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Genetic diversity of chickpea (*Cicer arietinum* L.) cultivars from Ethiopia by using ISSR markers

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Inter Simple Sequence Repeat (ISSR) markers were employed to reveal genetic diversity and relatedness among 27 chickpea (*Cicer arietinum* L.) cultivars in Ethiopia. Four di-nucleotide repeat primers amplified 24 clear and reproducible bands of which 22 were polymorphic (91.67%). The genetic variation among 27 chickpea cultivars including 12 kabuli and 15 desi verities is high; in which desi type exhibited a genetic diversity of 75% with Shannon index of 0.47, while the kabuli type chickpea had 91.67% genetic diversity and Shannon index of 0.50. Unweighted Pair Group of Arithmetic mean (UPGMA) dendrogram and NJ trees with Jaccard's similarity coefficient showed three major clusters. This was also recovered by 3D principal coordinates analysis, although some cultivars were intermixed. Analysis of molecular variance (AMOVA) demonstrated highly significant ($p < 0.001$) genetic diversity within cultivars (97.71%) than among cultivars (2.29%). The distinct cultivars (Aererti, Tejie, Fetenech and Maryie) can serve as parents for future genetic resources conservation and Chickpea breeding program in Ethiopia.

Key word: Chickpea cultivars, genetic diversity, ISSR markers, Ethiopia.

INTRODUCTION

Chickpea (*Cicer arietinum*) belongs to family Leguminosae and comprises 43 species; of which nine are annuals while others are perennial (Gautam et al., 2016). It is originated in south-eastern Turkey and adjoining Syria as primary centre of diversity at the Fertile Crescent, but now it is cultivated throughout the semi arid regions of the world (Thudi et al., 2016).

Chick pea, the only cultivated self pollinated species within the genus Cicer, is a diploid plant with 2n = 2x = 16. It ranks third among food legume production after common bean (*Phaseolus vulgaris*) and field pea (*Pisum sativum*), and second in area coverage among pulses grown in Ethiopia proceeded by faba bean (Varshney et al., 2013). There are two main types of chickpea cultivars: desi type and kabulitype chickpea grown in temperate regions and semi-arid tropics, respectively. In Ethiopia approximately, more than 85% of the area is covered with desi type; whereas the rest of 15% is kabuli

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type (FAO, 2013). The cultivated species is found in West Asia and North Africa covering Turkey in the north to Ethiopia in the south, and Pakistan in the east to Morocco in the west (Aggarwal et al., 2015). Chickpea is valued for its high dietary protein content, ability to fix atmospheric nitrogen and the absence of major anti-nutritional factors (Gautam et al., 2016). This makes it an important component of cropping system and considered as a nutritious and healthy food.

Analysis of genetic diversity and population structure relatedness among germplasm is the key for successful breeding program, effective plant conservation and, for elucidating the genetic relatedness of crop species. This can be carried out by using different markers like morphological characters, biochemical and molecular methods (Atnaf et al., 2017). Molecular markers are widely used to study the genetic diversity, identify redundancies in germplasm collections, test accession stability and integrity, and resolve taxonomic relationships (Zulhairil et al., 2015). Molecular markers for assessments of genetic variation in plants have shown many advantages. They are neutral, not related to age and tissue type, and not influenced by environmental conditions, have feasibility and lower costs, and are more informative than morphological markers. Thus, molecular markers can be considered to be a more effective approach compared to morphological markers, to identify plant genotypes (Choudhary et al., 2013).

Moreover, polymerase chain reaction (PCR) based markers are currently available for genetic study this includes amplified fragment length polymorphism (AFLP), random amplified polymorph DNA (RAPD), simple sequence repeat (SSR) and inter simple sequence repeat (ISSR) (Aggarwal et al., 2015). ISSR-PCR technique is a simple and quick method that combines the advantages of AFLP and RAPD. These sequences are abundantly dispersed throughout the genome and highly polymorphic in comparison with other markers. Due to their reproducibility, no gene sequence information required prior genetic studies and ISSR markers were used in this study (Deepankar et al., 2014).

The genetic diversity of released chickpea cultivars were not studied in Ethiopia at molecular level beyond their productivity through conventional breeding, this may lead to erosion of novel genes used for biotic and abiotic stresses tolerance. Therefore, this study aimed to assess the genetic diversity and relationship among Ethiopian chickpea cultivars, in order to provide basic information for future genetic resource conservation and chickpea breeding program.

MATERIALS AND METHODS

Plant materials

In this study, 29 cultivated chickpea cultivars consist of 14 kabuli type and 15 desi type varieties collected from 3 High Land Pulse Crop Improvement Programs / DebrZiet, Holeta and Sirinka/ in EIARC were used (Table 1). Among these Holeta ARC varieties were breeding lines. Ten chickpea seeds of each cultivar were sown in 20-25 cm diameter plastic pots containing black vertisol soil with organic manure and watered daily. Unfortunately, two of the kabuli type cultivars (Akuri and ICC-4973) spoiled by powdery mildew were excluded from the analysis. Five grammes (5 g) of healthy and young leaf samples were harvested and dried in silica gel.

DNA extraction

DNA was extracted using Invisorb® spin plant mini kit extraction prot ocol (Robert, 2009) with slight modifications in concentrations, incubation and centrifugation period at genetic research laboratory of Addis Ababa University. The isolated DNA quality was checked on 0.83% agarose gel electrophoresis under UV light trans illuminator BioDoc Analyzer apparatus and the gel picture was taken. Genomic DNA samples with high band intensity were selected for PCR analysis as seen in Table 2.

ISSR analysis

A total of 21 ISSR primers were tested for ISSR amplification on five representative cultivars. The total volume of PCR reaction was 25 µl containing, 14.6 µl ddH_20, 1.5 µl dNTPs (0.2 mmol/L), 2.6 µl buffer (10x), 1.2 µl $MgCl_2$ (2 mmol/L), 1µl primer (20 pmol/µl), 0.5 µl Taq Polymerase (5 µ /µl) and 1µl template DNA. The profile of PCR reaction program was 4 min preheating and initial denaturation at 94°C, followed by 40 cycles at 94°C for 15 s transition, 1 min annealing at 48°C, 1½ min initial chain elongation at 72°C and ended with 7 min extension phase at 72°C. The amplified PCR products were resolved on 1.67% agarose gel with 1X TBE buffer at 100 V for 2 h. The resultant gel and bands were visualized and acquired under UV light Biodoc Analyzer documentation system. The size of amplified product was estimated using 500 bp DNA ladder on each side of the gel as a marker.

Statistical analysis

ISSR reproducible amplified fragments which appear and are distinct were scored as ‘1’ for presence, ‘0’ for absence. The genetic diversity for varieties was measured by a number of polymorphic loci, percent of polymorphism, gene diversity (*h*) and Shannon diversity index (*i*) with POPGENE ver 1.32 software. Analysis of molecular variance (AMOVA) was used to calculate variation among and within cultivars using Areliquin ver 3.01. It was done by computation of the distance between “haplotypes”of each individual`s data pattern (Excoffier et al., 2006). The Jaccard’s similarity coefficient for clustering analysis was calculated as follow (Jaccard, 1908):

$$Sij = \frac{a}{a+b+c}$$

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 individual `j` but not in individual`i`.

The Cluster analysis was performed based on Unweighted Pair Group of Arithmetic mean (UPGMA) using NTSYS- pc ver 2.02 (Rohlf, 2005), and Neighbor Joining (NJ) method (Saitou and Nei, 1987) to compare individual cultivars clustering pattern using

Table 1. Twenty nine (29) chickpea varieties collected for this study.

Kabuli varieties	Year of release	Breeder/maintainer	Source	Seed color
DZ-104	1974	DZARC/EIARC	Ethiopia	White cream
Arerti	1999	DZARC/EIARC	ICARDA	White cream
Shasho	1999	DZARC/EIARC	ICRISAT	White cream
Chefe	2004	DZARC/EIARC	ICRISAT	White cream
Habru	2004	DZARC/EIARC	ICARDA	White
Ejeri	2005	DZARC/EIARC	ICARDA	White
Teji	2005	DZARC/EIARC	ICARDA	White
Acos Dubie	2009	DZARC/EIARC	Mexico	White cream
Yelibie	2006	SRARC/ARARI	ICRISAT	Yellowish
Kobo	2010	SRARC/ARARI	ICRISAT	Yellowish
Kaseche	2010	SRARC/ARARI	ICRISAT	White
Akuri	2011	SRARC/ARARI	ICRISAT	Cream
ICC-4973	Breeding line	HARC	India	White
ICC-19180	Breeding line	HARC	ICRISAT	White
DZ-10-11	1974	DZARC/EIARC	Ethiopia	Brown
Dubie	1978	DZARC/EIARC	Ethiopia	Grey
Mariye	1985	DZARC/EIARC	ICRISAT	Brown
Worku	1994	DZARC/EIARC	ICRISAT	Golden
Akaki	1995	DZARC/EIARC	ICRISAT	Brown
Naatolii	2007	DZARC/EIARC	ICRISAT	Light Golden
Minjar	2010	DZARC/EIARC	ICRISAT	Golden
Mastewal	2006	DZARC/ EIARC	ICRISAT	Golden
Fetenech	2006	SRARC/ ARARI	ICRISAT	Reddish
Kutaye	2005	SRARC/ ARARI	ICRISAT	Red
ICC- 4948	Breeding line	HARC	India	Dark Brown
ICC- 15996	Breeding line	HARC	ICRISAT	Reddish
ICC- 5003	Breeding line	HARC	India	Dark Brown
1CC- 4918	Breeding line	HARC	India	Brown
Pm- 233	Breeding line	HARC	ICARDA	Light Brown

DZ: Debre Ziet, SR: Sirinka, H: Holeta, EIAR: Ethiopian Institute of Agricultural, ICARDA: International Center for Agricultural Research in the Dry Area Research Center and ICRISAT: International Crops Research Institute for the Semi Arid Tropic.

FreeTree 0.9.1.50 Software (Pavlicek et al., 1999). The principal coordinated analysis method (PCO) was performed based on Jaccard's similarity coefficient to further examine the patterns of variation among individual cultivars (Jaccard, 1908). The Jaccard's similarity coefficient was calculated with PAST software ver 1.18 for 2D ordination and the first three axes were latter used to plot the 3D PCO with STATISTICA ver 6.0 software (Hammer et al., 2001).

RESULTS AND DISCUSSION

Out of 21 ISSR primers tested initially, only 4 di-nucleotide repeat primers were screened for the analysis. They were able to produce polymorphic banding pattern and were chosen to distinguish chickpea cultivars (Figure 3). But 17 primers did not amplify any of the genomic DNA and as such, non-amplifying primers are also reported in other crop plants (Bhagyawant and Srivastava, 2008). A total of 24 markers were generated in polymorphism in 22 bands (91.67%). The number of polymorphic loci ranged from 2 (UBC-840) to 8 (UBC-849) where $(GA)_8$ repeat motif gave the least and $(GT)_8$ gave the most amplification in chickpea genome. The size of amplified fragments ranges from 500 to 3500 bp. UBC-830 and UBC-849 showed 100% polymorphism means that all loci of the scored bands detected to be diversified in the genome of chickpea (Table 2).

In this study, Gene diversity and Shannon diversity index was also calculated using 27 *Cicer arietinum* cultivars, where UBC-849 showed the highest values (0.46 and 0.65) and UBC-842 showed the least values

Table 2. List of 4 di-nuclotide ISSR primers Selected for polymorphism detection.

ISSR primers		Number of loci		Percent of variation	With individual primers	
Name	**Repeat motif**	**Total**	**Polymorphic**	**polymorphism**	**H±SD**	**I±SD**
UBC–830	(TG) 8 G	7	7	100	0.39±0.12	0.57±0.13
UBC–840	(GA) 8 YT	3	2	66.67	0.27±0.25	0.40±0.35
UBC–842	(GA) 8 YG	6	5	83.33	0.25±0.18	0.39±0.24
UBC–849	(GT) 8YA	8	8	100	0.46±0.04	0.65±0.04
Average	-	6	5.5	87.5	0.34±0.15	0.50±0.19
Total	-	24	22	91.67	-	-

H: gene diversity, SD: Standard deviation and I: Shannon diversity index for each selected primers.

Table 3. AMOVA for detecting genetic diversity within and among chickpea cultivars.

Source of variation	d.f	SS	Variance components	% of variation	Fixation indices	P
Among cultivars	1	1.293	0.02304 Va	2.29	0.02285	0.00
Within cultivars	25	24.633	0.98533 Vb	97.71	-	0.00
Total	26	25.926	1.00838	100	-	-

SS: sum of square, d.f: degrees of freedom and P: P-Valuefor each cultivar types.

(0.25 and 0.39) respectively. The study showed higher genetic diversity in kabuli type (*h*=0.33 and *i*=0.50) than desi cultivars (*h*=0.30 and *i*= 0.47) even though kabuli chickpea cultivars were originated from the desi type in the Mediterranean basin through natural mutation and selection (Hawtin and Singh, 1981). As chickpea plant is highly self-pollinated species, higher genetic diversity was expected among cultivars than within cultivars. However, the AMOVA analysis showed larger genetic diversity within cultivars (97.71%) than among cultivars (2.29%). This is attributed to the fact that the multiple evolutionary forces like mating types, gene flow, genetic drift, evolutionary history, mode of reproduction and natural selection (Table 3).

Similarly, Aggarwal et al. (2015) work on Indian chickpea and Edossa et al. (2010) on lentil support the higher genetic diversity within populations than among population. The morphological and molecular diversity of Ethiopian lentil using 4 ISSR primers found 56.28% diversity within population than among population (43.72%). This could be attributed to mutation of SSR loci, random genetic drift and differential selection pressure on the loci. Dendrogram resulted from cluster analysis of UPGMA and Neighbour Joining by similarity coefficient which revealed three distinct clusters and sub clusters in each to discriminate all varieties each other (Figure 1).

Cluster - I forked into two sub-clusters at about 76% similarity coefficient and consisted of almost all the desi type chickpea cultivars except Arerti and Kobo cultivars, in which they showed about 66% and 79% similarity with the second sub cluster respectively. This might be due to the fact that kabuli cultivars are hybrids of wild and cultivated desi chickpea and hence these cultivars might have close genetic similarity with desi type chickpea.

Cluster - II consisted of all Kabul cultivars and spliced into two distinct sub-clusters around 60% similarity coefficient, where Worku and Mariye show 82% and 70% closely relatedness with second sub-cluster of kabuli cultivars. Clustering of Fetenech in the third group negates the expectation that it would be grouped in desi type cluster.

Individuals escaped from each cultivar type in dendrogram might have accumulated adaptive gene complexes for environmental change and can serve as a parent in future chickpea breeding program. PCO analysis confirm the result of UPGMA clustering, where the lengths of coordinates axis given by Eigen values and predict how individual cultivars are related to each other. The first three coordinates with of 2.43, 1.93 and 1.31Eigen values accounted 13.23, 10.52 and 7.14% variation respectively. This used to construct the three dimensional (3D) representation for better grouping of individuals in to their respective cultivars type (Figure 2).

Conclusion

Generally, ISSR markers showed higher genetic diversity in kabuli type than desi type chickpea cultivars. But the total genetic diversity is narrower in Ethiopia. Therefore, the National Breeding Program should target on kabuli cultivars (Arerti and Tejie) for selecting individuals with desirable traits to broaden the genetic base of the chickpea in the country. This will support future conservation and improvement programs.

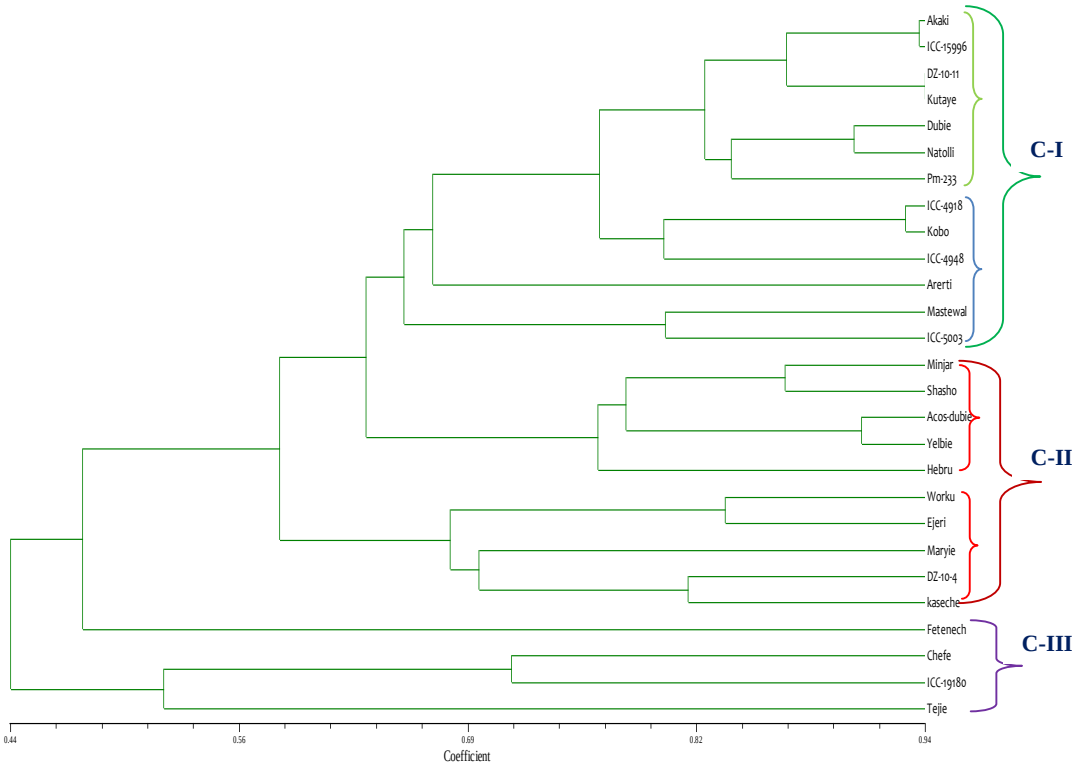

Figure 1. Dendrogram of 27 Chickpea cultivars based on UPGMA cluster analysis depicting their genetic relationship.

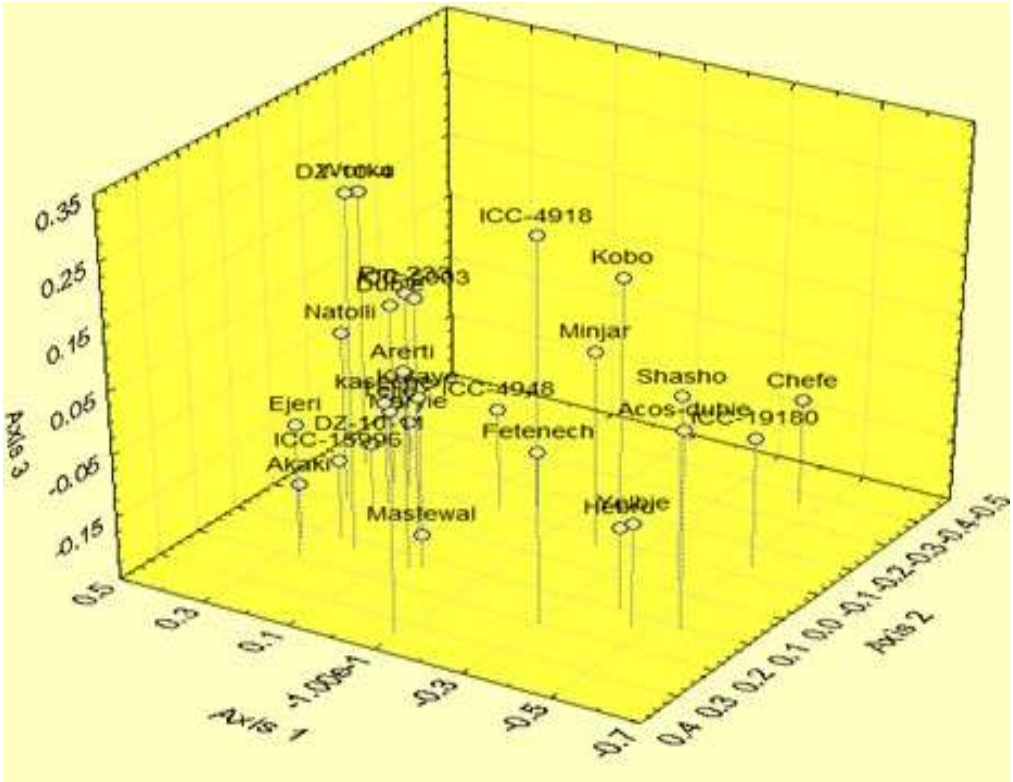

Figure 2. 3D representation PCO analysis of genetic relationship among 27 chickpea cultivars.

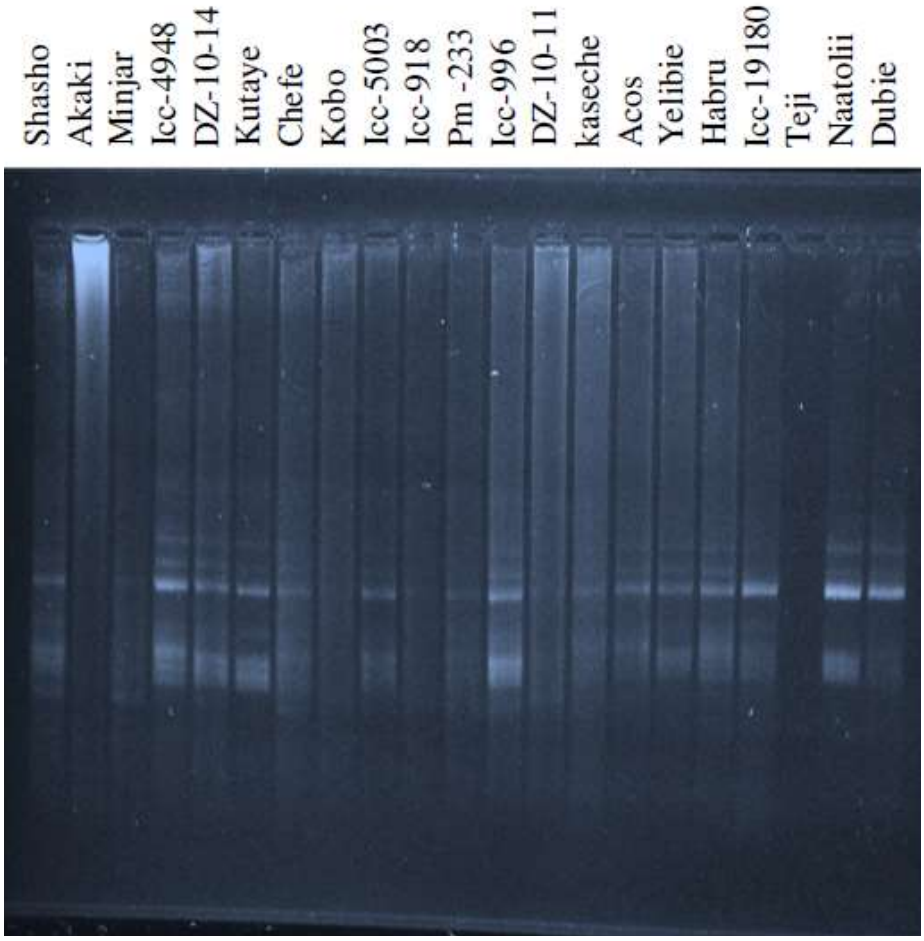

Figure 3. Bands generated from 21 representative chickpea cultivars using primer UBC–849.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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