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Genetic diversity and population structure of Ethiopian finger millet (*Eleusine coracana* (L.) Gaertn) genotypes using inter simple sequence repeat (ISSR) markers

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Eleusine coracana **is an annual allotetraploid (2n=4x=36) that belongs to grass family, Poaceae. This research aimed to investigate level of genetic diversity of 80 accessions using ISSR markers. DNA was extracted from a bulk of three plants per accession using a modified CTAB method. Six ISSR primers amplified a total of 45 clear and reproducible bands. The total genetic diversity (H) and Shannon's diversity information index (I) for the entire populations was 0.28 and 0.41, respectively. Analysis of molecular variance in both grouping and without grouping revealed larger genetic diversity within the populations (58.54%) than among populations (41.45%). Of the total genetic diversity, 5.88% was attributed to populations within groups, 38.33% was attributed to among groups and 55.79% was attributed to differences within populations. Both unweighted pair- group method with arithmetic average phenograms and a neighbor joining trees were constructed for the individuals and populations using Jaccard's similarity coefficient. Most individuals from all populations tended to form their own cluster, while only few of the individuals were distributed all over the tree. Generally, the result of the present study confirmed the presence of genetically diversified accessions that can be used to improve the productivity, collection, conservation and sustainable use.**

Key words: *Eleusine coracana*, gene flow, genetic diversity, inter simple sequence repeat (ISSR) marker, unweighted pair group method with arithmetic mean (UPGMA).

INTRODUCTION

Eleusine coracana, commonly called finger millet, is an annual allotetraploid (2n=4x=36; genome constitution AABB) cultivated plant that belongs to the grass family Poaceae, subfamily Chloridoideae. There are about nine species under the genus, *Eleusine* Gaertn. Two species, *Eleusine indica* and *Eleusine floccifolia,* are believed to be the genome donors to the cultivated species, *E. coracana* (Bisht and Mukai, 2001).

It is extensively cultivated in the tropical and sub-tropical regions of Africa and India and is known to save

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the lives of poor farmers from starvation at times of extreme drought (Kotschi, 2006). It is adapted to a wide range of environments and grown mainly by subsistence farmers. Finger millet serves as a food security crop because of its high nutritional value, excellent storage qualities and its importance as a low input crop (Dida et al., 2007). Ethiopia is one of the major producers of finger millet in addition to Uganda, India, Nepal and China and it is also native to the highlands of the country. Finger millet plays an important role in both the dietary needs and incomes of many rural households like other African countries due to its richness in fiber, iron and calcium (Babu et al., 2007).

Assessment of genetic diversity on the basis of morphological traits is not very reliable, as it may be influenced by the environment, and the number of traits with known inheritance is small. Molecular markers have the distinct advantages of being independent of climatic variables and very numerous, and ISSR-PCR is one of the most convenient popular method to identify and study intraspecific genetic polymorphism (Zietkiewicz et al., 1994). The number of finger millet genotypes used in various molecular genetic diversity studies so far include 2 by ribosomal DNA (Hilu, 1995), 22 by RFLP, RAPD and ISSR (Salimath et al., 1995), 12 by RAPD (Fakrudin et al., 2004), 32 by RAPD (Babu et al., 2007), 79 by SSR (Dida et al., 2008), 3 by RAPD and ISSR (Gupta et al., 2010), 83 by RAPD, SSR and mitochondrial DNA (Panwar et al., 2010), 52 using RAPD, 64 by RAPD (Bezaweletaw, 2011), 52 by SSR and RAPD (Kumar et al., 2012), 67 by SSR (Arya et al., 2013), 32 by RAPD (Karad et al., 2013), 103 by SSR (Nirgude et al., 2014) and 190 by SSR (Babu et al., 2014). Even though it is an important crop as a food security, production of the crop is inconsistent due to cultivation; genetic improvement and utilization remain far behind the other crops. The unavailability of detailed information on the current uses, genetic status and potential, etc. are limitations of finger millet. The aim of this study is therefore, to investigate the level of genetic diversity of *E. coracana* populations collected from different parts of Ethiopia, using ISSR markers. This will provide information on the overall genetic variability of *E. coracana* populations which may assist in the identification and selection of the genetic materials for conservation and further breeding.

MATERIALS AND METHODS

Plant material and DNA extraction

Eighty (80) accessions collected from different regions of Ethiopia, Zimbabwe and India obtained from Ethiopian Institute of Biodiversity were germinated and grown in a greenhouse for six weeks. Total genomic DNA was extracted from approximately equal amount (0.2g) of three silica gel dried leaves per accession by using cetyl trimethyl ammonium bromide (2% cetyltrimethyl ammonium bromide, 1% polyvinylpyrolidone, 100 mM Tris: pH = 8, 20 mM EDTA, 1.4 M NaCl) and 0.03 M β-mercaptoethanol extraction protocol based on Borsch et al. (2003) with minor modifications. Approximately, equal amounts (0.2 g) of the dried leaf samples were bulked and ground with Mix and Mill grinding machine, MM 400. The quality and amount of genomic DNA was tested using 1% agarose and NanoDrop (NanoDrop™2000/2000c) spectrophotometer, respectively. Working solution was made by diluting the genomic DNA in a 1:5 ratio, tested using the same procedure and diluted DNA stored at -20°C until used.

ISSR-PCR amplification and gel electrophoresis

Three individuals were selected from each population with 1:5 dilutions to screen primers for their amplification, polymorphism and reproducible bands. A total of six out of seven polymorphic and reproducible ISSR primers were selected after testing and screening. Polymerase chain reaction (PCR) amplifications were performed in Biometra 2003 T3 Thermo cycler programmed to run the following temperature profile: a preheating and initial denaturation for 4 min at 94°C, then 15 s denaturation at 94°C, 1 min primer annealing at 45/48/55°C(based on primers used), 1.30 min extension at 72°C for 40 cycles and the final extension for 7 min at 72°C with holding temperature at 4°C. Each PCR reaction of ISSR markers had a final reaction volume of 25 µl, containing 1 µl dNTPs, 2.5 µl PCR buffer, 3 µl $MgCl_2$, 0.4 µl primer, 0.4 µl Taq polymerase and 10to 50 ng template DNA. A negative control, in which the template DNA was replaced by double distilled water, was also included in each round of reactions to check for absence/presence of contamination. The PCR products were stored at 4°C until loading on the gel for electrophoresis. Amplification products were separated by electrophoresis in 1.67% (w/v) agarose gels. A total of 8 µl PCR product of each sample and 6x loading dye was loaded onto the ISSR gel. Fifteen wells comb was used for each ISSR gel slab. The first lane was loaded with 100 bp ladder by loading 2 µl (peq gold range mix) with loading dye in that well as a size standard and the last lane was control (without DNA template). The ISSR electrophoreses were done for about 3 h at constant voltage of 100 V. After electrophoresis, the gels were stained in 50 µl (10 mg/ml) ethidium bromide mixed with 450 ml distilled water for 30 min and distained with distilled water for 30 min then banding patterns were visualized under UV light and photograph using canon camera in the Biometra Biodoc Analyzer (Biosens SC750) and documented for band scoring and analysis.

Data recording and statistical analysis

Inter simple sequence repeat (ISSR) markers were treated as dominant markers and each locus was considered as a bi-allelic locus with one amplifiable and one null allele. Scoring was performed manually for each primer based on presence (1) and absence (0) or ambiguous (?), and each band was regarded as a locus. POPGENE version1.32 software (Yeh et al., 1999) was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, gene diversity (h) and Shannon-Weaver diversity index (I). Analysis of molecular variance (AMOVA) was used to calculate variation among and within population using Areliquin 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) softwares were used to calculate Jaccard's similarity coefficient which is calculated with the 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 individual i but not in individual j and 'c' is the total number of bands present in

Table 1. List of ISSR primers used with their repeat motifs, amplification fragment, amplification pattern and total number of scorable bands.

S/N	Primers		Amplified Fragment size (bp)	NSB	Amplification pattern
	Name	Sequence			
1	UBC 810	(GA)8C	300-1000	7	Very good
2	UBC 811	(GA)8T	400-1000	4	Very good
3	UBC 848	(CA)6RG	200-1000	5	Excellent
4	UBC 866	(CTC)6	300-1500	10	Excellent
5	UBC 873	(GACA)4	300-1500	9	Excellent
6	UBC 880	(GGAGA)3	200-1000	10	Excellent

Single-letter abbreviations for mixed base positions: R = (A, G), NSB: number of scorable bands.

individual *j* but not in individual *i*.

The unweighted pair group method with arithmetic average (UPGMA) (*S*neath and Sokal, 1973) was used in order to determine the genetic relationship among accessions and generates 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 accessions and evaluate patterns of accession clustering using Free Tree 0.9.1.50 Software (Pavlicek et al., 1999).

A principal coordinated (PCOA) analysis was performed based on Jaccard's coefficient (Jaccard, 1908) to further examine the patterns of variation among individual samples. The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer et al., 2001). The first three axes were later used to plot with STATISTICA version 6.0 software (Hammer et al., 2001; Statistica (StatSoft, Inc.) 2001).

RESULTS AND DISCUSSION

Inter simple sequence repeat primers and their banding patterns

Among the seven primers tested initially, six of them gave clear, reproducible and polymorphic bands selected as informative markers. The fragment size amplified with these primers was in the range of 200 to 1000 bp. A total of 45 fragments were amplified by the six ISSR primers of which 35 were polymorphic. The highest number of bands was amplified with primer UBC-880, while the lowest number was amplified with UBC-811 (Table 1).

Polymorphism and genetic diversity

The number of polymorphic loci ranges from 2 for UBC-811 to 9 for UBC-880, where they are SSR with di- and penta- nucleotide repeat motif, respectively, (Table 2). Penta-nucleotide primer (UBC 880) generated high number of percent polymorphism as compared to the di-, tri- and tetra-nucleotide, showing that the penta-nucleotides interestingly contributed more polymorphism. UBC-811 showed the least polymorphism with 50.0%, while UBC-880 showed the highest with 90.0% polymorphism. The overall gene diversity (h) by the six primers was 0.28, while Shannon's information index was 0.41. The highest gene diversity (h) 0.37 and Shannon's information index (0.54) were obtained from UBC- primer

Table 2. Number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (h) and Shannon's information index (I) of finger millet with each population across all primers tested.

	NPL	PP (%)	h	I
All primers				
Gojam	25	55.56	0.19 ± 0.20	0.28 ± 0.29
Gondar	18	40.00	0.17 ± 0.21	0.24 ± 0.30
Awi	20	44.44	0.19 ± 0.22	0.28 ± 0.31
Wellega	21	46.67	0.19 ± 0.21	0.28 ± 0.31
Ilu Ababora	7	15.56	0.06 ± 0.15	0.08 ± 0.21
Adwa	11	24.44	0.10 ± 0.19	0.15 ± 0.27
Shire	8	17.78	0.06 ± 0.14	0.09 ± 0.21
Hadiya	10	22.22	0.10 ± 0.19	0.14 ± 0.28
Omo	6	13.33	0.05 ± 0.14	0.07 ± 0.20
India	9	20.00	0.09 ± 0.18	0.12 ± 0.26
Zimbabwe	20	44.44	0.19 ± 0.23	0.28 ± 0.32
Overall	35	77.78	0.28 ± 0.19	0.41 ± 0.26
Groups				
Oromia	21	46.67	0.19 ± 0.21	0.28 ± 0.31
Amhara	27	60.00	0.24 ± 0.21	0.35 ± 0.30
Tigray	13	28.89	0.11 ± 0.19	0.17 ± 0.27
SNNP	11	24.44	0.10 ± 0.19	0.15 ± 0.28
Exotic	23	51.11	0.21 ± 0.22	0.30 ± 0.31
Overall	35	77.78	0.29 ± 0.19	0.43 ± 0.26
Individual primer				
810	6	77.78	0.31 ± 0.18	0.46 ± 0.25
811	2	50.00	0.17 ± 0.22	0.25 ± 0.32
848	4	80.00	0.29 ± 0.18	0.43 ± 0.26
866	7	70.00	0.18 ± 0.19	0.29 ± 0.27
873	8	88.89	0.30 ± 0.17	0.46 ± 0.24
880	9	90.00	0.37 ± 0.16	0.54 ± 0.22
Overall	35	77.78	0.28 ± 0.19	0.41 ± 0.26

Table 3. AMOVA of finger millet populations.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	Fixation Index (FST)	P-value
A						
Among populations	10	187.56	2.49	41.45	0.41	P<0.001
Within populations	63	204.28	3.52	58.54	-	P<0.001
Total	73	391.84	6.01	-	-	
B						
Among geographic groups	4	153.75	2.42	38.33		P<0.001
Among populations with in geographic groups	6	33.81	0.37	5.88	0.44	P<0.001
Within populations	63	204.28	3.52	55.79	-	P<0.001
Total	73	391.84	6.31	-	-	-

A: Without grouping; B: with groups.

880, while the least gene diversity of (0.17) and Shannon's information index of (0.25) from UBC-811. Moreover, the choice of appropriate primer motives in ISSR fingerprint is critical to detect high polymorphism and reveal relationship within and among populations. The abundance and distribution of SSRs in the genomes of finger millet could be another factor that determines the levels of polymorphism.

This study found high percentage of polymorphism (77.78%) using six ISSR primers with inclusion of large number of finger millet accessions from diverse ecological condition and large geographical range such as Ethiopia, Zimbabwe and India. Similarly, Ramakrishnan et al. (2016) reported 76.48% polymorphism on 128 genotypes using RAPD marker, Babu et al. (2007) reported 91% polymorphism in 32 genotypes using RAPD marker; Bezaweletaw (2011) reported 72.35% percentage of polymorphism on 66 genotypes of finger millet from Ethiopia and Eritrea, and Fakrudin et al. (2004) reported 85.82% percentage of polymorphism on 32 germplasms from Indian, while Panwar et al. (2010) reported 56.17% polymorphisms in 83 genotypes using RAPD marker, Babu et al. (2014) reported only 46% polymorphism in 190 genotypes of finger millet using SSR makers; Salimath et al. (1995) reported 26% percentage of polymorphism on 17 genotypes of finger millet from Africa, Asia and Brazil. It may be because finger millet is a highly self-pollinated crop which resulted in low level of polymorphism by SSR marker analysis (Dida et al., 2007).

Among all the populations, that from Gojam showed higher percentage of polymorphism with 55.56%, while the least percentage was detected from the Omo population with 13.33%. Generally, Amhara region finger millet population has highest percent of polymorphism (60.0%) than other regional populations (24.44 to 51.11%) (Table 2). The overall genetic diversity (h) by the six primers was 0.28, while Shannon's information index (I) was 0.41. The highest gene diversity (h) 0.19 and Shannon's information index (0.29) were obtained from Gojam, Wellega, Awi and Zimbabwe populations, while Omo population showed the least gene diversity of 0.05 and Shannon's information index of 0.07. Generally, the Amhara region populations showed the highest variability (0.35), while the least was obtained from SNNP (0.15) (Table 2). Amhara region farmers are active in finger millet seed exchange with other regions via market channels and also through seed dispersal.

Analysis of molecular variance (AMOVA)

AMOVA revealed that higher percent of variation (58.54%) is attributed to the within population variation, while 41.45% is due to the among population variation (Table 3). On the other hand, of the total genetic diversity, 5.88% was attributed to populations within groups, 38.33% to among groups and 55.79% to differences within populations. This is in agreement with previous report by Babu et al. (2014) who reported 73% variance within populations and 27% variance among populations in 190 finger millet genotypes. Similar results were found in 12 genotypes of cherry plum (Wo¨hrmann et al., 2011) in which 96.8% of the total variance was identified and attributed to divergence within populations and only 3.2% was attributed to divergence among populations and Tsehay (2012) reported high variation (90.59%) attributed to the within species component, while the remaining variation (9.41%) was due to among species variation. Gichuki et al. (2003) also found that in 74 genotypes of sweet potato, 93.4% of total variance was within populations and 6.6% of the variance was among the various geographical regions which implies high genetic exchange or gene flows among populations by the dispersal of the seeds and seed exchange via market channels. There were highly significant genetic

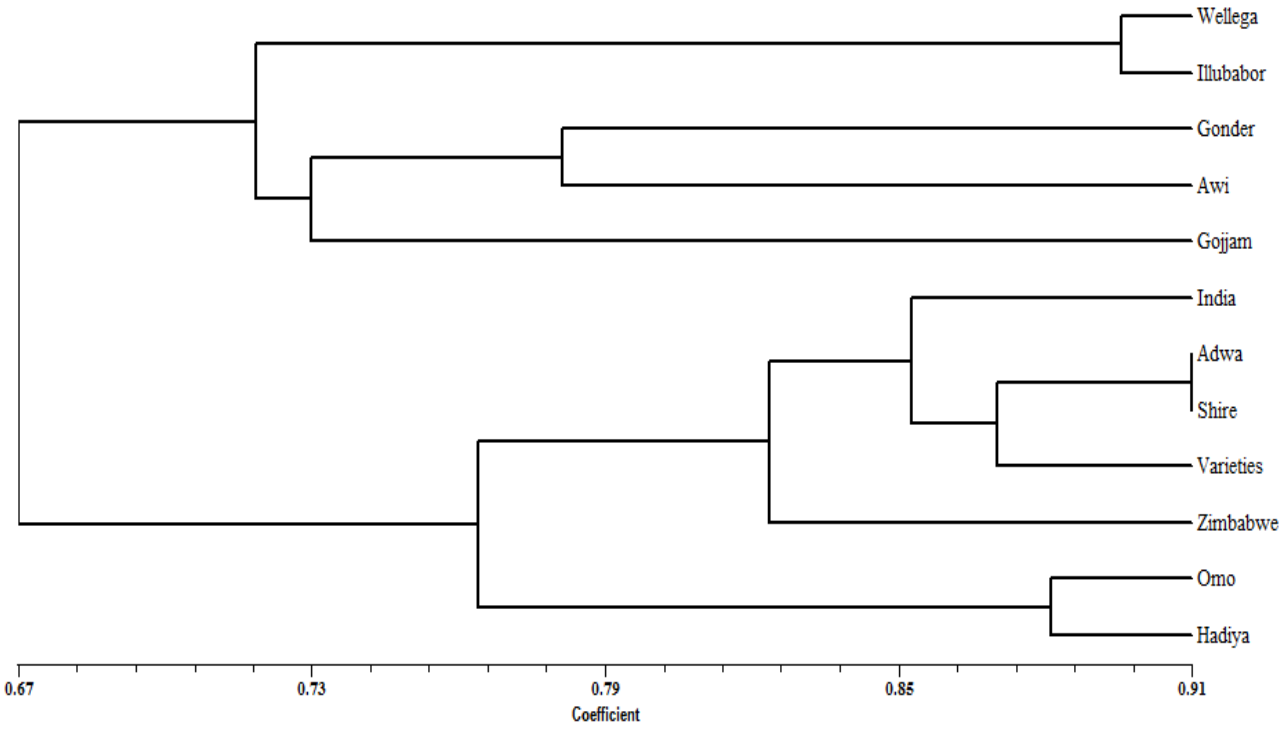

Figure 1. UPGMA based dendrogram for twelve populations of *E. coracana* collected from different regions of Ethiopia and exotic (India and Zimbabwe) using six ISSR primers.

differences between the five groups as well as between the eleven populations of *E. coracana* ($p<0.001$).

Cluster analysis

UPGMA and NJ analysis was carried out to construct a dendrogram for the 12 populations and 80 individuals based on 45 PCR bands amplified by six ISSR primers (Figure 1). The genetic similarity coefficients between pairs of populations (Table 4) varied from 0.581 (between Hadiya and Gojam) to 0.905 (between Shire and Adwa). Besides this, individual based UPGMA and PCOA clustering of an overall analysis (Figure 2) showed strong clustering of individuals with respect to their population except few intermixed populations. The present study is in agreement with the finding of Fakrudin et al. (2004) who found a clear apportionment of finger millet accessions in concordance with geographical origin using RAPD marker, while in contrast with Bezaweletaw (2011), no clear-cut clustering of accessions to their geographic origin was reported. Though the accessions assessed in this study mainly represented landraces from different geographical regions of Ethiopia, the analysis of UPGMA tree showed a clear-cut pattern of variation in relation to geographical region, which could be due to domestication and cultivation of finger millet that might result from development of local landraces limited to a particular location coupled with limited gene flow.

Conclusions

The genetic diversity data generated by six ISSR primers revealed that high genetic diversity exists in finger millet germplasms. The assessed genetic diversity level varied among populations, which could be due to different environmental conditions in which they are growing, naturally distributed and human selection pressure. Moreover, Gojam and Wellega populations showed relatively high genetic diversity than others. The AMOVA analysis showed that, high genetic variation within populations than among populations shows the existence of high gene flow and low genetic differentiation among populations. The UPGMA cluster analysis supported the grouping of accessions to the defined geographical location and the respective population in the analysis of the total accessions. The findings of this study indicate that ISSR markers could be good tools to assess the genetic diversity and relationship at inter and intra population level of finger millet.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Table 4. Pair-wise Jaccard's similarity coefficient based comparisons among eleven populations of *E. coracana* collected from Ethiopia and exotic.

Population	Wellega	Illuababora	Gonder	Gojam	Awi	India	Zimbabwe	Adwa	Shire	Omo	Hadiya	Ethiopian Varieties
Wellega	1.00											
Ilu Ab	0.891	1.00										
Gonder	0.703	0.763	1.00									
Gojam	0.718	0.716	0.718	1.00								
Awi	0.703	0.713	0.78	0.743	1.00							
India	0.661	0.658	0.715	0.682	0.723	1.00						
Zimb	0.653	0.684	0.754	0.661	0.755	0.83	1.00					
Adwa	0.657	0.641	0.693	0.665	0.727	0.84	0.83	1.00				
Shire	0.672	0.67	0.707	0.695	0.725	0.85	0.82	0.905	1.00			
Omo	0.585	0.596	0.68	0.644	0.674	0.78	0.76	0.757	0.79	1.00		
Hadiya	0.598	0.591	0.682	0.581	0.663	0.78	0.76	0.745	0.749	0.68	1.00	
Ethiopian varieties	0.669	0.668	0.721	0.639	0.741	0.857	0.802	0.840	0.892	0.748	0.767	1.00

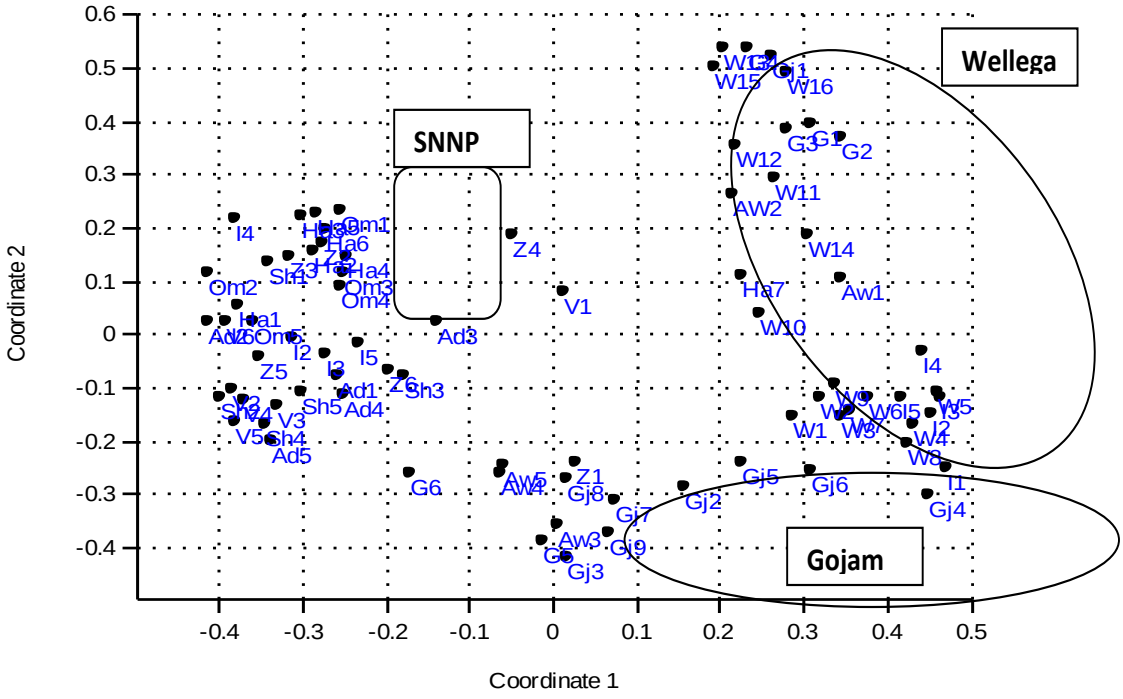

Figure 2. Two-dimensional plot obtained from PCOA of 80 *E. coracana* accessions using six ISSR primers with Jaccard's coefficient similarity. W1-16 = Wellega; Aw1-5=Awi; S1-5= Shire; Gj1-9= GojamIlu; 1-5= Ilu Ababora; I1-5= India; O1-5 = Omo; Ad1-5=AdwaG1-6= Gondar; Z1-5= Zimbabwe; H1-7= Hadiya.

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ABBREVIATIONS

AFLP, Amplified fragment length polymorphism; **AMOVA,** analysis of molecular variance; **CTAB,** cetyl trimethyl ammonium bromide; **2D,** two dimensions; **3D,** three dimensions; **EDTA,** ethylenediamine tetraacetic acid; **H,** Nei"s genetic diversity; **I,** Shannon information index; **ISSR,** inter simple sequence repeat; **NJ,** neighbor joining; **NPL,** number of polymorphic loci; **PCO,** principal coordinate; **PCR,** polymerase chain reaction; **PPL,** percent of polymorphic loci; **RAPD,** random amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **SSR,** simple sequence repeat; **TBE,** tris

borate EDTA; **TE,** tris EDTA; **UPGMA,** unweighted pair group method with arithmetic mean.

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