DNA MARKER BASED HYBRIDITY TEST OF DIFFERENT EGGPLANT (*Solanum melongena* L.) HYBRIDS

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DNA MARKER BASED HYBRIDITY TEST OF DIFFERENT EGGPLANT (*Solanum melongena* L.) HYBRIDS

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This is to certify that the thesis entitled ***"DNA MARKER BASED HYBRIDITY TEST OF DIFFERENT COMMERCIAL EGGPLANT (Solanum melongena L.) hybrids"*** *submitted to the Department of Biotechnology, Sher-e-BanglaAgricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE (MS) in BIOTECHNOLOGY,*** *embodies the result of a piece ofbonafide research work carried out by* ***MAHBUBA HOSSAIN,*** *Registration No.* ***16-07553*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed during the course of this investigation has been duly acknowledged and style of this thesis have been approved and recommended for submission.

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ABBREVIATIONS

FULL WORD	ABBREVIATION
Advance Chemical Industries	ACI
Amplified Fragment Length Polymorphism	AFLP
And others (at elli)	*et al.*
Bangladesh Agricultural Research Institute	(BARI)
Base pair	bp
Centimeter	cm
CetylTrimethyl Ammonium Bromide	CTAB
Continued	Cont"d
Degree celsius	^{0}C
Deoxyribonucleic acid	DNA
Deionized water	dH_2O
Diversity Arrays Technology	DArT
Ethylene Diamine Tetra Acetic Acid	EDTA
Etcetera	etc.
Ethidium Bromide	Et-Br
Government	Govt.
Gram	g
Grow Out Test	GOT
Gram per Liter	g/L
Genetic diversity index	GDI
Inter Simple Sequence Repeats	ISSRs
Marker assistant breeding	MAS
Metal Eggplant Genotype	MEG
Metal Eggplant Hybrid	MEH
Micro liter	µl
Micro mole	µM
Mili liter	ml
Namely	*viz.*
Negative logarithm of hydrogen ion concentration (-log[H+])	pH
Percent	%

ABBREVIATIONS (Cont'd)

FULL WORD	ABBREVIATION
Polymorphic Information Content	PIC
Polymerase Chain Reaction	PCR
Random Amplified Polymorphic DNA	RAPD
Research and Development	R&D
Restriction Site associated DNA Markers	RAD
Restriction Fragment Length Polymorphism	RFLP
Rotation per minute	rpm
Seed Certification Agency	SCA
Short Tandem Repeat	STR
Simple Sequence Length Polymorphism	SSLP
Single Feature Polymorphism	SFP
Single Nucleotide Polymorphism	SNP
Simple Sequence Repeat	SSR
Sodium chloride	NaCl
Sodium Dodecyl Sulphate	SDS
Species	spp.
Solanum	*S.*
Thermophilusaquaticus	Taq
Tris Boric Acid EDTA	TBE
Tris-EDTA	TE
tons	t
Unweighted Pair Group of Arithmetic	UPGMA
Ultra Violet	UV
Volt	V

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The Author

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DNA MARKER BASED HYBRIDITY TEST OF DIFFERENT EGGPLANT (*Solanummelongena*L.) HYBRIDS

ABSTRACT

An experiment was carried out to identify the hybrid variety of eggplant through SSR marker. Four eggplant hybrids *viz*MEH5003, MEH5006, MEH5009, MEH5012 and their corresponding parents were used as experimental materials. The hybrid varieties of eggplant were developed by Metal Seed Ltd., Dhaka, Bangladesh. The experiment was conducted at the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. Initially different SSR primerswere used to establish polymorphism among the parents of each hybrid. The identified polymorphic primers were used to screen the hybrid variety together with corresponding parents. It was noticed that only one primer *viz* MEH SSR-04 showed polymorphism in the parents of MEH5009 hybrid. This primer was used for the detection of hybrid variety. A 150bp DNA fragment was amplified in male parent and hybrid variety which was absent in female genotype. This DNA band can be used for large scale screening of that specific hybrid variety for seed purity in commercial purpose. The parents of other hybrid showed monomorphic DNA band and some of the SSR primerswere not amplified in any eggplant genotype under investigation. The confirmation of genetic purity in seeds is very important for identification of genetic contamination and hybrid purity for farmers and plant breeders before sale of the seed to public domain. The genetic purity test by conventional method called Grow Out Test (GOT) is very tedious and time taking, therefore molecular marker technique may be used as alternative approach for its accuracy and rapid detection method.

CHAPTER I

INTRODUCTION

Eggplant (*Solanum melongena* L., 2n = 2x = 24) is a species belonging to the Solanaceae family. It isalso referred to as brinjal (Ali *et al.,*2011), is one of the most common vegetable crops grown in different parts of the world (Bletsos*et al.,* 2003). In production terms, eggplant is the third most important *Solanaceae*crop species (after potato and tomato;) and is cultivated all over the world, but most intensively in China and India. It showed a wide variation according to their morphological structure (Cerciola*et al.,* 2013). A large number of cultivars have been cultivated throughout the world according to market needs and consumer demands (Sidhu*et al.,* 2005).

It is (*Solanum melongena L.*) is an annual and herbaceous plant that has large diversity for fruit color, shape and size etc. (Sidhu*et al.,* 2005).It is a good source of vitamins and minerals, with nutritional value comparable to tomatoes (Kalloo, 1993).The high yielding hybrid cultivars have been developed specifically to overcome problems derived from out of season production under adverse environmental conditions in Mediterranean climate areas (Prohens*et al.,* 2005). These commercial hybrids are usually less affected by adverse weather conditions (Rodríguez *et al.,* 2008) and some of them are parthenocarpic cultivars (Rotino*et al.,* 1997; Donzella*et al.,* 2000; Spena and Rotino 2001; Kikuchi *et al.,* 2008; Saito *et al.,* 2009). In the last thirty years, many different commercial F1 hybrids have been developed and offered to the market (Sekara*et al.,* 2007). An F1 hybrid is the first filial generation of offspring of distinctly different parental types. A hybrid vegetable is produced when plant breeders intentionally cross-pollinate two different varieties of a plant, aiming to produce an offspring or hybrid that contains the best traits from the parents. F1 hybrids are used in genetics and in selective breeding, where it may appear as F1 crossbreed. Hybrid seed production is predominant in modern agriculture for food security. It is one of the main contributors to the dramatic rise in agricultural output during the last half of the 20th century.

Bangladesh is slowly coming out of a seasonal barrier in vegetables production mainly, helped by consistent growth in sales and cultivation of hybrid seeds that offer farmers scope to profit from off-season vegetables. Vegetables seeds, mainly hybrid, now meet almost 40 percent of the annual demand of over 2,600 tones with various imported and locally innovated hybrid varieties capable of growing in off-season. Almost all the vegetables including brinjal, bitter gourd, cabbage, cauliflower, cucumber, lemon, gourd, bean, okra, papaya, ridge gourd, snake bean, green chilli, ginger, onion, garlic, tomato, radish and pumpkin which are now available in the country belong to the hybrid variety. Different public and private sectors (BRAC Seeds, Metal Seed Ltd., LalTeer Seeds, Advanced Chemical Industries (ACI), Supreme Seed Company, Giant Agro processing Ltd.etc) are involved in producing hybrid seeds. Seed Certification Agency (SCA) is the only legal authority to certify seeds. More than 20 different eggplant hybrids are available in this country out of them Surobhi, Gaint green, BARI hybrid brinjal-3 and BARI hybrid brinjal-4 etc are the example of hybrid variety of brinjal in Bangladesh.

Hybridity can be confirmed by two methods. One is conventional method another is molecular technique. The conventional method for estimating hybrid seed purity or hybridity is known as Grow Out Test (GOT).The hybridity test done in natural field condition. The seed of parent1 (male), parent2 (female) and hybrids are sown in ideal condition. The morphological data are recorded in all the hybrid samples and then it is compared with parental line.There are some disadvantages involved in grow-out test such as some morphological characteristics need to be assessed in adult stage and are influenced by environmental factors,take more time to complete. Seeds have to be stored safely before released to farmers. The investment used in producing the seeds is high. Huge amount of land, labor and cultivation cost are involved. Seed viability is reduced due to over the period of time.

Hybridity test through molecular technique for assessing purity of parental lines is done by DNA markers. It has several advantages over conventional method, such as not influenced by environment,high power of discrimination among closely related individual,polymorphism establishment within DNA level,more accurate andexact blue print of genetic make upcan be achieved.

A molecular marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like minisatellites. Markers can exhibit two modes of inheritance, i.e. dominant/recessive or co-dominant. If the genetic pattern of homozygotes can be distinguished from that of heterozygotes, then a marker is said to be co-dominant. Generally co-dominant markers are more informative than the dominant markers (Manikanda, 2012).

Some commonly used molecular markers are;RFLP (Restriction fragment length polymorphism),SSLP (Simple sequence length polymorphism), AFLP (Amplified fragment length polymorphism), RAPD (Random amplification of polymorphic DNA), VNTR (Variable number tandem repeat), SSR Microsatellite polymorphism (Simple sequence repeat), SNP (Single nucleotide polymorphism), STR (Short tandem repeat), SFP (Single feature polymorphism), DArT (Diversity Arrays Technology), RAD markers (Restriction site associated DNA markers).Among the available molecular markers, microsatellites or simple sequence repeats (SSRs) which are tandem repeats of two/four or six nucleotide long DNA motifs, have gained considerable importance in plant genetics and breeding owing to many desirable genetic attributes including hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage including organellar genomes, chromosome specific location and amenability to automation and high throughput genotyping (Kalia*et al*., 2011)

SSR markers are non-isotype based precise PCR technology. Stretches of DNA, consisting of randomly repeating small nucleotide units and conserved regions flanking the repeats are suitable for designing PCR primer pairs. Used for amplifyingthe intervening repeat loci were first referred to as Microsatellites by Litt and Luty (1989) and later as Simple Sequence Repeats (SSRs) by Jacob *et al*. (1991).

SSRs provide a powerful means to link the genetic maps of related species and since many of them are located within genes of known, any allelic variation present can be exploited to generate perfect markers (Andersen *et al.,* 2003).

Polymorphism or genetic variation among the parents is pre-requisite for hybridity test through molecular marker. Variation at DNA levelis used as an indicator for hybridity. Hence polymorphism study among the parent is essential. SSR primers are used for polymorphism study. After establishment of polymorphism among the parents using SSR markers those specific SSR primerscan be used for screening of the hybrid as well as its corresponding parents.

Considering the above facts, the present study has been undertaken to provide DNA fingerprinting and molecular diversity of different hybrid eggplant varieties. The major objectives of this study are given below:-

1. Polymorphism study among different commercial eggplant hybrids.
2. Molecular diversity analysis of different parent of commercial metal eggplant hybrids.
3. Hybridity test among the collected materials.
4. Assessment of genetic purity.

CHAPTER II

REVIEW OF LITERATURE

Literature review of hybridity evaluation using molecular marker is recently in Bangladesh.Few works also done in other country. Therefore, few available literature are cited below:

2.1 The concept of molecular marker

Molecular markers are reliable tools to characterize the DNA profile of plant genotypes to study the genetic diversity. According to Datta*et al*., (2011) molecular markers are specific fragments of DNA that can be identified within the whole genome. Molecular markers are found at specific locations of the genome.

Molecular markers have been found to be more dependable than the phenotypic observations for evaluating the variations and in the assessment of the genetic stability (Leroy *et al.*, 2000) and provide an efficient means to link phenotypic and genotypic variation (Varshney*et al*., 2005).

These methods are being very rapidly adopted by the researchers all over the world for the crop improvement. The molecular marker techniques are diverse and vary in principle, application and amount of polymorphism observed and in time requirements. Molecular markers present an efficient tool for fingerprinting of cultivars, and assessment of genetic resemblance and relationships.

With the advent of molecular biology techniques, molecular markers are effective because they identify an abundance of genetic linkage between identifiable locations within a chromosome and are able to be repeated for verification. A molecular marker is a DNA sequence that can be readily detected and whose inheritance can easily be monitored. Amin *et al*.(2010) stated that, the use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes.

Molecular markersare able to identify particular locations on a chromosome, allowing creation of physical maps. Molecular marker can identify many alleles an organism for a particular trait (bi allelic or poly allelic).

Molecular marker can identify small changes within the mapping population enabling distinction between a mapping species, allowing for segregation of traits and identity. Some studies which were conducted during the last decade of the 20th century reported numerous DNA markers that have been utilized in plant breeding programs. Apart from the application of molecular markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the genetic variations within cultivars and germplasms (Henry,1997).

The most interesting application of molecular markers is marker-assisted selection (MAS). They have proved to be excellent tools for assessment of genetic diversity in a wide range of plant species (Madhumati, 2014).

Suitable DNA markers should be polymorphic in the DNA level and can be expressed in all tissues, organs, and various developmental stages. The molecular approach for identification of plant genotypes seems to be more effective as it allows direct access to the hereditary material (Paterson *et al.,* 1991) unlike the morphological markers.

Molecular markers can be used for molecular characterization and detecting genetic variation and relationship of plants. These markers can detect the variation that arises from deletion, duplication, inversion, and/or insertion in the chromosomes. Such markers themselves do not affect the phenotype of the traits of interest because they are located only near or linked to genes controlling the traits (Mondini*et al.,* 2009).

Amin *et al.* (2010) reported the use of molecular markers in identification of vegetables crop varieties like tomato, potato, onion, garlic and related species and linked to major disease resistance in tomato like *Meloidogyne incognita* and tomato mosaic virus. These markers are selectively neutral because they are usually located in non-coding regions of DNA. Usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors. Apart from the use of DNA markers in construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within cultivars and fingerprinting the germplasms. DNA markers are accepted widely as potentially

valuable tools for crop breeding such as rice (Mackill*et al.,* 1999 and McCouch*et al*., 1988), wheat and forage species(Jahufer*et al.,* 2003).

According to Krap*et al.* (1997), DNA based markeris classified into three categories depending on technique used. Hybridization based DNA markers, arbitrarily primed polymerase chain reaction (PCR)-base markers, and sequence targeted and single locus DNA marker. Restriction Fragment Length Polymorphism (RFLP) is a hybridization based marker in which DNA polymorphism is detected by digesting DNA with restriction enzyme followed by DNA blotting and hybridizations with probes. Sequence Tagged Sites (STS), Sequence Repeat (SSRs), Single Nucleotide Polymorphism (SNPs) markers belongs to sequence targeted and single locus PCR based DNA markers. Of these, RFLP and micro satellites are co-dominant markers, while RAPD and AFLP markers are largely dominant markers.

2.2 Microsatellite or Simple Sequence Repeats (SSRs) marker

Microsatellites (SSRs) are short tandem repeats of simple (1–6 nt) motifs. Their value for genetic analysis lies in their multi-allelism, co-dominant inheritance, relative abundance, genome coverage and suitability for high-throughput PCR-based platforms. They can serve as highly informative genetic markers, and in conjunction with the use of polymerase chain reaction (PCR) technology enable the detection of length variation (Powell, 1996).

Microsatellite primers developed for one species can be used to detect polymorphism at homologous sites in related species. They have become one of the most useful molecular marker systems in plant breeding. The development of SSR markers from genomic libraries is expensive and inefficient (Squirrell*et al.*, 2003).

It was long assumed that SSRs were primarily associated with non-coding DNA, but it has now become clear that they are also abundant in the single and low-copy fraction of the genome (Toth*et al.,* 2000). SSRs are commonly referred to as "genic SSRs" or "EST-SSRs" and are present in 1 to 5% of the expressed plant DNA sequence (Varshney*et al.,* 2005).

SSR markers are non-isotype based precise PCR technology. Stretches of DNA, consisting of randomly repeating small nucleotide units and conserved regions flanking the repeats are suitable for designing PCR primer pairs. Used for amplifyingthe intervening repeat loci were first referred to as Microsatellites by Litt*et al.* (1989) and later as Simple Sequence Repeats (SSRs) by Jacob *et al*. (1991).

Cericola*et al*. (2013) phenotyped 238 eggplant breeding lines, heritage varieties and selections within local landraces from Asia and the Mediterranean Basin with respect to key plant and fruit traits, and genotyped using 24 microsatellite loci distributed uniformly throughout the genome.

Bryan *et al*. (1999) developed some PCR-based markers from mononucleotide simple-sequence repeats in the chloroplast genome of *Nicotianatabacum* and applied to the analysis of genetic diversity and were found to detect high levels of polymorphism at three taxonomic levels in Solanaceous plants.

Smith *et al.* (1997) made a comparison of SSR with data from RFLP and pedigree in maize. They stated that SSR revealed co-dominantly inherited multi-allelic product of loci that can be readily mapped. SSR profiles can be interpreted genetically without the need to repeatedly map amplified bands to marker loci in the different populations. They anticipated that SSR profiling will replace RFLP and PCR based arbitrary primer methods. Munoz-Falcon *et al*. (2011) have shown that genomic SSR markers are more polymorphic than EST-SSRs in eggplant.

On the other hand, SSRs provide a powerful means to link the genetic maps of related species, and since many of them are located within genes of known, any allelic variation present can be exploited to generate perfect markers (Andersen *et al.,* 2003).So transfer of SSR markers is a very efficient approach for DNA marker development.

2.3 Hybridity test in eggplant by SSR markers

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 hybridity testing. 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 twelve eggplant hybrids and their respective parental lines based on generated Genetic purity of hybrid is an essential requirement for its commercial success, conventionally genetic purity testing was done through grow-out test (GOT), which was laborious and required one full season and expenditure in storage and hence increased hybrid seed cost. In the context of disadvantages of conventional method of hybrid identification methods, molecular marker based method is much better for purity testing. ISSR molecular marker have been successfully used to estimate the analysis of genetic diversity at inter- and intra-specific level in a wide range of crop species which include rice, wheat, fingermillet, Vigna and sweet potato. The aim of present study to identify the molecular marker for genetic purity analysis of brinjal hybrids and their parental line.

Munoz-Falcon *et al.* (2011) studied 42 eggplant accessions, which included 25 *Striped* accessions, of which 19 were of the*Listada*type (renowned spanish eggplant)and 6 of the Other Non-Spanish Striped group and 17 Non-Striped accessions were characterized with 17 genomic SSRs and 32 EST-SSRs. Genomic SSRs had a greater polymorphism and polymorphic information content (PIC) than EST-SSRs. Listadade Gandíaproved to be genetically diverse, specific and universal alleles for two SSRmarkers were found for this landrace. All the Listada accessions cluster together in the multivariate PCoA and UPGMA phenograms performed, and are separated from the Other Non-Spanish Striped and Non-Striped accessions. SSR markers revealed of great utility to obtain a specific fingerprint for the Listada de Gandía eggplant as well as to establish the uniqueness and distinctness of this landrace.

Tumbilen*et al.* (2011) identified genic microsatellite (SSR) markers from an expressed sequence tag library of *S. melongena* and used for analysis of 47 accessions of eggplant

and closely related species. The markers had very good polymorphism in the 18 species tested including 8*S. melongena* accessions. Moreover, genetic analysis performed with these markers showed concordance with previous research and knowledge of eggplant domestication. These markers are expected to be a valuable resource for studies of genetic relationships, fingerprinting, and gene mapping in eggplant.

Ali *et al.* (2011) analyzed the diversity of eggplant using inter-simple sequence repeat (ISSR) and RAPD procedures to subdivide 143 Chinese-cultivated eggplants based on coefficient of parentage, genetic diversity index (GDI) and canonical discriminant analysis. ISSR markers were more effective than RAPD markers for detecting genetic diversity, which ranged from 0.10-0.51. Their ISSR/RAPD data provide molecular evidence that coincides with morphological-based classification into three varieties and further subdivision into eight groups, except for two groups. The mean coefficient of parentage and proportional contribution increased from 0.05 to 0.10% and from 3.22 to 6.46% during 1980-1991 and 1992-2003, respectively.

Ge*et al.* (2013) conducted an experiment in where 100 simple sequence repeat (SSR) markers were used to examine the genetic diversity and relationships among 92 eggplant accessions collected from seven areas in China. These analysis revealed a moderate amount of polymorphism with an average polymorphism information content (PIC) value of 0.285. The average value of number of effective loci (Ne), expected heterozygosity (He) and Shannon's Information index (I) were 1.631, 0.323 and 0.570, respectively. The levels of genetic diversity observed in the seven areas were not evenly distributed and decreased from south to north. The results will be useful for eggplant germplasm management and will lead to more efficient use of germplasm in eggplant breeding.

Caguiat*et al.* (2012) showed the first report on genetic diversity assessment of Philippine eggplant accessions and landraces, six wild species and six cultivated variaties among the traditional varieties (improved cultivars and hybrids) were characterized using 17 simple sequence repeat (SSR) markers. The results showed high genetic variation among the traditional varieties and landraces of *S. melongena*. In contrast, low genetic variation was observed among the cultivated varieties and hyrids. Low genetic variation was also observed among the wild species analyzed using SSRs

markers. This result is in contrast to the high genetic variation observed among the wild species used using morphological traits. Among the three groups of eggplant genetic resources analyzed, the traditonal eggplant and landraces were the most diverse. Implications of these findings for eggplant breeding programs and germplasm management were discussed.

Therefore, in order to confirm genetic variations, studies based on other molecular methods are necessary. The regional genetic populations include a wide eggplant genetic diversity which can be good source for the breeding studies performed in the future.

CHAPTER III

MATERIALS AND METHODS

The chapter focused on the materials and methods of the experiment. The details of different materials and methodologies followed for the study have been described in this chapter.

3.1 Experimental site and time duration

The experiment was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh. The period of the experiment was July 2016 to December 2017.

3.2 Name and source of study materials

Four eggplant hybrid and their eight corresponding parents were used as experimental materials(Table 1). All the genotypes were collected from Metal Seed Ltd., Dhaka, Bangladesh. The list of hybrid and its parents are given in Table no. 1

Table 1: Name of hybrids and of different Eggplant

Sl. no.	Hybrid Name	Parents		Source
		Female	Male	
1.	MEH 5003	MEG5001	MEG5002	Metal Seed Ltd., Dhaka, Bangladesh.
2	MEH 5006	MEG5004	MEG5005	Metal Seed Ltd., Dhaka, Bangladesh.
3.	MEH5009	MEG5007	MEG5008	Metal Seed Ltd., Dhaka, Bangladesh.
4.	MEH5012	MEG5010	MEG5011	Metal Seed Ltd., Dhaka, Bangladesh.

3.3. Collection of leaf sample

All the hybrid and its parental seeds are grown in research field of Metal Seed at Salna, Gazipur.In order to carry out SSR marker analysis, young, fresh and tender leaves from the seedlings of about one-month-old were collected from research field of Metal Seed. Those leafs are used as source of genomic DNA extraction.

3.4. Genomic DNA extraction

DNA extraction from young leaves were performed according to a modified Doyle and Doyle (1990) method by using SDS as detergent. The following reagents and methods were used for the isolation of total genomic DNA.

3.4.1. Reagents required

1. Extraction buffer, p^H=8.0

Composition of extraction buffer is as follows:

- 1M TrisHCl
- 0.5M EDT A (Ethylene diamine tetra-acetic Acid) (p^H=8.0)
- 5MNaCI
- D. H_20
- 1 % SDS (Sodium Dodecyl Sulphate)

2. Phenol : Chloroform: Isoamyl Alcohol = 25: 24 : 1

3. TE (Tris EDT A) buffer, p^H =8.0

- 1M Tris HCI
- 0.5 MEDTA
- D. H_20

4. Isopropanol

5. 0.3 M Sodium Acetate

6. Absolute (100%) ethanol

7. Ethanol (70%)
8. RNAase

9. Ethidium Bromide Solution

3.4.2. Reagent preparation for DNA extraction

Extraction buffer (1000ml)

For the preparation of 1000ml DNA extraction buffer, 100ml1M Tris HCL (p^H =8.0) was mixed with 40ml of 0.5M EDTA and added to 100ml 5M NaCl in a 1000ml measuring cylinder. Finally, sterilized dH_20 was added to make the volume up to 1000 ml, then mixed well and autoclaved.

1M Tris HCI (p^H 8.0) (250 ml)

Initially, 30.28 g Triswas taken in a volumetric flask (500 ml). 100ml dd H_20 was added and p^H was adjusted to 8.0 by adding HCI. Then sterilized dH_20 was added to make the volume up to 250 ml. The solution was autoclaved.

0.5M EDTA (p^H 8.0) (250ml)

46.53 g EDTA.$2H_20$ was added in a volumetric flask (500 ml) and 100ml dd. H_20 was added. Then 4 g NaOHwas added and p^H was adjusted to 8.0 with NaOH. Sterilized dH_20 was added to make its volume up to 250 ml. Finally the solution was autoclaved.

5M NaCl (250ml)

For the preparation of 5M NaCl, 73.05 g of NaCIwas added in 250 ml dd. H_20 in a 500 ml volumetric flask, mixed well and autoclaved.

1 % SDS (Sodium Dodecyl Sulphate) (100 ml)

1g of SDS was added in 100ml dH_20 in a 250 ml beaker. As SDS is hazardous, so the proper mixing was done by a hot top magnetic stirrer and was not autoclaved.

2 x CTAB (for plant DNA extraction)

To make 2% CTAB solution, 2g of CTAB was added in 100ml 0.5 M NaCl. Finally the solution was autoclaved.

1x TE buffer (100ml)

1 ml Tris (p^H 8.0) was taken in a volumetric flask (250ml). Then 0.2 ml EDTA (p^H 8.0) was added. Sterilized dH_20 was added to make the volume upto 100ml.

Composition of 5x TBE buffer (1 litre)

- 54 g Tris HCI
- 27.5 g of Boric Acid
- 4.65 g of EDTA
- p^H=8.0

Phenol: Chloroform: Isoamyl Alcohol = 25: 24 :1 (100 ml)

At first 50ml phenol was taken in a volumetric flask (250ml). Then 48ml Chloroform was added. 2ml Isoamyl Alcohol was also added and mixed well. The solution was stored at 4°C.

RNAase

l0mg of RNAase was added to 1 ml of d. H_20. Then it was dissolved completely with the help of necessary heat (at 50°C in water bath for 30 minutes).

70% Ethanol (1000 ml)

700 ml absolute ethanol was mixed with 300 ml dH_20.

3.5. Sequential steps for DNA extraction

1. For Isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from 12 different eggplant hybrid varieties. Total DNA was isolated by using Phenol: Isoamyl Alcohol and ethanol precipitation method.
2. Initially, healthy youngest leaves were washed thoroughly by tap water followed by deionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
3. Approximately 200mg of young leaves were cut into small pieces and then taken in morter. 600µl of extraction buffer was added to it. The ground samples were

takeninto the 1.5ml eppendorf tube and then it was vortexed for 20 seconds in a vortex mixture and then incubated at 65°C for 20 minutes in hot water bath.

4. Equal volume (600µl) of Phenol: Chloroform: Isoamyl Alcohol (25: 24 : 1) was added to the tube. Then it was vortexed for 20 seconds.

5. The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a pipette tip without disturbing the lower portion and transferred into a new eppendorf tube. Approximately 400-450 µl was taken and then equal volume of Chloroform: Isoamyl Alcohol (24: 1) was added to it. The solution was vortexed for 10 seconds.

6. Again the solution was centrifuged at 13000 rpm for 10 minutes.

7. The supernatant was taken in a separate eppendorf tube and the lower layer was discarded.

8. The amount of the solution was multiplied with 0.6 and then same volume of Isopropanol (0.6 volume of the liquid) was added.

9. It was then tapped by finger for 20-30 seconds (The genomic DNA was visible as cotton like structure).

10. After tapping the sample was again centrifuged at 13000 rpm for 15 minutes. The liquid was discarded completely and re-precipitation of the DNA solution was done by adding 500µl of absolute (100%) cold ethanol plus 20µl 0.3 M Sodium acetate.

11. It was shaken gently. Tapping was done to separate pellet. The sample was centrifuged at 13000 rpm for 15 minutes. The liquid was removed completely by pouring and blotting the open tube end on fresh tissue paper.

12. The DNA pellet was then air dried for 2-3 hours. It was then dissolved in an appropriate volume (30 to 40µl) of TE buffer and treated with 3µl of RNAase for removing RNA. Then it was spinned for 4-5 seconds.

13. Finally, the DNA samples were stored in freezer at -20°C.

3.6. Confirmation of extracted DNA

To confirm the extracted DNA sample 1% Agarose gel, working sample of each genomic DNA, 2x loading dye and deionized H_20 is needed.

Table 2. DNA confirmation reagents with amount

Components	**Amount (µI)**
Working DNA sample	3.0
$D.H_20$	2.5
2x loading dye	2.5
Total	8.0

Working solution of DNA samples preparation

DNA concentration were adjusted to 25 ng/µl for doing PCR using the following formula: $V_1 \times S_1 = V_2 \times S_2$

Where,

V_1 = Initial volume of DNA solution (µl)

S_1 = Initial DNA concentration (ng/µl)

V_2 = Final volume of DNA solution (µl)

S_2 = Final DNA concentration (ng/µl)

3.6.1. 1 % Agarose gel preparation

Reagents

- Agarose powder
- 5x TBE buffer (p^H 8.3)
- Ethidium Bromide

Procedure

1g of agarose powder was taken in a 500 ml Erlenmeyer flask containing 100 ml electrophoresis buffer (1x TBE buffer) prepared by adding 20 ml of 5x TBE buffer in 80 ml of de-ionized water. The flask was enclosed with aluminum foil paper to prevent excessive evaporation. The flask was heated in a microwave oven for about 3 minutes with occasional swirling to generate uniform suspension until no arose particle was seen to generate homogenous and crystal clear suspension. The agarosesolution was cooled to about 45-50^0C (flask was cool enough to hold comfortably with bare hand) and 1µl ethidium bromide (DNA stain) was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Tran illuminator) . The molten gel was poured immediately on to a clean gel bed (l5×15×2 cm^3 n size). That was placed on a level bench and appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth about 2 mm above the plate. After 25 minutes, gel was completely cooled at room temperature and solidified and the comb was removed gently. The gel was then ready for loading the DNA samples.

3.6.2. Preparation of DNA samples for electrophoresis

The samples were all in the same concentration in buffer. For each sample, 3µl dH_20 and 3µl 2x loading dye (0.255 xylene ethanol, 0.255 bromophenol blue, 30% glycerol and 1mM EDTA) was taken in an eppendorf tube using 0.5-10µl adjustable micropipette. Loading dye was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally, 8.0µl expected DNA was added to it and mixed well. The sample was, then loaded into the well of the gel and allowed them to sink to the bottom of the well. The gel was placed in the electrophoresis chamber keeping the gel horizontal and submerged in 1x TBE buffer (running buffer). The gel tank was covered and the

electrophoresis power supply was connected and turned on to move DNA from negative to positive (black to red) through the gel. Electrophoresis was carried out at 75volt for about 60 minutes.

3.7 Documentation of the DNA samples

The gel was carried out from the gel chamber and was placed on an ultraviolet light box (UV trans-illuminator) to examine and photographed by a GelCam camera, DNA samples showing better quality bands were taken for quantification and working solution preparation for next process.

3.8. Amplification of SSR markers by PCR

3.8.1. Principle of the amplification of SSR marker

Microsatellites or SSR are tandem repeats of 1 - 6 nucleotides. For example, $(A)_n$, $(AT)_n$, $(ATG)_n$, $(GATT)_n$, $(CTACG)_n$, $(TACGAC)_n$, and so on. They are abundant in genomes of all organisms. The sequence of unique flanking regions of SSR can be used to design primers and carry out PCR to amplify SSR containing sequences. The polymorphism can be detected by agarose gel electrophoresis if differences are large enough (agarose gels can detect differences greater than 10 base pair). SSRs were first used as markers for use in genetic mapping in humans (Litt*et al*., 1989).

3.8.2. List of SSR primers

Five SSR primers *viz*. MEHSSR-01, MEHSSR-02, MEHSSR-03, MEHSSR-04, MEHSSR-05, were selected for PCR reaction on 12 eggplant genotype for their ability to produce polymorphic band.

Table 3. The list of SSR primers and their sequences and GC content

Name of SSR primer (Trade Name)	Sequences of the primer (5'-3')	%of (G+C) content	Annealing temperature (0^0C)
MEHSSR-01	For. GTGACTACGGTTTCACTGGT Rev. GATGACGACGACGATAATAGA	46	65
MEHSSR-02	For. AATGAGTCAGAAACCACGCC Rev. CGTTT AACCTTTGGCTCGGAA	49	63
MEHSSR-03	For. ACATGCCACTCATGTTGGTG Rev. CTTCAGCCATGGACCACATT	50	64
MEHSSR-04	For. AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCA G	43	65
MEHSSR-05	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCAC A	44	65

3.8.3 PCR amplification and reactions

PCR reactions were performed on each DNA sample. 2xTaq ready Master Mix was used. DNA amplification was per formed in oil-free thermal cycler (Esco Technologies swift™ Mini Thermal Cyclers) and Q-cycler Korea. To prepare a 10.50 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table.

Table 4. Reaction mixture composition for PCR for each eggplant genotype

Reagents	Amount (µl)
2xTaq Master Mix	5.00
SSR Forward primer	1.25
SSR Reverse primer	1.25
De- ionized water	1.00
Sample DNA	1.50
Total Reaction volume	10.00

From frozen stocks of the PCR reagents i.e., 2xTaq Master Mix, primer and DNA working samples were melt, mixed by vortexing and kept on ice for maintaining good

quality. DNA samples were pipetted first into PCR tubes compatible with the thermo-cycler used (0.2 ml). A pre-mixture was then prepared in the course of the following order; reaction mixture, DNA sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermo cycler and the cycling was started immediately.

3.8.4.PCR amplification thermal profile

DNA amplification was performed in a thermal cycler (Esco Technologies Swift™ Mini Thermal cyclers). Polymerase chain reaction (PCR) technique is used to selectively amplify a specific segment of the total genomic DNA based on the selected SSR primers. SSRs were amplified under the following PCR reaction conditions: Pre-denaturation with 95^0C for 4 min; denaturation with 95^0C for 40 sec, annealing at 61^0C for 33 sec, extension at 72°C for 40 sec ,final extension at 72°C for 5min continuing with 30 cycles and finally stored at 4°C.

3.8.5. Electrophoretic separation of the amplified products

PCR products for each sample were confirmed by running it in 2% agarose gel containing 1µl ethidium bromide in IX TBE buffer at 80 V for I hour. 5 ul Loading dye was added to the PCR product and spinned them well. Then loaded them in the wells and 100 bp DNA ladder (Bioneer) was also placed in both left and right side of the gel. Under ultra-violet light on a trans-illuminator SSR bands were observed.

3.8.6 Documentation of PCR amplified DNA products:

The gel was taken out carefully from the gel chamber and was placed on high performance ultra-violet light box (UV trans-illuminator) of gel documentation for checking the DNA band and photographed by a Gel Cam Polaroid camera.

Since SSR markers are co dominant hence, each band represented the phenotype at a single allelic locus (Williams *et al.,* 1990). One molecular weight marker, 100 bp (BIONEER, Cat. No. M-1070-Bio Basic, Canada) DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each

fragment with known sized fragments of molecular weight markers. All distinct bands or fragments were thereby given identification numbers according to their band and size scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer.

This was done separately for individual fragments and primers. The band size for each markers was scored. The scores obtained for the SSR primers were then used to assess the polymorphism of parents and to test their F1 hybridsNei, M. *et al.* (1972).

3.9 Precautions

- To maintain a strategic distance from all types of contaminations and keep DNA pure, all dishes, micropipette tips, eppendorf tubes, glass pipettes, deionized water and buffer solutions were legitimately autoclaved. Metal supplies i.e., scissors, forceps were cleaned with absolute ethanol.

- Since Ethidium Bromide (Et-Br) is an intense mutagen and carcinogenic in nature, hand gloves were utilized when taking care of anything that has been presented to Et-Br.

- Always power pack was kept tum off and the leads were unplugged before opening the electrophoresis unit to avoid electrical hazard.

- Eye protector was used while working with trans-illuminator as it produces UV radiation of 254 nm range which can cause eye damage.

- The common safety measures were kept up when performing PCR responses.

All the disposables such as PCR tubes, tips, eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary. Hand gloves were worn amid treatment of PCR segments. Contamination of PCR segments was maintained a strategic distance from.

CHAPTER IV

RESULTS AND DISCUSSION

The present study was conducted to identify eggplant hybrids and to establish polymorphism among the parents of individual hybrid through molecular markers.This chapter comprises the presentation and discussion of the results of the experiment. In the SSR analysis significant genetic variation and polymorphisms for characterization of different eggplant cultivars were identified. The results of the experiment are presented and expressed in table 5 to 9 and Plate 1 to 7 for ease of understanding.

4.1. DNA extraction and confirmation

Genomic DNA extraction of 12 eggplant genotypes were done by using the SDS method with minor modification. RNA sharing was removed by applying RNAase treatment. Finally, the purified DNA was stored at - 20°C freezer for further use.

The extracted genomic DNA of 12 samples were loaded on 1% agarose gel for conformation quantification of DNA sample. It revealed that, all the samples showed clear DNA band in each well (Plate 1.). Hence, the genomic DNA of each sample was diluted on the basis ofconcentration. The working DNA sample was prepared for PCR amplification.

DNA confirmation

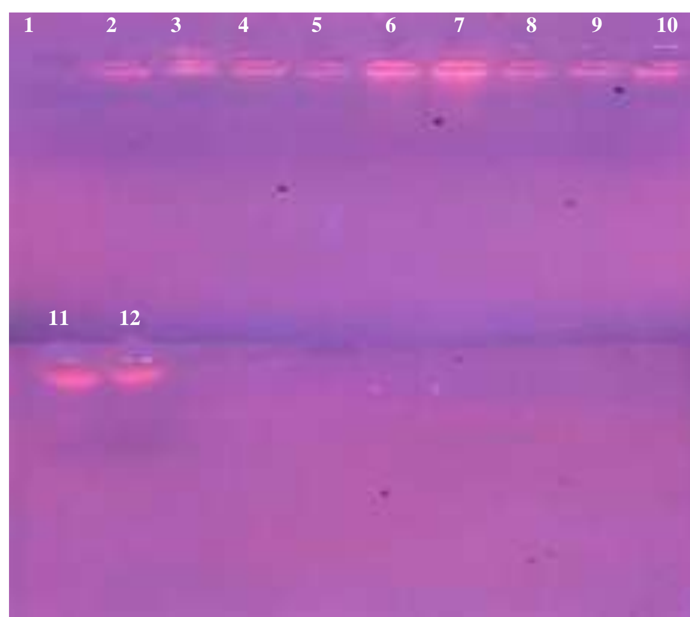

Plate 1. Genomic DNA from different eggplant genotypes

Lane 1 = MEG5001, Lane 2 = MEG5002, Lane 3 =MEH5003, Lane 4 = MEG5004, Lane 5 = MEG5005, Lane 6 = MEH5006, Lane 7 = MEG5007, Lane 8 =MEG5008, Lane 9 = MEH5009, Lane 10 = MEG5010, Lane 11= MEG5011, Lane 12=MEH5012 .

4.2Principle of Hybridity test through molecular marker

Hybridity test through molecular marker need two important steps-

Step 1. Establishment of Polymorphism Among the Parents.

To achieve this objective huge number of molecular marker has toscreened between two parents of a specific hybrid. SSR primer is the best for the study because it will give co-dominant band among the studied genotypespolymorphism can be easily identifiedfrom the DNA amplification pattern. A demo or model amplification pattern is given below.

Two parents of an eggplant hybrid was amplified by amolecular marker named as MEHSSR-04 in the following pattern.

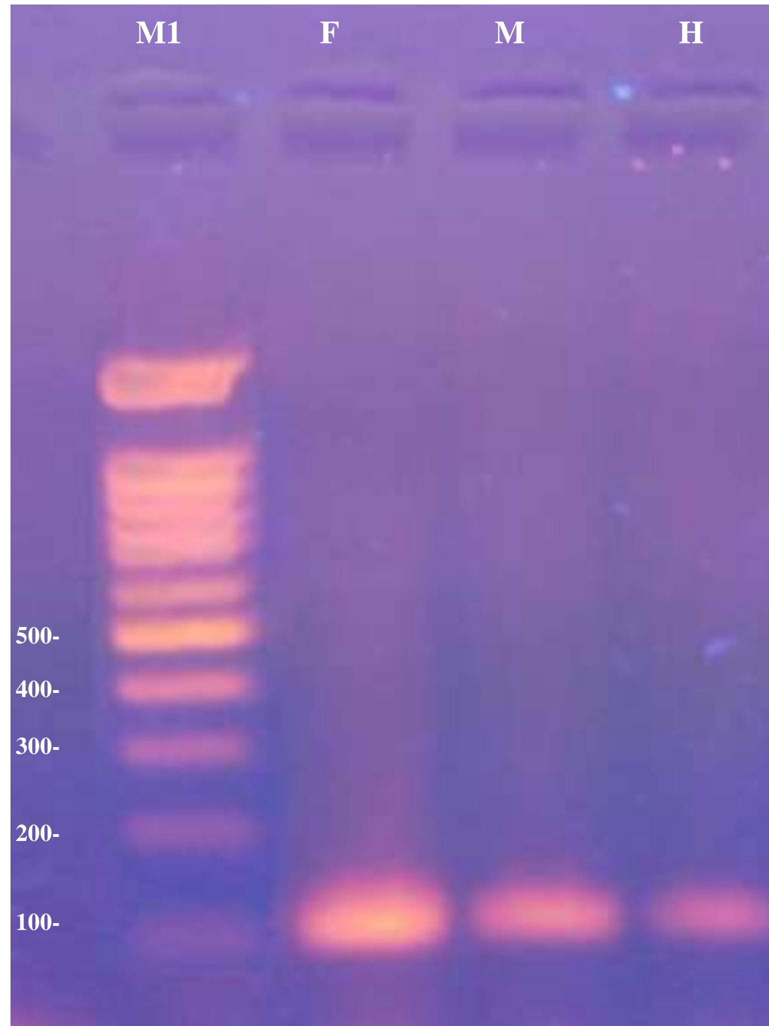

Plate 2. The SSR primer MEH SSR-04 was used for polymorphism study among parents (Female and male) and corresponding hybrid variety.

M1=100bp DNA ladder, Lane1=MEG5007, Lane2=MEG5008, Lane3=MEH5009

The above DNA amplification reveals that, the 100bp DNA fragment is common in both the genotypes. Hence, in this respect, their is no polymorphism among the parents.

Step 2.The Second Step of Hybridity Test through Molecular Marker will Include Male and Female Parent and Also the Corresponding Hybrid Sample.

Only the polymorphic SSR primer is used that was identified in previous step. The DNA banding pattern is given below:

It indicates that, 150bp DNA fragment was amplified by the molecular marker MEHSSR03 in male parent and hybrid offspring and it was absent in female parent. It was the most important DNA band which we are looking for the hybridity test. It proved that, this portion of genome

has come from male parent in hybrid variety. Hence, it is amplified only in male parent and in hybrid offspring. Normally hybrid seed is collected from the female parent. If any DNA amplification present in female parent and hybrid offspring but not in male parent than it is indicator of female genome. Hence, this type of DNA band will be confirmation of female parent. It should be discarded for hybridity test.

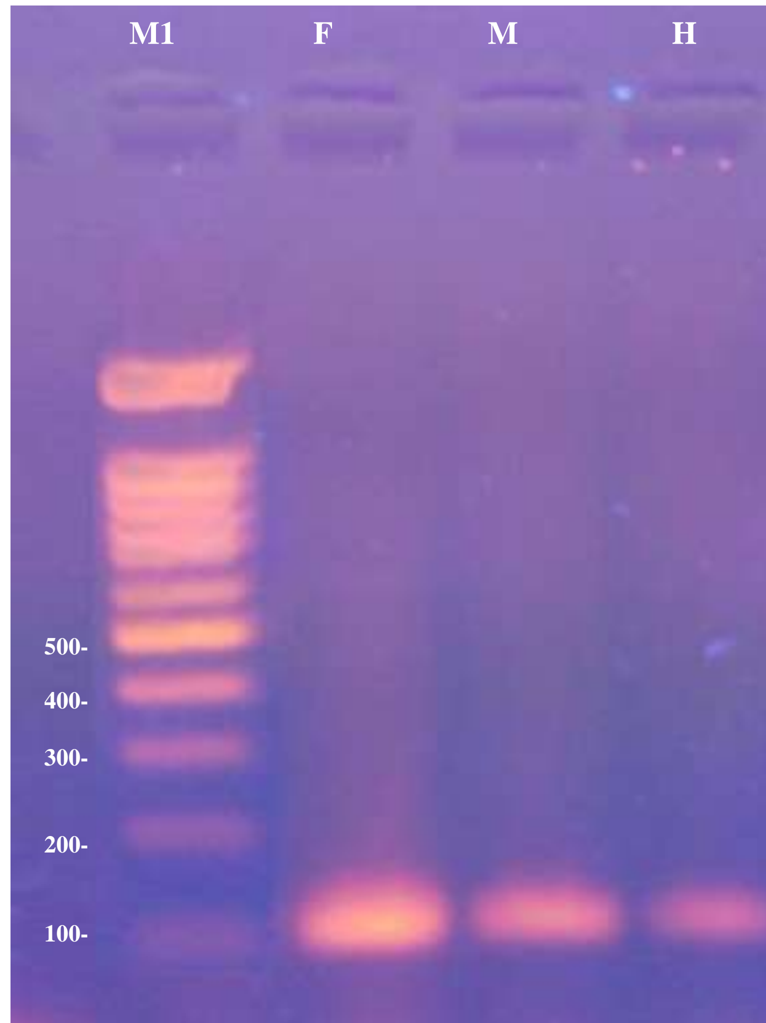

Plate 3. The molecular marker MEHSSR03 used for polymorphism study among the parents (female and male) and their corresponding hybrid variety.

M1=100bp DNA ladder, Lane1=MEG5010, Lane2=MEG5011, Lane3=MEH5012

The above discussion reveals that, for hybridity test through molecular marker need specific DNA amplification which is present in male and hybrid offspring but not in female parent. By applying this principle we done this research workhas been done in four different hybrid and its corresponding parents. The individual result is given below.

4.3 Polymorphism survey, band size and banding pattern of eggplant hybrid and their corresponding parents

Five molecular markers*viz*MEHSSR-01, MEHSSR-02, MEHSSR-03, MEHSSR-04, and MEHSSR-05 were used for hybridity detection in four eggplant hybrids and their corresponding parents. The DNA amplification status of each primer and hybrid are given in Table 5 to 9.

Table 5.DNA amplification Status of the hybrid MEH5003 and its corresponding parents.

Sl No	Primer Name	DNA amplification of the hybrid MEH5003 and its corresponding parents.			
		Primer sequence (5'-3')	Female Parents	Male Parents	Hybrid-Name MEH5003
1	MEHSSR01	For. GTGACTACGGTTTCACTGGT Rev.GATGACGACGACGATAATAGA	Not Amplified	Not Amplified	Not Amplified
2	MEHSSR02	For. AATGAGTCAGAAACCACGCC Rev.CGTTTAACCTTTGGCTCGGAA	Not Amplified	Not Amplified	Not Amplified
3	MEHSSR03	For. ACATGCCACTCATGTTGGTG Rev.CTTCAGCCAPGGACCACATT	Not Amplified	Not Amplified	Not Amplified
4	MEHSSR04	For.AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCAG	Not Amplified	Not Amplified	Not Amplified
5	MEHSSR05	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCACA	NotAmplified	Not Amplified	Not Amplified

Table 6: DNA amplification status of the hybrid MEH5006 and its corresponding parents.

Sl No	Primer Name	DNA amplification status of the hybrid MEH5006 and its corresponding parents.			
		Primer sequence (5'-3')	Female Parent	Male Parent	Hybrid Name MEH5006
1	MEHSSR01	For. GTGACTACGGTTTCACTGGT Rev.GATGACGACGACGATAATAGA	Not Amplified	Not Amplified	Not Amplified
2	MEHSSR02	For. AATGAGTCAGAAACCACGCC Rev.CGTTTAACCTTTGGCTCGGAA	Not Amplified	Not Amplified	Not Amplified
3	MEHSSR03	For. ACATGCCACTCATGTTGGTG Rev.CTTCAGCCAPGGACCACATT	Amplified	Amplified	Amplified
4	MEHSSR04	For.AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCAG	Not Amplified	Not Amplified	Not Amplified
5	MEHSSR05	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCACA	Not Amplified	Not Amplified	Not Amplified

Table 7.DNA amplification Status of the hybrid MEH5009 and its corresponding parents.

Sl No	Primer Name	DNA amplification status of the hybrid MEH5009 and its corresponding parents.			
		Primer sequence (5'-3')	**Female Parent**	**Male Parent**	**Hybrid Name MEH5009**
1	MEHSSR01	For. GTGACTACGGTTTCACTGGT Rev.GATGACGACGACGATAATAGA	Not Amplified	Not Amplified	Not Amplified
2	MEHSSR02	For. AATGAGTCAGAAACCACGCC Rev.CGTTTAACCTTTGGCTCGGAA	Not Amplified	Non Amplified	Not Amplified
3	MEHSSR03	For. ACATGCCACTCATGTTGGTG Rev.CTTCAGCCAPGGACCACATT	Not Amplified	Not Amplified	Not Amplified
4	MEHSSR04	For.AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCAG	Amplified	Amplified	Amplified
5	MEHSSR05	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCACA	Not Amplified	Not Amplified	Not Amplified

Table 8.DNA amplification Status of the hybrid MEH5012 and its corresponding parents.

Sl No	Primer Name	DNA amplification status of the hybrid MEH5012 and its corresponding parents.			
		Primer sequence (5'-3')	**Female Parent**	**Male Parent**	**Hybrid Name MEH5012**
1	MEHSSR01	For. GTGACTACGGTTTCACTGGT Rev.GATGACGACGACGATAATAGA	Not Amplified	Not Amplified	Not Amplified
2	MEHSSR02	For. AATGAGTCAGAAACCACGCC Rev.CGTTTAACCTTTGGCTCGGAA	Not Amplified	Not Amplified	Not Amplified
3	MEHSSR03	For. ACATGCCACTCATGTTGGTG Rev.CTTCAGCCAPGGACCACATT	Amplified	Amplified	Amplified
4	MEHSSR04	For.AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCAG	Not Amplified	Not Amplified	Not Amplified
5	MEHSSR05	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCACA	Not Amplified	Not Amplified	Not Amplified

Table 9. Summary of DNA amplification pattern in eggplant hybrid

Primer Number	Primer Name	Sequence of Primer (5'-3')	(G+C) %	Number of DNA band(s)	Number of Hybrid	Band size ranges (bp)
1	MEHSSR01	For. GTGACTACGGTTTCACTGGT Rev.GATGACGACGACGATAATAGA	46			
2	MEHSSR02	For. AATGAGTCAGAAACCACGCC Rev.CGTTTAACCTTTGGCTCGGAA	49			
3	MEHSSR03	For. ACATGCCACTCATGTTGGTG Rev.CTTCAGCCAPGGACCACATT	50	1	MEH5006 MEH5012	150,100
4	MEHSSR04	For.AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCAG	43	1	MEH5009	100
5	MEHSSR05	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCACA	44			

It reveals that, only two primer *viz* MEHSSR-03 and MEHSSR-04 showed DNA amplification in three hybrids and their corresponding parents.

The molecular marker MEHSSR-03 produced 100bp DNA fragement in both male and female parents of the hybrid MEH5006 and MEH5012. The same amplification was also noticed in the hybrid variety (Plate 4).

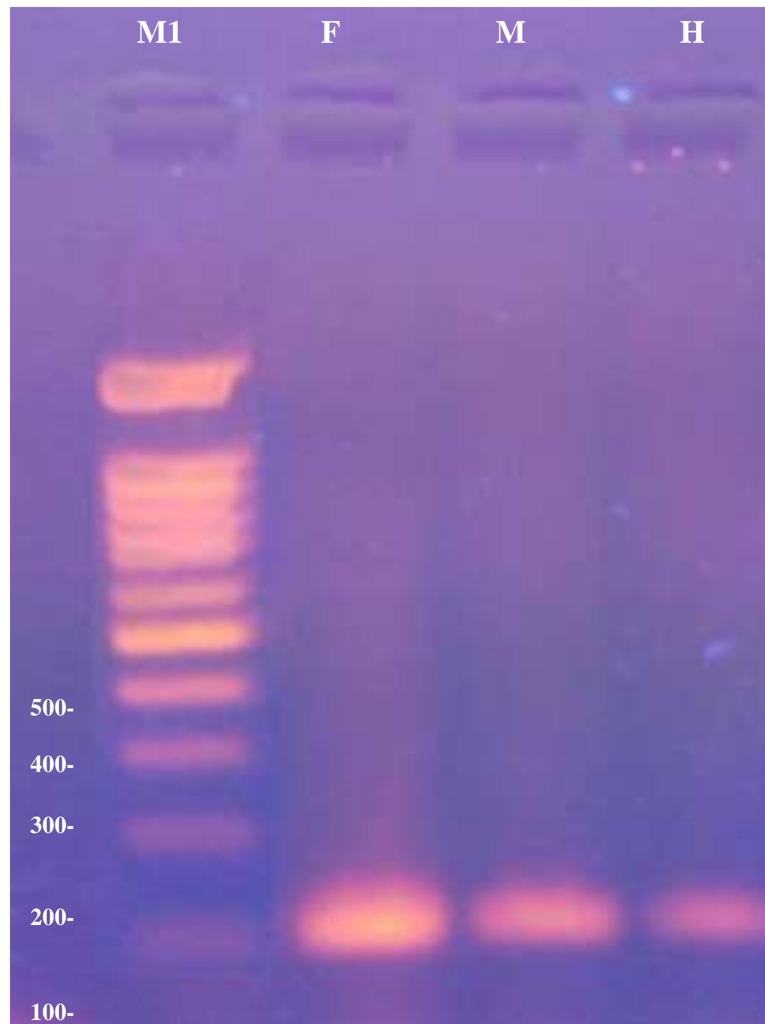

Plate 4. The molecular marker MEHSSR03 used for polymorphism study among the parents (female and male) and their corresponding hybrid variety.

M1=100bp DNA ladder, Lane1=MEG5010, Lane2=MEG5011, Lane3=MEH5012

It indicates that, the monomorphic DNA band present in all three genotypes. It proved that, this primer was not suitable for hybridity test for the specific hybrid MEH5012. No polymorphism also occured in the parent of the hybrid MEH5009 by the same primer. Hence, it can not be used for the hybridity detection of the hybrid MEH-5006.

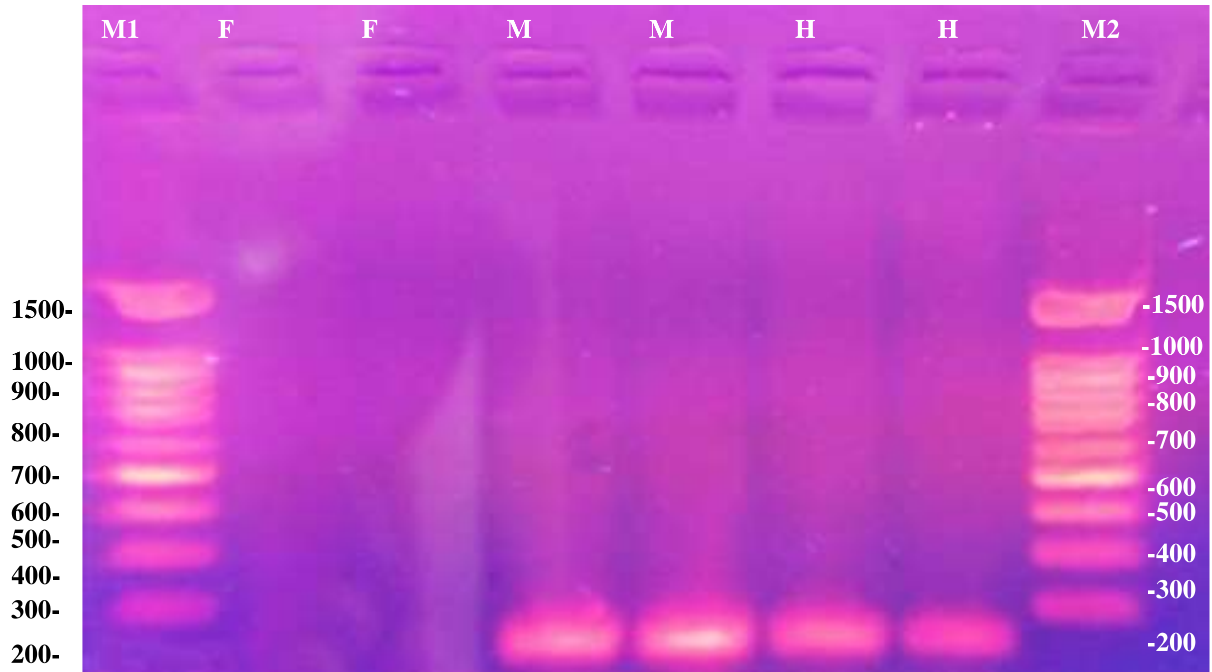

Plate 5.PCR amplification with SSR primer MEHSSR-03.

M1 & M2 = 100 bp DNA ladder, Lane 1= MEG5004, Lane 2 =MEG5004, Lane 3 =MEG5005, Lane 4 = MEG5005 Lane 5 = MEH5006, Lane 6 =MEH5006.

Lane 1 & 2 is female parent, lane 3 & 4 is male parent and lane 5 & 6 is hybrid variety of eggplant.

It is interesting to note that, the primer MEHSSR-04produced polymorphic DNA fragment among the parent of the hybrid MEH5006 (Plate 6). The primer gave 150bp DNA band in male parent and it was absent in female parent. The hybrid variety also showed the male parent type of DNA band. The replicated trial also showed the same pattern of DNA amplification.

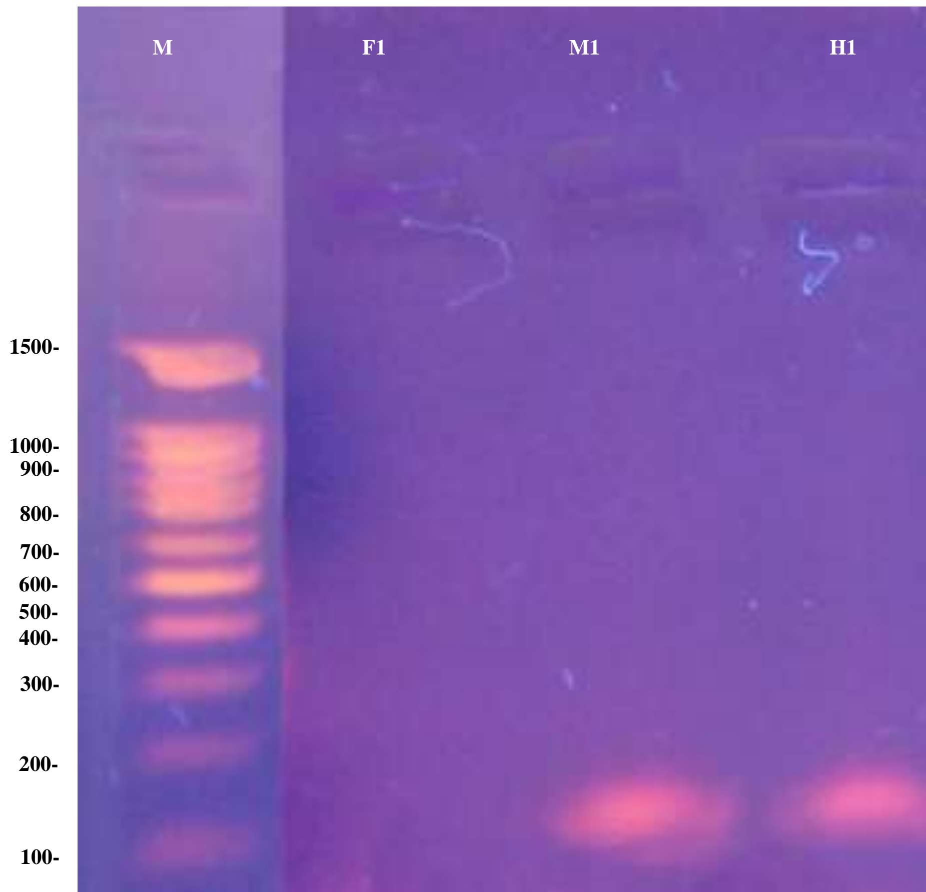

Plate 6. PCR amplification with SSR primer MEHSSR-04

M = 100 bp DNA ladder, Lane 1= MEG5007, Lane 2 =MEG5008, Lane 3 =MEH5009

It is discussed in the principle of hybridity test that, if a primer produces DNA band in the male parent but not in the female parent and same band in hybrid genome this type of amplification can be used for hybridity test. The detail justification and scientific reason is mentioned in previous part. Fifty percent (50%) of male genome was transferred to hybrid variety through pollen grain. Hence, the DNA fragment which was present in male and hybrid genotype but not in female genotype is the indicator of hybrid variety. Therefore, the primer MEHSSR-03 will be used for hybrid detection of the specific hybrid MEH5006 (Plate 7).

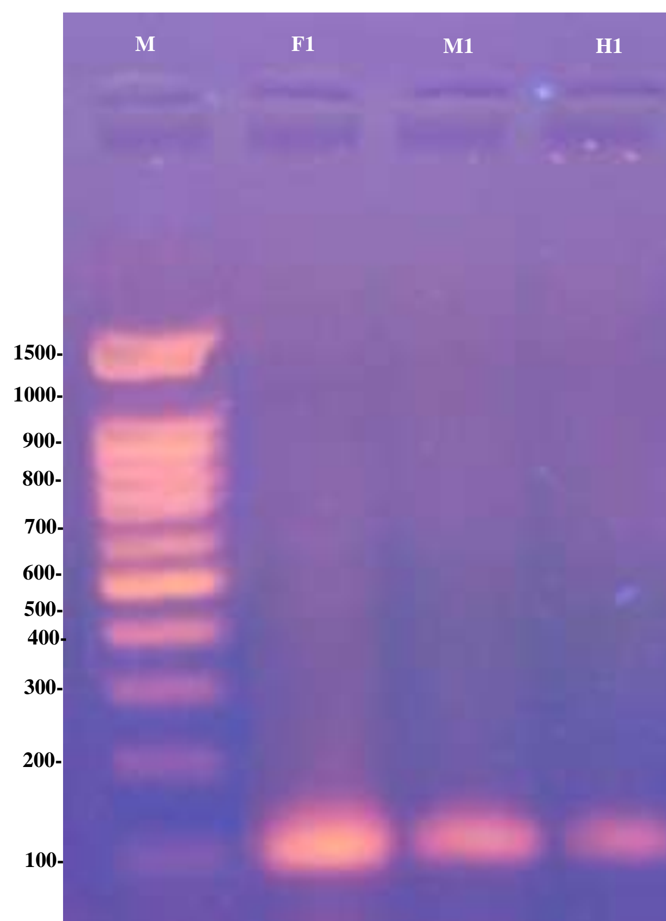

Plate 7. DNA banding pattern of the hybrid MEH5012 and its parent by the molecular marker MEHSSR-03

M = 100 bp DNA ladder, Lane 1= MEG5010, Lane 2 =MEG5011, Lane 3 =MEH5012

CHAPTER V

SUMMARY AND CONCLUSION

Eggplant (*Solanummelongena*L.)is an important crop in Bangladesh as well as in all over the world for its economical and medicinal values. Being a poorly studied genome, little information is available on the molecular characterization of eggplant hybrid.This is why, there is a need to conduct studies to evaluate the genetic diversity,genetic purity, polymorphism study and molecular diversity analysis of eggplants for breeding and conservation purposes. This research investigation presented mainly the hybridity test of eggplant hybrid through molecular marker. The present experiment was conducted at Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207.

Seed is the most important input which contributes a lot for higher production. Seed security is an important issue for sustainable agriculture development. The seed which has high yielding capacity, extra purity and having more than 90% germination capacity is known a quality seed. Till late eighty, BADC was the only organization to supply quality seeds to the farmers. By the passage of time many private organizations have been developed to supply seeds among the farmers. Vegetables seeds get more attention by the private sector due to its more profitability. Hybrid seed technology is a new dimension in seed industry. Hybrid seeds show better performance in respect of size, vigor, yield and resistance to pests and diseases compared to traditional seeds. Hence, it got rapid popularity to the farmers. It captures the market in such a way that "farmer known quality seed means hybrid seed". In last two decades a big market was developed on hybrid seed importing, trading and other seed delivery systems. Many private organizations have established their Research and Development (R&D) for hybrid varieties. Among them LalTeer Seed Ltd. (LTSL), Supreme Seed, ACI Seed, Metal Seed etc. are promising seed industries in Bangladesh.

Hybridity test through molecular maker is a new concept in the field of applied molecular biology in agriculture. The present finding indicated that SSR primer can be used for detection of polymorphism among the parents of any hybrid. The identified polymorphic primer can be used for hybrid identification for commercial purpose.

The scientific aspect of research proposal is original and till now not a single seed company expresses this idea to identify the hybrid seed. All the seed companies producing and selling hybrid seeds can use this molecular technique. It will save money, time and manpower than traditional methods of hybridity test. The idea would be accepted by policy makers and govt. of authority to ensure quality seed. The farmers will not be cheated by the seed companies and they will be able to get pure seeds of hybrid variety.

This study shows that SSR markers can be used to successfully detect hybrids in eggplants. Screening SSR markers for polymorphism showed that different eggplant genotypes can be represented by different alleles which is helpful in identifying true hybrids among progenies. The use of SSR markers provided an early detection method to select and screen out plants even at an early stage of development.

Different SSR primers (MEHSSR01, MEHSSR02,MEHSSR03,MEHSSR04,MEHSSR05) were used to establish polymorphism among the parents of each hybrid. The identified polymorphic primers were used to screen the hybrid variety together with corresponding parents. It was found that only one primer *viz* MEH SSR-04 showed polymorphism in the parents of MEH5009 hybrid. This primer was used for the detection of hybrid variety. It was noted that, a 150bp DNA fragment was amplified in male parent and hybrid variety which was absent in female genotype. This DNA band can be used for large scale screening of that specific hybrid variety for seed purity in commercial purpose. The parents of other hybrid showed monomorphic DNA band and some of the SSR primers were not amplified in any eggplant genotype under investigation.

Molecular techniques for identification of hybrid seed has great potentiality in respect of commercialization under Bangladesh condition. The developed protocol can be used for hybridity test in any crop. Molecular technique for hybridity test is safer, more reliable and less time consuming. The commercial aspects of new innovation may be viable for all the hybrid seedinstitute of Bangladesh.

RECOMMENDATION

Larger number of samples and higher number of primers would be necessary to generate and construct an appropriate genetic relationship, sample identification and analysis of genetic variation among different varieties and cultivars.

The present work was the preliminary study to assess hybridity, genetic variation of eggplant genotypes and it had some limitations in term of limited number of individuals and varieties as well as number of primers used. The results indicate that the present study might be used as a guideline for further study and the following points might be considered for sustaining the genetic qualities of eggplant in Bangladesh:

1. Large number of hybrid varieties and higher number of primers should be studied with adequate replications to increase accuracy.
2. Details survey work should be conducted using more molecular markers for obtaining polymorphism among the parents.
3. Other molecular markers such as RAPD, AFLP, etc. should be used for further confirmation.

Molecular techniques for identification of hybrid seed has great potentiality in respect of commercialization under Bangladesh weather condition. The developed protocol can be used for hybridity test in any crop.Molecular technique for hybridity test is safer, more reliable and less time consuming. The commercial aspects of new innovation may be viable for all the hybrid seedinstitute of Bangladesh.

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