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SSR markers based DNA fingerprinting and it's utility in testing purity of eggplant hybrid seeds

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RESEARCH ARTICLE

Abstract

Conventionally, the genetic identity and purity of seeds is assessed by field plot test. However, field plot test method is time consuming, requires large area of land and involves skilled personnel often making subjective decisions. These limitations and the environmental dependency of the entire procedure can be overcome effectively by employing DNA fingerprinting technology. Therefore, the present study was undertaken to identify simple sequence repeat (SSR) markers that could be used to test the genetic identity and purity of six brinjal hybrids and their respective parental lines based on generated DNA fingerprint. In the present study, four markers were found to be polymorphic between parental lines of four respective hybrids (viz. PH-5, PH-9, NDBHL-20 and Kashi Komal) and were found to be suitable for ensuring the genetic identity and purity of these hybrids. Among the identified markers, a set of three markers (emg01B17, emd05F05 and CSM31) could be used for ensuring the identity of the hybrids. Utility of SSR marker based DNA fingerprinting in ensuring the seed purity has been further demonstrated in comparison with that of field plot test.

Keywords: field plot test, genetic identity, genetic purity

1. Introduction

Eggplant (*Solanum melongena* L.) is one of the common vegetable crops of India, which attributes 8.23% of the total vegetable production (Government of India, 2014). In order to meet the ever-growing market demand and to increase productivity, the hybrid technology has been exploited in this crop since 1970's. At present many hybrids of public and private industries are ruling the market and their number is expected to increase due to ever changing breeding objectives that are being evolved to meet the current and future demands of the producers and consumers. The new hybrids attain acceptance when the farmer gets genetically pure seeds. Apart from this, the crop raised from such pure seeds will be uniform that gives uniform, yielding uniform harvested product, which is a prerequisite for farm mechanisation and also to compete in open markets of the World Trade Organization regime.

As hybrids contribute greatly in crop production through increased yields, reduced application of pesticides due to inbuilt pest resistance and enhanced keeping quality of fruits, their price as well as demand is consequently very high as compared to that of open pollinated varieties, in the seed market. This high cost and huge demand in the market has in turn triggered the sale of spurious seeds to the farmers. Besides this, as the eggplant hybrid seeds are produced using 'Hand Emasculation and Pollination' technique, where the flowers of female parent are emasculated manually by removing the anthers before their dehiscence and pollen collected from flowers of male parent are used to pollinate emasculated female flowers to get hybrid seeds, there is every chance for the presence of admixtures (selfed/off-types seeds) in the produced hybrid seed lots due to manual/mechanical error associated with the technology. Hence, to safeguard the farmers' interests and to ensure that the farmers obtain true value for the money spent on purchase of seeds, it is necessary to confirm the identity and purity of the hybrid seeds before it reaches the farmers' fields.

Conventionally, genetic identity and purity of seed lot is assessed by field plot test, which is a well-accepted testing procedure based on plant morphological characters. The field plot test involves the comparison of plants raised from seeds of test sample with that of an authentic sample, for the phenotypic characters, throughout the crop's growing season. Since the number of seed lots to be tested in each season is quite large, field plot test method becomes time consuming and expensive, requiring large areas of land. Many of the characters used are multigenic or quantitative and their expressions are altered by environmental factors, thereby necessitating replication of observations. Further, as it takes a complete season, many seed lots will be sold off before the field plot test results are out from seed testing labs. In case if the seeds are stored, it locks up the capital invested on hybrid seed production, besides adding the additional expenditure incurred on storage to the cost of production, which ultimately increases the hybrid seed cost. Thus there are compelling reasons to find more rapid and cost effective procedures to augment this process.

One such alternative is the molecular markers. Molecular markers assay variation in the nucleotide sequence of DNA. Such difference remains unaffected across different growth stages, seasons, locations and agronomic practices. In brinjal, different molecular markers are being used for phylogenetic interpretations, fingerprinting of cultivars and marker assisted selection (Furini and Wunder, 2004; Tiwari *et al.* 2009; Verma *et al.* 2012). Among molecular markers, simple sequence repeat (SSR) markers were reported to be the best for testing genetic identity and purity of seeds, because, are being co-dominant in nature, they determine the heterozygosity of the hybrid by presence of polymorphic parental alleles, which facilitates in testing the hybrid identity and purity against the admixture of selfed seeds as well as off-types. Suitability of SSR markers for ensuring the seed purity and identity of hybrid seeds has been well established in different crops. However, when it comes to establishing the identity of the hybrid as well as assessing the extent of off type admixtures in the seed lot, a single SSR marker may not always serve the purpose because narrow genetic base between the parental lines leads to similar DNA fingerprint between the hybrids. As demonstrated by Kumar *et al.* (2014), DNA fingerprint of SSR marker emk03O04 was similar for eggplant hybrids PH9 and Kashi Komal and that of SSR marker emh11O01 was similar for hybrids PH5 and Kashi Komal. Hence, there is a need to identify as many suitable SSR markers for assessing the hybrid seed purity and establishing the identity of the hybrid. In view of this, the present work was undertaken to evaluate and identify the simple sequence markers, which can be used for establishing the genetic identity as well as purity of brinjal hybrids based on generated DNA fingerprint. Further, the utility of SSR marker based DNA fingerprinting for ensuring the seed purity has been demonstrated in commercial seed lot of brinjal hybrid PH-9.

2. Materials and methods

Plant material

Seeds of six brinjal hybrids (PH-5, PH-6, PH-9, NDBHL-20, Kashi Sandesh and Kashi Komal) and their respective parental lines (male and female parent) that are in active seed multiplication chain were collected from the respective breeders and used in the present study. Commercial seeds of brinjal hybrids PH-9 sold in the market were used to assess the utility of SSR marker based DNA fingerprinting to assess the genetic purity in comparison with that of grow out test/field plot test.

DNA fingerprinting

Eighty SSR markers were randomly selected from the sequence information available in literature (Nunome *et al.* 2009; Vilanova *et al.* 2012) and were custom synthesised from Integrated DNA Technologies (IDT®, Coralville, IA, USA) (Table 1). DNA was isolated from the bulk of leaf samples from 10 individual plants of respective hybrid and parental lines by following the method of Doyle and Doyle (1990). Quantity and quality of extracted DNA was estimated by using Bio-Photometer (Eppendorf, Hamburg, Germany) as well as by analysing the DNA on 0.8% agarose gel using diluted uncut lambda DNA as standard.

Targeted DNA amplification was carried out as per Kumar *et al.* (2014) in a 25 μl reaction mixture containing 1X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$), 100 μM each of dNTPs, 0.4 μM each of forward and reverse primers, one unit of Taq DNA polymerase (Bangalore Genie Pvt. Ltd., Bangalore, India) and 20 ng of genomic DNA template. The DNA amplification was carried out in a thermal cycler (QB96; Biotron Healthcare (India) P. Ltd., Mumbai, India). The first cycle consisted of denaturation of template DNA at 94 °C for five min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for one min and extension at 72 °C for one min. The last cycle consisted only of primer extension at 72 °C for seven min. Amplified DNA fragments were separated in 3% agarose (Agarose SFRTM; amresco®, Solon, OH, USA) gel containing the nucleic acid stain ethidium bromide using 1X TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA pH=8.0; Sigma-Aldrich®, Bengaluru, India). The size of the amplified fragments was determined by using a size standard (20 bp DNA ladder; GeNie™, Bengaluru, India). DNA fragments were visualised under UV light and photographed using gel documentation system (Alphaimager-HP; ProteinSimple™, San Jose, CA, USA).

Table 1. List of simple sequence repeat markers used in the present study.

Primer	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Primer	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
emk02K24	agtaggctaaacgacctctaaatttgc	gtttggtgttgacaaaaagaacctgac	emg01B17	acaaggctcaaagtcacaagtcaa	gtttggctctgcccctaacatctacaaa
emj04D04	attgacctagccctcttaggcgac	gtttatgttggaaggtttaaccgcagc	emf21O06	agattaccttcttccgcttcatgc	gtttcaaatgccaaacatgaaccac
emf01O01	aggaattggatttccactcatacg	gtttggaagatgagattcctttcttga	emh21G04	atgcaaccaccaggaacacataaa	gtttccttcatgtgaacgtcatcacc
emg11M21	atagcctactgccttcaagaccat	gtttcctacgtccagtccccttaggt	emf01J09	atagcacccacactaaaccttggg	gtttcacttcttggtccattgttcaga
emb01O09	gtcctacgtccagtccccttaggt	tcccttggaatgccatctctttta	emk04N11	atctccccctcaactttgaacaat	gtttgtgtgatatagcccaacaattcac
eme03A05	attttatgctgctcctcggattgat	gtttgtgcatctcttgttggtaggagc	eme02B08	attgggaaaagcaaaagtcaggaga	gtttgggttcctcataattgtaatggc
emh11G09	atctctgtcatttatttttgggtcc	gtttcatccacttgggcttaaaatgct	emf21I08	actatccaaggaccaaaagtgtcca	gtttggcagtcagaaaggtgatcttg
eme01B01	atgtgcatctcttgttggtaggagc	gtttctcggattgatcccaaagggtact	emb01F20	tgggaaaagcaaaagtcaggagaa	gtttcattgcaatctcatggagtagtgttc
emd04E10	atggttatccggcctcctatacctc	gtttaggaattgaaggtccaaagcatga	emb0048	tcattgcaatctcatggagtagtgttc	gtttgggaaaagcaaaagtcaggagaa
emf21N12	agtcaataacctcccacatcccac	gtttgcttgagcaccatgtgtttgat	emf11B07	acgagagttgctacagttaagggg	gtttggggaccaaagtgtattttcaagg
emf21K08	atcaatgacacccaaaacccattt	gtttgaaaacccaatacaaatccga	emf01C03	agtccaccatgagtgagtgagtga	gtttacgtgttgggcctccaaaatatc
emb01G19	aattaaggctgagaggggaagacg	aaaggaggaaagggaaagggaaag	emk04H07	atttggctgggttgttggtctagt	gtttggcccaattactcaaataccctg
emg11B20	atcattgccgtatcaggttcactc	gtttgggaaagttgagaatttcttgggg	emf01A03	aggagaaggattaggaagatatccca	gtttgagaaatcgaaatgcctcact
emf01B07	ataccagataaaacgaagcacggc	gtttgatgagtgtgggacttgaaga	emj03E23	agcacaaaggagagaaagcaattt	gtttagaagctgttatcgaatcgttgcc
emd01D08	atcaacaggtggctaggtttcacaa	gtttccagcttagggcctccatctactt	emf11N23	atgttcttcccttttttccccttttt	gtttccaagaaagaagaaaaccccaca
emg11D05	acgtgtgaacttaagcagaatgctc	gtttgcaaaaagtttctgtcatgctcca	emd13H06	atagtggttttccaattaccccca	gtttacattctaccctccccagaccc
emd01B12	atcaatagcttgccacttggcttaac	gtttaggaaacctaacccaaacctggaa	emf21N03	accagaggagcaaagggaaaaata	gtttacgctactggaccaaaccaacaat
emf21M05	atcccaagacctggaagtcaccta	gtttagaagccttgccacttggcttaac	emj01G23	attaactggccatgaacacctgtc	gtttgacctcaataaagggggtttgcat
emh05B12	ataattggaccaagtgtggtgtgg	gtttaggggcctcgatatagtaggttgg	emf21I04	agagaggtgactgaaagcaaaacg	gtttggaaaataccaaccaagccaatca
emh21L21	atccataactacccacacccaacc	gtttgatctttttgaccccaacc	emg11D19	acatggtaggtggagacagaacca	gtttgcaaagagcctatgaccaggtt
emf21M21	atgtcttggaccccgctaagtatc	gtttcgcggaagcaattaaaaatgac	emb01A21	tcatggtaggtggagacagaacca	gtttggattagcatgtggaggactgaa
emb01F16	aaaacagaagcaaagtcggcagtc	gtccaccaacaccttaccatcctc	emf21H22	atcaagatgaacaagactaaggagtgc	gtttcttcaacctgtctttagccca
emi06A04	atttgggacaaatgtgggtgagac	gtttccacgctacttaggggactcaa	eme08D09	atggattagcatgtggaggactgaa	gtttcatggtaggtggagacagaacca
emf01M12	acgcacctcagactgaagagtttt	gtttgaaagggaattgaatggtgcttca	emg21A08	atggcaaggactgaggtatcacaa	gtttccgcttatttgatggatcttgc
emf01M06	agccacttacactgccaacattca	gtttagtggtagaagcgatggtggtga	emh11B18	atcaaaaccaacctccagttctcg	gtttcaaatcgcagagttcatccttcct
emb01A03	cggatttagaggacgtttggattg	gtttggtggagctcagctgttagtttg	emd03D09	acagcactgctctaatggctttggtc	gtttcaagtgtgggggtggactacactta
ecm031	accaaaggagaaggagatgtgcta	gtttgcttcccttccaaaatgacaag	emi03K06	atgttttgtggtgccacgtagatg	gtttaaggtgcagggtaattgtcattgc
em256	tcttaaatgtgccagccctctcat	gctgctgtttgttgacaaaattgg	emg11I03	attaggcacaagtgccacctgaat	gtttcagccgggagtctgataggtaaaa
emh21M11	attggtgaacgatgatcctgaatg	gtttagagaatgggatgagattgcttcg	emi06F08	ataatgaaccaaagcgagagcaac	gtttcaggtccataggggtggatctatg
eme03H10	atggaattgttccctgaagtcctgt	gtttcccaagcctgcaatctttaacatc	eme03F04	atatgacgacagacgtaaagcgacc	gtttcagagttttgccatctgtgtcgag
emd18E02	agtgctctgaactcctttccttca	gtttccctaaaaggaatatgtgctctgg	emb01D19	cgaccccagatccagaaataaaga	cccaagagttgtactcgtcaacca
emk01A16	agaccttttaagaagaggagcagca	gtttaaacatctcgacttcccattggtg	emd05F05	acgggggtgtctcattacactactgg	gtttacccgttcctcagcttatagaccc
eme10H05	acaagacgaaagtgtgcagaccag	gtttgaaagtgaagagtccgtgcagt	emd01C11	acgactaaggaggcattttcttgc	gtttcacttggcaaagctagctatattcttg
ecm001	accttacgcaatttacacttcccc	gtttcaatggcgtcacctctctctct	emi04H10	atcggagcaagagacattagatgc	gtttccaacacagtccccaatacaacaa
emg11M09	atacattgaaattggctgagcttg	gtttggatcttcgctagaactttggc	emh02A04	attgatttctaagcgcactcgcac	gtttagggattgttcaattctgggtctg
emg11K23	atgactgcaatgtcctcacttggt	gtttgggggctatttttaaaactggatag	eme25D01	agtcccaaccaaaatcgtagaggc	gtttcactgaaggatgtggagtgtga
emf11N03	atatcgttgaagcaaacatctgcc	gtttcgctatatcaccccaatca	eme11f04	acccccaaatcaaatcatttaccc	gtttggcatggttagggtttttagcgtt
emf11O07	acttgaaggttggtcttcacttgag	gtttctttttgagaaccttcttggcttgg	CSM27	tgtttggaggtgagggaaag	tccaactcaccggaaaaatc
emk03H16	agcttggaaatcacaaaacccttg	gtttgatcgatgaatggtgaattgg	CSM31	caaccgatatgctcagatgc	gccctatggtcatgttttgc
emb01M15	gcaaggctcaaagtcacaagtcaa	ggctctgcccctaacatctacaaa	CSM32	tcgaaagtacagcggagaaag	gggggtttgattttcattttc

Utility SSR marker based DNA fingerprinting in seed purity testing

Two hundred plants were raised in the field using randomly selected seeds from commercial brinjal hybrid PH9 following recommended agronomic package of practices. During the cropping season, selfed or off-type plants were identified and tagged based on phenotypic characters, which are given at Supplementary Table S1. Genetic purity was calculated as percentage of plants that were true to the type over a count of 200 plants. Similarly, SSR marker (emb01M15) based DNA fingerprint of all respective two hundred plants was generated using DNA extracted from leaf samples of individual plants raised in the field as detailed above and genetic purity was calculated as percentage of plants that were true to the type amongst the entire 200 plants.

3. Results and discussion

Among the eighty markers studied, four markers (viz. emk02K24, emb01M15, emd05F05 and CSM31) were found to be polymorphic among parental lines of respective hybrids (viz. PH5, PH9, NDBHL20 and Kashi Komal), which could be used for ensuring the genetic purity of respective parental lines and hybrids. Size of each allele detected by using identified primers for each loci and lines combination is given at Supplementary Table S2. Marker emk02K24 amplified four alleles in the range of 290 to 220 bp across the brinjal genotypes studied and was found useful in assessing the genetic purity of two hybrids (viz. PH5 and PH9) and their respective parental lines (Figure 1A). Marker emb01M15 amplified two alleles each in the size range of 260 to 230 bp across the genotypes (Figure 1B) and was found useful for ensuring the genetic purity of three hybrids (viz. PH9, NDBHL20 and Kashi Komal). emd05F05 had heterozygous profile in three hybrids (viz. PH5, NDBHL20 and Kashi Komal), amplifying two alleles of size 250 and 210 bp (Figure 1C). CSM31 amplified three alleles of size 250 and 220 bp across the genotypes studied and was effective in ensuring the genetic purity of PH9 and Kashi Komal (Figure 1D). None of the SSR markers screened was found to be suitable for ensuring the genetic purity of brinjal hybrids PH6 and Kashi Sandesh. This could be because of the fact that the SSR markers used in the present study are less in number and does not provide genome wide coverage, due to which they failed in capturing the observed phenotypic variation at DNA level. This calls for further screening of more number of brinjal SSR markers providing uniform coverage across the genome. In brinjal, the available genomic markers (genomic SSRs and EST-SSRs) are limited in number in comparison to other crops like rice where 5,700 to 10,000 markers are available which are distributed uniformly throughout the genome (Temnykh *et al.*, 2000). Hence, there is a need for development of marker resources providing high-density coverage, which can be utilised for testing genetic purity as well as marker assisted selection applications in brinjal. Though, the SSR marker emb01M15 has given a polymorphic profile between the parental lines of hybrids PH6 and Kashi Sandesh, it is not suitable for testing the purity of respective hybrids, due to its heterozygous state in the female parent, which skews the decision on whether the seed/plant is selfed or hybrid, based on generated DNA fingerprint. Similar was the case with SSR marker emk02K24 for hybrid Kashi Komal. In the present study, sets of three identified markers (emb01M15, emd05F05 and CSM31) alone are sufficient to precisely distinguish four hybrids (PH5, PH9, NDBHL20 and Kashi Komal) from each other, even though all the nine markers were informative (Figure 2). Thus emb01M15, emd05F05 and CSM31 can be used as referral markers for unambiguous identification and protection of these hybrids. However the relevance of the entire set of four markers cannot be undermined as they can be effectively used to differentiate future eggplant hybrids from these existing hybrids.

Field plot test results revealed 18% of selfed seed contamination in commercial hybrid PH9 (Supplementary Table S3) and off-types or outcrossed plants were not detected, thereby reflecting the stringency executed during hybrid seed production in terms of isolation and timely field inspections and rogueing. In order to examine the utility of SSR marker based DNA fingerprinting for seed purity assessment, DNA fingerprint of all 200 plants raised in the field were generated using SSR marker emb01M15 (Figure 3). In the SSR marker analysis, all the identified selfed plants matched with those identified through field plot test and no additional off-types/outcrossed plants/male parent plants were found. Thus the present study could successfully establish the utility of the SSR marker based DNA fingerprinting ensuring the genetic identity and purity of brinjal hybrids and parental lines. The high percentage of selfed seeds could be attributed to self-pollination due to missed emasculation or due to the mechanical damage of anthers that occurred during emasculation of flowers in female parental line.

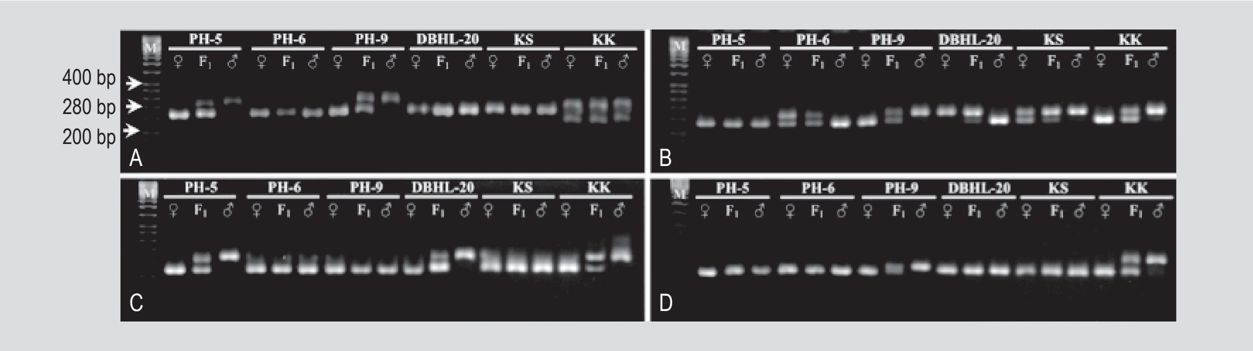
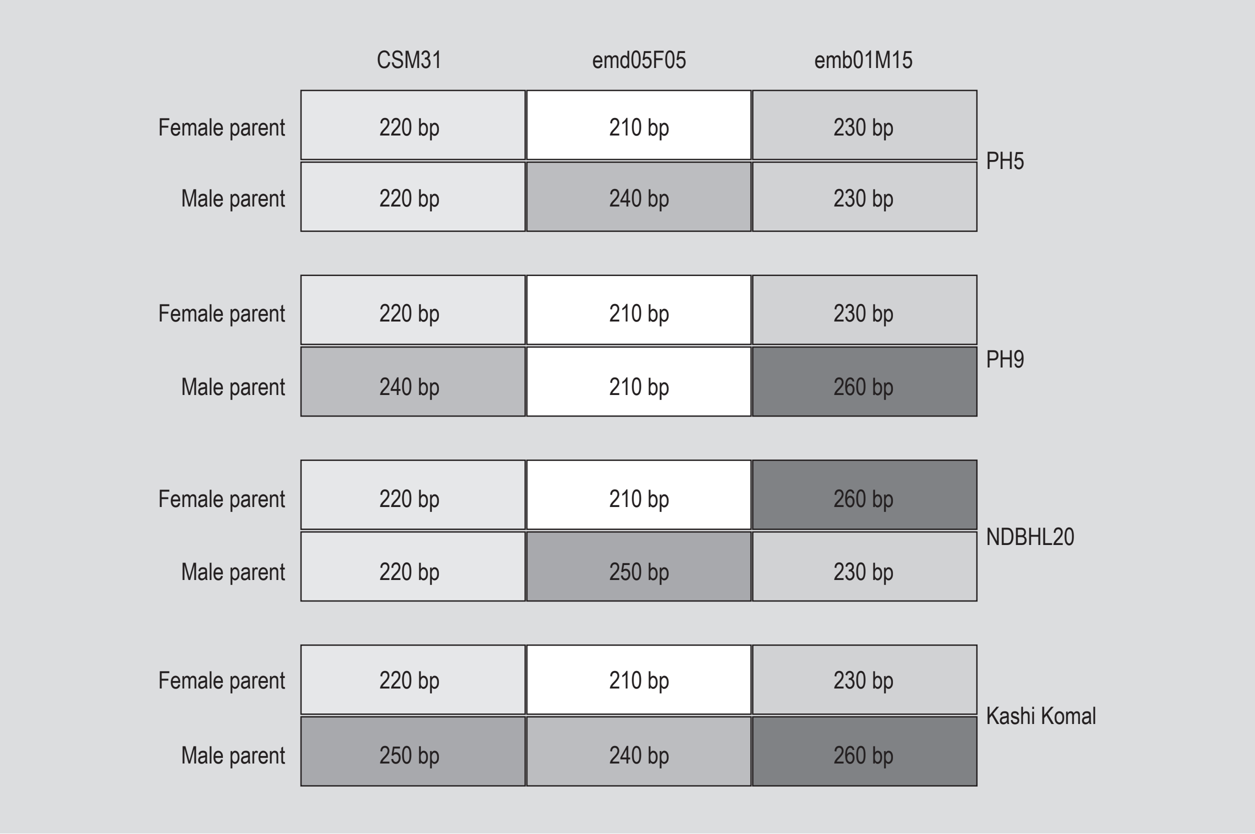


Figure 1. Simple sequence repeat (SSR) profiles of six brinjal hybrids (F_1) and respective parental lines (♀ and ♂) obtained from different markers. (A) SSR profile of marker emk02K24. (B) SSR profile of marker emb01M15. (C) SSR profile of marker emd05F05. (D) SSR profile of marker CSM31. M = 20 bp DNA Ruler from GeNie™.



Hybrid	Parent	CSM31	emd05F05	emb01M15
PH5	Female parent	220 bp	210 bp	230 bp
	Male parent	220 bp	240 bp	230 bp
PH9	Female parent	220 bp	210 bp	230 bp
	Male parent	240 bp	210 bp	260 bp
NDBHL20	Female parent	220 bp	210 bp	260 bp
	Male parent	220 bp	250 bp	230 bp
Kashi Komal	Female parent	220 bp	210 bp	230 bp
	Male parent	250 bp	240 bp	260 bp

Figure 2. Diagrammatic representation of DNA fingerprints of four brinjal hybrids based on three SSR markers. In each hybrid the columns represent the allelic profile of female and male parent, respectively. The shades indicate different alleles amplified by the respective SSR marker in the hybrids with the given size of the amplified DNA fragment.

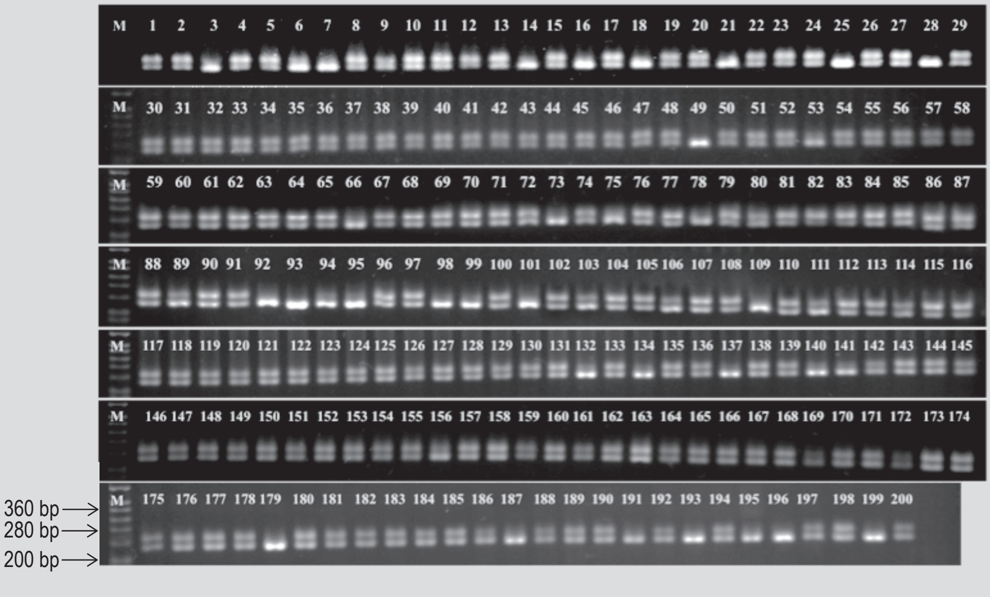


Figure 3. Assessment of genetic purity in hybrid seeds of PH-9 using the simple sequence repeat marker emb01M15. M = 20 bp DNA ruler from GeNie™, lane 1-200 = individual F_1 plants representing a random sample from commercial seeds of hybrid PH9, lane 3, 6, 7, 14, 16, 18, 21, 25, 28, 49, 53, 66, 73, 75, 78, 92, 93, 94, 95, 98, 99, 101, 109, 132, 134, 137, 140, 141, 179, 187, 191, 193, 195, 196, and 199 represents selfed plant, a contaminant.

4. Conclusions

In the present study four SSR markers were identified that could be effectively used to assess the genetic identity and purity of four hybrids out of the total six hybrids studied. Among the four markers, a set of three markers differentiated four of the six hybrids from each other. Extending this study with more number of markers providing genome wide coverage would reveal better information on the genetic relatedness between the eggplant hybrids, which would be possible, when more genomic resources for eggplant are developed through the use of advanced techniques such as genome sequencing. Genetic purity testing of the commercial seeds of hybrid PH9 through field plot test as well as SSR marker based DNA fingerprinting revealed the contamination of female line to the extent of 18%, which emphasises the utility of DNA fingerprinting in ensuring the seed purity and need for further stringency in the conduct of seed production procedures.

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Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/QAS2015.0689.

Table S1. Phenotypic characters used to identify the selfed/offtypes during the grow out test.

Table S2. Size of each allele detected by using different primers for each loci and lines combination.

Table S3. Results of the field plot test.

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