Plant Archives Vol. 25, No. 1, 2025 pp. 1926-1932 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org

DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2025.v25.no.1.281

ASSESSMENT OF GENETIC DIVERSITY IN CHICKPEA (*CICER ARIETINUM* L.) GENOTYPES USING SSR MARKERS

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(Date of Receiving-03-02-2025; Date of Acceptance-10-04-2025)

ABSTRACT

Genetic diversity is prerequisite to start breeding programme in any crop. Genetic base of self-pollinated crops is very narrow so assessing the extent of genetic variability present among developed varieties/lines and germplasm is critical step for their exploitation in breeding programme. Keeping this in view, a systematic effort was made to characterize the 54 chickpea genotypes including germplasm, advanced breeding lines and released varieties to assess the existing diversity using SSR marker. Molecular characterization of chickpea genotypes with 32 SSR primers revealed the use of 19 primers for diversity analysis as they produced polymorphic banding pattern. A total of 52 alleles were produced with an average of alleles varying from 2 to 4 per primer. The size of the amplified bands ranged from 125 to 300 bp and PIC values of primers ranged from 0.42 (CAGP407) to 0.74 (TA71 & TA 72) with an average value of 0.63. Assessment of dissimilarity index was done based on Jaccard's similarity coefficient arising from the SSR marker data. Dissimilarity index was used to estimate the genetic diversity and relatedness among the chickpea genotypes. The dendrogram based on total SSR polymorphism, grouped 54 chickpea genotypes into three major clusters which were further grouped into different sub-clusters. Highest dissimilarity index of 0.87 was recorded between Vijay and DCP 92-3 followed by 0.81 between ICC 10945 and DCP 92-3 indicating the presence of wide genetic base in the material and their use in breeding programme.

Key words: Chickpea, SSR, diversity, alleles, PIC, dissimilarity index.

Introduction

Chickpea (*Cicer arietinum L.*) is one of the most important food legumes cultivated and consumed worldwide. It is cultivated mostly in South Asia, Middle East, and parts of Africa. Among chickpea growing countries, India ranks first followed by Turkey and Pakistan at second and third position respectively (FAOSTAT, 2023). It plays a vital role in human nutrition. It serves as one of the most important sources of protein for millions of people who are vegetarian either by choice or due to cultural, religious, or economic reasons (Saeed *et al.*, 2011; Jannatabadi *et al.*, 2014). Chickpea is also rich in other essential nutrients, including dietary fibre, vitamins and minerals, such as folate, iron, magnesium, potassium, and zinc. Chickpea have low glycemic index as it releases sugar, gradually into the bloodstream thus controlling blood sugar levels and is beneficial for diabetic person. Chickpea is essential not only for its nutritional value, but also for its ecological importance, contributing to soil nitrogen fixation and sustainable agriculture. Kabuli (white seeded) and desi (brown seeded) are two main types of cultivated chickpea which represents two diverse gene pools in chickpea genome. Modern plant breeding programme is focused on uniformity of genotype which gives rise to narrow genetic base of cultivated chickpea (Robertson *et al.*, 1997). Success of plant breeding programme depends on the extent of availability of genetic diversity which promotes the search for new sources of variation (allele) in the genome (Upadhyaya *et al.*, 2008). Diverse genetic backgrounds are utilised to create

favorable new gene combinations among parental lines which provide allelic variation necessary for hybridization programme. Assessment of the extent of genetic diversity within chickpea genotypes is obtained either based on morphological attributes or with the help of molecular markers for the effective quantification of genetic variation. Morphological traits have several limitations, including limited polymorphism, low heritability, late expression, and epistatic and pleiotropic gene effects (Eivazi *et al.*, 2008). In contrast, molecular markers serve as a valuable supplement to morphological characterization as they are abundant, unaffected by plant tissue or environmental factors, and enable early identification of cultivars during plant development (Manifesto *et al.*, 2001).

A number of molecular markers including RFLPs (Udupa *et al.*, 1993), AFLPs (Vos *et al.*, 1995,) and RAPDs (Ahmad 1999; Sudupak *et al.*, 2002) have been used to determine genetic relationships among the chickpea accessions (Singh *et al.*, 2003). Molecular genotyping through RFLP and AFLP markers is costly and time-consuming, while the use of RAPD is restricted due to its low reproducibility and dominant nature. SSR markers are highly informative markers and are used for genetic diversity studies because of their simplicity, high levels of polymorphism, high reproducibility, and co-dominant inherit-ance patterns (Powell *et al.*, 1996). SSR markers has been used effectively for gene mapping and diversity fingerprinting of chickpea (Winter *et al.*, 2000; Saeed *et al.*, 2011; Jannatabadi *et al.*, 2014; Ghaffari *et al.*, 2014). Therefore, present study was done to find the existing genetic diversity among chickpea genotypes using SSR markers in order to identify diverse genotypes that could be utilized in chickpea improvement programme.

Materials and Methods

Present experiment was carried out at Research laboratory of the Department of Molecular Biology and Genetic Engineering of Bihar Agricultural University, Sabour Bhagalpur. The experimental material includes 54 chickpea genotypes including germplasm, advanced breeding lines and released varieties (Table 1).

DNA Extraction

Genomic DNA was extracted from chickpea genotypes through CTAB method given by Doyle and Doyle 1990 with few modifications. Chickpea seed was germinated on germination paper and 200 mg of tissue was collected and homogenized in 2 ml DNA extraction buffer containing 100mM Tris-Cl (pH 8.0), 1.4M NaCl, 20mM EDTA (pH 8.0), 2% (w/v) CTAB), Poly vinylpyrrolidone (1%) and 20µl Beta-mercaptoethanol. Homogenized leaf extract was incubated in water bath at 65ºC for 45 minutes with 3 to 4 times shaking at regular interval. Digested tissue was centrifuged at 10,000 rpm for 5 minute and supernatant was collected in fresh tube. Equal volume of chloroform and isoamyl alcohol (24:1) solution was added, to the digested extract, mixed well and centrifuged for 5 minutes at 10,000 rpm. Aqueous supernatant was collected and equal volume of chloroform and isoamyl alcohol (24:1) was added once again and centrifuged for 5 minutes at 10,000 rpm. Supernatant was collected and equal volume of isopropanol was added, mixed and centrifuged for 10 minutes at 10,000 rpm. Supernatant was discarded and DNA pellet was washed with 70% chilled ethanol and air dried. The pellet was dissolved in TE buffer (50mM Tris, 10 mM EDTA). The purity of the DNA was estimated by spectrophotometry through estimation of A260/A280 ratio and visualization on 0.8% Agarose gel. Yield was estimated by measuring absorbance at 260 nm. DNA sample was further diluted to a uniform concentration of 50 ng/µl.

Primers

SSR primers distributed throughout the chickpea genome were selected and got synthesized using the forward and reverse primer sequence information available in the public database. Annealing temperature of markers was optimized before utilization. List of primers along with SSR repeats, sequence of primer and annealing temperature is presented in Table 2.

PCR Amplification using SSR primers

PCR amplification using SSR primers was done in automated thermal cycler (Applied Biosystems). PCR reaction was carried out in PCR tubes using 50ng of extracted genomic DNA, PCR premix having taq polymerase and other PCR components (Excelris) along with forward and reverse primer. Template DNA was initially denatured at 94ºC for 4 minutes followed by 35 cycles (30 sec denaturation at 94ºC, 40 sec annealing at 55ºC, 40 sec of primer extension at 72ºC) of PCR amplification, and final extension of 72ºC for 5min. The amplified fragments were separated on 2.5% Agarose gel containing ethidium bromide and run for 2 hours in 1X TAE buffer followed image acquisition under UV gel documentation system.

Data analysis

PCR amplicon obtained from SSR primers were scored in binary format, presence of band was scored as 1 and the absence of a band scored as 0. The analysis was done using Darwin software tool, coefficient of genetic dissimilarity (GD) was calculated. Based on the

Table 1: List of chickpea genotypes used in study.

S. No.	Name of Genotype	Type of Genotype	Source
1.	Vishal	Released variety	MPKV, Rahuri
2.	BGD 72	Released variety	IARI, New Delhi
3.	IPC 09-09	Advanced breeding line	IIPR, Kanpur
4.	SAKI-9516	Released variety	JNKVV, Jabalpur
5.	AP-5	Released variety	AAU, Gujarat
6.	BARWAN	Germplasm/landrace	Exotic landrace
7.	DEON	Germplasm/landrace	Exotic landrace
8.	IPC 10-135	Advanced breeding line	IIPR, Kanpur
9.	IPC 11-20	Advanced breeding line	IIPR, Kanpur
10.	BG 391	Released variety	IARI, New Delhi
11.	JG 30	Released variety	JNKVV, Jabalpur
12.	PUSA 240	Released variety	IARI, New Delhi
13.	IPC-2000-02	Advanced breeding line	IIPR, Kanpur
14.	ICC-37	Germplasm/landrace	ICRISAT, Hyderabad
15.	DCP 92-3-(4)	Advanced line	IIPR, Kanpur
16.	PDE 2	Released Variety	PAU, Ludhiana
17.	ICC 4958	Germplasm/landrace	ICRISAT, Hyderabad
18.	ICCV 07-118	Released variety	ICRISAT, Hyderabad
19.	PUSA 244	Released variety	IARI, New Delhi
20.	IPC 92-72	Advanced Breeding line	IIPR, Kanpur
21.	VIJAY	Released variety	MPKVV, Rahuri
22.	PUSA 351	Released variety	IARI, New Delhi
23.	IPC 92-39	Advanced breeding line	IIPR, Kanpur
24.	KWR-108	Released variety	CSAUAT, Kanpur
25.	RSG-991(Aparna)	Released variety	Durgapur, West Bengal
26.	ICCV-07-117	Released variety	ICRISAT, Hyderabad
27.	PUSA 256	Released variety	IARI, New Delhi
28.	JG 2004-3	Advanced breeding line	JNKVV, Jabalpur
29.	JG 74	Released variety	JNKVV, Jabalpur
30.	ICC 10945	Germplasm/landrace	ICRISAT, Hyderabad
31.	JG 14-16	Advanced breeding line	JNKVV, Jabalpur
32.	JG 16	Variety	JNKVV, Jabalpur
33.	MPG 2003-115	Advanced breeding line	MPKVV, Rahuri
34.	JG 31	Released variety	JNKVV, Jabalpur
35.	JG 18	Released variety	JNKVV, Jabalpur
36.	ICCV 07-119	Released variety	ICRISAT, Hyderabad
37.	ICCL 81248	Advanced breeding line	ICRISAT, Hyderabad
38.	JG 22	Released variety	JNKVV, Jabalpur
39.	JG 33	Released variety	JNKVV, Jabalpur
40.	JG 14	Released variety	JNKVV, Jabalpur
41.	IPC 09-171	Advanced breeding line	IIPR, Kanpur
42.	IPC 09-161	Advanced breeding line	IIPR, Kanpur
43.	ICC-67	Germplasm/landrace	ICRISAT, Hyderabad
44.	PG 186	Released variety	GBPAUT, Pantnagar
45.	IPC 10-59	Advanced breeding line	IIPR, Kanpur
46.	IPC 05-66	Advanced breeding line	IIPR, Kanpur
47.	IPC 05-59	Advanced breeding line	IIPR, Kanpur
48.	PUSA 372	Released variety	IARI, New Delhi
49.	RSG-963	Released variety	RARI, Durgapura
50.	JG 14-11	Released variety	JNKVV, Jabalpur
51.	JG 32	Released variety	JNKVV, Jabalpur
52.	IPC 09-152	Advanced breeding line	IIPR, Kanpur
53.	IPC 06-11	Advanced breeding line	IIPR, Kanpur
54.	ICCV 07-102	Released variety	ICRISAT, Hyderabad

Table 2: List of primers used for molecular diversity analysis.

S. No.	Primer name	Primer Sequence (5'-3')	Repeats	Annealing Temp ($_{Tm}$)
1.	TA64	FP- ATATATCGTAACTCATTAATCATCCGCRP- AAATTGTTGTCATCAAA TGGAAAATA	(TAA)39	55^{0}C
2.	TA113	FP- TCTGCAAAAACTATTACGTTAATACCARP- TTGTGTGTAATGGATT GAGTATCTCTT	(TAA)26	56^{0}C
3.	CAGP38	FP-CTGTTACGTGCAATGGATGCRP-TCGGTATGACACAAAAATGTGA	(AT)11	55^{0}C
4.	CAGP28	FP-ATCGCGTTAAATACTTGGACTRP- CATATGTGGAATGGACTATGC	(AT)19	55^{0}C
5.	CAGP296	FP-TGATGTTCTCTGTCTTTCCTTRP- TCCATATGCAAGAGTAAAAGC	(TTA)5	55^{0}C
6.	CAGP318	FP-TGGCCCAATATTCAGATTCCRP-TCACCAAACCAGGTACTGCTC	(GTT)5	55^{0}C
7.	TA46	FP-TTTATTGCAATAAAACTCATTTCTTATCRP- TTCTTTTTGTGTGAAA AAAAAATATAGTGA	(TAA)22	57^{0}C
8.	TA72	FP-GAAAGATTTAAAAGATTTTCCACGTTARP- TTAGAAGCATATTGTT GGGATAAGAGT	(ATT)36	55^{0}C
9.	CAGP407	FP-GAGCACCAATTAAAGAGGGCRP- GGCATTTAAAACATAAATCCCAA	TA)13	55^{0}C
10.	CAGP458	FP-TCAAAGGCAATTTTGATTGAARP- TTTTTGATAGTCGCAGGACCTT	(TA)9	55^{0}C
11.	TA71	FP-CGATTTAACACAAAACACAAARP- CCTATCCATTGTCATCTCGT	(AAT)32	55^{0}C
12.	TR29	FP-GCCCACTGAAAAATAAAAAGRP- ATTTGAACCTCAAGTTCTCG	(TAA)8n (TAA)32	55^{0}C
13.	CAGP504	FP-TCACTTTTACATTACACACAAGGATTRP- CATGTCTTTTGATATAC TACCTCCACA	(ATA)21	55^{0}C
14.	CAGP521	FP-AATGACGCAGGTTCAACACARP- ATGTCATTATGTTGCATTAAGA TCTAC	(TA)10	55^{0}C
15.	CAGP527	FP-TCTTCACGCTCCCTACAAAARP- TGAGCGTTATAGGGGAGAAAAA	(AT)11	55^{0}C
16.	CaSTMS2	FP-ATTTTACTTTACTACTTTTTTCCTTTCRP- AATAAATGGAGTGTAAA TTTCATGTA	(TAT)25	55^{0}C
17.	TA21	FP-GTACCTCGAAGATGTAGCCGATARP-TTTTCCATTTAGAGTAGGAT CTTCTTG	(TAA)51	55^{0}C
18.	TA28	FP-TAATTGATCATACTCTCACTATCTGCCRP- TGGGAATGAATATATTT TTGAAGTAAA	(TAA)37n (TAA)30	55^{0}C
19.	CAGP834	FP-AACGCTTTCCTACCAATTTTTRP- GGAGGAAGTCGTTAACAAAAGA	(TAA)8	55^{0}C
20.	CAGP897	FP-GTCCCAAAGCAATTTATTATCRP- CACTGCCACCGTATTATTAAA	(AT)17	55^{0}C
21.	CAGP1024	FP-AACCACATTTGTTGTCGCTGRP- CAAAATAAATGAAGGAGAGAAT AAAGC	(AT)13	55^{0}C
22.	CAGP1067	FP-GAGCTCAATAATTGGATTAGATTTATGRP- TGGTACAAAATACCAG GATCAA	ATA)29	55^{0}C
23.	CAGP1090	FP-CATGCGAATTTAGGAAGAGAGARP- AGACATGGAGAGACCAAACAA	(AT)15	55^{0}C
24.	CaSTMS21	FP-CTACAGTCTTTTGTTCTTCTAGCTTRP- ATATTTTTTAAGAGGCTT TTGGTAG	(CT)9n (CTTT)2	56^{0}C
25.	TA27	FP-GATAAAATCATTATTGGGTGTCCTTTRP- TTCAAATAATCTTTCATC AGTCAAATG	(CT)4	57^{0}C
26.	TA117	FP-GAAAATCCCAAATTTTTCTTCTTCTRP- AACCTTATTTAAGAATAT GAGAAACACA	(TAA)21	55^{0}C
27.	TA142	FP-TGTTAACATTCCCTAATATCAATAACTTRP- TTCCACAATGTTGTA TGTTTTGTAAG	(ATT)52	56^{0}C
28.	CAGP1359	FP-GCCTAAAAGATATACAATTCACTRP- TCAAAGTGTTCGATGATTGTC	(TTA)15	55^{0}C
29.	CAGP106	FP-CCTACGTGGACCCTCTTATTARP- GCTTGTGTGGTTGATTTTATC	(TA)15	55^{0}C
30.	CAGP765	FP-GGTGAAAATCAAAAGTAGGAGARP- GGTATTCCTTCCATCCTCTT	(TAT)28	55^{0}C
31.	TA118	FP-ACAAGTCACATGTGTTCTCAATARP- GGAAAGGTTAAGAAATTT TACAATAC	(AG)11	56^{0}C
32.	NCPGR6	FP-TATGTCTACACCTATGCATCRP- GACCAAGATTAGTAGAACCT	(TAA)45	55^{0}C

Table 3: Polymorphism information content of polymorphic primers.

Marker name	Chromosome location	No. of alleles	PIC value
CAGP38	1	3	0.64
CAGP106	8	3	0.66
CAGP521	4	2	0.63
CAGP897	5	2	0.58
CAGP1067	6	2	0.53
CAGP1359	7	3	0.65
CASTM2	4	4	0.71
TA-71	3	4	0.74
TA117	7	2	0.70
TA21	5	2	0.55
TA72	2	4	0.74
CAGP527	4	3	0.68
CAGP504	4	3	0.67
CAGP458	3	2	0.55
CAGP765	8	2	0.58
CAGP1024	6	3	0.69
TA113	1	2	0.56
CAGP296	2	3	0.66
CAGP407	3	3	0.42
Mean		**2.73**	**0.63**

dissimilarity matrix, a dendrogram showing genetic relationships between genotypes was constructed using the Unweighted Pair Group Method Arithmetic Average (UPGMA).

Polymorphism information content (PIC value) of SSR primers was calculated using equation given by Botstein *et al.*, (1980). PIC value gives an account of a locus or loci keeping in mind not only the number of alleles but also the frequency distribution of those alleles.

$$PIC=1-\Sigma(Pi)^2$$

Pi depicts the proportion of samples carrying the i^{th} allele.

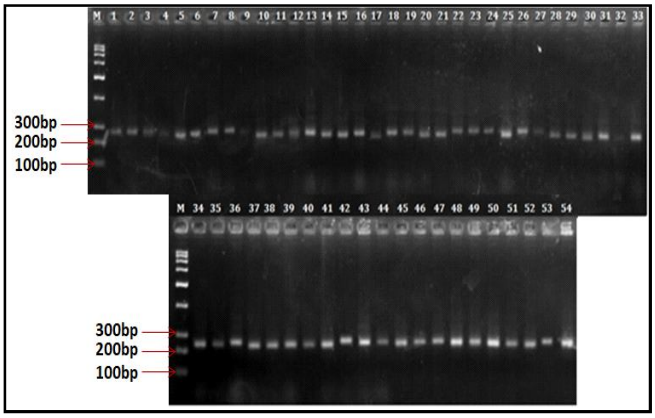

Fig. 1: Amplification profile of TA72 SSR detected among chickpea genotypes: M-100bp ladder and 1-54 is chickpea genotypes enlisted in Table 1.

Results and discussion

Knowledge of genetic diversity existing among cultivated and wild relatives has been successfully utilized for fingerprinting, germplasm management and genotype selec-tion. For this purpose, a systematic categorization of targeted plant material and knowledge of the genetic relationships among them is required (Makwana *et al.*, 2023; Yadav *et al.*, 2022; Tomar *et al.*, 2022). Studies on genetic diversity and relationships among landraces and improved varieties facilitate use of the genetic resources in crop improvement programme (Imtiaz *et al.*, 2008, Saeed *et al.*, 2011, Choudhary *et al.*, 2012). Characterization of the genetic diversity present in landrace, advanced breeding lines and released varieties; using SSR marker and determining the potential utility of these markers was main goal of the present study. Efficiency of SSR markers used in molecular diversity analysis depends on a number of parameters like number of alleles per locus, genetic diversity and PIC values. In present investigation 32 SSR markers were initially tested for polymorphic nature out of which 19 SSR were found polymorphic and reproducible among 54 chickpea genotypes (Fig. 1). A total of 52 alleles were identified by 19 SSR markers across the genotypes. The number of alleles per locus ranges from 2 to 4 with an average of 2.73 alleles per locus in the total collection. The allele size varied from 150 to 300bp. Similar observation was reported by Yadav *et al.*, (2016) while using SSR markers for genetic diversity analysis among chickpea cultivars. Polymorphism information content (PIC) ranged from 0.42 (CAGP407)) to 0.74 (TA71 & TA 72) with an average value of 0.63 (Table 2). Higher PIC value of marker indicates more informative nature of the marker. Similar range of PIC values was reported by Ghaffari *et al.*, (2014); Samyuktha *et al.*, (2018); Shanmugam and Kalaimagal (2019) in their studies on use of SSR markers for genetic diversity analysis in chickpea genotypes. PIC value of SSR marker is also affected by the existing diversity among the studied genotypes (Table 3). Lower PIC value depicts the close relatedness of the genotypes under study.

Assessment of dissimilarity based on Jaccard's similarity coefficient arising from the SSR marker data was used to estimate the genetic relatedness and diversity among the 54 chickpea genotypes. Dendrogram was prepared through UPGMA which grouped the genotypes into three major clusters and three sub clusters (Cluster I: Included 19 genotypes *i.e.*, ICC 10945, Vijay, JG 74, JG 14-16, JG 14, BG 391, JG 22, RSG 991, JG 2004-3, AP-5, SAKI 9516, JG 30, BARWAN, IPC 09-09, BGD 72, IPC 11-20, DEON, IPC 10-135, Vishal. These

genotypes were further distributed into two sub clusters Ia and Ib comprising 12 and 7 genotypes respectively. Cluster II contained 28 genotypes which were further sub grouped into cluster IIa and IIb having 19 and 9 genotypes respectively. Cluster IIa contained 19 genotypes including IPC 09-171, JG 33, IPC 09-161, IPC10-59, PUSA 372, IPC 05-66, ICC 4958, DCP 92-3, MPG 2003-115, IPC 92-72, ICCL 81248, JG 31, JG 18, JG 16, JG 14-11, RSG 963, ICCV 07-119, IPC 2000-02, JG 32 whereas cluster IIa comprises of genotypes IPC 09-152, IPC 05-59, PG186, ICCV 07-102, IPC 06-11, ICC 67, PDE-2, ICC 37, ICCV 07-118. Cluster III contained 7 genotypes including IPC 92-39, PUSA 240, PUSA 351, PUSA 256, ICCV 07-117, KWR 108, PUSA 244. Cluster III was also further grouped two sub clusters including IIIa and IIIb having 6 and 1 genotype respectively.

Grouping of PUSA cultivars released from IARI, New Delhi together in cluster III with minimum intra cluster distance confirms their close relationship with each other. Similarly, most of the JG genotypes were grouped in cluster IIa showing close resemblance. Similar results have been reported by various research groups (Rizvi *et al.*, 2014 and Solanki *et al.*, 2022). Dissimilarity index was found to be maximum (0.87) between Vijay and DCP 92-3 followed by 0.81 between ICC 10945 and DCP 92-3 indicating the presence of wide genetic variability between these genotypes. For success of breeding programme genetic diversity is needed. Parent should be selected from different clusters.

Genotypes closely related to each other and from same cluster should not be used in extensive breeding programme (Ghafoor *et al.*, 2003; Afzal *et al.*, 2018). In present study ample amount of genetic diversity was detected in chickpea genotypes that may help in the selection of genetically diverse parents for the success of breeding programme.

Conclusion

Result of present study indicates the existence of genetic diversity among the chickpea genotypes thus providing an opportunity to the breeders to plan hybridization program for chickpea improvement programme. Nineteen SSR primers were found to be polymorphic out of the 32 SSR markers used in the current study. Maximum allelic variation was depicted in TA71 and TA72 SSRs, suggesting their utility in marker assisted breeding. Through crossing of genetically diverse genotypes, it will be possible to improve chickpea for yield and other traits.

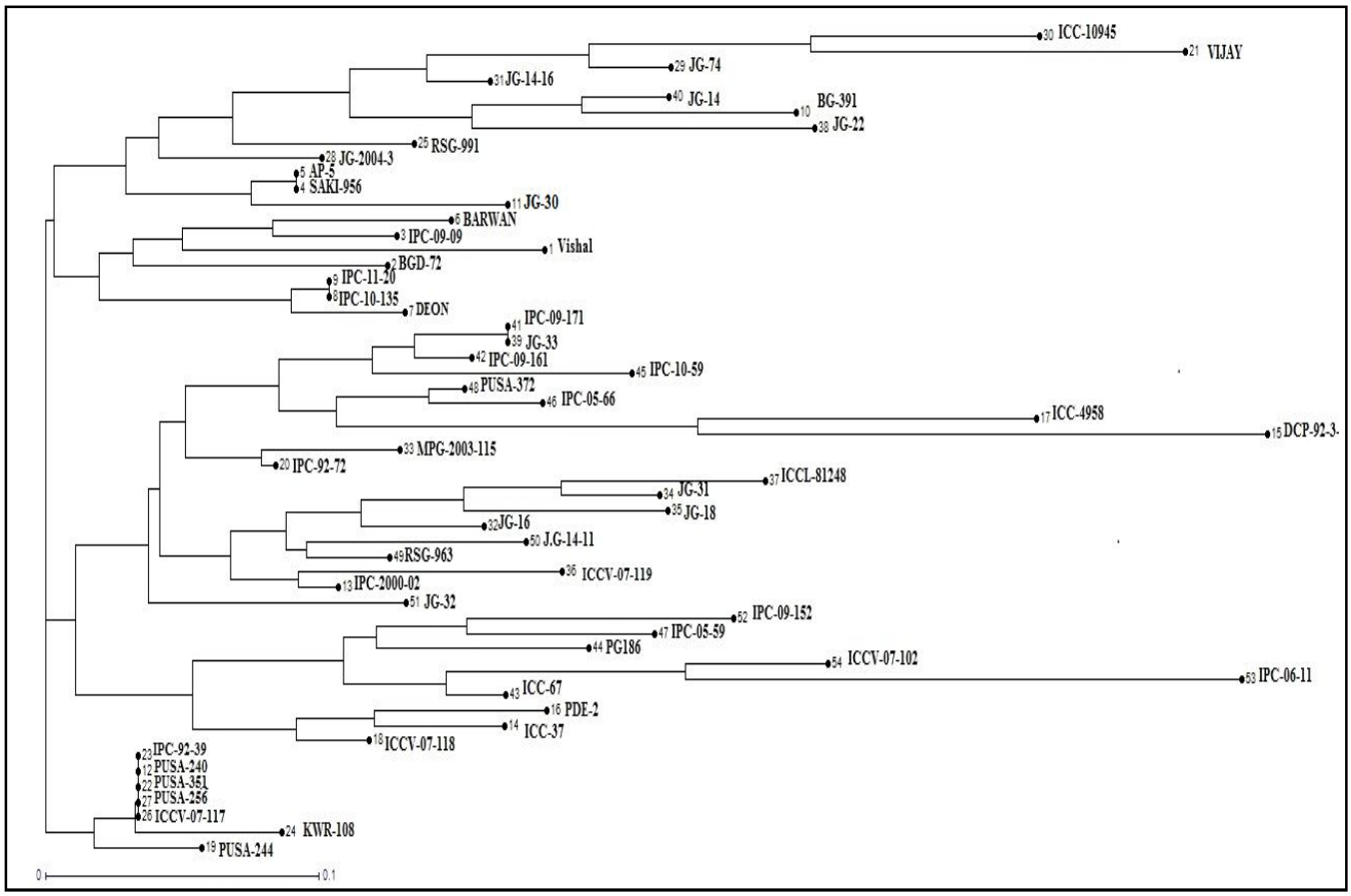

Fig. 2: Dendrogram showing clustering of 54 chickpea genotypes constructed using UPGMA based on Jacquard's similarity coefficient obtained from SSR marker analysis.

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