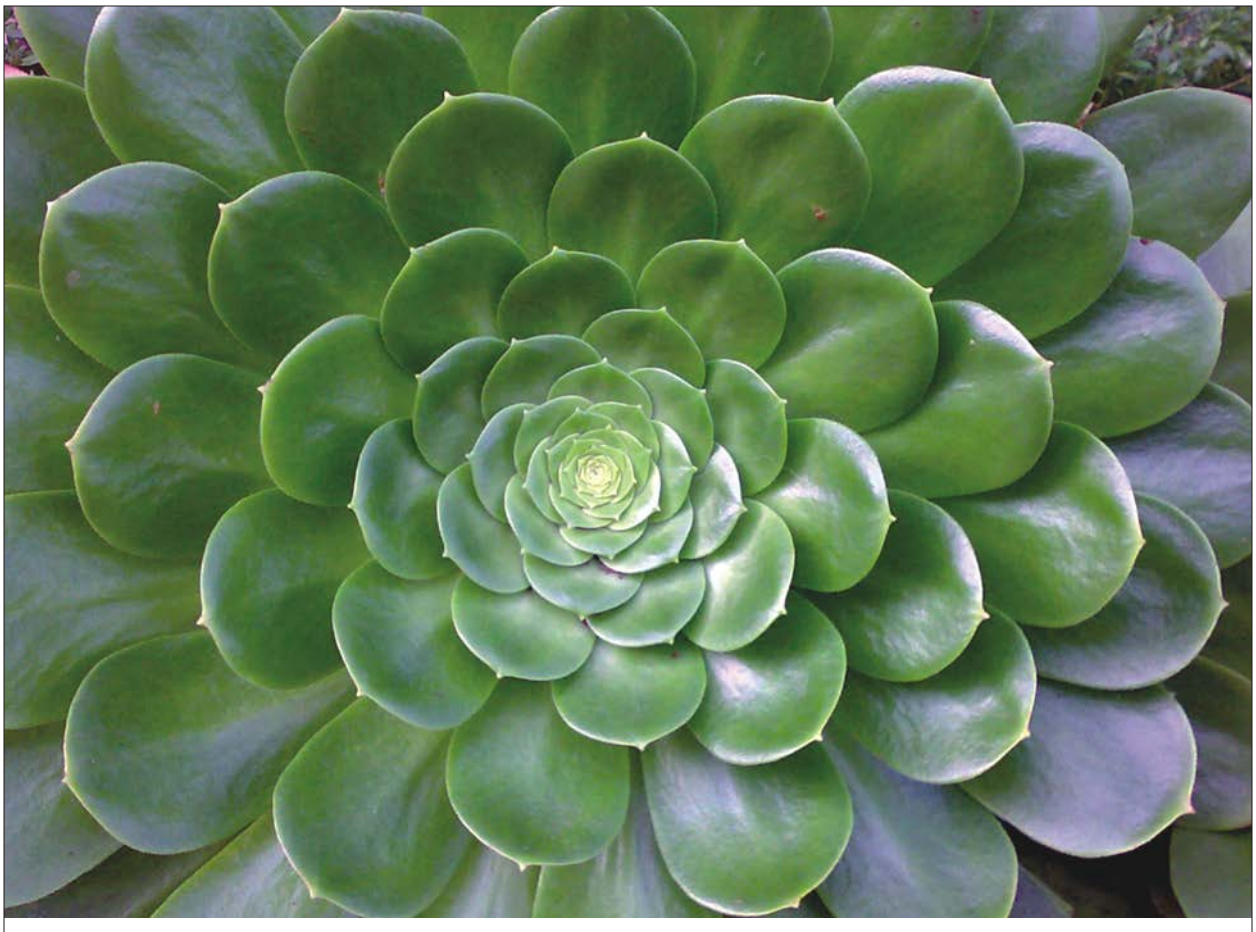

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Inter Simple Sequence Repeats Reveal Significant Genetic Diversity Among Chickpea (*Cicer arietinum* L.) Genotypes

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ABSTRACT

The present investigation was carried out to access the suitability and reliability of ISSR analysis for inferring genetic diversity among chickpea genotypes. A total of six ISSR markers were used to study genetic diversity among 115 genotypes of chickpea. Out of six ISSR markers $(GTGC)_4$ $(GTG)_5$ and $(TCC)_5$ were found to generate reproducible DNA fragments of size ranging from 0.15-3, 0.40-2.5 and 0.35-2 kb and yielded 2-9, 3-10 and 4-11 number of fragments, respectively. Clustering analysis separated all the genotypes into five genetically similar groups. The value of cophenetic correlation coefficient (r) is 0.91 which is considered as a good fit. The genetic similarity coefficient among 115 chickpea genotypes ranged from 0.01 to 0.90. These values indicated high genetic variability among chickpea cultivars and can be used efficiently for gene tagging and genome mapping of crosses to introgress the favourable traits such as high yield potential, disease and insect resistance into the cultivated genotypes.

Key words: Chickpea, genetic diversity, ISSR, polymorphism, NTSYS-pc

INTRODUCTION

Chickpea (*Cicer arietinum* L., 2n = 2x = 16) is an autogamous annual cool season grain legume cultivated in arid and semi-arid areas across the world (Iruela *et al*., 2007). It stands between 20 cm and 1 m tall (Muehlbauer and Tullu, 1997; Farshadfar and Farshadfar, 2008), with a genome size of 750 Mbp (Arumuganathan and Earle, 1991), slightly less than the well-characterized tomato genome (950 Mbp). Chickpea is being valued for its high dietary protein content, its ability to fix atmospheric nitrogen making it an important component of the cropping system and absence of specific major anti-nutritional factors means that it is considered nutritional and healthy (Williams and Singh, 1987; Gill *et al*., 1996; Mohammed *et al*., 2011).

India is the largest producer of chickpea, accounting for 66% of the world production (http://faostat.fao.org; Arfaoui *et al*., 2005). The crop is the second most important pulse in terms of area under cultivation all over the world after dry beans but ranks third in terms of production, following dry beans and peas (http://faostat.fao.org). The yield potential of present-day chickpea

varieties exceeds 4 t ha^{-1}; however, actual yield is less than 0.8 t ha^{-1} (Madrid *et al.*, 2008). The gap between potential and actual average yield is mainly due to diseases and poor management. The most important chickpea diseases on a global scale are fusarium wilt and ascochyta blight (Nene and Reddy, 1987; Iqbal *et al.*, 2003). Both these diseases are very damaging and account for major (10 to 90%) crop losses every year. In many national and international programmes, improving resistance to fusarium wilt and ascochyta blight is a major objective. The identification and tagging of resistance genes for ascochyta blight and fusarium wilt would be invaluable tools for the development of resistant chickpea cultivars through Marker Assisted Selection (MAS).

Quantitative Trait Loci (QTL) mapping is a highly effective approach to identify and tag disease resistance genes in plants (Young, 1996). QTL mapping may be appropriate for identifying the number and position of genes conferring disease resistance, because previous reports suggest that resistance is controlled by multiple genes (Tekeoglu *et al.*, 2000). Knowledge of the extent of genetic diversity within a species is a critical factor when selecting parents for QTL mapping, because sufficient DNA polymorphism must exist between parents for segregation analysis and genetic mapping (Collard *et al.*, 2003). Due to obligatory self-pollination and thousands of years of selection, the genome of chickpea (*Cicer arietinum* L.) has been streamlined and became extensively monotonous. Therefore, little or no genetic diversity has been detected using allozymes (Ahmad and Slinkard, 1992; Oram *et al.*, 1987; Tuwafe *et al.*, 1998; Gaur and Slinkard, 1990a,b), RFLP (Udupa *et al.*, 1993; Simon and Muehlbauer, 1997) and RAPD (Simon and Muehlbauer, 1997; Singh *et al.*, 2002). Recently, Inter Simple Sequence Repeats (ISSR) have become the molecular marker system of choice for many areas of genome analysis and genetic mapping. ISSR-PCR is a simple, quick and efficient technique. It has high reproducibility. The primers are not proprietary (as in SSR-PCR) and can be synthesized by anyone. Variations in primer length, motif and anchor are possible. The primers are long (16-25 bp) resulting in higher stringency. The amplified products (ISSR markers) are usually 200-2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. These sequences are abundant, dispersed throughout the genome and are highly polymorphic in comparison with other molecular markers (Akkaya *et al.*, 1992; Morgante and Olivieri, 1993; Wang *et al.*, 1994). Rao *et al.* (2007) also investigated genetic relationship between 19 chickpea cultivars and five accessions of its wild progenitor *Cicer reticulatum* L. using RAPD and ISSR markers. On an average, six bands per primer were observed in RAPD analysis and 11 bands per primer in ISSR analysis. Low level of genetic polymorphism were reported in chickpea genotypes using RFLP and this limits the number of loci to be scored in a cross based on interspecific accessions of *Cicer arietinum* L. (Kazan and Muehlbauer, 1991; Ahmad *et al.*, 1992; Udupa *et al.*, 1993; Labdi *et al.*, 1996; Tayyar and Waines, 1996). Because of their short repeat length and limited interaction at individual loci, ISSR were used in the present study to investigate the genetic diversity among chickpea genotypes.

MATERIALS AND METHODS

Plant materials and DNA extraction: One hundred fifteen chickpea varieties (Table 1) were grown in the randomized blocks designed in three replicates at the Research Farm of the Department of Plant Breeding, CCSHAU, Hisar. Genomic DNA was isolated from leaves of 3-4 weeks old seedlings using modified CTAB method of Murray and Thompson (1980). The quality and concentration of DNA were measured on Nano-Drop spectrophotometer (ND-1000) and electrophoresis using 0.8% agarose gel.

Table 1: List of chickpea genotypes used in present study

Sr. No.	Genotype	Sr. No.	Genotype	Sr. No.	Genotype	Sr. No.	Genotype
1	ICCV 92944	30	RSG 963	59	GNG 469	88	RSG 888
2	ICCV 4958	31	RSG 807	60	JG 315	89	Sadabahar
3	Katila	32	Pusa 261	61	Pusa 1053	90	RSG-11
4	BG 396	33	Annegiri	62	GCP 101	91	Pusa 329
5	BG 1006	34	Pusa 209	63	BGM 413	92	HC-1
6	IPC 92-39	35	CSJD-844	64	Virat(K)	93	Dohad yellow
7	IPC 98-12	36	RSG 931	65	L 550(K)	94	Pusa 1003
8	PDG 84-16	37	GNG 146	66	PBG-5	95	JKG-1 (K)
9	ICCV 14880	38	BGM 408	67	ICCV-2	96	RSGK-6 (K)
10	IPC 99-18	39	Pusa 267	68	BGD 72	97	JG 130
11	IPC 2000-33	40	GG-2	69	PBG-1	98	B 108
12	IPC 2001-2	41	RS-10	70	PDG 4	99	Pusa 256
13	BG 276	42	Pusa 244	71	RSG 44	100	BGD 75
14	Tyson	43	GPF-2	72	Pusa 212	101	Pusa 362
15	IPC 95-1	44	Vijay	73	GL 769	102	Vishal
16	H-208	45	JGG-1	74	Vaibhava	103	C 214
17	ICCV 96030	46	PG 12	75	Avrodhi	104	C 15
18	HC-3	47	RSG-2	76	JG 74	105	C 20
19	PG 96006	48	Chaffa	77	KPG 59	106	C 16
20	IPC 97-67	49	PDG-3	78	ICCV 37	107	M 1
21	IPC 94-94	50	GNG 1292	79	CSG 8962	108	M 2
22	IPC 2000-41	51	JG 11	80	Pusa 372	109	H04 -57
23	E 100 Ym	52	KWR 108	81	Pusa 391	110	H04 -44
24	IPC 2000-45	53	HK 94-134	82	SAKI 9516	111	H04 -45
25	GNG 663	54	JG 218	83	GCP 105	112	HC-5
26	C-235	55	Phule G-5	84	HK 98-155	113	H04 -87
27	DCP 92-3	56	Pant G-114	85	RAU 52	114	H04 -11
28	L 551	57	Pusa 312	86	Pusa 240	115	H03 -56
29	Radhey	58	K 850	87	RSG 973		

DNA amplification: DNA amplification was carried out in thermal cycler (MJ Research) using ISSR molecular markers. The PCR reaction was carried out in a reaction volume of 15 μL which consists of 1x PCR Buffer, 200 μM of each dNTPs, 2.5 mM $MgCl_2$, 0.4 μM of primer, 1 unit of Taq DNA polymerase and about 20 ng of template DNA. PCR conditions were an initial denaturation at 94°C for 5 min., 35 cycles at 94°C for 1 min, 50-55°C (different for each primer) for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. Amplified PCR products were separated on 1.5% (w/v) agarose gel in 1x Tris-Borate EDTA (TBE) buffer at 90 V for 1 h, stained with ethidium bromide and photographed under ultraviolet light using Gel Documentation System (SynGene) Fig. 1. One kb and 100 bp ladders were used as molecular size markers. All PCR reactions were run in duplicate and only reproducible and clear bands were scored.

Data analysis: The ISSR bands were scored as present (1) or absent (0), each of which was treated as an independent character in spite of its intensity. Data analysis was performed using NTSYS-pc (Numerical Taxonomy System, version 2.02 (Rohlf, 1990). The SIMQUAL program was used to calculate the Jaccard's coefficient, a common estimator of genetic similarity. Correlation was estimated by means of the Mantel matrix correspondence test (Mantel, 1967). This test yields a product moment correlation (r) greater than 0.5 will be statistically significant.

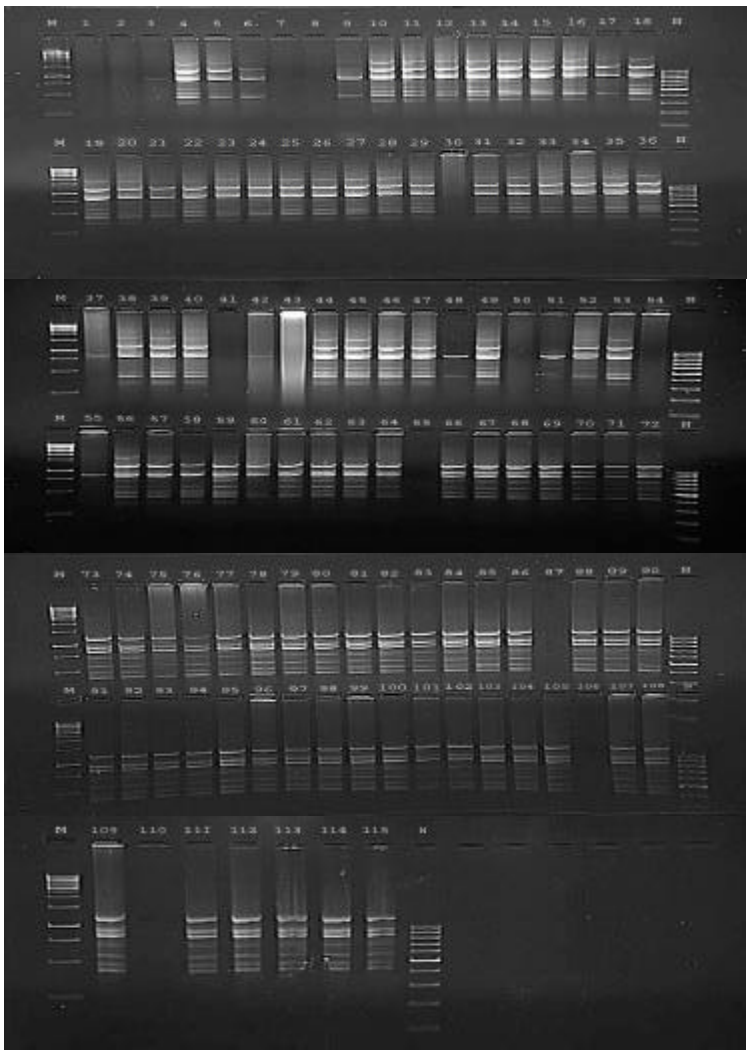

Fig. 1: Agarose gel pattern of PCR products of 115 chickpea genotypes amplified with the primer $(TCC)_5$. M represents 1 kb DNA ladder while N represents 100 bp DNA ladder

Using the binary data, a similarity matrix was constructed using the Jaccard's coefficient, which was further subjected to UPGMA (unweighted pair group method with arithmetic average) clustering analysis and a dendrogram was generated. Statistical stability of the branches in the cluster was estimated by bootstrap analysis with 2,000 replicates, using the Winboot software program (Yap and Nelson, 1996). Finally, a two and three dimensional principal component analysis (PCA) was performed in order to emphasize on the resolving power of the ordination.

RESULTS AND DISCUSSION

Variability among genotypes with regard to agronomic characters, morphological characters, biochemical properties (i.e., storage proteins, isozymes) and molecular characteristics are either indirect or direct representations of differences at the DNA level. Therefore, assessment of genetic

diversity is important not only for crop improvement but also for competent management and conservation of germplasm. For this purpose, the genetic fingerprinting of 115 chickpea (*Cicer arietinum* L.) genotypes was performed using 6 ISSR primers. The primers tested were three tetranucleotide $(GTGC)_4$ $(GCAT)_4$ $(GGAT)_4$ and two trinucleotide $(GTG)_5$ $(TCC)_5$ 3' anchored repeat primers. One dinucleotide $(CT)_8$ repeat primer was also included in present study. Out of these, 3 markers $(GTGC)_4$ $(GTG)_5$ and $(TCC)_5$ were found to generate reproducible DNA fragments of size ranging from 0.15-3, 0.40-2.5 and 0.35-2 kb and yielded 2-9, 3-10 and 4-11 number of fragments, respectively. A total of 1527 scorable gel positions having product sizes ranging from 150 bp to 3 kb were detected on agarose gel. Out of 24, 13 loci were polymorphic, showing a total of 54.1% polymorphism. Amplification with $(GTG)_5$, results 20 polymorphic loci out of 21 while $(GTGC)_4$ score only 8 polymorphic loci out of 14. Third primer i.e. $(TCC)_5$ results a total of 18 different loci as shown in pictures, out of which 14 are polymorphic, thus showing 77.7% polymorphism. The quality data was combined for all primers and used to generate dendrogram that clustered into genetically similar groups.

The Dendrogram shows that genotypes ICCV 92944, ICCV 4958, IPC 98-12, RS-10, GPF-2, GNG-1292, JG-218 and L-550 (Sr. No. 1, 2, 7, 41, 43, 50, 54 and 65) are 100% similar and forms cluster I (Fig. 2). Genotypes Pusa 1053, GCP 101, PBG-5, ICCV-2 and PBG-1 (Sr. No. 61, 62, 66, 67 and 69) formed cluster II with 100% similarity while genotypes GL-769, Vaibhava, KPG-59, ICCV-37 and CGS-8962 (Sr. No. 73, 74, 77, 78 and 79) formed cluster III with 100% similarity. Genotypes RSG-11, JKG-1, RSGK-6, JG-130, B-108, Pusa-256, C-20 and M-1 (Sr. No. 90, 95, 96, 97, 98, 99, 105 and 107) formed cluster IV and genotypes Pusa-329, Dohad yellow, BGD-75 and C-214 (Sr. No. 91, 93, 100 and 103) formed cluster V also having 100% similarity.

PCA is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called 'principal components'. Principal components are assured to be independent only if the data set is jointly normally distributed. PCA is sensitive to the relative scaling of the original variables. The results of PCA analysis were comparable to the cluster analysis (Fig. 3, 4). There is mainly one dense cluster in PCA and these results are based on Eigen values which are generated by binary data. Two genotypes ICCV-96030 and PUSA 362 appears to be distinct from other genotypes in the PCA.

The goodness of the clustering method was assessed by calculating cophenetic correlation coefficient (r), using COPH modules. The Jaccard's coefficient of similarity or cophenetic correlation coefficient (r) is 0.91 which is considered as a good fit according to Mantel (1967). The bootstrap value comes out to be 68. The genetic similarity coefficient among 115 chickpea genotypes ranged from 0.01 to 0.90. These values indicated high genetic variability among chickpea cultivars under study and contrary to the observation of Singh *et al.* (2002) which studied genetic diversity in 23 genotypes using RAPD markers and found that genetic similarity based on Jaccard's coefficient ranged from 0.92 to 0.99 indicating narrow genetic variability.

Earlier, Oram *et al.* (1987) studied isozyme variability for 27 isozyme loci in 20 cultivated chickpea accession and concluded that as a species, chickpea was relatively poor in genetic variation at isozyme loci. Tuwafe *et al.* (1998) surveyed isozyme variability in 1392 accession of cultivated chickpea from 25 countries and found polymorphism for only 4 isozyme loci. Gaur and Slinkard (1990a,b) did not find any genetic variation for isozyme loci in *Cicer arietinum*. All these observation indicates limited variability present at isozyme loci in cultivated chickpea.

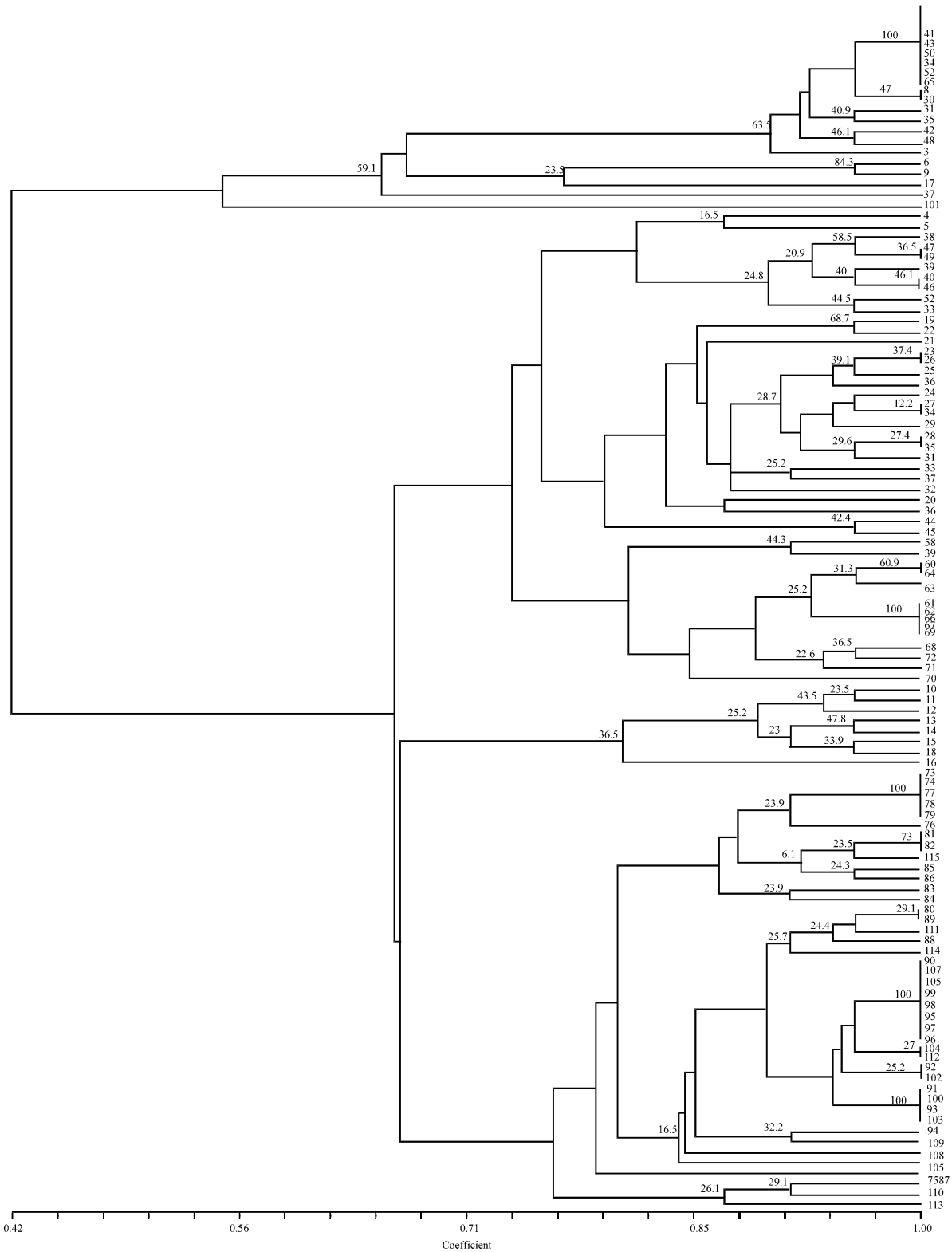

Fig. 2: Dendrogram generated using unweighted pair group method with arithmetic average analysis, showing relationships between chickpea genotypes using ISSR data

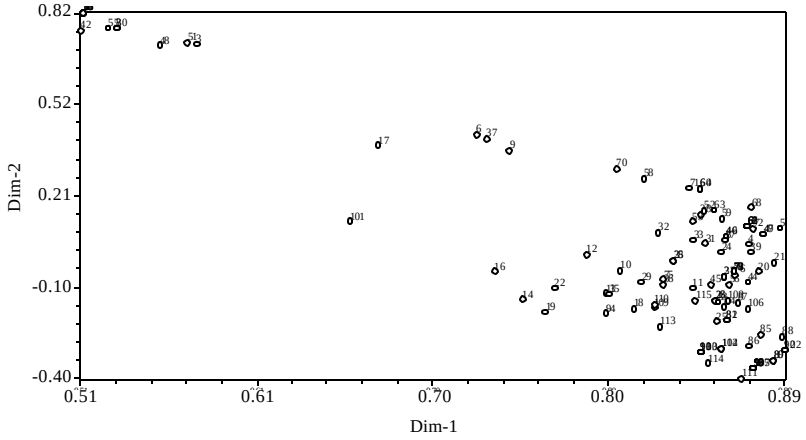

Fig. 3: Two dimensional plot of principal component analysis of using selected chickpea genotypes ISSR analysis. The numbers plotted represents individual cultivars

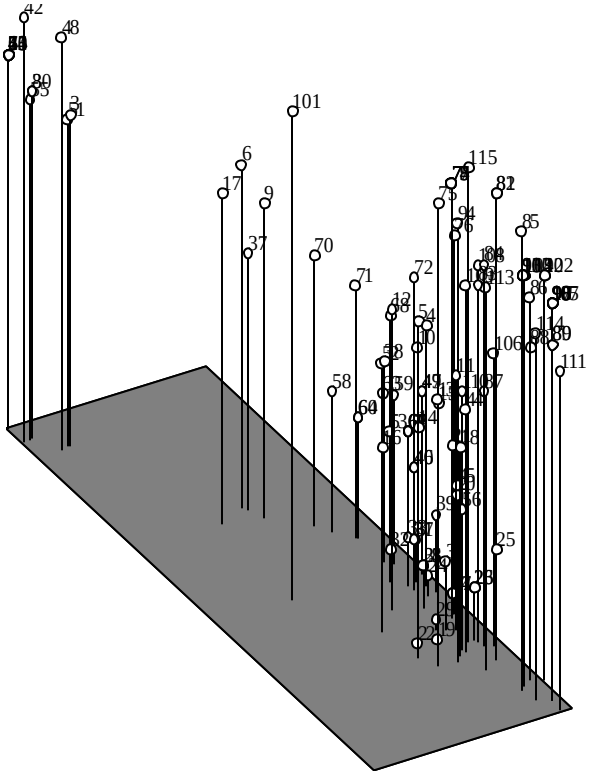

Fig. 4: Three dimensional plot of principal component analysis of using selected chickpea genotypes ISSR analysis. The numbers plotted represents individual cultivars

Ratnaparkhe *et al*. (1998) studied the inheritance of an Inter Simple Sequence Repeat (ISSR) polymorphism in a cross of cultivated chickpea (*Cicer arietinum* L.) and closely related wild species (*Cicer reticulatum* L.) using primer that anneal to a simple repeat of the various lengths. The ISSR

loci showed virtually complete agreement with expected Mendelian ratios. Twenty two primers were tested for analysis and yield a total of 31 segregating loci. They reported an ISSR marker linked to the gene for resistance to fusarium wilt race 4. Further, the number of accessions and genetic diversity in the wild annual *Cicer* germplasm was very limited as compared to other major collections, such as wheat, barley and rice (Virk *et al*., 1995; Abbo *et al*., 2003).

Rajesh *et al*. (2003) using ISSRs screened 13 *Cicer* species consisting of 6 annuals and 7 perennials with two accessions of each species and four cultivars of *C. arietinum*: Vijay, JG62, ICC4958 and V65R. Among 40 ISSR primers representing di, tri, tetra and penta repeats and 25 primers showed good amplification but no polymorphism while 15 primers revealed reproducible polymorphic patterns. With 15 primers, a total of 115 bands were scored giving an average of 7.6 bands amplified per primer. Among 115 bands, 100 were polymorphic leading to an average of 6.6 polymorphic bands per primer. In our study, among 53 bands, 42 are polymorphic resulting in an average of 14 polymorphic bands per primer which shows much higher polymorphism than previous reports.

Ten primers were taken for ISSR analysis and six primers were found to be polymorphic as reported by Rao *et al*. (2007). On an average 11 bands per primer and 80% polymorphic bands were observed in a total of 64 bands. The wild species shared 20.3% common bands and 79.6% polymorphic bands with ISSR markers. 43.75% common bands and 56.25% polymorphic bands were found among cultivated chickpea varieties and 34.37% common bands and 65.63% polymorphic bands were found among wild accessions while in present investigation, we found 95.2% polymorphic bands with the primer $(GTG)_5$.

The genetic fingerprinting of chickpea genotypes was performed using ISSR primers by Bhagyawant and Srivastava (2008). Twelve *Cicer* genotypes were screened using 10 ISSR primers for PCR-studies. The primers tested were six dinucleotide [$(TC)_8C$ $(AC)_7T$ $(AC)_8TT$ $(AC)_8GG$ $(TG)_8GA$ $(TG)_8AA$] and three trinucleotide $(ATG)_6$ $(CTC)_6$ and $(GAA)_6$ 3' anchored repeat primers. One pentanucleotide $(GGAGA)_3$ repeat primer was also included in the ISSR-PCR studies. Among the dinucleotide repeats $(TC)_8C$ $(AC)_7T$ and $(AC)_8TT$ produced better amplification patterns. A total of 492 bands were amplified across 12 cultivars with 7 primers, revealing an average of 70.28 bands per primer and 5.85 bands per primer per genotype.

Chickpea has been characterized as a species with poor genetic variability but our reports showed that ample genetic diversity exists for short sequence tandem repeats. Thus, it is expected that the utilization of a large number of genotypes and increased resolution associated with ISSR-PCR may provide effective marker system to access the genetic diversity. Also, the large amount of genetic variation can be used efficiently for gene tagging and genome mapping of crosses to introgress the favourable traits such as high yield potential, disease and insect resistance into the cultivated genotypes. However, there is need of effective collaboration between plant breeders and molecular biologists for marker assisted selection. Some chickpea genotypes used in present study like ICCV-2, ICCV-4958 possess agronomically important traits i.e., resistance to fusarium wilt and early maturity.

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