International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 6, Issue2, 2015, pp203-210
http://www.bipublication.com

Research Article

Standardization of DNA isolation and PCR protocols for ISSR analysis in Cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.] Genotypes

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[Received-18/04/2015, Published-07/05/2015]

ABSTRACT

Cluster bean [*Cyamopsis tetragonoloba* (L) Taub.] is one of the most important cash legume crop has played an increasingly important role in wide range of industries. Owing to the significance of molecular marker studies in numerous applications including in genetic improvement of crops, there is an obvious need to undertake such studies in cluster bean. Present research work was carried out with the genomic DNA optimization and PCR standardization for ISSR analysis in cluster bean genotypes. The standard CTAB method for isolation of genomic DNA from cluster bean have their own limitation as it produces gel image with high contamination of yellowish, sticky and viscous matrix. The commonly used CTAB method was modified by giving Polyvinylpyrrolidone (PVP) treatments after grinding fresh young leaves of cluster bean. The yield of genomic DNA was increased in ranged from120 ng/µl to 220 ng/µl of 0.2 g leaf tissue and the purity (ratio) was between 1.5-1.7 indicating minimal levels of contaminating metabolites. ISSR protocol was standardized after implementing various modifications. Based on above findings of present experiment work this modified genomic DNA isolation and PCR protocol may be utilized for ISSR profiling to produce sharp and clear bands in cluster bean genotypes.

Keywords: Standardization, DNA isolation, PCR protocols, ISSR analysis, cluster bean *Cyamopsis tetragonoloba*, genotypes, CTAB, PVP

INTRODUCTION

Cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.] commonly known as guar is a native plant of India principally grown for its green fodder and pods that are used for food and feed. Cluster bean is grown mainly in India, Pakistan, United States, Sudan, South Africa, Brazil, Malawi, Zaire and also in some parts of Australia [1]. India is a major producer of cluster bean and contributes to 80% of the world's total production of cluster bean [12], which is 7.5 to 10 lakh tonne annually. In India, Rajasthan alone contributes 70% of India's total production.

Cluster bean is widely grown in dry, warm and arid regions on account of its system sustaining properties, particularly in enhancing the soil fertility and eking out poultry and animal husbandry under harsh growing conditions where other crops fall short even to negotiate and produce biomass [11]. Though, it is traditionally cultivated under poorly endowed situations yet, it has high industrial value and export potentials**.** In cluster bean selection for industrially important biochemical attribute i.e. galactomanan is very difficult in any breeding program due to high extrapolations of the environment and its interaction with genotypes (Sharma *et al*., 2014). Molecular markers offer several advantages over the conventional breeding tools for selection of diverse parents. Inter simple sequence repeat DNA (ISSR) uses arbitrary 10-base primers to amplify the random portion of the genome (Williams *et al*., 1990). The data from ISSR analysis have indicated greater diversity than allozymes in plant species [6,7]. It is high-throughput marker technology, which allows the analysis of large number of individuals with a large number of markers in relatively short time, as only a few primers allow the generation of sufficient data to obtain a robust estimate of diversity index. ISSR markers are based on amplified arbitrary sequences and sample a wider part of the genome than RAPD primers and have allowed the resolution of complex taxonomic relationships [3,4]. Inter-simple sequence repeats (ISSRs) is a type of molecular marker, proposed by [19] for fingerprinting. ISSRs fingerprinting has been commonly used to study population genetics, taxonomy and phylogeny of many plant species [18]. ISSR primers can confirm specific amplified DNA polymorphic fragments within the variety [9]. ISSR have been widely used in the genotype identification, genetic mapping and marker-assisted selection of plants, due to their simplicity, low cost and lower infra-structure requirements. These markers have been extensively used for identifying relationships at the cultivar and species level [3,4]. Although reproducibility is a major concern in such markers [18], they explore the whole genome and sometime generate information which might be of interest for molecular breeders such as amplicons co-segregates with a particular trait or an amplicon specific for a particular genotype.

On the other hand very limited research has been conducted at molecular level, even up to simple marker techniques in cluster bean. Therefore, it becomes pertinent to standardize the genomic DNA extraction and PCR protocol for molecular marker analysis in cluster bean.

MATERIALS AND METHODS

Plant Material

The plant material was collected from National Bureau of Plant Genetic Resources (NBPGR), CAZRI, Jodhpur (Rajasthan) and PAU, Ludhiana, India that included genotypes from India. A total of 30 cluster bean genotypes were analyzed in the Research Laboratories of the Horticulture and Department of Biotechnology at Babasaheb Bhimrao Ambedkar University, Vidya-Vihar, Rae Bareli Road, Lucknow during March, 2013 to September, 2013 and March, 2014 to September, 2014. Similarly PCR amplification of both the year was done at Central Soil Salinity Research Institute- Biotechnology Lab, Lucknow, Uttar Pradesh. The collected germplasm is now maintained in the Horticulture Research Farm– Department of Applied Plant Science (Horticulture), Babasaheb Bhimrao Ambedkar University, Lucknow, India for further studies.

Extraction of Genomic DNA

For isolation of genomic DNA, young leaves (0.2 g) which should be 2–3 week old of 30 superior genotypes of cluster bean were employed. Crush leaf with liquid nitrogen and wash with 2-3 times by wash buffer (0.5 % PVP) then after add extraction buffer.

ISSR-PCR Amplification

A total of 21 single-10-mer oligonucleotide random primers (SBS Genentech Company

Limited, China) were used for molecular DNA analysis. The samples were incubated in a thermal cycler (Eppendorf Master Cycler Nexus Gradient, Germany). The results in discrete, well-separated fragments on agarose gel were selected for further amplification of entire set of the cluster bean genotypes.

The final PCR reaction was carried out in 20 µl of reaction mixture containing 50 ng of template genomic DNA, 100 mM of each dNTP, 3.0 µl of primer (50 ng/µl), 2.0 units of Taq DNA polymerase, 2.0 µl of 10 X PCR buffer and 1.5 mM $MgCl_2$. Amplification was carried out in master cycler-384 thermal cycler (Eppendorf Master Cycler Nexus Gradient, Germany). PCR conditions for ISSR analysis included an initial pre denaturation step of 5 min at 94^0C and following 30 cycles of amplification. The reaction mixture was subjected to denaturation at 94^0C for 1 min, followed by annealing at 38-50^0C for 1 min. Extension was performed at 72^0C for 2 min then final extension was carried out at 72^0C for 10 min.

RESULTS AND DISCUSSION

In cluster bean morphological and biochemical markers are most frequently utilized for determination of genetic diversity. Nevertheless, these markers are highly influenced by the environment and showed limited polymorphism. Such, drawback of morphological and biochemical markers can be overcome by DNA based molecular markers. In molecular marker based analysis, quality of genomic DNA play a very important role.

ISSR analysis, only those primers were selected that showed reproducible and contrasting amplification patterns. Using this scale, finally, 21 ISSR primers were selected for the diversity analysis of the current set of cluster bean genotypes.

In present investigation first of all genomic DNA was extracted by the normal CTAB method [5] and it gave very low concentrations of genomic DNA and the yield of genomic DNA was 25ng/µl per 0.2g of leaf tissue. Such genomic DNA was contaminated with polysaccharides and phenolics as shown by the nano-drop spectrophotometer readings which gave a 250/280 nm ration of 1.29. However, electrophoresis evinced sheared bands which clearly indicate the presence of polysaccharides as shown in Figure 1. Consequently, different modifications were tried to remove the impurities of polysaccharides and increase the concentration of genomic DNA [13]. In the present study such, modification done in after grinding leafs tissues giving the Polyvinylpyrrolidone (PVP) wash for two-three times.

However, modification was equally effective for both conditions in cluster bean such as leaf tissue collected from the field as well as leaf tissue collected from controlled tissue culture conditions. The modified CTAB procedure gave higher concentration of genomic DNA as indicated by 180ng/µl to 240ng/µl per 0.1g of leaf tissue as shown in Figure 2.Which exhibited clear, sharp bands as compare to sheared bands. In order to check the impact of storage on genomic DNA with modified method, the extracted genomic DNA was stored for eight months at -80◦C and after eight months of storage same quality and quantity of genomic DNA was observed as shown in Table 3.

Therefore, the results of the present experiment clearly established the advantage of the modified CTAB methods for extraction of genomic DNA from cluster bean leaf tissue. Moreover, genomic DNA was isolated by this method yielded strong and reliable amplification products as shown in Figure 3 and its fragment size were ranged from 300 bp to 1800 bp. Therefore, the standardized genomic DNA isolation and PCR protocol for ISSR analysis may serve as an proficient tool for determination of genetic diversity in cluster bean at the molecular level.

DISCUSSION

Besides the use of a particular type of molecular marker, molecular characterization also depends

on successful isolation of quality DNA. Problems are reported for the isolation of plant DNA. Isolated DNA contains colored substances, polysaccharides and phenolic compounds [2,16,17]. The use of DNeasy Plant Mini Kit allowed the isolation of DNA from the plants studied, which we found suitable for ISSR-PCR amplification.

In this study, we not only grouped the genotypes for a particular trait but further sub-divided these already grouped genotypes with reference to the next trait and so on. This kind of characterization is morphological as well as biochemical provided a flow chart (Figs. 1&2) which can be used for characterization and identification of the genotypes using various combinations of traits, whereas, in earlier studies, the grouping provided the genetic similarity within the genotypes and cannot be used for dividing or subdividing the genotypes based on such combination of traits.

CONCLUDING REMARK

The breakthrough of PCR (polymerase chain reaction) was a milestone in this endeavor and proved to be a distinctive process that brought about a new class of DNA profiling markers. This facilitated the development of marker based gene tags, map based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping, marker assisted selection of desirable genotypes, etc. Thus giving new dimensions to concerted efforts of breeding and marker aided selection that can reduce the time span of developing new and better varieties and will make the dream of super varieties come true. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively.

REFERENCES

1. Anonyms. (2006). “Guar Production” Vernon Agricultural Research & Extension Center. Texas A & M University.
2. Aras, S., Duran, A. and Yenilmez, G. (1993). Isolation of DNA for RAPD analysis from dry leaf material of some Hesperis L. specimens. *Plant Mol. Biol. Rep*., 21: 461a-461f.
3. Casiva, P. V., Saidman, B. O., Vilardi, J. C. and Cialdella, A. M. (2002). First comparative phenetic studies of Argentinean species of Acacia (Fabaceae), using morphometric isozymal and RAPD approaches. *Am. J. Bot*., 89: 843–853.
4. Cottrell, J. E., Forrest, G. I. and White, M. S. (1997). The use of random amplified DNA markers to identify and estimate the relatedness of clones belonging to the genus Populus. *Bot. J. Scot*., 49: 89– 102.
5. Doyle, J. and Doyle, J.L. (1987). Genomic plant DNA preparation from fresh tissue-CTAB method. *Phytochem. Bull*., 19: 11-15.
6. Esselman, E. J., Crawford, D., Brauner, S., Stussy, I. F., Anderson, G. J. and Silva, M. O. (2000). RAPD marker diversity within and divergence among species of Dendroseris (Asteraceae: Lactuceae). *Am. J. Bot*., 87: 591–596.
7. Esselman, E. J., Jianqiamg, L., Crawford, D. J., Windus, J. L. and Welfe, A. D. (1999). Clonal diversity in the rare Calamagrostis porteri spp. Insperata (Poaceae): comparative results for allozymes and random amplified polymorphic DNA and inter-simple sequence repeat markers. *Mol. Ecol*., 8: 443–453.
8. Kumar, V. and Sharma, S.N. (2009). Assessment of genetic diversity of sesame (*Sesamum indicum* L.) genotypes using morphological and RAPD markers. *Indian Journal of Genetics and Plant Breeding*, 69: 209-218.
9. Leian, P., Bordallo, P. and Colova, V. (2005). Tracing the pedigree of Cynthiana grape by DNA microsatellite markers. *Proc. Fla. State Hort. Soc*., 118: 200-204.
10. Masuzaki, S., Yaguchi, S., Yamauchi, N. and Shigyo, M. (2007). Morphological characterization of Allium multiple alien

addition lines reveals chromosomal locations of gene(s) related to bulb formation in *Allium cepa*. *The Journal of Horticultural Science and Biotechnology*, 82: 393-396.

11. Mistra, B.S., Yadav, V. and Vishal, M., *et al*. (2013). Physiological and molecular characterization of cluster bean (*Cymopsis tetragonoloba* L.) rhizobia isolated from different areas of Rajasthan, India, *Legume Research*, 36: 299-305.
12. Pathak, R., Singh, M. and Henry, A. (2011). Genetic diversity and interrelationship among cluster bean (*Cyamopsis tetragonoloba*) genotypes for qualitative traits. *Indian Journal of Agricultual Science*, 81: 402-406.
13. Porebski, S.L., Grant, B. and Bernard, R.B. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter*. 15: 8-15.
14. Rao, L.S., Usha Rani, P., Deshmukh, P.S., Kumar, P.A. and Panguluri, S.K. (2007). RAPD and ISSR fingerprinting in cultivated chickpea (*Cicer arietinum* L.) and its wild progenitor *Cicer reticulatum* Ladizinsky. *Genetic Resources and Crop Evolution*, 54: 1235-1244.
15. Sharma, P., Sharma, V. and Kumar, V. (2014). Genetic diversity analysis of Cluster bean (*Cyamopsis tetragonoloba* L.) genotypes Using RAPD and ISSR Markers. *Journal of Agricultural Science and Technology*.16: 433-443.
16. Temiesak, P., Ponpim, Y. and Harada, T. (1993). RAPD analysis for varietal identification in Brassica. *Kasetsart. J. Nat. Sci.*, 27: 37-42.
17. Vanijajiva, O., Sirirugsa, P. and Suvachittanont, W. (2005). Confirmation of relationships among Boesenbergia (Zingiberaceae) and related genera by RAPD. *Biochem. Syst. Ecol*., 33: 159-170.
18. Wolf, A. D. and Randle, C. P. (2001). Relationships within and among species of the holoparasitic genus Hyobanche (Orobanchaceae) inferred from ISSR banding patterns and nucleotide sequences. *Systematic Bot*., 26:120-130.
19. Zietkiewicz, E. A., Rafalshi and Labuda, D. (1994).Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification, *J. Genomics*, 20: 176-183.

Tables- 1. List of 21 ISSR primer with their source in the present study

S. No.	Primer code	Primer sequence	Source
1.	UBC-808	AGAGAGAGAGAGAGAGC	SBS Genentech Co., Limited, China
2.	UBC-818	CACACACACACACACAG	SBS Genentech Co., Limited, China
3.	UBC-820	GTGTGTGTGTGTGTGTC	SBS Genentech Co., Limited, China
4.	UBC-854	TCTCTCTCTCTCTCTCRG	SBS Genentech Co., Limited, China
5.	UBC-856	ACACACACACACACACYA	SBS Genentech Co., Limited, China
6.	UBC-868	GAAGAAGAAGAAGAAGAA	SBS Genentech Co., Limited, China
7.	UBC-879	CTTCACTTCACTTCA	SBS Genentech Co., Limited, China
8.	ISSR-1	GAGAGAGAGAGAGAGAC	SBS Genentech Co., Limited, China
9.	ISSR-2	CACACACACACACACAC	SBS Genentech Co., Limited, China
10.	ISSR-5	CACACACACACAGG	SBS Genentech Co., Limited, China
11.	ISSR-8	GAGAGAGAGAGACC	SBS Genentech Co., Limited, China
12.	ISSR-9	GTGTGTGTGTGTCC	SBS Genentech Co., Limited, China
13.	IS-5	AGAGAGAGAGAGAGAGT	SBS Genentech Co., Limited, China
14.	IS-7	GAGAGAGAGAGAGAGAT	SBS Genentech Co., Limited, China

15.	IS-14	GTGTGTGTGTGTGTGTC	SBS Genentech Co., Limited, China
16.	IS-25	AGCAGCAGCAGCAGCAGC	SBS Genentech Co., Limited, China
17.	UBC 814-11	CTCTCTCTCTCTCTCAT	SBS Genentech Co., Limited, China
18.	UBC 826-11	ACACACACACACACACC	SBS Genentech Co., Limited, China
19.	UBC 829-11	TGTGTGTGTGTGTGTGT	SBS Genentech Co., Limited, China
20.	UBC 841	GAGAGAGAGAGAGAGAYC	SBS Genentech Co., Limited, China
21.	UBC 855	ACACACACACACACACYT	SBS Genentech Co., Limited, China

Table- 2. DNA synthesis report of 21- 10 mer ISSR random primers used in PCR analysis

Primer Code	Name of Sequence (5'- 3')	Length of Primer (nm)	Molecular Weight (MW)	Tm (°C) (%)	GC Content (%)
UBC-808	AGAGAGAGAGAGAGAGC	17	5366.58	55	52.94
UBC-818	CACACACACACACACAG	17	5086.37	56	52.94
UBC-820	GTGTGTGTGTGTGTGTC	17	5294.50	57	52.94
UBC-854	TCTCTCTCTCTCTCTCRG	18	5335.50	49	55.56
UBC-856	ACACACACACACACACYA	18	5367.06	57	50.00
UBC-868	GAAGAAGAAGAAGAAGAA	18	5671.82	44.5	33.33
UBC-879	CTTCACTTCACTTCA	15	4437.95	47	40.00
ISSR-1	GAGAGAGAGAGAGAGAC	17	5366.58	52	52.94
ISSR-2	CACACACACACACACAC	17	5046.34	52	52.94
ISSR-5	CACACACACACAGG	14	4210.80	52	57.14
ISSR-8	GAGAGAGAGAGACC	14	4370.92	40	57.14
ISSR-9	GTGTGTGTGTGTCC	14	4316.86	40	57.14
IS-5	AGAGAGAGAGAGAGAGT	17	5381.60	50	47.06
IS-7	GAGAGAGAGAGAGAGAT	17	5381.60	50	47.06
IS-14	GTGTGTGTGTGTGTGTC	17	5294.50	54	52.94
IS-25	AGCAGCAGCAGCAGCAGC	18	5527.64	60	66.67
UBC 814-11	CTCTCTCTCTCTCTCAT	17	4998.29	52	47.10
UBC 826-11	ACACACACACACACACC	17	5046.34	54	52.90
UBC 829-11	TGTGTGTGTGTGTGTGT	17	5309.52	52	52.90
UBC 841	GAGAGAGAGAGAGAGAYC	18	5663.27	52	55.56
UBC 855	ACACACACACACACACYT	18	5358.05	56	50.00

Table- 3. Usefulness of the genomic DNA quantification before and after storage of eight month period

S. No.	Genotypes	Quantification (ng/µl)		Quantification(ng/µl)	
		Before	**After**	**Before**	**After**
1.	IC- 258087	1.88	1.72	110	95
2.	IC-258092	1.89	1.91	120	110
3.	IC-28272	1.72	1.94	170	165
4.	IC-311440	1.85	1.96	180	175
5.	IC-311441	1.77	1.93	175	180
6.	IC-325757	1.79	1.88	200	195
7.	IC-329038	1.82	1.86	130	130
8.	IC-369789	1.83	1.87	143	135
9.	IC-369868	1.86	1.83	145	140

10.	IC-370478	1.89	1.84	146	132
11.	IC-370490	1.91	1.81	148	136
12.	IC-373427	1.77	1.89	159	120
13.	IC-373480	1.81	1.75	155	145
14.	IC-402293	1.74	1.78	160	142
15.	IC-415137	1.93	1.81	170	180

16.	IC-415142	1.85	1.72	156	138
17.	IC-415157	1.73	1.83	165	146
18.	IC-415159	1.92	1.86	178	155
19.	IC-421242	1.79	1.93	150	172
20.	IC-421798	1.97	1.83	160	166
21.	IC-421806	1.65	1.76	135	147
22.	IC-421809	1.75	1.94	168	157
23.	IC-421812	1.61	1.79	176	125
24.	IC-421815	1.76	1.81	155	165
25.	IC-421820	1.93	1.78	190	186
26.	IC-421828	1.66	1.95	183	192
27	IC-421834	1.80	1.86	169	181
28.	IC-421838	1.74	1.92	174	183
29.	IC-421855	1.63	1.76	164	171
30.	HG-365 (Durgajay × Hisar Local)	1.99	1.92	170	176

FIGURES

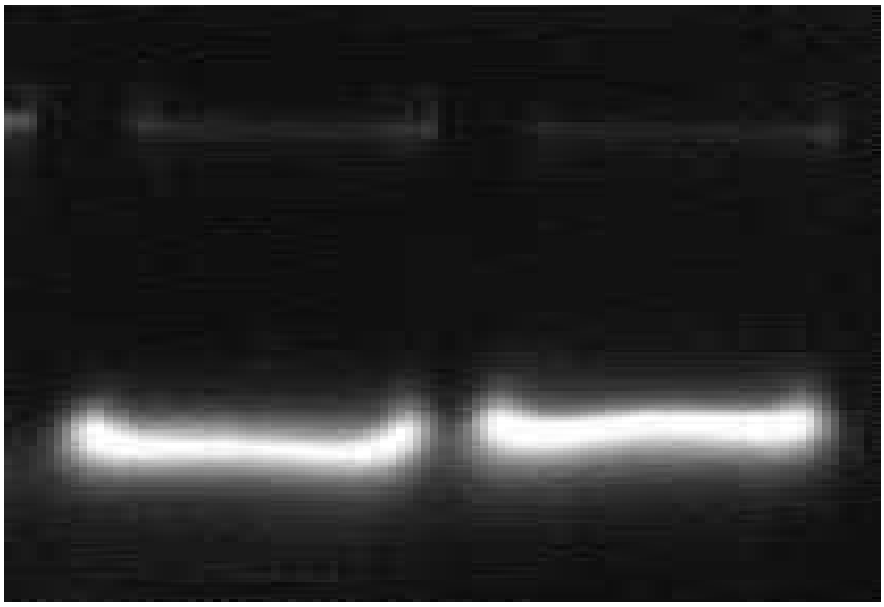

Figure 1. Genomic DNA shearing before modification.

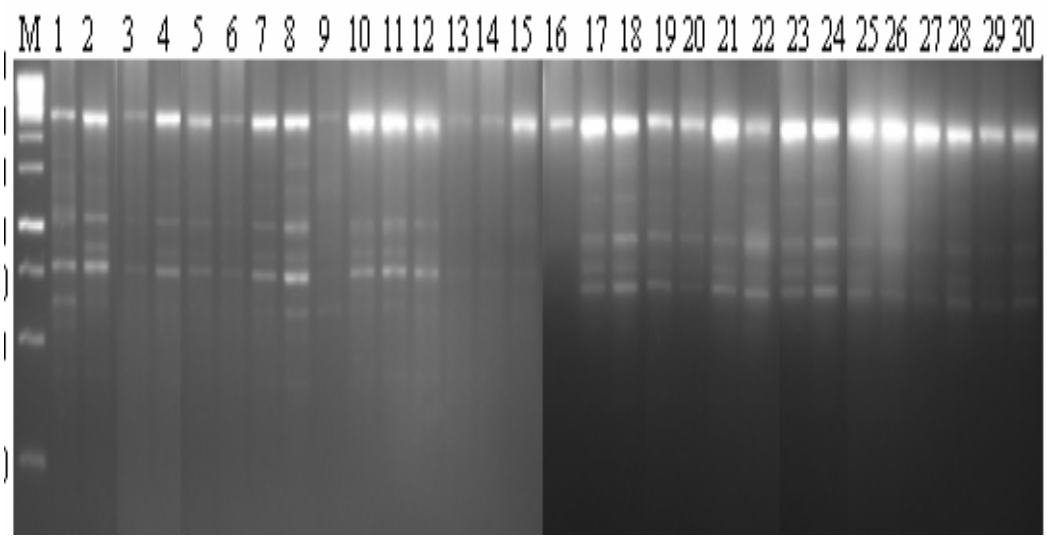

Figure 2. Gel profiling of genomic DNA in thirty diverse genotypes of cluster bean.

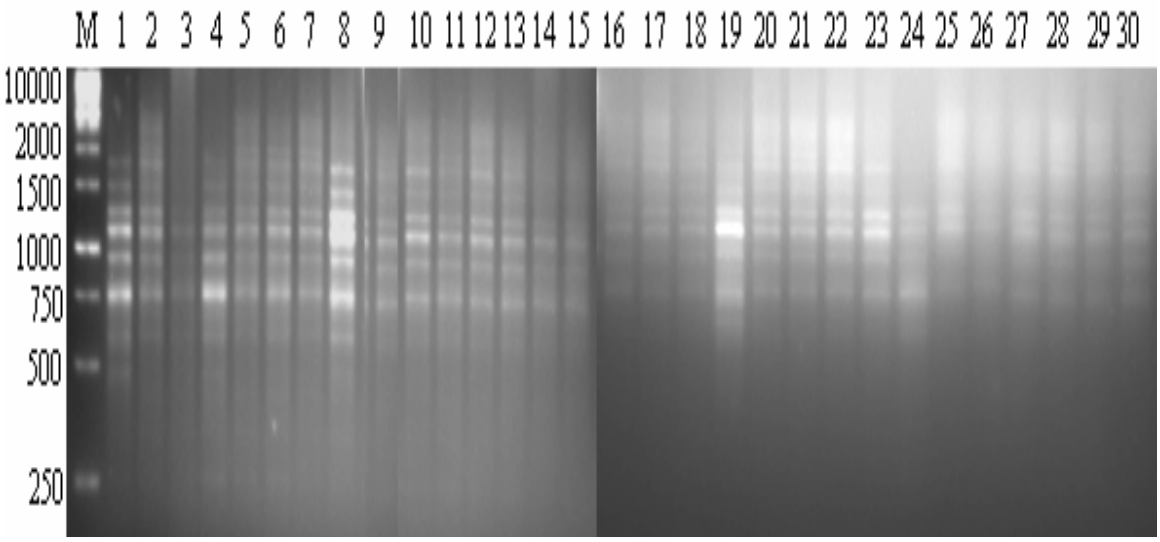

Figure 3. Gel profiling of IS (Add) SR-PCR amplification in thirty diverse genotypes of cluster bean.