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Genetic Diversity Study of the *Brachiaria brizantha* (A. Rich.) stapf collected from Ethiopia using Inter Simple Sequence Repeat (ISSR) Markers

By

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This is to certify that the thesis prepared by Fentahun Meheret, entitled: Genetic Diversity Study of the *Brachiaria brizantha* (A.Rich) stapf collected from Ethiopia using Inter Simple Sequence Repeat (ISSR) Markers and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology complies with the regulations of the University and meets the standard with respect to originality and quality.

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ABSTRACT

Genetic Diversity Study of the *Brachiaria brizantha* (A.Rich) stapf collected from Ethiopia using Inter Simple Sequence Repeat (ISSR) Markers

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Addis Ababa University, 2013

Brachiaria brizantha is a C4 grass commonly used as forage in the tropics. Leaf samples from seven populations originating from Welega, Jimma, Omo, Gondar, Gojam, Borena and Ilu Ababora were collected from Zeway Regeneration and Conservation Site of ILRI, Ethiopia. Genomic DNA was extracted from 79 accessions each from three individuals using CTAB extraction method. A diluted genomic DNA was subjected to PCR amplification. From the ISSR primers tested, only six amplified 80 scorable bands from the 79 accessions used. 96.25% Percentage of polymorphic loci (PPL), gene diversity (h) = 0.366 and Shannon information's index (I) = 0.539 were detected at species level. The number of bands identified by each primer ranged from 8 to 19, with an average of 13.3. The highest genetic diversity was generated from accessions collected from Ilu Ababora (PPL= 58.75 %, h=0.210 and I =0.315) while the least was from those collected from Gondar region (PPL=28.75%, h=0.124 and I =0.183). Jaccard's similarity coefficients ranged from 0.302 to 0.431 and analysis of molecular variance indicated the presence of higher proportion of variation within population (64.66%) than between populations (35.34 %). Cluster analysis using the un-weighted paired group method with arithmetic average (UPGMA) and Jaccard's similarity coefficient (0.38) clustered the accessions into three major (I, II and III) clusters in their respective regional collection. Principal Component Analysis (PCO) showed accessions in populations formed their own cluster. Thus ISSR markers detected a range of genetic diversity from *Brachiaria brizantha* germplasm collections from Ethiopia.

Key words: Forage, Genetic variability, Molecular markers

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
CTAB	Cetyltrimethyl Ammonium Bromide
ISSR	Inter Simple Sequence Repeats
M.a.s.l	meter at sea level
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SSR	Simple Sequence Repeat

1. INTRODUCTION

Brachiaria brizantha (Palisade grass) is a tussocky perennial C4 grass belongs to the family Poaceae, genus *Brachiaria* which encompasses more than 100 species. It is native to Africa (Renvoize *et al*., 1996) and is widely grown in tropical America and Southeast Asia (De Valle *et al*., 2008). It grows in various regions of Ethiopia (Phillips, 1995).

Brachiaria brizantha is a variable grass known to exist in diploid to polyploid forms (Valle and Savidan, 1996). Both sexual and apomixis reproduction systems were observed in this species (Mendes-Bonato *et al*., 2002;Daniela *et al*., 2005; Risso-Pascotto *et al*., 2006). Polyploid accessions reproduce primarily by apomixis that severely complicates crossing /hybridization efforts (Daniela *et al*., 2005). However, there was success with Mulato, which is the first cultivar that was produced from crossing of *Brachiaria ruziziensis* with this species in1988 (CIAT, 2001).

Livestock production plays significant role to the economy of smallholder farmers and the national economy. In Ethiopia, both farming and pastoral households are largely dependent on livestock for their livelihood. Livestock also generate more than 85% of the farm cash income that contributes about 13-16% of total gross domestic product(GDP) and the share to total exports is about 16% (Yayneshet Tesfaye, 2010). However, there are a number of constraints such as inadequate feed resource, diseases and low genetic potential that hamper animal production and lead to low outputs. The availability of feed resources is the most important factor that determines productivity of livestock (Mengistu Alemayehu, 2006; Yihalem Denekew *et al*., 2012). For instance, forages grass like

B.brizantha with low crude protein content (Goes *et al*., 2005) coupled with inadequate pasture management and overgrazing can limit its economic importance. Therefore, sustainable forage production and genetic improvements strategies on locally available forages will alleviate critical problems due to their tolerance to drought, disease and poor management practices.

Among *B. brizantha*, there is vast natural variability across tropical ecosystems which could be identified and characterized with the use of genetic markers. However, morphological and molecular markers analysis were performed on limited number of accessions with the initiation of the Tropical Forages Breeding Program conducted by the Brazilian Agricultural Research Corporation (Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA))(De Assis *et al*., 2002; Machado Neto *et al*. 2002;Vigna *et al*., 2011).

Ethiopia, with its enormous variations in climate and relief and its heavily dissected landscape contributes different types of biodiversity. Large area of the country is covered with great diversity of forage grasses, forage legumes and browses indigenous to the country. Studies show that 736 species of grasses, 358 species of legumes and 179 species of browse trees are recorded so far. They are adapted to different ecosystems throughout the country and are key source of animal feed. Some of the most common forages include those in the genera *Aeschynomene*, *Alysicarpus*, *Indigofera*, *Lablab*, *Lotononis*, *Macrotyloma*, *Neonotonia*, *Trifolium*,*Vigna*,and *Brachiaria* (IBC, 2005). But the wide genetic diversity of locally available forages species are not exploited yet in the

country. Therefore, research on molecular diversity of the indigenous forage species and their genetic improvement through breeding programs is vital. To that end, this study is carried out to estimate genetic diversity and population structure of *Brachiaria brizantha* in Ethiopia using by ISSR markers.

2. LITERATURE REVIEW

2.1. Geographical Distribution and Taxonomy

Several important grass species are native to Africa. Among these, *Brachiaria brizantha* is natural being distributed in tropical and Southern Africa (Figure 1) (Keller-Grein *et al*., 1996). It grows extensively in humid and sub-humid zones of the tropics such as in tropical America and Southeast Asia (De Valle *et al*., 2008). Their natural habitats accommodate the open and wooded grasslands, margins of woodlands and thickets, highland grasslands and valleys. They are found in lowland as warm-season grass, from sea level up to 2000 m in the tropics and up to 1000 m in higher latitudes (Renvoize *et al*., 1996; Cook *et al*., 2005). Similarly in Ethiopia, its habitats encompass open grassy slopes and woodland clearings from 1100-2100 m above sea level in various regions: Tigray, Gondar, Gojam, Shewa, Ilu Ababora, Kefa, Gamo Gofa, Welega, Sidamo, Bale and Harerge based on the former administrative demarcation of the country (Phillips, 1995).

Taxonomically, *B.brizantha* is positioned in the family Poaceae and genus *Brachiaria* which is synonymous to *Panicum brizantha* and *Urochloa brizantha* (Phillips, 1995). Its basic morphological structures are characterized by tough culms (50-200cm high), glabrous or pilose leaves (7-15mm wide), inflorescence, elliptical spikelets supports florets (born as single row on a crescentic rhachis, 1 mm wide), cartilaginous lemmas and glumes. The inflorescence structure is one of the most easily observed arrangements of panicle that is consisting of 1- 10 often gently nodding racemes on an axis 4-15 cm long (Phillips, 1995; Cook *et al*., 2005).

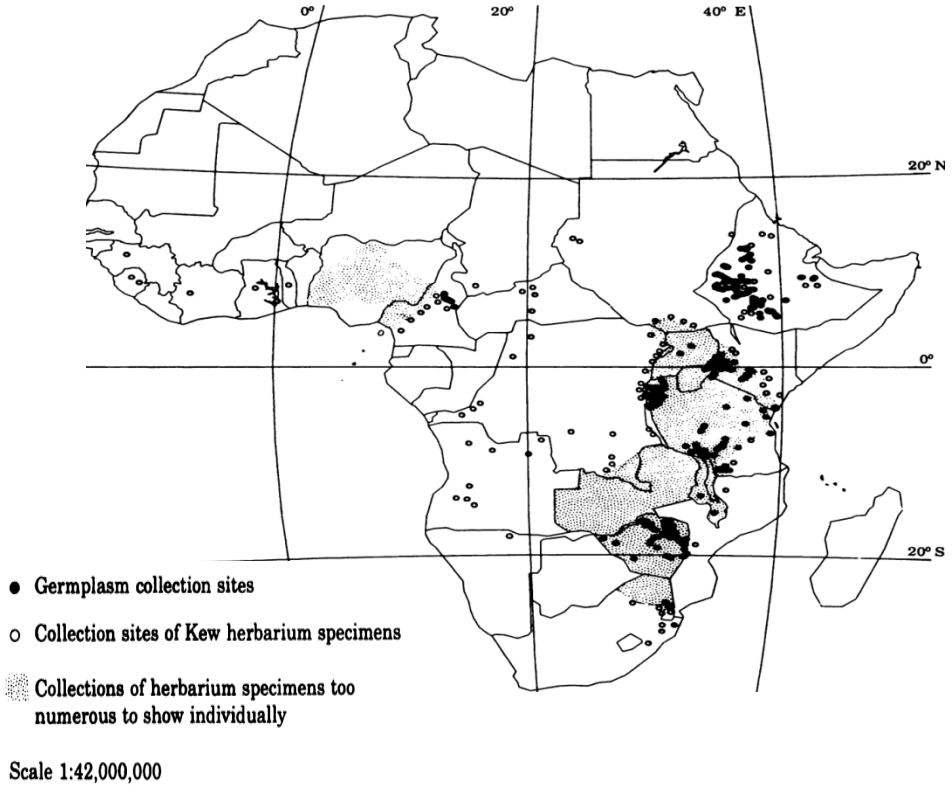

Figure 1. Africa map showing natural distribution of *Brachiaria brizantha* (Keller-Grein *et al*., 1996).

2.2. Propagation and Mode of Reproduction

Brachiaria brizantha is propagated sexually by seeds and vegetatively by clump and culms in tropical areas. Sexual propagation is limited with an unsuccessful seed production (seed abortion) and dormancy of the seeds (Ellis, 1988; Hopkinson *et al*., 1996). But a number of self and open-pollinated pistils are fully developed into viable seeds at a rate of 2% and 6% respectively, if floral structures, anthesis and gametophytes develop normally (Araujo *et al*., 2007). Beside this, *Brachiaria brizantha* is predominantly a polyploid form that reproduces by apomixis (Mendes-Bonato *et al*., 2002; Daniela *et al*., 2005; Risso-Pascotto *et al*., 2006). It differs from sexual one that is observed in diploid forms by formation of an unreduced embryo sac due to circumvented

or altered meiosis during Megasporogenesis and by embryo development without fertilization of the egg cell (Nogler, 1984).

2.3. Polyploidy and improvement in *Brachiaria brizantha*

In genus *Brachiaria*, the majority of species are polyploid (Valle and Savidan 1996; Mendes Bonato *et al*., 2002). Cytological analysis shows the following ploidy levels in *B.brizantha*: diploid (2x= 18), tetraploid (4x=36) and hexaploid (6x=54) (Mendes-Bonato *et al*., 2002). Diploid level is rare and is correlated with sexual reproduction while Polyploidy is correlated with apomixis (Valle and Savidan, 1996).

The first genetic improvement of *Brachiaria* species depended entirely on selection among naturally existing genotypes. But the creation of a tetraploid *B.ruziziensis* and understanding the genetics and cytogenetic of *Brachiaria* species have opened the way for controlled genetic manipulation (Miles and Do valle, 1996). Besides, evidence of natural hybridization in accessions of *Brachiaria brizantha* with genomes not closely related was first observed (Mendes *et al*., 2006). Cultivar Mulato with desirable traits has been developed from *B. ruziziensis* and other closely related species (*B. brizantha* and *B. decumbent*) after several artificial crossing (CIAT, 2001).

2.4. Economic importance

The importance of *Brachiaria brizantha* can be seen from its extensive use as pasture grass in the tropics where it plays an important role as the major livestock feed. However, its feeding value is inadequate for performance of livestock production. Evaluation of chemical composition showed that it has high ash and neutral detergent fiber (NDF) but

low crude protein (CP) concentration compared to moringa and sugar cane molasses *(*Sanchez *et al*., 2005). Therefore, supplementations viz soybean, maize of livestock grazing with *Brachiaria brizantha* pasture could be economically feasible during the rainy and dry season (Goes *et al*., 2005;Sampaio *et al*., 2010; Valente *et al*., 2012).

Unlike Ethiopia where modern cultivation of the grass is limited, in northeastern Brazil, a total herbage accumulation from *Brachiaria brizantha* can reach up to 28 metric tons of DM per hectare (ha) during the grazing season (Santos *et al*., 2003). It is responsible for almost 90% of beef and large proportion of about 25.7 billion liters of milk produced annually in Central Brazil (Souza Sobrinho *et al*., (2009) as cited in Inyang, 2009). Hence, it has high contribution for development of agricultural sectors due to its tolerance to environmental constraints (grown infertile and acidic soil, insects attack, and invasiveness). Ethiopia could also exploit this valuable grass to maximize its cattle production to meet the current increasing demand for the leather and other related agro-industries.

2.5. Genetic Diversity and Application of Genetic Markers

Genetic diversity being referred to as the sum total of genetic variations found in a species or population is source for ecological biodiversity. Measuring genetic diversity is significant to examine variations present among the organisms on the basis of genetic markers at phenotypic, biochemical and genotypic level. It has been used for comparison of the genetic composition of members of different species over a wider taxonomic range (Demeke, *et al*., 1992; Dale and Schanz, 2002). It is a prerequisite for efficient breeding

plans, collection expeditions or germplasm exchange in order to acquire specific characteristic (Demeke, *et al*., 1992; Abebe Demissie and Bjornstrand, 1996).

There are two main classes of genetic markers that are commonly used for measurement of genetic diversity at species levels, namely morphological and molecular markers. But recent advancement in biotechnology has led to the development of a number of powerful and novel tools called DNA based markers that offer the promise of making plant breeding more precise and faster. DNA based markers are an easily identifiable piece of genetic material that distinguish the presence or absence in experimental plants of specific alleles of interest that could define the position of the genes controlling a particular trait and detect a selection traits in artificial or natural populations (Gupta and Varshney, 2004; Mackill and McNally, 2005). They are heritable as simple Mendelian traits which represent large number of agronomic and disease resistance traits available in major crop species (Gupta and Varshney, 2004; Jain *et al*., 2010).

Moreover, these markers have had significant roles in studies of phylogeny, species evolution and to increase our understanding of the distribution and extent of genetic variation within and among species (Bonato *et al*., 2006; Mondini *et al*., 2009).

Genetic diversity at phenotypic level

Phenotypes simply describe the observable characteristics (traits) of grasses that are expressed in terms of their genotypic and environmental interactions. Examples for direct measurable complex traits include leaf area, plant height/width, seed number, seed size,

flowering time, germination time, etc. These parameters have been used for characterization and identification of species in taxonomy and variety classification.

The type and structure of the genetic variation underlying phenotypic traits determines the potential for and rate of an adaptive response to natural selection. Genetic causes of phenotypic variance are attributable to many sources, mutation being the primary one. It also occurs through random evolutionary processes. These phenotypic variations include variation in size, shape, coloration, behavior, physiology, etc. Genetic diversity of species is evaluated on the basis of these parameters assembly called as morphological markers. They were among the earliest genetic markers used in germplasm management (Stanton *et al*., 1994).

When the traits are highly heritable, morphological based markers are simple, cheap and easy for monitoring without biochemical or molecular techniques (Taba *et al*., 1998; Chittaranjan and Albert, 2008). However, several drawbacks limit their application such as low polymorphism, low heritability, late expression, and are influenced by environmental factors. Moreover, Chittar- anjan and Albert (2008) reported the uncertainty to distinguish homozygous dominant and heterozygous individuals because of dominant intra-allelic interactions.

Genetic diversity at molecular level

Biochemical Diversity: Plants are inexhaustible sources of a wide range of proteins and isoenzymes. These could be used for identification and differentiation of species on the basis of biochemical markers. This technique can be carried out on crude extracts. It

involves protein extraction and electrophoresis separation of protein molecules into specific banding pattern by polyacrylamide gel electrophoresis in the presence of sodium dodecyle sulphate (SDS-PAGE) (Schulman *et al*., 2004). Beside this, purification of the protein is not required (Mukhlesur and Hirata, 2004).

Most economically important traits have been interrelated with proteins and isozymes polymorphism. As a result, biochemical markers have been extensively used to study genetic variations between and within different species of plants (Machado- Neto *et al*., 2002; Barakat, 2004; Salimi and Rashid Abdola, 2013). However, this type of analysis is no powerful to discriminate genetic diversity between closely related varieties (Barakat, 2004; Patra and Chawla, 2010).

The major limitation of the biochemical markers are representing only a very small and biased fraction of the total genome (coding regions), comparative instability in the face of environmental variation, labor intensive and not well adapted to automation and high throughput analysis for breeding. Additionally, the application of isozymes markers is inconvenient due to the fact that each of the proteins that are being scored might not be expressed in the same tissue and at the same time in development.

Genetic diversity at DNA level: In nature, considerable variation in nuclear DNA content occurs within and among species. These differences occur due to insertions, deletions, translocations, duplications, point mutations, etc. DNA based markers have

been used for better detection of these differences between two or more individuals at molecular level.

It works by highlighting polymorphisms (differences) as the presence or absence of bands across individuals. They could be providing a complementary technology to augment morphological measures of plant identity and genetic diversity. DNA based markers may be tightly linked to loci of agronomic importance trait (Winter and Kahl, 1995) and offer an easily quantifiable measure of genetic variation at any growth stage of plant species. Besides, these markers are cost effective than the other characterization techniques in conventional breeding which would require several crosses, several generations, careful phenotypic selection and the tight linkage of dominance and recessive alleles in loci that may make it further difficult for selection of outstanding traits.

Overview of commonly used nuclear DNA markers

The advent of PCR has favored the development of DNA based markers. This is because, it requires very low amounts of DNA extracted rapidly from different plant material with high throughput methods (Paris and Carter, 2000). The most commonly used DNA based markers are restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR) and inter simple sequence repeats (ISSR).

Restriction fragment length polymorphisms (RFLPs) are one of the earliest non-PCR based molecular markers which are used to differentiate organisms by investigating the size variability of restriction fragment length spread across the genome (Chittaranjan and Albert, 2008). The techniques employ gel electrophoresis resolution in gel matrix and molecular hybridization of cDNA or genomic DNA probes with genomic DNA fragmented by restriction enzymes.

These are often co-dominant and very informative markers used in plants and were commonly used for detection of polymorphism, genome mapping and genetic disease analysis in breeding programs. However, generating RFLPs data are time consuming, requires a relatively large amount of DNA and involves expensive radioactive materials (Kumar *et al*., 2009; Mondini *et al*., 2009). Due to the above limitations, more efficient molecular markers, such as RAPDs and AFLPs, were developed.

Random amplified polymorphic DNAs (RAPDs) are the earlier most widely used PCR-based, multilocus and dominant molecular markers that came after the advent of RFLP markers. This marker is convenient in its performance and does not require any information about the DNA sequence to be amplified (Zhivotovsky, 1999; Bardakci, 2001; Weder, 2002). These techniques involve the use of a single short and arbitrary primer in a PCR reaction and result in amplification of several discrete DNA products due to variability in primer binding sites and in DNA length between those sites (Powell *et al*., 1996). The amplified fragments are separated on agarose gels, detected by ethidium bromide when visualized under ultraviolet light.

RAPDs are more sensitive, cheap, easy and more effective in discriminating between closely related varieties because they can successfully produce variety of specific finger prints. However, its limitation such as irreproducibility, dominancy and inaccurate estimates of inter-specific similarities led to a replacement by a more robust DNA fingerprinting technique termed as amplified fragment length polymorphism (AFLP) (Scheirwater and Ender, 1993; Powell *et al*., 1996; Bardakci, 2001).

Amplified fragment length polymorphisms (AFLPs*)* are among PCR based molecular markers that are attractive multi-locus DNA fingerprinting techniques which efficiently identify DNA polymorphisms without prior information (Vos *et al*., 1995; Hartl *et al*., 1999). They involve restriction of genomic DNA followed by ligation of adaptors to restricted fragments. A pre-selection step is performed using magnetic beads followed by a round of selective PCR which include steps from DNA digestion, ligation, and pre-amplification to selective amplification (Zabeau and Vos, 1993; Vos *et al*., 1995). Finally, DNA polymorphisms are detected through the complex process in agarose gel matrix.

Given that AFLPs technologies are fast, efficient, reliable, cost-effective, reproducible, sensitive and with better resolution compared with RFLP or other PCR - based markers (e.g. RAPD and ISSR), they are commonly used in genomic mapping, evolutionary genetics and ecology. However, these techniques are tedious, time-consuming, require high quality DNA, dominancy nature and limitation in versatility to be applied to species

with complex genomes (e.g. tree and wheat) (Paglia and Morgante, 1998; Peng *et al*., 2000).

Simple sequence repeats (SSRs) are also known as microsatellites are co-dominant and multi-allelic markers that represent DNA sequences of tandemly repeated short motifs generally between 2 and 6 base-pairs long that are widely dispersed through the whole eukaryotic genomes with an accumulation in repetitive DNA and untranslated 3'- and 5'-regions of genes. They are often highly polymorphic (Powell *et al*., 1996; La Rota *et al*., 2005). The unique sequences of SSR primers are flanked and amplified tandemly in repeated region of template DNA. Amplified fragment can be easily visualized in gels matrix. On the other hand, forward SSR primers could be chemically attached to a fluorescent dye to serve for automation of the technology (Rakoczy-Trojanowska and Bolibok, 2004).

SSRs are currently one of the most widely used types of molecular markers for constructing genetic maps, marker assisted selection and analysis of relationships in a number of crop species (Barakat *et al*., 2011; Pei *et al*., 2010). These potential applications have been justified attributable to their abundance, co-dominance, greater reliability, high reproducibility, hypervariabilty, specificity and transportability between species (Rakoczy-Trojanowska and Bolibok, 2004; Varshney *et al*., 2005). Additionally, these markers have potential applications in plant variety rights and seed certification (Jahufer *et al*., 2003). However, they are expensive, time consuming and require involvement of specific sequence data for an individual species (Zane *et al*., 2002).

ISSR markers and application in plants

The inter-simple sequence repeats (ISSR) markers are regions of the nuclear DNA; located at specific sequences between two inverted SSR regions (Figure 2). ISSR markers are PCR based markers using SSRs as primers anchored at 3' or 5' which can be di-, tri-, tetra- or penta-nucleotides (Blair *et al.*, 1995). When primers are annealing on two microsatellites sequences of DNA strands, the PCR reaction generates a band of a particular size for that locus. Each locus is scored for an individual in a binary way, as "1" or "0" (presence or absence of a product) respectively. Then, they allow detection of size polymorphism between microsatellites repeats (Zietkiewicz *et al*., 1994). DNA variability at different levels, from single base changes to deletions and insertions are detected through these markers (Tileye Feyisa *et al*., 2007; Naik *et al*., 2009; Chezhian *et al*., 2010).

ISSR markers may be associated with biochemical traits, highly polymorphic and dominant markers that follow simple Mendelian inheritance (Ratnaparkhe *et al*., 1998; Wang *et al*., 1998; Li *et al*., 2009). These markers have been successfully used for the assessment of genetic diversity in Bermuda grass (Farsani *et al*., 2012), *B.* ruziziensis (Azevedo *et al*., 2011), ryegrass (Ghariani *et al*., 2003) and other species (Tileye Feyisa *et al*., 2007; Li *et al*., 2009). They are also useful in the studies of phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al*., 2002).

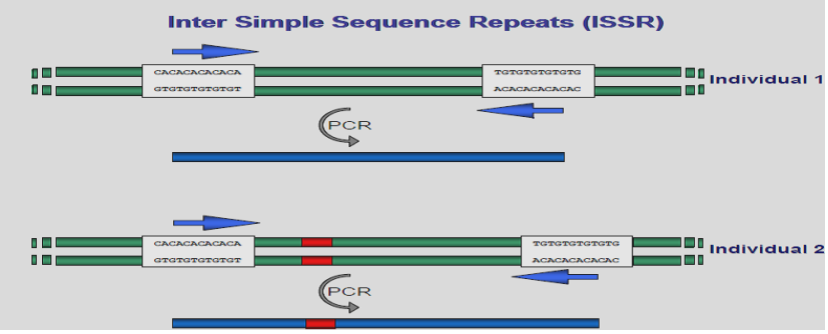

Figure 2. Principles of the amplification of DNA with a single oligo-nucleotide primer in ISSR marker system. DNA segments delimited by the inverted simple sequence repeats (SSR) (Individual 1 and 2) are amplified with a single ISSR primer (green). ISSR Variation that may result from insertion or deletion (Red mark) in different individuals produces PCR fragments of different sizes (blue) of the segment (Kassahun Tesfaye *et al*., 2005).

ISSR markers have been more specific than RAPD markers to study genetic diversity due to its longer SSR-based primers (16-25bp) with higher primer annealing temperature (45-60)℃ which enable for higher-stringency and band reproducibility (Wolfe *et al*., 1998; Reddy *et al*., 2002). In addition to this, the major limitations such as low reproducibility of RAPD, high cost of AFLP, the need to know the flanking sequences to develop species specific primers for SSR polymorphism and large quantities of high quality genomic DNA (for RFLP)(Reddy *et al.*, 2002) are overcome by this markers (Zietkiewicz *et al*., 1994;Huang *et al*., 2010).

Generally, technically these markers are efficient, quick, reliable, very reproducible, highly discriminative and informative, independent of sequence information and require small quantity of sample DNA. These facts suggest that ISSR could be used extensively for assessing the molecular genetic variability within and among many living organisms (Kol and Lazebny, 2006; Lalhruaitluanga and Prasad, 2009). However, the major

limitation of these makers is analysis of dominant data in population genetic studies. It is not as simple as co- dominant markers such as isozymes and SSR marker (Cekic *et al*., 2001; Potter *et al*., 2002).For the present study, ISSR markers are selected because of their availability, purpose of the study, skill in molecular techniques and cost-effectiveness.

2.6. Genetic diversity in *Brachiaria brizantha*

A number of accessions of *Brachiaria brizantha* were collected from various tropical ecological zones. However, limited accessions were utilized for morphological and molecular studies. Morphological analysis of this species on the basis of morphologic characters with other six *Brachiaria* species viz *B. decumbens*, B.*ruziziensis*, *B. humidicola*, B. *jubata*and and *B. dictyoneura* demonstrated genetic diversity among accessions and species. It was grouped with *B. decumbens* and *B.ruziziensis* species (De Assis *et al*., 2002). But it could be distinguished from the two species by its erect, tufted clumps, often much longer leaf blades and with slightly different spikelets and shorter roots (Phillips, 1995; Renvoize *et al*., 1996).

On the other hand, molecular analysis (uni-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), RAPD markers) showed significant genetic variation among accession of *Brachiaria* species (Machado Neto *et al*., 2002; Guaberto, 2009). But there is not a considerable genetic variability among *B.brizantha* germplasm as revealed by simple sequence repeat (SSR) markers. This marker identified only presence of three distinct allelic pools (Vigna *et al*., 2011).

3. OBJECTIVE OF THE STUDY

3.1.General objective

The general objective of this study is to investigate genetic diversity and population structure of *Brachiaria brizantha* from various administrative regions of Ethiopia.

3.2.Specific objectives

The specific objectives of this study were:

- To estimate the genetic diversity in *B. brizantha* populations collected from different parts of Ethiopia,
- To reveal genetic structure within and among populations of *B. brizantha*,
- To identify populations of *B. brizantha* with high genetic diversity for future improvement and conservation programs in the country.

4. MATERIALS AND METHODS

4.1. Plant materials

Plant samples were collected from Zeway Forage Conservation Site of ILRI that was previously collected from various regions of Ethiopia for regeneration and maintenance purposes. This site is located about 165km south of Addis Ababa at 7°53′ 9 " N, and 38°44′ 68" E, at altitude of 1640 m.a.s.l close to Zeway lake western shore with 700 mm annual average rainfall, 26℃ and 20℃ maximum and minimum average temperature, respectively. The soil type is sandy loam with pH of 8.04. List of accessions used in this study are shown in Table 1(appendex1.).

Three young leaves were harvested separately from three randomly selected individual grasses per accession after two weeks of cutting from seven different populations and dried in silica gel for genomic DNA extraction. The populations were Welega, Jimma, Ilu Ababora, Borena, Omo, Gojam and Gondar. Moreover, populations were grouped in to four geographical regions viz Northwest Ethiopia (Gondar and Gojam), West Ethiopia (Welega), South west Ethiopia (Jimma and Ilu Ababora) and South Ethiopia (Omo and Borena).

Table 1. Number of accessions of *B.brizantha* collected from each region

No	Populations	Regions	No of accessions	Remark
1	Welega	Oromiya	14	
2	Jimma	Oromiya	12	
3	Ilu Ababora	Oromiya	14	
4	Borena	Oromiya	9	
5	Omo	South Nations	11	
6	Gojam	Amhara	11	
7	Gondar	Amhara	8	

4.2.Genomic DNA extraction

In this study, bulk sampling approach was chosen as it permits representation of an accession by optimum number of plants (Gilbert *et al.*, 1999). Approximately equal amount of three silica dried leaves per accession were bulked and ground with pestle and mortar using sand. Genomic DNA was extracted in 1.5ml Eppendorf tubes using about 0.3 to 0.5g powder leaves following Borsch *et al*., (2003) with slight modification. Then well crushed leave samples were mixed with 700µl cethyltrimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 5 M NaCl, 2% PVP, 0.5M EDTA at pH 8.0, 1M Tris-HCl at pH 8.0 and 98% mercaptoethanol) pre-warmed in water at 65℃ for 30min. The mixture was incubated for up to 30min by thoroughly mixing at about 10 min interval in electrical constant temperature water box. Then centrifugation at 13,000 rpm for 7min was followed in Mikro-20-zentrifugen and the same is repeated to attain quality DNA in the second extract. The supernatant of crude DNA extraction (second) was transferred to new 1.5ml Eppendorf tubes using cut blue tips.

This crude DNA in the second extract was purified using 600µl chloroform and followed by centrifugation at 13,000 rpm for 7min. The supernatant for the second extraction was treated with isopropanol (2/3 of the solution volume) and incubated for more than 2h at 20℃. Then, it was centrifuged at 13,000 rpm for 12min, followed by aspiration and washed with 200µl ethanol (70%). For further purification a pellet was dissolved with 100µl×TE buffer and half of the solution volume of 7.5MNH_4Ac was added first, then 3MNa Ac in the second round. Each salt treatment was followed by 100% ethanol (50µl) treatment. Then each mixture was incubated for more than 2h at - 20℃, centrifuged at

13,000 rpm for 35min, followed by aspiration the fluid, washed pellet with 200µl ethanol (70%) and aspirated using yellow tips. Finally DNA was re-suspended in 100 µl 1×TE buffer (1M Tris-Hcl and 0.5 M EDTA pH 8.0) and stored at 4℃.

4.3. Electrophoresis and Gel documentation

The genomic DNA was tested using 1% agarose gel by applying 2µl genomic DNA loaded after mixing with 1µl 6×loading dye and 3µl deionized water. About 0.5g agarose and 100ml 1×TBE buffer (1M Tris-HCl, 0.5 M EDTA and 0.88M boric acid pH 8.0) were boiled using microwave oven for about 1min. It was casted on casting stand (Gel tray) after mixing with 2µl ethidium bromide for solidification. Subsequently, the DNA in agarose gel was subjected to electrophoresis in BIORAD min-sub® cell GT at 80V (from Biometra® standard power pack P25) for 40min and visualized on UV using gel documentation system. The gel was photographed using Canon camera attached to gel documentation system and documented for later use.

4.4. ISSR Primers screening and Genomic DNA amplification

For efficient genetic diversity analysis of *Brachiaria brizantha*, initially three individuals were randomly selected for ISSR primer screening and optimization. A total set of 13 ISSR primers were acquired from Genetic Teaching and Research Laboratory (originally obtained from University of British Colombia, Primer kit UBC 900) of the Microbial, Cellular and Molecular Biology Department, College of Natural Sciences, Addis Ababa University. Primer names and sequences are listed in Table 2.

Diluted genomic DNA (1:5) amplification was executed in Biometra 2003 T3 Thermo Cycler following standard PCR procedures for screening primers and final ISSR amplification. Each PCR- ISSR reaction was optimized in a mixture of 17µl sterile deionized H_2O, 0.8µl dNTP (25mM), 2.5µl Taqbuffer (10X reaction buffer S), 3µl MgCl2 (25mM), 0.4 µl primer (13pmol/µl), 0.3µl Taqpolymerase (5unit/µl) and 2µl diluted template DNA. The final reaction volume was 26µl and was subjected to PCR programmer settings run from pre-heating at 99℃, initial denaturation 94℃ for 4min, 40 cycles of denaturation at 94℃ for 15 seconds, primer annealing at (45℃/ 48℃) base on primer used for 1 min and extension at 72℃ for 1.30 min, and final extension at 72℃ for 7 min. The PCR product was stored at 4℃ (paused and storage temperature). The PCR products were visualized with UV light in 1.67% agarose gels, stained by immersion in 50µl ethidium bromide diluted with 450ml H_2O and destained with distilled H2O. The image was photographed with gel documentation system and documented for scoring.

ISSR primers that were used in genus *Brachiaria* to estimate the genetic variation of *B. ruziziensis* genotypes by Azevedo *et al*., (2011) and in other grasse family (Ghariani *et al*., 2003: Farsani *et al*., 2012) were used initially to screen primers. The PCR products such as preliminary screening, producing a higher level of polymorphism and reproducible fragment patterns were used as guidelines for the screening of the best top (reproducible) primers. Six best top (reproducible) primers (ISSR-812,ISSR-818,ISSR-841, ISSR-844, ISSR-873, and ISSR-880) were screened and optimized for detecting genetic diversity in *Brachiaria brizantha* samples out of 13 candidate primers (Table 2).

List of primers, primer sequence, annealing temperature, amplification pattern and repeat motives used for optimization and screening are indicated in Table 3.

Table 2. List of primers, primer sequence, annealing temperature, amplification pattern and repeat motives used for optimization and screening

ISSR-Primer	Primer motif	T°(℃)	Amplification quality	Repeat motif
848	(CA)8NG	48	X	Dinucleotide
818	(CA)8G	48	Polymorphic, reproducible	Dinucleotide
844	(CT)8RC	48	Polymorphic, reproducible	Dinucleotide
816	(CA)8T	48	X	Dinucleotide
841	(GA)8YC	48	Polymorphic, reproducible	Dinucleotide
812	(GA)8A	45	Polymorphic, reproducible	Dinucleotide
824	(TC)8G	48	X *	Dinucleotide
810	(AG)8T	45	X	Dinucleotide
813	(CT)8T	45	X	Dinucleotide
826	(AC)8C	48	X	Dinucleotide
834	(AG)8YT	45	X	Dinucleotide
880	(GGAGA)3	45	Polymorphic, reproducible	Penta-nucleotide
873	(GACA)4	48	Polymorphic, reproducible	Tetra-nucleotide

X: Less reproducible and less polymorphism, X*: no amplification, T° (℃): annealing temperature
Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T)
Source: Primer kit 900 (UBC 900)

4.5. Scoring and analysis of PCR-ISSR products

From a total of 242 accessions of *Brachiaria brizantha* at Zeway forage regeneration and conservation site, 79 accessions were randomly sampled for extraction of genomic DNA. ISSR-PCR amplification was carried out for these diluted DNA samples.

Agarose gel photographs that have shown clear PCR-ISSR product patterns (amplified bands) were scored as binary digit matrix (“1” for presence, “0” for absence and “?” for ambiguous) for all 79 accessions. A total of 80 bands were scored between 250 - 8000 bp

molecular weight of DNA ladders markers(10kbp). The matrix was fed and analyzed by different statistical software packages. POPGENE version1.32 software (Yeh *et al.*, 1999) was used to determine percentage of polymorphism, gene diversity (h), Shannon's information index (I). A genetic variation partitioned among and within seven population of this species was performed using Analysis of Molecular Variance (AMOVA), with Areliquin version 3.01 software (Excoffier *et al*., 2006).

To illustrate the genetic relationships among the 79 accessions, a dendrogram was constructed on the basis of Jaccard's similarity(S_{ij}) coefficient with one possible tie found between the closest pairs using un-weighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) and Neighbor Joining (NJ) (Saitou and Nei, 1987; Studier and Keppler, 1988) cluster analysis. Both cluster analysis were done using a software package of NTSYS (Numerical Taxonomy and Multivariate Analysis System) - pc version 2.02 algorism (Rohlf, 2000) and Free Tree 0.9.1.50 Software (Pavlicek *et al*., 1999) respectively. These softwares were used for calculation of Jaccard's similarity coefficient (S_{ij}). It was calculated by using the following formula:-

$$S_{ij} = \frac{a}{a + b + c}$$

Where, 'a' is the total number of bands shared between individuals i and j

'b' is the total number of bands present in individuals i but not in individuals j

'c' is the total numbers of bands present in individuals j but not in individuals i

The relationship of individual accessions on the scatter diagram was established using principal coordinated analysis (PCO) based on the results of Jaccard's similarity coefficient (Jaccard, 1908). It was computed from setting of PAST software version 1.18 (Hammer *et al.,* 2001). This software also performed two dimensional plots whereas three dimensional plots was done using three axes with the use of configuration of STATISTICA version 6.0 software (Hammer *et al.,* 2001; Sta. soft, Inc., 2001).

5. RESULTS

5.1. ISSR- primers and their banding patterns

Among the tested ISSR primers, only six primers, viz four di-nucleotide, one tetra-nucleotide and one penta nucleotide repeat motif, which produced relatively clear, reproducible and polymorphic bands, were selected as informative primers. The other seven ISSR primers gave either smeared, failed to give amplification products, poor or non-reproducible bands were excluded from the analysis. Thus, the ISSR primers selected for genetic diversity estimation based on preliminary evaluation using 3 representative individuals of 79 accessions of *Brachiaria brizantha* were ISSR-812, ISSR-818, ISSR-841, ISSR-844, ISSR-873, and ISSR-880(Table 3).

The six primers selected generated a total of 80 scorable and reproducible bands in 79 individuals. The molecular weight of the bands amplified using the primers were in the range of 750 bp to 8000 bp. The numbers of scorable bands that were generated by each primer ranges from 8 to 19 with an average of 13.3. The highest numbers of scorable bands (19) were generated with primer ISSR-812 while the only tetra-nucleotide primer ISSR-873 produced eight scorable bands. None of the bands was unique to a particular accession or population. Out of these ISSR primers, gel electrophoresis pattern obtained using primer ISSR-812 is illustrated in Figure 3. For the rest of the primers, gel pictures are shown in appendix 2.

Table 3. List of ISSR primers used showing their repeat motifs, amplification pattern and number of scorable bands (NSB).

ISSR-Primers	Sequence	Repeat motif	Amplification pattern	NSB
812	(GA)8A	Di-nucleotide	polymorphic, reproducible	19
818	(CA)8G	Di-nucleotide	polymorphic, reproducible	15
841	(GA)8YC	Di-nucleotide	polymorphic, reproducible	16
844	(CT)8RC	Di-nucleotide	polymorphic, reproducible	12
873	(GACA)4	Tetra-nucleotide	polymorphic, reproducible	8
880	(GGAGA)3	Penta-nucleotide	polymorphic, reproducible	10

Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T)

NSB: number of scorable bands

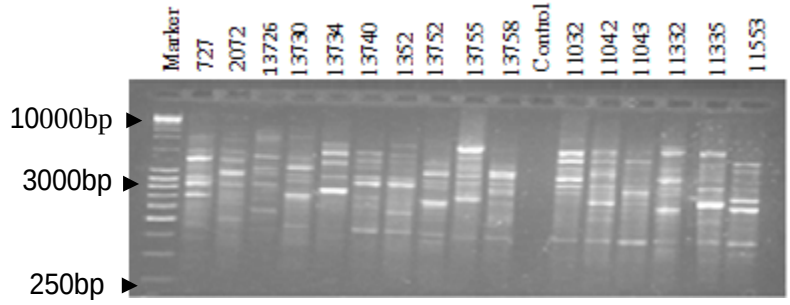

Figure 3. Gel picture of ISSR bands generated from accessions collected from Gojam and Jimma using ISSR-812 primer.

5.2. Level of genetic diversity

Seven populations of *B. brizantha* were subjected to genetic diversity analysis to evaluate the level of polymorphisms within populations and divergence among populations. PCR amplification with selected six ISSR primers generated a total of 80 scorable bands. The di-, tetra- and penta-nucleotide repeat motif primer employed in the analysis, did not equally contribute for levels of polymorphism in terms of number and percentage of variation within the populations (Table 4). Out of the total 80 bands, 77 fragments were polymorphic which accounts for 96.25%, with only 3 loci being monomorphic (Table 5).

Among these, maximum percentage of polymorphic loci (80%) was generated by ISSR-880 primer in Welega population and followed by ISSR-873 primer which revealed 75% variability in Ilu Ababora population. ISSR-841 primer generated the least percentage of polymorphic loci (6.25%) for Jimma population. Moreover, penta-nucleotide primer (ISSR-880) also generated high number of polymorphism loci within population as compared to the di- and tetra-nucleotides, indicating that the penta-nucleotides interestingly contributed more polymorphisms.

Table 4. Number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), gene diversity (h) and Shannon information index (I) calculated for each population per primer.

	ISSR- primers											
	812		818		841		844		873		880	
	NPL	PPL	NPL	PPL	NPL	PPL	NPL	PPL	NPL	PPL	NPL	PPL
Populations												
Welega	8	42.11	6	40	5	31.25	7	58.33	4	50	8	80
Jimma	8	42.11	4	26.67	1	6.25	7	58.33	4	50	6	60
Ilu Ababora	11	57.89	8	53.33	9	59.25	6	50	6	75	7	70
Borena	8	42.11	4	26.67	7	43.75	7	58.33	5	62.50	7	70
Omo	5	26.32	8	53.33	10	62.50	7	58.33	5	62.50	6	60
Gojam	4	21.05	9	60	7	43.75	6	50	5	62.50	7	70
Gondar	5	26.32	3	20	5	31.25	6	50	0	0	4	40
Overall												
NPL	17	89.47	15	100	16	100	12	100	7	87.00	10	100
h±	0.312±		0.401±		0.411±		0.371±		0.323±		0.373±	
SD	0.184		0.093		0.120		0.129		0.156		0.129	
I ±	0.464±		0.587±		0.595±		0.550±		0.483±		0.553±	
SD	0.243		0.104		0.139		0.149		0.217		0.146	

SD= Standard deviation

On the other hand, three di-nucleotide (ISSR-818, ISSR-841, and ISSR-844) and penta-nucleotide (ISSR-880) repeat motif primers generated 100% polymorphism among populations (Table 4). In the case of genetic diversity estimation due to gene diversity (h) and Shannon's information index (I), the highest values were achieved from ISSR-841 primer and the least from ISSR-812 primer (Table 4

Based on aggregate data from all the six ISSR Primers, the highest level of percent polymorphism was obtained from samples of Ilu Ababora (population), followed by Omo, Borena, Welega, Gojam and Jimma with 58.75% , 51.25%, 47.50%, 47.50, 46.50%, and 37.50 respectively. The least level of polymorphism were obtained from accessions in Gondar that had only 23 polymorphic loci which accounted for 28.75% percent polymorphism. Beside this, high level of polymorphism was detected when populations of *B. brizantha* were analyzed for by grouping based on geographical origins. Accordingly, the South West Ethiopia showed highest percent polymorphism (81.25%), while the least level of polymorphism were accounted by West Ethiopia with only 47.50%. The South and North West Ethiopia populations showed 76.25% and 71.25% percent polymorphism, respectively (Table 5).

The gene diversity (*h*) index of the seven populations of *B. brizantha* with overall primers analysis (Table 5), were calculated and the values ranged from 0.12 for Gondar to 0.21 (Ilu Ababora) populations and 0.37 at species level. Furthermore, when populations of *B. brizantha* samples collected from Ethiopia were grouped into their geographical origin, gene diversity (h) was least in West (0.16) and highest (0.30) in the south west of

Ethiopia. In addition, the same diversity patterns were also observed for Shannon diversity index whereby the least value was obtained from Gondar (0.18) in the north, while the highest value was achieved from Ilu Ababora (0.32) in the southwest. In case of geographical regions based analysis, south west Ethiopia showed the highest variability (0.45) while the least was obtained from west Ethiopia (0.25).

Table 5. The Number of polymorphic loci (NPL), Percent polymorphic loci (PPL), gene diversity(h) and Shannon's information(I) Index, using data generated from all the six primers. The analysis was carried out at population level and grouping made based on geographic origin

	NPL	PPL (%)	h±SD	I±SD
Populations				
Welega	38	47.50	0.162±0.183	0.247±0.272
Jimma	30	37.50	0.147±0.200	0.216±0.289
Ilu Ababora	47	58.75	0.210±0.195	0.315±0.282
Borena	38	47.50	0.184±0.218	0.272±0.294
Omo	41	51.25	0.183±0.198	0.274±0.295
Gojam	37	46.50	0.184±0.203	0.266±0.295
Gondar	23	28.75	0.124±0.187	0.183±0.274
Overall	77	96.25	0.366±0.140	0.539±0.177
Groups				
West Ethiopia	38	47.50	0.162±0.183	0.247±0.272
South west Ethiopia	65	81.25	0.303±0.182	0.449±0.250
South Ethiopia	61	76.25	0.250±0.174	0.383±0.247
North west Ethiopia	57	71.25	0.273±0.199	0.402±0.280
Overall	77	96.25	0.366±0.140	0.539±0.177

SD = Standard deviation

5.3. Analysis of Molecular Variance (AMOVA)

To assess the overall distribution of genetic diversity within and among population, the AMOVA analysis was carried out using the 80- ISSR bands generated. The AMOVA

results (Table 6) revealed highly significant genetic differences (P<0.001) between the four groups as well as between the seven populations of *B. brizantha* samples collected from Ethiopia. Of the total genetic diversity, 23.22% was attributed to populations within geographical groups, 13.37% to among groups and 63.52% to differences within populations. But the *F*ST value showed a relatively higher differentiation (*F*ST = 0.36) among geographical regions than at population level (*F*ST = 0.35). The genetic variation at population level accounted 35.34% of the total variation, while the within populations component accounted for 64.66% without creating grouping. Both analytical approaches revealed higher within population variation compared to among populations.

Table 6. Analysis of Molecular Variance (AMOVA) among and within seven populations of *B.brizantha*. The analysis was carried out by grouping population based on geographic origin (sample collected from West, Southwest, South, Northwest Ethiopia) and without grouping populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Indices (FST)	p-value
Among groups	3	144.28	1.07	13.37		p<0.001
Among populations within groups	3	75.73	1.88	23.22		p<0.001
Within populations	72	370.22	5.14	63.52		p<0.001
Total	78	590.23	8.10		0.36	
Among populations	6	220.01	2.81	35.34		p<0.001
Within populations	72	370.22	5.14	64.66		p<0.001
Total	78	590.22	8.10		0.35	

5.4. Cluster analysis

Dendrograms (Figure 4-6) were constructed by using (UPGMA) algorithm and neighbor joining (NJ) methods cluster analysis based on Jaccard's similarity coefficient matrixes with one possible tie found between the closest pairs. The dendrogram constructed from UPGMA clearly identified three major clusters (I, II and III) using Jaccard's similarity coefficient of around 0.38 (Figure 5). The first cluster was composed of 12 accessions collected from Jimma region. The second major cluster consisted of 19 accessions which were collected from Northwest Ethiopia (Gojam and Gondar regions). The third major cluster was composed of three sub-clusters, with the first sub-cluster consisting of 14 accessions collected from Welega region, the second sub-cluster consisting of 20 accessions collected from Borena and Omo regions and the third sub- cluster composed of 14 accessions came from Ilu Ababora region (Figure 5). Moreover, NJ tree also generated similar numbers of major and sub- clusters like that of UPGMA with similar distribution over groups (Figure 6). However, accessions collected from Welega and Ilu Ababora regions were formed new major clusters and associated to I and II respectively. The third major cluster in NJ tree was composed of accession collected from three regions (Gojam, Gondar and Jimma), Omo and Borena in first, second and third sub-clusters, respectively. In addition, Jaccard's similarity coefficient for populations ranged from 0.302 to 0.431, with the highest similarity value (0.431) was being between Gojam and Jimma populations and the least similarity value (0.302) being among Gondar and Jimma populations (Table 7).

Table 7. Pair wise Jaccard's similarity coefficient based comparisons among seven populations of *B. brizantha* collected from Ethiopia

Populations	Welega	Jimma	Ilu Ababora	Borena	Omo	Gojam	Gondar
Welega	1.00						
Jimma	0.335	1.00					
Ilu Ababora	0.407	0.345	1.00				
Borena	0.405	0.375	0.419	1.00			
Omo	0.385	0.321	0.339	0.409	1.00		
Gojam	0.352	0.431	0.369	0.348	0.348	1.00	
Gondar	0.344	0.302	0.395	0.322	0.378	0.423	1.00

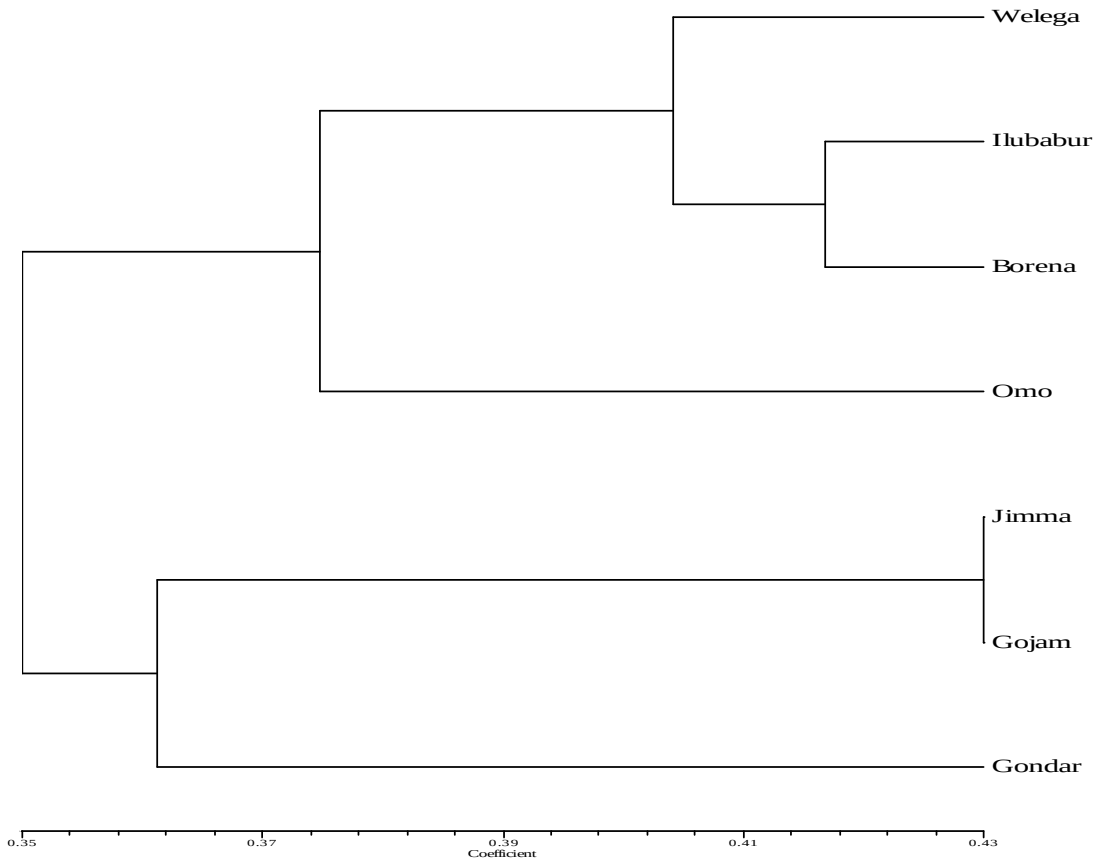

Figure 4. UPGMA based dendrogram for seven populations of *Brachiaria brizantha* collected from different regions of Ethiopia.

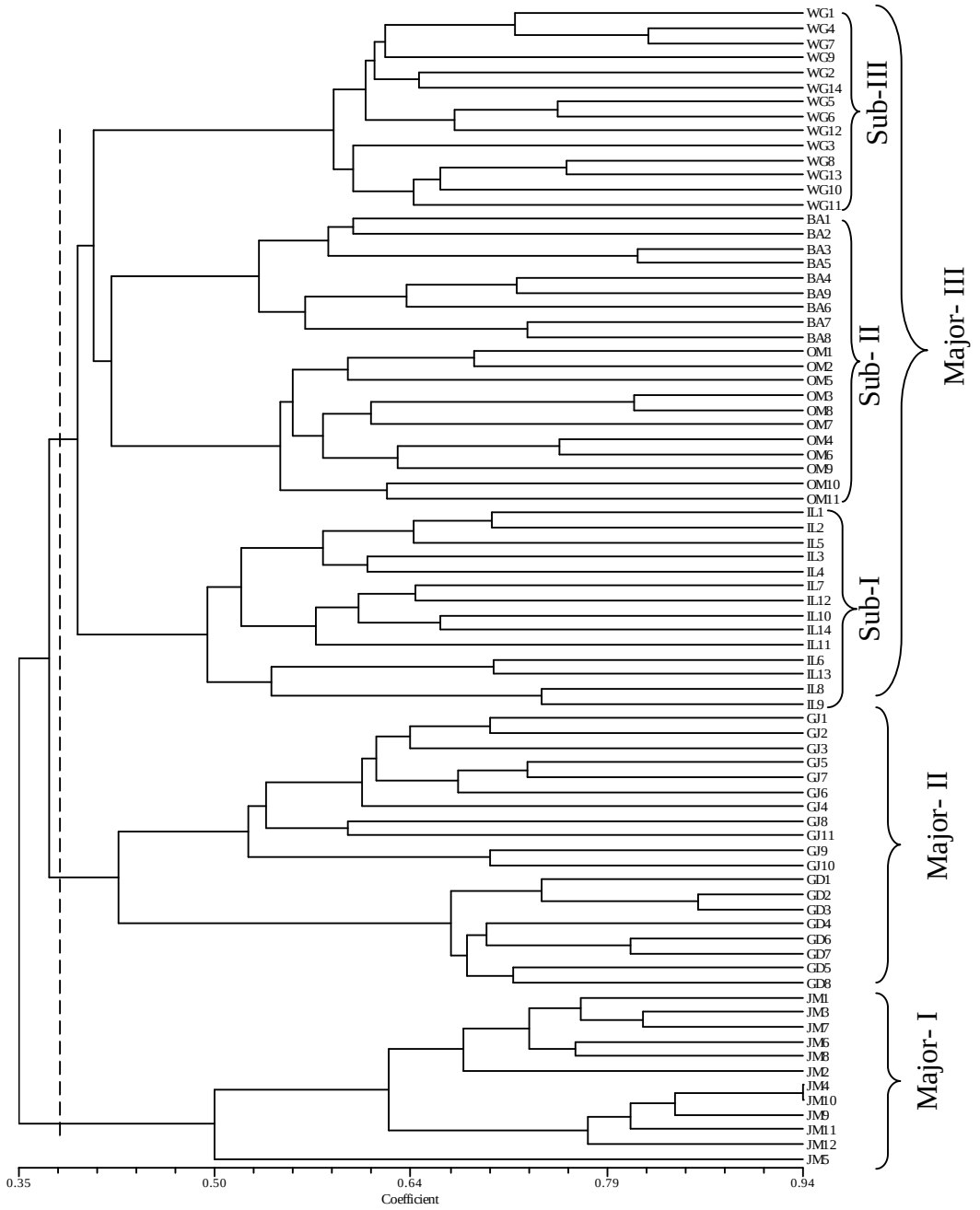

Figure 5. UPGMA based dendrogram for 79 accessions of seven populations of *Brachiaria brizantha using* 6 ISSR primers. Key: WG- Welega, JM-Jimma, IL-Ilu Ababora, GJ- Gojam, BA- Borena, GD-Gondar and OM-Omo; See appendix 1 for accessions symbols

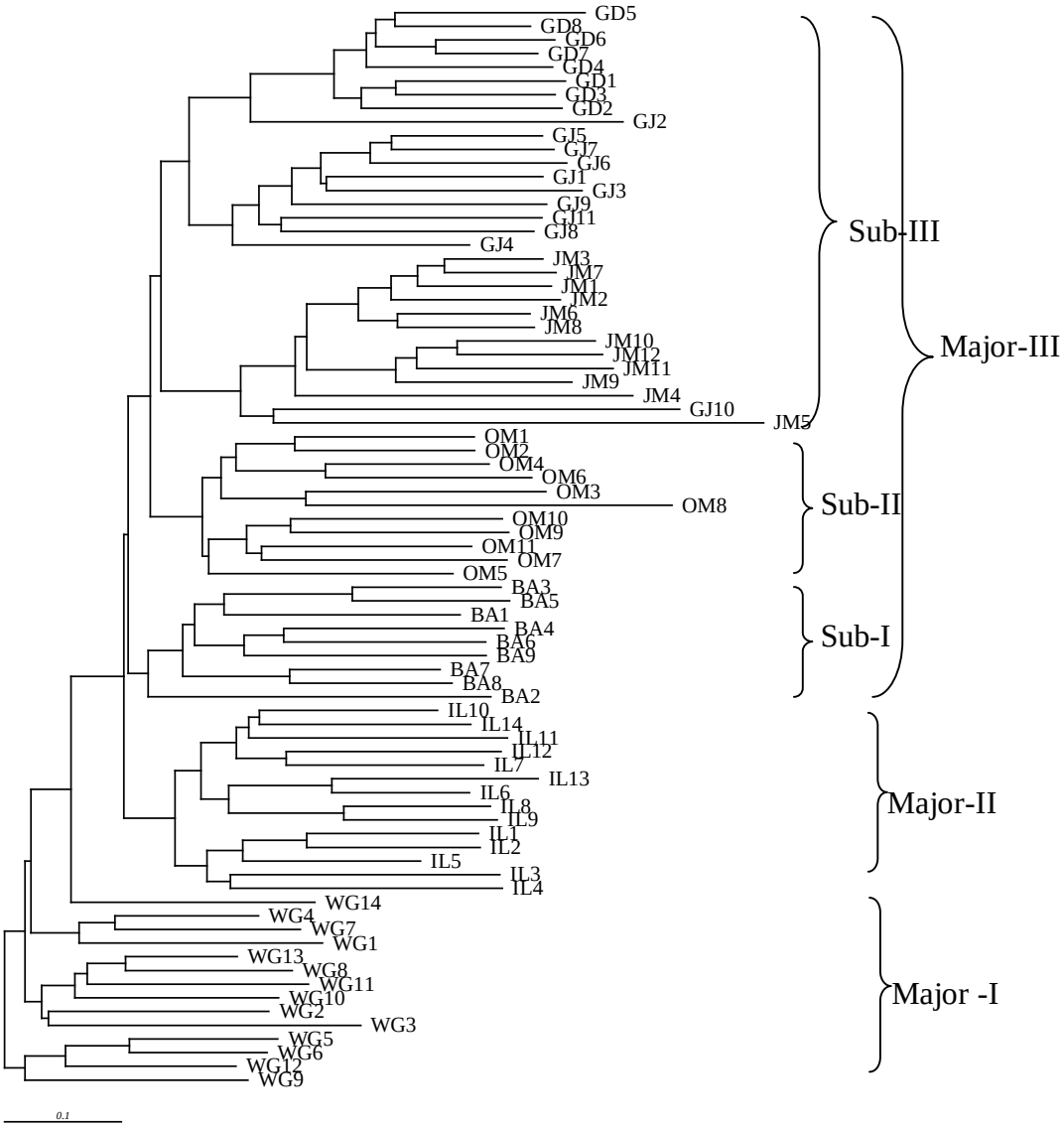

Figure 6. Neighbor-joining dendrogram for 79 accessions of seven populations of *B.brizantha* collected from various regions of Ethiopia using six primers. The NJ algorithm is based on Jaccard's coefficients obtained after pair wise comparison 80 ISSRs data. Key: WG-Welega, JM-Jimma, IL-Ilu Ababora, GJ-Gojam, BA- Borena, GD-Gondar and OM-Omo; See table 1 for accessions symbols

5.5. Principal Coordinate Analysis (PCO)

Principal coordinate analysis was carried out using Past and Statistica software packages by employing Jaccard's coefficients of similarity to plot the ISSR products (80 scored

bands) of 79 accessions of *B. brizantha* on 2D and 3D coordinate planes. The first three coordinates of the PCO had eigen-values of 11.44, 7.22 and 3.60 with percentage of 38.71%, 24.44% and 12.17%, respectively. The analysis of this study showed that the accessions of the seven populations examined tend to form clusters based on their geographic origin. Moreover, two dimensional representations showed samples from Jimma, Welega, Gojam and Gondar formed strict distinct grouping (Figure 7-8). Likewise, the result of three coordinate analyses showed similar pattern.

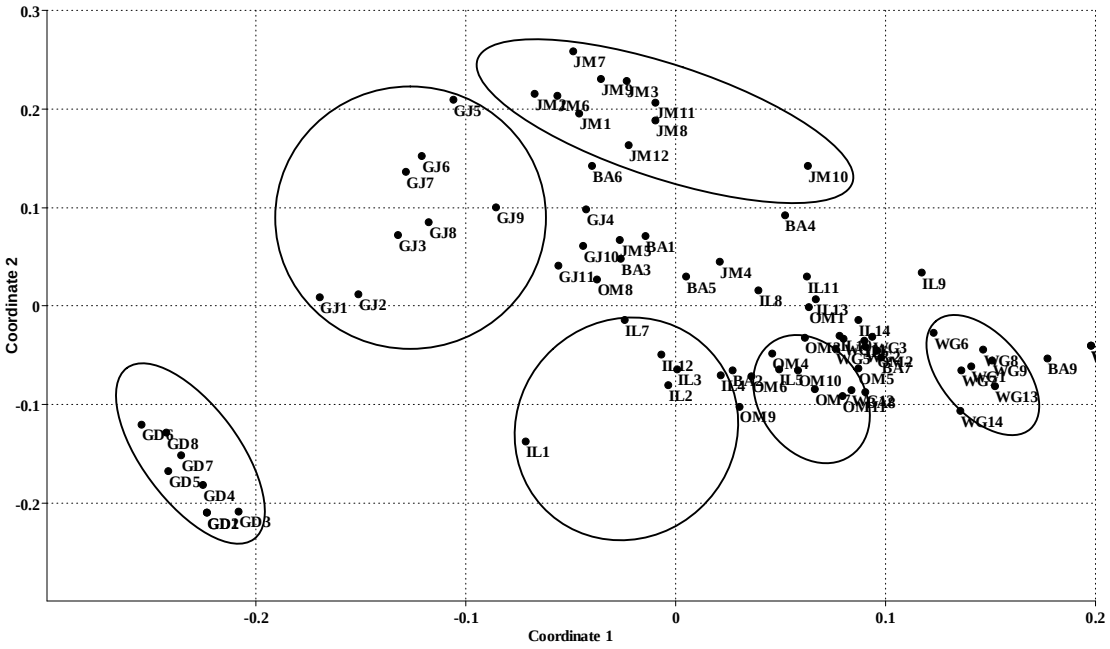

Figure 7. Two dimensional representations of 79 *Brachiaria brizantha* accessions based on Jaccard's similarity coefficients. Key: WG-Welega, JM-Jimma, IL-Ilu Ababora, GJ- Gojam, BA- Borena, GD-Gondar, and OM-Omo

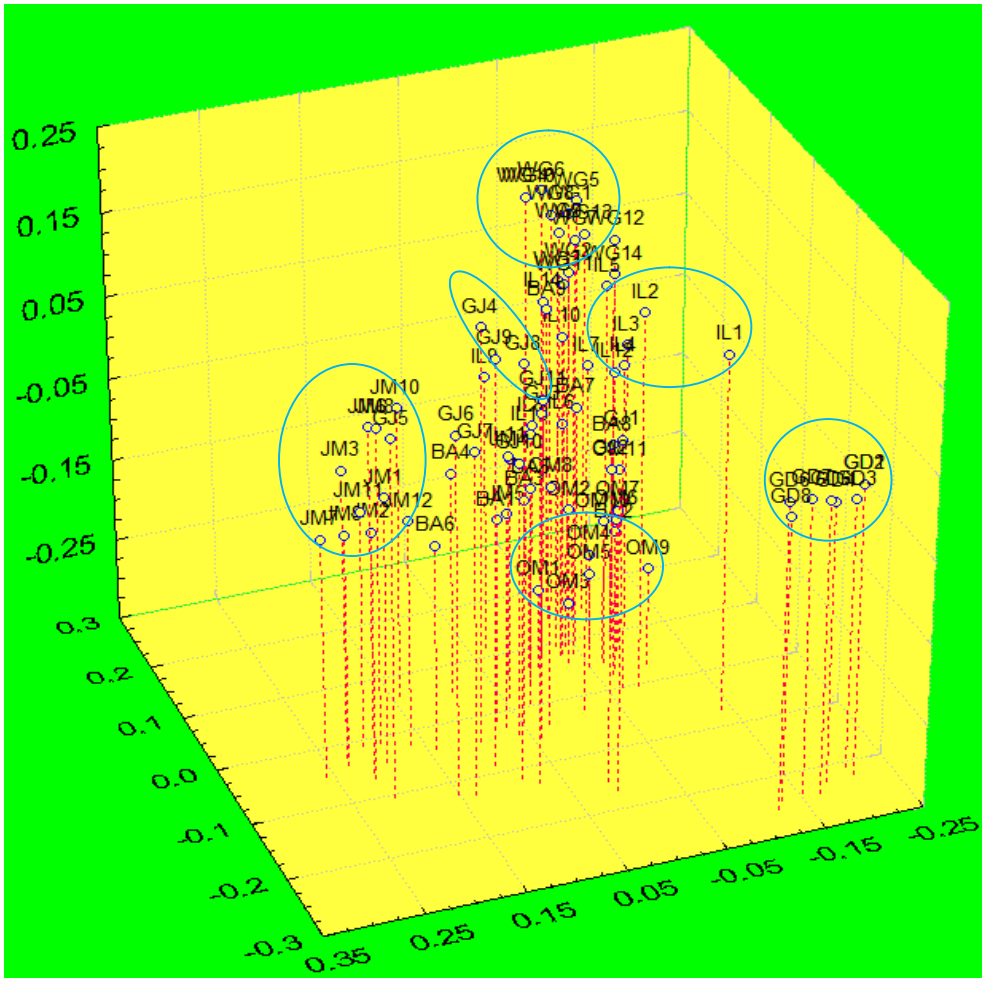

Figure 8.Three dimensional representation of principal coordinate analysis of genetic relationships among 79 accessions of *Brachiaria brizantha* accessions as revealed by ISSR marker generated by six primers. Key: WG-Welega, JM-Jimma, IL-Ilu Ababora , GJ- Gojam, BA- Borena, GD-Gondar, OM-Omo See table 1 for accessions symbols

6. DISCUSSION

6.1. ISSR markers and its use in *Brachiaria brizantha*

In the present investigation, ISSR markers have been employed to assess the genetic diversity of seven populations of *Brachiaria brizantha* collections in Ethiopia for the first time. Due to their simplicity, reliability, reproducibility and ability to detect high level of polymorphism, ISSR technologies are useful for analysis of the molecular genetic variability in plants (Zietkiecz *et al*, 1994; Morgante *et al*., 2002). For instance, ISSR markers were used to investigate variability within and among populations of lentil (Edossa Fikru *et al*, 2010) and *B.ruziziensis* (Azevedo *et al*., 2011). However, effective analysis of genetic diversity by ISSR markers depends on the variety and frequencies of microsatellites within the specific genomes (Chunjiang *et al*., 2005).

Bulk sampling strategies are important to represent enormous accession for genetic diversity analysis of a species (Gilbert *et al*., 1999). Bulking may be performed at the level of plant material or genomic DNA template. Hence, three leaves form three individual per accessions were bulked in this study following other parallel studies in lentil (Edossa Fikru *et al*, 2010) and sesame (Dagmawi Teshome, 2011) that have reported that bulking at the leaf stage is effective in producing representative profiles. The optimal bulk size has been estimated in several studies. In the case of RAPD and AFLP analysis, relevant bulk size of 20 and 30 individuals were reported for perennial ryegrasses (Sweeney and Danneberger, 1994; Guthridge *et al*., 2001). However, for detection of rare alleles and effective measurement of genetic variation within populations, 3-5 individuals are optimal (Gilbert *et al*., 1999; Kraft and Sall, 1999).

Studies indicated that primers with arbitrary sequence have produced detectable levels of amplifications (Williams *et al*., 1993). ISSR primers were observed to be useful to detect polymorphism in grasses including *Brachiaria* spp. (Ghariani *et al*., 2003, Azevedo *et al*., 2011, Farsani *et al*., 2012). In the present study, each ISSR primers (four di-, tetra-and penta-nucleotide repeat motif primer) detected high level of polymorphism, gene diversity and Shannon information's index at species level. Pent nucleotide repeat (ISSR-880) and tetra-nucleotide repeat (ISSR-873 motif primers detected higher percentage of polymorphic loci in Welega (80%) and Ilu Ababora (75%) than di-nucleotide repeat motif primers did. However, only tetra-nucleotide repeat primer (ISSR-873) did not detect any polymorphism among accessions of Gondar, while di-nucleotide repeat primer ISSR- 841 identified the least polymorphism (6.25%) within Jimma population.

Four Primers viz ISSR-818, ISSR- 841, ISSR-844 and ISSR-880 detected 100% polymorphism among populations collected from Ethiopia. The results suggest that primers with di-nucleotide repeat motif detected high level of genetic diversity. The comparable analysis of 15 samples in perennial ryegrass and 93 genotypes in *B. ruziziensis* by 13 and 12 ISSR primers respectively, high level of polymorphisms were detected by di-nucleotide repeats primers and penta-nucleotide repeats motif primers (Pivoriene and Pasakinskiene, 2008, Azevedo *et al.*, 2011).

6.2. Genetic diversity in *Brachiaria brizantha* from Ethiopia

The overall analysis of the seven *B. brizantha* populations in this study using six ISSR primers generated 80 scorable and reproducible bands with the average being 13.3. The

ISSR bands generated in this study were subjected to genetic diversity analysis parameter and identified the availability of a range of genetic diversity indexes at population level, with 96.25% of bands displaying polymorphism at species level. However, there was considerable variation in percent polymorphism; with values ranging from 28.75% for Gondar population to 58.75% for Ilu Ababora population. The comparable analysis of 42 samples in *Brachiaria* species (*B. brizantha, B. decumbens, B. ruziziensis, B. jubata, B. nigropedata and B. humidicola*) using 10 RAPD markers generated 114 polymorphic bands with an average of 11.4 bands (Ambiel *et al*., 2010). Similarly, 184 polymorphic and 18 monomorphic bands with average of 14 using 14 RAPD markers from 37 samples in *B. ruziziensis* were reported by Guaberto, (2009). Azevedo *et al*., (2011) also identified 89 polymorphic bands (7.4bands/primer) using 12 ISSR markers for 93 genotypes of *B. ruziziensis*.

In line with this, Shannon index at species level (0.54) in the present study was higher than the average values for long lived perennial herbs (0.116) and widespread species (0.202) (Hamrick and Godt, 1989).

The percentage of polymorphism, gene diversity and Shannon's information index were consistently higher for accessions from Ilu Ababora as compared to those from other regions, indicating that a relatively greater genetic diversity lies among the accessions from Ilu Ababora (14). In contrast, sample from Gondar (8) were the least polymorphic with low genetic diversity parameter. This variation in polymorphism could probably be

a consequence of geographical isolation which limited the extent of gene flow that could be caused by over grazing of grasses including *B. brizantha*.

Analysis on the basis of geographical regions, viz northwest, west, south west and south Ethiopia, showed a range of value of percent polymorphism, gene diversity and Shannon information's index. Among the four geographic groups, southwest Ethiopia accommodated more genetic diversity as compared to others. The population based analysis revealed high and moderate intra-population molecular genetic diversity for Ilu Ababora and Jimma populations, respectively, where both are located in southwest part of Ethiopia. This could be explained by the presence of natural forest which is rich in biodiversity/biodiversity hotspot. Moreover, the area is also recognized as the center of origin and diversity for several plant species (IBC, 2007). However, populations from west Ethiopia showed low level of genetic variability than other groups which could be explained by overgrazing by livestock. Samples of accession for northwest and south Ethiopia exhibited nearly comparable pattern of genetic diversity. The results suggest that high value of genetic diversity correlated with sample size and coverage of geographic area (26 samples from southwest Ethiopia viz from Ilu Ababora, Jimma and 14 samples west Ethiopia (Welega) showed genetic diversity (h) of 0.303 and 0.162 respectively). The results appear to be in agreement with the fact that the larger the population size the higher will be variability observed (Prober and Brown, 1994; Nageswara Rao *et al*., 2007; Ravikanth *et al*., 2008).

Genetic diversity is important for ecological and evolutionary processes ranging from individual fitness to ecosystem functions (Rezvani *et al*., 2012). In this study; each genetic diversity parameters confirm that there is moderate to high gene diversity in population of *B.brizantha* collected from Ethiopia. Therefore, the diverse populations such as Ilu Ababora and Omo populations should be targeted for conservation and further breeding programs as genetic variation is a key for successful breeding program to develop suitable cultivars that can be adapted to different environments and management systems.

The AMOVA revealed a higher share of genetic variation within population of *B. brizantha*. This is in agreement with the partitioning of a variation of 84% that was observed within this species based on 15 SSR markers (Vigna *et al*., 2011). A similar result was obtained in the analysis of genetic variation of other *Brachiaria* species. Azevedo *et al*., (2011) found about 73.43% of variation within population in *B.ruziziensis* using 12 ISSR markers. Ambiel *et al*., (2010) also reported that 68.79% of total diversity in different *Brachiaria* species (*B. brizantha, B. decumbens, B. ruziziensis, B. jubata, B. nigropedata* and *B. humidicola*) was intra-species based on 10 RAPD markers. *B. ruziziensis* had largest proportion of variation (63.93%) within population as revealed by 14 RAPD markers (Guaberto, 2009).

The population genetic structure of a species is determined by a number of evolutionary factors including reproductive behaviors, natural selections and seed dispersal mechanisms (Hamrick and Godt, 1990). Therefore, AMOVA analysis of the seven

populations of *B. brizantha* from Ethiopia revealed higher proportion of genetic variations within population than among population, which could be explained by higher level of gene flow. Moreover, pollen flow by insects and wind, and seed dispersion during hay transportation, migration of animal and birds could explain this observation. The observed large genetic variability reduces the chance of genetic drift and inbreeding depression and hence increases the survival chance of the population under various biotic and abiotic stresses including the current risk of climate change.

6.3. Genetic relationship of *Brachiaria brizantha* populations

To better visualize the genetic relationship of accessions of *Brachiaria brizantha*, cluster analysis (UPGMA, NJ) and PCO were performed on the basis of Jaccard's similarity coefficient. UPGMA has showed clear clustering of accession of *B. brizantha* on the basis of their regions of collection and respective populations (Figure 5). The result revealed that the individuals collected from the same geographic region showed close genetic relationships and were grouped into the same cluster. On the other hand, NJ analysis revealed a slight different pattern of cluster compared to UPGMA. For instance, samples collected from Welega were separated as major cluster I. However, it was a member of major cluster III as sub-cluster in the case of UPGMA. It was also clustered a sample (GJ10) with Jimma population as UPGMA did not. Hence, UPGMA analysis is corroborated with Jaccard's similarity coefficients value which was not close to 1.0 between any two different populations, indicating no redundant accessions among populations that were sampled in this study. Besides, PCO analysis based on two and three coordinates showed that populations of Jimma, Gondar, Gojam and Welega clearly

formed their own clusters while others tend to scatter on the 2D and 3D space. The results suggest that there were clear divergence among accession of the seven populations that should be exploited for breeding via heterosis effect (Gallais, 1988; Patrick *et al*. 2013).

7. CONCLUSION

The ISSR products were investigated through genetic diversity parameters and detected various level of genetic diversity within seven populations of *Brachiaria brizantha*. Accessions collected from Ilu Ababora regions revealed high degree of genetic diversity than other regions warranting that they should be protected and safeguarded while the least diverse from Gondar region should get special attention in order to balance genetic resources of such beneficial genes across the various ecosystems. To sum up, genetic diversity was detected at species level by ISSR markers in *Brachiaria brizantha* population from Ethiopia. This genetic diversity will serve as inputs for further successful genetic improvement and breeding program in the case of *Brachiaria brizantha* to provide farmers with suitable new cultivars of forage adapted to different environments and management systems. It implies that wide range of genetic diversity in forage crop can contribute for sustainable growth and development of the livestock sector in Ethiopia by increasing the productivity of the livestock.

8. RECOMMENDATIONS

In this study a wide range of genetic diversity were identified. It suggested that:

- Conservation attention should be given to areas with higher diversity such as Ilu Ababora and Omo which could serve as source of diverse individuals for forage improvement.
- Crossing program should focus on divergent accession from Ilu Ababora to exploit heterosis effect.
- Populations from Gonder were observed to show low diversity and hence, special attention should be give to conserve these rare accessions.
- In this study, only 79 accessions *Brachiaria brizantha* were evaluated. Hence, future evaluation of genetic diversity with better representation of regions across Ethiopia should be carried out to compare the level of diversity at different agro-ecologies.
- The DNA based diversity analysis should be supplemented with field based morphological markers to better contribute to the greater understanding of this species diversity under different environments.
- Molecular genetic markers have their own advantages and limitations. Hence, an integrated analysis should be done using more advanced molecular markers such as AFLP, SSR and chloroplast markers for assessment of genetic diversity and gene flow within and among populations of *Brachiaria brizantha* in Ethiopia.

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10. APPENDICES

Appendix 1. List of accession of *Brachiaria brizantha* species used for genetic diversity analysis

Accessions	AS		**State**	**Zones**	**Wereda/ district**	**Altitude**
2072	GJ1	-	B. Gumuz	Metekel	Dangur	1200
13726	GJ2	-	Amhara	Gojam	Machakel	1920
13730	GJ3	Apo	Amhara	Gojam	Guzamn	1690
13734	GJ4	Apo	Amhara	Gojam	Guzamn	1490
13740	GJ5	-	B. Gumuz	Metekel	Mandura	1280
13751	GJ6	-	B. Gumuz	Metekel	Mandura	1150
13753	GJ7	-	Amhara	Gojam	Merawi	1940
13755	GJ8	-	Amhara	Gojam	Merawi	2050
13758	GJ9	-	Amhara	Gojam	BD Zuria	1900
13762	GJ10	-	Amhara	Gojam	BD Zuria	1850
13826	GJ11	Apo	Amhara	Gojam	Kuarit	1800
9677	JM1	Apo	Oromiya	Jimma	Kersa	1450
11032	JM2	-	Oromiya	Jimma	Kersa	1840
11042	JM3	-	Oromiya	Jimma	Kersa	1840
11043	JM4	Apo	Oromiya	Jimma	Kersa	1740
11332	JM5	-	Oromiya	Jimma	S. cherorsa	1410
11335	JM6	Apo	Oromiya	Jimma	S. cherorsa	1520
11553	JM7	Apo	Oromiya	Jimma	Sokoru	1850
13584	JM8	Apo	Oromiya	Jimma	S. cherorsa	-
13591	JM9	Apo	Oromiya	Jimma	S. cherorsa	1870
13594	JM10	Apo	Oromiya	Jimma	Goma	1670
13598	JM11	Apo	Oromiya	Jimma	Goma	1750
13600	JM12	Apo	Oromiya	Jimma	Goma	1750
13346	WG1	-	Oromiya	Welega	Bila Sey	1750
13352	WG2	Apo	Oromiya	Welega	Diga Leka	1900
13353	WG3	Sex	Oromiya	Welega	Diga Leka	1500
13356	WG4	-	Oromiya	Welega	Gimbi	1300
13365	WG5	Apo	Oromiya	Welega	Boji	2000
13368	WG6	Apo	Oromiya	Welega	Nejo	1990
13375	WG7	-	Oromiya	Welega	Mena Sibu	1900
13377	WG8	Apo	Oromiya	Welega	Diga Leka	1470
13379	WG9	-	Oromiya	Welega	Diga Leka	2160
13382	WG10	Apo	Oromiya	Welega	Jimma Arjo	1440

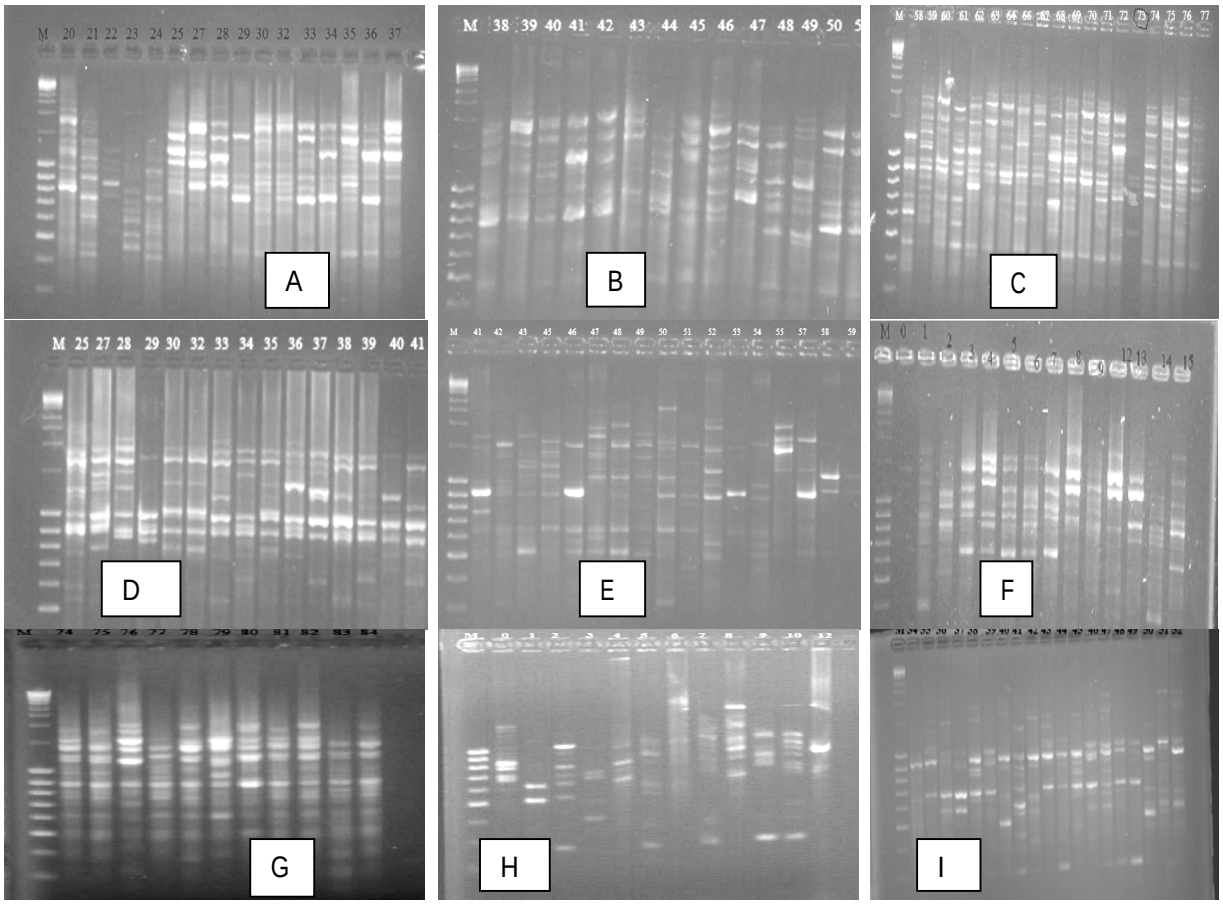
13635	WG11	-	Oromiya	Welega	Hawa Welele	1760
13640	WG12	-	Oromiya	Welega	Dale Lalo	1500
13643	WG13	Apo	Oromiya	Welega	Dale Lalo	1560
13648	WG14	Apo	Oromiya	Welega	Yubdo	1640
13386	IL1	Apo	Oromiya	Ilu Ababora	Bedele	1570
13389	IL2	Apo	Oromiya	Ilu Ababora	Bedele	1990
13391	IL3	-	Oromiya	Ilu Ababora	Bedele	2010
13602	IL4	Apo	Oromiya	Ilu Ababora	Dedesa	2100
13604	IL5	Apo	Oromiya	Ilu Ababora	Gechi	2010
13606	IL6	-	Oromiya	Ilu Ababora	Bedele	1990
13610	IL7	-	Oromiya	Ilu Ababora	Chora	1620
13614	IL8	Apo	Oromiya	Ilu Ababora	Yayu	1610
13617	IL9	Apo	Oromiya	Ilu Ababora	Ale	1610
13619	IL10	Apo	Oromiya	Ilu Ababora	Bure	1610
13620	IL11	Apo	Oromiya	Ilu Ababora	Bure	1720
13622	IL12	Apo	Oromiya	Ilu Ababora	Bure	1510
13623	IL13	Apo	Oromiya	Ilu Ababora	Bure	1510
13629	IL14	Apo	Oromiya	Ilu Ababora	Soya	1320
13420	BA1	-	Oromiya	Borena	Adolana wedera	1800
13423	BA2	-	Oromiya	Borena	Adolana wedera	2000
13429	BA3	Apo	Oromiya	Borena	Adolana wedera	1960
13434	BA4	-	Oromiya	Borena	Liben	1850
13436	BA5	Apo	Oromiya	Borena	Liben	1750
13448	BA6	Apo	Oromiya	Borena	Adolana wedera	1800
13452	BA7	Apo	Oromiya	Borena	O. Shakiso	1970
13456	BA8	-	Oromiya	Borena	hagermariam	1920
13462	BA9	-	Oromiya	Borena	Yabelo	1610

13467	OM1	Apo	SN	Omo	Damot Gale	1940
13469	OM2	Apo	SN	Omo	Sodo Zuria	1950
13492	OM3	Apo	SN	Omo	Sodo Zuria	1660
13499	OM4	-	SN	Omo	Offa	1840
13482	OM5	Apo	SN	Omo	AMZ	1690
13484	OM6	-	SN	Omo	Chencha	2080
13502	OM7	Apo	SN	Omo	Kucha	1420
13506	OM8	Apo	SN	Omo	Gofa Zuria	1510
13518	OM9	Apo	SN	Omo	Selamago	1740
13522	OM10	Apo	SN	Omo	Selamago	1990
13525	OM11	-	SN	Omo	Gofa Zuria	1070
13790	GD1	-	Amhara	Gondar	Dembia	1850
13799	GD2	-	Amhara	Gondar	Dembia	1910
13802	GD3	Apo	Amhara	Gondar	Gondar	2180
13809	GD4	Apo	Amhara	Gondar	Chilga	2050
13810	GD5	Apo	Amhara	Gondar	Chilga	2080
13815	GD6	Apo	Amhara	Gondar	Chilga	2080
13777	GD7	Apo	Amhara	Gondar	Fogera	1820
13786	GD8	Apo	Amhara	Gondar	Gondar Zuria	2040

Keys:

YSW: Yem Special Wereda SN: Southern Nation
BD Zuria: Barhirdar Zuria
B. Gumuz: Benishangul Gumuz
S. cherorsa: Sera Cherorsa,
AS: accessions symbols
MHB: Mennana Herena Buluk
O. Shakiso: Odu Shakiso
A MZ: Arba Minch Zuria
RM: Reproductive mode
G. Gofa: Gamo Gofa
K. A. and T: Kembata Alaba and Tembaro
G J* and Omo: former administrative demarcation

Appendix 2. ISSR fingerprint generated from seven populations of *Brachiaria brizantha* using six primers. Primer 812 (A-C), primer 818 (D), Primer 841 (E), primer 844 (F-G), primers 873 (H) and Prime 880 (I) respectively



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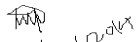
Addis Ababa, Ethiopia

This thesis has been submitted for examination with my approval as the research supervisor of the candidate.

Advisor: fentahun meheret Signature: _____  Date: 11/02/2014

Advisor: _____Signature: _____Date: _____

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