

Full Length Research Paper

Analysis of genetic diversity in chickpea (*Cicer arietinum* L.) cultivars using random amplified polymorphic DNA (RAPD) markers

Zahid Mahmood¹, Mohammad Athar^{2*}, Muhammad Aurangzeb Khan¹, Muhammad Ali¹,
Shahzadi Saima³ and Altaf A. Dasti³

¹Institute of Biotechnology, Bahauddin Zakariya University, Multan-60800, Pakistan.

²California Department of Food and Agriculture, 3288 Meadowview Road, Sacramento, CA 95832, USA.

³Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan-60800. Pakistan.

Accepted 10 September, 2010

Genetic diversity of seven chickpea (*Cicer arietinum* L.) cultivars of Pakistani origin was analyzed by using random amplified polymorphic DNA (RAPD) markers, an extremely effective method to determine the variations among the chickpea cultivars. Polymerase chain reaction (PCR) conditions were optimized for RAPD and the conditions which gave the optimized results were selected for further amplifications. Using nine random decamers for seven genotypes of chickpea, 63 bands were amplified. Out of 63 bands, 50 were polymorphic in all the seven chickpea cultivars. The numbers of RAPD fragments generated per primer ranged from 3 to 11. However, majority of the primers amplified 7 to 11 fragments. The Jaccard's similarity coefficients ranged from 0.333 to 0.651. Maximum similarity (65.1%) was observed between PK G-3 and PK G-4 and the lowest similarity (33.3%) was observed between PK G-3 and PK G-7. A dendrogram was constructed by using the unweighted pair group arithmetic mean arrangement (UPGMA) that was based on similarity coefficients. Seven chickpea cultivars were clustered in two distinct groups of which two cultivars (PK G-6 and PK G-7) stood separately in the dendrogram. The results from this study may be useful to maximize the selection of diverse parent cultivars and to broaden the germplasm base in the future for chickpea breeding programs. The information generated from this study can also be used in identifying efficient strategies for the sustainable management of the genetic resources of chickpea crop.

Key words: Random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR), chickpea cultivars, genetic diversity.

INTRODUCTION

The genus *Cicer* L. (Family Fabaceae) consists of 44 species including 35 perennial and eight wild species, and one domesticated chickpea, *Cicer arietinum* L. (van der Maesen et al., 2007). Chickpea is an edible legume and is high in proteins and is one of the earliest cultivated grain legumes (Athar and Bokhari, 2006; Redden and

Berger, 2007). Its remains of about 7,500-year-old have been found in the Middle East (Duke, 1981; Redden and Berger, 2007; Tanno and Wilcox, 2006). According to Food and Agriculture Organization statistics, the cultivated chickpea produces 8.7 million tons annually from 10.6 million hectares with an average seed yield of 819 kg per hectare and is listed in the first rank among the cool season food legumes based on harvested areas (FAO, 2006). It is cultivated on large areas in the world, yet with a relatively low productivity, notably due to lime induced Fe deficiency (Cani and Toker, 2009; Toker, 2009; Upadhyaya et al., 2001).

Chickpea with 17-24% proteins, 41-50.8% carbohydrates and high percentage of other mineral nutrients and un-

*Corresponding author. E-mail: atariq@cdfa.ca.gov. Tel: 916-262-0855. Fax: 916-262-2059.

Abbreviations: RAPD, Random amplified polymorphic DNA; PCR, polymerase chain reaction; UPGMA, unweighted pair group arithmetic mean arrangement.



Figure 1. Growth conditions of chickpea plant cultivar PK G-7.

saturated linoleic and oleic acid is one of the most important crops for human consumption (Huisman and van der Poel, 1994; Hulse, 1991; Kerem et al., 2007). Chickpea, with low production cost, wide climate adaptation and an ability for it to be used in crop rotation and atmospheric nitrogen fixation, is one of the most important legume plants in sustainable agriculture system (Cani and Toker, 2009; Singh and Ocampo, 1997; Smithson et al., 1985). Chickpea has high variation for different quality and quantity traits, including ideal plant type (tall type), shape and color of grain, flower color, podding, color of seed coat, earliness, resistance to diseases and pests, which helps breeders to release improved and advanced lines and varieties (Collard et al., 2007; Dasgupta et al., 1987; Singh and Ocampo, 1997).

Identification of different genotypes of crop species is essential when diverse accessions of crop germplasm are to be characterized, newly developed cultivar is to be registered and purity of the variety is to be determined. Among numerous techniques available for assessing the genetic variability and relatedness among crop germplasm, DNA-based markers provide very effective and reliable tools for measuring genetic diversity in crop germplasm and studying evolutionary relationships (Iruela et al., 2002). Molecular genetic techniques using DNA polymorphism have been increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process (O'Neill et al., 2003). Unlike the morphological and biochemical markers which may be affected by environmental factors and growth practices, DNA markers depict genome sequence composition, thus enabling the

detection of differences in the genetic information carried by the different individuals (Iruela et al., 2002).

The present study was undertaken to evaluate the pattern and the existence of genetic variability and relatedness among traditional varieties to improve cultivars of chickpea which were conducted using RAPD markers. This would help in the identification and differentiation of various cultivars being grown for local consumption and/or for export purposes. This will also contribute to maximize the selection of diverse parent cultivar and to broaden the germplasm base in the future of chickpea breeding programs. The information generated from this study will be used in identifying efficient strategies for the sustainable management of the genetic resources of chickpea crops.

MATERIALS AND METHODS

Genetic diversity of seven chickpea (*C. arietinum* L.) cultivars collected from Arid Agriculture Research Institute, Chakwal, Pakistan was analyzed by using RAPD markers. These chickpea cultivars were Channay Balkasar (PK G-1), 6KCC-120 (PK G-2), 5CC-109 (PK G-3), Channay Wanhar (PK G-4), Wanhar 2000 (PK G-5), Bittle-98 (PK G-6) and 5CC-108 (PK G-7). Chickpea cultivars were grown in Bio Park Bahauddin Zakariya University, Multan. Earthen pots (30 cm diameter) were filled with soil and 15 - 20 seeds of each cultivar were sown in a pot in March 2009 (Figure 1). Pots were labeled and placed in the open ground and watered regularly as required.

A modified cetyl trimethyl ammonium bromide (CTAB) procedure based on the protocol of Doyle and Doyle (1990) is the method of choice for obtaining good quality total 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. After cell disruption and incubation with hot CTAB isolation buffer, proteins were extracted using chloroform isoamyl-alcohol, and the CTAB-DNA complex was precipitated with isopropanol. The DNA pellet was washed, dried and re-dissolved in TE buffer.

Primers were obtained from Operon Technologies, Alameda, California. They are supplied lyophilized as 0.5 OD (approximately 15 µg). The lyophilized primers in 1000 µl 1X TE (pH 7.5) were left overnight in the refrigerator to ensure that the entire DNA has dissolved. Each sample was aliquoted into 10 x 100 µl in 0.5 ml eppendorf tubes and kept one aliquot at -20°C for current use. A small hole was punched in the lids of the remaining nine tubes. The tubes were lyophilized and stored at -20°C. The samples were re-dissolved in 100 µl of sterile distilled water for use. The final concentration of primers used was approximately 1 µl/25 µl of RAPD reaction.

The RAPD reaction

The method of Williams et al. (1990) was optimized for the amplification reaction. Most amplification reactions were performed in a 25 µl volume overlaid with 50 µl of light mineral oil (BDH) using a gene amp® thermocycler (GA-9700). Each reaction contained 500 mM KCl, 100 mM Tris-HCl (pH 9.1 at 20°C), 0.1% triton, 2 µl gelatin, 200 µM of each of the dNTPs, 0.25 µM primer, 100 ng template DNA, 1.0 U *Taq* polymerase and 2.5 mM MgCl₂.

The amplification profile for RAPD of chickpea consisted of

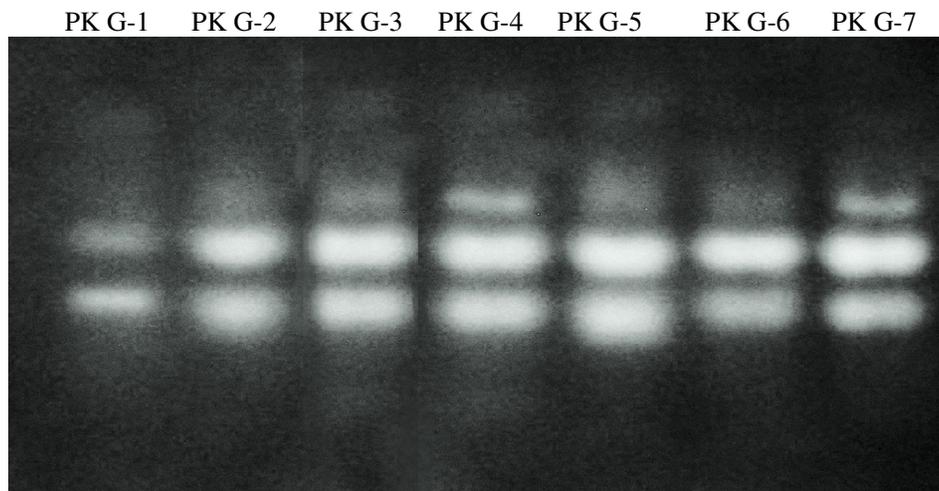


Figure 2. RAPD generated from seven cultivars of chickpea by using primer OPA-03.

denaturation at 94°C for 5 min, followed by 45 cycles comprising of a denaturation step at 94°C for 45 sec, an annealing step at 37°C for 1 min and an extension step at 72°C for 2 min. The cycling program was terminated by a final extension step at 72°C for 9 min.

The amplification products were separated by loading 12.5 µl of each reaction onto a 1.5% ultra pure agarose gel containing ethidium bromide (0.17 ng/ml) in TBE buffer (0.089 M Tris-borate, 0.002 M EDTA, pH 7.8) and running at 5 V/cm for 4 h. The sizes of the fragments obtained were estimated by running alongside, a 1 kb ladder marker (Gibco-BRL). Bands were viewed using a UV transilluminator (Maniatis et al., 1982).

Data analysis

DNA fragments banded on agarose were scored for all tested cultivars of chickpea as 1 for presence and 0 for absence of the band. The resulting matrix was used to calculate the Jaccard's similarity coefficient (Sokal and Sneath, 1963). Un-weighted pair group arithmetic mean average (UPGMA) analysis were used to construct the dendrogram. All statistical analysis was carried out with MVSP, computer package (Version 2.1).

RESULTS AND DISCUSSION

The generated RAPDs were used to determine the genetic diversity among seven Pakistani cultivars of chickpea. Numerous polymorphisms were observed among the cultivars of chickpea. Amplification of genomic DNA was extracted from these cultivars using 20 decamer primers out of which nine decamer primers produced a variety of RAPD patterns as presented in Figure 2. With primer OPA-3, most of the amplified bands among chickpea cultivars were monomorphic (Figure 2). Only PK G-4 and PK G-7 have one polymorphic band. Maximum number of polymorphism and monomorphism among chickpea cultivars were observed by using primer OPA-4 (Figure 3). Several polymorphic bands were amplified among the chickpea cultivars by using OPA-07 primer.

Out of nine, only one band was observed to be monomorphic and the rest were observed as polymorphic (Figure 4)

A total of 63 bands were amplified from nine random decamers in seven genotypes of chickpea. Out of nine random primers used, almost all of them showed polymorphisms among the cultivars of chickpea. The monomorphic bands are constant bands and cannot be used to study diversity, while polymorphic bands revealed differences and can be used to examine and establish systematic relationships among the genotypes (Hadrys et al., 1992).

The number of RAPD fragments generated per primer ranged from 3 fragments to a maximum of 11 fragments. The result is supported by the findings of Kernodle et al. (1993) who reported that the variation in the number of bands amplified by different primers is influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome. Similar findings were also observed by Mahmood et al. (2009) in *Gossypium* spp. Sensitivity of the working environment and equipment used can influence the RAPD amplification (Devos and Gale, 1992).

The use of markers in a PCR is a powerful tool that reveals extensive DNA polymorphism, and it has become valuable in genetic analysis (Davierwala et al., 2000; Neeraja et al., 2002; Porreca et al., 2001; Saker et al., 2005). Since RAPD-PCR does not require prior sequence information and an arbitrary chosen short primer is used at lower annealing temperature than routine PCR to amplify one or multiple DNA segments from genomic DNA, a large number of polymorphic DNA markers may be easily generated.

The similarity matrix is based on Jaccard's similarity coefficients presented in Table 1. The similarity coefficients ranged from 0.333 to 0.651. Maximum similarity (65.1%) was observed between PK G-3 and PK G-4 and

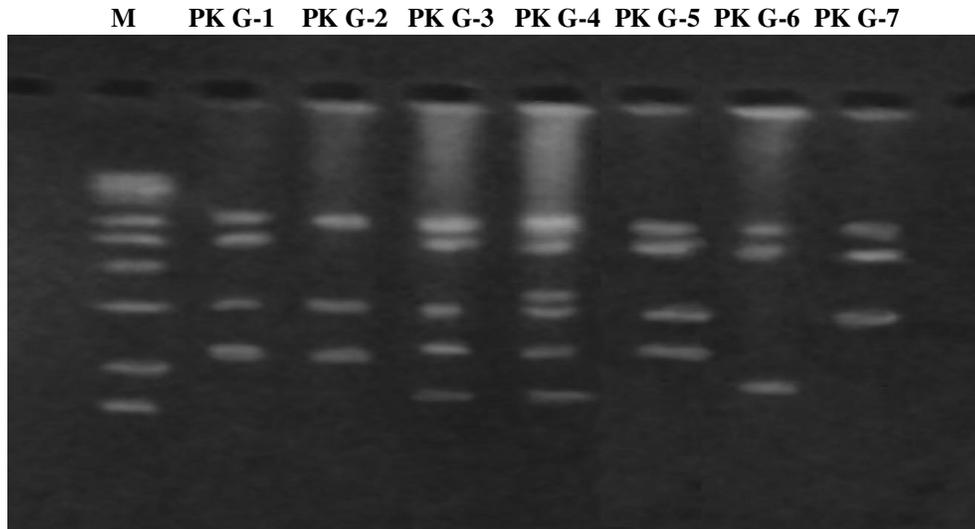


Figure 3. RAPD generated from seven cultivars of chickpea by using primer OPA-04, M is 1kb ladder.

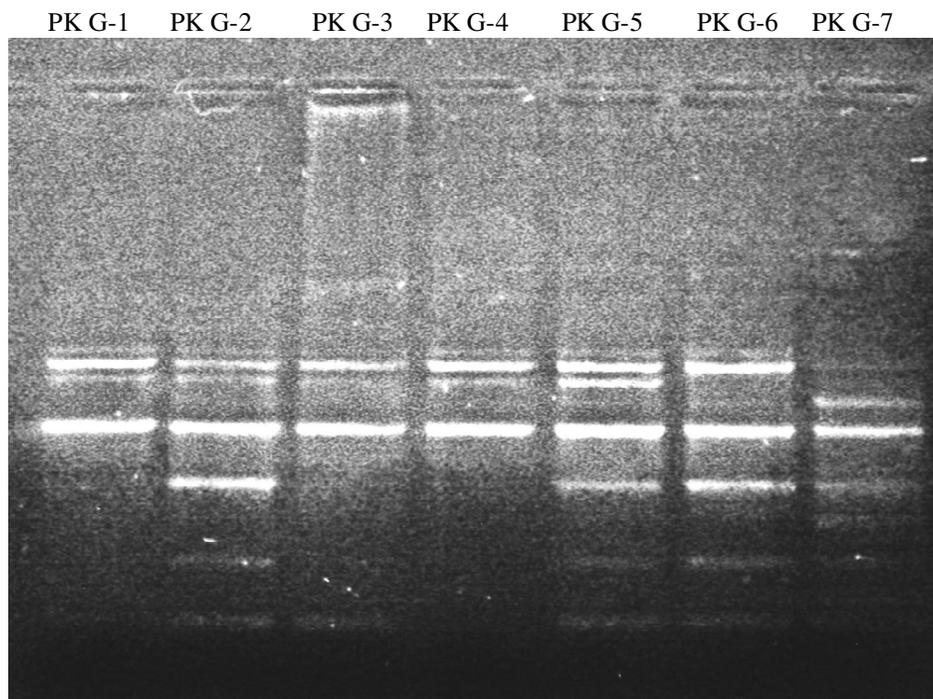


Figure 4. RAPD generated from seven cultivars of chickpea by using primer OPA-07.

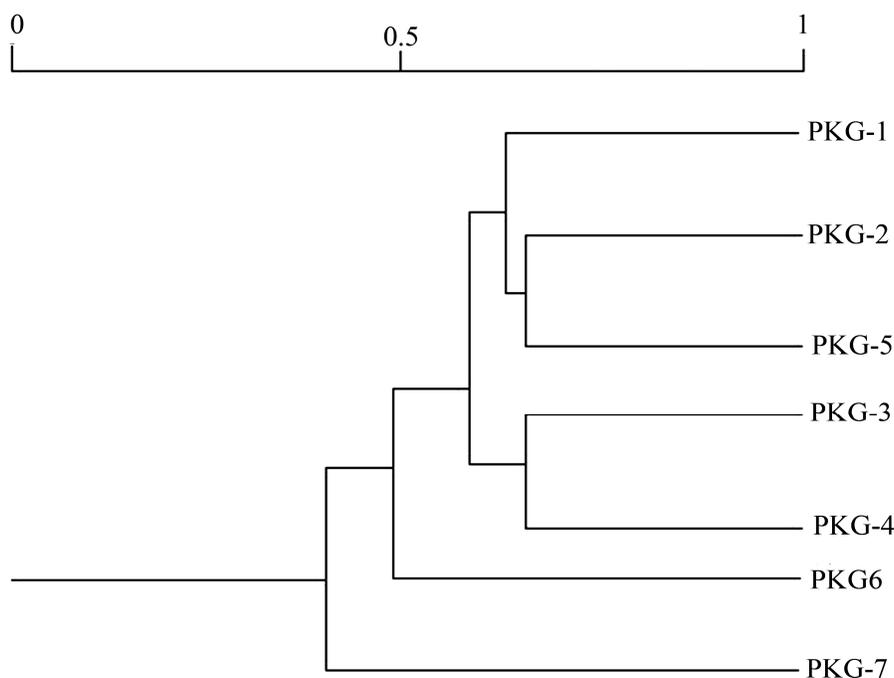
lowest similarity (33.3%) was observed between PK G-3 and PK G-7. The values of similarity coefficients in the present study are almost similar to the observation of Rasul et al. (2007) who reported similarity coefficient ranged of 0.36 to 0.86, with an average similarity value of 0.72 among the teasel gourd cultivars and this value indicated a narrower genetic variability in the improved varieties. The results are also similar to the results of

Talebi et al. (2008a,b) who reported that the average polymorphic information content (PIC) was 0.43, ranging from 0.68 to 0.12 among elite lines of chickpea.

Genetic erosion and habitat destruction by modern agriculture has increased the significance of collection and preservation of plant germplasm. In many countries, the landraces carrying a vast amount of genetic diversity were distributed in remote villages. The number of land-

Table 1. Similarity coefficients of seven chickpea using Jaccard's analysis.

Cultivar	PK G-1	PK G-2	PK G-3	PK G-4	PK G-5	PK G-6	PK G-7
PK G-1	1						
PK G-2	0.619	1					
PK G-3	0.556	0.644	1				
PK G-4	0.512	0.643	0.651	1			
PK G-5	0.634	0.651	0.553	0.545	1		
PK G-6	0.468	0.521	0.500	0.400	0.532	1	
PK G-7	0.435	0.429	0.333	0.340	0.468	0.449	1

**Figure 5.** UPGMA clusters analysis-based dendrogram depicting genetic relationships among seven chickpea cultivars.

ances began to decline in 1970s when high-yielding varieties were introduced (Berger et al., 2003). Most of the old landraces are now available in certain gene banks only, not in the hands of farmers. Wide spectrum of a biological and economical index could be useful for the modeling of varieties corresponding to modern requirements and conditions.

The dendrogram based on similarity coefficients was constructed by using the unweighted pair group of arithmetic means (Figure 5). Seven cultivars clustered into two distinct groups (1 and 2) and two cultivars PK G-6 and PK G-7 stand separately in the dendrogram. The positions of PK G-6 and PK G-7 are both present at the end of the dendrogram and showed maximum variation with the rest of the cultivars. This shows that large amount of genetic variation exists among chick pea genotypes and this can be utilized in the breeding program to develop high yielding cultivars.

Conclusion

The general conclusion from this study is that RAPD profiling is efficient in revealing usable level of DNA polymorphisms among the cultivars of chickpea. These results promote the initiative to integrate these RAPD markers in chickpea breeding applications and DNA fingerprinting. Limited evaluation of chickpea lines in this study led to the identification of genetic variation, which exists in the accessions. It is expected that when such diverse lines are involved in breeding programs, as a result of reshuffling of the alleles due to recombination, there are better chances for the appearance of transgressive segregation with beneficial traits that can be selected to extract high yielding lines with desirable trait combination. Further, large amount of genetic variation which exists between chickpea genotypes can be used efficiently for gene tagging and genome mapping of crosses to

introgress the favorable traits such as high yield potential, disease and insect resistance into the cultivated genotypes.

It may be inferred from the results that RAPD profiling is efficient in revealing useable level of DNA polymorphism among the cultivars of chickpea. By this study, we can show how much diversity and similarity level is present in seven cultivars of chickpea. We can also be able to identify genetic makeup of the variety to protect the breeder's right. We can estimate the genetic distance among the cultivars of chickpea and calculate the similarity coefficient among chickpea cultivars. It is suggested on the basis of the present study that chickpea cultivars PK G-3 and PK G-7 will produce higher hybrid vigor when they are utilized in breeding programs because they are genetically distinct from other cultivars of the chickpea and have minimum genetic similarity. Information obtained from this study may be utilized for developing high yielding chickpea cultivars with desirable characters such as disease and insect resistance, nutritionally rich and enhanced vigor.

REFERENCES

- Athar M, Bokhari TZ (2006). Ethnobotany and production constraints of traditional and commonly used vegetables of Pakistan. *J. Vegetable Sci.* 12: 27-38.
- Berger J, Abbo S, Turner NC (2003). Ecogeography of annual wild *Cicer* species: the poor state of the world collection. *Crop Sci.* 43: 1076-1090.
- Cani H, Toker C (2009). Evaluation of annual wild *Cicer* species for drought and heat resistance under field conditions. *Genet. Resour. Crop Evol.* 56: 1-6.
- Collard BCY, Ades PK, Pang ECK, Brouwer JB, Taylor PWJ (2007). Prospecting for sources of resistance to ascochyta blight in wild *Cicer* species. *Australasian Plant Pathol.* 30: 271-276.
- Dasgupta T, Islam MO, Gayen P, Sarkak KK (1987). Genetic divergence in chickpea (*Cicer arietinum* L.). *Exp. Genet.* 3: 15-21.
- Daviewala AP, Chowdari KV, Kumar S, Reddy APK, Ranjekar PK, Gupta VS (2000). Use of three different marker systems to estimate genetic diversity of Indian elite rice varieties. *Genetica*, 108: 269-284.
- Devos KM, Gale MD (1972). The use of random amplified polymorphic DNA markers in wheat. *Theor. Appl. Genet.* 84: 194-200.
- Doyle JJ, Doyle JH (1990). Isolation of plant DNA from fresh tissue. *Focus*, 12: 13-15.
- Duke JA (1981). *Handbook of Legumes of World Economic Importance*. Plenum Press, New York.
- FAO (2006). <http://www.faostat.fao.org>
- Hadrys H, Balck M, Schierwater B (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* 1: 55-63.
- Huisman J, van der Poel AFB (1994). Aspects of the nutritional quality and use of cool season food legumes in animal feed. In: Muehlbauer FJ, Kaiser WJ (eds.). *Expanding the Production and Use of Cool Season Food Legumes*. Kluwer Acad. Publ. Dordrecht, The Netherlands. pp. 53-76.
- Hulse JH (1991). Nature, composition and utilization of grain legumes. In: *Uses of tropical Legumes: Proceedings of a Consultants' Meeting*, 27-30 March 1989, ICRISAT Center. ICRISAT, Patancheru, AP, India, pp. 11-27.
- Iruela M, Rubio J, Cubero JI, Gil J, Millan T (2002). Phylogenetic analysis in the genus *Cicer* and cultivated chickpea using RAPD and ISSR markers. *Theor. Appl. Genet.* 104: 643-651.
- Kerem Z, Lev-Yadun S, Gopher A, Weinberg P, Abbo S (2007). Chickpea domestication in the neolithic levant through the nutritional perspective. *J. Archaeol. Sci.* 34: 1289-1293.
- Kernodle SP, Cannon RE, Scandalios JG (1993). Concentration of primer and template qualitatively affects product in RAPD-PCR. *Biotechniques*, 1: 362-364.
- Mahmood Z, Raheel F, Dasti AA, Saima S, Athar M (2009). Genetic diversity analysis of the species of *Gossypium* by using RAPD. *Afr. J. Biotechnol.* 8: 3691-3697.
- Maniatis T, Fritsch EF, Sambrook J (1982). *Molecular Cloning Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, New York. Muehlbauer FJ, Kaiser WJ (eds.) *Expanding the Production and Use of Cool Season Food Legumes*. Kluwer Acad. Publ. Dordrecht, The Netherlands.
- Neeraja CN, Sarla N, Siddiq EA (2002). RAPD analysis of genetic diversity in Indian landraces of rice (*Oryza sativa* L.). *J. Plant Biochem. Biotechnol.* 9: 93-97.
- O'Neill R, Snowdon RJ, Kohler W (2003). Population genetics aspects of biodiversity. *Prog. Bot.* 64: 115-137.
- Porreca P, Sabina MR, Martelli G, Sunseri F, Greco I, Pruneddu G, Spanu A (2001). Genetic variability among Italian rice (*Oryza sativa* L.) cultivars investigated by RAPDs analysis. *J. Genet. Breed.* 55: 349-355.
- Rasul MG, Hiramatsu M, Okubo H (2007). Genetic relatedness (diversity) and cultivar identification by randomly amplified polymorphic DNA (RAPD) markers in teale gourd (*Momordica dioica* Roxb.). *Sci. Hort.* 111: 271-279.
- Redden B, Berger J (2007). History and origin of chickpea. In: Yadav SS, Redden B, Chen W, Sharma B (eds.). *Chickpea Breeding and Management*. CAB International, Wallingford. pp.1-13.
- Saker MM, Youssef SS, Abdullah NA, Bashandy HS, EL-Sharkawy AM (2005). Genetic analysis of some Egyptian rice genotypes using RAPD, SSR and AFLP. *Afr. J. Biotechnol.* 4: 882-890.
- Singh KB, Ocampo B (1997). Exploitation of wild *Cicer* species for yield improvement in chickpea. *Theor. Appl. Genet.* 95: 418-423.
- Smithson JB, Thompson JA, Summerfield RJ (1985). Chickpea (*Cicer arietinum* L.). In: Summerfield RJ and Roberts EH (eds.). *Grain Legume Crops*, pp. 312-390. Collins, London, UK.
- Sokal RR, Sneath PHA (1963). *Principles of Numerical Taxonomy*: Freeman, San Francisco, California.
- Talebi R, Fayaz R, Mardi M, Pirsyedi SM, Naji AM (2008a). Genetic relationships among chickpea (*Cicer arietinum*) elite lines based on RAPD and agronomic markers. *Int. J. Agric. Biol.* 8:1560-8530.
- Talebi R, Naji AM, Fayaz F (2008b). Geographical patterns of genetic diversity in cultivated chickpea (*Cicer arietinum* L.) characterized by amplified fragment length polymorphism. *Plant Soil Environ.* 54: 447-452.
- Tanno K, Willcox G (2006). The origins of cultivation of *Cicer arietinum* L. and *Vicia faba* L. early finds from Tell el-Kerkh, north-west Syria, late 10th millennium B.P. *Veg. Hist. Archaeobot.* 15: 197-204.
- Toker C (2009). A note on the evolution of kabuli chickpea as shown by induced mutations in *Cicer arietinum* Ladizinsky. *Genet. Resour. Crop Evol.* 56: 7-12.
- Upadhyaya HD, Bramel PJ, Singh S (2001). Development of a chickpea core subset using geographic distribution and quantitative traits. *Crop Sci.* 41: 206-210
- Weising K, Ramser J, Kaemmer D, Kahl G, Epplen JT (1991). Oligonucleotide fingerprinting in plants and fungi, pp. 312-329. In: Burke T, Dolf G, Jeffreys AJ and Wolff R (eds.). *DNA Fingerprinting: Approaches and Applications*, pp. 312-390. Birkhauser Verlag, Basel, Switzerland.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.