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Genetic Diversity of Wild Sorghum (*Sorghum bicolor* ssp. *verticilliflorum* (L.) Moench) Germplasm from Ethiopia as Revealed by ISSR Markers

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Abstract: Ethiopia is one of the center of origin and diversity of *Sorghum bicolor* (L.) Moench. The genus *Sorghum* has been divided into three subspecies, namely, *bicolor*, *verticilliflorum* and *drummondii*. The study of the genetic diversity of the wild species of sorghum significantly contributes to the improvement of *Sorghum*. The aim of study was to investigate the genetic diversity of wild *Sorghum* from Ethiopia using Inter-Simple Sequence Repeat markers (ISSR) markers. ISSR were used to estimate genetic diversity among and within 12 populations of wild sorghum that were collected from different ecological zones of Ethiopia. Seven selected ISSR primers yielded 43 reproducible bands from 96 individuals, eight individuals representing each population. Jimma 1 population exhibited highest gene diversity of 0.2885. The least gene diversity (0.1192) was observed in East Wollega 1. Shannon diversity index also showed the same diversity pattern where Jimma 1 population showed the highest value (0.4067) and the least Shannon diversity index was exhibited by East Wollega 1 population (0.1757). Analysis of molecular variance showed that within population variation was higher (54.68%) than among population variation (45.32%). Un-weighted pair group method with arithmetic mean (UPGMA) based dendrogram showed Gambella 1 and Jimma 2 populations showed highest similarity. Individual based UPGMA clustering of overall analysis showed five major clusters and some of individuals were clustered with respect to their populations except few intermixed individuals from other populations. In two dimensional (2 D) Principal coordinate analysis (PCO), most of West Wollega 1 and West Wollega 2 individuals formed their separate group except few individuals from other populations mixed with them.

Key words: Ecological zone, Ethiopia, molecular marker, populations

INTRODUCTION

Sorghum bicolor (L.) Moench is divided into three subspecies (ssp.), namely, *bicolor* (the domesticated forms) *verticilliflorum* (the closest wild relative of the domesticated spp.) and *drummondii* (the weedy forms which are hybrids between wild relatives and cultivated forms) (Doggett, 1988). Subspecies *bicolor* have four botanical races, *bicolor*, *kafir*, *caudatum*, *durra* and *guinea* and ten intermediate races (Harlan and de Wet, 1972). The four wild races, *verticilliflorum*, *arundinaceum*, *virgatum* and *aethiopicum* are recognized in the subspecies *Verticilliflorum* (James *et al.*, 2012). Subspecies, *Verticilliflorum* differ from each other by leaves, inflorescence and ecogeographical distribution of the plant. *Verticilliflorum* is widely spread race in sub-Saharan Africa (De Wet and Huckabay, 1967; De Wet *et al.*, 1970; De Wet, 1978).

The depletion of wild sorghum populations in Ethiopia is due to the growing human population pressure

and recurrent drought that forced farmers to cultivate the most marginal lands formerly occupied by wild sorghum and its habitat is used for agriculture, grazing, human settlement, construction, etc. The increase in human population and consequently increase in agricultural activities are the main threat that affected the distribution of wild sorghum in Ethiopia (Ayana *et al.*, 2000). In addition, farmers' practices in relation to their management of sorghum wild relatives largely contributed to the decrease in wild populations (Ayana *et al.*, 2000; Tesso *et al.*, 2008). Hence, the conservation of these wild sorghum populations should be given due attention. For effective conservation of these wild sorghum populations, Evaluation of its genetic diversity and frequent monitoring of its genetic change is needed (Muraya *et al.*, 2010). The methods of genetic diversity study extend from analysis of morphological character to biochemical and molecular traits (Muthusamy *et al.*, 2008). Traditional classification mainly based on morphological characteristics. However, these traits may not be distinct

and usually require growing plants to maturity prior to identification. Morphological characters are unstable because they are easily influenced by environment. Wild sorghum has a great potential both from economic and ecological perspectives. However, there have been only few researches on its genetic diversity. Ayana *et al.* (2000) studied the molecular genetic diversity of wild sorghum from Ethiopia using RAPD whereas Adugna and Bekele (2013) studied phenotypic diversity. Moreover, the former study using RAPD was carried out by pooling samples of accessions and no report on within accessions genetic diversity. The objective of present study is to investigate the genetic diversity of wild sorghum from Ethiopia using ISSR markers.

MATERIALS AND METHODS

Plant material: Twelve accessions of wild sorghum that were collected from different ecological zones of Ethiopia (Fig. 1) were obtained from Institute of Biodiversity Conservation (IBC), Ethiopia. Eight individuals from each accession were planted in glasshouse to collect young leaves for DNA extraction.

DNA extraction and PCR amplification: Total genomic DNA was isolated by using modified CTAB method of Wang *et al.* (1996) with minor modifications. Quality of the DNA was checked by electrophoresis of the samples on 1% agarose gel and staining with ethidium bromide. DNA concentration was determined by nanodrop.

A total of 20 primers (Sigma-Aldrich) were screened to get the primers that are polymorphic and reproducible. Two individuals were selected from each population to screen the primers. A total of seven polymorphic and reproducible ISSR primers were selected (Table 1). The polymerase chain reaction was conducted in a TC-412 version 34.11 block 96×0.2 mL Thermocycler. PCR amplification was carried out in a 25 μ L volume containing 1 μ L of 20 ng μ L⁻¹ template DNA, 17.97 μ L H₂O, 100 mM of 0.2 μ L dNTPs, 2.5 μ L of 10x buffer (25 mM of MgCl₂, 100 mM of Tris-Cl, 500 mM KCl, pH at 9.1, 0.1% Triton), 2.5 μ L of 25 mM MgCl₂, 0.5 μ L primer (20 pmol μ L⁻¹) and 0.33 μ L (5 U) Taq Polymerase. The amplification program was initial denaturation at 94°C for 5 min, denaturation at 94°C, primer annealing at (45/49°C) depending on primers for 1 min followed by 2 min extension at 72°C with a final extension of 7 min at 72°C. The amplified DNA fragments

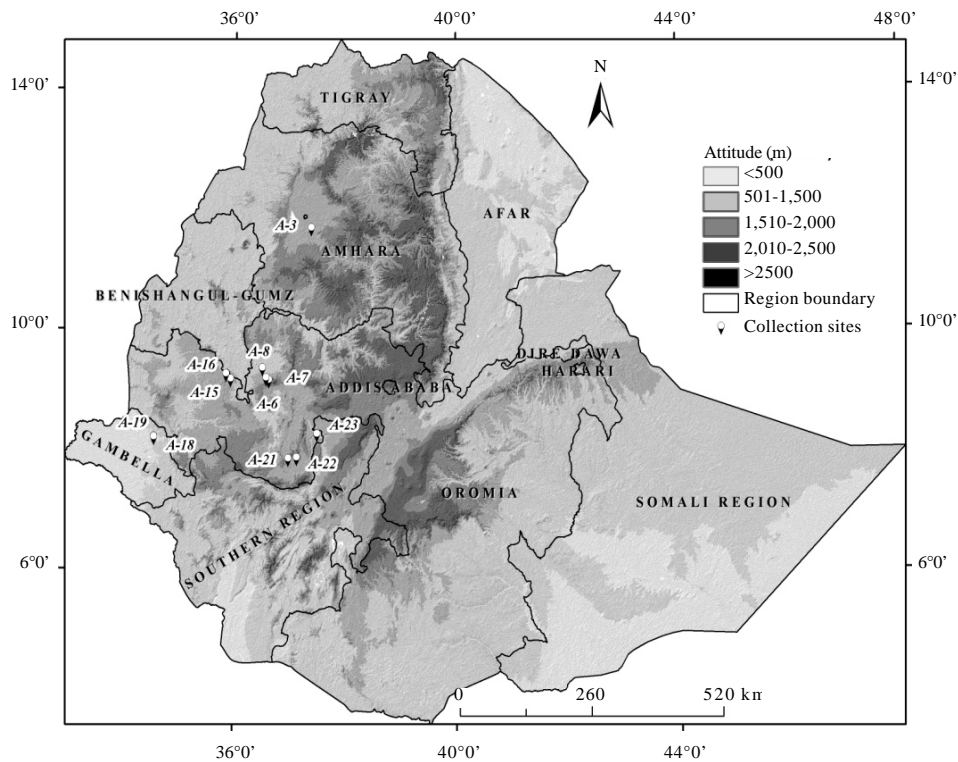


Fig. 1: Map of Ethiopia showing the collection sites of wild sorghum A-3: West Gojam, A-6: East Wollega 1, A-7: East Wollega 2, A-8: East Wollega 3, A-15: West Wellega 1, A-16: West Wellega 2, A-18: Gambella 1, A-19: Gambella 2, A-21: Jimma 1, A-22: Jimma 2 and A-23: Jimma 3

Table 1: Primers selected and used, their sequence, annealing temperature (°C), amplification pattern, number of bands scored and repeat motives

ISSR primer code	ISSR primer sequence (5'-3')	Annealing temp. (°C)	Amplification pattern	No. of scored bands	No. of polymorphic loci
810	GAGAGAGAGAGAGAT	45	Good	8	8
824	TCTCTCTCTCTCTCG	45	Good	6	5
825	ACACACACACACAC T	49	Good	6	8
827	ACACACACACACAC G	49	Good	6	2
841	GA AG GAGAGAGAGAYC	49	Good	7	6
873	GACAGACAGACA GACA	45	Good	5	7
880	GGAGAGGAGAGGAGA	45	Good	5	6
Total				43	42

Single-letter abbreviations for mixed base positions: Y: (C,T), inter simple sequence repeat (ISSR)

(10 µL) was mixed with 2 µL of 6x loading dye and separated by electrophoresis on 1.7% agarose in 1x TAE buffer. DNA ladder of 100 bp was used to estimate molecular weight. Electrophoresis was conducted for 3 h at constant voltage of 100 V. DNA was stained with 50 µL of 10 mg mL⁻¹ Ethidium Bromide in 250 mL of double distilled H₂O for 30 min and washed for 30 min and the picture was taken using gel documentation system.

Data scoring and analysis: ISSR bands were scored visually/manually for each individual sample from the gel photograph. The bands were recorded as discrete characters, presence '1' or absence '0' and '?' for missing data. Data analysis was conducted using only the polymorphic bands. Different softwares were used for data analysis. POPGENE version 1.32 software (Yeh *et al.*, 1997) was used to calculate genetic diversity for each population as number of polymorphic loci (NPL), Percent Polymorphism (PP), Gene Diversity (GD) and Shannon diversity index (I). Analysis of molecular variance (AMOVA) was used to calculate variation among and within populations using Arlequin version 3.01 (Excoffier *et al.*, 2006). NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.*, 1999) software's were used to calculate Jaccard's similarity coefficient. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the population and generate phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The Neighbor Joining (NJ) method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.*, 1999).

To further examine the patterns of variation among individual samples on 3D, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer *et al.*, 2001). The first three axes were used to plot the three dimensional PCO with STATISTICA version 6.0 software (Hammer *et al.*, 2001; Statistica Soft, 2001).

RESULTS

Banding patterns of the ISSR primers: The size of the bands amplified was in the range of 200 and 800 bp but most of them have size range between 300 and 500 bp. A total of 43 clear and reliable bands were scored. Primer 810 produced the eight polymorphic bands which is the largest number whereas primers 873 and 880 exhibited five polymorphic bands which is the least. Primers 824, 825 and 827 produced six polymorphic bands whereas primer 841 produced seven polymorphic bands. Average number of bands amplified per primer was 6.143.

Genetic diversity: The highest genetic diversity was exhibited by Jimma 1 (A-21) population with gene diversity value of 0.2885, followed by Gambella 2 (A-19) population with gene diversity value of 0.2594. The least gene diversity was exhibited by East Wollega 2 (A-7) population. Shannon diversity index also showed the same diversity pattern where Jimma 1 and Gambella 2 populations showed the highest value with 0.4067 and 0.3694, respectively and the least Shannon diversity index was exhibited by East Wollega 2 population with the value of 0.1757 (Table 2).

Analysis of molecular variance: Analysis of molecular variance (AMOVA) was carried out on overall ISSR data scored from wild sorghum populations without grouping. AMOVA without grouping the populations revealed that higher percentage of variation (54.68%) is attributed to the within population variation while the remaining variation is due to among population variation which was 45.32%.

Clustering analysis: UPGMA cluster analysis formed two clusters, I and II. Cluster I further formed two sub clusters and cluster II formed three sub clusters. In addition, three populations, West Gojam (A-3), West Wollega 1 (A-15) and West Wollega 2 (A-16) failed to cluster with other groups. While from the rest nine populations seven were grouped together, East Wollega 1 (A-6), Gambella 1 (A-18), Gambella 2 (A-19), Jimma 1 (A-21), Jimma 2 (A-22), Jimma 3 (A-23) and East Shewa (A-24) and the remaining two populations, East Wollega 2 (A-7) and East Wollega

Table 2: Number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (GD) and Shannon index (I) for each population

Populations	Code	NPL	PP (%)	GD	I
West Gojam	A-3	21	46.67	0.1941	0.2812
East Wollega	A-6	20	44.44	0.2075	0.2925
East Wollega	A-7	14	31.11	0.1192	0.1757
East Wollega	A-8	18	40.00	0.1647	0.2411
West Wollega	A-15	25	55.56	0.2398	0.3427
West Wollega	A-16	21	46.67	0.1915	0.2778
Jimma	A-21	28	62.22	0.2885	0.4067
Jimma	A-22	18	40.00	0.1634	0.2363
Jimma	A-23	16	35.56	0.1655	0.2338
East Shewa	A-24	14	31.11	0.1388	0.1980
Gambella 1	A-18	14	31.11	0.1419	0.2016
Gambella 2	A-19	26	57.78	0.2594	0.3694

Table 3: Pairwise Jaccard similarity coefficient based comparisons among 12 populations of wild sorghum

	A-3	A-6	A-7	A-8	A-15	A-16	A-21	A-22	A-23	A-24	A-18	A-19
A-3	-											
A-6	0.661	-										
A-7	0.521	0.574	-									
A-8	0.450	0.483	0.641	-								
A-15	0.561	0.530	0.562	0.566	-							
A-16	0.564	0.544	0.482	0.520	0.558	-						
A-21	0.598	0.644	0.512	0.482	0.548	0.531	-					
A-22	0.642	0.763	0.641	0.511	0.569	0.582	0.703	-				
A-23	0.544	0.655	0.644	0.534	0.525	0.555	0.599	0.708	-			
A-24	0.632	0.755	0.652	0.543	0.563	0.591	0.731	0.804	0.755	-		
A-18	0.587	0.741	0.594	0.491	0.498	0.538	0.668	0.815	0.677	0.7551	-	
A-19	0.530	0.659	0.579	0.470	0.457	0.511	0.639	0.735	0.693	0.7069	0.745	-

A-3: West Gojam, A-6: East Wollega , A-7: East Wollega 2a, A-8: East Wollega 3, A-15: West Wellega 1, A-16: West Wellega 2, A-18: Gambella 1, A-19: Gambella 2, A-21: Jimma , A-22: Jimma 2, A-23: Jimma 3, A-24: East Shewa

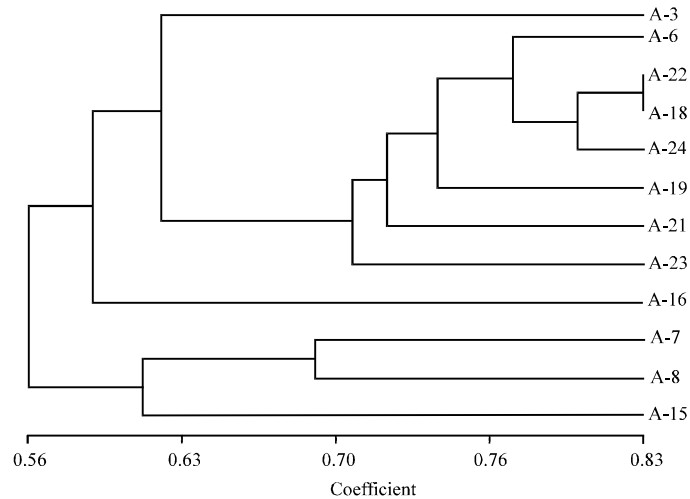


Fig. 2: UPGMA based dendrogram for 12 populations of wild sorghum collected from different ecological zones of Ethiopia A-3: West Gojam, A-6: East Wollega 1, A-7: East Wollega 2a, A-8: East Wollega 3, A-15: West Wellega 1, A-16: West Wellega 2, A-18: Gambella 1, A-19: Gambella 2, A-21: Jimma 1, A-22: Jimma 2, A-23: Jimma 3, A-24: East Shewa

3 (A-8) formed separate group. Gambella 1 (A-18), Jimma 2 (A-22) were not separated (Fig. 2). On the other hand, individual based NJ clustering of overall analysis showed individuals were clustered with respect to their populations except few intermixed individuals from other populations (Fig. 3). Pairwise Jaccard similarity

coefficient-based comparisons of the 12 populations showed Gambella 1 (A-18) and Jimma 2 (A-22) are closest with similarity coefficient of 0.815, whereas West Gojam (A-3) and East Wollega 2 (A-8) are the most distant populations with similarity coefficient of 0.45 (Table 3).

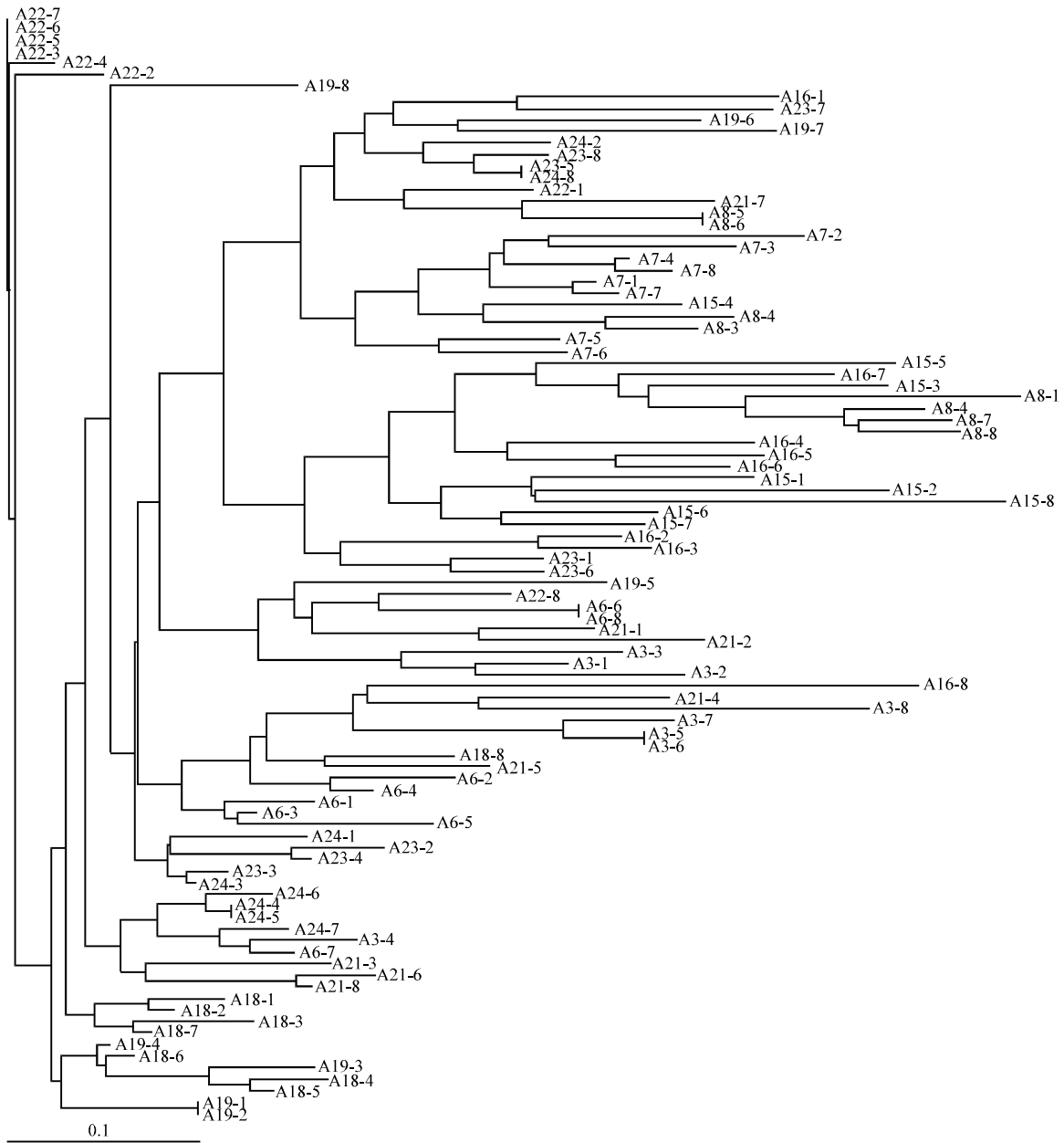


Fig. 3: Neighbor-joining analysis of 96 individuals of wild sorghum amplified by seven ISSR primers A-3: West Gojam, A-6: East Wollega 1, A-7: East Wollega 2, A-8: East Wollega 3, A-15: West Wellega 1, A-16: West Wellega 2, A-18: Gambella zone 1, A-19: Gambella zone 2, A-21: Jimma 1, A-22: Jimma 2, A-23: Jimma 3 and A-24: East Shewa

Principal coordinate analysis: The first three coordinates of the PCO having eigen values of 7.3199, 4.1255 and 3.0232 with variance of 19.595, 11.044 and 8.093%, respectively were used to reveal the grouping of individuals using two and three coordinates. In 2D, most of West Wollega 1 (A-15) and West Wollega 2 (A-16) individuals formed their separate group except few

individuals mixed with them. In 3D, most of East Wollega 2 (A-7), West Wollega 2 (A-16) Gambella 1 (A-18) individuals tended to form their own grouping and the rest were inter-mixed with each other and formed separate group with other populations. Except populations from West Gojjam, PCO in 2D showed similar result with that of NJ and UPGMA (Fig. 4).

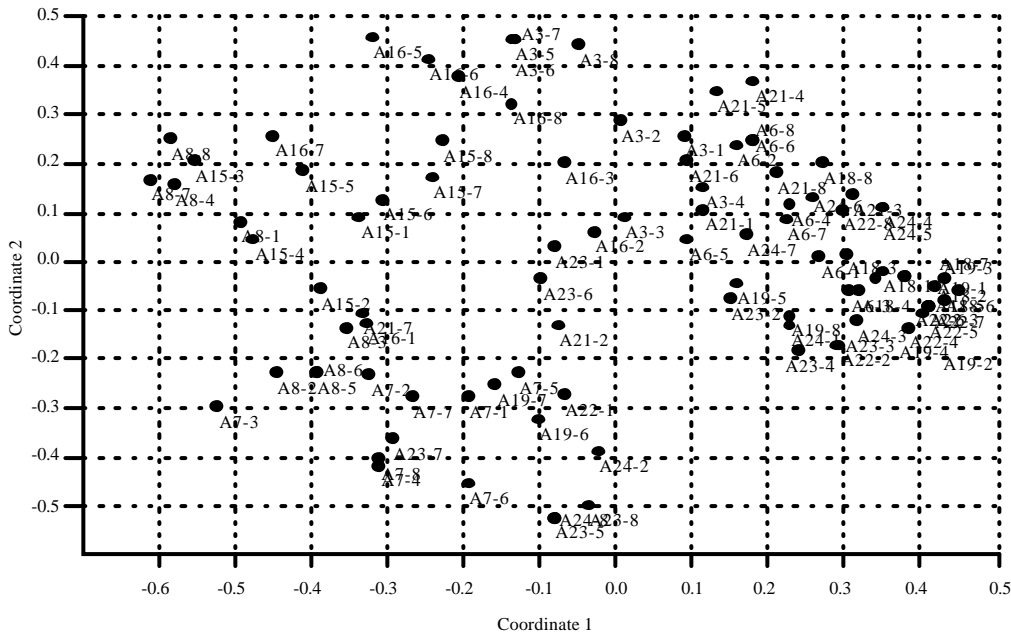


Fig. 4: 2D representation of principal coordinate analysis of genetic relationships among 96 individuals of 12 wild sorghum populations

DISCUSSION

The present study generated 43 loci using seven primers where 42 of the loci (93.33%) were polymorphic. The percent polymorphism for each primer ranged from 40-100%. This is in agreement with previous studies on the cultivated sorghum with 84.0% polymorphism reported by Fang *et al.* (2008). In the present study, primer 880 (penta-nucleotide repeat), showed the least percent polymorphism. Fang *et al.* (2008) reported among 22 primers selected for cultivated sorghum analysis, di-nucleotide repeats were found to be more abundant in sorghum.

When ecological zone based analysis was considered, Jimma 1 (A-21) showed highest percent polymorphism (62.22%) followed by Gambella 2 (A-19), (57.78%). Both gene diversity and Shannon diversity index exhibited the same pattern of diversity. Jimma 1 (A-21) and Gambella 2 (A-19) populations showed higher diversity and East Wollega 2 (A-7) East Shewa (A-24) and Gambella 1 (A-18) populations showed less gene diversity and Shannon diversity index. Previous study by Ayana *et al.* (2000) on wild sorghum from Ethiopia using RAPD marker indicated that populations from Jimma area exhibited 0.47% Shannon diversity index and those from Gambella showed 0.5 gene diversity. The present study agrees with this previous one as populations from these

two areas exhibited higher Shannon diversity index and gene diversity. The difference in gene diversity among population is due to evolutionary forces that include genetic drift, mutation and selection (Papa, 2005). Process of migration is driving the changes in gene frequencies in natural populations.

Gene flow affects genetic diversity in populations of crop landraces and progenitors/the wild relatives (Gepts and Papa, 2003). With regard to genetic diversity, the wild progenitors of crops are potentially containing more diversity than their respective crops. Domestication has induced marked bottlenecks in genetic diversity of crops (Gepts, 1993). Higher levels of genetic diversity in the wild compared to its cultivated congener which suggests that the process of domestication reduced levels of genetic variation in sorghum. Wild sorghum is thus a potential source of novel genes for broadening the genetic base of cultivated sorghum (Evan, 2009). But in our study in some areas such as East Wollega 2 (A-7) the genetic diversity is very low probably due to the reduction in wild sorghum population as a result of farm land expansion.

Wild sorghum species grow in and around sorghum crop fields in all regions of Ethiopia (Tesso *et al.*, 2008). Generally, gene flow, migration and genetic drift may be the possible reason for difference in genetic diversity. Samples from East Wollega 2 (A-7), East Shewa (A-24)

and Gambella 1 (A-18) showed low genetic diversity. This could be due to the reason that samples were collected from cultivated sorghum field. Geographic differences in the distribution of genetic diversity are common. Populations may differ with respect to all aspects of diversity and show variation in the number of alleles, the identity of those alleles and the effect they have on the characteristics in the population (Rao and Hodgkin, 2002).

AMOVA analysis resulted in higher genetic diversity within populations (54.68%) and lower genetic diversity among populations (45.32%). Genetic diversity analysis of wild sorghum by RAPD (Ayana *et al.*, 2000) showed similar result with the present study that is within population diversity is higher than among population. Within population variation is greater due to (1) Selection for adaptation to microclimatic conditions, (2) The evolutionary advantage of broad genetic variation in a long-lived species growing under varying weather and climatic conditions and (3) High gene flow from other populations, mainly via., the long-distance transfer of pollen (Eriksson, 1998). Migration of individuals (e.g., seeds) or gametes (e.g., pollen) between populations counteracts this divergence between populations, causing a reduction in the genetic diversity among populations and increasing the level of genetic diversity within populations (Papa, 2005).

UPGMA group dendrograms showed samples from East Wollega 1 (A-6), Jimma 1 (A-21), Jimma 2 (A-22), Jimma 3 (A-23), Gambella 1 (A-18), Gambella 2 (A-19) and East Shewa (A-24) clustered together. Samples from Jimma 2 (A-22) and Gambella 1 (A-18) merged together and were not separated and (Ayana *et al.*, 2000) also obtained similar results. This may be due to samples from these two different areas have the same origin. In addition, samples from East Wollega 2 (A-7) and East Wollega 3 (A-8) also clustered together. However, samples from West Gojam (A-3) West Wollega 1 (A-15) and West Wollega 2 (A-16) formed separate groups.

Both UPGMA and NJ indicated that most of the individuals clustered within their groups except few individuals which were distributed throughout other populations. These cosmopolitan alleles may be due to ancestral characters shared by most population through common descent from the progenitor of *Sorghum bicolor*. Wild sorghum has a great potential both from economic and ecological perspectives. However, continued pressure due to expansion of agricultural land, grazing and human settlement have negatively affected the genetic diversity of the species. Therefore, researchers, policy makers and other stakeholders need to come-up with sustainable conservation strategies.

CONCLUSION

From the 12 populations, samples from Jimma 1 (A-21) and Gambella 2 (A-19) populations showed higher gene diversity and Shannon diversity index while a populations from East Wollega 1 (A-7) has the least gene diversity and Shannon diversity index. Analysis of molecular variance showed that the higher proportion of genetic variation was attributed to within population than among population. UPGMA based dendrogram for 12 populations of wild sorghum from 12 different ecological zone of Ethiopia by using seven ISSR primers showed samples from Gambella 1 (A-18) and Jimma 2 (A-22) populations were closely related than others and in addition Pairwise Jaccard Similarity Coefficient-based comparisons of the 12 populations showed Gambella 1 (A-18) and Jimma 2 (A-22) are closest whereas West Gojam (A-3) and East Wollega 2 (A-7) are the most distant populations.

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