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Molecular characterization of chickpea (*Cicer arietinum* L.) Genotypes using ISSR markers

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Abstract
Chickpea (*Cicer arietinum* L., 2n=16) is a legume of family *Fabaceae*, subfamily *Faboideae.* Chickpea is the second largest grown food legume of the world (Gaur *et al*., 2008). Genetic divergence among 28 genotypes of *Cicer arietinum*L.was assessed through ISSR molecular markers.ISSR analysis showed 85.09 per cent of polymorphism. A total of 109 amplified bands were obtained that ranged between 150 bp to 2100 bp using 20 ISSR primers. The 92 out of 109 bands were polymorphic. The average PIC (polymorphic information content) of ISSR was found 0.257.
Jaccard's similarity coefficient for ISSR lay between 0.54 to 0.94 (54% to 94%). The similarity coefficients values were significant for this marker.Dendogram based on genetic distance calculated by UPGMA method segregated the 28 chickpea genotypes into three ISSR major clusters, respectively. Two and three dimension principal component analysis (PCA) showed similar clustering of 28 genotypes as evident from cluster tree analysis with dice similarity coefficients ranged from 0.77 to 0.91 (77% to 91%) for ISSR analysis.
Based upon ISSR analysis of 28 genotypes with 20 primers genotype PBC-509, PBC-511, Pratap channa-1, Dohad yellow and Pratap Rajchana were identified as genetically diverse. Therefore, the results of the present study can prove helpful for implementation of chickpea improvement programmes.

Keywords: *Cicer arietinum*, molecular markers, ISSR, PIC, PCA, Dendrogram

1. Introduction
Chickpea (*Cicer arietinum* L., 2n=16) is a legume of family *Fabaceae*, subfamily *Faboideae.* Chickpea cultivation in India covers an area of about 81.17 lakh hectares with a production of 59.01 lakh tonnes grains (FAOSTAT data, 2016) [6]. Characterization of variability is a first step in breeding programme for developing high yielding varieties. A large amount of diversity is found among all cultivated chickpea germplasm for different agronomic, morphological and physiological attributes (Stalker, 1992) [11]. If diversity among genotypes is huge than possibilities of crop improvement, heritability and genetic advance will be higher.
Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and population. Various molecular markers are being used for fingerprinting such as restriction fragment length polymorphism (RFLP) (Dubreuil and Charcosset, 1998) [5], randomly amplified polytmorphic DNA (RAPD) (Williams *et al*., 1990) [12], microsatellites (Smith *et al*., 1997) [10] and amplified fragment length polymorphism (AFLP) (Agrawal *et al*., 2011).
ISSR technique was reported by Zietkiewicz*et al.* (1994) [13] are DNA sequences of about 100–3000 bp present between adjacent, oppositely oriented microsatellite sequences. These primers based are used to amplify inter-SSR DNA regions. ISSRs are amplified by PCR technique using SSR core regions as primers with minute selective nucleotides as anchors into the non-repeat adjacent regions (16–18 bp). The generated fragments of multiple loci are separated by gel electrophoresis. Techniques relevant to ISSR investigation are Single Primer Amplification Reaction that uses a single primer consists of core motif of SSR and Directed Amplification of SSR sequence DNA. Nearly all the proprieties of SSR, AFLP and RAPD are covered by ISSR markers (Carvalho *et al.*, 2009) [2].
The present study was proposed using 28 diverse elite genotypes to analyze genetic diversity critically at molecular level with by using ISSR analysis because chickpea is very importance crop of the State in terms of area stability, production, productivity and its large consumption.

2. Materials and Methods

2.1. Plant material

In the present investigation, leaf samples of 21 days old plants from 28 genotypes of chickpea (*Cicer arietinum* L.) were procured from field of Agricultural Research Station (ARS), MPUAT, Borwat Farm, Dahod Road, Banswara (Raj.) – 327001.

2.2. ISSR analysis

The genomic DNA was extracted from 21 days old leaves of 28 genotypes of chickpea by CTAB extraction method of Doyle and Doyle (1987), with slight modifications. Twenty ISSR primers were used in the present investigation out of which 15 primers showed amplification in all the genotypes. Expected outcomes of amplification were detected with an ideal concentration of DNA template (25 ng/µl) for a reaction volume (20 µl). Samples of 10 µl ISSR–PCR product were analyzed by electrophoresis on 1.5% agarose gel in 1X TAE buffer. The sizes of DNA fragments were estimated by comparison with standard ladder (100bp and1 kb; Bangalore Genei, India) containing 5 µg/ml of Ethidium bromide. Then the gels were visualized, photographed and analysed. ISSR patterns were analysed by scoring presence (1) or absence (0) of bands for estimation of similarity among all tested samples. The matrix of similarity (Jaccard) and similarity of coefficients were calculated and the dendrogram obtained by clustering according to the Unweighted Pair-Group Method with Arithmetic averages (UPGMA) using NTSYS-pc software. Principal component analysis along with 2D and 3D plots were constructed using eigen values and vectors.

3. Results and Discussions

Isolated and purified DNA was subjected to PCR based molecular marker (ISSR) for assessment of genetic diversity in the genotypes of *C. arietinum*. A total of 109 amplified bands were obtained from the 20 primers, out of which 92 were polymorphic. The total number of amplified bands varied between 4 (UBC-845) and 11 (UBC-810, UBC-824 and UBC-836) with an average of 7.26 per primer (Table 1 and Fig. 1).

The polymorphism percentage ranged from 50.00% (UBC-813) to 100% (UBC-817, UBC-818, UBC-820, UBC-826 and UBC-845).Average polymorphism across the 28 genotypes of*Cicer arietinum*was found to be 85.09%. Overall size of PCR amplified products ranged between 150 bp to 2100 bp. The PCR amplification using ISSR primers gave rise to reproducible amplification products. The number of potential ISSR markers depends on the variety and frequency of microsatellites, which tends to change with species and the SSR motifs that are targeted (Despeiger*et al.,* 1995) [3]. The average PIC was 0.257 ranging from 0.101 to 0.446. The lowest and highest PIC values were recorded for primers UBC-815 and UBC-818, respectively.

Three unique bands were detected in three genotypes *viz.,* PBC-503 (800 bp) with ISSR primer UBC-810; PBC-508 (1250 bp) with ISSR primer UBC-811 and PBC-505 (400 bp) with ISSR primer UBC-820 respectively (Table 2).

ISSR similarity matrix data, the value of similarity coefficient ranged from 0.54 to 0.94, *i.e.* 54-94%. The 28 genotypes could be divided into three major clusters at a similarity coefficient of 0.68 (Fig. 2). Two and three dimension principal component analysis based on ISSR data (Fig. 3) showed similar clustering of 28 genotypes as evident from cluster tree analysis. Dice similarity coefficients ranged from 0.77 to 0.91, indicative of an average degree of variation among the genotypes.

In conclusion the ISSR patterns obtained from our study can serve as a vital input to the conventional method of varietal identification, future germplasm management, and marker assisted selection to improve the efficiency of new cultivar development in future breeding programs that relies solely on morphological characters. Interestingly collections originating from various parts of the country did not form well defined distinct groups and were interspersed with each other, indicating no association between RAPD pattern and the geographic origin of accession.

Appendices

Table 1: DNA amplification pattern and polymorphism generated in *Cicer arietinum*using 15 ISSR primers

S. No.	Primer Code	Molecular weight range (bp)	Total numberof scorable bands (a)	Total Number of polymorphic band (b)	Polymorphism (%)b/a X 100	PIC Value
1.	UBC-810	150-2100	**11**	9	81.81	0.1605
2.	UBC-811	400-1200	9	7	77.78	0.1574
3.	UBC-813	350-1500	6	3	50.00	0.1301
4.	UBC-814	150-1100	7	6	85.71	0.2202
5.	UBC-815	150-1600	5	4	80.00	0.1011
6.	UBC-817	150-1600	7	7	100	0.3472
7.	UBC-818	350-1300	6	6	100	0.4465
8.	UBC-820	300-1000	5	5	100	0.3868
9.	UBC-822	200-1250	8	7	87.50	0.2428
10.	UBC-824	200-2000	11	9	81.81	0.2051
11.	UBC-826	450-1500	7	7	100	0.2759
12.	UBC-834	175-900	4	3	75.00	0.2965
13.	UBC-836	200-1300	11	9	81.81	0.2935
14.	UBC-840	300-1600	8	6	75	0.2035
15.	UBC-845	350-1200	4	4	100	0.4003
	Total		109	92	85.09%	0.2578

Table 2: Molecular Weight Range and Unique Alleles Obtained Using ISSR Primers

S. No.	Primer code	Total number of unique bands	Genotype	Size of bands (bp)
1.	UBC-810	1	PBC-503	800
2.	UBC-811	1	PBC-508	1250
3.	UBC-820	1	PBC-505	400
Total		3		

Plate 20: ISSR profile generated through UBC-811 [5' (GA)8C 3']

Plate 21: ISSR profile generated through UBC-813 [5' (CT)8T 3']

M_1= 100 bp DNA Ladder **M_2= 1000 bp DNA Ladder**

G_1- G_{28} represents following *Cicer arietinum* genotypes

G_1- PBC-501	G_2- PBC-502	G_3- PBC-503	G_4- PBC-504	G_5- PBC-505	G_6- PBC-506
G_7- PBC-507	G_8- PBC-508	G_9- PBC-509	G_{10}- PBC-510	G_{11}- PBC-511	G_{12}- PBC-512
G_{13}- PBC-513	G_{14}- PBC-514	G_{15}- PBC-515	G_{16}- PBC-516	G_{17}- PBC-517	G_{18}- PBC-518
G_{19}- PBC-519	G_{20}- PBC-520	G_{21}- PBC-521	G_{22}- PBC-522	G_{23}- PBC-523	G_{24}- PBC-524
G_{25}- PBC-525	G_{26}- Pratap Channa-1		G_{27}- Dohad Yellow		G_{28}- Pratap Rajchana

Fig 1: ISSR Profiles Generated by Primer UBC-811 and UBC-813. The Numbers (1-28) Corresponding to Genotypes at the Top of Each Lane.Lane M1 (100bp) & M2 (1000bp) are the Molecular Weight Markers

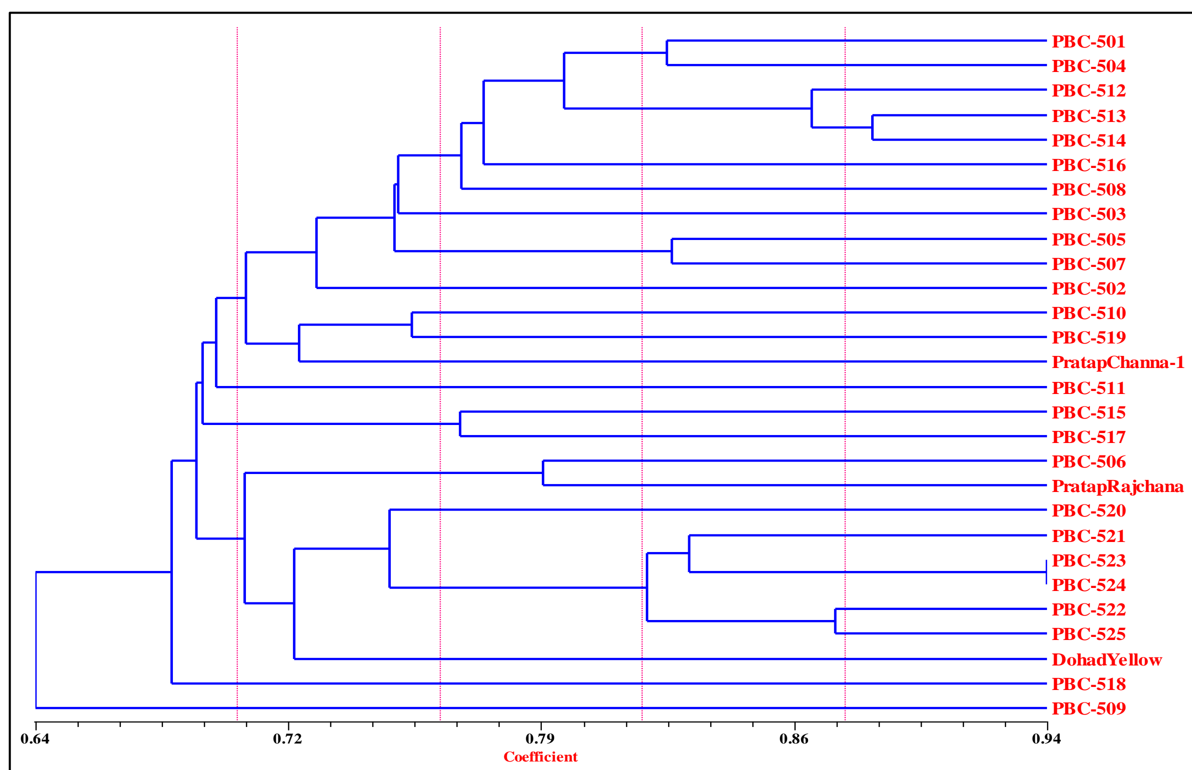
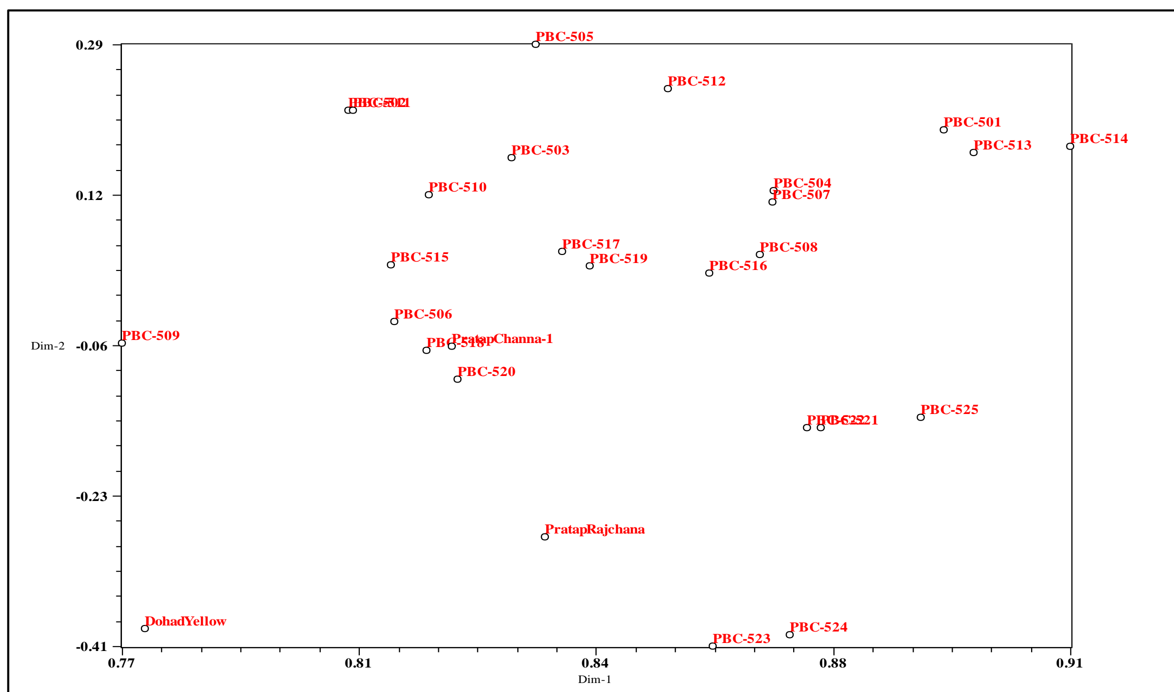
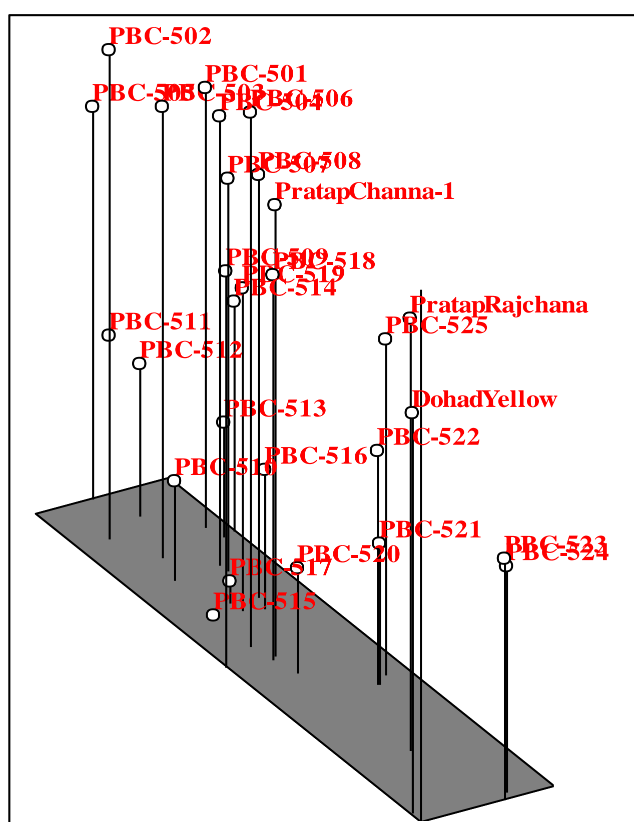


Fig 2: Dendrogram generated for *Cicer arietinum* genotypes for ISSR using UPGMA cluster analysis based on Jaccard Similarity Coefficient



(a)



(b)

Fig 3(a, b): A two dimensional and three dimensional plot of Principal component analysis based on ISSR primer in chickpea genotypes

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