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RESEARCH ARTICLE

ANALYSIS OF GENETIC DIVERSITY IN FIVE GENOTYPES OF *CICER* BASED ON PROTEIN PROFILING AND RAPD

Jahangir, A.D*., Vaseem, R. and Kudesia, R.

Institute of Basic Sciences, Department of Botany, Bundelkhand University, Jhansi (U.P.)-284128, (India)

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ABSTRACT

Electrophoretic SDS-PAGE and RAPD analyses were performed to estimate genetic diversity in five *Cicer* cultivars. In SDS-PAGE, a total 36 polypeptide bands were detected, only 4 were monomorphic and the rest 32 were polymorphic with an average polymorphism of 88.88%. Jaccard's similarity ranged from 0.25000 to 0.56000. A dendrogram constructed based on UPGMA clustering method revealed two major clusters, cluster 1 and cluster 2, comprising of two accessions each. The accession Shubhra occupies a distinct place as revealed in the dendrogram. In case of RAPD, primer OPM-05 was used and led a total of 21 bands, all of them were polymorphic. Dice's coefficient ranged from 0.00000 to 0.25000. A dendrogram constructed based on UPGMA revealed two major clusters. Accession DCP-92-3 and IPC-04-20 are genetically dissimilar, hence it is recommended that these should be used for future breeding programs to create higher amount of genetic variability in chickpea.

INTRODUCTION

Chickpea is the most important pulse crop in India. It is native of South Europe, now commonly grown in Uttar Pradesh, Panjab, Maharastra, Rajasthan, Bihar and Madhya Pradesh. These together accounts for more than 90% of the total area under it. There are two broad groups of gram-brown and white. The brown or desi type is most widely grown. The white or kabuli is characterized by larger seeds. The genus *Cicer* includes 33 perennials, eight annuals and one unspecified wild species as well as the cultivated chickpea (Van der Maesen 1987). Chickpea is the second most important cool season pulse crop in the world and is grown in atleast 33 countries including central and west Asia, South Europe, Ethiopia, North Africa, North and South America and Australia (Ladizinsky and Adler 1976; Singh and Ocampo 1997). In India, gram is sown as Rabi crop at the end of the rainy season. Sowing takes place from September to November, and harvesting from February to April. India is the largest producer of chickpea, accounting for 66% of the world production (FAO 2004). The average annual yield world wide (0.78 ton/ hectare) is considered to be somewhat lower than its potential yield (Singh *et al.,* 1994; Sudupak *et al.,* 2002). Genotyping of different species is necessary for characterization of different accessions of crop gemplasm, testing varietal purity and registration of newly developed cultivars (Chowdhury *et al.,* 2002). Among numerous techniques available for assessing the genetic variability and relatedness, seed storage protein analysis represents a valid alternative to varietal identification (Manella *et al.,* 1999).

Corresponding author: jahangirdar53@gmail.com

It is a useful tool for studying genetic diversity via Sodium dodecyl sulphate polyacrylamide gel electrophoreseis (SDS-PAGE) (Sadia *et al.,* 2009). Seed storage protein markers are highly polymorphic and environmntal influence on their electrophoretic pattern is limited (Gepts *et al.,* 1986; Sadia *et al.,* 2009). Seed storage protein profiling based on SDS-PAGE can be employed for various purposes, such as characterization of germplasm (Javid *et al.,* 2004; Iqbal *et al.,* 2005), varietal identfication, biosystematic analysis, determination of phylogenetic relationship between different species (Sammour 1991; Isemura *et al.,* 2001; Ghafoor *et al.,* 2002). Genetic diversity of seed storage proteins has been reported for lima bean (Lioi *et al.,* 1999), *Phaseolus vulgare* (Ferreira *et al.,* 2000) and chickpea (Ghafoor *et al.,* 2003). Phylogenetic relationship among *Cicer* species based on SDS-PAGE data has suggested that *Cicer reticulatum* is the wild progenitor of cultivated chickpea (Ahmad and Slinkard 1992). Assessment of the extent of genetic variability within chickpea is fundamental for chickpea breeding and the conservation of genetic resources and is particularly useful as a general guide in the choice of parents for breeding hybrids. Criteria for the estimation of genetic diversity can be different which include morphological traits (Upadhaya *et al.,* 2007) or molecular markers (Sharma *et al.,* 1995). Molecular markers have proved to be useful tools in the characterization and evaluation of genetic diversity within and between species and populations. A no. of DNA based markers is now available for the effective quantification of genetic variation in plant species. Restriction fragment length polymorphism (RFPL) and Amplified fragment length polymorphism (AFLP) have been applied successfully and have provided considerable genetic information in a no. of plant species (VOS *et al.,* 1995, Xu *et*

al., .2000). These techniques are slow and expensive and are not amenable for assessment of genetic variation in large scale population genetic studies. More recently PCR based RAPD and simple sequence repeat (SSR) markers requiring small amounts of DNA have also been developed (Williams *et al.,* 1990). SSR markers have been proved to be polymorphic but require nucleotide information for primer design (Sun *et al.,* 1998). RAPD methodology overcomes this limitation; considerable polymorphic markers can be obtained with relative ease from minute amounts of genomic DNA without prior knowledge of sequence information RAPD is a PCR based technique first developed by Williams *et al.,* (1990) widely used in molecular biology. Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus don't require prior knowledge of a DNA sequence. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate (Bardakcl F 2001).

MATERIALS AND METHODS

The germplasm of five accessions of *Cicer* were obtained from Indian Institite of Pulse Research, Kanpur (U.P.) India. During the present study, five accessions were used to estimate genetic diversity by using SDS-PAGE and RAPD analysis. The details of five accessions are given in Table 1.

Protein extraction

For total seed protein extraction from indivisual seed samples, 1gm seed of each accession was taken and ground into fine powder using pestle and mortar. Five hundred ml of protein extraction buffer was added to 0.01g of seed flour and vortexed thoroughly to homogenize. The homogenate samples were centrifuged at 6,000 rpm for 10 minutes at room temperature. The extracted crude proteins were recovered as clear supernatant and were transferred to a new 1.5ml eppendorf tubes and stored at 4°C until they were run on the polyacrylamide gel.

Protein profiling

Protein profiling of extracted samples were analyzed through SDS-PAGE using 12% polyacrylamide gel. Electrophoresis was carried out at 75V for 3 hrs. A protein marker was loaded as standard along with the samples with equal quantities of protein (4ml) into each well of the gel. The gels were then fixed in solution (10% acetic acid and 40% ethanol) for 15 minutes with constant shaking and then stained with 0.2% (W/V) Commassie brilliant blue R 250 overnight on an electric shaker using Double Shaker Mixer Model DH-10. Destaining was carried out for a couple of hours followed by gel preservation, scanning and photography.

Protein imaging and data analysis

Gel photographing and documentation were carried out. With regard to variation in protein banding pattern, electrophorogram of each accession was scored for the presence or absence of bands and used to construct a dendrogram by the unweighted pair group mean and arithmetic average method (UPGMA).

RAPD analysis

For RAPD, 1g seed of each accession was used to isolate the genomic DNA.

Genomic DNA isolation

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Various plants contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification (Zidani *et al.,* 2005). CTAB procedure based on the protocol of Doyle and Doyle (1990) is the method of choice for obtaining good quality DNA from many plant species and also from fungi (Weising *et al.,* 1991). CTAB is a cationic detergent which solubilizes membranes and forms a complex with DNA. One gram seed of each accession was ground to a fine powder in liquid nitrogen then transferred to PCR tubes and an equal volume of hot (65^0C) 2X CTAB was added to it. The mixture was incubated at 65^0C for 10 minutes and mixed it with an equal volume of chloroform/isoamyl glcohol (24: 1) and centrifuged at 13000 g for 5 minutes. The aqueous phase was then transferred to another tube and 1/10 volume of 10 X CTAB was added to it, mixed and treated with an equal volume of chloroform/isoamyl alcohol. An equal volume of CTAB precipitation buffer was added to the aqueous phase to precipitate the DNA. The DNA pallet was rehydrated in high salt TE buffer and the DNA reprecipitated with 2 volumes of chilled ethanol. Finally the DNA pallet was air dried rehydrated in 0.1 X TE buffer and treated with RNase.

Selection of the primer

The decamer primer OPM-05 (5'GGGAACGTGT 3') used for RAPD analysis in chickpea was selected on the basis of prior study. It showed high polymorphism in the earlier studies also as revealed by Talebi et el 2009 and other workers.

Polymerase Chain reaction

PCR stand for polymerase chain reaction is a technique used to selectively amplify in-vitro a specific segment of the total genomic DNA a billion fold. The most essential requirement of PCR is the availability of a pair of short oligonucleotide called primers has sequences complementary to either end of the target DNA segment to be synthesized in large amounts (Ahmad *et al.,* 2010). Amplification are carried out in a themocycler for about 35-40 cycles with an initial strand separation at 94^0C, 1 minute at 37^0C and 2 min. at 72^0C. Amplification products were electrophoresed in 2% agarose gels and detected by staining with ethiduim bromide. After electrophoresis, the RAPD patterns were visualized with UV transilluminator. RAPD markers were scored as DNA fragments present or absent. The bands scored were used for the construction of dendrogram by using UPGMA

Table1: Five experimental accessions of *Cicer*

S.NO.	Accession name	Sample name	Source	Seed size
1	Dhawal	S1	IIPR, Kanpur	Extra Bold
2	Ujjwal	S2	Same	Bold
3	Shubhra	S3	Same	Bold
4	DCP-92-3	S4	Same	Small
5	IPC-04-20	S5	Same	Small

Table 2: Showing the presence and absence of bands of different molecular weights in different samples

Band No.	R.F. value	M.W.K.D.	S1	S2	S3	S4	S5
1.	0.01	99	+	+	+	+	+
2.	0.02	98	+	+	+	+	+
3.	0.03	97	+	+	+	−	+
4.	0.05	95	+	−	−	+	+
5.	0.06	94	+	+	+	−	+
6.	0.07	93	+	+	−	+	+
7.	0.08	92	+	−	+	−	−
8.	0.10	90	−	+	−	+	+
9.	0.11	89	+	−	+	+	−
10.	0.12	88	+	−	−	−	−
11.	0.13	87	−	+	−	−	+
12.	0.15	85	+	+	+	+	+
13.	0.16	84	+	+	+	−	−
14.	0.17	83	−	−	+	+	−
15.	0.18	82	+	+	−	+	−
16.	0.20	80	+	−	+	+	−
17.	0.21	79	+	+	−	−	−
18.	0.22	78	−	−	+	−	−
19.	0.23	77	−	+	−	+	+
20.	0.25	75	+	+	−	−	+
21.	0.27	73	−	+	+	+	+
22.	0.30	70	+	−	−	+	−
23.	0.31	69	−	+	+	−	+
24.	0.33	67	−	−	−	+	−
25.	0.35	65	+	+	+	+	+
26.	0.36	64	−	+	+	−	−
27.	0.37	63	−	+	−	+	−
28.	0.38	62	+	−	−	−	+
29.	0.41	59	+	−	−	−	−
30.	0.42	58	+	−	−	+	−
31.	0.45	55	−	−	−	−	+
32.	0.46	54	−	−	−	+	−
33.	0.48	52	+	+	+	−	+
34.	0.50	50	−	+	−	−	−
35.	0.52	48	−	−	−	−	+
36.	0.60	40	−	−	−	−	+

The symbols (+) and (−) indicate the presence and absence of a band respectively

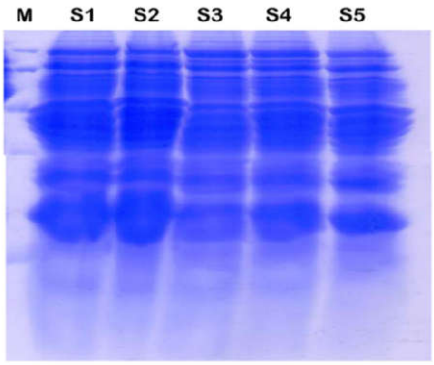

Fig. Protein profile of five accessions of Cicer produced using SDS-PAGE

(Unweighted Pair Group Mean and Arithmetic Average) method to know the phylogenetic relationships among the five accessions of *Cicer*.

RESULTS AND DISCUSSION

The SDS-PAGE of seed proteins of five genotypes was carried out to investigate the genetic diversity. Seed storage protein profiling showed distinct polymorphism in electrophoretic banding patterns and led to detection a total of 36 polypeptide bands. Out of 36 bands only four were monomorphic and the rest 32 were polymorphic. The average polymorphism was 88.88 %. Similar results were also reported by Nisar et al (2007) in Chickpea. Polymorphism was evident in all seed proteins of diverse molecular weights among all accessions but major diversity was found in low molecular weight region. The molecular weights of peptides ranged from 99 to 40 KD with the presence or absence of particular band. The maximum protein bands generated in case of Dhawal (21) and the minimum (16) in Shubhra. The protein band for highest molecular weight (i.e. 99 KD) was generated in all the five accessions while that of lowest molecular weight (i.e. 40 KD) was generated in IPC-04-20 (Table 2, Figure 1).The data obtained from SDS-PAGE analysis was used for construction of dendrogram using UPGMA (Unweighted Pair Group Mean and Arithmetic average) Procedure is presented in Figure 2. Five accessions were grouped in two clusters. Cluster I and cluster II, comprising of two accessions each. The cluster analysis revealed that Ujjwal and IPC-04-20 are very close to each other. Accession Shubhra occupies a distinct place as

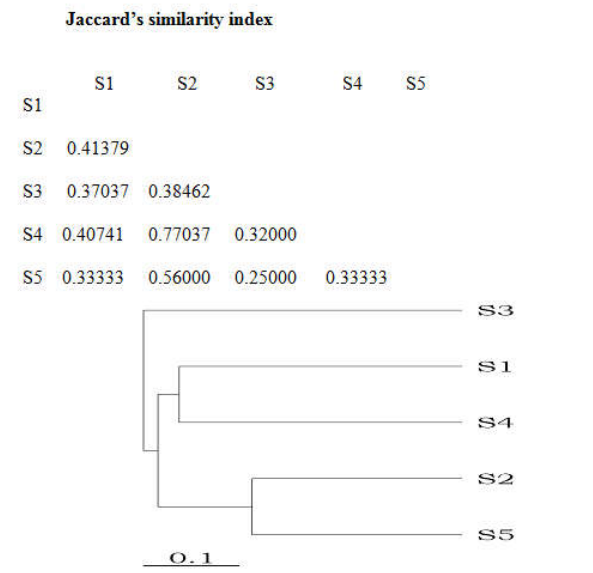

Figure 2: UPGMA dendrogram depicting phylogenetic relationships among five *Cicer* accessions

(Parker *et al.,* 1998). As storage proteins are not affected by environmental fluctuation, their profiling using SDS-PAGE technology is particularly considered as a reliable tool for economic characterization of germplasm (Javid *et al.,* 2004; Iqbal *et al.,* 2005). Comparison of seed storage proteins have been found to provide no biological basis for separating closely related small and large seeded lentils (Ladizinsky 1979). Similar was true for chickpea genotypes evaluated in the present study as no difference in seed storage proteins of bold and small seeded genotypes was observed. To confirm the findings of protein profiling, RAPD markers were used to estimate genetic diversity in chickpea at molecular level. RAPD markers represent an efficient and inexpensive way to generate molecular data, thus have been used successfully in various taxonomic and phylogenetic studies (Aboelwafa *et al.,* 1995; Sharma *et al.,* 1995; Friesen *et al.,* 1997., Wolff and Morgan-Richards 1998). RAPD primer (OPM-05) was used to analyze the genetic diversity among five accessions. Only clearly scorable bands were included in the analysis.

Table 3 : Binary scoring of bands in five accessions of *Cicer* using RAPD

Band No.	Rf value	Base pairs	S1	S2	S3	S4	S5
1	0.21	1185	0	0	1	0	0
2	0.26	1110	1	0	0	0	0
3	0.37	945	0	0	0	1	0
4	0.41	885	1	0	1	0	0
5	0.42	870	0	0	0	0	1
6	0.43	855	0	1	0	0	0
7	0.47	795	1	0	1	0	1
8	0.50	750	0	0	0	1	0
9	0.53	705	1	0	0	0	0
10	0.55	675	0	1	0	0	0
11	0.58	630	0	0	1	0	0
12	0.60	600	1	1	0	0	0
13	0.63	555	1	0	1	0	0
14	0.65	525	0	0	0	1	0
15	0.70	450	0	0	1	1	1
16	0.73	405	1	0	0	0	0
17	0.81	285	0	0	0	0	1
18	0.83	255	1	1	0	1	0
19	0.90	150	0	0	0	0	1
20	0.96	60	0	0	0	0	1
21	0.98	30	1	0	0	0	0

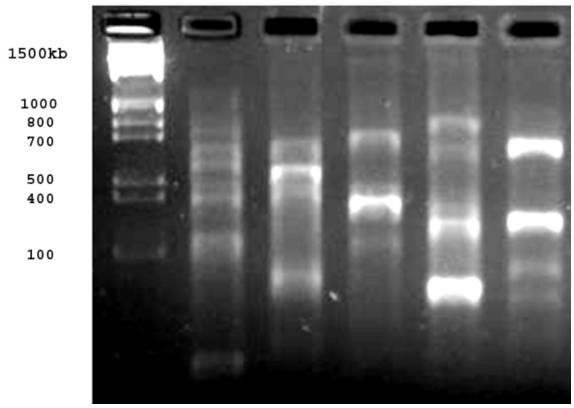

Figure 3: RAPD profile of five *Cicer* accession

revealed in the dendrogram. The results of present studies further strengthened previous finding of Ghafoor *et al.,* (2003), Yasmin *et al.,* (2010), Asgar *et al.,* (2003), Ferreira *et al.,* (2000) and Dasgupta and Singh (2003) who reported high genetic diversity in various legume species. Protein electrophoresis is a powerful tool for population genetics

Minor bands which could not be scored reliably were not included in the analysis. In total 21 visible bands were scored in all accessions corresponding to an average of 4.2 bands per accessions. All bands were polymorphic (Table 3,Figure 3). Similar results were observed by Ahmad F (1999) in chickpea by using 75 random decamer primers. Several other workers also observed high polymorphism by using different random primers in different species. Sudupak *et al.,* (2002) used seven decamer primers in chickpea and observed 96.82% polymorphism. Sharma *et al.,* (2000) also recorded 100% polymorphism in *Podophylum haxandrum* by using seven decamer primers and SDS-PAGE. Jaya prakash *et al.,* (2006) recorded 97.4% polymorphism in sorghum genotypes by using 64 RAPD primers. Aboelwafa *et al.,* (1995) used 40 primers and recorded 90% polymorphism in Lens.Verma *et al.,* (2009) used 20 random primers and observed 96.8% polymorphism in *Trichodesma indicum*. Iqbal *et al.,* (2010) worked on *Jatropa curcas* L. and recorded 93.90% polymorphism by using 50 random primers. Chickpea genotype Dhawal produced the maximum number of bands (9), while Ujjwal gave the minimum number (4) of bands (Table 3, Figure 3).

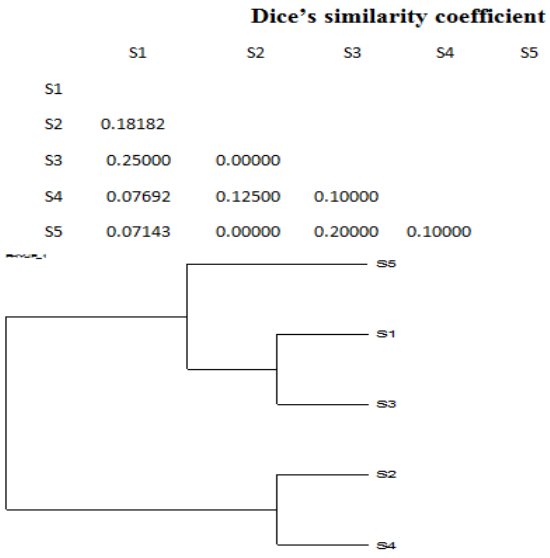

Figure 4: UPGMA dendrogram depicting genetic relationships among *Cicer* accessions based on RAPD

The dendrogram obtained from the UPGMA method is shown in Figure 4. Cluster analysis divided the five accessions into two clusters. Cluster I comprising three accessions (Dhawal, Shubhra and IPC-04-20) while as the cluster II consists of two accessions (Ujjwal and DCP-92-3). It is evident from the dendrogram that Dhawal and Shubhra and Ujjwal and DCP-92-3 are very close to each other while as IPC-04-20 and DCP-92-3 are genetically dissimilar. Closely related species commonly lose the ability to interbreed and become genetically isolated due to chromosomal structural mutations (Tayyar and Wainess 1996). IPC-04-20 and DCP-92-3 are genetically distant, hence it is recommended that these two accessions should be used in future breeding programs to create higher amount of genetic variability in chickpea. Our results show that RAPD markers successfully identified genetic variation in *Cicer*.The variation identified was greater than that reveled by the seed storage proteins used in previous studies of genetic relationships among annual *Cicer* species (Tuwafe *et al.,* (1988), Kazan and Muehlbaur (1991), Ahmad and Slinkard (1992), Ahmad *et al.,* (1992), Labdi *et al.,* (1996), Tayyar and Wainess (1996). In general electrophoretic SDS-PAGE and PCR based RAPD analysis were performed to establish fingerprints in five *Cicer* cultivars and to elucidate their genetic relationships. The resulted protein banding pattern showed 88.88% polymorphism among five cultivars. On the other hand RAPD-PCR profile revealed the high levels of polymorphism (i.e.100%). Jaccard's coefficient ranged from 0.25000-0.56000 in SDS-PAGE while in RAPD it ranged from 0.00000 -0.25000. The variation might be due to use of single decamer primer. If more than one random decamer primers would have been used, the results might resemble with that of the protein profiling. The general conclusion from the study is that RAPD profiling is efficient in revealing usable level of DNA polymorphism among the cultivars of chickpea.

Conclusion

Considering all the data of the present study, it can be concluded that the biochemical markers like seed protein analysis are not powerful enough to discriminate between closely related accession/varieties. RAPD markers on the other hand are much more sensitive and reveal complete identification at the DNA level and can successfully be used to produce specific fingerprints.

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