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DNA fingerprinting and genetic diversity analysis of chickpea genotypes using SSR, scot and DAMD markers

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Abstract
Chickpea is the major leguminous crop in India as it is cheapest source to obtain nutrition. India is world leader in chickpea production by producing 15 times higher yield than second producer. There are many varieties of chickpea which can be improved through breeding programs, for this DNA fingerprinting is an important tool. Also DNA fingerprinting is most prominent method for protection of university and farmers released varieties from biopiracy. DNA fingerprinting includes the analysis by using molecular markers. Three different types of marker systems i.e. SSR, DAMD and SCoT were used in this study to generate fingerprint of selected genotypes. For six chickpea genotypes screening, 78 primers were used. Among them 33 primers were polymorphic and they produced 290 bands. Out of that, 103 bands were polymorphic and the amplicons size ranged from 100bp – 1107bp, by all marker systems across the genotypes. CESSR 42, CESSRDB 54, SSR 22, SSR 60, SSR 63, TA-96, GA-20, ICCM65a, SCoT 11 and URP4R markers effectively discriminate chickpea genotypes by producing unique amplicons. For the genetic diversity analysis dendrogram and similarity matrix was prepared by using software. The genetic similarity was ranged from 0.775 – 0.906 with an average 0.840, which indicates high diversity in genotypes studied. The data of genetic diversity analysis will be very useful for protection of the genotypes.

Keywords: fingerprinting, varieties, genotypes, molecular markers, ssr, damd, scot, etc
SSR: Simple Sequence Repeats
SCoT: Start Codon Targeted
DAMD: Directly Amplified Minisatellite DNA

Introduction
Chickpea (*Cicer arietinum* L.), is the third most important cool season legume in the world, is a diploid with 2n=16 (Arumuganathan *et al*., 1991) [1] with genome size 931 Mbp. India is the world leader in chickpea (Bengal gram) production produces some 15 times as much as the second largest producer, Australia. In India major states growing chickpea are Madhya Pradesh, Rajasthan, Bihar, Maharashtra, Uttar Pradesh, West Bengal, Gujarat, Punjab, Haryana, Karnataka, Andhra Pradesh etc.

Chickpea is a nutrient-dense food, providing rich content (>20% of the Daily Value, DV) of protein, dietary fiber, folate and certain dietary minerals such as iron and phosphorus (Jukanti *et al.*, 2012) [2] Thiamin, vitamin B_6, magnesium, and zinc contents are moderate, providing 10 – 16% of the daily value. Chickpea is good and cheap source of protein for people in developing countries. Chickpea is rich in minerals (phosphorus, calcium, magnesium, iron and zinc), fiber, unsaturated fatty acids and β-carotene.

DNA fingerprinting is a tool which has a variety of applications with animal studies and recently is being used in plants. It is used to identify genetic diversity within breeding populations, to positively identify and differentiate accessions, cultivars, and species that might be difficult to characterize due to similar morphological characteristics. Different molecular marker systems have been used as per their efficiency in detecting polymorphism and assessing genetic diversity. Among numerous techniques available for accessing 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) [3]. A variety of molecular marker systems are

used for cultivars identification such as SSR, ISSR, SCoT and DAMD. Paksereshtet *et al*. in 2013 [4] worked on ISSR, SCoT and DAMD to generate polymorphic fragments in chickpea and to identify the relationships of different types of molecular fingerprinting with morphological based clustering of genotypes. Simple Sequence Repeats (SSR) is a potentially powerful technique for DNA fingerprinting is which have long been known to be polymorphic and wide in plant genomes (Morgante *et al*., 1993) [5]. SSR primers have shown high levels of polymorphism in many important crops (Varshney *et al*., 2007) [6]. With initiating a trend away from random DNA markers towards gene-targeted markers, a novel marker system called Start Codon Targeted (SCoT) (Collard and Mackill, 2009) [7] was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT markers are generally reproducible, and it is suggested that primer length and annealing temperature are not the sole factors determining reproducibility. Minisatellite DNAs are tandemly repeated regions of genomes, many of which show high levels of length differences due to variations in the number of repeat units (Jeffreys *et al*., 1985) [8]. This technique was first reported by Heath *et al*. (1993) and termed as directed amplification of minisatellite region DNA (DAMD). The efficiency of SCoT and DAMD for fingerprinting of genotypes was more than ISSR marker (Paksereshtet *et al*., 2013) [4].

Materials and Method

Collection of experimental material

Seeds of various elite genotypes of chickpea were supplied by Senior Research Scientist, Pulses research unit, Dr. PDKV, Akola. The six genotypes namely JAKI-9218, PKV-K-2, Vijay, Vihar, Digvijay and AKG-46 are used in present investigation.

Procedure for DNA isolation

DNA isolation based on the protocol of Doyle and Doyle, 1990 was followed to obtain good quality DNA from the chickpea varieties. Leaf sample was taken and grinded with liquid nitrogen in mortar and pestle. After fine grinding preheated extraction buffer containing β- mercaptoethanol was added. The grinded extraction was taken in eppendorf tube, and incubated in water bath at 60ºC for 1hr with periodic inverting the tubes. The tubes were centrifuged supernatant was collected into another tube and equal volume of Chloroform: Isoamyl alcohol (24:1) was added. Then the tubes were centrifuged, the aqueous layer was collected in another tube and equal volume of ice-cold isopropanol was added, the tubes were slightly vertexed and incubated at –20°C, then centrifuged to get the pellet. The pellet collected and washed twice with 70% ethanol. The pellet was dissolved in TE Buffer and stored in –20°C temperature for further studies. The RNA was removed by treating with RNase A (10 mg/ml) in Thermo mixture. The DNA obtained after extraction had been confirmed in agarose gel electrophoresis. Extracted DNA was quantified by using Nano-spectrophotometer at 260/280nm wavelength, the purity was between 1.7 - 1.8. The concentration of aliquot was made up to 50ng for the PCR reaction.

Markers used

About 53 SSR, 15 SCoT and 10 DAMD markers were used for screening of six chickpea genotypes. List of total 78 primers used for this study are given in Table 1 and Table 2.

Table 1: List of SSR markers used for study

SSR markers			
Sr. No.	**Primer name**	**Primer Sequence (5' → 3')**	**Primer Sequence (5' → 3')**
1.	CESSR14	GGCACAAGGTATCTCCACAA	ATGCTTGCCTCAACCTCAGA
2.	CESSR15	CATGACATCCTCAATCCTTGG	TAGCGACAAATCTTAGCCGTAG
3.	CESSR20	CGAAACTCGAACGTGCAAT	TTTGGCGAATTTGAAAGGAG
4.	CESSR30	TCGGACCACAAGAGCATCTA	CGTGGAAGAAAGGAATGTTG
5.	CESSR31	ACGTAGGTTAAGGTTGCTGGTC	TTCAACGTGTTCGAAAGCTC
6.	CESSR42	TGGTTGAAGAAAAGAAGGTAGTG	CGGTTCACTAATGCAAAAACCT
7.	CESSR43	CATTAAAGCTAGGAGTTTGTGCTG	ACGGTACCATACCCGACTACAT
8.	CESSR61	CACTCTTCCCTCCCTTTCTTTA	GAATCAGGGTAGGTTTGTTTGC
9.	CESSR62	ACCAGCTGCTAGACCTGATGTT	GCAATAAAACAAAATCCTCACACC
10.	CESSR65	CTCCTCCACTCATCT TCATCTTC	GAGAAGGTGTTTCCGGTAAAAGT
11.	CESSR68	AATGGCCACCATTTTCTCATC	AAACGTTCTTTCCATCCTTCTG
12.	CESSR71	TTGTAGTTCTCCTCTCTCTCTCTC	CATCAAAACCAAACCTATGGAG
13.	CESSR72	ATTTCACTCCTCACTTCTCACC	CACGAAAATCGGATGATTCAG
14.	CESSR77	CCAACTTAAACTCATTTCGTCTCA	CCAAGATGTGTTTTTGATGATG
15.	CESSR78	ATTGCTGAGGCTGTGAATTGTA	CCCAATACATCAAAGATAGATCG
16.	CESSRDB13	ATCTGGGAGCTTGTGAGTTA	TTGTATCTCCTTCAGATGGC
17.	CESSRDB23	GTGTGGACCTGAAATTGAGT	GAATATGGGAACAAGTGCAT
18.	CESSRDB27	GGTGAGATTAGGAAGCAATG	TATCCAATCCCCATAAGATG
19.	CESSRDB29	TTTAGTTGCACAACAACAGC	AAATCCACATCCAAAAAGGT
20.	CESSRDB35	TCTAGAGCTAGCCAAAGGAA	GCATCGTAATCATCGGTACT
21.	CESSRDB45	AGATGGTTTGAATGTTGAGG	CACTTGACCCTTTGATTGTT
22.	CESSRDB54	AGTGTTGTGGGTTTCATTTC	TTGATTTGCCAAAGTACACA
23.	CESSRDB56	TGTCTGGAACAACAAGTGAG	GCCAATCAGATTTCCTCTTA
24.	SSR 1	TGAATTTTGTTTACCACCCCTC	TTTGGCTTATTCTGTTCTTCCC
25.	SSR 2	GACAAAACAACCTCCCAAGAAA	GACGACAACAACAACAACAACA
26.	SSR 4	GACAAAACAACCTCCCAAGAAA	AACAACGACAACAACAACAACG
27.	SSR 5	GAGCCCTGAAATGAAGAAAGAA	CACCTTTGAGCCCTAGTCTGTT
28.	SSR 7	CACACACACAGACACACACACA	TGGTTCAGACATCACACCAAAT
29.	SSR 14	ACCTCCGTCCACACATTTCTAC	GTCGAAGCCATTGTTTTGTTG
30.	SSR 22	GCTTTCCCTTTACTTCTTGGGT	TGCTATTCAAGTCTCCCTCCTC
31.	SSR 24	TGTCAGTGGATCACCAATTAGC	CAATCCCCATAAGATGAACTCC

32.	SSR 25	GGCACATGGTTCCTCTTAAACT	CCATCACACCTTATTGCTTTCA
33.	SSR 31	TAACGACAACGACAACAACAGC	GCCATTCCAGAGAGCCTTG
34.	SSR 35	TGCTCATGCTTACTTCTTCCTG	GTCGGCTTGGCTCATGTAAT
35.	SSR 38	GCGAAGGCTGTTAAGTGGAG	GACGACGAGGATGAGGACTT
36.	SSR 39	CCTGTGCATAAAGAAAACCTCC	GGTAGAAACGACGAATAGGGC
37.	SSR 42	CCTTTGGGTGGTTCATAGAAAA	CATCGTAATCATCGGTACTCCA
38.	SSR 56	GTGTGAGGAAAATTGAGGGAAG	ATGATTACGCCAAGCTCAGAAT
39.	SSR 58	GATTCGCCCTTTCGAGCG	TGGTGAGAGAAGCAAGACCCA
40.	SSR 60	GGTCATGTTGATTTCTCACCAA	GAACTTTCCGCACACGTTATG
41.	SSR 61	GTTACAAGTCGCCATTCCAAA	CATTTGTCTCGTTCACATACCG
42.	SSR 62	CATGCTCCCCTAATTGACATA	AGGCATAAATCCATCTGCATAC
43.	SSR 63	TCCGATGGAACCTTCTCTTTTA	CTCTTCGGGGTCGTATTGATT
44.	ICMM0065a	CATTCCCGCAAAGCTTGTAT	GGTGCCTTGGAGAAAATCAA
45.	ICMM0127	TGTTGAACGAATTTACTCATCG	GGTGGGCTCCTATTGTTTGA
46.	ICMM0293	AGTGATGCCACGAGAATTGC	CTGGTTCGGAATTGTCATCC
47.	ICMM0249	TTTCTTCGCATGGGCTTAAC	GGAGATTTGTTGGGTAGGCTC
48.	TR-20	ACCTGCTTGTTTAGCACAAT	CCGCATAGCAATTTATCTTC
49.	TA-96	TGTTTTGGAGAAGAGTGATTC	TGTGCATGCAAATTCTTACT
50.	GA-20	TATGCACCACACCTCGTACC	TGACGGAATTCGTGATGTGT
51.	GA-16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC
52.	TR-19	TCAGTATCACGTGTAATTCGT	CATGAACATCAAGTTCTCCA
53.	TA-2	AAATGGAAGAAGAATAAAAACGAAA	TTCCATTCTTTATTATCCATATCACTACA

Table 2: List of DAMD and SCoT markers used for study

SCoT markers			DAMD markers		
Sr. No.	**Primer Name**	**Primer sequence**	**Sr. No.**	**Primer Name**	**Primer sequence**
1.	SCoT1	CAACAATGGCTACCACCA	1.	URP1F	ATCCAAGGTCCGAGACAACC
2.	SCoT2	CAACAATGGCTACCACCC	2.	URP2F	GTGTGCGATCAGTTGCTGGG
3.	SCoT11	AAGCAATGGCTACCACCA	3.	URP6R	GGCAAGCTGGTGGGAGGTAC
4.	SCoT12	ACGACATGGCGACCAACG	4.	URP4R	AGGACTCGATAACAGGCTCC
5.	SCoT13	ACGACATGGCGACCATCG	5.	URP9F	ATGTGTGCGATCAGTTGCTG
6.	SCoT14	ACGACATGGCGACCACGC	6.	URP13R	TACATCGCAAGTGACACAGG
7.	SCoT16	ACCATGGCTACCACCGAC	7.	URP17R	AATGTGGGCAAGCTGGTGGT
8.	SCoT20	ACCATGGCTACCACCGCG	8.	URP25F	GATGTGTTCTTGGAGCCTGT
9.	SCoT22	AACCATGGCTACCACCAC	9.	URP30F	GGACAAGAAGAGGATGTGGA
10.	SCoT24	CACCATGGCTACCACCAT	10.	URP38F	AAGAGGCATTCTACCACCAC
11.	SCoT25	ACCATGGCTACCACCGGG			
12.	SCoT28	CCATGGCTACCACCGCCA			
13.	SCoT33	CCATGGCTACCACCGCAG			
14.	SCoT35	CATGGCTACCACCGGCCC			
15.	SCoT36	GCAACAATGGCTACCACC			

PCR amplification

Amplification was carried out in reaction mixture (20µl) containing, 2.5µl of 10X buffer, 1µl of 25mM $MgCl_2$, 1.5µl of 50ng template DNA, 0.5µl of forward primers, 0.5µl of reverse primers, 0.5µl dNTP mix (10mM each) and 0.2µl Taq polymerase. PCR was performed in Thermal Cycler of Applied Biosystems. The reaction used had initial denaturation 94°C for 3min; Denaturation 94°C for 45secs; annealing at 50-60°C for 1min; extension at 72°C for 1min for 30 cycles then final extension at 72°C for 10min and hold at 4°C. The amplified products were analyzed by 8% Polyacrylamide gel electrophoresis stained with Silver Nitrate and observed with a white light illuminator. For determination of the approximate size of bands a 100bp DNA marker was run along with the amplified PCR products. The gel after being visualized under white light with was then photographed using Gel Documentation Unit (Bio-Rad). The scoring of all markers amplicons were recorded for each genotype.

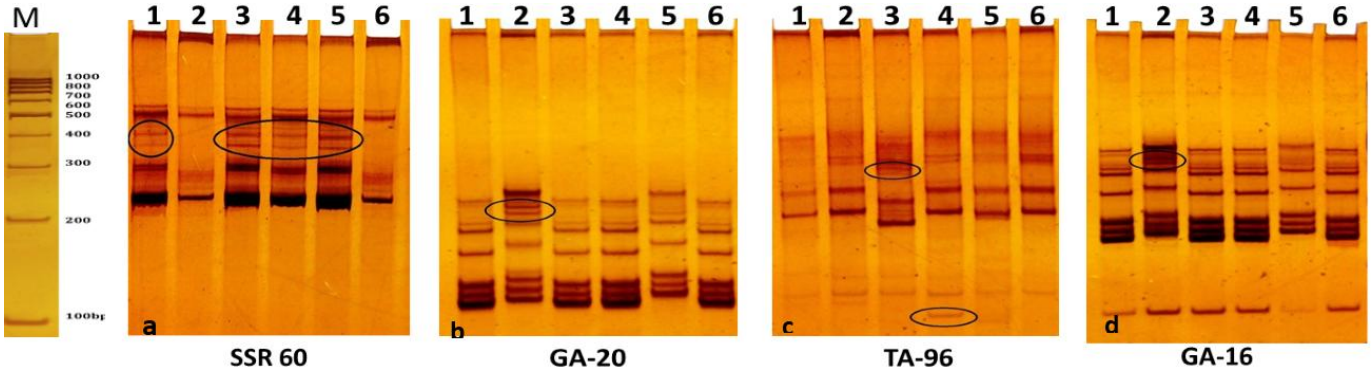

Fig 1: Amplification of chickpea by primer a. SSR 60, b. GA-20, c. TA-96 and d. GA-16

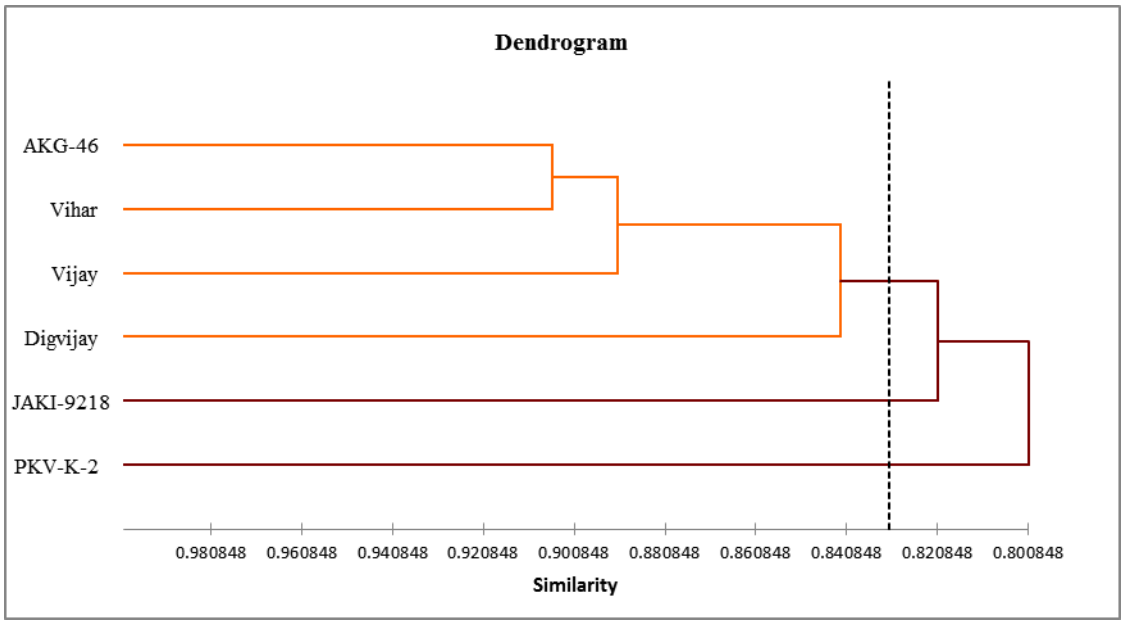

Fig 2: Dendrogram of Chickpea genotypes constructed using XL-STAT

Result and Discussion

Screening and validation of various markers systems used for Chickpea

Six university released genotypes of chickpea were screened by 53 SSR primers. From that 25 primers produced polymorphic alleles. The number of amplified fragments varied from 5 to 14 with an average nine bands per primer. Maximum polymorphism of 75% was produced by primer GA-20. Out of 222 bands, 78 bands were polymorphic and the amplicons size ranged from 100bp – 990bp. An average polymorphism of 33% observed in all primers across the six genotypes of chickpea. CESSR and CESSRDB primers were used by Choudhary *et al*., 2008 [11] for allelic variation in 30 chickpea accessions and reported the band size ranged from 173bp – 650bp whereas Shukla *et al*., 2011 [12] used SSR primers for genetic diversity analysis in chickpea reported 3-18 number of bands for SSR primers used.

Among 15 SCoT primers used, four primers produced polymorphic alleles. The number of amplified fragments for these four primers varied from seven to ten with an average eight bands per primer. Maximum number of polymorphic bands was produced by primer SCoT 25. SCoT 25 showed amplification of total eight bands in size 247bp – 467bp in Vijay, Vihar, Digvijay and AKG-46 whereas out of eight bands only 358bp band was amplified in JAKI-9218 and PKV-K-2. Out of 33 bands, 15 bands were polymorphic and the amplicons size ranged from 115bp – 1107bp with an average polymorphism of 45% by polymorphic SCoT primers across the genotypes. Pakseresht *et al*., 2013[4] studied comparative assessment of ISSR, DAMD and SCoT for evaluation of genetic diversity among 40 chickpea genotypes collected from north-west.

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In present investigation 10 DAMD primers were used for screening of chickpea from which four primers produced polymorphic alleles. The number of amplified fragments by these four primers varied from 7 to 12 with an average of nine bands per primer. URP17R produced only one polymorphic allele from seven amplified alleles, whereas remaining three primers produced three polymorphic bands each. Out of 35 bands, 10 bands were polymorphic and the amplicons size ranged between 121bp – 800bp with an average polymorphism of 29% by four polymorphic DAMD primers across the genotypes. Pakseresht *et al*., 2013 reported genetic diversity among 40 chickpea genotypes collected from north-west regions of Iran. In their study they got 8-12 fragments produced by 10 DAMD primers. The statistics of all polymorphism is given in Table 3.

Table 3: Statistics of chickpea polymorphic primers used for analysis

Sr. No.	Primer name	Total number of amplified bands	No of poly-morphic bands	No of mono-morphic bands	Polymorphism %	Band size Range (bp)
SSR primers						
1.	CESSR 42	12	08	04	66	220-520
2.	CESSR 62	10	06	04	60	310-480
3.	CESSR 65	05	01	04	20	210-355
4.	CESSR 71	10	04	06	40	180-990
5.	CESSRDB 13	05	01	04	20	285-536
6.	CESSRDB 23	10	01	09	10	186-970
7.	CESSRDB 27	05	01	04	20	167-281
8.	CESSRDB 45	09	04	05	44	222-445
9.	CESSRDB 54	11	07	04	63	187-560
10.	SSR 2	14	01	13	07	164-517
11.	SSR 7	09	02	07	22	178-585
12.	SSR 22	04	01	03	25	277-521
13.	SSR 25	08	02	06	25	167-566
14.	SSR 35	07	01	06	14	286-700
15.	SSR 60	07	04	03	57	265-540
16.	SSR 62	12	01	11	08	123-678
17.	SSR 63	10	01	09	10	186-970
18.	ICCM 0065a	12	01	11	08	175-987
19.	ICCM 0127	08	05	03	62	167-635
20.	ICCM 0249	08	01	07	12	168-336
21.	ICCM 0293	07	02	05	28	218-498
22.	TR-20	07	02	05	28	121-319
23.	TA-96	08	04	04	50	174-310
24.	GA-20	12	09	03	75	118-221
25.	GA-16	12	07	05	58	100-279
SCo T primers						
1.	SCoT 1	08	03	05	37	115-535
2.	SCoT 11	10	04	06	40	150-1107
3.	SCoT 12	07	01	06	14	175-750
4.	SCoT 25	08	07	01	87	247-555
DAMD primers						
1.	URP 1F	09	03	06	33	160-710
2.	URP 4R	12	03	09	25	121-720
3.	URP 17R	07	01	06	14	138-380
4.	URP 30F	07	03	04	42	100-800

Validation of polymorphic markers for molecular fingerprinting

All the polymorphic primers were considered for presence and absence of bands among the genotypes. The 33 polymorphic markers were validated further for the JAKI-9218. It is recorded that out of 33 markers, in JAKI-9218, 14 primers showed amplification. They produced 31 polymorphic alleles of band size ranged from 158bp – 487bp. Among them two alleles discriminate JAKI-9218 from other five genotypes under study. SSR 22 and SCoT 11 each produced a unique band of size 277bp and 174bp respectively. For PKV-K-2, 21 primers showed amplification which produced 51 polymorphic alleles. Out of these 51 alleles of band size between 142bp – 680bp. CESSR 42 has produced three alleles of size 222bp, 230bp and 280bp whereas GA-20 has produced an allele of size 201bp which was unique for PKV-K-2. Vijay has produced 53 polymorphic alleles by 23 amplified primers from total 33 polymorphic primers.

The band size ranged from 158bp – 586bp. Amplicons of size 193bp and 247bp were found to be unique for Vijay produced by primer TA-96 and SSR 63 respectively. Vihar showed amplification by 22 primers. A total of 48 polymorphic alleles of size between 158bp – 487bp were obtained. Amplicons of size 167bp and 185bp produced by SSR 25 were unique which discriminate Vihar from other genotypes of chickpea under study. Digvijay showed amplification by 22 primers which produced 59 polymorphic bands of size ranged from 142bp – 560bp. A band of size 278bp, 278bp and 551bp was unique produced by CESSRDB 54, ICCM 065a and URP 4R respectively in Digvijay. Out of 33 polymorphic primers, 19 primers showed amplification in AKG-46. 44 polymorphic bands were produce of size from 158bp – 467bp.

Genetic similarity and dendrogram analysis

Amplified bands obtained with all the molecular markers were scored visually for the presence (1) and absence (0) of bands for all the genotypes. The estimates of genetic similarity for all marker systems used in chickpea based on Jaccard's coefficient ranged between 0.775 - 0.906. Vihar and AKG-46 showed closest relationship with 0.906 similarity (Table 4). A main cluster of four genotypes was found which include AKG-46, Vihar, Vijay and Digvijay. PKV-K-2 and JAKI-9218 were found to be genetically dissimilar from the group of four genotypes forming the cluster. PKV-K-2 showed similarity with the other genotypes ranging from 0.788 – 0.828 and JAKI-9218 showed similarity with other genotypes ranging between 0.788 – 0.844.

Table 4: Genetic similarity matrix of chickpea genotypes

	JAKI-9218	PKV-K-2	Vijay	Vihar	Digvijay	AKG-46
JAKI-9218	1					
PKV-K-2	0.775	1				
Vijay	0.844	0.788	1			
Vihar	0.826	0.793	0.892	1		
Digvijay	0.788	0.821	0.844	0.835	1	
AKG-46	0.825	0.828	0.891	0.906	0.848	1

Conclusion

The DNA fingerprinting and genetic diversity analysis is essential for further breeding ddevelopment programs on chickpea. It is also plays an important role in reducing biopiracy of various varieties. Diversity between the varieties based on morphological characters which vary due to environment and its evaluation requires plants with full maturity. Markers based on expressed gene products, proteins or isozymes are also influenced by the environment and reveal a low level of polymorphism and low abundance (Ram *et al*., 2007) [13]. While molecular markers are based on DNA acts as powerful tools in the genetic variation assessment and in the evaluation of genetic relationships within and among species. The molecular markers are also untouched by environmental factors (Powel *et al*., 1996) [14]. Because of the high level of polymorphism in microsatellite markers they particularly useful for developing unique DNA profiles and such profiles would be especially valuable to unambiguously distinguish cultivars in order to obtain plant variety protection.

In this study, SSR, SCoT and DAMD these four types of marker systems were used for analysis of DNA fingerprinting. Among them SSR was more efficient as compared to SCoT and DAMD. Total 25 SSR primers showed polymorphic amplification. And for the unique varietal identification 8 primers produce unique amplification they are as CESSR 42, CESSRDB 54, SSR 22, SSR 60, SSR 63, TA-96, GA-20 and ICCM65a. From 15 SCoT and 10 DAMD markers only two primers showed unique amplicons, one by SCoT and one by DAMD. As per the results obtained from this study, microsatellite markers (SSR) are most prominent, accurate and effective method for DNA fingerprinting and varietal identification. Also SSR showed high polymorphism and reproducibility than other two marker systems under study.

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