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GENETIC VARIABILITY STUDIES OF CLUSTER BEAN VARIETIES BY RAPD ANALYSIS, OPTIMIZATION AND INFERENCE OF PCR CONDITIONS

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SUMMARY

Cyamopsis tetragonoloba (L.) Taub, commonly known as guar belongs to family Fabaceae. Guar seed has an important place in industry because of its galactomannan rich endosperm. Good quality genomic DNA was isolated from 48 independent cluster bean varieties and conditions were optimized for further amplification by PCR using random one hundred and thirty decamer operon primers. An evaluation was made for the application of RAPD as a genetic marker system in commercially important cluster bean varieties and a dendrogram was prepared. Various factors influencing the RAPD amplification were optimized and the data revealed that 50ng of template DNA, 1.5mM Mg^{2+} ion concentration, 4.0 U/reaction of *Taq* DNA polymerase and annealing temperature of 400C was found to be essential for reproducible RAPD banding pattern. Cluster bean cultivars revealed significant polymorphism with reference to RAPD markers showing authentic genotypic diversity among its races. Out of a total of one hundred thirty operon primers employed; ninety-seven random primers showed amplified products with 91.34% polymorphism, yielding 644 polymorphic and 61 monomorphic alleles. All the genotypes could be grouped in two major and three minor sub-clusters, when binary matrix was subjected to NTSYS-pc software analysis and clustered dendrogram was constructed. The results of the present study can be used for molecular breeding and improvement of cluster bean for various desired traits through hybridization in future.

Key words: Guar, *Cyamopsis tetragonoloba*, DNA extraction, molecular markers, RAPD, PCR

Cluster bean is an important crop in India having agro-industrial potential and is a major source of guar gum in the world enabling export to 65 countries. This crop thrives well in rainfall range of 300-450 mm with 3-4 spells, at temperature 25-40°C, relative humidity 50-65%, and longer warmer days with 8-9 h sunshine (Satpal *et al*., 2018). It is a rich source of high quality galactomannan and protein. Galactomannan polysaccharide (guar gum) is a substance which forms gel in cold water and have innumerable end uses and helps the country to earn foreign exchange. The remaining part of seed *i.e*. a byproduct of guar gum industry called as guar meal, having light grayish color with beany flavour, is rich source of protein and amino acids but limiting in methionine, tryptophan and threonine. It consists of 4-6 per cent oil and its fatty acid composition is well comparable to common edible oils. Toxic factors present in guar meal are residual guar gum, trypsin inhibitor, polyphenols and saponins. Guar seed or guar meal can be detoxified by various processing technologies. Guar meal contains 40-45% protein and used as animal feed (Panchta *et al*., 2016). The potential of this crop can further be extended by making various products such as stabilizing agents, food additives etc. as per need of the global market (Singh and Dahiya, 2004). The detoxified protein concentrate and protein isolate can be used for human or animal consumption. For more rewardable use of guar, after exporting guar gum, guar meal could be processed and it could be used for supplementation of livestock food. Guar gum is used in dairy products like ice cream and as a stabilizer in cheese and cold-meat processing. Matured seeds are crushed and turned into flour, which are used as a thickening agent in food, paper and textile industries. The thickening power of cluster bean is more than 5 times of corn starch (Saeed *et al.,* 2011). After being partially hydrolyzed it is completely soluble in water makes a soft food. Guar gum itself and its derivatives are in great demand all over the world. The stability of cluster bean production relies on its ability to adapt to natural stresses and pathogens.

The molecular markers have been used to scrutinize DNA sequence variation(s) in various crops. Markers can aid selection for target alleles, minimize linkage drag around the target gene, and reduce the number of generations required to recover a very high percentage of the recurrent parent genetic background. Restriction fragments length polymorphism (RFLP) markers have been the basis for most of the work in crop plants. Earlier, valuable markers have been generated from random amplification polymorphic DNA (RAPD) and amplified fragments length polymorphism (AFLP). Simple sequence repeats (SSR) (microsatellite) markers have been utilized extensively to tag the genes/QTLs and genome mapping of major crop plants (Ashwani, 2009).

MATERIALS AND METHODS

Plant materials

The present investigation was carried out on 48 genotypes of cluster bean as given in Table 1. The seeds of these genotypes were procured from Forage section, Department of Genetics and Plant Breeding CCS HAU, Hisar. The crop was raised in dryland area of Forage Section. Fresh leaves from 3-4 week old plants were taken for the present research.

Chemicals

Taq DNA polymerase, magnesium chloride and PCR buffer (10x) were obtained from Biolabs Pvt. Ltd. India, dNTPs were obtained from Fermentas. All other chemicals used in the present investigations were of analytical grade and procured from Sigma Chemicals Co™. USA, Merck Ltd. India, Himedia, Life Technologies Pvt. Ltd. India and SISCO Research Laboratories, India.

Molecular Markers

A total of 130 RAPD primers were used in the present investigation (Table 2) All these primers were got synthesized from Life Technologies Inc. India, Biolabs England.

DNA Extraction

Leaf samples were taken from 3-4 week old seedlings. Five gram of the fresh leaf tissue was homogenized to fine powder in liquid nitrogen using sterilized pre-chilled mortar and pestle. The leaf powder was mixed with 15 ml of pre-warmed 2.0% CTAB extraction buffer (65ºC) in sterilized 50 ml polypropylene tubes. The samples were thoroughly mixed with the extraction buffer by inverting the tubes several times and were incubated in water bath at 65ºC for 90 minutes. Occasional mixing of the contents was done by gently inverting the tubes at an interval of 15-20 min. After incubation, the samples were cooled to room temperature followed by addition of 15 ml of chloroform: isoamyl alcohol (24:1) solution. Samples were again mixed thoroughly by gently inverting the tubes several times and centrifuged for about 15 min. at 10,000 rpm. After centrifugation, the upper aqueous phase was transferred to a pre-sterilized centrifuge tube followed by addition of 10 ml of chloroform: isoamyl alcohol (24:1) solution and mixed well. The upper aqueous phase after second centrifugation at 10,000 rpm for 15 minutes was transferred to another pre-sterilized centrifuge tube. Equal volume of ice-cold iso-propanol was subsequently added and mixed

TABLE 1
List of 48 Guar genotypes used for Genetic diversity analysis

S. No	Genotype	S. No	Genotype	S. No.	Genotype	S. No	Genotype
1.	FS 277	13.	IVTG 22	25.	GP 97	37.	GP219
2.	HG182	14.	IC 116607	26.	GP 98	38.	GP220
3.	HG258	15.	GP31	27.	GP 99	39.	GP 181
4.	HG119	16.	GP32	28.	GP 100	40.	GP182
5.	HG365	17.	GP33	29.	GP211	41.	GP183
6.	HG 867	18.	GP34	30.	GP212	42.	GP184
7.	HG 884	19.	GP 91	31.	GP213	43.	GP185
8.	AVTG 1	20.	GP 92	32.	GP214	44.	GP186
9.	AVTG 6	21.	GP 93	33.	GP215	45.	GP187
10.	PRTF 5277	22.	GP 94	34.	GP216	46.	GP188
11.	HFG119	23.	GP 95	35.	GP217	47.	GP189
12.	IVT 24	24.	GP 96	36.	GP218	48.	GP190

TABLE 2
List of RAPD primers used in genetic diversity analysis.

Primer	Primer Sequence (5'-3')	Primer	Primer Sequence (5'-3')
RAPD 1	TCTTTTATCA	RAPD 14	CCCAGCTGTG
RAPD 2	GTCTCCGCAA	RAPD 15	CCTAGTCGAG
RAPD 3	CCAGCTTAGG	RAPD 16	CACAGGCGAG
RAPD 4	CCGCCCAAAC	RAPD 17	GTGTCGCGAG
RAPD 5	TCTGTCGAGG	RAPD 18	GGCATGACCT
RAPD 6	CACCTTTCCC	RAPD 19	TGGGCGTCAA
RAPD 7	AGCGAGCAAG	RAPD 20	CCAGCAGCTT
RAPD 8	GAACACTGGG	RAPD 21	GACTGCACAC
RAPD 9	CCCTACCGAC	RAPD 22	ACGCAGGCAC
RAPD 10	AATGCCCCAG	RAPD 23	GAGGGAAGAG
RAPD 11	TGGCCCTCAC	RAPD 24	AGGCGGGGAAC
RAPD 12	CCCGCTACAC	RAPD 25	AGCAGGTGGA
RAPD 13	GAGCGTCGAA	RAPD 26	TGGGAGATGG
RAPD 27	GTTTCGCTCC	RAPD 49	AGTCAGCCAC
RAPD 28	TGATCCCTGG	RAPD 50	AATCGGGCTG
RAPD 29	CATCCCCCTG	RAPD 51	AGGGGTCTTG
RAPD 30	GGACTGGAGT	RAPD 52	GGTCCCTGAC
RAPD 31	TGCGCCCTTC	RAPD 53	GAAACGGGTG
RAPD 32	TGCTCTGCCC	RAPD 54	GTGACGTAGG
RAPD 33	GGTGACGCAG	RAPD 55	GGGTAACGCC
RAPD 34	GTCCACACGG	RAPD 56	GTGATCGCAG
RAPD 35	TGGGGGACTC	RAPD 57	CAATCGCCGT
RAPD 36	CTGCTGGGAC	RAPD 58	TCGGCGATAG
RAPD 37	GTAGACCCGT	RAPD 59	CAGCACCCAC
RAPD 38	CCTTGACGCA	RAPD 60	TCTGTGCTGG
RAPD 39	TTCCCCCGCT	RAPD 61	TTCCGAACCC
RAPD 40	TCCGCTCTGG	RAPD 62	AGCCAGCGAA
RAPD 41	GGAGGGTGTT	RAPD 63	GACCGCTTGT
RAPD 42	TTTGCCCGGA	RAPD 64	AGGTGACCGT
RAPD 43	AGGGAACGAG	RAPD 65	CAAACGTCGG
RAPD 44	CCACAGCAGT	RAPD 66	GTTGCGATCC
RAPD 45	ACCCCCGAAG	RAPD 67	TTCGAGCCAG
RAPD 46	GGACCCTTAC	RAPD 68	GTGAGGCGTC
RAPD 47	CAGGCCCTTC	RAPD 69	GGGGGTCTTT
RAPD 48	TGCCGAGCTG	RAPD 70	CCGCATCTAC
RAPD 71	GATGACCGCC	RAPD 101	AGCGCCATTG
RAPD 72	GAACGGACTC	RAPD 102	CACCGTATCC
RAPD 73	GTCCCGACGA	RAPD 103	GGGGTGACGA
RAPD 74	TGGACCGGTG	RAPD 104	CTTCCCCAAG
RAPD 75	CTCACCGTCC	RAPD 105	CATCCGTGCT
RAPD 76	TGTCTGGGTG	RAPD 106	AGGGCGTAAG
RAPD 77	AAAGCTGCGG	RAPD 107	TTTCCCACGG
RAPD 78	TGTCATCCCC	RAPD 108	GAGAGCCAAC
RAPD 79	AAGCCTCGTC	RAPD 109	CTGGGGACTT
RAPD 80	TGCGTGCTTG	RAPD 110	ACCCGGTCAC
RAPD 81	GACGGATCAG	RAPD 111	ACGGATCCTG
RAPD 82	CACACTCCAG	RAPD 112	GAGGATCCCT
RAPD 83	TTCCCCCCAG	RAPD 113	CCTGATCACC
RAPD 84	GGGATATCGG	RAPD 114	GGTGATCAGG
RAPD 85	CCAAGCTTCC	RAPD 115	CCGAATTCCC
RAPD 86	GGAAGCTTGG	RAPD 116	GGGAATTCGG
RAPD 87	TTGGTACCCC	RAPD 117	CCGATATCGG
RAPD 88	ACGGTACCAG	RAPD 118	GGGATATCGG
RAPD 89	GGCTGCAGAA	RAPD 119	CCAAGCTTCC
RAPD 90	TGCTGCAGGT	RAPD 120	GGAAGCTTGG
RAPD 91	CCAGTACTCC	RAPD 121	TTGGTACCCC
RAPD 92	GGAGTACTGG	RAPD 122	ACGGTACCAG
RAPD 93	AACCCGGGAA	RAPD 123	GGCTGCAGAA
RAPD 94	TTCCCGGTT	RAPD 124	TGCTGCAGGT
RAPD 95	CCTCTAGACC	RAPD 125	CCAGTACTCC
RAPD 96	GGTCTAGAGG	RAPD 126	GGAGTACTGG
RAPD 97	TTGGCACGGG	RAPD 127	AACCCGGGAA
RAPD 98	GTGTGCCCCA	RAPD 128	TTCCCGGGTT
RAPD 99	CTCTGGAGAC	RAPD 129	CCTCTAGACC
RAPD 100	GGTCTACACC	RAPD 130	GGTCTAGAGG

to precipitate DNA and tubes were kept undisturbed for 15 min. DNA was spooled out using sterile glass hooks and washed in Wash-I solution for 20 minutes followed by 2 minutes washing in Wash-II solution. DNA was then air-dried overnight at room temperature and subsequently dissolved in appropriate volume of TE buffer and samples were stored at -20°C till further use. To remove RNA contamination, DNA samples were treated with 1 µl of RNase A solution (10 mg/ml) per 50 µl of DNA sample and incubated in water bath at 37ºC for 3 h. An equal volume of chloroform: iso-amyl alcohol (24:1) solution was then added and contents were centrifuged at 10000 rpm for 15 min. at room temperature. Aqueous phase was transferred to fresh sterilized centrifuge tube. DNA from the aqueous phase was precipitated by adding two volumes of ice-cold ethanol. Centrifugation was done at 10,000 rpm for 10 min. at 4ºC to pellet down DNA. Supernatant was carefully removed and pellet was washed with 70% ethanol. The DNA pellet was dried overnight at room temperature and then dissolved in appropriate volume of TE buffer (stock solution) and stored at -20ºC till further use.

Qualitative and Quantitative Estimation of DNA

Quality and quantity of DNA was estimated by UV spectrophotometer and agarose gel electrophoresis. For Quantitative estimation of DNA using UV spectrophotometer analysis, an aliquot of DNA samples was suitably diluted and absorbance (A) was determined at 260 nm and 280 nm wavelength in spectrophotometer. Using the relationship of O.D. unit of 1.0 at 260 nm equivalent to 50 µg DNA per ml, the quantity of DNA was estimated from the formula:

Concentration of DNA (µg/ml) = A_{260} x 50 x dilution factor.

Quality of DNA samples was checked both by UV-spectrophotometer and on agarose gel electrophoresis. Using spectrophotometer, the ratio of the absorbance at 260 nm and 280 nm was noted. Samples with a ratio of 1.8 were considered of good quality.

$$A_{260}/A_{280} = 1.8 \text{ (pure DNA)}$$

Optimization of PCR amplification

PCR amplification was carried out in PTC - 100, programmable thermal cycler from MJ Research.

Conditions for PCR amplification were optimized using RAPD primers. PCR reactions were performed using varying concentrations of template DNA (50,100,150 ng), primers (15 µM, 20 µM and 25µM), $MgCl_2$ (1.5mM and 2.0 mM), dNTPs mix (200 µM, 500 µM and 1mM), Taq DNA polymerase (2 units, 3 units and 5 units) were used in a reaction volume of 10µl. Different annealing temperatures (34, 40, 45ºC) were employed during PCR amplification. PCR was done in PTC-100 thermal cycler. After initial denaturation for 4 min. at 94°C, PCR was run for 40 cycles consisting of a denaturation step at 94°C for 1 min., annealing at 34°, 40°, 45°C for 1 minute each, Extension step at 72°C for 2 min., and Final Extension was appended at 72°C for 8 min. Amplified DNA fragments were resolved by submerged horizontal electrophoresis in 1.5 per cent (w/v) agarose gel and visualized by staining with ethidium bromide. Agarose solution was prepared in 1X TBE and ethidium bromide was added in the gel at a concentration of 0.5 µg/ml and then mixed. It was poured in gel casting plate with appropriate comb with required well number and size. DNA samples were mixed with one µl of 6X loading dye solution and loaded on wells using micropipette (Eppendorf). Electrophoresis was done using 1X TBE as running buffer at constant voltage (3 v/cm of gel). PCR amplification products were visualized under UV light and photographed using Alfa Imager gel documentation system.

Data analysis

Binary character matrix was obtained by positions of scorable RAPD bands. The symbol 1 denotes the presence of a band, whereas, 0 for the absence of band at a particular position. The presence of an amplified band (amplicon) in each position was recorded as 1 and the absence as 0. Based on presence/ absence of bands, genetic similarity was calculated to estimate all pair wise differences in the amplification product for all genotypes. The genetic associations between varieties were evaluated by using NTSYS-pc (Rohalf, 1990) and dendrograms were constructed by unweighted pair group method with arithmetic averages (UPGMA) subprogram of NTSYC-pc. The data generated from polymorphic fragments were analyzed according to Nei and Li (1979) formula given below:

$$\text{Similarity (F)} = \frac{2Mx}{My+Mz}$$

Dissimilarity = 1 – F

Where,

Mx = Number of shared fragments between genotypes y and z

My = Number of scored fragments of genotype y

Mz = Number of scored fragments of genotype z

2D and 3D Principal Component Analysis

The two dimensional and three dimensional Principal Component Analysis (PCA) was done for providing suitable means of testing the relationship among 48 cluster bean genotypes using NTSYS-pc.

RESULTS AND DISCUSSION

The application of DNA based molecular markers has ushered in a new era of genome wide analysis together with tremendous progress in structural and functional genomics. Polymerase chain reaction (PCR) based molecular markers like RAPD; allow the rapid detection of DNA polymorphism from many individuals or pooled samples in order to avoid erratic amplifications, good quality of genomic DNA free of contaminants and optimization of PCR reaction conditions is a perquisite for developing strategies for crop improvement programs in future.

DNA extraction. Total genomic DNA was extracted from fresh leaves of cluster bean varieties by employing various DNA extraction methods such as CTAB method (Thompson and Murray 1980), SDS potassium acetate method (Tai and Tanksley, 1990) and SDS phenol chloroform method (Zhao *et al*., 1989) with some modifications. The quality and quantity of genomic DNA extracted by CTAB method was highest as compared to the other two methods (data not shown) which could be successfully employed for DNA based molecular marker analysis of cluster bean.

Optimization of the genomic DNA concentration

In order to determine the optimal concentration of template, genomic DNA was varied from 25 ng to 100 ng. The reactions were replicated twice to examine the possible influence of DNA concentration on the fidelity of PCR amplifications. The results presented in Fig. 1 clearly reveal that only the concentration range of 40-50 ng yielded reproducible patterns, too little template DNA gave variable banding patterns while excess amounts of genomic DNA resulted in non-specific amplifications.

Variability at low template concentrations may be as a result of a reduced probability in initiating amplification reactions and reflects the inefficiency of the priming events as reported by Welsh and McClelland (1990).

Optimization of the Mg2+ ion concentration

In order to examine the influence of Mg2+ ions on the efficiency and fidelity of the RAPD amplifications a concentration of 1.5mm and 2.0mm was used for optimization of PCR conditions. Specific and reproducible results were obtained only in the presence of 1.5mM Mg2+ ion concentration with higher concentration of template DNA (100ng) and $MgCl_2$ (2.5mM), smearing in DNA banding pattern was observed. This clearly demonstrates relationship between Mg2+and DNA concentrations and suggests that the stringency of annealing process is decreased at higher Mg2+concentrations. Using suboptimal concentrations of MgCl2 can result in reduced product yield (Zangenberg *et al*., 1999). MgCl2 concentration has been found to alter the amount and type of product produced in PCR reaction (Ellsworth *et al*., 1993).

Effect of concentration of Taq polymerase

Different concentrations of the Taq DNA polymerase (0.5, 1.0, 1.5, 2.0 units) were used for optimization. Amplification with 0.5U Taq DNA polymerase consistently gave simple and reliable banding patterns that lacked the minor bands generated under conditions with higher enzyme concentrations

Effect of Denaturing Temperature

A much larger effect was observed when the standard denaturing temperature of 94ºC with initial pre-denaturation of 5 min was lowered to 90ºC (data not shown). A comparison of the pattern reveals major bands produced under a denaturing temperature of 94ºC disappeared and additional minor bands were formed when denaturing was carried out at 90ºC. Zangenberg *et al*. (1999) observed that the lower annealing temperatures often results in overall increase in non-specific amplification while the higher annealing temperature results in more specific amplification. On the contrary, Pammi *et al*., (1994) observed little amplification at 36ºC, whereas, the temperature optimum was at 48ºC and significant reduction was found in amplification patterns at a temperature above 48ºC.

RAPD as molecular marker

In the present investigation 48 diverse cluster bean genotypes were subjected for RAPD profile analysis. The results of the present study revealed 130 primers in cluster bean varieties which represents detailed response of genotypes amplified, total bands produced, polymorphic and monomorphic bands obtained and the Percent polymorphism detected in various genotypes.

RAPD Analysis

To study the polymorphism between genotypes, a total of 130 RAPD primers, with high G+C content were used. The list of primers used in this study, with their sequences is given in table 3.2. Out of 130 primers, 97 primers produced amplification of different alleles. All primers which showed amplification were polymorphic with one or more genotypes. The DNA amplification and polymorphism generated among various cluster bean genotypes using RAPD markers are summarized in Table 3.

The amplification of alleles obtained with different primers in all the 48 genotypes of cluster bean were scored on the basis of presence (1) or absence (0) of bands on agarose gel (1.6%). Sharp, clear, reproducible and unambiguous bands were observed.

A total of 705 sharp and reproducible alleles were obtained from 97 primers, out of which 644 alleles were polymorphic while 61 alleles were monomorphic showing 91% polymorphism among the genotypes. The size of the alleles ranged from 200 to 2500 bp. Size range was found to differ with different primers.

Allele Scoring and Data Analysis

The amplification of alleles observed was scored. Presence of an allele was scored as 1 and absence of an allele was scored as 0. The scored data was used to calculate similarity genetic distance using NTSYS-pc Software. Dendrogram was constructed by using distance matrix by the unweighted pair-group method with arithmetic average (UPGMA) sub-programme of NTSYS-pc. The genetic associations between varieties were evaluated by calculating the Jaccard similarity coefficient. Similarity coefficients were used for cluster analysis of varieties, performed using the 'SAHN' (sequential, agglomerative, hierarchical nested clustering method) sub program

of NTSYS-pc (Rohalf, 1990) and dendrograms were constructed by unweighted pair group method with arithmetic averages (UPGMA) subprogram of NTSYC-pc.

Polymorphic Information Content (PIC)

Polymorphic information content (PIC) was calculated for all the primers used in this study. For RAPD markers used in the study the PIC values ranged from 0.12 to 0.87 (Table 3). Out of 97 primers, 65 primers showed PIC scores ≥ 0.30. The mean PIC for primers found to be 0.37.

Similarity/Dissimilarity analysis

To find out the similarity between the genotypes, 0/1 binary matrix data was used to prepare the dendrogram revealing genetic relationships among selected cluster bean genotypes by NTSYS-pc using Jaccard similarity coefficient analysis.

TABLE 3
Amplification profile of RAPD primers used for screening polymorphism among 48 genotypes of cluster bean.

Total number of Primers used	130
Primers which showed amplification	97
Primers which produced polymorphism	97
Primers which produced monomorphism	0
Number of alleles amplified	705
Number of monomorphic alleles	61
Number of polymorphic alleles	644
Percent Polymorphism	91.34

Cluster Tree Analysis

Dendrogram was constructed using similarity matrix value as determined from RAPD data for 48 genotypes using UPGMA (unweighted pair group method of arithmetic averages) subprogram of NTSYS-PC programme. The Dendrogram (Fig. 4.1) constructed depicts the relationship among all *Cyamopsis tetragonoloba* genotypes. The association amongst different genotypes presented in the form of dendrogram prepared by using Jaccard similarity coefficient. The genotypes which are lying nearer to each other in the dendrogram are most similar to one another than those lying apart. Similarity matrix data (Figure 4.2), of 48 genotypes revealed the genetic relationship among them. The resemblance coefficient between the two genotypes is the value at which their branches join. The dendrogram showed the relative magnitude of resemblance among different clusters. Genetic distance ranged from 0.11 to 0.97. Genotypes with maximum genetic similarity (97%) are GP 215 and GP 216. The next similar (96%) genotypes are GP 212 and GP 213. Third most similar genotypes were GP 212 and GP 100 (95%). Among all 48 genotypes analyzed, GP 96 and FS 277 were most diverse (89%) with minimum value of similarity coefficient. The second most dissimilar genotypes (88%) were FS277 and GP100, GP217. Third most diverse genotypes were FS277 and GP32, GP213 with dissimilarity index of 87%. Similarity index analysis resulted in a good level of DNA polymorphism among the various genotypes.

All the 48 guar genotypes were divided into 2 major clusters at a similarity coefficient of 0.68. Variety FS 277 is out grouped. Major cluster I was occupied by 34 cluster bean varieties and was further divided into three sub-clusters. Subcluster 1a constituted of genotype HG-258 and subcluster 1c consists of genotype HG182 whereas subcluster 1b constituted of 32 genotypes. Major-cluster II comprised of 13 varieties. GP 190 (sub cluster D) diverged from other varieties and 10 varieties are grouped together in sub cluster C, which is further divided into two subgroups. Through UPGMA cluster analysis, the similarity

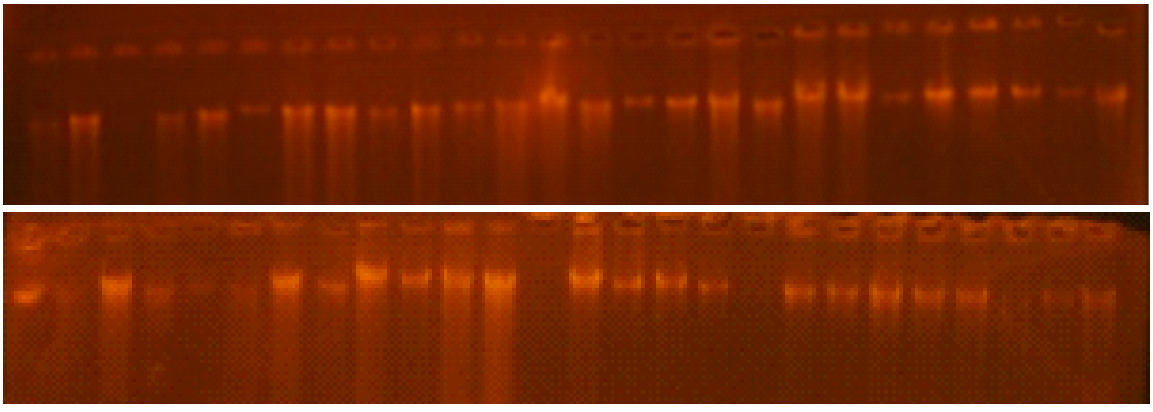

Fig. 1. RAPD as molecular marker.

TABLE 4
Codes given to varieties for diversity analysis

Code	Variety	Code	Variety	Code	Variety	Code	Variety
C1	FS 277	V13	IVTG22	V25	GP97	V37	GP219
C2	HG182	V14	IC1166007	V26	GP98	V38	GP220
C3	HG258	V15	GP31	V27	GP99	V39	GP181
C4	GP221	V16	GP32	V28	GP100	V40	GP182
V5	HG365	V17	GP33	V29	GP211	V41	GP183
V6	HGS867	V18	GP34	V30	GP212	V42	GP184
V7	HG884	V19	GP91	V31	GP213	V43	GP185
V8	HGS04-875	V20	GP92	V32	GP214	V44	GP186
V9	RGC 986	V21	GP93	V33	GP215	V45	GP187
V10	HGS 818	V22	GP94	V34	GP216	V46	GP188
V11	HFG119	V23	GP95	V35	GP217	V47	GP189
V12	IVT24	V24	GP96	V36	GP218	V48	GP190

coefficient for variety HG-258 in subcluster 1a was found to be 0.68 and variety HG182 in subcluster 1c was found clustered at similarity coefficient 0.70 whereas subcluster 1b was ranged in similarity coefficient 0.69 to 0.97. In major cluster II, subcluster II a, GP 190 was found with similarity coefficient 0.69 and rest 13 genotypes fall in a similarity coefficient from 0.78 to 0.95.

Principle Component Analysis (PCA)

Principal Component Analysis produced the two dimensional (Fig. 3) and three dimensional (fig 4.4) scale among the various clusterbean genotypes. In present investigation, PCA analysis was done using software programme NTSYS-pc. The binary data obtained from scoring was used for the analysis.

Two and Three dimensional PCA

Two dimensional analysis was done using 2D. GP218, GP219, GP220, GP181, GP182, GP183, GP184, GP185, GP186, GP187, GP188, GP189 and GP190 whereas Cluster II comprised of 34 varieties. It showed that all the three methods of grouping the genotypes on the basis of their diversity are highly reliable and produced the same results. PCA subprogram of NTSYS-pc. Two main clusters were formed, first cluster at PCA value of 0.20 (Dim. 2) to 0.95 (Dim-1) was occupied by the varieties as clustered by NTSYS-pc dendrogram. Second main cluster at PCA value of 0.41 (Dim-2) to 0.95 (Dim-1) clubbed the rest of genotypes. The genotype FS 277 was out grouped from rest of 47 genotypes. Thirteen genotypes grouped in cluster I and 34 genotypes grouped in cluster II. The

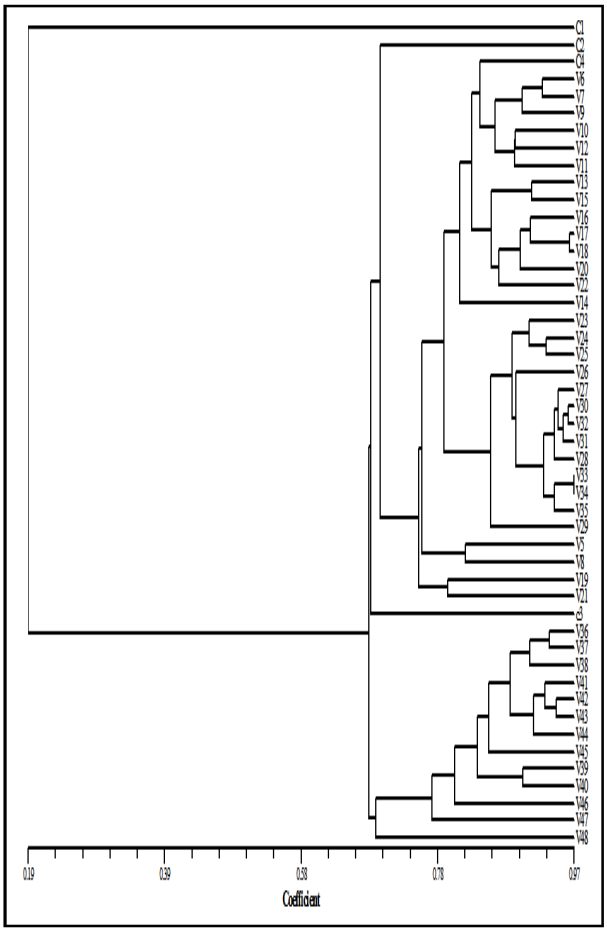

Fig. 2. Genetic similarity among 48 Cluster bean genotypes revealed by UPGMA cluster analysis.

grouping obtained by PCA analysis was almost similar to that obtained by NTSYS-pc clustering. The dendrogram, two dimensional and three dimensional analysis revealed that genotype FS-277 was outgrouped. Cluster I comprised of 13 genotypes (Fig. 4).

TABLE 4
Amplified band details of RAPD primers used in genetic diversity analysis

S. No.	Primer	Amplified bands	Monomorphic	Polymorphic	PIC value
1.	TCTTTTATCA	9	0	9	0.32
2.	GTCTCCGCAA	6	0	6	0.46
3.	CCAGCTTAGG	7	1	6	0.34
4.	CCGCCCAAAC	6	2	4	0.42
5.	TCTGTCGAGG	11	0	11	0.37
6.	CACCTTTCCC	8	0	8	0.57
7.	AGCGAGCAAG	6	1	5	0.53
8.	GAACACTGGG	3	0	3	0.50
9.	CCCTACCGAC	7	0	7	0.30
10.	GGGGTGACGA	9	0	9	0.57
11.	CTTCCCCAAG	5	0	5	0.21
12.	TTTCCCACGG	6	0	6	0.53
13.	GAGAGCCAAC	6	0	6	0.25
14.	CTGGGGACTT	5	0	5	0.44
15.	ACCCGGTCAC	11	0	11	0.50
16.	ACGGATCCTG	9	2	7	0.44
17.	GAGGATCCCT	5	0	5	0.26
18.	CCTGATCACC	8	1	7	0.44
19.	GGTGATCAGG	5	0	5	0.16
20.	CCGAATTCCC	9	1	8	0.21
21.	GGGAATTCGG	7	0	7	0.58
22.	CCGATATCCC	4	0	4	0.35
23.	AGGCGGGGAAC	9	0	9	0.41
24.	AGCAGGTGGA	6	1	5	0.30
25.	TGGGAGATGG	9	2	7	0.25
26.	GTTTCGCTCC	5	1	4	0.17
27.	TGATCCCTGG	5	0	5	0.39
28.	CATCCCCCTG	9	1	8	0.38
29.	GGACTGGAGT	7	2	5	0.22
30.	TGCGCCCTTC	10	0	10	0.35
31.	TGCTCTGCCC	9	0	9	0.28
32.	GGTGACGCAG	9	2	7	0.12
33.	CCTTGACGCA	12	2	10	0.47
34.	TTCCCCCGCT	9	1	8	0.30
35.	GGAGGGTGTT	7	0	7	0.22
36.	TTTGCCCGGA	8	0	8	0.82
37.	AGGGAACGAG	6	0	6	0.55
38.	CCACAGCAGT	4	0	4	0.41
39.	ACCCCCGAAG	7	0	7	0.55
40.	GGACCCTTAC	3	0	3	0.45
41.	CAGGCCCTTC	6	0	6	0.21
42.	TGCCGAGCTG	10	0	10	0.49
43.	AGTCAGCCAC	11	0	11	0.33
44.	AATCGGGCTG	10	1	9	0.26
45.	GTGACGTAGG	8	0	8	0.44
46.	GGGTAACGCC	8	0	8	0.30
47.	GTGATCGCAG	10	0	10	0.25
48.	CAATCGCCGT	10	4	6	0.17
49.	TCGGCGATAG	8	2	6	0.39
50.	CAGCACCCAC	10	0	10	0.38
51.	TCTGTGCTGG	10	1	9	0.32
52.	TTCCGAACCC	9	2	7	0.35
53.	AGCCAGCGAA	9	1	8	0.28
54.	GACCGCTTGT	10	0	10	0.11
55.	AGGTGACCGT	5	1	4	0.47
56.	GTCCCGACGA	9	0	9	0.40
57.	TGGACCGGTG	6	1	5	0.32
58.	CTCACCGTCC	9	2	7	0.23
59.	TGTCTGGGTG	5	1	4	0.18
60.	AAAGCTGCGG	5	0	5	0.37
61.	TGTCATCCCC	9	1	8	0.41

cont...

TABLE 4 contd.

62.	AAGCCTCGTC	6	0	6	0.57
63.	TGCGTGCTTG	4	0	4	0.38
64.	GACGGATCAG	7	0	7	0.52
65.	CACACTCCAG	3	0	3	0.45
66.	TTCCCCCCAG	6	0	6	0.21
67.	TGAGTGGGTG	7	0	7	0.20
68.	GTTGCCAGCC	8	0	8	0.82
69.	ACTTCGCCAC	6	0	6	0.55
70.	ACCGCGAAGG	4	0	4	0.43
71.	GGACCCAACC	7	0	7	0.56
72.	GTCGCCGTCA	3	0	3	0.47
73.	TCTGGTGAGG	6	0	6	0.23
74.	TGAGCGGACA	10	0	10	0.45
75.	ACCTGAACGG	7	1	6	0.18
76.	TTGGCACGGG	5	3	2	0.53
77.	GTGTGCCCCA	10	1	9	0.43
78.	GGGGTGACGA	7	0	7	0.22
79.	CTTCCCCAAG	12	4	8	0.87
80.	CATCCGTGCT	6	0	6	0.55
81.	AGGGCGTAAG	4	0	4	0.41
82.	TTTCCCACGG	7	0	7	0.55
83.	GAGAGCCAAC	3	0	3	0.45
84.	CTGGGGACTT	9	2	7	0.23
85.	ACCCGGTCAC	5	1	4	0.18
86.	ACGGATCCTG	5	0	5	0.37
87.	GAGGATCCCT	6	1	5	0.32
88.	CCTGATCACC	9	2	7	0.23
89.	GGGATATCGG	9	2	7	0.26
90.	CCAAGCTTCC	5	1	4	0.15
91.	GGAAGCTTGG	5	0	5	0.38
92.	TTGGTACCCC	9	1	8	0.41
93.	ACGGTACCAG	9	0	9	0.41
94.	GGCTGCAGAA	6	1	5	0.30
95.	TGCTGCAGGT	9	2	7	0.25
96.	CCAGTACTCC	5	1	4	0.17
97.	GGAGTACTGG	5	0	5	0.36

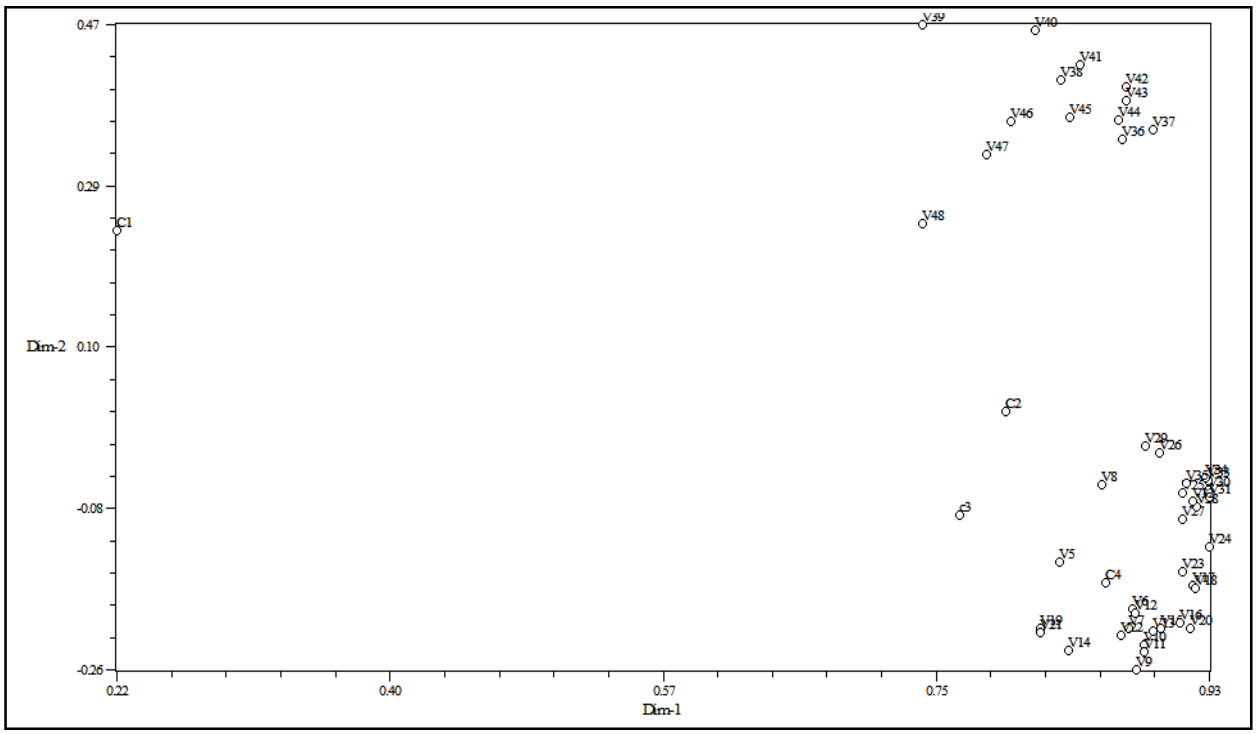

Fig. 3. Two dimensional PCA (Principal component analysis) scaling of 48 genotypes using RAPD markers.

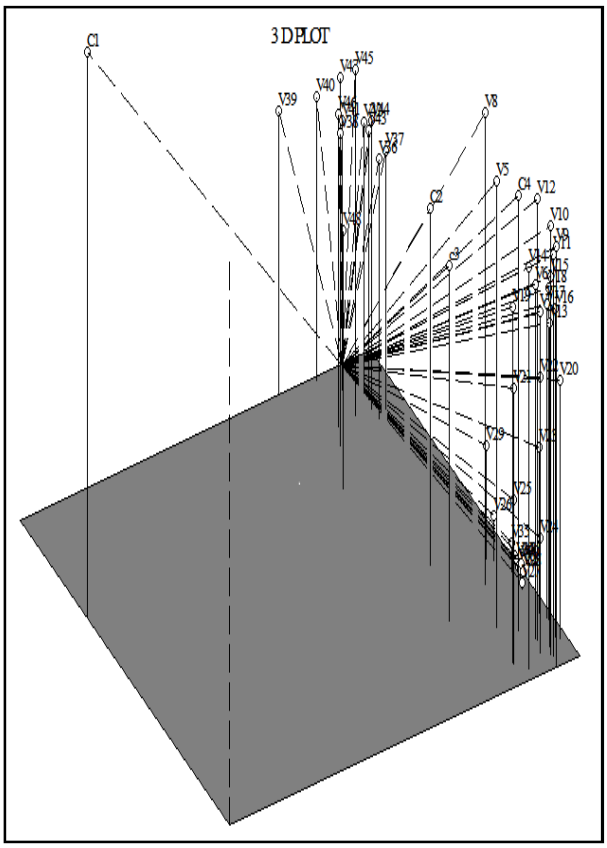

Fig. 4. Three dimensional PCA (Principal component analysis) scaling of 48 genotypes using RAPD markers.

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