International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 4, Issue 1, 2013, pp 158-166
http://www.bipublication.com

MOLECULAR CHARACTERIZATION OF CLUSTER BEAN (*CYAMOPSIS TETRAGONOLOBA*) CULTIVARS USING PCR-BASED MOLECULAR MARKERS

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[Received-01/12/2012, Accepted-29/01/2013]

ABSTRACT

Molecular markers are being widely used in various areas of plant breeding as an important tool for evaluating genetic diversity and determining cultivars identity. The present study was undertaken to evaluate the genetic diversity among fifteen genotypes of Cyamopsis tetragonaloba Taub. by using RAPD technique. A total of three primers generated 19 amplicons with an average of 6.33 amplicons per primer. Of the total amplicons, 15 amplicons were found polymorphic and showed 78.94% polymorphism. Dendrogram constructed by cluster analysis of RAPD markers using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) showed two major and four minor clusters. The maximum genetic similarity up to 98% was observed between the genotypes AVTG-2 and IVTG-8. Overall, the present study revealed the existence of considerable genetic variations among the genotypes. In conclusion, the genotypes used in this study could be tapped in breeding programs aimed at crop improvement.

Keywords: *Cyamopsis tetragonaloba*, Genetic diversity, Dendrogram, RAPD analysis, QTL mapping, Primer, RAPD profile, genotypes

[I] INTRODUCTION

Clusterbean or guar (*Cyamopsis tetragonaloba* Taub.) is a widely grown crop but not on commercial basis on large scale. Basically cluster bean is a draught hardy, deep rooted, summer annual legume. It is an important self pollinated, multipurpose and restorative leguminous vegetable crop. It belongs to the tribe Galagae (Indigoferae) of the Leguminosae family. The haploid and diploid chromosome number of all the three genus of species cyamopsis reported to be n=7 and n=14.Its centre of origin is in the Tropical Africa. The crop is mainly grown in the dry habitats of Rajasthan, Haryana, Gujarat and Punjab and to a limited extent in Uttar Pradesh, Madhya Pradesh, Andhra Pradesh, Tamil Nadu, Karnataka and Kerala. The crop is known for exceptionally high adaptation towards poor and erratic rains, low inputs and less care, soil enrichment properties. These qualities have made it the most favored crop of marginal farmers in the arid areas. Mainly used in paints, pharmaceuticals, cosmetics, textiles, jewelry, handicraft, and insulation. Guar gum is

commonly used as a stabilizer in many food products like ice-cream fruit beverages. Chocolate, milk and milk products cake toppings etc.

The analysis of genetic diversity and relatedness between individuals within a species or between different species of populations are phylogenetic studies which were initially conducted using qualitative and quantitative traits, which are mostly morphological. These analyses are mostly based on quantitative traits that are highly influenced by environmental effects and require tedious statistical procedures. Molecular markers are being widely used in various areas of plant breeding as an important tool for evaluating genetic diversity and determining cultivars identity (molecular fingerprinting). Establishment of a molecular marker and phenotypic assessment database of crop germplasm has helped breeders to trace down the origins and degrees of relatedness of many land races and cultivars. Considering the potential of molecular markers, the crop breeders extended their hands to use these to supplement other tools currently exploited in their crop breeding activities**.**

There are different types of DNA markers those have been developed viz., AFLP-amplified fragment length polymorphism; RFLP-restriction fragment length polymorphism; STS-sequence tagged sites; ASAP-allele specific associated primers; EST- expressed sequence tag markers; SSCP- single strand conformation polymorphism; RLGS-restriction landmark genomic scanning ; VNTRs-variable number of tandem repeats; HVRs-hypervariable regions; RAPD- random amplified polymorphic DNA; SCAR-sequence characterized amplified regions for amplification of specific band; RAMPO- randomly amplified microsatellite polymorphisms; CAPs- cleaved amplified polymorphic sequences (Panella and Gepts, 1992; Ouedraogo *et al*., 2002).

[II] MATERIALS AND METHODS

The present investigation was carried out at Department of Plant Biotechnology. Genetic diversity analysis of Cluster Bean (Cyamopsis tetragonaloba) by using RAPD molecular markers. RAPD marker is a PCR based molecular Marker.

RAPD analysis involves the following four steps:-

a) Total genomic DNA extraction
b) DNA quantification
c) PCR amplification using 10-base random primers
d) Electrophoresis and visualization of amplification products

2.1 Plant Material

A total of Fifteen genotypes of Guar (*Cyamopsis tetragonaloba* (Linn.) Taub.) Collected from Food science and Technology College, M.A.U. Parbhani, The seeds of guar germplasm lines were washed with mild detergent teepol, followed by 3 washings of sterile water and then given treatment of 0.01 % mercuric chloride to avoid contamination and subsequently were washed with sterile distilled water. These treated seeds were kept carefully on previously wetted germination paper blotters under laminar air flow. These blotters were folded properly, tied with rubber bands with appropriate labels according to genotypes. These folded germination papers were arranged on germination trays. The trays were then incubated at $37^{O}C$ in BOD incubator for 7 days and adequate water was sprayed at an interval of 12 hours to maintain sufficient moisture for germination. Germinated seeds of 1.0 to 2.0 cm length from shoot portion (after removing cotyledons) were used for DNA extraction.

2.2 Chemicals and Solutions

Molecular biology grade chemicals used in the present study were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, Qualigens Fine Chemicals, Mumbai, Merck [India] Limited, Mumbai. Taq Polymerase, Dexyribonucleotides, assay buffer, $MgCl_2$ and Agarose used for PCR were obtained from Bangalore Genei Pvt. Ltd. The primers used were obtained from Bioserve Biotechnologies (India) Pvt. Ltd. Hyderabad. For all the molecular biology experiments, sterile double distilled water was used.

2.3 Sterilization

The chemical reagents, tips (20-200 µl and 100 µl), eppendorf tubes (1.5 ml and 0.5 ml), PCR tubes, DNA extraction buffer, mortar and pestle and all other related equipments were autoclaved at 121^{O}C for 15 min. under 1.06 Kg/cm^2 pressure. The glassware's were also autoclaved after washing and rinsing with double distilled water.

Table 1. : List of 15 Genotypes

1	AVT-G-1	9	IVT-G-12
2	AVT-G-2	10	IVT-G-14
3	G-6	11	IVT-G-16
4	G-7	12	G-17
5	IVT-G-8	13	IVT-G-18
6	G-9	14	G-19
7	IVT-G-11	15	G-20
8	G-12		

Table 2: Components of 2 % extraction buffer

Sr. No.	Components	Quantity
1.	10 % CTAB	20 ml
2.	4 M NaCl	35 ml
3.	1M Tris HCl (pH 8.0)	10 ml
4.	0.5M EDTA (pH 8.0)	4 ml
	Sub Total	**69 ml**
5.	Double distilled sterile water	31 ml
	Total	**100 ml**

2.4.2 Isolation of Genomic DNA and purification

The protocol of cetyl trimethyl ammonium bromide (CTAB) DNA extraction method of Saghai-Maroof (1984) was used with some modifications. The different steps in the method included the following:

1. Around 2.00 g of the leaf tissue was grounded to fine powder in liquid nitrogen (-196^{0}C) with mortar and pestle.
2. To the powdered tissue, 20 ml of 2 per cent DNA extraction buffer (with 0.2 % β mercaptoethanol) was added and mixed well by gentle inversion and incubated at 65^{O}C for 30 min in a water bath.
3. The mixture was then subjected to centrifugation at 10,000 rpm at 4^{0}C temperature for 10 min.
4. The supernatant was taken with wide bored pipette and then mixed with equal volumes of freshly prepared Chloroform: Isoamyl alcohol mixture, mixed well and recentrifuged at 10,000 rpm for 10 min. at 4^{0}C temperature and then collected the supernatants into a fresh tube.
5. Repeated the above steps till no white interface was visible and went on collecting supernants.
6. To the collected supernants, 0.7 vol. of chilled isopropanol was added mixed well and the DNA was allowed to precipitate at 20^{O}C for overnight.
7. The DNA was pelleted by centrifugation at 10,000 rpm for 10 min at room temperature. The collected pellet was washed with 70 per cent alcohol by adding 500 µl of 70 per cent ethanol, briefly centrifuging it in the centrifuge machine and then the supernatant was discarded. The washing procedure was repeated twice and pelleted by centrifugation at 10,000 rpm.
8. The collected pellet was air dried in laminar airflow and dissolved in required quantity of TE buffer.DNase free RNase-A was added at a final concentration of 20 µg/ml and incubated at 37^{O}C for 1 hour in hot water bath.
9. To the incubated sample equal volume of phenol: chloroform (1:1) mixture wash added and centrifuged at 10,000 rpm for 10 minutes.
10. The aqueous phase was collected in fresh tube and the above step was repeated till no white interface was seen.
11. To the aqueous phase 0.1 volume of sodium acetate and 2 volumes of ice cold absolute ethanol was added, mixed well and kept it at -20^{O}C for 1 hour.
12. The DNA was pelleted by centrifugation at 10,000 rpm for 10 min at room temperature. The collected pellet was washed with 70 per

cent alcohol by adding 100 µl of 70 per cent ethanol, briefly centrifuged and then the supernatant was discarded. The washing procedure was repeated twice and re-pelleted by centrifugation at 10,000 rpm. The collected pellet was air dried in laminar flow and dissolved in required quantity of TE buffer and stored at 20^{O}C.

2.4.3 DNA quantification

Quality and quantity of DNA was checked by comparing DNA samples with known amount of DNA. The DNA concentration thus estimated and rechecked qualitatively by running on 0.8 per cent Agarose gel. For preparation of Agarose gel, Agarose @ 0.8 gm/100 ml was melted in 1 x TAE buffer by heating to boiling. After cooling the solution to about 45^{O}C, ethidium bromide @ 3-5 µg/ml was added. To the DNA samples, 6 x gel loading dye were added so that the final concentration was 1 x before loading. The gel was run at 60 V for 1 hour.

2.5 Dilution of DNA samples

Part of DNA samples were diluted with appropriate quantity of sterilized distilled water to yield a working concentration of 25ng/µl and stored at 4^{0}C until PCR amplification.

2.6 Primer screening

There are three primers mainly used for molecular characterisation is, OPG-5, OPQ-7, OPQ-11. Primers from different series were screened on the basis of reproductive and scroreable amplifications for the analysis of all the sixteen genotypes of guar.List of the primers used along with their sequences are given in the Table-3.

Table 3: Primers selected for RAPD analysis of the selected genotypes

Sr. No	Primer	Primer sequence
1	OPQ-5	5'-CCGCGTCTTG-3'
2	OPQ-7	5'-CCCCGATGGT-3'
3	OPQ-11	5'-TCTCCGCAAC-3'

2.7 PCR optimization and RAPD Amplification

2.7.1 PCR Optimization for RAPD Analysis

PCR amplification reactions were set in a 25 µl reaction volume. The different components of PCR were optimized to develop an appropriate DNA profile by employing RAPD technique. Different concentrations of genomic DNA (25, 50, 75 ng per 25 µl reaction mixture), $MgCl_2$ (1.5, 2.5 and 3.5 mM) and *Taq* DNA polymerase (0.5 and 0.6 U) were tried to obtain an optimum reaction mixture. The different concentrations of the primers (5, 10, 15, 20 and 25 pm/µl) were also tried for the complete amplification of the genomic DNA. In case of optimization of primer annealing, the different temperatures (34, 35, 36 and 37^{O}C) were tried. The final concentrations of the reagents and enzymes in the final reaction mixture were taken as follows:

Table 4

	Reagents	Volume per reaction
1	Sterile double distilled water	17.7 µl
2	10X PCR buffer (with KCL)	2.5 µl
3	dNTPs (10 mM)	0.5 µl
4	$MgCl_2$ (25mm)	1.5µl
5	Primer (20 pm/µl)	1.5 µl
6	Taq DNA polymerase (1.5U/µl)	0.3 µl
7	Template DNA (25 ng)	1.0 µl
	Total volume:	**25**µl

2.7.2 RAPD amplification

A master mix in sterile double distilled water having all the above mentioned compounds in required quantities was prepared, while preparing the mix, buffer was added to the water first followed by other components to avoid degradation of any of them and all operations were carried out on ice. Twenty five ng of genomic DNA from different samples was taken into individual 0.5 ml PCR tubes and the master mix was added to them to a final concentration of 25 µl. Amplifications were performed in a thermal cycler using the following cyclic parameters.

Table 5

Steps	Temperature	Time	Cycles
Initial Denaturation	**94˚C**	**4**	
Denaturation	**94˚C**	**1**	**Repeat 40 times**
Annealing	**35˚C**	**1**	
Primer Extension	**72˚C**	**2**	
Final Extension	**72˚C**	**10**	**1**
Hold	**4˚C**		

2.8 Agarose Gel Electrophoresis

The amplified products were resolved on 1.2 % Agarose gel in 1 x TAE buffer at 100V for 90 min. The gel was stained in ethidium bromide. After electrophoresis the gel was carefully taken out of the casting tray and photograph was taken on a gel documentation system (Alphamager ^{TM} 2200).

2.9 Scoring and Data Analysis

Each amplification product was considered as RAPD marker and was scored across all samples. Bands were scored as present (1) or absent (0). Missing and doubtful cases were scored as (9). Molecular weights of the bands were estimated by using 1kb DNA ladder (MBI, Fermentas, U.K.) as standards. All amplifications were repeated at least twice and only reproducible bands were considered for analysis. The data was used for similarity based analysis using the programme NTSYS-Pc (Version 2.02) developed by Rohlf, (1990). Dice's similarity coefficients (F') was calculated using the programme SIMQUAL. Similarity coefficients were used to construct UPGMA (unweighted pair group method with average) to generate Dendrogram.The polymorphic percentage of the obtained bands were calculated by using following formula,

Polymorphic % = (no. of polymorphic bands /Total bands) X 100

[III] RESULTS

The genus *Cyamopsis* belongs to the family *Fabales* (subfamily: Faboideae) Cluster bean major importance for live hood of millions of people as vegetable. The addition of small amount of Cluster bean in normal diet can improve the nutritional balance of normal diet by providing high protein and lysine.

The present study entitled **"Molecular characterization of Cluster bean *(Cyamopsis tetragonaloba)* using molecular markers"** was undertaken. In this study, 15 germplasm lines of Cluster bean were subjected to amplification by RAPD primers in PCR master cycler. The banding pattern thus obtained by RAPD markers clearly distinguished germplasm into different clusters showing genetic diversity.

The study was undertaken to focus the following objectives:

1. Isolation of genomic DNA from sample.
2. Amplification of Genomic DNA in PCR and,
3. To carry out Cluster bean analysis.

Observations were recorded considering the above objectives and data were analyzed. The results are presented under following headings:

DNA was extracted using CTAB method of DNA extraction described by Saghai-Maroof (1984) with some modifications. Polyvinyl pyrolidon was added to extraction buffer which resulted in extraction of pure DNA. The results of Agarose gel electrophoresis showed good quality of DNA. The quantity of DNA was checked by comparing DNA samples with known amount of DNA i.e. lambda DNA. Quantity of all samples was found to be in the range of 70-200 ng /µl. These samples were used as stocks and working samples of 30 ng /µl by were used by diluting with sterile double distilled water.

The RAPD primers were used to amplify genomic DNA and amplified product was resolved on 1.2 % Agarose gel by staining with ethidium bromide.

3.1. Genetic diversity analysis using RAPD markers

After isolation of genomic DNA from all 15 germplasm lines of Cluster bean DNA samples were subjected to PCR using RAPD primers. A total of three primers generated 19 amplicons with an average of 6.33 amplicons per primer (Table7). Of the total amplicons, 15 amplicons were found polymorphic. They showed 78.94 per cent polymorphism and the average number of polymorphic bands per primer were 5.0 (Table7).

Different primers produced a different level of polymorphism among the different germplasm.

The primer OPQ-11 was the most informative as it generated highest number of polymorphic amplicons i.e. 7. The primer OPQ-11 stood least informative and was able to generate only 2 polymorphic bands. The average size of fragments was between 100 to 6000 bp (Figure 1).

The data obtained by RAPD markers was analyzed by NTSYS-PC and Dendrogram was generated by using Jaccard's similarity coefficient. Dendrogram is presented in Figure 4. To estimate the genetic similarity of the Cluster bean germplasm a similarity matrix has been shown in Table 6, obtained by Jaccard's similarity coefficient.

The maximum similarity was observed between these two germplasm were found highly similar with similarity coefficient.

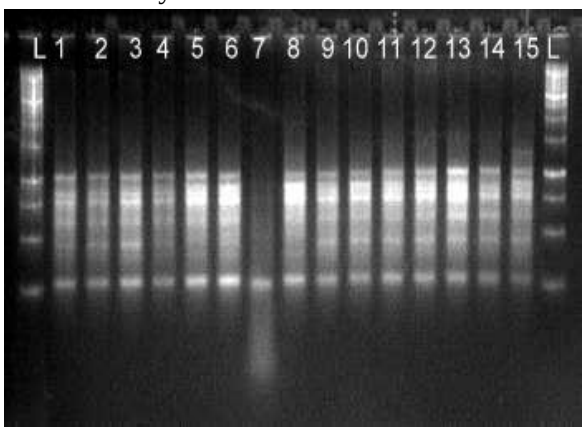

Figure 1: RAPD profile of Guar germplasm lines generated with primer OPQ-5

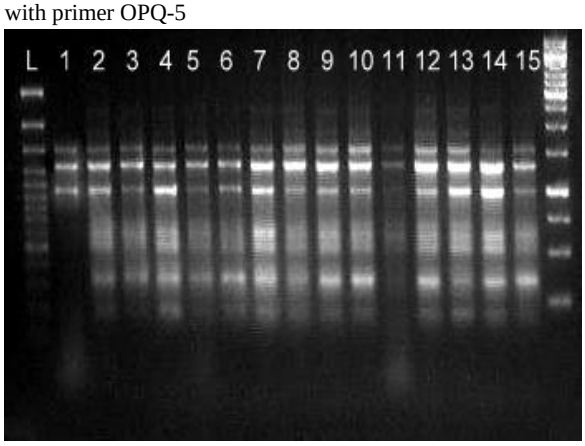

Figure 2: RAPD profile of Guar germplasm lines generated with primer OPQ-07

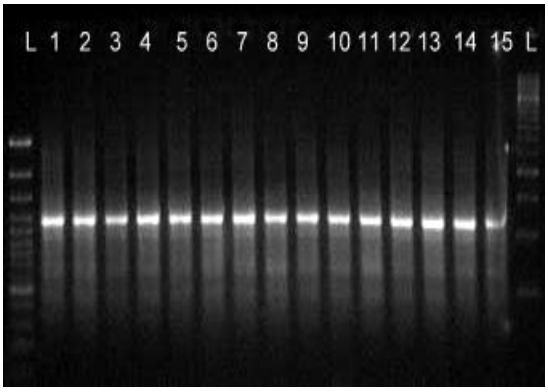

Figure 3: RAPD profile of Guar germplasm lines generated with primer OPQ-11

Table 6: Average Jaccard's similarity coefficient values of each germplasm line obtained by RAPD markers

1	**AVT-G-1**	**9**	**IVT-G-12**
2	**AVT-G-2**	**10**	**IVT-G-14**
3	**G-6**	**11**	**IVT-G-16**
4	**G-7**	**12**	**G-17**
5	**IVT-G-8**	**13**	**IVT-G-18**
6	**G-9**	**14**	**G-19**
7	**IVT-G-11**	**15**	**G-20**
8	**G-12**		

Sr. No.	Name of genotypes	Similarity coefficient value
1.	IVT-G-1	0.74
2.	IVT-G-2	0.80
3.	G-6	0.74
4.	G-7	0.78
5.	IVT-G-8	0.73
6.	G-9	0.79
7.	IVT-G-11	0.75
8.	G-12	0.71
9.	IVT-G-12	0.70
10.	IVT-G-14	0.76
11.	IVT-G-16	0.77
12.	G-17	0.74
13.	IVT-G-18	0.77
14.	G-19	0.79
15.	G-20	0.72
Average Similarity coefficient value		**0.75**

Table 7: List of RAPD primers and polymorphic amplicons generated

Sr. No	Primer code	Total No. of amplicons	No. of polymorphic amplicons	Percent polymorphism
1.	OPQ-5	7	6	85.67
2.	OPQ-7	8	7	87.50
3.	OPQ-11	4	2	50.00
TOTAL		19	15	78.94

Average number of amplicons per primer = 6.33
Average number of polymorphic amplicons per primer = 5
Total percent polymorphism by RAPD marker =78.94%

3.2. Cluster analysis of RAPD markers:

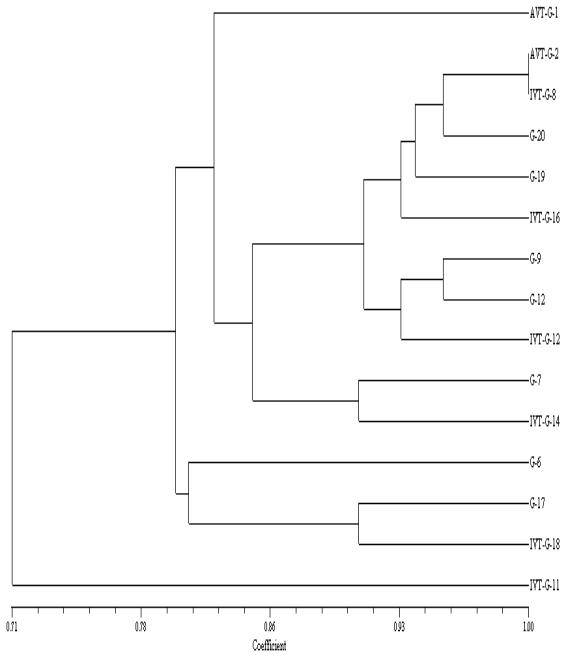

Figure 4: Dendrogram generated by RAPD markers

The UPGMA dendrogram obtained from the cluster and using Jaccard's coe ffi cient showed two major and four minor clusters (figure 4) while AVTG-1,G-6 and IVTG-11 did not fall in any cluster. Minor cluster A1 comprised of Five genotypes AVTG-2, IVTG-8, G-20, G-19 and IVTG-16 Minor cluster A2 comprised of three genotypes G-9,G-12 and IVTG-12 . showing their geographical relationship. The analysis of grouping pattern of this cluster for the genotypes did not correspond to their geographical regions. Minor clusters A3 and A4 included two and two genotypes, respectively. The maximum genetic similarity up to 98% was observed be-tween the genotype AVTG-2 and IVTG-8.Cluster B which comprised of 1 genotypes.

[IV] DISCUSSION

The use of molecular markers enables Cluster bean breeders to connect the gene action underlying a specific phenotype with the distinct regions of the genome in which the gene reside. Molecular markers allow the direct selection for genotypes, thereby providing a more efficient means of selection. Molecular markers provide an opportunity to identify and isolate the gene relating to character by map-based cloning.

Genetically diverse line provide ample opportunity to create a favourable gene combination and probability of producing a unique genotype increases in proportion to the number of genes by which the parents differ.

Genetic diversity analysis of cluster bean germplasm can provide practical information for selection of parental material and thus, assist in forecasting breeding strategies.

4.1. Analysis of cluster bean genetic diversity based on RAPD markers.

Improvement of crop through utilization of available genetic diversity among the germplasm is the key to successfully breeding programmes.

Hence, it is essential to characterize cluster bean germplasm using markers like PCR based markers such as RAPDs, ISSR, RFLPs, and microsatellites.

In present study average similarity coefficient calculated by RAPD marker was found to be 0.75.

In the present study, genetic diversity among *guar* species were analyzed by 3 RAPD primers among 15 cultivars, total 15 polymorphic DNA fragments were generated. The similarity coefficient among all cultivars ranged from 0.70-0.98

Kaga *et al*. (2000) studied comparative molecular mapping in *Ceratotropis* species using interspecific crosses between azukibean *(Vigna*

angularis) and rice bean *(Vigna umbellate).* They have developed a genetic linkage map with 86 F_2 derived plants from inter specific cross between azukibean *(Vigna angularis)* and rice bean *(Vigna umbellate).* Total 14 linkage groups, each containing more than 4 markers were constructed with one phenotypic, 114 RFLP and 74 RAPD markers. The total map size was 1720 cM and the average distance between markers was 9.7 cM. Although the present is not fully saturated, it may facilitate gene tagging, QTL mapping and further useful gene transfer for azukibean breeding.

Bushehry (2003) evaluated genetic diversity in soybean as determined by RAPD and DAF markers. The relationships of several soybean cultivars were evaluated using simple matching coefficients, random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) markers. Genetic similarities among cultivars obtained by using DAF and RAPD markers were 0.704-0.899 and 0.561-0.854, respectively. Better cultivar classification was obtained by using both DAF and combined DAF and RAPD data, enabling the grouping of Iranian cultivars with the highest relationships. DAF marker technology could also be used to evaluate soybean genomic DNA with greater resolution and more informative content without the inherent disadvantages of RAPD.

[V] CONCLUSION

- RAPD markers method effectively classifies the cultivars belonging to *Cluster* species.
- Grouping results from cluster analysis based on NTSYS-PC showed that, the cultivars chosen had narrow genetic variation to a considerable extent.
- The cultivars with distinct DNA profiles provided useful information for selection of parents to develop new cluster bean hybrids.
- RAPD primer OPQ-7 generated maximum number of amplicons *i.e.* 8 and OPQ-7 primer showed highest polymorphism *i.e.* 87.50%.

[VI] ACKNOWLEDGEMENTS

The authors are sincerely grateful to the College of Agricultural Biotechnology, Loni, India for their encouragement and support.

[VII] REFERENCES

[1] Anita Punia, Pooja Arora, Rakesh Yadav, and Ashok Chaudhury (2009) Optimization and Inference of PCR Conditions for Genetic Variability Studies of Commercially Important Cluster Bean Varieties by RAPD Analysis.

[2] Bushehry, A.A.S. (2003). Genetic diversity in soybean as determined by RAPD and DAF markers. Iranian J. of Agril. Sci., 34 (3) : 625-633.

[3] Chowdhury, A.K., P. Srinivas, P. Tongpamnak and P. Saksoong (2001). Genetic diversity based on morphology and RAPD analysis in vegetable

[4] Chowdhury, A.K., P. Srinivas, P. Tongpamnak, and P. Saksoong, (2000). Identification of cultivars of vegetable soybean (*Glycine* max (L.) by RAPD markers. SABRAO Journal of Breeding and Genetics. 32(2):63-72.

[5] Kaga, A., Yoon, M.S., Tomooka, N. and Vaughan, D.A. (2000). Collection of *Vigna spp*. and other legumes from the islands of southern Okinawa prefecture, Japan. In: Report to IPGRI and East Asia Plant Genetic Resources Coordinators.National Institute of Agrobiological Resources, Japan: 2-25.

[6] Karp A and Edward K.J. (1997). Molecular techniques in the analysis of the extent and distribution of genetic diversity, "In molecular genetic techniques for plant genetic resources" IPGRI, *Rome, pp* 11-22.

[7] Li, C. A. Fatokun, B. Ubi, B. B. Singh and Scoles G. J. (2001). Determining Genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. *Crop Sci*. 41: 189-197.

[8] Lin JhyJhu, Kuo, J. Ma Jin, J.A. Saunders, H.S. Beard, M.H. Mac Donald, W. Kenworthy, G.N. Ude and B.F. Matthews (1996). Identification of molecular markers in soybean comparing RFLP, RAPD and AFLP DNA mapping tchniques. Plant mol. Bio. Repor. 14(2):156-169.

[9] Mohamoud, Z., Chris, C. S., and Wlfgand, L. (2004). Hybrid performance and AFLP- Based genetic similarity of Faba bean Euphytica, 139:207- 216.

[10] Ntundu, W. H., Bach, I. C., Christiansen, J. L. and Andersen, S. B. (2004). Analysi of genetic diversity in bambara groundnut [*Vigna subterranea* (L.)Verdc] landraces using amplified fragment length polymorphism (AFLP) markers. *African J. Biotechnol.*, 3: 220-225.

.[11] Qiu, LiJuan, R.L. Nelson, and L.O. Vodkia (1997). Evaluation of soybean germplasm with random amplification polymorphic DNA (KADD) markers. *Acta Agronomica Sinica*, 23 (4) : 408-417.

[12] Rakesh pathak, Manjit singh, A.,Henry ,and S.K.,Singh (2005) genetic divergence in guar (Cyamopsis tetragonaloba) genotype using RAPD markers. *Legumes in dry areas* 2:164-169.

[13] Robertson, L. D., Ocampo, B. and Singh, K. B. (1997). Morphological variation in wild annual *Cicer* species in comparison to the cultigen. *Euphyti* 95: 309-319.

[14] Saiki, R.K., Fatoona, F., Mullis, K.B., Horn, G.P., Erlich, H.A ,and Arnhein, N. (1985). enzymatic amplification of thirteen globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia Science. 230:1350-1354.

[15] Sonia chanda ,S. Lakhanpaul and K.V. Bhat (2004) Random amplified polymorphic DNA (RAPD) analysis in guar (Cyamopsis tetragonoloba) cultivars. *Genetica* 109:227-234

[16] Souframanien J. and T. Gopalakrishna (2004) A comparative analysis of genetic diversity in guar genotypes using RAPD and ISSR markers. *Theor Appl. Genet.*1687-1693.

[17] Venkataramana, V. Bhat, Prashant P. B. and Suman Lakhanpaul (1999) Study of genetic diversity in Indian and exotic sesame (Sesamum indicum L.) germplasm using random amplified polymorphic DNA (RAPD) markers Euphytica 110: 21-34.

[18] Wilkes, G. (1983). Current status of crop plant germplasm. Plant Sci., 1: 133-181.