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Vinay Kumar
Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Parades, India

Vinay Kumar
Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Parades, India

SK Singh
Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Parades, India

Pooran Chand
Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Parades, India

Corresponding Author:
Vinay Kumar
Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Parades, India

Genetic diversity analysis in chickpea (*Cicer arietinum*) genotypes using morphological and SSR markers

Vinay Kumar, Mukesh Kumar, SK Singh and Pooran Chand

Abstract
Genetic diversity and relationships of 37 chickpea genotypes were studied using 10 SSR markers and 10 morphological characters. High diversity and coefficients of variation were recorded for all morphological characters. Considerable diversity was observed with high PCV in comparison to GCV. High heritability (>80%) was observed for most of the characters like 100-seed weight followed by number of seeds per pod, number of pods per plant, harvest index, biological yield per plant and number of primary branches per plant. The moderate (60-80%) heritability revealed by grain yield per plant and plant height. The analysis of genetic divergence through Mahalanobis D^2 statistics revealed considerable genetic diversity among genotypes. PIC values ranged from 0.053 (Primer 7) to 0.876 (primer 4) with an average of 0.497. The resolving power (RP) varies between 0.702 (Primer 4) to 1.942 (Primer 7) with an average value of 1.311. Results showed that the introduction of genetic materials from exotic sources broadened the genetic base of the national chickpea breeding programme. Further implications of the findings of this study can be useful for selective breeding of specific traits and in enhancing the genetic base of breeding programmes.

Keywords: Chickpea, morphology, SSR markers, genetic diversity

Introduction
Chickpea (*Cicer arietinum* L.) is a cool season grain legume with high nutritive value and is the third most important pulse crop in the world after soybean and beans. In India, chickpea cultivated on 8.19 million hectares area and production contributed 7.17 million tonnes with the productivity of 875.30 kg/ha in 2014-2015. In U.P, chickpea production was 475.45 tons from an area of 558 hectare with the productivity 852.1 kg/ ha (Anonymous, 2015). It is cultivated throughout the country except in high altitudes and coastal region. In addition to being a major source of dietary protein for humans in semiarid tropical regions, chickpea plays an important role in the maintenance of soil fertility, particularly in dry rain fed areas (Choudhary *et al.* 2012) [4]. For effective utilization of germplasm collections in breeding programmes, genetic characterization in terms of measure of the extent and pattern of genetic diversity within and between populations (Rubenstein *et al.* 2005) [22] is essential. This characterization is not only to unveil the magnitude of genetic diversity available in the germplasm for conservation purposes, but also to determine genes useful for possible progress in future breeding programmes. Screening and selection would more likely result in better and promising genotypes if germplasm sources were genetically diverse (Keneni *et al.* 2011) [14].
Genetic characterization can be made by different methods, ranging from conventional methods like the use of descriptor lists of morphological characters, as well as biochemical and molecular methods (Carvalho 2004; de Vicente *et al.* 2005; Keneni *et al.* 2011) [2, 5, 14]. Morphological characters are the strongest determinants of the agronomic value and taxonomic classification of plants. Compared with other methods, morphological evaluations are direct, inexpensive and easy. However, error scanarise; furthermore, morphological estimations are more dependent on the environment (Jannatabadi *et al.* 2014) [12]. Additionally, some genetically related cultivars are morphologically very similar and it is difficult to distinguish between them by visual comparison. Also, genetically distant material can show very similar morphology due to cultivation selection/ pressure.
DNA analysis could help to differentiate genotypes accurately and may be used in cultivar identification (Castro *et al.* 2011) [3]. For chickpea, various marker systems such as amplified fragment length polymorphism (AFLP; Talebi *et al.* 2008b) [26], random amplified polymorphic DNA (RAPD; Talebi *et al.* 2008a) [26] and microsatellite markers like simple-sequenced repeats (SSR) or sequenced tagged microsatellite sites (STMS; Saeed *et al.* 2011; Keneni *et al.* 2011; Ghaffari *et al.* 2014) [23, 14, 10] have been used for diversity analysis.

The present study was aimed to characterize different germplasm of chickpea by the use of microsatellite and morphological markers, as well as to determine the potential utility of these markers for cultivar characterization.

Materials and Methods

Plant material and field evaluation

Thirty seven chickpea (*Cicer arietinum* L.) accessions from different geographical locations were considered for the study of genetic variation using morphological and SSR markers (Table 1). Field experiments were laid out in randomized complete block design with three replications in 2015 – 2016. Experiment was sown on in three row plot of four meter length. The row to row and plant to plant distance maintain at 30 and 10 cm respectively. Five plants were randomly chosen from each plot to measure the Days to 50% flowering, Days to maturity, plant height, Number of primary branches per plant, number of pods per plant, number of seeds per pod, biological yield per plant, harvest index, 100-seed weight and seed yield per plant.

Diversity analysis by morphological markers

Analysis of variance

The mean values of genotypes in each replication were used for statistical analysis. The data were analyzed for a randomized block design to test the significance of differences between the genotypes for various characters. The steps involved in the analysis of the randomized block design were as described by Panse and Sukhatme (1969) [19].

Genetic divergence through D^2 analysis and molecular markers

The genetic divergence in forty five genotypes was estimated using Mahalanobis D^2 statistic (1936) following Rao (1952) [21].

DNA extraction and SSR analysis

Total genomic DNA was extracted from 2 g fresh leaves of each genotype following a CTAB extraction protocol by Doyle and Doyle (1987) [7]. A total of 10 SSR markers were screened in the genotypes. The primers were dissolved in appropriate amount of 1 X TE buffer according to the concentration of supplied primers.

SSR markers used in this study were developed by Winter *et al*. (2000) [29] and distributed through the all linkage groups of the chickpea genetic linkage map. PCR was performed in a total reaction volume of 20 µL containing 1U Taq DNA polymerase (Bangalore Genei) 10 mMTris–HCl pH8.0 (Himedia), 50 mM NaCl (Himedia), 1.5 mM MgCl2, 0.25 mM of each dNTPs (Bangalore Genei), 10 pmol of each primer and 20 ng of template DNA, using a Eppendorf ThermoCycler. Amplifications were programmed for an initial step at 94 °C for 5 min, followed by 38 cycles of denaturation at 94 °C for 1 min, annealing at the required Tm for 1 min and elongation at 72 °C for 2 min, followed by a final elongation step at 72 °C for 10 min.

PCR products were analyzed using 2% Methaphor agarose electrophoresis gels stained with ethidium bromide. Frequencies of incidence of all polymorphic alleles for each SSR markers were calculated and used for determining statistical parameters. Number of alleles, effective number of alleles, gene diversity and polymorphism information content were calculated by GENALEX 6.1 software (Peakal, Smouse 2006) [20].

Table 1: Details of 37 chickpea genotypes tested

Sr. No.	Germplasm	Source
1	ICCV-14510	JNKVV, Jabalpur
2	BG-362	IARI, New Delhi
3	Pusa-1053	IARI, New Delhi
4	BGD-72	IARI, New Delhi
5	Pusa-2024	IARI, New Delhi
6	DCP-92-3	JNKVV, Jabalpur
7	HC-05	JNKVV, Jabalpur
8	C-910	IIPR, Kanpur
9	Pusa-1003	IARI, New Delhi
10	GNG-1958	JNKVV, Jabalpur
11	PKV-4	JNKVV, Jabalpur
12	Pusa-256	IARI, New Delhi
13	Pusa-1103	IARI, New Delhi
14	HK-4	JNKVV, Jabalpur
15	ICCV-13309	JNKVV, Jabalpur
16	BGD-1005	IARI, New Delhi
17	C-927	IIPR, Kanpur
18	Pusa-2085	IARI, New Delhi
19	JG-62	JNKVV, Jabalpur
20	Pusa-5023	IARI, New Delhi
21	Pusa-547	IARI, New Delhi
22	Pusa-372	IARI, New Delhi
23	ICCV-95334	IARI, New Delhi
24	Pusa-3022	IARI, New Delhi
25	Pusa-1108	IARI, New Delhi
26	Pusa-1105	IARI, New Delhi
27	JGK-01	JNKVV, Jabalpur
28	GNG-1581	JNKVV, Jabalpur
29	Pusa-5028	IARI, New Delhi
30	C-925	IIPR, Kanpur
31	BGD-112	IARI, New Delhi
32	RSG-931	JNKVV, Jabalpur
33	ICCV-14508	NBPGR, New Delhi
34	GNG-1969	JNKVV, Jabalpur
35	C-905	IIPR, Kanpur
36	ICCV-14512	NBPGR, New Delhi
37	ICCV-07102	NBPGR, New Delhi

Results and Discussion

Diversity of morphological characteristics

In this study, 37 chickpea genotypes were characterized using ten morphological characteristics of the chickpea map. The mean values of thirty seven genotypes observed for ten characters studied along with their range and critical differences are presented in Table 2. Analysis of variance for the randomized block design with respect to thirty seven genotypes of chickpea exhibited significant differences used in the present investigation for all studied characters *viz.*, days to 50% flowering, days to maturity, plant height, number of primary branches per plant, number of pods per plant, number of seed per pod, biological yield per plant, harvest index, 100 seed weight, grain yield per plant.

The results of variance analysis of ten morphological traits showed significant differences among the examined genotypes, indicating the presence of variability that can be exploited through selection (Table 2). For each of the traits evaluated, descriptive statistics, including the extreme genotype mean values along with the corresponding genotypes, the mean, median, range, variance with their coefficient of variation are summarized in Table 3. Among traits, grain yield (g per plant) ranged from 17.60 to 38.60 with a mean value of 27.08 g per plant. High differences between the maximum and minimum mean values were found for all other traits. All the 37 genotypes were divided into 07 different clusters. The first cluster contained eleven genotypes

and most of them were released varieties from Pusa institute.the genotypes falls in this cluster are similar in maximum plant height, lower harvest index and higher grain yield. The 6th cluster contained eight genotypes representing different locations and these are similar in minimum number of branches per plant, less number of pods per plant and bold seeded which had the maximum contribution towards the genetic diversity (Table 5).

The present study aimed at characterizing the genetic diversity of landrace and advanced chickpea germplasm using SSR and morphological attributes and determining the potential utility of these markers. Studies on genetic diversity and relationships among landraces and improved varieties are not only useful for germplasm conservation, but also facilitate use of the genetic resources in crop improvement programmes (Imtiaz *et al*. 2008; Saeed *et al*. 2011; Choudhary *et al*. 2012) [11, 23, 4].

Inter and Intra- cluster distance

The average intra and inter cluster D^2 values are presented in table 8. The maximum (202.07) intra cluster distance were observed for cluster IV followed by cluster VII (129.87), cluster I (124.46), cluster VI (88.51), cluster V (72.23), cluster III (70.97) and minimum (51.74) for cluster II

The maximum inter cluster distance (968.771) exhibited between cluster VIand cluster VII, followed by cluster II and VII (674.956) and cluster III and IV (651.273). The minimum inter cluster distance (147.919) revealed between cluster I and II. The maximum inter cluster distance indicated that genotypes of cluster VI and VII are not so closely related whereas the inter cluster distance indicated that the genotypes of these cluster are closely related. The genotypes of cluster I and II showed minimum inter clusters distances, hence these genotypes are closely related.

It is suggested that, crosses among the parents belonging to most divergent clusters would be expected to manifest maximum heterosis and also wide variability of genetic architecture. Thus the crosses between the genetically diverse genotypes of cluster IV characterized by days to 50% flowering, plant height, number of primaty branches per plant, number of pods per plant, and number of seed per pod.

The results showed that the inter-cluster distances between the different clusters of chickpea genotypes differed widely. The inter-cluster distances were larger than the intra-cluster distance suggesting wider genetic diversity among the chickpea genotypes of different groups. Similar results were also shown by Lokere *et al.* (2007) [16], Dwevedi and Lal (2009) [8], Thakur and Sirohi (2009) [27], Sial *et al.* (2010) [24], Ojha *et al*. (2011) [18], Babber *et al*. (2012) [1] and Gaikwad *et al.* (2014) [9].

SSR allelic polymorphism and genetic diversity

Diversity analysis using 10 SSR markers produced 11 alleles, This suggested the presence of considerable polymorphism at the studied microsatellite loci and revealed a moderate level of genetic diversity in the existing chickpea germplasm, which is similar to the results obtained by Khan *et al.* (2010) [15] and Ghaffari *et al*. (2014) [10].

In total, 11 SSR loci covering various bin locations on different linkage groups were used for genetic diversity analysis in 37 chickpea genotypes (Table 6). The PIC values ranged from 0.053 (Primer 7 to 0.876 (Primer 4) which showed the genetic difference among them, however all the primers produce monomorphic banding pattern. Cluster analysis using the un-weighted neighbor joining clustering algorithm clearly delineated the genotypes in four major clusters (Fig. 1). Cluster I contained ten genotypes of which most of them were exotic genotypes. Cluster IV included sixteen genotypes of which diverse nature from other germplasm. All the accessions of the landrace and cultivated cluster have relatively high (>80%) membership in their clusters.

Narrow genetic variation had been reported in chickpea germplasm by various researchers (Singh *et al.* 2003; Upadhaya *et al.* 2012), but it was now possible to conduct an extensive molecular diversity study in chickpea using large number of SSR markers to identify genetically diverse germplasm with potentially beneficial traits for chickpea improvement programmes.

Comparison of morphological and molecular study:

The traditional morphological characterization is though essential but has its own drawbacks, as it is descriptive and error- prone. Therefore, several biochemical and molecular tools are being adopted for plant variety characterization and identification of plant. These laboratory based techniques are essential, very precise, easy to adopt and highly unambiguous in nature (Karp *et. al.* 1997) [13]. The data collected are comparable. Unlike morphological traits the information obtained and not affected by the environmental or physiological factors. The qualitative nature and precision of the data gathered also makes it most appropriate for the description of the distinctiveness of the accessions. Lack of information on specific plant characteristics and genetic diversity is one of major constraints in the use of germplasm by breeders and others users. Morphological and molecular characterization the germplasm should be characterize for abiotic and biotic stresses and resulted germplasm should be grouped into different categories of abiotic and biotic stresses. Progress in crop breeding requires a continuous supply of genes or gene complexes to meet needs that may or may not be foreseen.

Genetic diversity analysis conducted through morphological and molecular approaches suggests that chick pea released varieties from different research institutes across India posses low genetic diversity as they were placed in same clusters when analysed through different morphological (D^2 analysis) and molecular markers as compared to the other none released genotypes, even these released varieties are from geographically diverse origin which indicated that the geographical diversity of the germplasm has no association with the morphological and molecular diversity. Accessions namely RSG-931, JGK-1 and ICCV-143309 which were found to be more diverse in both morphological and molecular levels, should be used for future chick pea breeding programme.

Cluster analysis using morphological and SSR markers separated all chickpea genotypes into six and four distinct groups, respectively. Most Indian landraces accessions studied in present research were grouped relatively close together and this close relation between molecular genetic variability may be reflected to close geographic sources of these accessions.

Table 2: Analysis of variance (ANOVA) for ten characters in chickpea (*Cicerarietinum* L.)

Source of Variation	d. f.	Days to 50% flowering	Days to maturity	Plant height	No. of. primary branch per plant	No. of. Pod per plant	No. of Seed per pod	Biological yield/plant	Harvest index	100 seed weight	Grain Yield per plant
Replication	2	21.56	29.59	17.07	0.043	94.15	0.0002	5.57	19.52	1.13	5.26
Treatment	36	764.55**	581.69**	4406.21**	30.54**	44768.58**	10.53**	21761.61**	40180.19**	8620.26**	2801.23**
Error	72	897.76	481.90	945.90	2.75	1691.21	0.18	1670.99	265.69	104.02	539.53

Table 3: Mean performance of genotypes combination for ten characters in Chickpea (*Cicerarietinum* L.*)*

S. No.	Genotype	Days to 50% flowering	Days to maturity	Plant height	No. of primary branch per plant	No. of. Pod per plant	No. of Seed per pod	Biological yield per plant	Harvest index	100 seed weight	Grain Yield per plant
1	ICCV-14508	90.66	146.00	53.20	2.53	31.56	1.32	53.66	53.03	43.86	27.60
2	C-910	97.00	152.66	56.93	3.46	61.30	1.85	42.73	45.76	28.40	19.60
3	C-927	98.66	155.00	62.83	3.13	31.23	1.70	43.06	54.00	34.30	23.33
4	C-905	99.66	158.00	46.86	4.53	76.26	2.14	78.53	49.23	15.06	30.86
5	ICCV-14512	98.0	155.00	48.90	3.13	36.46	1.76	45.86	52.83	34.13	24.20
6	ICCV-95334	95.00	150.66	41.76	3.66	90.86	1.84	91.93	42.00	15.80	38.60
7	ICCV-14510	92.33	151.33	39.00	3.40	31.10	2.18	71.73	52.66	21.46	37.80
8	ICCV-07102	96.66	156.33	54.16	2.80	29.96	1.90	45.26	53.23	32.26	24.13
9	C-925	98.33	156.33	49.13	3.20	36.13	1.90	55.86	48.26	29.90	27.00
10	ICCV-13309	94.00	155.66	55.46	3.46	65.10	2.18	69.06	46.46	27.43	32.13
11	PKV-4	94.66	154.00	55.73	2.66	15.36	1.78	61.66	49.50	44.30	30.53
12	DCP-92-3	93.33	156.66	40.90	4.00	33.80	1.49	50.00	54.86	26.63	27.46
13	GNG-1969	93.00	153.33	64.90	3.33	67.00	1.40	48.60	50.73	31.16	24.66
14	BGD-72	96.66	152.66	45.30	3.40	35.70	1.60	44.60	39.43	27.63	17.60
15	JG-62	91.66	152.33	51.86	3.40	32.76	1.56	47.00	59.66	30.73	28.06
16	Pusa-1103	93.00	156.66	52.20	3.60	36.06	1.64	56.13	53.66	41.46	30.13
17	Pusa-3022	98.33	155.00	45.23	3.06	35.90	2.20	41.00	52.30	23.86	21.46
18	BGD-112	98.33	154.00	41.43	2.73	32.26	1.60	53.60	53.23	25.66	28.53
19	HK-4	95.66	152.00	54.63	3.06	33.26	1.78	48.20	49.83	42.10	24.06
20	Pusa-2085	99.33	157.00	44.63	3.40	35.13	1.52	56.96	46.70	19.66	26.60
21	BG-362	94.33	153.00	57.70	3.20	37.23	1.90	52.33	41.43	42.70	21.73
22	Pusa-1053	94.33	154.33	59.46	3.53	66.16	1.90	45.60	47.73	31.40	26.33
23	Pusa-2024	98.33	157.33	46.80	3.53	38.60	1.70	90.13	55.36	14.86	27.13
24	Pusa-256	98.66	153.33	50.90	3.66	61.43	1.48	47.53	45.76	25.26	21.80
25	HC-5	91.66	156.00	56.76	3.53	37.30	1.69	52.93	54.86	41.20	29.06
26	GNG-1958	92.66	156.00	51.23	3.00	34.66	1.80	48.40	44.70	33.40	21.66
27	Pusa-1105	97.00	154.00	59.23	4.53	82.33	2.42	49.53	46.13	33.43	27.73
28	Pusa-5028	97.00	152.00	53.63	3.60	50.83	1.59	73.53	46.03	41.53	33.86
29	Pusa-372	92.00	152.33	52.53	3.53	90.06	1.79	98.80	37.16	17.83	36.73
30	Pusa-1003	97.00	156.33	54.23	3.00	36.20	2.06	42.93	59.20	38.20	25.33
31	GNG-1581	100.00	154.33	53.76	3.40	33.10	2.65	46.73	44.13	39.56	20.66
32	Pusa-1108	98.33	154.33	56.33	3.60	88.46	2.24	83.00	42.73	19.43	35.46
33	Pusa-5023	96.33	151.66	44.16	3.00	30.36	1.37	52.60	49.70	15.66	26.00
34	BGD-1005	96.33	154.66	52.86	2.46	20.96	1.37	45.73	52.76	31.00	24.13
35	Pusa-547	95.66	154.00	54.93	3.06	49.16	2.16	53.06	59.50	28.40	31.60
36	RSG-931	91.33	152.66	43.83	4.40	65.26	1.64	43.53	57.96	33.26	25.20
37	JGK-1	96.66	155.33	42.76.	2.66	30.56	1.49	65.73	42.86	41.86	28.20
	Mean	95.72	154.14	51.25	3.37	45.94	1.80	55.58	49.60	30.40	27.08
	Range	90.66	146.00	39.00	2.46	15.36	1.32	41.00	37.16	14.86	17.60
		100.00	158.00	64.90	4.60	90.86	2.65	98.80	59.66	44.30	38.60
	C.D.	5.74	5.58	7.83	0.42	10.47	0.10	10.40	4.15	2.59	2.59
	SE(d)	2.038	1.49	2.09	0.11	2.79	0.02	2.78	1.10	0.69	1.38
	C.V.	3.68	1.67	7.07	5.80	10.54	2.77	8.66	3.87	3.95	10.10

Table 4: Clustering pattern of 37 chickpea (*Cicer arietinum* L.) genotypes on the basis of D^2 cluster analysis

Clusters	No of genotypes	Genotype name
I	11	BG-362, Pusa-372, ICCV 14512, Pusa-256, Pusa 2085, HK-4,Pusa 3022,Pusa 1053, ICCV 13309, GNG-1969,Pusa 1103
II	5	Pusa 2024, C-910, Pusa-1003,Pusa 1105, C-925
III	3	Pusa 5023, ICCV 14508, ICCV-95334
IV	3	GNG-1958, JGK-1, BGD-112
V	3	C-927, C-905, HC-5
VI	8	PKV-4,Pusa 547, BGD-1005,Pusa 1108, JG-62, GNG-1581, ICCV 7102, ICCV-14510
VII	4	BGD-72, RSG-931, DCP-92-3,Pusa 5028

Table 5: Contribution (%) of 10 characters of 37 genotypes in chickpea

Characters	% contribution
Days to 50% flowering	0.00
Days to maturity	0.00
Plant height	0.45
No. of primary branch per plant	3.15
No. of pod per plant	6.76
No. of seed per pod	28.23
Biological yield per plant	0.45
Harvest index	0.45
100 seed weight	47.75
Grain Yield per plant	12.76

Table 6: Primer code, amplified bands, polymorphic bands, monomorphic bands, % polymorphism and PIC values of chickpea by using SSR Markers

S. No.	Primer code	Product size (bp)	Amplified bands	Monomorphic bands	Polymorphic bands	Polymorphism %	PIC	RP
1.	Primer 1	960	2	2	0	0	0.616	1.215
2.	Primer 2	420	1	1	0	0	0.543	1.35
3.	Primer 3	120	1	1	0	0	0.835	0.81
4.	Primer 4	135	1	1	0	0	0.876	0.702
5.	Primer 5	260	1	1	0	0	0.763	0.972
6.	Primer 6	280	1	1	0	0	0.109	1.89
7.	Primer 7	285	1	1	0	0	0.053	1.942
8..	Primer 8	180	1	1	0	0	0.646	1.188
9.	Primer 9	670	1	1	0	0	0.427	1.152
10.	Primer 10	130	1	1	0	0	0.105	1.89
	Total		11	11	0			
	Average		1.1	1.1	0	0	0.497	1.311

Table 7: Distribution of 37 chickpea genotypes into different clusters based on 10 SSR marker analysis

Cluster	No. of Genotypes	Genotypes
I	12	ICCV-14508, PKV-4, C-910, ICCV-14510, Pusa-1053, GNG-1581, Pusa-2085, Pusa-2024, Pusa-1003, BG-362, Pusa-372, Pusa-256
II	4	ICCV-07102, DCP-92-3, BGD-1005, Pusa-5023
III	10	C-927, GNG-1969, BGD-72, JG-62, Pusa-1103, C-925, HK-4, Pusa-3022, BGD-112, GNG-1958
IV	8	ICCV-14512, C-905, HC-5, ICCV-95334, Pusa-1105, Pusa-5028, Pusa-1108, Pusa-547
V	1	ICCV-13309
VI	2	RSG-931, JGK-1

Table 8: Estimation of average Inter and Intra Cluster distance for seven Clusters in Chickpea (*Cicer arientum* L.)

Clusters	I	II	III	IV	V	VI	VII
I	124.462						
II	147.919	51.745					
III	218.578	321.705	70.971				
IV	460.557	260.046	651.273	202.075			
V	314.437	188.502	291.670	244.532	72.236		
VI	256.455	181.654	608.381	517.610	570.857	88.518	
VII	640.648	674.956	520.328	563.885	537.182	968.771	129.871

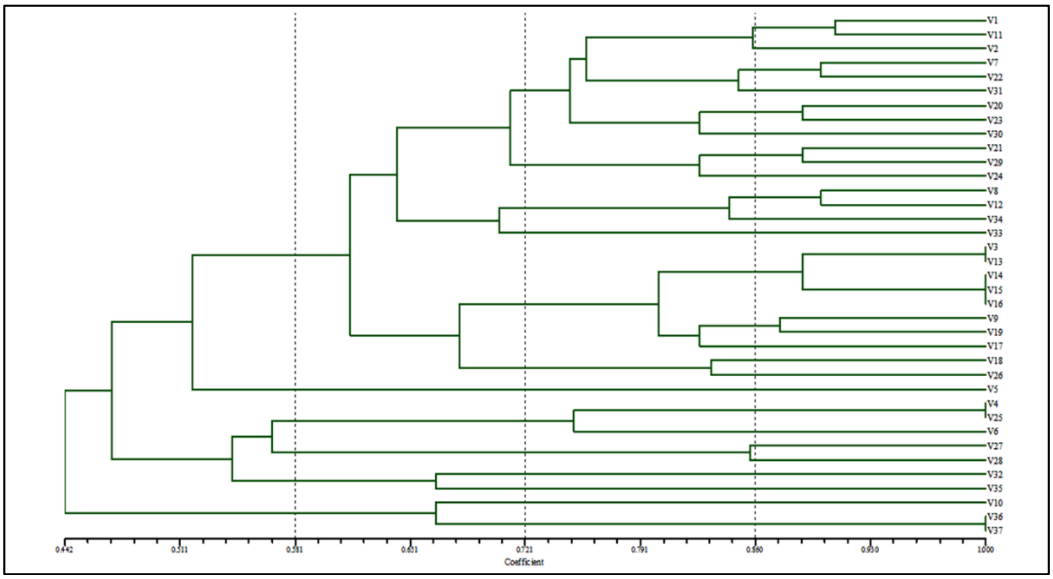

Fig 1: Dendrogram showing clustering of 37 chickpea varieties constructed using UPGMA based on Jacquard's similarity coefficient obtained from SSR marker analysis

Conclusion

In conclusion, results of the present study indicate that the extent of genetic variability in the germplasm studied seem to have remained quite constant. Information about the current genetic diversity permits the classification of our available germplasm into various/ heterotic groups, like RSG-931, JGK-1 and ICCV-143309 are particularly important to hybrid/cross-breeding programmes for chickpea.

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