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# Characterization of chickpea (*Cicer arietinum* L.) genotypes through morphological and RAPD analysis

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**Abstract**
Fifteen Chickpea (*Cicer arietinum* L.) genotypes were evaluated for genetic diversity using morphological traits and RAPD markers. Eight morphological traits were studied and subjected to analysis of variance. Mean squares due to genotypes were highly significant as well as wide mean range performance was observed for germination percentage, plant height, effective pods per plant, test weight and seed yield. Ward's cluster analysis based on morphological traits separated the accessions into two groups. RAPD analysis was carried out with 16 primers. They were screened but only 11 primers produced amplification. These 11 primers produced 76 bands, in which 64 were polymorphic bands. Average polymorphism was 84.2 percent. Based on the RAPD markers, a dendrogram was constructed using the UPGMA method. The similarity coefficient ranged from 0.51 to 0.88 with an average of 0.70. Cluster analysis based on RAPD data separated the accession into five main groups. The trends of genotypes relationship amongst the chickpea determined by RAPDs were consistent with their morphological traits.

**Keywords:** Chickpea, genetic diversity, morphological traits, RAPD

## Introduction
Chickpea (*Cicer arietinum* L.) is an edible legume belongs to family Fabaceae, subfamily Faboideae and commonly known as "Bengal gram". Chickpea with 17-24% proteins, 41-50.8% carbohydrates, high percentage of mineral nutrients and unsaturated linloeic and oleic acid, is one of the most important crops for human consumption (Kerem *et al*., 2007) [19]. 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) [7]. As chickpea has high nutritive value, it is popularly said as "meat" of the poor. Recent studies by government agencies have shown that their consumption can assist in lowering of cholesterol in the blood stream. India is the largest producer of chickpea in the world but its productivity is very low when compared with cultivation in other countries like Italy, Iran, and Turkey. In India, chickpea is grown on about 6.67 million ha area producing 5.3 million tonnes, which represents 30% and 38% of the national pulse acreage and production, respectively. Chickpea production has gone up from 3.65 to 5.63 million tonnes between 1950-51 and 2004-05, registering a growth of 0.58% annually. In India, Rajasthan, Madhya Pradesh, Uttar Pradesh, Gujarat, Andhra Pradesh, Karnataka, Haryana and Bihar are the major chickpea growing states.
Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. It has been shown that different markers might reveal different classes of variation (Bayraktar *et al*., 2007; Powell *et al*., 1996) [6, 26]. It is correlated with the genome fraction survived by each kind of marker, their distribution throughout the genome and the extent of DNA target which is analysed by each specific assay (Davila *et al*., 1999) [11]. Molecular markers are practically unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant (Winter and Kahl, 1995) [39]. Various molecular markers are being used for fingerprinting such as restriction fragment length polymorphism (RFLP) (Dubreuil and Charcosset, 1998) [13], random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) [38], microsatellites (Smith *et al*., 2000) [30] and amplified fragment length polymorphism

(AFLP) (Agrawal *et al*., 1999) [1]. The enormous attraction of RAPDs is because of there is no requirement for DNA probes, or any sequence information for the design of specific primers. The procedure involves no blotting or hybridizing steps. RAPD markers offer many advantages such as higher frequency of polymorphism, rapidity (Fahima *et al*., 1999) [16], technical simplicity, requirement of a few nanograms of DNA, no requirement of prior information of the DNA sequence and feasibility of automation (Subudhi and Huang, 1999) [32]. RAPD technique which was developed by Williams *et al*. (1990) [38] has been widely applied in either identification of cultivars, analysis of seed purity or estimating genetic relationships and diversity among crop germplasm (Crockett *et al*., 2000; Chowdhury *et al*., 2017; Baruah *et al*., 2017) [9, 8, 5]. Primers can also assessed capable of generating high polymorphism, higher polymorphic information content (PIC) values and higher marker index.

## Materials and Methods

Present investigation was conducted on 15 chickpea genotypes viz., C-201,C-204,C-207,C-212,C-213,C-216,C-217,C-218,C-219,C-222,C-224,C-225,C-228,C-230 and LC (Table 1). All the facilities related to present study were made available by the Department of Molecular Biology and Biotechnology and Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, MPUAT, RCA, Udaipur.

**Table 1:** List of the 15 different chickpea genotypes with their pedigree used in this study

| S. No. | Entry | Pedigree | Source | S. No. | Entry | Pedigree | Source |
|---|---|---|---|---|---|---|---|
| 1. | C-201 | ICCV 88506 x ICCV 90201 | Dhaulakuan | 9. | C-219 | (JAKI 9226 x DCP 20) x JK 412 | Sehore |
| 2. | C-204 | C 235 x ICCV 90201 | Dhaulakuan | 10. | C-222 | ICCV 10 x ICCL 87322 | Rahuri |
| 3. | C-207 | ICCC 37 X K 1189 | Jabalpur | 11. | C-224 | GNG 1490 x SG 703 | Sriganga nagar |
| 4. | C-212 | KPG 143-1 X IPC 92-1 | IIPR, Kanpur | 12. | C-225 | Dahod yellow x ICCV 2 | Banswara |
| 5. | C-213 | (ICCV 91902 x ICCV 10) x ICCV 89230 | Sehore | 13. | C-228 | GJG 9707 x IPC 97-7 | Junagarh |
| 6. | C-216 | KWR 108 x JG 315 | IIPR, Kanpur | 14. | C-230 | GNG 1477 x CSG 8962 | Sriganga nagar |
| 7. | C-217 | GCPZ x ICCV 2 | IIPR, Kanpur | 15. | LC* | Pratap Chana | MPUAT Udaipur |
| 8. | C-218 | ICCV 10 x ICCL 87322 | Rahuri | | | | |

* Local Check genotype.

### DNA isolation, quality testing and quantification

DNA extracted from the 15 chickpea genotypes were compared by RAPD marker analysis. In this methodology, DNA was extracted from young leaves (3 weeks old plantlets) using CTAB method (Doyle and Doyle 1990) [12]. DNA was amplified by using random oligonucleotide primers in a DNA thermo cycler (Eppendorf). The amplified samples were separated on agarose gel electrophoresis (1.2%). The bands were scored for their presence or absence. DNA samples were quantified in nano-spectrophotometer. All chemicals used in DNA isolation and PCR technique were of analytical grade and purchased from Hi-media.

### Optimization of PCR conditions and RAPD analysis

Random amplification of polymorphic DNA was done by using 15 primers obtained from Bangalore Genei Pvt. Ltd., Bangalore. PCR reaction was performed in a final volume of 20 µl containing 10X Reaction Buffer, 1 unit of Taq. DNA polymerase, 200 µM each of dNTPs mix, 0.5 µM/reaction of random primers and 50 ng of template DNA. The PCR was performed in PCR eppendorf thermo cycler using the following cycling parameters: Initial denaturation step at 94 ºC for 5 min followed by 44 cycles at 94 ºC for 1 min, annealing at 36 ºC for 1 min and extension at 72 ºC for 2 min and final extension for 5 min at 72 ºC. The amplified products were separated by 1.2% agarose gel electrophoresis containing 0.5µ/ml ethidium bromide. The gel was viewed under UV transilluminator and photographed with the help of gel documentation system (Alpha DG DOC). A 100 bp DNA ladder was included in the gel as standard molecular weight marker. A set of 15 decanucleiotide RAPD primers were used for PCR amplification. The sequences of primers were selected from literature and purchased from Genei Pvt Ltd, Bangalore. Scoring of the RAPD products were done as-The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored, but a major band corresponding to faint bands was considered for scoring. The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient. Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationship of the genotypes using computer program NTSYS pc version 2.02 (Rohlf, 1997) [28].

### Morphological Characters Under Investigation

The experiment was laid out in randomized block design with three replications. Each entry was planted in 3 rows of 4 m length with a spacing of 30 x 10 cm. All the recommended agronomic practices and plant protection measures were adopted to raise a healthy crop. Characters under investigation were [1] Days to 50 percent flowering [2], Days to maturity [3], Germination Percentage [4] Plant height (cm) [5], Number of effective pods per plant [6], Test Weight in gram (The weight of 100 seeds) [7] Seed yield (kg/ha) and [8] Total Protein content. The observations were recorded on 5 randomly selected plants for each entry (genotype), from all three replications. Total Protein content (Snell and Snell, 1955) [34] of the seeds were examined as follows: 100 mg of chickpea seeds were digested in concentrated $H_2SO_4$ in Kjeldahl flask. 0.5 ml of 30 percent H2O2 was added to support complete digestion till the disappearance of colour. Volume was made upto 100 ml with distilled water after cooling. 5.0 ml of aliquot was mixed with 2.0 ml NaOH (10%, w⁄v) and 1.0 ml sodium metasilicate (10%, w⁄v) and diluted to 45.0 ml with distilled water. To it 1.6 ml of Nessler's reagent was added and final volume was brought to 50.0 ml with distilled water. Absorbance at 540 nm was recorded.

## Results and Discussion

In the present studyan attempt had made to estimate various morphological parameters, which would help in framing an

effective breeding programme. Isolated and purified DNA was subjected to PCR based marker (RAPD) for assessment of genetic diversity at molecular level. DNA fingerprinting has become essential for evaluation and identification of crop germplasm, since this technological intervention is more precise, economical and least affected by the environmental factors. Work on both morphological and molecular marker was already done by Talebi *et al.* (2008) [36] in chickpea, Ali *et al.* (2007) [3] in pea, Shrivastava *et al*. (2008) [29] in oat, Subramanian *et al.* (2000) [31] in groundnut and Mahasi *et al.* (2010) [21] in safflower. This was a complementary approach using information from both morphological traits and RAPDs, shown to generate more accurate estimates of genetic diversity and of relationships between genotypes, than either data set alone.

**Analysis of experimental design**

The data of 8 morphological characters were subjected to analysis of variance for Randomized Block Design (RBD). The mean squares due to genotypes were significant for all the traits thereby indicating substantial amount of variability among the genotypes. The mean squares due to replications were also significant for plant height, No. of effective pods per plant and test weight (Table 2).A perusal of mean performance revealed that narrow mean range was found for the characters such as days to 50% flowering (61.33-68.67), total protein content (18.10-22.77) and days to maturity (91.33-102.33). Moderate mean range was found for characters such as test weight (12.46-26.85) and germination percentage (73.33-93.33), while most of the characters *viz.* plant height (46.33-68.33cm), seed yield (1284.70-1805.53) and number of effective pods per plant (53.33-113) had wide mean range. The number of effective pods per plant, plant height and seed yield had high value of variance indicating that the diversity existed, which can contribute to improvement of the crop. Similar results were observed by Ali *et al.* (2007) [3] for a set of pea (*Pisumsativum* L.) germplasm showing the consistency of the traits in the germplasm. The average protein content was 20.80 per cent and ranged from 18.10 -22.77 per cent. The genotype C-217 (22.77%) was found most superior in protein content, followed by C-201 (22.17%). The genotype C-228 (18.10%) had minimum protein content. Similar results were reported by Esmat *et al.* (2010) [15] and theirprotein content ranged from 17 to 21.56 per cent.

**Table 2:** Analysis of variance of chickpea genotypes for 8 characters

| Sr. No. | Characters | Replication | Genotype | Error |
|---|---|---|---|---|
| | **Degree of Freedom** | **(2)** | **(14)** | **(28)** |
| 1 | Days to 50% Flowering | 5.49 | 21.09** | 3.04 |
| 2 | Days to Maturity | 0.60 | 26.23** | 5.89 |
| 3 | Germination Percentage (%) | 60.00 | 132.38* | 55.24 |
| 4 | Plant Height (cm) | 22.16** | 156.69** | 13.44 |
| 5 | Number of effective pods per plant | 171.27** | 955.48** | 26.67 |
| 6 | Test Weight (g) | 7.09** | 51.84** | 2.12 |
| 7 | Seed Yield (kg/ha) | 630.84 | 59973.09** | 9174.09 |
| 8 | Total Protein content (%) | 0.10 | 4.33** | 0.19 |

*Values significant at 5%

** Values significant at 1%

**Classifying the Genotypes using Ward's Cluster Analysis (On the basis of morphological characters)**

Ward's hierarchical cluster analysis was carried out on the basis of 8 morphological characters. It was used to measure genetic distance between 15 chickpea genotypes (Fig. 1). Cluster analysis grouped the genotypes into two clusters, cluster I and II were apart at 25 rescaled values. Cluster analysis was found useful by Malik *et al.* (2010) [23] in grouping chickpea genotypes.

Cluster I included 13 genotypes. This cluster was further subdivided into two sub clusters, A and B at 13 rescaled values. Sub cluster A included eight genotypes which was further divided into two groups A' and A". Group A' included six genotypes with similar germination percentage, no. of effective pods per plant, seed yield and protein content. Whereas days to 50 per cent flowering, days to maturity, plant height and test weight were significantly different in these genotypes (CD at 5%). Group A" included two genotypes having days to maturity, germination percentage, seed yield and protein content at par for both genotypes. Whereas difference was significant for days to 50 percent flowering, plant height, number of effective pods per plant and test weight. Sub cluster B included five genotypes, days to maturity, germination percentage, seed yield and protein content were at par for these genotypes. Whereas difference was significant for days to 50 percent flowering, plant height, number of effective pods per plant and test weight. Cluster II included two genotypes having similardays to maturity, plant height, test weight, seed yield and protein content. Whereas days to 50 percent flowering, germination percentage and no. of effective pods per plant were significantly different in these genotypes (CD at 5%).

The results were in conformation with observation of Talebi *et al.* (2008) [36]. They studied genetic relationships among 36 accessions of chickpea. Cluster analysis based on morphological traits separated the accessions into 3 clusters (Sultana *et al*., 2006) [33]**.** Lentil genotypes used intheir study were grouped in three clusters (I, II and III), comprised of 6, 6 and 5 genotypes, respectively**.** Cluster analysis indicates the extent of genetic diversity that is of practical use in plant breeding.

**Assessment of amplified fragments obtained from RAPD**

All the 15 chickpea genotypes were examined for DNA polymorphism using 16 oligonucleotide primers showing high (G+C) content. Out of 16 primers, 11 primers produced amplification whereas 5 primers *viz.* OPA-01, OPC-08, RKAT-2, RKAT-8 and RKAT-14 did not show any amplification. Out of 11, all the primers showed variable degree of polymorphism ranging from 25 per cent (RKAT-4) to 100 per cent [OPK-09, OPK-9(C), RKAT-5 and OPJ-04]. Overall polymorphism was found to be 84.21 per cent. Similar results were reported by Tahir *et al.* (2011) [35]. They reported that 5 primers produced polymorphic amplification.

On average, 5.8 bands per primer were observed by RAPD, the varieties shared 55.17% polymorphic bands. The DNA amplification and polymorphism generated among various Chickpea genotypes using random primers are presented in Table 2.

The maximum number of amplicons was produced by the primers OPK9(c) and OPJ-04. Band pattern with primer OPK-09 is shown in figure 1. The minimum number of amplicons was produced by the primer RKAT-4 and OPK-10. Among all the primers tested, OPK9(c) and OPJ-04 proved to be the best primers as scorable bands were polymorphic with average polymorphism 100 percent. The results obtained were in conformity with the earlier reported by Mahmood *et al.* (2011) [22] and Talebi *et al.* (2008) [36]. Thus, it is opined that RAPD assays can be efficient in identifying DNA polymorphism provided suitable primers are used.

**Table 2:** DNA Polymorphism generated using 16 RAPD Primers in 15 chickpea genotypes

| S. No. | Primers code | Sequences (5'→3') | Total No. of bands (a) | Total No. of polymorphic bands (b) | Polymorphism % (b/a × 100) |
|---|---|---|---|---|---|
| 1. | OPA-01 | CAGGCCCTTC | NA | NA | NA |
| 2. | OPC-08 | TGGACCGGTG | NA | NA | NA |
| 3. | OPK-09 | GTGGTCCGCA | 9 | 9 | 100 |
| 4. | OPK9(A) | CCCTACCGACA | 6 | 5 | 83 |
| 5. | OPK9(C) | CCCTACCGACC | 10 | 10 | 100 |
| 6. | RKAT-2 | CAGGTCTAGG | NA | NA | NA |
| 7. | RKAT-4 | TTGCCTCGCC | 4 | 1 | 25 |
| 8. | RKAT-5 | ACACCTGCCA | 8 | 8 | 100 |
| 9. | RKAT-6 | CCGTCCCTGA | 6 | 5 | 83 |
| 10. | RKAT-8 | TCCTCGTGGG | NA | NA | NA |
| 11. | RKAT-9 | CCGTTAGCGT | 5 | 4 | 80 |
| 12. | RKAT11 | CCAGATCTCC | 7 | 6 | 86 |
| 13. | RKAT12 | CTGCCTAGCC | 7 | 4 | 57 |
| 14. | RKAT14 | GTGCCGCACT | NA | NA | NA |
| 15. | OPK-10 | GTGCAACGTG | 4 | 2 | 50 |
| 16. | OPJ-04 | CCGAACACGG | 10 | 10 | 100 |
| | | **TOTAL** | 76 | 64 | 84.21 |

NA – Not amplified

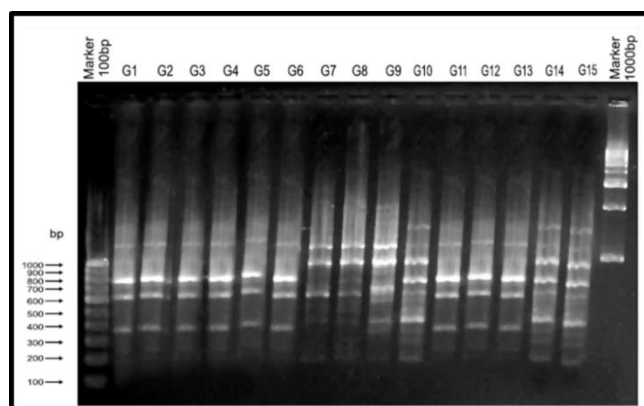


**Fig 1:** RAPD profile of chickpea genotypes (G1-G15) generated with primer OPK-09

**Assessment of relationship between genotypes based on chickpea morphological characters and cluster analysis based on RAPD**

The banding pattern generated and polymorphism reflected through RAPD was used to calculate the genetic similarity among the 15 chickpea genotypes taken for the present study. Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard's coefficient analysis (Jaccard, 1908). The similarity coefficient matrix generated was subjected to algorithm UPGMA and dendrogram was generated using NTSYSpc 2.02 program (Rohlf, 2004) (Fig. 2).

The RAPD data were used to obtain a similarity matrix. The similarity coefficients for different genotypes ranged from 0.51 to 0.88. The average similarity across all the genotypes was found to be 0.70 indicating a high level of genetic similarity among the genotypes. The maximum similarity coefficient (0.88) was observed between C-212 and C-213, C-224 and C-225, followed by C-207 and C-212 and C-213 to C-216 with the similarity of 0.87 and 0.86, respectively. The minimum similarity coefficient (0.51) was observed between C-201 and C-230 and C-201 and LC. The results obtained were in accordance with the earlier reported by Datta and lal (2011) [10]. A total of 121 amplicons ranging from 0.2 kb to 11 kb was obtained with an average polymorphism of 87%. The number of amplicons per primer varied from 4 (RKAT-4 and OPK-10) to 10 (OPK9(C) and OPJ-04); each primer generated an average of 6.9 RAPD markers. The similarity coefficients based on 694 RAPD amplicons ranged from 0.51 to 0.88. Genotypes C-201 and C-230 showed the lowest similarity index (0.30) while genotypes C-224 and C-225 showed highest similarity index (0.96) with RAPD primers. Similar results were also reported by Dongre *et al.* (2004) [14]

Cluster I included two genotypes *viz.* C-201 (G1) and C-204 (G2) at similarity coefficient of 0.802. Out of 8 morphological characters, 5 *viz.* days to 50 per cent flowering, days to maturity, No. of pods per plant, seed yield and protein content were similar in these genotypes. Similar result was reported by Wang *et al.* (2011) [40]. They studied RAPD based genetic diversities and correlation with morphological character in *Camellia* (*Theaceae*) cultivars in China. Cluster II which was the major cluster included four genotypes *viz.* C-207 (G3), C-212 (G4), C-213 (G5) and C-228 (G13). Cluster II was divided into two subclusters, A and B. Sub cluster A included three genotypes *viz.* C-207 (G3), C-212 (G4) and C-213 (G5) which were related to each other at similarity coefficient of 0.837. Sub cluster A was further sub divided into subgroups A' and A''. A' included only one genotype C-207 (G3). Second sub group A'' included two genotypes *viz.* C-212 (G4) and C-213 (G5) at similarity coefficient 0.88 and morphologically also both were similar. Out of eight morphological characters, five *viz.* Days to 50% flowering, Days to maturity, Germination percentage, Test weight and protein contents were similar in these two genotypes. Sub cluster B included only one genotype *i.e.* C-228 (G13) which

joined with sub cluster A at similarity coefficient 0.78.Cluster III included three genotypes *viz.* C-216 (G6), C-224 (G11) and C-225 (G12) at similarity coefficient of 0.822. Cluster III joined cluster II at similarity coefficient of 0.757. Cluster III was divided into two sub clusters, A and B. Sub cluster B included two genotypes *i.e.* C-224 (G11) and C-225 (G12), with similarity coefficient of 0.88. Out of 8 morphological characters, 4 *viz.* days to 50 per cent flowering, test weight, seed yield and protein content were similar in these genotypes. Sub cluster A included only one genotype C-216 (G6) which joins cluster B at similarity coefficient 0.822. C-216 (G6) showed much morphological divergence from C-224 (G11) and C-225 (G12).

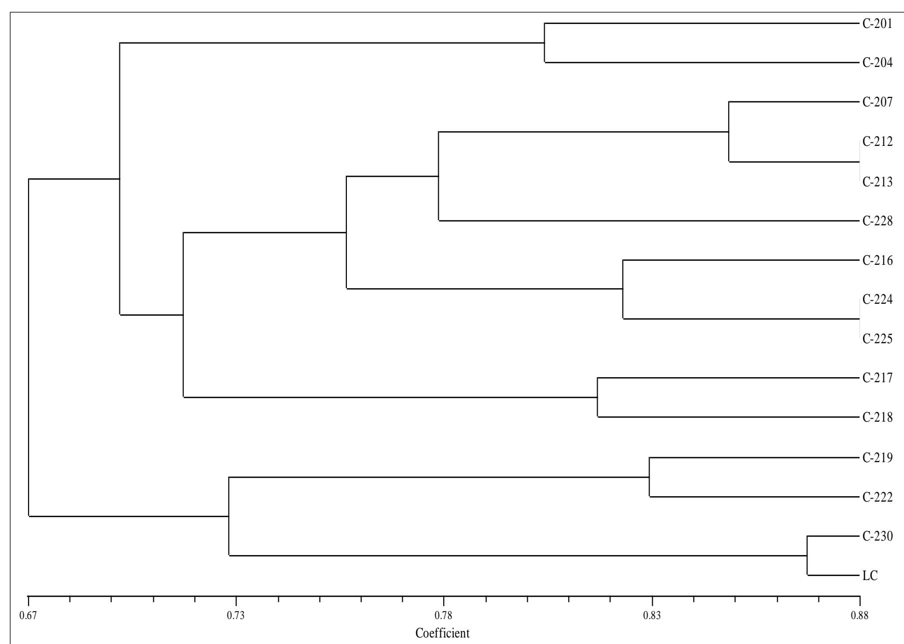


**Fig 2:** Dendrogram generated for fifteen chickpea genotypes using UPGMA cluster analysis based on Jaccard similarity coefficient

Cluster IV included two genotypes *i.e.* C-217 (G7) and C-218 (G8). Cluster V which was one of the major clusters included four genotypes *viz.* C-219 (G9), C-222 (G10), C-230 (G14) and LC (G15). Cluster V was divided into two subclusters, A and B. Subcluster A included two genotypes *viz.* C-219 (G9) and C-222 (G10) at similarity coefficient of 0.83 and they were similar in four morphological characters out of eight viz. days to 50% flowering, germination percentage, no. of effective pods per plant and protein content. Sub cluster B included two genotypes *viz.* C-230 (G14) and LC (G15) at similarity coefficient 0.877. Subcluster A joined Subcluster B at similarity coefficient 0.729. Cluster V joined rest of the clusters at similarity coefficient 0.692. Therefore, the cluster tree, revealed a similar result about 15 genotypes. The association amongst different genotypes was presented in the form of dendrogram, the genotypes which were lying close to each other in the dendrogram were genetically closer to each other than those lying apart.

In general, there was an association between the dendrogram obtained by RAPD markers and morphological characteristics. This was in accordance with the results reported by Nebauer *et al.* (2000) [24] in the genus *Digitalis***,** Parentoni *et al.* (2001) [25] and Thakur *et al.* (2008) [37] in maize, Raza *et al.* (2018) [27] in Sunflower.

Hence, study at morphological and molecular level, comprising fifteen chickpea genotypes showed that the variation at morphological level was more. Some genotypes categorized in same group showed different morphological character indicating the impact of environment in the expression of characters. Thus RAPD marker is a good indicator of morphological divergence. Hence, it is recommended that genetically distant lines observed among the 15 genotypes of chickpea, should be used in future breeding programmes for improving yield and quality characteristics. Further, it was observed that PCR based assay like RAPD can be used effectively to estimate genetic variability of chickpea and considering easy handling of the technique, they are especially suitable for breeding programmes, where large number of lines/accessions have to be analyzed. Therefore, it could be concluded that RAPD profiles were more efficient in detecting polymorphism and distinguishing genotypes at varietal and species level as well as successfully used for identification and phylogenetic relationship among and within the species.

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