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Genetic diversity analysis of Lima bean (*Phaseolus lunatus* L.) Landrace from Ethiopia as reveled by ISSR marker

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ABSTRACT: Lima bean (*Phaseolus lunatus* L.) is one of the five most important legume crops from the genus *Phaseolus* though it is one of the neglected crops in Ethiopia. There is no national breeding program for its improvement. As it is true for all crops, understanding the genetic diversity of Lima bean is useful for its improvement and sustainable use. Therefore, the objective of the current study was to investigate genetic diversity of Lima bean landrace collected in Ethiopia using eight ISSR markers. A total of 96 landrace were collected from five administrative zones of Ethiopia from which 106 clear bands were detected. About 95 (88.7%) of the bands were polymorphic. The genetic diversity analyses result revealed that landrace collected from West Wellega showed the highest (0.1864) genetic diversity. AMOVA demonstrated highly significant (P=0.00) genetic diversity among and within populations. A considerable proportion (66%) of the total genetic diversity was distributed among populations and 34% within populations. In addition, the calculated Fst value was high (Fst = 0.66), associated with a low gene flow value (Nm=0.27) indicating lower differentiation of the populations, which, in turn, implied no significant exchange of planting materials among farmers in the studied populations and the nature of the crops that Lima bean is 52% self pollinated crop. UPGMA, STRUCTURE and PCoA analysis showed very strong grouping among individuals collected from the same zones and geographically distinct zones. Overall, genetic diversity achieved from this study could be used as pioneer information about the existing genetic resource for future Lima bean conservation and improvement strategy in Ethiopia.

Key words/phrases: Accessions, Ethiopia, Genetic diversity, Landraces, Lima bean, Molecular markers

INTRODUCTION

Legume seeds are an important staple food and source of dietary minerals that potentially provide all of the 15 essential minerals required by humans such as manganese, copper phosphorus and thiamine (vitamin B1) (Heuze *et al*., 2013). Lima bean (*P. lunatus*) is one of the five domesticated species of the genus *Phaseolus* and the second most cultivated species after common bean (*P. vulgaris*) (Baudoin *et al*., 2004). Lima bean is believed to be originated in the general area of Guatemala, Mexico and Peru. The distribution routes were from the Pacific foothills of Mexico, through the Central and South Americas to Southern Canada (Long *et al*., 2014). It was later spread to Southern Asia and through the slave trade, extended to West and Central Africa. Fofana *et al*. (2001) asserted that Lima bean entered the African continent through the Portuguese explorers, particularly to the Central and Western parts of Africa from Brazil during the slave trade (Amoatey *et al*., 2000). Lima bean is diploid (2n=22) and propagated by seed (Martinez *et al*., 2004). The seeds are different from each other in their size, shape, color and appearance of eye and having high protein (210-260 g/kg) and high carbohydrate contents (550-640 g/kg), low fat (10-23 g/kg) and fiber levels (32-68 g/kg), high levels of minerals such as K, Zn, Ca and Fe, and low levels of Na and P (Oshadi, 1993). Lima bean sometimes called butter bean is one of an important grain legume grown in South Western Ethiopia. The dried seeds are eaten after getting them boiled with other cereals and pulses, a dish locally known as "*nifro*". It is cultivated in the home gardens or intercropped with cereals in the field. Seeds are variable in size, shape and color (EBI, 2012).

Genetic erosion, which is defined as the reduction or loss of genetic diversity within and among populations of the same species over time (Jarvis *et al*., 2000) is a major concern today particularly in the cases of neglected and underutilized/orphan crops like Lima bean that is there is no or little research done to improve the crop at list in Ethiopia. This research was designed to analyze the genetic diversity of Lima bean collections from five zones of Ethiopia using inter simple sequence repeat (ISSR) marker. The findings from this research will be an important input for breeders by improving different traits like grain yield, fodder quality, and household nutrition among many other traits. This will ultimately support farmers and better use of Lima bean in Ethiopia.

MATERIALS AND METHODS

Plant materials

Seeds of 96 Lima bean landrace accessions that represent five populations were collected from five administrative zones of Ethiopia namely Guragea, Jimma, Gamo Gofa, West Wellega and Kelem Wellega (Figure. 1) from farmers' traditional seed storage containers, from local markets and from farmers' fields by using the seed collecting format of Ethiopian Biodiversity Institute (EBI) protocol. The seeds were planted 3:1 (3x soil and 1x burn compost) soil composition in open field at College of Natural Sciences, Addis Ababa University to obtain young leaves for DNA extraction.

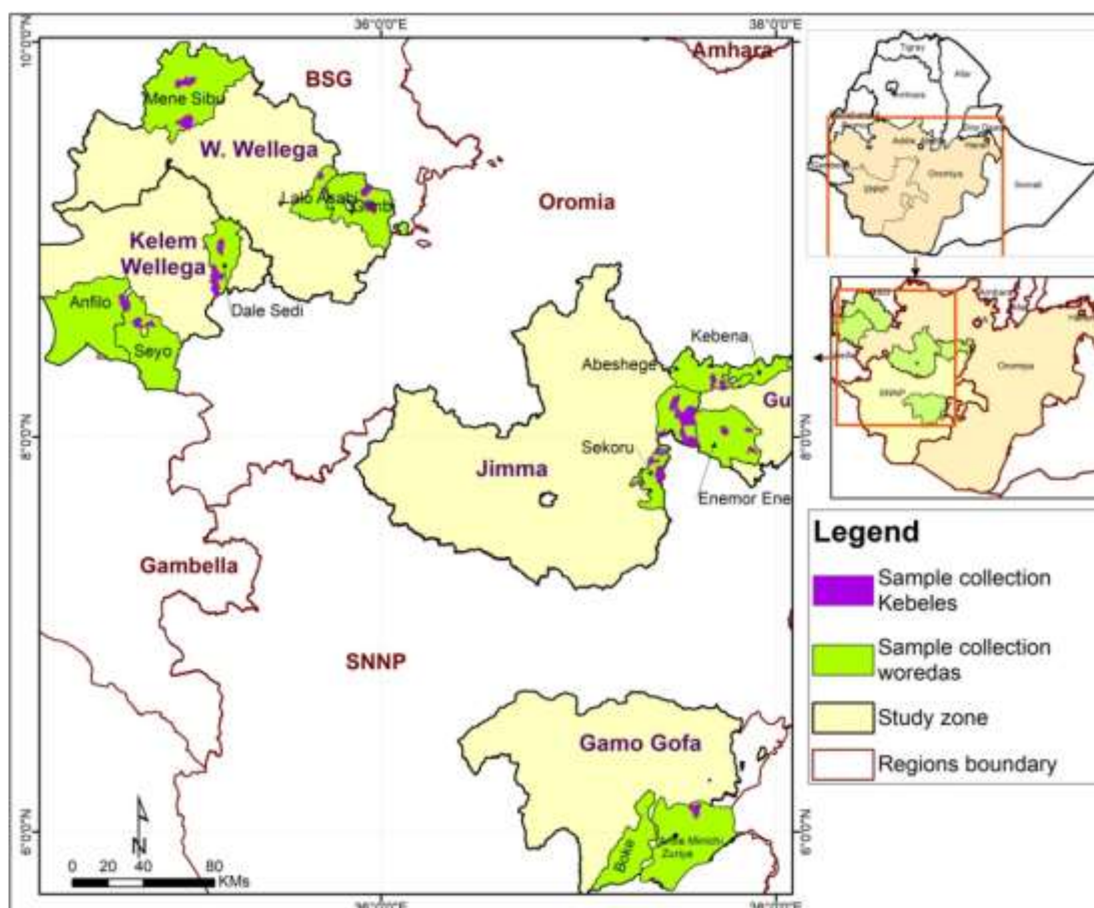

Figure 1. Lima bean sample collection Sites.

DNA *extraction and electrophoresis*

DNA was extracted using modified CTAB extraction of Borsch *et al.* (2003). Approximately 100 mg young leaves from each individual plant were ground in liquid nitrogen using mortar and pestle. To estimate the concentration and quality of the DNA, 4 µL DNA was mixed with 2µL loading dye and electrophoresed using 0.8% agarose gel at 85Vconstant voltage for 45 min. The gel was stained with ethidium bromide for 30 min. Gel picture was taken by Bio-Rad Gel Doc™ EZ system imager. Furthermore, the concentration and purity of the isolated genomic DNA was quantified by using NanoDrop (NanoDrop™2000/2000c) spectrophotometer to estimate the quality and quantity of the DNA. Finally, DNA samples were adjusted to concentration of 100 ng /µl by diluting with sterilized double distilled water and later it was stored at –20°C until used for PCR.

Primer screening

A total of 13 primers were screened for polymorphism and reproducibility using DNA of two individuals from each population (Table 1).

Table 1.List of primers used for screening.

Primer	Annealing	Primer sequence (5′ to 3′)
DPISR1	55°C	AGGAGGAGGAGGAGGAGG
DPISR2	52 °C	AGAGAGAGAGAGAGAGAGAGG
DPISR3	52°C	AGAGAGAGAGAGAGAGAGAGC
DPISR4	57°C	AGAGAGAGAGAGAGAGAGAGT
DPISR6	52°C	CTCTCTCTCTCTCTCTCTCTG
DPISR7	57°C	CTCTCTCTCTCTCTCTCTCTT
DPISR8	55°C	GACACGACACGACACGACAC
DPISR9	45°C	ACTGACTGACTGACTG
DPISR11	55°C	AGAGAGAGAGAGAGAGAGAG
DPISR12	45°C	GACAGACAGACAGACA
UBC834	45°C	AGAGAGAGAGAGAGAGYT
UBC810	42°C	GAGAGAGAGAGAGAGAT
UBC812	45°C	GAGAGAGAGAGAGAGAA

PCR *and gel electrophoresis*

PCR amplification was carried out in a 20 µL reaction mixture containing 100ng/µL of 1.5 µL template DNA, 12.7 µL H_2O, 0.4 µL dNTPs (0.25mM), 2µL PCR buffer ($MgCl_2$ free),1.6µL $MgCl_2$ (50 mM), 1.3 µL primer (50pM) and 0.5 µL Taq Polymerase (5U/µL). The amplification program was initial denaturation at 94 °C for 4 min followed by 40 cycles of 94 °C denaturation for 15 sec, annealing at 42 °C to 55 °C depending on the primers used for 1 min, extension at 72 °C for 30 sec or 1 min and the final extension at 72 °C for 5 minutes with a final holding temperature of 4 °C using Biometra 2003 T3 Thermo Cycler. The PCR products (8 µl) mixed with 2 µl of 6x loading dye were electrophoresed using 1.6% agarose gel Mixed with 100 ml of TAE buffer and stained with 3 µl ethidium bromide for 1 h and 30 min at constant voltage of 80 V.

Data analyses

Each fragment that was amplified using ISSR primers was treated as a unit character and scored as '0' for absence, '1' for presence and '?' for missing data. Based on recorded bands, different software was used for analysis. POPGENE 3.0 was used to calculate genetic diversity and Shannon diversity for each population and for each primer as number of polymorphic loci and percent of polymorphism.

The Pair wise Nei's genetic distances between geographical populations of *P. lunatus* were assessed. Based on such data the geographic location-based population cluster was constructed by using NTSYSpc2.0 version.

The genetic relatedness among the Ethiopian *P. lunatus* samples was further investigated using Principal Coordinate analysis (PCoA) using GenAlEx 6.503.

To examine the genetic structure, the genotypic data for ISSRs marker was analyzed using a model-based approach implemented in STRUCTURE. Applies a model to the data of K=5 that represent the number of populations.

The Analysis of Molecular Variance (AMOVA) was used to analyze among and within population genetic diversity by using Arlequin 3.5.2.2 version.

RESULTS

Banding patterns and ISSR primers

Out of the 13 screened primers, eight of them produced reproducible and polymorphic bands and used in this study (Table 2). The molecular weight of the amplified fragments ranged from 200bp to 3400 bp (Fig. 2). A total of 106 fragments were amplified by the eight ISSR primers of which 95 (88.79%) were polymorphic. The highest number of bands (19) was amplified by primer UBC 812 followed by DPISR1 and DPISR9 which generated 17 and 16 scorable bands, respectively. The least number of bands (8) were amplified by primer DPISR12.

Table 2. List of scorable bands generated by each primer.

Primers	Repeat motif	Number of scorable bands
DPISR1	(AGG)6	17
DPISR9	(ACTG)4	16
DPISR12	(GACA)4	8
DPISR4	(AG)10T	15
DPISR3	(AG)10C	12
DPISR2	(AG)10G	11
UBC812	(GA)8A	19
UBC834	(AG)10YT	9
Total		**106**

Genetic diversity and Shannon Weaver's diversity indices

Among the five Lima beans population evaluated using 8 ISSR markers, samples from West Wellega exhibited the highest genetic diversity and Shannon diversity index (H = 0.1864 and I = 0.2753). The least gene diversity and Shannon diversity index was exhibited by Jimma population (H = 0.1614 and I = 0.2328). Primer DPISR2 showed the highest gene diversity and Shannon diversity index (0.21 and 0.29, respectively) and primer 812 revealed the least (0.09 and 0.10) gene diversity and Shannon diversity index as shown in (Table 3).

Table 3. Number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (H) and Shannon Information Index (I) of 96 Lima bean accessions based on all primers used.

Population	With all primer			
	NPL	PP	H+SD	I+SD
West Wellega	53	49.53	0.1864	0.2753
Kelem Wellega	34	31.78	0.1677	0.2468
Jimma	26	24.3	0.1614	0.2328
Guragea	32	29.91	0.1848	0.2660
Gamo Gofa	[illegible]7	[illegible].58	[illegible].1703	0.2480
Sum	**182**	**170.1**	**0.8706**	**1.2689**
Average	**36.4**	**34.02**	**0.17412**	**0.25378**
For individual primer				
DPISR1	17	100	0.1482	0.1908
DPISR9	17	100	0.1482	0.1908
DPISR12	4	50	0.1532	0.2179
DPISR4	11	78.67	0.1642	0.2384
DPISR3	11	91.67	0.1886	0.2498
DPISR2	8	72.73	0.2100	0.2936
UBC812	19	100	0.0906	0.1015
UBC834	9	100	0.1230	0.1439
Total	**96**	**693.07**	**1.226**	**1.627**
Average	**12**	**86.63**	**0.1532**	**0.203338**

Number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (H) and Shannon Information Index (I)

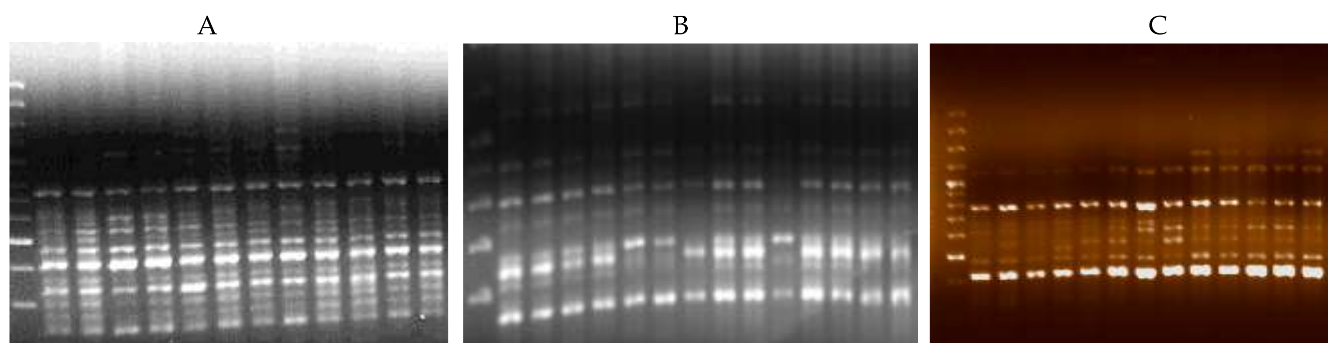

Figure 2.PCR **amplification products using** ISSR **markers.** DPISR1 **(A),** DPISR12 **(B) and** UBC834 **(C) from 96 Lima bean samples amplified fragments ranged from 200 bp to 3400 bp using 100 bp** DNA **ladder.**

Analysis of molecular variance (AMOVA)

Partitioning of genetic diversity of the five populations using AMOVA revealed that out of the total genetic diversity, 66% was among the population 34% was within the population with a gene flow of Nm=0.2739 (Table 4).

Table 4. AMOVA **of Lima bean populations.**

Source of variation	d.f	Sum of squares	Variance Components	Percentage of variation	Fixation Indices	P
Among Populations	4	1008.346	12.80809 Va	66.00	0.66000	0.00
Within Populations	91	600.425	6.59808 Vb	34.00		0.00
Total	95	1608.771	19.40617			

Nm (Gene flow) *=0.2739* ***P value*** *<0.001 represent statically highly significant difference*

Genetic similarity

The highest similarity was observed between West Wellega and Kelem Wellega Lima bean populations (0.741) followed by Gurage and Jimma (0.70). The least similarity was observed between Kelem Wellega and Gurage populations. The Kelem Wellega and West Wellega populations share relatively high similarity values than Gurage and Jimma populations (Table 5).

Table 5. Similarity matrix for Jaccard's coefficients for 5 Lima bean populations.

Population name	**Guragea**	**Jimma**	**Gamo Gofa**	**West Wellega**	**Kelem Wellega**
Guragea	1.000				
Jimma	0.8536	1.000			
Gamo gofa	0.7711	0.7384	1.000		
West Wellega	0.6487	0.6030	0.6800	1.000	
Kelem Wellega	0.6621	**0.6011**	0.7087	**0.8611**	1.000

Cluster analysis

Jaccard's similarity coefficients were also used to construct UPGMA dendrogram for 96 individuals based on the bands obtained with the eight primers. Accordingly, individuals collected from West Wellega and Kelem Wellega tended to form strong separate group from the rest in UPGMA. However, populations collected from Gurage, Gamo Gofa and Jimma formed the same grouping based on their place of origin (Fig. 3).

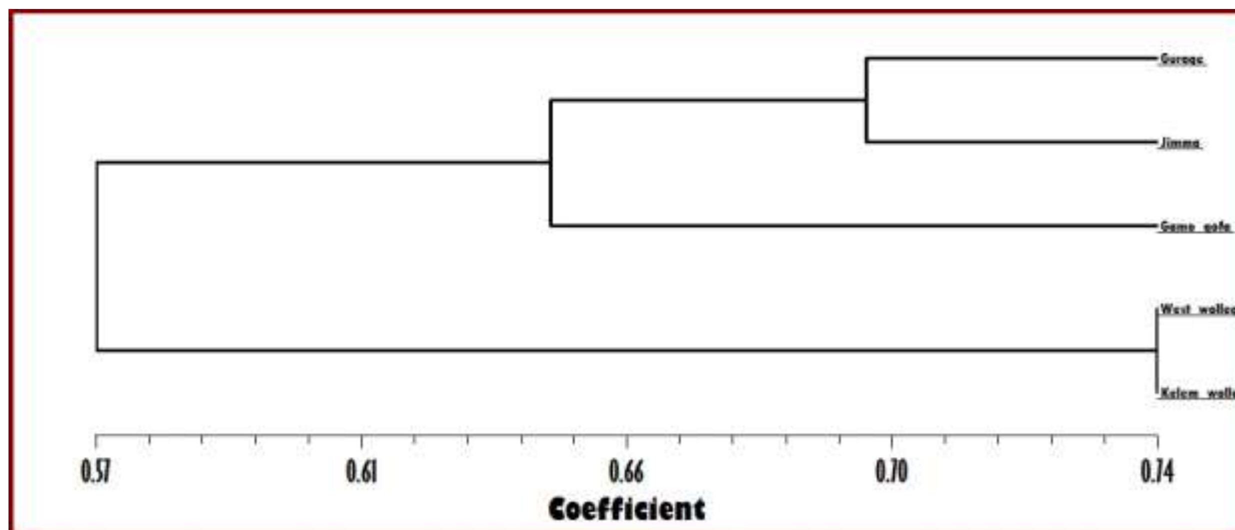

Figure 3. UPGMA **based dendrogram for 5 Lima bean populations using 8 ISSR primers**

Genetic distance

Genetic distance matrix revealed that the highest genetic distance was observed between Kelem Wellega and Jimma populations (0.5089) followed by West Wellega and Jimma populations (0.5059). The least genetic distance was observed between Kelem Wellega and West Wellega (0.1496) populations (Table 6).

Table 6. Genetic distance matrix for Jaccard's coefficients for 5 Lima bean populations.

Population name	Guragea	Jimma	Gamo gofa	West Wellega	Kelem Wellega
Guragea	0.00				
Jimma	0.1583	0.00			
Gamo gofa	0.2599	0.3033	0.00		
West Wellega	0.4328	0.5059	0.3857	0.00	
Kelem Wellega	0.4123	**0.5089**	0.3444	**0.1496**	0.00

Structure analysis

The result obtained from analysis formed three groups, Kelem Wellega and West Wellega in the same group, Guragea and Jimma in the other group and Gamo Gofa formed their own group. The groupings showed clear association with geographical origins of the populations (Fig 4).

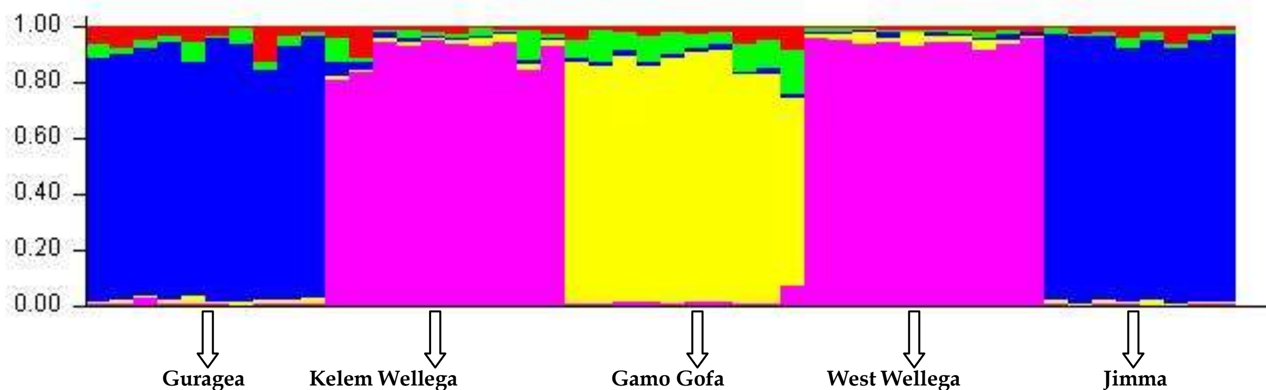

Figure 4. Structure analysis of 96 individuals of the five populations of Lima beans.

Principal coordinates (PCoA) analysis

Individuals collected from West Wellega and Kelem Wellega clustered in the same group tend to form strong separate group. However, populations collected from Gurage, Gamo Gofa and Jimma were observed to form their own group based on their geographic origin (Fig. 5)

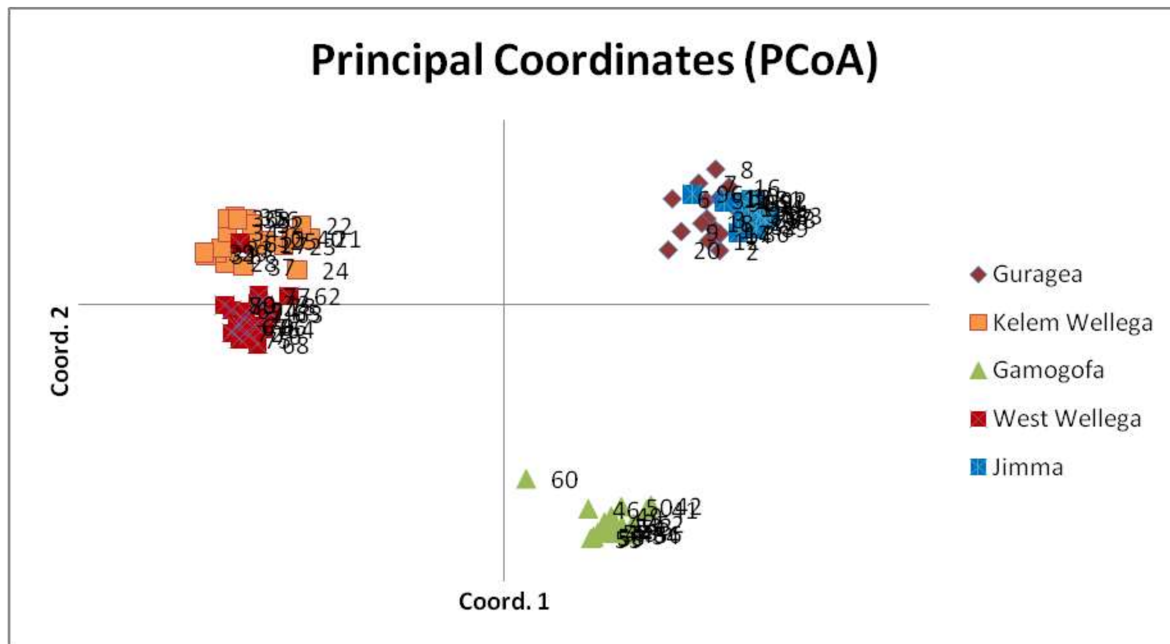

Figure 5. Dimensional representation of 96 individuals belonging to five Lima beans. Population based on Jaccard's similarity coefficient.

DISCUSSION

In the present study, ISSR marker was used for the first time to assess genetic variation of Lima bean populations from Ethiopia. The goal of the study was to know the existing genetic resources with the likelihood of contributing to development strategies for effective conservation and sustainable use of *P. lunatus* of Ethiopia.

This study investigated the genetic diversity and relationship among 96 accessions of Lima bean using eight ISSR primers. The utilization and effectiveness of ISSR markers to determine genetic polymorphisms have been confirmed to be efficient for diversity studies in other leguminous crops from the genus *Phaseolus* such as common bean (*P. vulgaris* L.), runner bean (*P. coccineus* L.), tepary bean (*P. acutifolius* A. Gray) and in Lima bean (*P. lunatus* L.).

Out of 106 loci generated by eight primers 95 (88.2%) of them were polymorphic which is in consistent with Martinez *et al.* (2017) who reported on the genetic structure of Lima bean landraces grown in the Mayan area using four ISSR generated 75 bands, where all them (100%) were polymorphic, Martinez *et al*. (2008) worked on the genetic erosion and *In situ* conservation of Lima bean landraces in its Mesoamerican diversity center and Out of the 90 bands analyzed 71 (78.8%) were polymorphic. Cabral *et al*. (2018) reported on the genetic diversity of common bean cultivars using 20 ISSR primers and generated 51 bands of which 39 (76.4%), were polymorphic. Maria *et al*. (2015) reported on the Genetic variability analysis of faba bean accessions using Inter-simple sequence repeat (ISSR) markers and generated 142 bands out of which 134 (79%) were polymorphic. However, Variations in the percent of polymorphism could be due to differences in the genotypes, the number of examined samples, the number of primer used and the nature of primers among others.

AMOVA analysis result showed high among population's genetic diversity (66%) and

remaining diversity was distributed within populations (34%) with Fst value of 0.66 and Nm (gene low) of 0.27. Nm estimates, indicate that the average number of successful immigrants per generation should be estimated in one to favor the reduction of population structure (Slatkin and Barton, 1989). This result is due to the occupation of Lima bean in restricted area unlike many other cultivated crops grown in Ethiopia which is due to the climbing nature of the crop. Farmers grow Lima bean along a fence line and use the crop mostly for home consumption which significantly decreased seed exchange between the communities through the National Market. Lima bean is 52% a self pollinated plant as a result gene flow among populations is low. Our results are in consistent with Martinez *et al*. (2017) who reported the low level of long term gene flow found with Nm = 0.29. Martinez *et al*. (2007) reported Nm = 0.28 between wild and cultivated populations of Lima beans from the Yucatan Peninsula. This could be due to low rate of seed exchange among different regions and markets which could lead to low intermix among populations. Jiang *et al*. (2012) reported that the mating system, life history traits of a species, Gene flow, genetic drift and evolutionary history might have an important influence on genetic structure and diversity of a given population.

The clustering pattern observed using PCoA, UPGMA and STRUCTURE analysis of Jaccard's coefficient of similarity was supported by the high level of genetic structure and low level of intermix between a population observed in that Guragea and Jimma population makes one group, West Wollega and Kelem Wollega make the other group relatedness based on geographic distance. On the other hand, accessions from Gamo Gofa tend to form their own clustering. However, in the UPGMA dendrogram, accessions from Gamo Gofa were closely related with the accession collected from Guragea and Jimma. Similar trend was observed between West Wollega and Kelem Wollega which are also relatively found at geographically shorter distance. This clustering may indicate absence of massive seed movements in distant geographical areas. The separation and formation of a cluster between accessions may suggest that accessions may have been isolated from each other for a longer period in time and as a result there was limited gene flow due to long distance. This result is in agreement with Camacho *et al*. (2017) who reported on the genetic structure of Lima bean landraces grown in the Mayan area using ISSR markers and Martinez *et al*. (2006) who used SSR markers and found that the wild and domesticated populations of Lima Bean in the Yucatan Peninsula are grouped according to the region where they were collected. Genetic variation is useful for heterotic patterns in hybrid breeding and for relating the observed pattern with presence of certain economically important qualitative and quantitative traits.

Such information can be used to design effective germplasm conservation and for setting germplasm collection task as well as to guess or predict the risk of genetic erosion in certain areas. So, the knowledge and understanding of the genetic diversity and molecular characterization of *Lima Bean* can help in identifying potential elite genotypes. Genetic diversity study will offer significant aid for targeted genetic improvement of nutritional and other quality traits in Lima bean which may give rise to a high quality, legume-based protein diet to those areas with high protein deficiency. Moreover, this result can serve as a baseline information for further improvement of Lima Bean through breeding.

CONCLUSIONS

The present study highlighted significant information on the genetic relationship among the 96 land races of Lima bean and confirmed that there was low level of gene flow and high level of genetic differentiation between the populations and correlation of genetic distance with geographic distance of the locations where seed samples were collected. This is an encouraging result for further collection activities so as to capture more variability from other agro ecologies of the country. Although the study was based on a limited number of markers No single method is adequate for assessing genetic variation in germplasm collections, because different methods of sampling genetic variation allow sampling at different levels and differ in their power of genetic resolution as well as the quality of information content. However, generating more information on the genetic diversity is an important parameter in the future

efforts of Lima bean genetic resources conservation and sustainable utilization.

Currently only five samples of Lima bean collection found at Ethiopian Biodiversity institutes (gene bank). Thus, it would be useful to increase representative samples from all parts of the country to capture the maximum diversity ad minimize the genetic erosion of this bean. Furthermore, the present study showed Land races from West Wellega and Gamo gofa exhibited higher genetic diversity than the other three populations. Those areas should be targeted for improvement of this crop by breeders.

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