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GENETIC DIVERSITY AS REVEALED BY RAPD ANALYSIS AMONG CHICKPEA GENOTYPES

SAIEMA RASOOL

Department of Botany, Jamia Hamdard, New Delhi, India-110062
Corresponding author's e-mail: saimu083@yahoo.co.in

Abstract

The objectives of this study were to estimate the genetic diversity and to evaluate the relationship between eight chickpea genotypes that is the valuable source for breeding. Chickpea seed is a good source of carbohydrates and proteins, constituting 80% of the total dry seed weight. The variable response of chickpea genotypes were characterized by using the 15 RAPD primers. The total numbers of amplification products generated were 915, and among them 898 were found to be polymorphic. The number of amplification products ranged from 28-81 from 15 arbitrary primers. The molecular weight of the generated bands in the present study ranged from 100-2968 bp. Primers BG-30, C and OPA-02 generated the maximum number of amplified products. Minimum number of 28 RAPD products were obtained with OPA-04. 16 RAPD products were recorded as unique or species specific and resulted in 98.1% of polymorphism.

Introduction

Chickpea (*Cicer arietinum* L.) is the world's third most important pulse crop after bean and pea. It is widely distributed pulse crop across the tropics, sub-tropics and temperate regions (Singh, 1997). It accounts for about 15% (9.3 million tons) of the world's total pulse production (Anon., 2007). Chickpea is important because it provides food for humans as well as for livestock. Moreover, chickpea pod covers and seed coats can also be used as fodder. The chickpea seed is a good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight. West Asia and Iran is known to be a genetic diversity centre and rich in both landrace and wild relatives of chickpea (Singh & Ocampo, 1997). Modern plant breeding and agricultural systems have narrowed the genetic base of cultivated chickpea (Robertson *et al.,* 1997). In addition, the genetic erosion of chickpea resources due to biotic and abiotic stresses as well as economic and strategic reasons is a persistent process. It has been known that interspecific hybridization will increase the variation and can be useful for plant breeding purposes in a 'recalcitrant' crop like chickpea (Singh *et al.,* 1994; van Rheenen *et al.,* 1993).

Evaluation 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 the genetic diversity can be different, which include molecular markers (Sharma *et al.,* 1995a). Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations (Bakht *et al.,* 2011). More recently, PCR-based RAPD markers requiring small amounts of DNA have been developed (Williams *et al.,* 1990). The development and application of randomly amplified polymorphic DNA (RAPD) markers generated by the polymerase chain reaction (PCR) using arbitrary primers has resulted in alternative molecular markers for the detection of nuclear DNA polymorphisms. The technical simplicity of the RAPD technique has facilitated its use in the analysis of phylogenetic relationships, cultivar identification, genetic diversity, parentage determination and marker-assisted selection in several plant genera (Kawchuk *et al.,* 1994; Aboelwafa *et al.,* 1995; Sharma *et al.,* 1995; Friesen *et al.,* 1997; Wolff & Morgan, 1998; Bakht *et al.,* 2012).

The aim of this study was to asses the possibility of PCR based amplification in all the eight genotypes of chickpea and to detect the genetic variation for understanding the species relationships in the genus.

Materials and Methods

Plant material: Eight chickpea genotypes procured from Shere Kashmir University of Agricultural Science and Technology, Kashmir were used for molecular study. The chickpea genotypes were SKUA-01, SKUA-02, SKUA-03, SKUA-04, SKUA-05, SKUA-06, SKUA-07 and SKUA-08. Plants were grown hydroponically and DNA was isolated separately from each genotype.

DNA isolation: Leaves of 15 days old seedlings were used for DNA extraction. Plant DNA was extracted by using CTAB method of Saghai-Maroof *et al.,* (1984). 1.0g of fresh leaf tissue was weighed and ground to a fine powder with the help of mortar and pestle (pre-cooled). The frozen leaf powder was transferred to eppendroff tube containing 1 mL of extraction buffer and 0.2% β-mercapto-ethanol. The tubes were then kept in incubator at 65˚C for 45 min and swirled in between to ensure efficient extraction from cell content. After incubation an equal volume of chloroform: isoamyl alcohol in the ratio of 24:1 was added and the content of the tubes were mixed gently for few min. The tubes were then centrifuged at 10000 x g for 10 min at 4˚C. The resultant supernatant was collected in fresh tube with the help of wide bored tip and equal volume of pre-chilled isopropanol was added to precipitate the DNA. The tubes were incubated on ice for 20 min and again centrifuged at 10,000 x g for 10 min at 4ºC. The supernatant was discarded and the resultant pellet was washed with 95% ethanol and then by 70% ethanol. The pellet was finally dissolved in TE buffer and kept overnight for complete dissolution.

DNA purification: In order to purify extracted DNA co-precipitated RNA was eliminated by adding 0.7 units of RNAase per sample (Bangalore Genei, India). About 5 µl of RNAase was added to the tubes and incubated at 37˚C for an hour. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1 was added to the tubes. The tubes were gently stirred and centrifuged at 8000 x g for 10 min at 4˚C. The aqueous phase was collected in fresh tubes in which one tenth part of sodium acetate and 3 times of volume absolute alcohol was added. The mixture was again centrifuged to collect the pellet. The pellet was then washed with 95% ethanol and air dried. The dried pellet finally dissolved in TE buffer and stored at 4˚C.

DNA quantification: The isolated genomic DNA was quantified by using nano-drop spectrometer (λBIO 20, Perkin Elmer, Germany). Stock DNA was diluted in TE buffer to make a working solution of 15 ng/µl for PCR reaction.

RAPD primers: Nineteen primers from Qiagen (Operon technologies, USA) and five primers from Bangalore Genei (India) were procured for initial screening of repeatable amplification with eight genotypes.

Isolated DNA of different accessions was used as a template for Polymerase chain reactions (PCR). PCR was carried out in 25 µl reaction volumes containing 10mM Tris-HCl pH 9.0; 50mM KCl; 0.1% TRITON X-100; 1.5mM MgCl; 0.1mMdNTP; 2mM primer; 0.5 unit of Taq DNA polymerase(Bangalore Genei, India) and 25ng template DNA. Amplification were carried out in a thermo-cycler programmed for 35 cycles with an initial melting at 94˚C for 4 min, followed by denaturation at 94˚C for 1 min. The annealing was performed at 37˚C for 1 min, which was then followed by polymerization at 72˚C for 2 min. Final extension step was at 72˚C for 7min.

Amplification products were resolved on 1.0% (w/v) agarose gel in 1X TBE buffer and stained with ethidium bromide. The gel was visualized and photographed under UV light by using gel documenting system (UVi Tec). High molecular weight DNA ladder (Bangalore Genei, India) was used as molecular weight marker.

Data analysis: DNA fragment positions in comparative RAPD profiles for each accession and primer combinations were scored from photographic films of gels. A few bands, which were not reproducible, were excluded. The RADP profiles of only those accession and primer combinations was included in the study, which gave positive amplification for all the accessions and for which no blank lane/ unclear bands were observed. Band was scored '1' for its presence and `0' for its absence. These binary data were then utilised to generate genetic similarity data among accessions. Genetic similarity index for all the genotypes of chickpea was obtained with RAPD patterns generated utilising different random primers. Twenty four primers were analysed in the present study of which fifteen were found to be useful. The Jaccard's similarity coefficient values (Jaccard, 1908) for each pair-wise comparison between genotype were calculated and similarity coefficient matrix was constructed (Table 1). This matrix was subjected to unweighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure (Fig. 1). All these computation was carried out using NTSYS-PC software (Rohlf, 1998).

Table 1. Genetic similarity matrix of eight chickpea genotypes by 15 RAPD primers using Jaccard's analysis.

	SKUA-01	SKUA-02	SKUA-03	SKUA-04	SKUA-05	SKUA-06	SKUA-07	SKUA-08
SKUA-01	1.00							
SKUA-02	0.09	1.00						
SKUA-03	0.06	0.10	1.00					
SKUA-04	0.03	0.07	0.17	1.00				
SKUA-05	0.03	0.05	0.07	0.15	1.00			
SKUA-06	0.03	0.05	0.04	0.08	0.13	1.00		
SKUA-07	0.03	0.05	0.06	0.07	0.15	0.26	1.00	
SKUA-08	0.03	0.04	0.03	0.05	0.11	0.16	0.21	1.00

Results

The variable response of chickpea genotypes were characterized by using the 15 RAPD primers. The total numbers of amplification products generated were 915, and among them 898 were found to be polymorphic. The number of amplification products ranged from 28-81 from 15 arbitrary primers. The molecular weight of the generated bands in the present study ranged from 100-2968bp. Maximum number of fragments (83) were amplified by BG-30 (Fig. 2). Minimum numbers of 28 RAPD products were obtained with OPAA-04 (Fig. 3). 16 RAPD products were recorded as unique or species specific and resulted in 98.1% of polymorphism. The RAPD cluster analysis showed three major clusters namely cluster-I, cluster-II and cluster–III comprising of 2, 2 and 4, respectively. Cluster-I includes two genotypes SKUA-01, SKUA-02. Cluster –II also includes two genotypes SKUA-03 and SKUA-04. Cluster-III includes the 4 genotypes SKUA-05, SKUA-06, SKUA-07 and SKUA-08. The cluster-III is further sub-divided into cluster-IIIa comprising only one genotype SKUA-05 and cluster-IIIb having three genotypes SKUA-06, SKAU-07 and SKUA-08. The genotypes SKUA-06 and SKUA-07 occupies a distinct place as revealed in the dendrogram constructed with the maximum similarity co-efficient of 26%.

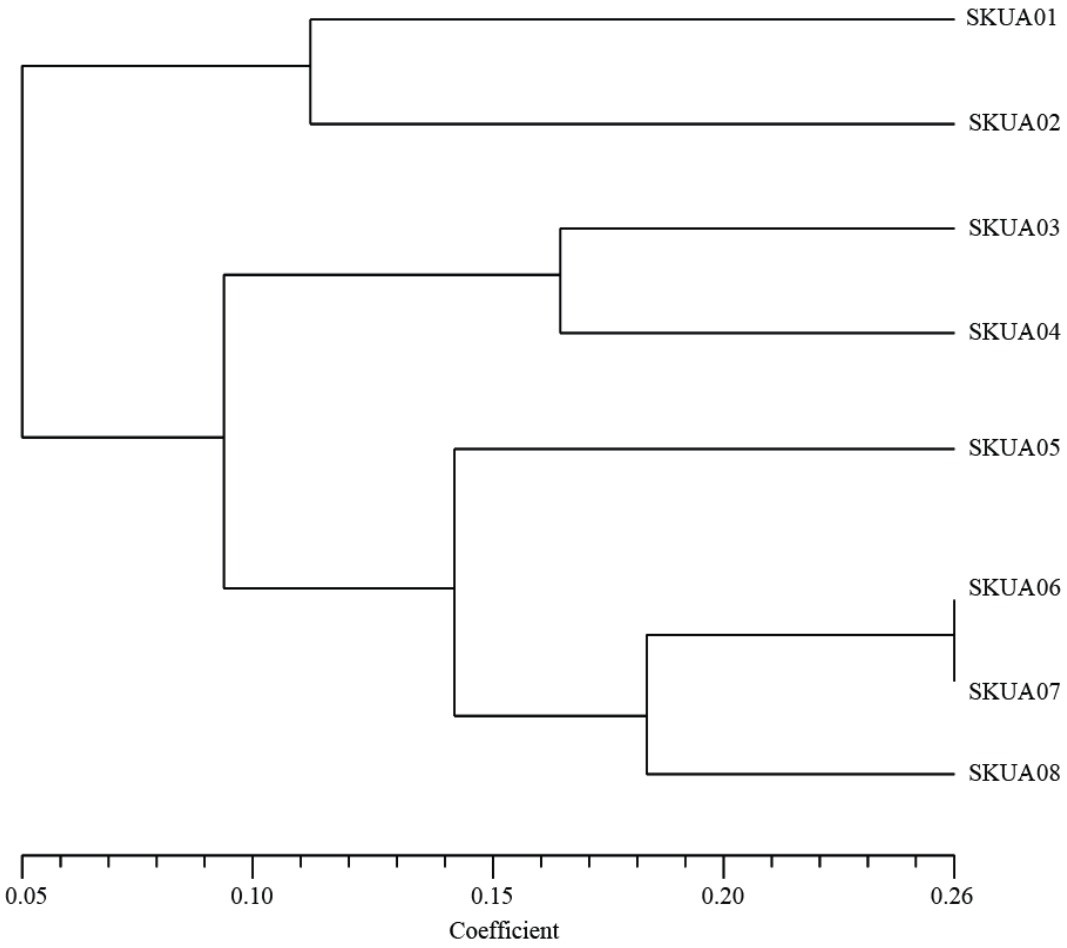

Fig. 1. UPGMA clusters analysis-based dendrogram depicting genetic relationships among eight chickpea genotypes.

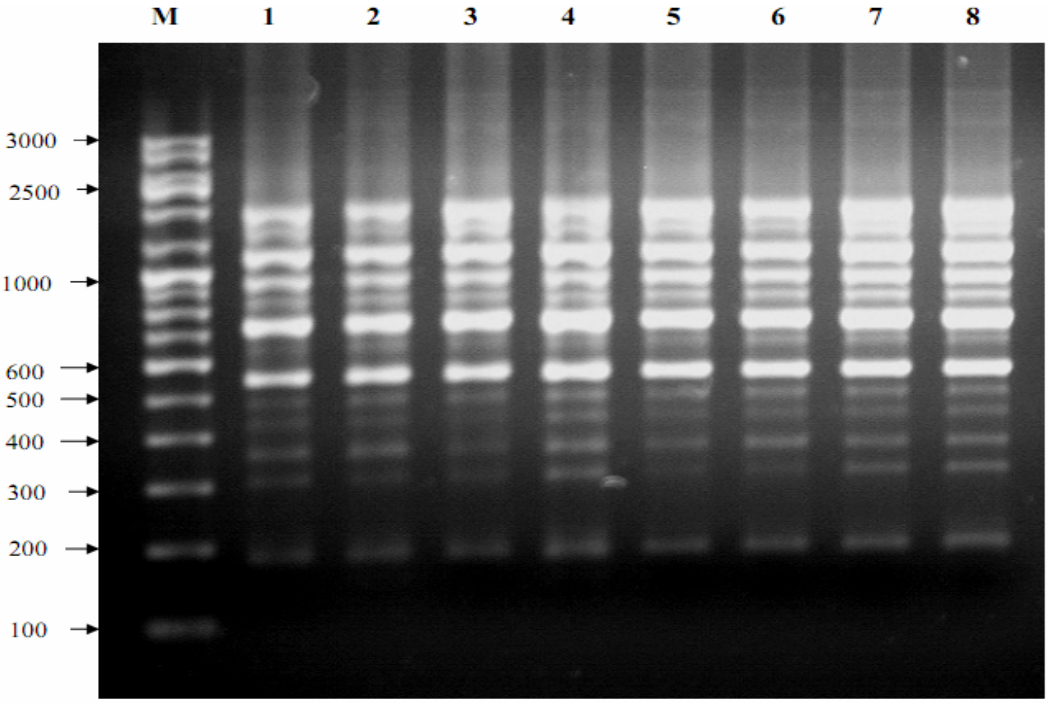

Fig. 2. RAPD profiles of eight chickpea genotypes obtained with primer BG-30.

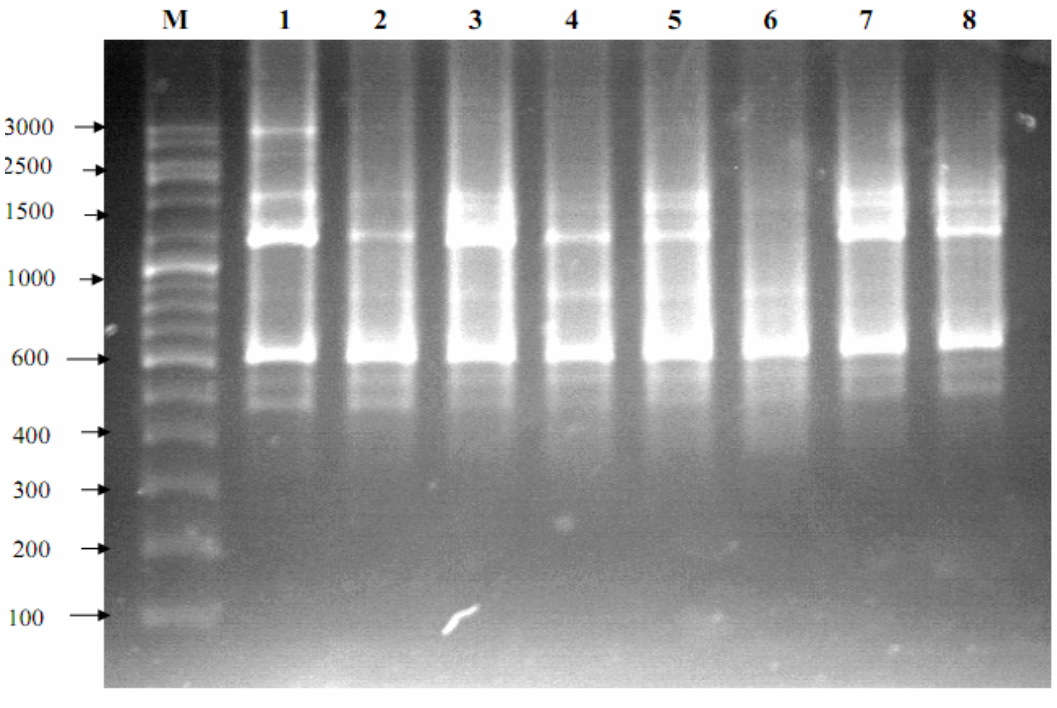

Fig. 3. RAPD profiles of eight chickpea genotypes obtained with primer OPAA-04.

Discussion

RAPD (Random Amplified Polymorphic DNA) markers represent an efficient and economical way to generate molecular data and have been used successfully in various taxonomic and phylogenetic studies (Abo-elwafa *et al.,* 1995; Sharma *et al.,* 1995b; Friesen *et al.,* 1997; Wolff & Morgan, 1998; Fernandes *et al.,* 2011). RAPD (Williams *et al.,* 1990) analysis can be used to characterize DNA variation patterns within species and among closely related taxa. Within grain legume crops alone, RAPD markers have been widely used for the identification of genetic relationships among cultivars (Brown-Guedira *et al.,* 2000; Subramanian *et al.,* 2000; Amadou *et al.,* 2001; Dwivedi *et al.,* 2001; Galvan *et al.,* 2001; Li & Nelson, 2001; Maciel *et al.,* 2001; Sonnante & Pignone 2001; Tosti & Negri 2002), among wild forms (Cattan-Tou-pance *et al.,* 1998), or between cultivars and wild forms (Mimura *et al.,* 2000; Raina *et al.,* 2001). Genetic diversity is normally measured as the average sequence divergence between any two individuals for a given loci. Some of this variation in the context of polymorphism reflects the choice of genotype, but major differences are also observed for random genes within a single genome. The high degree of polymorphism in this study compared to other reports appears to be due to more diverse material, which belonged to different chickpea genotypes. The polymorphism in RAPD is due to a single-base change. In this study, RAPD produced a higher number of bands because RAPDs are random in nature and can anneal anywhere in the genome.

In the present study, the variation among the chickpea genotypes was also assessed with random amplified polymorphic DNA (RAPD) markers. Fifteen RAPD primers amplified a total of 915 DNA fragments with an average of 61 fragments per primer. Out of the total amplified fragments, only 16 were monomorphic and the remaining 899 (98.1%) were polymorphic. This polymorphism was an indication of prevalence of moderate diversity among these eight chickpea genotypes (Punitha & Raveendran, 2004). RAPD markers showed a high level of polymorphism and a high number of clearly amplified bands. Extensive DNA polymorphism has also been reported using RAPD markers in several other crops plants (Hou *et al.,* 2005). The RAPD-based dendrogram of chickpea genotypes displayed the genetic relationships between these genotypes, which accorded with previous studies of chickpea (Tayyar & Waines, 1996; Iruela *et al.,* 2002). This analysis has proven to be successful in revealing the diversity among the genotypes of chickpea as also reported in *Curcuma* spp. (Syamkumar & Sasikumar, 2007), *Crocus* spp. (Grill-Caiola *et al.,* 2004), *Vigna* spp. (Betal *et al.,* 2004) and in Wheat (Bibi *et al.,* 2012). Genotypes SKUA-06 and SKUA-07 showed the similarity co-efficient of 26% and the genotypes SKUA-03 and SKUA-04 with similarity of 16.5%, SKUA-05 showed 14.5% of similarity co-efficient respectively.

Genetic diversity is normally measured as the average sequence difference between any two individuals for a given loci. Some of this variation in the extent of polymorphism reflects the option of genotype, but major differences are also observed for random genes within a single genome. The high degree of polymorphism in this study compared to other reports appears to be due to more diverse material, which belonged to different chickpea genotypes. The polymorphism in RAPD is due to a single-base change. In this study, RAPD produced a higher number of bands because RAPDs are random in nature and can anneal anywhere in the genome. PCR technology has promoted the development of a range of

molecular assay systems that detect polymorphisms at the DNA level. The past limitations associated with pedigree data and morphological, physiological and cytological markers for assessing genetic diversity in cultivated and wild plant species have been largely circumvented by the development of DNA markers such as RAPD, SSR and AFLP. However, these markers have technical differences in terms of cost, speed and amount of DNA needed.

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