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Genetic diversity of chickpea (*Cicer arietinum* L.) germplasm in Pakistan as revealed by RAPD analysis

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ABSTRACT. Genetic diversity analysis of chickpea germplasm can provide practical information for the selection of parental material and thus assist in planning breeding strategies. Chickpea seed is a good source of carbohydrates and proteins, constituting 80% of the total dry seed weight. Released cultivars and advanced lines of 30 chickpea genotypes were subjected to RAPD analysis for assessment of genetic diversity. We used 16 RAPD primers. Amplification of genomic DNA of the 30 genotypes yielded 62 fragments that could be scored. The number of amplification products produced per primer varied from two to four, with a mean of three bands. The total number of bands amplified by 16 anchored primers varied from 16 to 34. The primer GLK-15 produced the largest number (N = 4) of fragments, whereas primers GLK-19 and GLD-19 produced the smallest number (N = 1) of fragments. The single

band produced by the GTGTGCCCCA primer in the PB-2000 and 07005 genotypes may be attributed to temperature tolerance phenotypes.

Key words: Chickpea; Polymerase chain reaction; Random amplified polymorphic DNA; Genetic diversity

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the world's third most important pulse crop after bean and pea. It is an important pulse crop with a wide distribution across the tropics, subtropics and temperate regions (Singh, 1997). It accounts for about 15% (9.3 million tons) of the world's total pulse production (Food and Agriculture Organization, 2007). In many areas of production, including West and Central Asia, North Africa, Southern and Eastern Europe, North America and Australia, the chickpea is affected by the foliar fungal disease of chickpea. However, improved genetics can be packaged in a seed and be more easily adapted than improved agricultural practices that depend more heavily on input availability, infrastructure, access to market, and skill in crop and soil management (Campos et al., 2004).

In Pakistan, there are two kinds of gram crop, namely desi and kabuli. A good gram crop has a yield of 15 to 20 quintals per hectare, and in the case of kabuli, varieties give about 20 quintals. Chickpea (gram) crop is mostly grown as a single crop or combined with barley, linseed, mustard, pea, sweet potato, wheat, or sorghum, etc. The utility of DNA markers for marker-assisted selection is the current trend in modern agriculture. These molecular (DNA) markers allow the construction of a complete genome map and can be integrated with conventional linkage maps, which play an important role in plant breeding strategies (Simon and Muehlbauer, 1997). Improvement of crops through the utilization of available genetic diversity in the germplasm is a key factor for a successful breeding program (Renganayaki et al., 2001). Variance of relatively highly heritable quantitative genetic markers provides estimates of genetic diversity, and thus, genetic variation among diverse genotypes could be a practical way to select parents to be crossed.

Random amplified polymorphic DNA (RAPD) is a DNA polymorphic assay, based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequence. RAPD produces DNA profiles of varying complexity, depending on the primer and template used. Polymorphisms could be caused by the difference of a single-nucleotide sequence at the priming sites (such as point mutations), or by structural arrangements within the amplified sequence (e.g., insertions, deletions, inversions) (Williams et al., 1990; Welsh and McClelland, 1990). RAPD analysis has been used in many applications and various organisms, especially in plant science (Caetano-Anolles, 1994; Sharma and Mohapatra, 1996).

These techniques differ in their principles and generate varying amounts of data. RFLPs are well suited for the construction of linkage maps because of their high specificity and their co-dominant nature, although they have also been used for the analysis of genetic diversity. However, RFLP analysis is labor intensive, time-consuming and expensive. Later, with the development of polymerase chain reaction (PCR)-based RAPD and simple sequence repeats (SSRs), most of the problems associated with RFLP were overcome. SSRs, which are based on microsatellite sequences, have been shown to detect very high levels of polymorphism. How-

ever, prior information about the genome is necessary before SSR markers can be exploited to their fullest potential. On the other hand, the technique of RAPD gained importance due to its simplicity, efficiency, relative ease to perform, and non-requirement of sequence information.

Based on the above considerations, the aim of this research was to determine the genetic relationship between different accessions of inbred chickpea based on RAPD marker data, and to use these results to contribute to germplasm bank management, conservation programs, and breeding purposes.

MATERIAL AND METHODS

Plant material

Thirty chickpea accessions were used in this study, including 7 cultivars and 23 advanced lines. Five selected cultivars were collected from the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, while other genotypes (2 approved varieties and 23 advanced lines) of chickpea were collected from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. The genotypes were CM-72, CM-88, CM-98, CM-2000, CM-2008, PB-2000, PAIDAR-91, 07001, 07002, 07005, 07007, 07008, 07009, 07010, 07012, 07017, 07018, 07019, 07020, 07021, 07022, 07023, 07025, 07041, 07044, 07046, 07053, 07056, 07058, and 07059. RAPD analysis was performed at the Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad.

DNA extraction

Small protruding leaves were taken from 2-week-old young seedlings and stored immediately at -40°C for DNA extraction. DNA was extracted by the modified CTAB method (Doyle and Doyle, 1990). DNA concentration was determined spectrophotometrically, and the quality of DNA was determined by 0.8% agarose gel electrophoresis. Concentrations of $MgCl_2$, *Taq* DNA polymerase and template DNA was optimized for PCR.

RAPD analysis

PCR was run in a thermal cycler (Eppendorf AG No. 533300839; Germany) with 15 ng DNA as a template. Amplification reactions contained 1.0 *Taq* DNA polymerase (MBI, Fermentas, Vilnius, Lithuania), 50 mM KCl, 3 mM $MgCl_2$, 100 mM of each dNTP, 0.2 M decamer primer (GeneLink Company, Hawthorne, NY, USA). The DNA amplification protocol was 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, and finally 72°C for 10 min. All amplification products were electrophoresed on 1.2% (w/v) agarose gels at 80 V for 2 h, stained with ethidium bromide, visualized in a UV transilluminator at 300 nm and photographed in a gel documentation system (SynGen, Synoptics Ltd., UK).

Data analysis

The fingerprints were examined under the ultraviolet transilluminator and pho-

tographed using the Syngene Gel Documentation System (GDS). The RAPD bands were counted and designated as present (1) or absent (0). The data were collected and aligned for the construction of the similarity matrix and cluster analysis. Coefficients of similarity among genotypes were calculated according to Nei and Li (1979). A dendrogram based on these similarity coefficients was constructed using the unweighted pair group method with arithmetic mean (UPGMA). This similarity matrix was analyzed using NTSYS-PC 2.01 and clustered with an UPGMA average algorithm to determine the genetic relationships between the 30 chickpea genotypes.

RESULTS

Thirty genotypes of chickpea were analyzed by RAPD using 16 RAPD primers. Each primer-template yielded distinct, easily detectable bands of variable intensities. Indistinct bands produced by nonspecific amplification were ignored. The bands used for fingerprinting were reproducible over repeated runs, with sufficient intensity to detect the presence or absence with confidence; considering all the primers and accessions, a total of 61 bands were polymorphic. The number of amplification products produced per primer varied from 2 to 4, with a mean of 3 bands. The number of bands per primer was not similar to that obtained by other authors (Noli et al., 1999). However, the mean number of polymorphic bands per primer was lower than that obtained by Carelli et al. (2006). This level of polymorphism observed in chickpea is comparable to the reports of several RAPD studies by various workers. The low degree of similarity (monomorphic bands) indicated high divergence between the genotypes evaluated. Number of bands produced per genotype ranged from 16 to 33 with an average of 25 bands per genotype. Chickpea genotype 07012 produced the maximum number of bands, while CM-72 gave the minimum number of bands.

The data from the similarity matrix show a clear picture of genetic similarities and dissimilarities within cultivars and advanced lines. CM-88 and 07017 showed high similarities while the most diverse genotypes were 07053 and 07019, as well as the 07053 and 07046 genotypes, also showing high diversity. The maximum number of fragments were produced by the primer GLK-15 while a lower frequency of fragments were produced by the primers GLK-19, GLD-16, GLD-8 and GLD-19.

Multivariate analysis was conducted to generate a similarity matrix using the Popgene software, version 1.44 (Yeh et al., 2000) based on Nei's UPGMA to estimate genetic distance and relatedness of chickpea germplasm (Figure 1). Cluster analysis defined four main groups, which were further classified into eight distinct groups. Group A consisted of 2 genotypes, namely CM-72 and CM-88, showing close similarity between them. Group B consisted of 16 chickpea strains, namely CM-98, 07041, CM-2000, 07023, 07002, 07044, 07025, 07058, 07020, 07053, 07019, 07046, 07012, 07001, 07009, and PB-2000. In this group, five further groups were formed. In one, a further cluster was formed by CM-98, CM-2000 and 07041, a second cluster formed by 07023 and 07002, a third cluster formed by 07044, 07025, 07058, a fourth cluster formed by CM-88, 07019, 07046 and 07012, and a fifth cluster formed by 07009 and PB-2000, which are highly diverse.

The main group, C, consisted of 7 chickpea genotypes, namely 07007, 07008, 07056, 07059, 07005, CM-2000, and 07010. In this group, 07008, 07056, 07059, and 07005 were

clustered together, while 07007, CM-2008 and 07010 remained unclustered, showing a distinct behavior. Group D included four genotypes, i.e., 07022, 07021, PAIDAR-91, and 07017, where 07021 and PAIDAR-91 were clustered together, while 07022 and 07017 remained unclustered showing a distinct behavior from other strains of Group D.

Genotype 07018 did not cluster with any of the other genotypes showing a distinct behavior from all other genotypes of all groups A, B, C, and D.

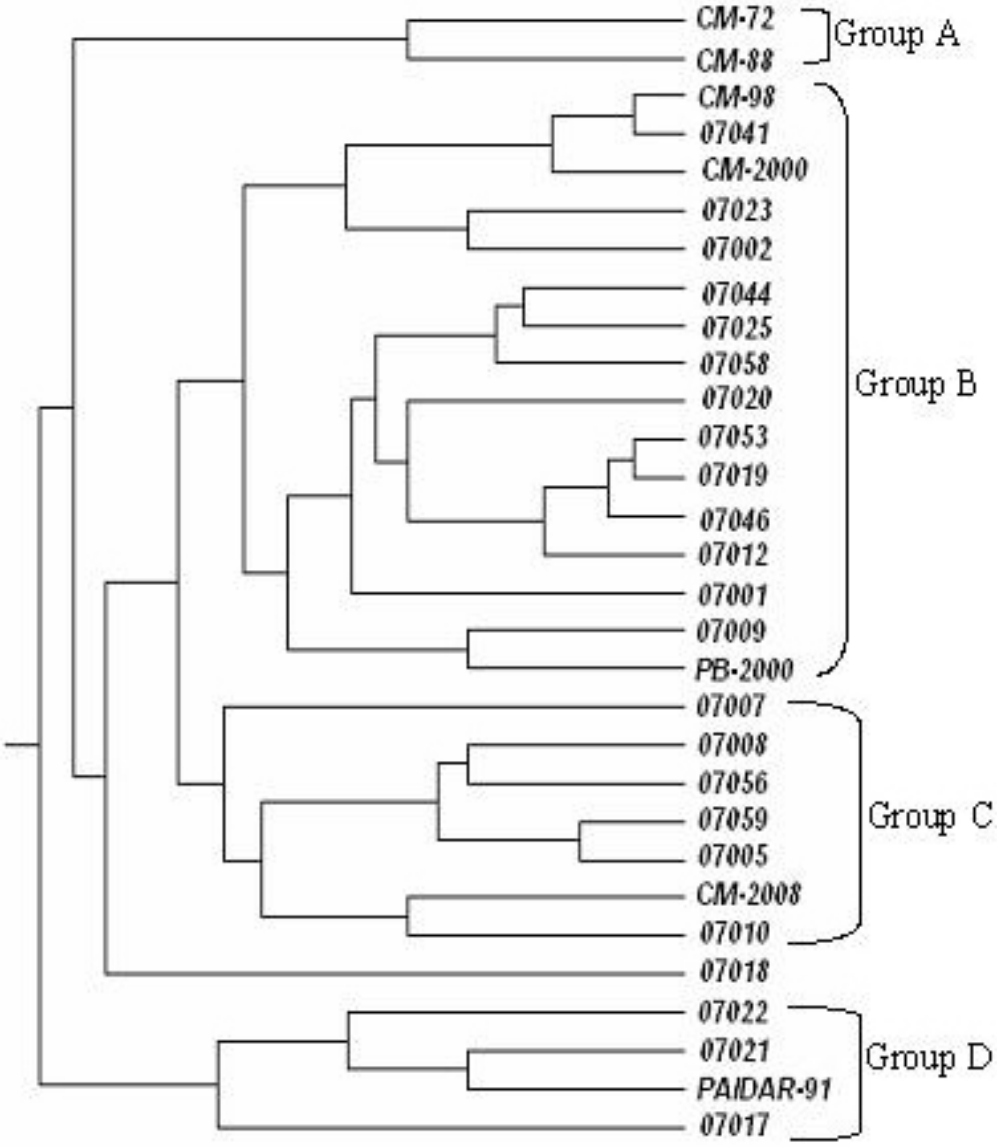

Figure 1. Genetic distance of 30 chickpea accessions developed from RAPD data using unweighted pair group method with arithmetic mean (UPGMA).

DISCUSSION

As previously observed by Villand et al. (1998), the percentage of the presence of an RAPD marker shows a strongly bimodal distribution with most RAPD markers amplified with either a high or a low frequency. These data confirm the need to sample a large number of accessions to ensure the inclusion of low-frequency alleles in chickpea breeding programs. With the exception of 25 of 804 amplification products, no significant differences were observed between the mean percentage of the presence of RAPD markers between commercial cultivars and landraces. The 16 primers allowed the discrimination of all the possible pair-wise comparisons between genotypes. Although there are bands that were monomorphic, most cultivars or landraces produced unique amplification profiles sufficient to distinguish them from the other genotypes tested. These results confirm the efficiency of RAPD markers for the identification of plant genotypes (Williams and Clair, 1993; Weising et al., 1995; Villand et al., 1998; Noli et al., 1999). The combined analysis of the amplification products generated by the primers GLC-4, GLC-2, GLD-1, GLK-20, GLK-19, GLD-2, GLD-4, GLK-15, GLD-19, GLD-7, GLJ-10, GLD-1, GLJ-12, GLC-12, GLI-6, and GLD-6 was enough to assess the genetic diversity among the genotypes.

Genetic diversity is normally measured as the average sequence divergence between any two individuals for a given loci. Some of this variation in the extent 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 a different chickpea germplasm. 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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