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MOLECULAR CHARACTERISATION OF GREEN GRAM (*VIGNA RADIATE* L., *WILZECK*) GENOTYPES BY USING ISSR MARKERS.

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Abstract

Assessing the genomic diversity in green gram genotypes plays an essential role in germplasm selection and multiplication. Ten ISSR primers were used to assess molecular characterisation of twenty green gram genotypes, all the primers resulted in amplification of genotypes. DNA amplification with 10 ISSR markers produced, a total of 1277 scorable bands and 176 were polymorphic bands which recorded 14.79 percentage polymorphism. The primer UBC 854 recorded highest polymorphic percentage of 46.72% followed by UBC 848 which recorded 20.68%. Three unique bands were generated by the primer UBC 836 which could identify different genotypes. Among, the ten ISSR primers amplified, 8 primers could identify genotypes individually. Construction of dendrogram and clustering of green gram genotypes resulted into eight main clusters done by using ISSR data and the similarity coefficient among the genotypes varied with the range of 50% to 94%. Thus, the ISSR technique found to be easy, cost effective and a convenient method for varietal identification of green gram genotypes.

Key words: Green gram, Molecular characterisation, ISSR markers and Varietal identification.

Introduction

Green gram (*Vigna radiata* L.Wilzeck) (2n=22) belongs to Leguminosae or Fabaceae family as it has of 579 Mbp (Arumuganathan and Earle, 1991). It is cultivated in tropics and sub tropics and it's a autogamy crop and a rainy season crop; however, with the development of early maturing varieties, it has proved to be an ideal crop for spring and summer seasons. Pulses are the enrich sources of high quality digestable protein (24%) and quantity which is about two third of the protein content of soybean, twice that of wheat and thrice that of rice (Thirumaran and Seralathan, 1988) and with high nutritional benefits. Due to it's high protein content it is consumed in different forms such as dal, halwa and snack. Sprouted seeds synthesize ascorbic acid (Vitamin C) which increase the riboflavin and thiamine content. It is a short duration crop and has the capacity to fix atmospheric nitrogen and it can be used as green manure and excellent green fodder to animals. It fits well for multiple and intercropping cultivation.

India is a largest producer of pulse in the world with 25% share in global production and it contributes 11% to the total pulse production around the world. The area under coverage of pulses cultivation in India is 29.99 Million hectares, the production is about 25.23 MT and yield is about 841 kg/hectares. In Tamilnadu, the actual area under green gram cultivation is 0.87 Million hectares, the overall production is about 0.5 MT and the yield of green gran is about 635 kg/hectares according to "Agricultural Statistics at a Glance" (2018).

Varietal identification and development is the significant aspects of seed quality and seed genetic purity assessment. All the variety must possess the distinctiveness, uniformiy and stability as per "The Protection of Plant Varieties and Farmer's Rights" (PPV & FR Act, 2001), which pave a way to maintain variety for seed multiplication, seed certification and seed quality control. The conventional GOT (grow out test) is based on the identification of morphological characteristics at different stages of plant growth. This is affected by environmental factors and is time-consuming. Thus, the molecular based seed purity test could be a better alternate in receiving more attention since, as these are not influenced by the genotype and environment interactions, making DNA fingerprinting as the most straight forward method (Krawczak and Schmidtke, 1994) for cultivar

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identification or seed purity assessments.

In the recent years ISSR (INTER SIMPLE SEQUENCE REPEAT) have been utilized for molecular characterisation in black gram, rice, chick pea, chilli and tomato. ISSR primers produce more information in terms of total number of loci and polymorphic bands. This method uses microsatellites, generally 16-25bp long as primers. It uses single PCR reaction pointing manifold genomic loci to augment the inter-SSR sequence of different sizes. Being a smart breeding method it has been used to identify germplasms, revolve uncertain parentage and study genetic diversity, population genetics, taxonomy and phylogeny of many plant species (Reddy *et al*., 2002). Hence, the present study was carried out for molecular characterisation and identification of the green gram genotypes.

Materials and Methods

Plant Material

In this study, seeds of twenty green gram genotypes were collected from different sources (Table 1) were used for the study. All the twenty green gram genotypes were raised in pots, in green house, Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University. A random selection of young and healthy leaves were from 15 days old plant and the collected leaf samples were sterilized and placed on blotter paper for drying.

Table 1: List of twenty greengram genotypes and their source.

Geno-types	Name of the Genotypes	Source
G1	Chidambaram Local-1	Chidambaram, TamilNadu
G2	Maruvathur Local-1	Thanjavur, TamilNadu
G3	Kambam Local-1	Theni, TamilNadu
G4	Paiyur 1	Regional Research Station, Paiyur
G5	ADT 2	TNAU, Coimbatore
G6	ADT 3	TNAU, Coimbatore
G7	VBN 2	National Pulse Research Centre, Vamban
G8	VBN 3	National Pulse Research Centre, Vamban
G9	CO 1	National Pulse Research Centre, Vamban
G10	CO 2	National Pulse Research Centre, Vamban
G11	CO 7	TNAU, Coimbatore
G12	CO 8	TNAU, Coimbatore
G13	VRM 1	TNAU, Coimbatore,
G14	KM 2	Kolliyanur block, Villupuram
G15	DGGV2	UAS, Dharwad
G16	DGGV 7	UAS, Dharwad
G17	Pusa Vishal	IARI, New Delhi
G18	AKM 8803	Pulse Research Unit, Akola
G19	TAP 7	Pulse Research Unit, Akola
G20	MH- 421	HAU, Haryana

Table 2: Quantification of isolated DNA of *V. radiata* L. purified by Liquid Nitrogen method.

Genotypes	Concentration (ng/µl)	260/280
Chidambaram Local-1	241	1.35
Maruvathur Local-1	248	1.36
Kambam Local-1	291	1.38
Paiyur 1	260	1.37
ADT 2	251	1.36
ADT 3	325	1.42
VBN 2	998	1.7
VBN 3	314	1.43
CO 1	299	1.41
CO 2	333	1.43
CO 7	244	1.34
CO 8	296	1.39
VRM 1	274	1.39
KM 2	308	1.43
DGGV2	339	1.45
DGGV 7	239	1.34
Pusa Vishal	306	1.31
AKM 8803	281	1.37
TAP 7	326	1.43
MH -421	324	1.38

Plant genomic DNA Isolation and Quantification

Extraction and Isolation of plant genomic DNA was carried out by using the method described by Doyle and Doyle, (1987). The extraction of plant genomic DNA was carried out by liquid Nitrogen method followed by addition of 2-mecapto ethanol, chloroform : isoamyl alcohol and 2-propanol where the thread formation occurs and DNA purification was done by adding RNase. Expression of polymorphic and monomorphic bands depends upon the concentration and quality of the isolated genomic DNA, which were analysed by electrophoresis by an agarose gel and quantification of genomic DNA was done using UV spectrophotometer at a ratio of A260/ A280 which provides an estimate of DNA nucleic acid purity level of isolated genomic DNA. (Table 2).

ISSR marker and PCR analysis

A total of ten ISSR primers synthesised by Biogene Life Science Pvt. Ltd. Chennai, were used for PCR analysis. The list of ISSR primers used for the present study are given in table 4.

PCR analysis

Polymerase Chain Reaction is a technique of enzymatic synthesis of multiplying specific DNA sequences into million copies, developed by Kary Mullis in 1983. It is a simple and in expensive technique used for the characterizing, analysing and synthesizing specific portion of DNA or RNA. It utilizes the natural function of the polymerases, present in all living things to copy genetic material or perform "Molecular Photocopying". PCR amplification was done first by adding 1 µl of genomic DNA to the 0.2ml thin walled PCR tube. Then add 14 µl of the cocktail mix to make a final reaction volume of 15 µl. Mix gently and briefly centrifuge it. The

Table 3: Thermo cycler condition for ISSR primers.

S. No	Step	Temp. (°C)	Duration (min)	Number of cycles
1.	Initial denaturation	94	5	1
2.	Final denaturation	94	1	29
3.	Annealing	52	1	
4.	Extension	72	1	
5.	Final extension	72	10	1

Table 4: Details of ISSR markers used for the analysis of 20 green gram genotypes.

S. No	ISSR Primers	Sequences (5'-3')	Band size	Total number of bands	Number of polymorphic bands	Percentage Polymorphism
1.	**UBC-810**	GAGAGAGAGAGAGAGAT	280-1300	112	11	9.82
2.	**UBC-811**	GAGAGAGAGAGAGAGAC	310-1300	126	12	9.52
3.	**UBC-817**	CACACACACACACACAA	230-1000	149	26	17.44
4.	**UBC-818**	CACACACACACACACAG	300-1400	151	7	4.63
5.	**UBC-820**	GTGTGTGTGTGTGTGTT	250-1500	73	13	17.80
6.	**UBC-822**	TCTCTCTCTCTCTCTCA	300-1600	178	22	12.35
7.	**UBC-836**	AGAGAGAGAGAGAGAGYA	260-1300	155	4	2.58
8.	**UBC-848**	CACACACACACACACARG	280-1400	116	24	20.68
9.	**UBC-854**	TCTCTCTCTCTCTCTCRG	300-1600	107	50	46.72
10.	**UBC-878**	GGCGGCGGCGGCGGCAT	210-1600	110	7	6.36
Total				**1277**	**176**	**147.9**
Average				**127.7**	**17.6**	**14.79**

Table 5: Similarity Coefficient of 20 green gram genotypes.

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20
G1	1																			
G2	**0.94**	1																		
G3	0.74	0.71	1																	
G4	0.65	0.64	0.62	1																
G5	0.71	0.7	0.68	0.75	1															
G6	0.77	0.75	0.82	0.62	0.67	1														
G7	0.74	0.71	0.73	0.69	0.73	0.79	1													
G8	0.85	0.82	0.85	0.69	0.75	0.89	0.82	1												
G9	0.83	0.78	0.8	0.63	0.71	0.86	0.78	0.89	1											
G10	0.76	0.71	0.76	0.64	0.73	0.82	0.86	0.82	0.85	1										
G11	0.8	0.8	0.78	0.6	0.7	0.77	0.71	0.84	0.78	0.69	1									
G12	0.78	0.75	0.73	0.69	0.75	0.81	0.75	0.82	0.8	0.8	0.75	1								
G13	0.77	0.72	0.74	0.63	0.81	0.78	0.81	0.81	0.84	0.87	0.74	0.76	1							
G14	0.79	0.77	0.77	0.7	0.79	0.76	0.77	0.81	0.81	0.82	0.71	0.74	0.88	1						
G15	0.76	0.75	0.73	0.66	0.65	0.79	0.7	0.77	0.71	0.66	0.7	0.7	0.66	0.74	1					
G16	0.78	0.81	0.71	0.71	0.75	0.77	0.73	0.82	0.78	0.71	0.78	0.78	0.76	0.82	0.81	1				
G17	0.75	0.77	0.7	0.68	0.69	0.76	0.7	0.79	0.77	0.68	0.72	0.72	0.71	0.76	0.79	**0.93**	1			
G18	0.77	0.79	0.72	0.75	0.74	0.78	0.79	0.81	0.79	0.7	0.72	0.77	0.71	0.73	0.82	0.85	0.83	1		
G19	0.74	0.74	0.74	0.65	0.66	0.82	0.76	0.83	0.78	0.71	0.73	0.73	0.72	0.75	0.78	0.83	0.87	0.8	1	
G20	0.68	0.65	0.65	**0.5**	0.55	0.68	0.65	0.67	0.68	0.67	0.69	0.61	0.7	0.7	0.64	0.64	0.64	**0.6**	0.75	1

G1 - Chidambaram Local-1; **G2** - Maruvathur Local-1; **G3** - Kambam Local-1; **G4** - Paiyur 1; **G5** - ADT 2; **G6** - ADT 3; **G7** - VBN 2; **G8** - VBN 3; **G9** - CO 1; **G10** - CO 2; **G11** - CO 7; **G12** - CO 8; **G13** - VRM 1; **G14** - KM 2; **G15** - DGGV 2; **G16** - DGGV 7; **G17** - Pusa Vishal; **G18** - AKM 8803; **G19** - TAP 7; **G20** - MH -421

tubes are placed in the thermal cycler and PCR is carried out under the following temperature conditions (Table 3).

Agarose gel electrophoresis

It is a technique used to separate DNA fragments based on their molecular weight and it is an underlying part of all experiments carried out in molecular biology. Amplified products were separated by gel electrophoresis on a 0.8 % of agarose gel stained by containing 0.5 µg/ml ethidium bromide in 100ml of 0.5x TBE (Tris-Borate-EDTA) buffer, pH 8.0. The template was mounted on an appropriate electrophoresis tank is filled with 0.5x TBE buffer until the gel was immersed up to 1mm. Load 2 µl of DNA sample mixed with 2 µl of 6x bromophenol (loading dye). Run the gel at 70 V for one hour and visualization of the DNA was done on a UV transilluminator and photographs were taken using gel documentation system (BIORAD Gel. Doc. XR imaging system).

Scoring and analysis of ISSR data

In ISSR analysis, the scoring of bands has been indicated as presence and absence (presence=1 and absence= 0) of character for subsequent analysis using the software NTSYS-pc version 2.02. (Rohlf, 1998). PIC value of calculated for each of ISSR loci using the formula developed by Roldan-Ruiz *et al*., (2000). PIC=2fi (1-fi) Where, fi is frequency of marker bands which were present and 1-fi frequency of markers bands which were absent. The scoring data in the form of binary values was used for the construction of dendrogram.

Similarity matrix was generated using the SIMQUAL programme of NTSYS-pc software, version 2.02 reported by (Rohlf, 1998). The similarity coefficients were used for cluster analysis and dendrogram was constructed by the Unweighted Pair-Group Method Arithmetic mean (UPGMA) by Sneath and Sokal, (1973).

Result and Discussion

The result obtained from twenty green gram genotypes using ten ISSR primers were detailed below (Table 4). From the above study, isolation of plant genomic DNA was isolated by liquid nitrogen method Doyle and Doyle, (1987). The quantification of plant genomic DNA isolated from various genotypes of *Vigna radiata* L. ranged from 239 to 998 ng/µl. The genotype VBN 2 yielded the highest amount of DNA (998 ng/µl). Whereas the lowest amount of DNA (239 ng/ µl) was obtained from genotype DGGV 7.

The ratio of absorbance (A260/A280) ranged from 1.31 ng/ µl to 1.7 ng/ µl and the quality of DNA was also checked by gel electrophoresis in all genotypes, showing that genomic DNA was intact with high molecular weight and free from RNA contamination (Table 2).

By using ten ISSR primers all genotypes resulted amplification (Fig. 1 to 4). An overall of 1277 amplified bands were obtained from the 10 primers and all were polymorphic. The primer UBC-822 generated high number of 178 scorable bands followed by UBC-836

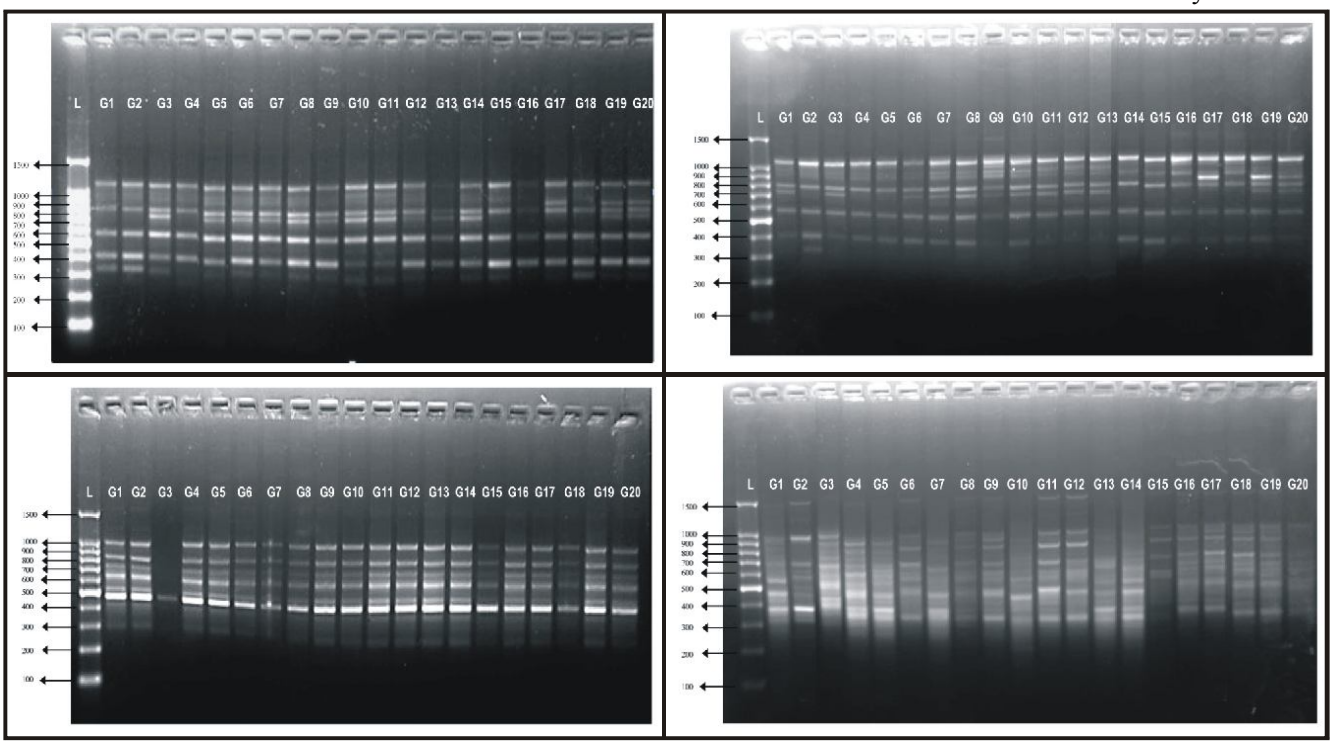

Fig 1: ISSR analysis on 20 green gram genotypes with the primer- a) UBC –810, b) UBC –811, c) UBC –817, d) UBC –854.

primer produced 155 reproducible bands. The total number of amplified bands varied between 73 and 178 (Table 4). The resultant of average number of polymorphic bands was found to be 14.79%.

Overall size of PCR amplified products ranged between 200 bp to 1600 bp. The PCR amplification using ISSR primers gave rise to reproducible amplification products. Similar results were shown by Das *et al.*,

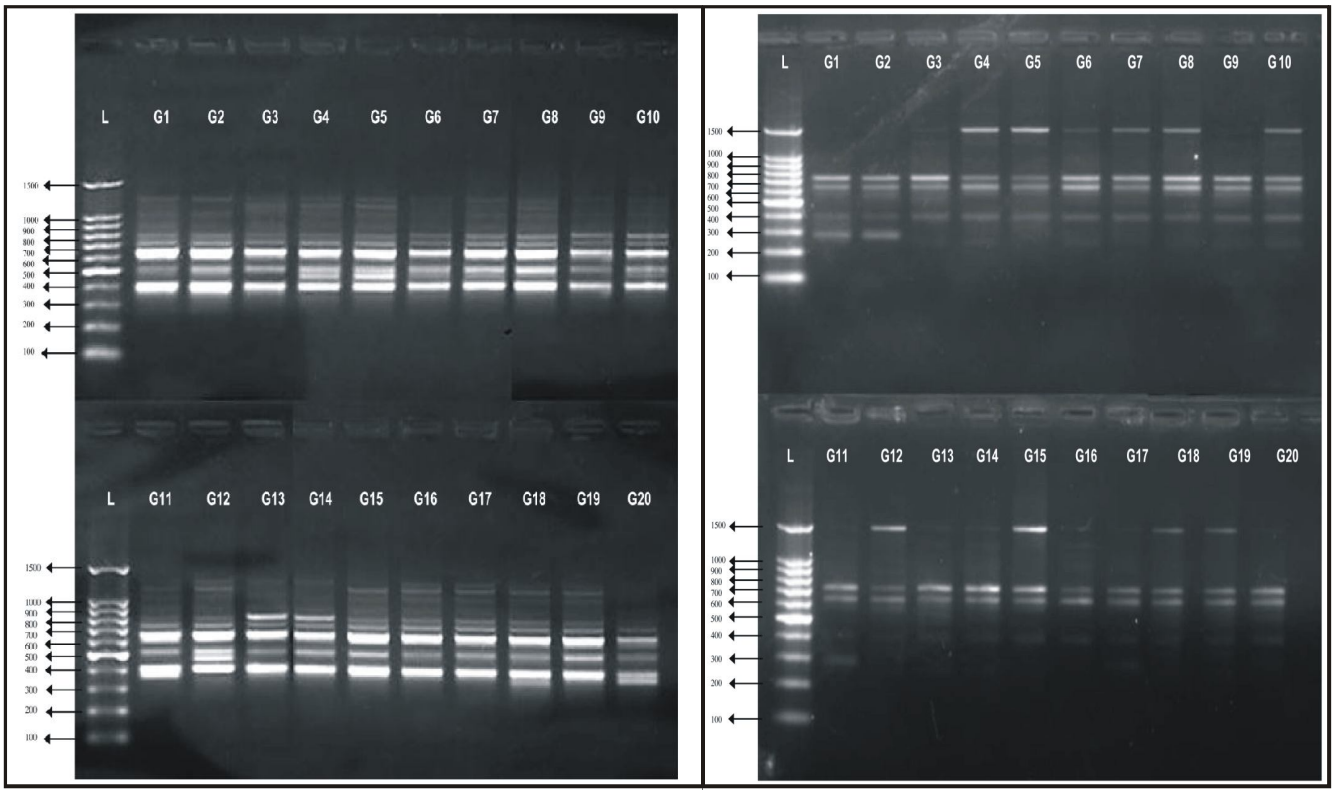

Fig 2: ISSR analysis on 20 green gram genotypes with the primers - e) UBC –818, f) UBC –820.

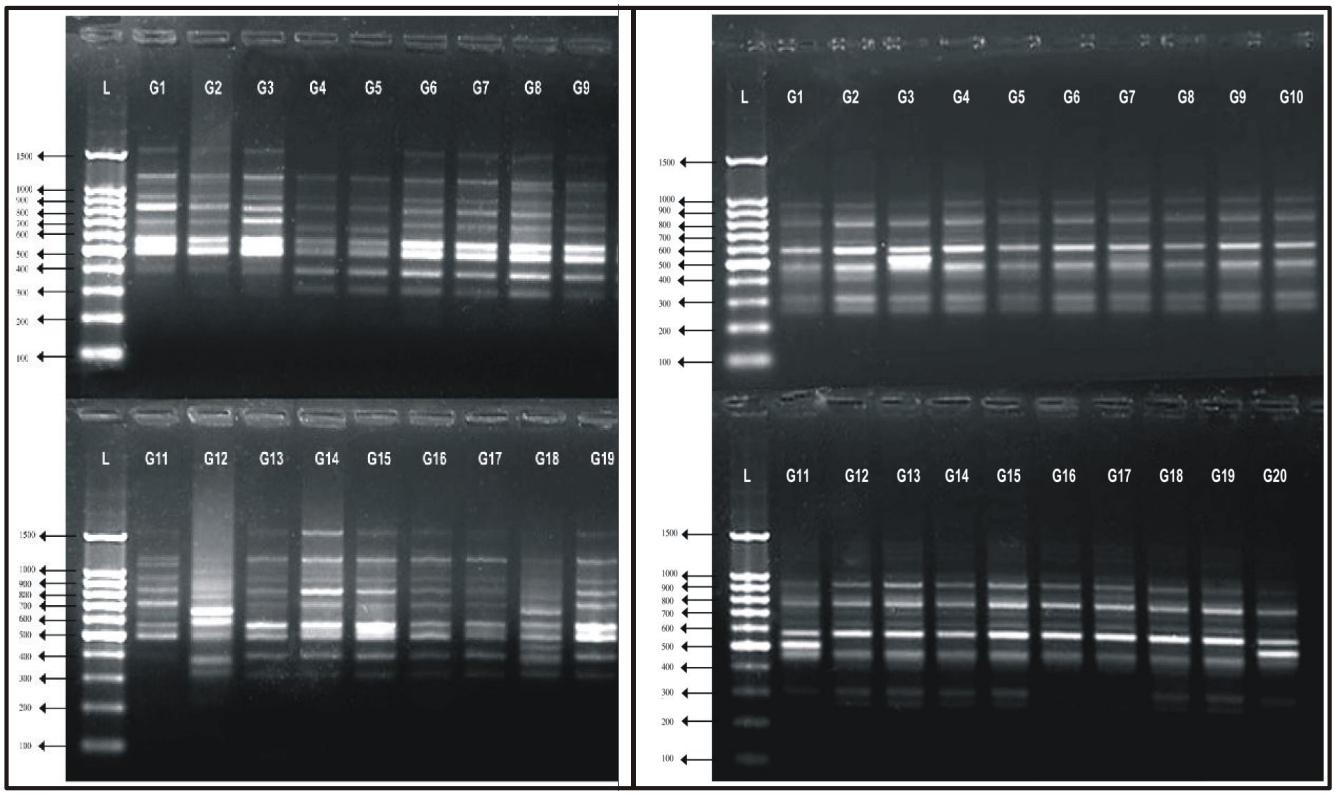

Fig. 3: ISSR analysis on 20 green gram genotypes with the primers - g) UBC –822, h) UBC –836.

Table 6: Identification of unique bands in *V. radiata* L. genotypes by ISSR primers.

Markers	Present/ Absent	Genotypes Identified	Base pairs (bp)
UBC-811	+	Maruvathur Local-1	320
		CO 1	900
UBC-818	+	CO 8	450
		MH-421	320
UBC-820	+	CO 7	300
UBC-822	+	CO 8	630
		AKM 8803	450 ,680
UBC-836	+	Kambam Local-1	550
		CO 7	500
		MH -421	250
UBC-848	+	TAP 7	900
UBC-854	+	Paiyur 1	580
		Pusa Vishal	790
UBC-878	+	Chidambaram Local-1	550
		CO 7	430

(2014); Hardik *et al.,* (2015); Singh *et al*., (1991) and Tantasawat *et al*., (2010).

Similarity matrix

Based on ISSR similarity matrix data, the value of similarity coefficient ranged from 0.5 to 0.94 (Table 5). The green gram genotypes Chidambaram Local -1 with Maruvathur Local-1 showed maximum similarity (94%) followed by DGGV 7 with Pusa Vishal (93%) among the ten genotypes, while the genotypes Paiyur 1 with MH-421 showed least pair wise similarity (50%) followed by AKM 8803 with MH-421 (60%) among the twenty green gram genotypes.Similar findings were reported by Anamika Nath *et al.,* (2017); Harsukh *et al.,* (2014); Hardik *et al*., (2015) and Swati Sharma *et al*., (2018), in green gram cultivars.

Cluster tree analysis

The dendrogram was constructed for twenty genotypes of green gram using UPGMA method to group the genotypes based on Jaccard's similarity coefficient matrices calculated from 10 ISSR markers. The coefficient value ranged from 0.94 to 0.5 indicated the genetic diversity among the 20 genotypes. The dendrogram clearly indicated eight main clusters (Fig. 5). Cluster I consisted of four genotypes ADT 3, Kambam Local-1, VBN 3 and CO 1.Cluster II also consisted of four genotypes AKM 8803, DGGV 2, DGGV 7 and Pusa Vishal, the Cluster III consisted of two genotypes such as TAP 7 and MH-421.Cluster IV consist of three genotypes namely Chidambaram Local-1, Maruvathur Local-1 recorded similarity coefficient of 0.94 and CO 7,Cluster V consist of VRM 1 and KM 2 genotypes, Cluster VI consist of ADT 2 and Paiyur 1 genotypes, Cluster VII consist of CO 2 and VBN 2 genotypes and Cluster VIII consist of CO 8 genotype. The UPGMA distributed the 20 genotypes into eight main clusters; similarity coefficient values ranging from 0.94 to 0.5. One genotype namely, CO 8 stands out and forms a separate cluster. The genetic variation amongst advanced lines of all the genotypes, also found the same result by Tantasawat

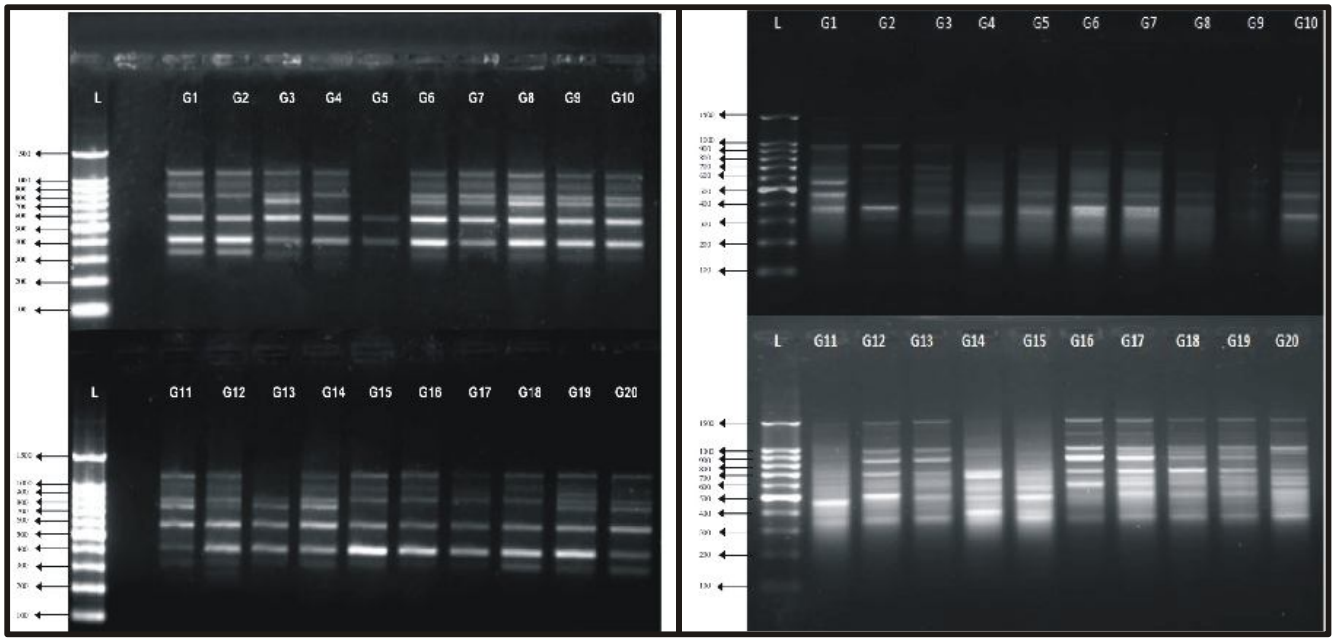

Fig. 4: ISSR analysis on 20 green gram genotypes with the primers - i) UBC –848, j) UBC –878. (**G1** - Chidambaram Local-1; **G2** - Maruvathur Local-1; **G3** - Kambam Local-1; **G4** - Paiyur 1; **G5** - ADT 2; **G6** - ADT 3; **G7** - VBN 2; **G8** - VBN 3; **G9** - CO 1; **G10** - CO 2; **G11** - CO 7; **G12** - CO 8; **G13** - VRM 1; **G14** - KM 2; **G15** - DGGV 2; **G16** - DGGV 7; **G17** - Pusa Vishal; **G18** - AKM 8803; **G19** - TAP 7; **G20** - MH -421).

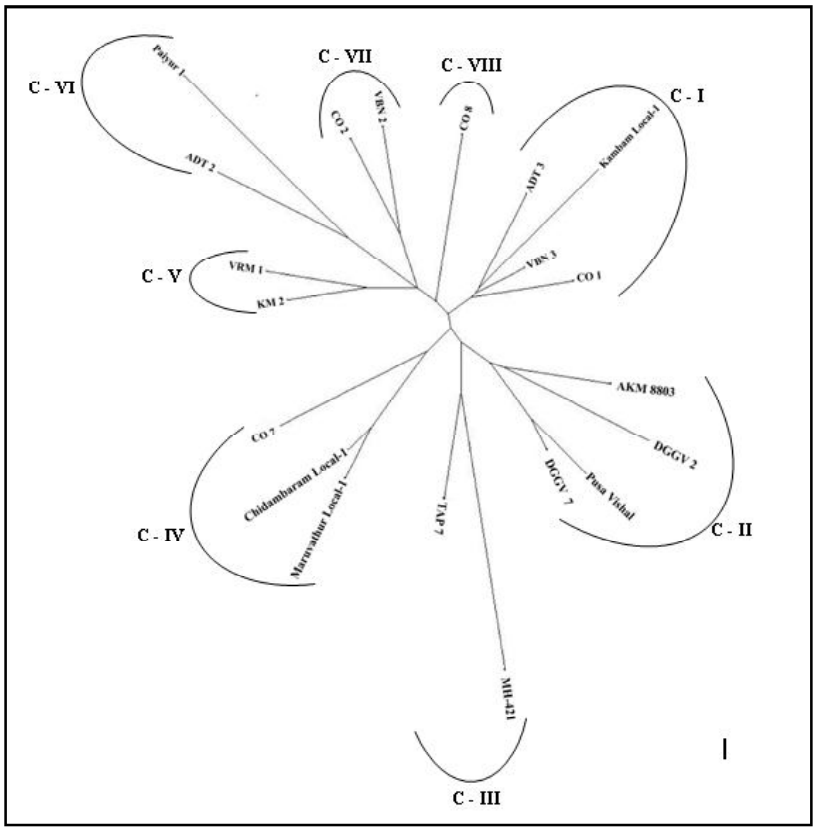

Fig. 5: Construction of dendrogram between 20 green gram genotypes using UPGMA method from 10 ISSR markers scoring data.

et al., (2010) and Bharati *et al*., (2012).

Unique identification of green gram genotypes

ISSR markers were used for the unique identification of 20 genotypes of green gram. The unique markers were located across all the primers that individually identified each of the genotypes. Details of the genotypes, specific marker generated by different primers are given in table 6. The identification is based on presence and absence of unique marker. Eleven genotypes could be identified on the basis presence of single unique marker. The ISSR primer UBC-811 had identified Maruvathur Local -1 at 320 bp and CO 1 genotype at 900 bp. The ISSR primer UBC-818 identified CO 8 genotype at 450 bp and MH-421 at 320 bp. The ISSR primer UBC-820 identified CO 7 at 300bp and the UBC-822 primer had identified CO 8 at 630 bp and AKM 8803 at 450 and 680 bp. The primer UBC-836 had identified three genotypes namely Kambam Local- 1 at 550 bp, CO 7 at 500 bp and MH-421 at 250 bp. The primer UBC-848 had identified the TAP 7 genotype at 900 bp. UBC 854 primer identified Paiyur-1 genotype at 580 bp and Pusa Vishal at 790 bp. The primer UBC 878 identified Chidambaram Local -1 at 550 bp and CO 7 at 430 bp.

Conclusion

1. ISSR markers were useful in characterization and identification of green gram genotypes. Hence the high similarity was observed between Chidambaram Local-1 and Maruvathur Local -1 (0.94) followed by DGGV 7 and Pusa Vishal (0.93).
2. Least similarity was found between Paiyur 1 and MH-421 (0.5) followed by AKM8803 and MH421(0.6), as they have distinct parental characters among the 20 green gram genotypes.
3. 10 ISSR primers produced very low level of polymorphic percentage (14.79 %) is due to narrow genetic base of the genetic material used.
4. The present study identified some of the unique primers for identification of green gran genotypes. The ISSR primer UBC 836 generated 3 different unique bands in Kambam Local-1, CO 7 and MH-421.Hence, ISSR primer UBC-836 can be used for varietal identification on these green gram genotypes.
5. Hence forth these elite genotypes would be used for seed technological and breeding purposes and it can be efficiently utilized.

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