DNA FINGERPRINTING OF SELECTED BLACK PEPPER (*Piper nigrum* L.) VARIETIES

By

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(2009-11-157)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"DNA fingerprinting of selected black pepper (*Piper nigrum* L.) varieties"** 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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CERTIFICATE

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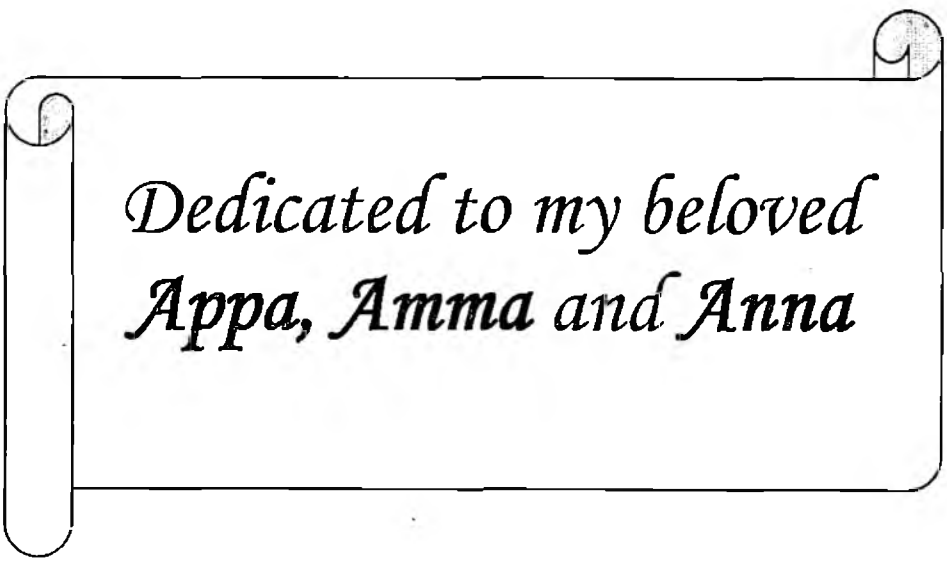
Dedicated to my beloved
Appa, Amma and Anna

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ABBREVIATIONS

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
Bp	Base pair
β	Beta
C	Cytosine
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^{0}C	Degree Celsius
cm	Centimeter
Cm	Centi Morgan
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
DAF	DNA Amplification Fingerprinting
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine TetraAcetic Acid
g	Gram
G	Guanine
HCl	Hydrochloric Acid
ISSR	Inter Simple Sequences Repeat
Kb	Kilo basepairs
KAU	Kerala Agricultural University
L	Litre
M	Molar
MAS	Marker-Assisted Selection
Mg	Milligram
mA	Milli Ampere
Ml	micro litre
µM	micro molar
Mb	Mega base pairs
Min	Minutes
ml	Millilitre

Mg	Magnesium
$MgCl_2$	Magnesium Chloride
Mm	Milli mole
Mwt	Molecular weight
µg	Microgram
µl	Microlitre
NaCl	Sodium Chloride
ng/µl	Nanogram per micro litre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
PVP	Poly vinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RNA	Ribo Nucleic acid
RNase	Ribonuclease
RFLP	Restriction Fragment Length Polymorphism
Rpm	Revolutions per minute
sec	Second (s)
SCAR	Sequence Characterized Amplified Region
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
t/ha	tons per hectare
U	Unit
UV	Ultra violet
UgV	Ugandan variant XV
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume


Introduction

1. INTRODUCTION

Universally acclaimed as the 'King of Spices', black pepper (*Piper nigrum* L.,) enjoys a unique position as a commercial crop of pre-history and is of great economic importance to several nations of the world (Pillai *et al.*, 1985). Until 18th century, black pepper cultivation and production had been the monopoly of India; the major share contributed by the state of Kerala. However, during the past two centuries, pepper cultivation has been taken up on a commercial scale by several other nations too. The total number of pepper producing countries in the world is ten.

Historical evidences show that black pepper cultivation and trade has been existing in India for the past 6000 years or more (Pillai, 1997) . The species *Piper nigrum* (L.) has been accepted as a native of India (Hooker, 1885 and Baber, 1906) The area under pepper cultivation in Kerala is approximately 2.38 lakh ha with a production of nearly 65,990T (Madan , 2000). Despite these facts, the yield of the pepper vine in our country is alarmingly low (238g/vine); when compared to other countries (eg. Thailand- 3.6kg per vine: Ghosh *et al.*, 1999). The estimated area under pepper in India is 2,36,180 hectares (2006-07) and the estimated production is 50,000 tons (2007-08).

More than 15 high yielding varieties have been released for cultivation among which two are hybrids Panniyur 1 and Panniyur 3 developed at the Pepper Research Station, Panniyur, Kerala. Panniyurl is still the ruling variety in all the states in India. The variety has special distinguishing characters such as long spike, high fruit setting, bold berries and high yield. After the release of the popular variety Panniyur-1, Kerala Agricultural University has released Panniyur-2, Panniyur-3, Panniyur-4, Panniyur-5, Panniyur-6 and Panniyur-7. IISR Girimunda and IISR Malabar Excel are the two recent high yielding varieties released from Indian Institute of Spices Research, Calicut.

Our forefathers might have adopted this plant on realizing the medicinal and culinary properties of this queer vine. Through a long and patient process of selection and rejection over several centuries, around 70 cultivars were adopted for cultivation in the different tracts of the state. These cultivars were highly variable in their morphological characters and yield potential (Mathai *et al.*, 1981; Ibrahim *et al.*, 1984) and this variability has been preserved due to the vegetative means of propagation, which is the accepted practice in the crop. The non- availability of sufficient quantity of planting materials is,
spread popularization of these high yielders

DNA fingerprinting for cultivar or
important tool in germplasm management and plant registration system. Different molecular marker techniques are accessible today for fingerprinting plant germplasm but information on their relative efficacy in particular crop is not clear.

At this context, the present study was taken up with the following specific objectives.

1. To characterize the released black pepper varieties of Kerala Agricultural University using different molecular marker like- RAPD, ISSR and SSR.

2. To develop a DNA fingerprint with which the variety could be identified and its fidelity detected.

The DNA fingerprints would be an efficient tool for assessing clonal fidelity and for proving ownership claims.

Review Of Literature

REVIEW OF LITERATURE

Kerala has records of more than hundred black pepper cultivars with highly variable characters. Only a few of them have been identified and reported to be economically productive. Although India holds the prime position in production, consumption, and export of black pepper (George *et al.*, 2005), the productivity is less (368 kg/ha), compared to that of Malaysian and Indonesian black pepper. Distinct variations in morphological traits among Indian landraces have been reported (Mathew *et al.*, 1999). Variations in yield traits among Indian cultivars have been reported based on spike length, floral composition, fruit number and size (Ibrahim *et al.*, 1985a; Ravindran and Babu, 1994; Amma *et al.*, 2001).

2.1 General background

2.1.1 Based on morphological characters investigation in black pepper

Piper nigrum is a unique crop and attempts to compare its genetic behavior with that of any other crop may not be quite proper. Even though it belongs to the class dicotyledons, the anatomical characters are intermediate between those of dicotyledons and monocotyledons (De Waard and Zeven, 1969). It belongs to the family *piperaceae* under the order piperales, which is one of the most primitive branches originated from Ranales. The plant, a weak stemmed climbing vine, is a native of evergreen forests of Western Ghats of Kerala and is said to have been domesticated several thousand years ago (Perseglove, 1969).

Krishnamurthy (1969) has reported that pepper vine in its wild state is mostly dioecious, but most of the cultivated types are monoecious, a condition which probably originated from the wild ones as a result of continuous selection and vegetative propagation by man through ages. Another reason for the wide variability observed is its suspected polyploid nature (De Waard and Zeven, 1969).

Mathai *et al.* (1981) reported more than 70 cultivated varieties of pepper. These local varieties were broadly divided into Malabar and Travancore cultivars (Ibrahim *et al.*, 1984). Kanakamany (1985) formulated a key for identification of black pepper types based on morphological characters. Based on this key, the 45 selected were found to fall into 42 groups.

Panniyur1 (P1), the first hybrid of black pepper, hybridization of Malabar type Uthirenkotta as female parent and the Travancore type Cheriakaniakkadan as the male parent, was released in 1966. By virtue of the vegetative propagation method, heterosis could be fixed in crop. The high yield and other desirable characters of P1 have been reported by Ramankutty (1977), Nambiar *et al.*, (1978), Kannan (1985) and Ibrahim *et al.*, (1986a). Pillai *et al.*, (1987) assessed the heterosis of P1. During the following years, several high yielding varieties have been released in black pepper, the salient features of which, as reported by PRS, Panniyur (Vanaja *et al.*, 2006) are tabulated in Table 1.

2.1.2 Important characters for morphological scoring

Several vegetative and reproductive characters were given importance for characterization of vines and assessing the extent of variability in clonally propagated black pepper varieties. Many of these were of quantitative nature enabling statistical analysis for interpretation where as several others were of qualitative nature, effective enough only to discriminate the varieties.

2.1.2.1 Vegetative characters

Presence or absence of anthocyanin pigmentation on the leaf sheath covering the young growing tip was reported to be controlled by a dominant-recessive intra-allelic interaction where in the alleles governing pigmentation was observed to be dominant (Ibrahim *et al.*,1986b).

Table 1. Salient features of the high yielding pepper varieties

Variety	Parentage	Green yield (kg/vine)	Oleoresin%	Average yield (Kg/ha)	Dry-yield potential (kg/ha)	Other specific features
P1	UthirenkottaX Cheriyakaniakkadan	2.2	11.8	35.3	8800	Prefer open condition
P2	O.P.* progeny of Balakotta	4.9	10.9	35.8	3313	Prefer open or partial shade robust vine
P3	UthirenkottaX Cheriyakaniakkadan	4.9	12.7	27.8	3269	Prefer open conditions
P4	Selection from Kuthiravaly types	2.6	11.3	34.7	2443	Prefer open or partial shade
P5	O.P.* progeny of Perumkodi	3.2	12.33	35.7	2248	Stable yielding shade tolerant
P6	Selection from Karimunda types	3.3	8.27	32.93	3359	Tolerates shaded and unfavorable conditions
P7	O.P.* progeny of Kalluvally-IV	2.7	10.61	33.57	2770	Tolerates shaded and unfavorable conditions

*O.P-Open Pollinated

Shape of leaf, leaf area, petiole characters, texture and colour of leaf etc. were reported to help in rapid identification of varieties in pepper (Perseglove, 1969; George and Mercy, 1978; Nambiar *et al.*, 1978; Kanamany, 1982). Ibrahim *et al.* (1985) have worked out a constant which when multiplied with the dimension of the leaf would yield an estimate of leaf area.

Stem characters like internodal length, branching nature, direction of growth of branches etc. were reported to vary with cultivars (Kanakamany, 1982; Ibrahim *et al.*, 1986a; Sujatha and Namboothiri, 1995).

Five pepper varieties were evaluated for growth and yield under coffee based cropping system during the period 1997-2002 at Zonal Agricultural Research Station, Mudigere, Karnataka. Out of five cultivars, Panniyur-3 has recorded highest mean cumulative yield and Panniyur-5 recorded next best yield. The varieties did not differ in plant height. The study revealed that Panniyur–3 and Panniyur-5 are two best varieties for coffee based cropping system in hill zone of Karnataka (Bhagavantagoudra et *al.*, 2008).

2.1.2.2. Reproductive characters

Flowering in black pepper occurs in the month of May- June (Anandaraj *et al.*, 2005). Following the monsoon rain vegetative bud differentiation occurs. Some reports show that the *Piper* vines can be induced to flower by copious irrigation for about 20 days (KAU, 1996). The process of spike formation and differentiation of florets occur during July. During flowering, the shoot apical meristem elongates and transforms into an inflorescence meristem. Spikes originate, enclosed in a prophyll, and it takes three to four weeks for the full spike emergence. The main inflorescence which generally grow indeterminately, gives rise to hundreds of floral meristems (Cohen *et al.*, 2003).

Inflorescence characters like number and length of spike, sex types of flower, stamen and stigmatic characters etc. were reported to be of economic importance as they influence yield (Nambiar *et al.*, 1978; Chandy *et al.*, 1979;

Kanakamany, 1982). Similarly the number of berries per spike, percentage of developed and underdeveloped berries per spike, 100 berry weights, 100 berry volumes, drying percentage as well as chemical composition also manifested pronounced variation (Nambiar *et al.*, 1978; Kanamany, 1982; Raja *et al.*, 1983; Sujatha and Namboothiri, 1995).

The reproductive transition in black pepper starts at shoot apex by forming the dome shaped apical meristem, leaf initials and axillary bud initials (Ravindran and johny, 2000). The terminal buds of the plagiotropic branches produced the spike and growth was continued by axillary buds. The growing axillary bud pushed the spike aside and inflorescence appeared to be leaf opposed (Ravindran *et al.*, 1992).

Flower composition of the spike varied among cultivars (Ravindran and John, 2000). Some are completely bisexual as in Karimunda, while in other, varying number of unisexual flower occurs. In panniyur-1, 97 percent bisexual flower and the rest are pistillate. Spike branching in black pepper is very rare, though, considerable variation in spike length, floral composition, fruit number and size are reported (Ravindran and John, 2000).

Mutation in the floral meristem of black pepper could also result in branching of inflorescence. Scientists from Indian Institute of Spices Research have reported a new spike variant with hundred percent of its spikes, proliferating (Sasikumer *et al.*, 1992).

2.3 DNA based molecular characters

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 variation 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) technique

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 on 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) while Caetano-Anolles *et al.* (1991) reported on DNA Amplification Fingerprinting (DAF). The PCR reaction requires deoxynucleotides to provide both energy and nucleotides for synthesis of DNA polymerase primer, template and buffer containing magnesium (Taylor, 1991). Typical PCR amplification utilises oligonucleotide primers that hybridise to opposite 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 by DNA polymerase leading to amplification of the target sequence. The result is an exponential increase in the number of copies of the region amplified by the primer (Saiki *et al.*, 1988; Mullis, 1990). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi (Williams *et al.*, 1990).

The introduction of the PCR technique has revolutionized standard molecular techniques and has allowed for the proliferation of new tools to detect

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 (Tommercup *et al.*, 1998). Polymorphism between two individuals is generally scored as a 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 relatively low cost. The main advantage of this technique over other techniques is its inherent simplistic analysis (a single reaction can contain all reagents) and the ability to conduct PCR test with extremely, small quantities of tissue for DNA extraction (Welsch *et al.*, 1991). On the other hand PCR is limited in its usefulness because of the time and cost required to obtain the DNA sequence information required for primer design (Samec and Nasinec, 1995; Thottappilly *et al.*, 2000).

2.5 Molecular marker for varietal characterization

Markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and proteins based on markers and constitute a new generation of genetic markers (Bostein *et al.*, 1980; Tanksley *et al.*, 1989). Hence, varietals profiling methods that directly utilize DNA could potentially address all of 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.*, 1999).

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 be used to assay a large number of samples. These include Randomly Amplified Polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al.*, 1990), Amplified Fragment Length of Polymorphisms (AFLP) (Vos *et al.*, 1995), Simple Sequences

Repeats (SSR)(Tautz,1989),Inter Simple Sequences Repeats (ISSR),DNA Amplification Fingerprinting(DAF), Sequence Tagged Sites(STS) Sequences Characterized Amplified Regions(SCAR) and Expression Sequences Tags(EST). Of these, RFLP and micro satellites are co-dominant markers and their map positions on the rice genome are well known, while RAPD and AFLP markers are largely dominant markers.

2.6 Randomly Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA is a PCR-based technique. RAPD markers are generated by the use of short (10-mer) synthetic oligonucleotides in single strand primer (Williams *et al.*, 1990). In this technique, a decamer primer of arbitrary sequence is allowed to anneal at a relatively low temperature priming the amplification of DNA fragments distributed at random in the genome (Williams *et al.*, 1990).

Amplification products are visualised by separation on agarose and stained with ethidium bromide. They usually result in DNA fragment patterns that are polymorphic between genotypes, there by detecting diversity within them (Tommercup *et al.*, 1998). However, a key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation. There are several advantages of RAPDs compared to other DNA based techniques. It is simple, rapid and does not involve radioactivity and costs less (Varghese *et al.*, 1997). Another advantage of the RAPD method is that a universal set of random primers can be used for genomic analysis of any organism (Welsch and McClelland, 1990). Short random primers have been used to reproducibly amplify segments of genomic DNA from a wide variety of species including plants (Williams *et al.*, 1990; Quiros *et al.*, 1991; Fregene *et al.*, 1997). Polymorphisms detected by RAPDs are inherited in a Mendelian fashion as dominant markers (William *et al.*, 1990; Welsch *et al.*, 1991). This polymorphism has been proved to be useful for identifying variation at different levels. RAPD analysis enables differentiation between very closely related organisms due to high resolution of the technique (Tommercup *et al*, 1998). The polymorphic fragments

generated by RAPDs are useful as genetic markers to identify organisms (Williams *et al.*, 1990) and the relative degree of similarity between individual populations and species (Yang and Quiros, 1993; Tonukari *et al.*, 1997).

Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics.

Identification of hybrids in black pepper was reported by using the male parent-specific RAPD markers. Total DNA from the leaves of black pepper was prepared by a modified CTAB extraction method using increased concentration of CTAB (4%) and β-mercaptoethanol (0.5%). Thirty-five decamer oligonucleotide primers were screened by PCR. RAPD banding pattern among parents and their hybrid populations was compared to assess hybridity at the DNA level (George *et al.*, 2005).

DNA finger printing of *Piper nigrum* L. and *Piper longum* L. cultivars using RAPD markers was reported by Keshavachandran *et al.* (2005). Fourteen land races and three advanced cultivars of *P. nigrum* and eleven land races and one advanced cultivar of *P.longum* were studied. Forty decamer primers were used for screening, among which ten primers were used for final analysis. These generated 119 amplification products. Cultivar specific single bands were reported for a few land races and accessions of both the *Piper* species. They observed the influence of random primers on the uniformity of RAPD fingerprints developed from different tissues of a particular variety.

RAPD analysis was conducted in 22 cultivars of *P. nigrum* (black pepper) and one accession each of *P. longum* and *P. colubrinum* (Pradeepkumar *et al.*, 2003) Twenty-four primers generated 372 RAPD markers of which 367 were polymorphic. The existence of wide genetic diversity as revealed in the present study is supported by earlier reports of extensive inter- and intra population morphological variability in pepper cultivars from South India. Genetic proximity among *P. nigrum* cultivars could be related to their phenotypic similarities or geographical distribution. Greater divergence was observed among landraces than among advanced cultivars.

RAPD assay has been used for the identification of cultivars and genotypes and for genetic fingerprinting and related studies in a variety of crop spices. Detection of DNA sequence polymorphism among closely related lines of common wheat (*Triticum aestivum*) has been reported by (He *et al.*, 1992). A high level polymorphism was observed among a number of commercial varieties and breeding lines of wheat. Over 38 percent of the 65 primers, used for PCR amplification, produced readily detectable and reproducible DNA polymorphism.

Ratnaparkhe *et al.* (1995) used RAPD markers for the identification of pigeon pea (*Cajanus Cajan* (L.) Mill sp.) cultivars and its wild spices. They showed extremely high level of polymorphism among the wild spices while little variation was detected within the accessions. The cultivars and wild species studied were distinguished with help of different primers.

Raychoudhary *et al.* (2001) used 58 random decamer primers for identification and classification of aromatic rice based on DNA fingerprinting and found that 96.5% of the primers detected polymorphism among the genotypes.

RAPD markers, in particular, have been successfully employed for determination of intra-species diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami and Ranade, 1999).

Raina *et al.* (2001) illustrated the use of RAPD and ISSR fingerprints as the genetic marker analysis of genetic diversity, varietal identification and phylogenetic relationships in peanut cultivars and wild species.

Pradeepkumar *et al.* (2001) reported molecular characterization of *Piper nigrum* L. cultivars using RAPD markers. Thirteen land races and nine advanced cultivars of *P.nigrum* were used. DNA was extracted according to the CTAB method with minor modifications. A total of 34 decamer primers (series OPA, OPB etc.) were screened using three representative genomic DNA samples of black pepper. Of these, 24 primers that yielded consistent and clear band patterns were selected for the final analysis of the 22 accessions. The 24 selected decamer primers generated 372 amplicons and the total number of markers per primer ranged from 4 (OPV05) to 21 (OPF09). The range of polymorphic markers per primer was 3 (OPV05) to 21 (OPF09) with a mean of 15.3.

Sujatha (2001) studied the characterization of field established tissue culture derived black pepper (*Piper nigrum* L.) plants using morphological, cytological and molecular markers using four promising varieties P1, P2, P4 and Subhakara. The intervarietal polymorphism brought out by morphological and molecular marker is useful in discriminating the varieties and to asses the genetic distances between them, which is an important criterion in the selection of parents for hybridization. Among the three makers used, RAPD markers were found to be most effective in understanding the genetic constitution of individual vines.

Naghia *et al.* (2002) demonstrated use of RAPD markers to study genetic diversity among rice parental lines. The cluster analysis based on this marker revealed close genetic relationship among rice genotypes used in the hybrid rice breeding programs.

Horn *et al.* (2002) used RAPD, RFLP and AFLP markers to study molecular diversity of CMS sources and fertility restoration in the genus *Helianthus.*

Papavo *et al.* (1998) examined genetic diversity of 30 sunflower inbreds by RAPD and isozyme analysis. High polymorphism was observed by RAPD markers and analyzed the line distribution on the genetic similarity dendrograms. The high effectiveness of RAPD analysis for differentiating genotypes of inbred lines was demonstrated.

RAPD was used to estimate the genetic relationships among 20 selected banana cultivars from different regions of Kenya. Nineteen random primers were used. The polymorphisms in PCR amplification products were subjected to the UPGMA and plotted in a phenogram. The dendogram constructed from the similarity data showed that all the 20 cultivars analysed were related (Ongusoa *et al.*, 2002).

Seven high yielding, new, promising lines of black pepper, (*Piper nigrum*) were characterized using RAPD and morphological features. Out of the 14 random decamer primers studied, 9 could generate unique bands in a 6 lines.16 unique bands were produced by the nine primers making an average of 1.7 bands per primer (Sreedevi *et al.*, 2005).

George *et al.* (2005) reported identification of true hybrids in black pepper (*Piper nigrum* L.) at the juvenile stage using male parent-specific RAPD markers. Eleven black pepper accessions and their hybrid populations were evaluated out of 35 decamer primers tested 13 yielded good amplification products. These primers were useful in generating at least one band to select true hybrids based on shared bands in male parent and offspring.

Deepamala *et al.* (2005) reported that 14 sunflower cultivars have been fingerprinted by RAPD, ISSR and AFLP markers utilizing 361, 21 and four primer combinations, respectively. On an individual assay basis, AFLP was proven to be the best marker system as compared with the other two markers. To understand genetic relationships among these cultivars, Jaccards similarity coefficient and UPGMA clustering algorithm were applied to the 3 marker data sets. However, strong correlation was observed between RAPD and ISSR marker systems.

Research was conducted to make clear the kinship among them.20 primers selected from 80 RAPD primers had been screened out and were used to amplify the total DNA of the 28 test materials, and all of them could generate the clear bands (Jiang *et al.*, 2005). The amplifications with the 20 primers were repeated, and the results were reliable. The result analysis showed that, at 0.36 of the genetic similarity coefficient, the 28 germplasms were clustered into 6 different groups, in which *Piper methysticum* was clustered into a single group. This indicated those kavas were distantly related to the others.

Uses of RAPD and peroxidase markers for fingerprinting of black pepper (*Piper nigrum* L.) varieties and micropropagated clones were reported by Sujatha *et al.* (2006). In this study, the clonal fidelity and varietal diversity was assessed in black pepper using RAPD and peroxidase polymorphism exhibited by 20 conventional clones and 38 tissue culture derived clones of the varieties Panniyur-1, Panniyur-2, Panniyur-4 and Subhakara. Within conventionally propagated plants no polymorphism was detected and within micro propagated clones one or two TC plants in each variety exhibited some polymorphism with respect to the presence or absence of some bands.

Multani and Lyon, (2006) Random amplified polymorphic DNA markers generated by 30 random decamer primers were used to fingerprint 12 released cultivars and a breeding line of *Gossypium hirsutum* and 1 cultivar of *G. barbadense* presently under cultivation in Australia. Among a total of 453 developed markers, 69 (15.2%) were only present in the *G. barbadense* cultivar Pima S-7. Of the remaining markers, 128 (33.3%) were fixed in all 13 *G. hirsutum* cultivars. In pairwise comparisons of the degree of band sharing, nine closely-related cultivars showed 92.1 to 98.9 per cent genetic similarity. Cluster analysis of genetic distance estimates between each of the cultivars. Ten of the *G. hirsutum* cultivars can be characterized individually based upon cultivar-specific RAPD markers.

Genetic fidelity was assessed in micro propagated *Chlorophytum arundinaceum* using random amplified polymorphic DNA (RAPD), karyotype

analysis and meiotic behavior of *in vitro* and *in vivo* plants. Five arbitrary decamers displayed same banding profile within all the micropropagated plants and *in vivo* explant donor. The cytological and molecular analysis complemented and compared well and showed no genomic alterations in the plants regenerated through shoot bud differentiation (Lattoo *et al.* 2006).

Budiguppe *et al.* (2007) Random Amplified Polymorphic DNA (RAPD) for the evaluation of relatedness among six *Piper* species to estimate the genetic relatedness of these six morpho-agronomically contrasting species. Six *Piper* species were screened using RAPD with decamer primer of arbitrary sequence. Out of 100 primers screened 12 were selected which gave clear and bright fragments. DNA banding patterns generated by RAPD were recorded as '1' for presence and '0' for absence. Genetic distance between these 6 species was calculated based on the RAPD data set as per Squared Euclidean distances. Based on the number of bands all the species were grouped into 3 clusters and the dendrogram revealed maximum similarity between *P. betel* and *P. longum* and also in between *P. nigrum* and *P. mullesua* species, altogether forming one cluster. The method can identify any of the single hybrid or species tested.

Dhanya *et al.* (2007) used random decamer primers and inter simple sequence repeats primers for PCR amplification. RAPD and ISSR banding patterns of DNA isolated from dried powdered berries of black peppers using the RAPD primers OPC-05, OPC-11, OPC-16, and OPA-18 and ISSR primers IDT-01 and IDT-12. The absorbance ratio of the isolated DNA at 260/280 (1.7-1.8) and restriction digestion pattern of the DNA compared to the standard DNA along with the consistent amplification pattern in PCR using RAPD and ISSR markers. The protocol may be also applicable to other dry plant tissues rich in polysaccharides and polyphenolic compounds.

Foot rot disease caused by *Phytophthora capsici* is one of the major production constraints in black pepper in India. RAPD profile of moderately resistant and susceptible lines indicated the presence a unique band of 360 base pair in moderately resistant lines with the primer OPA-01. The DNA fragment

associated with these lines was cloned, sequenced and converted into a SCAR marker. The SCAR primer was tested on plants that were classified as moderately resistant and susceptible. SCAR primers amplified DNA invariably from resistant plants only. The sequence of the unique band matched with stress related gene sequences reported in several eukaryotes including *Arabidopsis thaliana* (Anandaraj *et. al.*, 2005).

Kizhakkayil *et al.* (2008) reported molecular characterization of traded black pepper (*Piper nigrum* L.) from India, Indonesia, Vietnam and Malaysia. Molecular profiling (RAPD) and clustering of these samples revealed a comparatively high genetic similarity with in the samples from a particular country than between any two countries. The UPGMA dendrogram constructed based on the similarity coefficient revealed a total of four groups in two different clusters. The two Indonesian samples formed cluster I, while others form clusters II. The genuine Indian varieties and the traded pepper from India formed separate group in cluster II. The aspect of genetic similarity was discussed in relation to the origin and spread of black pepper.

The protein profiles of a relatively tolerant and a susceptible black pepper (*Piper nigrum* L.) variety to foot rot caused by *Phytophthora capsici* along with that of a resistant wild species (*Piper colubrinum*) were evaluated to detect variations in the defense related proteins/enzyme expression in response to *P. capsici* infection (Nazeem *et. al.*, 2008). The SDS-PAGE analysis revealed two additional polypeptides of 16.5 and 8 KD in the leaves of the tolerant variety ('Kalluvally') and 'Panniyur-1'. However, *P. colubrinum* with 16 distinct bands had an altogether different banding pattern. The native protein profile obtained also indicated the expression of two additional proteins in *P. nigrum*. The over-expressed protein was characterized as β-1, 3 glucanase. The resistant genotype *P. colubrinum*, possessed higher enzyme activities than the *P. nigrum* varieties tested.

In order to compare the genetic relationships among kava pepper (*Piper methysticum* Forst.), black pepper (*Piper nigrum* L.,) and its wild relatives as well as to distinguish between kava pepper from black pepper, RAPD and SCAR

markers were used by Jiang *et al.* (2009). 20 primers selected from 80 random primers were used for RAPD amplification to identify the genetic relationships among the different genotypes. Total 170 bands were amplified by 20 random primers, in which 20 bands were polymorphic.

Random amplified polymorphic DNA (RAPD) analysis was carried out in 17 cultivars of sugarcane (Kawar *et al.*, 2009). Selected 40 primers generated 325 bands, 134 of which were found to be polymorphic. The genetic similarity among sugarcane cultivars ranged from 0.77 to 0.99. The study revealed the limited genetic base of the current Indian commercial varieties and the need to diversify the genetic base by using new sources from the germplasm.

RAPD markers were developed for the authentication of *Piper nigrum* (L.) and during the study RAPD was employed for authentication of *Piper nigrum* from its adulterant *Carica* papaya (Khan *et al.*, 2010). Eight decamer oligonucleotide primers were used for screening among these 5 gave species specific reproducible unique amplicons, which is used to distinguish the genuine and adulterant samples having similar morphology.

Genetic diversity analysis in *Piper* species using RAPD markers was reported (Sen *et al.*, 2010). The study revealed the genetic diversity among eight species of *Piper* by RAPD. Among 22 decamer RAPD primer screened, 11 were selected for comparative analysis of different species of *Piper*. High genetic variations were found among different species of *Piper*.

The genetic diversity and phylogenetic relationships of eight different varieties of rice (*Oryza sativa)* viz., MO 16, Ptb 43, Palakkaddan Matta, Ptb 40, MO 17, MO 6, Ptb 39 and KTR 2 from Kerala state, South India were analyzed by RAPD. Out of twenty 10-mer RAPD primers screened initially, eleven were chosen and used. Of the 101 total RAPD fragments amplified, 28 were found to be shared by individuals of all eight varieties. The remaining 73 fragments were found to be polymorphic. This Study offered a rapid and reliable method for the estimation of variability between different accessions which could be utilized by

the breeders for further improvement of the local rice varieties (Skaria *et al.*, 2011).

2.7 Inter Simple Sequence Repeats (ISSR)

PCR based technique involves amplification of DNA segments in between two identical micro satellite repeat regions on the complementary strands. The inter simple sequence repeat (ISSR-PCR) (Zietkiewiez *et al.*, 1994) is another newly developed method which relies on one primer for PCR and holds promise for variety identification. It involves the amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeats (SSR) containing primer and can be undertaken for any spices that contains a sufficient number and distribution of SSR Motifs and has the advantage that genomic sequence data is not required (Gupta *et al.*, 1994; Goodwin *et al.*, 1997). This technique amplifies large numbers of DNA fragments per reaction, representing multiple loci from across the genome; it is an ideal method for fingerprinting varieties and a useful alternative to single-locus or hybridization – based methods. This method was found useful in fingerprinting varieties of rice (Parsons *et al.*, 1997; Blair *et al.*, 1999).

This technique amplifies larger number of DNA fingerprints per reaction, representing multiple loci from across the genome. It is an ideal method for fingerprinting varieties and is useful in fingerprinting varieties of corn (Kantety *et al.*, 1995) and finger millet (Salimath *et al.*, 1995).

ISSRs have high reproducibility possible due to the use of longer primer(16-25 mers) as compared to RAPD primers (10-mers) which permits the subsequent use of high annealing temperature (45°C-60°C) leading to higher stringency. The amplified products are usually 200-2000bp long and amenable to detection by both agarose gel electrophoresis and polyacryamide gel electrophoresis. ISSR segregate mostly as dominant markers following simple Mendelian inheritance (Gupta *et al.*, 1994; Tsumura *et al.*, 1996; Ratnaparkhe *et al.*, 1998; Wang *et al.*, 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus

enabling distinction between homozygote and heterozygote (Wang *et al.*, 1998; Sankar and Moore, 2001).

ISSR markers are effective multilocus markers for application such as diversity analysis, fingerprinting and genome mapping, gene tagging and marker assisted selection. as no prior sequence knowledge is required, they are more rapidly applied than SSR markers, and they are more reliable and robust than RAPD markers, mainly due to the method of detection, and possibly also to the fact that primers are longer, and hence PCR condition are more stringent.

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 rice (Joshi *et al.*, 2000), wheat (Nagaoka and Ogihara, 1997), finger millet (Salimath *et al.*, 1995) vigna (Ajibade *et al.*, 2000), sweet potato (Huang and Sun, 2000) and plant ago (Wolff and Morganrichard, 1998).

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

Nonanchored inter simple sequence repeats (ISSRs) are arbitrary multiloci markers produced by PCR amplification with a microsatellite primer by Borent and Branchard (2001). They are advantageous because no prior genomic information is required for their use. We found the technique stable across a wide range of PCR parameters. Polymorphisms were abundant among 7 dicot species tested with 2 tri-nucleotide and 2 tetra-nucleotide primers. Thus, nonanchored ISSR markers are a good choice for DNA fingerprinting.

Goulao *et al.* (2001) used two micro satellite-based methologies (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 indicates that the results obtained based on the RAPD, AFLP, SSR and ISSR techniques are 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, namely RAPD and RFLP.

Genetic relationships and fidelity among the cultivars and three micropropagated banana varieties (robusta, Giant Governor, Martaman) were assessed by RAPD and ISSR markers. Total of 5330 RAPD and 2741 ISSR bands were generated with 21 RAPD and 12 ISSR primers in micro propagated plants. The percentage of polymorphic loci by RAPD and ISSR found to be 1.75, 5.08 in Robusta and 0.83, 5.0 in Gaint Governor respectively. Among the two marker systems used ISSR fingerprinting detected more polymorphism than RAPD in Robusta and Giant Governor, martaman revealed complete genetic stability (Ray, *et al.,* 2006).

The sex of *Carica papaya*, an angiosperm and *Cycads cardinals*, a gymnosperm using ISSR and RAPD techniques in pre-flowering stage. One female-specific band generated from ISSR profile using primer (GACA) 4 was detected in papaya, which seems to have importance from agricultural point of view. Sequencing of a male-specific RAPD band (PCR with primer OPB 01) in *C. cardinals* revealed homology with putative retro elements of diverse plants, probably indicating its use in the detection of male *C. circinalis.* (Gangopadhyay, *et al.*, 2007)

The genetic analysis of micro propagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers (Sreedhar *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 analysis of micro propagated and regenerated plantlets of banana as 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 homogenous RAPD and ISSR patterns. Based on these results they confirmed their monomorphic nature with no genetic variation among the plantlets analyzed.

Li *et al.* (2008) Inter-simple sequence repeat (ISSR) was used to conduct DNA fingerprint analysis of thirty-four major cultivated strains of *Auricularia auricula* in China. On the base of ISSR analysis, the 34 strains were clustered by UPGMA and two specific ISSR bands from strain 173 and 186 were converted into two sequences 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* is practicable and significative.

Genetic fidelity of *in vitro* raised 45 plants of gerbera (*Gerbera jamesonii* Bolus) derived from three different explants, *viz.*, and capitulum, leaf and shoot tips, was assessed by 32 ISSR markers, for their genetic stability. Out of 32 ISSR markers, 15 markers produced clear, distinct and scorable bands with an average of 5.47 bands per marker. The markers designed from AG motif amplified more number of bands. The markers anchored at 3′ ends produced high number of consistent bands than unanchored markers. Fifteen ISSR markers generated a total of 3773 bands, out of which 3770 were monomorphic among all the clones. The Jaccard's similarity coefficient revealed that out of 45 clones derived from different explants, 44 were grouped into a single large cluster along with the mother plant

with a similarity coefficient value of 1.00, whereas one clone (C38) remained ungrouped. The clones derived from capitulum and shoot tip explants did not show any genetic variation, whereas, one of the leaf-derived clones exhibited some degree of variation (Bhatia, *et al.,* 2009).

The use of ISSR marker for identification of different varieties of banana and detection of genetic uniformity of micro propagated plantlets was done by (Rout *et al.*, 2009). Fifteen ISSR primers were used to differentiate the four cultivated banana. Based on these identity markers, the genetic distance between varieties was estimated and their genetic relationship was found out. The banana varieties having genome constitution AAA were grouped together whereas 'Bantala' variety is out grouped with genome constitution BBB. A homogenous amplification Profile was observed for all the micro propagated plants of 'Bantala' when compared to mother plant. In case of 'Grand Naine', few plants showed variation at the DNA level in primer IG-13. But, morphologically they were identical when compared with original mother plant. The results confirmed that the clonal fidelity of *in vitro* raised plantlets and corroborate the fact that *in vitro* multiplication is the safest mode for multiplying true to type plants.

The genetic fidelity of *in vitro* raised gerbera clones was assessed by using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers by (Bhatia *et al.*, 2010) . Out of 35 RAPD and 32 ISSR primers screened, only 12 RAPD and 10 ISSR primers produced clear, reproducible and scorable bands. The 12 RAPD primers produced 54 distinct and scorable bands, with an average of 4.5 bands per primer. The number of scorable bands for ISSR primers varied from 3 (ISSR-14) to 9 (ISSR-07), with an average of 5.5 bands per primer. The number of bands generated per primer was greater in ISSR than RAPD. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother and the *in vitro* raised plantlets was 1, indicating 100 per cent similarity. This confirmed the true-to-type nature of the *in vitro* -raised clones.

To identify different cultivars and monitor somaclonal variations of banana during rapid mass Micro propagation Inter simple sequence repeats (ISSR) marker study was done. DNA templates from 30 Banana cultivars were evaluated using 45 primers. Genetic diversity was analyzed. Total number of bands varied between the various cultivars from 5 to 9.The percentage of total polymorphism is about 85.1 per cent. Four large group were obtained. Furthermore, a homogenous amplification profile was observed for all the micro propagated plants of 'Brazil' when compared to their mother plants; the developed profiles of different micro propagated clones were typical to that of the donor mother plants (Lu and Ying, 2011).

2.8 Simple Sequence Repeats (SSRs) Or Microsatellites

The fingerprinting of accessions and analysis of genetic diversity in collections and natural populations are important aspects in the management and utilization of plant collections. Conventional characterization has relied on morphological and phenological characteristics. In recent years, SSRs have become the markers of choice to fingerprint accessions. An important advantage of SSRs is their ability to detect genetic diversity at a higher level of resolution than other methods. Furthermore, they are robust, information may be obtained from a small amount of plant material and at any stage of development, results can be obtained quickly, and the data are not affected by environmental conditions (Lanteri and Barcaccia, 2005). Also SSR primers and data can be shared among labs.

SSR markers are found in all eukaryotic genomes. They are short tandem repeat motifs usually consisting of 1 to 6 bp of nucleotides (Powell *et al.*, 1996). They were first referred to as microsatellites by Litt and Lutty (1989) and later as Simple Sequence Repeats (SSRs) by Jacob *et al.* (1991). Conserved regions flanking the repeats are suitable for designing PCR primer pairs to be used for amplifying the intervening repeat loci. These loci are highly variable on account of the number of repeat units found for each locus in any given population (Morgante and Oliveri, 1993). The high levels of heterozygosity, the co-dominant and PCR-

based nature of these repeat loci have made SSRs the molecular markers of choice for genetic mapping and diversity studies (Wang *et al.*, 1994; Gupta *et al.*, 1996).

SSR markers are advantageous to applied plant breeding because they are co-dominant, easily assayed and detect high levels of polymorphism (Morgante and Olivieri, 1993) and for these reasons SSR markers have become highly valuable markers to breeders for the purposes of genome and QTL mapping. SSR markers have, thus, become the marker class of choice for the molecular mapping of many crop species (Roa *et al.*, 2000).

Prior to their use in such studies, SSR marker loci must first be isolated and characterized. Different methods have been used for the development 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).

PCR-based methodologies provide an alternative method for isolation of microsatellite loci. 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 pentanucleotide 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. There is estimated to be a total of

5X 10^3 to 3X10^5 microsatellites per plant genome (Condit and Hubbell, 1991). SSRs occur in many plant genomes such as maize (Shattuck-Eidens *et al.*, 1991; Senior and Heun, 1993), soybean (Akkaya *et al.*, 1992), Brassica (Poulsen *et al.*, 1994; Kresovch *et al.*, 1995), rice (Wu and Tanksley, 1993; Zhao and Kochert, 1993) and barley (Saghai-Maroof *et al.*, 1994).

The first amplification of microsatellites in plants has been in cultivar identification and they are the markers of choice in genotyping in cultivars (Weising *et al.*, 1991; Weising *et al.*, 1990; Beyermann *et al.*, 1992).

Rongwen *et al.* (1995) used SSRs to develop unique DNA profiles or fingerprints for 96 soybean cultivars. Seven SSR loci clearly differentiated all but two closely related individuals. Similar results are being 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; and Guilford *et al.*, 1997) and barley (Russel *et al.*, 1997). The screening of microsatellite alleles in varieties would generate a database useful for variety identification and the development of molecular markers for marker assisted selection (Garland *et al.*, 1999).

Mackill *et al.* (1995) found that microsatellite have average polymorphism at least 1.5 times higher than AFLP and RAPD markers in a comparison of 12 *japonica* cultivars.

Provan *et al.* (1997) using SSR detected intra and inter cultivar polymorphism between the cultivated and wild rice and the extent of chloroplast genomic differentiation was quantified. Olufowote *et al.* (1997) found microsatellites to be more powerful for the identification within cultivar variation.

Sebastian *et al.* (1998) studied the molecular diversity of Philippine landraces and cultivars and found that diversity is more in land races compared to modern cultivars. Use of microsatellite polymorphisms for the identification of

Australian breeding lines of rice was investigated (Garland *et al.*, 1999) and most of the cultivars could be uniquely identified by atleast 6 one microsatellite marker.

Vander *et al.* (2000) conducted a study to develop a simple sequence repeat (SSR) markers in Eucalyptus from amplified inter-simple sequences repeat (ISSR). Two *Eucalyptus grandis* and 36 progeny of them, *E. urophylla, E. nitens, E.globulus and E. comaldulensis* were studied. The result shows the spices specific banding pattern. He also reported the feasibility of the microsatellite for eucalyptus germplasm characterization.

Narve *et al.* (2000) assessed the simple sequence repeat (SSR) diversity of 39 elite soybean genotypes and 40 plant introductions (PIs). A total of 397 alleles were detected among the 79 genotypes at 74 SSR marker loci.

Alvaez *et al.* (2001) used 17 microsatellite loci to study diversity among 31 tomato accessions comprising nine spices of the genus *lycopersicon.* The microsatellite polymorphisms 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.

Molecular genetic diversity analysis in rice using RAPD and SSR markers by Ravi *et al.* (2003) revealed that the cluster produced based on RAPD and SSR markers were not conserved, since matrix correlation value was 0.582 as against the minimum required value of 0.800. Further, it was also noted that the two marker systems contrasted most notably in pair by pair comparison of relationship.

Guohao *et al.* (2003) developed 56 SSR markers, out of which 19 could detect polymorphism among 24 cultivated peanut genotypes. The average number of alleles per locus was 4.25. They could identify 14 alleles at PM50 locus when 48 genotypes were surveyed. Using five such highly polymorphic markers they could differentiate 24 cultivated ground nut genotypes.

Among various PCR based markers, SSR markers are more popular in rice because they are highly informative, mostly monolocus, co dominant, easily

analyzed and co effective (Gracia *et al.*, 2004). SSR markers are class of repetitive DNA sequences usually 2.6 bp that are distributed through out the whole genome and are flanked by highly conserved region (Chambers and Avoy, 2000).

Viruel *et al.* (2005) studied SSRs which allowed the unambiguous identification of all the mango genotypes. A total of 88 fragments were detected with the 16 simple sequence repeats (SSRs) with an average of 5.5 bands. Two primer pairs amplified more than a single locus. The mean expected and observed heterozygosities over the 14 single-locus SSRs averaged 0.65 and 0.69 respectively. The total value for the probability of identity was 2.74 $x10^{-9}$. UPGMA cluster analysis and Principal coordinate's analysis group the genotypes according to their origin and their classification as monoembryonic or polyembryonic types reflecting the pedigree of the cultivars and the movement of mango germplasm.

Galli *et al.* (2005) fingerprinted a collection of 66 apple cultivars with 14 SSR markers and were able to distinguish all apple genotypes except for somatic mutants.

Comparative evaluation of SSR and ISSR markers for polymorphism in land races and varieties of rice indicated that both the SSR (0.51) and ISSR (0.46) primers showed almost similar values for Polymorphic Information Content (PIC). Maximum PIC values were observed with trinucleotide ISSR primer followed by dinucleotide ISSR primer (Prasad *et al.*, 2005).

Vouillamos *et al.* (2006) genotyped 116 accessions of traditional grape cultivars from Armenia, Georgia and Turkey using 12 nuclear microsatellite markers, and found 17 identical genotypes and six cases of homonymy. They also stated that the origin of grape appears to be Georgia since four outgroup varieties from Western Europe was more closely related to Georgian cultivars than other germplasm.

Li *et al.* (2007) used Simple sequence repeat (SSR) markers to detect genetic variation of 60 elite maize inbred lines. Twenty-three SSR primers giving

stable amplified detected 96 alleles among the inbred lines tested. The average number of allele per SSR locus was 3.96 with an range from 2 to 7.The value of polymorphism information content (PIC) for each SSR locus varied from 0.15 to 0.80 with an average of 0.60.The UPGMA analysis classified 60 inbred lines into 6 clusters, which correspond to the heterotic groups determined by their pedigree information and breeders experiences.

Yan *et al.* (2008) reported that a total fifteen polymorphic microsatellite loci from a population of 22 individuals using a modified biotin-capture method. The isolated loci provided microsatellite markers with polymorphism of 3 to 11 alleles per locus. The expected and observed heterozygosities ranged from 0.392 to 0.855 and from 0.136 to 1.000, respectively. These markers were suggested to be the useful tools for analyzing questions concerning population genetic structure and mating system of *Primula merrilliana.*

Menezes *et al.* (2009) reported nine microsatellite markers from an enriched library of *Piper nigrum L.* Twenty varieties clonal from the Brazilian germplasm collection were analyzed and observed and expected heterozygosity values ranged over 0.11 to 1.00 and 0.47 to 0.87, respectively. The nine microsatellite loci characterized would contribute to the studies of genetic diversity and conservation of *Piper nigrum.*

Pei-chun *et al.* (2009) developed eleven primer sets of polymorphic microsatellite DNA loci for *P.polysyphonum.* Allele numbers ranged from two to ten, with observed heterozygosities ranging from 0.222 to 0.889. Four loci exhibited a departure from Hardy–Weinberg equilibrium, possibly due to population admixture. No loci pairs revealed significant linkage disequilibrium. Among the eleven loci, two with extremely high numbers of TCG repeats were obtained. The polymorphic microsatellite DNA markers reported here should provide a helpful means to address questions concerning population structure and demographic history of *P. polysyphonum* for conservation efforts.

Pritesh *et al.* (2010) reported twenty five determinate and indeterminate cultivars of tomato from different geographical locations of India were screened with twenty three SSR primers in order to determine genetic identities, genetic diversity and genetic relationships among these cultivars. 40 alleles were amplified using SSR primers with scorable fragment sizes ranging from approximately 150 to 1000 bp. UPGMA clustering grouped the cultivars into five groups with the USA cultivars forming a distinct group. The genetic distance information obtained in this study might be useful to breeder for planning crosses among these cultivars.

2.3.6 Amplified Fragment Length Polymorphisms (AFLP)

Amplified Fragment Length Polymorphism (AFLP) technique combines principles of RFLP analysis with PCR technology (Vos *et al.*, 1995). Total genomic DNA is digested with two restriction enzymes. Adaptors of known sequence are then ligated to the DNA fragments. Primers complementary to the adaptors, with additional 1 to 3 selective nucleotides on the 3'end, are used to amplify the restriction fragments. The PCR amplified fragments can then be separated by gel electrophoresis and the banding patterns visualized. A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit the application. The care is needed in the selection of primers with selective bases. AFLP profiles require no prior DNA sequence information and the number and nature of amplified fragments are altered by the choice of primer pair. The technique also has the advantage of sampling many loci simultaneously and, in addition, it is more robust than arbitrary priming techniques such as RAPD, because more stringent conditions are used in the PCR. AFLP has predominantly been applied in genetic mapping studies (Ballvora *et al.* 1995; Becker *et al.* 1995; Meksem *et al.* 1995; Van Eck *et al.* 1995). According to Van Eck *et al.* (1995) the technique is semi-quantitative because the intensity of AFLP bands can be used to determine zygosity. AFLP has been used to analyze varieties of various species, including cereals, potatoes, sunflowers, Brassicas, beans and lentils (Cooke and Reeves 1998; Law *et al.* 1998).

Recently, a new multiplex PCR based method (AFLP) has been developed in which a subset of restriction fragments' are selectively amplified using oligonunucleotide primers complementary to sequences that have been ligated to each end (Zabeau and Vos, 1993; Vos *et al.* , 1995). AFLP analysis allows the reliable identification of over 50 loci in a single reaction (Zabeau and Vos, 1993; Vos *et al.*, 1995). This technique combines the reliability of the RFLP and ease of the PCR and thus AFLP is a new typing method for DNA of any origin or complexity (Janssen *et al.*, 1996; Lin *et al.*, 1996).

The high diversity obtained with SSR is consistent with the known characteristics that they are more variable and high expected heterozygosity than the RAPDs or AFLPs (Powell *et al.*, 1996). The high level of polymorphism associated with SSRs are expected because of unique evolution of unique of these genomic region; replication slippage (Tautz *et al.*, 1986) rather than mutation, insertion or deletions.

The ability of AFLPs to distinguish among genotypes is not hindered by their bi-allelic nature (presence or absence) and thus polymorphisms can be identified between very closely related genotypes. AFLP as a tool for DNA fingerprinting is used in *lactuca* species (Hill *et al.*, 1996) barley (Ellis *et al.*, 1997), rice (Zhu *et al.*, 1999a and Singh *et al.*, 1999) and Bermuda grass (Zhang *et al.*, 1999).

Zhu *et al.* (2000) used four pairs of primers with high polymorphism and powerful distinctiveness has been selected from 68 pairs of primer after constructing the AFLP analysis system in apple varieties. Genetic diversity of P32M46 AFLP fingerprinting of 25 important apple varieties in China was analyzed, difference bands for each varieties were determined, and all apple varieties tested were identified based on the P32M46 AFLP fingerprinting. Key points and application for testing trueness and purity of apple varieties of AFLP techniques.

De Riek, (2001) used AFLP based alternatives for the assessment of Distinctness, uniformity and stability of sugar beet varieties. AFLP data were obtained for three consecutive seed deliveries of 15 sugar beet varieties. In total, 696 AFLP markers were scored on 1350 plants. As a first approach a cluster analysis based on Nei's standard genetic distance between varieties was made.

AFLP is considered as a molecular technique for next generation. Based on the introduction of AFLP technique, the research advances in fruit tree were reviewed, including genetic diversity detection, variety taxonomy, variety identification, pedigree analysis and genetic tagging. The number of trees used by AFLP was counted up and the applied prospect of AFLP in fruit tree (Xu and Qin, 2003).

The genetic diversity of 23 maize inbred lines and one teosinte accession was analyzed based on microsatellite (SSR) and AFLP markers. With 40 pairs of SSR primers, totally 202 polymorphic fragments were detected. Twelve AFLP primer combination were used and totally 444 polymorphic bands were produced. Both SSR and AFLP were highly polymorphic. The average of polymorphic information content (PIC) was 0.6 for SSR. Percentage of polymorphic AFLP bands was 72 per cent. The accessions assessed could be clustered into five groups in both marker systems. This is partly in accordance with the previous classification based on conventional methods but with some difference. It was suggested that both system can be used to analyze the genetic diversity of maize inbred lines (Jin *et al.*, 2003).

Genetic maps of random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and inter simple sequence repeats (ISSR) markers in pineapple (2n=*2x*=50) .On the basis of a segregating population of 46 F1 individuals from a cross *Ananas comosus* x *A. bracteatus*, genetic maps of these two species were constructed using the two-way pseudo-test cross approach. The *A. bracteatus* map consists of 335 markers (60 RAPDs, 264 AFLPs and 11 ISSRs) assembled into 50 linkage groups, 26 of them with at least four markers. The *A. comosus* map consists of 157 markers (33 RAPDs, 115 AFLPs, eight

ISSRs and the 'piping' trait locus) organized into 30 linkage groups, 18 of them with at least four markers. These maps cover, respectively, 57.2 per cent of the *A. bracteatus* genome and 31.6 per cent of the *A. comosus* genome .A rough estimate of 120 and 127 kbp/ cM on average was found for the relationship between physical and genetic distance for *A. bracteatus* and *A. comosus*, respectively (Carlier, *et al.*, 2004).

Nazeem *et al.* (2005) studied the variability and relatedness among 49 varieties of black pepper (*Piper nigrum* L.) using molecular markers RAPD and AFLP. The similarity matrix was subjected to cluster analysis and dendrogram generated using the software NTSYS. The dendrogram revealed an average similarity of 63 per cent among accessions. The dissimilarity observed between the varieties Panniyur 1 and Panniyur 3 the progenies of the same parentage, Uthirankotta and Cheriyakanyakadan was only 18 per cent.

Sami *et al.* (2005) studied the genetic relationships among 21 cotton genotypes from two different species *G. barbadense* and *G. hirsutum*. 16 AFLP primer combinations produced 940 bands among which 474 were polymorphic, thus, representing a level of polymorphism of 50.4 per cent among the 21 cotton genotypes. The amplification of AFLP templates resulted in a number of reproducible fragments ranging from 31 to 90 per primer with a size range of 60 bp to 780 bp. Fifteen primer combinations detected unique specific markers identifying 8 out of the 21 genotypes. A dendrogram was generated from the AFLP information that revealed two main clusters. All the genotypes belonging to *G. barbadense* except one were grouped in one cluster, while the accessions representing *G. hirsutum* constituted the second cluster, thus, confirming the results previously obtained by RAPD, ISSR and SSR analysis on the same cotton genotypes.

A highly resistant primitive cultivated species *Ralstonia solanacearum* was employed to generate a mapping population to perform the bulked segregant analysis for screening of AFLP markers linked with the resistance. Another population which had genetic similarity to the mapping population was used for

testing of the markers achieved. A novel strategy named common AFLP marker was used to identify the genomic position of the molecular markers in the linkage map. Several informative primer combinations were employed in the detection and the common AFLP markers ATG/CTC 307.0, ATG/CTC 246.0, ATG/CTC 191.0 and AAC/CAC 79.0 were considered as markers that associated with the resistance and located on the relevant chromosome maps (Gang *et al*, 2005).

Tae-Jin *et al.* (2006) adopted AFLP to study 55 germplasm materials, 656 bands were detected with 20 pairs of AFLP primers and 487 of them were polymorphic. On average each primer combination could be used to detect 24.4 polymorphic bands. The 55 materials were clustered into 6 groups, which were identical to the groups divided by their origins. These results showed that AFLP was conductive to analyzing the genetic diversity of corn. Further analysis of the relation of the Pioneer population of American origin to Reid and BSSS populations indicated that many materials of Pioneer populations had genetic relations to the Reid populations.

Yang and Yu, (2006) compared AFLP (Amplified Fragment Length Polymorphism) and RAPD (Randomly Amplified Polymorphism DNA) techniques, which were used to study the genetic diversity of *Oreochromis niloticus*. The AFLP marker system had lower polymorphism information content and less effective alleles per locus than RAPD, but the former had much higher assay efficiency index than the latter detected by 7 AFLP primer combinations and 20 RAPD primers. The similarity index of fragments of the two markers was similar. The results showed that AFLP is better than RAPD and both fit for genetic detection of genomic DNAs.

Finger printing analysis with Amplified Fragment Length Polymorphic DNA (AFLP) was reported to be an ideal tool for cultivar identification and phylogenetic studies in black pepper (Joy *et al.*, 2007). The analysis was performed in 30 popular cultivars of black pepper and found considerable variability among cultivars of black pepper. It shows the high level of polymorphism and the unique characteristics of the major cultivars.

Jiang *et al.* (2007) reported that the genetic relationship between Kava and *Piper nigrum* has been studied by the AFLP molecular markers. 31 test materials were used in the pepper, including 6 Kava genotypes, 23 cultivated and wild peppers, 1 *Peperomia pellucida* and 1 tobacco. Based on the fluorescence-labeled AFLP, the 31 germplasms were divided into 5 different groups at 0.52 of the genetic similarity coefficient. The genetic distance between Kava and other *Piper* germplasms was far, and the fluorescence-labeled AFLP was applicable to the identification and DNA fingerprint construction of Kava.

An amplified fragment length polymorphism (AFLP) technique was used to analyze the DNA fingerprints of 3 types of *Fructus evodiae* including *Evodia rutaecarpa var.officinalis*, *E.rutaecarpa var.bodinieri* and *E.rutaecarpa* (Juss.) Benth. Three primer combinations (E-AGC/M-CAG, E-AGC/M-CTG and E-ACG/M-CTG) screened out from eighteen primer pairs were used to conduct genetic analysis and variety identification of nineteen materials of *Fructus evodiae*. 93 bands were generated and 57 fragments appeared to be polymorphic (61.3% on average). The results showed that AFLP was an efficient method to identify the germplasms of *Fructus evodiae*. UPGMA cluster analysis was also performed. Genetic similarity coefficient (SC) ranged from 0.235 to 0.941, indicating that *Fructus evodiae* is rich in genetic diversity. Dendrogram revealed that *E.rutaecarpa var.officinalis* and *E.rutaecarpa var.bodinieri* from Yuqing region were clustered into one group, which suggested that human cultivation activities have influenced on the genetic characteristics of *Fructus evodiae* (Zhang, 2007).

Jiang *et al.* (2009) adopted AFLP to study 28 germplasms including 6 materials of *P. methysticum*, 21 materials of cultivated and wild pepper, 1 material of *Peperomia pellucida* belonged to different genus as tested materials, were screened from 64 pairs of primers for AFLP amplification and the clustering analysis was conducted with MVSP3. 191 bands were amplified by 4 pairs of primers, 189 of which had polymorphism being 98.6 per cent. 28 germplasms were clustered into 6 different groups at the genetic similarity coefficient of 0.52 by silver staining AFLP, in which 6 materials of *Piper methysticum* were clustered into a single group, indicating that *P. methysticum* belonged to *Piper* L.; the research

provided the basis for molecular identification and the fingerprint construction of *P. methysticum.*

Zheng *et al.* (2009) analyzed the genetic diversity of *Kobresia* accessions at the molecular level and further obtained the necessary information for breeding and germplasm evaluation. Genomic DNA of *Kobresia* was amplified with four E+3 and M+3 primer combinations with AFLP. AFLP analysis produced 164 scorable bands, of which 154 (93.96%) were polymorphic. The mean Nei's gene diversity index (H) was 0.2430, and the Shannon's information index (I) was 0.4012, indicating the abundant genetic diversity of *Kobresia.* The 11 *Kobresia* accessions from Tibetan Plateau, China, can be classified into five groups after cluster analysis based on the UPGMA method. In general, there was abundant genetic diversity among *Kobresia* accessions resources, and the genetic coefficient was unrelated to their geographic latitude. Natural habitats influenced genetic differentiation of *Kobresia.*

Liu *et al.* (2010) Amplified fragment length polymorphism (AFLP) technique was used to analyze the genealogical relationship of 7 introgressed cotton varieties from the hybridization of upland (*Gossypium hirsutum* L.) and sea-island cottons (*Gossypium barbadense* L). Ten pairs of primer combinations with high polymorphism and resolution were selected from 64 primers. These 10 primer combinations resulted in a total of 480 bands, of which 374 bands (77.9%) were polymorphic and 51 bands (10.6%) were specific. Cluster analyses showed that 7 varieties of cottons were divided into two groups. Our results suggested that interspecific hybridization is feasible to broaden germplasm of upland cotton and AFLP technique could be applied for the identification of variety purity and genealogical relationship.

Materials and Methods

3. MATERIALS AND METHODS

The study on"DNA Fingerprinting of selected Black Pepper (*Piper nigrum* L.) varieties" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2009-2011. The materials used and methodologies adopted are discussed in this chapter.

3.1 Materials

3.1.1 Plant materials

Morphological features of seven black pepper varieties Panniyur 1 to Panniyur 7 released by KAU were recorded for the plants maintained at Pepper Research Station, Panniyur. Rooted cuttings of each variety was collected from PRS, Panniyur and 10 plants in each variety maintained as potted plants in the green house at CPBMB were used for the present study (Plate 1).

3.1.2 Laboratory chemicals, glassware and equipment items:

The chemicals used in the present 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 marker (λDNA/*Hind*III+ *Eco*RI double digest) were supplied by Bangalore Genei Ltd. All the plastic ware used were obtained from Axygen and Tarson India Ltd. The decamer primers were obtained from Operon Technologies Inc. (Alamedda, Calif.), ISSR, SSR and AFLP primers were obtained from Sigma Aldrich Chemical 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 Eppendorf Master Cycler (Eppendorf, USA), agarose gel electrophoresis done in horizontal gel electrophoresis systems (BIO-RAD, USA).

Plate 1: Seven black pepper varieties maintained as source plant

3.2 Methods

Molecular analysis of the seven black pepper varieties (Panniyur 1-Panniyur 7) was carried out with four different marker systems- RAPD, ISSR, SSR and AFLP.

3.2.1 Genomic DNA extraction

Young tender, pale green leaves (first to third from the tip) were collected on ice from individual plants in each accession grown in the green house conditions. 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 that is very high in black pepper.

Among the most commonly used protocols, CTAB method developed by Rogers and Benedich (1994) was used for the extraction of genomic DNA.

Reagents:

I. CTAB buffer: 2X

- 2% CTAB (w/v)
- 100mM Tris (pH8)
- 20mM EDTA (pH8)
- 1.4M Nacl
- 1% PVP

Modified:

- 4%CTAB (w/v)
- 100mM Tris (pH8)
- 20mM EDTA (pH8)
- 1.4M Nacl
- 1% PVP

II. 10%CTAB solution:

- 10% 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% and 100%

VII. Sterile distilled water

Procedure:

- Ground one gram of cleaned leaf tissues in a pre-chilled mortar and pestle in the presence of liquid nitrogen.
- Added 4ml of extraction buffer (2x/4x) and 50µl of β-mercaptoethanol
- Transferred the homogenized sample into an autoclaved 50 ml centrifuge tube and 3 ml of pre-warmed extraction buffer (total 7 ml).
- Mixed well and incubated the mixture at 65°C for 20 to 30 minutes with occasional mixing by gentle inversion.
- Added equal volume (7 ml) of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify. Spun at 10,000 rpm for 15 minutes at 4°C
- After centrifugation the contents got separated into three distinct phases.

  Aqueous topmost layer - DNA
  Interphase - fine particles, cell debris, emulsified protein
  Lower layer - Chloroform and pigments
- Transferred the top aqueous layer to a clean centrifuge tube and added $1/10^{th}$ volume of 10 percent CTAB solution and equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion.
- Centrifuged at 10,000 rpm for 15 minutes at 4°C.
- 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 -20°C for half an hour for complete precipitation.
- Centrifuged at 10,000 rpm for 15 minutes at 4°C. 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 TE buffer or sterilized water and stored at -20°C.
- Loaded the samples on 0.8 per cent agarose to observe the quantity and quality of DNA.

3.2.2 Purification of DNA

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

Reagents

- Phenol: chloroform mixture (24:1, v/v)
- Chilled isopropanol
- 70 per cent ethanol
- TE buffer
- Chloroform: Isoamyl alcohol (24:1, v/v)
- 1% 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, added RNase solution (2 µl) 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 -20°C 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 TE buffer.
- Loaded the samples on 0.8 percent agarose gel at constant voltage of 100V to test the quality and to find whether there was any shearing during RNase treatment.

3.2.3 Electrophoresis of DNA:

Reagents and Equipment

1. Agarose – 0.8 percent (for genomic DNA)
   – 1.3 percent (for PCR RAPD samples)
   – 2 percent (for PCR ISSR and SSR samples)
2. 50X TAE buffer (pH 8.0)
   - Tris buffer
   - 0.5mM EDTA
3. Tracking/loading dye (6X)
   -Bromophenol blue
   -Glycerol
4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml)
5. Electrophoresis unit, power pack, gel casting tray, comb
6. UV transilluminator (Herolab ^{R})
7. Gel documentation and analysis system

Composition of reagents is provided in Annexure II.

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. Microwaved 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. (Ethidium bromide was added at this point to a concentration of 0.5 µg/ml)
- 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.
- 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 per well. Loaded suitable molecular weight marker (λDNA *Eco*RI/ *Hin*dIII double digest) in one lane.
- Electrophoresed at 100 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 one or two bases.

3.2.4 Gel documentation

Gel documentation was done with BioRad Gel Documentation System using PDQuest software. PDQuest is a software package for imaging, analyzing, and data basing 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 your computer screen.

Procedure:

To use the basic mode of the acquisition window, select Basic in the Options dialog.

- Position Gel: The Gel Doc XR camera will be in Live/Focus mode. While focussing the position of the gel should be within the frame.
- Select an Illumination Mode: Select the type and scale Gel Doc XR data using the Image Mode option buttons.
- Acquire Image: Click on Auto Expose. After the automatic exposure time has been reached, fine-tune the exposure by entering slightly different times (in seconds) in the Exposure time field. Alternatively, click on Manual Expose to enter an exposure time directly. Once the image is acquired click on Freeze.
- Optimize the Display: The Display controls are useful for quickly adjusting the appearance of image for output to a video printer.
- Analyze the Image: The Analysis step of the Gel Doc XR acquisition window allows adding annotations and analyzing the newly acquired image.
- Select Output: The image can be video printed or saved to a file.

3.2.5 Assessing the quality and quantity of DNA

The purity of DNA was further checked by using NanoDrop ND-1000 spectrophotometer. Nucleic acid shows absorption maxima at 260nm whereas proteins show peak absorbance at 280nm. Absorbance recorded at both wavelength and purity 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.

1OD at 260 nm = 50 µg DNA/ml

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

Procedure for Nanodrop spectrophotometry:

- Connect the Nanodrop spectrophotometer to the System and open the operating software ND-100.
- Select the option Nucleic acid.
- With the sampling arm open, pipette 1µl distilled water onto the lower measurement pedestal.
- Close the sampling arm and initiate 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 pipetted onto measurement pedestal and select measure.
- When the measurement is complete, open the sampling arm and wipe 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

Four different types of markers were used for the study which includes RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequences Repeats) and SSR (Simple Sequence Repeats). Under each marker analysis system, each of the seven varieties 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 analysis system could be obtained simultaneously.

3.3.1 DNA amplification conditions

The PCR condition required for effective amplifications in RAPD, ISSR, SSR and AFLP 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 0.5 ml or 0.2 ml PCR tubes. The PCR was carried out in an Eppendorf Master Cycler (Eppendorf, USA).

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

3.3.2 RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA (40 to 50 ng/µl) were isolated from leaf samples of seven black pepper cultyivars and subjected to RAPD as per the procedure. Random decamer primers supplied by 'Operon Technologies' USA with good resolving power was used for amplification of DNA. The decamer primers for RAPD assay were selected after an initial screening study of primers.

The amplification was carried out in an Eppendorf Master Cycler Eppendorf, USA). The PCR reaction was performed using a 20 µl reaction mixture, consisting of

a) Genomic DNA (45 ng)	- 2.0 µl
b) 10X Taq assay buffer B	- 2.0 µl
c) $MgCl_2$	- 1.5 µl
c) dNTP mix (10mM each)	- 1.0 µl
d) Taq DNA polymerase (1U)	- 0.3 µl
e) Decamer primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 11.7 µl
Total volume	- 20.0 µl

The thermocycler was programmed as follows:

94^0C for 4 minutes	-	Initial denaturation	
92^0C for 1 minute	-	Denaturation	40 cycles
37^0C for 1 minute	-	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 random primers for RAPD analysis

Thirty decamer primers (Operon Technologies) were screened with bulked DNA samples by PCR for RAPD analysis and are listed in Table 2.

3.3.2.2 Random primers selected for DNA fingerprinting

The amplified products were run on 1.3 per cent agarose gel using 50X TAE buffer stained with ethidium bromide along with marker (λDNA/ *Eco*RI+H*ind*III double digest). Electrophoresis was performed at 80 V for one hour twenty minutes. The profile was visualized under UV transilluminator and documented using gel documentation system (Biorad, USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb/bp of bases was recorded in comparison with marker.

Out of 30 decamer primers screened for RAPD analysis, 10 primers (Listed in Table 3) yielded the best amplification products for each black pepper variety.

Table 2: List of Operon decamer primers used for screening black pepper samples

Sl. No	Primer	Nucleotide Sequence
1	OPA10	5'-GTGATCGCAG-3'
2	OPA 11	5'-CAATCGCCGT-3'
3	OPA12	5'-TCGGCGATAG-3'
4	OPA-13	5'-CAGCACCCAC-3'
5	OPA-14	5'-TCTGTGCTGG-3'
6	OPA-15	5'-TTCCGAACCC-3'
7	OPA-16	5'-AGCCAGCGAA-3'
8	OPA-18	5'-AGGTGACCGT-3'
9	OPA-19	5'-CAAACGTCGG-3'
10	OPA-20	5'-GTTGCGATCC-3'
11	OPA22	5'-TGCCGAGCTG-3'
12	OPA23	5'-AGTCAGCCAC-3'
13	OPA26	5'-GGTCCCTGAC-3'
14	OPA 27	5'-ATCGGATTCA-3'
15	OPC 01	5'-TTCGAGCCAG-3'
16	OPU 01	5'-ACGGACGTCA-3'
17	OPU 02	5'-CTGAGGTCTC-3'
18	OPU 03	5'-CTATGCCGAC-3'
19	OPU 09	5'-CCACATCGGT-3'
20	OPU 13	5'-GGCTGGTTCC-3'
21	OPP 08	5'-ACATCGCCCA-3'
22	OPP 14	5'-CCAGCCGAAC-3'
23	OPAH 06	5'-GTAAGCCCCT-3'
24	OPA28	5'-GTGACGTAGG-3'
25	OPA 29	5'-GGGTAACGCC-3'
26	OPA30	5'-AGGTGACCGT-3'
27	OPA32	5'-TCGGCGATAG-3'
28	OPA 36	5'-AGCCGGGAA-3'
29	OPA 38	5'-AGTGCATTCA-3'
30	OPA 39	5'-CAAACGTCGG-3'

Table 3: Details of selected for RAPD primers

Sl. No	Name of Primer	Sequence
1	OPU 01	5'-ACGGACGTCA- 3'
2	OPU 02	5' -CTGAGGTCTC- 3'
3	OPU 03	5' -CTATGCCGAC- 3'
4	OPU 09	5' -CCACATCGGT- 3'
5	OPU 13	5' -GGCTGGTTCC- 3'
6	OPP 08	5' -ACATCGCCCA- 3'
7	OPP 14	5' -CCAGCCGAAC- 3'
8	OPAH 06	5' -GTAAGCCCCT- 3'
9	OPA30	5'AGGTGACCGT 3'
10	OPA28	5'-AGTGCATTCA -3'

3.3.3 ISSR (Inter Simple Sequence Repeats) analysis

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

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consists of

a) Genomic DNA (25 ng)	- 1.5 µl
b) 10X Taq assay buffer B	- 2.0 µl
c) $MgCl_2$	- 2.0µl
c) dNTP mix (10mM each)	- 1 µl
d) Taq DNA polymerase (1U)	- 0.3 µl
e) Primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 11.7 µl
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
51^0C for 1 minute	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 8 minutes	-	Final extension	
4^0C for infinity to hold the sample			

3.3.3.1 Screening of ISSR primers for analysis

Thirty four primers (ISSR Technologies) were screened for ISSR analysis and are listed in Table 4.

Table 4: List of ISSR primers used for screening black pepper samples

Sl. No	Name of Primers	Sequence
1	UBC 811	5'-GAGAGAGAGAGAGAGAC-3'
2	UBC812	5'-GAGAGAGAGAGAGAGAA-3'
3	UBC 814	5'-CTC TCT CTC TCT CTC TA -3'
4	UBC 813	5'- CTCTCT CTC TCT CTC TT -3'
5	UBC 817	5'- CAC ACA CAC ACA CAC AA -3
6	UBC 820	5'- GTG TGT GTG TGT GTG TC -3'
7	UBC 826	5'- ACA CAC ACA CAC ACA CC -3'
8	UBC 834	5' AGA GAG AGA GAG AGA GYT -3'
9	UBC 836	5'-AGAGAGAGAGAGAGAGYA -3'
10	UBC 840	5'-GAGAGAGAGAGAGAGAYT -3'
11	UBC 845	5'- CTCTCTCTCTCTCTCTRG -3'
12	UBC 848	5'- CACACACACACACACARG – 3'
13	UBC 863	5'- AGTAGTAGTAGTAGTAGT -3'
14	UBC 890	5'- VHVGTGTGTGTGTGTGT -3'
15	UBC 825	5'- ACACACACACACACA CT -3'
16	UBC 847	5'- CACACACACACACACARC -3'
17	UBC 857	5'-ACACACACACACACACYG -3'
18	UBC 865	5'-CCGCCGCCGCCGCCGCCG -3'
19	UBC 866	5'-CTC CTC CTC CTC CTC CTC -3'
20	UBC51	5'-GTG TGT GTG TGT GTGTCTC -3'
21	UBC 815	5'- CTC TCT CTC TCT CTC TG-3'
22	UBC 835	5'- AGAGAGAGAGAGAGAGYC- 3'
23	UBC 840	5'- GAGAGAGAGAGAGAGAYT -3'
24	UBC 843	5'- CTC TCT CTC TCT CTC TRA -3'
25	UBC 844	5'- CTC TCT CTC TCT CTC TRC -3'
26	ISSR1	5'-TGTGTGTGTGTGTGTGTGGCACATCATCAT -3'
27	ISSR2	5'-GTGTGTGTGTGTGTGAT -3'
28	ISSR3	5'-CTCTCTCTCTCTCTCTTG -3'
29	ISSR4	5'- GAGAGAGAGAGAGG-3'
30	ISSR5	5'- CACACACACACACAC-3'
31	ISSR6	5'- GTGTGTGTGTGTCC-3'
32	ISSR7	5'-CACACACACACACAyGCTAGTGCTCACACA-3'
33	ISSR8	5'-TGTGTGTGTGTGTGyCATGCACATTGTGTG -3'
34	ISSR9	5'-TGTGTGTGTGTGTGGCACATGCARTGT-3'

3.3.3.2 ISSR analysis selected primers

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA / *Eco*RI+H*ind*III double digest). Electrophoresis was performed at 40 V for four hours. The profile was visualized under UV transilluminator and documented using gel documentation system (Biorad, USA). The documented ISSR profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb/bp of bases was recorded in comparison with the marker.

Out of 34 primers screened for ISSR analysis, 10 primers (Listed in Table 5) yielded the best amplification products for each of the seven black pepper variety.

3.3.4 SSR (Simple Sequence Repeat) analysis

The good quality genomic DNA (20 to 25 ng/µl) were isolated from leaf samples of seven black pepper and were subjected to SSR analysis as per the standard procedure. SSR primers supplied by Sigma, USA were used for amplification of DNA.

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). It was performed in a 20 µl reaction mixture consisting of,

a) Genomic DNA (20 ng)	-	1.5µl
b) 10X Taq assay buffer A	-	2.0µl
c) dNTP mix (10mm each)	-	1µl
d) Taq DNA Polymerase (1U)	-	0.3µl
e) Forward Primer (10pM)	-	0.75µl
f) Reverse Primer (10pM)	-	0.75µl
g) Autoclaved Distilled Water	-	13.7µl
Total volume	-	20.0µl

Table 5: Details of selected for ISSR primers

Sl. No	Name of Primers	Sequence
1	UBC51	5'-GTG TGT GTG TGT GTGTCTC -3'
2	UBC812	5'-GAGAGAGAGAGAGAGAA-3'
3	UBC 811	5'-GAGAGAGAGAGAGAGAC-3'
4	UBC 835	5'- AGAGAGAGAGAGAGAGYC- 3'
5	UBC 825	5'- ACACACACACACACACT -3'
6	UBC 836	5'-AGAGAGAGAGAGAGAGYA -3'
7	UBC 843	5'- CTC TCT CTC TCT CTC TRA -3'
8	ISSR3	5'- CTCTCTCTCTCTCTCTTG-3'
9	ISSR4	5'- GAGAGAGAGAGAGG-3'
10	ISSR6	5'- GTGTGTGTGTGTCC-3'

The amplification was carried out with the following programme

94^0C for 1 minute	-	Initial denaturation	
94^0C for 1 minute	-	Denaturation	30 cycles
Primer annealing temperature denoted in Table 7 for 1 min			
72^0C for 1 minute	-	Primer extension	
72^0C for 5 minutes	-	Final extension	
4^0C for infinity to hold the sample			

3.3.4.1 Screening of SSR primers for SSR analysis

Twenty nine primers were screened by PCR for SSR analysis and are listed in Table 6.

3.3.4.2 SSR primers selected for DNA fingerprinting

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA / *Eco*RI+*Hind*III double digest and 100bp ladder). Electrophoresis was performed at 60 V for one hour ten minutes. The profile was visualized under UV transilluminator and documented using gel documentation system. The size of polymorphic band in kb/bp of bases was recorded in comparison with the marker. The documented SSR profile was carefully examined for amplification of DNA as bands and was used for developing fingerprints.

Out of 29 primers screened for SSR analysis, 8 primers (Listed in Table 7) yielded the best amplification products.

Table 6: List of SSR primers used for screening black pepper Samples

Sl. No	Name of Primers	Sequence
1	PN A5	**F** 5'-CTTCCAGACCAATAATCAACTT-3' **R** 5'-ATCCCAAAATACACAACAATTC-3'
2	PN B5	**F** 5'-GTTTTGAATGGGTCGGTGAT-3' **R** 5'-ATTGTTCTGATTTCTTCGTTATTG-3'
3	PN B9	**F** 5'-AGTATTGGTTGTTTCTCTC-3' **R** 5'-ATGTAAAATCGATAGTCCTCA-3'
4	PN E3	**F** 5'-TTTGTGTCCTCTCCCTCTCC-3' **R** 5'-AAGACTAAATAGGCAAGGCAAA-3'
5	PN F1	**F** 5'-ACTTCAGTGCTATTTTTATCTTCC-3' **R** 5'-CCAACGCCCACTCTCAT-3'
6	PN G11	**F** 5'-TTACTAGTGTCCACCCCCACT-3' **R** 5'-TCGATGGAAAGTCACCCTCT-3'
7	PN H4	**F** 5'-CTTTTCCCACAATTCAGTCTCG-3' **R** 5'-ACCCATGCGTGTATCTTCTCAG-3'
8	PN H8a	**F** 5'-TGTGTCTTTTATATTTTTGATG-3' **R** 5'-TATTAGTAGTTCTCCCTTTTGA-3'
9	PN D10	**F** 5'-GTGTTACCTTTGGGGCATTCA-3' **R** 5'-TGTGTCAGGGCATCAAACC-3'
10	Pc-b8	**F** 5'-AGCAGATACGCATGACCGTA-3' **R** 5'-CTTGCTAGTCTCGATTGCAC-3'
11	Pc-b12	**F** 5'-GCTGGTGCTGGTGCCCCTTA-3' **R** 5'-TGCTGCCGCTGCTTTTGACG-3'
12	Pc-b13	**F** 5'-AACGCGTTGAACGATGCATC-3' **R** 5'-CATATAGTCGTGCTGAGAGG-3'
13	SSR306	**F** 5'-ACATGAGCCCAATGAACCTC-3' **R** 5'-AACCATTCCGCACGTACATA-3'
.14	SSR286	**F** 5'-AGCTATGGAGTTTCAGGACCA-3' **R** 5'-ATTCAGGTAGCATGGAACGC-3'

15	SSR557	**F** 5'-GCCACAAGAAACATTGCTGA-3' **R** 5'-TACGCGCACGTGCATAAATA-3'
16	SSR565	**F** 5'-GAGGTGATGAGAACTCGCC-3' **R** 5'-TCAGAGGCTTCTGGGTCAGT-3'
17	SSR326	**F** 5'-TTAGGCAGCTTACGACTGGA-3' **R** 5'-GAGCCAGAGGTCCTTCAGTG-3'
18	SSR47	**F** 5'-TCCTCAAGAAATGAAGCTCTGA-3' **R** 5'-CCTTGGAGATAACAACCACAA-3'
19	SSR72	**F** 5'-GGTTCCCTTCTCTCTTTGTCC-3' **R** 5'-GCGTGTTCTTCGATTTGACA-3'
20	SSR34	**F** 5'-TTCGGATAAAGCAATCCACC-3' **R** 5'-TCGATTGTGTACCAACGTCC-3'
21	SSR122	**F** 5'-ACGGGATTGTACCCAATCAA-3' **R** 5'-AGGGTTTGAAGAGGAGGAGG-3'
22	SSR350	**F** 5'-GGAATAACCTCTAACTGCGGG-3' **R** 5'-CGATGCCTTCATTTGGACTT -3'
23	SSR578	**F** 5'-ATTCCCAGCACAACCAGACT-3' **R** 5'-GTTGGTGGATGAAATTTGTG-3'
24	SSR128	**F** 5'-GGTCCAGTTCAATCAACCGA-3' **R** 5'-TGAAGTCGTCTCATGGTTCG-3'
25	SSR45	**F** 5'-TGTATCCTGGTGGACCAATG-3' **R** 5'-TCCAAGTATCAGGCACACCA-3'
26	SSR04	**F** 5'-TTCTTCGGAGACGAAGGGTA-3' **R** 5'-CCTTCAATCCTCCAGATCCA-3'
27	SSR296	**F** 5'-CCGGAACAAGTCCCTTCATA-3' **R** 5'-TCAGCCAAGTTCATGGTACATC-3'
28	SSR593	**F** 5'-TGGCATGAACAACAACCAAT-3' **R** 5'-AGGSSGTTGCATTAGGCCAT-3'
29	SSR603	**F** 5'-GAAGGGACAATTCACAGAGTTTG-3' **R** 5'-CCTTCAACTTCACCACCACC-3'

Table 7: Details of selected for ISSR primers

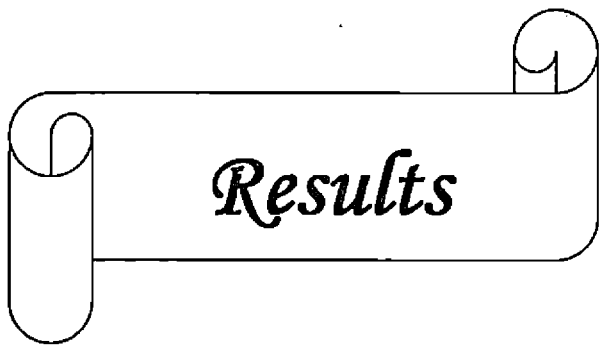
Sl. No	Name of Primers	Annealing temperature	Sequence
1	PN A5	58°C	**F** 5'CTTCCAGACCAATAATCAACTT3' **R** 5'ATCCCAAAATACACAACAATTC3'
2	PN B5	58°C	**F** 5'GTTTTGAATGGGTCGGTGAT3' **R** 5'ATTGTTCTGATTTCTTCGTTATTG3'
3	PN E3	58°C	**F** 5'TTTGTGTCCTCTCCCTCTCC3' **R** 5'AAGACTAAATAGGCAAGGCAAA3'
4	PN F1	58°C	**F** 5'ACTTCAGTGCTATTTTTATCTTCC3' **R** 5'CCAACGCCCACTCTCAT3'
5	PN G11	58°C	**F** 5'TTACTAGTGTCCACCCCCACT3' **R** 5'TCGATGGAAAGTCACCCTCT3'
6	PN H4	53°C	**F** 5'CTTTTCCCACAATTCAGTCTCG3' **R** 5'ACCCATGCGTGTATCTTCTCAG3'
7	PN H8a	48°C	**F** 5'TGTGTCTTTTATATTTTTGATG3' **R** 5'TATTAGTAGTTCTCCCTTTTGA3'
8	PN D10	58°C	**F** 5'GTGTTACCTTTGGGGCATTCA3' **R** 5'TGTGTCAGGGCATCAAACC3'

3.4.4 Scoring of bands and data analysis

Scoring of bands on agarose was done with the Quantity one software loaded in Gel Doc. λDNA marker (*Eco*RI+H*ind*III double digest) and 100bp ladder from Invitrogen was used as a molecular weight size marker for each gel alongside the DNA samples. The bands were scored as 1 and 0 for the presence and absence respectively and their size recorded in relation to the molecular weight markers used. The fingerprint results obtained from RAPD, ISSR and SSR were transformed into data matrix as discrete variables. Jaccard's coefficient of similarity was measured and a dendrogram based on similarity coefficients was generated by using Unweighted Pair Group Method with Arithmetic Mean (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 a primer was calculated as the sum of 'band informativeness' of the bands produced by the primer. Band informativeness (Ib) is = 1(2(0.5-p)), where p is the proportion of accessions containing the band. Resolving power of the primer is represented as: $Rp=\sum Ib$.

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


Results

4. RESULTS

The study on "DNA fingerprinting of selected black pepper varieties" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2010-11. The results of different experiments are described in this chapter.

4.1. Source of DNA

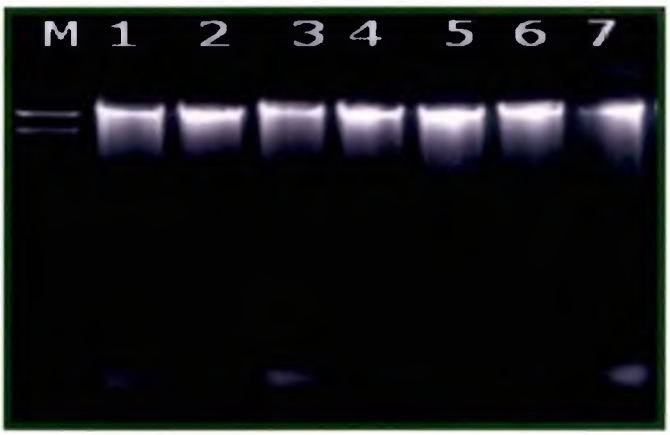
Morphological parameters of the seven released black pepper varieties of Kerala Agricultural University viz., Panniyur 1 to Panniyur 7 recorded for the plants maintained at Pepper Research Station, Panniyur provided in Table 8. As reported in many other crops, young leaves were selected as the ideal part for extraction of total genomic DNA of the plant. In black pepper, very tender, fresh, pale green leaves (0.5 to 1g) yielded good quality DNA in sufficient quantity. There was no browning of the extract if suitable antioxidants were added.

4.1.1. Isolation, purification and quantification of DNA

Genomic DNA isolated through the CTAB method reported by Roger and Benedich 1994 was not pure and had protein contamination (Plate 2a). However the genomic DNA isolated with modified CTAB buffer (3.2.1) gave good quality DNA. The agarose gel electrophoresis indicated clear discrete band without RNA contamination and spectrophotometric analysis gave ratio of UV absorbance ($A_{260}/_{280}$) between 1.8 and 2.0. Quality of DNA isolated through the modified method for selected black pepper varieties are indicated in Plate 2b and in Table 9.

Table 8: Morphological parameters of seven black pepper varieties

Characters	P1	P2	P3	P4	P5	P6	P7
Leaf shape	Large heart shaped leaves	Large heart shaped leaves	Heart shaped with slight wavy margin	heart shaped leaves	Large oval shaped leaves	Small oval shaped leaves	Heart shaped with slight wavy margin
Plant height (m)	6.1	5.6	5.2	5.4	6.1	2.9	5.3
Length of leaf (cm)	14.8	14.33	12.6	13.6	15.2	11.3	17.5
Breadth of leaf (cm)	9.5	10.5	9.4	10.27	8.5	4.5	10.2
Internodal length (cm)	7	5	5.5	6.1	5.8	5.8	5.8
Colour of shoot tip	Pale yellow	Pale purple	greenish yellow	Dark pink	faint violet	Light pink	Light purple
Length of spike (cm)	17	12.3	14.5	9.3	13.1	7.92	19.4
No of spikes/m^2	19	34	20	46	33	47	20
No of berries/spike	107	84.5	68.1	37.8	51	53	133
Setting percentage	96	74.2	89.2	85.7	85.7	99	91.5
1000 green berry weight (g)	155	127	153	116	110	130	150



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
1: Panniyur1, 2: Panniyur2, 3: Panniyur3, 4: Panniyur4, 5:Panniyur5, 6: Panniyur6, 7: Panniyur7
a. Rogers and Benedich method

M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
1: Panniyur1, 2: Panniyur2, 3: Panniyur3, 4: Panniyur4,
5: Panniyur5, 6: Panniyur6, 7: Panniyur7
b. Rogers and Benedich modified method after RNase treatment

Plate 2: DNA isolated through different methods from tender leaves of black pepper

Table 9: Quality and quantity of DNA isolated from *Piper nigrum* genotypes as assessed by Nano Drop spectrophotometer method

Varieties	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260/280}$	Quantity (ng/µl)
Panniyur 1	18.294	9.786	1.86	914.7
Panniyur 2	18.724	9.922	1.89	936.2
Panniyur 3	22.101	12.058	1.83	1105.05
Panniyur 4	22.878	12.434	1.84	1143.9
Panniyur 5	18.321	9.850	1.86	916.05
Panniyur 6	20.672	11.468	1.80	1033.6
Panniyur 7	22.017	12.017	1.83	1100.85

4.2 Molecular marker analysis:

The protocol for different marker assays- RAPD, ISSR and SSR were validated with bulked DNA of black pepper varieties. Different primers were screened with the genomic DNA of two selected varieties utilizing the validated protocols.

4.2.1 Random Amplified Polymorphic DNA (RAPD) analysis:

RAPD analysis with the thermal settings identified (3.3.2) gave good amplification. The amplification pattern was different corresponding to the decamer primer (OPA, OPAH, OPU and OPC) used (Plate 3a).

Based on the amplification pattern 10 decamer primers were selected out of 30 primers for RAPD assay of seven black pepper varieties and the details are provided in Table 10.

4.2.2 Inter Simple Sequences Repeat (ISSR) analysis:

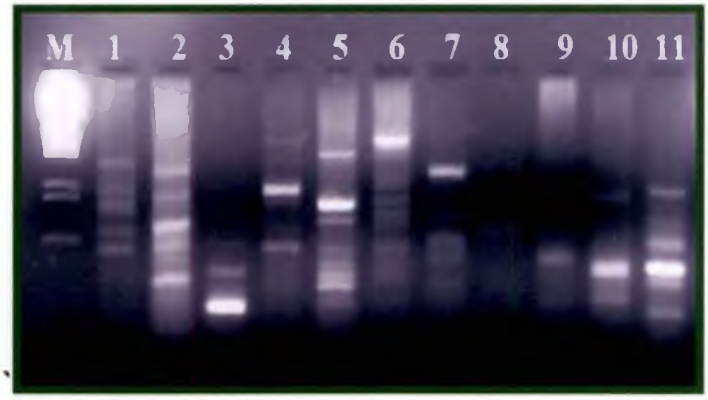
Thirty three ISSR primers used for amplification of the genomic DNA with thermal settings mentioned earlier (3.3.3) gave different amplification pattern for the two selected varieties of black pepper (Plate 3b). Based on the amplification pattern ten primers were selected for ISSR analysis of seven varieties and the details are provided in Table 11.

4.2.3 Simple Sequences Repeat (SSR) analysis:

Twenty nine primer sets were screened for amplification of SSR region in the genomic DNA of black pepper with thermal settings mentioned earlier (3.3.4). The amplification observed is presented in (Plate 3c). Based on the amplification pattern eight primer sets were selected for SSR fingerprinting of black pepper varieties and the details are provided in Table 12.

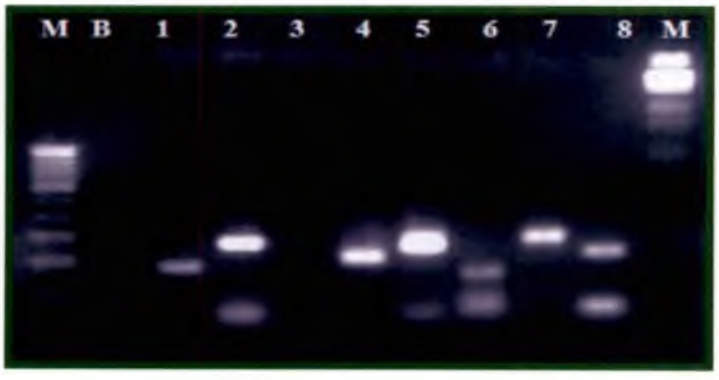
M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
B: Control
1-10: Amplification pattern with primers

a. Screening of RAPD primers for amplification of black pepper DNA



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
1-11: Amplification pattern with primers

b. Screening of ISSR primers for amplification of black pepper DNA



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
B: Control
1-8: Amplification pattern with primers

c. Screening of SSR primers for amplification of black pepper DNA

Plate 3: Screening of RAPD, ISSR and SSR primers for amplification of black pepper genomic DNA

Table 10: Details of amplification with the 30 primers screened for RAPD assay in black pepper

S.No	Primers	Amplification pattern			
		No. of Bands	type of bands		remarks
			Distinct	faint	
1	OPA10	3	1	2	---
2	OPA 11	2		2	---
3	OPA12	2		2	---
4	OPA-13	3	1	2	---
5	OPA-14	0			---
6	OPA-15	0			---
7	OPA-16	0			---
8	OPA-18	2		2	---
9	OPA-19	0			---
10	OPA-20	0			---
11	OPA22	4	2	2	---
12	OPA23	0			---
13	OPA26	0			---
14	OPA 27	3	1	2	---
15	OPC 01	0			---
16	OPU 01	4	4		Selected
17	OPU 02	5	4	1	Selected
18	OPU 03	3	3		Selected
19	OPU 09	4	3	1	Selected
20	OPU 13	4	4		Selected
21	OPP 08	6	4	2	Selected
22	OPP14	5	5		Selected
23	OPAH 06	8	7	1	Selected
24	OPA28	6	6		Selected
25	OPA 29	0			---
26	OPA30	4	2	2	Selected
27	OPA32	2		2	---
28	OPA 36	2	1	1	---
29	OPA 28	3	2	1	---
30	OPA 39	0			---

Table 11: Details of amplification with the 33 primers screened for ISSR assay in black pepper

Sl. No	Primers	Amplification pattern			
		No. of Bands	type of bands		remarks
			Distinct	faint	
1	UBC 813	2	1	1	---
2	UBC812	5	4	1	Selected
3	UBC 814	0			---
4	UBC 811	6	6	0	Selected
5	UBC 817	0			---
6	UBC 820	0			---
7	UBC 826	0			---
8	UBC 834	1		1	---
9	UBC 836	5	4	1	Selected
10	UBC 840	0			---
11	UBC 845	0			---
12	UBC 848	0			---
13	UBC 863	0			---
14	UBC 890	3	1	2	---
15	UBC 825	6	5	1	Selected
16	UBC 847	0			---
17	UBC 857	0			---
18	UBC 865	0			---
19	UBC 866	0			---
20	UBC51	4	4		Selected
21	UBC 815	0			---
22	UBC 835	6	5	1	Selected
23	UBC 843	3	2	1	Selected
24	UBC 844	0			---
25	UBC840	0			---
26	ISSR1	2	2		---
27	ISSR2	0			---
28	ISSR3	3	3		Selected
29	ISSR4	5	4	1	Selected
30	ISSR5	0			---
31	ISSR6	6	4	2	Selected
32	ISSR7	0			---
33	ISSR8	0			---
34	ISSR9	0			---

Table 12: Details of amplification pattern with the 29 primers screened for SSR assay in black pepper

Sl. No.	Primers	Amplification pattern			
		No. of Bands	type of bands		remarks
			Distinct	Faint	
1	PN A5	1	1	0	Selected
2	PN B5	2	2	0	Selected
3	PN B9	0		0	---
4	PN E3	1	1	0	Selected
5	PN F1	2	2	0	Selected
6	PN G11	2	2	0	Selected
7	PN H4	1	1	0	Selected
8	PN H8a	2	2	0	Selected
9	PN D10	1	1	0	Selected
10	Pc-b8	0	--	--	--
11	Pc-b12	0	--	--	--
12	Pc-b13	0	--	--	--
13	SSR306	0	--	--	--
14	SSR286	0	--	--	--
15	SSR557	0	--	--	--
16	SSR565	0	--	--	--
17	SSR326	0	--	--	--
18	SSR47	0	--	--	--
19	SSR72	0	--	--	--
20	SSR34	0	--	--	--
21	SSR122	0	--	--	--
22	SSR350	0	--	--	--
23	SSR578	0	--	--	--
24	SSR128	0	--	--	--
25	SSR45	0	--	--	--
26	SSR4	0	--	--	--
27	SSR296	0	--	--	--
28	SSR593	0	--	--	--
29	SSR603	0	--	--	--

4.3 DNA fingerprinting for the seven black pepper varieties

4.3.1 Panniyur 1

A. RAPD profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 1 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 4a and Table 13.

Forty eight clear distinct loci were observed with the 10 RAPD primers.The amplicons ranged in size from 200bp to 1400bp (Table13). Most of the primers amplified five distinct bands for the variety Panniyur 1 while it was only 4 for the primer OPU 01 and OPA 30. The primer OPP14 gave 6 distinct bands. Fingerprint was developed based on clear distinct bands (Fig.1a).

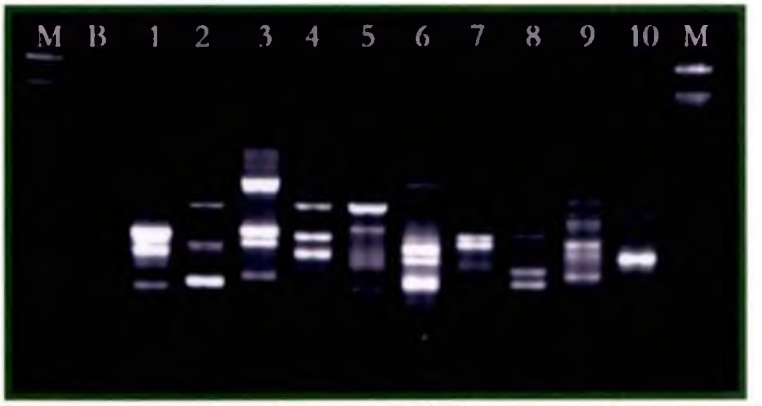
B. ISSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 1 with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 4b and Table 14.

Fifty two clear distinct loci were observed with the 10 ISSR primers.The amplicons ranged in size from 200bp to 1600bp (Table14). Most of the primers amplified five and six distinct bands for the variety Panniyur1 while it was only two for the primer UBC 843. The primer ISSR 6 gave seven distinct bands. Fingerprint was developed based on clear distinct bands (Fig.1b).

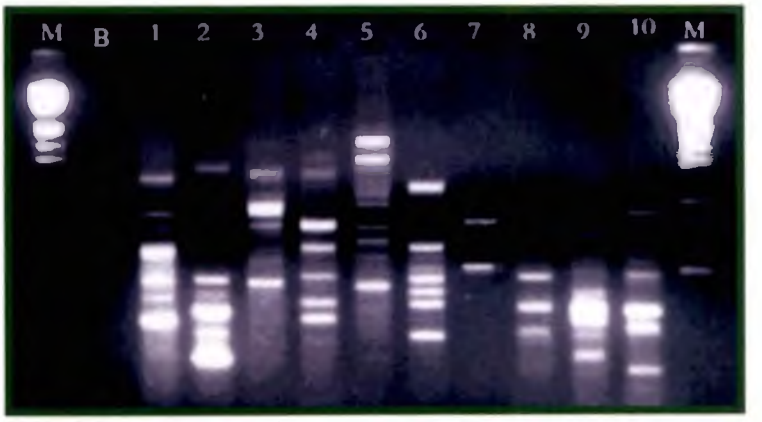
C. SSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 1 with the eight selected primers in SSR analysis is presented in Plate 4c and Table 15.



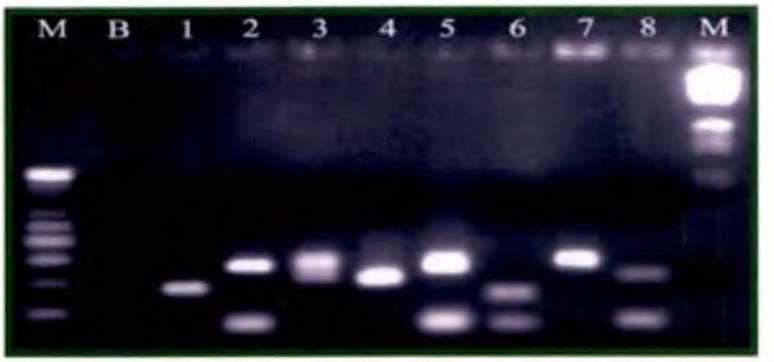
M: Marker Lambda DNA (*Eco* RI/ *Hin*d III digest), B: Control
1: OPU1, 2: OPU2, 3:OPU3, 4: OPU9, 5: OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker, B: Control,
1:UBC51, 2:UBC812, 3:UBC811, 4:UBC835, 5:UBC825, 6:UBC836, 7:UBC843, 8:ISSR3, 9:ISSR4, 10:ISSR6

b. Amplification with ISSR primers



M: 100bp ladder /Marker, B: Control
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate 4: Amplification pattern of black pepper variety Panniyur 1 with the selected RAPD, ISSR and SSR primers

Table 13: Amplification pattern depicted for the black pepper variety Panniyur 1 with the 10 selected RAPD primers

	1	2	3	4	5	6	7	8	9	10
Primers	**OPU 01**	**OPU 02**	**OPU 03**	**OPU 09**	**OPU 13**	**OPP 08**	**OPP 14**	**OPAH 06**	**OPA 28**	**OPA 30**
Total Amplicons	5	8	7	6	6	6	8	7	6	6
Distinct Amplicons	4	5	5	5	4	5	6	5	5	4
Distribution (Mol. Size/ bp)										
1400			—			—				
1300				—			—			—
1200								—		
1100		—		—	—		—		—	—
1000	—									
900								—		
800			—		—				—	
700	—	—	—	—			—	—		
600	—			—		—	—		—	—
500		—		—	—		—	—	—	
400	—	—	—		—	—		—	—	
300		—	—			—	—			—
200						—				
100										

Table 14: Amplification pattern depicted for the black pepper variety Panniyur1 with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	9	7	5	6	9	7	2	4	8	7
Distinct Amplicons	6	6	5	6	6	5	2	3	7	6
Distribution (Mol. Size/ bp)										
1600					—					
1500										
1400										
1300		—								
1200				—	—					
1100	—		—							
1000						—				
900		—	—		—		—			
800	—		—		—				—	—
700				—	—		—		—	
600	—			—		—			—	—
500	—	—	—	—	—	—		—		—
400	—	—		—		—		—	—	—
300	—	—		—		—		—	—	—
200		—							—	—
100										

Table 15: Amplification pattern depicted for the black pepper variety Panniyur 1 with the 8 selected SSR primers

	1	2	3	4	5	6	7	8
Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	2	2	1	2
Distinct Amplicons	1	2	2	1	2	2	1	2
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100		—			—	—		—

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1600										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

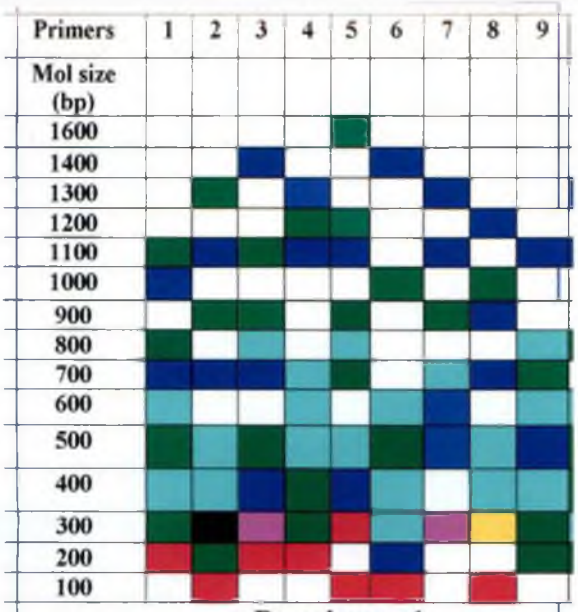
b. ISSR Fingerprint

Primers	1	2	3	4	5	6	7	8
Mol size (bp)								
300								
200								
100								

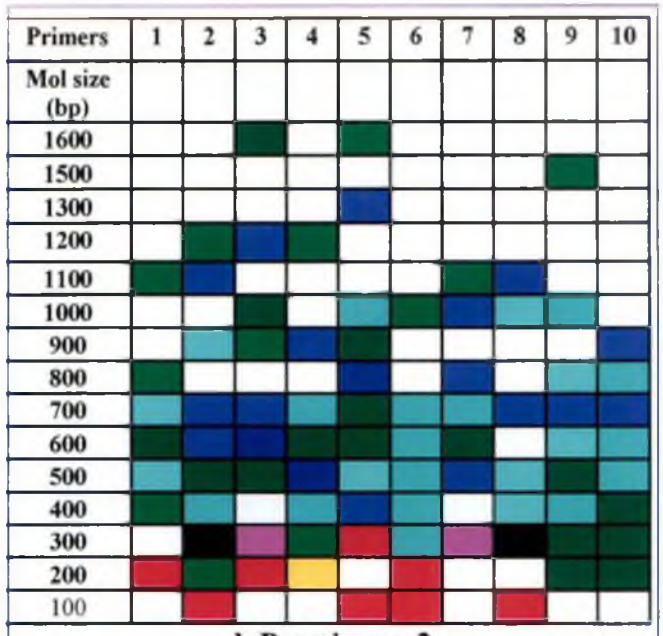
c. SSR Fingerprint

Colour code for sharing of band among varieties

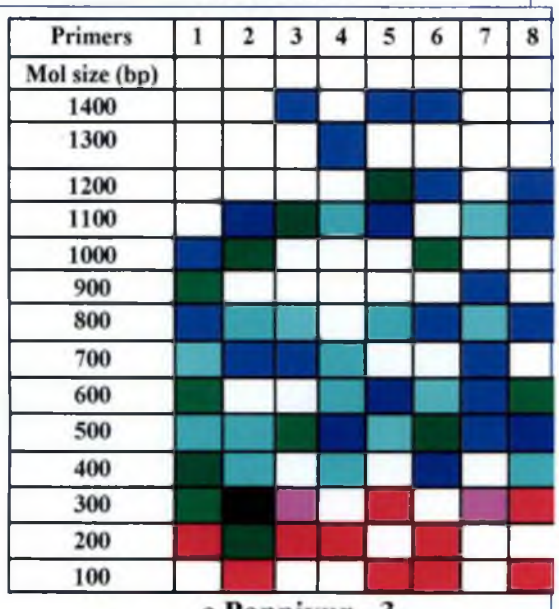
Fig.1: Fingerprint for variety Panniyur 1 through RAPD, ISSR and SSR analysis



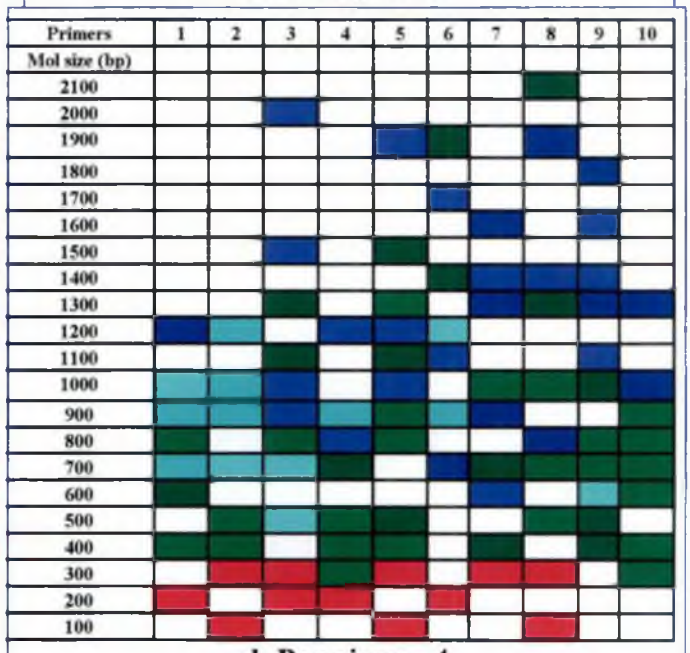
a.Panniyur - 1



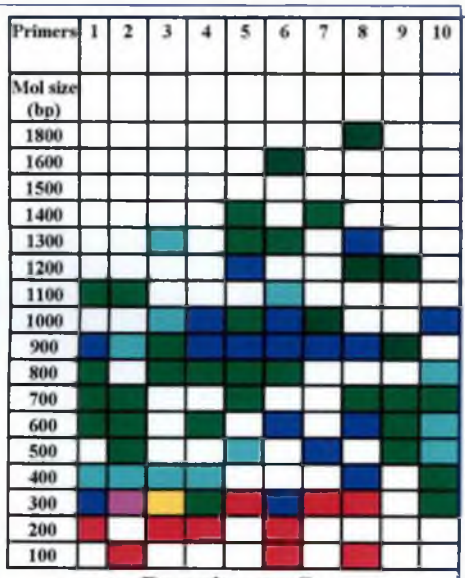
b.Panniyur - 2



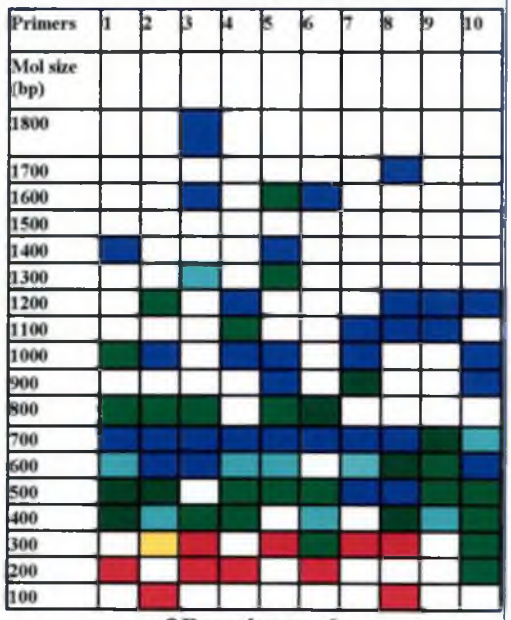
c.Panniyur - 3



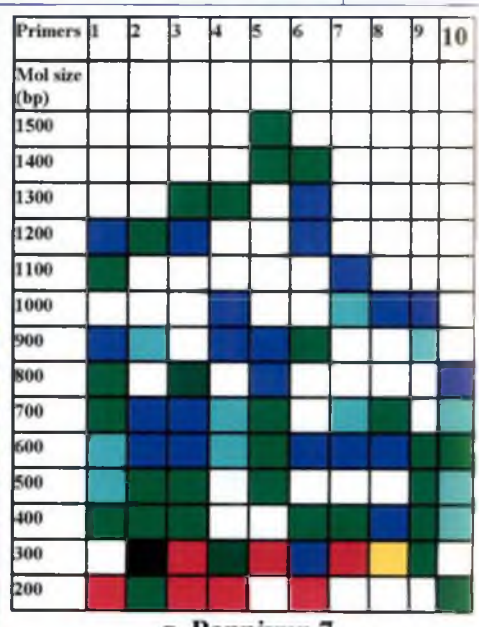
d. Panniyur - 4



e. Panniyur - 5



f.Panniyur - 6



g. Panniyur-7

RAPD ISSR SSR RAPD+ISSR
RAPD +ISSR+SSR ISSR+SSR RAPD+SSR

Fig. 8.Combined finger print of seven black pepper varieties

Thirteen clear distinct loci were observed with the eight SSR primers (Table 15). The amplicons ranged in size from 100bp to 300bp. Fingerprint was developed based on clear distinct bands (Fig. 1c). DNA fingerprint for variety Panniyur 1 through combined RAPD, ISSR and SSR analysis is provided in Fig. 8a.

4.3.2 Panniyur 2

A. RAPD profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 2 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 5a and Table 16.

Forty eight clear distinct loci were observed with the 10 RAPD primers. The amplicons ranged in size from 300bp to 1300bp (Table16). Most of the primers amplified five distinct bands for the variety Panniyur 2 while it was only three for the primer OPU 01. The primer OPU 02 and OPAH 09 gave six distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 2a).

B. ISSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 2 with the 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 5b and Table 17.

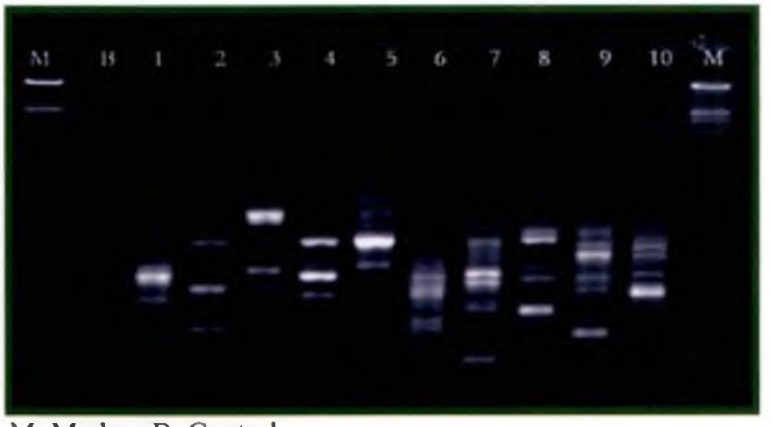
Fifty three clear distinct loci were observed with the 10 ISSR primers. The amplicons ranged in size from 200bp to 1700bp (Table17). Most of the primers amplified five distinct bands for the variety Panniyur 2 while it was only three for the primer UBC 843 and ISSR 3. The primer UBC825 gave seven distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 2b).

C. SSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 2 with the 8 selected primers in SSR analysis is presented in Plate 5c and Table 18.

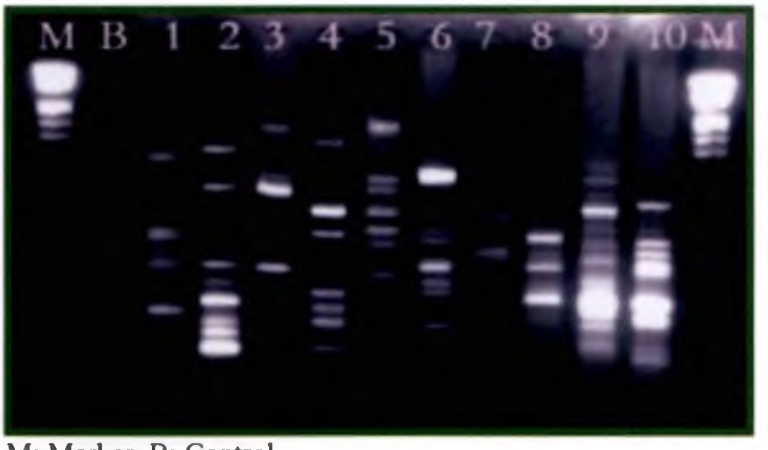
Thirteen clear distinct loci were observed with the eight SSR primers (Table 18). The amplicons ranged in size from 100bp to 300bp. Fingerprint was developed based on clear distinct bands (Fig. 2c).

DNA fingerprint for variety Panniyur 2 through combined RAPD, ISSR and SSR analysis is provided in plate Fig. 8b.



M: Marker, B: Control
1: OPU1, 2: OPU2, 3:OPU3, 4: OPU9, 5: OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker, B: Control
1:UBC51, 2:UBC812, 3:UBC811, 4:UBC835, 5:UBC825, 6:UBC836, 7:UBC843, 8:ISSR3, 9:ISSR4, 10:ISSR6

b. Amplification with ISSR primers

M: 100bp ladder /Marker, B: Control
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate 5: Amplification pattern of black pepper variety Panniyur 2 with the selected RAPD, ISSR and SSR primers

Table 16: Amplification pattern depicted for the black pepper variety Panniyur2 with the 10 selected RAPD primers

	1	2	3	4	5	6	7	8	9	10
Primers	OPU 01	OPU 02	OPU 03	OPU 09	OPU 13	OPP 08	OPP 14	OPAH06	OPA 28	OPA 30
Total Amplicons	7	7	5	6	7	8	7	9	7	7
Distinct Amplicons	3	6	4	4	5	5	5	6	**5**	**5**
Distribution (Mol. Size/ bp)										
1300					—					
1200			—							
1100		—						—		
1000					—		—	—	—	
900	—	—		—						—
800					—		—		—	—
700	—	—	—	—		—	—	—	—	—
600		—	—			—			—	—
500	—			—	—	—	—	—		—
400		—		—	—	—		—	—	
300		—	—			—	—	—		

Table 17: Amplification pattern depicted for the black pepper variety Panniyur2 with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	8	8	6	7	9	9	3	4	9	8
Distinct Amplicons	5	5	5	5	7	6	3	3	8	6
Distribution (Mol. Size/ bp)										
1600			—		—					
1500									—	
1400										
1300										
1200		—		—						
1100	—						—			
1000			—		—	—			—	
900		—	—		—					
800	—								—	—
700	—			—	—	—	—			
600	—			—	—	—	—	—	—	—
500	—	—	—		—	—		—	—	—
400	—	—		—		—		—	—	—
300		—		—		—			—	—
200		—		—					—	—
100										

Table 18: Amplification pattern depicted for the black pepper variety Panniyur 2 with the 8 selected SSR primers

Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	1	2	1	2
Distinct Amplicons	1	2	2	1	1	2	1	2
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100		—				—		—

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1700										
1600										
1500										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

b. ISSR Fingerprint

Primers	1	2	3	4	5	6	7	8
Mol size (bp)								
300								
200								
100								

c. SSR Fingerprint

Colour code for sharing of band among varieties

Fig.2: Fingerprint for variety Panniyur 2 through RAPD, ISSR and SSR analysis

4.3.3 Panniyur 3

A. RAPD profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 3 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 6a and Table 19.

Fifty two clear distinct loci were observed with the 10 RAPD primers. The amplicons ranged in size from 300bp to 1400bp (Table 19). Most of the primers amplified five and six distinct bands for the variety Panniyur 3 while it was only four for the primer OPU 01, OPU 03 and OPA 28. The primer OPP 14 gave seven distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 3a).

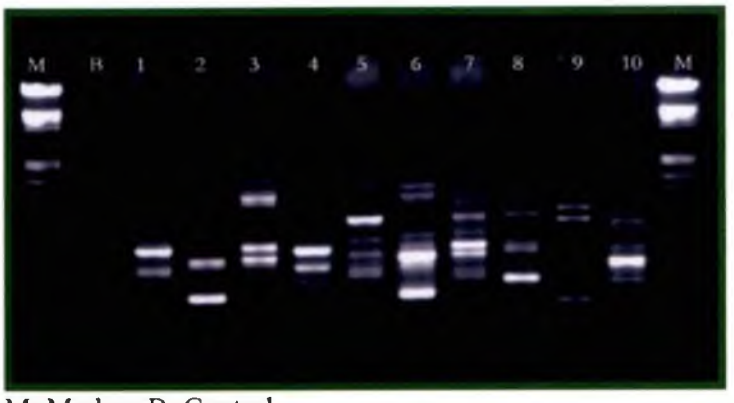
B. ISSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 3 with 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 6b and Table 20.

Thirty six clear distinct loci were observed with the 10 ISSR primers. The amplicons ranged in size from 200bp to 1200bp (Table 20). Most of the primers amplified three and five distinct bands for the variety Panniyur 3 while it was only two for the primer UBC 843 and ISSR 3. The primer UBC 812 gave six distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 3b).

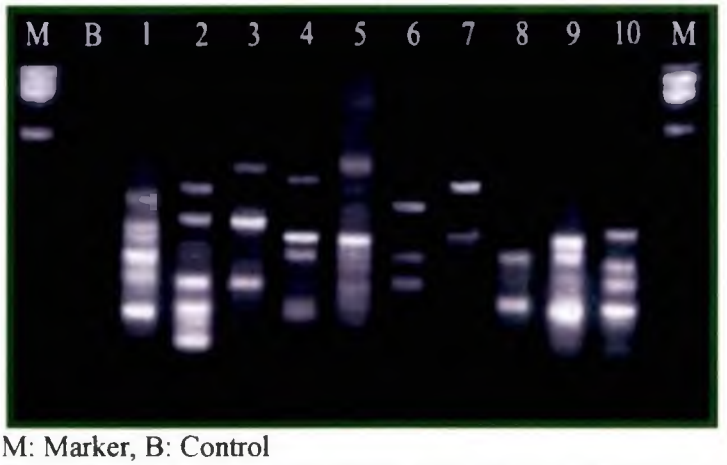
C. SSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 3 with the 8 selected primers in SSR analysis with the thermal settings identified is presented in Plate 6c and Table 21.



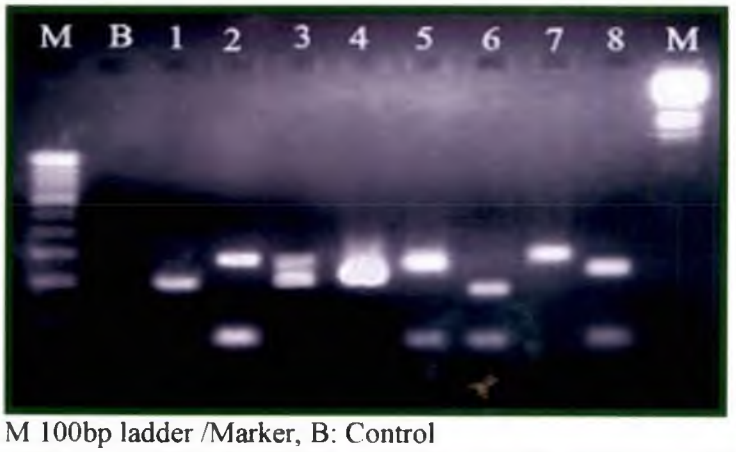
M: Marker, B: Control
1: OPU1, 2:OPU2, 3:OPU3, 4:OPU9, 5: OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker, B: Control
1:UBC51, 2:UBC812, 3:UBC811, 4:UBC835, 5:UBC825, 6:UBC836, 7:UBC843, 8:ISSR3, 9:ISSR4, 10:ISSR6

b. Amplification with ISSR primers



M 100bp ladder /Marker, B: Control
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate 6: Amplification pattern of black pepper variety Panniyur 3 with the selected RAPD, ISSR and SSR primers

Table 19: Amplification pattern depicted for the black pepper variety Panniyur 3 with the 10 selected RAPD primers

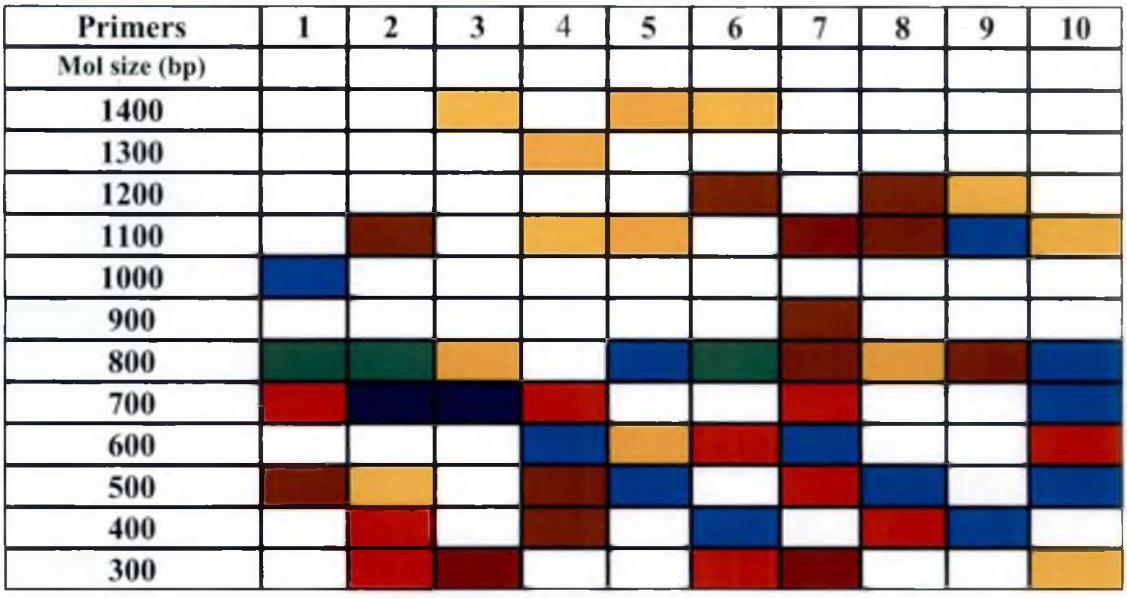
	1	2	3	4	5	6	7	8	9	10
Primers	OPU 01	OPU 02	OPU 03	OPU 09	OPU 13	OPP 08	OPP 14	OPAH06	OPA 28	OPA 30
Total Amplicons	6	8	7	7	7	8	9	7	5	7
Distinct Amplicons	4	6	4	6	5	5	7	5	4	6
Distribution (Mol. Size/ bp)										
1400			—		—	—				
1300				—						
1200						—		—	—	
1100		—		—	—		—	—	—	—
1000	—									
900							—			
800	—	—	—		—	—	—	—	—	—
700	—	—	—	—			—			—
600				—	—	—	—			—
500	—	—		—	—		—	—		—
400		—		—		—		—	—	
300		—	—				—			—

Table 20: Amplification pattern depicted for the black pepper variety Panniyur3 with the 10 selected ISSR primers

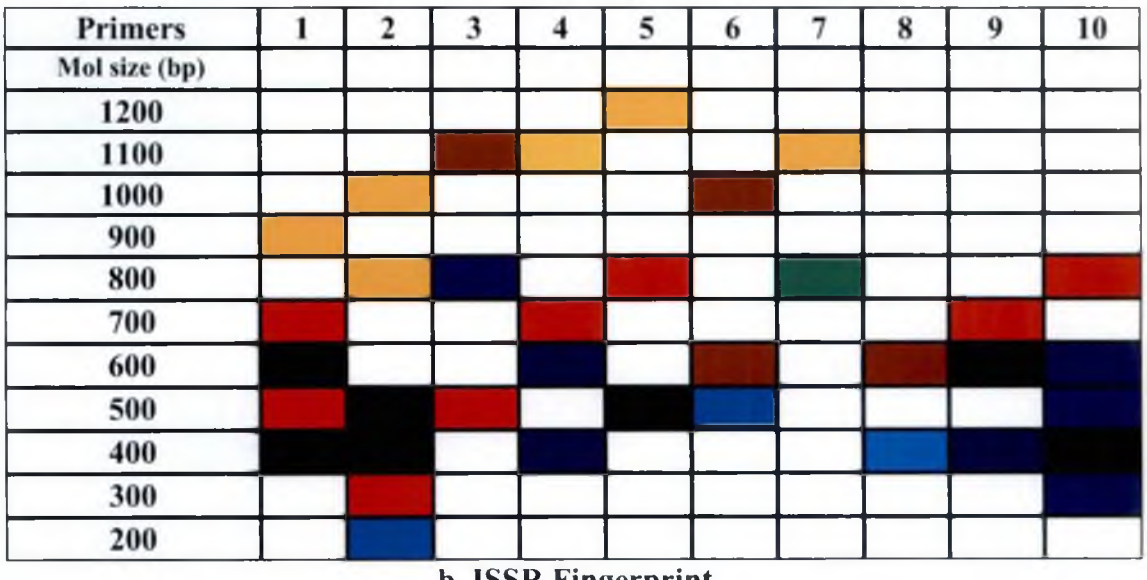
	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	7	7	4	7	9	7	3	4	6	6
Distinct Amplicons	5	6	3	4	3	3	2	2	3	5
Distribution (Mol. Size/ bp)										
1200					—					
1100			—	—			—			
1000		—				—				
900	—									
800		—	—		—		—			—
700	—			—					—	
600	—			—		—		—	—	—
500	—	—	—		—	—				—
400	—	—		—				—	—	—
300		—								—
200		—								

Table 21: Amplification pattern depicted for the black pepper variety Panniyur3 with the 8 selected SSR primers

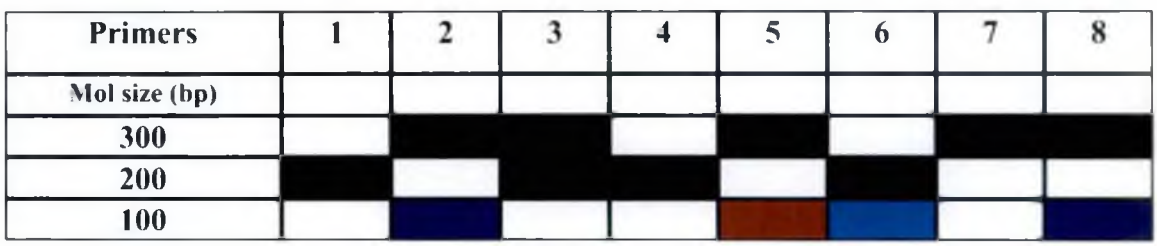
	1	2	3	4	5	6	7	8
Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	2	2	1	2
Distinct Amplicons	1	2	2	1	2	2	1	2
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100		—			—	—		—



a. RAPD Fingerprint



b. ISSR Fingerprint



c. SSR Fingerprint

Colour code for sharing of band among varieties

Fig.3: Fingerprint for variety Panniyur 3 through RAPD, ISSR and SSR analysis

Thirteen clear distinct loci were observed with the eight SSR primers (Table 21). The amplicons ranged in size from 100bp to 300bp. Fingerprint was developed based on clear distinct bands (Fig. 3c).

DNA fingerprint for variety Panniyur3 through combined RAPD, ISSR and SSR analysis is provided in Fig. 8c.

4.3.4 Panniyur 4

A. RAPD profile

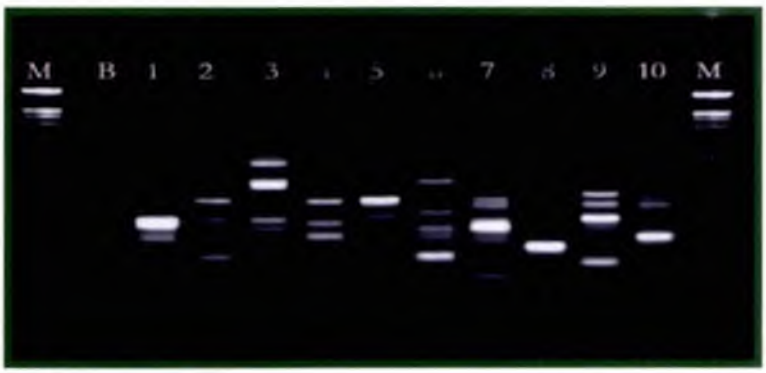
The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 4 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 7a and Table 22.

Forty two clear distinct loci were observed with the 10 RAPD primers. The amplicons ranged in size from 500bp to 2000bp (Table 22). Most of the primers amplified four distinct bands for the variety Panniyur 4 while it was only two for the primer OPA 30. The primer OPU 03 and OPA 28 gave six distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 4a).

B. ISSR profile

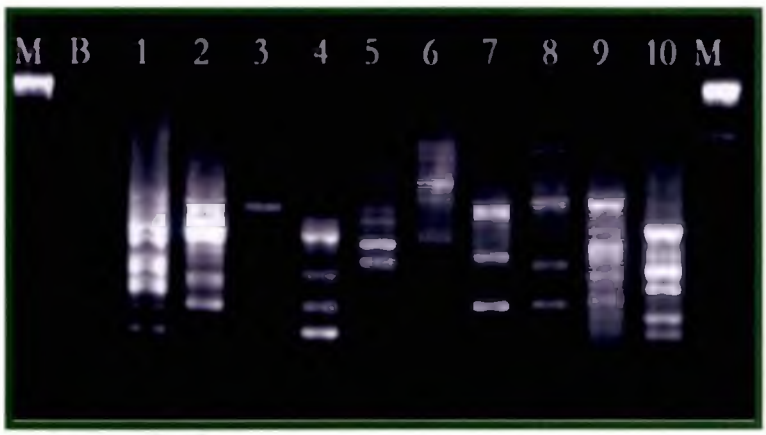
The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 4 with 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 7b and Table 23.

Fifty three clear distinct loci were observed with the 10 ISSR primers. The amplicons ranged in size from 300bp to 2100bp (Table 23). Most of the



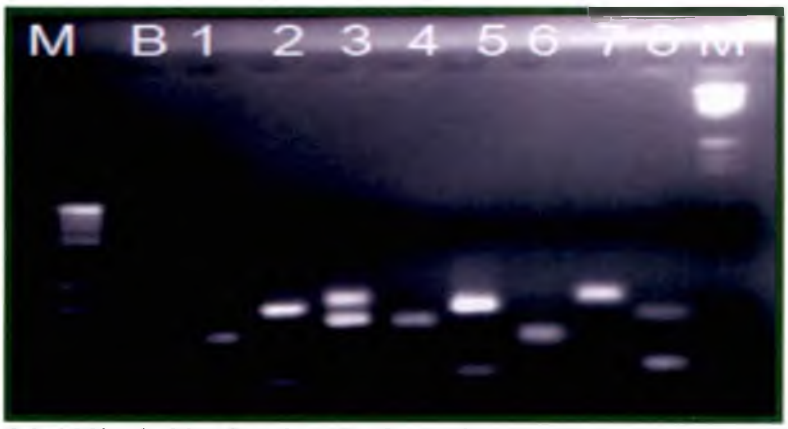
M: Marker, B: Control
1: OPU1, 2: OPU2, 3:OPU3, 4: OPU9, 5: OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker B: Control
1:UBC 51, 2:UBC 812, 3:UBC 811, 4:UBC 835, 5:UBC 825, 6:UBC 836, 7:UBC 843, 8:ISSR 3, 9:ISSR 4, 10:ISSR 6

b. Amplification with ISSR primers



M: 100bp ladder /Marker, B: Control
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate 7: Amplification pattern of black pepper variety Panniyur 4 with selected RAPD, ISSR and SSR primers

Table 22: Amplification pattern depicted for the black pepper variety Panniyur4 with the 10 selected RAPD primers

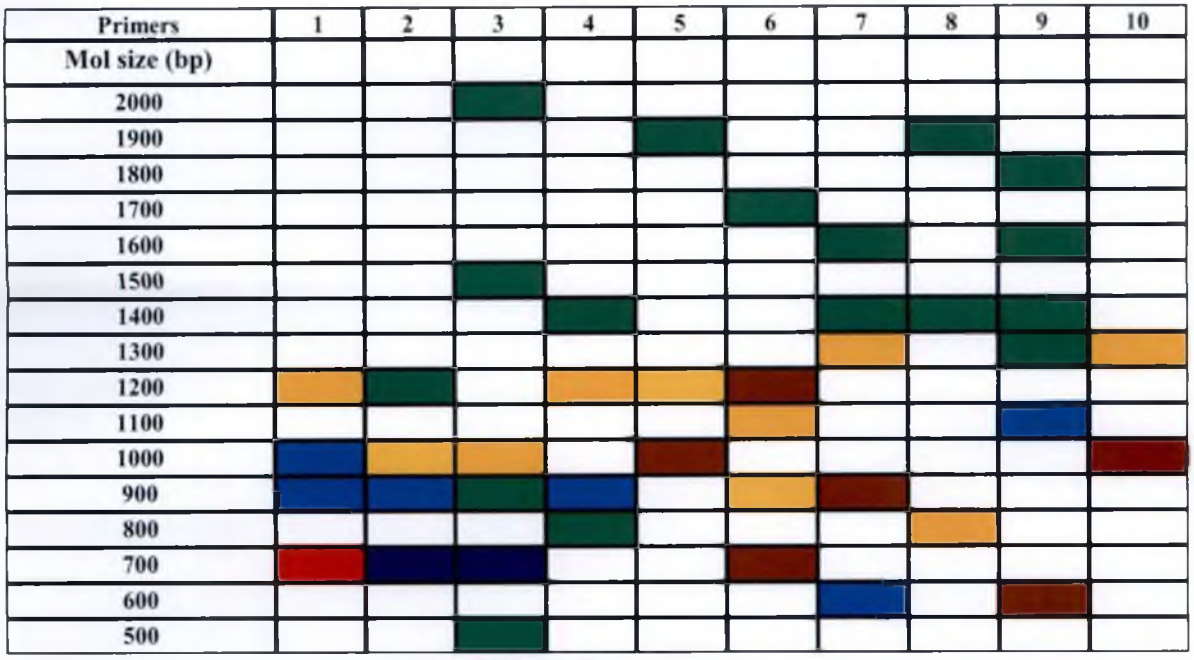
Primers	OPU 01	OPU 02	OPU 03	OPU 09	OPU 13	OPP 08	OPP 14	OPAH06	OPA 28	OPA 30
Total Amplicons	4	5	6	5	6	4	5	7	6	6
Distinct Amplicons	4	4	6	4	3	5	5	3	6	2
Distribution (Mol. Size/ bp)										
2000			—							
1900					—			—		
1800									—	
1700						—				
1600							—		—	
1500			—							
1400				—			—	—	—	
1300							—		—	—
1200	—	—		—	—	—				
1100						—			—	
1000	—	—	—		—					—
900	—	—	—	—		—	—			
800				—				—		
700	—	—	—			—				
600									—	
500			—				—			
400										
300										
200										
100										

Table 23: Amplification pattern depicted for the black pepper variety Panniyur4 with the 10 selected ISSR primers

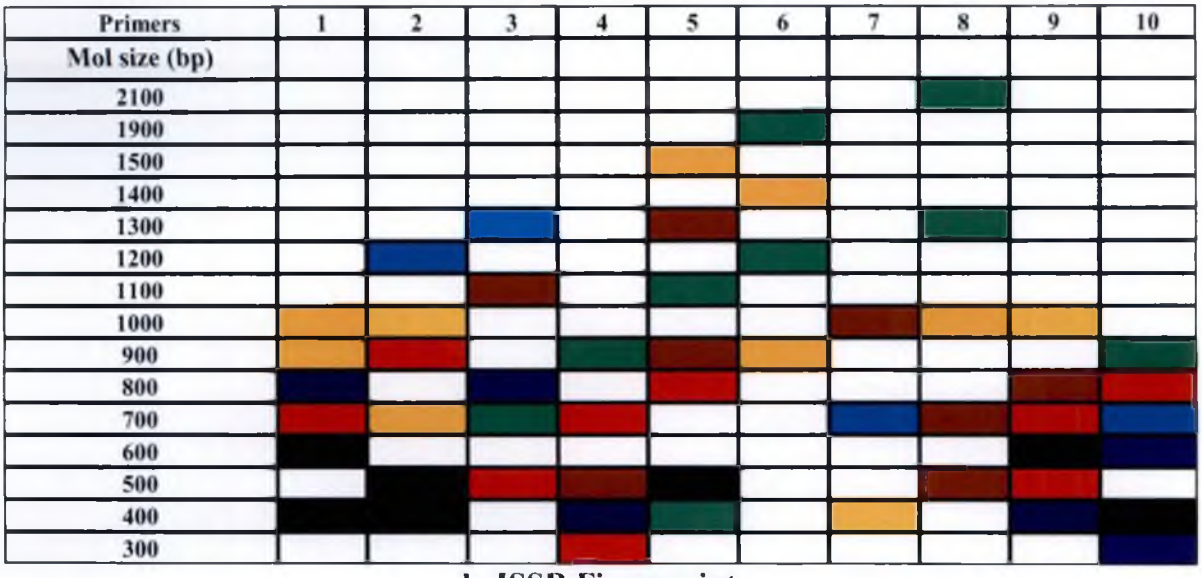
	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	8	9	6	6	7	7	3	8	9	7
Distinct Amplicons	6	6	5	5	7	4	3	5	7	6
Distribution (Mol. Size/ bp)										
2100								—		
2000										
1900						—				
1800										
1700										
1600										
1500					—					
1400						—				
1300			—		—			—		
1200		—				—				
1100			—		—					
1000	—	—					—	—	—	
900	—	—		—	—	—				—
800	—		—		—				—	—
700	—	—	—	—			—	—	—	—
600	—								—	—
500		—	—	—	—			—	—	
400	—	—		—	—		—		—	—
300				—						—
200										

Table 24: Amplification pattern depicted for the black pepper variety Panniyur4 with the 8 selected SSR primers

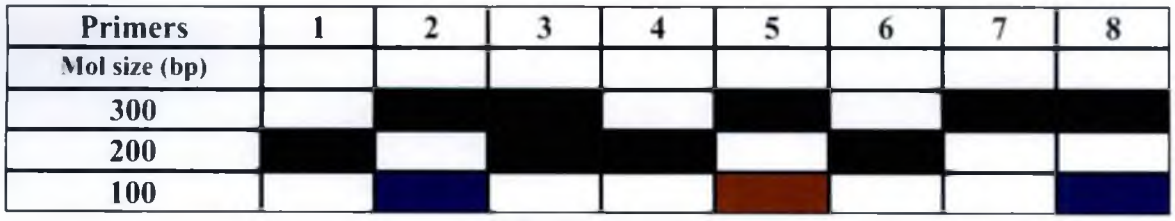
	1	2	3	4	5	6	7	8
Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	2	1	1	2
Distinct Amplicons	1	2	2	1	2	1	1	2
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100		—			—			—



a. RAPD Fingerprint



b. ISSR Fingerprint



c. SSR Fingerprint

Colour code for sharing of band among varieties 

Fig.4: Fingerprint for variety Panniyur 4 through RAPD, ISSR and SSR analysis

primers amplified five and six distinct bands for the variety Panniyur 4 while it was only three for the primer UBC 843. The primer UBC 825 gave seven distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 4b).

C. SSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 4 with the 8 selected primers in SSR analysis with the thermal settings identified is presented in Plate 7c and Table 24.

Thirteen clear distinct loci were observed with the eight SSR primers (table 24). The amplicons ranged in size from 100bp to 300bp. Fingerprint was developed based on clear distinct bands (Fig. 4c).

DNA fingerprint for variety Panniyur 4 through combined RAPD, ISSR and SSR analysis is provided in Fig. 8d.

4.3.5 Panniyur 5

A. RAPD profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 5 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 8a and Table 25.

Thirty one clear distinct loci were observed with the 10 RAPD primers. The amplicons ranged in size from 200bp to 1300bp (Table 25). Most of the primers amplified three distinct bands for the variety Panniyur 5 while it was only two for the primer OPP 14. The primer OPP 08 gave seven distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 5a).

B. ISSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 5 with 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 8b and Table 26.

Forty seven clear distinct loci observed with the 10 ISSR primers. The amplicons ranged in size from 300bp to 1800bp (Table 26). Most of the primers amplified five distinct bands for the variety Panniyur 5 while it was only two for the primer UBC 843. The primer UBC 811, UBC 812, UBC 825 and ISSR 6 gave six distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 5b).

C. SSR profile

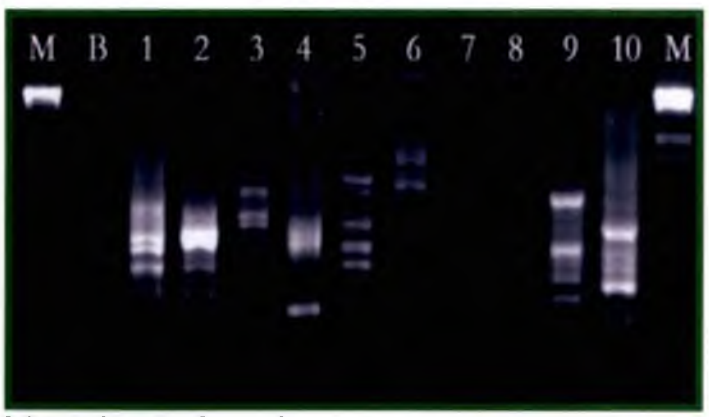
The amplification pattern observed for the genomic DNA of black pepper variety Panniyur5 with the 8 selected primers in SSR analysis with the thermal settings identified is presented in Plate 8c and Table 27.

Thirteen clear distinct loci were observed with the eight SSR primers (Table 27). The amplicons ranged in size from 100bp to 300bp. Fingerprint was developed based on clear distinct bands (Fig. 5c).

DNA fingerprint for variety Panniyur 5 through combined RAPD, ISSR and SSR analysis is provided in Fig. 8e.

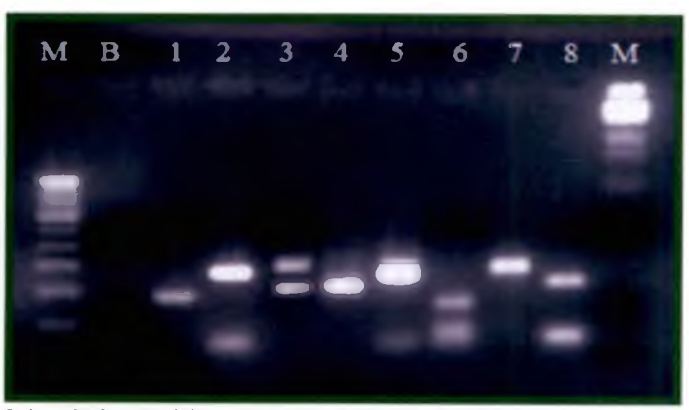
M: Marker, B: Control
1: OPU1, 2: OPU2, 3:OPU3, 4: OPU9, 5: OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker, B: Control
1:UBC51, 2:UBC812, 3:UBC811, 4:UBC835, 5:UBC825, 6:UBC836, 7:UBC843, 8:ISSR3, 9:ISSR4, 10:ISSR6

b. Amplification with ISSR primers



M: 100bp ladder /Marker, B: Control
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate 8: Amplification pattern of black pepper variety Panniyur 5 with selected RAPD, ISSR and SSR primers

Table 25: Amplification pattern depicted for the black pepper variety Panniyur 5 with the 10 selected RAPD primers

	1	2	3	4	5	6	7	8	9	10
Primers	OPU 01	OPU 02	OPU 03	OPU 09	OPU 13	OPP 08	OPP 14	OPAH 06	OPA 28	OPA 30
Total Amplicons	4	5	4	4	4	7	5	5		5
Distinct Amplicons	3	4	3	3	3	6	2	4	0	4
Distribution (Mol. Size/ bp)										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

Table 26: Amplification pattern depicted for the black pepper variety Panniyur 5 with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	7	7	6	7	6	4	3	4	8	7
Distinct Amplicons	5	6	6	4	6	4	2	3	5	6
Distribution (Mol. Size/ bp)										
1800										
1700										
1600										
1500										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										

Table 27: Amplification pattern depicted for the black pepper variety Panniyur 5 with the 8 selected SSR primers

	1	2	3	4	5	6	7	8
Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	1	2	1	1
Distinct Amplicons	1	2	2	1	1	2	1	1
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100		—				—		—

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1800										
1600										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										

b. ISSR Fingerprint

Primers	1	2	3	4	5	6	7	8
Mol size (bp)								
300								
200								
100								

c. SSR Fingerprint

Colour code for sharing of band among varieties

Fig.5: Fingerprint for variety Panniyur 5 through RAPD, ISSR and SSR analysis

4.3.6 Panniyur 6

A. RAPD profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 6 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 9a and Table 28.

Forty three clear distinct loci were observed with the 10 RAPD primers. The amplicons ranged in size from 400bp to 1800bp (Table 28). Most of the primers amplified four distinct bands for the variety Panniyur 6 while it was only three for the primer OPP 08 and OPA 28. The primer OPU 03, OPU 13, OPP 14, OPAH 06 andOPA 30 gave five distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 6a).

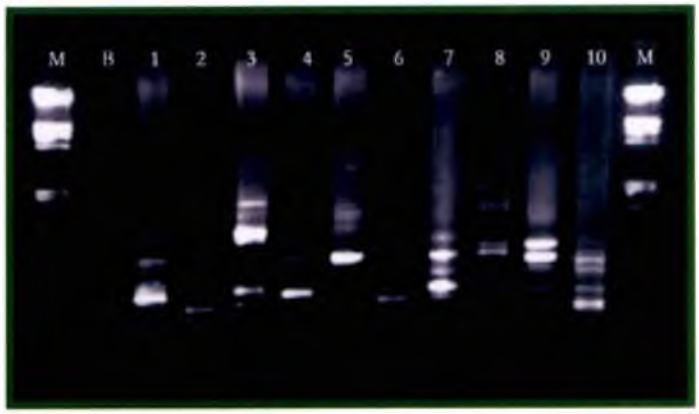
B. ISSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 6 with 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 9b and Table 29.

Thirty nine clear distinct loci observed with the 10 ISSR primers. The amplicons ranged in size from 200bp to 1600bp (Table 29). Most of the primers amplified four distinct bands for the variety Panniyur 6 while it was only two for the primer UBC 843 and ISSR 3. The primer UBC 51, UBC 812, UBC 825 and ISSR 6 gave five distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 6b).

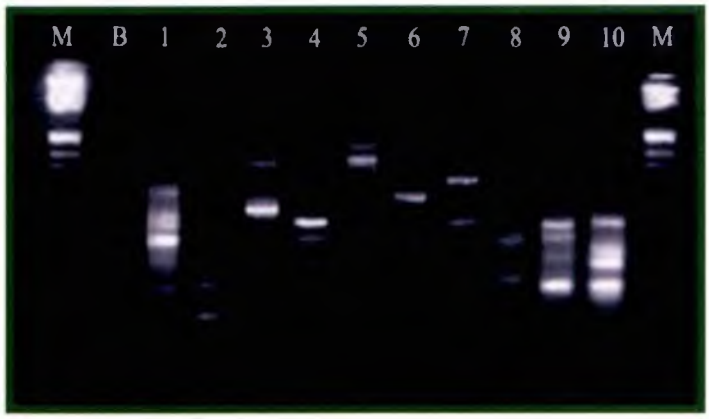
C. SSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 6 with the 8 selected primers in SSR analysis with the thermal settings identified is presented in Plate 9c and Table 30.



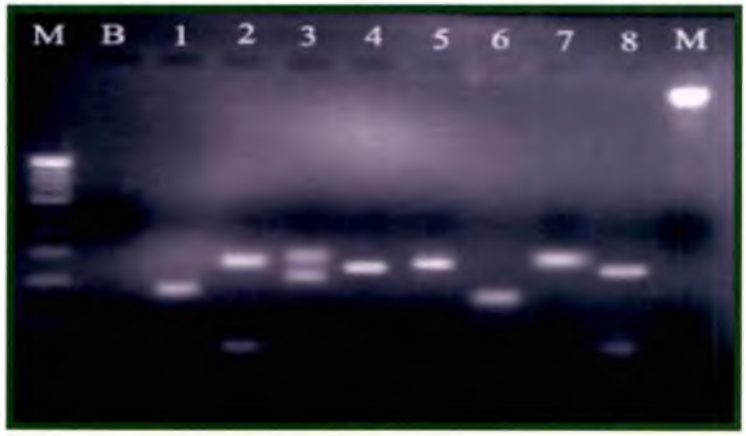
M: Marker, B: Control,
1: OPU1, 2: OPU2, 3:OPU3, 4: OPU9, 5: OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker, B: Control,
1:UBC51, 2:UBC812, 3:UBC811, 4:UBC835, 5:UBC825, 6:UBC836, 7:UBC843, 8:ISSR3, 9:ISSR4, 10:ISSR6

b. Amplification with ISSR primers



M: 100bp ladder /Marker, B: Control,
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate 9: Amplification pattern of black pepper variety Panniyur 6 with selected RAPD, ISSR and SSR primers

Table 28: Amplification pattern depicted for the black pepper variety Panniyur 6 with the 10 selected RAPD primers

	1	2	3	4	5	6	7	8	9	10
Primers	OPU 01	OPU 02	OPU 03	OPU 09	OPU 13	OPP 08	OPP 14	OPAH 06	OPA 28	OPA 30
Total Amplicons	5	5	5	4	7	3	6	6	6	5
Distinct Amplicons	4	4	5	4	5	3	5	5	3	5
Distribution (Mol. Size/ bp)										
1800			—							
1700								—		
1600			—			—				
1500										
1400	—				—					
1300			—							
1200				—			—	—	—	—
1100								—	—	
1000	—	—		—	—		—			—
900					—					—
800							—			
700	—	—	—	—	—	—	—	—		—
600	—	—	—	—	—					—
500							—	—		
400		—				—			—	
300										
200										

Table 29: Amplification pattern depicted for the black pepper variety Panniyur6 with the 10 selected ISSR primers

	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	7	6	4	6	9	5	2	3	8	5
Distinct Amplicons	5	5	3	4	5	4	2	2	4	5
Distribution (Mol. Size/ bp)										
1600					—					
1500										
1400										
1300			—		—					
1200		—								
1100				—						
1000	—									
900							—			
800	—	—	—		—	—				
700									—	—
600	—			—	—		—	—	—	
500	—	—		—	—	—			—	—
400	—	—	—	—		—		—	—	—
300		—				—				—
200										—
100										

Table 30: Amplification pattern depicted for the black pepper variety Panniyur6 with the 8 selected SSR primers

	1	2	3	4	5	6	7	8
Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	1	2	1	2
Distinct Amplicons	1	2	2	1	1	1	1	2
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100		—						—

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1800										
1700										
1600										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10
Mol size (bp)										
1600										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

b. ISSR Fingerprint

Primers	1	2	3	4	5	6	7	8
Mol size (bp)								
300								
200								
100								

c. SSR Fingerprint

Colour code for sharing of band among varieties

Fig.6: Fingerprint for variety Panniyur 6 through RAPD, ISSR and SSR analysis

Twelve clear distinct loci were observed with the eight SSR primers (Table 30). The amplicons ranged in size from 100bp to 300bp. Fingerprint was developed based on clear distinct bands (Fig. 6c).

DNA fingerprint for variety Panniyur 6 through combined RAPD, ISSR and SSR analysis is provided in fig. Fig.8f

4.3.7 Panniyur 7

A. RAPD profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 7 with the 10 selected primers in RAPD analysis with the thermal settings identified is presented in Plate 10a and Table 31.

Thirty three clear distinct loci were observed with the 10 RAPD primers. The amplicons ranged in size from 300bp to 1300bp (Table. 31). Most of the primers amplified three distinct bands for the variety Panniyur 7 while it was only two for the primer OPU 13 and OPA 28. The primer OPU 01, OPU 02, OPU 09, OPP 14 and OPA 30 gave four distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 7a).

B. ISSR profile

The amplification pattern observed for the genomic DNA of black pepper variety Panniyur 7 with 10 selected primers in ISSR analysis with the thermal settings identified is presented in Plate 10b and Table 32.

Forty four clear distinct loci observed with the 10 ISSR primers. The amplicons ranged in size from 200bp to 1500bp (Table 33). Most of the primers amplified four and five distinct bands for the variety Panniyur 7 while it was only three for the primer UBC 836, UBC 843, ISSR 3. The primer UBC 51 and UBC812 gave six distinct bands. Fingerprint was developed based on clear distinct bands (Fig. 7b).

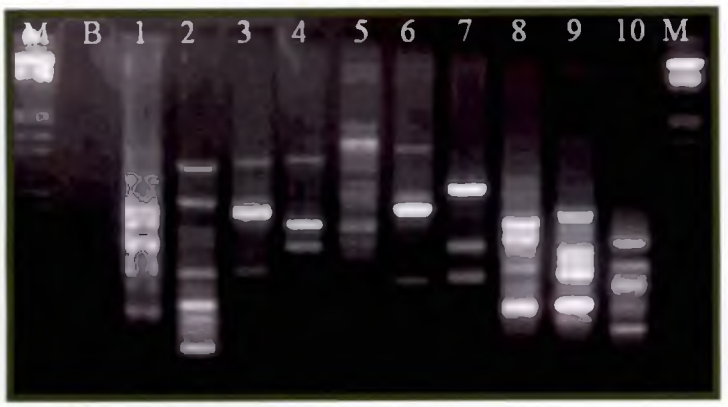
C. SSR profile

A total of ten clear distinct amplification sites were observed for the genomic DNA of black pepper variety Panniyur 7 by using eight primers (Plate 10c and Table 33). The size of these amplified products ranged from 100 to 300 bp and was evenly distributed. Fingerprint was developed based on clear distinct bands (Fig. 7c).

DNA fingerprint for variety Panniyur 7 through combined RAPD, ISSR and SSR analysis is provided in Fig. 8g

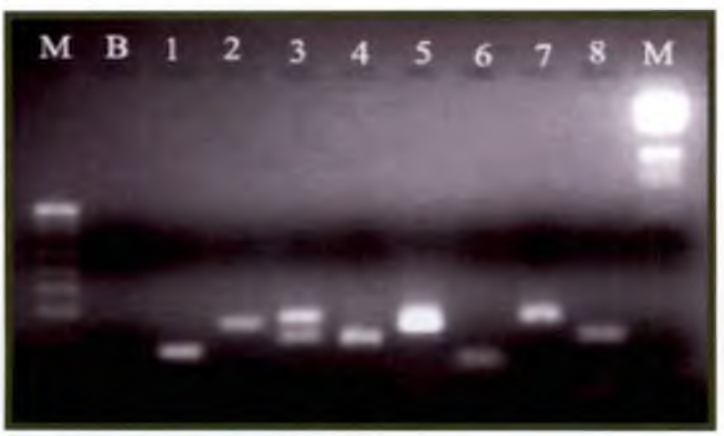
M: Marker, B: Control,
1: OPU1, 2: OPU2, 3:OPU3, 4: OPU9, 5:OPU13, 6:OPP08, 7:OPP14, 8:OPAH06, 9:OPA28, 10:OPA30

a. Amplification with RAPD primers



M: Marker, B: Control,
1:UBC51, 2:UBC812, 3:UBC811, 4:UBC835, 5:UBC825, 6:UBC836, 7:UBC843, 8:ISSR3, 9:ISSR4, 10:ISSR6

b. Amplification with ISSR primers



M: 100bp ladder /Marker, B: Control,
1:PNA5, 2:PNB5, 3:PND10, 4:PNE3, 5:PNF1, 6:PNG11, 7:PNH4, 8:PNH8a

c. Amplification with SSR primers

Plate.10: Amplification pattern of black pepper variety Panniyur 7 with selected RAPD, ISSR and SSR primers

Table 31: Amplification pattern depicted for the black pepper variety Panniyur7 with the 10 selected RAPD primers

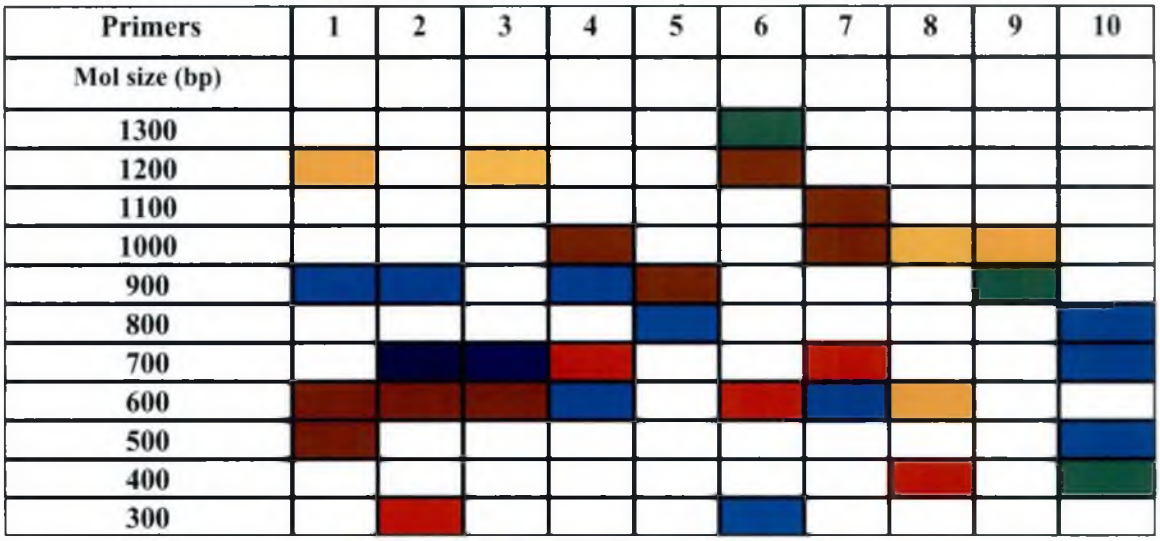
	1	2	3	4	5	6	7	8	9	10
Primers	OPU 01	OPU 02	OPU 03	OPU 09	OPU 13	OPP 08	OPP 14	OPAH 06	OPA 28	OPA 30
Total Amplicons	4	5	5	5	7	6	4	6	6	6
Distinct Amplicons	4	4	3	4	2	3	4	3	2	4
Distribution (Mol. Size/ bp)										
1300						—				
1200	—		—			—				
1100							—			
1000				—			—	—	—	
900	—	—		—	—				—	
800					—					—
700		—	—	—			—			—
600	—	—	—	—		—	—	—		
500	—									—
400								—		—
300		—				—				
200										
100										

Table 32: Amplification pattern depicted for the black pepper variety Panniyur7 with the 10 selected ISSR primers

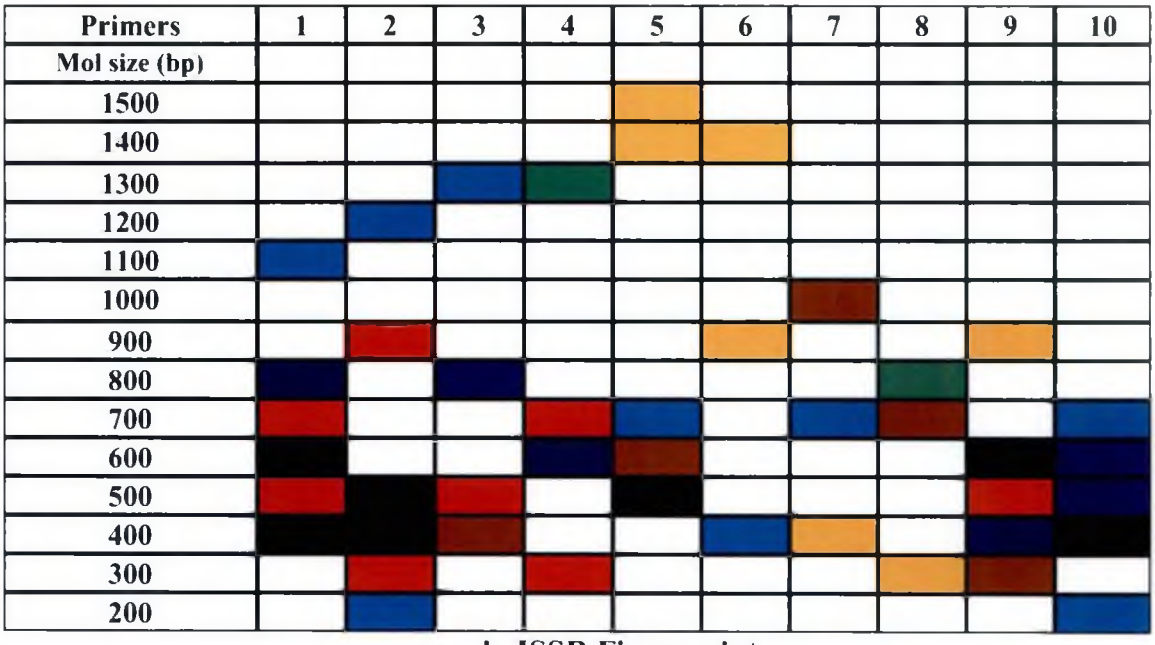
	1	2	3	4	5	6	7	8	9	10
Primers	UBC 51	UBC 812	UBC 811	UBC 835	UBC 825	UBC 836	UBC 843	ISSR 3	ISSR 4	ISSR 6
Total Amplicons	7	8	6	6	7	4	4	5	7	7
Distinct Amplicons	6	6	4	4	5	3	3	3	5	5
Distribution (Mol. Size/ bp)										
1500					—					
1400					—	—				
1300			—	—						
1200		—								
1100	—									
1000							—			
900		—				—			—	
800	—		—					—		
700	—			—	—		—	—		—
600	—			—	—				—	—
500	—	—	—		—				—	—
400	—	—	—			—	—		—	—
300		—		—				—	—	
200		—								—

Table 33: Amplification pattern depicted for the black pepper variety Panniyur 7 with the 8 selected SSR primers

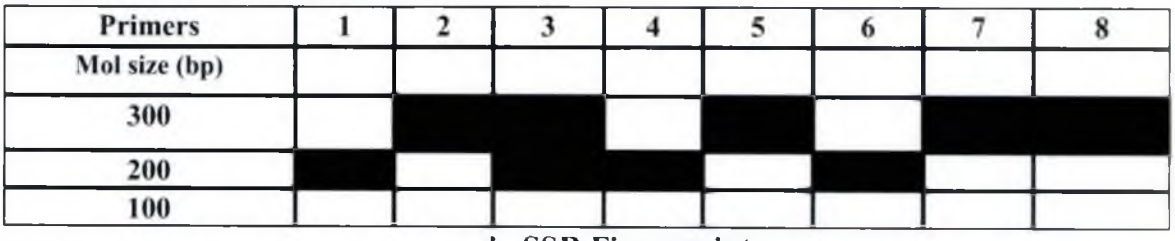
	1	2	3	4	5	6	7	8
Primers	PN A5	PN B5	PN E3	PN F1	PN G11	PN D10	PN H4	PN H8a
Total Amplicons	1	2	2	1	2	2	1	2
Distinct Amplicons	1	1	2	1	1	1	1	2
Distribution (Mol. Size/ bp)								
300		—	—		—		—	—
200	—		—	—		—		
100								



a. RAPD Fingerprint



b. ISSR Fingerprint



b. SSR Fingerprint

Colour code for sharing of band among varieties

Fig.7: Fingerprint for variety Panniyur 7 through RAPD, ISSR and SSR analysis

4.4. Cluster analysis for the seven black pepper varieties

Genetic similarity coefficient for the varieties ranged from 0.52 to 0.76 (Fig. 9). Highest similarity 76 per cent was observed between black pepper varieties Panniyur 1 and Panniyur 3. The variety Panniyur 4 was distinct from the other with 48 per cent variability.

4.4.2 Resolving power (Rp) of 10 selected RAPD and ISSR primers

The Resolving power (Rp) calculated for the random primer is presented in Fig. 10a. It ranged between 7.42 (OPA 28) and 9.42 (OPU 02 and OPP 14) with an average of 8.42 for RAPD primers. ISSR primers (Fig. 10b) recorded values ranging between 5.42 (UBC 843) and 12.28 (UBC 811 and UBC825) with an average of 8.61.

4.4.3 Polymorphic Information Content (PIC) value for the 10 selected RAPD and ISSR primers

The Polymorphic Information Content (PIC) value calculated for the 10 selected RAPD primers (Fig. 11a) varied from 0.86 (OPU 01) to 0.90 (OPU 03 and OPP 08) with a mean of 0.88. The 10 selected ISSR primers (Fig. 11b) recorded values ranging from 0.80 (UBC 843) to 0.89 (UBC811, UBC812, UBC 825 and UBC836) with an average of 0.84.

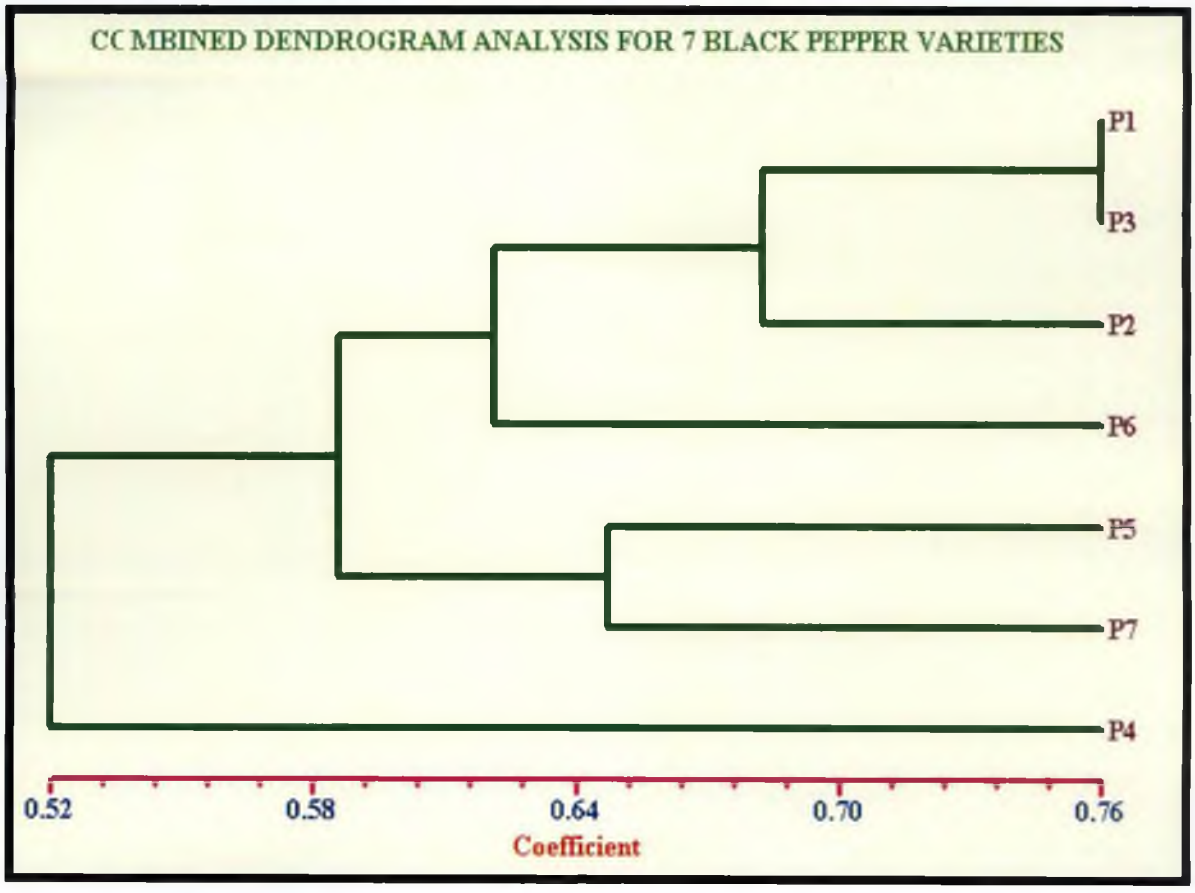
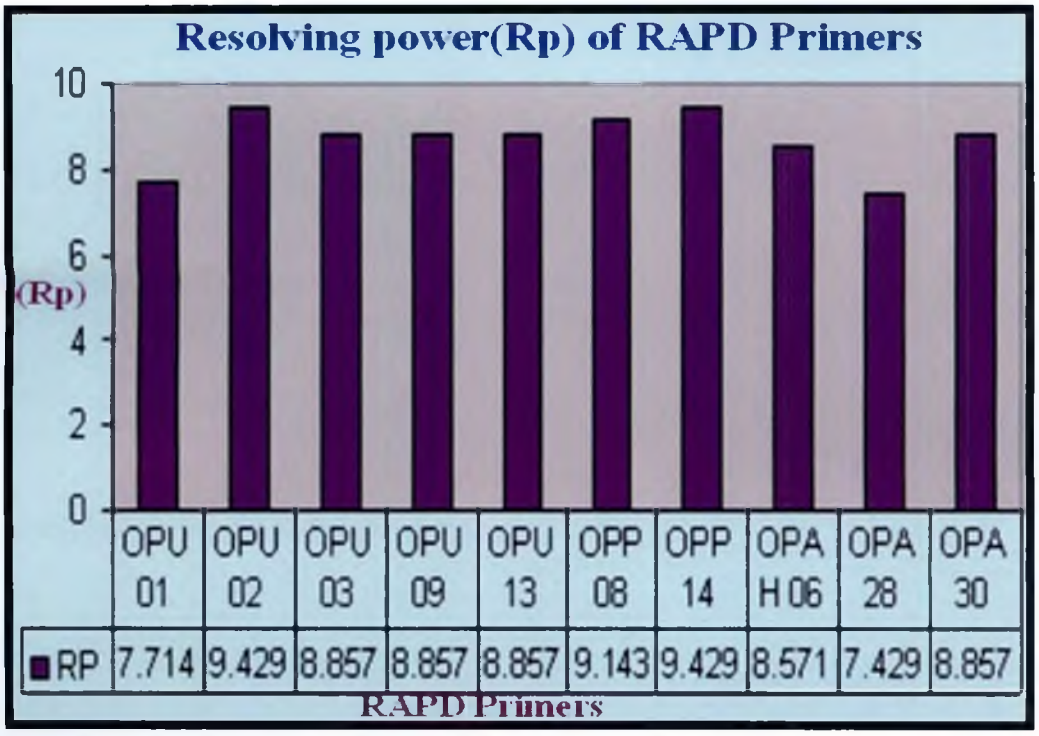
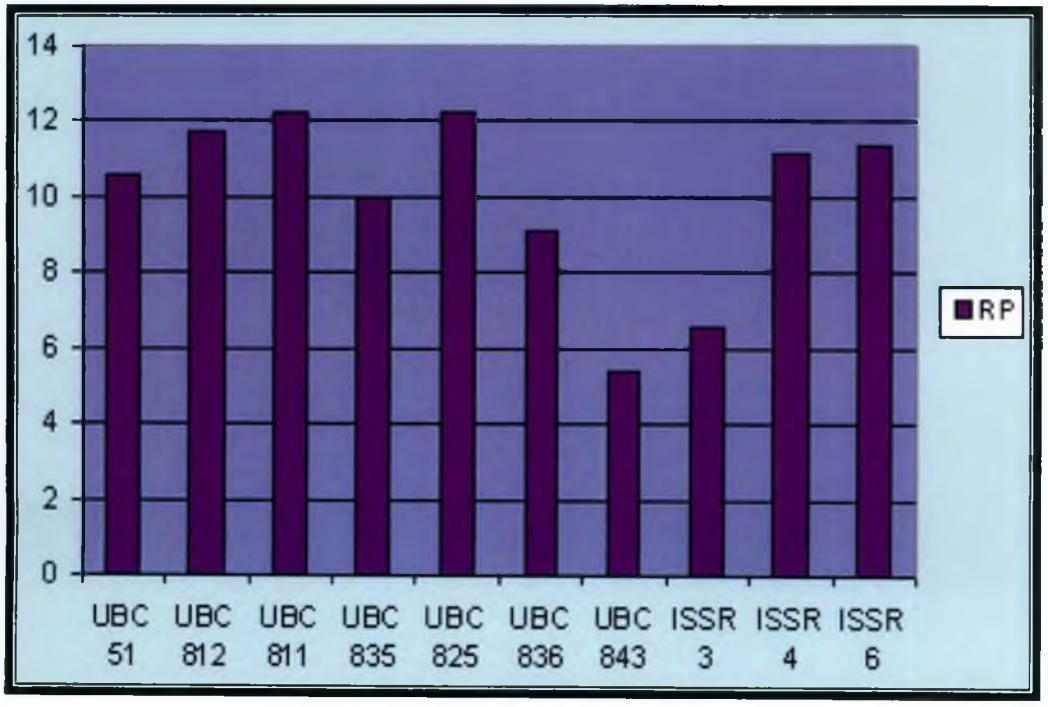


Fig.9. Dendrogram with RAPD, ISSR and SSR analysis for the 7 black pepper varieties

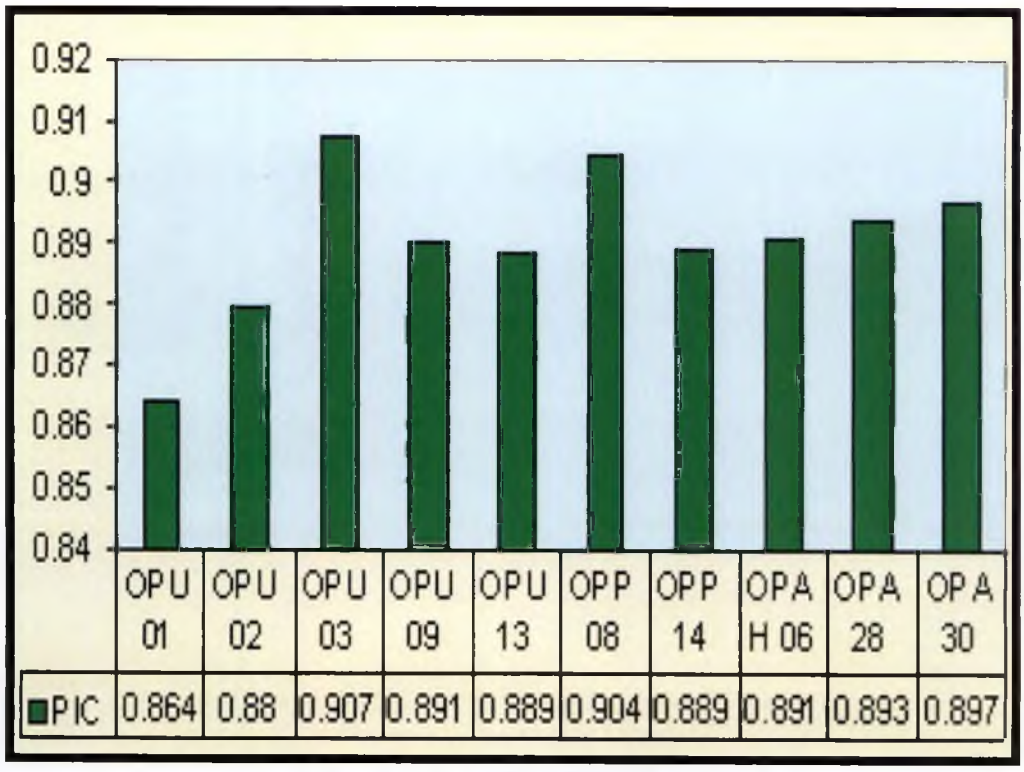


a. 10 selected RAPD primers

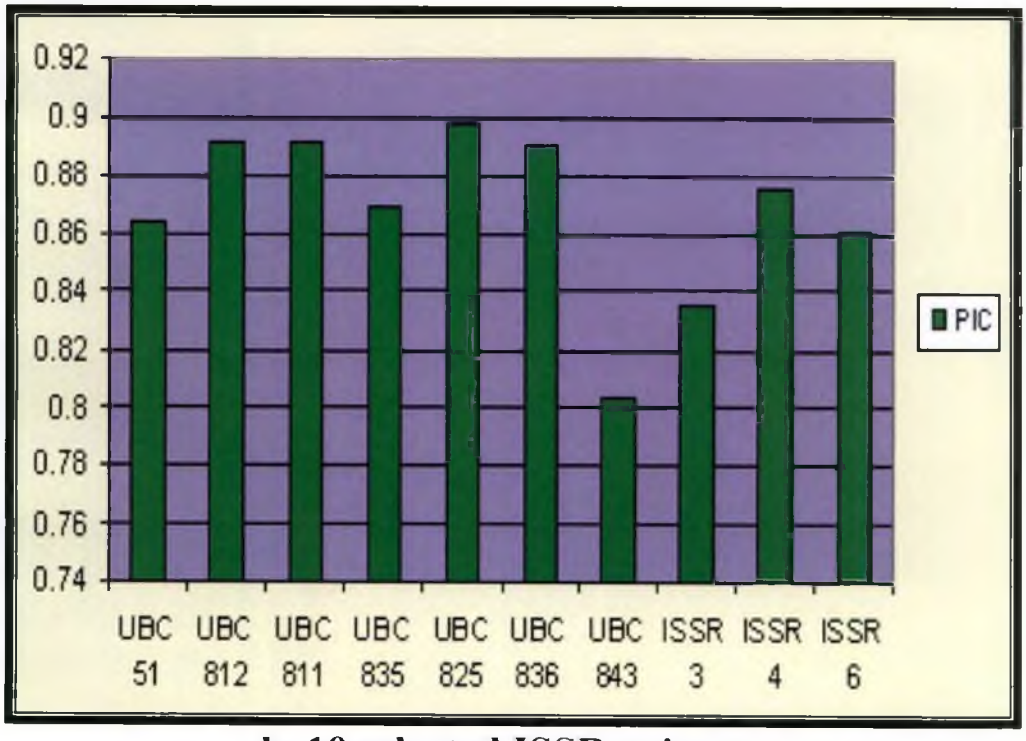


b. 10 selected ISSR primers

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

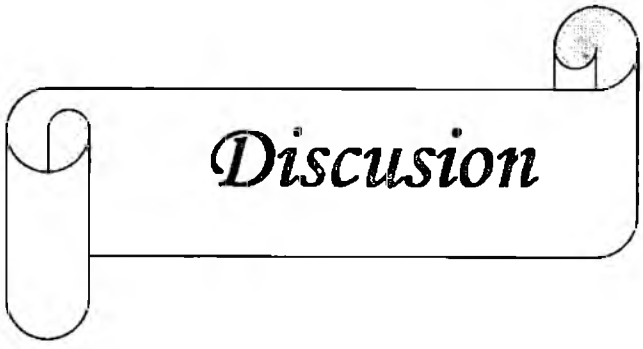


a. 10 selected RAPD primers



b. 10 selected ISSR primers

Fig 11: Polymorphic Information Content (PIC) value for 10 selected RAPD and ISSR primers


Discusion

5. DISCUSSION

The genus *Piper* includes the most valuable economically important spice crop, Black Pepper (*Piper nigrum* L.) the 'King of Spices'. Apart from Asian countries such as Malaysia, India, Indonesia, Thailand, Vietnam, China and Srilanka, it is also cultivated in Brazil and Madagascar. The Western Ghats of Indian peninsula is the primary centre of origin of black pepper. By habit, it is a perennial woody climber. Kerala the southernmost state of India occupies a considerable portion of the Western Ghats and is a rich source of wild relatives of this spice crop. The hot and humid climate of the sub-mountainous tracts of Western Ghats is ideal for its cultivation and hence Kerala is the major producer of black pepper in India.

Being the centre of origin, natural variability available in this crop is very high. Selection and habituation has resulted in evolving large number of cultivars and over 200 such genotypes are available with the farmers for cultivation. Efforts have been made to characterise these cultivars and varieties through molecular tools (Pradeepkumar *et al.,* 2003; Nazeem *et al.*, 2005; Keshavachandran *et al.*, 2005); based on which similarity/divergence among many of the genotypes have been worked out.

In black pepper efforts to develop varieties with high yield and quality through selection as well as hybridization were initiated as early as in 1953 (Nambiar *et al.*, 1978) at Pepper Research Station, Panniyur, which released the first ever hybrid variety of pepper Panniyur 1. Ever since this classic achievement, attempts were and are being made all over the world to generate high yielding black pepper varieties. More than 15 high yielding varieties have been released for cultivation in black pepper among which two are hybrids Panniyur 1 and Panniyur 3 developed at the Pepper Research Station, Panniyur, Kerala (Vanaja *et al.*, 2006). Panniyur 1 is still the ruling variety in India and have gained popularity in all the pepper producing countries. The variety has special distinguishing characters such as long spike, high fruit setting bold berries and high yield. After

the release of the popular variety Panniyur 1, the station has released Panniyur 2, Panniyurb 3, Panniyur 4, Panniyur 5, Panniyur 6 and Panniyur 7.

Piper nigrum L. cultivars have a high level of polymorphism (96.6%) indicating extensive genetic variation of the Indian germplasm (Pradeepkumar *et al.*, 2003). It is also reported that increased divergence among landraces, compared to the advanced cultivars that have been derived mostly by clonal selection from land races, though a few are of hybrid origin. Morphologically divergent intraspecific variants of this species occur both in wild forms and in the cultivated varieties.

Many of the reports on molecular characterisation include a combination of two or more molecular markers for overcoming the drawback of individual systems and thus to generate a wholesome information about the material under study (Parani *et al.*, 1997; Papavo *et al.*, 1998). In the present investigation a combination of three molecular marker systems *Viz.*, RAPD, ISSR and SSR analysis were used to develop a DNA fingerprint with which the variety could be identified and its fidelity detected.

5.1 Isolation, purification and quantification of DNA

The samples were young leaves from seven black pepper varieties. The protocol suggested by Roger and Benedich (1994) modified with 4X CTAB extraction buffer yielded good quality DNA. The electrophoresed DNA showed distinct bands without shearing.

The grinding in liquid nitrogen was found to improve the quality of DNA isolated. On grinding, the leaves of black pepper turned brown immediately due to phenolic oxidation. The quinones produced were known to be powerful oxidising agents, which damage DNA and proteins (Weising *et al.*, 2005). The addition of antioxidant like β-mercaptoethanol and sodium metabisulfite in the extraction buffer or during grinding was found effective. Similar results were also reported in coffee, which is a crop with high phenols (Sreenath *et al.*, 1992).

The detergent used in the extraction buffer of CTAB (Cetyl Trimethyl Ammonium Bromide), which helps in the disruption of the cell membrane thereby releasing nucleic acid into the extraction buffer and prevents co-precipitation of polysaccharides with nucleic acid by acting as a selective precipitant of nucleic acids. CTAB is a cationic detergent, which solubilises membrane and form a complex with DNA (Sghaier and Mohammed, 2005).

The advantageous effect of the CTAB along with PVP on the quality of DNA was also reported by Gallego and Martinez (1996) and (Sreenath *et al.*, 1992). It effectively disrupts the cell membrane and together with NaCl separates the polysaccharides. The EDTA in the extraction buffer protects the DNA from endonuclease by chelating the Mg^{2+} ions of DNA. Double treatment with chloroform: isoamylalcohol mixture and centrifugation effectively removes the pigment and proteins. The addition of chilled isopropanol precipitates the DNA and washing the pellet with 70 percent alcohol followed by absolute alcohol removes the traces of CTAB. TE buffer rehydrates the DNA and dissolves it (Wettasinghe and Peffley, 1998; Babu, 2000).

Problems encountered in the isolation and purification of high molecular weight DNA from certain plant species include: degradation of DNA due to endonuclease, polyphenols and other secondary metabolites that directly and indirectly interfere with subsequent enzymatic reactions as reported by Weishing *et al.* (1999) and Matasyoh *et al.* (2008). EDTA is also a major component of TE buffer in which the DNA was dissolved and stored. RNase treatment was given in order to remove RNA contamination from the isolated DNA samples.

A DNA sample was reported as high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasinghe and Peffley, 1998). Use of Rnase A was reported by several workers (Raval *et al.* (1998), Wettasinghe and Peffley (1998) and 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 indicated that the DNA under test was of good quality.

The absorbance ratio was calculated as OD at 260/280, for the various samples, using spectrophotometer. Those samples with ratio between 1.8 and 2.0 were considered to be of high quality DNA. If the value goes beyond 2.0, it indicates RNA contamination and if less than 1.8, it indicates protein contamination. All molecular analysis was done with high quality DNA.

5.2 Molecular marker analysis

Molecular marker technology provides novel tools for DNA fingerprinting. Most of the molecular marker are developed by the PCR (Polymerase Chain Reaction) technology and amplifies unique regions on the genomic DNA based on the primers designed for DNA amplification. Three such PCR based marker systems (RAPD, ISSR SSR) were utilized for fingerprinting black pepper varieties.

5.2.1 RAPD analysis:

The RAPD technique was developed by Williams *et al.,* (1990) and the technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary decamer primers. In RAPD markers, polymorphism results from the changes in the sequence of the primer binding site. Usually RAPD markers are dominant in nature (Waugh and Powell, 1992) because polymorphisms are detected as the presence or absence of bands.

The RAPD technique (Parani *et al.*, 1997) is a simple technique which detects polymorphism and has been used for molecular characterisation in several crop plants such as *Oryza sativa* L. (Chakravarthi and Naravaneni, 2006), *Piper nigrum* L.(Keshavachandran *et al.*, 2005), *Manihot esculenta crantz.* (Santha, *et all.,* 2005), *Ficus carica* L. (Khadari *et al.*, 1995), Hybrid rice (Seyyed *et al.*, 2010) and *Mangifera indica* L. (Adato *et al.*, 1995). Therefore, RAPD seems to be a useful tool for identification of black pepper cultivars.

5.2.3 ISSR analysis:

The choice of a molecular marker technique depends on its reproducibility and simplicity. The best marker for genome mapping, marker assisted selection, phylogenic studies, and crop conservation should have low cost and labour requirements and high reliability. Since 1994, a molecular marker technique called Inter Simple Sequence Repeat (ISSR) has been available and is being exploited (Zietkiewicz *et al.*, 1994). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Like RAPD, ISSR markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers.

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 *Oryza spices* (Joshi *et al.,* 2000), *Solanum lycopersicon* Mill.(Aguilera *et al.,* 2011) and *Mangifera indica* L. (Luo *et al.,* 2011).

5.2.4 SSR analysis:

Simple Sequence Repeats, also referred as microsatellites observed in eukaryotic genome are unique to individuals with respect to the number of repeats and regions flanking the repeats. They are highly informative molecular markers, with high degree of polymorphism, codominant in nature and exhibiting mendelian inheritance. Menezes *et. al.,* (2009) have reported nine microsatellite loci in *Piper nigrum* which could be utilized for characterisation of black pepper germplasm. SSR markers are also reported and exploited in various other crops for hybridity testing (Muhammad *et al.,* 2009), evaluation of mapping population (Lee *et al.,* 2004) and marker assisted breeding (Francia *et al.,* 2005).

The suitability of the primers selected for analysis were evaluated by calculating the Resolving power (Rp) and Polymorphic Information Content (PIC). The Rp values ranged from 7.43 to 9.43 for the RAPD primers and it was from 5.21 to 12.01 for ISSR primers. The Rp values reported for variability

analysis in various crops ranged between 2.59 to 12.50 (Prevost and Wilkinson, 1990, Kashavachandran *et al.*, 2005, Sarla *et al.*, 2005).

The PIC values ranged between 0.86 and 0.91 for RAPD primers and it was between 0.80 and 0.90 for ISSR primers. The PIC values indicate the extent of polymorphism detected by the primer among the varieties studied. The PIC values reported in various other crops for the primers used for characterisation is in conformity to the values observed in the present study (Karihaloo *et al.*, 2003; Kelley *et al.*, 2004; Dongre and Kharbikar, 2004, Hollman *et al.*, 2005); thus confirming the suitability of the primers selected for DNA fingerprinting in the present study.

5.3 Fingerprinting of black pepper varieties

The black pepper varieties selected for the study are the seven released by Kerala Agricultural University. These varieties have different parentage and are unique in their morphological parameters. However, varietal identification is difficult, observing the phenotypic characters. The morphological characters as described by the Pepper Research Station, Panniyur, Kerala; from where these varieties originated, are provided in table 8.

The amplification pattern observed in RAPD, ISSR and SSR assay were utilized for developing finger print for the selected varieties.

5.3.1 Variety, Panniyur 1:

Panniyur 1 is a high yielding hybrid variety of black pepper with Uthirankotta and Cheriyakaniakadan as its parents. Long spike, yellowish green growing tip and large heart shaped leaves are the morphological characters to distinguish the variety.

Molecular assay recorded distinct banding pattern for the variety Panniyur 1. Each of the primer in RAPD and ISSR assay gave polymorphic banding pattern

with the bands ranging in size from 200bp to 1600bp. Each primer gave two to six amplicons. SSR analysis also gave distinct pattern for the variety with one or two amplicons of 100bp to 300bp size for each primer (Fig.1).

The gel profile of Panniyur 1 when compared with the other varieties indicated that most of the amplicons are shared with some other variety for the same primer. Different colour codes were provided to indicate the sharing of bands with other varieties. RAPD primer 500bp (OPA 28) and ISSR primers 300bp (UBC 51) and 1300bp (UBC 812) gave unique amplicons (depicted in green) for Panniyur 1 (Fig.1). SSR analysis indicated that most of the amplicons for all the primers were shared with all the other varieties studied (depicted in black colour). However, this sharing of bands with other varieties does not make the variety loose its identity. The combined fingerprint developed for variety Panniyur 1 (Fig. 8) is unique and different from all others.

5.3.2 Variety, Panniyur 2:

The variety Panniyur 2 is a selection from open pollinated progeny of 'Balankotta'. It is unique for its high piperine (6.6%) content. The molecular profile developed through RAPD, ISSR and SSR analysis is depicted in Fig.2. The banding pattern observed was good enough to characterise the variety. RAPD primers 1300bp (OPU 13), 500bp (OPP 8), 300bp (OPAH 6) and 700bp (OPA28) and ISSR primers 1600bp (UBC 811), 200bp (UBC 835), 700bp (UBC 836) and 1500bp (ISSR 4) gave unique bands for the variety. SSR banding pattern was almost the same as for other varieties.

The combined fingerprint developed for the variety Panniyur 2 through RAPD, ISSR and SSR analysis is depicted in Fig.8 and it is unique when compared to that for other varieties.

5.3.3 Variety, Panniyur 3:

Panniyur 3 is another hybrid variety with the same parentage as for Panniyur 1 and morphological features are almost same as variety Panniyur 1 but leaves having wavy margin and spikes shorter in length.

The fingerprint developed through RAPD, ISSR and SSR analysis is depicted in Fig.3. The banding pattern observed was good enough to characterize the variety with RAPD primers 800bp (OPU 01), 800bp (OPU 02) and 800bp (OPP 08) and ISSR primers 800bp (UBC 843). Most of the bands observed were found shared with the other varieties.

The combined fingerprint developed for the variety Panniyur 3 through RAPD, ISSR and SSR analysis is depicted in Fig.8 and it is unique when compared to that for other varieties.

5.3.4 Variety, Panniyur 4:

The variety Panniyur 4 is a clonal selection from cultivar 'Kuthiravaly' as its parent. Dark pink growing tip and large heart shaped leaves are the morphological characters to distinguish the variety. The molecular profile developed through RAPD, ISSR and SSR analysis is depicted in Fig.3. The banding pattern observed was good enough to characterise the variety. RAPD primers 1200bp (OPU 02), 2000bp,1500bp, (OPU 03), 1400bp, 800bp (OPU 09), 1900bp (OPU 13), 1700bp (OPP 08), 1600bp, 1400bp (OPP 14), 1900bp, 1400bp (OPAH 6) and 1800bp, 1600bp, 1400bp, 1300bp (OPA 28) and ISSR primers 700bp (UBC 811), 900bp (UBC 835), 1100bp, 400bp (UBC 825), 1900bp, 1200bp (UBC 836), 2100bp, 1300bp (ISSR3) and 900bp (ISSR 6) gave unique bands for the variety. SSR banding pattern was almost the same as for other varieties.

The combined fingerprint developed for the variety Panniyur 4 through RAPD, ISSR and SSR analysis is depicted in Fig.8 and it is unique when compared to the other varieties.

5.3.5 Variety, Panniyur 5:

Panniyur 5 variety is a selection from open pollinated progeny of 'Perumkodi'. It is unique for its high essential oil (3.8%) and oleoresin (12.33%) content. Long spike, purple growing tip and large oval shaped leaves are the morphological characters to distinguish the variety.

The molecular profile developed through RAPD, ISSR and SSR analysis is depicted in Fig.5. The banding pattern observed was good enough to characterise the variety. RAPD primers 300bp (OPU 01), 100bp (OPP 08) and 1300bp (OPAH 06) and ISSR primers 1100bp, 600bp (UBC 812), 300bp (UBC 811), 800bp (UBC 835), 1600bp, 1300bp, 1100bp (UBC 836), 1400bp (UBC 843), 1800bp, 1200bp (ISSR 3) and 1200bp (ISSR 4) gave unique bands for the variety. SSR banding pattern was almost the same as for other varieties.

The combined fingerprint developed for the variety Panniyur 5 through RAPD, ISSR and SSR analysis is depicted in Fig.8 and it is unique when compared to the other varieties.

5.3.6 Variety, Panniyur 6:

The variety Panniyur 6 is a clonal selection from cultivar of 'Karimunda' as its parent. Light pink growing tip, bold attractive berries, large number of spikes from unit area and small oval shaped leaves are the morphological characters to distinguish the variety (Vanaja *et al.,* 2006). The molecular profile developed through RAPD, ISSR and SSR analysis is depicted in Fig.6. The banding pattern observed was good enough to characterise the variety. RAPD primers 1400bp (OPU 01), 1800bp, 1600bp (OPU 03), 700bp (OPU13), 1600bp (OPP 08), 1200bp (OPP 14), 1700bp (OPAH 06) and 1200bp (OPA 30) gave unique bands for the variety and ISSR primers indicated that most of the amplicons for all the primers were shared with all the other varieties. SSR banding pattern was almost the same as for other varieties.

The combined fingerprint developed for the variety Panniyur 6 through RAPD, ISSR and SSR analysis is depicted in Fig.8 and it is distinct for this variety.

5.3.7 Variety, Panniyur 7:

Panniyur 7 variety is a selection from open pollinated progeny of 'Kalluvally'. Long spikes hidden under the leaves, light purple growing tip and heart shaped leaves with wavy margin are the morphological characters to distinguish the variety. The molecular profile developed through RAPD, ISSR and SSR analysis is depicted in Fig.7. The banding pattern observed was good enough to characterise the variety. RAPD primers 1300bp (OPP 08), 900bp (OPA 28) and 400bp (OPA 30) and ISSR primers 1300bp (UBC 835) and 800bp (ISSR 3) gave unique bands for the variety and most of the bands observed were found shared with the other varieties. SSR banding pattern was almost the same as for other varieties.

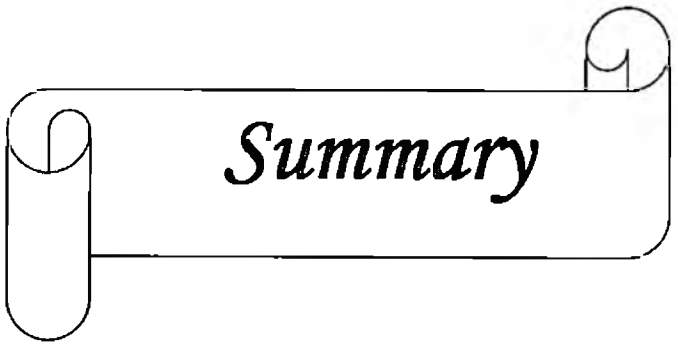
The combined fingerprint developed for the variety Panniyur 7 through RAPD, ISSR and SSR analysis is depicted in Fig.8 and it is unique when compared with the other varieties.

The fingerprints for the varieties Panniyur 1 to Panniyur 7 were unique and distinct. Though individual primers of each marker system showed several bands shared among varieties, the pattern obtained when all the bands irrespective of the marker system (*i.e.*, RAPD, ISSR and SSR) are considered together; is unique for each variety which forms the fingerprint of the particular variety. They could be very well utilized to prove varietal identity.

5.4 Genetic variability analysis

The amplification pattern observed in RAPD, ISSR and SSR analysis was scored and analysed for relatedness/variability among the varieties. The computer package NTSYS-PC (Rohlf, 2005) was used for cluster analysis. Maximum variability observed was 48 percent and it was for the variety Panniyur 4. The varieties Panniyur 1 and Panniyur 3 having the same parentage indicated 76 percent similarity. Similar reports were made by Onguso *et al.* (2004) for banana in which the highly related cultivars had a similarity coefficient of 0.85 while the lowest value was 0.42. In the analysis of genetic relatedness in *Gossypium* species, Gomes *et al.* (2004) observed a similarity range from 0.54 to 0.86 while Dongre and Khanuja *et al.*, (2004) obtained 67 to 87 percent similarity among 25 accessions of *Gossypium hirsutum* from different origins.

Future line of work include developing fingerprints for more varieties, including supporting data of parents from which the variety actually originated and utilization of unique molecular profile obtained in relation to specific traits in the identified varieties.


Summary

6. SUMMARY

The study entitled "DNA fingerprinting of selected black pepper (*Piper nigrum* L.) varieties" was carried at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture during the period 2009-2011. The objectives of the study were to characterise the released black pepper varieties of KAU using different molecular markers - RAPD, ISSR and SSR and to develop DNA fingerprint with which the variety could be identified and its fidelity detected. The Seven released black pepper varieties (Panniyur I to Panniyur 7) collected from Pepper Research Station, Panniyur and maintained at CPBMB, COH were used for the study.

The salient findings are as follows:

1. The protocol for genomic DNA isolation was standardised. The protocol suggested by Rogers and Benedich (1994) with slight modification (4% CTAB) was found to be the most appropriate for isolation of DNA from black pepper varieties. The RNA contamination was completely removed through RNase treatment.
2. The quality and quantity of DNA was analyzed by NanoDrop® ND-1000 spectrophotometer. The absorbance ratio ranged from 1.80-1.89, which indicated good quality DNA and the recovery was high. This DNA was suitable for RAPD, ISSR and SSR analysis.
3. Protocol for RAPD, ISSR and SSR assay in black pepper varieties were standardized with the various quantities of DNA, PCR mixtures and conditions for DNA amplification.
4. 30 RAPD, 34 ISSR primers and 29 SSR primer pairs were screened for their ability to amplify DNA fragments. Out of these 10 RAPD, 10 ISSR primers and 8 SSR primer pairs were selected based on the number of the bands and nature of amplicons.
5. The ISSR primers (ISSR3, ISSR4 and ISSR6) and all the SSR primer pairs (PNA5, PNB5, PND10, PNE3, PNF1, PNG11, PNH4 and PNH8a) are specific for black pepper varieties.

6. The Resolving power (Rp) of the RAPD and ISSR primers was calculated and the values ranged between 7.42 and 9.42 for RAPD primers and 5.42 to 12.28 for ISSR primers. The RAPD primers OUP 02 and OPP 14 and ISSR primers UBC 811 and UBC 825 showed high resolving power.

7. The Polymorphic Information Content (PIC) calculated ranged between 0.86 to 0.91 for RAPD primers and 0.80 to 0.89 for ISSR primer indicating the suitability of primers to detect polymorphism. RAPD primers OPU 03 and OPP 08 and ISSR primers UBC 825 reported highest PIC values.

8. Distinct bands were used to develop DNA fingerprint of black pepper varieties Panniyur 1 to Panniyur 7 through RAPD, ISSR and SSR analysis.

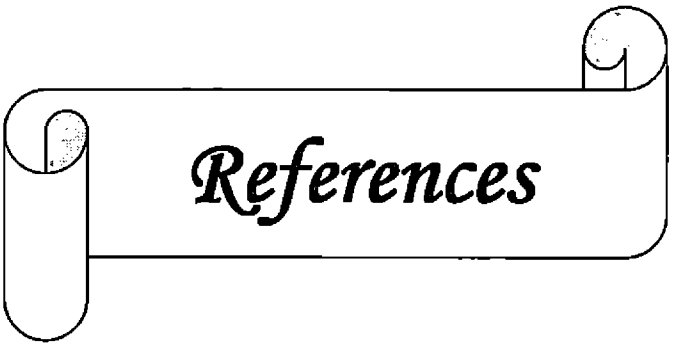
9. Sharing of amplicons developed for each primer with other varieties was analysed and demarcated with different colour codes in the fingerprints developed. Most of the amplicons were found shared among the varieties. However, the pattern of sharing was different and good enough to separate out the varieties.

10. Combined DNA fingerprint of each variety with RAPD, ISSR and SSR data was also developed.

11. The scored data based on RAPD, ISSR and SSR banding was used to construct a dendrogram using the NTSYS pc. (ver. 2.1) software. Similarity coefficient ranged from 0.52 to 0.76. The highest similarity (76 percent) was observed between black pepper varieties Panniyur 1 and Panniyur 3. Panniyur 4 was the most distant one among the seven varieties analysed.

12. The fingerprint developed was good enough to provide varietal identity and the analysis could reveal variability/ relatedness among the seven varieties.

Future line of work include developing fingerprints for more varieties, including supporting data of parents from which the variety actually originated and utilization of unique molecular profile obtained in relation to specific traits in the identified varieties.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Annexure

ANNEXURE-I

Details of laboratory equipment items used for the study

High speed refrigerated centrifuge	: Kubota, Japan
Horizontal electrophoresis system	: BIO-RAD
Thermal cycler	: Master cycler personal, Eppendorf
Gel documentation system	: Biorad, USA
NanoDrop^{R} ND-1000 Spectrophotometer	: NanoDrop^{R} Technologies Inc.USA

ANNEXURE-II

Composition of buffers and dyes used for agarose gel electrophoresis

1. **TAE Buffer 50X**

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

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 1mg/ml in water and was stored at room temperature in a dark bottle.

ABSTRACT

Black pepper *(Piper nigrum L.)* famous as "Black Gold" and also known as "King of Spices" is one of the important agricultural commodities of commerce and trade in India since pre-historic period. The crop is the major source of income and employment for rural households in the predominantly pepper growing State of Kerala where more than 2.5 lakh farm families are involved in pepper cultivation. Karnataka, Tamil Nadu are the other major pepper producing States in the country. Kerala accounts for 80-90% of the total pepper production in the country. Idukki and Wynadu are the two major pepper producing districts in Kerala. Different cultivars/varieties are popular among the farmers and there phenotypic identity is not very distinct.

The study entitled "DNA fingerprinting of selected black pepper (*Piper nigrum* L.) varieties" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture during the period 2009-2011. The objectives of the study were to characterize the released black pepper varieties of KAU using different molecular markers - RAPD, ISSR and SSR and to develop DNA fingerprint with which the variety could be identified and its fidelity detected.

Seven black pepper varieties (Panniyur 1 to Panniyur 7) collected from Pepper Research Station, Panniyur and maintained at CPBMB, COH were used for the study. DNA extraction was done with CTAB (Rogers and Benedich, 1994) method with slight modification. The RNA contamination was completely removed through RNase treatment. Good quality DNA with UV absorbance ratio (A_{260}/A_{280}) 1.80 - 1.89 was used for further analysis.

The PCR conditions were optimized for RAPD, ISSR and SSR assay. 30 RAPD, 34 ISSR and 29 SSR primers were screened with bulked DNA of black pepper varieties for amplification and those which gave reliable distinct banding pattern were selected for further amplification and fingerprinting. The PCR products obtained from RAPD, ISSR and SSR analysis were separated on 1.3 to 2 percent agarose gel and the amplification patterns recorded.

The genomic DNA from each variety was amplified with 10 each of selected RAPD and ISSR primers and 8 SSR primer pairs. The amplification pattern was scored and depicted to develop fingerprint for each variety.

The Resolving power (Rp) worked out for the different primers ranged between 7.42 to 9.42 in RAPD and 5.42 to 12.28 in ISSR analysis; indicating the capacity of the primers selected to distinguish the varieties. The Polymorphic Information Content (PIC) varied from 0.86 to 0.90 for RAPD analysis and it was between 0.80 and 0.89 in ISSR analysis indicating the variability among the genotypes.

Distinct bands were used to develop DNA fingerprint of black pepper varieties Panniyur 1 to Panniyur 7 through RAPD, ISSR and SSR analysis. Sharing of amplicons developed for each primer with other varieties was also analyzed and demarcated with different colour codes in the fingerprints developed. Most of the amplicons were found shared among the varieties. However, the pattern of sharing was different and good enough to separate out the varieties. Combined DNA fingerprint of each variety with RAPD, ISSR and SSR data was also developed.

The amplification pattern observed in RAPD, ISSR and SSR analysis was scored and analyzed for quantifying the variability among the varieties. The computer package NTSYS-Pc was used for cluster analysis. Maximum variability observed was 48 percent for the variety Panniyur 4. The varieties Panniyur 1 and Panniyur 3 having the same parentage indicated 76 percent similarity. The fingerprint developed was good enough to provide varietal identity and the analysis could reveal variability/relatedness among the seven varieties.

Separate and combined fingerprints were developed for all the seven varieties through RAPD, ISSR and SSR analysis. The DNA fingerprints thus developed could be utilized for the variety registration and settling IPR issues.