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Molecular Characterisation and Genetic Diversity Analysis through SSR Markers in Germplasm Lines of Green gram [*Vigna radiata* (L.) Wilczek]

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Abstract

Fifty-six germplasm lines of Green gram [*Vigna radiata* (L.) Wilczek] were used to determine the extent of genetic diversity through simple sequence repeats (SSR) markers. Molecular characterization of 56 green gram genotypes was done with fifteen standardized SSR primers. All primers showed scorable polymorphism. DNA bands generated from SSR-PCR amplification were scored using binary system and the information was used to calculate Jackard's similarity matrix using NTSYS-pc version 2.1 The genetic similarity between genotypes ranged from 0.35 to 1.00. A minimum similarity coefficient of 0.35 was observed between genotypes LGG-585 and LGG-573 and maximum was between KKM-3 and TM-962 (1.00). The average genetic similarity value of 0.85 that existed among the germplasm lines indicate moderate level of genetic diversity within the self-pollinated crop green gram, which is possibly due to accumulation of novel gene combinations in response to dynamic pressures of natural selection. The UPGMA dendrogram based on SSR results divided the 56 green gram genotypes into eight main clusters under which the cluster I was highly diverse compared to all other clusters and consisted of 15 genotypes, followed by cluster II with 9 genotypes, cluster VI with 7 genotypes, cluster IV and V with 6 genotypes, cluster III with 5 genotypes, and cluster VII and VIII with 4 genotypes each. The present study revealed that SSR markers may be successfully utilized for determining genetic diversity and relationships among germplasm lines of green gram.

Keywords: Green gram germplasm, genetic diversity, dendrogram, SSR markers

1. Introduction

Green gram [*Vigna radiate* (L.) Wilczek] also known as mung bean is a diploid (2n) legume with chromosome number 22 belonging to the family Fabaceae. It is an important short duration food legume of the tropical and sub tropical countries of the world. It is third most important pulse crop of India providing vegetable protein for people. India is the largest producer and consumer of green gram in the world accounting for 55% of total world acreage and 45% of total production (Rishi, 2009; Singh et al., 2013). For better use of germplasm collections, assessment of phenotypic or genotypic diversity is essential since germplasm are the

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basic material for crop improvement programmes.

Conventional plant breeding has tremendously contributed for increase in crop yields through genetic improvement which primarily relies on phenotypic selection hence considerable difficulties are associated with this process, primarily due to genotype–environment interactions. Besides, screening procedures many a times are difficult, unreliable or expensive owing to the nature of the biotic and abiotic stress target traits. The development of DNA (or molecular) markers has irreversibly changed the disciplines of plant genetics and plant breeding. It is very much essential to precisely know the genetic architecture of traits considered for genetic improvement hence it is evident to estimate genetic variability of these traits. DNA-based molecular markers such as simple sequence repeats (SSRs) have been proven as powerful tools in studying the genetic diversity and population structure of the species (Yuliasti and Reflinur, 2015). Phenotypic/Morphological markers used for diversity studies are not adequate, because these markers are subject to environmental influences unlike DNA based molecular markers (Nath et al., 2018) DNA-based markers are less affected by age, the physiological condition of samples, and environmental factors. Involvement of DNA markers in conventional plant breeding improves the efficiency of selection because selection is based on genotypes and not on phenotypes.

To characterize and identify novel genotypes for uses in the crop breeding programmes, molecular techniques using DNA polymorphism are increasingly used. Assessment of the genetic variation in green gram has been carried out using different types of molecular markers including random amplified polymorphic DNA (RAPD) (Santalla et al., 1998; Lakhanpaul et al., 2000), amplified fragment length polymorphism (AFLP) (Bhat et al., 2005) and simple sequence repeat (SSR) (Gwag et al., 2010). Among various marker systems available, SSRs are the markers of choice for plant breeders because of their reproducibility, cost effectiveness, multi-allelic nature, co-dominant inheritance, cross species transferability, relative abundance, good genetic coverage and their hyper-variable nature (Singh et al., 2013). The main objective of this study was to carry out molecular characterization of green gram genotypes using SSR markers leading to estimation of genetic diversity and genetic relatedness among germplasm lines representing different agro-climatic regions.

2. Materials and Methods

The experiment was conducted in an Augmented Design with 56 genotypes of green gram [*Vigna radiata* (L.) Wilczek] grown in 8 Blocks during *summer* 2017 at experimental plot of College of Agriculture, Hassan, University of Agricultural Sciences, Bengaluru, Karnataka, (India). The gross area of experiment was 302.5 m^2 and each block size was 3×3 m^2. The row spacing was 30 cm and inter plant distance was 10 cm. Observations were also recorded for 20 metric characters viz., days to 50% flowering, days to 50% maturity, plant height, plant diameter, number of primary branches$plant^{-1}$, number of clusters $plant^{-1}$, leaf area, specific leaf weight, leaf area ratio, number of pods $cluster^{-1}$, number pods $plant^{-1}$, pod length, number of seeds pod^{-1}, pod yield $plant^{-1}$, seed yield $plant^{-1}$, seed yield $plot^{-1}$, threshing %, biological yield, harvest index and 100 seed weight. The observations recorded on agronomic traits would help us to assess genetic diversity based on phenotypic data so that one can have a comparison between genetic diversity as explained by phenotypic data and genotypic data.

2.1. Study material

The study material comprised of 56 germplasm lines of green gram [*Vigna radiata* (L.) Wilczek] obtained from different Research Institutions and Agricultural Research Stations of India (Table 1). Newly emerged leaf samples of the genotypes were used for DNA extraction.

2.2. Genomic DNA extraction and quantification

Total genomic DNA was isolated from 56 genotypes using acetyltrimethyl ammonium bromide (CTAB) extraction protocol (Doyle and Doyle, 1987) and was then quantified spectrophotometrically on a nano spectrophotometer (Implen, Germany).

2.2.1. SSR-PCR amplification

Fifteen SSRs or microsatellite repeat primers were used to screen germplasm lines of green gram presented in Table 2. PCR amplification was carried out in a 20-µL reaction volume containing 200 µM dNTP mix, 1.5 mM $MgCl_2$, 1 U of Taq polymerase,1X reaction buffer, 0.5 µM primer, double-distilled water, and 20 ng of genomic DNA. The amplification was performed with reaction conditions of pre-denaturation at 94 °C for 4 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 48–56.7 °C for 1 minute, extension at 72 °C for 45 seconds and final extension was done for 10 minutes at 72 °C with a hold temperature of 4 °C. 3% metaphor agarose (Sigma-Aldrich, India) was used for separation of amplification products by electrophoresis and gel images were captured.

2.3. Data analysis

DNA bands generated from SSR-PCR amplification were scored using binary system where a scoring of 1 was given for presence of band and 0 for absence of bands for each primer and were used to calculate Jackard's similarity matrix using NTSYS-pc version 2.1. Cluster analysis was performed on molecular data. Similarity matrix were compiled for all pairs of accessions using Jackard's similarity coefficient and dendrogram for genetic diversity was constructed using un-weighted pair-group method with arithmetic mean (UPGMA) analysis.

3. Results and Discussion

Molecular characterization of 56 green gram genotypes was attempted with 15 standardized primers. All of the

Table 1: List of germplasm used in the study and their source

Sl. No.	Genotype	Source	Sl. No.	Genotype	Source
1.	Selection- 4- Check	UAS, Raichur	29.	LGG-572	RARS, Guntur
2.	DGG-1- Check	ARS, Bidar	30.	PM-110	RARS, Guntur
3.	Barimung- Check	UAS, Raichur	31.	LGG-577	RARS, Guntur
4.	KKM-3	UAS, Banglore	32.	IC-436624	IIPR, Kanpur
5.	Harsha	UAS, Raichur	33.	IC-436723	IIPR, Kanpur
6.	VBN-1	Coimbatore	34.	IC-413316	IIPR, Kanpur
7.	BGS-9	ARS, Bidar	35.	IC-436746	IIPR, Kanpur
8.	KM13-16	ARS, Bidar	36.	VGG10-010	Coimbatore
9.	KM13-19	ARS, Bidar	37.	VGG04-011	Coimbatore
10.	KM13-39	ARS, Bidar	38.	VGG04-007	Coimbatore
11.	GG13-7	ARS, Bidar	39.	COGG-93	Coimbatore
12.	GG13-6	ARS, Bidar	40.	VBNGG-2	Coimbatore
13.	KM13-44	ARS, Bidar	41.	TARM-2013	Coimbatore
14.	GG13-10	ARS, Bidar	42.	VGG04-005	Coimbatore
15.	SML-668	ARS, Bidar	43.	COGG-920	Coimbatore
16.	KM13-9	ARS, Bidar	44.	VGG07-003	Coimbatore
17.	IPM99-125	ARS, Bidar	45.	VGG10-002	Coimbatore
18.	LGG-596	RARS, Guntur	46	VGG-112	Coimbatore
19.	LGG-572	RARS, Guntur	47.	IC-92048	NBPGR, Akola
20.	LGG-450	RARS, Guntur	48.	AKL-103	NBPGR, Akola
21.	LGG-583	RARS, Guntur	49.	AKL- 39	NBPGR, Akola
22.	LGG-590	RARS, Guntur	50.	AKL-106	NBPGR, Akola
23.	LGG-588	RARS, Guntur	51.	AKL-225	NBPGR, Akola
24.	LGG-589	RARS, Guntur	52.	AKL-95	NBPGR, Akola
25.	LGG-579	RARS, Guntur	53.	AKL-194	NBPGR, Akola
26.	LGG-562	RARS, Guntur	54.	AKL-212	NBPGR, Akola
27.	LGG-582	RARS, Guntur	55.	AKL-195	NBPGR, Akola
28.	LGG-585	RARS, Guntur	56.	AKL-211	NBPGR, Akola

Table 2: List of SSR primers and their details

Sl. No	Seq Id.	Seq Name	Sequence 51→31	Length	Tm °C
1.	1B28022	CEDG204	CCTTGGTTGGAGCAGCAGC	19	55.4
	IB28023	CEDG204	CACAGACACCCTCGCGATG	19	55.4
2.	IB28030	CEDG092	TCTTTTGGTTGTAGCAGGATGAAC	24	54
	IB28031	CEDG092	TACAAGTGATATGCAACGGTTAGG	24	54
3.	IB28036	CEDG275	CACACTTCAAGGAACCTCAAG	21	52.4
	IB28037	CEDG275	GTAGGCAACCTCCATTGAAC	20	51.8
4.	IB28038	CEDG020	TATCCATACCCCAGCTCAAGG	20	51.8
	IB28039	CEDG020	GCCATACCAAGAAAGAGG	18	48
5.	IB28040	CEDG264	GATTCCCTTCCTAGCTATGG	20	51.8
	IB28041	CEDG264	CTGCTGGACATGAAGATTCAG	21	52.4

Table 2: Continue...

Sl. No	Seq Id.	Seq Name	Sequence 51→31	Length	Tm °C
6.	IB28042	CEDG271	GCACTAAAGTTAGACGTGGTTC	22	53
	IB28043	CEDG271	CACTCCCACTGCCAAACAAGG	21	56.3
7.	IB28046	CEDG056	TTCCATCTATAGGGGAAGGGAG	22	54.8
	IB28047	CEDG056	GCTATGATGGAAGAGGGCATGG	22	56.7
8.	1B28054	CEDG016	TTAGTTCACTCCGCTTGGTC	20	51.8
	IB28055	CEDG017	CACGTCATCCTCTGTTAGAC	20	51.8
9.	IB28058	CEDG022	AGGAATGTGAGATTTG	16	38.3
	IB28059	CEDG022	AATCGCYYCAAGGTCAAGCC	20	51.8
10.	IB28062	CEDG198	CAAGGAAGATGGAGAGAATC	20	49.7
	IB28063	CEDG198	CCTTCTAAGAACAGTGACATG	21	50.5
11.	IB28064	CEDG225	GAGGAAGTGTTGCAGCACC	19	53.2
	IB28065	CEDG225	GTAGACTCTGCAGAGGGATG	20	53.8
12.	IB28070	CEDG112	GCAATATTCGCATTATTCATTCA	23	48.1
	IB28071	CEDG112	GTGTTTCAAAGCACTATACTTAA	23	48.1
13.	IB28072	DMB-SSR182	TAGAGCCTTCTGGTTTTTCACA	22	51.1
	IB28073	DMB-SSR182	AGGAGGAGGATTTTGATGATGA	22	51.1
14.	IB28076	DMB-SSR217	TCCTTGCCTTATGATTCTGTGA	22	51.1
	IB28077	DMB-SSR217	TTTGGCCACTTCCAAACTTTA	21	48.5
15.	IB28080	LR738A	CGCAAAGAGAGAGAGAGAG	19	51.1
	IB28081	LR738A	CCCCCATCTGAAAGAAAGAG	20	51.8

primers showed scorable polymorphism by presence and absence of bands. Jaccard's similarity coefficient values for SSR primers ranged from 0.35 to 1.00 with an average of 0.85. Based on the dendrogram generated through the UPGMA (Figure 1), genotypes were divided into eight main clusters as presented in the Table 3. Cluster I consisted of 15 genotypes, followed by cluster II with 9 genotypes, cluster VI with seven genotypes, cluster IV and V with six genotypes, cluster III with five genotypes, and cluster VII and VIII with four genotypes each. A minimum similarity coefficient of 0.35

Table 3: List of different clusters formed from 56 genotypes using SSR markers

Cluster number	No. of genotypes	Name of genotypes
Cluster I	15	KKM-3, KK13-16, LGG-572, LGG-450, AKL-103, BGS- 9, IC-92048, AKL-212, AKL-39, AKL-106, AKL-225, AKL-95, AKL-194, AKL-195, AKL-211
Cluster II	9	Harsha, LGG-596, IPM99-125, LGG-583, PM-110, VGG10-010, VGG01-011, VGG04-007, Barimung
Cluster III	5	GG13-6, KM13-44, GG13-7, LGG-585, LGG-573
Cluster IV	6	VBN-1, VBNGG-2, DGG-1, LGG-577, COGG-93, IC-413316
Cluster V	6	TARM-2013, VGG04-005, VGG-112, COGG-920, VGG07-003, VGG10-002
Cluster VI	7	KM13-39, KM13-9, KM13-19, LGG-590, LGG-582, GG13-10, SML-668
Cluster VII	4	IC-436624, IC-436723, Selection-4, IC-436746
Cluster VIII	4	LGG-588, LGG-579, LGG-589, TM-962

was observed between genotypes LGG-585 and LGG-573 and maximum was between KKM-3 and TM-962 (1.00) exhibiting minimum genetic divergence.Absence and presence of bands represented as monomorphic and polymorphic in Table 4. The genotype AKL-211 showed presence of polymorphic band at 100bp for the primer CEDG-092. CEDG-225 primer amplified 250bp polymorphic band in the genotype VGG04-005 (Figure 2). The primer CEDG-275 showed presence of polymorphic band at 250bp and 200bp, for the genotypes KM13-9 and LGG-582 respectively. The genotype KM13-9 showed presence

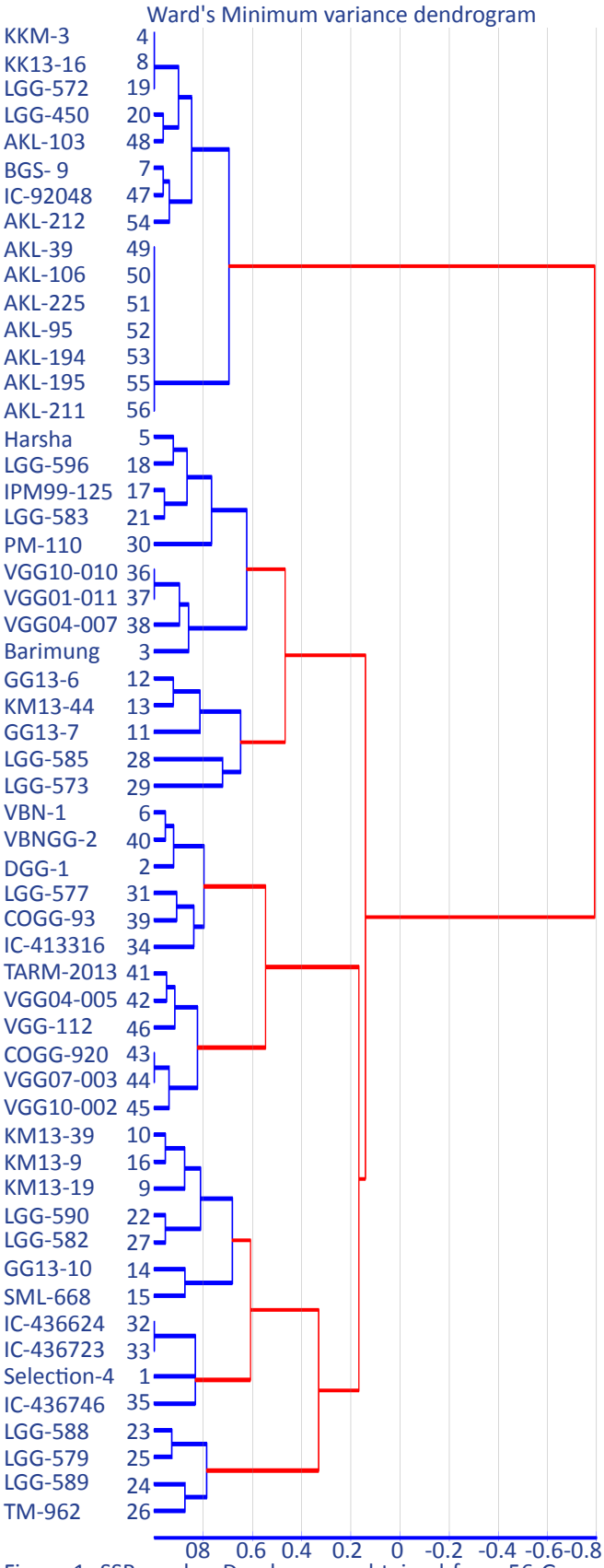


Figure 1: SSR marker Dendrogram obtained from 56 Green gram genotypes

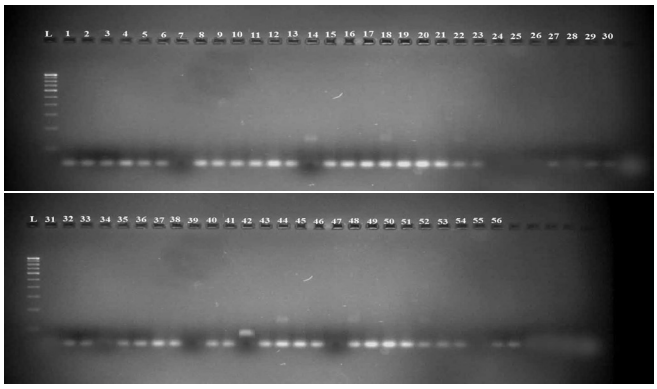
Table 4: List of number of monomorphic and polymorphic green gram genotypes

Primers	NMG	NPG	NPG*
CEDG204	38	18	-
CEDG092	14	41	1 (AKL-211 at 100bp)
CEDG275	50	4	2 (KM13-9 at 250bp) & LGG (582 at 200bp)
CEDG020	33	23	-
CEDG264	40	16	-
CEDG271	44	12	-
CEDG056	44	12	-
CEDG016 & 17	25	31	-
CEDG022	33	23	-
CEDG198	53	3	-
CEDG225	44	11	1 (VGG04-005 at 100bp)
CEDG112	49	7	-
DMB-SSR182	43	12	1 (KM-13 at 300bp)
DMB-SSR217	52	4	-
LR738A	40	16	-

NMG: No. of monomorphic genotypes (Presence of band); NPG: No. of Polymorphic genotypes (Absence of band); NPG*: No. of Polymorphic genotypes (Presence of band at different length)

of 300bp polymorphic band for the primer DMB-SSR-182.

The cluster I was the biggest, comprising 15 genotypes, and was subdivided into I-A and I-B. Sub-cluster I-A comprised 8 genotypes namely; KKM-3, KM13-16, LGG-572, LGG-450, AKL-103, BGS-9, 1C-92048 and AKL-212. Within this sub-cluster, KKM-3, KM13-16 and LGG-572 showed highest similarity value of 1.00 followed by genotypes LGG-450, AKL-103, BGS-9 and 1C-92048 (0.95). The genotypeAKL-212 had lowest similarity value of 0.90. Sub-cluster I-B comprised 7 genotypes; AKL-39, AKL-106, AKL-225, AKL-95, AKL-194, AKL-195 and AKL-211. The cluster II, comprised 9 genotypes, and was subdivided into II-A and II-B. Sub-cluster II-A was comprised of 5 genotypes namely; Harsha, LGG-596, IPM99-125, LGG-583 and PM110. Within this sub-cluster, genotype IPM99-125 and LGG-583 registered similarity value of 0.95 and genotype Harsha and LGG-596 recorded similarity value of 0.90 and the genotype PM110 was somewhat distinct from other genotypes with a similarity value of 0.75. Sub-cluster II-B consisted4 genotypes; VGG10-010, VGG04-011, VGG04-007 and Barimung. Within this sub-cluster, genotype VGG10-010 and VGG04-011 had a similarity value of 1.00. The cluster III, comprised 5



L=Ladder

Lane 1 to 30: Selection-4, DGG-1, Barimung, KKM-3, Harsha, BGS-9, KM13-16, KM13-19, KM13-39, GG13-7, GG13-6, KM13-44, GG13-10, SML-668, KM13-9, IPM99-125, LGG-596, LGG-572, LGG-450, LGG-583, LGG-590, LGG-588, LGG-589, LGG-579, TM-962, LGG-582, LGG-585, LDD-572, PM-110

Lane 31 to 56: LGG-577, IC-436624, IC-436723, IC-413316, IC-436746, VGG10-010, VGG04-011, VGG04-007, COGG-93, VBNGG-2, TARM-2013, VGG04-005, COGG-920, VGG07-003, VGG10-002, VGG-112, IC-92048, AKL-103, AKL-39, AKL-106, AKL-225, AKL-95, AKL-194, AKL-212, AKL-195, AKL-211

Figure 2: SSR gel profiling of Green gram for CEDG225 Primer

genotypes, and was subdivided into III-A and III-B. Sub-cluster III-A comprised 3 genotypes; GG13-6, KM13-44 and GG13-7. Within this sub-cluster, genotype GG13-6 and KM13-44 exhibited similarity value of 0.90 and the genotype GG13-7 was distinct from other genotypes with a similarity value of 0.80. Sub-cluster III-B comprised2 genotypes; LGG-585 and LGG-573 showing similarity value of 0.70.The cluster IV, comprised 6 genotypes, and was subdivided into IV-A and IV-B. Sub-cluster IV-A comprised 3 genotypes; VBN-1, VBNGG-2 and DGG-1. Within this sub-cluster, genotype VBN-1 and VBNGG-2 recorded similarity value of 0.95. Sub-cluster IV-B comprised 3 genotypes; LGG-577, COGG-93 and IC-413316. Within this sub-cluster, genotype LGG-577 and COGG-93 recorded similarity value of 0.90 and the genotypes IC-413316 was distinct from other genotypes with a similarity value of 0.83.The cluster V, comprised 6 genotypes, and was subdivided into V-A and V-B. Sub-cluster V-A comprised 3 genotypes; TARM-2013, VGG04-005 and VGG-112. Within this sub-cluster, genotype TARM-2013 and VGG04-005 registered similarity value of 0.94. Sub-cluster V-B comprised 3 genotypes; COGG-920, VGG07-003 and VGG10-002. Within this sub-cluster, genotype COGG-920 and VGG07-003 exhibited similarity value of 1.00. The cluster VI, consisted of 7 genotypes, and was subdivided into VI-A and VI-B. Sub-cluster VI-A

Comprised of 5 genotypes; KM13-39, KM13-9, KM13-19, LGG-590, LGG-582, GG13-10 and SML-668. Within this sub-cluster, genotype KM13-9 and KM13-19 and genotype LGG-590 and LGG-582 had similarity value of 0.95. Sub-cluster VI-B comprised of 3 genotypes; COGG-920, VGG07-003 and VGG10-002 exhibiting similarity value of 0.85. The cluster VII, comprised 4 genotypes; IC-436624, IC-436723, Selection-4 and IC-436746. The genotype IC-436624 and IC-436723 had similarity value of 1.00. The cluster VIII, comprised of 4 genotypes, and was subdivided into VIII-A and VIII-B. Sub-cluster VIII-A consisted of 2 genotypes; LGG-588 and LGG-579 showing similarity value of 0.93. Sub-cluster VIII-B comprised 2 genotypes; LGG-589 and TM-962 with a similarity value of 0.88.

The SSR markers showed a high level of polymorphism. Similar finding is reported by Singh et al. (2013) and Chattopadhyay et al. (2008) who assessed polymorphism in green gram with combined RAPD, ISSR and SSR markers. Jaccard's genetic similarity values of SSR markers were found in the ranges of 0.35 to 1.00 (average: 0.85) revealing low level of diversity observed through SSR within green gram clusters. Similar finding is reported by Wang et al. (2018). This moderate level of genetic diversity within the self-pollinated members of green gram genotypes from the genus *Vigna* suggests its moderate genetic base, which is possibly due to accumulation of novel gene combinations in response to dynamic pressures of natural selection (Kaur et al., 2016). Comparative studies in *Vigna* species involving RAPD, AFLP, ISSR, and SSR marker systems were successfully used and reported by researchers (Souframanien and Gopalakrishna, 2004; Gillaspie et al., 2005; Dikshit et al., 2007; Muthusamy et al., 2008; Lestari et al., 2014; Zia et al., 2014; Changyou et al., 2017; Wang et al., 2017; Kanimoli et al., 2018); however, the reports on green gram are very limited. The genotypes from different clades show genetic proximity because of genome conservation depending on their phylogenetic relationships and such orthologous or conserved regions play a pivotal role in exploiting the genomic resources (Souframanien and Dhanasekar, 2014). The potentiality of SSRs in green gram diversity analysis has also been reported by many earlier workers (Dikshit et al., 2007; Gupta et al., 2013 ; Saiful et al., 2014; Sanghani et al., 2015; Honglin et al., 2015; Kaur et al., 2018 ; Naveenkumar et al., 2018). Ranade and Gopalakrishna, 2009 revealed that using of more sensitive techniques for DNA fragment size analysis like PAGE or capillary electrophoresis (Dutta et al., 2011) may give better results.

4. Conclusion

The results revealed presence of moderate to high genetic similarity between genotypes, thus indicating lower genetic variability among the green gram genotypes. The UPGMA dendrogram based on SSR results divided the 56 green gram genotypes into eight main clusters. SSR markers are useful in molecular characterization and assessment of green gram diversity which forms basis for crop improvement programmes. The information generated on marker data is of great significance to plant breeders to be utilised in marker assisted breeding programmes.

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