

Genetic Diversity Study of *Brachiaria brizantha* (A.Rich) Stapf Collected from Ethiopia Using Inter- Simple Sequence Repeat (ISSR) Markers

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Abstract

Brachiaria brizantha is a grass that instigate the economical growth in livestock sectors. However, it is orphan forage species where genetic degradation due to poor rangeland management practices, particularly overgrazing combine with climate changes highly affect its contribution in Ethiopia. Research centers have been confused to re-introduce the germplasm. It is because of lack of species profile documentation. Genomic DNA was extracted from leaf by using a modified CTAB method. A total of 80 bands were amplified by the six ISSR primers. Percentage of polymorphic loci (PPL), gene diversity (h) and Shannon information's index (I) were 96.25%, 0.366 and 0.539, respectively among population. Analysis of molecular variance indicated the presence of higher proportion of variation within population (64.66%) than among populations (35.34 %). Cluster analysis using the un-weighted paired group method with arithmetic average (UPGMA) at Jaccard's similarity coefficient of around 0.38 clustered the accessions into three major (I, II and III) clusters in their respective origin of collection. Principal Component Analysis (PCO) showed accessions in populations formed their own distinct and clear cluster. Thus ISSR markers detected a range of genetic diversity in *Brachiaria brizantha* germplasm collections from Ethiopia.

Keywords: *Brachiaria brizantha*, Genetic diversity, ISSR markers

Introduction

Brachiaria brizantha is a forage grass belongs to the family Poaceae, genus *Brachiaria* which encompasses more than 100 species. It grows in various regions of Ethiopia (Phillips, 1995). It is known to exist in diploid to polyploidy 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; Riso-Pascotto *et al.*, 2006). *Brachiaria brizantha* is naturally variable grass across tropical ecosystems which could be identified and characterized with the use of genetic markers. Characterization with morphological and molecular (SSR and biochemical) markers 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).

Studies showed that 736 species of grasses are recorded so far in Ethiopia which are adapted to different ecosystems throughout the country and are key sources of animal feed (IBC, 2005). However, these ample genetic resource is not exploited yet in the country. Therefore, research on molecular diversity of forage species for their genetic improvement through breeding is vital. To that end, this study was carried out to estimate genetic diversity, variability within and among populations of *Brachiaria brizantha* in Ethiopia by using ISSR markers.

Materials and Methods

Plant materials

Plant samples were collected from Zeway Forage Conservation Site of ILRI that was previously collected from various regions of Ethiopia. This site is located about 165km south of Addis Ababa at 7°53' 9" N, and 38°44' 68" E, at altitude of 1640 meter above sea level with 700 mm annual average rainfall, 26°C and 20°C maximum and minimum average temperature, respectively. The soil type is sandy loam with pH of 8.04. List of number of accessions used in this study are shown in Table 1.

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	

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. The leaves powder was incubated with warmed 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% β -mercaptoethanol) for 30min followed by centrifugation at 13,000 rpm for 7min and decanted the supernatant. The pellet was re-dissolved with CTAB extraction buffer again to attained quality DNA and centrifuge with similar revolutions. The second supernatant was treated with chloroform and centrifuge at 13,000 rpm for 7min. The crude DNA in supernatant solution was precipitated at 20°C. With treatment of isopropanol for 2 hours, then it was centrifuge at 13,000 rpm for 12min, followed by aspiration and washed with ethanol (70%). For further purification a pellet was dissolved with TE buffer and NH_4Ac was added first, then Na Ac in the second round. Each salt treatment was followed by 100% ethanol, 2hour incubation, centrifugation at 13,000 rpm for 35min, followed by aspiration the fluid, washed pellet with ethanol (70%) and aspirated. Finally DNA was re-suspended in TE buffer.

Genomic DNA amplification and Data analysis

Out of 13 candidate primers 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, six best top (reproducible) primers (812,818,841,844, 873, and 880) were screened and optimized for detecting genetic diversity in *Brachiaria brizantha*.

Each PCR reaction was optimized in a mixture of 17 μl sterile deionized H_2O , 0.8 μl dNTP (25mM), 2.5 μl Taqbuffer (10X reaction buffer S), 3 μl MgCl_2 (25mM), 0.4 μl primer (13pmol/ μl), 0.3 μl Taqpolymerase (5unit/ μl) and 2 μl diluted template DNA. The final reaction volume was 26 μl and was subjected to PCR programmer settings run from pre-heating at 99°C, initial denaturation 94°C for 4min, 40 cycles of denaturation at 94°C for 15 seconds, primer annealing at (45°C/ 48°C) base on primer used for 1 min and extension at 72°C for 1.30 min, and final extension at 72°C for 7 min. The PCR product was stored at 4°C (paused and storage temperature). The PCR products were separated in 1.67% agarose gels. Then it was stained by immersion in ethidium bromide and visualized with UV light. The image was photographed with gel documentation system and documented for scoring.

Agarose gel photographs that have shown clear PCR product patterns (amplified bands) were scored as binary digit matrix (“1” for presence, “0” for absence and “?” for ambiguous). A total of 80 bands were scored between 250-8000 bp molecular weight of DNA ladders markers (10kbp). The matrix data was fed and analyzed by different statistical software packages such as POPGENE version1.32 (Yeh *et al.*, 1999) and Areliquin version 3.01(Excoffier *et al.*, 2006) for analysis of percentage of polymorphism (PPL), gene diversity (h) and Shannon’s information index (I), and Analysis of Molecular Variance (AMOVA), respectively. Un-weighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) analysis was done using NTSYS (Numerical Taxonomy and Multivariate Analysis System) - pc version 2.02 algorism (Rohlf, 2000). Furthermore, 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).

Results

ISSR- primers and their banding patterns

In this study, six ISSR primers viz four di-nucleotide (812, 818, 841, 844),one tetra-nucleotide (873) and one penta nucleotide (880) repeat motif produced relatively clear, reproducible and polymorphic bands were used

for genetic diversity analysis (Table 2). Figure 1 illustrated the banding pattern of ISSR primers products obtained from accessions in Gojam and Jimma population.

The six ISSR primers generated a total of 80 scorable and reproducible bands in 79 accessions. 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 812, while the only tetra-nucleotide primer 873 produced eight scorable bands. None of the bands was unique to a particular accession or population.

Table 2. List of ISSR primers, sequence, number of scorable bands (NSB), Number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), gene diversity (h) and Shannon information index (I)

ISSR-Primers	Sequence	NSB	NPL	PPL	H	I
812	(GA)8A	19	17	89.47	0.312	0.464
818	(CA)8G	15	15	100	0.401	0.587
841	(GA)8YC	16	16	100	0.411	0.595
844	(CT)8RC	12	12	100	0.371	0.550
873	(GACA)4	8	7	87	0.323	0.483
880	(GGAGA)3	10	10	100	0.373	0.553

Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T) NSB: Number of scorable bands

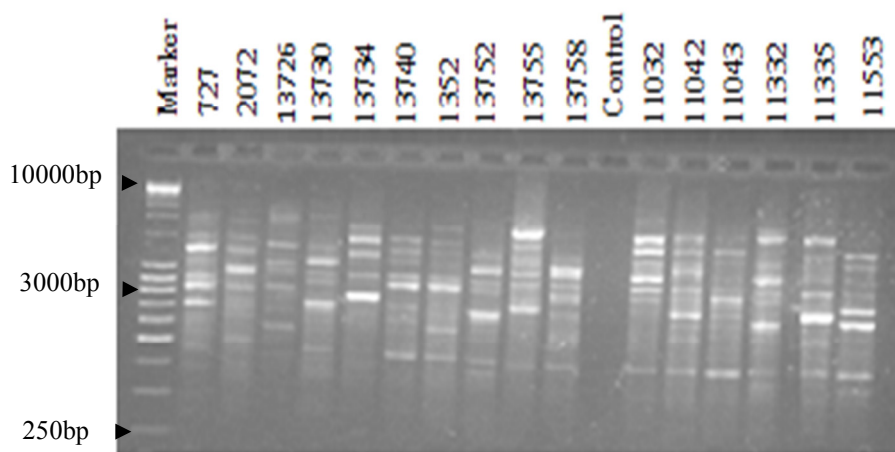


Figure 1. Gel picture of ISSR bands generated from accessions collected from Gojam and Jimma using ISSR primer.

Level of genetic diversity

Out of the total 80 scorable bands, 77 fragments were polymorphic which accounts for 96.25%, with only 3 loci being monomorphic (Table 3). Among these, maximum percentage of polymorphic loci (100%) was generated by four primers viz 818,841,844 and 880 whereas primer (873) amplified the least percent of polymorphism loci (87%) among seven populations. In the case of genetic diversity estimation due to gene diversity (h) and Shannon's information index (I), the highest values were obtained from 841 primer and the least from 812 primer (Table 3).

In the case of population analysis, the highest level of percentage of polymorphic loci (PPL) 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 which accounted for 28.75% PPL. Beside this, high level of polymorphism was detected when populations of *B. brizantha* were analyzed via grouping based on geographical origins. Accordingly, the South West Ethiopia showed the highest PPL (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% PPL, respectively (Table 3).

The gene diversity (*h*) index ranged from 0.12 for Gondar to 0.21 (Ilu Ababora) population and 0.37 at species level (Table 3). 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 (I) 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 3. The Number of polymorphic loci (NPL), Percent of polymorphic loci (PPL), gene diversity (h) and Shannon's information Index (I) of population and groups

	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

Analysis of Molecular Variance (AMOVA)

AMOVA results 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 (Table 4). 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. 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 4. Analysis of Molecular Variance (AMOVA) among and within seven populations of *B. brizantha* (Population was grouped based on geographic origin)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	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		
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		

Cluster analysis

Dendrograms were constructed by using (UPGMA) algorithm 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 2). 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.

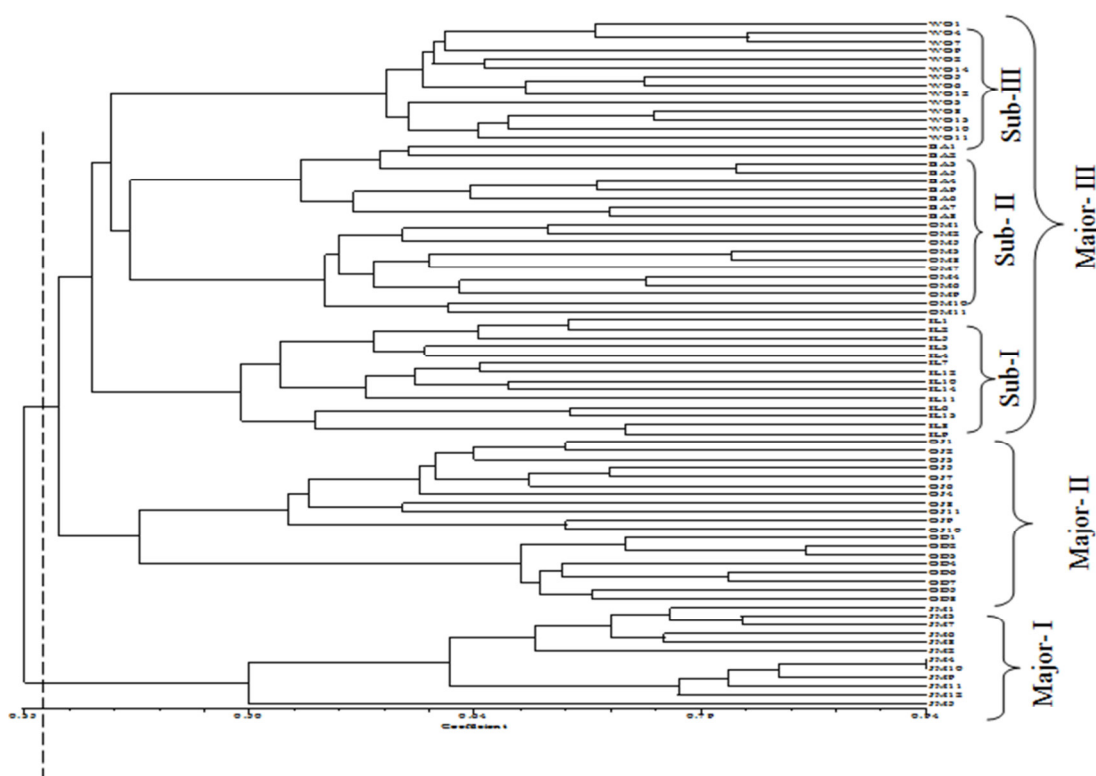


Figure 2. UPGMA based dendrogram for 79 accessions of seven populations of *Brachiaria brizantha* using 6 ISSR primers. Key: WG- Welega, JM-Jimma, IL-Ilu Ababora, GJ- Gojam, BA- Borena, GD-Gondar and OM-Omo

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 3). Likewise, the result of three coordinate analyses showed similar pattern (Figure 4).

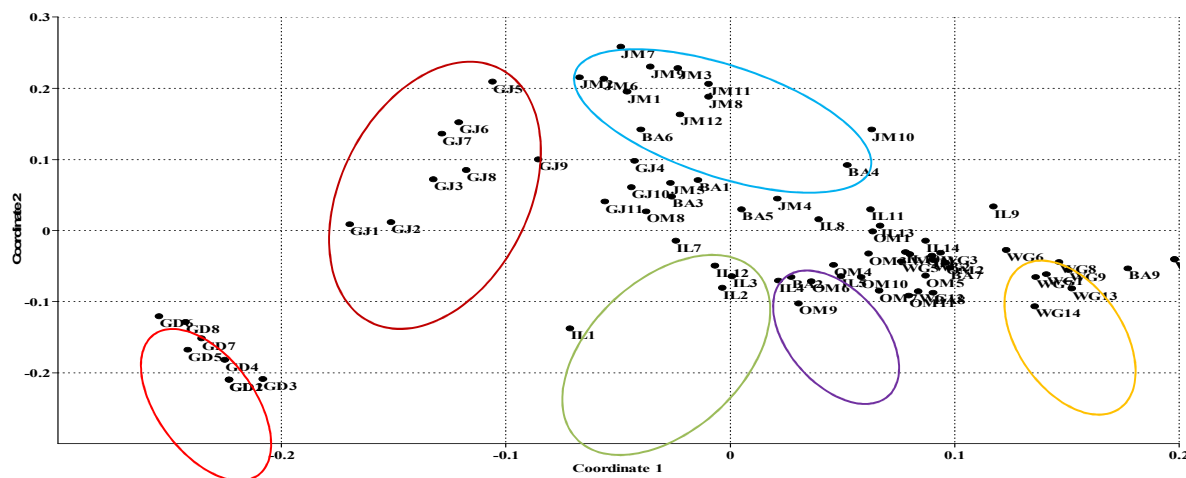


Figure 3. Two dimensional representations of 79 *Brachiaria brizantha* accessions based on Jaccard's similarity coefficients. Key: WG-Welega, JM-Jimma, IL-Ilu Ababora, GJ- Gojam, BA- Borena, GD-Gondar, and OM-Omo

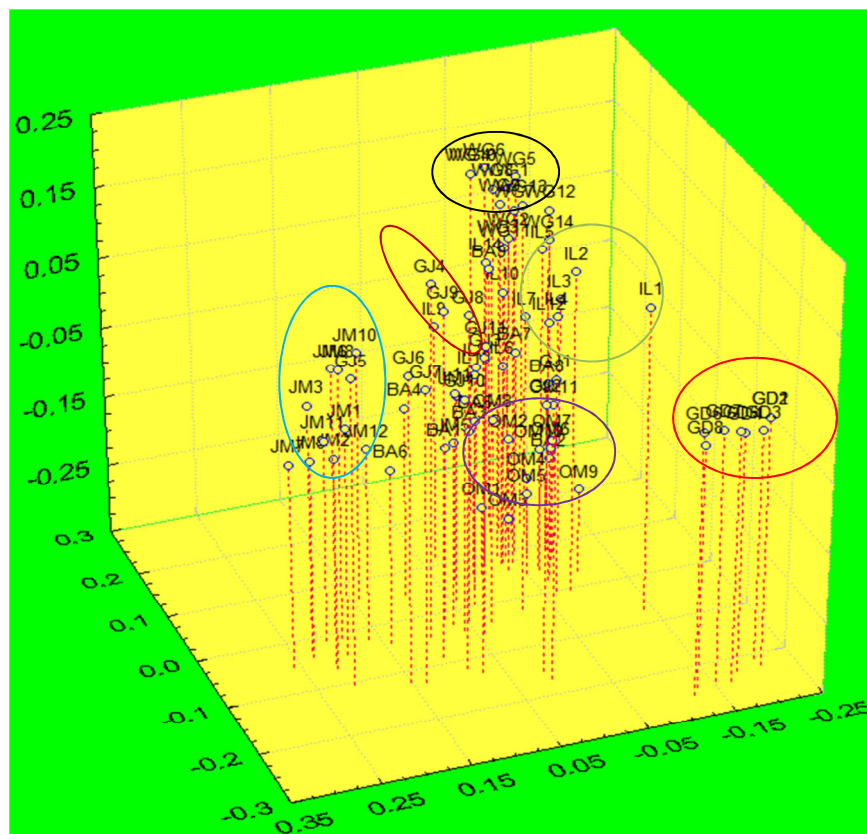


Figure 4. Three dimensional representation of principal coordinate analysis of genetic relationships among 79 accessions of *Brachiaria brizantha* accessions as revealed by ISSR marker generated by six primers. Key: WG-Welega, JM-Jimma, IL-Ilu Ababora , GJ- Gojam, BA- Borena, GD-Gondar, OM-Omo

Discussion

ISSR markers and its use in *Brachiaria brizantha*

In the present study, 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 (Zietkicz *et al.*, 1994; Morgante *et al.*, 2002). 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).

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 a range of polymorphism, gene diversity and Shannon information's index at population level. Hence, four Primers viz 818, 841, 844 and 880 detected 100% polymorphism among populations. 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, indicated that 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).

Genetic diversity in *Brachiaria brizantha* from Ethiopia

A total of 80 scorable bands generated by six primers were subjected to genetic diversity analysis parameters. They identified a range of genetic diversity indexes at population level, with 77 polymorphic bands which accounted 96.25% of bands displaying polymorphism. However, there was considerable variation in percentage of polymorphic loci; 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 (7.4 bands/primer) using 12 ISSR markers for 93 genotypes of *B. ruziziensis*.

In line with this, Shannon index at population 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, samples from Gondar (8) were the least polymorphic with low genetic diversity should get special attention. 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 *B. brizantha*.

Analysis on the basis of geographical regions, viz northwest, west, south west and south Ethiopia, showed a range of 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.

Genetic relationship of *Brachiaria brizantha* populations

To better visualize the genetic relationship of accessions of *Brachiaria brizantha*, cluster analysis (UPGMA) 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 3). The result revealed that the individuals collected from the same geographic region showed close genetic relationships and were grouped into the same cluster. 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).

Conclusion

A range of genetic diversity was detected via ISSR markers in *Brachiaria brizantha* populations collected 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 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.

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