Genetic Diversity Analysis of Kabuli Chickpea (*Cicer Arietinum* L.) Promising Genotypes Using Molecular Marker

Tajender Kumar [1], Priyanka N Timbadiya [2], U K Kandoliya [3]

[1]*Student, Dept of Biotech, College of Agri, Junagadh Agril University, Junagadh, Gujarat, India.*

[2]*SRF, Dept of Biotech, College of Agri, Junagadh Agril University, Junagadh, Gujarat, India.*

[3]*Associate Professor, Dept of Biotech, College of Agri, Junagadh Agril University, Junagadh, Gujarat, India.*

Corresponding Author: piya.timbadiya@gmail.com

Abstract: - The present investigation was carried out to study molecular diversity of different 20 kabuli Chick pea genotypes using PCR based molecular markers to find out the phylogenic relationship among them. In this study, 21 RAPD, 15 ISSR and 15 SSR primers were used to observe the relationship and polymorphism of kabuli Chick pea genotypes. Out of 21 RAPD primers 10 has generated 68 polymorphic bands with an average of 1.64 bands with 100% polymorphism per primer. The 15 ISSR primers engendered 82 bands in which 80 bands were polymorphic with 75 shared and 5 unique bands that were equal to 100% polymorphism with an average of 5.3 bands per primer. Total of 20 SSR primers were tested, with 12 of them amplifying a total of 14 bands out of them 7 polymorphic and 7 monomorphic bans having 22 shared and 1 unique band with 75.55% polymorphism with an average of 1.5 bands per primer. The similarity coefficient of clusters analysis ranged from 69 to 94% for RAPD, 65 to 97 % for ISSR and 66 to 75% for SSR. dendrogram which divided the genotypes into two main clusters I and II with an average resemblance of 77 %. Cluster I consist of all 19 Genotypes while cluster II consist only one genotype (ICCV-191308) which is most variable from all the genotypes. The pooled data of RAPD, ISSR and SSR generated clustering pattern appeared to be similar as RAPD clustering pattern. Based on the molecular markers associated with coconut genotypes, it was concluded that all molecular markers like RAPD, ISSR and SSR are most reliable to distinguish kabuli Chick pea genotypes.

Key Words: ***Cicer arietinum L., Kabuli chickpea, RAPD, ISSR, SSR, PCR, Molecular marker.***

I. INTRODUCTION

Chickpea (Cicer arietinum L.), also known as Gram, Chana, Bengal gramme, and Garbanzo beans, is the world's second most significant food legume after soybean, widely farmed as a source of protein in tropical, sub-tropical, and temperate climates (Gaur et al., 2012).

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It is a self-pollinated crop from the Fabaceae family (Kupitcha, 1977), with a basic chromosome number of 2n=2x=16 and a genome size of 738 Mb (Varshney and Tuberosa, 2013). Although the pulses and legume crops are self-pollinated crops, it showed remarkable variability in biochemical composition of seeds (Saba et al., 2015). It also showed very wide variety of response to altered environmental condition like disease stress (Kandoliya and Vakhariya, 2013 a; Patel et al., 2015), abiotic stresses (Patel et al., 2019 a; Shaikh et al., 2021; Trivedi et al. 2018) as well as application of various chemicals and hormones (Patel et al., 2019 b; Shaikh et al.,2022; Solanki et al., 2018). Chick pea (Cicer arietinum L.) also being a self-pollinated pulse crop showed significant degree of genetic variability (Kandoliya and Vakhariya, 2013 b) among the different varieties. The large-scale production and the development of

better varieties of kabuli chickpea in this area are restricted by the lack of information about their genetic diversity. In India there are problems in improving a self-pollinated crop like chickpea productivity, due to lack of efficient genetic variability. Therefore, attempts were made with an objective to analyze molecular diversity of different promising genotypes of chickpea using PCR based molecular markers.

II. MATERIALS AND METHODS

The experiment was carried out in Department of Biotechnology, Junagadh Agricultural University, Junagadh during 2020-21. Genomic DNA isolated from fresh root tips by following modified CTAB (Cetyl Trimethyl Ammonium Bromide) method as described by (Doyle and Doyle, 1987). In order to perform PCR (Polymerase Chain Reaction) based analysis, the DNA concentration was determined by picodrop (Qiagen). The concentration of DNA was adjusted to ng.µl^{-1}for further work. Various molecular marker techniques such as Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) were used for fingerprinting of kabuli chickpea genotypes. Primers required for the above techniques were synthesized at sigma aldrich chemicals pvt ltd.

RAPD analysis: Amplification of RAPD fragments was performed according to (Williams *et al.* 1990) with some modification using arbitrary primers The PCR reaction mixture (15µl) contained 10x PCR buffer (10 X Tris – HCL, pH 8.3), 2.5 mM each dNTPs, 25 pmoles primer, 50 ng of genomic DNA and 3 unit of Taq DNA polymerase (Promega, Madison, WI). The samples were subjected to 40 repeats of the following cycle: 94° C 1 min, 35° C for 1 min, 72°C for 2 min following a final extension of 10 minutes.

ISSR analysis: Genomic DNA was amplified using PCR reactions for ISSR according to method given by (Palai and Rout 2011). with some modifications. Fourteen ISSR primers were randomly selected for the study. The PCR reaction mixture (**15 µl**) contained 10x PCR buffer (10mM Tris-HCL, pH 8.3), 2.5 mM each dNTPs, 25 pmoles primer, (Promega, Madison, WI). Initial denaturation was for 1 minutes at 94°C, 45 s at 48°C and 2min at 72°C; with a final extension at 72°C.

SSR analysis: Twelve SSR primers were used for the PCR reaction as given by (Wani, *et. al.*, 2020) with modifications. The 25il reaction mixture contained 10x PCR buffer (10 m M Tris-HCL, pH 8.3), 2.5m M ach dNTPs, 25 pmoles each for Forward and Reverse primers, 50 ng.µl^{-1} of genomic DNA and 3 unit of Taq DNA polymerase (Promega, Madison, WI). All SSR amplification were performed by initially incubating at 94°C for 0.30 min, 60°C for 1 min, 72°C for 2 in; with final 5 min extension a 72°C.

All the above PCR amplification was performed in 0.2 ml thin-walled tubes placed in a thermal cycler (LightCycler, Eppendroff). The amplified products were analyzed by electrophoresis in 1.2% (RAPD), 1.5% (ISSR) and 1.8% (SSR,) agarose gel stained in ethidium bromide (10mg/ ml) and run 1x Tris borate EDTA buffer at 80 V for 45 minutes, The separated bands were visualized under UV transilluminator and photographed using a gel documentation system (BioRad).

Statistical analysis: Polymorphic information content (PIC) for RAPD, ISSR and SSR was calculated on the basis of allele frequency (Anderson *et al.,* 1993).

$$PIC_i = 1 - \sum_{j=1}^{n} Pij^2$$

Where,

Pij is the frequency of jth allele for marker i and summation extends over n

alleles.

PIC values were used to calculate a RAPD primer index (RPI), ISSR primer index (IPI) and SSR primer index (SPI) which were generated by multiplying the PIC values of all the markers amplified by the same primer.

Dendrogram analysis: Clear and distinct bands amplified by RAPD, ISSR and SSR primers were scored for the presence (1) and absence (0) for the corresponding band among the genotypes. The data were entered in to MS-Excel data sheet and subsequently analyzed using NTSYS-pc version 2.02 (Rohlf, 1998). The data matrix was read by NTSYS-pc version 2.2 (Numerical Taxonomy and Multivariate Analysis System for Personal Computers, Exeter Software) and analyzed by the SIMQUAL (similarity for qualitative data) program with Jaccard's similarity coefficient. SIMQUAL is a program for computing a variety of similarity and dissimilarity coefficients for qualitative data. The qualitative nature of the absence (0) or presence (1) state of a RAPD marker was used as the basis for similarity analysis among various sesame genotypes. A matrix of 0 and 1 act as the input, and the output is a matrix of similarity or dissimilarity coefficients. The resultant similarity matrix was entered into SAHN (sequential, agglomerative, hierarchical, and nested clustering method) clustering program, a tree matrix was produced and a dendrogram constructed using UPGMA (Unweighted Pair-Group Method with Arithmetic Averages). The assumption underlying the use of UPGMA

clustering is the equal rate of evolution along all dendrogram branches. Dendrogram of publication quality were produced from the output tree file of SAHN by TREE (tree display) program in graphics mode.

III. Result And Discussion

Out of 21 RAPD primers screened, 11 primers amplified a total of 68 bands. The RAPD markers OPY-25 produced maximum number of 8 bands, while OPY-5 produced minimum number of 3 bands. Out of 68 bands, all the bands were polymorphic with an average of 6 bands per primer. all the 68 polymorphic bands, shared polymorphic within one variety, (Table 1).

Table.1. Size, number of amplified bands, percent polymorphism and PIC obtained by RAPD primers

Sr.no.	Primer	Allele/Band Size(bp)	Total number of bands	Numberof Poly-morphic bandS	Number of Mono-morphic bands	% Polymor-phism	PIC value	RPI
1.	OPA-10	350-1300	7	7	0	100	0.39	2.76
2.	OPA-16	300-1250	6	6	0	100	0.25	1.51
3.	OPA-17	150-1350	7	7	0	100	0.20	1.38
4.	OPA-18	350-1300	7	7	0	100	0.33	2.33
5.	OPC-05	350-950	3	3	0	100	0.25	0.74
6.	OPC-06	400-1350	7	5	0	100	0.30	2.11
7.	OPC-08	400-1300	6	6	0	100	0.27	1.59
8.	OPD-20	400-1250	4	4	0	100	0.21	0.83
9.	OPE-19	200-1150	7	7	0	100	0.14	0.99
10.	OPY-20	400-1200	6	6	0	100	0.32	1.90
11.	OPY-25	250-1300	8	8	0	100	0.24	1.95
		Total	**68**	**68**	**0**	**100**	**0.26**	**1.64**
		Average		**5.46**				

The percent polymorphism obtained for RAPD primers were 100% with an average value of 100% per primer. The Polymorphism Information Content (PIC) values for RAPD marker were ranged from 0.14 to 0.39 with an average value of 0.26 per primer and RAPD primer index (RPI) differed from 0.74 to 2.76 with an average value of 1.64 as presented in Table 2. The results of present study results are quiet supported by the following research done by several scientists. By using OP series primers for genetic relationship in Chick pea genotypes (Bhagyawan *et al.* 2015) with Genetic diversity of twelve germplasm accessions was determined by RAPD analysis. Ten operon primers selected and out of ten RAPD primers, one was found to be non-amplifying (OPAC-06). Such kind of non-amplifying primers were also reported earlier in chickpea (Bhagyawant et al., 2008). Rest of the RAPD primers yielded a total of 59 bands in a molecular weight range of 500-2000 bps. The average number of bands per primer per accession accounted to 5.9. Maximum number of 9 bands was scored by OPA-18 while least number of bands were shown by OPG-11 and OPZ-10. The total polymorphic bands as generated by these RAPD primers are 16. Highest no. of polymorphic bands was obtained with primer OPG-04 and lowest polymorphic bands were obtained in primer OPZ-06 and OPA-11.

Total 10 chick pea varieties were subjected to ISSR with 20 primers. Out of 20 primers 14 primers amplified a total of 51 bands, out of which 43 bands were polymorphic with an average of 3.07 bands per primer while 8 bands were monomorphic. The average percentage of polymorphism was about 82.14% for the 14 ISSR primers. The highest 100% polymorphism was obtained with 6 primers. The amplified fragments were in range of 100-1300 bp.. The smallest fragment of 100 bp was amplified by UBC-810 demonstrated a shortest distance between two adjacent microsatellites and the largest fragment of 1300 bp was amplified by UBC-822 represented the longest distance between two microsatellites among all the studied varieties. UBC- 834 primer produced highest 5 bands (allele) while UBC-816 and UBC-853 produced lowest 2 bands. The polymorphic information content (PIC) was calculated for each primer. The highest PIC value calculated was 0.87 which was with UBC-810, while lowest PIC value was recorded with prime UBC-823 (0.09) with an average of 0.38 per primer. ISSR primer index (IPI) differed from 0.36 to 2.61 with an average of 1.35 per primer (Table 2).

Table.2. Size, number of amplified bands, percent polymorphism and PIC obtained by ISSR primers in the 28 coconut genotypes and hybrids

Sr. No.	Primer	Allele/Band Size	Total Number of Bands	Number of Poly-morphic BandS	Number of Mono-morphic Bands	% Poly-morphism	PIC value	IPI
1.	UBC -810	100-1100	3	3	0	100	0.87	2.61
2.	UBC-811	950-1190	4	4	0	100	0.19	0.76
3.	UBC-816	1000-1150	2	1	1	50	0.47	0.95
4.	UBC-822	500-1300	4	3	1	75	0.38	1.52
5.	UBC-823	300-650	4	4	0	100	0.09	0.37
6.	UBC-825	280-730	4	3	1	75	0.47	1.87
7.	UBC-826	350-800	4	3	1	75	0.53	2.12
8.	UBC-841	600-1000	4	3	1	75	0.59	2.38
9.	UBC-842	300-900	4	3	1	75	0.36	1.42
10.	UBC-853	500-600	2	1	1	50	0.18	0.36
11.	UBC-855	500-900	3	3	0	100	0.19	0.57
12.	UBC-880	400-1100	4	3	1	75	0.25	1.01
13.	UBC-834	350-1200	5	5	0	100	0.28	1.39
14.	UBC-873	700-1200	4	4	0	100	0.40	1.61
			51	**43**	**8**	**82.14**	**0.38**	**1.35**

The lowest IPI value was with UBC- 853 and highest was with UBC-810.

All the 20 SSRs primers were amplified a total of 12 bands (Table3). The SSR marker CkPea_SS_11 and CkPea_SS_12 produced maximum number of 2 bands, while CkPea_SS_1, CkPea_SS_3, CkPea_SS_4, CkPea_SS_5, CkPea_SS_9,

CkPea_SS_10, CkPea_SS_13, CkPea_SS_16, CkPea_SS_17 and CkPea_SS_18 produced minimum number of 1 band. Out of 14 bands, 7 bands were polymorphic with an average of 05 bands per primer and 0.5 bands were monomorphic. Among the (Table 4). The amplified fragments ranged from 100-300 bp. The largest amplicone of 300 bp was amplified by SSR primer CkPea_SS_9, CkPea_SS_10, CkPea_SS_17 and smallest fragment of 100 bp was found with CkPea_SS_1, The percent polymorphism obtained for SSR primers were ranged from 0 % to 100% with an average value of 50 % per primer. The primer CkPea_SS_1, CkPea_SS_9, CkPea_SS_13, CkPea_SS_16, and CkPea_SS_17 did not give any polymorphic fragment, it gave only 1 monomorphic fragment. The polymorphic information content (PIC) was calculated for each primer and it was ranged from 0.10, 0.10 to 0.44 for CkPea_SS_3, CkPea_SS_10 and CkPea_SS_4 primers respectively with an average value of 0.10 for each primer. The SSR primer index (SPI) also same as PIC data (Table 3).

Table.3. Size, number of amplified bands, percent poymorphism and PIC obtained by SSR primers in the 28 coconut genotypes and hybrids

Sr. No.	Primer	Allele/Band Size	Total Number of Bands	Number of Poly-morphic BandS	Number of Mono-morphic Bands	% Poly-morphism	PIC value	IPI
1.	CkPea_SS_1	100	1	0	1	0	0.00	0.00
2.	CkPea_SS_3	150	1	1	0	100	0.10	0.10
3.	CkPea_SS_4	150	1	1	0	100	0.44	0.44
4.	CkPea_SS_5	290	1	1	0	100	0.19	0.19
5.	CkPea_SS_9	300	1	0	1	0	0.00	0.00
6.	CkPea_SS_10	300	1	1	0	100	0.10	0.10
7.	CkPea_SS_11	100-290	2	1	1	50	0.00	0.00
8.	CkPea_SS_12	100-300	2	1	1	50	0.00	0.00
9.	CkPea_SS_13	200	1	0	1	0	0.00	0.00
10	CkPea_SS_16	200	1	0	1	0	0.00	0.00
11	CkPea_SS_17	300	1	0	1	0	0.00	0.00
12	CkPea_SS_18	250	1	1	0	100	0.36	0.36
		Total	**14**	**7**	**7**	**50**	**0.10**	**0.10**
		Average		**0.5**	**0.5**			

Based on molecular marker study through RAPD, ISSR and SSR kabuli chickpea genotypes can be differentiated from one another to some extent. The reliability of one particular marker does not fulfill the goal of identification of genotypes. So, the pooled cluster analysis using three molecular markers to confirm the difference and similarity between genotypes of present study.

Genetic similarity of all three molecular markers were determined for each pair of twenty kabuli chickpea entries which revealed that the lowest similarity of 69% was noticed between ICCV-191312 and GJGK-1828, while highest of 94% was noticed between ICCV-191311 and ICCV-191310 genotypes (Table 4).

Table.4. Jaccard's similarity coefficient of 20 kabuli chickpea genotypes based on pooled data study.

	ICCV-191306	ICCV-191305	ICCV-181314	ICCV-191313	ICCV-191318	ICCV-191312	ICCV-191302	ICCV-191310	ICCV-191311	ICCV-191303	ICCV-191316	ICCV-191308	ICCV-191301	ICCV-191317	ICCV-191315	KAK-2	PKV-4	GJGK-1828	GJGK-1826	GJGK-1812
ICCV-191306	1																			
ICCV-191305	0.86	1																		
ICCV-181314	0.88	0.81	1																	
ICCV-191313	0.83	0.75	0.93	1																
ICCV-191318	0.94	0.84	0.82	0.85	1															
ICCV-191312	0.86	0.76	0.81	0.81	0.95	1														
ICCV-191302	0.91	0.79	0.93	0.87	0.89	0.90	1													
ICCV-191310	0.86	0.79	0.93	0.91	0.92	0.90	0.95	1												
ICCV-191311	0.91	0.77	0.91	0.89	0.92	0.90	0.93	0.93	1											
ICCV-191303	0.86	0.84	0.89	0.82	0.92	0.84	0.91	0.91	0.89	1										
ICCV-191316	0.91	0.81	0.95	0.89	0.85	0.81	0.93	0.93	0.91	0.89	1									
ICCV-191308	0.78	0.82	0.83	0.76	0.85	0.72	0.86	0.86	0.79	0.83	0.88	1								
ICCV-191301	0.84	0.92	0.79	0.76	0.89	0.81	0.81	0.86	0.83	0.83	0.83	0.81	1							
ICCV-191317	0.82	0.85	0.82	0.82	0.88	0.78	0.84	0.88	0.86	0.84	0.86	0.82	**0.97**	1						
ICCV-191315	0.73	0.83	0.75	0.75	0.80	0.71	0.75	0.8	0.77	0.8	0.77	0.77	0.88	0.87	1					
KAK-2	0.75	0.80	0.81	0.79	0.84	0.76	0.84	0.88	0.86	0.84	0.81	0.77	0.87	0.90	0.91	1				
PKV-4	0.81	0.83	0.83	0.78	0.77	0.69	0.83	0.86	0.83	0.83	0.81	0.75	0.87	0.91	0.87	0.94	1			
GJGK-1828	0.69	0.74	0.76	0.79	0.73	**0.65**	0.73	0.76	0.79	0.76	0.76	0.67	0.79	0.82	0.79	0.85	0.82	1		
GJGK-1826	0.76	0.81	0.82	0.82	0.92	0.84	0.85	0.90	0.87	0.92	0.82	0.75	0.85	0.87	0.83	0.86	0.83	0.82	1	
GJGK-1812	0.77	0.79	0.85	0.82	0.78	0.72	0.82	0.87	0.85	0.78	0.89	0.81	0.86	0.88	0.8	0.84	0.86	0.82	0.80	1

Jaccard's similarity coefficient and UPGMA method were used to develop a dendrogram (Table 5 and Fig 1) which divided the genotypes into two main clusters I and II with an average resemblance of 77 % (Fig 1). The cluster-I was divided into two sub clusters A and B both contained a total of 19 genotypes (Fig. 1).

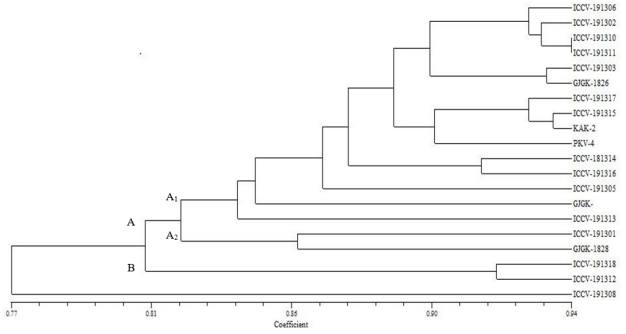

Fig.1. Dendrogram cluster for the pooled data analysis from RAPD, ISSR and SSR

Subcluster-A was further divided into two groups A1 and A2 which had nearly 82 % likeness. Group A1 consists of 15 genotypes and having nearly 84 % similarity, while group A2 consisted of only 2 genotypes such as ICCV-191301 and GJGK-1828 having nearly 86 % similarity. Sub cluster B consisted two genotypes ICCV-191312 and ICCV-191318 having 92% similarity. The cluster -II consist only one genotype i.e., ICCV-191308 which is most variable from other genotypes which could be useful for marker assisted selection in crop improvement programs.

IV. CONCLUSION

Based on the present study, it was concluded that the collective data of different markers used to generate mixed clustering pattern to confirm the difference and similarity between

varieties as the reliability of one particular marker does not fulfill the goal of identification of varieties. From the clustering pattern and genetic relationship obtained using these markers, breeders can identify the diverse genotype from different clusters and employ them in their future breeding programme for a self-pollinated crop like chickpea.

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