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"ESTIMATION OF GENETIC DIVERSITY OF MUNGBEAN (*VIGNA RADIATA* L.) CULTIVAR USING SSR MOLECULAR MARKER ANALYSIS"

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ABSTRACT

Thirty-two genotypes of Mungbean were screened against MYMV disease during summer 2020-21 to identify tolerant/resistant genotypes for MYMV. Only one genotype showed highly resistant (HR) reaction, four genotypes showed moderately resistant reaction (MR). Only one genotype showed resistant (R) reaction while seven genotypes showed highly susceptible (HS), remaining twelve genotypes showed moderate susceptible reaction (MS). Total nineteen SSR markers were utilized for the validation of resistant genes on thirty-two mungbean genotypes. The study revealed that the average percentage of major allele frequency ranged between 0.000% (DMB SSR-008, DMB SSR-158 and DMB SSR- 059) to 100% (CEDG-133, CEDG-275 and VES-0503). Heterozygosity is defined as the probability that two randomly chosen alleles from the population are different. Out of nineteen markers, seven were polymorphic while, three were found to be monomorphic and remaining nine markers were not produced any bands. The total number of alleles amplified was 26 with a mean value of 2.60.

Keyword: *SSR, polymorphism, genetic diversity, mungbean.*

I. INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek], also known as green gram or mungbean is a self pollinated crop that belongs to the subgenus *Ceratotropis* (2n=2x=22) with a genome size of 579 Mbp. It is one of the important pulse crops of India and it ranks third important legume crop after chickpea and pigeonpea. It is grown on an area of 3.7 million hectares in India with a production of 1.57 million tonnes and productivity of 406.98 Kg/ha which is insufficient for internal consumption and highlights the need for new varieties with higher yields. The crosses between the parents with maximum genetic divergence are generally the most responsive for genetic improvement (Arunachalam 1981). The success of any crop improvement programme depends on available genetic diverse cultivars for understanding the progress made in any breeding programme. The methods of detection and assessment of genetic diversity have extended from analysis of discrete morphological traits to molecular traits. The power of discrimination of DNA based markers is so high that very closely related varieties can be differentiated. Several molecular markers have been used in mungbean including AFLP (Bhat *et al.*, 2005), RAPD (Singh *et al.*, 2013; Lakhanpaul *et al*., 2000), SSR (Gwag *et al*., 2010) and ISSR (Reddy *et al.*, 2008). Among several classes of available DNA markers, Microsatellites or Simple Sequence Repeats (SSR) have become major molecular marker after its emergence as a Polymerase Chain Reaction (PCR)-based genetic marker (Chen *et al*., 2002) because of their reproducibility, multi-allelic nature, co-dominant inheritance and good genetic coverage. SSRs are very popular because of their abundant distribution and hyper variable nature. SSR analysis involves the PCR amplification of the regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat motif (Reddy *et al*., 2008). Although, SSR is based on microsatellite sequences

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but it does not need flanking sequence information, therefore easy to develop. It produces more information in terms of number of loci, polymorphic bands and are highly reproducible in nature.

II. MATERIALS AND METHOD

Plant Material

The experimental materials used in the present investigation consisted of thirty two mungbean genotypes and nineteen SSR molecular markers. The source of accession of different *Vigna* species is given in Table 1 and list of molecular markers given in Table 2. The present investigation was carried out at Dept. of Molecular Biology and Genetic Engineering, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut.

Table 1: List of mungbean genotypes used in this study with characteristics

S. No.	GENOTYPES USED	GRADE	MYMV REACTION
1	Dasapalla green local	5	Moderately Susceptible
2	Saline 7	9	highly Susceptible
3	Jagatsinghpur local-1	5	Moderately Susceptible
4	Gania local	9	Highly Susceptible
5	Dhenkanal local-1	3	Moderately Resistant
6	Bilipara local	5	Moderately Susceptible
7	Saline 2	5	Moderately Susceptible
8	Jharsuguda local-1	4	Moderately Resistant
9	Paralakhemadi local	5	Moderately Susceptible
10	Odogaon brown local	9	Highly Susceptible
11	Kamakhya local	5	Moderately Susceptible
12	Bhapur brown local	7	Susceptible
13	Saline 1	9	Highly Susceptible
14	Mayurbhanja local-1	5	Moderately Susceptible
15	Mahimunda local	8	Highly Susceptible
16	Nayagarh local	6	Moderately Susceptible
17	Nayagarh local-a	5	Moderately Susceptible
18	Hinjili (durabandha)	7	Susceptible
19	Bargarh local-3	7	Susceptible
20	Boudh local-2	5	Susceptible
21	Makarjhola local	3	Moderately Resistant
22	Bargarh local-3	9	Highly Susceptible
23	IPM-02-3	1	Highly Resistant
24	IPM 02-14	3	Moderately Resistant
25	Bargarh local-1	7	Susceptible
26	Kamadev	5	Moderately Susceptible
27	OBGG-52	2	Resistant
28	Sundergarh local	7	Susceptible
29	MH-903	6	Moderately Susceptible
30	ML-1299	5	Moderately Susceptible
31	IPM-430-1	8	Highly Susceptible
32	PM-1623	7	Susceptible

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Isolation of genomic DNA

Extraction of total genomic DNA was carried out as described by Saghai-Maroof et al., (1984) with minor modifications to suit the materials under consideration. The leaves were ground with CTAB extraction buffer, which was already maintained at 60ºC in water bath incubated for 1hour at 65°C with 4-5 times intermittent shaking for complete mixing and formation of emulsion. The DNA pellet was dissolved in appropriate volume of TE buffer and stored at -20°C until further use. Part of the stock DNA samples were diluted with appropriate amount of TE buffer and used for PCR reaction.

Polymerase Chain reaction (PCR) experiment

DNA templates would be amplified using a set of ten SSR primers listed in Table 2. The PCR reactions with the specific primers were carried out as per the following stated cycle regime except for the annealing temperatures, which would be standardized based on temperature gradient PCR. The annealing temperatures of the primers will work out at different temperature i.e.; 50-60ºC. SSR primers were tested for amplification and the ones which gave better resolution was chosen for the present study.

SSR amplification

SSR amplifications were carried out in 20µl volume containing 2.0µl 10X PCR Buffer (with MgCl2), 3.0µl sterile distilled water, 4.0 µl of 1mM each dNTPs Mix, 100ng/µl of 1.0 µl Forward and Reverse Primer each, 8.0µl of genomic DNA and 1U/µl of 1.0 µl Red Taq Polymerase (Genei, Banglore). Amplification conditions were 1 cycle for 4min at 94°C, 35 cycles of 1min at 94°C, 1 min at 35°C and 1min at 72°C and final extension for 7 min at 72°C. Amplified products were electrophorised on 2.4% agarose gel and separated in 1X TAE buffer at 75V. The gels were visualized under UV after staining with Ethidium Bromide and were documented using a Gel documentation and image analysis system.

Table 2. Primers used for this experiment.

Marker	Forward Sequence	Reverse Sequence
CEDG 180	GGTATGGAGCAAAACAATC	GTGCGTGAAGTTGTCTTATC
CEDG 044	TCAGCAACCTTGCATTGCAG	TTTCCCGTCACTCTTCTAGG
CEDG 116	TTGTATCGAAACGACGACGCAGAT	AACATCAACTCCAGTCTCACCAAA
CEDG 275	CACACTTCAAGGAACCTCAAG	GTAGGCAACCTCCATTGAAC
CEDG 006	AATTGCTCTCGAACCAGCTC	GGTGTACAAGTGTGTGCAAG
CEDG 041	GCTGCATCTCTATTCTCTGG	GCCAACTAGCCTAATCAG
VES0503	CGCTTTTGTAGGATTGGAACA	TGAAGGATGAGGGGAAGATG
CEDG 013	CGTTCGAGTTTCTTCGATCG	ACCATCCATCCATTCGCATC
CEDG 284	GGTGCTAACGTTGGAAACTGAG	CACTCCATTCTGAGGATCAATCC
CEDG 198	CAAGGAAGATGGAGAGAATC	CCTTCTAAGAACAGTGACATG
CEDG133	GCATACATAATGTGGTGAGATG	GTCTCGTGCCTTTCACAC
CEDG 014	GCTTGCATCACCCATGATTC	AAGTGATACGGTCTGGTTCC
DMB 158	TGGAAAATTTGCAGCAGTTG	ATTGATGGAGGGCGGAAGTA
VrD1	CAGCTTCTTGTTCTTGCTCC	CGAATGTGCACAGGTGGTGT
CEDG 228	GTCGTTTCCGGAAACTGTTC	GATCCGAACCTCTTTCTGC
CEDG 293	GGATGGTAATGGTAGTTGCTG	CTTCTAGAAACCCGTCACTG
DMB 008	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC
DMB 059	TGCCAGATTTGAGAAGAAAGGT	CATGCATGTGGATAAGAATTCAG
DMB063	TTCCCTGTGTCCTTATATGTCC	GAGGATAGTGAATTTTGAAGGC

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Data analysis

In our molecular analysis using SSR markers, alleles were designated based on fragment size; bands would be scored as diallelic (1 = band present, 0 = band absent) and stored in an excel spreadsheet (Microsoft) file as well as in NEXUS format. The agarose gel electrophoresis data was used to calculate the number of bands produced by each primer, the percent of bands shared by all varieties of mungbean, as well as the percent of bands which would be unique to only one variety. The coefficients of genetic similarity for all pair-wise comparisons were computed using Jaccard's13 coefficient, and then the distance matrix was subjected to cluster analysis by the unweighted paired group method using arithmetic average (UPGMA) to produce a dendrogram.

III. Result & Discussion

Screening results of Mungbean genotypes against YMV

Thirty-two genotypes of Mungbean were screened against MYMV disease during Summer 2020-21 to identify tolerant/resistant genotypes for MYMV. In the present study, only one genotypes showed highly resistant (HR) reaction with a scale of 1 viz., IPM-02-3 (Table 3). So, these genotypes can be used as donors for development of MYMV resistant lines. Out of Thirty-two, four genotypes showed moderately resistant reaction (MR) with a scale of three and four viz., Dhenkanal local-1, Jharsuguda local-1, Makarjhola local and IPM 02-14. Only one genotypes showed resistant (R) reaction with a scale of two viz., OBGG-52. Out of thirty-two genotypes only seven genotypes showed highly susceptible (HS) with scale of eight and nine viz., Saline 7, Gania local, Odogaon brown local, Saline 1, Mahimunda local, Bargarh local-3 and IPM-430-1. Only seven genotypes showed susceptible (S) with scale of seven viz., Bhapur brown local, Hinjili (durabandha), Bargarh local-3, Boudh local-2, Bargarh local-1, Sundergarh local and PM-1623. The remaining twelve genotypes showed moderate susceptible reaction (MS) with scale fiveviz., Dasapalla green local, Jagatsinghpur local-1, Bilipara local, Saline 2, Paralakhemadi local, Kamakhya local, Mayurbhanja local-1, Nayagarh local, Nayagarh local-a, Kamadev, MH-903 and ML-1299. Evaluation of Mungbean genotypes for disease resistance is a crucial step in controlling plant diseases through host plant resistance. Identification of resistant lines is essential in the ambit of integrated disease management. Earlier studies indicated that identification of resistant sources to YMV is reliable option for controlling the viral disease.

SSR based molecular analysis

Genetic markers are used to portray diversity within the cultivated germplasm and to identify grouping of cultivars which are adapted to particular regions (Paterson et al. 1991). DNA-based markers are ubiquitous, repeatable, stable and highly reliable (Virk et al., 2000; Singh et al., 2013). They are less affected by age, physiological condition of samples and environmental factors. Polymorphisms at DNA level can be studied by numerous approaches like polymorphism information content etc (Singh et al., 2013). Among several classes of available DNA markers, ISSRs (Reddy et al., 2008) and SSRs (Gwag et al., 2010) have become major molecular markers for a wide range of studies in plants and animals after their emergence as a Polymerase Chain Reaction markers (Chen et al., 2002) because of their co-dominant segregation and their ability to detect large number of discrete alleles repeatedly, accurately and efficiently (Rekha et al., 2015). The assessment of diversity of mungbean germplasm has been carried out by many researchers in the past (Lakhanpal et al., 2000; Chattopadhyay et al., 2008; Nirmal Bharti, 2016; Datta et al., 2012; Molla et al., 2016; Kaur et al., 2016; Rekha et al., 2015; Sao et al.,2015).

In the present study, total nineteen SSR markers were utilized for the validation of resistant genes on thirty-two mungbean genotypes. The study revealed that the average percentage of major allele frequency ranged between 0.000% (DMB SSR-008, DMB SSR-158 and DMB SSR- 059) to 100% (CEDG-133, CEDG-275 and VES-0503). Heterozygosity is defined as the probability that two randomly chosen alleles from the population are different. It varied from 0.0000 (DMB SSR-008, DMB SSR-158 and DMB SSR- 059) to 0.6367 (CEDG-116) with an average of 0.2865. The homozygosity was found to be high with an average of 0.6080. The genetic diversity for a specific locus/ marker can be evaluated by Polymorphic Information Content (PIC) value. The PIC value ranged between 0.0000

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(DMB SSR-008, DMB SSR-158 and DMB SSR- 059) to 0.5874 (CEDG-116) with a mean value of 0.2528.

Out of nineteen markers, seven were polymorphic while, three were found to be monomorphic and remaining nine markers were not produced any bands. The total number of alleles amplified was 26 with a mean value of 2.60. Marker CEDG-014 and CEDG-116 amplified the four alleles, which is highest number of alleles among the ten amplified markers followed by CEDG-006 and DMB SSR-008 amplified three alleles. It was noted that the linked markers for the gene resistance to Mungbean yellow mosaic virus (MYMV) produced very good information as specific banding patterns on resistance genotypes. Some markers produced banding patterns in both resistance and susceptible genotypes of different allele size. SSR marker CEDG-014 produced 180 and 240 bp banding pattern. It was recorded that this marker differentiates resistant genotypes with 240 bp and susceptible with 180 bp band size. This marker also produced specific allele in resistant genotypes (IPM-02-3, IPM 02-14 and OBGG-52) of 200 bp allele size.

Molecular marker CEDG- 006 also produced 140 bp banding pattern in the susceptible genotypes (Dasapalla green local, Bilipara local, Paralakhemadi local, Saline 1, Nayagarh local, Bargarh local-3, Bargarh local-2, Bargarh local-1, Kamadev, Sundergarh local, ML-1299, IPM-430-1 and PM-1623) and 220 bp allele sizes in the resistant genotypes (Saline 7, Jagatsinghpur local-1, Dhenkanal local-1, Jharsuguda local-1, Odogaon brown local, Kamakhya local, Bhapur brown local, Nayagarh local, Bargarh local-3, Bargarh local-2, Bargarh local-1 and Sundergarh local). In the same time this marker also produced specific banding pattern in genotype Gania local and Saline 2 of the allele size (260 bp). According to Li et al., (2001), polymorphism in the SSR could also be due to changes in the SSR region itself, caused by the expansion or contraction of SSRs, or interruption.

Molecular marker CEDG- 116 also produced 90 bp -100 bp banding pattern in the susceptible genotypes (Saline 7, Gania local, Odogaon brown local, Saline 1, Mahimunda local, Bargarh local-3 and IPM-430-1) and 300 bp allele sizes in the resistant genotypes (Dhenkanal local-1, Jharsuguda local-1, Makarjhola local, IPM-02-3, IPM 02-14, OBGG-52). In the same time this marker also produced specific banding pattern in genotype Bilipara local of the allele size (120 bp).

SSR marker CEDG-145 also produced two different allelic banding patterns of the size 100 bp and 130 bp. This marker produced 130 bp banding pattern in resistant genotypes (Jharsuguda local-1, Makarjhola local, IPM-02-3, IPM 02-14 and OBGG-52) and 100 bp (Dasapalla green local, Saline 7, Jagatsinghpur local-1, Gania local, Bilipara local, Saline 2, Paralakhemadi local, Odogaon brown local, Kamakhya local, Bhapur brown local, Saline 1, Mayurbhanja local-1, Mahimunda local, Nayagarh local, Nayagarh local-a, Hinjili (durabandha), Bargarh local-3, Boudh local-2, Bargarh local-3, Bargarh local-1, Sundergarh local, MH-903, ML-1299, IPM-430-1 and PM-1623) in the susceptible genotypes of another origin.

In this study it is concluded that SSR markers CEDG- 006, CEDG- 116 and CEDG-145 found to be tightly linked with resistant gene/genes of different origin. This is also concluded that by the way this marker technology we can easily be identify the different genetic origin of resistant genes against MYMV. These three SSR markers may be utilised to differentiate the resistant and susceptible genotypes in the group of mungbean population. This technology may also be very fruitful in the Mungbean improvement programme for development/ identification of resistant genotypes in the segregating generations. These markers will also be utilized in the Marker Assisted Selection programme in the development of resistant Mungbean genotypes against MYMV.

Table 3. DNA polymorphic result by markers utilized on mungbean

S. No.	SSR Primer code	No. of Alleles	No. of polymorphic Loci	% Polymorphism	H (Heterozygosity)/ Gene diversity	H' (Homozygosity)	PIC (Polymorphic information content)
1	**CEDG-014**	4	3	75.00	0.5879	0.4121	0.5439
2	**CEDG-006**	3	2	66.67	0.4178	0.5822	0.3696
3	**CEDG-116**	4	2	50.00	0.6367	0.3457	0.5874
4	**CEDG-145**	2	1	50.00	0.375	0.6250	0.3047

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5	**CEDG-133**	2	2	100.0	0.2188	0.7812	0.1948
6	**CEDG-275**	2	2	100.0	0.2706	0.3457	0.234
7	**DMB SSR-008**	3	1	33.33	0.0000	1.0000	0.0000
8	**DMB SSR-158**	2	1	50.00	0.0000	0.3457	0.0000
9	**VES-0503**	2	2	100.0	0.3578	0.6422	0.2938
10	**DMB SSR- 059**	2	1	50.00	0.0000	1.0000	0.0000
Total		**26**	**17**	675.0	2.8646	6.0798	2.5282
Mean		**2.6**	**1.7**	**67.50**	**0.2865**	**0.608**	**0.2528**
Min		**2.0**	**1.0**	**33.33**	**0.0000**	**0.3457**	**0.0000**
Max		**4.0**	**3.0**	**100.0**	**0.6367**	**1.0000**	**0.5874**

Fig 1: Electrophoretic banding pattern of MYMV, linked SSR markers amplified with thirty-two Mungbean genotypes.

The data collected from SSRs were combined for UPGMA cluster analysis. The combined cluster analysis of thirty two genotypes grouped into two major clusters showing correlation between phylogenetic divergence and geographical distribution. It is difficult to accurately identify varieties by their morphological characteristics; however, SSR markers used in the study detected a high level of polymorphism as well as monomorphism and were successful in distinguishing accessions. Group 1 was the most diverse among all consisting of 30 genotypes viz., Dasapalla green local, Saline 7, Jagatsinghpur local-1, Gania local, Dhenkanal local-1, Saline 2, Jharsuguda local-1, Paralakhemadi local, Odogaon brown local, Kamakhya local, Bhapur brown local, Mayurbhanja local-1, Mahimunda local, Nayagarh local, Nayagarh local-a, Hinjili (durabandha), Bargarh local-3, Boudh local-2, Makarjhola local, Bargarh local-3, IPM-

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02-3, IPM 02-14, Bargarh local-1, Kamadev, OBGG-52, Sundergarh local, MH-903, ML-1299, IPM-430-1, PM-1623. The second cluster includes two genotypes each i.e., Saline 1and Bilipara local. It is concluded from the study that assessment level of diversity and species relationship among genotypes of Vigna species have a great significance for designing breeding strategies which allows selection of the desired accessions for crossing. Many of the accessions included in the study are morphologically similar but lack the pedigree information. Thus, identification of the genetic distance among accessions will be important to maximize their use in breeding strategies.

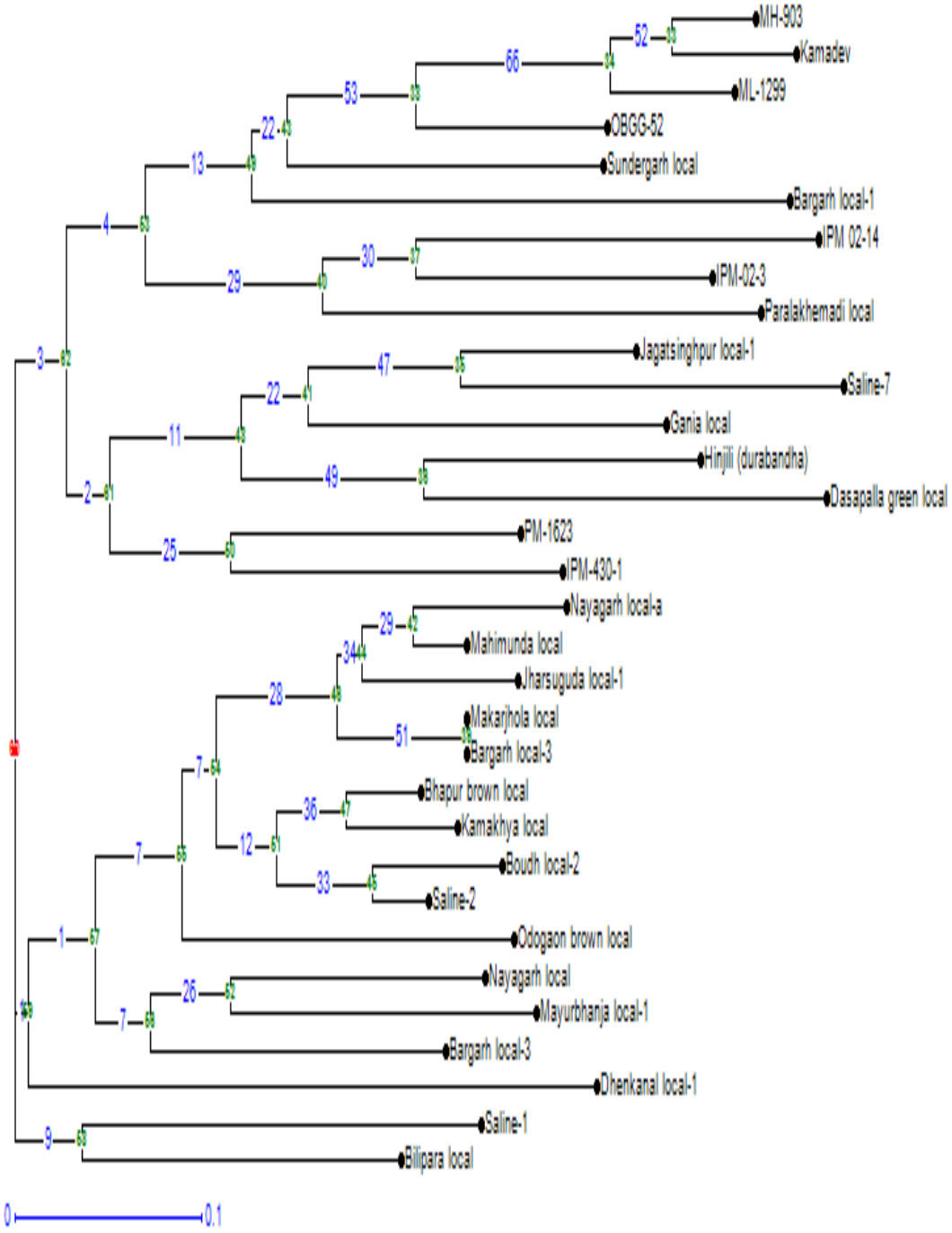

Fig 2: Dendogram generated using UPGMA analysis showing relationships among mungbean genotypes obtained by SSR data.

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