Mangal et al., Vegetos 2016, 29:2
http://dx.doi.org/10.5958/2229-4473.2016.00016.1

Vegetos- An International Journal of Plant Research

Research Article A SCITECHNOL JOURNAL

Characterization of Cultivated and Wild Genotypes of Brinjal (*Solanum melongena* L.) and Confirmation of Hybridity using Microsatellite Markers

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Abstract

Knowledge of brinjal genome organization is rather limited compared to other solanaceous crops, especially tomato and potato. There is a strong brinjal breeding programme at Indian Agricultural Research Institute (IARI) since its inception which led to release of several varieties and hybrids in this crop. In the present investigation, the diversity of twenty genotypes of brinjal representing nine open pollinated varieties, four hybrids, seven parents of hybrids (one parent common for two hybrids) and three wild relatives namely *S. integrifolium, S. incanum* and *S. aethiopicum* has been analyzed using 47 microsatellite loci distributed uniformly throughout the genome. These 47 simple sequence repeat (SSR) loci amplified a total of 135 alleles among the 23 genotypes with one to seven alleles per loci. The average number of alleles per loci was found to be 2.87. The highest polymorphism information content (PIC value) was observed to be 0.75 for the marker emf11F24 located on linkage group 11. Our study also utilized the SSR marker technique for confirming the hybridity of four IARI brinjal hybrids. Four markers viz. emg11104, eme08D09, ecm009, and emf11F24 confirmed the hybridity of three hybrids namely, PH-5, PH-6 and DBHL-20.

Keywords

Brinjal; Genetic diversity; Microsatellite markers; Hybridity

Introduction

Brinjal (*Solanum melongena*L., 2n=2x= 24.), also known as aubergine or eggplant, belongs to the family *Solanaceae*, but unlike most of the solanaceous crop species, it is endemic to the old world. Its progenitor is presumed to have been the African species *S. incanum* [1], but its centre of domestication and genetic diversity lies in the Indo-Burma region, where it has been grown for at least 1,500 years [2]. The production of brinjal is highly concentrated, in five countries, namely China, India, Egypt, Iran and Turkey with 90 percent of output coming from them [3]. India is the second (25%) largest producer of brinjal in the world (Annonymous, 2014). Eggplant is the third most important solanaceous crop worldwide after potato and tomato [3].

The evaluation of genetic resources is crucial for breeders to produce new cultivars or to further improve the existing ones, according to changing consumers preferences or challenges during growth conditions [4]. The genetic diversity in breeding materials can be assessed by biochemical, and horticultural traits, pedigree record analysis and DNA fingerprinting with molecular markers [5,6,7]. SSR or microsatellites are tandemly repeated short nucleotide units of one to five nucleotides and their value for genetic analysis lies in their multi-allelism, codominant inheritance, relative abundance, genome coverage and suitability for high throughput PCR-based platforms [8]. A number of workers have developed SSRs for eggplant, including genomic SSRs by Nunome et al. [9], Barchi et al. [10] and genic SSRs by Stagel et al. [11] which they tested primarily on eggplant cultivars. The current study was undertaken to utilize genomic SSRs for determining the diversity among twenty three genotypes vis *a vis* to evaluate the use of markers to confirm the hybridity of four IARI hybrids.

Material and Methods

Plant material

The germplasm used in this study consisted of 23 genotypes representing nine open pollinated varieties, four hybrids, seven parents of hybrids (one parent common for two hybrids) and three wild relatives. All these 23 genotypes were maintained through selfing at the research farm of Division of Vegetable Science, IARI, New Delhi, India. The morphological features of the different genotypes are mentioned in Table 1. Young, healthy and uninfected leaves from each genotype were collected and brought to the laboratory in liquid nitrogen (-196°C) where they were kept in deep freezers at -80°C for further use.

DNA extraction

Genomic DNA was extracted from young leaf tissue following the C-TAB procedure [12]. DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide (Sigma Aldrich Chemical Pvt. Ltd, Bangalore, India) and also by using a NanoDrop® ND-1000 spectrophotometer.

Selection of the primer

A total of 47 SSR primers were selected from already published genomic SSRs by Nunome et al. [9] and were custom synthesized (SBS Genetech Co.Ltd., Beijing, China). The markers were selected in such a way to get an approximate coverage of brinjal genome and at least four markers were selected per chromosome (except Chromosome 1 and 10). The details of the primers mentioned in Table 2.

Polymerase chain reaction (PCR) analysis

All the SSR markers were amplified by PCR in 15 µl volumes with 50ng genomic DNA, 1.0 U *Taq* DNA polymerase (Hi media Laboratories, Mumbai, India), 1.0 µM of each primer, 0.6 µl of 10 mM dNTP mix (Hi media Laboratories, Mumbai, India), and 1.5 µl of 10X PCR buffer having 17.5 mM $MgCl_2$ (Hi media Laboratories, Mumbai, India). All the primers were amplified using touchdown PCR in an Eppendorf Mastercycler. Amplification conditions used were, one cycle of 94°C for 3 min; 10 cycles of 94°C for 0.5 min, 65–55°C decreasing by 1°C per cycle for 1 min, and 72°C for 1 min; 30 cycles of 94°C for 0.5 min, 55°C for 1 min, and 72°C for 1 min; and

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Received: March 03, 2016 **Accepted:** May 24, 2016 **Published:** May 30, 2016

Table 1: Characteristic traits of various genotypes used in the study.

S. No.	Name of genotype	Characters	Type of genotype
1	Pusa Bhairav	Purple, long fruit	Open pollinated variety
2	Pusa Kranti	Fruits are oblong, dark purple in colour.	Open pollinated variety
3	G-190	Green, round fruit	Open pollinated variety
4	Pusa Bindu	Purple, small, round fruit	Open pollinated variety
5	Pusa Purple Cluster (PPCl)	Fruits are small, dark purple in colour and borne in clusters.	Open pollinated variety
6	Selection 195	White, round, small fruits in clusters	Open pollinated variety
7	Pusa Purple Long (PPL)	Fruits are long, glossy, light purple in colour, smooth and tender	Open pollinated variety
8	Pusa Ankur	Fruits are oval-round, small-dark purple.	Open pollinated variety
9	Pusa Anupam	Purple long fruit	Open pollinated variety
10	PH-5	Fruits are medium sized, long, dark purple.	Hybrid
11	NDB25	Thick skin, purple long fruit	Female parent of PH-5
12	129-5	Long fruit, Purple	Male parent of PH-5
13	PH-6	Fruits round medium sized, glossy, purple, partially pigmented peduncle	Hybrid
14	BR112	Light purple oblong fruits with thin skin	Female parent of PH-6
15	91-2	Dark purple round fruit	Male parent of PH-6 and female parent of PH-9
16	PH-9	Fruit are oval round, glossy attractive, dark purple, peduncle partially pigmented	Hybrid
17	190-10-12	Dark purple oblong fruit	Male parent of PH-9
18	DBHL-20	Long purple fruits in clusters	Hybrid
19	Pusa Shymla	Dark purple glossy, long fruit.	Female parent of DBHL-20
20	DBL02	Light purple long fruit	Male parent of DBHL-20
21	S. integrifolium	Fruits are small, green and with ridges	Wild relative of S. melongena
22	S. incanum	Oval, light green fruits	Wild relative of S. melongena
23	S. aethiopicum	Very small, green fruits	Wild relative of S. melongena

a final cycle of 72°C for 5 min [9]. Amplified products were resolved on 3.0% agarose gels with Tris/Acetate /EDTA (TAE) stained with ethidium bromide, at a constant voltage of 60 V for 3 h using a horizontal gel electrophoresis system (BioRad, USA) and visualized and photographed under UV light in a gel documentation unit (Alpha imager, Cell bioscinces, Santa Clara, CA). On the agarose gel, twennty three genotypes of brinjal were loaded in the same order as they are mentioned in the Table 1.

Data analysis

The amplification products were scored for each genotypes based on presence and absence of band using binary code one and zero for the presence and absence of band, respectively. Molecular size (bp) of amplified DNA fragment was determined by comparison with 50 bp ladder (BR Biochem, Bioscience, Life Sciences) using image acquisition analysis software of alpha imager gel documentation system. The binary matrix was used to estimate Jaccard's genetic similarity coefficients for SSR. Statistical analysis was performed using NTSYS-pc 2.02 analytical package with cluster analysis using the SHAN module [13].

For single-locus evaluations of the SSR data, all DNA fragments were scored as allele sizes at each locus. The polymorphism information content (PIC) of each microsatellite locus, which combines the number of alleles and their frequency distribution within a population [14] and serves as a measure of allele diversity at a locus, was evaluated by applying the following equation, as suggested by Anderson [15].

$$PIC = 1-\sum_{i=1} P_i^2$$

where Pi is the frequency of the i-th allele among a total of n alleles [16].

Results

Allelic variations

A total of 47 SSR markers were used for diversity analysis of the 23 brinjal genotypes. Out of which, 13 markers were found to be monomorphic across all the genotypes selected and hence were unable to differentiate between these genotypes. Out of the remaining 34 markers, 21 showed polymorphism only in the wild genotypes of brinjal viz. *S. integrifolium, S. incanum and S. aethiopicum* and not in the cultivated brinjal genotypes, whereas 13 markers were found to be highly polymorphic across all the genotypes. The genotypes used in the study comprised open pollinated varieties and hybrids and some of the OP genotypes are also parents of IARI released brinjal hybrids included in the study. The potential four markers out of the 13 highly polymorphic markers which differentiated the hybrids from the parents were again used on separate sets of each hybrid and its parents and results of that experiments are presented in the section on confirmation of hybridity.

These 47 SSRs amplified a total of 135 alleles (Table 2) and the number of alleles per loci ranged from one to seven. Maximum seven alleles were observed for two markers viz. ecm001 located on linkage group 3 and emb01H07 located on linkage group 5. Furthermore, when just the cultivated brinjal genotypes were considered, it was found that allele number ranged from one to five. Maximum number of alleles in the cultivated genotypes of *S. melongena* were observed to be five for markers ecm009 (located on linkage group 8) and emf 11F24 (located on linkage group 11). A total of 82 alleles were identified in the twenty *S. melongena* genotypes (Table 2), which

Table 2: Allelic variations in 47 SSR loci.

S. No	Marker	No of alleles in total 23 genotypes	No of alleles in 20 cultivated genotypes	Allele Sizes observed in Cultivated Genotypes	Allele Sizes observed in wild genotypes	Product size range (bp)	PIC in All the 23 Genotypes	PIC in 20 *S. melongenea* Genotypes	Polymorphism status
1	emg11P03	1	1	195		195	0	0	M
2	em245 [b]	3	1	148	195	128-158	0.17	0	P (WR)
3	emg11B20	2	1	217	128, 158	185-217	0.10	0	P (WR)
4	emf01B07	1	1	227	185, 217	227	0	0	M
5	em4_1 [a]	4	2	120, 158	227	108-158	0.56	0.5	P (WR)
6	eme03H04	5	3	112, 135, 181	108, 120, 143	110-181	0.58	0.52	P (All)
7	ecm031	6	3	193, 258, 237	110, 112, 135, 140	193-258	0.60	0.52	P (All)
8	em256 [b]	2	1	250	193, 210, 231, 237, 243, 258	241-250	0.08	0	P (WR)
9	ecm001	7	4	244, 228, 209, 203	241, 250	189-244	0.74	0.69	P (All)
10	ecm090	2	2	145,155	189, 200, 222	145-155	0.5	0.5	M
11	em155 [a]	4	3	211, 250, 264	145,155	204-264	0.49	0.43	P (ALL)
12	emd16C09	2	1	283	204, 250, 264	283-336	0.19	0	P (WR)
13	em119 [a]	3	2	159, 205	283, 336	154-205	0.54	0.5	P (WR)
14	eme02B08	4	1	300	154, 169, 205	290-370	0.23	0	P (WR)
15	emf01A03	3	1	250	290, 300, 350, 370	200-250	0.16	0	kP (WR)
16	emb01H07	7	4	333,490, 296, 400	200, 240, 250	275-490	0.75	0.71	P (All)
17	em117 [a]	3	1	142	275, 289, 400, 104, 127, 142	104-142	0.16	0	P (WR)
18	emh11I06	4	1	336	258, 290, 294, 336	258-336	0.24	0	P (WR)
19	ecm070	2	1	270	260, 270	260-270	0.08	0	P (WR)
20	emf01K21	1	1	162	162	162	0	0	M
21	em134 [a]	4	1	178	158, 170, 178, 182	158-182	0.23	0	P (WR)
22	emf21N03	3	2	184, 194	178, Not amplified in two genotypes	178-194	0.40	0.41	P (All)
23	emg11D22	3	2	288, 300	288, 300, 315	288-315	0.52	0.5	P (WR)
24	emg11I04	4	2	285, 312	280, 290	280-312	0.58	0.46	P(All, hybridity)
25	emf21I04	3	2	193, 197	157, 193	157-197	0.57	0.50	P (WR)
26	eme08D09	5	2	210, 215, 238, 250, 255	210, 255	210-255	070	0.69	P (All- hybridity)
27	ecm023	2	1	250	230, 250	230-250	0.08	0	P (WR)
28	em114 [a]	2	1	211	185, 211	185-211	0.08	0.24	P (WR)
29	emg01L21	2	2	243, 300	243, 300	243-300	0.5	0.5	M
30	em120 [a]	2	1	171	156, 171	156-171	0.08	0	P (WR)
31	ecm009	5	5	185, 195, 210, 236, 246	195, 236	185-246	0.69	0.67 0	P (All- hybridity)
32	ecm032	2	1	285	285,310	285-310	0.08		P (WR)
33	emd22G11	3	3	150, 183, 194	194, Not amplified in two genotypes	150-194	0.61	0.60	P (all)
34		emf11M07	1	1	178	178	0	0	M
35	emg01D17	3	2	417, 428	410, 417	410-428	0.41	0.33	P (All)
36	emi02F16	3	1	191	156, 170, 191	156-191	0.16	0.22	P (WR)
37	eme09E09	3	1	150	137, 150, 187	137-187			P (WR)
38	em140 [a]	3	3	214, 286, 319	214, 329	214-329	0.55	54	P(All)
39	emf11F24	6	5	172, 181, 185, 191, 197	110, 181	110-197	0.75	0.73	P (all-hybridity)

40	eme03F04	2	1	326	212, 326	212-326	0.23	0	P (WR)
41	emb01D19	1	1	172	172	172	0	0	M
42	emi02E15	1	1	321	321	321	0	0	M
43	emb01E03	2	2	337	368	337-368	0.5	0.5	M
44	emf21A12	1	1	189	189	189	0	0	M
45	emg21I10	1	1	227	227	227	0	0	M
46	emi02E20	1	1	232	232	232	0	0	M
47	emf01A06	1	1	256	256	256	0	0	M
Total		**135**	**82**						
Average		**2.87**	**1.74**				**0.30**	**0.24**	

P (WR): Polymorphism observed only in wild relatives.
P (All): Polymorphism observed in all the genotypes
P (all- hybridity): Polymorphism observed in all the genotypes and marker also confirmed hybridity.

represent nine open pollinated varieties, four hybrids, seven parents of hybrids .

The highest value of PIC was observed to be 0.75 across all the 23 genotypes and 0.73 among the 20 cultivated genotypes for marker emf11F24 located on linkage group 11 (Table 2). Marker emb01H07 located on linkage group 5 had the same PIC value (0.75) when the whole set of 23 genotypes was considered, however, it had a slightly lower (0.71) PIC value among the cultivated genotypes.

Cluster analysis

The dendrogram graphically represent the information regarding genotypes which are grouped together at various levels of (dis) similarity (figure 1). Jaccard's similarity coefficient was used to generate this dendrogram using UPGMA clustering option of NTSYS pc 2.02 software package [13]. In this dendrogram, twenty three genotypes taken in this study have been shown to be separated in such a way that all the cultivated genotypes were clustered together in one major group while the three wild relatives were outliers. The scale of the dendrogram constructed from the data generated was between 0.27 and 0.97 with a mean value of 0.62.

Confirmation of hybridity

Attempts were made to identify SSR markers which could be used to confirm the hybridity of four hybrids (PH-5, PH-6, PH-9 and DBHL-20) used in the present study. Out of the 47 SSR markers used, four primers viz. emg11104, eme08D09, ecm009 and emf11F24 were able to confirm the hybridity of three of the four hybrids used in the study, viz. PH-5, PH-6 and DBHL-20, whereas none of the markers used could confirm the hybridity of PH-9. Marker emf11F24which is located on Linkage group-11, although confirmed the hybridity of three hybrids, PH-5, PH-6 and DBHL-20, however it could not differentiate between these three hybrids as all the these had same two bands of 197 bp and 181bp while their respective female parents possessed only single band of 197 bp and male parents had a single band of 181bp (Figure 2). In addition, same type of bands could be observed in other genotypes not involved in hybrid production eg. Pusa Kranti, G-190, Pusa Purple long had a single, female parent specific band of 197 bp and Pusa Bhirav, Pusa Bindu, Pusa Purple Cluster, Pusa Ankur, Pusa Anupam and Selection 195 had a single, male parent specific band of 181bp. All the three wild relatives also had single band of 181 bp (Figure 2).

The marker ecm009 (linkage group-8) was found to be polymorphic across all the 23 genotypes studied, more over this marker could also confirm the hybridity of DBHL-20 as this hybrid had two alleles at 221bp and 247 bp, respectively, and its female parent Pusa Shyamla was homozygous for allele at 221 bp and the male parent DBL-02 had homozygous allele at 247 bp (Figure 3). None of the other hybrid used in the study was heterozygous for thismarkers, suggesting thereby that this marker can be used specifically for confirming the hybridity of hybrid DBHL-20. In both Figure 2 and 3, although several bands are visible, however only those bands were considered for confirmation of hybridity where alternate alleles are present in male and female parents. The marker emg 11104 which is located on linkage group-7 confirmed the hybridity of two hybrids (PH-5 and DBHL-20), although it could not differentiate between these hybrids as both of them had same two bands of 285bp and 312 bp while their respective female parents possessed single band of 312 bp and male parents had a single band of 285bp (Figure 4). Similar observation was recorded in marker eme08D09 located on linkage group 7, it also confirmed the hybridity of PH-5 and DBHL-20 and both the hybrids possessed two alleles of 210 bp and 255 bp, respectively, while parents had single allele of alternate type (Figure 5).

Discussion

The assessment of genetic dissimilarity or similarity is not only important for crop improvement efforts but also for efficient management and protection of germplasm resources as well as for breeding purposes, to predict the ability to combine or to rapidly verify the breeding material. The availability of diversity is crucial for genetic improvement and elite gene exploitation, such as genes for tolerance resistance to biotic and abiotic stresses. This information can be generated through DNA fingerprinting approaches capable of exhibiting large number of loci for extensive variability. For breeding, it is necessary to detect polymorphisms among cultivars and lines. However, in solanaceous plants, a low frequency of polymorphism among cultivars and intraspecific lines has been reported [17,18,19], probably due to its autogamous nature.

During recent past, SSR also known as microsatellites have become the most popular source of genetic markers owing to their high reproducibility, multi-allelic nature, co-dominant inheritance, abundance, and wide genome coverage. SSR markers have been successfully adopted to analyze genetic diversity in a variety of different plant species [20,21,22]. Several studies have been performed to determine the genetic diversity of eggplant using random amplified polymorphic DNA (RAPD) [22], RAPD and AFLP [23], simple sequence repeats [33,24,25], RFLP [26] and STMS [27].

Genomic SSRs selected on the basis of high polymorphism information content [9] in our present investigation successfully helped to discriminate 23 genotypes comprising open pollinated varieties, hybrids, parents of hybrids and 3 wild relatives. Successful

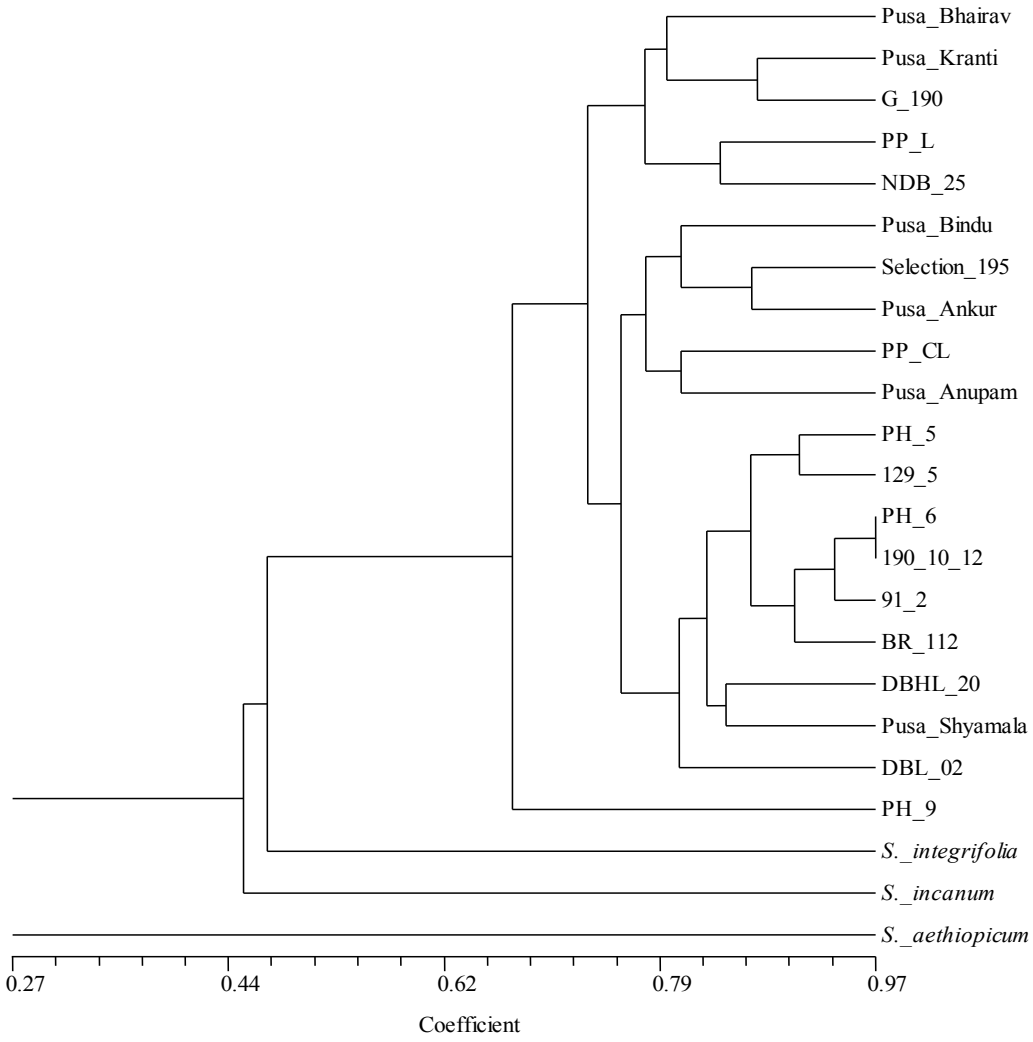

Figure 1: UPGMA dendrogram analysis of the 23 genotype set, based on 47 SSR markers.

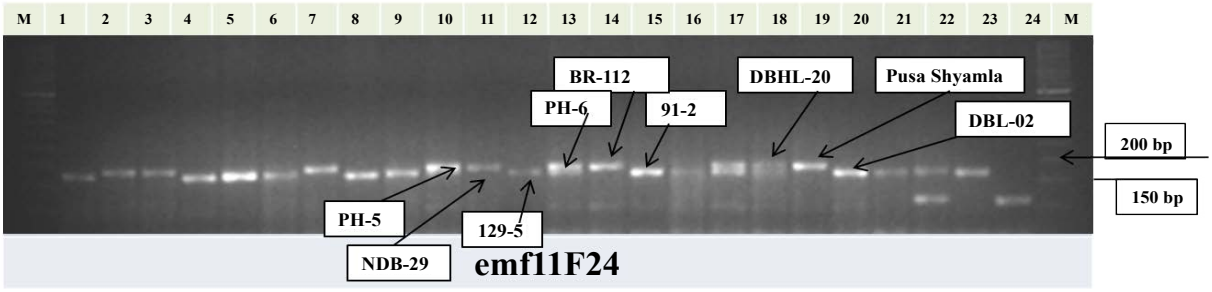

Figure 2: Polymorphism observed in 23 brinjal genotypes using marker emf11F24. Arrows indicate that hybrids (PH-5, PH-6 and DBHL-20) have two bands while their respective female and male parents have alternate alleles.

amplifications of expected sizes were obtained. The genetic similarity estimated according to SSR data scaled between 0.24 and 1, suggesting discriminatory potential of SSR markers among plants of close or distant genetic backgrounds. Dendrogram prepared using UPGMA clustering based on Jaccard's similarity coefficient revealed that the wild relative of brinjal (*S. aethiopicum* or scarlet eggplant) formed a separate cluster while the cultivated genotypes along with two wild relatives (*S. incanum* and *S. integrifolium*) clustered together. This indicated that *S. aethiopicum* might not have contributed in the ancestry of cultivated brinjal genotypes used in the study, while *S. incanum* and *S. integrifolium* might have close relation to cultivated *S. melongena*. Several other molecular genetic studies of eggplant

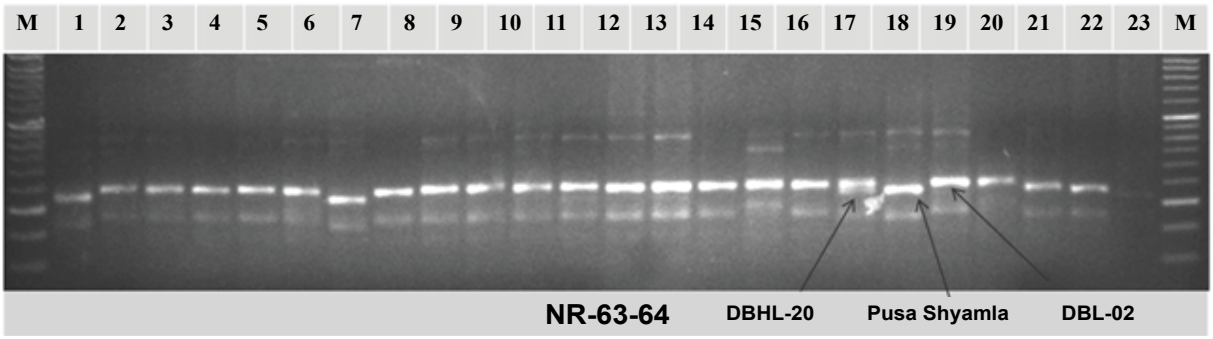

Figure 3: Polymorphism observed in 23 brinjal genotypes using marker ecm009. Hybrid DBHL-20 has two bands while the respective female and male parents have alternate alleles.

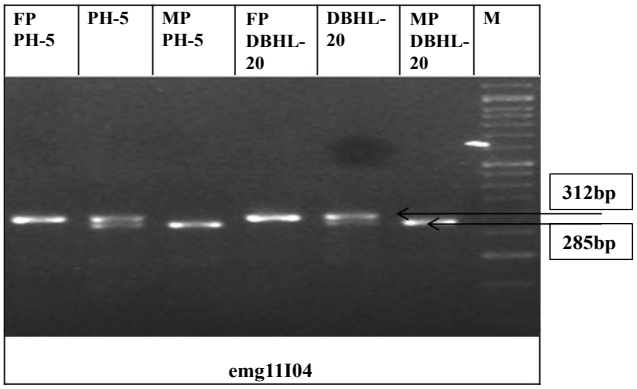

Figure 4: Banding pattern in Hybrids PH-5 and DBHL-20 and their parental lines using marker emg 11I04.

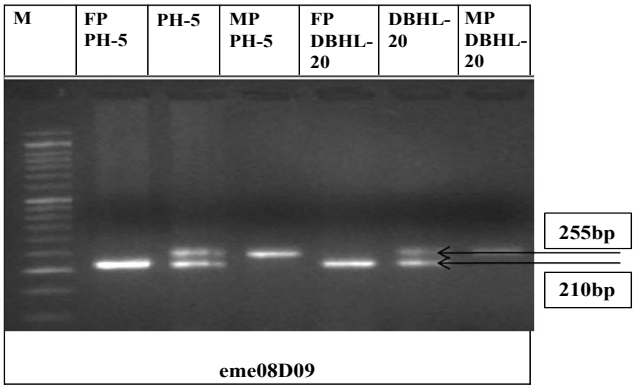

Figure 5: Banding pattern in Hybrids PH-5 and DBHL-20 and their parental lines using markr eme08D09. **FP- female parent, MP-male parent.*

have also shown that *S. incanum* is a putative progenitor of brinjal and its genetic relationship with *S. melongena* has been established using various marker systems including chloroplast DNA, isozymes, RAPD, and AFLP [28,29]. Similar observation regarding genetic distance between the cultivated *S. melongena* and *S. aethiopicum* which is mainly cultivated in Africa for its fruits and leaves was made by Tumbilen et al. [29]. In general, higher diversity was observed when cultivated and wild genotypes were taken together as 21 markers had shown polymorphism only in the wild relatives and not in the cultivated genotypes suggesting the utility of wild relatives as a source of unique genes with strong potential for involvement in breeding programmed. On an average, 2.88 alleles per marker were detected in these 23 genotypes, while 1.74 alleles per marker were detected in the twenty cultivated genotypes and these results are in agreement with those obtained in previous studies of microsatellites in eggplant [25]. The number of alleles per locus was found to vary between 1 and 9 (mean 3.1) [30] in the study by Stagel et al. [25] which was a bit higher than what was obtained in the present study probably because they used more number of wild genotypes than used in this study [31]. All the primers used in the present study were taken from those developed by Nunome et al. [9] from SSR-enriched genomic library of eggplant and amplicon [32] size range of the alleles in general was in agreement with those reported by Nunome et al. [9].The difference is that Nunome et al. [9] mentioned a single product size [33,34], while the present study is reporting the range of amplicon sizes as well as actual amplicon sizes observed in cultivated and total genotypes as multiple alleles were observed in most of the markers [35,36,37]. Further, the only exception we have observed in the amplicon size is with respect to marker emg 01D17 in which Nunome et al. [9] have reported an amplicon of 280 bp while we observed three alleles of 410, 417, and 428 bp. In addition this marker was polymorphic among all the genotypes tested. The difference presumably is because the amplicon included an intron [38,39,40].

The purity of hybrid is one of the most important characteristics of good quality seed [41,42]. Characterization and identification of cultivars are crucial to varietal improvement, release and in seed production programme. It is mandatory to maintain the genetic purity of hybrid seed for the successful crop production. Farmers can harness the full potential of any hybrid only when they get genetically pure seeds of the hybrid. Hence, ensuring the genetic purity of certified seeds of brinjal hybrids is mandatory in India, which is done through field grow out test (GOT) based on the morphological characters of plants grown to maturity [43]. GOT being land and labor intensive, time consuming and influenced by the environment [27], unequivocal characteristic pattern of hybrids can be obtained using DNA markers [28,44]. The present study utilized the SSR marker technique for confirming the hybridity of four brinjal hybrids along with their parental lines. Four SSR primers viz. emg11104, eme08D09, ecm009 and emf11F24 were able to confirm the hybridity of three hybrids (PH-5, PH-6 and DBHL-20) [45]. Further it was also observed that the marker ecm009 (linkage group-8) can be used specifically for confirming the hybridity of DBHL-20. However, it was also felt that more markers need to be screened to provide an unequivocal characteristic banding pattern of hybrids used in the

study [46]. Alternatively, multiplexing of two or more polymorphic primers for these hybrids could provide characteristic banding pattern for each hybrid as has been observed by Kumar et al. [24]. SSR technique has been successfully applied to distinguish and identify the hybrids from their parental lines in a number of crops like Brassica [47] and sunflower [35].

Thus, given the relative dearth of eggplant specific markers as compared to those available for other solanaceous species, the information generated in the present study regarding SSR markers of brinjal would be valuable for eggplant breeders and germplasm conservationists.

Acknowledgements

The work was supported by IARI institutional project on "Molecular breeding of Vegetable crops".

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