphic DNA (RAPD); amplified fragment length polymorphism (AFLP); and variable number tandem repeats (VNTR), that is, both minisatellites and microsatellites (simple sequence repeats or SSR). These markers can be used for characterization of plant genetic resources. A marker is said to be polymorphic when it presents differences in molecular weight, enzymatic activity, structures, or restriction sites (Pineda *et al*., 2007).

2.4. Molecular/Genetic Markers

A molecular marker is DNA sequence that is readily detected and whose inheritance can be easily be monitored. The uses of molecular markers are based on the naturally occurring DNA polymorphism, which forms basis for designing strategies to exploit for applied purposes. A marker must to be polymorphic i.e. it must exist in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by a marker it also carries. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two discontinuous variants or genotypes. DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Unlike protein marker, DNA marker segregate as single gene and it is not affected by the environment. DNA is easily extracted from plant materials and its analysis can be cost and labor effective. Genetic markers are simply landmarks on chromosomes that serve as reference points to the location of other genes of interest when a genetic map is constructed. Breeders are interested in knowing the association (linkage) of markers to genes controlling the traits they are trying to manipulate. The rationale of markers is that an easy-to-observe trait (marker) is tightly linked to a more difficult-to-observe and desirable trait. Hence, breeders select for the trait of interest by indirectly selecting for the marker (that is readily assayed or detected or observed). When a marker is observed or detected, it signals that the trait of interest is present (by association) (Kumar *et al*., 2009).

Genetic markers can be detected at both the morphological level and the molecular or cellular level – the basis for classification of markers into two general categories as morphological markers and molecular markers. Morphological markers are manifested on the outside of the organism as a product of the interaction of genes and the environment (i.e., an adult phenotype). On the other hand, molecular markers are detected at the sub-cellular level and can be assayed before the adult stage in the life cycle of the organism. Molecular markers of necessity are assayed by chemical procedures and are of two basic types – protein and DNA markers. For the generation of molecular markers based on protein polymorphisms, the most frequently used technique is the electrophoretic separation of proteins, followed by specific staining of a distinct protein subclass. Less commonly, specific proteins are detected by monoclonal antibodies with an attached fluorescent label. Although some earlier studies focused on seed storage protein patterns, the majority of protein markers are derived from allozymes (Weising *et al*., 2005).

Allozyme analysis is relatively straight forward and easy to carry out. A tissue extract is prepared and electrophoresed on a non- denaturing starch or polyacrylamide gel. The proteins of this extract are separated by their net charge and size. After electrophoresis, the position of a particular enzyme in the gel is detected by adding a colorless substrate that is converted into a dye under appropriate reaction conditions. Depending on the number of loci, their state of homo- or heterozygosity, and the enzyme configuration (i.e., the number of separable subunits), from one to several bands are visualized. The positions of these bands can be polymorphic and thus informative (Weising *et al*., 2005).

According to Acquaah (2007) Chemical assays for isozymes (multiple forms of enzymes) marked the beginning of the practical application of molecular markers. The assays detect variations in protein products (products of translation) not variation in DNA. Their use is limited by the insufficient number of assays available (only about several dozen protocols exist), and their uneven distribution on the genetic map. DNA markers arrived on the scene with the discovery of restriction fragment length polymorphisms (RFLPs) in the 1980s, and are superior to both morphological and isozyme markers. Since then, several other molecular markers have been developed. DNA markers that are currently widely used include RFLP, AFLP (amplified fragment length polymorphism), SNPs (single nucleotide polymorphisms), and microsatellites or simple sequence repeats (SSRs). Molecular markers have been taken, in

recent years, to refer to assays that allow the detection of specific sequence differences between two or more individuals. However, it should be recognized that iso-enzyme and other protein-based marker systems also represent molecular markers and were in wide use long before DNA markers became popular. One of the earliest types of DNA-based molecular markers, restriction fragment length polymorphisms (RFLPs), was based around the detection of variation in restriction fragment length detected by Southern hybridization. The types of sequence variation detected by this procedure could be caused by single base changes that led to the creation or removal of a restriction endonuclease recognition site or through insertions or deletions of sufficient size to lead to a detectable shift in fragment size. This technique has been largely superceded by microsatellite or simple-sequence repeat (SSR) markers and is now rarely used in screening material for breeding programs, but it remains an important research tool. Molecular markers are classified in various ways, including a genetic basis and an operational basis. Various systems are used to assay molecular markers. There are enzyme-based markers (isozymes) and DNA-based markers (require hybridization between a probe and homologous DNA segment(s) within the genome). PCR-based molecular markers have the advantage of requiring small amounts of DNA and being relatively quick to assay. On the basis of genetic characteristics, molecular markers may be grouped into two general categories:

1. Single-locus, multi-allelic, co-dominant markers. Examples are RFLPs and microsatellites (SSRs). Microsatellites are capable of detecting higher levels of polymorphisms than RFLPs.
2. Multi-locus, single-allelic, dominant markers. Examples are AFLPs and RAPD.

The analysis of genetic diversity and relatedness between or within different populations, species, and individuals is a central task for many disciplines of biological science. During the last three decades, classical strategies for the evaluation of genetic variability, such as comparative anatomy, morphology, embryology, and physiology, have increasingly been complemented by molecular techniques. These include, for example, the analysis of chemical constituents (so-called metabolomics), but most importantly relate to the development of molecular markers. Marker technology based on polymorphisms in proteins or DNA has catalyzed research in a variety of disciplines such as phylogeny, taxonomy, ecology, genetics, and plant and animal breeding. The following properties would generally be desirable for a molecular marker: Moderately to highly polymorphic (It must be polymorphic as it is polymorphism that is measured for genetic diversity studies); Co- dominant inheritance

(which allows the discrimination of homo- and heterozygous states in diploid organisms); Unambiguous assignment of alleles; Frequent occurrence in the genome; Even distribution throughout the genome; Selectively neutral behavior (i.e., no pleiotropic effects); Easy access (i.e., by purchasing or fast procedures); Easy and fast assay (e.g., by automated procedures); High reproducibility; Easy exchange of data between laboratories and Low cost for both marker development and assay (Maheswaran, 2004).

No single type of molecular marker fulfills all of these criteria. However, one can choose between a variety of marker systems, each of which combines some or even most of the above-mentioned characteristics (Weising *et al*., 2005).

2.4.1. Applications of Molecular Markers

Molecular markers produced a greater impact on genome mapping, gene tagging and evolutionary studies of crop plants. As far as mapping genomes and genes is concerned, the success depends on the availability of suitable base populations of F2s, backcross progenies, Doubled Haploids (DH), Recombinant Inbred Lines (RIL), and Near Isogenic Lines (NIL). Exploiting the available populations in conjunction with molecular marker techniques, molecular linkage maps have been constructed for several crop species and very many major and minor genes have been mapped with molecular markers. These "molecular and gene tags" are to be used to exercise marker aided selection, map based cloning and physical mapping of genes of agronomic importance. There are success stories on cloning genes based on their map positions. Apart from genome/gene mapping, molecular markers are employed in assessing the extent of genetic diversity in plant populations (Maheswaran, 2004).

Applications of these markers for genetic studies of cereals have been so much diverse. Main uses include: Assessment of genetic variability and characterization of germplasm; Identification and fingerprinting of genotypes; Estimation of genetic distance between population, inbreeds, and breeding materials; Detection of monogenic and quantitative trait loci (QTL); Marker assisted selection; Identification of sequences of useful candidate genes; etc. (Korzun, 2003).

2.4.2. Common Types of DNA Markers

2.4.2.1. Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of particular *Restriction Endonucleases*, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. This technique is mainly based on the special class of enzyme i.e. Restriction Endonucleases (Kumar *et al*., 2009).

In RFLP, DNA polymorphism is detected by hybridizing a chemically labelled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profile. This differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms. The RFLP markers are relatively highly polymorphic, co-dominantly inherited and highly reproducible. Because of their presence throughout the plant genome, high heritability and locus specificity the RFLP markers are considered superior. The method also provides opportunity to simultaneously screen numerous samples (Agarwal *et al*., 2008).

2.4.2.2. Random Amplified Polymorphic DNA

RAPD is a PCR-based technology. The method is based on enzymatic amplification of target or random DNA segments with arbitrary primers. In 1991 Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermo cyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals. However, due to the stoichastic nature of DNA amplification with random sequence primers, it is important to optimize and

maintain consistent reaction conditions for reproducible DNA amplification. RAPDs are DNA fragments amplified by the PCR using short synthetic primers (generally 10bp) of random sequence. These oligo-nucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1–10 genomic sites simultaneously. Amplified products (usually within the 0.5–5 kb size range) are separated on agarose gels in the presence of Ethidium bromide and view under ultraviolet light and presence and absence of band will be observed (Kumar *et al*., 2009).

2.4.2.3. Amplified Fragment Length Polymorphism

AFLP is a multiplex PCR based method in which a subset of restriction fragments are selectively amplified using oligo-nucleotide primers complementary to sequences that have been ligated to each end. AFLP analysis allows the reliable identification of over 50 loci in a single reaction. This technique combines the reliability of the RFLP and ease of the PCR and thus AFLP is a new typing method for DNA of any origin or complexity (Maheswaran, 2004).

To overcome the limitation of reproducibility associated with RAPD, AFLP technology was developed. It combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers (Agarwal *et al*., 2008).

Thus, the technique involves four steps: (1) restriction of DNA and ligation of oligo-nucleotide adapters (2) pre-selective amplification (3) selective amplification (4) gel analysis of amplified fragments. AFLP is a DNA fingerprinting technique, which detects DNA restriction fragments by means of PCR amplification. AFLP involves the restriction of genomic DNA, followed by ligation of adaptors complementary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments (Kumar *et al*., 2009).

2.4.2.4. Single Nucleotide Polymorphism

Single nucleotide variations in genome sequence of individuals of a population are known as SNPs. They constitute the most abundant molecular markers in the genome and are widely

distributed throughout genomes although their occurrence and distribution varies among species (Agarwal *et al*., 2008).

SNPs are bi-allelic markers, which unravels polymorphism between individuals due to change of a single nucleotide. These single nucleotide variations arise because of point mutations. The detection of SNPs needs only a plus/minus assay with automation. Both gel-based and non-gel-based methods are being used to detect these abundant polymorphisms. However, adequate sequence information is necessary. In case of gel-based methods, specific primers are designed, which would cause amplification of a positive allele due to exact match of primer. Mismatches in primer design and/or DNA sequence fail to give amplification of a negative allele. In case of non-gel-based methods, appropriate regions are amplified and then mismatches detected by techniques such as denaturing High Performance Liquid Chromatography (HPLC) or Matrix- Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDITOFMS). Both these techniques are extremely sensitive and can detect mismatches from a few down to only one nucleotide (Maheswaran, 2004).

SNPs (pronounced “snips”) are single-base pair positions in the genomes of two (or more) individuals, at which different sequence alternatives (alleles) exist in populations. Per definition, the least frequent allele should have an abundance of at least 1%. Among the many types of mutations naturally occurring in genomes, single nucleotide exchanges (i.e., base substitutions) stand out by their sheer numbers per genome, their relatively low mutation rates (as opposed to, e.g., microsatellites of all types; their even distribution across the genomes, and their relative ease of detection). In addition to SNPs, the presence vs. absence of small insertions and deletions (indels) are receiving increasing attention as potential bi-allelic markers (Weising *et al*., 2005).

2.4.2.5. Microsatellites or Simple Sequence Repeat

Molecular markers responsible for various revolutions in the field of molecular genetics revolution are micro satellites arrays of tandemly repeated di, tri, tetra and penta-nucleotide DNA sequences which occur dispersed throughout the genomes of all eukaryotic organisms investigated to date. The micro satellites are otherwise called as Sequence Tagged Micro satellite Sites (STMS) or Simple Sequence Repeats (SSR). SSRs are currently considered the molecular markers of choice within the genome mapping community and are rapidly being

adopted by plant researchers as well. SSRs consist of around 10- 50 copies of motifs from 1 to 5 base pairs that can occur in perfect tandem repetition, as imperfect (interrupted) repeats or together with another repeat type. These repeated motifs are flanked by unique or single copy sequences, which provide a foot hold for specific amplification *via* PCR. Primers complimentary to the unique sequences in those flanking regions can be designed to amplify single copy products (Maheswaran, 2004).

Recent developments that have occurred in molecular markers for many crop species have major implications for the future of the technology. Of particular importance was the development of microsatellite or SSR markers that now form the basis for analysis and allow highly multiplexed SSR screens. The technological advances have improved our capacity for whole genome screens (Langridge and Chalmers, 2004).

Duwayri *et al*. (2007) demonstrated the use of SSR markers for characterizing cultivated durum wheat and its naturally occurring hybrids with wild wheat and demonstrated the efficiency of SSR technology for polymorphism detection and introgression assessment in wheat, thereby offering wide scope of applications in marker aided breeding programs of wheat.

Emon *et al*. (2010) conducted characterization and genetic diversity of wheat genotypes in relation to boron use efficiency using SSR markers and identified the most boron efficient genotypes and suggested those varieties could be used for developing boron efficient varieties.

Sehgal *et al*. (2012) conducted molecular characterization and genetic diversity of wheat genotypes using SSR markers and suggested the identification of rust resistant genes in wheat germplasm that will help in accelerating the breeding program in future, including pyramiding of different rust resistant genes in wheat genotypes and varieties.

Furthermore, different works have been conducted by various authors using SSR markers. Such as; Screening of drought tolerance wheat genotypes (Bousba *et al*., 2012); Identification and mapping of alien segments carrying genes for effective resistance to leaf rust in bread wheat (Iqbal *et al*., 2007); Identification of Fusarium head blight resistance QTLs in wheat population (Najaphy *et al*., 2006); Assessment on genetic diversity of wheat to improve the

yellow rust resistant breeding programs (Jamalirad *et al*., 2012); Association mapping of agronomic traits (Liu *et al*., 2010); and Parental selection (Casassola *et al*., 2013).

SSR markers were markers of choice because of their reproducibility, their even distribution throughout the genome, their co-dominance nature and their availability in Holetta biotechnology laboratory.

3. MATERIALS AND METHODS

3.1. Plant Materials

Seeds of 41 bread wheat and 24 durum wheat varieties were obtained from different breeding centers of Ethiopia (Table 1 and 2). Seedlings of these varieties were raised in small pots in the green house of Holetta Agricultural Research Center (HARC) - Biotechnology Laboratory. Young, vigorously growing fresh leaf samples were collected from two weeks old seedlings to extract genomic DNA.

Table 1: List of released and advanced bread wheat varieties of Ethiopia used for the study

Variety	Year of Release	Breeder/Maintainer	Production Status
ETBW 6008	Advanced Line	KARC/EIAR	----------
ETBW 6006	Advanced Line	KARC/EIAR	----------
ETBW 5496 (Hulluka)	2012	KARC/EIAR	Under Production
ETBW 5520 (Ogolcho)	2012	KARC/EIAR	Under Production
Hoggana	2011	KARC/EIAR	Under Production
Shorima	2011	KARC/EIAR	Under Production
Kekeba	2010	KARC/EIAR	Under Production
Danda'a	2010	KARC/EIAR	Under Production
Millennium	2007	KARC/EIAR	Under Production
Gassay	2007	ADARC/ARARI	Under Production
Dinknesh	2007	ARARC/ARARI	Under Production
Alidoro	2007	KARC/EIAR	Under Production
Menze	2007	DBARC/ARARI	Under Production
Mararo	2005	KARC/EIAR	Under Production
Tay	2005	ADARC/ARARI	Under Production
Senkegna	2005	ADARC/ARARI	Under Production
Digalu	2005	KARC/EIAR	Under Production
Bobicho	2002	KARC/EIAR	Under Production
Densa	2002	ADARC/ARARI	Under Production
KBG-01	2001	KARC/EIAR	Under Production
Dure	2001	SARC/OARI	Under Production
Sirbo	2001	KARC/EIAR	Under Production
Doddota	2001	KARC/EIAR	Under Production
Guna	2001	ADARC/ARARI	Under Production
Simba	1999/00	KARC/EIAR	Under Production

Sofoumar	1999/00	SARC/OARI	Under Production
Madda walabu	1999/00	SARC/OARI	Under Production
Hawi	1999/00	KARC/EIAR	Under Production
Katar	1998/99	KARC/EIAR	Under Production
Shina	1998/99	ADARC/ARARI	Under Production
Tuse	1997	KARC/EIAR	Under Production
Abola	1997	KARC/EIAR	Under Production
Galema	1995	KARC/EIAR	Under Production
Kubsa	1995	KARC/EIAR	Under Production
Mitike	1994	KARC/EIAR	Under Production
Dashen	1984	KARC/EIAR	Under Production
Pavon-76	1982	KARC/EIAR	Under Production
Et-13A2	1981	KARC/EIAR	Under Production
K6295-4A	1980	KARC/EIAR	Under Production
K6290 bulk	1977	KARC/EIAR	Under Production
Dereselgn	1974	KARC/EIAR	Under Production

Source: MoARD

Table 2: List of released and advanced durum wheat varieties of Ethiopia used for the study

Varieties	Year of Release	Breeder/Maintainer	Production Status
Candidate#4	Advanced Line	DZARC/EIAR	----------
Candidate #5	Advanced Line	DZARC/EIAR	----------
DW/SR	Advanced Line	DZARC/EIAR	----------
Werer	2009	DZARC/EIAR	Under Production
Hitosa	2009	DZARC/EIAR	Under Production
Denbi	2009	DZARC/EIAR	Under Production
Mosobo	2004	ADARC/ARARI	Under Production
Metaya	2004	ADARC/ARARI	Under Production
Selam	2004	ADARC/ARARI	Under Production
Megenagna	2004	ADARC/ARARI	Under Production
Yerer	2002	DZARC/EIAR	Under Production
Ude	2002	DZARC/EIAR	Under Production
Ginchi	1999/00	DZARC/EIAR	Under Production
Asassa	1997	DZARC/EIAR	Under Production
Robe	1997	DZARC/EIAR	Under Production
TOB-66	1996	DZARC/EIAR	Under Production
Quamy	1996	DZARC/EIAR	Under Production
Bichena	1995	DZARC/EIAR	Under Production
Kilinto	1994	DZARC/EIAR	Under Production
Foka	1993	DZARC/EIAR	Under Production
Boohai	1982	DZARC/EIAR	Under Production

Cocorit/71	1976	----------	Out of Production
Gerardo	1976	----------	Out of Production
Arendato	1966	DZARC/EIAR	Out of Production

Source: MoARD

3.2. DNA Extraction and PCR

Genomic DNA of 60 wheat varieties 5 advanced lines (41 bread and 24 durum wheat) were extracted from fresh leaves following the CTAB protocol (Doyle and Doyle, 1990) (Annex 1). The quantity and quality of the genomic DNA was determined using Nanodrop spectrophotometer and agarose gel electrophoresis. Finally, the DNA samples were diluted to a concentration of 25ng/µl.

PCR was performed using a total of 15 primer pairs which covers 2 chromosomes from the sub - genome A (1A & 7A), 5 chromosomes from the sub-genome B (2B, 3B, 4B, 5B, and 6B) and 3 chromosomes from the sub-genome D (2D, 3D & 5D).

As optimized for the present study each PCR mixture was prepared in 10µl reaction volume; containing 7.22 µl of RNase/DNase free water, 1.0 µl of 10X Reddy mix Buffer with $MgCl_2$ (15mM), 0.2 µl of dNTPs (conc. 20mM), 0.2 µl of each primer pair, 0.18 µl of thermo prime Taq DNA polymerase (conc. 5U/µl), and 1 µl template DNA (conc. 25ng/µl). The master mix was prepared in 2ml tube using the following steps: RNase/DNase free water, 10X Reddy mix Buffer with $MgCl_2$, dNTPs, forward (1 µmol/µl) and reverse primer (10 µmol/µl), and Taq DNA polymerase were added in 2ml tube. The mixture was then vortexed briefly and centrifuged. Next 9 µl from the mixture was distributed in to wells on the PCR plate. Finally, 1 µl of template DNA from each genotype was pipetted in to PCR plate wells containing the mixture (one well for one genotype) for amplification. The PCR plate was then tightly sealed, and placed on Techne Thermal Cycler (TC-4000) for amplification.

The PCR amplification protocol was optimized by running gradient PCR starting from 51 to 65^{o}C for each primer pairs. After identification of best annealing temperatures working for most of the primer pairs (55 and 62^{o}C) the following program was prepared for the normal PCR and saved on the thermo cycler (TC- 4000).

There was an initial denaturation at 94^{o}C for 5 min, followed by three stages. Stage one had 12 cycles of denaturation at 94^{o}C for 30 sec followed by annealing at 62^{o}C for 30 sec and an extension at 72^{o}C for 30 sec. The second stage had 35 cycles of denaturation at 94^{o}C for 5 min, followed by annealing at 55^{o}C for 30 sec, and extension at 72^{o}C for 30 sec. There was a final extension period at 72^{o}C for 10 min.

Table 3: List of wheat SSR primer pairs, locus names, chromosome location, repeat types, annealing temperature and fragment length

Locus	Chromosome-Location	Forward Primer	Reverse Primer	Repeat Type	Annealing Temp.	Opata. (bp)	Synth. (bp)
*Xgwm*114	3D	ACA AAC AGA AAA TCA AAA CCC G	ATC CAT CGC CAT TGG AGT G	(GA)53	60^{o}C	134	181
*Xgwm*136	1A	GAC AGC ACC TTG CCC TTT G	CAT CGG CAA CAT GCT CAT C	(CT)58	60^{o}C	278	321
*Xgwm*192	5D	GGT TTT CTT TCA GAT TGC GC	CGT TGT CTA ATC TTG CCT TGC	(CT)46	60^{o}C	191	232
*Xgwm*251	4B	CAA CTG GTT GCT ACA CAA GCA	GGG ATG TCT GTT CCA TCT TAG	(CA)28	55^{o}C	110	109
*Xgwm*257	2B	AGA GTG CAT GGT GGG ACG	CCA AGA CGA TGC TGA AGT CA	(GT)30	60^{o}C	190	192
*Xgwm*269	5D	TGC ATA TAA ACA GTC ACA CAC CC	TTT GAG CTC CAA AGT GAG TTA GC	(CA)29	60^{o}C	148	126
*Xgwm*285	3B	ATG ACC CTT CTG CCA AAC AC	ATC GAC CGG GAT CTA GCC	(GA)27	60^{o}C	222	227
*Xgwm*292	5D	TCA CCG TGG TCA CCG AC	CCA CCG AGC CGA TAA TGT AC	(CT)38	60^{o}C	214	188
*Xgwm*311	2D	TCA CGT GGA AGA CGC TCC	CTA CGT GCA CCA CCA TTT TG	(GA)29	60^{o}C	157	143
*Xgwm*332	7A	AGC CAG CAA GTC ACC AAA AC	AGT GCT GGA AAG AGT AGT GAA GC	(GA)36	60^{o}C	290	211
*Xgwm*383	3D	ACG CCA GTT GAT CCG TAA AC	GAC ATC AAT AAC CGT GGA TGG	(GT)27	60^{o}C	188	199
*Xgwm*484	2D	ACA TCG CTC TTC ACA AAC CC	AGT TCC GGT CAT GGC TAG G	(CT)29	55^{o}C	153	143
*Xgwm*499	5B	ACT TGT ATG CTC CAT TGA TTG G	GGG GAG TGG AAA CTG CAT AA	(GA)32	60^{o}C	131	177
*Xgwm*518	6B	AAT CAC AAC AAG GCG TGA CA	CAG GGT GGT GCA TGC AT	(CA)34	55^{o}C	166	154
*Xgwm*583	5D	TTC ACA CCC AAC CAA TAG CA	TCT AGG CAG ACA CAT GCC TG	(CA)27	60^{o}C	165	161

Source: Roder *et al*., 1998.

3.3. PAG Electrophoresis and Band Scoring

Fragment separation was done using vertical gel electrophoresis apparatus on six percent polyacrylamide gel. The polyacrylamide gel was prepared from mixture of acrylamide:bis-acrylamide (19:1) powder, double distilled autoclaved water, 10X TBE buffer, TEMED and APS.

For preparation of 6% polyacrylamide gel: 6g acrylamide:bis-acrylamide powder, 89ml double distilled autoclaved water and 10ml 10X TBE buffer were poured in to a beaker with magnet and put on the surface of hot plate stirrer (heat off). Then the mixture was allowed to stir till the powder totally mixes in the solution. Later 1000 µl APS and 60 µl TEMED were added into the solution under horizontal laminar flow hood, respectively.

Immediately after preparation of the mixture, the solution was poured into the already prepared vertical electrophoresis glasses. After that a comb with 21 well producing fingers was inserted at the top of the solution in the glass to produce wells.

The PCR product was later mixed with 5X loading dye (previously mixed with gel red) at a ratio of 5:1 for the vertical electrophoresis. The first well was used for DNA ladder (Hyper ladder II) mixed with loading dye and the other 20 were used for 20 different genotypes. 1X TBE was used as a running buffer during electrophoresis.

The PCR products were electrophoresed on vertical electrophoresis apparatus (PS 305 LTI, France) containing 6% polyacrylamide gels under 150 V for 3 hour (sometimes more), and observed under a UV transilluminator. Bands were scored as present (1) or absent (0) at each locus.

3.4. Method of Data Analysis

For computation of number of allele pre locus, number of polymorphic loci and its percentage, PIC and Nei's gene diversity the software program POPGENE (version 1.32) was used.

PIC was calculated using the formula;

$$PIC = 1 - \sum_{i=1}^{N} p^2 i$$

, where *pi* is the population frequency of the *i*th allele.

Nei's Genetic Diversity (H) was calculated using the formula;

H= 1- J, where J is probability of gene identity. $\mathbf{J= \sum_{K} X^2 k}$, where *x*k is the frequency of the kth allele.

The genetic variability among varieties was estimated by computing a distance matrix based on all reliable bands detected and on all possible pair-wise comparisons between varieties. The application of the software program R (version 3.0.2) was used for the calculation of genetic dissimilarities (UPGMA), construction of dendrograms and visualization of clusters. The dendrograms were constructed using the distance matrix calculated from the SSR data generated using 41 bread and 24 durum wheat varieties.

4. RESULTS AND DISCUSSION

4.1. Number of Alleles and Polymorphic Loci

A total of 116 and 131 alleles were found in bread and durum wheat varieties, respectively (Table4). The allele number detected by a primer ranged from 4 (*Xgwm*583) to 16 (*Xgwm*136) with an average of 7.73 among bread wheat varieties and 2 (*Xgwm*583) to 15 (*Xgwm*269) with an average of 8.73 among durum wheat varieties.

Observed and effective number of alleles according to Kimura and Crow (1964) was 1.94 and 1.58 among bread wheat varieties and 1.94 and 1.56 among durum wheat varieties, respectively.

The number of polymorphic loci and its percentage were 109 (93.97 %) and 123 (93.89 %) among bread and durum wheat varieties, respectively. The lowest and the highest markers were recorded as 16 and 4 (Fig 1 and 2).

The number of alleles detected per locus ranged from 4-16 with an average of 7.73 among bread wheat varieties and 2-15 with an average of 8.73 among durum wheat varieties (Table 4). Various authors (Naghavi *et al*., 2009; Desta *et al*., 2013; Rao and Gerdezi, 2013) also reported similar results were they found 2-27 alleles per locus.

By comparing the sub-genomes, it was found that the largest number of alleles per locus occurred in the A sub-genome (16 alleles per locus) as compared to D sub-genome (15 alleles per locus) and B sub-genome (10 alleles per locus).

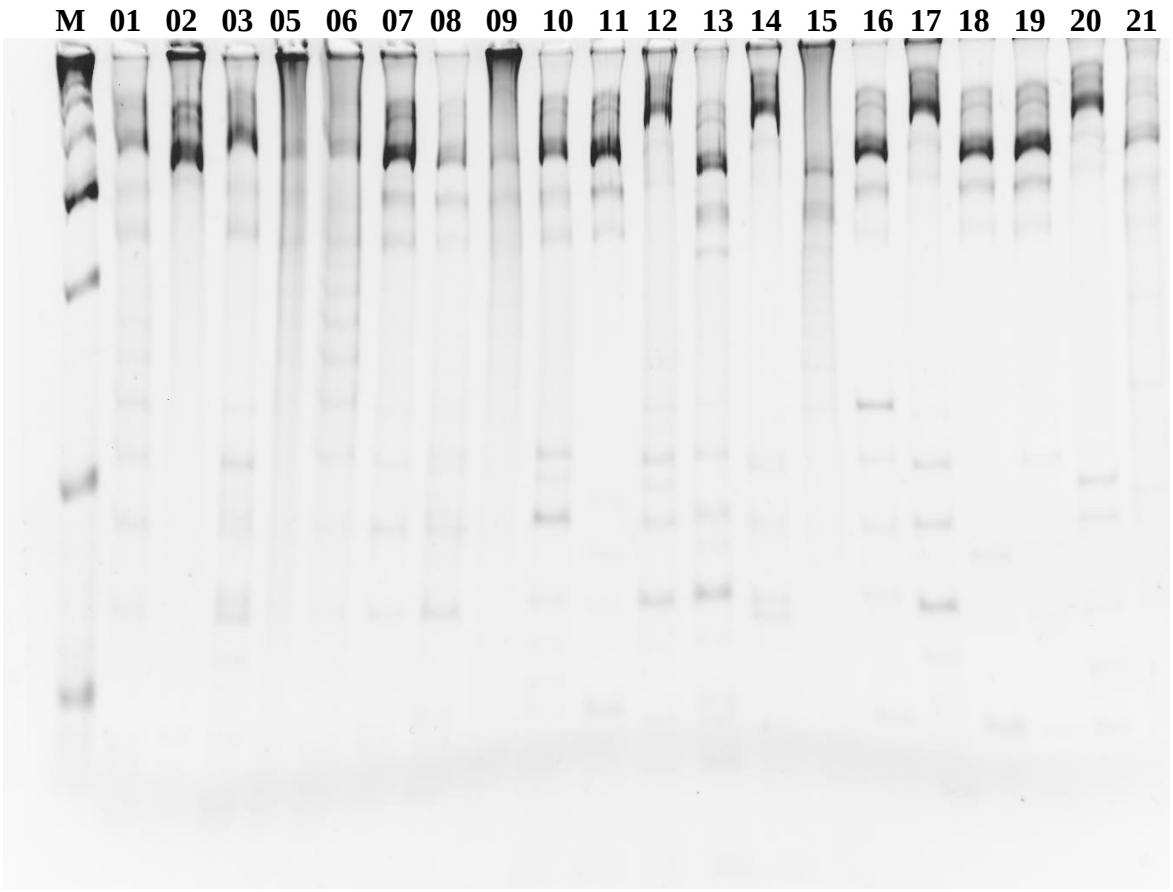

Figure 1: SSR patterns in durum wheat population using primer *Xgwm*136. From left to right, lanes are: M, correspond to 100 bp DNA ladder; and each number indicates different durum wheat varieties.

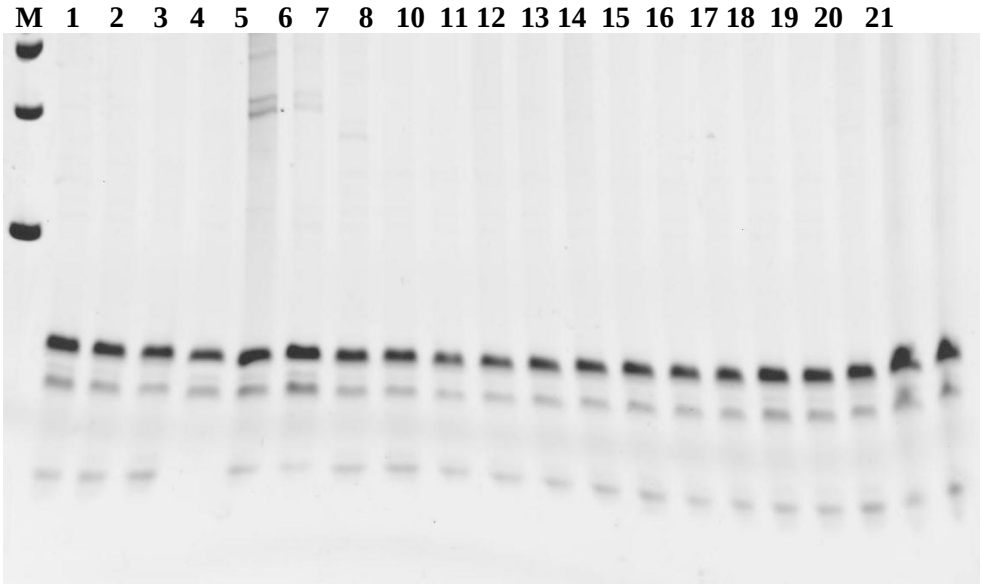

Figure 2: SSR patterns in bread wheat population using primer Xgwm583. From left to right, lanes are: M, correspond to 100 bp DNA ladder; and each number indicates different bread wheat varieties.

4.2. Polymorphic Information Content (PIC)

As a measure of microsatellite informativeness, the PIC value ranged from a low of 0.16 to a high of 0.83 with an average of 0.56 among bread wheat varieties, and from a low of 0.36 to a high of 0.89 with an average of 0.63 among durum wheat varieties (Table 4).

The lowest PIC was observed in locus *Xgwm*257 and the highest was observed in locus *Xgwm*136 among bread wheat population, which is close or similar to the average PIC reported by various authors (Naghavi *et al*., 2009; Pan Dong *et al*., 2009; Akfirat and Uncuoglu, 2013; Rao and Gerdezi, 2013).

On the other hand, the PIC value of markers among durum wheat varieties ranged from a low of 0.36 (*Xgwm*257) to a high of 0.89 (*Xgwm*292) with an average of 0.63 (Table 4), which is close to the average PIC reported by (Spanic, 2012). The average PIC values showed that the markers were highly informative as described by (Botstein *et al*., 1980).

From bread wheat SSR data 10 out of 15 primers found to have a PIC value greater than or equal to 0.50. In durum wheat varieties 11 out of 15 primers were having PIC value greater than 0.50. Except primer *Xgwm*383 which was less polymorphic in bread wheat varieties (Table 4).

The total "number of alleles per locus" and "PIC" was calculated by adding the values from each marker accordingly to each sub-genome where they belong and an average was calculated for each sub-genome. The average number of alleles and average PIC per "sub-genome" were calculated for both bread and durum wheat varieties. From the bread wheat SSR data, it was found that the "A" sub-genome had the highest "average" number of alleles (14) and "average" PIC (0.74) per locus.

On the other hand, the SSR primers from the "D" sub-genome had the highest "average" number of alleles (8.88) and "average" PIC (0.68) as calculated from the SSR data of durum wheat varieties.

4.3. Nei's Genetic Diversity

Nei's (1973) gene diversity for each locus among bread wheat varieties ranged from 0.18 to 0.44 with an average of 0.34, and for each locus among durum wheat varieties it ranged from 0.10 to 0.43 with an average of 0.33 (Table 4).

The highest level of gene diversity (0.44) was observed in locus *Xgwm*311 and the lowest level (0.18) was observed in locus *Xgwm*484. In durum wheat the highest level of gene diversity value (0.43) was observed in locus *Xgwm*518 and the lowest value (0.10) in locus *Xgwm*484. Similar results were reported by (Roy and Chakraborty, 2009) where they found an average gene diversity of 0.33 and 0.30.

Based on the computational results of bread wheat SSR data a strong positive correlation (0.70) was observed between number of alleles per locus and number of repeat motifs of SSR markers. Similarly there was a positive correlation (0.32) between the above two values in durum wheat too. In addition, the number of alleles per locus and the PIC values showed positive correlations of 0.52 and 0.53 in bread and durum wheat varieties, respectively.

Table 4: Number of Alleles per Locus, PIC and Nei's GD calculated for bread and durum wheat varieties

Locus name	Bread wheat			Durum wheat		
	Number of Alleles	PIC	Nei's GD (h)	Number of Alleles	PIC	Nei's GD (h)
*Xgwm*114	8	0.72	0.34	8	0.83	0.32
*Xgwm*136	16	0.83	0.30	14	0.60	0.40
*Xgwm*192	12	0.50	0.41	8	0.66	0.41
*Xgwm*251	9	0.76	0.34	7	0.79	0.37
*Xgwm*257	5	0.16	0.28	7	0.36	0.17
*Xgwm*269	6	0.52	0.39	15	0.75	0.34
*Xgwm*285	6	0.44	0.30	6	0.40	0.28
*Xgwm*292	6	0.50	0.38	11	0.89	0.23
*Xgwm*311	9	0.55	0.44	14	0.71	0.36
*Xgwm*332	12	0.64	0.36	9	0.57	0.40
*Xgwm*383	7	0.70	0.37	8	0.72	0.38
*Xgwm*484	5	0.35	0.18	5	0.41	0.10
*Xgwm*499	5	0.71	0.21	10	0.72	0.28
*Xgwm*518	6	0.73	0.38	7	0.61	0.42
*Xgwm*583	4	0.38	0.25	2	0.44	0.15
Total	116			131		
Mean	7.73	0.56	0.34	8.73	0.63	0.33

4.4. Genetic Distance among Varieties

The value of pair-wise comparison of genetic distance between varieties that was computed using the 15 primer pairs ranged from 0.07 to 0.12. A low genetic distance 0.07 was observed between Galema and K-6290 bulk and Hoggana and Dure. A high GD 0.12 was observed between Shina and Bobicho, Shina and Digalu, Shina and KBG-01, Shina and Hawi, Shina and Galema, and Shina and Mitikie etc. (Table5). The average distance among bread wheat varieties was 0.10.

On the other hand, on durum wheat the genetic distance ranged from 0.07 (between Werer and Gerardo; Candidate#4 and Denbi) to 0.14 (between Selam and Candidate#4/Candidate#5/Denbi/Bichena/Werer/Gerardo/Ginchi) (Table6). The average genetic distance was 0.11. A variety pair with higher value was more diverse than a pair with low value. The average values indicated that bread and durum wheat varieties included in this study shared 90.2% and 89.5% of their marker alleles, respectively.

Comparison between the two wheat types indicated that durum wheat varieties had relatively wider genetic base than bread wheat varieties. This could be associated with the fact that durum wheat varieties were mainly selections from the local landraces, in which Ethiopia is the center of diversity for tetraploid wheat. On the other hand, bread wheat varieties released in the country are introductions from abroad, and mainly from a single source, i.e. CIMMYT. Hence, the narrow genetic base in bread wheat was expected.

Even if durum was more diverse than bread wheat, the overall genetic base of the varieties in the two wheat types in Ethiopia is very narrow. This was in line with the finding of Jemanesh et al. (2013) that clearly described the diversity of Ethiopian durum wheat varieties that were released over the last 43 years to be relatively low.

Low genetic diversity of improved durum wheat varieties, where Ethiopia is the center of diversity for the species, could be a result of genetic erosion of landraces (Bayush T. and Berg, 2007) that were used as parents for broadening the genetic base of released varieties. As identified by Geleta and Grausgruber (2013) one of the causes of genetic erosion of landraces was low yield. Low yield leads to replacement by high yielding local or improved varieties (van de Wouw *et al*., 2009).

This suggests that with the decrease in the number of landraces in the country (Teklu and Hammer, 2006; FAO, 1997), the number of local varieties which were used to raise diverse varieties had decreased, which in turn led for usage of some varieties repeatedly as parent materials. The decrease in genetic diversity of durum wheat varieties in Ethiopia was conditioned by breeders' activities and natural selections (Fu *et al*., 2006; Laido *et al*., 2013; Jemanesh *et al*., 2013). This is because breeding programs in developing countries used wild relatives and landraces less frequently in crossing blocks. Breeders use these materials, particularly when they make crosses for resistance to biotic stresses, tolerance to abiotic stresses, or quality (Smale, 1997). This showed that use of wild relatives and landraces in breeding programs was low leading to narrowed genetic base of released varieties. But, wild relatives and genes found in various wheat forms have special values to increase the potential for wide crosses, because wide crosses are crucial for expanding the genetic diversity of wheat (Yang and Smale, 1996).

Jemanesh *et al*. (2013) also described most of released Ethiopian durum wheat varieties were bred, directly or indirectly, from CIMMYT germplasms which may share one or few common lines in their pedigrees. This was supported by van Ginkel and Tanner (1988) that showed three out of four high yielding durum wheat varieties released from 1967 to 1982 in Ethiopia were introductions from CIMMYT.

Table 5: Genetic distance of 41 bread wheat varieties based on 15 SSR markers

	Hoggana	Shorima	Kekeba	Danda'a	Millennium	Gassay	Dinknesh	Alidoro	Menze	Mararo	Tay	Senkegna	Digalu	Bobicho	Simba
Hoggana	0.00														
Shorima	0.08	0.00													
Kekeba	0.09	0.08	0.00												
Danda'a	0.10	0.10	0.10	0.00											
Millennium	0.10	0.10	0.09	0.10	0.00										
Gassay	0.10	0.10	0.11	0.10	0.10	0.00									
Dinknesh	0.10	0.11	0.10	0.11	0.11	0.10	0.00								
Alidoro	0.09	0.08	0.09	0.10	0.10	0.10	0.10	0.00							
Menze	0.09	0.10	0.08	0.10	0.10	0.09	0.10	0.10	0.00						
Mararo	0.09	0.10	0.10	0.11	0.10	0.09	0.08	0.10	0.09	0.00					
Tay	0.10	0.11	0.10	0.11	0.10	0.09	0.10	0.10	0.09	0.09	0.00				
Senkegna	0.10	0.11	0.10	0.10	0.10	0.09	0.10	0.11	0.09	0.09	0.08	0.00			
Digalu	0.10	0.10	0.09	0.10	0.09	0.10	0.10	0.10	0.09	0.09	0.09	0.10	0.00		
Bobicho	0.10	0.11	0.10	0.12	0.10	0.08	0.09	0.10	0.09	0.09	0.08	0.08	0.10	0.00	
Simba	0.09	0.11	0.11	0.10	0.11	0.10	0.11	0.08	0.10	0.11	0.10	0.10	0.11	0.10	0.00
KBG-01	0.10	0.11	0.10	0.11	0.10	0.09	0.11	0.11	0.09	0.09	0.08	0.09	0.09	0.08	0.10
Dure	0.07	0.10	0.10	0.10	0.09	0.10	0.11	0.10	0.08	0.09	0.10	0.10	0.09	0.10	0.09
Sofoumar	0.09	0.10	0.08	0.10	0.10	0.09	0.10	0.10	0.08	0.09	0.08	0.09	0.09	0.09	0.10
Madda-walabu	0.10	0.09	0.09	0.10	0.09	0.09	0.11	0.09	0.09	0.10	0.10	0.10	0.08	0.10	0.10
Hawi	0.10	0.10	0.09	0.11	0.10	0.10	0.11	0.10	0.10	0.10	0.09	0.10	0.10	0.09	0.09
Sirbo	0.10	0.10	0.10	0.11	0.10	0.10	0.10	0.10	0.09	0.09	0.10	0.10	0.09	0.10	0.10
Tuse	0.10	0.11	0.10	0.10	0.08	0.10	0.11	0.11	0.10	0.09	0.09	0.09	0.09	0.10	0.11
Abola	0.10	0.12	0.10	0.10	0.09	0.09	0.10	0.11	0.09	0.10	0.09	0.09	0.09	0.09	0.10
Galema	0.10	0.11	0.11	0.11	0.10	0.09	0.10	0.11	0.09	0.10	0.09	0.08	0.09	0.09	0.10
Katar	0.10	0.11	0.10	0.10	0.10	0.09	0.10	0.11	0.09	0.10	0.08	0.09	0.09	0.10	0.11
Kubsa	0.10	0.10	0.10	0.10	0.10	0.09	0.10	0.10	0.10	0.09	0.10	0.09	0.10	0.10	0.10
Mitike	0.10	0.11	0.09	0.11	0.09	0.08	0.10	0.10	0.09	0.09	0.09	0.09	0.08	0.08	0.11
Pavon-76	0.11	0.12	0.11	0.11	0.10	0.09	0.11	0.12	0.09	0.10	0.10	0.09	0.09	0.09	0.11
Et-13A2	0.09	0.10	0.10	0.11	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.09	0.10	0.10
K6290 bulk	0.10	0.11	0.10	0.11	0.11	0.09	0.11	0.11	0.08	0.10	0.10	0.09	0.09	0.08	0.10
Dereselgn	0.10	0.10	0.10	0.11	0.08	0.10	0.10	0.10	0.10	0.09	0.11	0.10	0.09	0.10	0.11
ETBW 5496	0.10	0.09	0.10	0.09	0.09	0.09	0.10	0.09	0.10	0.09	0.10	0.10	0.09	0.10	0.11
K6295-4A	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.09	0.10	0.10
Doddota	0.09	0.10	0.11	0.09	0.09	0.10	0.11	0.10	0.10	0.10	0.11	0.10	0.08	0.12	0.10
Dashen	0.09	0.09	0.10	0.09	0.10	0.10	0.10	0.10	0.09	0.09	0.10	0.10	0.10	0.11	0.10
Densa	0.10	0.10	0.10	0.10	0.10	0.08	0.10	0.11	0.10	0.10	0.11	0.09	0.10	0.10	0.11
Guna	0.11	0.10	0.11	0.10	0.10	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.10
Shina	0.10	0.11	0.11	0.10	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.12	0.11
ETBW 6008	0.11	0.11	0.11	0.10	0.09	0.09	0.10	0.11	0.10	0.09	0.09	0.08	0.10	0.09	0.11
ETBW 6006	0.10	0.11	0.11	0.11	0.11	0.08	0.09	0.11	0.10	0.10	0.11	0.10	0.11	0.10	0.11
ETBW 5520	0.10	0.11	0.11	0.10	0.11	0.08	0.11	0.11	0.10	0.10	0.10	0.09	0.10	0.10	0.09

Table 5 (Continued)

	KBG-01	Dure	Sofoumar	Maddawalabu	Hawi	Sirbo	Tuse	Abola	Galema	Katar	Kubsa	Mitike	Pavon-76	Et-13A2	K6290 bulk
KBG-01	0.00														
Dure	0.10	0.00													
Sofoumar	0.09	0.09	0.00												
Maddawalabu	0.09	0.09	0.09	0.00											
Hawi	0.09	0.10	0.09	0.09	0.00										
Sirbo	0.09	0.10	0.09	0.10	0.09	0.00									
Tuse	0.10	0.09	0.10	0.10	0.11	0.09	0.00								
Abola	0.08	0.10	0.10	0.09	0.11	0.10	0.09	0.00							
Galema	0.08	0.09	0.09	0.10	0.09	0.09	0.10	0.09	0.00						
Katar	0.09	0.09	0.10	0.10	0.11	0.09	0.08	0.08	0.08	0.00					
Kubsa	0.10	0.10	0.10	0.09	0.11	0.10	0.09	0.10	0.10	0.09	0.00				
Mitike	0.09	0.10	0.09	0.09	0.10	0.10	0.09	0.08	0.10	0.08	0.09	0.00			
Pavon-76	0.08	0.10	0.10	0.10	0.10	0.09	0.10	0.09	0.08	0.10	0.10	0.10	0.00		
Et-13A2	0.09	0.09	0.10	0.10	0.11	0.09	0.10	0.10	0.09	0.08	0.09	0.10	0.09	0.00	
K6290 bulk	0.09	0.09	0.09	0.10	0.10	0.09	0.10	0.09	0.07	0.09	0.10	0.09	0.08	0.08	0.00
Dereselgn	0.10	0.10	0.09	0.09	0.10	0.09	0.10	0.09	0.10	0.10	0.10	0.09	0.10	0.10	0.10
ETBW 5496	0.10	0.10	0.10	0.09	0.11	0.10	0.09	0.10	0.10	0.09	0.08	0.09	0.10	0.10	0.10
K6295-4A	0.09	0.10	0.09	0.08	0.10	0.09	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.09	0.10
Doddota	0.10	0.09	0.11	0.09	0.11	0.10	0.09	0.09	0.10	0.09	0.10	0.10	0.10	0.08	0.10
Dashen	0.10	0.10	0.09	0.10	0.11	0.10	0.10	0.11	0.11	0.11	0.09	0.10	0.10	0.10	0.11
Densa	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.09	0.09	0.08	0.10	0.09	0.10	0.09	0.09
Guna	0.10	0.10	0.10	0.10	0.11	0.10	0.09	0.10	0.09	0.10	0.08	0.10	0.10	0.10	0.10
Shina	0.12	0.11	0.11	0.11	0.12	0.11	0.10	0.11	0.12	0.11	0.10	0.12	0.11	0.10	0.11
ETBW 6008	0.09	0.10	0.10	0.10	0.10	0.10	0.08	0.09	0.09	0.09	0.09	0.08	0.09	0.10	0.09
ETBW 6006	0.10	0.11	0.11	0.11	0.11	0.10	0.10	0.09	0.10	0.09	0.10	0.09	0.09	0.09	0.10
ETBW 5520	0.08	0.09	0.10	0.09	0.11	0.09	0.10	0.09	0.08	0.09	0.08	0.10	0.09	0.08	0.08

Table 5 (Continued)

	Dereselgn	ETBW 5496	K6295-4A	Doddota	Dashen	Densa	Guna	Shina	ETBW 6008	ETBW 6006	ETBW 5520
Dereselgn	0.00										
ETBW 5496	0.10	0.00									
K6295-4A	0.09	0.09	0.00								
Doddota	0.09	0.09	0.09	0.00							
Dashen	0.10	0.09	0.09	0.11	0.00						
Densa	0.09	0.10	0.10	0.08	0.11	0.00					
Guna	0.10	0.09	0.10	0.10	0.08	0.10	0.00				
Shina	0.11	0.10	0.11	0.11	0.09	0.10	0.08	0.00			
ETBW 6008	0.10	0.09	0.10	0.10	0.10	0.09	0.09	0.11	0.00		
ETBW 6006	0.10	0.10	0.10	0.10	0.11	0.08	0.10	0.09	0.09	0.00	
ETBW 5520	0.10	0.09	0.09	0.09	0.10	0.10	0.09	0.10	0.10	0.10	0.00

Table 6: Genetic distance of 24 durum wheat varieties based on 15 SSR markers

	Mosobo	Metaya	Selam	Megenagna	Yerer	Werer	Candidate -4	DW/SR	Foka	Hitosa	Denbi	Ude	TOB-66	Asassa
Mosobo	0.00													
Metaya	0.10	0.00												
Selam	0.10	0.12	0.00											
Megenagna	0.11	0.10	0.13	0.00										
Yerer	0.12	0.12	0.13	0.10	0.00									
Werer	0.12	0.11	0.14	0.11	0.09	0.00								
Candidate-4	0.12	0.11	0.14	0.10	0.09	0.08	0.00							
DW/SR	0.11	0.11	0.13	0.10	0.09	0.09	0.08	0.00						
Foka	0.11	0.10	0.11	0.11	0.11	0.10	0.11	0.10	0.00					
Hitosa	0.12	0.11	0.13	0.11	0.08	0.09	0.08	0.08	0.10	0.00				
Denbi	0.12	0.11	0.14	0.10	0.08	0.08	0.07	0.08	0.11	0.08	0.00			
Ude	0.12	0.11	0.13	0.10	0.10	0.10	0.09	0.10	0.11	0.10	0.09	0.00		
TOB-66	0.12	0.11	0.13	0.10	0.10	0.12	0.11	0.11	0.11	0.10	0.11	0.10	0.00	
Asassa	0.11	0.10	0.13	0.10	0.10	0.10	0.11	0.10	0.11	0.10	0.11	0.09	0.09	0.00
Candidate-5	0.12	0.12	0.14	0.10	0.08	0.09	0.09	0.10	0.11	0.10	0.09	0.09	0.10	0.10
Boohai	0.11	0.11	0.13	0.10	0.10	0.11	0.11	0.10	0.11	0.10	0.10	0.11	0.08	0.09
Cocorit/71	0.11	0.10	0.12	0.10	0.11	0.10	0.10	0.11	0.10	0.11	0.11	0.10	0.11	0.10
Ginchi	0.11	0.11	0.14	0.10	0.11	0.11	0.11	0.11	0.12	0.11	0.10	0.10	0.09	0.09
Bichena	0.12	0.11	0.14	0.10	0.10	0.10	0.09	0.10	0.11	0.10	0.99	0.09	0.09	0.09
Quami	0.11	0.11	0.12	0.11	0.10	0.11	0.11	0.10	0.10	0.10	0.11	0.11	0.09	0.09
Kilinto	0.12	0.11	0.12	0.11	0.11	0.11	0.12	0.12	0.12	0.11	0.11	0.11	0.10	0.11
Gerardo	0.13	0.11	0.14	0.10	0.09	0.07	0.09	0.10	0.10	0.10	0.09	0.10	0.11	0.11
Arendato	0.10	0.10	0.11	0.10	0.11	0.11	0.11	0.11	0.11	0.10	0.11	0.11	0.10	0.11
Robe	0.11	0.11	0.12	0.10	0.12	0.11	0.11	0.11	0.11	0.12	0.12	0.10	0.10	0.10

Table 6 (Continued)

	Candidate-5	Boohai	Cocorit/71	Ginchi	Bichena	Quami	Kilinto	Gerardo	Arendato	Robe
Candidate-5	0.00									
Boohai	0.09	0.00								
Cocorit/71	0.11	0.11	0.00							
Ginchi	0.10	0.08	0.11	0.00						
Bichena	0.09	0.09	0.11	0.08	0.00					
Quami	0.10	0.08	0.10	0.10	0.09	0.00				
Kilinto	0.10	0.10	0.11	0.09	0.10	0.11	0.00			
Gerardo	0.09	0.11	0.11	0.10	0.09	0.11	0.11	0.00		
Arendato	0.11	0.11	0.10	0.11	0.11	0.11	0.10	0.11	0.00	
Robe	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.00

4.5. UPGMA Clustering

A dendrogram was constructed for each population. Accordingly, bread and durum wheat varieties were clustered into three major groups (Fig. 3 and Fig. 4). In addition, four durum wheat varieties stood alone in the cluster.

The correlation values (of the original distance matrix and co-phenetic value taken from the dendrogram) were 0.62 and 0.86 for bread and durum wheat varieties, respectively. These values indicated that the data matrix and the co-phenetic values correlated with high significance, so that each dendrogram has reflected the original distance matrix of each population, therefore there is no distortion due to the grouping method.

The result based on the dendrogram of bread wheat varieties showed that the samples were clearly separated into 14 main clusters containing 33 varieties and 8 standalones. Cluster 1 up to 8 and cluster 12 up to 14 had two varieties each. Namely: Kubsa and Hulluka; Guna and Shina; Shorima and Kekeba; Madda Walabu and Hawi; Alidoro and Simba; Hoggana and Dure; Et 13-A2 and Doddota; Dinknesh and Mararo; Tuse and ETBW 6008; Abola and Katar; and Digalu and Mitikie, respectively.

The ninth cluster included three varieties: ETBW 6006, Gassay and Densa. The tenth cluster included five varieties, namely: Pavon-76, Galema, K 6290 bulk, KBG-01 and Ogolcho. Cluster eleven included three varieties, namely: Senkegna, Tay and Bobicho. The standalone varieties are Danda'a, Dashen, sirbo, K 6295-4A, Menze, Sofoumar, Millennium and Dereselgn.

On the other hand, the dendrogram of durum wheat varieties showed that the samples were clearly separated into three main groups and four standalone varieties; namely Mosobo, Selam, Foka and Cocorit/71. The first cluster contained nine varieties: Ude, Werer, Gerardo, Candidate#4, Denbi, DW/SR, Hitosa, Yerer and Candidate#5. The second cluster included eight varieties: Kilinto, Robe, Asassa, Quamy, TOB-66, Bichena, Boohai, and Ginchi and the third cluster contained three varieties namely; Arendato, Metaya and Megenagna.

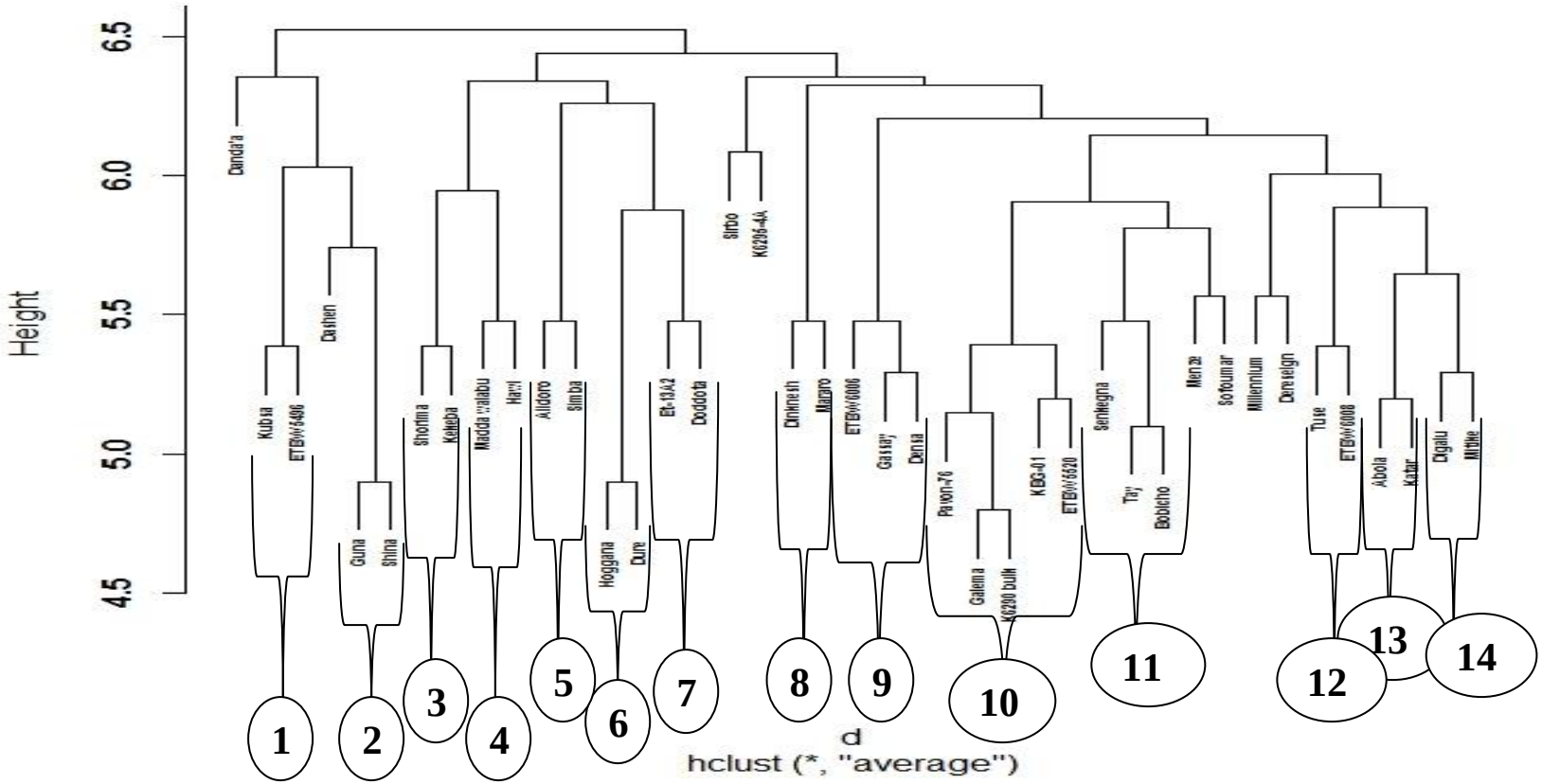

Figure 3: UPGMA dendrogram of 41 bread wheat varieties and advanced lines based on allelic profile generated using 15 SSR markers

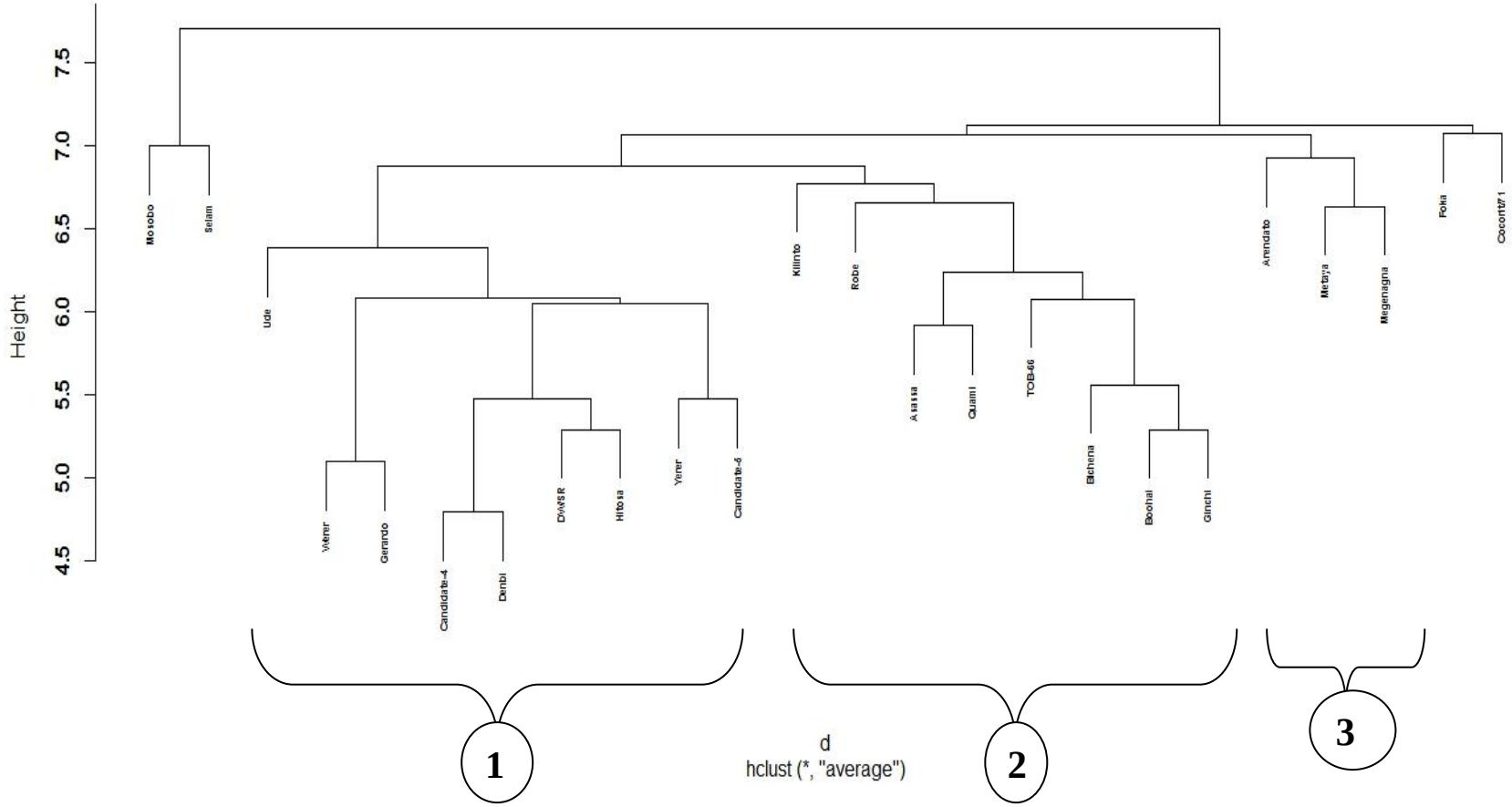

Figure 4: UPGMA dendrogram of 24 durum wheat varieties and advanced lines based on allelic profile generated using 15 SSR markers

5. SUMMARY AND CONCULUSIONS

5.1. Summary

Wheat (*Triticum spp*.) is a cereal crop commonly cultivated as a grain crop and is mostly grown in highlands of Ethiopia. For the purpose of improvement of this valuable crop conventional breeding has been playing major role. Therefore, different breeding programs have been taking place in different research centers of the country using introduced varieties from CIMMYT and landraces, which have been cultivated for long time by indigenous farmers of the country.

There are 58 bread and 34 durum wheat varieties which were released up to the year 2012. But, not enough diversity assessment has been done with these released varieties. Hence, the need for assessment of genetic diversity of released bread and durum wheat varieties initiated the present study.

For this study seeds of 41 bread wheat and 24 durum wheat varieties were used for extraction of total genomic DNA following CTAB protocol. PCR was performed using 15 polymorphic SSR primer pairs within 10µl reaction volume for the purpose of amplification. Separation of amplified fragments was performed using PAGE on vertical electrophoresis apparatus.

Number of alleles per locus were ranged from 4 (*Xgmw*583) to 16 (*Xgmw*136) and an average of 7.73 among bread wheat and ranged from 2 (*Xgmw*583) to 15 (*Xgmw*269) and an average of 8.73 among durum wheat varieties, suggested that marker *Xgmw*583 appears to be the one with the lowest number of alleles per locus for both wheat populations.

PIC values ranged from 0.16 (*Xgmw*257) to 0.83 (*Xgmw*136) among bread wheat and ranged from 0.36 (*Xgmw*257) to 0.89 (*Xgmw*292) among durum wheat varieties, revealed that marker *Xgmw*257 was the one with the lowest PIC for both populations. On the other hand, locus *Xgmw*136 and *Xgmw*292 were markers with the highest PIC for bread and durum wheat varieties, respectively. Therefore, locus *Xgmw*136 appear to be the only marker with the highest number of alleles per locus

and PIC, and could be suggested as the most polymorphic marker. Locus *Xgmw*136 and *Xgmw*269 were markers with the highest number of alleles per locus for bread and durum wheat varieties, respectively.

Bread wheat varieties were clearly separated into fourteen groups with eight standalones and durum wheat varieties were separated into three main groups and two outliers consisting four varieties.

In general, the results obtained from this study showed low genetic diversity among bread as well as durum wheat varieties. Therefore, it was recommended to apply Marker Assisted Breeding (MAB) in addition to conventional breeding to create molecular profile of elite varieties, in turn to avoid the problem of release of new varieties close to each other and/or to varieties released before.

5.2. Conclusions

Based on the results of the present study the following conclusions can be drawn;

This experimental result confirmed that SSRs are powerful tools to evaluate genetic diversity among different wheat genotypes.

The PIC values showed that markers used for this study ranged from less polymorphic to highly polymorphic, but the average values suggested that those markers were highly informative. There was a positive correlation between number of alleles per locus and PIC; 0.52 for bread wheat and 0.53 for durum wheat population, indicated that there was a direct relationship between the number of alleles per locus and the value of PIC.

There was also a strong positive correlation (0.70) between number of alleles per locus and number of repeat motifs of SSR markers for bread wheat varieties, suggesting direct relationship between number of alleles per locus and number of repeat motifs.

Based on the average results of Nei's gene diversity; 0.34 (bread wheat) and 0.33 (durum wheat), it can be concluded that the average heterozygosity in the Ethiopian wheat varieties is low.

In general, the results obtained from this study showed that there was low genetic diversity among bread and durum wheat varieties. Because the average distance among bread wheat (9.76) revealed all bread wheat varieties share more than 90% of their marker information. On the other hand, the average distance among durum wheat (10.49) revealed all durum wheat varieties share more than 88% of their marker information. Therefore, it could be concluded that wheat varieties released in the country have a very narrow genetic base risking the sector for potential devastation by biotic and abiotic stresses as has been observed recently by loss of resistance of most commercial wheat varieties to stem rust. Hence, the breeding program needs to put an extra effort to develop wheat varieties with diverse genetic background across the country.

5.3. Recommendations

It is recommended to conduct further analysis including the pedigree information of the varieties used in this study.

It is also recommended to use landraces and cultivated varieties of the country to cross with newly introduced varieties. Because it helps to increase the genetic information in the gene pool, thereby, broadening the genetic base of varieties which will be released in the future.

It is useful to apply Marker Assisted Breeding (MAB) in addition to conventional breeding to create molecular profile of elite varieties, in turn to avoid the problem of release of new varieties close to each other and/or to varieties released before.

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7. APPENDIXES

Annex 1: DNA Extraction from Fresh Leaves using CTAB Method

Procedure:

1. Take 200mg of fresh leaves and put in to liquid nitrogen for freeze drying.
2. Pulverize the freeze dried leaves in to fine powder using microfuge (supplemented with metal beads) or using mortar and pestle.
3. Put the pulverized leaves in to 2ml tubes and add 800µl CTAB pre heated in 60oc water bath. Make sure the leaf tissue is in solution and not in a clump at the bottom of the tube.
4. Add 800µl chloroform–isoamyl alcohol (24:1) in fume hood and incubate the tubes in 60oc water bath for 30 min (Borges, 2009). Whirl mix can be used for mixing solutions. Be careful not to drip chloroform onto the tubes, it has a low viscosity and drips out of the tip – it will make the label bleed off of the tube.
5. Centrifuge at 12,000rpm for 10 min.
6. Take the aqueous part (top layer) and transfer to new, labeled tubes. Be careful to avoid transferring any chloroform (bright green liquid).
7. Add 2/3 by volume isopropanol pre cooled in -20oc and gently mix with hand (follow up the formation of pellet).
8. Centrifuge at 13,000rpm for 10 min.
9. Remove the supernatant completely, leaving the pellet at the bottom of the tube. Be careful not to dislodge pellet.
10. Add 100µl Washing buffer (wash the pellet), mix gently and carefully remove the washing buffer completely. (pipetting can be used to remove the washing buffer).
11. Re-suspendin 100µlTE buffer supplemented with RNaseA (final conc. 10mg/ml) and incubate the tubes in 37oc water bath for 30 min. This step should be included if clean, RNA free extractions are needed.
12. Add 100µl ammonium acetate (7.5M) and 750µl absolute alcohol and mix gently.
13. Centrifuge at 13,000rpm for 10 min and Remove the solution completely leaving the pellet only. Invert tubes and allow the pellet to dry for 5-10 minutes.
14. Re-suspend the pellet in 100µl reinst water and allow DNA to dissolve overnight.