DNA FINGERPRINTING OF BRINJAL (*Solanum melongena* L.) VARIETIES AND RELATED SPECIES

By

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(2011-11-102)

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KERALA, INDIA
2013

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THESIS

Submitted in partial fulfillment of the requirement

for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

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KERALA, INDIA

2013

DECLARATION

I hereby declare that the thesis entitled **"DNA fingerprinting of brinjal (*Solanum melongena* L.) varieties and related species."** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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ACKNOWLEDGEMENT

I bow to the lotus feet of ***Almighty*** *whose grace had endowed me the inner strength and confidence and blessed me with a helping hand at each step during my long sojourn at Kerala.*

At this moment of accomplishment, my heart is overwhelmed with gratitude and I wish if these words could convey the subtle feelings.

I am ineffable in expressing my deep sense of gratitude to my Major Advisor ***Dr. P. A. Nazeem,*** *Professor & Co-ordinator (DIC), CPBMB, College of Horticulture, Vellanikkara, for her inspiring guidance, critical comments, constant supervision, support and encouragement throughout the course of my research work. I am really grateful for the keen interest taken by her in the prompt correction of the manuscript.*

My sincere thanks are due to, ***Dr. P. A. Valsala,*** *Professor and Head, CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for the valuable guidance, timely suggestions and help rendered in the conduct of the experiment.*

I also owe my deep sense of gratitude and sincere thanks to ***Dr. R. Keshavachandran,*** *Professor, CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for the his timely help, critical scrutiny of the manuscript and valuable guidance for the successful completion of my research work.*

I am extremely thankful to ***Dr. Deepu Mathew,*** *Assistant Professor, CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for his support, suggestions and guidance throughout the study.*

I take this opportunity to express my indebtedness with veneration and devotion to ***Dr. P. Indira,*** *Professor, Department of Olericulture, College of Horticulture, Vellanikkara,, member of my advisory committee for her constant support, suggestions, valuable guidance and timely help rendered in the conduct of the experiment.*

Words fall short as I place on record my indebtedness to ***Dr. Abida, and Dr. Shylaja*** *of CPBMB for their untiring support and valuable guidance during my course of study.*

My sincere thanks to ***Shailaja, Simi, Divya, Priya, Anu, Sreela, Githu*** *and* ***Nithin*** *of CPBMB, who helped me in several ways for the completion of this venture.*

I wish to express my sincere thanks to all the non-teaching staff members and labourers of CPBMB for their whole-hearted cooperation and timely assistance. I also appreciate and acknowledge the facilities provided at ***CPBMB*** *and* ***Bioinformatics Centre*** *for the successful completion of my research work.*

My heartfelt gratitude cannot be captured in words for the unflinching support, constant encouragement, warm concern, patience and valuable advice of my friends ***Ravindra, Subhasini, Sharda, Pujaita, Mani, Shiny, Subbareddy, Ningaraju & Naveen,*** *whose prayers, love and affection rendered me a successful path which propped up my career all along. I wish to express my sincere gratitude to my senior friends* ***Amar, Pawan, Promod, Narender, Rohit, Razzaq, Meera, Renju, Rasmi, Achintya, Ashok, Rakesh, Manjunath, Rajesh, Varun, Alex, Rohini, Sreeja, Jusna & Aj*** *for their whole hearted support.*

My duty is incomplete if I forget my junior friends ***Shital, Vaishali, Renuka, Shriram, Sachin, Aoudumbar, Yogesh, Datta, Yashwant, Purushottam and Dheemant*** *whose helping hands, love and affection fetched a remarkable place in my days in Kerala.*

The award of ***DBT fellowship*** *is greatly acknowledged.*

Above all, I am forever beholden to ***my beloved parents, my brothers, my sisters and my family members*** *for their unfathomable love, boundless affection, personal sacrifice, incessant inspiration and constant prayers, which supported me to stay at tough tracks.*

Vikhe Parimal Laxman

Dedicated to my beloved parents and guru

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ABBREVIATIONS

%	Percentage
>	Greater than
µg	Microgram
A	Ampere
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
bp	Base pair
cc	*cubic centimetre*
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DAF	DNA amplification fingerprinting
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed sequence tags
g	Gram
GD	Genetic Distance
ha	Hectare
ISSR	Inter Simple Sequence Repeat
kb	Kilo basepairs
L	Litre
M	Molar
MAS	Marker Assisted Selection
mg	Milligram
ml	Millilitre
mM	Milli mole
NBPGR	National Bureau of Plant Genetic Resources
ng	Nanogram

QTL	Quantitative trait loci
ºC	Degree Celsius
OD	Optical Density
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SRAP	Sequence Related Amplified Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsatellite Sites
STR	Short Tandem Repeat
STS	Sequence Tagged Sites
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Brinjal also known as eggplant (*Solanum melongena* L., 2n=2x=24) is one of the few cultivated solanaceous species originated in the old world. India along with Indo-China is considered to be the centre of its diversity (Vavilov, 1951; Lester and Hasan, 1991). It is a major vegetable crop in several countries like India, Japan, Indonesia, China, Bulgaria, Italy, France, USA and many African countries. It is an economically important vegetable crop of India with the production of 12,634.13 MT from an area of 691.54 ha and average productivity of 18.2 MT/ha (NHB, 2012).

The consumption of fruits and vegetables is important in improving the defence mechanism in human body against various ailments, and thus there is a growing interest among consumers for the health benefits from foods. Brinjal fruit serves as a good source of minerals and vitamins, and hence, it is important for human nutrition (Kalloo, 1988). It is also compared with tomato in terms of total nutritional value. It has been used in traditional medicines for treatment of asthma, bronchitis, cholera, dysuria and for lowering blood cholesterol (Khan, 1979).

Various cultivars of brinjal and its wild, weedy forms found in India and Indo-China are grouped in *S. melongena* complex, and *S. insanum* has been regarded as the progenitor of cultivated brinjal (Lester and Hasan, 1991). The cultivated brinjal germplasm, including a wide range of landraces and cultivars, is further divided into three sub-heads based on fruit shape *i.e.,* var. *depressum* (dwarf, early types), var. *esculenta* (round or egg-shaped fruits) and var. *serpentinum* (long slender forms) (Choudhury, 1976).

Solanum is a large genus. Among 22 Indian species, there is a group of five related species namely, *S. melongena*, *S. incanum* sobriquet *S. coagulans*, *S. xanthocarpum*, *S. indicum* and *S. maccani* (Ram, 2012). *S. incanum*, a wild form of *S. melongena* is found in southern India and it has also been described as *S. cumingii*. Under domestication process this wild form gave rise to *S. ovigerum* (small round/oblong fruits with white green or violet colour) which evolved progressively into advanced cultivars with large fruits. *S. insanum*, widespread in India is probably

a form of *S. ovigerum* which reversed to the wild state with strong prickliness. Later on all these taxa were brought under the umbrella of *S. melongena* (Ram, 2012). Another important wild relative of *S. melongena* is *S. macrocarpon* also known as tomato brinjal, is one among the 22 Indian species having thick leaves, blue/violet flowers, white coloured fruits (Behera and Singh, 2002).

Till now, a large number of brinjal cultivars are known and are characterized by variation in morphology (growth habit and plant vigour, hairiness and prickliness, fruit colour, size and shape), physiology (earliness of flowering, water need and uptake) and biochemical features (Daunay *et al.,* 2001). Conventionally, morphological descriptors are used for establishing the identity of varieties in various crops but these suffer from many drawbacks such as influence of environment on trait expression, epistatic interaction etc. Electrophoresis of seed protein and Isozyme analysis has overcome these limitations to some extent in recent past. Nevertheless, many powerful DNA based techniques are now available for identifying genotypes since all genetic differences between individuals are laid down in the primary sequence of their genomic DNA. Different methods *viz*., RFLP, RAPD, AFLP, SSR, ISSR assay, are now reported for identification of many crop varieties (Kumar, 2005).

Enforcement of trade related aspect of intellectual property rights agreements (TRIPs) under Word Trade Organisation (WTO) has resulted in worldwide shift from free exchange and unhindered exploitation to controlled access to plant genetic resources. Intellectual property rights of plant breeders and farmers need to be protected either by adaptation of patent system or some form of effective *sui generis* system. United Nation (UN) convention on biological diversity (CBD) (1993) recognizes the sovereignty of nations over their plant genetic resources and rights of farming community to receive compensation for direct and indirect commercial exploitation of traditional varieties.

To comply with these international developments, India has enacted Protection of Plant Varieties and Farmer's Rights Act (PPVFR) to provide legal framework for plant breeders and farmers rights. Enforcement of this act and eventual increased private sector investment would mean greater ownership related

disputes in future (Bhat, 2008). DNA fingerprinting through its high precision in indentifying plant genotypes, holds considerable promise as a reliable tool for intellectual property protection of crop varieties and germplasm. Different molecular marker techniques are accessible today for fingerprinting plant germplasm but information on their relative efficacy in particular crop is not clear.

Kerala Agricultural University is a pioneer public sector institute in breeding and crop improvement and several varieties including hybrids of vegetables have been released so far (Rajan and Prameela, 2004). In brinjal crop, four varieties including one F1 hybrid have been released and several others are in pipeline. Molecular data have not been developed for these varieties and since genetic variability detected through molecular markers in Solanaceous crops is relatively low, the relationship of cultivated species with its wild relatives also need to be elucidated for further exploitation.

In this context the present study was taken up with the following specific objectives:

1. To characterize four brinjal varieties, three accessions and two wild relatives using molecular markers.

2. To develop DNA fingerprint specific to the four varieties released by KAU.

Review of Literature

2. REVIEW OF LITERATURE

2.1 General background

Brinjal along with potato, tomato, pepper and tobacco belongs to the Solanaceae family and are economically important crops. *Solanum melongena* L., the cultivated brinjal, was originally described by Linnaeus, (1753) on the basis of plants which were cultivated in Asia, Africa and America. A large number of cultivars are now known and characterized by their variability in morphology, physiology and biochemical features (Daunay *et al.*, 1991). At the beginning of the 20th century, a very refined study on the family Solanaceae, focusing on the largest genus *Solanum* was done for its characterization (Daunay *et al.*, 2001). Other taxonomists have also contributed to the characterization of this genus, considering specific characters of taxonomic significance (Correll 1962; Seithe and Anderson 1982; Whalen 1984; Bohs, 1999). The vast amount of available literature has however, led to a considerable confusion surrounding the genus, for which 1,000 to 1,400 *Solanum* species have been associated to more than 3,000 binomial names (Daunay and Lester, 1988).

India and Indo-China region represent the centre of brinjal diversity (Vavilov, 1951; Lester and Hasan, 1991), but the affinities of brinjal (*S. melongena*) to related species remain uncertain. Taxa that are morphologically similar to brinjal are difficult to classify (Karihaloo and Gottlieb, 1995), and the delimitation of the cultivated brinjal from the weedy forms *Solanum insanum* and its wild progenitor *Solanum incanum* is unclear (Lester and Hasan, 1990). It is also recognized that *S. incanum* taxa described for Indian lines are distinct from those in Africa and the Middle East (Lester and Hasan, 1991). Furthermore, Lester and Hasan (1991) noted that both *S. melongena* and *S. incanum* have been frequently confused with the less closely related scarlet brinjal *Solanum aethiopicum* L., the gboma brinjal *Solanum macrocarpon* L. and with other wild species.

Kerala Agricultural University (KAU) has released three varieties of brinjal *viz*., Surya, Swetha, Haritha and one F_1 hybrid Neelima, having different yield and

morphologically important traits. In addition to these varieties, KAU is also having a large collection of brinjal accessions, having resistance to bacterial wilt (Rajan and Prameela, 2004).

2.1.1 Important characters for morphological scoring

Wide morphological diversity has been reported in brinjal and their wild types for plant morphology (inflorescence, leaf and fruit), physiology and biochemical properties (Collonier *et al.,* 2001). Fruit shape, size, colour and taste are the most noticeable characters that vary among the individuals (Fray *et al.,* 2007). Morphological diversity among the Solanaceae family members which cover genus, species and cultivars is really noteworthy (Knapp *et al.,* 2004). Flowers, fruits and leaves are important plant parts used commonly in taxonomy as they were targets of the domestication process (Doganlar *et al.,* 2002, Knapp *et al.,* 2004).

2.1.1.1 Vegetative characters

S. melongena has a wide diversity in plant morphology such as plant growth habit, vigour, hairiness, prickliness, fruit shape, size, colour and yield potentials. Fruit colour, size and shape are the most distinctive characters that vary between the cultivated *solanum* species and their wild types (Kumar *et al*., 2008).

2.1.1.2. Reproductive characters

Brinjal is an annual herbaceous plant. Inflorescence is often solitary but sometimes it constitutes a cluster of 2 to 5 flowers. Solitary or clustering of inflorescence is a varietal character. Flower is complete, actinomorphic and hermaphrodite. Calyx is five lobed, gamosepalous and persistent. It forms cup like structure at the base, corolla is five lobed gamopetalous with margins of lobes incurved. There are five stamens which are free and inserted at the throat of corolla, anthers are cone shaped free and apical dehiscence. Ovary is hypogynous, bicarpellary, syncarpous and with basal placentation. In brinjal, heterostyly is a common feature. Four types of flowers have been reported depending on the length of styles, *viz.,* (i) long-styled with large ovary, (ii) medium-styled with medium size

ovary, (iii) Pseudoshort-styled with rudimentary ovary and (iv) true short-styled with very rudimentary ovary (Krishnamurthi and Subramaniam, 1954).

Brinjal is usually self-pollinated but the extent of cross pollination is reported as high as 29 per cent and hence, it is classified as often cross pollinated or facultative cross pollinated crop. Out crossing takes place with the help of insects.

Flowers generally emerge 40 to 45 days after transplanting. Anthesis occurs at about 6 to 8 a.m. and usually between 9.30 and 11.15 a.m. during winter. Stigma receptivity is highest during anthesis. Anthers usually dehisce 15 to 20 min. after the flowers have opened. The receptivity of the stigma can be observed from its plump and shiny appearance which gradually becomes brown with the loss of receptivity. The period of effective receptivity ranges from a day prior to flower opening until about four days after opening. Pollen usually remains viable for a day during summer and 2-3 days in winter under field conditions (Nothmann *et al.*, 1983).

2.1.1.3 Characteristics of the genotypes identified for the study

Surya (SM 6-7) is a high yielding variety developed by single plant selection method from an Annamalai collection SM 6. This variety was released in the year 1992. It is also resistant to the bacterial wilt caused by *Ralstonia solanacearum* E. F. Smith. Its morphological futures are non prickly stem and leaves, green leaves with violet tinged vein, glossy, oval and medium sized purple coloured fruits.

The variety Swetha (SM 6-6) is developed from Annamali collection (SM 6) by single plant selection method. It is a high yielding variety released in the year 1997. It is having bushy growth habit with light purple tinge on the leaf stalk, violet flowers, plant non prickly, fruits white, medium to long (12.67 cm), born solitary or occasionally in clusters. It is resistant to bacterial wilt and tolerant to fruit borer under field condition.

The variety Haritha (SM 141) is a local collection from Cochin. It was developed by single plant selection method and released in 1998. Spreading habit, non prickly stem, green leaves and vein, white flowers, light green, long and fleshy

fruits with less seeds are the key characters. This variety is also resistant to bacterial wilt caused by *Ralstonia solanacearum* E. F. Smith.

Neelima is the high yielding hybrid with Surya and SM 116 as its parents. It was released in 1998. Spreading habit, non prickly stem, green leaves with violet veins, purple flowers, large oval to round, glossy violet fruits are the important morphological characters and it is also resistant to bacterial wilt (Rajan and Prameela, 2004).

The accessions SM 396 and SM 397 are the better performing genotypes identified at KAU for their high yield and disease resistance. The genotupes are under multi locational trials prior to release as variety.

S. melongena var. *insanum* are shrubs of 75 cm size, leaves alternate, angulate, prickly along the nerves, base obliquely truncate, apex (sub)acute; petiole also prickly, flowers 1-4, extra-axillary, corolla purple to violet, acute, berry oblong-globose, small and solitary, 3 cm across, fruiting calyx enlarging, seeds minutely pitted (Martin and Rhodes, 1979).

S. macrocarpon is a wild relative grown for its large, glabrous leaves, used as a green vegetable. Fruits have large, often clasping calyx. They are sub spherical and large (3-10 cm in diameter, 2-6 cm long), cream white, green-white or green. Fruits are sweeter in taste and most preferred. At full maturity fruits turn yellow, orange or brown with cracked surface (Bukenya and Carasco, 1994).

2.2 Genetic markers in plants

Genetic markers are measurable inherited genetic variations, used to understand genetic components. There are different types of genetic markers with different properties, each having its own advantages and disadvantages to assess the genetic variations among natural populations. Currently, the most commonly used genetic markers are molecular markers. Due to the rapid developments in the field of molecular genetics, a variety of different techniques have emerged to analyze genetic variation during the last few decades (Whitkus *et al.,* 1994; Karp *et al*., 1996, 1997a, b; Parker *et al.*, 1998; Schlötterer, 2004). These genetic markers may differ with

respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate and important genetic marker will depend on its specific application, the presumed level of polymorphism, the presence of sufficient technical facilities or know-how, time constraints and financial limitations. Generally markers are divided in to three broad classes: those based on visually assessable traits (morphological and agronomic traits), those based on gene product (biochemical markers), and those relying on a DNA assay (molecular markers). Genetic markers are widely used by breeders and conservationists to study genetic diversity and to assist in crop improvement.

2.2.1 Morphological markers

The earliest studies of genetic characterization and divergence are based on morphological markers such as qualitative and quantitative traits (Arriel *et al.,* 2007). These markers are inexpensive and simple to score and are based on distinct phenotypes such as plant colour, plant height, seed characteristics, etc. The apparent disadvantage of such markers is prominent in studies of genetic diversity where the expression of the phenotype is highly influenced by environmental conditions. Lack of adequate genome coverage because of limitation of the number of markers and problems of dominance can also be mentioned as weaknesses of morphological markers (Brown, 1978). Furthermore, the expression of these characters which are influenced by the environment may require that plants be grown to a suitable stage before certain characters can be scored.

2.2.2 Biochemical markers

Biochemical markers are markers derived from study of the chemical products of gene expressions. These are also termed as isozyme/allozyme markers or protein markers. According to Karp *et al*. (1997), the agro-morphological markers resulted in the development of biochemical markers that complement its drawbacks. According to Gottlieb (1981) the oldest biochemical technique used to study variations is isozyme analysis. It has the power to reveal polymorphism of alleles at

particular locus on the basis of protein mobility (Brown *et al.,* 1978). Isozyme technique is fast, cheap and simple. However, isozyme markers are not as plentiful as DNA markers; these underestimates the level of genetic diversity (Dudnikov, 2003) and sometimes interpretation of bands become difficult due to complex banding profiles arising from polyploidy or duplicate genes. In addition, proteins with identical electrophoretic mobility (co-migration) may not be homologous (Morell *et al.,* 1995). Isozyme studies in plants have demonstrated differences in pattern and band intensities by tissue types and developmental stages (Montarroyos *et al*., 2003). Although isozymes have limitations but the technique was used for genetic diversity analysis in many species (Dudnikov, 2003) and it appeared to be more informative at lower taxonomic levels, particularly at species and population level characterization (Brown, 1990). Isozyme markers were applied to characterise *Coffea Arabica* (Berthaud *et al.,* 1988). However, their use on wild *C. arabica* species characterizations from Ethiopia had failed to reveal polymorphism, indicating that isozyme technique is not appropriate for diversity study in *Coffea arabica* due to the small number of isozyme systems available (Berthaud *et al.,* 1988). Paillard *et al*., (1996) also tried to construct isozyme based genetic map for coffee and were unsuccessful due to the low polymorphism level.

2.2.3 Molecular markers

Molecular markers are fragments of nuclear, mitochondrial or chloroplast DNA, with specific sequences such as RFLP, RAPD, AFLP, ISSR, SSR etc. These technologies based on polymorphism in DNA, can be considered as objective measures of variations and have catalyzed research in a variety of disciplines such as phylogeny, taxonomy, ecology, genetics and plant breeding. Markers are informative only if, they are polymorphic in populations. Level of polymorphism is an important determinant of what a marker is useful for. Different types of molecular markers with different properties exist, each with its own advantages and disadvantages (Karp *et al.,* 1997; Weising *et al.,* 2005). However, it is extremely difficult to find molecular markers which could adequately meet all the ideal properties (Lowe *et al*., 2004). Depending upon the type of the study to be undertaken, one can identify between varieties of marker systems that could fulfill the objective of the study

(Weising *et al*., 2005). Many authors also suggest the use of more than one type of molecular marker in a single experiment (Karp *et al.,* 1997). The DNA based marker systems are generally classified as hybridization-based (non-PCR) markers and PCR based markers (Joshi *et al*., 1999).

2.3 PCR- based molecular marker techniques

DNA fingerprinting is a technique, which has been widely adapted to differentiate organisms at the species and subspecies levels (McClean *et al*., 1994). The techniques used for cultivar identification are designed to detect the presence of specific DNA sequences or combination of sequences that uniquely identify the plant. Cultivar identification can be achieved more accurately using DNA fingerprinting data, especially in materials characterized by high genetic variations between cultivars. The most closely related cultivars are usually distinguished with the DNA fingerprinting methods (Beckmann and Soller, 1986). The application of DNA fingerprinting could be very valuable in the identification of cultivars and species and could help to create more efficient breeding programs through the detection of genetic linkages between DNA fingerprinting bands and agriculturally important quantitative trait loci (QTL). The high variability of DNA fingerprinting described in humans, animals and plants allows the identification of different individuals, genotypes, and species (Lin *et al*., 1993).

2.4 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) technique has been the basis of a growing range of new techniques for genome analysis based on the selective amplification of genomic DNA fragments (Saiki *et al*., 1988). Williams *et al.* (1990) reported the use of PCR with short oligonucleotide primers of arbitrary (random) sequence to generate markers, the basis of the Random Amplified Polymorphic DNA (RAPD). Welsh and McClelland (1990) also reported on Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). DNA Amplification Fingerprinting (DAF) was also reported as another technique of PCR used in various experiments (Caetano-Anolles *et al*., 1991). The PCR reaction requires deoxynucleotides, DNA polymerase, primer, template and buffer containing magnesium (Taylor, 1991).

Typical PCR amplification utilises oligonucleotide primers which hybridise to complementary strands. The product of DNA synthesis of one primer serves as template for another primer. The PCR process requires repeated cycles of DNA denaturation, annealing and extension with DNA polymerase enzyme, leading to amplification of the target sequence. This results in an exponential increase in the number of copies of the region amplified by the primer (Saiki *et al*., 1988). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi.

The introduction of the PCR technique has revolutionized standard molecular techniques and has allowed for the proliferation of new tools for detecting DNA polymorphism (Hu and Quiros, 1991). The electrophoresis pattern of fragments generated by each primer for one isolate can be used as DNA fingerprints for assaying diversity (Tommerup *et al*., 1998). Polymorphism between two individuals is generally scored as presence or absence (non-amplification) of a particular DNA fragment. The absence may result from deletion of a priming site or insertion rendering site too distant for successful amplification. Insertion can change the size of a DNA fragment without preventing its amplification (Williams *et al*., 1990). PCR is simple, fast, specific, sensitive and the main advantage of this technique over others is its inherent simplistic analysis and the ability to amplify extremely, small quantities of DNA (Welsch *et al*., 1991).

2.5 Molecular markers for varietal characterization

Markers based on differences in DNA sequences between individuals generally detect more polymorphism than morphological and protein based markers and thus constitutes a new generation of genetic markers (Bostein *et al*., 1980; Tanksley *et al*., 1989). Hence, varietal profiling methods that directly utilize DNA could potentially address all the limitations associated with morphological and biochemical data. Cultivar identification using DNA fingerprinting is currently being investigated in a number of laboratories using different methods (Weising *et al*., 1995).

Polymerase chain reaction (PCR) based techniques make use of random or specific primers to amplify random or specific DNA fragments from the genome. They are simple to perform, easily amenable for automation and used to assay a large number of samples. These include Randomly Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995), Simple Sequence Repeats (SSR) (Tautz,1989), Inter Simple Sequence Repeats (ISSR), DNA Amplification Fingerprinting (DAF), Sequence Tagged Sites (STS), Sequence Characterized Amplified Regions (SCAR), Expressed Sequence Tags (EST) etc. Of these, RFLP and SSR are co-dominant markers, while others are largely dominant markers. Out of the several molecular marker systems available, ISSR and SSR markers were utilized in the present study.

2.7 Inter simple sequence repeats (ISSR) markers

ISSRs are DNA fragments of about 100-3000 base pairs located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). About 10-60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. The ISSR primer is composed of microsatellite sequences either unanchored (Gupta *et al*., 1994; Wu *et al*., 1994) or anchored at the 5` or 3` end by two or four arbitrary nucleotides (Zietkiewicz *et al.*, 1994; Fang *et al*., 1997). The sequence between the two binding sites in opposite orientation within suitable distance is amplified and loss or gain of binding sites are detected as band polymorphism (Yang *et al*., 1996). The addition of a different base at the 5` or 3` end renders their binding sites more specific and reproducible (Barth *et al.*, 2002).

ISSR method has been used extensively to identify and determine relationships at the species and cultivar levels (Martins *et al.*, 2003). This method is widely applicable because it does not need sequence data for primer construction and is rapid, inexpensive and randomly distributed throughout the genome. The ISSR method has been reported to produce more complex marker patterns than the RAPD approach, which is advantageous when differentiating closely related cultivars

(Parsons *et al*., 1997; Chowdhury *et al*., 2002). In addition, ISSR markers are more reproducible than RAPD markers (Goulão and Oliveira, 2001), because ISSR primers, designed to anneal to a microsatellite sequence, are longer than RAPD primers, allowing higher annealing temperatures to be used. Also because of the multilocus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Godwin *et al*., 1997).

ISSR markers have proven valuable for fingerprinting studies and genetic diversity investigations in several crops such as corn (Kantety *et al*., 1995) finger millet (Salimath *et al*., 1995) rice (Blair *et al*., 1999; Joshi *et al*., 2000; Girma *et al*., 2007), tea (Assefa *et al*., 2003) and coffee (Aga, 2005; Tesfaye, 2006). ISSRs have been successfully used to estimate the extent of genetic diversity at inter and intra specific level in a wide range of crop spices which include wheat (Nagaoka and Ogihara., 1997), plantago (Wolff and Morgamrichard, 1998), vigna (Ajibade *et al*., 2000) and sweet potato (Huang and Sun, 2000).

ISSR markers linked to the traits of agronomic importance have been sequenced and used as STS markers in marker aided selection. An attractive possibility is thus the use of ISSR as probes for in-situ hybridization for physical mapping of homologous chromosome sites (Pasakinskiene *et al*., 2000). Another advantage in the use of ISSR markers lies in their linkage to SSR loci. Although microsatellites themselves are non functional and selectively neutral, they are known to be linked to coding regions and to mark gene rich regions (Kojima *et al*., 1998).

Non anchored ISSRs are arbitrary multiloci markers produced by PCR amplification with a microsatellite primer (Borent and Branchard, 2001). They are advantageous because no prior genomic information is required for their use. The technique is reported to be stable across a wide range of PCR parameters. Polymorphisms were abundant among seven dicot species tested with two tri-nucleotide and two tetra-nucleotide primers. Thus, non anchored ISSR markers are a good choice for DNA fingerprinting.

Goulao *et al*. (2001) used two microsatellite based methodologies (SSR and ISSR) for fingerprinting and determination of similarity degree between 41 commercial cultivars of apple. A total of 13 SSR primer sets were used and 84 polymorphic alleles were amplified. Seven ISSR primer yielded a total of 252 bands, of which 176 (89.1%) were polymorphic. This study indicated that the results obtained based on the RAPD, AFLP, SSR and ISSR techniques were significantly correlated. The SSR and ISSR markers were found to be useful for cultivar identification and assessment of phenotypic relationship revealing advantages, due to higher reproducibility over other commonly employed PCR-based methods.

For molecular characterisation of cultivated chestnut, 125 RAPD and 157 ISSR polymorphic markers were amplified using 28 and seven primers respectively. High level of congruence among the two marker systems was obtained from comparison of phenetic similarities based on the percentage of shared fragments. ISSR markers revealed important advantages over RAPDs, due to a high effective multiplex ratio (12.5 for ISSR compared with 2.2 for RAPD analysis) and reproducibility (Goulao *et al*., 2001).

Zehdi *et al.* (2004) used 14 microsatellite primers to examine the genetic diversity in Tunisian date-palm germplasm showing a high level of polymorphism in 49 accessions from three oases, with little geographic structure within Tunisia.

The genetic makeup of micropropagated and regenerated plantlets of banana was assessed by RAPD and ISSR markers (Venkatachalam *et al*., 2007). The plantlets were analyzed for their genetic stability using RAPD and ISSR markers. A total of 50 RAPD and 12 ISSR primers resulted in 625 distinct and reproducible bands showing homogeneous RAPD and ISSR patterns. Based on these results they confirmed their monomorphic nature with no genetic variation among the plantlets analyzed.

The genetic variability of the Moroccan landraces of sorghum has been reported by Medraoui *et al.* (2007). Thirty-eight RAPD primers and four ISSR primers were used. The percentage of polymorphic fragments revealed with ISSR (98%) was higher than the one revealed with RAPD (85%). The level of the variability obtained

through the two techniques was very high. Nevertheless, ISSR markers revealed more diversity than RAPD (0.995 ± 0.006 against 0.946 ± 0.031).

Utilizing inter simple sequence repeats (ISSRs), the genetic diversity of 33 Greek tomato (*Solanum lycopersicum* L.) landraces and three cultivars, three cherry tomato (*S. lycopersicum* var. *cerasiforme*) cultivars and two accessions of *Solanum pimpinellifolium* L. were studied (Terzopoulos and Bebeli, 2008). Furthermore, 12 ISSR primers were also used to identify 27 morphotypes derived from seven landraces. Based on Jaccard's coefficient, an average genetic similarity of 0.797 (ranged from 0.56 to 0.95) was found among the accessions. Cluster analysis using the UPGMA method placed all tomato landraces and cultivars into a single group, while the cherry hybrids and the *S. pimpinellifolium* accessions were placed in a second group. The ISSR data distinguished all the 27 morphotypes from each other and grouped the morphotypes derived from the same landrace together. The ISSR technology proved useful in describing genetic diversity among Greek tomato landraces and was capable of distinguishing the closely related morphotypes.

Liu *et al.* (2008) used three molecular marker systems, RAPD, ISSR and SRAP, for identification and genetic diversity analysis of 35 elite late-bolting radish cultivars. Detected by 35 RAPD primers, 22 ISSR primers and 17 SRAP primer combinations, the proportions of polymorphic bands were 85.44 per cent, 85.20 per cent and 85.41 per cent, respectively, and the mean genetic similarity coefficients between pairs of genotypes were 0.781, 0.787 and 0.764, respectively. Each of the three molecular marker systems could identify all the cultivars. Five sets of three RAPD primers, three sets of three ISSR primers and 16 sets of three SRAP primer combinations were able to distinguish all the cultivars. A linear relationship was observed between Resolving power (Rp) of a primer and its ability to distinguish genotypes. The 35 cultivars were clustered into three major groups based on the RAPD, ISSR and marker combination data with UPGMA, which were in high accordance with their own origins and main characteristics. The results demonstrated that these three marker systems could be useful for identification and genetic diversity analysis of radish cultivars.

Li *et al*. (2008) used Inter simple sequence repeat (ISSR) for DNA fingerprint analysis of thirty four major cultivated strains of *Auricularia auricula* in China. On the basis of ISSR analysis, the 34 strains were clustered by UPGMA and two specific ISSR bands from strain 173 and 186 were converted into two sequence characterized amplified region (SCAR) markers which were used to rapid strain identification. The genetic background of cultivated strains of *A. auricula* was similar and nomenclature of the cultivated strains was confused and full of synonyms. Utilization of ISSR fingerprint and its SCAR marker for rapid identification of cultivated strains in *A. auricula* was thus made practicable and significant.

Diversity and genetic relationship in 100 cashew germplasm accessions were analyzed by Thimmappaiah *et al.* (2009) using RAPD and ISSR markers. Using 10 selected RAPD primers 60 bands were generated, of which 51 were polymorphic (85%), and with 10 selected ISSR primers 67 amplified bands were observed with 58 polymorphic bands (86.6%). By combining markers, a total of 127 bands were detected, of which 109 bands (85.8%) were polymorphic and produced on an average of 5.45 polymorphic bands per primer. Primers with high polymorphic information content and marker index were identified for discriminating accessions. High percentage of polymorphism (>85%) observed with different markers indicated high level of genetic variation existing among the accessions. Genetic relationship estimated using similarity co-efficient (Jaccard's) values between different pairs of accessions varied from 0.43 to 0.94 in RAPD, 0.38 to 0.89 in ISSR and 0.43 to 0.87 with combined markers suggested a diversity (dissimilarity) ranging from 6 to 57 per cent, 11 to 62 per cent and 13 to 57 per cent respectively and the diversity skewed around 50 per cent indicated moderate diversity. The cluster analysis with UPGMA method separated the accessions broadly into 13 clusters and in that three into smaller clusters.

Kalpana *et al.* (2012) reported genetic variations and relationships between 14 *Morus alba* and two *Morus lhou (ser) koidz* cultivars using the RAPD and ISSR markers with a set of 40 RAPD primers and 10 ISSR primers. Polymorphism exhibited by RAPD, ISSR and RAPD + ISSR primers among the sixteen samples were 66.67 per cent, 55.05 per cent and 64.11 per cent respectively. The genetic relationship between mulberry varieties and genetic dissimilarity coefficients were

estimated for each pair of accessions. The dissimilarity coefficients ranged between 0.123–0.378 and 0.095–0.285 for RAPD and ISSR markers respectively. Dendrogram generated by UPGMA clustering method clustered the genotypes into three and four groups by RAPD and ISSR fingerprint.

Liu and Wu (2013) reported ISSR markers in forty nine potato onion cultivars (*Allium cepa* L. var. *aggregatum* G. Don). Several polymorphic amplified bands, with an average of 8.41 per primer, were obtained from seventeen ISSR primers. The percentage of polymorphism was 93.5 per cent. Genetic similarity varied from 0.4969 to 0.8616, the average value of the effective number of alleles, Nei's genetic diversity and Shannon's information index was 1.4716, 0.3072 and 0.4590, respectively. Forty nine varieties were classified into six groups according to the ISSR ($D = 0.72$). These results showed that combined methods were more accurate for characterization of the genetic diversity and identification of potato onion cultivars.

2.7.1 ISSR markers in brinjal

ISSR analysis was performed by Isshiki *et al.* (2008) in eight cultivars of brinjal and 12 accessions in eight related *Solanum* species to evaluate the applicability of this analysis for assessing the phylogenetic relationships and identifying cultivars. A total of 552 polymorphic amplified bands were obtained from 34 of the 100 primers tested, and the percentage of polymorphism was 99.1 per cent. Cluster analysis based on the ISSR markers classified the *Solanum* species into seven groups. Combining the ISSR markers obtained by a few of the 34 primers was enough for distinguishing the eight cultivars of brinjal. This ISSR analysis was demonstrated to be available for the phylogenetic study and the cultivar identification.

Molecular characterization of 19 advanced cultivars and landraces of brinjal was carried out using RAPD and ISSR markers. Twenty nine RAPD primers generated a total of 240 amplified fragments, while 23 anchored and non-anchored ISSR primers produced 299 fragments. Of these, 66 (27.5%) RAPD and 56 (18.73%) ISSR fragments were polymorphic (Tiwari *et al.*, 2009).

Ali *et al.* (2011) analyzed the diversity of brinjal using ISSR and RAPD procedures and subdivided 143 Chinese-cultivated brinjal based on 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, slightly lower than what is known from other crops.

Genetic relationships between ten genotypes of brinjal were studied by Mahmoud *et al.* (2012) using ISSR markers. Seven out of 20 ISSR primers were used to assay the levels of polymorphism among the Egyptian cultivars of brinjal. Variations in banding patterns were observed among these ten genotypes where 24 monomorphic and 47 polymorphic distinct fragments (61% of polymorphism) accounted for high level of polymorphism between them. The genetic relationships based on ISSR markers were developed using SPSS computer program.

Kumchai *et al.* (2013) used RAPD and ISSR markers for identification of interspecific hybrids between cultivars of brinjal and its wild relative *S. torvum*, which has disease resistance and desirable traits for crop improvement, population were developed through hybridization and embryo rescue. Twenty one hybrid progenies were obtained and examined based on morphological traits, RAPD and ISSR markers. Five of them were confirmed to be true interspecific hybrids. Eighteen and 14 bands from seven RAPD and 14 ISSR primers, respectively, were polymorphic and present in all the five hybrid seedlings and their parents.

2.8 Simple sequence repeats (SSR) or Microsatellite markers

Simple sequence repeat or microsatellite is the term used to refer tandemly repeated short nucleotide units between 1-5 bp in the genome (Staub and Serquen, 1996; Powell *et al.*, 1996). These repeats show a genome-wide distribution and can be placed in either genes or non-coding regions of the nuclear genome or else in extra-nuclear genomes (Nunome *et al.*, 2003ab; Varshney *et al.*, 2005). In the genome, this distribution was reported to be collected around particular regions of the chromosomes such as centromeric areas. For the generation of SSR markers, sequence data would be required. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the

number of repeat units of a microsatellite, resulting in polymorphism that can be detected by gel electrophoresis.

SSR is a PCR based molecular method (Staub and Serquen, 1996) and the principle is the detection of polymorphism resulting from different numbers of repeat units in different individuals and is observed co-dominantly (Powell *et al.,* 1996; Jones, *et al.,* 1997). The level of polymorphism is very high which makes SSR an ideal marker for mapping, diversity studies, fingerprinting and population genetics (Jones *et al.,* 1997; Mohan *et al.,* 1997). However, the major disadvantage is related with development of SSR primers.

An important advantage of SSRs is their ability to detect genetic diversity at a higher level of resolution than other methods. Furthermore, they are co-dominant, information may be obtained from a small amount of plant material at any stage of development, and the data are not affected by environmental conditions (Lanteri and Barcaccia, 2005). In addition, SSR primers and data can be shared among labs.

Different methods have been used for the characterisation of SSR loci. Initially, microsatellites were isolated from partial genomic libraries with small insert size. Large numbers of clones were screened by colony hybridization with repeat containing probes. Later, in order to reduce the time invested in microsatellite isolation and to significantly increase yield, library enrichment techniques were developed (Holton, 2001; Zane *et al*., 2002). Microsatellite loci have been developed using RAPD-PCR of genomic DNA (Ender *et al*. 1996; Lunt *et al*. 1999; Liu, 2008) and an AFLP-PCR approach called FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) (Zane *et al*., 2002; Sun *et al.,* 2008; Zang *et al*., 2008).

Among the classes of repetitive DNA sequences that have proven amenable for PCR amplification, SSRs remain the best choice of markers (Jacob *et al*., 1991). SSRs include a variety of di-, tri-, tetra- and penta-nucleotide tandem repeats (Hamada *et al*., 1982; Tautz, 1989; Weber and May, 1989) that can detect high levels of polymorphism at multiple loci and which can serve as a major source of genetic variation thus aiding individual identification.

SSR loci can be amplified by PCR using primers, which are complimentary to the region flanking repeats. It is estimated to have a total of 5×10^3 to 3×10^5 microsatellites per plant genome (Condit and Hubbell, 1991). Rongwen *et al*. (1995) used SSRs to develop unique DNA profiles or fingerprints for 96 soybean cultivars. Seven SSR loci clearly differentiated all except two closely related individuals. Similar results were reported for other crop species *viz*., grape (Thomas and Scott, 1993; Botta *et al*., 1995, avocado (Lavi *et al*.,1994), sorghum (Brown *et al*.,1996), hops (Brady *et al*., 1996), cucumbers and melons (Katzir *et al*.,1996), apple (Szewc-McFadden *et al*., 1996; Guilford *et al*., 1997), barley (Russell *et al*., 1997) etc. The screening of microsatellite alleles in varieties generated a database useful for variety identification and the development of molecular markers for marker assisted selection (Garland *et al*., 1999)

Struss *et al*. (1998) estimated genetic diversity among 163 barley genotypes. 15 barley microsatellite markers detected a total of 130 alleles. The number of alleles per microsatellite marker varied from 5 to 15. On an average 8.6 alleles per locus were observed.

Vander *et al*. (2000) Conducted a study to develop a simple sequence repeat (SSR) markers in Eucalyptus from amplified inter simple sequences repeat (ISSR). *Eucalyptus grandis* and its 36 progenies, *E. urophylla*, *E. nitens*, *E. globulus and E. comaldulensis* were studied. The result showed the species specific banding pattern. They also reported the feasibility of the microsatellite for eucalyptus germplasm characterization.

Alvaez *et al*. (2001) used 17 microsatellite loci to study diversity among 31 tomato accessions comprising nine species of the genus *Lycopersicon*. The microsatellite polymorphism were used to estimate the distribution of diversity throughout the genus to evaluate the efficiency of microsatellites for establishing species relationships in comparison with existing phylogeny reconstructions.

Huang *et al*. (2002) used a set of 24 wheat microsatellite markers, representing at least one marker from each chromosome, for assessment of genetic diversity in 998 accessions of hexaploid bread wheat (*Triticum aesrivum* L.) which

originated from 68 countries of five continents. A total of 470 alleles were detected with an average allele number of 18.1 per locus.

He *et al.* (2003) developed 56 SSR markers, out of which 19 had detected polymorphism among 24 cultivated peanut genotypes. The average number of alleles per locus was 4.25. They identified 14 alleles at PM50 locus when 48 genotypes were surveyed. Using five such highly polymorphic markers they differentiated 24 cultivated groundnut genotypes.

Meerow *et al.* (2003) studied genetic variation with in *Cocos nucifera* germplasm collections at two locations in South Florida, representing eight cultivars with SSR microsatellite DNA loci. A total of 67 alleles were detected, with eight the highest number at any one locus.

Benor *et al*. (2008) determined the genetic diversity of 39 determinate and indeterminate tomato inbred lines collected from China, Japan, S. Korea, and USA. Using 35 SSR polymorphic markers, a total of 150 alleles were found with moderate levels of diversity, and a high number of unique alleles existing in these tomato lines. The mean number of alleles per locus was 4.3 and the average polymorphic information content (PIC) was 0.31. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering at genetic similarity value of 0.85 grouped the inbred lines into four groups.

To provide simple sequence repeat (SSR) based DNA fingerprinting information for sugarcane cultivars that were developed in Yunan Province, China. (Xin-Long *et al.*, 2010), genomic DNAs from 27 cultivars were amplified with 8 pairs of polymorphic SSR primers. A total of 129 bands were obtained, including 123 polymorphic bands (95.35%). The mean value of polymorphic information content (PIC) was 0.9445, and the genetic similarity coefficient ranged from 0.269 to 0.767.

RAPD and SSR were applied to assess genetic diversity in 61 tomato varieties from different species (Meng *et al.,* 2010). Two thousand sixty two and 869 clear fragments were amplified by RAPD and SSR, respectively. On the other hand,

more polymorphic products were found with SSR as compared to RAPD, *i.e.,* 100 and 43.84 per cent, respectively. In addition, a higher value of the average similarity coefficient and lower PIC value were reflected in RAPD (0.79, 0.407) compared to SSR (0.56, 0.687) and concluded that SSR was a higher effective marker than RAPD to assess genetic diversity in tomato accessions.

2.8.1 Simple sequence repeats (SSR) markers in brinjal

Nunome *et al.* (2009) constructed simple sequence repeat (SSR) enriched genomic libraries in order to develop SSR markers, and sequenced more than 14,000 clones. From these sequences, they designed 2,265 primer pairs to flank SSR motifs and identified 1,054 SSR markers from amplification of 1,399 randomly selected primer pairs. The markers have an average polymorphic information content of 0.27 among eight lines of *S. melongena*. Of the 1,054 SSR markers, 214 segregated in an intraspecific mapping population. They constructed cDNA libraries from several brinjal tissues and obtained 6,144 expressed sequence tag (EST) sequences. From these sequences, they designed 209 primer pairs, seven of which segregated in the mapping population. On the basis of the segregation data, they constructed a linkage map, and mapped 236 segregating markers to 14 linkage groups. The linkage map spans a total length of 959.1 cM, with an average marker distance of 4.3 cM. These markers proved to be a useful resource for qualitative and quantitative trait mapping and for marker-assisted selection in brinjal breeding.

Molecular characterization of brinjal genotypes collected from different geographical regions of Turkey was carried out using SSR and RAPD markers (Demir *et al.,* 2010). With amplification of five SSR loci, the number of alleles per microsatellite locus ranged from 2 to 10, with a total of 24 alleles. The greatest number of alleles was found at the *emf21H22* locus (10 alleles); followed by *emh11O01* and *emf21C11* as five and four alleles, respectively. The average number of alleles per locus was 4.8. Using 11 decamer RAPD primers, 100 bands were amplified, among which 29 were polymorphic. The number of bands per primer ranged from 7 to 14. Primer OPB07 was the most polymorphic, generating 64 per cent polymorphic bands; the rest of the primers gave less than 50 per cent

polymorphism. UPGMA dendrograms were used to examine the genetic relatedness of the genotypes.

Yan *et al.* (2011) used 101,270 brinjal expressed sequence tag (EST) sequences at public databases to search for simple sequence repeats (SSRs) and 405 potential SSR loci were identified from 388 sequences. The highest proportion (34.07%, 138) was represented by tri-nucleotide, followed by di-nucleotide (19.51%, 79) and hexa-nucleotide (15.8%, 64). Among the di-nucleotide repeats, AG/CT was the most common (55.69%), followed by AT/AT (31.64%) and AC/GT (12.66%). Further, 288 pairs of primers were developed from these sequences. A random set of 100 EST-SSR primers were amplified in 12 brinjal accessions and 88 successfully amplified PCR products. Thirty two markers revealed 83 polymorphic alleles among the 42 cultivated accessions and the number of allelles per locus varied between two and six (mean 2.6). Polymorphic information content (PIC) values among the 42 cultivated types were calculated and varied from 0.045 to 0.701 (mean 0.289). The markers showed low frequency transferability in *Solanaceae*. The 32 SSRs were used to evaluate genetic diversity. These SSRs proved valuable markers for future genetic study, such as genetic diversity estimation, linkage mapping, association mapping and molecular breeding.

Muñoz-Falcón *et al.* (2011) used forty two eggplant accessions, which included 25 *Striped* accessions, of which 19 were of the *Listada* type (six accessions of *Listada de Gandía*, eight of *Other Spanish Listada*, and five of *Non-Spanish Listada*) and six 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, as a mean, a greater polymorphism and polymorphic information content (PIC) than EST-SSRs. For two SSR markers, *Listada de Gandía* accessions were proved to be genetically diverse, specific and universal alleles. All the *Listada* accessions clusterd together in the multivariate PCA and UPGMA phenograms performed, and are separated from the *Other Non-Spanish Striped* and *Non-Striped* accessions. Also, *Listada de Gandía* accessions were clearly differentiated from the *Other Spanish Listada* and *Non-Spanish Listada* accessions in these analyses. SSR markers revealed great utility to obtain a specific fingerprint for the *Listada de*

Gandía brinjal as well as to establish the uniqueness and distinctness of this landrace. This information was very helpful for the enhancement and protection from imitation of *Listada de Gandía*, and contributed to support its potential recognition with a Protected Designation of Origin (PDO) status.

The use of AFLP and SSR markers confirmed the existence of genetic variation within each group of brinjal. In this respect, SSRs were more reliable than AFLPs to distinguish closely related brinjal materials and for allowing detection of specific universal SSR markers for the *Almagro* and *Listada de Gandía* landraces. Correlation coefficients between distances based on morphological, AFLP and SSR markers were moderate, reflecting the different genetic information provided by the three markers. The results showed that morphological and molecular markers provided complementary information, and when used in combination were very useful for the protection of local materials of brinjal (Prohens *et al.*, 2011).

Tümbilen *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 brinjal and closely related species. The markers had very good polymorphism in the 18 species tested including eight *S. melongena* accessions. Moreover, genetic analysis performed with these markers showed concordance with previous research and knowledge of brinjal domestication. These markers were valuable resource for studies of genetic relationships, fingerprinting, and gene mapping in brinjal.

Molecular markers specific for *Solanum elaeagnifolium* (silverleaf nightshade) were not available. Zhu *et al*. (2012) identified 35 simple sequence repeat (SSR) primer pairs from potato, tomato and brinjal and tested these for cross-species transferability in *S. elaeagnifolium*. Among them, 13 primer pairs successfully produced bands representing different alleles. The polymorphic information content ranged from 0 to 0.84. The transferable rate of SSR from potato, tomato and eggplant to *S. elaeagnifolium* was 20 per cent, 40 per cent and 46 per cent, respectively. SSR analysis revealed high level of genetic diversity among 40 individuals collected within a paddock. Highly polymorphic and transferable cross-

species SSR markers were useful for determining the extent of genetic diversity in *S. elaeagnifolium* populations.

Wen *et al.* (2012) used 105 brinjal SSR markers to analyze the genetic polymorphism of 34 brinjal high generation materials. The result indicated that there were 47 markers showing polymorphisms among the tested materials which had 148 polymorphism sites. The polymorphic information content (PIC) varied from 0.025 to 0.909, showed high genetic polymorphism. The similarity coefficient of the tested materials was between 0.63 to 0.93, implied little genetic differences and relatively narrow genetic base. By UPGMA cluster analysis, they classified the tested materials into two clusters and four sub-clusters. The result of SSR molecular marker was almost the same with that of fruit trait cluster analysis.

Chinnappareddy *et al.* (2012) in the Indian Institute of Horticulture Research (IIHR) brinjal breeding program produced five genotypes with increased yield and excellent producer acceptance. Molecular profiles of these genotypes were developed using 39 EST-SSR primers for maximum discrimination and repeatability at 35 loci. In total, there were 181 alleles with the number of alleles per locus ranging from 9.0 (EEMS 24) to 3.0 (EEMS 46 and EEMS 20), with a mean of 5.02 alleles per locus ranging from 85 to 300 bp. Polymorphic information content of the markers ranged from 0.343 to 0.794. Expected heterozygosity ranged from 0.560 to 0.880 and the probability of identity ranged from 0.010 to 0.376. Pair-wise comparison of microsatellite data led to development of an unweighted pair group method with arithmetic mean dendrogram and DNA barcodes for easy and accurate identification of brinjal genotypes with combinations of their morphological traits. The results indicated that 'IIHR-3' and 'IIHR-7' originated from a single source and 'Arka Anand' is a cross between 'IIHR-3' and 'SM-6-6', indicated a good fit with genetic similarity values. The DNA barcode fingerprint was deposited and used for clear identification of brinjal genotypes and to address intellectual property rights related issues.

Hurtado *et al.* (2012) assessed the diversity and relationships of 52 accessions of brinjal from three geographically distant secondary centers of diversity (China, Spain, and Sri Lanka) using 28 morphological descriptors and 12 highly polymorphic genomic SSRs. A wide variation was found for most morphological traits, and

significant differences among the three centers of diversity were detected for 22 of these traits. The SSR characterization identified 110 alleles and allowed obtaining a unique genetic fingerprint for each accession. Many alleles were found to be private to each origin, but no universal alleles were found for any of the origins. The PCA analysis showed that the genetic differentiation among origins was less clear than for morphological traits, although the analysis of the population structure showed that accessions mostly group according to the origin, but also provides evidence of migration among the three secondary centers of diversity. The genetic diversity (HT) within each origin was high, ranging between HT = 0.5400 (Sri Lanka) and HT = 0.4943 (China), while the standardized genetic differentiation (G'ST) among origins was moderate (G'ST = 0.2657). The correlation between morphological and SSR distances was non-significant (r = 0.044), indicating that both data were complementary for the conservation of germplasm and breeding of brinjal.

Adeniji *et al.* (2012) used seven *Solanum* species (brinjal) for molecular diversity. Thirty nine *Solanum* accessions, a landrace and tomato variety (LBR 48) were molecularly analyzed by simple sequence repeat (SSR) marker technique. A dendrogram was obtained based on the Jaccard's coefficient of similarity and unweighted pair group method with arithmetic mean (UPGMA) clustering. A total of 417 alleles were amplified with the number of alleles ranging from 5 to 38. Polymorphism was fairly high (0.05 to 0.92) among SSR markers with high number of repeats. Findings indicated that entries originated from different parts of the world did not form a distinct cluster, and there was no association between SSR marker pattern and geographical origin.

Hence, in conclusive summary of the available reviewed literature pertaining to DNA fingerprinting in brinjal necessitates the utility of proposed research work. Earlier work done in this regard is very limited and thus indicates the suitability of ISSR and SSR markers for DNA fingerprinting brinjal genotypes.

Material and Methods

3. MATERIAL AND METHODS

The study on "DNA fingerprinting of brinjal (*Solanum melongena* L.) varieties and related species" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2011-2013. The material used and methodologies adopted are presented in this chapter.

3.1 Materials

3.1.1 Plant Materials

The brinjal varieties/accessions selected for the study included *Solanum melongena* genotypes- Surya, Swetha, Haritha, Neelima, SM 116, SM 396, SM 397 and two wild relatives *S. melongena* var. *insanum* and *S. macrocarpon*. Breeder seeds were obtained from the Department of Olericulture, College of Horticulture, Kerala Agricultural University. Seeds were sown in pots and one month old seedlings (ten seedlings per variety) were transplanted and maintained in pot culture at open condition.

3.1.2 Laboratory chemicals, glasswares and equipment items

The chemicals used in the study were of good quality (AR grade) procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, Taq buffer and molecular markers used were supplied by Bangalore Genei Ltd. All the plasticware used were obtained from Axygen and Tarson India Ltd. ISSR and SSR primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Veriti Thermal Cycler (Applied Biosystem, USA) and agarose gel electrophoresis was performed in horizontal gel electrophoresis unit (BIO-RAD, USA). The details of laboratory equipment items used are given in Annexure I.

3.2 METHODS

3.2.1 Morphological analysis

Morphological characters of brinjal varieties/accessions maintained at CPBMB Vellanikkara in pots were recorded as per the minimal descriptor of NBPGR (Srivastava *et al.,* 2001). The characters observed were as follows:

a) Plant growth habit
b) Leaf and petiole colour
c) Presence of prickles on leaves, stem and calyx.
d) Flower colour
e) Fruit colour
f) Fruit shape
g) Fruit curvature
h) Fruit position

3.2.2 Molecular analysis

Molecular analysis of the varieties/accessions were carried out with two different marker systems- Inter simple sequence repeats (ISSR) and simple sequence repeats (SSR).

3.2.2.1 Genomic DNA extraction

Young tender, pale green leaves (first to third from the tip) were collected on ice from individual plants in each accession. The surface was cleaned by washing with sterile water and wiping with 70 percent alcohol. The fresh leaves were ground into a fine powder in liquid nitrogen along with β -mercaptoethanol and PVP using ice-cold mortar and pestle in order to prevent browning due to phenol oxidase activity.

Among the most commonly used protocols, CTAB method developed by Rogers and Bendich (1994) was used for the extraction of genomic DNA. The reagents required for DNA isolation are given in Annexure II.

Reagents used are,

I. CTAB buffer (2X):

- 2 per cent CTAB (w/v)
- 100mM Tris (pH8)
- 20mM EDTA (pH8)
- 1.4M NaCl
- 1 per cent PVP

II. 10 per cent CTAB solution:

- 10 per cent CTAB (w/v)
- 0.7M NaCl.

III. TE buffer:

-10mM Tris (pH8)

-1mM EDTA (reagent 1 and 3 autoclaved and stored at room temperature)

IV . Chloroform: isoamyl alcohol (24:1 v/v)

V. Isopropanol

VI. Ethanol 70 per cent and 100 per cent

VII. Sterile distilled water

Procedure:

- One gram of clean leaf tissues was ground in pre-chilled mortar and pestle in the presence of liquid nitrogen.
- 4ml of extraction buffer (2x), 50µl of β-Mercaptoethanol and a pinch of Poly Vinyl Pyrolidone (PVP) were added to the mortar.
- The homogenized sample was transferred into an autoclaved 50ml centrifuge tube and 3ml of pre-warmed extraction buffer was added (total 7ml).

- The contents were mixed well and incubated at 65ºC for 20 to 30 minutes with occasional mixing by gentle inversion.
- Equal volume (7ml) of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion to emulsify. Spun at 10,000 rpm for 15 minutes at 4ºC
- After centrifugation the contents got separated into three distinct phases.

Aqueous topmost layer	-	DNA and RNA
Interphase	-	fine particles and proteins
Lower layer	-	Chloroform, pigments and cell debris

- Transferred the top aqueous layer to a clean centrifuge tube and added $1/10^{th}$ volume of 10 per cent CTAB solution and equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion.
- Centrifuged at 10,000 rpm for 15 minutes at 4ºC.
- Transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitated. Kept at -20ºC for half an hour for complete precipitation.
- Centrifuged at 10,000 rpm for 15 minutes at 4ºC. Gently poured off the supernatant.
- Washed the DNA pellet with 70 per cent ethanol followed by 100 per cent ethanol.
- Spun for 5 min at 10,000 rpm and decanted the ethanol.
- Air dried the pellet, dissolved in 50µl of sterilized water and stored at -20ºC.
- Loaded the samples on 0.8 per cent agarose to observe the quantity and quality of DNA.

3.2.3 Purification of DNA

The DNA which had RNA as contaminant was purified by RNase treatment and further precipitation.

Reagents used are,

- Phenol: chloroform mixture (24:1, v/v)
- Chilled isopropanol
- 70 per cent ethanol
- TE buffer
- Chloroform: Isoamyl alcohol (24:1, v/v)
- 1 per cent RNase

One per cent solution was prepared by dissolving RNase (Sigma, USA) in TE buffer at 100^0C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^0C.

Procedure

- To 100 µl DNA sample, RNase solution (2 µl) was added and incubated at 37^0C in dry bath for 1 hour.
- The volume was made up to 250 µl with distilled water.
- Added equal volume of chloroform: isoamyl alcohol (24: 1) mixture and mixed gently.
- Centrifuged at 10,000 rpm for 15 minutes at 4^0C.
- Transferred the aqueous phase into a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24: 1)
- Centrifuged at 10,000 rpm for 15 minutes at 4^0C.
- Transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA precipitated. Kept at -20ºC for half an hour for complete precipitation.
- Incubated the mixture at -20^0C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4^0C.

- Washed the DNA pellet with 70 per cent ethanol
- Air dried the pellet, dissolved in 50 to 100 µl sterilized water.
- Loaded the samples on 0.8 per cent agarose gel at constant voltage of 100 V to test the quality and to find whether there was any shearing during RNase treatment.

3.2.4 Electrophoresis of DNA

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al*., 1989).

Reagents and Equipments

1. Agarose - 0.8 per cent (for genomic DNA)
   - 2 per cent (for PCR ISSR and SSR samples)
2. 50X TAE buffer (pH 8.0)
   - Tris buffer
   - Acetic acid
   - 0.5mM EDTA
3. Tracking/loading dye (6X)
   -Bromophenol blue
   -Glycerol
4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml)

Composition of reagents is provided in Appendix III.

Procedure

- The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

- Prepared 0.8 per cent agarose (0.8 g in 100ml) in a glass beaker or conical flask with 100 ml 1X TAE buffer. Micro waved for 45 to 60 seconds until agarose was dissolved and solution was clear.

- Solution was allowed to cool to about 42 to 45 ^{0}C before pouring. (4µl Ethidium bromide was added at this point to a concentration of 10 µl/ml) and mixed well.

- Poured this warm gel solution into the tray to a depth of about 5 mm. Allowed the gel to solidify for about 30 to 45 minutes at room temperature.

- To run, gently removed the comb and the tape used for sealing, placed the tray in electrophoresis chamber, and covered (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose).

- To prepare samples for electrophoresis, added 1 µl of 6x gel loading dye for every 5µl of DNA solution. Mixed well and loaded 6µl DNA sample per well. Loaded suitable molecular weight marker (λDNA *Eco*RI/ *Hind*III double digest) in one lane.

- Electrophoresed at 70 volts until dye has migrated two third the length of the gel.

- Intact DNA appears as orange fluorescent bands. If degraded, it appears as a smear because of the presence of a large number of bands, which differ in base length. The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.2.5 Gel Documentation

Gel documentation was done with BioRad Gel Documentation System using PDQuest™ software. PDQuest is a software package for imaging, analyzing, and databasing 2-D electrophoresis gels. PDQuest can acquire images of gels using any of several Bio-Rad imaging systems. An image of a gel is captured using the controls in the imaging device window and displayed on computer screen.

3.2.6 Assessing the quality and quantity of DNA by spectrophotometer

The purity of DNA was further checked by using NanoDrop ND-1000 spectrophometer. Nucleic acid shows absorption maxima at 260nm whereas proteins show peak absorbance at 280nm. Absorbance is recorded at both wavelength and purity is indicated by the ratio OD_{260}/OD_{280}. The values between 1.8 and 2.0 indicate that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} equivalent to 50µg double stranded DNA/ml sample.

1 OD at 260 nm = 50 µg DNA/ml

Therefore $OD_{260} \times 50$ gives the quantity of DNA in µg/ml.

Procedure for quantity detection using Nanodrop

- Connected the Nanodrop spectrophotometer to the System and open the operating software ND-100.
- Selected the option Nucleic acid.
- With the sampling arm open, pipetted 1µl distilled water onto the lower measurement pedestal.
- Closed the sampling arm and initiated a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement is made.
- Set the reading to zero with sample blank.
- 1µl sample was pipetted onto measurement pedestal and select measure.
- When the measurement was complete, opened the sampling arm and wiped the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.3 Molecular Markers used for the study

Two types of markers were used for the study which includes ISSR (Inter Simple Sequences Repeats) and SSR (Simple Sequence Repeats). Under each marker

analysis system, DNA from all of the nine genotypes of brinjal were amplified separately with all the selected primers. This would help to obtain amplification pattern for a particular variety with different primers in a marker system simultaneously.

3.3.1 DNA amplification conditions

The PCR condition required for effective amplification in ISSR and SSR analysis include appropriate proportions of the component of the reaction mixture. The reaction mixture includes template DNA, assay buffer A or B, $MgCl_2$, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2ml PCR tubes. The PCR was carried out in Veriti Thermal Cycler (Applied Biosystem, USA).

Another important factor, which affect amplification rate is the temperature profile of thermal cycle. The thermocycler was programmed for desired times and temperature for denaturation, annealing and polymerization.

3.3.2 ISSR (Inter Simple Sequence Repeat) analysis

The good quality genomic DNA (25-30 ng/µl) isolated from brinjal leaf samples were subjected to ISSR analysis. ISSR primers with good resolving power were used for amplification of DNA. The primers for assay were selected after an initial screening.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA (30 ng)	- 2 µl
b) 10X Taq assay buffer B	- 2.0 µl
c) $MgCl_2$	- 2.0µl
c) dNTP mix (10 mM each)	- 1.5 µl
d) Taq DNA polymerase (3U)	- 0.4 µl
e) Primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 10.6µl
Total volume	- 20.0 µl

The amplification was carried out with the following programme

94^0C for 4 minutes	-	Initial denaturation	
94^0C for 45 seconds	-	Denaturation	35 cycles
43^0C to 55^0C for 1 minutes	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 8 minutes	-	Final extension	
4^0C for infinity to hold the sample			

3.3.2.1 Screening of ISSR primers and analysis

Thirty six primers (ISSR Technologies) were screened for ISSR analysis and are listed in Table 1. Primers were selected from literatures based on previous studies in ISSR analysis in brinjal.

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100-bp DNA ladder). The profile was visualized under UV (312 nm) transilluminator and documented for further analysis. The documented ISSR profiles were carefully examined for amplification of bands.

Table 1: List of ISSR primers screened with brinjal samples

Sl. No	Primer	Nucleotide Sequence
1	UBC 811	5’GAGAGAGAGAGAGAGAC3’
2	UBC 813	5’CTCTCTCTCTCTCTCTT3’
3	UBC 814	5’CTCTCTCTCTCTCTCTA3’
4	UBC 815	5’CTCTCTCTCTCTCTCTG3’
5	UBC 834	5’AGAGAGAGAGAGAGAGYT3’
6	UBC 835	5’AGAGAGAGAGAGAGAGYC3’
7	UBC 836	5’AGAGAGAGAGAGAGAGYA3’
8	UBC 840	5’GAGAGAGAGAGAGAGAYT3’
9	UBC 844	5’CTCTCTCTCTCTCTCTRC3’
10	UBC 890	5’VHVGTGTGTGTGTGTGT3’
11	UBC 866	5’CTCCTCCTCCTCCTCCTC3’
12	UBC 807	5’AGAGAGAGAGAGAGAGT3’
13	UBC 843	5’CTCTCTCTCTCTCTCTRA3’
14	UBC S2	5’CTCTCTCTCGTGTGTGTG3’
15	UBC 820	5’GTGTGTGTGTGTGTGTC3’
16	UBC 854	5’TCTCTCTCTCTCTCTCRG3’
17	UBC 845	5’CTCTCTCTCTCTCTCTRG3’
18	UBC 817	5’CACACACACACACACAA3’
19	UBC 826	5’ACACACACACACACACC3’
20	UBC 818	5’CACACACACACACACAG3’
21	ISSR 04	5’ACACACACACACACACC3’
22	ISSR 05	5’CTCTCTCTCTCTCTTG3’
23	ISSR 06	5’GAGAGAGAGAGAGAGAC3’
24	ISSR 07	5’CTCTCTCTCTCTCTTG3’
25	ISSR 08	5’GAGAGAGAGAGAGAGAT3’
26	ISSR 09	5’CTCTCTCTCTCTCTCTCG3’
27	ISSR 10	5’ACACACACACACACACG3’
28	ISSR 15	5’TCCTCCTCCTCCTCC3’
29	SPS 03	5’GACAGACAGACAGACA3’
30	SPS 08	5’GGAGGAGGAGGA3’
31	UBC 868	5’GAAGAAGAAGAAGAAGA3’
32	UBC 895	5’- AGAGTTGGTAGCTCTTGATC-3’
33	UBC 899	5’-CATGGTGTTGGTCATTGTTCCA -3’
34	UBC 880	5’- GGAGAGGAGAGGAGA-3’
35	UBC 892	5’ - TAGATCTGATATCTGAATTCCC-3’
36	UBC 900	5’ - ACTTCCCCACAGGTTAACACA-3’

3.3.3 SSR (Simple Sequence Repeat) analysis

The good quality genomic DNA (20 to 25ng/µl) isolated from brinjal leaf samples were subjected as per the procedure reported by Demir *et al.* (2010). SSR primers supplied by sigma, USA were used for amplification of DNA. So SSR primers for the assay were selected after an initial screening of primers.

The amplification was carried out in Veriti Thermal Cycler (Applied Biosystem, USA). PCR amplification was performed in a 20 µl reaction mixture which consisted of,

a) Genomic DNA (30ng)	-	2.0µl
b) 10X Taq assay buffer A	-	2.0µl
c) dNTP mix (10mm each)	-	1.5µl
d) Taq DNA Polymerase (1U)	-	0.3µl
e) Forward Primer (10pM)	-	0.75µl
f) Reverse Primer (10pM)	-	0.75µl
g) Autoclaved Distilled Water	-	12.7µl
Total volume	-	20.0µl

The thermocycler was carried out with the following programme

94^0C for 3 minute	-	Initial denaturation	
94^0C for 1 minute	-	Denaturation	35 cycles
53^0C to 55^0C for 1 minute	-	Primer annealing	
72^0C for 1 minute	-	Primer extension	
72^0C for 5 minutes	-	Final extension	
4^0C for infinity to hold the sample			

3.3.3.1 Screening of SSR Primers and analysis

Sixty one primer combinations were screened by PCR for SSR analysis and are listed in Table 2. The amplified products were run on two per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100bp ladder). The profile was visualized under UV (312 nm) transilluminator and documented. The documented SSR profiles were carefully examined for amplification of DNA as bands.

Table 2: List of SSR primers screened with brinjal samples

Sl. No.	Name of Primers	Sequence
1	LE aac001	**F** 5'- AGGAAGAGCGTGAGTCTGAAC-3' **R** 5'-TCCTGCGCCACTTTAGAG-3'
2	LE aat001	**F** 5'-GATGGACACCCTTCAATTTA-3' **R** 5'-TCCAAGTATCAGGCACACC-3'
3	LE aat004	**F** 5'- CAGGATCAGAACAGCGATG-3' **R** 5'-CCACTGGTATCCATCTTTCAC-3'
4	LE aat005	**F** 5'- GGTCATGCAGGTTGGATTAC-3' **R** 5'-AACCTTCCTTCCTATTGGC-3'
5	LE aat006	**F** 5'- GCCACGTAGTCATGATATACATAG-3' **R** 5'-GCCTCGGACAATGAATTG-3'
6	LE aat007	**F** 5'-CAACAGCATAGTGGAGGAGG-3' **R** 5'-TACATTTCTCTCTCTCCCATGAG-3'
7	LE aat008	**F** 5'-GAGTCAACAGCATAGTGGAGGAGG-3' **R** 5'-CGTCGCAATTCTCAGGCATG-3'
8	LE ac001	**F** 5'-TGCCTTCCATCTAACCAATC-3' **R** 5'-CTGTGGCAAATATGTCCCTAAG-3'
9	LE act001	**F** 5'- AATCATCAACTTTAAACTGTGACAC-3' **R** 5'-TGCATTGAGATGATGAGTCGTTGG-3'
10	LE agat001	**F** 5'- TCCAGATAGTCAGTCAGTCAGACAGC-3' **R** 5'-TCTCTATCTTTAAGAGTGGGAGAAC-3'
11	LE aga001	**F** 5'-TTCTTCACTGTTGACAGAGAGAC-3' **R** 5'-CATTAGTTGAGAGTGATACCGC-3'
12	LE ag001	**F** 5'- GCTCGAGCACATATAGAAGAGAATCA-3' **R** 5'-CCATTTCATCATATCTCTCACCTTGC-3'
13	LE ag002	**F** 5'- AGACGCTTCGACGGGGTTTA-3' **R** 5'-AGGACAGGTGAATGGGTCAAAGA-3'
14	LE ag003	**F** 5'-ACCCTAAAACTAACGACATTCAACG-3' **R** 5'-TTCGTGGACTAATGTATGAAGTGTACC-3'
15	LE at001	**F** 5'-GCGCGAGCTCTCTCTGATCTCT-3' **R** 5'-TTGTAATTGCATCGGCCACG-3'
16	LE at002	**F** 5'-TACTGCATTTCAGGTACATACTCATC-3' **R** 5'-ATAAACTCGTAGACCATACCCTC-3'
17	LE at003	**F** 5'- GAGAAGTTGGTGCATTCATAAC-3' **R** 5'-AAACAGTAAACCAAACACTTGC-3'
18	LE at004	**F** 5'- GCCAGTTGATCATCATCATGAGTACA-3' **R** 5'-AGAAGCCAATGAAGTGAGTGTTGC-3'
19	LE at005	**F** 5'- TGCAGCCTTTGGGTAAAC-3' **R** 5'-ATAGTTTGAAGAGAGGGAGAAAG-3'
20	LE at006	**F** 5'- CATAATCACAAGCTTCTTTCGCCA-3' **R** 5'-CATATCCGCTCGCTCGTTTCGTTAGTAAT-3'
21	LE at007	**F** 5'-GCCCTAGATCTCACAAGCC-3' **R** 5'-CACAAAGCTGAATGATACGAAG-3'

22	LE at008	**F** 5’-AAGCGCGAGCTCTCTCTGATCTC-3’ **R** 5’-CCACGATCTCCGCCATATGC-3’
23	LE at011	**F** 5’- TGGGCTGACTTCGAGTTTG-3’ **R** 5’-CGAGAAAGGGCAGAGAATG-3’
24	LE at012	**F** 5’-CGGCAAAGGGACTCGAATTC-3’ **R** 5’-GTGGCGGAGTAGAAACCTTAGGA-3’
25	LE at013	**F** 5’- ATCACAAGCTTCTTTCGCCACA-3’ **R** 5’-ACCCATATCCGCTCGTTTCG-3’
26	LE at014	**F** 5’-TGTGTTGCGTCATTACCACTAAC-3’ **R** 5’-CCCAACCACCAATACTTTCC-3’
27	LE at015	**F** 5’-GGATTGTAGAGGGTGTTGTTGG-3’ **R** 5’-TTTGATAATGACTTTGTCGATG-3’
28	LE at016	**F** 5’- CCCAAATGCTATGCAATACA-3’ **R** 5’-AGTTCAGGATTGGTTTAAGGG-3’
29	LE at017	**F** 5’- TGAGAACAACGTTTAGAGGAGCTG-3’ **R** 5’-GGGGCAGAATCTCGAACTC-3’
30	LE at019	**F** 5’- TGCCTCTCTTCAAAGATAAAGC-3’ **R** 5’-CGGAAAGTTCTCTCAAAGGAG-3’
31	LE aat001	**F** 5’-GATGGACACCCTTCAATTTA-3’ **R** 5’-TCCAAGTATCAGGCACACC-3’
32	LE at010	**F** 5’- TGGCTCTGCTCAACTCAAGAACTAC-3’ **R** 5’-CACGTGAGGTTAGCCAGTGCATC-3’
33	LE at018	**F** 5’- CGGCGTATTCAAACTCTTGG-3’ **R** 5’-GCGGACCTTTGTTTTGGTAA-3’
34	SSRKAU1	**F** 5’-AATTCACCTTTCTTCCGTCG-3’ **R** 5’-GCCCTCGAATCTGGTAGCTT-3’
35	SSRKAU2	**F** 5’-TTCAGGTATGTCTCACACCA-3’ **R** 5’-TTGCAAGAACACCTCCCTTT-3’
36	SSRKAU3	**F** 5’-GGGTTATCAATGATGCAATGG-3’ **R** 5’-CCTTTATGTCAGCCGGTGTT-3’
37	SSRKAU4	**F** 5’-TGCCAATCCACTCAGACAAA-3’ **R** 5’-TGGATTCACCAAGGCTTCTT-3’
38	SSRKAU7	**F** 5’-GAAGGGACAATTCACAGAGTTTG-3’ **R** 5’-CCTTCAACTTCACCACCACC-3’
39	SSRKAU8	F 5’-AATGAAGAACCATTCCGCAC-3’ R 5’-ACATGAGCCCAATGAACCTC-3’
40	SSRKAU9	**F** 5’-ACATGAGCCCAATGAACCTC-3’ **R** 5’-AACCATTCCGCACGTACATA-3’
41	SSRKAU10	**F** 5’-GCGATGAGGATGACATTGAG-3’ **R** 5’-TTTACAGGCTGTCGCTTCCT-3’

42	SSRKAU11	**F** 5’-TGTTGGTTGGAGAAACTCCC-3’ **R** 5’-AGGCATTTAAACCAATAGGTAGC-3’
43	SSRKAU12	**F** 5’-TCCTCAAGAAATGAAGCTCTGA-3’ **R** 5’-CCTTGGAGATAACAACCACAA-3’
44	SSRKAU13	**F** 5’-GGAATAACCTCTAACTGCGGG-3’ **R** 5’-CGATGCCTTCATTTGGACTT-3’
45	SSRKAU15	**F** 5’-TGTTGCTCGAACTCTCCAAA-3’ **R** 5’-CATAGGAGAGGTAACCCGCA-3’
46	SSRKAU16	**F** 5’-GTTTCTATAGCTGAAACTCAACCTG-3’ **R** 5’-GGGTTCATCAAATCTACCATCA-3’
47	SSRKAU17	**F** 5’-TTCGTTGAAGAAGATGATGGTC-3’ **R** 5’-CAAAGAGAACAAGCATCCAAGA -3’
48	SSRKAU18	**F** 5’-CCGTTACCTTGGTCCATCAC-3’ **R** 5’-GGGAGATGCCACATCACATA-3’
49	SSRKAU6	**F** 5’-TGGCATGAACAACAACCAAT-3’ **R** 5’-AGGAAGTTGCATTAGGCCAT-3’
50	SSRKAU19	**F** 5’-ATTGTACAAAGACCCGTGGC-3’ **R** 5’-GTTGCACACTGGATCAATGC-3’
51	SSRKAU20	**F** 5’-AGGGTCCTTCGTTTGGAACT-3’ **R** 5’-GCATTCCACTTGTGAAGCAT-3’
52	SSRKAU21	**F** 5’-GGTCCAGTTCAATCAACCGA-3’ **R** 5’-TGAAGTCGTCTCATGGTTCG-3**’**
53	SSRKAU22	**F** 5’-GCAGAGGATATTGCATTCGC-3’ **R** 5’-CAAACCGAACTCATCAAGGG-3’
54	SSRKAU23	**F** 5’-TGGCTGCCTCTTCTCTGTTT-3’ **R** 5’-TTTCTTGAAGGGTCTTTCCC-3’
55	SSRKAU24	**F** 5’-CCGAGGCGAATCTTGAATAC-3’ **R** 5’-GCACCATCTCTTGTGCCTCT-3’
56	SSRKAU25	**F** 5’-CTCGTCTTTAGGTATCAATGGAGAT-3’ **R** 5’-TCAATGCTACTCAATGGCTCA-3’
57	SSRKAU5	**F** 5’-GATCGGCAGTAGGTGCTCTC-3’ **R** 5’-CAAGAAACACCCATATCCGC-3’
58	SSR 306	**F** 5’-ACATGAGCCCAATGAACCTC-3’ **R** 5’-AACCATTCCGCACGTACATA-3’
59	SSR 45	**F** 5’-TGTATCCTGGTGGACCAATG-3’ **R** 5’-TCCAAGTATCAGGCACACCA-3’
60	SSR 48	**F** 5’-ATCTCCTTGGCCTCCTGTTT-3’ **R** 5’-GTCATGGCCACATGAATACG-3’
61	SSR350	**F** 5’-GGAATAACCTCTAACTGCGGG-3’ **R** 5’-CGATGCCTTCATTTGGACTT-3’

3.4 Diversity analysis using ISSR assay

Fifteen different ISSR primers were selected from the previous screening (Table 1) for diversity analysis of the selected genotypes.

3.5 Scoring of bands and data analysis

Scoring of bands on agarose was done with the Quantity one software (BioRad) in the Gel Doc imagination system. 100 bp ladder was used as molecular weight size marker for each gel along with DNA samples. The fingerprint was developed from clear distinct band for each genotype. The bands were scored as 1 for presence and 0 for absence respectively and their size recorded in relation to the molecular weight marker used. The bands were also depicted in an word excel format based on molecular weight. Different colour codes were provided to the amplicons of same size with respect to a particular primer in different genotypes ie. bands of same size unique to a genotype was given green colour, those shared by two genotypes were given yellow colour and so on. The fingerprint results obtained from ISSR and SSR were transformed into data matrix as discrete variables. Jaccards coefficient of similarity was measured and a dendrogram based on similarity coefficient was generated by using Unweighted pair Group Method with Arithmetic means (UPGMA). Only distinct and well resolved fragments were scored. The resulting data were analysed using the software package NTsys (Rohlf, 2005).

Resolving power (Prevost and Wilkinson, 1999) was used to identify the primers that would distinguish the accessions most efficiently. Resolving power (Rp) of ISSR primers was calculated as the sum of 'band informativeness' of the bands product by the primer. Band informativeness (Ib) is = 1(2(0.5-p)), where p is the proportion of accession containing the band. Resolving power of the primer is represented as : $Rp = \Sigma Ib$.

Polymorphic information content (PIC) value of the primer is represented as $PIC= 1- \Sigma pi^2$, where *pi* is the frequency of the i^{th} allele. Thus confirming the suitability of the primer selected for the DNA fingerprinting. The PIC value (Hollman *et al.,* 2005) of the marker detects polymorphism within a population depending on the detectable alleles and their frequency.

Results

4. RESULTS

The results of different experiments carried out for DNA fingerprinting of four varieties, three accessions and the related species are described in this chapter.

4.1 Morphological characterisation

Morphological characters were recorded as per the minimal descriptor list of NBPGR (Srivastava *et al*., 2001). Morphological parameters observed for the different genotypes are listed in Table 3. Among the genotypes studied the main morphological variations were observed for plant growth habit, flower colour, fruit shape and colour. The specific characteristics of the genotypes can be observed in the Plate 1 and 2.

4.1.1 Vegetative characters

1. There are three type of growth habit in brinjal *viz.*, prostrate, intermediate and erect. Most of the varieties/accessions had intermediate growth habit except Swetha, SM 116 and *S. macrocarpon* which were erect in stature. The SM 396 accession was having a prostrate growth habit.
2. In petiole, four types of colors were observed among the genotypes *viz.*, violet, greenish violet, green and dark violet. Among all the genotypes, Surya, *S. melongena* var. *insanum* and *S. macrocarpon* were having violet petiole. Neelima, SM 116 and SM 397 were dark violet colored. Haritha and SM 396 having green and Swetha was the only one accession observed which was having greenish violet petiole colour.
3. Two main leaf colours observed were green and dark green. Among the genotypes, Haritha and SM 396 were green and others were dark green colored.
4. SM 397 and *S. melongena* var. *insanum* were the only accessions having prickles on leaves, stem and calyx. In other genotypes, prickles were completely absent.

4.1.2 Reproductive characters

1. Brinjal flowers are basically of two colours either white or violet. Among the varieties/accessions under investigation Haritha was the only variety with white flowers.

2. Varieties/accessions varied greatly in there fruit characteristics like fruit colour and fruit shape. There were purple, white, green and striated fruits. Haritha, SM 396 and *S. melongena* var. *insanum* were having green fruits. Swetha and *S. macrocarpon* were having white fruits. Surya, Neelima, SM 116 and SM 397 were purple fruited.

3. Swetha and Haritha were having the long fruit shape, other genotypes were oval shaped. The wild relatives *S. melongena* var. *insanum* and *S. macrocarpon* belonged to the round fruited category.

4. Swetha and Haritha were the only two varieties having slightly curved fruit.

5. *S. macrocarpon* was having semi pendent fruit position and others were pendent in nature for fruit position.

Table 3: Morphological characters of brinjal varieties/accessions and related species

Morphological characters	**varieties / accessions/related species**								
	Surya	Swetha	Haritha	Neelima	SM 116	SM 396	SM 397	*S. melongena* var. *insanum*	*S. macrocarpon.*
Plant growth habit	Intermediate	Erect	Intermediate	Intermediate	Erect	Prostrate	Intermediate	Intermediate	Erect
Leaf colour	Dark green	Dark green	Green	Dark green	Dark green	Green	Dark green	Dark green	Dark green
Petiole colour	Violet	Greenish violet	Green	Dark Violet	Dark Violet	Green	Dark Violet	Violet	Violet
Presence of prickles on leaves	Absent	Absent	Absent	Absent	Absent	Absent	Present	Present	Absent
Presence of prickles on stem	Absent	Absent	Absent	Absent	Absent	Absent	Present	Present	Absent
Presence of prickles on calyx	Absent	Absent	Absent	Absent	Absent	Absent	Present	Present	Absent
Flower colour	Light Violet	Bluish violet	White	Bluish violet	Bluish violet	Light Violet	Bluish violet	Light Violet	Bluish violet
Fruit colour	Purple	Milky white	Green	Purple	Purple	Green	Purple	Green	Milky white
Fruit shape	Oval	Long	Long	Oval	Oval	Oval	Oval	Round	Round
Fruit curvature	None	Slightly curved	None	None	None	None	None	None	None
Fruit position	Pendent	Pendent	Pendent	Pendent	Pendent	Pendent	Pendent	Pendent	Semi pendent

a. Surya

b. Swetha

c. Haritha

d. Neelima (hybrid)

Plate 1: Morphological characters of the brinjal varieties and hybrid released by KAU

a. SM 116

b. SM 397

c. SM 396

d. S. melongena* var. *insanum

e. S. macrocarpon

Plate 2: Morphological characters of the three accession and two wild relatives

4.2 Molecular characterisation

4.2.1 Isolation, purification and quantification of DNA

As reported in many other crops young leaves were selected as the ideal part for extraction of genomic DNA. The leaves were collected in the morning (8 to 9 am) from potted plants. Young tender, pale green leaves (1g) yielded good quality DNA in sufficient quantity.

Genomic DNA isolated through the CTAB method reported by Roger and Bendich (1994) was not pure and had RNA contamination (Plate 3a). There was no browning of the extract when β-mercaptoethanol was added. RNase treatment and further precipitation gave sufficient quantity of good quality DNA from leaf sample. The agarose gel electrophoresis indicated clear discrete band without RNA contamination (Plate 3b) and spectrophotometric analysis gave ratio of UV absorbance ($A_{260}/_{280}$) between 1.8 and 2.0 (Table 4).

4.3 Molecular Marker Analysis:

The protocol for different marker assays- ISSR and SSR were validated with bulked DNA of brinjal varieties. Different primers were screened with the genomic DNA of three selected varieties utilizing the validated protocols.

4.3.1 Inter Simple Sequence Repeat (ISSR) analysis:

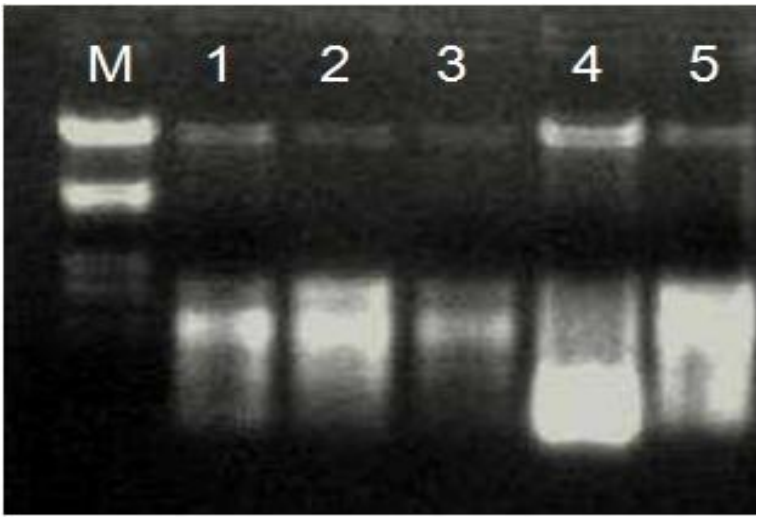
4.3.1.1 Primer screening for ISSR assay:

Thirty six ISSR primers used for amplification of the genomic DNA with thermal settings mentioned earlier (3.3.2) gave different amplification pattern (Plate 4a and Table 5) for the bulked DNA. Based on the amplification pattern, 10 primers were selected for ISSR analysis. Details are provided in Table 6.

4.3.2 Simple Sequence Repeat (SSR) analysis:

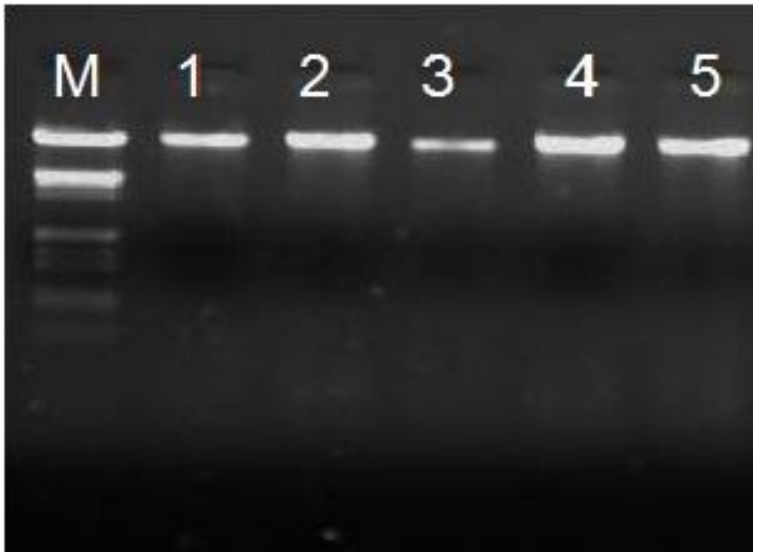
4.3.2.2 Primer screening for SSR assay:

Sixty one primer sets were screened for amplification of SSR region in the genomic DNA of brinjal with thermal settings mentioned earlier (3.3.3). The amplification observed is presented in Plate 4b and Table 7. Based on the best amplification pattern, 10 primer sets (Table 8) were selected for SSR fingerprinting of brinjal genotypes.



M: Molecular weight marker λ DNA(Eco RI/ *Hind* III digest) Lane 1 to 5 brinjal DNA samples

a. Rogers and Bendich method before RNase treatment



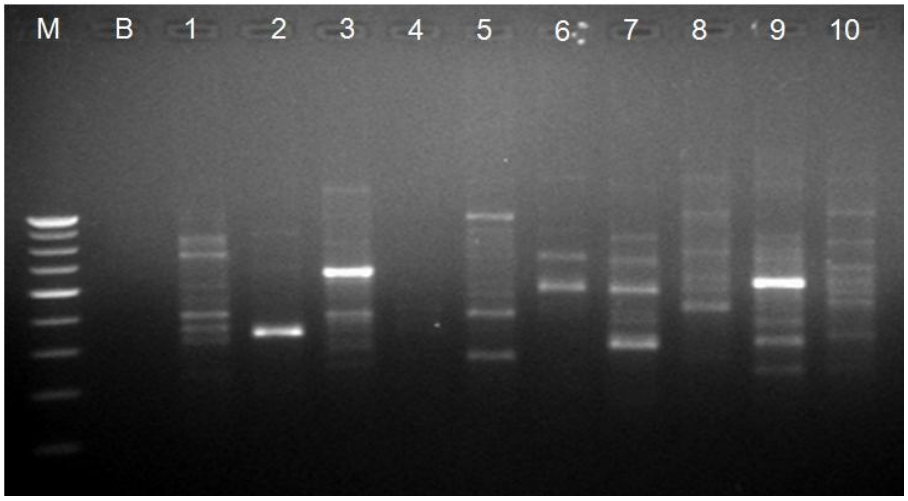
M: Molecular weight marker λ DNA(Eco RI/ *Hind* III digest) Lane 1 to 5 brinjal DNA samples

b. Rogers and Bendich method after RNase treatment

Plate 3 : Isolation of DNA from tender leaves in brinjal

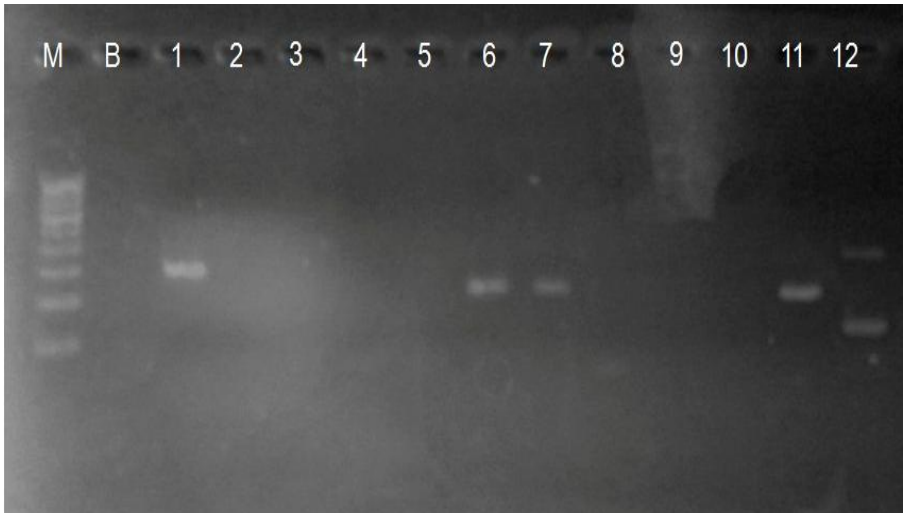
Table 4: Quality and quantity of DNA isolated from brinjal genotypes assessed by Nano Drop spectrophotometer

Genotype	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
Surya	4.956	2.588	1.82	247.81
Swetha	10.422	5.669	1.84	521.01
Haritha	10.422	5.569	1.85	540.90
Neelima	43.277	22.501	1.89	2163.85
SM 116	23.314	12.950	1.80	1165.69
SM 396	14.150	7.707	1.84	707.52
SM 397	26.473	14.337	1.85	1323.64
S. melongena var. *insanum*	9.584	4.914	1.87	478/99
S. macrocarpon	25.062	14.248	1.87	1253.11



M : Marker 100bp ladder, B: Control, 1-10: Amplification pattern with different primers

a. Screening of ISSR primers for amplification of brinjal DNA



M : Marker 100bp ladder, B: Control, 1-12: Amplification pattern with different primers

b. Screening of SSR primers for amplification of brinjal DNA

Plate 4: Screening of ISSR and SSR primers for amplification of brinjal genomic DNA

Table 5: Details of amplification with the 36 primers screened for ISSR assay in brinjal genotypes

l. No	Primers	Amplification pattern			
		No. of Bands	type of bands		Remarks
			Distinct	Faint	
1	UBC 811	8	7	1	Selected
2	UBC 813	1	1	0	Not selected
3	UBC 814	0	0	0	Not selected
4	UBC 815	2	1	1	Not selected
5	UBC 834	6	5	1	Not selected
6	UBC 835	4	2	2	Not selected
7	UBC 836	3	0	3	Not selected
8	UBC 840	6	4	2	Not selected
9	UBC 844	5	4	1	Selected
10	UBC 890	4	2	2	Not selected
11	UBC 866	7	5	2	Selected
12	UBC 807	9	6	3	Selected
13	UBC 843	0	0	0	Not selected
14	UBC S2	8	7	1	Selected
15	UBC 820	2	1	1	Not selected
16	UBC 854	7	6	1	selected
17	UBC 845	1	0	1	Not selected
18	UBC 817	0	0	0	Not selected
19	UBC 826	8	5	3	Selected
20	UBC 818	0	0	0	Not selected
21	ISSR 04	7	5	2	Selected
22	ISSR 05	3	1	1	Not selected
23	ISSR 06	7	4	3	Not selected
24	ISSR 07	0	0	0	Not selected
25	ISSR 08	10	3	7	Selected
26	ISSR 09	4	2	2	Not selected
27	ISSR 10	6	4	2	Selected
28	ISSR 15	6	5	1	Selected
29	SPS 03	6	1	5	Not selected
30	SPS 08	6	0	6	Not selected
31	UBC 868	7	5	2	Selected
32	UBC 895	7	6	1	Selected
33	UBC 899	8	5	3	Selected
34	UBC 880	8	6	2	Selected
35	UBC 892	0	0	0	Not selected
36	UBC 900	0	0	0	Not selected

Table 6: Details of ISSR primers selected for fingerprinting

Sl. No.	Primer	Annealing temperature (°C)	Nucleotide Sequence
1	UBC 868	43	5’GAAGAAGAAGAAGAAGA3’
2	UBC 807	45	5’AGAGAGAGAGAGAGAGT3’
3	ISSR 08	45	5’GAGAGAGAGAGAGAGAT3’
4	ISSR 15	45	5’TCCTCCTCCTCCTCC3’
5	UBC 811	47	5’GAGAGAGAGAGAGAGAC3’
6	ISSR 04	47	5’ACACACACACACACACC3’
7	ISSR 10	47	5’ACACACACACACACACG3’
8	UBC S2	51	5’CTCTCTCTCGTGTGTGTG3’
9	UBC 826	52	5’ACACACACACACACACC3’
10	UBC 866	55	5’CTCCTCCTCCTCCTCCTC3’

Table 7: Details of amplification pattern with the 61 primers screened for SSR assay in brinjal genotypes

Sl. No	Primer	Amplification pattern			
		No. of Bands	type of bands		Remarks
			Distinct	Faint	
1	LE aac001	1	0	1	Not selected
2	LE aat001	0	0	0	Not selected
3	LE aat004	1	1	0	Not selected
4	LE aat005	0	0	0	Not selected
5	LE aat006	0	0	0	Not selected
6	LE aat007	0	0	0	Not selected
7	LE aat008	0	0	0	Not selected
8	LE ac001	1	1	0	Selected
9	LE act001	0	0	0	Not selected
10	LE agat001	0	0	0	Not selected
11	LE aga001	0	0	0	Not selected
12	LE ag001	0	0	0	Not selected
13	LE ag002	0	0	0	Not selected
14	LE ag003	0	0	0	Not selected
15	LE at001	0	0	0	Not selected
16	LE at002	0	0	0	Not selected
17	LE at003	0	0	0	Not selected
18	LE at004	0	0	0	Not selected
19	LE at005	0	0	0	Not selected
20	LE at006	0	0	0	Not selected
21	LE at007	0	0	0	Not selected
22	LE at008	0	0	0	Not selected
23	LE at011	0	0	0	Not selected
24	LE at012	0	0	0	Not selected
25	LE at013	0	0	0	Not selected
26	LE at014	0	0	0	Not selected
27	LE at015	0	0	0	Not selected
28	LE at016	0	0	0	Not selected
29	LE at017	0	0	0	Not selected
30	LE at019	0	0	0	Not selected
31	LE aat001	1	1	0	Not selected

32	LE at010	0	0	0	Not selected
33	LE at018	0	0	0	Not selected
34	SSRKAU1	1	1	0	Selected
35	SSRKAU2	0	0	0	Not selected
36	SSRKAU3	0	0	0	Not selected
37	SSRKAU4	0	0	0	Not selected
38	SSRKAU7	0	0	0	Not selected
39	SSRKAU8	1	1	0	Not selected
40	SSRKAU9	1	1	0	Selected
41	SSRKAU10	0	0	0	Not selected
42	SSRKAU11	0	0	0	Not selected
43	SSRKAU12	0	0	0	Not selected
44	SSRKAU13	1	1	0	Selected
45	SSRKAU15	0	0	0	Not selected
46	SSRKAU16	0	0	0	Not selected
47	SSRKAU17	1	1	0	Selected
48	SSRKAU18	0	0	0	Not selected
49	SSRKAU6	0	0	0	Not selected
50	SSRKAU19	0	0	0	Not selected
51	SSRKAU20	0	0	0	Not selected
52	SSRKAU21	0	0	0	Not selected
53	SSRKAU22	0	0	0	Not selected
54	SSRKAU23	1	1	0	Selected
55	SSRKAU24	0	0	0	Not selected
56	SSRKAU25	0	0	0	Not selected
57	SSRKAU5	0	0	0	Not selected
58	SSR 306	1	1	0	Selected
59	SSR 45	1	1	0	Selected
60	SSR 48	1	1	0	Selected
61	SSR350	1	1	0	Selected

Table 8: Details of SSR primers selected for fingerprinting

Sl. No.	Name of Primer	Annealing temperature (°C)	Sequence
1	SSRKAU1	53	**F** 5'-AATTCACCTTTCTTCCGTCG-3' **R** 5'-GCCCTCGAATCTGGTAGCTT-3'
2	SSR 306	53	**F** 5'-ACATGAGCCCAATGAACCTC-3' **R** 5'-AACCATTCCGCACGTACATA-3'
3	SSR 45	54	**F** 5'-TGTATCCTGGTGGACCAATG-3' **R** 5'-TCCAAGTATCAGGCACACCA-3'
4	LE ac001	53	F 5'-TGCCTTCCATCTAACCAATC-3' R 5'-CTGTGGCAAATATGTCCCTAAG-3'
5	SSR 48	54	**F** 5'-ATCTCCTTGGCCTCCTGTTT-3' **R** 5'-GTCATGGCCACATGAATACG-3'
6	SSRKAU17	55	**F** 5'-TTCGTTGAAGAAGATGATGGTC-3' **R** 5'-CAAAGAGAACAAGCATCCAAGA-3'
7	SSRKAU9	53	**F** 5'-ACATGAGCCCAATGAACCTC-3' **R** 5'-AACCATTCCGCACGTACATA-3'
8	SSR350	53	**F** 5'-GGAATAACCTCTAACTGCGGG-3' **R** 5'-CGATGCCTTCATTTGGACTT-3'
9	SSSKAU23	53	**F** 5'-TGGCTGCCTCTTCTCTGTTT-3' **R** 5'-TTTCTTGAAGGGTCTTTCCC-3'
10	SSRKAU13	53	**F** 5'-GGAATAACCTCTAACTGCGGG-3' **R** 5'-CGATGCCTTCATTTGGACTT-3'

4.4. DNA fingerprinting of the brinjal varieties/accessions and wild relatives

4.4.1. Surya

A. ISSR profile

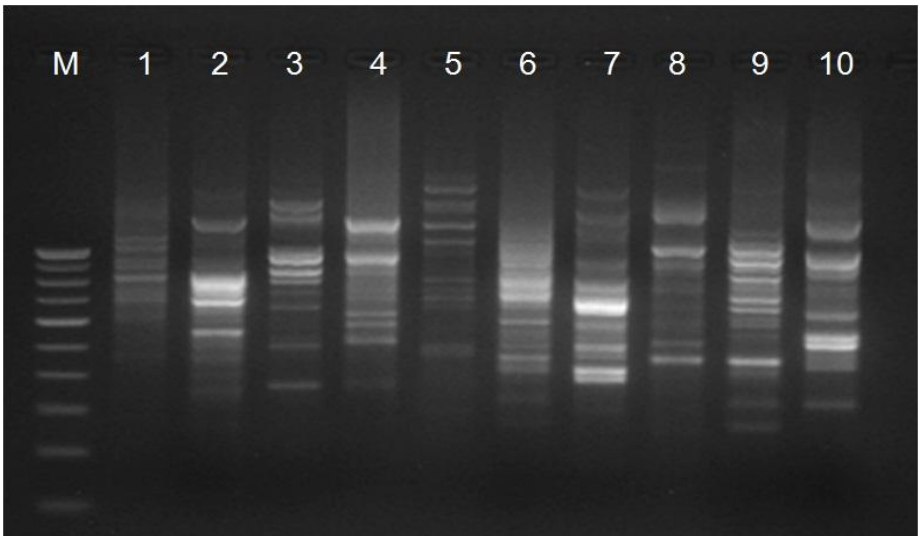
The amplification pattern observed for the genomic DNA of brinjal variety Surya with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 5a and Table 9.

Seventy four different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1800bp (Table 9). Most of the primers amplified eight distinct bands for the variety Surya while it was only five for the primer UBC 868 and UBC S2. The primer UBC 826 gave 10 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.1a.

B. SSR profile

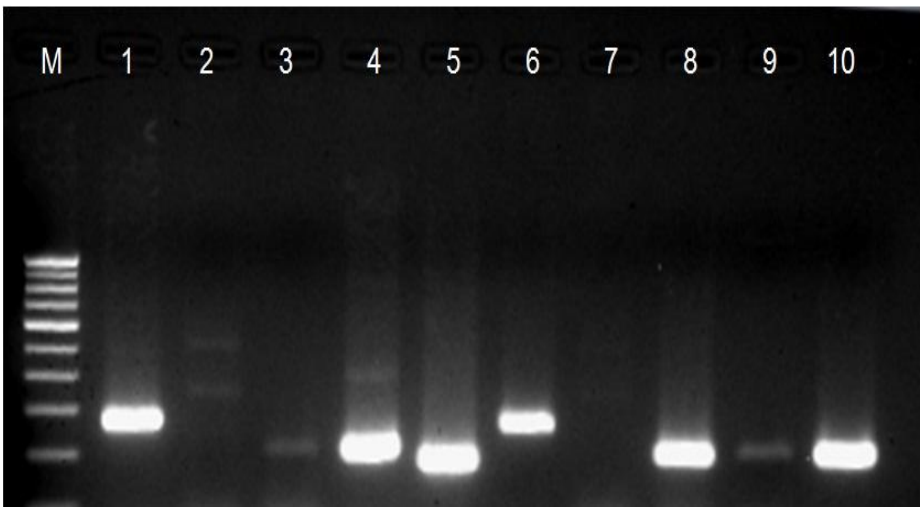
The amplification pattern observed for the genomic DNA of brinjal variety Surya with the 10 selected primers in SSR analysis is presented in Plate 5b and Table 10.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 10). The amplicons ranged in size from 200bp to 300bp. Fingerprint developed based on clear distinct bands is provided in Fig.1b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp),
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 5: Amplification pattern of brinjal variety Surya with the selected ISSR and SSR primers

Table 9: Amplification pattern depicted for the brinjal variety Surya with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	7	11	11	9	11	10	11	8	13	9
Distinct Amplicons	5	8	9	6	9	9	8	5	10	8
Distribution (Mol. Size/ bp)										
1800					—					
1700					—					
1600										
1500			—		—					
1400										
1300		—	—	—	—			—		—
1200										
1100	—								—	
1000	—		—		—	—		—	—	—
900	=		—	—		—	—	—	—	—
800	—	=	—	—	=	—	—		—	
700		=			=	—	—		=	—
600		=		=		—	—		=	=
500		—	—	—		=	=	=	=	=
400						=	=			

Table 10: Amplification pattern depicted for the brinjal variety Surya with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. Size/ bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 1: Fingerprint for brinjal variety Surya through ISSR and SSR analysis

4.4.2. Swetha

A. ISSR profile

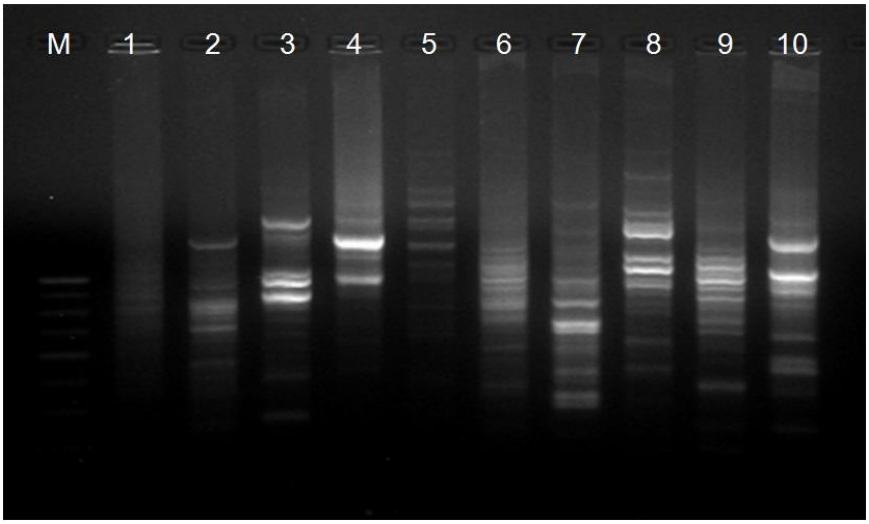
The amplification pattern observed for the genomic DNA of brinjal variety Swetha with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 6a and Table 11.

Seventy three different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1800bp (Table 11). Most of the primers amplified eight distinct bands for the variety Swetha while it was only three for the primer ISSR 15. The primer UBC S2 and UBS 826 gave 10 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.2a.

B. SSR profile

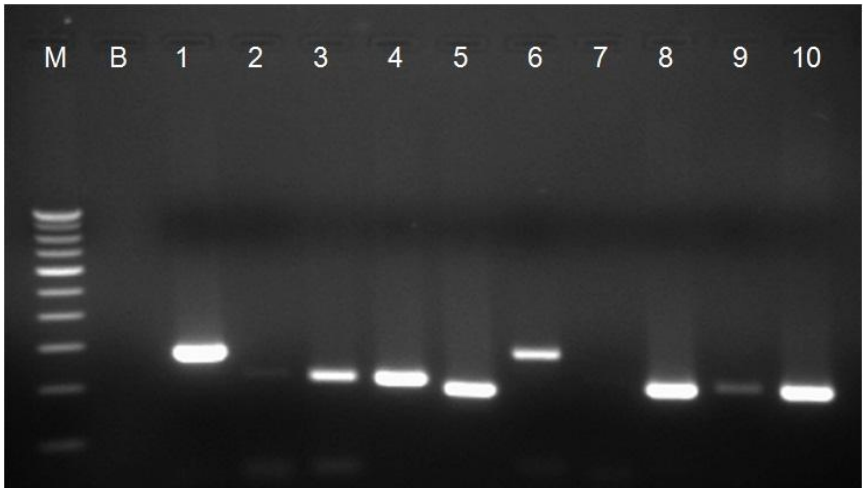
The amplification pattern observed for the genomic DNA of brinjal variety Swetha with the 10 selected primers in SSR analysis is presented in Plate 6b and Table 12.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 12). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.2b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04,
7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17,
7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 6: Amplification pattern of brinjal variety Swetha with the selected ISSR and SSR primers

Table 11: Amplification pattern depicted for the brinjal variety Swetha with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	4	8	9	8	11	9	12	10	12	9
Distinct Amplicons	4	6	7	3	9	8	9	10	10	7
Distribution (Mol. Size/ bp)										
1800					—					
1700					—			—		
1600										
1500			—	—	—			—		
1400					—					
1300		—	—	—	—	—			—	—
1200						—		—	—	
1100	—				—	—		—	—	
1000	—		—		—	—	—	— —	— —	—
900	—	—	—	—	—	—			—	—
800	—	— —	—		—	—	—	— —	— —	—
700		—	— —			—	— —		—	—
600		—				—	—	—		— —
500							— —	—	—	
400							— —			

Table 12: Amplification pattern depicted for the brinjal variety Swetha with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. Size/ bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

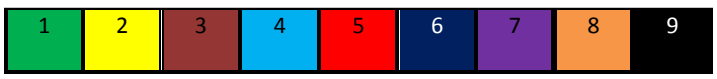


Fig 2: Fingerprint for brinjal variety Swetha through ISSR and SSR analysis

4.4.3. Haritha

A. ISSR profile

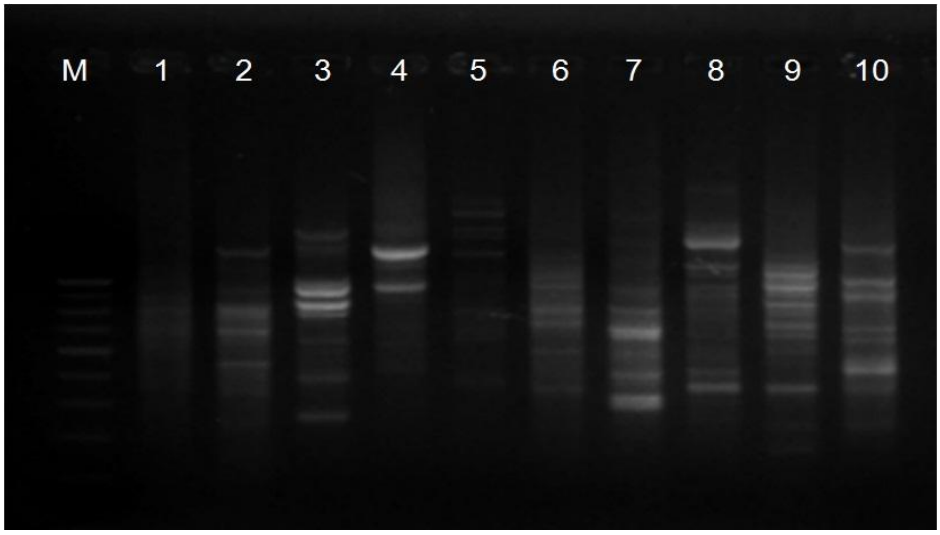
The amplification pattern observed for the genomic DNA of brinjal variety Haritha with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 7a and Table 13.

Seventy five different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 2000bp (Table 13). Most of the primers amplified seven distinct bands for the variety Haritha while it was only three for the primer ISSR 15. The primer UBC S2 and UBC 826 gave 12 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.3a.

B. SSR profile

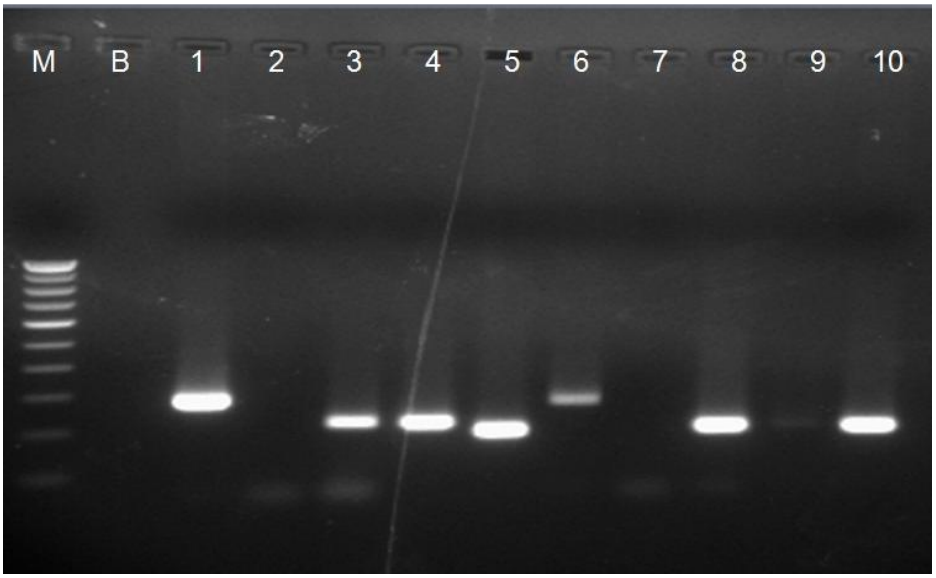
The amplification pattern observed for the genomic DNA of brinjal variety Surya with the 10 selected primers in SSR analysis is presented in Plate 7b and Table 14.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 14). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.3b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 7: Amplification pattern of brinjal variety Haritha with the selected ISSR and SSR primers

Table 13: Amplification pattern depicted for the brinjal variety Haritha with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	5	7	10	7	11	8	9	14	13	7
Distinct Amplicons	5	6	10	3	7	7	6	12	12	6
Distribution (Mol. Size/ bp)										
2000										
1900										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

Table 14: Amplification pattern depicted for the brinjal variety Haritha with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. Size/ bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
2000										
1900										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 3: Fingerprint for brinjal variety Haritha through ISSR and SSR analysis

4.4.4. Neelima

A. ISSR profile

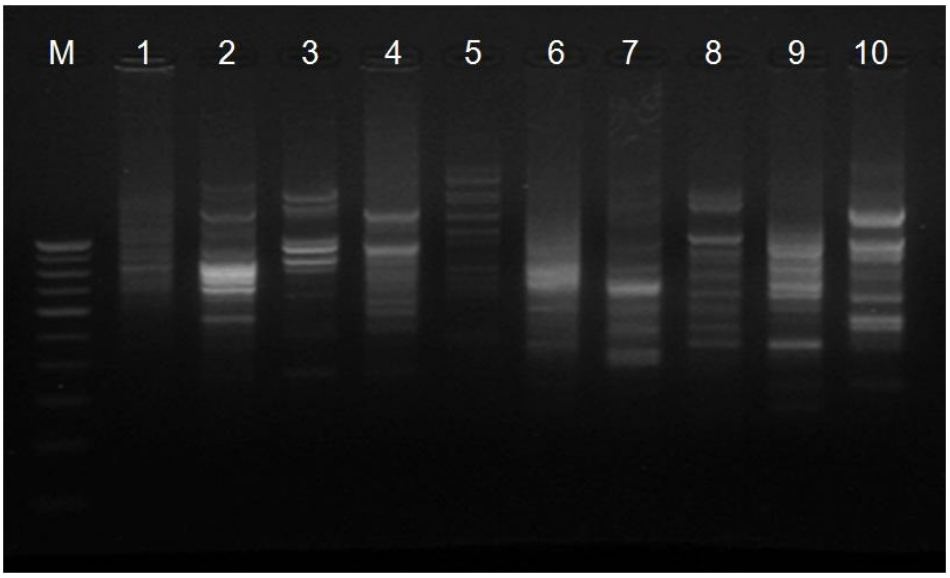
The amplification pattern observed for the genomic DNA of brinjal F1 hybrid Neelima with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 8a and Table 15.

Eighty one different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1800bp (Table 15). Most of the primers amplified nine distinct bands for the F1 hybrid Neelima while it was only five for the primer UBC 868. The primer UBC 826 gave 10 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.4a.

B. SSR profile

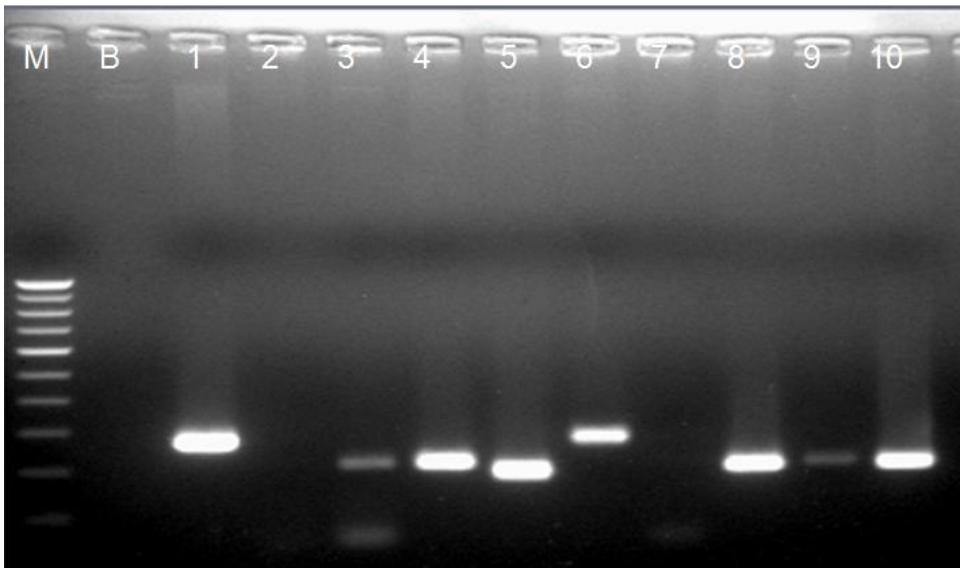
The amplification pattern observed for the genomic DNA of brinjal F1 hybrid Neelima with the 10 selected primers in SSR analysis is presented in Plate 8b and Table 16.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 16). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.4b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 8: Amplification pattern of brinjal F1 hybrid Neelima with the selected ISSR and SSR primers

Table 15: Amplification pattern depicted for the brinjal F1 hybrid Neelima with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	10	10	11	13	12	13	5	12	8	9
Distinct Amplicons	7	4	4	7	8	6	2	6	3	5
Distribution (Mol. Size/ bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

Table 16: Amplification pattern depicted for the brinjal F1 hybrid Neelima with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. size/bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 4: Fingerprint for brinjal hybrid Neelima through ISSR and SSR analysis

4.4.5. SM 396

A. ISSR profile

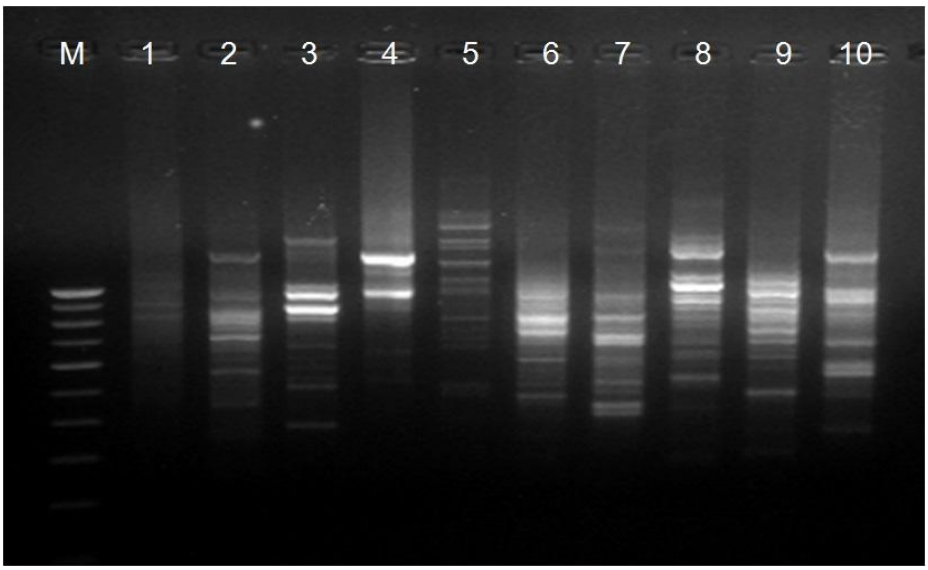
The amplification pattern observed for the genomic DNA of brinjal accession SM 396 with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 9a and Table 17.

Eighty four different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1800bp (Table 17). Most of the primers amplified ten distinct bands for the accession SM 396 while it was only two for the primer ISSR 15. The primer ISSR 08 gave 12 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.5a.

B. SSR profile

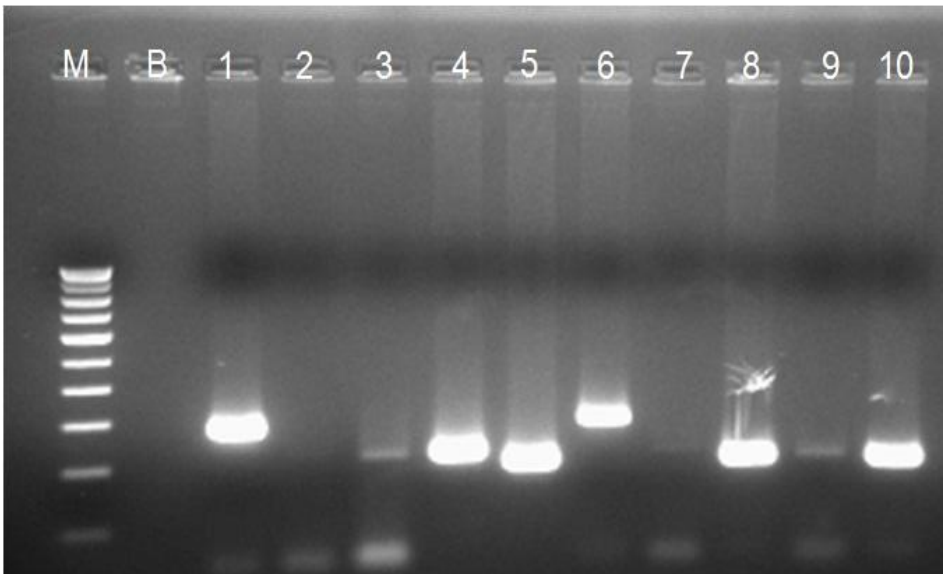
The amplification pattern observed for the genomic DNA of brinjal accession SM 396 with the 10 selected primers in SSR analysis is presented in Plate 9b and Table 18.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 18). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.5b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker(100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 9: Amplification pattern of brinjal accession SM 396 with the selected ISSR and SSR primers

Table 17: Amplification pattern depicted for the brinjal accession SM 396 with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	4	9	12	6	13	10	11	12	13	9
Distinct Amplicons	4	8	12	2	11	10	9	11	10	7
Distribution (Mol. Size/ bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

Table 18: Amplification pattern depicted for the brinjal accession SM 396 with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. Size/ bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 5: Fingerprint for brinjal accession SM 396 through ISSR and SSR analysis

4.4.6. SM 397

A. ISSR profile

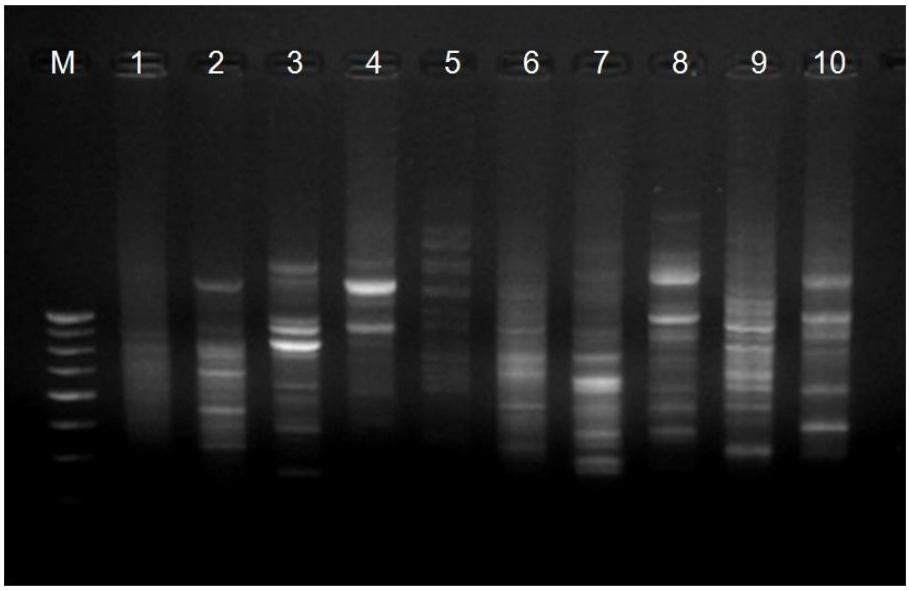
The amplification pattern observed for the genomic DNA of brinjal accession SM 397 with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 10a and Table 19.

Eighty three different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1800bp (Table 19). Most of the primers amplified nine distinct bands for the accession SM 397 while it was only three for the primer ISSR 15. The primer UBC 826 andUBC 811 gave 11 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.6a.

B. SSR profile

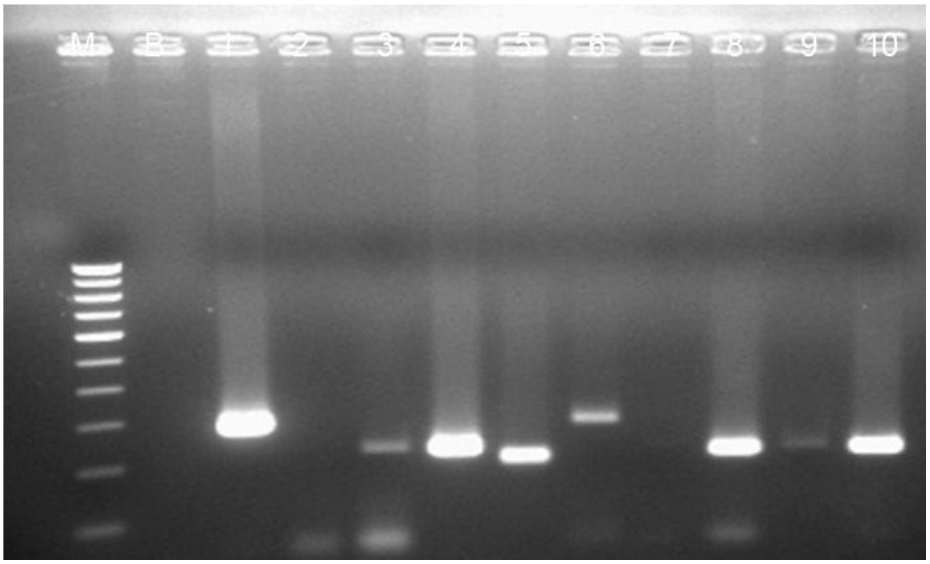
The amplification pattern observed for the genomic DNA of brinjal accession SM 397 with the 10 selected primers in SSR analysis is presented in Plate 10b and Table 20.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 20). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.6b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 10: Amplification pattern of brinjal accession SM 397 with the selected ISSR and SSR primers

Table 19: Amplification pattern depicted for the brinjal accession SM 397 with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	5	10	11	7	12	10	9	12	11	6
Distinct Amplicons	5	9	10	3	11	10	9	9	11	6
Distribution (Mol. Size/ bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

Table 20: Amplification pattern depicted for the brinjal accession SM 397 with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. Size/ bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 6: Fingerprint for brinjal accession SM 397 through ISSR and SSR analysis

4.4.7. *S. melongena* var. *insanum*

A. ISSR profile

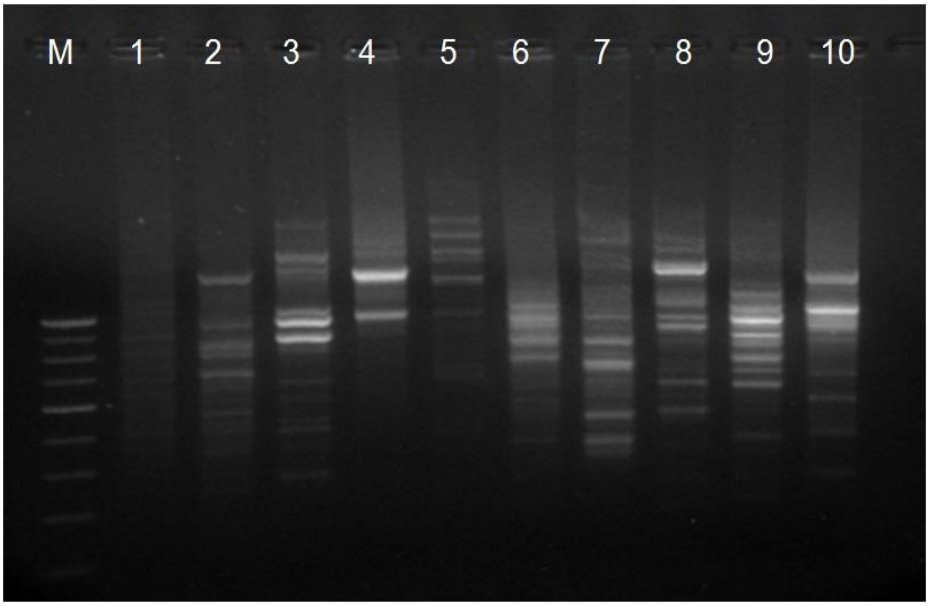
The amplification pattern observed for the genomic DNA of *S. melongena* var. *insanum* with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 11a and Table 21.

Fifty two different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1900bp (Table 21). Most of the primers amplified nine distinct bands for the *S. melongena* var. *insanum* while it was only two for the primer ISSR 15. The primer ISSR 08 and UBC 820 gave 10 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.7a.

B. SSR profile

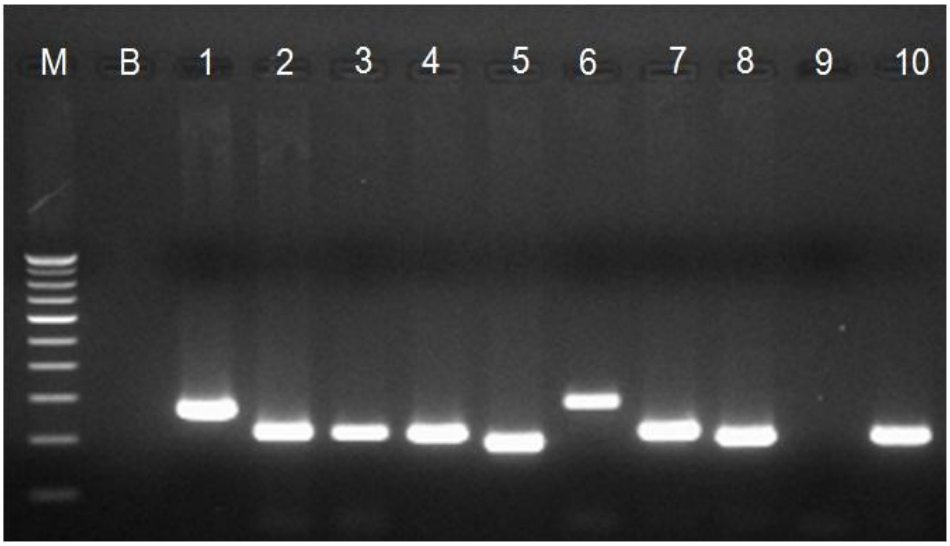
The amplification pattern observed for the genomic DNA of *S. melongena* var. *insanum* with the 10 selected primers in SSR analysis is presented in Plate 11b and Table 22.

Nine different clear distinct loci were observed with the 10 SSR primers (Table 22). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.7b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 11: Amplification pattern of *S. melongena* var. *insanum* with the selected ISSR and SSR primers

Table 21: Amplification pattern depicted for the *S. melongena* var. *insanum* with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	7	11	12	5	11	6	10	10	10	8
Distinct Amplicons	7	5	10	2	6	5	8	8	10	7
Distribution (Mol. Size/ bp)										
1900										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

Table 22: Amplification pattern depicted for the *S. melongena* var. *insanum* with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. size/ bp)										
300	—					—				
250		—	—	—			—	—		—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	**UBC 868**	**UBC 807**	**ISSR 08**	**ISSR 15**	**UBC 811**	**ISSR 04**	**ISSR 10**	**UBC S2**	**UBC 826**	**UBC 866**
Mol size (bp)										
1900										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 7: Fingerprint for *S. melongena* var. *insanum* through ISSR and SSR analysis

4.4.8. *S. macrocarpon*

A. ISSR profile

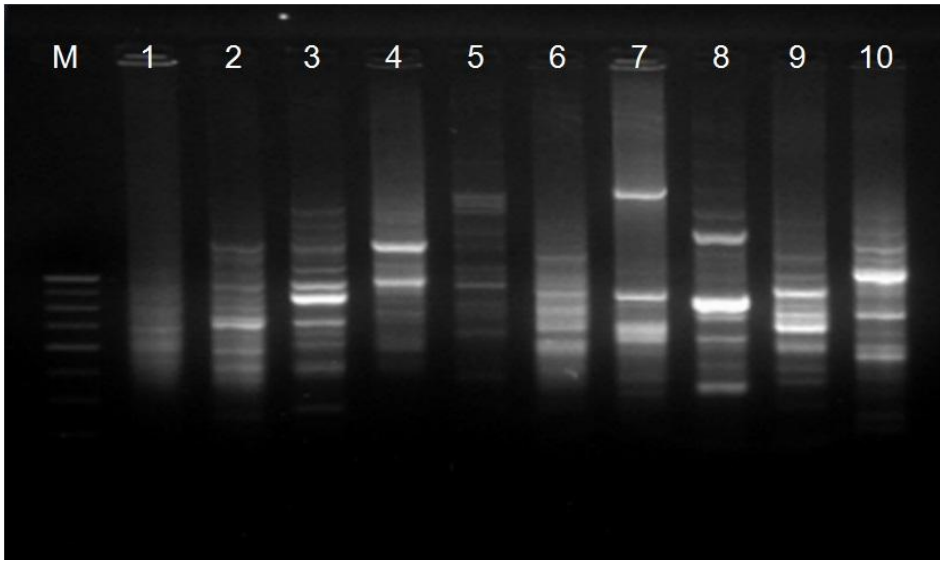
The amplification pattern observed for the genomic DNA of *S. macrocarpon* with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 12a and Table 23.

Seventy different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 2000bp (Table 23). Most of the primers amplified six distinct bands for the *S. macrocarpon* while it was only three for the primer UBC 868. The primer ISSR 08 gave 12 distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.8a.

B. SSR profile

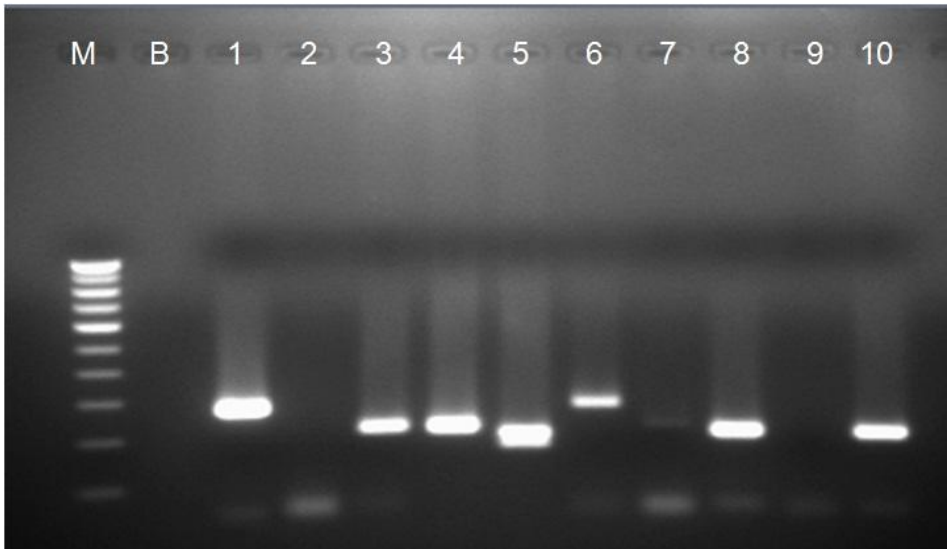
The amplification pattern observed for the genomic DNA of *S. macrocarpon* with the 10 selected primers in SSR analysis is presented in Plate 12b and Table 24.

Seven different clear distinct loci were observed with the 10 SSR primers (Table 24). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.8b.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8-UBCS2, 9- UBC826, 10- UBC866.

a. Amplification with ISSR primers



M- marker (100bp), B- blank
1-SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9- SSR KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers

Plate 12: Amplification pattern of *S. macrocarpon* with the selected ISSR and SSR primers

Table 23: Amplification pattern depicted for the *S. macrocarpon* with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Total Amplicons	8	7	5	4	11	8	3	9	9	9
Distinct Amplicons	5	6	5	3	4	4	3	7	6	6
Distribution (Mol. Size/ bp)										
2000					—		—			
1900					—					
1800										
1700			—		—					
1600										
1500			—		—			—		
1400			—							—
1300		—	—	—	—					—
1200		—	—			—				
1100			—		—					—
1000		—			—	—			—	
900		—	—	—	—	—	—	—		
800	—	—	—	—	—	—		—	—	—
700	—	—	—	—	—	—	—	—	—	
600	—	—	—		—	—		—	—	—
500		—	—					—	—	
400										

Table 24: Amplification pattern depicted for the *S. macrocarpon* with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU 1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. Size/ bp)										
300	—					—				
250			—	—				—		—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
2000										
1900										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

Fig 8: Fingerprint for *S. macrocarpon* through ISSR and SSR analysis

4.4.9 SM 116

A. ISSR profile

The amplification pattern observed for the genomic DNA of brinjal accession SM 116 with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 13 and Table 25.

Sixty eight different clear distinct loci were observed with the 10 ISSR primers and the amplicons ranged in size from 400bp to 1800bp (Table 25). Most of the primers amplified eight distinct bands for the accession SM 116 while it was only two for the primer ISSR 15. The primer UBC 807 and UBC 826 gave nine distinct bands. Fingerprint was developed based on clear distinct bands and is provided in Fig.9a.

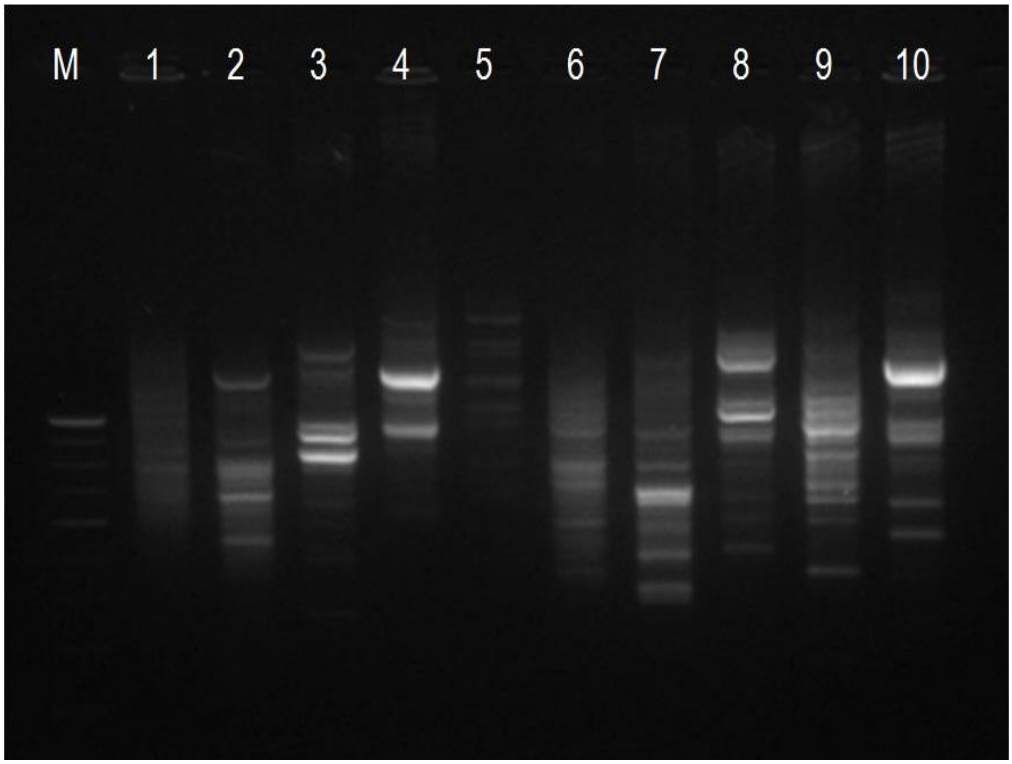
B. SSR profile

The amplification pattern observed for the genomic DNA of brinjal accession SM 116 with the 10 selected primers in SSR analysis is presented in and Table 26.

Eight different clear distinct loci were observed with the 10 SSR primers (Table 26). The amplicons ranged in size from 200bp to 300bp. Fingerprint was developed based on clear distinct bands and is provided in Fig.9b.

4.5 SSR profile for the F1 hybrid Neelima and its parents

The F1 hybrid Neelima and its parents Surya and SM 116 were further analysed together with the 10 selected primers to detect polymorphism so as to prove the hybridity (Plate 14a and 14b). All the 10 primers gave monomorphic bands in all the three genotypes- Neelima and its parents Surya and SM 116. Thus the primers selected were not found good enough to prove hybridity of Neelima.



M- marker (100bp),
1- UBC868, 2- UBC807, 3- ISSR08, 4- ISSR15, 5- UBC811, 6- ISSR04, 7- ISSR10, 8- UBCS2, 9- UBC826, 10- UBC866.

Plate 13: Amplification pattern of brinjal accession SM 116 with the selected ISSR primers

Table 25: Amplification pattern depicted for the brinjal accession SM 116 with the 10 selected ISSR primers.

	1	2	3	4	5	6	7	8	9	10
Primers	UBC868	UBC807	ISSR08	ISSR15	UBC811	ISSR04	ISSR10	UBCS2	UBC826	UBC866
Total Amplicons	6	9	9	7	8	9	11	10	10	9
Distinct Amplicons	5	9	5	2	7	8	8	8	9	7
Distribution (Mol. Size/ bp)										
1800					—					
1700					—					
1600					—			—		
1500			—		—			—		
1400										
1300		—	—	—	—	—				—
1200						—				
1100	—				—	—		—	—	
1000	=		—		—	—		—	—	—
900	—	—	—	—		—	—	—	=	—
800	—	=	—			—	—	=	=	=
700		=				—	=		=	—
600		=				—	—			—
500		—					—	—	—	
400							=			

Table 26: Amplification pattern depicted for the brinjal accession SM 116 with the 10 selected SSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac001	SSR 48	SSR KAU 17	SSR KAU 9	SSR 350	SSR KAU 23	SSR KAU 13
Total Amplicons	1	0	1	1	1	1	0	1	1	1
Distinct Amplicons	1	0	1	1	1	1	0	1	1	1
Distribution (Mol. size/ bp)										
300	—					—				
250			—	—				—	—	—
225					—					
200										

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
Mol size (bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

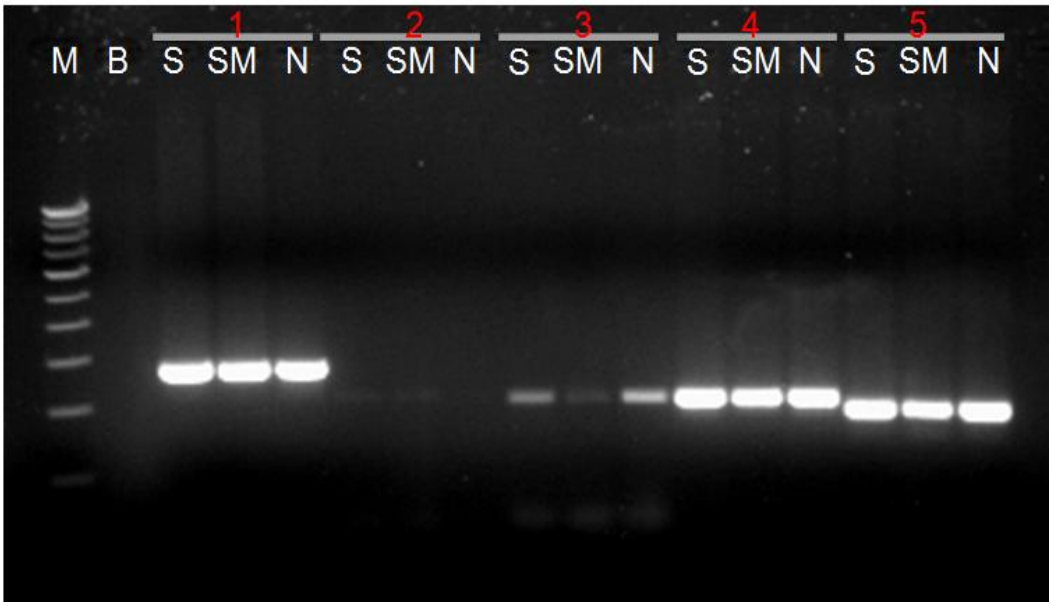
a. ISSR fingerprint

	1	2	3	4	5	6	7	8	9	10
Primers	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13
Mol size (bp)										
300										
250										
225										
200										

b. SSR fingerprint

Colour code for sharing of bands among genotypes

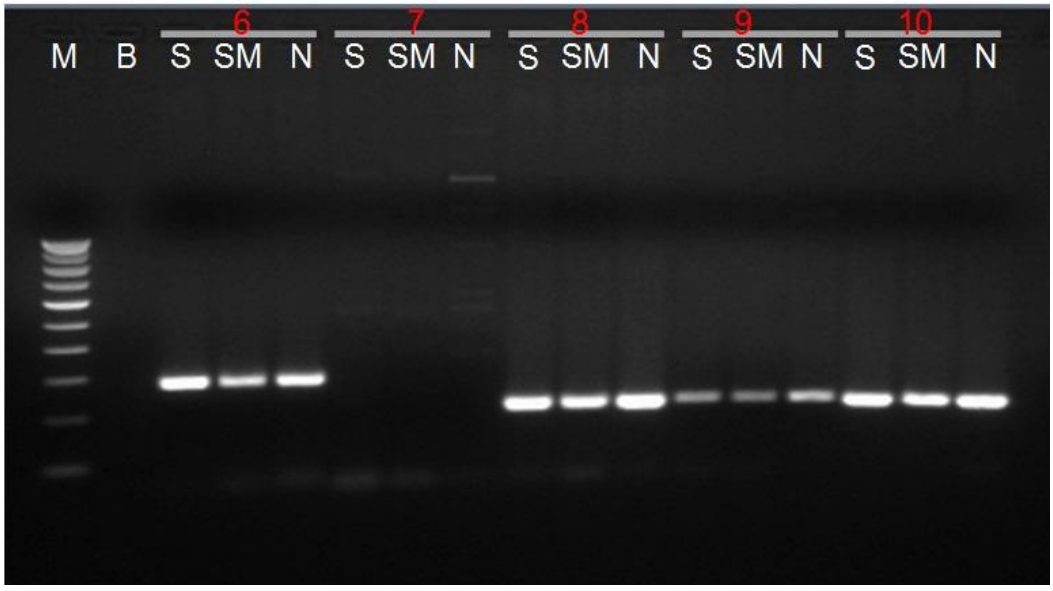
Fig 9: Fingerprint for brinjal accession SM 116 through ISSR and SSR analysis



S- Surya, SM- SM 116, N- Neelima

M- marker(100bp), B- blank 1- SSR KAU1, 2- SSR 306, 3- SSR 45, 4- LEac001, 5- SSR 48.

a. Amplification with SSR primers (1 to 5)



S- Surya, SM- SM 116, N- Neelima

M- marker(100bp), B- blank, 6- SSR KAU17, 7- SSR KAU 9, 8- SSR 350, 9 KAU 23, 10- SSR KAU 13

b. Amplification with SSR primers (6to 10)

Plate 14: Amplification pattern of brinjal F1 hybrid Neelima and its parents Surya and SM116 with the selected SSR primers

4.6 Diversity analysis of nine brinjal genotypes using 15 ISSR primers

In order to perform the diversity analysis, characterisation was done with five additional primers (Plate 15) so as to generate the ISSR data with 15 primers (Table 27). The details of amplification with the five additional primers are as follows.

UBC 844

The primer UBC 844 generated a total of thirteen clear, distinct and reproducible bands and it could detect four polymorphic bands (Plate15.1). The per cent of polymorphism was 69.23.

UBC 854

The ISSR primer UBC 854 was able to generate eighteen amplicons. They were distinct and reproducible. It could generate nine polymorphic bands out of eighteen amplicons (Plate15.2) and the per cent of polymorphism was 50.

UBC 880

A total of fifteen amplicons were produced by the primer UBC 880. They were clear, distinct and reproducible (Plate15.3). It could generate eight polymorphic bands out of fifteen amplicons and the per cent of polymorphism was 53.33.

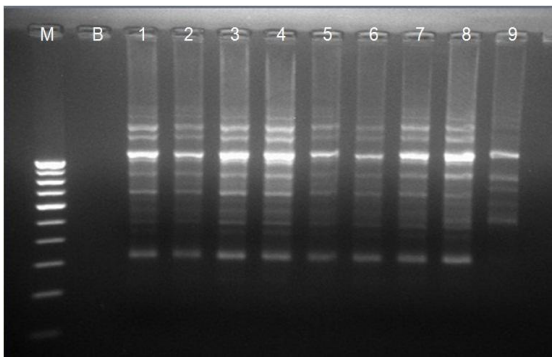
UBC 895

The gel profile with the amplification of UBC 895 gave a total of fourteen amplicons and all amplicons were clear and distinct. It could generate nine polymorphic bands (Plate15.4), giving a polymorphism percentage of 64.28.

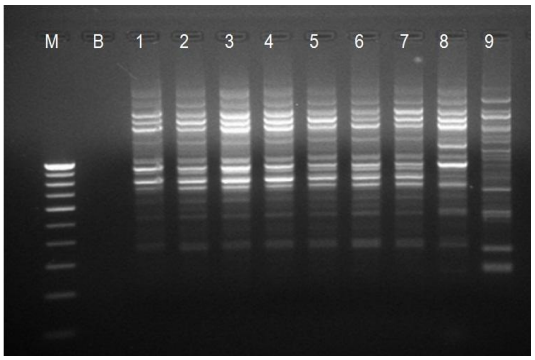
UBC 899

The ISSR primer UBC 899 was able to generate a total of thirteen amplicons. It detected five polymorphic amplicons (Plate15.5) giving a polymorphism percentage of 38.46.

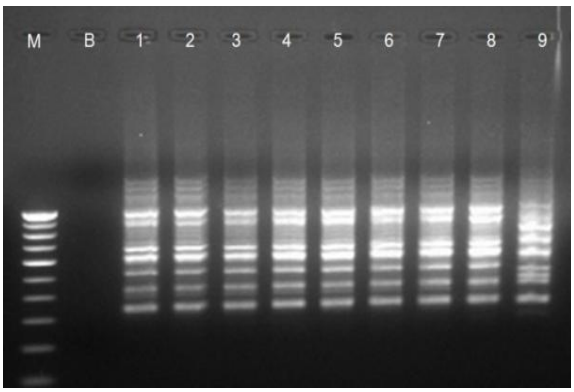
The consolidated data for diversity analysis with the 15 primers is presented in Table 28.



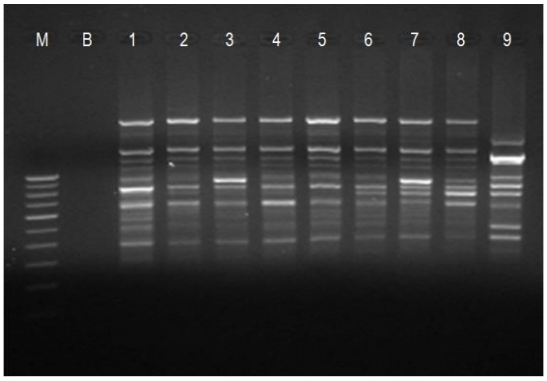
1. UBC 844



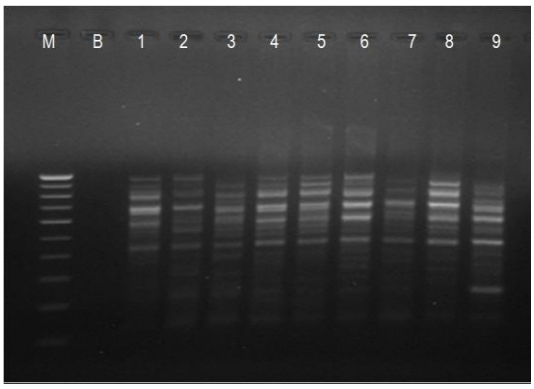
2. UBC 854



3. UBC 880



4. UBC 895



5. UBC 899

M- marker (100bp), B- blank, 1- Surya, 2- SM 116, 3- Neelima, 4- Swetha, 5- Haritha, 6- SM 396, 7- SM 397, 8- *S. melongena* var. *insanum*, 9- *S. macrocarpon*

Plate 15: Amplification pattern of brinjal accessions with the five ISSR primers.

Table 27: Details of selected ISSR primers used for diversity analysis

Sl. No.	Name of Primer	Annealing temperature (ºC)	Sequence
1	UBC 868	43	5'GAAGAAGAAGAAGAAGA3'
2	UBC 807	45	5'AGAGAGAGAGAGAGAGT3'
3	ISSR 08	45	5'GAGAGAGAGAGAGAGAT3'
4	ISSR 15	45	5'TCCTCCTCCTCCTCC3'
5	UBC 811	47	5'GAGAGAGAGAGAGAGAC3'
6	ISSR 04	47	5'ACACACACACACACACC3'
7	ISSR 10	47	5'ACACACACACACACACG3'
8	UBC S2	51	5'CTCTCTCTCGTGTGTGTG3'
9	UBC 826	52	5'ACACACACACACACACC3'
10	UBC 866	55	5'CTCCTCCTCCTCCTCCTC3'
11	UBC 844	46	5'CTCTCTCTCTCTCTCTRC3'
12	UBC854	49	5'TCTCTCTCTCTCTCTCRG3'
13	UBC880	43	5'- GGAGAGGAGAGGAGA-3'
14	UBC895	53	5'- AGAGTTGGTAGCTCTTGATC-3'
15	UBC899	59	5'-CATGGTGTTGGTCATTGTTCCA -3'

Table 28: Details of amplification with the 15 primers for ISSR assay in brinjal genotypes

Sl No	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons	Polymorphism (%)	Resolving power(Rp)	Polymorphic information content (PIC)
1	UBC 868	8	7	1	87.00	9.99	0.83
2	UBC807	15	11	4	73.33	18.44	0.91
3	ISSR 08	18	11	7	38.88	20.66	0.92
4	ISSR 15	15	10	5	66.66	14.44	0.95
5	UBC811	20	15	5	75.00	21.77	0.96
6	ISSR 04	14	12	2	85.71	17.10	0.91
7	ISSR 10	18	15	3	83.33	19.77	0.92
8	UBC S2	22	18	4	81.81	21.10	0.93
9	UBC826	18	13	5	72.22	22.22	0.93
10	UBC866	16	11	5	68.75	17.33	0.94
11	UBC 844	13	9	4	69.23	28.44	0.93
12	UBC 854	18	9	9	50.00	15.77	0.88
13	UBC 880	15	8	7	53.33	20.88	0.91
14	UBC 895	14	9	5	64.28	23.99	0.92
15	UBC 899	13	5	8	38.46	22.22	0.91
Total		237	163	74	1007.93	294.20	13.81
Average		15.8	10.8	4.9	67.19	19.61	0.92

4.6.1 Resolving power (Rp) of 15 selected ISSR primers

The resolving power (Rp) calculated for random primers is presented in Fig. 11. It ranged between 9.9 (UBC 868) and 28.44 (UBC 854) with an average of 19.15.

4.6.2 Plolymorphic Information Content (PIC) value for the 15 selected ISSR primers

The Plolymorphic Information Content (PIC) value calculated for the 15 selected ISSR primers (Fig. 12) varied from 0.83 to 0.96 and hence were found good enough for evaluating the genotypes.

4.6.3 Cluster analysis for the brinjal varieties

Genetic similarity coefficient for the varieties ranged from 0.51 to 0.84 (Fig.10). Highest similarity (84%) was observed between brinjal varieties Surya and the hybrid Neelima (Table 29). The variability observed among the *S. melongena* genotypes studied was 29 per cent (Table 21). The genotypes Surya, Swetha, SM 396, SM 397 and the hybrid Neelima indicated a similarity of 73 per cent. The accession SM 116 was little more distinct among the other genotypes. *S. melongena* var. *insanum* was found to group together with the melongena genotypes with 37 per cent variability and *S. macrocarpon* showed the maximum variability of 49 per cent.

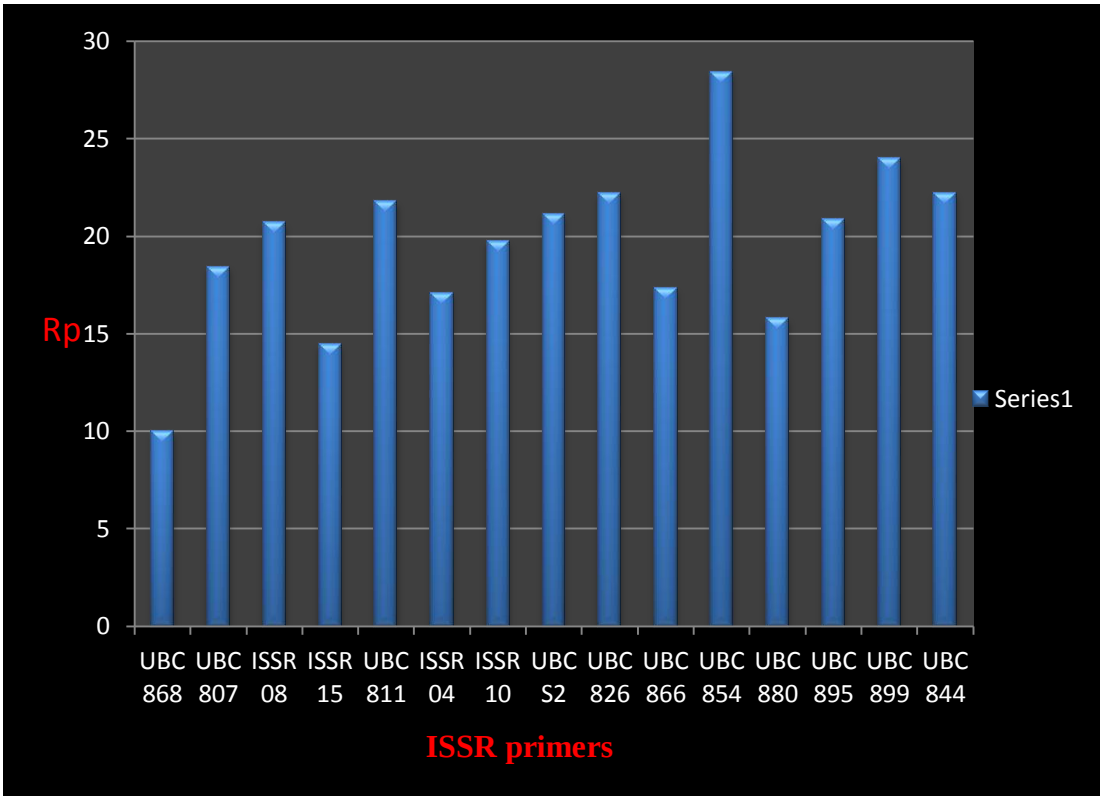


Fig 10: Resolving power (Rp) of 15 selected ISSR primers

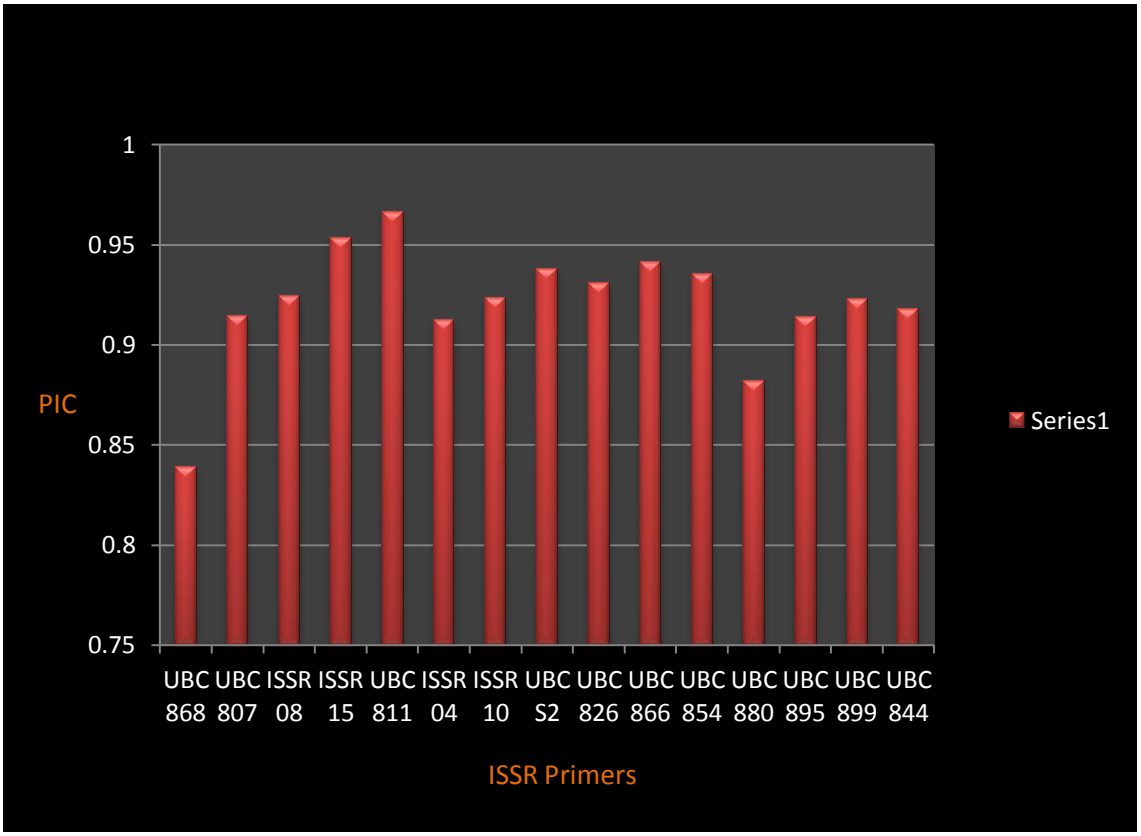


Fig 11: Polymorphic Information Content (PIC) value for 15 selected ISSR primers

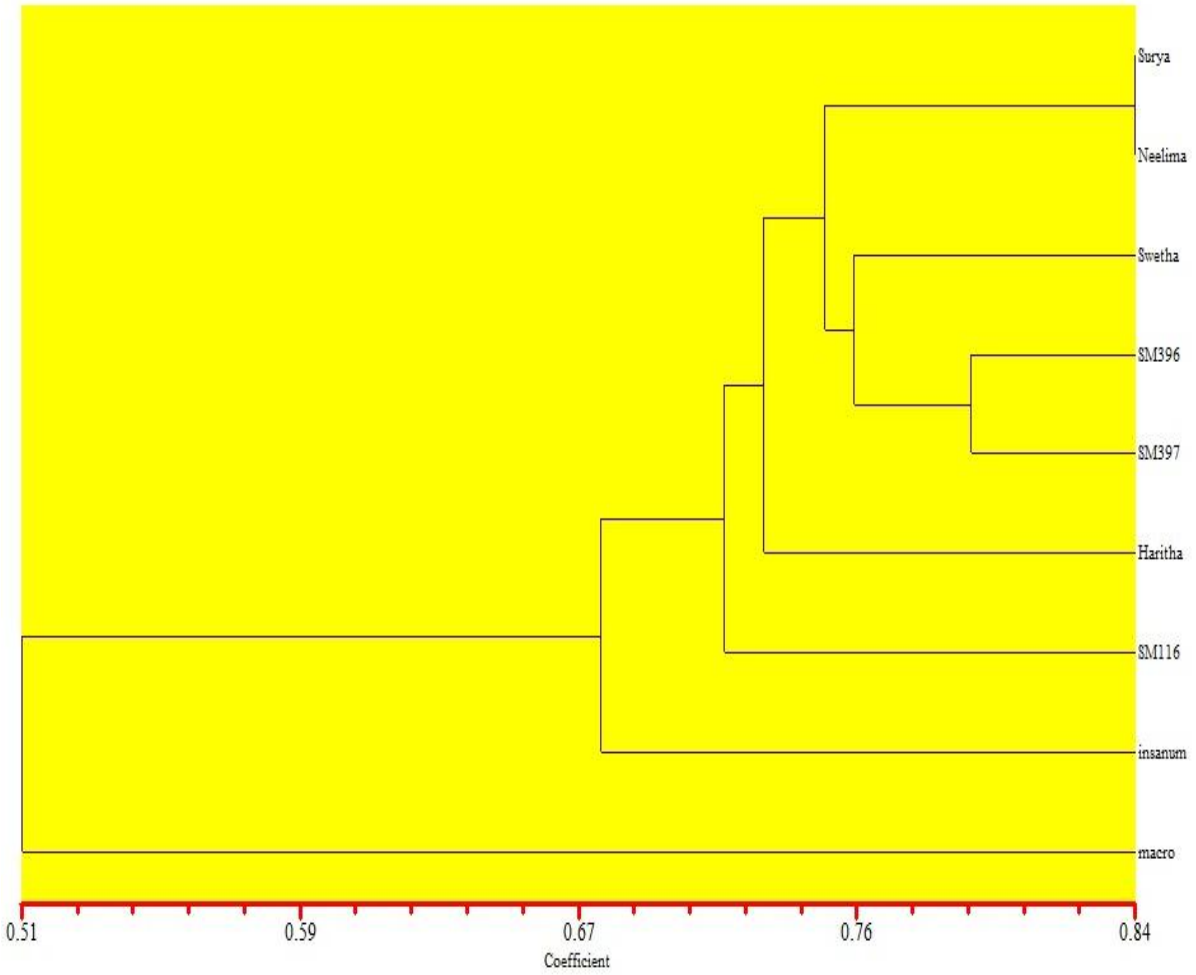


Fig 12: Dendrogram for brinjal genotypes with ISSR and SSR markers

Table 29: Similarity matrix of different brinjal genotypes based on the proportion of shared ISSR and SSR fragments

	Surya	**SM116**	**Neelima**	**Swetha**	**Haritha**	**SM396**	**SM397**	***S. melongena* var. *insanum***	***S. macrocarpon***
Surya	1.0000								
SM116	0.6828	1.0000							
Neelima	0.8391	0.7056	1.0000						
Swetha	0.7366	0.7416	0.7322	1.0000					
Haritha	0.7059	0.7191	0.7104	0.7268	1.0000				
SM396	0.7826	0.7500	0.7500	0.7473	0.7166	1.0000			
SM397	0.7312	0.6978	0.7459	0.7624	0.7797	0.7901	1.0000		
S. melongena var. *insanum*	0.6327	0.7151	0.6440	0.6862	0.7011	0.6684	0.7081	1.0000	
S. macrocarpon	0.5000	0.5026	0.5231	0.5306	0.4949	0.4951	0.5101	0.4925	1.0000

Discussion

5. DISCUSSION

Brinjal (*Solanum melongena* L.) is an important Solanaceous vegetable crop of the sub-tropics and tropics. The name brinjal is popular in Indian subcontinent and is derived from Arabic and Portuguese terms, binjan and birinjala. The name eggplant has been derived from the shape of the fruit of some varieties, which are white and resembling chicken eggs. It is also called aubergine (French) in Europe.

Brinjal is an important vegetable in the warm areas of Far East, being grown extensively in India, Bangladesh, Pakistan, China, Philippines and is also popular in Egypt, France, Italy and United States. In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except at higher altitudes. It is a versatile crop adapted to different agro-climatic regions and can be grown throughout the year. Though a perennial, it is grown commercially as an annual crop. A number of cultivars are grown in India, consumer preference being dependent upon fruit colour, size and shape.

Being originated in India/Indochina; wide genetic variability occurs in the natural population and is being exploited in crop improvement programmes. Efforts have been made to develop varieties with high yield and quality through selection as well as hybridization. The genotypes evaluated in the study include three released varieties, one F1 hybrid, three selected accessions and two wild relatives. Kerala Agricultural University has released three brinjal varieties *viz.*, Surya, Swetha, Haritha and one F1 hybrid Neelima which are high yielding and wilt resistant (Rajan and Prameela, 2004). Among these, Surya (SM 6-7) is the first variety of brinjal which was released at national level by Kerala Agricultural University having purple and oval fruits. Surya and Swetha (SM 6-6) were developed by single plant selection from an Annamalai collection SM 6. Haritha was developed from SM 141, which is a local collection from Cochin with white flowers, long and green fruits. Neelima (Surya x SM 116) is the hybrid released for cultivation having high yield and wilt resistance. The accessions SM396 and 397 selected for the study are the ones identified for their robust nature and high yield which are in pipeline for variety release. The two wild relatives selected are *S. macrocarpon and S. melongena* var. *insanum*. Both are unique for their morphological characters. *S. macrocarpon* is also known as tomato brinjal and is characterised with spiny leaves and tomato like green fruits. The *S. melongena* var. *insanum* included in the study is a perennial with small,

solitary fruits commonly referred as ‘temple chunda’ and is mainly used for rituals and ayurvedic medicines.

There exist wide genetic diversity within the cultivated species as well as between the wild relatives. Efforts have been made to characterize these cultivars and wild relatives through molecular tools (Sunseri *et al*., 2010; Ali *et al.,* 2011; Adeniji *et al.,* 2012; Hurtado *et al*., 2012) based on which, similarity/divergence among many of the genotypes have been worked out.

There are many reports on molecular markers in which two or more combinations were used for overcoming the drawback of individual marker systems and thus to generate wholesome information about the material under study (Parani *et al*., 1997; Popov *et al.,* 1998). In the present investigation, a combination of two molecular marker systems *viz.,* ISSR and SSR analysis were used to establish a comprehensive DNA fingerprint data with which the variety could be identified.

5.1 DNA isolation

Pale green tender leaves (1^{st} or 2^{nd} leaf from tip) were used for DNA extraction from brinjal genotypes. The protocol suggested by Rogers and Bendich (1994) using 2x CTAB extraction buffer yielded good quality DNA. The electrophoresed DNA showed distinct bands without smear.

The major problem encountered in the isolation and purification of high molecular weight DNA from plant species is the degradation of DNA due to endonuclease, polyphenols and other secondary metabolites that directly and indirectly interfere with subsequent enzymatic reactions. The addition of antioxidant, β-mercaptoethanol and PVP (poly vinyl pyrrolidone) during grinding was found effective in obtaining good quality DNA.

5.1.1 Purification and Quantification of DNA

The quality of DNA was tested by subjecting it to agarose gel electrophoresis as well as spectophotometric method. DNA was visualized on 0.8 per cent agarose gel under UV light by ethidium bromide staining. A DNA sample is reported as of high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasingf and Peffley, 1998). The DNA extracted showed high amount of RNA as a smear below it. To remove RNA, RNase A was used. Use of

RNase A has been reported by several workers (Raval *et al*., 1998; Gallego and Martinez, 1996). In the present investigation, the RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicted that the DNA under test was of good quality.

In the spectrophotometeric method, the ratio of optical density at 260 and 280 nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samples. Those samples with ratio between 1.8 and 2.0 were considered to be of high quality. All the samples under the study recorded a ratio between 1.8 and 2.0.

5.2 Molecular Marker Analysis

Molecular marker technology provides novel tools for DNA fingerprinting of varieties, establishing the fidelity of progenies, germplasm characterization, diversity and population structure analysis etc. Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based markers. Most of the molecular markers are developed by PCR (Polymerase Chain Reaction) technology and amplifies unique regions on the genomic DNA based on the primers designed for DNA amplification. In the present study, two such PCR based marker systems, ISSR and SSR were utilized for DNA fingerprinting of brinjal varieties/accessions and wild relatives.

5.2.1 ISSR analysis

The marker system called ISSR (Inter Simple Sequence Repeats) is a PCR based method, that asses variation in the numerous microsatellite regions dispersed throughout the genome. In this technique reported by Zietkiewicz *et al*., (1994), primers based on microsatellites are utilized to amplify inter simple sequence repeat sequences in the DNA. When the primer successfully locates two microsatellite regions within an amplifiable distance away on the two strands of the template DNA, the PCR reaction generates a band of a particular molecular weight for that locus representing the intervening stretch of DNA between the microsatellites. The method uses a single oligonucleotide primer composed of 4 to 10 tri or di nucleotide repeats and ending with 3’- or 5’- anchor sequence.

ISSR is highly sensitive, reproducible marker system and has been successfully applied in genetic and evolutionary studies of many species, including rice (Joshi *et al.,* 2000), wheat (Nagaoka & Ogihara, 1997), fingermillet (Salimath *et*

al., 1995), *Vigna* (Ajibade *et al.*, 2000). Few studies have been performed in brinjal to determine the genetic diversity using ISSR markers (Isshiki *et al.*, 2008; Ali *et al.*, 2011; Kumchai *et al.*, 2013). The ISSR marker requires small amounts of DNA and does not require information on DNA sequence. ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome (Gupta *et al*., 1996; Buhulikar *et al*., 2004). ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.*, 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al*., 2005).

The ISSR markers are found useful in the fingerprinting of cultivated and wild species germplasm, and in understanding the evolutionary relationships of various crops such as apple (Goulao *et al*., 2001), *Solanum lycopersicum* L. (Terzopoulos and Bebeli 2008) and *Auricularia auricula* (Li *et al*., 2008).

The Rp values for the ISSR primers selected in the study ranged from 9.99 to 28.44. The Rp values reported for variability analysis in various crops is in conformity to the values observed in the present study. The PIC values ranged between 0.83 and 0.96 for ISSR primers. The PIC values indicate the extent of polymorphism detected by the primer among the genotypes studied.

5.2.2 SSR analysis

Microsatellites, or simple sequence repeats (SSRs), are becoming increasingly attractive markers in molecular breeding, diversity assessment and fingerprinting (Morgante and Olivieri, 1993; Powell *et al*., 1996). SSRs are short tandemly repeated sequence motifs of approximately 1-8 bp in length, which are scattered throughout the genome and can vary between individuals in repeat length. High frequencies of polymorphism have been described for SSRs in brinjal (Nunome *et al.*, 2009; Sunseri *et al.*, 2010; Meng *et al.*, 2010) and several other plant species (Saghai-Maroof *et al*., 1994; Gupta *et al*., 1996; Chase *et al*., 1996). Primer pairs designed for the flanking sequences can be used in PCR reactions for site-specific amplification of the microsatellite, thereby producing sequence-tagged microsatellite markers (Powell *et al*., 1996).

Simple sequence repeats or microsatellites provide an ideal tool for diversity studies due to their high information content, ease of genotyping through PCR, co-dominant and multi allelic nature and high discriminating power (Russell *et al*., 1997). In addition, only small amounts of DNA are required and the quality of the DNA need not to be as high as for most of the other advanced DNA assay methods (Rafalski *et al*., 1996).

5.3 Fingerprinting of brinjal varieties/accessions and wild relatives

Four released varieties of brinjal from KAU three accessions and two wild relatives were selected for the study. These varieties have different parentage and are not unique in their morphological parameters. The morphological characters of the genotypes observed as per the minimal descriptor of NBPGR is provided in Table 3. The genotypes selected were with diverse characters, the flower colour varying from white to violet, the fruit colour-green to purple, fruit shape round to long and with striking difference in presence of prickles, petiole colour, plant growth habit etc.

The amplification pattern observed in ISSR and SSR assay were utilized for developing fingerprint for the selected varieties, accessions and wild relatives.

This study has helped to characterize the genotypes and make fingerprint data useful for the variety registration and detecting infringement of breeder's right and biopiracy.

5.3.1 Surya

Surya (SM 6-7) is a high yielding variety developed by single plant selection method from an Annamalai collection SM 6, having intermediate growth habit, non-prickly stem and leaves, violet petiole colour, light violet flower and purple fruit colour (Plate 1a) which can be morphologicaly evaluated to distinguish the variety. (Rajan and Prameela, 2004).

The molecular fingerprint developed for the variety 'Surya' indicated 58 amplicons of which 21 (36.2 %) were monomorphic and shared with all the other eight genotypes (Fig 1a, Table 30). Among the ten primers, UBC 811 amplified one unique band (500 bp) for this variety. It indicated 84 per cent similarity with the hybrid Neelima for which Surya is the maternal parent and thus substantiating the high similarity. However, the fingerprint developed through ISSR assay was unique

Table 30: Details of amplifications with the selected 10 ISSR primers and sharing of bands with the other genotypes

Primers	Amplicons	Surya	Swetha	Haritha	Neelima	SM 396	SM 397	SM 116	SI*	SM**
UBC 868	Total	4	4	5	4	4	5	4	5	3
	Monomorphic	1	1	1	1	1	1	1	1	1
	Unique	0	0	0	0	0	0	0	0	1
UBC 807	Total	5	5	5	7	6	6	6	4	8
	Monomorphic	3	3	3	3	3	3	3	3	3
	Unique	0	0	0	1	0	0	0	0	2
ISSR 08	Total	7	6	8	6	9	8	5	8	11
	Monomorphic	4	4	4	4	4	4	4	4	4
	Unique	0	0	0	0	0	0	0	1	4
ISSR 15	Total	5	3	3	4	2	3	2	2	4
	Monomorphic	2	2	2	2	2	2	2	2	2
	Unique	1	1	1	0	0	0	0	0	1
UBC 811	Total	7	9	7	8	9	9	7	6	11
	Monomorphic	3	3	3	3	3	3	3	3	3
	Unique	0	0	0	0	0	0	0	0	0
ISSR 04	Total	7	8	7	5	7	9	8	5	6
	Monomorphic	2	2	2	2	2	2	2	2	2
	Unique	0	0	0	0	0	0	0	0	0
ISSR 10	Total	6	6	5	7	6	6	6	6	3
	Monomorphic	1	1	1	1	1	1	1	1	1
	Unique	0	0	0	1	0	0	0	0	1
UBC S2	Total	4	8	10	8	9	7	7	7	6
	Monomorphic	0	0	0	0	0	0	0	0	0
	Unique	0	2	2	0	0	0	0	0	0
UBC 826	Total	7	8	9	7	7	9	6	8	5
	Monomorphic	4	4	4	4	4	4	4	4	4
	Unique	0	0	1	0	0	0	0	0	0
UBC 866	Total	6	7	5	7	6	6	6	7	5
	Monomorphic	1	1	1	1	1	1	1	1	1
	Unique	0	0	0	0	0	0	0	0	1
Grand total	Total	58	64	64	63	65	68	57	58	62
	Monomorphic	21	21	21	21	21	21	21	21	21
	Unique	1	3	4	2	0	0	0	1	10

SI***-** *S. melongena* var. *insanum*; SM****-** *S. macrocarpon*

for the variety Surya (Fig. 13). The molecular profile developed through SSR assay was not good enough to distinguish the genotypes. The amplification pattern observed with the 10 selected SSR primers were uniform among the *S. melongena* genotypes.

5.3.2 Swetha

The variety Swetha (SM 6-6) was developed from Annamali collection (SM 6) by single plant selection method. It is a high yielding variety with erect growth habit, non-prickly plant, bluish violet flower colour, long and white coloured fruits (Plate 1b) are the key characters to distinguish the variety (Rajan and Prameela, 2004).

The ISSR fingerprint developed for the variety Swetha was distinct for the genotype. The ten primers selected provided 64 amplicons of which 21 were monomorphic (Table 30, Fig. 13). Three unique amplicons were observed with the primers ISSR 15 (1500 bp) and UBC S2 (1700 bp, 1200 bp). These bands of relatively higher molecular size could be characteristic to this variety. The long white coloured fruits is a distinguishing feature of this variety among the other genotypes and the unique bands could be associated with this character.

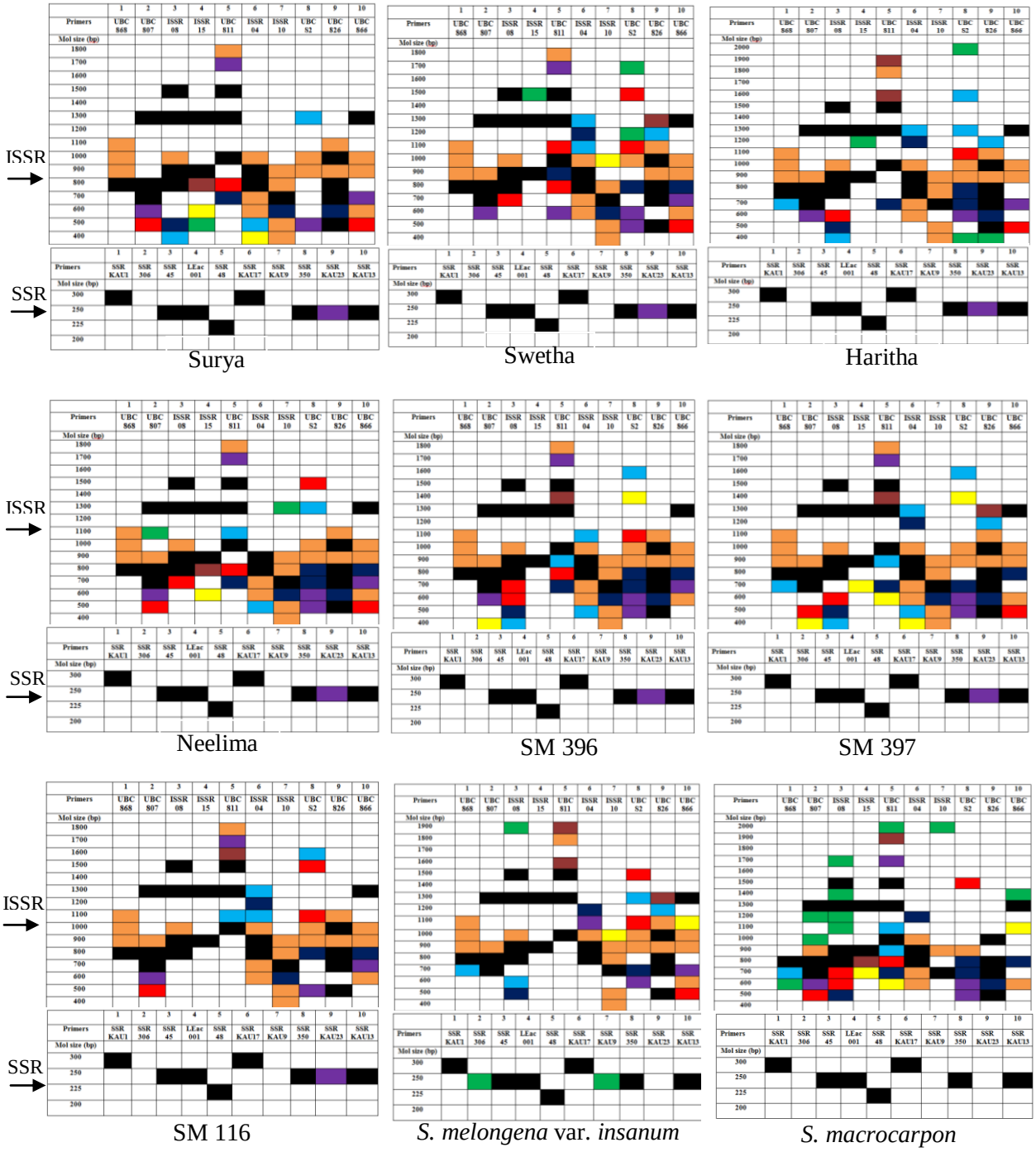
The SSR profile developed for the variety Swetha was not distinct and was same as the other *S. melongena* varieties/accessions.

The similarity matrix (Table 29) indicated that this variety is closer to the accession SM 397 (76%) and the variability observed was maximum with the wild relative *S. macrocarpon* (47%).

5.3.3 Haritha

The variety Haritha (SM 141) is the local collection from Cochin and developed by single plant selection method. Intermediate growth habit, green leaf and petiole colour, white flowers, long and green coloured fruits with less seeds (Plate 1c) are the main morphological characters to distinguish the variety. (Rajan and Prameela, 2004).

The ISSR profile for the variety Haritha indicated 64 amplicons of which 32.8 per cent were monomorphic among the nine genotypes studied (Table 30).



	1	2	3	4	5	6	7	8	9	10
ISSR primer	UBC 868	UBC 807	ISSR 08	ISSR 15	UBC 811	ISSR 04	ISSR 10	UBC S2	UBC 826	UBC 866
SSR primer	SSR KAU1	SSR 306	SSR 45	LEac 001	SSR 48	SSR KAU17	SSR KAU9	SSR 350	SSR KAU23	SSR KAU13

Fig 13: ISSR/SSR fingerprint developed for the nine *solanum* genotypes

Unique amplicons to distinguish this genotype (Fig. 13) was observed with the primers ISSR 15 (1200 bp), UBC S2 (2000 bp, 400 bp) and UBC 826 (400 bp). White flowers and long, green coloured fruits were the distinguishing morphological characters for this variety and the unique bands may be the markers linked to these characters. The SSR profile was same as the other seven melongena genotypes. The fingerprint developed with the ISSR profile was good enough to identify the variety (Fig. 13).

5.3.4 Neelima

Neelima is a high yielding hybrid of brinjal developed with Surya and SM 116 as its parents. Hybrid vigour, intermediate growth habit, non-prickly stem, dark violet petiole colour, bluish violet flower colour, large and oval fruits (Plate 1d) are the key characters to distinguish this hybrid (Rajan and Prameela, 2004).

The fingerprint developed for the hybrid Neelima through ISSR and SSR analysis is depicted in Fig. 4. The ten selected ISSR primers provided 63 amplicons of which 33.3 per cent were monomorphic among the genotypes studied (Table 30). Two unique bands were observed (Fig.13) for Neelima with the primers UBC 807 (1100bp) and ISSR 10 (1300 bp). It shared maximum amplicons with the maternal parent Surya and the similarity coefficient worked out was 84 per cent (Fig. 10, Table 29). Among the seven *Solanam melongena* genotypes studied, Surya and SM 116 were observed to have maximum variability (32%), thus substantiating the selection of parents and the hybrid vigour reported for this genotype.

The SSR profile developed for hybrid Neelima was same as for the other *melongena* genotypes.

5.3.5 SM 116

SM 116 is a high yielding accession of brinjal, collected from IARI, New Delhi. It has erect growth habit, non-prickly stem and fruit, dark violet petiole colour, bluish violet flowers, purple and oval fruits (Plate 2a) are the morphological characters distinguish the accession (Srivastava *et al*., 2001).

The fingerprints developed through ISSR and SSR analysis are depicted in Fig. 9. In ISSR assay, no unique band was observed for this accession but the

fingerprint developed was unique (Fig. 13). It can be used to distinguish this accession from the other brinjal genotypes. The ISSR primers provided 57 amplicons of which 36.8 per cent were monomorphic (Table 30). The SSR profile developed was same as for the other melongena genotypes.

5.3.6 SM 396

SM 396 is a high yielding accession of brinjal from the local collection. The morphological characters which distinguish the accession (Plate 2c) are prostrate growth habit, green petiole colour, light violet flowers, green and oval fruits (Table 3).

The fingerprints developed through ISSR and SSR analysis are provided in Fig. 5. No unique band was noticed in the fingerprints generated by both ISSR and SSR marker systems. In ISSR assay out of the total 65 bands 32.3 per cent were monomorphic with all the genotypes studied (Table 30). It indicated maximum similarity (79%) with the accession SM 397 (Fig. 10, Table 29). However, the fingerprint generated by ISSR assay was unique for the accession.

5.3.7 SM 397

SM 397 is the local collection and high yielding accession of brinjal, having intermediate growth habit, dark violet petiole colour, oval and purple fruits, prickles on stem, leaves, and calyx (Table 3, Plate 2b).

The fingerprints developed through ISSR and SSR analysis are depicted in Fig. 6. No unique band was noticed in the fingerprints generated by both the marker systems. In ISSR assay, total 68 amplicons were observed of which 30.8 per cent were shared with all the other genotypes (Table 30). However, the fingerprint generated by ISSR marker systems was unique enough to identify this genotype (Fig. 13).

5.3.8 *S. melongena* var. *insanum*

S. melongena var. *insanum* is the wild relative of brinjal having intermediate growth habit, violet petiole colour, round and green small fruits, prickles on stem, leaves, and calyx (Table 3, Plate 2d).

The fingerprints developed through ISSR assay (Fig. 13) was good enough to characterise the genotype. Out of the total 58 amplicons observed in the ISSR assay, 36.2 per cent were monomorphic among the genotypes studied (Table 30). One unique band (1900 bp) was observed with the primer ISSR 08. The dendrogram created (Fig. 10) indicated the clear relation of this genotype with the others. Though it clustered along with the other *melongena* genotypes it stood separately with 37 per cent variability (Table 29).

The SSR primers 306 and SSR KAU 9 gave two distinct bands to identify this genotype. None of the other genotypes including *S. macrocarpon* did not amplifying there two loci with the selected primers. SSR being a co dominant marker system, these two primers which gave amplicons unique to the genotype are of great value in breeding programmes including *S. melongena* var. *insanum*.

5.3.9 *S. macrocarpon*

S. macrocarpon is another wild relative of brinjal, with erect growth habit, thick and dark green coloured leaves, violet coloured petiole, bluish violet flowers, round and milky white fruits (Table 3, Plate 2e).

The fingerprints developed through ISSR and SSR analysis were observed to be distinct for this genotype (Fig. 13). The ten selected ISSR primer gave 62 amplicons of which 33.8 per cent were monomorphic. Since it is a different species, the variability observed was also high. Six out of the 10 primers gave unique bands for this genotype (Fig. 13) and the variability observed was over 50 per cent (Table 29). This wild relative occupied a distant distinct position in the dendrogram (Fig. 10) as compared to the other genotypes.

The SSR profile developed for this genotype was also unique. The primer SSR KAU 23 which amplified all *melongena* genotypes did not amplify *S. melongena* var. *insanum* and *S. macrocarpon*. However the primers SSR 306 and SSR KAU 9 clearly distinguished *S. melongena* var. *insanum* and *S. macrocarpon* (Fig. 13).

The fingerprints for the brinjal genotypes Surya, Swetha, Haritha, Neelima, SM 116, SM 396, SM 397, *S. melongena* var. *insanum* and *S. macrocarpon* were unique and distinct. Though individual primers of each marker system gave several

amplicons shared among the genotypes, these were unique when put together, forming specific fingerprints of the particular genotype. Thus the fingerprint developed can be used effectively to prove identity of the genotypes.

The SSR primers tested did not distinguish between the *solanum* genotypes and thus was not good enough to prove the hybridity of Neelima.

5.4 Genetic variability analysis

The amplification pattern observed in ISSR and SSR analysis was scored and analysed for relatedness/ variability among the genotypes. The computer package NTSYS-PC (Rohlf, 2005) was used for cluster analysis. The dendrogram and the similarity matrix obtained by UPGMA cluster analysis of the ISSR and SSR markers are given in Fig. 10 and Table 29. The dendrogram clearly distinguished the *Solanum* genotypes into three groups. All the cultivars of *S. melongena* were categorized into a single group. Hybrid Neelima and its maternal parent Surya showed maximum (84 %) similarity. The accession SM 116 was 79 per cent similar and separated from other *melongena* genotypes.

All the varieties and accessions of brinjal were categorized into a single group with 79 per cent similarity in the present dendrogram. Low level of molecular polymorphism between cultivars of brinjal were also observed using allozyme markers (Isshiki *et al.,* 1994a and Isshiki *et al.,* 1994b) and microsatellite markers (Nunome *et al.,* 2003). This low level of the polymorphism was assumed to be due to the narrow genetic background of brinjal.

Maximum variability observed in the study was 49 per cent and it was for the genotype *S. macrocarpon.* The data indicating that the taxonomic position of *S. macrocarpon* should be outside the section *Melongena,* have been obtained by the several studies of AFLP (Furini and Wunder, 2004 and Mace *et al.,* 1999), seed protein (Karihaloo *et al.,* 2002). In the present study also, *S. macrocarpon* is clearly separated from the other genotypes.

The previous studies of crossability (Daunay *et al.,* 1991), isozymes (Isshiki *et al.,* 1994a), mtDNA (Isshiki *et al.,* 2003) and seed protein (Karihaloo *et al.,* 2002) recognized relationships of *S. melongena* var. *insanum* with the other related *Solanum* species. In the present study it showed 71 per cent similarity with

the *S. melongena* varieties and accessions and was grouped in between *S. macrocarpon* and *S. melongena.*

The results of the present study throw light upon the low detectable genetic variability in brinjal through the selected molecular marker systems. Though the genotypes selected for the study were quite distinct with respect to morphological characters, very low variability at molecular level was observed between *S. melongena* genotypes studied. The markers developed were not found linked to any distinct morphological character except in case of variety Swetha. The marker systems studied relate to the repeats (SSR) and its flanking regions (ISSR) which need not be part of expressed regions (exons) in the genome. Markers based on the transcriptome and single nucleotide polymorphism might give better information on the genetic variability in brinjal observed at expression level. However, the data generated based on 10 different primers would be novel information to use as a fingerprint for a particular genotype. Among the marker systems studied, ISSR was found to be better than SSR to characterise the brinjal genotypes. The unique amplicons observed for the *S. melongena* var. *insanum with* the primers SSR 306 and SSR KAU 9 are of great value for use in the MAS programe involving cultivated melongena genotypes. Similarly, the primer SSR KAU 23 is good enough to detect all *S. melongena* varieties/accessions. Further characterisation of the unique bands observed in the ISSR and SSR assay would be effective in developing trait related markers for molecular breeding in brinjal.

Summary

6. SUMMARY

The study entitled "DNA fingerprinting of brinjal (*Solanum melongena* L.*)* varieties and related species" was carried out at the Center for Plant Biotechnology and Molecular Biology, College of Horticulture Vellanikkara during the period 2011-2013. The objective of the study was to characterize the four brinjal varieties released by Kerala Agricultural University using molecular markers such as ISSR and SSR and to develop a DNA fingerprint specific to each variety, with which the variety could be identified and its fidelity could be detected. Three brinjal accessions selected for their better performance (SM 116, SM 396, SM 397) and two wild relatives - *S. melongena* var. *insanum* and *S. macrocarpon* were also characterised in the study. Breeder seeds were obtained from the Department of Olericulture, College of Horticulture, Kerala Agricultural University and maintained at CPBMB, for characterisation.

The salient findings of the study are as follows:

1. Morphological characters were recorded for all the nine genotypes with respect to six vegetative and five reproductive characters. The data recorded was used to validate the genotypes which was used for fingerprinting. All the genotypes exhibited their true characters described earlier.

2. The protocol suggested by Rogers and Bendich (1994) was found ideal for isolation of DNA from the brinjal genotypes. The RNA contamination was completely removed through RNase treatment.

3. Two molecular marker systems namely, ISSR and SSR were employed for characterisation of the selected genotypes. A total of 36 ISSR primers and 61 SSR primer pairs were screened for their ability to amplify the DNA fragments. Out of these, 10 ISSR primers and 10 SSR primer pairs were selected based on the number of bands and nature of amplicons.

4. The Resolving power (Rp) of the ISSR primers was calculated and the values ranged between 9.9 and 28.44. The ISSR primers UBC 854 and UBC 899 showed high resolving power.

5. The Polymorphic Information Content (PIC) was calculated and it ranged between 0.83 and 0.96 for ISSR primers indicating the suitability of primers to detect polymorphism. The ISSR primers UBC 811 and ISSR 15 recorded highest PIC values.

6. Distinct bands were used for developing DNA fingerprint of brinjal varieties/accessions and wild genotypes through ISSR and SSR analysis.

7. The amplicons for each primer shared among the genotypes were analyzed and demarcated with different colour codes in the fingerprints developed. Most of the amplicons were found shared among the genotypes. However, the pattern of sharing was different and good enough to separate out the genotypes. Certain primers amplified unique bands for different genotypes and these could be further characterized to develop trait specific markers.

8. The brinjal varieties and accessions were monomorphic with respect to the SSR analysis performed in the study. In order to prove the hybridity of Neelima, it was compared along with its parent Surya and SM 116. The SSR primers evaluated could not distinguish the hybrid and its parents.

9. The SSR primers SSR 306 and SSR KAU 9 were found to be good enough to distinguish the *Solanum* species.

10. The scored data based on ISSR and SSR amplicons was used to construct a dendrogram using the NTSYS pc (version 2.02i) software. Similarity coefficient ranged from 0.51 to 0.84. The highest similarity (84 %) was observed between brinjal hybrid Neelima and its maternal parent Surya.

11. The variability observed among the *Solanum melongena* genotype was only 29 per cent in ISSR analysis.

12. The *S. macrocarpon* was the most distinct one from other brinjal genotypes with 49 per cent variability.

13. The fingerprint developed is good enough to provide varietal identity and the analysis could reveal variability/ relatedness among the selected genotypes.

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Annexures

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	:	Kubota, Japan
Horizontal electrophoresis System	:	Biorad, USA
Thermal cycler	:	Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	:	Biorad, USA
Nanodrop® ND-1000 spectrophotometer	:	Nanodrop®Technologies lnc. USA

ANNEXURE II

Reagents required for DNA isolation

Reagents:

1. 2x CTAB extraction buffer (100 ml)

CTAB	:	2g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. CTAB (10 %, 100 ml)

CTAB	:	10 g
NaCl	:	4.09 g

3. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0ºC and was used for the study.

5. Ethanol (70 %)

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml)

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE III

Composition of Buffers and Dyes used for Gel electrophoresis

1. TAE Buffer 50X

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8.0)	:	100 ml

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

DNA FINGERPRINTING OF BRINJAL (*Solanum melongena* L.) VARIETIES AND RELATED SPECIES

By

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(2011-11-102)

ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirement

for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

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2013

ABSTRACT

ABSTRACT

Brinjal (*Solanum melongena* L.) also known as aubergine or eggplant, is a member of the family Solanaceae. It is an important vegetable in central, southern and south-east Asia, and in a number of African countries. In India, it is one of the most common, popular and principal vegetable crop grown throughout the country except in higher altitudes.

The study entitled "DNA fingerprinting of brinjal (*Solanum melongena* L.*)* varieties and related species" was carried out at the Center for Plant Biotechnology and Molecular Biology, College of Horticulture Vellanikkara during the period 2011-2013. The objective of study was to characterize the four brinjal varieties released by Kerala Agricultural University using molecular markers like ISSR and SSR and to develop a DNA fingerprint specific to each variety. Three superior accessions and two wild relatives were also characterized to detect the level of variability.

The brinjal varieties/accessions selected include three *Solanum melongena* varieties- Surya, Swetha, Haritha and the hybrid Neelima; three accessions- SM 116, SM 396, SM 397 and the two wild relatives *S. melongena* var. *insanum* and *S. macrocarpon*. Breeder seeds were obtained from the Department of Olericulture, College of Horticulture, Kerala Agricultural University and maintained at CPBMB, COH and further used for the study.

Morphological characters were recorded for six vegetative and five reproductive characters according to the descriptor of NBPGR. The data recorded was used to validate the genotypes which was used for fingerprinting. DNA extraction was done according to the method described by Rogers and Bendich 1994. The RNA contamination was completely removed through RNase treatment. Good quality DNA with UV absorbance ratio (OD_{260}/OD_{280}) 1.80 – 1.89 was used for further analysis.

Thirty six ISSR primers and sixty one SSR primer pairs were screened with DNA of brinjal genotypes for amplification and those which gave reliable distinct banding pattern were selected for further amplification/fingerprinting. The PCR

product obtained from ISSR and SSR analysis were separated on 2 per cent agarose gel and amplification patterns recorded.

The genomic DNA from each genotype was amplified with 10 each of selected ISSR and SSR primers. The amplification pattern was scored and depicted to develop fingerprint for each brinjal genotype.

The Resolving power (Rp) of the ISSR primers was calculated and the values ranged between 9.9 and 28.44 indicating the capacity of the primers selected to distinguish the genotype. The Polymorphic Information Content (PIC) was also calculated and it ranged between 0.83 and 0.96 for ISSR primers, further indicating the suitability of primers to detect polymorphism. The PIC value for SSR primers were zero and not suitable to detect polymorphism.

Distinct amplicons developed through ISSR and SSR analysis were used to develop the DNA fingerprint of brinjal genotypes. Sharing of amplicons for each primer with other genotypes was also analyzed and demarcated with different colour codes in the fingerprint developed. Most of the amplicons were found shared among the genotypes indicating their genetic uniformity. However, the pattern of sharing was different and good enough to separate out the varieties and distinct amplicons were observed in the genotypes.

The ISSR and SSR banding pattern was scored and analysed for their uniformity/variability using the software NTSYS pc (version 2.02i). Similarity coefficient ranged from 0.51 to 0.84. The highest similarity (84%) was observed between the brinjal hybrid Neelima and its maternal parent Surya. The *S. macrocarpon* was the most distinct one from other brinjal genotypes with 49 per cent variability.

Separate fingerprint were developed for all the four varieties, three accessions and two wild relatives through ISSR and SSR analysis. The DNA fingerprints thus developed could be utilised for the variety registration and settling IPR issues.