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Full Length Research Paper

Morphological and molecular variation in Ethiopian lentil (*Lens culinaris* Medikus) varieties

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Phenotypic and genetic diversity of ten improved lentil varieties of Ethiopia were assessed using nine agro-morphological characters and four ISSR primers. ANOVA of phenotypic data showed highly significant variation among the varieties for all traits. Number of pods and seeds, hundred seed weight, and number of secondary and primary branch showed high phenotypic coefficient of variation (PCV), while days to flowering, seed yield, plant height and days to maturity revealed low PCV. Ada'a, Chalew and R-186 were late in flowering and maturity, while Gudo was early flowering, but late maturing variety. Alemaya, Alemtena and Assano were early in flowering and maturity, while Chekol, EL-142 and Teshale were intermediate in flowering but early in maturity. The Euclidian distance between Ada'a and Assano was high, while the distance between Alemtena and Teshale was the least. Clustering analysis based on phenotypic data classified the varieties into two groups. ISSR analyses revealed that 75.93% of the amplified bands were polymorphic with average gene diversity (H) of 0.2734. Inter-varietal genetic distance between Gudo and Chekol was high, while that between Gudo and Alemaya was low. Clustering analysis based on ISSR marker also classified the varieties into two groups. The correlation (r=0.334) between morphological and ISSR dissimilarity matrices was significant. Results of the present study reveal the genetic variation between Ethiopian lentil varieties which would help in selection of varieties for specific agro-ecology and/or purpose, and for further breeding activities.

Key words: ISSR marker, genetic diversity, lentil varieties, morphological variation, correlation.

INTRODUCTION

Canada, India and Nepal are the leading lentil producing countries, contributing 1043200, 810000 and 161147 metric tones, respectively, to the world's total production. USA, Ethiopia, Bangladesh, Australia, Iran, Syrian Arab Republic and China are also important lentil producing countries. In 2008, Ethiopia produced 94103 metric tonnes of lentil from 107428 ha of land. In 2005, the country harvested 63357 metric tones of produce from 96385 ha of land. In these years, the acreage of land allocated to the crop increased by 11043 ha (11.46%), while the average seed yield increased by 218.63 kg/ha (33.26%). This drastic increase (greater than six fold) in Ethiopian lentil production would, therefore, primarily be attributed to the increased use of improved varieties and increment of acreage of land allocated to the crop. As compared to the average seed yield of Canada and USA in 2008, 1489.86 and 1027.66 kg/ha, respectively; however, Ethiopia's average seed yield is low indicating the need for further improvement and management activities (FAOSTAT, 2010).

Improved varieties are one of the most important components of modern agriculture in boosting up production and productivity. Various breeding activities, such as selection of genotypes with high and stable seed yield and resistance/tolerance to the most important biotic and abiotic stresses from indigenous landraces have been undergoing. As a result of such effort, EL-142 was obtained. However, since Ethiopian lentil landraces, in general, are said to be inferior for the most important traits, improvement activities based on selection alone appeared to be a great challenge. Therefore, it became necessary to introduce lentil germplasm from the International Center for Agricultural Research in the Dry Areas (ICARDA) and screen for the best ones. As a result,

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nine genotypes of exotic origin with high and stable seed yield and resistance/tolerance to the major biotic and abiotic stresses were registered and released for production. Nevertheless, information on the genetic variation/diversity of the varieties has not been generated.

The knowledge of genetic variation and relationships between varieties is important to understand the available genetic variability within and between varieties, and its potential use in breeding programs. Genetic variability could be studied using a number of marker systems such as morphological traits, molecular and physiological markers. Their specific advantages and disadvantages relevant to this study were briefly reviewed by Edossa etal. (2010).

Molecular analyses in conjunction with morphological and agronomic evaluation of germplasm are recommended, because these provide complementary information and increase the resolving power of genetic diversity analyses (Singh and Singh, 1991).

The present study was conducted with the following objectives: (1) to study the morphological and genetic diversity of Ethiopian lentil varieties, (2) to classify lentil varieties into groups based on both morphological traits and molecular profiles, and (3) to assess the level of correlation between phenotypic and genotypic distances in Ethiopian lentil varieties.

MATERIALS AND METHODS

Plant materials and DNA extraction

Seeds of ten released varieties (Table 1) obtained from Debre-Ziet (DZARC) and Sinana (SARC) Agricultural Research Centers were grown at SARC on station field plot (Bale-Robe, Ethiopia). Planting was done in two replications of 0.62 m^2 plot size (2 rows of 1.55 m length spaced at 0.2 m) during 'Bona/Meher' season (August to December). Seed rate was 65 kg/ha as per the recommendation for the area and no fertilizer was applied. All plants used in this study were generated from seeds under natural conditions. Fifteen individual plants were selected from each of the two replications and marked just before flowering. Morphological data were collected from all marked plants.

Young leaves were collected separately from fifteen randomly selected individual plants of each variety just before flowering and dried in silica gel. Total genomic DNA was isolated from about 0.2 g of the pulverized leaf sample, using a modified tripple cetyltrimethyl-ammoniumbromide (CTAB) extraction technique (Borsch et al., 2003). The isolated DNA samples were visualized using 1% ethidium bromide stained agarose gel under UV light. The second or third extractions were selected for PCR amplification based on DNA quantity and quality. The selected genomic DNA samples were diluted with sterile distilled water in 1:5 ratio.

ISSR analysis

A total of 12 ISSR primers (UBC primer set # 9, Vancouver, BC, Canada) were selected based on published results in lentil (Ford and Taylor, 2003; Kahraman et al., 2004) and related legume species such as common bean (de la Cruz et al., 2004; González et al., 2005), *Ammopiptanthus* (Ge et al., 2005) and chickpea (Flandez-Galvanez et al., 2003). These primers were screened for the amplification of unambiguously visible and polymorphic ISSR bands on samples from each variety. Finally, ISSR primers UBC 812 $[(GA)_8A]$, UBC 818 $[(CA)_8G]$, UBC 835 $[(AG)_8YC]$ and UBC 881 $[(G_3TG)_3]$, which produced unambiguously visible and polymorphic bands across the varieties were chosen for further analyses.

Optimization of PCR reaction components for ISSR genotyping was done using DNA extracted from representative plants of each variety used for screening primers. The optimum reaction components were 16.7 µl dH_2O, 250 µM of each dATP, dGTP, dCTP and dTTP, 2.6 µl 10X *Taq* buffer, 1 U *Taq* polymerase, 0.23 µM primer and 1 µl template DNA. The final reaction volume per sample was 26 µl. PCR amplification conditions were set as: initial denaturing at 94°C for 4 min followed by 40 cycles at 94°C for 15 s, 45/48°C for 1 min, and 72°C for 1½ min and ended with extension phase of 72°C for 7 min. The lid temperature was held at 105°C.

The amplified PCR products were stored at 4°C until the time of electrophoresis. PCR amplification was performed with Biometra® T3 thermorecycler.

The ISSR-PCR product was resolved on 1.7% agarose gel in 1X TBE buffer. Genomic DNA of 9 µl per sample was loaded with 2 µl of 6X loading dye and 100 bp DNA ladder (PEQLAB Biotechnologie GmbH) was used on each side of the gel as a marker. Electrophoresis was conducted at 100 V for about 2:00 h in 1X TBE buffer. The resultant gel was visualized by ethidium bromide staining under UV light and photographed with Biodoc Analyzer (035-300).

Phenotypic data

Phenotypic data were recorded on number of days to flowering and maturity, plant height (cm), number of primary and secondary branches, number of pods and seeds, 100-seed weight (g) and seed yield (g). All data were recorded on individual plant basis and the mean of fifteen plants was used to represent a variety at each replication.

Data analyses

Homogeneity of variance across accessions on phenotypic data was tested using Bartlett's (Snedecor and Cochran, 1983) homogeneity test. One-way analysis of variance (ANOVA) was conducted for all characters according to Gomez and Gomez (1984). Phenotypic coefficient of variation (PCV) was computed according to Burton and de Vane (1953) for each morphological trait. Euclidian distance between the varieties was calculated from the standardized trait mean values (mean of each trait was subtracted from the data values and the result divided by the standard deviation of the trait) over each accession using NTSYSpc Version 2.11T (Rohlf, 2004).

Similarly, the standardized trait mean values over each variety were used to perform cluster analysis with the same software. To group the varieties based on morphological dissimilarity, cluster analysis was conducted on Euclidian distance matrix with Unweighted Pair Group Method based on Arithmetic Averages (UPGMA) procedure of the Sequential, Agglomerative, Hierarchical and Nested (SAHN) clustering methods (Sneath and Sokal, 1973) with NTSYSpc2.11T. ISSR bands were scored manually for each individual variety from the gel photograph. Unambiguously scored bands were recorded as discrete characters, presence '1' or absence '0'. Matrix of binary data was constructed with rows equal to varieties and columns equal to distinct molecular marker band of the primers.

Genetic diversity measured by the percentage of polymorphic bands (P) (the ratio of the number of polymorphic bands to the total number of bands surveyed), Nei's gene diversity (H) (Nei, 1973) and Nei's standard genetic distance (D) (Nei, 1972) were computed

Table 1. List and related data of improved Ethiopian lentil varieties used in the study.

Local name	Origin	Source	Year of release (E.C.)
Chalew (NEL 358)	ICARDA	DZARC	1977
EL-142	Ethiopia	DZARC	1972
Ada'a (FLIP 86-14L)	ICARDA	DZARC	1987
Chekol (ENAL-2704)	ICARDA	DZARC	1986
Gudo (FLIP 84-78L)	ICARDA	DZARC	1987
Teshale (FLIP 96-46L)	ICARDA	DZARC	1986
R-186	ICARDA	DZARC	1972
Alemaya (FLIP 89-63L)	ICARDA	DZARC	1989
Alemtena (FLIP 96-49L)	ICARDA	DZARC	1986
Assano (FLIP 88-46L)	ICARDA	SARC	1994

DZARC = Debre-Zeit Agricultural Research Center; SARC = Sinana Agricultural Research Center; ICARDA = International Center for Agricultural Research in the Dry Areas.

with POPGENE ver 1.32 (Yeh et al., 2000). The haploid option of the software was used for analysis in accordance with the assumption of Ferguson et al. (1998) stating that each individual of a highly inbreeding species, such as *Lens* species, is homozygous. Since ISSRs are dominant markers, only the presence or absence of an allele can be determined and, therefore, each band position corresponds to a locus with two alleles represented by the presence or absence of a band (Powell et al., 1996).

Cluster analysis of the ISSR data was conducted based on the standard genetic distance matrix using UPGMA procedure of the SAHN clustering methods with NTSYSpc2.11T. The goodness of fit of clustering to the data set was tested with the same software, whereby the co-phenetic matrix generated from the UPGMA tree was compared with the dissimilarity matrix using the two-way Mantel (1967) test method.

RESULTS

Morphological variation

Bartlett's chi-square test revealed non-significant ($p<0.05$) difference among varieties for their variance in all traits, indicating homogeneity of variance among varieties. Analysis of variance showed highly significant ($p<0.01$) difference among varieties for all traits. Number of pods and seeds, hundred seed weight, and number of secondary and primary branch showed high phenotypic coefficient of variation (PCV). On the other hand, days to flowering, seed yield, plant height and days to maturity revealed low PCV (Table 2).

Intervarietal Euclidian distance was computed from the standardized trait mean value of each variety. High intervarietal Euclidian distance was observed between Ada'a/Assano, Assano/R-186, Ada'a/Chekol, Ada'a/Alemaya, Assano/Chekol, Ada'a/EL-142 and Assano/Chalew, while low intervarietal Euclidian distance was obtained between Chalew/EL-142, Alemaya/Assano, Alemaya/Chekol, Alemaya/EL-142, Alemtena/Teshale and Chekol/EL-142. Euclidian distance between the other pairwise varietal combinations was intermediate (Table 3).

A dendrogram was constructed from the standardized value of morphological traits. The varieties were grouped into two clusters at average Euclidian distance value of

Table 2. Analysis of variance and phenotypic coefficient of variation (PCV) for ten Ethiopian lentil varieties.

Traits*	Source of variation	Degrees of freedom	Sum of squares	Mean square	CV	PCV
DTF	Between varieties	9	1019.375	113.264**	2.59	15.020
	Within varieties	10	33.735	3.374		
	Total	19	1053.11			
DTM	Between varieties	9	7138.04	793.116**	2.44	22.166
	Within varieties	10	96.09	9.609		
	Total	19	7234.13			
PH	Between varieties	9	318.938	35.438**	7.72	18.144
	Within varieties	10	64.12	6.412		
	Total	19	383.058			
PB	Between varieties	9	52.762	5.862**	11.17	26.246
	Within varieties	10	10.615	1.062		
	Total	19	63.377			
SB	Between varieties	9	131.79	14.643**	10.95	26.884
	Within varieties	10	24.284	2.428		
	Total	19	156.074			
NP	Between varieties	9	22313.5	2479.278**	18.96	50.339
	Within varieties	10	3518.364	351.836		
	Total	19	25831.86			
NS	Between varieties	9	24608.83	2734.315**	19.09	43.796
	Within varieties	10	5196.595	519.659		
	Total	19	29805.43			
TSW	Between varieties	9	15.366	1.707**	5.90	39.164
	Within varieties	10	0.387	0.039		
	Total	19	15.753			
SY	Between varieties	9	11.749	1.305**	6.39	15.545
	Within varieties	10	2.206	0.221		
	Total	19	13.955			

*DTF = Number of days to flowering, DTM = Number of days to maturity, PH = Plant height (cm), PB = Number of primary branch, SB = Number of secondary branch, NP = Number of pods per plant, NS = Number of seeds per plant, TSW = 1000-seed weight (g), and SY = Seed yield (g/plant), CV = Coefficient of variation, PCV = Phenotypic coefficient of variation, ** = Significant at p = 0.01.

data produced two groups of Ethiopian lentil varieties at 0.32 average genetic dissimilarity (Figure 2). The first cluster consisted Ada'a, Chekol and EL-142, while the second one consisted Alemtena, Teshale, Assano, Alemaya, R-186, Gudo and Chalew. The correlations between cophenetic values (generated from the UPGMA tree) and genetic dissimilarity (r =0.829) showed good fit, interpreted according to Rohlf (2004), to the data set, and hence validated the observed clusters.

Correlation between morphological and molecular dissimilarity matrices

In order to compare the extent of agreement between dendrograms derived from morphology and ISSR markers, a distance matrix was constructed for each assay and compared using the Mantel (1967) matrix correspondence test. Accordingly, the correlation (r) between morphological and ISSR dissimilarity matrices was positive (0.334) and highly significant ($p<0.001$, 1000 random permutations). The average intervarietal Euclidian distance from the standardized morphological data (4.120) was greater than the average ISSR-based genetic dissimilarity (0.278) (Table 5).

DISCUSSION

Morphological variation

Analysis of variance showed highly significant difference

Table 3. Euclidian distance between ten improved Ethiopian lentil varieties.

Varieties	1	2	3	4	5	6	7	8	9	10
Ada'a (1)	0.000									
Alemaya (2)	**5.300**	0.000								
Alemtena (3)	3.372	3.311	0.000							
Assano (4)	**6.235**	2.892	4.955	0.000						
Chalew (5)	4.186	3.116	3.908	**5.063**	0.000					
Chekol (6)	**5.616**	2.671	3.768	**5.184**	3.363	0.000				
EL-142 (7)	**5.165**	1.890	3.149	4.395	2.993	1.333	0.000			
Gudo (8)	4.603	4.250	4.107	4.678	4.807	4.924	4.663	0.000		
R-186 (9)	4.086	4.598	4.672	**5.686**	3.760	4.767	4.864	4.755	0.000	
Teshale (10)	4.166	3.341	1.877	4.504	4.623	4.159	3.699	3.763	4.162	0.000

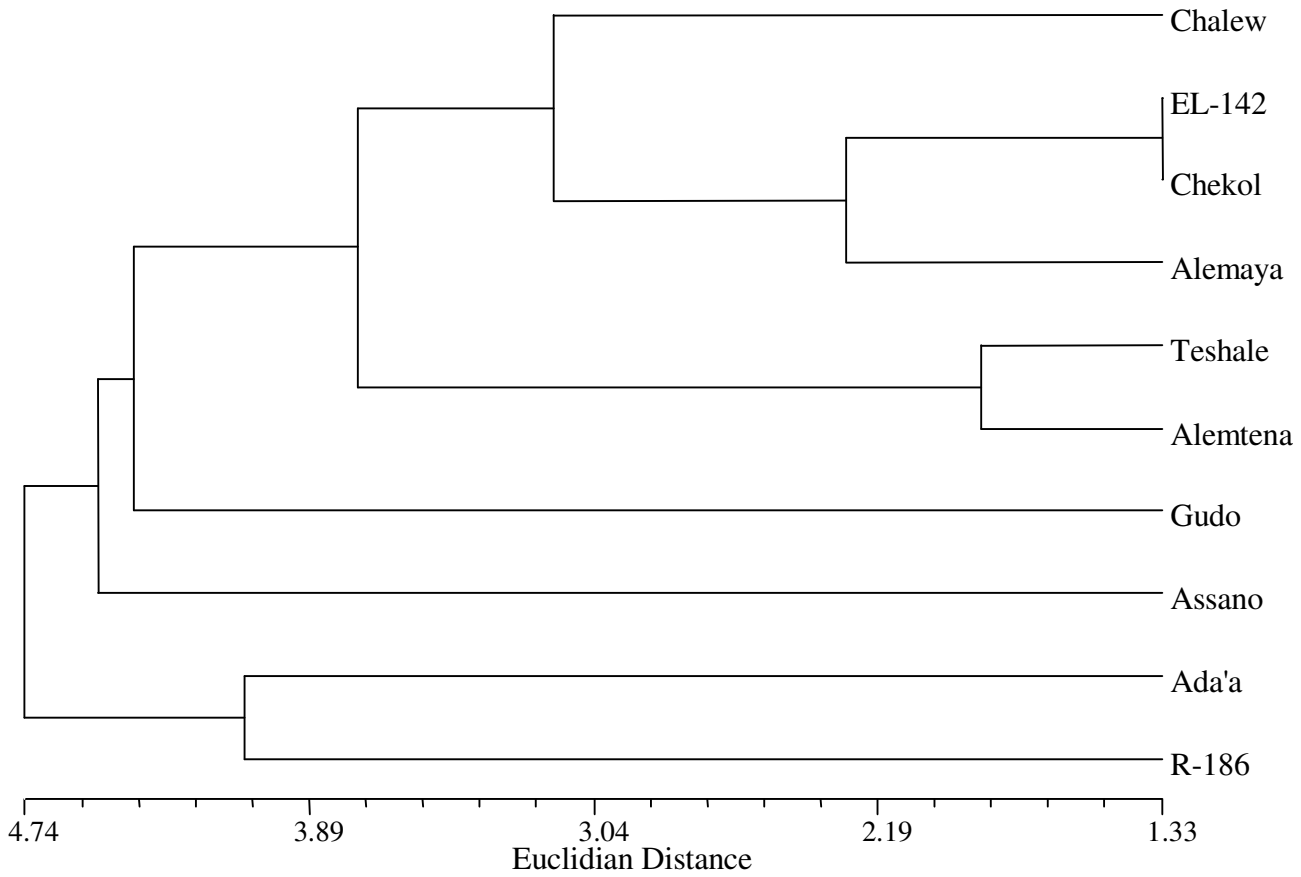

Figure 1. A dendrogram of ten improved Ethiopian lentil varieties derived by UPGMA from the Euclidian dissimilarity matrix of morphological data**.**

among varieties for all traits. This indicates the existence of high degree of phenotypic diversity in Ethiopian lentil varieties and implies great potential for improvement programs. Number of pods and seeds, hundred seed weight, and number of secondary and primary branch showed high phenotypic coefficient of variation (PCV) suggesting a wide opportunity for their improvement and/or to be used as a donor parent for these traits. On the other hand, days to flowering, seed yield, plant height and days to maturity revealed low PCV, suggesting low opportunity for improvement. This finding agrees with the result reported by Edossa et al. (2010) in Ethiopian lentil landraces for number of pods and seeds, number of secondary branches, plant height, and days to flowering and

Table 4. Genetic distance between improved lentil varieties of Ethiopia.

Varieties	1	2	3	4	5	6	7	8	9	10
Ada'a (1)	0.000									
Alemaya (2)	0.255	0.000								
Alemtena (3)	0.245	0.245	0.000							
Assano (4)	0.237	0.213	0.292	0.000						
Chalew (5)	0.377	0.229	0.274	0.267	0.000					
Chekol (6)	0.252	0.274	0.268	**0.437**	**0.405**	0.000				
EL-142 (7)	0.252	0.274	0.268	**0.437**	**0.405**	0.000	0.000			
Gudo (8)	0.333	0.171	0.230	0.223	0.106	**0.452**	**0.452**	0.000		
R-186 (9)	0.297	0.213	0.237	0.229	0.150	**0.449**	**0.449**	0.106	0.000	
Teshale (10)	0.343	0.287	0.061	0.314	0.331	0.287	0.287	0.287	0.290	0.000

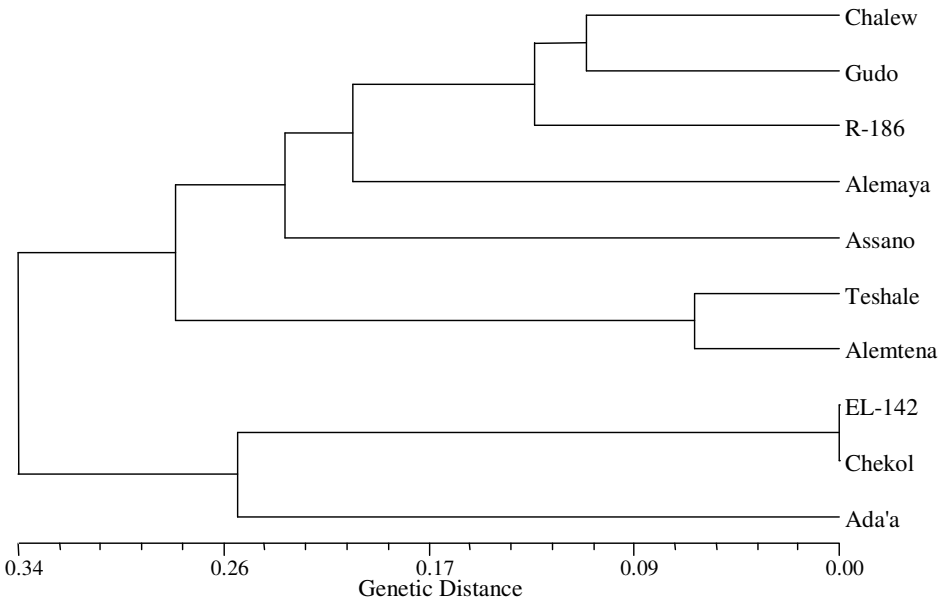

Figure 2. ISSR-based dendrogram of ten improved Ethiopian lentil varieties derived by UPGMA from Nei's (1972) genetic distance.

maturity, but not for hundred seed weight.

Time to maturity is one of the most important characters for a crop variety among the traits that determine its adaptation to a specific or wide range of agro-ecologies. Ada'a, Chalew, Gudo and R-186 were late in maturity, and, are expected to show good agronomic performance in areas with long growing season, and, therefore, these varieties may not perform well in areas with short rainy/moisture season/period. On the other hand,hand, Alemaya, Alemtena, Assano Chekol, EL-142 and Teshale were early maturing varieties and hence could be recommended for areas with short rainy season. This, however, does not mean that these varieties do not perform well in areas with long growing season. Assano, for instance, has been evaluated and released for its earliness and high seed yield in areas with long period of rainy season. Alemaya has been proved to be an exceptional variety in its wide range of adaptation to various agro-ecological conditions. Therefore, Alemaya could be used as a donor parent in improvement programs targeting to transfer this trait. Seed size/weight is among the economic traits for lentil, since large seeded lentil is generally, preferred, especially in the international market.

Comparatively, Alemtena, Assano and Gudo showed large seed weight, while Chalew, Chekol, EL-142 and R-186 were small seeded. Ada'a, Alemaya and Teshale were of medium seed size. Improvement programs may utilize this information in their breeding activities to produce a large seeded variety in addition to the other economic characters. The distance between two crop varieties is an important parameter for designing a successful crossing or hybridization program. Clustering analysis is also conducted based on the distance

Table 5. Mean, range, standard deviation and correlation coefficient of genetic dissimilarity (from ISSR marker) and Euclidian distance (from morphological traits) in Ethiopian lentil varieties.

	Morphological dissimilarity			ISSRs dissimilarity			Correlation
	Mean	Range	St. dev.	Mean	Range	St. dev.	
Varieties	4.120	1.333–6.235	1.026	0.278	0.000–0.452	0.102	0.334

between the varieties. Therefore, based on the results from phenotypic data, intervarietal crossing activities may be more successful, if conducted between varieties in different clusters and with high Euclidian distance between one another. Accordingly, crosses between Ada'a and Assano, Assano and R-186, Ada'a and Chekol, Ada'a and Alemaya, Assano and Chekol, and Assano and Chalew may produce good results for the phenotypic characters studied.

Molecular diversity

In this study, a total of (54) bands, of which 41 (P = 75.93%) were polymorphic, were amplified using four ISSR primers on ten Ethiopian lentil varieties. Yüzbaşıoğlu et al. (2006) from RAPD analysis of fourteen cultivars and thirteen breeding lines using nine 10-mer primers reported 54% polymorphism out of the total 41 reproducible bands produced. The present study revealed an average Nei's (1973) gene diversity (H) of 0.2734. This is by far greater than the genetic diversity reported by Edossa et al. (2007) for Ethiopian lentil landraces (P=59.57, H=0.175). This could be accounted primarily to the technique of the study and the pedigree of the materials. In the first place, a bulk of fifteen plants was used to represent each accession (which was expected to be a mixture of different genotypes) for diversity study in Ethiopian lentil landraces (Edossa et al., 2007). Use of bulk samples for genetic analysis does not reveal the genetic diversity within accessions, and hence does not indicate the overall genetic diversity in the constituent plants. Secondly, the lentil varieties used in the present study were obtained from a series of crossing activities and hence are expected to be composed of diverse genome. Thirdly, the improved varieties have originated from geographical regions that widely differ (as compared to that of Ethiopian lentil landrace accessions which were collected only from Ethiopia) and hence, are expected to reveal high genetic diversity.

Genetic distance is a measure of the allelic substitutions per locus that have occurred during the separate evolution of two populations or species. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Crosses between distantly related individuals are expected to be more heterotic than those between closely related genotypes. Therefore, prior knowledge of the genetic distance between genotypes or varieties is very important in designing successful hybridization program. Hence, hybridization activities between Gudo/Chekol, Gudo/EL-142, R-186/Chekol, R-186/EL-142, Chekol/Assano, Chekol/Calew, EL-142/Assano and EL-142/Chalew would be more heterotic than those between El-142/Chekol, Teshale/Alemtena, R-186/Gudo, Gudo/Chalew, R-186/Chalew and Gudo/Alemaya. This was further supported and strengthened by the results from cluster analysis.

Correlation of phenotypic and genetic diversity

The correlation (r) between morphological and ISSR dissimilarity matrices was positive (0.334) and significant ($p<0.001$). Edossa et al. (2010) also reported positive and significant correlation (r=0.279, $p<0.001$) between morphological and molecular dissimilarity matrices in Ethiopian lentil landraces. Piergiovanni and Taranto (2004) reported that the result from SDS-PAGE analysis agreed with that of the previous agronomic studies in lentil. Yoseph et al. (2005) working on 62 Ethiopian traditional maize accessions reported a correlation value of r=0.43 and r=0.39 between 15 morphological characters and SSR and AFLP, respectively. Furthermore, Roldan-Ruiz et al. (2001) working with 16 rye grass varieties reported a correlation value of r=-0.06 between AFLP and 15 morphological characters. In addition, Bolaric et al. (2005) working on 12 phenotypic characters of 22 perennial ryegrasses reported a correlation value of r=0.10 between morphological and molecular (RAPD) marker.

Therefore, in comparison with the lentil landraces and ryegrasses, Ethiopian lentil varieties appear to be more stable, as suggested by the higher agreement between phenotypic and ISSR based molecular distances. This indicates that the observed phenotypic variation was at least partly caused by genetic factors. However, when compared with the traditional Ethiopian maize accessions, Ethiopian lentil varieties seem to be less stable. Nonetheless, the positive and significant correlation obtained between the two dissimilarity matrices indicate that they likely reflect the same pattern of genetic diversity and validate the use of the data to calculate the different diversity statistics for Ethiopian lentil varieties. Nevertheless, the genetic relationship observed using molecular markers may provide information on the history and biology of cultivars, but it does not necessarily reflect what may be observed with respect to agronomic traits

(Métais et al., 2000).

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